1 **Protein Kinase Structure and Dynamics: Role of the αC-β4 Loop**

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Abstract

Although the αC-β4 loop is a stable feature of all protein kinases, the importance of this motif as a conserved element of secondary structure, as well as its links to the hydrophobic architecture of the kinase core, has been underappreciated. We first review the motif and then describe how it is linked to the hydrophobic spine architecture of the kinase core, which we first discovered using a computational tool, Local Spatial Pattern (LSP) alignment. Based on NMR predictions that a mutation in this motif abolishes the synergistic high-affinity binding of ATP and a pseudo substrate inhibitor, we used LSP to interrogate the F100A mutant. This comparison highlights the importance of the αC-β4 loop and key residues at the interface between the N- and C-lobes. In addition, we delved more deeply into the structure of the apo C-subunit, which lacks ATP. While apo C-subunit showed no significant changes in backbone dynamics of the αC-β4 loop, we found significant differences in the side chain dynamics of K105. The LSP analysis suggests disruption of communication between the N- and C-lobes in the F100A mutant, which would be consistent with the structural changes predicted by the NMR spectroscopy.

Introduction. Although the protein kinases, like the GTPases, have evolved to be highly regulated molecular switches, they transfer the γ-phosphate of adenosine triphosphate (ATP) to a protein substrate instead of to water. Understanding how mature and fully active protein kinases couple ATP binding to peptide/protein binding is especially challenging as it involves many sites that lie distal to the active site. This process of phosphorylating a heterologous protein substrate should be clearly distinguished from *cis*-autophosphorylation of the kinase core, which is usually a key initial step in the assembly of most active kinases. *Cis*-autophosphorylation of a protein kinase core is distinct and different from transferring the phosphate to a heterologous protein. In cells a fully active kinase transfers the γ-phosphate of ATP to a heterologous protein substrate that is typically tethered to a distal site that lies far from the site of phosphoryl transfer. To ask how a mature and fully active protein kinase couples ATP binding to peptide/protein binding we use the catalytic (C) subunit of cAMP-dependent protein kinase (PKA) as a model system. PKA activity is regulated by inhibitory regulatory (R) subunits and by heat-stable protein kinase inhibitors (PKIs). In addition, we have extensive NMR data and computational analyses of PKA that complement crystal structures and provide essential windows into dynamics.

The conserved motifs that define the kinase core were first recognized when Dayhoff aligned the cloned sequence of Src with the manually sequenced C-subunit of PKA in 1982 (1). By manually aligning a handful of protein kinases, Hanks, et al, subsequently showed that these motifs, which were scattered throughout the kinase core, were conserved in all protein kinases (2). When the first protein kinase structure was solved in 1991, these conserved sequence motifs became structural entities that correlated with β strands, α helices, and loops of the folded protein (3). That first structure, which was actually a binary complex of the PKA C-subunit and an inhibitory peptide (IP20) from PKI, also provided a detailed description of how the IP20 peptide was docked onto the kinase core (4). Subsequent structures of a ternary complex with ATP and IP20, solved in 1993, showed how the conserved motifs correlated with ATP and peptide binding and engaged the entire kinase core (5, 6). Although Johnson provided a more detailed description of these motifs (7), several essential points were not yet understood. Only later, using computational tools, did we come to appreciate how the conserved and non-linear hydrophobic core architecture was assembled and how hydrophobicity correlated with allosteric regulation of the kinase core (8-10). In addition, we did not initially appreciate the importance of the αC-β4 loop. Like helices and strands, the αC-β4 loop is also a well-defined element of secondary structure (11, 12), and this loop in the N-terminal lobe (N-Lobe) of the kinase core is essential for mediating the synergistic high-affinity binding of ATP and IP20.

73   Unlike other protein kinases such as PKC and the Leucine-rich repeat kinase 2 (LRRK2)

74   that are flanked by other domains that regulate activation and subcellular localization (13-15),

75   the PKA C-subunit represents a kinase domain that is flanked by relatively short N- and C-

76   terminal tails (16). In cells the C-subunit is then assembled as an inactive holoenzyme with

77   functionally non-redundant cAMP binding regulatory (RIα, RIβ, RIIα, and RIIβ) subunits allowing

78   the activity of the kinase domain to be unleashed rapidly and reversibly by allosteric binding of

79   cAMP to the cyclic-nucleotide binding domains (CNB) of the R-subunits (17). In addition, the

80   PKA C-subunit can be regulated by PKI. The R-subunits and PKI all share an inhibitor site that

81   docks to the active site cleft of the C-subunit; however, PKI and RIα/RIβ are unusual in that they

82   are pseudo substrates where high-affinity binding is synergistically coupled to the high-affinity

83   binding of ATP (~60nM). Even though the structure of the PKA C-subunit was solved over 30

84   years ago, we still are elucidating the mechanistic details of how the highly dynamic features of

85   the kinase core are regulated by a set of well-defined motifs and, in particular, how the high-

86   affinity binding of ATP and IP20 are coupled. Disease mutations are also providing key

87   mechanistic insights into how the synergistic binding of ATP and PKI can be uncoupled. Using

88   the PKA C-subunit as a prototype, we focus here, in particular, on the conserved hydrophobic

89   residues/motifs that define the core protein kinase architecture, which contributes so

90   significantly to entropy-driven allostery (18). We then describe how the αC-β4 loop is anchored

91   to the hydrophobic core architecture. Finally, we explore how a single mutation, F100A in the

92   αC-β4 loop, uncouples the synergistic binding of ATP and PKI using a computational approach

93   that first identified the hydrophobic spines (8, 9).

94

95   **Discovering the hydrophobic core architecture of protein kinases.** While we elucidated

96   conserved motifs in the kinase core early on (1, 2, 7) and identified, using affinity labeling,

97   specific residues that we hypothesized were associated with ATP binding (19, 20), we did not

98   have a thorough understanding of the bilobal kinase fold until the first protein kinase structure

99   was solved. This first structure defined the N-Lobe and the C-Lobe and validated the active site

100   localization of the regulatory triad that was identified by affinity labeling and cross-linking. This

101   triad consists of two residues in the N-Lobe - K72 in β3 and E91 in the αC-Helix and one in the

102   DFG motif in the C-Lobe - D184 (3). The subsequent structures in 1993 (5, 6) showed how ATP

103   was held into the active site cleft between the two lobes. Together, these structures validated

104   the affinity labeling experiments where the three motifs containing the regulatory triad residues

105   converged on the ATP phosphates and Mg^{2+} ions at the active site cleft. This structure of a fully

106   closed conformation also defined a novel ATP binding motif where the adenine ring of ATP was
107   buried under a Glycine-rich Loop (G-loop) in a pocket at the base of the cleft (5, 6). From these
108   early structures we gain an appreciation for what is now called the "Activation Loop". The
109   Activation loop is typically assembled by a key phosphorylation site (21, 22), and this loop is
110   very stable in the PKA C-subunit because T197 is constitutively phosphorylated in the purified
111   C-subunit.   At that time, however, we did not appreciate the highly dynamic features that
112   regulate kinase activity, including the Activation loop, nor did we appreciate the role of
113   hydrophobicity in driving dynamics. This would require both computational tools that were not
114   yet sufficiently robust as well as nuclear magnetic resonance (NMR) spectroscopy. In addition,
115   we would need structures of other kinases where the Activation loop is typically disordered (21,
116   22).

117   The importance of hydrophobic residues and the concept of non-contiguous but spatially
118   conserved hydrophobic motifs, some highly conserved and others just conserved as
119   hydrophobic residues, did not become obvious until we investigated structural differences with a
120   computational approach called Local Spatial Pattern (LSP) alignment. With LSP alignment we
121   first identified spatially conserved residues that revealed a conserved hydrophobic "spine"
122   architecture that was associated with active kinases but broken in inactive kinases (8). The
123   terminology of "spines" is related to the fluidity of these residues in contrast to hydrophilic
124   residues and ion pairs that are locked into a more rigid conformation by hydrogen bonds or
125   electrostatic bonds. The Regulatory Spine (R-spine) residues were identified first and
126   correspond to four spatially conserved residues - two in the C-lobe and two in the N-lobe that
127   were aligned in every active kinase but broken in inactive kinases (8). The assembly of the R-
128   Spine defines the switch mechanism of every active kinase. It reveals how kinases have
129   evolved to be dynamic molecular switches, similar to the GTPases. In contrast to metabolic
130   enzymes, they have not evolved to be efficient catalysts. The highly regulated R-Spine along
131   with the Regulatory Triad (K72/E91/D184) remain as hallmark signature motifs of every active
132   kinase that is capable of trans-phosphorylation of a heterologous protein substrate.

133   A second motif, referred to as the Catalytic Spine (C-Spine), defines the extensive and
134   fundamental core hydrophobic architecture of every kinase domain (9). The C-spine includes
135   motifs in both the N-Lobe and the C-Lobe, which are connected by the hydrophobic capping of
136   the adenine ring of ATP. Capping of the adenine ring is accomplished by two highly conserved
137   residues (A70 in β3 and V57 in β2) in the N-Lobe while the other surface of the adenine ring is
138   capped by a C-Lobe residue, L173 in β7, which is a conserved hydrophobic residue found in all
139   kinases. The dominant feature of the C-spine is the very unusual hydrophobic αF-Helix that

140   spans the C-lobe and is linked to all of the important elements of the C-Lobe (**Figure 1**). This
141   buried helix is unusual for several reasons. With a conserved glycine in the middle, it does not
142   have a strong helical propensity. In general, it is highly unusual to find a buried hydrophobic
143   helix in a globular domain; in many ways it is more like a trans membrane helix. The αF helix is
144   flanked by two charged residues. At the beginning of the αF Helix is the highly conserved D220
145   that couples the αF Helix to the catalytic machinery at the active site cleft (**Figure 2**).
146   Specifically, it hydrogen bonds to the backbone amides of Y164 and R165 in the Y/HRD motif
147   that precedes the catalytic loop. D220 is followed by another highly conserved residue, W222,
148   which faces the αH-αI loop, a tethering site on the bottom of the C-Lobe (23, 24). W222 also
149   shields the conserved ion pair between E208 at the end of the APE motif and R280 in the αH-αI
150   loop. At the other end of the αF Helix is E230 which in PKA recognizes the P-2 arginine in the
151   substrate peptide/protein. The remarkable features of the αF-Helix that allow it to be the central
152   organizing unit of the C-lobe, summarized in **Figure 1**, were clearly revealed in the LSP plots in
153   2008 (9). What was not fully appreciated in these first analyses, however, was the role of the
154   αE-Helix in the C-Lobe and another highly conserved motif in the N-Lobe, the αC-β4 loop.

