

# Is disrupted nucleotide-substrate cooperativity a common trait for Cushing's syndrome driving mutations of protein kinase A?

Walker, Caitlin; Wang, Yingjie; Olivieri, Cristina; V.S, Manu; Gao, Jiali; Bernlohr, David A.; Calebiro, Davide; Taylor, Susan S.; Veglia, Gianluigi

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1 **The allosteric E31V mutation disrupts the nucleotide-substrate cooper-**  
2 **ativity in protein kinase A: is there a common mechanism for Cushing's**  
3 **syndrome driving mutations?**

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6 Caitlin Walker<sup>1</sup>, Yingjie Wang<sup>1,2,†</sup>, Jiali Gao<sup>2,3</sup>, David A. Bernlohr<sup>1</sup>, Davide Calebiro<sup>4,5</sup>, Susan S.  
7 Taylor<sup>6,7</sup>, and Gianluigi Veglia<sup>1,2,\*</sup>  
8  
9  
10  
11

12 <sup>1</sup>*Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Min-*  
13 *neapolis, MN 55455, USA*

14  
15 <sup>2</sup>*Department of Chemistry and Supercomputing Institute, University of Minnesota, Minneapolis,*  
16 *MN 55455, USA*

17  
18 <sup>3</sup>*Institute of Systems and Physical Biology, Shenzhen Bay Laboratory, Shenzhen, 518055,*  
19 *China*

20  
21 <sup>4</sup>*Institute of Metabolism and Systems Research, University of Birmingham, B15 2TT, Birming-*  
22 *ham, UK*

23  
24 <sup>5</sup>*Centre of Membrane Proteins and Receptors, University of Birmingham, B15 2TT, Birmingham,*  
25 *UK*

26  
27 <sup>6</sup>*Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, CA*  
28 *92093*

29  
30 <sup>7</sup>*Department of Pharmacology, University of California at San Diego, La Jolla, CA 92093*  
31  
32  
33  
34

35 †Present Address: *Institute of Systems and Physical Biology, Shenzhen Bay Laboratory, Shen-*  
36 *zhen, 518055, China.*

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39  
40 \*Corresponding Author

41  
42 Gianluigi Veglia  
43 Department of Chemistry and Department of Biochemistry, Molecular Biology, and Biophysics,  
44 321 Church Street SE  
45 Minneapolis, MN 55455  
46 Telephone: (612) 625-0758  
47 Fax: (612) 625-5780  
48 e-mail: [vegli001@umn.edu](mailto:vegli001@umn.edu)

49 **ABSTRACT**

50 Somatic mutations in the *PRKACA* gene encoding the catalytic  $\alpha$  subunit of protein kinase A  
51 (PKA-C) are responsible for cortisol-producing adrenocortical adenomas. These benign neo-  
52 plasms contribute to the development of Cushing's syndrome. The majority of these mutations  
53 occur at the interface between the two lobes of PKA-C and interfere with the enzyme's ability to  
54 recognize substrates and regulatory subunits, leading to aberrant phosphorylation patterns and  
55 activation. Rarely, patients with similar phenotypes carry an allosteric mutation, E31V, located at  
56 the  $\alpha$ A-helix's C-terminal end and adjacent to the  $\alpha$ C-helix, a critical element in assembling the  
57 active conformation of kinases, but structurally distinct from the PKA-C interface mutations. Using  
58 a combination of solution NMR, thermodynamics, and kinetic assays, and molecular dynamics  
59 simulations, we show that the E31V allosteric mutation disrupts central communication nodes  
60 between the N- and C- lobes of the enzyme as well as the nucleotide-substrate binding coopera-  
61 tivity, a hallmark for kinases' substrate fidelity and regulation. For both orthosteric (L205R and  
62 W196R) and allosteric (E31V) Cushing's syndrome mutants the loss of binding cooperativity is  
63 proportional to the density of the intramolecular allosteric network. This structure-activity relation-  
64 ship suggests a possible common mechanism for Cushing's syndrome driving mutations in which  
65 decreased nucleotide/substrate binding cooperativity is linked to loss in substrate fidelity and dys-  
66 functional regulation.

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**KEYWORDS:** cAMP-dependent protein kinase A, Cushing's syndrome, allostery, cooperativity.

76 **INTRODUCTION**

77 Cushing's syndrome is defined by a collection of symptoms that result from prolonged  
78 exposure to high cortisol levels, with patients commonly presenting with abdominal obesity, met-  
79 abolic abnormalities, and hypertension [1]. Playing a fundamental role in regulating metabolism  
80 and cell proliferation in endocrine tissues, the cAMP signaling pathway and its aberrant activation  
81 are linked to several endocrine diseases [2-4]. The role of one component of this signaling cas-  
82 cade, cAMP-dependent protein kinase A (PKA), in Cushing's syndrome was not appreciated until  
83 recently when somatic mutations were identified in the *PRKACA* gene encoding the catalytic  $\alpha$   
84 subunit of PKA (PKA-C) [5-12]. To date, a total of eight mutations have been discovered. Except  
85 for one mutation, E31V, all mutations are located near the substrate-binding cleft adjacent to the  
86 catalytic/regulatory (R) subunit interface (**Figure 1A**).

87 PKA is the principal intracellular effector of the second messenger, cAMP. Inactive PKA  
88 exists as a holoenzyme ( $R_2:C_2$ ) containing an R-subunit dimer bound to two catalytic (C) subunits  
89 [13]. Each R-subunit contains an inhibitory sequence that occupies the active site of the enzyme.  
90 Following stimulation of adenylate cyclase, two cAMP molecules bind to each R-subunit, initiating  
91 a conformational change and releasing active PKA-C. While R-subunits are the primary intracel-  
92 lular regulator of PKA-C, an endogenous inhibitor (PKI) inhibits PKA-C activity within the nucleus  
93 and controls nuclear exportation [14]. Spatiotemporal regulation is controlled by various ancillary  
94 proteins such as A-kinase anchoring proteins (AKAPs) that, via interactions with R-subunits, lo-  
95 calize PKA-C near substrates [15].

96 PKA-C toggles between three major conformational states: open (apo), intermediate (nu-  
97 cleotide-bound), and closed (nucleotide/substrate-bound) [16]. This bean-shaped enzyme con-  
98 sists of a conserved catalytic core comprised of two lobes. The N-lobe of the kinase is smaller  
99 and contains mostly  $\beta$ -sheets and the  $\alpha$ C-helix and harboring the ATP binding site, while the C-  
100 lobe comprises mostly  $\alpha$ -helices and contains the substrate-binding cleft [17]. In contrast to other

101 Ser/Thr kinases, PKA-C contains an  $\alpha$ A-helix at its N-terminus, which anchors the N-lobe to the  
102 C-lobe and contributes to the tethering/positioning of the  $\alpha$ C-helix. This important structural motif  
103 is recognized for its role in the activation and inactivation of protein kinases [16]. E31V is located  
104 at the C-terminus of the  $\alpha$ A-helix and adjacent to the C-terminus of the  $\alpha$ C-helix. While other  
105 Cushing's syndrome mutations have been shown to disrupt R-subunit/PKA-C interactions, alter  
106 the enzyme's catalytic efficiency, and/or change its substrate specificity, the mechanism of dys-  
107 function for PKA-C<sup>E31V</sup> has remained elusive [18-20].

108 Recently, we discovered that the most common Cushing's syndrome mutation, PKA-  
109 C<sup>L205R</sup>, abrogates the nucleotide/pseudosubstrate binding cooperativity by reducing the intramo-  
110 lecular allostery between the small and large lobe [19]. Based on these findings, we suggested  
111 that this dysfunctional binding cooperativity and altered allostery disrupts substrate recognition  
112 and interactions with R-subunits, thereby altering canonical cAMP signaling. Despite E31V and  
113 L205R being spatially distant, our previous NMR analysis suggested they are allosterically cou-  
114 pled [19]. Therefore, we surmised that E31V may affect the kinase's function in a manner similar  
115 to L205R, *i.e.*, the non-conservative mutation may disrupt the allosteric network and the binding  
116 cooperativity.

117 To dissect this allosteric mutation's effects, we carried out solution NMR spectroscopy  
118 along with isothermal titration calorimetry (ITC), kinetic assays, and molecular dynamics (MD)  
119 simulations. We found that the E31V mutation ablates the canonical positive cooperativity, typi-  
120 cally seen for PKA-C while maintaining the kinase's catalytic efficiency. Specifically, the E31V  
121 mutation directly affects the allosteric node that connects the  $\alpha$ A-,  $\alpha$ C-helix, and activation loop,  
122 thereby disrupting nucleotide-substrate binding cooperativity. Finally, by comparing PKA-C<sup>WT</sup> with  
123 three drivers for Cushing's syndrome, PKA-C<sup>E31V</sup>, PKA-C<sup>L205R</sup>, and PKA-C<sup>W196R</sup>, we found a direct

124 relationship between the loss of binding cooperativity and the reduction of allosteric communica-  
125 tion within the enzyme. Altogether, our results suggest the existence of a common dysfunctional  
126 mechanism for PKA-C Cushing's mutations discovered thus far.