155

156   **The αC-β4 Loop.** The two spine residues in the N-Lobe, which lie at the end of the αC Helix
157   (RS3) and the beginning of β4 (RS4), stabilize a very important element of secondary structure
158   that is conserved in every protein kinase - the αC-β4 loop (residues 99-106) (**Figure 3**). The two
159   R-spine residues are brought together by a strategic β-turn motif (residues 100-103) where the
160   carbonyl of F100 is hydrogen bonded to the backbone amide of L103 (11). The other carbonyls
161   and amides of this turn, as well as those of the other residues in this motif, are filled by ordered
162   water molecules (**Figure 3B**). Structured water molecules highlight that this surface of the αC-
163   β4 loop is exposed to solvent, while the other buried surface of the αC-β4 motif is very
164   hydrophobic and anchored to the hydrophobic R-Spine and Shell residues (25). Only one
165   residue in the αC-β4 motif, V104, directly touches ATP through one of its methyl side chains
166   that is anchored to the adenine ring.

167   Although highly conserved and very stable, this β-turn is not traditionally recognized as a
168   stable element of secondary structure (12). The two N-lobe spine residues (RS3 and RS4)
169   anchor the αC-β4 motif to the R-Spine residues in the C-Lobe **(Figure 3B)** while the tip of the
170   loop is very hydrophobic **(Figure 3A)**. The critical features at the tip of the β-turn typically
171   include a proline. Even though the backbone amides and carbonyls of the β-turn are solvent
172   exposed, one can see in PKA that the hydrophobic side chain residues at the tip of the loop,

173 F100-P101-F102-L103, are buried in a hydrophobic pocket comprised primarily of residues in

174 the αE-Helix. The strategic importance of stabilizing the backbone of this αC-β4 loop is

175 highlighted by Y156 in the αE helix, which hydrogen bonds to the backbone amide of N99.

176 Although this residue (N99) is not conserved, its functional importance has been highlighted by

177 BRaf where replacement of this residue (R509) with histidine breaks the BRaf dimer interface

178 that is essential for BRaf activation (26, 27). The importance of this residue was also explored in

179 depth for the EGF receptor where this site is a hot spot for oncogenic mutations (28, 29).

180 Although the residue at the N99 position differs in every kinase, its spatial organization as well

181 as the hydrogen bonding of its backbone to the αE helix is conserved, and its strategic function

182 may also be conserved.

183   The first 600 picosecond Molecular Dynamics (MD) simulations of the PKA C-subunit,

184 published in 1999, not only demonstrated that the N-Lobe and the C-Lobe function as

185 independent rigid bodies but also showed that the αC-β4 loop (residues 99-106) is the only

186 piece of the N-lobe that remains anchored to the C-lobe whether the kinase is in an open or

187 closed conformation (30) (**Figure 4**). We now know from many kinase structures that positioning

188 and organizing the N-lobe for catalysis is complex and highly regulated. The analysis of LRRK2

189 supports the prediction that the N- and C-lobes of the kinase core function as independent rigid

190 bodies (31, 32). It also indicates that the αC-β4 loop remains as one of the most stable

191 elements of the kinase core whether LRRK2 is in an active or an inactive conformation (**Figure**

192 **4**). The αC-β4 loop is also stably anchored to the αE helix in the active and inactive Src as well

193 as in the active and inactive BRAF (**Figure 4**). Indeed, the αC-β4 loop is conserved as a stable

194 element of secondary structure in every protein kinase as it anchors to the αE Helix.

195

196 **The αE Helix.** Although the dynamic αC Helix, as well as the stable αF Helix described above

197 (**Figure 1**), have been long recognized as conserved elements of the kinase core, less attention

198 has been given to the αE Helix. A defining feature of the αE Helix, mentioned above, is Y156.

199 This conserved tyrosine, which can also be a histidine or phenylalanine in other kinases, is

200 anchored to the backbone amide of N99 in the αC-β4 loop. The importance of this residue is

201 highlighted in BRAF, as discussed previously. Two residues down from Y156 is H158 which in

202 PKA is firmly anchored to the conserved D220 in the αF Helix. H158 is one of three dually

203 protonated histidines in the C-subunit (33, 34). In the fully active C-subunit that is

204 phosphorylated on its Activation loop the side chain of His158 anchors the backbone amides of

205 the HRD residues (R165 and D166) in the Catalytic loop. In contrast to the Activation loop

206 (residues 184-pT197), which is dynamically assembled, the backbone of the catalytic machinery

207 in the C-Lobe (residues 162-182) is very stable. As seen in **Figure 2**, this interaction as well as

208 the hydrophobic anchoring of the catalytic loop to the αE and αF Helices makes the backbone of

209 the catalytic loop very stable. H158 is conserved as a histidine or phenylalanine in most

210 kinases and in the EGF receptor and in other tyrosine kinases this region is a hot spot for

211 oncogenic mutations (15).

212 I150 in the αE Helix is also a key residue. The importance of I150 as a critical player in

213 the hydrophobic core architecture was first highlighted by NMR (10). As seen in **Figure 2**, I150

214 is important for many reasons. It is anchored to L167 and P169 in the Catalytic loop and to L172

215 in β7, which connects the adenine capping residue, L173, to the hydrophobic core. On the other

216 side of L173, both I174 and I180 in β8 anchor the adenine capping residue to the αE Helix. In

217 contrast to the Activation Segment and the R-spine, which are dynamically assembled as part of

218 activation, the backbone residues of the catalytic machinery are always very stably anchored to

219 the hydrophobic core architecture. The two R-Spine residues in the C-Lobe, RS1 in the HRD

220 motif and RS2 in the DFG motif, are all highlighted in **Figure 2** where we can appreciate that the

221 catalytic machinery is anchored to the R-Spine, similar to the hydrophobic surface of the αC-β4

222 loop.

223

224 **Synergistic high affinity binding of ATP and pseudo-substrate inhibitors.** A highly unusual

225 allosteric feature of PKA that is perhaps unique to PKA is its heat-stable protein kinase inhibitor

226 (PKI). PKI was first identified as a high-affinity inhibitor of the PKA C-subunit in 1971 shortly

227 after the C-subunit was discovered (35). It is a classic Intrinsically Disordered Protein (IDP), a

228 prediction that was fully validated recently by NMR of full-length PKI (36). Once it was

229 sequenced (37), PKI was shown to be a pseudosubstrate, a feature that is shared by the type I

230 Regulatory (R) Subunits of PKA, RIα and RIβ. While the inhibitory properties of PKI were

231 localized to the N-terminus (residues 5-24) (38, 39), the unusual synergistic high-affinity binding

232 of ATP and PKI was characterized in detail by the peptide studies of Whitehouse and Walsh

233 (40). They confirmed that having a pseudo-substrate where the P-site in PKI is an alanine, is

234 essential for high affinity binding while the arginines that precede the P-site, as well as the P+1

235 hydrophobic residue, are also important for binding of both substrates and pseudosubstrates.

236 To achieve high-affinity binding, however, hydrophobic residues that preceded the inhibitor site

237 were required. Based on their peptide studies and subsequent biophysical studies, Walsh and

238 his colleagues predicted that this hydrophobic motif was an amphipathic helix (41-43).

239 Localization of the inhibitory region to the N-terminus of PKI and the rigorous characterization of

the inhibitor peptide (IP20, residues 5-24) enabled crystallization of that first PKA structure bound to IP20 (3). A second paper (4), which completely validated the peptide predictions, showed precisely how IP20 bound with high affinity through basic residues near the active site and a distal amphipathic helix (**Figure 5**). Showing how both charged and hydrophobic residues contribute to peptide binding at sites that are distal to the phosphoryl transfer site did not explain, however, how the synergistic high-affinity binding of ATP and IP20 was achieved. The RIα subunit is also a pseudosubstrate and displays the same synergistic high affinity binding with ATP (44, 45). Whether other kinases have such pseudosubstrate inhibitors that display this synergistic high-affinity binding with ATP is not clear, although two of the most important biological inhibitors of the PKA C-subunit, PKI and RIα, show this property, while RII subunits of PKA are actually substrates and not pseudo-substrates. The difference between substrate inhibitors and pseudo-substrate inhibitors is fundamental. Do other kinases have physiologically relevant pseudo-substrate inhibitors that binding with high affinity?

The specific features that convey high affinity binding to PKI are the amphipathic helix that docks into a hydrophobic groove on the C-Lobe while the high-affinity binding of RIα is due to the first CNB domain (46, 47), CNB-A, which docks onto the Activation loop and the αG-helix. These docking sites can be thought of as substrate tethering sites. While ATP binds with a 15 μM affinity (Km) when phosphorylating a small peptide, such as Kemptide (G-R-R-G-S-L), in the presence of PKI or IP20 (residues 5-25) it binds with an affinity of ~60 nM. Similarly, IP20 on its own has an affinity of ~200 nM, while in the presence of MgATP the affinity is ~1 nM. This high-affinity synergistic binding also requires two Mg^{2+} ions that bind to the phosphates of ATP and neutralize the negative charge of the phosphate (45), which allows the N- and C-lobes to fully close. In the fully closed conformation F54 in the G-loop is adjacent to F187 that follows the DFG motif. In this structure the γ-PO4 is well shielded from water, a phenomenon which is thought to facilitate the transfer of the phosphate (**Figure 5**). The second Mg^{2+} ion, in particular, bridges the D184 in the C-Lobe with K72 in the N-Lobe. allowing for full closure of the catalytic cleft. These unique features of IP20 allowed us to trap the fully closed conformation in our first structures of the PKA catalytic subunit (5).

Capturing Dynamics. Identifying allosteric sites that lie distal to the catalytic site where phosphoryl transfer takes place is one of the most important challenges facing the kinase signaling community, as these sites which may be only transiently sampled, can nevertheless be excellent therapeutic targets. While crystal structures and cryoEM structures provide us with high-resolution portraits of folded proteins, these structures are static snapshots. In contrast,

274   NMR provides a residue-specific window into dynamics. NMR studies of the PKA C-subunit, first
275   described three states that are associated with the fully phosphorylated protein - uncommitted,
276   committed and quenched states (48). It should be emphasized that these states are all active
277   conformations where the R-spine is intact; they simply correlate with the opening and closing of
278   the active site cleft. The apo state, which is in an open conformation, is uncommitted to catalysis
279   while the binding of nucleotide, which completes the C-Spine, commits the kinase to catalysis.
280   The intermediate states with bound nucleotide correspond to partially closed states where the
281   C-terminal tail and the G-loop are still dynamic. How these states correlate with the
282   "Communities" of the kinase core was demonstrated by McClendon, et al (49). The quenched
283   and fully closed conformational state is captured when both ATP and the pseudo-substrate,
284   IP20, bind with high affinity. While these initial NMR studies captured the backbone dynamics, it
285   is the side chains that report much of the entropy-driven dynamics (10), and to explore this
286   hydrophobic space required labeling the side chains. When side chains (Val, Leu, and Ile) were
287   labeled, one could for the first time observe the correlated motions of the hydrophobic core
288   architecture, which experimentally captured the entropy-driven allostery (50).

289   Defining and targeting the allosteric sites of protein kinases is a holy grail, and if we
290   delve into the architecture of the kinase core as well as the tails and domains that flank the
291   kinase core in the PKA C-subunit, there are many sites where the core can become "uncoupled"
292   from the flanking motifs. Within the core, many sites lie distal to the active site that can uncouple
293   ATP and peptide/protein binding. Which of these mechanisms can abolish the synergistic high-
294   affinity binding of ATP and IP20 or can all of them do it? Some of these "uncoupling motifs" such
295   as F327, Y204, and E230 are sites that we have experimentally queried, whereas others such
296   as W196R, L205R, and E31V are known disease mutations that correlate with Cushing's
297   Syndrome (51-55).

298   E230 is a critical residue for recognizing the P-2 arginine in PKA substrates. It couples
299   the peptide to Y204 in the P+1 loop and to E170 in the Catalytic loop. The E230Q mutant
300   uncouples the N- and C-lobes by "freezing" the enzyme into a stable open conformation that
301   can no longer bind ATP (56). Y204 is an example of an allosteric site that is not associated with
302   a change in conformation following replacement with Ala. Functionally this mutation reflects
303   instead an inability to transfer the γ-phosphate of ATP to a protein substrate while it is still fully
304   capable of transferring the γPO_4 to water. Even though there is not a change in structure in the
305   Y204A mutant, mechanistically the Y204A mutation reflects a change in dynamics that can lead
306   to significant changes in the communities that the residue interacts with. Although changes in
307   dynamics can be captured and validated by NMR, changes in dynamics that lead to changes in

308   stability can also be predicted by LSP alignment. LSP alignment identified this mutation, Y204A,

309   as part of a dynamics-driven allostery mechanism that can be described as a "Violin" model that

310   draws an analogy between distribution of thermal vibrations in proteins to vibrational patterns in

311   a violin (18, 57-59).

312   F327, which lies outside the kinase core, highlights another unique feature of PKA that is

313   conserved in all the AGC kinases (16). F327 is part of a C-terminal tail that wraps around both

314   lobes of the kinase core and is an essential feature of the ATP binding site (**Figure 5**). When all

315   of the residues comprising the hydrophobic shell around the adenine ring of ATP were mutated

316   to alanine, and analyzed in a yeast screen, only two were found to block viability, F327 and

317   L173 (60). We now know that L173 is a C-spine residue, but F327 lies outside of the kinase

318   core. Nevertheless, it is absolutely essential for binding of ATP. Replacing F327 with alanine

319   reduces the Km for ATP over 10-fold (~450 µM) and completely abolishes the high-affinity

320   binding of ATP.

321   Three Cushing syndrome mutations were also shown to "uncouple" high-affinity binding

322   of inhibitor proteins. L205R uncouples binding of both RI and RII subunits as well as PKI by

323   disrupting binding to the P+1 pocket (55), while W196R disrupts binding to the cyclic nucleotide

324   binding domain (CNB-A) of both RI and RII subunits (51, 61). E31V is an example of a mutant

325   that uncouples the αA-Helix from the Activation loop, and this mutation also abolishes the

326   synergistic high-affinity binding of ATP (53). Paul Herman identified a set of mutants on the C-

327   lobe of the C-subunit that selectively enhanced the docking of protein substrates (23, 24). He

328   defined these as tethering sites, and all of these are directly linked to the αF/αE-Helices. So,

329   there are a number of ways that one can uncouple the synergistic high-affinity binding of ATP

330   and IP20 or RIα by mutating sites that are distal to the catalytic site.

331

332   **F100A mutation highlights the importance of the αC-β4 Loop.** Another mutation that lies in

333   the αC-β4 loop, F100A, was recently described (23, 24). Adjacent residues in the αC-β4 loop

334   (V104I, L103F/I, and P101A) are actually oncogenic mutations in several protein kinases,

335   including PKA although none of these have been validated. The synergistic binding of ATP and

336   IP20 was completely abolished for the F100A mutant; even though the Kms for Kemptide and

337   ATP for the F100A mutant were similar to the wild type (wt) C-subunit (62). Peptide assays are

338   a good way to evaluate the kinetic machinery of a protein kinase, and biochemically, based on

339   the Kemptide peptide assay, the kinetic mechanism for the C-subunit was not altered

340   significantly by the F100A mutation. However, the peptide assay does not reflect how a protein

341   kinase works in cells where it phosphorylates other proteins, not peptides. One will never

342   replicate Michaelis Menton kinetics in cells where the assay depends on having a large excess

343   of a small peptide substrate. This is, of course, relevant for peptides or hormones binding to a

344   receptor on the surface of the cell, but inside cells the protein substrates are not typically in

345   huge excess of the kinase and the interactions are not diffusion limited. They are instead

346   dependent on co-localization where the protein substrate is tethered in close proximity to the

347   kinase. This can be achieved by tethering directly to sites such as those described by Paul

348   Herman on the C-Lobe of the PKA kinase core (23, 24) or by binding to a kinase scaffold protein

349   in a way that brings the P-site close to the active site of the kinase (63). With this mechanism

350   protein phosphorylation is not diffusion-limited; however, it still highly depends on Brownian

351   Dynamics (BD) (64, 65); the charged residues are now simply tethered in close proximity to the

352   active site.

353   The importance of the αC-β4 loop was actually initially hinted at by computational

354   analyses that involved Markov State modeling and NMR-restrained replica-averaged

355   metadynamics (RAM). These analyses suggested that there may be a flip of the αC-β4 loop in

356   the apo protein and in the mutant (62). As seen in **Figure 4**, no such flip has been observed so

357   far in any of the crystal structures of the C-subunit to date (wt, E230Q, unphosphorylated C, and

358   the apo C-subunit), regardless of closed or open conformation. The subsequent NMR studies of

359   the F100A mutant also highlighted V104 and I150 as critical residues. I150 is a critical part of

360   the hydrophobic core architecture of the C-Lobe that anchors the αE Helix to the C-spine (L172

361   and I174 in β7) and to L167and P169 in the Catalytic loop (see above discussion and **Figure 2**).

362   In contrast, V104, which is part of the αC-b4 loop, is one of three shell residues (SH1, SH2, and

363   SH3) that flank the R-spine residues in the N-lobe and V104 also touches ATP (25). Specifically,

364   one of the methyl side chains of V104 touches the adenine ring of ATP so it is one of the N-lobe

365   adenine capping residues for ATP (60) (**Figure 5**). All of the adenine capping residues in the C-

366   subunit come from the N-Lobe and the C-tail except for L173, which is the only capping residue

367   that comes from the C-Lobe. The C-spine is so important, because it links all of the hydrophobic

368   capping machinery in the N-Lobe with the hydrophobic core architecture in the C-Lobe. This

369   residue is not always conserved as a valine, but it is typically a small hydrophobic residue. This

370   hydrophobic residue also touches the well-studied gate-keeper residue, which in PKA is M120;

371   the gatekeeper is another shell residue (SH3) (66, 67).

372   The long computational studies (2 μsec) carried out by Veglia predicted that the αC-β4

373   loop undergoes a flip in the apo state, while the ITC results showed that the F100A mutant is

374   also more stable than wt C-subunit. Most importantly, the biochemical studies clearly

375   demonstrate that this mutation disrupts the synergistic binding of ATP and IP20. We thus asked

376 whether LSP could identify changes in dynamics that correlate with the NMR results. Could the

377 LSP analysis, which requires relatively short simulation times, predict residues or regions in the

378 C-subunit that could account for enhanced or reduced stability that results from this mutation

379 even if they cannot predict a conformational change?

380

381 **Comparing the LSP alignment of F100A with wt C-subunit.** Local Spatial Pattern (LSP)

382 alignment is a computational method developed in our laboratory for capturing conserved

383 patterns formed by Cα-Cβ vectors in proteins (**Figure 6A**). Initially, it was utilized to identify

384 conserved hydrophobic ensembles in protein kinases (8, 9). More recently, this technique was

385 applied to Molecular Dynamics (MD) simulations, in an effort to analyze stable regions in Protein

386 Kinase A (68). By comparing spatial patterns formed by Cα-Cβ vectors in differing

387 conformations generated via MD simulation it is possible to analyze thermal vibrations of

388 residues. These motions occur on a sub-nanosecond timescale and are considered to be the

389 foundation of dynamics-driven allosteric effects, which were predicted by Cooper and Dryden in

390 1984 (69) and have been observed in multiple proteins (70, 71).

391 LSP-alignment is a graph-theory based method that implements a Protein Residue

392 Network (PRN) approach (72). As we demonstrated, two major centralities of such PRNs can

393 contain important information on the local stability (Degree centrality, DC) and global

394 connectivity (Betweenness centrality, BC) of the protein (68) (**Figure 6B**). Our purpose here

395 was to identify changes in LSP-based PRNs associated with the F100A mutation, specifically

396 assessing whether these changes relate to the dynamic features of the αC-β4 loop and

397 correlate with the NMR results. **Figure 6C** shows the corresponding PRNs laid out by the

398 ForceAltlas2 algorithm (73). This algorithm treats the weights assigned to edges as attractive

399 forces that balance the imposed repulsion of the nodes. In LSP-based PRNs, compact and

400 highly interconnected nodes correspond to more ordered regions of the protein where Cα:Cβ

401 vectors move cohesively and preserve their mutual orientations. Both PRNs showed very similar

402 general structures, featuring dense groups of residues that correlate with the N and C-lobes.

403 These are linked by the Hinge, αC-β4 loop and αC-helix. The analysis also includes the ATP

404 molecule, that was described by N1-C8 vector. Notably, the darker color of the αC-β4 loop

405 residues in the F100A mutant indicates the increased level of BC.

406 To analyze changes of BC and DC in more detail we plotted them on scatterplots

407 (**Figure 7**). Residues from the αF-helix have the highest levels of DC in both the wt C-subunit

408 and F100A. This is consistent with the fact that this helix is the most stable structural element of

409 the kinase core and is known to be a major scaffold for the catalytic machinery of these

410   enzymes (**Figure 1**) (9). Several of the αF helix residues such as D220, W221, W222 and L227

411   from the C-spine, score high for both DC and BC. This indicates that these residues act both as

412   hubs and connectors. The most significant change in the F100A mutant is the increase of BC for

413   the five residues from the αC-β4 loop (highlighted in blue). Conversely, a set of highly

414   conserved residues (marked in red), previously noted for having the highest levels of BC (68),

415   show a reduction in their DC and this reduction is even more pronounced in the BC values in

416   the F100A mutant.