127

## 128 RESULTS

129 ***E31V mutation ablates nucleotide-substrate binding cooperativity in PKA-C.*** To evaluate  
130 the effects of E31V on the thermodynamics of nucleotide (ATP $\gamma$ N) and pseudosubstrate (PKI)  
131 binding, we used isothermal titration calorimetry (ITC) [21]. Values of  $\Delta G$ ,  $\Delta H$ ,  $-T\Delta S$ ,  $K_d$ , and co-  
132 operativity coefficients ( $\sigma$ ) obtained for PKA-C<sup>E31V</sup> are summarized in **Table S1 and S2** [19]. We  
133 found PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup> have similar binding affinities for ATP $\gamma$ N ( $K_d = 83 \pm 8$  and  $91 \pm 9$   
134  $\mu$ M, respectively). A 7-fold higher binding affinity is observed for PKA-C<sup>E31V</sup> compared to PKA-  
135 C<sup>WT</sup> when binding PKI<sub>5-24</sub> to their apo forms ( $K_d = 2.5 \pm 0.5$  and  $17 \pm 2$   $\mu$ M, respectively). In contrast  
136 to PKA-C<sup>WT</sup>, upon saturation with ATP $\gamma$ N, PKA-C<sup>E31V</sup> displays a 12-fold reduction in binding affinity  
137 ( $K_d = 0.16 \pm 0.02$  and  $2 \pm 1$   $\mu$ M, respectively). As previously determined, the binding of PKI<sub>5-24</sub> to  
138 PKA-C<sup>WT</sup> is highly cooperative ( $\sigma = 106 \pm 18$ ); in contrast, PKA-C<sup>E31V</sup> displays no cooperativity  
139 with  $\sigma = 1.3 \pm 0.7$ . To evaluate the effects of E31V on the kinase's catalytic efficiency, we carried  
140 out steady-state coupled enzyme assays using the standard substrate, Kemptide. Despite the  
141 dramatic effects on binding cooperativity, PKA-C<sup>E31V</sup> displayed only a slight increase in  $V_{max}$  and  
142 a slight decrease in  $K_M$ , resulting in similar catalytic efficiencies ( $k_{cat}/K_M = 0.41 \pm 0.05$  and  $0.46 \pm$   
143  $0.04$  for PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup>, respectively; **Figure 1B** and **Table S3**). Interestingly, mutagen-  
144 esis of residues adjacent to the E31 site has shown similar kinetic behavior [22].

145 ***NMR mapping of nucleotide/PKI binding response.*** To analyze the binding response of PKA-  
146 C<sup>E31V</sup> to nucleotide and pseudosubstrate, we mapped the amide backbone fingerprint of the en-  
147 zyme using [<sup>1</sup>H, <sup>15</sup>N]-TROSY-HSQC experiments [23]. The amide fingerprints of the kinase in  
148 different ligated forms are displayed in **Figure S1**. The global response of the two kinases to

149 ligand binding was determined using CONCISE (COordiNated Chemical Shifts bEhavior) [24],  
150 which performs a statistical analysis on linear chemical shift trajectories of amide resonances to  
151 identify the position of each state along the conformational equilibrium, shows that nucleotide and  
152 pseudosubstrate shift the overall populations from an open state to an intermediate and fully  
153 closed state. Upon binding the nucleotide, the probability density of the amide resonances from  
154 the apo shifts toward an intermediate state, and the subsequent saturation with PKI peptide fur-  
155 ther shifts toward the fully closed state (**Figure 1C**). Globally wild-type and mutant behave simi-  
156 larly; however, upon binding ATP $\gamma$ N, PKA-C<sup>E31V</sup> adopts a more open conformation compared to  
157 PKA-C<sup>WT</sup> and subsequent binding of PKI shifts the probability distribution toward a more closed  
158 state.

159 To further confirm the changes in the global response of PKA-C induced by E31V, we  
160 mapped the chemical shift perturbations (CSP,  $\Delta\delta$ ) of PKA-C<sup>E31V</sup>. Upon binding ATP $\gamma$ N, PKA-  
161 C<sup>E31V</sup> exhibits similar CSP patterns as wild-type (**Figure 2A,C**) with larger CSPs occurring  
162 throughout the N-lobe and in the c-terminal tail, though to a lesser extent than PKA-C<sup>WT</sup>. Further  
163 analysis of the  $\Delta$ CSP ( $\Delta\delta_{WT}-\Delta\delta_{E31V}$ ) shows regions of positive  $\Delta$ CSP confirming that upon binding  
164 ATP $\gamma$ N, PKA-C<sup>E31V</sup> does not adopt as closed of a conformation as the wild-type kinase (**Figure**  
165 **S2A**). Subsequent binding of PKI to ATP $\gamma$ N-saturated PKA-C<sup>E31V</sup> also exhibits similar CSPs com-  
166 pared to wild-type (**Figure 2B,D**), though to a larger extent as reflected in the negative  $\Delta$ CSP  
167 values (**Figure S2B**).

168 ***Rearrangement of the allosteric network of PKA-C<sup>E31V</sup> is linked to a decrease in nucleotide-***  
169 ***substrate binding cooperativity.*** Since cooperativity is often manifested as structural rearrange-  
170 ments upon ligand binding, we further analyzed the chemical shift perturbations of PKA-C<sup>WT</sup> and  
171 PKA-C<sup>E31V</sup> using CHEmical Shift Covariance Analysis (CHESCA). This statistical method identi-  
172 fies covariant residues networks involved in a concerted response upon ligand binding and help  
173 tracing allosteric pathways [25-27]. The [<sup>1</sup>H, <sup>15</sup>N]-TROSY-HSQC spectra of four forms of wild-type

174 and PKA-C<sup>E31V</sup> (apo, ATP<sub>γ</sub>N-bound, ADP-bound, and ATP<sub>γ</sub>N/PKI-bound) were used for  
175 CHESCA. When we analyzed the chemical shifts changes of PKA-C<sup>WT</sup>, we identified a well-orga-  
176 nized communication network in which spatially distinct clusters of residues responding to ATP  
177 and PKI binding in a coordinated manner [19]. In contrast, we observed a dramatic reduction in  
178 the intramolecular allosteric network of PKA-C<sup>E31V</sup> similar to PKA-C<sup>L205R</sup>, a Cushing's syndrome  
179 mutation with significantly higher occurrence [18, 19, 28]. In particular, highly correlated groups of  
180 residues in the N-lobe of PKA-C<sup>E31V</sup>, including the  $\alpha$ A-helix (K28, W30) and  $\alpha$ C-helix (A97), dis-  
181 play a dramatic reduction in the number of correlations for distal regions of the kinase, including  
182 the activation loop (R190, G193, L198),  $\alpha$ F-helix (K217, V219, G225), and C-terminal tail (E334,  
183 N340, E349) (**Figure 3**). Notably, the loss of correlations occurs in structural elements surround-  
184 ing the  $\alpha$ C-helix.

185 The typical CHESCA analysis gives pairwise correlations along the primary protein se-  
186 quences. Therefore, we adopted the definition of structural 'communities' introduced by McClen-  
187 don *et al.* [29] to obtain a three-dimensional view of the correlated structural changes. Using this  
188 analysis, we found strong correlations among the major communities in response to nucleotide  
189 and pseudosubstrate binding (**Figure 4**) [29]. In particular, ComA, ComB, and ComC show aver-  
190 age correlation coefficients higher than 0.95, indicating that these communities respond to ligand  
191 binding in a concerted manner. Notably, there are long-range correlations between ComA, ComB,  
192 and ComC with ComE, ComF and ComF1. ComC, which encompasses the  $\alpha$ A- and  $\alpha$ C-helix  
193 including E31, acts as a central hub, connecting six other communities as it is centered around a  
194 critical allosteric mediator the  $\alpha$ C-helix, which bridges both lobes of the kinase. The density of  
195 these correlations underscores the concerted response of the N- and C-lobe to ligand binding.  
196 While the E31V mutation exhibits some of the local and long-range correlations, the values of the  
197 correlation coefficients are lower. ComC and parts of the activation loop of the mutant exhibit the



198 most noticeable reduction in correlation to both local (ComA and ComB) and distal (ComE and  
199 ComF) communities.