417   The detailed values of DC and BC changes associated with the F100A mutation are

418   shown in **Figure 8**. Since both of these parameters have positive and negative values, we

419   visualize them separately, mapping them onto the PKA structure. Positive changes in DC

420   correspond to areas that lose stability of their Cα-Cβ vectors upon the mutation (**Figure 8A**, left,

421   red). These include the catalytic area of the kinase, and most importantly, universally conserved

422   K72, the DFG-motif, including D184, and the substrate binding site. Notably, the ΔDC value for

423   ATP is one of the highest (**Figure 8A**, center), indicating a significant loss of stability of the

424   adenine ring in the mutant. Negative changes in ΔDC (**Figure 8A**, right, blue) correspond to

425   areas of PKA that become more stable in the mutant. Significant changes were primarily

426   localized in the αC-β4 loop. Similarly, negative changes in ΔBC values (**Figure 8B**, right, blue)

427   were also found in the αC-β4 loop, signifying that the role of the major connecting area between

428   the lobes shifts from the catalytic area (**Figure 8B**, left, red) to this region. Overall, the F100A

429   mutation causes the rigidification of the αC-β4 loop, especially V104 at the tip becomes more

430   stable and rigid following mutation of F100. Our analysis suggests that the major connectors

431   between the N- and C-lobes such as K72 from β3 stand (N-lobe) and D184 from the DFG motif

432   (C-lobe) in the wt protein gets impaired and destabilized in response to the F100A mutation.

433   The rigidified αC-β4 loop loses the dynamic coupling between the two lobes and the

434   connectivity between both lobes changes, as seen by V104 and I150, which become the major

435   connector points following the mutation. Overall, the mutation at the αC-β4 loop changes the

436   allosteric communication between the two lobes.

437   In general, these results can be interpreted as a disruption of dynamic communication

438   between the two lobes where ATP binding in the N-Lobe comes together with the major catalytic

439   machinery in the C-Lobe (**Figure 2**). The prominent role of D184 in the C-lobe and K72 in the N-

440   Lobe, hallmark features of the conserved regulatory triad, are also altered. Upon the mutation, a

441   robustly stable region around F102-L106 becomes a dominant connector between the lobes,

442   leading to a significant disruption of correlated dynamics observable in the active site of the wt

443   C-subunit.

444

445   **Capturing side chain dynamics.** Our initial preliminary LSP alignment comparison of wt C-

446   subunit in the presence of ATP with the F100A mutant indicated significant destabilization of the

447   entire catalytic machinery in the C-lobe in parallel with enhanced stability of the αC-β4 loop, a

448   node in the C-tail and the αH-αI loop (**Figure 8A**). While a much more extended analysis is

449   needed to validate the LSP predictions, the overall results clearly show that the dynamic

450   features of the αC-β4 loop have changed in the F100A mutant. As discussed earlier and as

451   indicated in **Figure 4**, we did not see significant changes in the αC-β4 loops when we compared

452   our various crystal structures, which included open and closed conformations of the active

453   kinase as well as an inactive unphosphorylated C-subunit. Specifically, the tip of the αC-β4 loop

454   remained anchored to the αE Helix in all of these structures. Intrigued by our LSP results with

455   the F100A mutant, we thus looked more carefully at the side chain residues in the αC-β4 loop in

456   our various structures. We asked specifically if there were differences in any of the side chain

457   residues that could be predictive of subsequent conformational changes in the backbone as was

458   predicted by the NMR studies. The side chains of the αC-β4 loop in the ternary complex with

459   ATP and IP20 are shown in **Figure 9A**, and no significant differences were seen for the E230Q

460   mutant and the unphosphorylated C-subunit. In this structure the side chain of K105 was

461   toggling (~4.6A) between two carboxyl groups (E107 and E121), and its backbone carbonyl (the

462   first residue of β4) is hydrogen bonded to the backbone amide of E121 (the last residue of β5)

463   (**Figure 9B)**. While the apo protein showed no major changes in the backbone of the αC-β4

464   loop, the side chain of K105 has flipped and is now interacting with carbonyls in the β-turn

465   residues. It is also close to the side chain of N99. Based on the crystal structures, this space is

466   not sampled by K105 in the wt C-subunit; instead this space is filled by ordered water molecules.

467   To look more carefully at the space that is sampled by the side chain of K105, we looked

468   at the MD simulations that were used for the LSP analysis of the wt binary complex of the C-

469   subunit and the F100A mutant. As seen in **Figure 10A**, for the wt C-subunit there is one global

470   energy minimum, and the predominant structure for the global energy minimum correlates well

471   with our structure of the ternary complex (**Figure 9A**). Based on the simulations, however,

472   instead of toggling between E107 and E121, the side chain of K105 interacts predominantly with

473   the side chain of E107 (**Figure 10C**), while it rarely samples the side chain of E121 (**Figure**

474   **10D)**. In contrast to the wt C-subunit and the apo protein, the mutant shows two energy basins

475   (**Figure 10B**). The minor local minimum corresponds closely to the wt C-subunit with K105

476   interacting with E107. In contrast, the major global energy minimum shows the side chain of

477   E105 interacting with the side chain oxygen of N99 and far from the side chain of E107. The

478   probability distribution of this interaction with N99 is shown in **Figure 10E**, which confirms that

479   the mutant has a stronger propensity to interact with N99. As discussed above, N99 is thought

480   to be a critical residue based on the importance of the homologous residue in BRaf (R509) for

481   dimerization and because the backbone of this residue is always anchored to a key residue in

482   the αE Helix (Y156) in every kinase. To interrogate the strength of the Y156 hydrogen bond to

483   the backbone amide of N99, we also looked at its probability distribution in the simulations

484   (**Figure 10F**). While the H-bond is strong in the complex and in the mutant, it is surprisingly

485   destabilized in the apo structure.

486   After investigating the binary complex of the C-subunit for both wt and F100A, we next

487   analyzed the ternary complex. Using the MD simulations, we asked specifically if the F100A

488   mutation alters the dynamic properties of K105 when both ATP and PKI are present. The free

489   energy landscape (**Figure 11)** depicts the overall energy profile for the wt ternary complex and

490   the F100A mutant. The wt ternary complex, in general, behaves similarly to the binary complex.

491   Both exhibit a single global energy minimum (**Figure 11A**). In contrast, the F100A mutation

492   shows enhanced dynamics for both binary and ternary complexes, although there are multiple

493   energy basins in the ternary complex (**Figure 11B**). The side chain dynamics of K105 also show

494   different interaction profiles (**Figures 11 C**, **D** and **E)**, and differences are seen even when just

495   the binary and ternary complexes are compared. In the wt binary complex, the side chain of

496   K105 predominately interacts with the side chain of E107 (**Figure 10C**) and interacts more

497   transiently with the side chain of N99 (**Figure 10E**). This is in contrast to the wt ternary complex

498   where the interactions of K105 and N99 side chains increase (**Figure 11E**), while the interaction

499   with E107 decreases (**Figure 11C**). The side chain of K105 in the wt binary, ternary, and apo

500   states rarely samples the side chain of E121 (**Figures 10D and 11D**). The hydrogen bond

501   between Y156 and N99 is also maintain in the binary and ternary complexes, but broken in the

502   apo protein (**Figures 10F and 11F).**

503   Does the F100A mutation introduce additional changes in the crosstalk of the ternary

504   complex? The major difference is between K105 and E121**,** which correlates with the primary

505   energy minimum (**Figure 11D**). This interaction is not seen in any of the other complexes. In

506   addition, the side chain of K105 has a reduced propensity for interacting with the side chain of

507   E107 as shown in the second local minimum. Finally, there are no significant changes between

508   Y156 and the backbone amide of N99 in the mutant (**Figure 11F**). Hence, the enhanced side

509 chain dynamics of K105 have been observed following the F100A mutation where the side of

510 K105 toggles between E121 and N99, and to some extent E107 (**Figure 11G**). In general,

511 these results agree with the NMR experimental results and suggest that the F100A mutation

512 alters the dynamic interactions of the αC-β4 loop, leading to the hypothesis of the disruption in

513 the allosteric communication between both lobes.

514 The most striking difference, based on the MD simulations, is the enhanced interaction

515 of K105 with the side chain of E121, a key residue that binds directly to the adenine ring of ATP.

516 As seen in **Figure 12A**, in the crystal structure of the ternary complex the backbone amide of

517 E121 hydrogen bonds to the backbone carbonyl of K105, while the backbone carbonyl of E121

518 hydrogen bonds directly to the adenine ring of ATP. The hydrophobic packing between the ATP

519 adenine ring and V104, a shell residue, is extended to the R-spine residues, L95 (RS3) and

520 L106 (RS4), which is also highlighted. How these predicted changes in dynamics in the F100A

521 mutant correlate with enhanced stability and/or alter function need to now be validated

522 experimentally.

523 **Summary and future directions.** Based on the NMR analysis of the F100A mutant, we more

524 rigorously examined the αC-β4 loop showing first how it is firmly anchored to the hydrophobic

525 core architecture of the C-lobe. In addition, we reviewed the synergistic high-affinity binding of

526 ATP and PKI, a phenomenon that was described in detail many decades ago for PKI but never

527 fully explained in terms of its mechanism. In the F100A mutant, this synergy is uncoupled.

528 Finally, given that both NMR and the Markov State model suggested changes in the αC-β4 loop,

529 we carried out an LSP analysis of the mutant. The LSP analysis correlated remarkably well with

530 the NMR predictions. To explain this correlation, we compared various crystal structures of the

531 C-subunit to see if we could detect differences in side chain dynamics since the backbone did

532 not change significantly. LSP alignment is a tool that is perfectly poised to detect alterations in

533 side chain dynamics as it identifies differences in the geometry of the α carbon/β carbon vectors

534 (**Figure 6**), a property that likely precedes any conformational changes, which take a much

535 longer time to simulate with classical Molecular Dynamics. MD simulations of the binary and

536 ternary complexes of the wt C-subunit, the F100A mutant and the apo C-subunit were also used

537 to explore changes in side chain dynamics.

538 Although in these short MD simulations we did not detect a flip of any backbone residues

539 in the αC-β4 loop, we did see significant differences in the side chain dynamics of K105 in the

540 structure of the apo protein. This could, in principle, correlate with a weakening of the β-turn.