200 **MD simulations reveal altered conformational states of PKA-C<sup>E31V</sup> correlated to the reduc-**  
201 **tion in binding cooperativity.** To determine the effects of the E31V mutation on the conforma-  
202 tional energy landscape of nucleotide-bound PKA-C, we carried out parallel MD simulations in  
203 explicit water. We set up the simulations starting from the X-ray coordinates of PKA-C<sup>WT</sup> (PDB:  
204 4WB5 [30]) mutating E31 into a valine and removing PKI [17]. After initial equilibration, we pro-  
205 duced an MD trajectory and analyzed the backbone flexibility of PKA-C<sup>E31V</sup>. Relative to PKA-C<sup>WT</sup>,  
206 we observed increased root mean squared fluctuations (RMSF) of the backbone coordinates with  
207 effects that propagate to distal domains [31], including the N-lobe, activation loop, as well as the  
208 C-terminal tail (**Figure 5A**). The most noticeable effect of the E31V mutation is the increase in the  
209  $\alpha$ A-helix motions, presumably due to V31 moving towards the kinase's hydrophobic interior and  
210 disrupting the cation- $\pi$  interactions between W30, R93 and R190. These 'sandwiched' cation- $\pi$   
211 interactions have been shown to be partially responsible for positioning the indole ring of W30 in  
212 a conserved pocket that can be exploited to regulate kinases activity [22, 32]. As the  $\alpha$ A-helix is  
213 displaced, the indole ring of W30 undergoes a 180° flip, maintaining only one cation- $\pi$  interaction  
214 with R190 (**Figure 5B**). This motion is accompanied by the activation loop of PKA-C<sup>E31V</sup> adopting  
215 a *flipped* conformation similar to the L205R mutant in which an electrostatic node between the  
216 phosphate group of pT197, the guanidinium group of R194 of the activation loop, and the side  
217 chain of E86 of the  $\alpha$ C-helix is formed (**Figure 5C**) [19]. These new interactions cause the opening  
218 of the N-lobe and an outward tilt, involving the Gly-rich loop,  $\alpha$ B-, and  $\alpha$ C-helix. These motions  
219 can be inferred from the distance distributions of the conserved residues K72 and E91 residues,  
220 and the distance between the  $\alpha$ C-helix (E86) and the activation loop (R194) (**Figure 5D**). In con-  
221 trast to the stable K72-E91 salt bridge (~2.8 Å) observed for PKA-C<sup>WT</sup>, the distance between K72  
222 and E91 in PKA-C<sup>E31V</sup> varies between 2.8 and 4.8 Å, sampling more frequently a conformation

223 that resembles the  $\alpha C$ -out inactive state [33]. This large conformational change of the N-lobe is  
 224 corroborated by chemical shift changes observed for residues within the  $\alpha B$ -helix (K76) and  $\alpha C$ -  
 225 helix (Q96) (**Figure 5E**).

226 To link the MD simulations to the binding thermodynamics derived from the ITC experi-  
 227 ments, we computed the difference in free energy of binding ( $\Delta\Delta G_{\text{binding}}$ ) for PKA-C<sup>WT</sup> and PKA-  
 228 C<sup>E31V</sup> using the free energy perturbation (FEP) method [34], as detailed in the thermodynamic  
 229 cycle shown in **Figure S3**. The ratio of the cooperativity coefficients can be expressed in terms of  
 230 free energy:

$$231 \quad \frac{\sigma_{PKI}^{WT}}{\sigma_{PKI}^{E31V}} = \frac{K_{d Apo}^{WT} * K_{d Nucleotide}^{E31V}}{K_{d Nucleotide}^{WT} * K_{d Apo}^{E31V}} = \frac{K_{d Apo}^{WT}}{K_{d Apo}^{E31V}} \times \frac{K_{d Nucleotide}^{E31V}}{K_{d Nucleotide}^{WT}} = e^{-\frac{\Delta\Delta G_{Nucleotide} - \Delta\Delta G_{Apo}}{RT}}$$

$$232 \quad = e^{-\frac{(\Delta G_4 - \Delta G_3) - (\Delta G_2 - \Delta G_1)}{RT}}$$

233 Where  $\frac{\sigma_{PKI}^{WT}}{\sigma_{PKI}^{E31V}}$  represents the ratio of the cooperativity coefficients for wild-type and E31V, and the  
 234 free energy change of a mutation in different states  $\Delta G_1$  to  $\Delta G_4$  is illustrated in **Figure S3A**. Us-  
 235 ing this expression for the binding of PKI to apo PKA-C<sup>E31V</sup>, the FEP method calculates a free  
 236 energy difference between the free and bound state of  $-1.1 \pm 0.3$  kcal/mol, corresponding to a ~7-  
 237 fold reduction in the binding affinity of PKA-C<sup>E31V</sup> for PKI. This value is in excellent agreement  
 238 with the experimental results (**Table S5**). On the other hand, the binding of PKI to the nucleotide-  
 239 bound E31V mutant resulted in a free energy perturbation of  $0.3 \pm 0.2$  kcal/mol, indicating a  
 240 reduction in binding affinity. From the differences of these two values, it is possible to estimate  
 241 approximately a 11-fold reduction in the nucleotide/PKI binding cooperativity (**Figure S3B**), which  
 242 is in qualitative agreement with ITC experiments. We also calculated the change in cooperativity  
 243 for binding PKI first and then the nucleotide (**Figure S3C**). These calculations confirmed the re-  
 244 duction in cooperativity with a value that is approximately 24-fold lower for the ATP binding by

245 PKA-C<sup>E31V</sup>, further supporting the experimentally observed loss in cooperativity for PKA-C<sup>E31V</sup>  
246 (Table S5).

247 **Nucleotide-substrate binding cooperativity and extent of allosteric communication are di-**  
248 **rectly correlated.** Both thermodynamic and NMR data show that Cushing's syndrome mutants,  
249 PKA-C<sup>L205R</sup> and PKA-C<sup>E31V</sup>, exhibit reduced binding cooperativity and decreased intramolecular  
250 allosteric communication. Therefore, we hypothesized that the coordinated structural changes  
251 might be correlated to the nucleotide-substrate cooperative binding response. Hence, a disruption  
252 of the allosteric network would directly affect the nucleotide-substrate binding cooperativity. To  
253 test this, we analyzed the thermodynamics and structural response of PKA-C<sup>W196R</sup>, another mu-  
254 tant that was found in 3% of Cushing's patients [20]. This mutation is located in the activation loop  
255 and is adjacent to the T197 phosphorylation site. We repeated both ITC and NMR analysis for  
256 PKA-C<sup>W196R</sup>, and similarly to PKA-C<sup>E31V</sup> we found a significant attenuation in both binding coop-  
257 erativity and extent of intramolecular allosteric communication (Table S4, Figure S4). From the  
258 CHESCA matrices of these three mutants and PKA-C<sup>WT</sup>, we extracted the relative correlation  
259 score (see Material and Methods), which can be used to estimate the density of the intramolecular  
260 allosteric networks. We then plot the relative correlation scores versus  $\ln(\sigma)$ . We found that these  
261 parameters are linearly correlated ( $R^2 = 0.98$ , Figure 6). This relationship suggests that the extent  
262 of the nucleotide-substrate binding cooperativity depends on the coordinated structural changes  
263 of the two lobes of the enzyme upon nucleotide and substrate binding.

264

## 265 DISCUSSION

266 The genetic basis of adrenocortical adenomas (ACAs) has been known for the past decade, with  
267 the cAMP/PKA pathway playing a central role in adrenocortical growth steroidogenesis [3, 4].  
268 Although multiple components of the cAMP/PKA pathway have been implicated in various endo-  
269 crine disease states, it was not until recently that PKA-C was discovered to play a central role  
270 [35]. To date, eight mutations have been discovered in *PRKACA* as a rare genetic alteration in

271 cortisol-producing ACA's responsible for Cushing's syndrome [5-12]. Except for E31V, all muta-  
272 tions are positioned in the substrate binding cleft or at the R/C interface, providing a justification  
273 for the loss of substrate fidelity and regulation of the kinase [19, 20]. However, it has been difficult  
274 to rationalize why the E31V mutant results in the same phenotype of the other orthosteric Cush-  
275 ing's syndrome driving mutations. This present study shows that local conformational changes  
276 caused by the E31V mutation alter key allosteric interactions that link the terminal regions of the  
277 C-terminal tail,  $\alpha$ A- and  $\alpha$ C-helix. MD simulations revealed that the E31V mutation increases the  
278 conformational dynamics within the  $\alpha$ A-helix, causing it to dislodge from the kinase core and  
279 thereby disrupting canonical cation- $\pi$  interactions between W30 and R93 and R190 [36, 37].  
280 These structural alterations cause the N-lobe of the kinase to swing outward adopting a more  
281 open conformation, with the activation loop in a *flipped* conformation, forming a stable salt bridge  
282 with the  $\alpha$ C-helix and disrupting a critical allosteric node responsible for inter-lobe allosteric com-  
283 munication and binding cooperativity [36].

284 Cooperativity is fundamental factor for macromolecular assembly and signal amplification  
285 [38-40]. For PKA-C, binding cooperativity has been used to define the role of ATP as an allosteric  
286 effector, able to amplify the substrate's binding affinity [41]. However, PKA-C interacts with other  
287 binding partners including the R-subunits that keep its function under strict control. Notably, the  
288 R-subunits recognition sequences are highly homologous to those of substrates and PKI. There-  
289 fore, it is likely that the loss in nucleotide/PKI binding cooperativity we observed for these Cush-  
290 ing's syndrome mutants may negatively affect the assembly of the R:C complex and the entire  
291 cAMP signaling pathway.