541 Based on the MD simulations, the side chain of K105 is also flipped in the F100A mutant. In
542 both cases the side chain of K105 is exploring the space that is occupied by the ordered water
543 molecules in the other structures. These water molecules play an essential role in stabilizing the
544 β-turn motif. We thus predict that the enhanced flexibility of the side chain of K105 may be an
545 initial step that leads to subsequent changes in the backbone dynamics. This prediction needs
546 now to be validated with crystal structures to see if the mutation actually "freezes" an altered
547 conformation of the αC-β4 loop. The potential importance of both N99 and K105 in mediating
548 changes in dynamics also needs to be experimentally validated biochemically, by NMR, and by
549 further computational analyses. We predicted that N99 is important due to is conserved
550 hydrogen bonding to Y156 and the conserved packing of the side chain of Y156 with
551 hydrophobic residues in the β-turn. This, however, is the first potential indication of a direct role
552 for K105. The graphs of the hydrogen bond between the side chain of Y156 and the backbone
553 amide of N99 show that this bond is very stable in the binary and ternary complexes and in the
554 mutant but weakened in the apo protein (**Figure 10E**), and this would also be consistent with
555 the prediction that the β-turn may be more flexible in the absence of ligands but stabilized in a
556 different way in the F100A mutant.

557 Overall, our work suggests that the αC-β4 loop should be carefully examined in all
558 protein kinases as it is a focal point for linking ATP binding to peptide/protein binding and for
559 opening and closing of the catalytic cleft. Our early structure captured the high affinity binding of
560 both ATP and IP20, a pseudo-substrate inhibitor, in a fully closed conformation (**Figure 12B**). In
561 this conformation the IP20 peptide is firmly anchored to the catalytic machinery of the C-lobe,
562 while F327 and Y330 in the C-terminal tail are anchored to ATP. The side chain of the P-3
563 arginine in IP20 interacts directly with ATP, E127, and Y330 in the C-terminal tail, and all are
564 close to the αC-β4 loop. This convergence of the P-3 arginine side chain with Y330 may explain
565 the enhanced synergy that is seen when the PKA C-subunit interacts with its pseudo-substrate
566 inhibitors, PKI and RIα, and understanding this synergy is a major future challenge. E127 binds
567 to the other ribose hydroxyl moiety. E121 and E127 flank the linker that joins the N-and C-lobes
568 (**Figure 12A**), and this linker is likely a critical feature for opening and closing of the active site
569 cleft. K105 in the αC-β4 loop can obviously sense differences in the apo, binary and ternary
570 complexes, and can also sense mutations. This space now needs to be further explored. The
571 αC-β4 loop is also a good potential therapeutic target as the specific residues such as N99 and
572 K105 that are mechanistically important are different in all kinases in contrast to the conserved

573   residues such as K72 and D184 that position the phosphates of ATP. The importance of αC-β4

574   loop is also reinforced because the cancer mutations are located here (29).

575   Finally, given the extremely close correlation of the LSP analysis with the NMR results

576   and the Markov State model predictions, as well as our principal component analysis (PCA) of

577   the wt and F100A mutant, we suggest that LSP could be used as a predictor of dynamics for

578   any mutant or even for carrying out an alanine scan of any protein. These sites could then be

579   examined experimentally. Although purifying these proteins and especially labeling the

580   hydrophobic side chains is not only a major time investment but also very expensive, these

581   methods are essential to experimentally validate the importance of hydrophobic residues in

582   mediating entropy-driven allostery. In contrast, LSP is rapid and relatively inexpensive and could

583   easily be used as an initial screen to identify potentially important residues that contribute to

584   dynamics.

585

586   **Material and Methods**

587   **Molecular Dynamic (MD) Simulation.** The catalytic subunit of PKA was prepared using the

588   crystal structure (PDB: 1ATP) (5) for all-atom MD simulations using the AMBER16 suite (74). In

589   order to study the allostery dynamics of the aC-b4 loop and compare the effect of mutation of

590   F100A on structure-dynamics of the catalytic subunit, we prepared three systems: The wt C-

591   subunit (ATP/Mg^{2+}) where Mn^{2+} ions were replaced with Mg^{2+} and removed the PKI to form a

592   binary complex. In addition, we kept the PKI along with the ATP to form a ternary complex

593   (ATP/Mg^{2+}/PKI); the F100A mutant structure in both the binary and ternary complex by replacing

594   the Phe100 to Ala, and allowing the side chains to be added by the program LEaP module in

595   AMBER, and the Apo system, which was prepared by taking the coordinates from the PDB:

596   1J3H (75). Amber ff14SB (76) force field was used to describe proteins. Titratable residues

597   were protonated at pH 7.0 based on PROPKA3.0 (77, 78). Parameters for ATP and Mg^{2+} were

598   obtained from the Bryce Group AMBER parameters database (79, 80). The phosphorylated

599   serine and threonine residues were described using the phosaa10 force field (81). The

600   hydrogens and counter ions were added, and the system was solvated in the octahedron

601   periodic box using the TIP3P (82) water model and 150 mM NaCl with a 10 Å buffer in AMBER

602   tools. Systems undergo minimization, heating, and equilibration steps using the AMBER16.

603   Initially, systems were minimized by 1,000 steps of hydrogen-only minimization, 2,000 steps of

604   protein and ligand minimization, 2,000 steps of side chain minimization, 2,000 steps of

605   backbone minimization, and followed by removing all restraints for 5,000 steps of all-atom

606   minimization. Systems were gradually heated from 0° to 300° K over 50 ps with 2-fs time-steps

607   and 10.0 kcal mol^{-1}Å^{-2} position restraints on the protein. The temperature was maintained by the

608   Langevin thermostat (83, 84) while the pressure was maintained using the Barendsen barostat

609   (85). Constant pressure equilibration with a 10 Å non-bonded cutoff with Particle Mesh Ewald

610   (PME) (86) was performed with 1000 ps of unrestrained equilibration. Production simulations

611   were performed on Graphic Processing Unit-enabled AMBER16 as above in triplicate for 200 ns

612   each for an aggregate of 600 ns. Overall, 60,000 snapshots were generated for each system

613   and saved for analysis.

614

615   **LSP-alignment based Protein Residue Networks**. LSP-based PRNs were built as described

616   earlier (68). Five 10 ns intervals were taken from a 200 ns trajectory at specific intervals: 0-10

617   ns, 50-60 ns, 90-100 ns, 130-140 ns, and 170-180 ns. From each interval, 100 structures were

618   extracted with a step of 0.1 ns. The LSP-alignment between each set of 100 structures was

619   performed in an all-to-all manner. The resulting adjacency matrices were averaged for each set.

620   Degree centrality and betweenness centrality were calculated for five average matrices. Finally,

621   five values for the centralities were averaged, and the standard error of the mean was

622   calculated.

623

624

625   **Principal Component Analysis (PCA) and Free Energy Landscape (FEL).** PCA is a broadly

626   used method to extract the slow and functional motions of biomolecules (87-89). First, the

627   covariance matrix, C, was calculated based on the fluctuations of the Ca

628   atom of each residue. The elements C_{ij} in the matrix were obtained from the fluctuation of amino

629   acids and diagonalized as given in Equation 1

630

631    $$C_{ij} = < (x_i - < x_i >) (x_j - < x_j >) > \qquad (1)$$

632

633   where x_i and x_j are the ith or jth atom coordinates and $<x_i>$ and $<x_j>$ represent the mean average

634   coordinate of the ith or jth atom, respectively. The principal components (PCs) will be obtained by

635   diagonalizing the covariance matrix C. The corresponding eigenvalues and eigenvectors were

636   calculated.

637

638   The Free Energy Landscape (FEL) can be drawn based on PCA as a reaction coordinate using

639   the Equation 2 (90, 91)

640

641 $$G_i = -k_B T \, ln\frac{N_i}{N_m}$$ (2)

642   where k_B represents the Boltzmann constant and T represents the absolute temperature. N_i and

643   N_m are the ith bin populations and the maximum populated bin, respectively.

644

645

648

649   **Conflict of interest**. The authors declare no conflict of interest.

650

651   **Author contributions**. J.W. and S.S.T. reviewed the hydrophobic core architecture literature.

652   J.W., N.A.J., C.O., G.V., A.P.K., and S.S.T. contributed to the design of F100A analyses. J.W.,

653   N.A.J., C.O., G.V., A.P.K., and S.S.T. contributed to the critical analysis of the data and writing

654   of the manuscript. N.A.J. and A.P.K. performed the MD simulations and LSP analysis. J.W.,

655   A.P.K. and S.S.T. contributed to the formatting of the text and preparing figures. S.P.H., C.O.,

656   and G.V. contributed to the editing of the manuscript. All authors have given approval to the final

657   version of the manuscript.

658 **FIGURE LEGENDS.**

659 **Figure 1. Hydrophobic αF-helix serves as a central scaffold**. (A). αF-helix is very
660 hydrophobic and creates an interface with multiple motifs including the Catalytic machinery
661 (colored in tan) and tethering sites (in sand) of PKA C-subunit. Two key charged residues, D220
662 and E230, sit at the two ends of αF-helix. (B). αF-helix is a central scaffold for assembly of the
663 entire molecule. D220 forms two H-bonds to Y164 and R165 of YRD motif, and E230 salt-
664 bridges to P-2 Arg of PKI. (C). Sequence alignment of αF-helix segment of PKA with other
665 kinases. All share a very hydrophobic helix, the highly conserved residues, D220 are colored in
666 red and E230 in red box.

667 **Figure 2. Hydrophobic interface anchors the Catalytic machinery to the αE-helix and αF-
668 helix**. (A). I150 (in red) from αE-helix plays an important role by docking to αF-helix and
669 Catalytic loop. Three residues from β7, L172, L173, and I174 (in dark tan) assemble the
670 hydrophobic surface from αE-helix to ATP pocket. L173 and I174 are part of C-Spine. R-spine
671 are also shown in red. D220 bridges H158 from αE-helix to YRD motif. (B). The logo of spines
672 shows how important those hydrophobic residues are. The sequence of β6-β9 segment is also
673 shown.

674 **Figure 3. The buried surface of αC-β4 loop**. (A). αC-β4 loop (in teal) in PKA links the N-lobe
675 to its C-lobe. The hydrophobic residues on the loop docks to αE-helix, F18 and L19 from αA-
676 helix and Y306 from C-tail are also part of this surface. F100 is colored in red. (B). The H-bond
677 network of αC-β4 loop. Y156 from αE-helix H-bonds to N99 backbone amide. Three water
678 molecules also help to nucleus the network. (C). Sequence alignment of αC-β4 loop of PKA with
679 other kinases. The highly conserved residues are highlighted, E91 colored in red, L95 and L106
680 in red box.

681 **Figure 4. αC-β4 loop is a very stable element**. (A). Hydrophobic surface of αE-helix which
682 anchors to αC-β4 loop, αF-helix and tethering sites. (B). Superimposition of αC-β4 loop in
683 structures of PKA and other kinases. αC-β4 loop is very stable, not only in different
684 conformations of PKA, but also in other kinase structures including active and inactive Src. (C).
685 Sequence alignment of αE-helix of PKA with other kinases. Y156, in red box, is highly
686 conserved.

687 **Figure 5. Hydrophobic residues play key roles in PKA**. (A). High-affinity binding of PKI is
688 mediated by the hydrophobic surface of an amphipathic helix and P+1 inhibitor site, both
689 highlighted in red. The sequence of PKI is also shown. (B). Hydrophobic interface between the
690 αE and αF-helices. One side of αF-helix (in red) is shown in tan, and another side in sand,
691 which is the same color coding as Figure 1A. (C). Hydrophobic pocket surrounding ATP. L172,
692 L173, and I174 from β7 anchor this ATP pocket to αE-helix. F327 from C-tail is highlighted in
693 red.

694 **Figure 6. Local Spatial Pattern (LSP) alignment method.** (A). Spatial patterns detected by
695 the LSP-alignment are formed by Cα-Cβ vectors. Both mutual positions and orientation in space
696 of these vectors are taken into account. (B). Two major centralities that characterize graphs.
697 Degree centrality (DC) is the sum of connections for each node. The highlighted node in the
698 middle has the highest DC value of 8. Residues with high level of DC are local "hubs".
699 Betweenness Centrality (BC) of a node is the number of shortest paths between all other pairs
700 of nodes that pass through this node. Two highlighted nodes are the main connectors in the

701 graph with BC equal to 6. Residues with high level of BC are global connectors between local
702 hubs. **(**C) Visualization of Protein Residue Networks (PRNs) of PKA before and after the
703 mutation laid out by Gephi software package using ForceAtlase2 algorithm. The diameter of
704 nodes is proportional to the residues DC. Nodes with higher BC have darker color. Four
705 residues from the αC-β4 loop (103-106) are highlighted by oval. The Hinge region is indicated
706 by the dashed oval.

707 **Figure 7. Distribution of Betweenness centrality vs. Degree centrality for PKA residues:**
708 **wt C-subunit (A) and F100A (B)**. Residues with Degree centrality (DC) are local "hubs",
709 representing the most stable parts of the molecule. Residues with high Betweenness centrality
710 (BC) are global connectors and are "bottlenecks" between densely interconnected "hubs".
711 Residues of the central αF-helix are shown as brown circles. Their high levels of DC and BC
712 don't change upon the F100A mutation, meaning that the helix remains to be a central structural
713 element in the PKA mutant. Five residues from the αC-β4 loop are highlighted as blue dots. The
714 significant increase in BC and a certain increase in DC shows that in the mutant this group of
715 residues becomes a significant point of connectivity between the kinase lobes. On the contrary,
716 the highly conserved residues highlighted as red circles show a drastic decrease in BC. Error
717 bars represent standard error calculated for five 10 ns trajectories.

718 **Figure 8. Changes of DC and BC in PKA upon F100A mutation**. The middle graphs
719 represent changes in the corresponding parameters. Positive changes are mapped on the PKA
720 structure (left) with dark red color corresponding to the maximum changes. On the right the
721 negative values of the changes are mapped on the structure to illustrate their distribution. Dark
722 blue color corresponds to the most negative values. Error bars represent standard error
723 calculated for five 10 ns trajectories. The secondary structure of PKA is shown on top of the
724 sequence axis for reference.

725 **Figure 9**. **Dynamic feature of K105 on αC-β4 loop**. (A). In the wt PKA:ATP:Mg:PKI complex
726 structure, the side chain of K105 is likely interact with E107 and E121, and its main chain
727 carbonyl forms a H-bond to backbone amine of E121. (B). In the wt Apo PKA structure, the
728 K105 side chain interacts with β-turn, whereas its main chain carbonyl still H-bonds to backbone
729 amines of E107 and E121. The H-bonds are shown as dash line in black, and the non H-bond
730 are colored in green and the distance are labeled.

731 **Figure 10. Energy landscapes of wt C-subunit and F100A mutant in binary complex.** The
732 free energy landscapes (FEL) were generated based on the principal component analysis
733 (PCA) for the wt C-subunit (A) and F100A mutant (B). The H-bonds are shown in the black dash
734 line and the non H-bond in green along with the distance in Angstroms (Å). The probability
735 distribution plots for specific side chain dynamics are also shown; (C) K105 NZ to E107 OE2,
736 (D) K105 NZ to E121 OE2, (E) K105 NZ to N99 OD1, and (F) N99 N to Y156 OH. The
737 probability density function (PDF) is a relative measure of how densely data points are
738 distributed along the x-axis.

739 **Figure 11. Energy landscapes of wt C-subunit and F100A mutant in ternary complex.** The
740 free energy landscapes (FEL) were generated based on the principal component analysis
741 (PCA) for the wt C-subunit with PKI (A) and F100A mutant with PKI (B). The H-bonds are shown
742 in the black dash line and the non H-bond in green along with the distance in Angstroms (Å).
743 The probability distribution plots for specific side chain dynamics are also shown; (C) K105 NZ
744 to E107 OE2, (D) K105 NZ to E121OE2, (E) K105 NZ to N99 OD1, and (F). N99 N to Y156 OH.
745 (G) the enhanced side chain dynamics of K105 in the F100A ternary complex: the side chain of

746   K105 toggles to its neighboring residues, E121 (white, global energy minima), N99 (green, first
747   secondary minima), and E107 (sand, second secondary minima). The probability density
748   function (PDF) is a relative measure of how densely data points are distributed along the x-axis.

749   **Figure 12**. **High-affinity binding of ATP and PKI converge at the αC-β4 loop**. (A). V104
750   in αC-β4 loop (in teal) is hydrophobically anchored to the adenine ring of ATP. The main chain
751   carbonyl of K105 forms an H-bond to the backbone amide of E121, while its main chain
752   carbonyl hydrogen bonds to ATP. The distance between K105 and E121 side chains is
753   strengthened in the F100A mutant. Two spine residues, L95 and L106, in this loop are also
754   shown. The linker that joins the N- and C-lobes (red) is flanked by E121 and E127. (B).
755   Hydrophobic capping of the adenine ring of ATP is mediated mostly by N-lobe residues
756   including V104 in the αC-β4 loop, as well as F327 in the C-terminal tail. In contrast, the P-3 to
757   P+1 peptide is anchored to the catalytic machinery of the C-lobe. By binding to L173 in the C-
758   lobe, the adenine ring completes the C-Spine, and thus fuses the adenine capping motif in the
759   N-lobe with the extensive hydrophobic core architecture of the C-lobe. In the fully closed
760   conformation, the side chain of Y330, also in the C-terminal tail, is anchored to the ribose ring of
761   ATP. The only direct contact of the peptide/catalytic machinery with the N-lobe is mediated by
762   the P-3 arginine which binds to the ribose ring of ATP and to E127 in the linker. In the fully
763   closed conformation the P-3 arginine also binds to the side chain of Y330 in the C-terminal tail.
764   Some of the mutations that disrupt the synergistic high-affinity binding of ATP and
765   peptide/protein (E230Q, Y204A, F327A, L173A, and E31V) are highlighted. The hydrophobic
766   residues in the amphipathic helix and P+1 inhibitor site of PKI are shown in red.
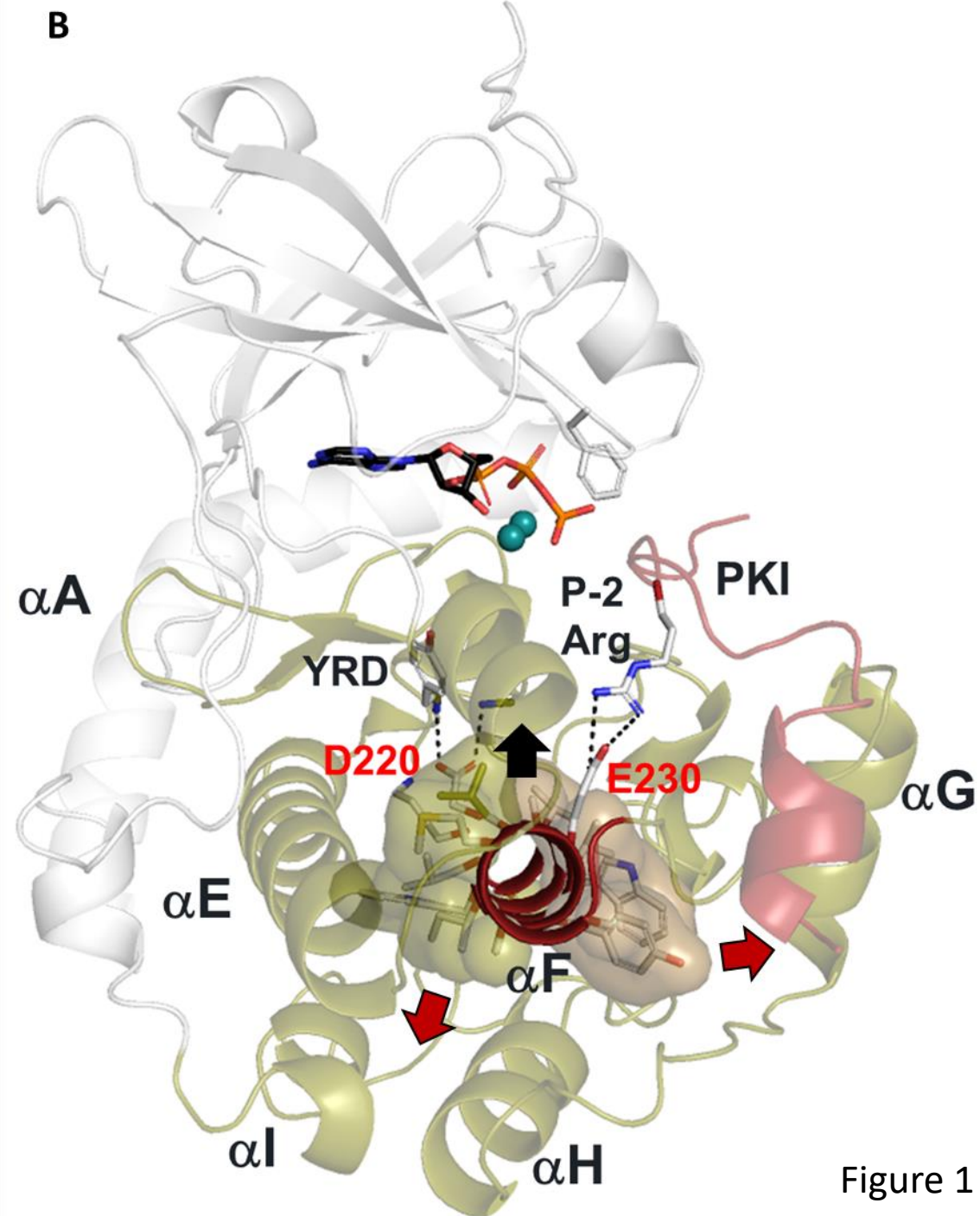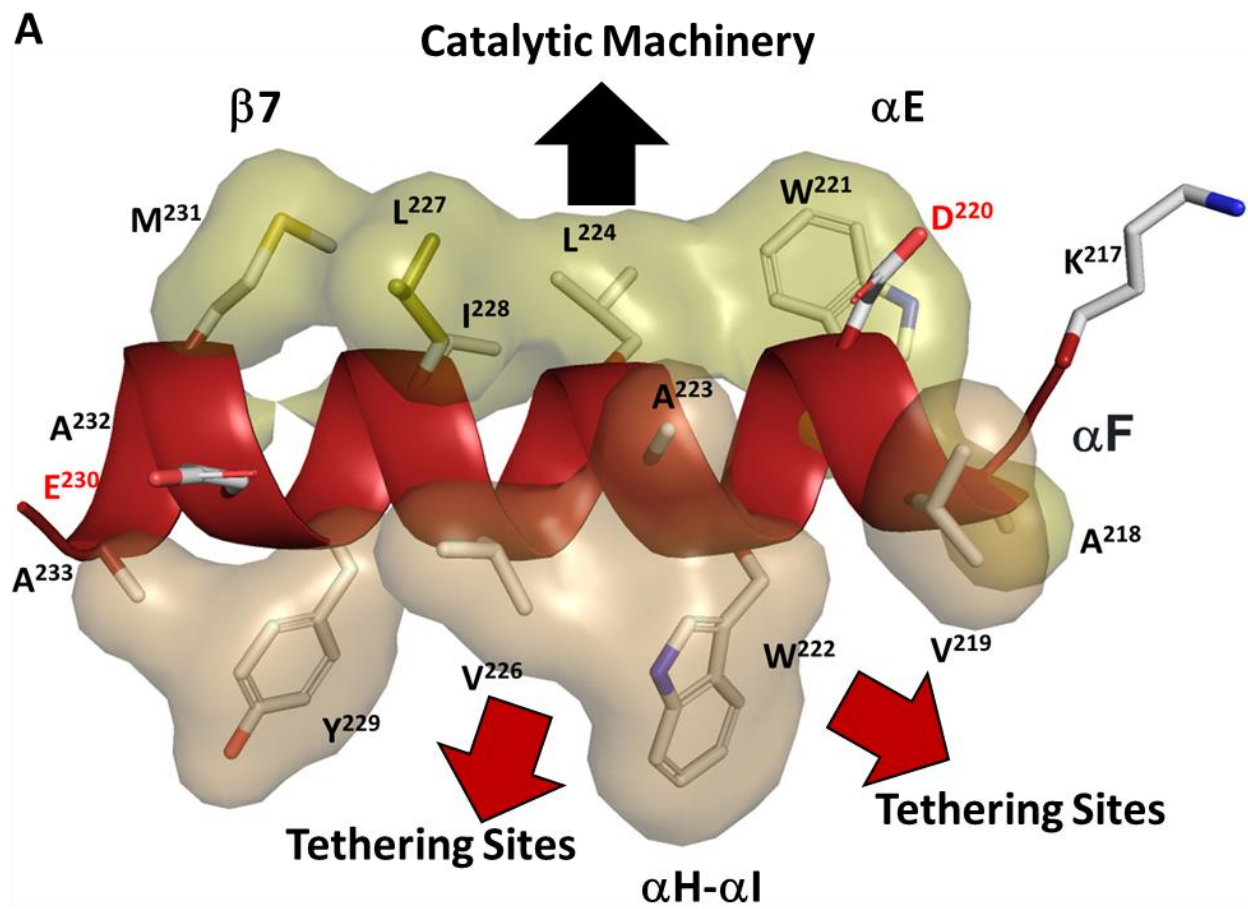
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REFERENCES.