292 In conclusion, we identified a common trait between orthosteric and allosteric mutations  
293 linked to Cushing's syndrome. These mutations display a reduced binding cooperativity with a  
294 concomitant loss in intramolecular allosteric communication. The effects derived by these events  
295 are manifested as a loss of substrate fidelity and regulation by the R-subunit, while the catalytic

296 activity of these mutants remains essentially unaltered. These results may explain how these  
297 aberrant enzymes give rise to anomalous phosphoproteomic profiles [20].

## 298 MATERIALS AND METHODS

299 **Sample Preparation.** Recombinant human C $\alpha$  subunit of cAMP-dependent protein kinase A  
300 cDNA (PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup>) was cloned into a pET-28a vector. A tobacco etch virus (TEV)  
301 cleavage site was incorporated via mutagenesis into the vector between the cDNA coding for the  
302 kinase and a thrombin cleavage site. The kinase was expressed in *Escherichia coli* BL21 (DE3)  
303 according to procedures previously published [19]. PKI (full-length) was expressed and purified  
304 according to procedures previously published [42]. Peptides (Kemptide/PKI<sub>5-24</sub>) were synthesized  
305 using standard Fmoc chemistry on a CEM Liberty Blue microwave synthesizer, cleaved with Re-  
306 agent K (82.5% TFA, 5% phenol, 5% thioanisole, 2.5% ethanedithiol, and 5% water) for 3 h and  
307 purified using a semipreparative Supelco C18 reverse-phase HPLC column at 3 mL/min. Molec-  
308 ular weight and the quantity of the peptides were verified by LC-MS and/or amino acid analysis  
309 (Texas Tech Protein Chemistry Laboratory).

310 **ITC Measurements.** ITC measurements were performed with a low-volume NanoITC (TA Instru-  
311 ments). PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup> were dialyzed into 20 mM MOPS, 90 mM KCl, 10 mM DTT, 10  
312 mM MgCl<sub>2</sub>, and 1 mM NaN<sub>3</sub> (pH 6.5). PKA-C concentrations for ITC measurements were between  
313 100-110  $\mu$ M as confirmed by A<sub>280</sub> = 53860 M<sup>-1</sup>cm<sup>-1</sup>. All measurements with ATP $\gamma$ N saturated PKA-  
314 C<sup>WT</sup> and PKA-C<sup>E31V</sup> were performed at 2 mM ATP $\gamma$ N. ITC measurements were performed at 300K  
315 in triplicates. Approximately 300  $\mu$ L of PKA-C was used for each experiment, and 50  $\mu$ L of 2 mM  
316 ATP $\gamma$ N and 0.6-0.8 mM PKI in the titrant syringe. The heat of dilution of the ligand into the buffer  
317 was taken into account for all experiments and subtracted. Curves were analyzed with the Nano-  
318 Analyze software (TA Instruments) using the Wiseman Isotherm [21]:

$$319 \frac{d[MX]}{d[X_{tot}]} = \Delta H^\circ V_0 \left[ \frac{1}{2} + \frac{1 - \frac{1-r}{2} - R_m/2}{(R_m^2 - 2R_m(1-r) + (1+r)^2)^{1/2}} \right] \quad (1)$$

320 where  $d[MX]$  is the change in total complex with respect to change in total protein concentration,  
321  $d[X_{tot}]$  is dependent on  $r$ , the ratio of  $K_d$  with respect to the total protein concentration, and  $R_M$ , the  
322 ratio between total ligand and total protein concentration. The free energy of binding was deter-  
323 mined using the following:

$$324 \quad \Delta G = RT \ln K_d$$

325 where  $R$  is the universal gas constant and  $T$  is the temperature at measurement (300K). The  
326 entropic contribution to binding was calculated using the following:

$$327 \quad T\Delta S = \Delta H - \Delta G.$$

328 Calculations for the cooperativity constant ( $\sigma$ ) were calculated as follows:

$$329 \quad \sigma = \frac{K_{d\ Apo}}{K_{d\ Nucleotide}}$$

330 where  $K_{d\ Apo}$  is the  $K_d$  of PKI<sub>5-24</sub> binding to the apoenzyme and  $K_{d\ Nucleotide}$  is the  $K_d$  of PKI<sub>5-24</sub> binding  
331 to the nucleotide-bound enzyme.

332 **Enzyme Assays.** Steady-state activity assays with Kemptide were performed under saturating  
333 ATP concentrations and spectrophotometrically at 298K as described by Cook et al [43]. The  
334 values of  $V_{max}$  and  $K_M$  were obtained from a nonlinear fit of the initial velocities to the Michaelis-  
335 Menten equation.

336 **NMR Spectroscopy.** Uniformly <sup>15</sup>N-labeled PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup> were overexpressed and  
337 purified as described above. NMR experiments were performed in 90 mM KCl, 20 mM KH<sub>2</sub>PO<sub>4</sub>,  
338 10 mM dithiothreitol (DTT), 10 mM MgCl<sub>2</sub>, and 1 mM NaN<sub>3</sub> at pH 6.5. Standard [<sup>1</sup>H-<sup>15</sup>N]-TROSY-  
339 HSQC experiments were carried out for PKA-C<sup>E31V</sup> and PKA-C<sup>WT</sup> on a 900-MHz Bruker Advance  
340 III spectrometer equipped with a TCI cryoprobe. Concentrations for samples were 0.2-0.3 mM as  
341 determined by A<sub>280</sub> measurements, 12 mM ATP<sub>γ</sub>N was added for the nucleotide-bound form, and

342 0.2-1.2 mM PKI for the ternary complex. Spectra were collected at 300K, processed using  
343 NMRPipe [44], and visualized using Sparky [45].

344 All [<sup>1</sup>H-<sup>15</sup>N]-TROSY-HSQC experiments were acquired with 2048 (proton) and 256 (nitrogen)  
345 complex points. Combined chemical shift perturbations were calculated using <sup>1</sup>H and <sup>15</sup>N chemi-  
346 cal shifts according to the following:

$$347 \Delta\delta = \sqrt{(\Delta\delta H)^2 + 0.154(\Delta\delta N)^2} \quad (1)$$

#### 348 **Chemical Shift Analyses.**

349 **COordiNated Chemical Shift bEhavior (CONCISE).** CONCISE was used to monitor chemical  
350 shift trajectories and measure the change in equilibrium position using each PKA-C construct  
351 (apo, ATP<sub>γ</sub>N, ADP, ATP<sub>γ</sub>N/PKI). This method uses principal component analysis to identify sets  
352 of residues whose chemical shifts respond linearly to a conformational transition (i.e. open, inter-  
353 mediate, and closed). Each residue provides a measure of the equilibrium position for each PKA-  
354 C construct in the form of scores along the first principal component (PC1). To identify the resi-  
355 dues whose chemical shifts follow a linear trajectory, a threshold of 3.0 for the ratio of the standard  
356 deviations of PC1 over PC2 was used, and residues not exhibiting a significant chemical shift  
357 were excluded based on the linewidth.

358 **CHEmical Shift Covariance Analysis (CHESCA).** CHESCA was used to identify and function-  
359 ally characterize allosteric networks of residues eliciting concerted responses to, in this case,  
360 nucleotide and pseudosubstrate. A total of four states were used to identify inter-residue correla-  
361 tions: apo, ADP-bound, ATP<sub>γ</sub>N-bound, and ATP<sub>γ</sub>N/PKI-bound. Identification of inter-residue cor-  
362 relations by CHESCA relies on agglomerative clustering (AC) and singular value decomposition  
363 (SVD). Pairwise correlations between chemical shift variations experienced by different residues  
364 are analyzed to identify networks of coupled residues and when plotted on a correlation matrix,  
365 allows for the identification of regions that are correlated to one another. A correlation coefficient

366 ( $R_{ij}$ ) cutoff of 0.96 was used to filter non-linear residues. Residues not exhibiting a significant  
367 chemical shift (small shifts in ppm) were excluded based on linewidth. For each residue the max  
368 change in chemical shift was calculated in both the  $^1\text{H}$  ( $x$ ) and  $^{15}\text{N}$  ( $y$ ) dimension ( $\Delta\delta_{x,y}$ ). Residues  
369 were included in CHESCA analysis only if they satisfied the following:  $\Delta\delta_{x,y} > \frac{1}{2} \Delta\nu_{xA,yA} + \frac{1}{2} \Delta\nu_{xB,yB}$   
370 , where  $A$  and  $B$  correspond to two different forms analyzed (note there is no dependence on  
371 which two forms satisfied this statement). Correlation scores were used to quantify the CHESCA  
372 correlation for each residue. Mathematically it is defined as the following: Corr. Score = number  
373 of residues where  $R_{ij} > 0.98$  / total number of  $R_{ij}$ . Community CHESCA analysis is a chemical shift  
374 based correlation map between various functional communities within the kinase. Each commu-  
375 nity is a group of residues (McClendon et al.<sup>35</sup>) associated with a function or regulatory mecha-  
376 nism. Mathematically, this community-based CHESCA analysis is a selective interpretation of  
377 CHESCA, where we evaluate a correlation score between residues in various communities as  
378 shown below. In order to represent community-based CHESCA analysis we lowered the correla-  
379 tion cutoff such that  $R_{\text{cutoff}} > 0.8$ . Suppose community A and community B has  $n_A$  and  $n_B$  number  
380 of assigned residues respectively, the correlation score between A and B is defined as,

$$381 \quad R_{A,B} = \text{Number of } (R_{ij} > R_{\text{cutoff}}) / (n_A * n_B).$$

382 Where  $R_{ij}$  is the CHESCA correlation coefficient between residue  $i$  (belongs to community A) and  
383 residue  $j$  (belongs to community B).  $R_{\text{cutoff}}$  is the correlation value cutoff.  $R_{A,B}$  can take values from  
384 0 (no correlation between residues in A and B) to 1 (all residues in A has correlation  $>$  cutoff with  
385 all residues in B).