1. W. C. Barker, M. O. Dayhoff, Viral src gene products are related to the catalytic chain of mammalian cAMP-dependent protein kinase. *Proc Natl Acad Sci U S A* **79**, 2836-2839 (1982).

2. S. K. Hanks, A. M. Quinn, T. Hunter, The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52 (1988).

3. D. R. Knighton, J. H. Zheng, L. F. Ten Eyck, V. A. Ashford, N. H. Xuong et al., Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**, 407-414 (1991).

4. D. R. Knighton, J. H. Zheng, L. F. Ten Eyck, N. H. Xuong, S. S. Taylor et al., Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**, 414-420 (1991).

5. J. Zheng, D. R. Knighton, L. F. ten Eyck, R. Karlsson, N. Xuong et al., Crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MgATP and peptide inhibitor. *Biochemistry* **32**, 2154-2161 (1993).

6. D. Bossemeyer, R. A. Engh, V. Kinzel, H. Ponstingl, R. Huber, Phosphotransferase and substrate binding mechanism of the cAMP-dependent protein kinase catalytic subunit from porcine heart as deduced from the 2.0 A structure of the complex with Mn2+ adenylyl imidodiphosphate and inhibitor peptide PKI(5-24). *EMBO J* **12**, 849-859 (1993).

7. D. A. Johnson, P. Akamine, E. Radzio-Andzelm, M. Madhusudan, S. S. Taylor, Dynamics of cAMP-dependent protein kinase. *Chem Rev* **101**, 2243-2270 (2001).

8. A. P. Kornev, N. M. Haste, S. S. Taylor, L. F. Ten Eyck, Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. *P Natl Acad Sci USA* **103**, 17783-17788 (2006).

9. A. P. Kornev, S. S. Taylor, L. F. Ten Eyck, A helix scaffold for the assembly of active protein kinases. *Proc Natl Acad Sci U S A* **105**, 14377-14382 (2008).

10. J. Kim, L. G. Ahuja, F. A. Chao, Y. Xia, C. L. McClendon et al., A dynamic hydrophobic core orchestrates allostery in protein kinases. *Sci Adv* **3**, e1600663 (2017).

11. G. D. Rose, From propensities to patterns to principles in protein folding. *Proteins* 10.1002/prot.26540 (2023).

12. G. D. Rose, W. B. Young, L. M. Gierasch, Interior turns in globular proteins. *Nature* **304**, 654-657 (1983).

13. A. C. Newton, Protein kinase C: perfectly balanced. *Crit Rev Biochem Mol Biol* **53**, 208-230 (2018).

14. S. S. Taylor, K. Soberg, E. Kobori, J. Wu, S. Pautz et al., The Tails of Protein Kinase A. *Mol Pharmacol* **101**, 219-225 (2022).

15. J. H. Weng, C. R. Trilling, P. Kaila Sharma, E. Stormer, J. Wu et al., Novel LRR-ROC Motif That Links the N- and C-terminal Domains in LRRK2 Undergoes an Order-Disorder Transition Upon Activation. *J Mol Biol* **435**, 167999 (2023).

16. N. Kannan, N. Haste, S. S. Taylor, A. F. Neuwald, The hallmark of AGC kinase functional divergence is its C-terminal tail, a cis-acting regulatory module. *Proc Natl Acad Sci U S A* **104**, 1272-1277 (2007).

17. S. S. Taylor, R. Ilouz, P. Zhang, A. P. Kornev, Assembly of allosteric macromolecular switches: lessons from PKA. *Nat Rev Mol Cell Biol* **13**, 646-658 (2012).

18. A. P. Kornev, S. S. Taylor, Dynamics-Driven Allostery in Protein Kinases. *Trends Biochem Sci* **40**, 628-647 (2015).

19. M. J. Zoller, S. S. Taylor, Affinity labeling of the nucleotide binding site of the catalytic subunit of cAMP-dependent protein kinase using p-fluorosulfonyl-[14C]benzoyl 5'-

818 adenosine. Identification of a modified lysine residue. *J Biol Chem* **254**, 8363-8368
819 (1979).
820 20. J. A. Buechler, S. S. Taylor, Dicyclohexylcarbodiimide cross-links two conserved
821   residues, Asp-184 and Lys-72, at the active site of the catalytic subunit of cAMP-
822   dependent protein kinase. *Biochemistry* **28**, 2065-2070 (1989).
823 21. B. Nolen, S. Taylor, G. Ghosh, Regulation of protein kinases; controlling activity through
824   activation segment conformation. *Mol Cell* **15**, 661-675 (2004).
825 22. L. N. Johnson, M. E. Noble, D. J. Owen, Active and inactive protein kinases: structural
826   basis for regulation. *Cell* **85**, 149-158 (1996).
827 23. S. J. Deminoff, S. C. Howard, A. Hester, S. Warner, P. K. Herman, Using substrate-
828   binding variants of the cAMP-dependent protein kinase to identify novel targets and a
829   kinase domain important for substrate interactions in Saccharomyces cerevisiae.
830   *Genetics* **173**, 1909-1917 (2006).
831 24. S. J. Deminoff, V. Ramachandran, P. K. Herman, Distal recognition sites in substrates
832   are required for efficient phosphorylation by the cAMP-dependent protein kinase.
833   *Genetics* **182**, 529-539 (2009).
834 25. H. S. Meharena, P. Chang, M. M. Keshwani, K. Oruganty, A. K. Nene et al., Deciphering
835   the structural basis of eukaryotic protein kinase regulation. *PLoS Biol* **11**, e1001680
836   (2013).
837 26. J. Hu, E. C. Stites, H. Yu, E. A. Germino, H. S. Meharena et al., Allosteric activation of
838   functionally asymmetric RAF kinase dimers. *Cell* **154**, 1036-1046 (2013).
839 27. A. S. Shaw, A. P. Kornev, J. Hu, L. G. Ahuja, S. S. Taylor, Kinases and pseudokinases:
840   lessons from RAF. *Mol Cell Biol* **34**, 1538-1546 (2014).
841 28. D. I. McSkimming, S. Dastgheib, E. Talevich, A. Narayanan, S. Katiyar et al., ProKinO: a
842   unified resource for mining the cancer kinome. *Hum Mutat* **36**, 175-186 (2015).
843 29. Z. Ruan, N. Kannan, Altered conformational landscape and dimerization dependency
844   underpins the activation of EGFR by alphaC-beta4 loop insertion mutations. *Proc Natl*
845   *Acad Sci U S A* **115**, E8162-E8171 (2018).
846 30. I. Tsigelny, J. P. Greenberg, S. Cox, W. L. Nichols, S. S. Taylor et al., 600 ps molecular
847   dynamics reveals stable substructures and flexible hinge points in cAMP dependent
848   protein kinase. *Biopolymers* **50**, 513-524 (1999).
849 31. A. Myasnikov, H. W. Zhu, P. Hixson, B. E. Xie, K. W. Yu et al., Structural analysis of the
850   full-length human LRRK2. *Cell* **184**, 3519-+ (2021).
851 32. H. Zhu, F. Tonelli, D. R. Alessi, J. Sun, Structural basis of human LRRK2 membrane
852   recruitment and activation. *bioRxiv* **2022.04.26.489605** (2022).
853 33. O. Gerlits, K. L. Weiss, M. P. Blakeley, G. Veglia, S. S. Taylor et al., Zooming in on
854   protons: Neutron structure of protein kinase A trapped in a product complex. *Sci Adv* **5**,
855   eaav0482 (2019).
856 34. D. C. Wych, P. C. Aoto, L. Vu, A. M. Wolff, D. L. Mobley et al., Molecular-dynamics
857   simulation methods for macromolecular crystallography. *Acta Crystallogr D Struct Biol*
858   **79**, 50-65 (2023).
859 35. D. A. Walsh, C. D. Ashby, C. Gonzalez, D. Calkins, E. H. Fischer et al., Purification and
860   characterization of a protein inhibitor of adenosine 3',5'-monophosphate-dependent
861   protein kinases. *J Biol Chem* **246**, 1977-1985 (1971).
862 36. C. Olivieri, Y. Wang, G. C. Li, S. M. V, J. Kim et al., Multi-state recognition pathway of
863   the intrinsically disordered protein kinase inhibitor by protein kinase A. *Elife* **9**, e55607
864   (2020).
865 37. J. D. Scott, E. H. Fischer, K. Takio, J. G. Demaille, E. G. Krebs, Amino-Acid Sequence
866   of the Heat-Stable Inhibitor of the Camp-Dependent Protein-Kinase from Rabbit
867   Skeletal-Muscle. *P Natl Acad Sci USA* **82**, 5732-5736 (1985).