386 **MD Simulations.** We used the crystal structure of PKA-C<sup>WT</sup> (PDB ID: 4WB5[30]) as the template.  
387 We further aligned the current structure with the full length PKA-C<sup>WT</sup> and added the missing resi-  
388 dues 1-12 at the N terminus. The protonation state of histidine residues followed our previous  
389 settings [31]. The protein was solvated in a rhombic dodecahedron solvent box with TIP3P water  
390 molecule layer extended approximately 10 Å away from the surface of the proteins. Counter ions



391 (K<sup>+</sup> and Cl<sup>-</sup>) were added to ensure electrostatic neutrality corresponding to an ionic concentration  
 392 of ~150 mM. All covalent bonds involving a hydrogen atom of the protein were constrained with  
 393 the LINCS[46]algorithm. and long-range electrostatic interactions were treated with the particle-  
 394 mesh Ewald [47] method with a real-space cutoff of 10 Å. Parallel simulations on the apo form,  
 395 the binary form with one Mg<sup>2+</sup> ion and one ATP, and the ternary form with two Mg<sup>2+</sup> ions, one ATP  
 396 and one PKI<sub>5-24</sub> were performed simultaneously using GROMACS 5.1.4 [48] with the  
 397 CHARMM36a1 force field [49]. Each system was minimized using the steepest decent algorithm  
 398 to remove bad contacts, and then gradually heated to 300K at a constant volume over 1 ns, using  
 399 harmonic restraints with a force constant 1000 kJ/(mol\*Å<sup>2</sup>) on heavy atoms of both proteins and  
 400 nucleotides. Over the following 12 ns of simulations at constant pressure (1 atm) and temperature  
 401 (300K), the restraints were gradually released. The systems were equilibrated for an additional  
 402 20 ns without positional restraints. The Parrinello-Rahman[50] barostat was used to keep the  
 403 pressure constant, while a V-rescale thermostat with a time step of 2 fs was used to keep the  
 404 temperature constant. Each system was simulated for 1.05 μs, with snapshots recorded every 20  
 405 ps.

406 **Relative change of cooperativity from free energy perturbation calculations.** The coopera-  
 407 tivity can be defined for both nucleotide and pseudosubstrate PKI, respectively. For nucleotide,  
 408 the change of cooperativity upon mutation can be rewritten as the difference in ΔΔG between  
 409 the apo and the PKI-bound state, as shown in the following equation and illustrated in **Figure**  
 410 **S3**:

$$411 \frac{\sigma_{Nucleotide}^{WT}}{\sigma_{Nucleotide}^{E31V}} = \frac{K_{d Apo}^{WT} * K_{d PKI}^{E31V}}{K_{d PKI}^{WT} * K_{d Apo}^{E31V}} = \frac{K_{d Apo}^{WT}}{K_{d Apo}^{E31V}} \times \frac{K_{d PKI}^{E31V}}{K_{d PKI}^{WT}} = e^{-\frac{(\Delta G_1 - \Delta G_3) + (\Delta G_4 - \Delta G_2)}{RT}}$$

412 The free energies ΔG due to amino acid mutations were determined following a protocol based  
 413 on the Bennett acceptance ratio (BAR) implemented in the GROMACS and PMX [34]. To avoid  
 414 the artifacts by introducing a charged mutation, the double-system/single-box setup was used.

415 The procedure employs dual protein topologies that include both residues of the wild-type ( $\lambda = 0$ )  
416 and the mutant protein ( $\lambda = 1$ ) coupled by the progressing variable  $\lambda$ . Of course, both the complex  
417 and unbound structures were used to obtain the change in binding free energies using standard  
418 thermodynamic cycle approach. Single-site mutations were performed based on the well-equili-  
419 brated structure of PKA-C<sup>WT</sup> from simulations. The computational details are identical to those  
420 detailed above, except that after 40 ns of equilibration of both initial and final states for each  
421 mutation, 200 additional trajectories, each lasting 100 ps, were initiated from the last 20 ns simu-  
422 lations both in the forward and in the backward transformations to accumulate statistical averages  
423 and fluctuations.

424

425 **ACKNOWLEDGMENTS**

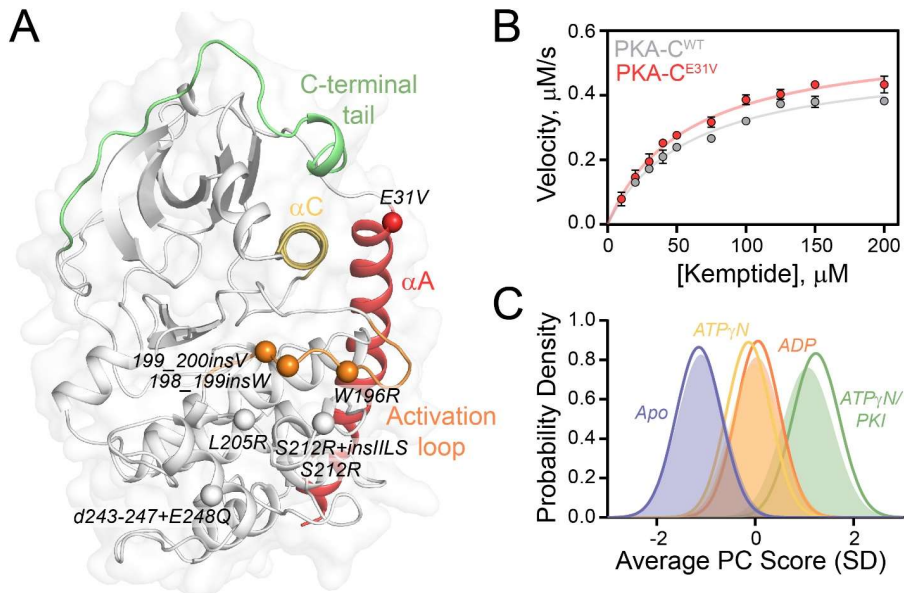
426 This work was supported by the National Institutes of Health, GM100310 (G.V.), S10 OD021536  
427 (G.V.) and GM046736 (J.G), and the American Heart Association, 20PRE35120253 (C.W). NMR  
428 experiments were carried out at the Minnesota NMR Center and MD calculations at the Minnesota  
429 Supercomputing Institute.

430

431 **AUTHOR CONTRIBUTIONS**

432 C.W. collected and analyzed activity assay, NMR, and ITC data and contributed to the writing of  
433 the manuscript. Y.W. carried out and analyzed MD simulations, with J.G. directing and assisting  
434 with analysis of the MD simulations. D.C, D.A.B and S.S.T contributed to critical analysis of the  
435 data and writing of the manuscript. G.V. conceived and directed the project, along with assisting  
436 with data analysis and writing the manuscript.

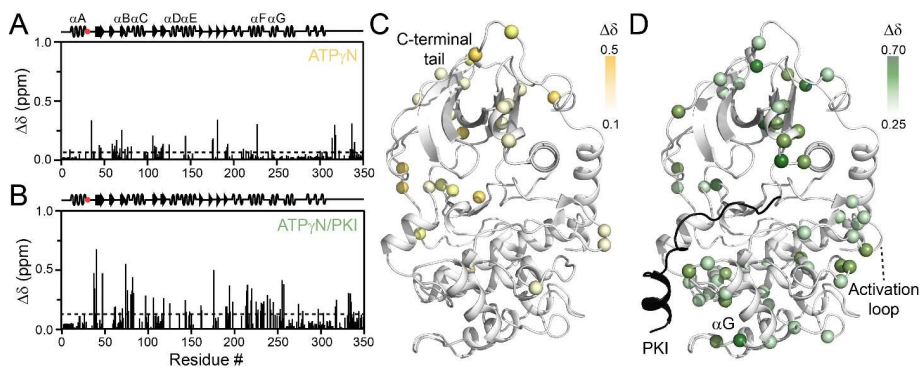
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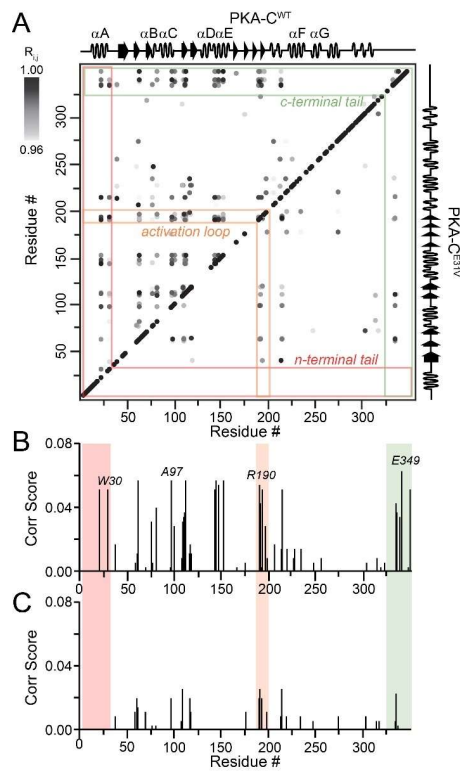
439 **Figure 1. Structural and kinetic characterization of PKA-C<sup>E31V</sup>.** (A) Structure of PKA-C bound  
 440 to endogenous inhibitor, PKI (PDB: 1ATP) highlighting important structural elements and locations  
 441 of Cushing's syndrome mutations in relation to E31V. (B) Steady state phosphorylation kinetics  
 442 of PKA-C<sup>E31V</sup> with Kemptide. (C) CONCISE analysis on the apo, ATP<sub>γ</sub>N-, ADP- and ATP<sub>γ</sub>N/PKI-  
 443 bound forms of PKA-C<sup>WT</sup> (opaque gaussian) and PKA-C<sup>E31V</sup> (outlined gaussian).

444



445  
 446 **Figure 2. Chemical shift perturbation of PKA-C<sup>E31V</sup>.** Chemical shift perturbation (CSP) of amide  
 447 fingerprint of PKA-C<sup>E31V</sup> upon binding (A) ATP<sub>γ</sub>N and subsequent binding of (B) PKI. The average  
 448 CSP is shown as a dashed line. CSPs of PKA-C<sup>E31V</sup> amide resonances mapped onto the structure  
 449 (PDB: 1ATP).

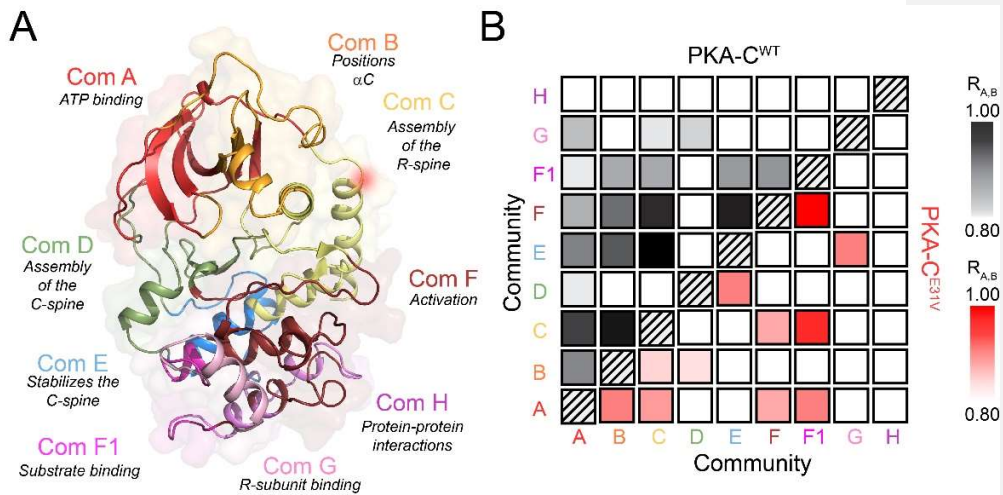
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451

452 **Figure 3. The reduction in binding cooperativity corresponds to a decrease of intramolec-**  
 453 **ular allosteric connectivities.** (A) CHESCA correlation matrix of PKA-C<sup>WT</sup> (top diagonal) and  
 454 PKA-C<sup>E31V</sup> (bottom diagonal) upon binding PKI highlighting the notable reductions in correlations  
 455 within the n-terminal tail (red), activation loop (orange), and c-terminal tail (green). Only correla-  
 456 tions with  $R_{ij} > 0.98$  are shown. Plot of correlation score vs. residue for (B) PKA-C<sup>WT</sup> and (C) PKA-  
 457 C<sup>E31V</sup> emphasizing the residues that show the largest reductions in correlation score that make  
 458 contacts with the  $\alpha C$ -helix. See material and methods for the calculation of correlation scores.

459



460

461 **Figure 4. NMR map of the structural responses to nucleotide and pseudo-substrate bind-**

462 **ing for wild-type and E31V mutants. (A)** Community map of PKA-C highlighting func-

463 tional/regulatory role of each community as defined by [29]. **(B)** Community CHESCA analysis

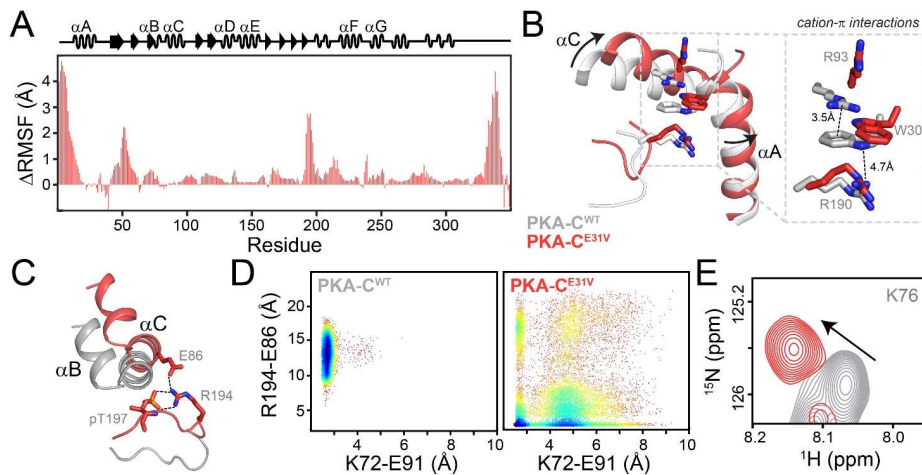
464 of PKA-C<sup>WT</sup> (top diagonal, black) and PKA-C<sup>E31V</sup> (bottom diagonal, red). Only correlations with

465  $R_{A,B} > 0.8$  are shown.

466



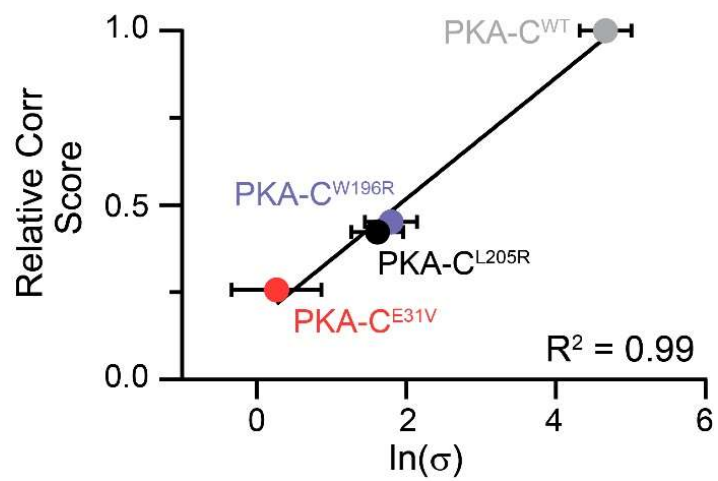
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468

469 **Figure 5. Comparison of MD simulations of PKA<sup>WT</sup> and PKA-C<sup>E31V</sup>.** (A) Changes in the root  
 470 mean square fluctuations ( $\Delta$ RMSF) of PKA-C<sup>E31V</sup> in the nucleotide-bound form over 1.0  $\mu$ s of  
 471 simulation. (B) Overlay of PKA-C<sup>WT</sup> (PDB: 1ATP; gray) and PKA-C<sup>E31V</sup> (from MD simulations; hot  
 472 pink) showing the structural rearrangements of the  $\alpha$ A- and  $\alpha$ C-helices caused by the E31V mu-  
 473 tation. The inset shows the cation- $\pi$  stacking interactions altered in response to the mutation. (C)  
 474 Overlay of PKA-C<sup>WT</sup> (PDB: 1ATP; gray) and PKA-C<sup>E31V</sup> (from MD simulations; hot pink) showing  
 475 the upward movement of the  $\alpha$ B- and  $\alpha$ C-helices and the rearrangements of the electrostatic  
 476 interactions between the activation loop and the  $\alpha$ C-helix. (D) Distinct conformational dynamics  
 477 of the  $\alpha$ C-helix in PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup>, as characterized by the two key salt bridges K72-E91  
 478 and E86-R194. (E) Portion of the [<sup>1</sup>H, <sup>15</sup>N]-TROSY-HSQC spectra showing the backbone amide  
 479 chemical shift changes of K76 (located in  $\alpha$ B-helix) in the PKA-C<sup>E31V</sup>.

480



481

482 **Figure 6. Relationship between coordinated structural changes identified by CHESCA and**  
483 **the nucleotide-substrate binding cooperativity ( $\sigma$ ) determined by ITC measurements.**

484

485 **Supplementary Information**

486  
487  
488 **The allosteric E31V mutation disrupts the nucleotide-substrate cooper-**  
489 **ativity in protein kinase A: is there a common mechanism for Cushing's**  
490 **syndrome driving mutations?**

491  
492  
493 Caitlin Walker<sup>1</sup>, Yingjie Wang<sup>1,2,†</sup>, Jiali Gao<sup>2,3</sup>, David A. Bernlohr<sup>1</sup>, Davide Calebiro<sup>4,5</sup>, Susan S.  
494 Taylor<sup>6,7</sup>, and Gianluigi Veglia<sup>1,2,\*</sup>

495  
496 <sup>1</sup>*Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Min-*  
497 *neapolis, MN 55455, USA*

498  
499 <sup>2</sup>*Department of Chemistry and Supercomputing Institute, University of Minnesota, Minneapolis,*  
500 *MN 55455, USA*

501  
502 <sup>3</sup>*Institute of Systems and Physical Biology, Shenzhen Bay Laboratory, Shenzhen, 518055,*  
503 *China*

504  
505 <sup>4</sup>*Institute of Metabolism and Systems Research, University of Birmingham, B15 2TT, Birming-*  
506 *ham, UK*

507  
508 <sup>5</sup>*Centre of Membrane Proteins and Receptors, University of Birmingham, B15 2TT, Birmingham,*  
509 *UK*

510  
511 <sup>6</sup>*Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, CA*  
512 *92093*

513  
514 <sup>7</sup>*Department of Pharmacology, University of California at San Diego, La Jolla, CA 92093*

515  
516  
517 †Present Address: *Institute of Systems and Physical Biology, Shenzhen Bay Laboratory, Shen-*  
518 *zhen, 518055, China.*

519  
520  
521  
522  
523 \*Corresponding Author

524  
525 Gianluigi Veglia  
526 Department of Chemistry and Department of Biochemistry, Molecular Biology, and Biophysics,  
527 321 Church Street SE  
528 Minneapolis, MN 55455  
529 Telephone: (612) 625-0758  
530 Fax: (612) 625-5780  
531 e-mail: [vegli001@umn.edu](mailto:vegli001@umn.edu)  
532

533 **Table of Contents:**

534 **Table S1.** Changes in enthalpy, entropy, free energy, and dissociation constant of binding ATP $\gamma$ N  
535 for PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup>.

536 **Table S2.** Changes in enthalpy, entropy, free energy, and dissociation constant for the binding of  
537 PKI<sub>5-24</sub> to apo and nucleotide-saturated PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup>.

538 **Table S3.** Kinetic parameters of Kemptide phosphorylation by PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup>.

539 **Table S4.** Changes in enthalpy, entropy, free energy, and dissociation constant for the binding of  
540 PKI<sub>5-24</sub> to apo and nucleotide-saturated PKA-C<sup>L205R</sup> and PKA-C<sup>W196R</sup>.

541 **Table S5.** Changes in relative binding free energy  $\Delta\Delta G$ , and cooperativity from PKA-C<sup>WT</sup> to  
542 PKA-C<sup>31V</sup> for the binding of PKI<sub>5-24</sub> as well as the binding of ATP in the apo and binary states.

543 **Figure S1.** [<sup>1</sup>H, <sup>15</sup>N]-TROSY-HSQC spectra for PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup> in the apo, ATP $\gamma$ N-,  
544 ADP-, and ATP $\gamma$ N/PKI-bound forms.

545 **Figure S2.** Change in chemical shift perturbation between PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup>.

546 **Figure S3.** Thermodynamic cycle linking the free energy perturbation (FEP) calculation to ratio  
547 of  $K_d$  and  $\sigma$ .

548 **Figure S4.**

549

550 **Table S1. Changes in enthalpy, entropy, free energy, and dissociation constant for the**  
551 **binding of nucleotide to PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup>.** All errors were calculated using triplicate  
552 measurements. Asterisk indicates data that has been previously published [19].

	$K_d$ ( $\mu\text{M}$ )	$\Delta G$ (kcal/mol)	$\Delta H$ (kcal/mol)	$-T\Delta S$ (kcal/mol)
*PKA-C <sup>WT</sup>	83 $\pm$ 8	-5.61 $\pm$ 0.06	-3.6 $\pm$ 0.1	- 2.0 $\pm$ 0.1
PKA-C <sup>E31V</sup>	91 $\pm$ 9	-5.56 $\pm$ 0.06	-3.7 $\pm$ 0.2	-1.9 $\pm$ 0.2

553

554 **Table S2. Changes in enthalpy, entropy, free energy, and dissociation constant for the**  
 555 **binding of PKI<sub>5-24</sub> to apo and nucleotide-saturated PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup>.** Errors in  $\Delta G$ ,  
 556  $\Delta H$ ,  $-T\Delta S$ , and  $K_d$  were calculated using triplicate measurements. Errors in  $\sigma$  were propagated  
 557 from error in  $K_d$ . Asterisk indicates data that has been previously published [19].

558 **Apo forms**

	$K_d$ ( $\mu\text{M}$ )	$\Delta G$ (kcal/mol)	$\Delta H$ (kcal/mol)	$-T\Delta S$ (kcal/mol)
*PKA-C <sup>WT</sup>	$17 \pm 2$	$-6.57 \pm 0.08$	$-10.8 \pm 0.5$	$4.2 \pm 0.5$
PKA-C <sup>E31V</sup>	$2.5 \pm 0.5$	$-7.7 \pm 0.1$	$-19.8 \pm 0.4$	$12.1 \pm 0.5$

559

560 **ATP $\gamma$ N saturated forms**

	$K_d$ ( $\mu\text{M}$ )	$\Delta G$ (kcal/mol)	$\Delta H$ (kcal/mol)	$-T\Delta S$ (kcal/mol)	$\sigma$
*PKA-C <sup>WT</sup>	$0.16 \pm 0.02$	$-9.33 \pm 0.07$	$-13.9 \pm 0.5$	$4.6 \pm 0.4$	$106 \pm 18$
PKA-C <sup>E31V</sup>	$2 \pm 1$	$-7.9 \pm 0.3$	$-17 \pm 1$	$9 \pm 1$	$1.3 \pm 0.7$

561

562

563 **Table S3. Kinetic parameters of Kemptide phosphorylation by PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup>.** Val-  
564 ues for  $K_M$  and  $k_{cat}$  were obtained from a non-linear least squares analysis of the concentration-  
565 dependent initial phosphorylation rates using a standard coupled enzyme activity assay. Error in  
566  $k_{cat}/K_M$  was propagated from error in  $K_M$  and  $k_{cat}$ .

	PKA-C <sup>WT</sup>	PKA-C <sup>E31V</sup>
$V_{max}$ ( $\mu\text{M}/\text{sec}$ )	$0.52 \pm 0.02$	$0.58 \pm 0.02$
$K_M$ ( $\mu\text{M}$ )	$59 \pm 7$	$56 \pm 5$
$k_{cat}$ ( $\text{s}^{-1}$ )	$24 \pm 1$	$26 \pm 1$
$k_{cat}/K_M$	$0.41 \pm 0.05$	$0.46 \pm 0.04$

567

568

569 **Table S4. Changes in enthalpy, entropy, free energy, and dissociation constant for the**  
 570 **binding of PKI<sub>5-24</sub> to apo and nucleotide-saturated PKA-C<sup>L205R</sup> and PKA-C<sup>W196R</sup>.** Errors in  $\Delta G$ ,  
 571  $\Delta H$ ,  $-T\Delta S$ , and  $K_d$  were calculated using triplicate measurements. Errors in  $\sigma$  were propagated  
 572 from error in  $K_d$ . Asterisk indicates data that has been previously published [19].

573 **Apo forms**

	$K_d$ ( $\mu\text{M}$ )	$\Delta G$ (kcal/mol)	$\Delta H$ (kcal/mol)	$-T\Delta S$ (kcal/mol)
*PKA-C <sup>L205R</sup>	$61 \pm 5$	$-5.79 \pm 0.04$	$-9.7 \pm 0.1$	$3.9 \pm 0.1$
PKA-C <sup>W196R</sup>	$5 \pm 2$	$-7.3 \pm 0.2$	$-21.1 \pm 0.8$	$13.8 \pm 0.5$

574

575 **ATP $\gamma$ N saturated forms**

	$K_d$ ( $\mu\text{M}$ )	$\Delta G$ (kcal/mol)	$\Delta H$ (kcal/mol)	$-T\Delta S$ (kcal/mol)	$\sigma$
*PKA-C <sup>L205R</sup>	$10 \pm 3$	$-6.9 \pm 0.2$	$-8.8 \pm 0.8$	$1.9 \pm 0.6$	$6 \pm 2$
PKA-C <sup>W196R</sup>	$0.95 \pm 0.05$	$-8.28 \pm 0.03$	$-19.2 \pm 0.5$	$10.9 \pm 0.5$	$5 \pm 2$

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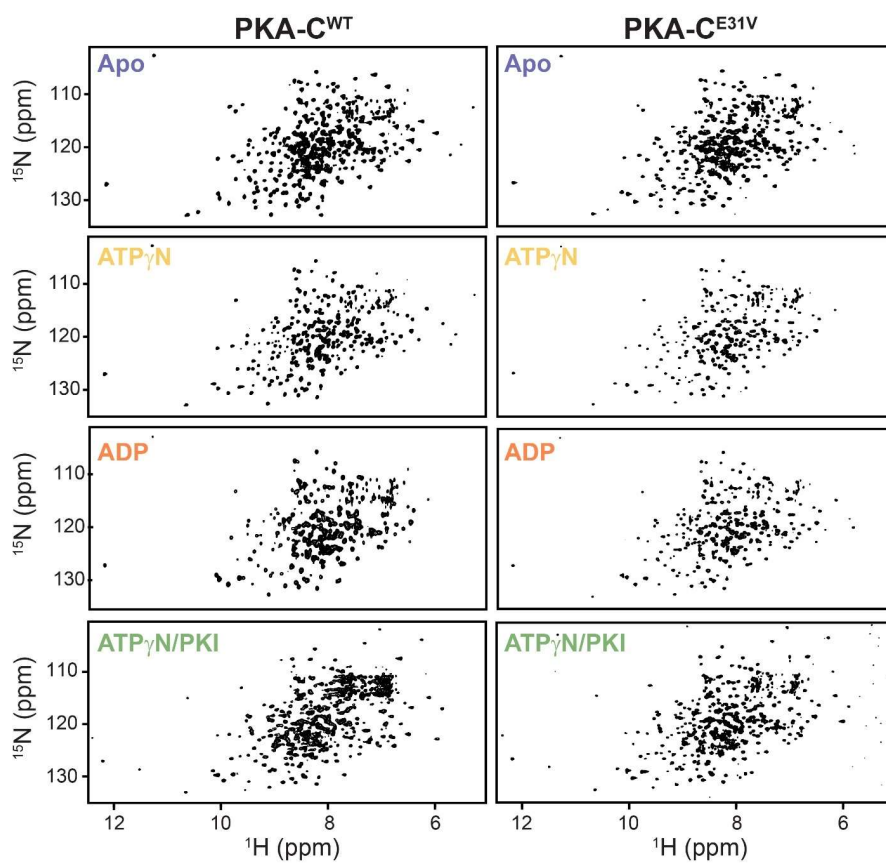
578 **Table S5. Changes in relative binding free energy,  $\Delta\Delta G$ , and cooperativity from PKA-C<sup>WT</sup>**  
579 **to PKA-C<sup>31V</sup> for the binding of PKI<sub>5-24</sub> and the binding of ATP in the apo and binary states.**

Commented [GV1]: Need to include W196R

	$\Delta\Delta G_{\text{apo}}$ (kcal/mol)	$\Delta\Delta G_{\text{binary}}$ (kcal/mol)	Reduction in $\sigma$
PKI <sub>5-24</sub> binding	-1.1 ± 0.3	0.3 ± 0.2	11 ± 5
ATP binding	-0.8 ± 0.2	1.1 ± 0.3	24 ± 9

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583 **Figure S1.** [ $^1\text{H}$ ,  $^{15}\text{N}$ ]-TROSY-HSQC spectra for PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup> in the apo, ATP $\gamma$ N-,  
584 ADP-, and ATP $\gamma$ N/PKI-bound forms.

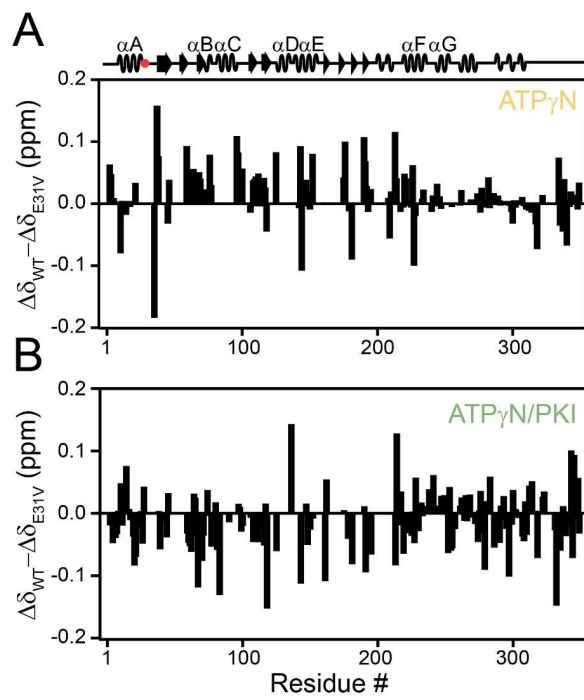


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587 **Figure S2. Change in chemical shift perturbation between PKA-C<sup>WT</sup> and PKA-C<sup>E31V</sup>.**

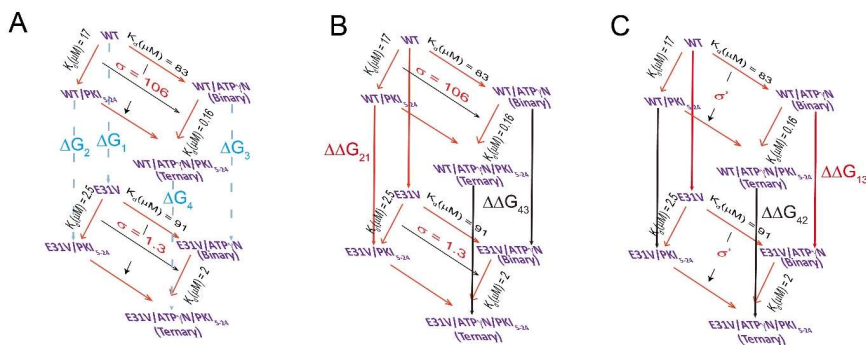
588 Change in CSP ( $\Delta\delta_{WT}-\Delta\delta_{E31V}$ ) upon binding (A) ATP<sub>γ</sub>N. and subsequent binding of (B) PKI.



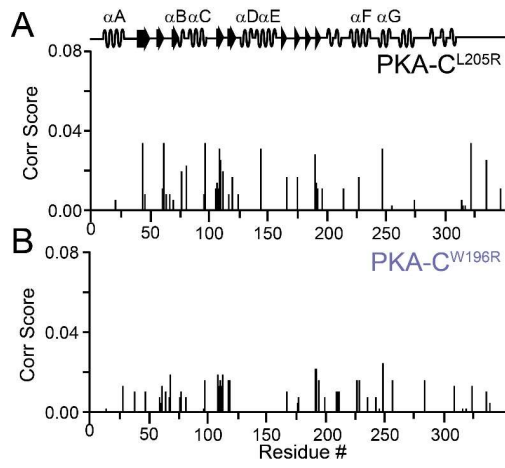
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591 **Figure S3. Thermodynamic cycle linking the free energy perturbation (FEP) calculation to**  
 592 **ratio of  $K_d$  and  $\sigma$ .** (A). Alchemical transition steps that determine the relative change of binding  
 593 free energy. The change in PKI-binding to the apo state upon mutation can either be obtained  
 594 from the experimental ratio  $\frac{K_d^{E31V}}{K_d^{WT}}$ , or be computed from the difference between the two alchemi-  
 595 cal transition  $\Delta G_2 - \Delta G_1$ . (B). Computational scheme used to determine the ratio of the coopera-  
 596 tivity coefficients ( $\sigma$ ).  $\Delta\Delta G_{21}$  is directly computed by grouping the two alchemical transitions in  
 597 the same simulation box with forward transition of WT  $\rightarrow$  E31V, along with the reverse transition  
 598 of E31V/PKI<sub>5-24</sub>  $\rightarrow$  WT/PKI<sub>5-24</sub>. The same computational scheme was used to determine  $\Delta\Delta G_{43}$ .  
 599 The ratio of  $\sigma$  was derived from the difference between  $\Delta\Delta G_{21}$  and  $\Delta\Delta G_{43}$ . (C). Computational  
 600 scheme used to determine the ratio of the cooperativity coefficients  $\sigma'$  calculating  $\Delta\Delta G_{13}$  and  
 601  $\Delta\Delta G_{42}$ , respectively.



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**Figure S4.**

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