868   38.   J. D. Scott, E. H. Fischer, J. G. Demaille, E. G. Krebs, Identification of an Inhibitory
869   Region of the Heat-Stable Protein Inhibitor of the Camp-Dependent Protein-Kinase. *P*
870   *Natl Acad Sci USA* **82**, 4379-4383 (1985).
871   39.   H. C. Cheng, S. M. van Patten, A. J. Smith, D. A. Walsh, An active twenty-amino-acid-
872   residue peptide derived from the inhibitor protein of the cyclic AMP-dependent protein
873   kinase. *Biochem J* **231**, 655-661 (1985).
874   40.   S. Whitehouse, D. A. Walsh, Mg X ATP2-dependent interaction of the inhibitor protein of
875   the cAMP-dependent protein kinase with the catalytic subunit. *J Biol Chem* **258**, 3682-
876   3692 (1983).
877   41.   D. B. Glass, H. C. Cheng, L. Mende-Mueller, J. Reed, D. A. Walsh, Primary structural
878   determinants essential for potent inhibition of cAMP-dependent protein kinase by
879   inhibitory peptides corresponding to the active portion of the heat-stable inhibitor protein.
880   *J Biol Chem* **264**, 8802-8810 (1989).
881   42.   D. B. Glass, L. J. Lundquist, B. M. Katz, D. A. Walsh, Protein kinase inhibitor-(6-22)-
882   amide peptide analogs with standard and nonstandard amino acid substitutions for
883   phenylalanine 10. Inhibition of cAMP-dependent protein kinase. *J Biol Chem* **264**,
884   14579-14584 (1989).
885   43.   J. Reed, J. S. De Ropp, J. Trewhella, D. B. Glass, W. K. Liddle et al., Conformational
886   analysis of PKI(5-22)amide, the active inhibitory fragment of the inhibitor protein of the
887   cyclic AMP-dependent protein kinase. *Biochem J* **264**, 371-380 (1989).
888   44.   F. W. Herberg, S. S. Taylor, Physiological inhibitors of the catalytic subunit of cAMP-
889   dependent protein kinase: effect of MgATP on protein-protein interactions. *Biochemistry*
890   **32**, 14015-14022 (1993).
891   45.   F. W. Herberg, M. L. Doyle, S. Cox, S. S. Taylor, Dissection of the nucleotide and metal-
892   phosphate binding sites in cAMP-dependent protein kinase. *Biochemistry* **38**, 6352-6360
893   (1999).
894   46.   C. Kim, N. H. Xuong, S. S. Taylor, Crystal structure of a complex between the catalytic
895   and regulatory (RIalpha) subunits of PKA. *Science* **307**, 690-696 (2005).
896   47.   C. Kim, C. Y. Cheng, S. A. Saldanha, S. S. Taylor, PKA-I holoenzyme structure reveals
897   a mechanism for cAMP-dependent activation. *Cell* **130**, 1032-1043 (2007).
898   48.   L. R. Masterson, L. Shi, E. Metcalfe, J. Gao, S. S. Taylor et al., Dynamically committed,
899   uncommitted, and quenched states encoded in protein kinase A revealed by NMR
900   spectroscopy. *Proc Natl Acad Sci U S A* **108**, 6969-6974 (2011).
901   49.   C. L. McClendon, A. P. Kornev, M. K. Gilson, S. S. Taylor, Dynamic architecture of a
902   protein kinase. *Proc Natl Acad Sci U S A* **111**, E4623-4631 (2014).
903   50.   L. G. Ahuja, P. C. Aoto, A. P. Kornev, G. Veglia, S. S. Taylor, Dynamic allostery-based
904   molecular workings of kinase:peptide complexes. *Proc Natl Acad Sci U S A* **116**, 15052-
905   15061 (2019).
906   51.   M. H. Omar, D. P. Byrne, K. N. Jones, T. M. Lakey, K. B. Collins et al., Mislocalization of
907   protein kinase A drives pathology in Cushing's syndrome. *Cell Rep* **40**, 111073 (2022).
908   52.   M. H. Omar, M. Kihiu, D. P. Byrne, K. S. Lee, T. M. Lakey et al., Classification of
909   Cushing's syndrome PKAc mutants based upon their ability to bind PKI. *Biochem J* **480**,
910   875-890 (2023).
911   53.   C. Walker, Y. Wang, C. Olivieri, S. M. V, J. Gao et al., Is Disrupted Nucleotide-Substrate
912   Cooperativity a Common Trait for Cushing's Syndrome Driving Mutations of Protein
913   Kinase A? *J Mol Biol* **433**, 167123 (2021).
914   54.   C. Olivieri, G. C. Li, Y. Wang, S. M. V, C. Walker et al., ATP-competitive inhibitors
915   modulate the substrate binding cooperativity of a kinase by altering its conformational
916   entropy. *Sci Adv* **8**, eabo0696 (2022).

55. C. Walker, Y. Wang, C. Olivieri, A. Karamafrooz, J. Casby et al., Cushing's syndrome driver mutation disrupts protein kinase A allosteric network, altering both regulation and substrate specificity. *Sci Adv* **5**, eaaw9298 (2019).

56. J. Wu, J. Yang, N. Kannan, Madhusudan, N. H. Xuong et al., Crystal structure of the E230Q mutant of cAMP-dependent protein kinase reveals an unexpected apoenzyme conformation and an extended N-terminal A helix. *Protein Sci* **14**, 2871-2879 (2005).

57. L. G. Ahuja, A. P. Kornev, C. L. McClendon, G. Veglia, S. S. Taylor, Mutation of a kinase allosteric node uncouples dynamics linked to phosphotransfer. *Proc Natl Acad Sci U S A* **114**, E931-E940 (2017).

58. L. G. Ahuja, S. S. Taylor, A. P. Kornev, Tuning the "violin" of protein kinases: The role of dynamics-based allostery. *IUBMB Life* **71**, 685-696 (2019).

59. L. K. Madan, C. L. Welsh, A. P. Kornev, S. S. Taylor, The "violin model": Looking at community networks for dynamic allostery. *J Chem Phys* **158**, 081001 (2023).

60. E. J. Kennedy, J. Yang, L. Pillus, S. S. Taylor, G. Ghosh, Identifying critical non-catalytic residues that modulate protein kinase A activity. *PLoS One* **4**, e4746 (2009).

61. R. M. Gibson, S. S. Taylor, Dissecting the cooperative reassociation of the regulatory and catalytic subunits of cAMP-dependent protein kinase. Role of Trp-196 in the catalytic subunit. *J Biol Chem* **272**, 31998-32005 (1997).

62. C. Olivieri, Y. Wang, C. Walker, M. V. Subrahmanian, K. N. Ha et al., The aC-b4 loop controls the allosteric cooperativity between nucleotide and substrate in the catalytic subunit of protein kinase A. *eLife*, submitted (2023).

63. T. Pawson, J. D. Scott, Signaling through scaffold, anchoring, and adaptor proteins. *Science* **278**, 2075-2080 (1997).

64. G. A. Huber, J. A. McCammon, Brownian Dynamics Simulations of Biological Molecules. *Trends Chem* **1**, 727-738 (2019).

65. S. H. Northrup, S. A. Allison, J. A. Mccammon, Brownian Dynamics Simulation of Diffusion-Influenced Bimolecular Reactions. *Journal of Chemical Physics* **80**, 1517-1526 (1984).

66. P. I. Poulikakos, C. Zhang, G. Bollag, K. M. Shokat, N. Rosen, RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature* **464**, 427-430 (2010).

67. A. L. Garske, U. Peters, A. T. Cortesi, J. L. Perez, K. M. Shokat, Chemical genetic strategy for targeting protein kinases based on covalent complementarity. *Proc Natl Acad Sci U S A* **108**, 15046-15052 (2011).

68. A. P. Kornev, P. C. Aoto, S. S. Taylor, Calculation of centralities in protein kinase A. *Proc Natl Acad Sci U S A* **119**, e2215420119 (2022).

69. A. Cooper, D. T. Dryden, Allostery without conformational change. A plausible model. *Eur Biophys J* **11**, 103-109 (1984).

70. N. Popovych, S. Sun, R. H. Ebright, C. G. Kalodimos, Dynamically driven protein allostery. *Nat Struct Mol Biol* **13**, 831-838 (2006).

71. C. M. Petit, J. Zhang, P. J. Sapienza, E. J. Fuentes, A. L. Lee, Hidden dynamic allostery in a PDZ domain. *Proc Natl Acad Sci U S A* **106**, 18249-18254 (2009).

72. L. Di Paola, A. Giuliani, Protein contact network topology: a natural language for allostery. *Curr Opin Struct Biol* **31**, 43-48 (2015).

73. M. Jacomy, T. Venturini, S. Heymann, M. Bastian, ForceAtlas2, a continuous graph layout algorithm for handy network visualization designed for the Gephi software. *PLoS One* **9**, e98679 (2014).

74. D. A. Case, T. E. Cheatham, 3rd, T. Darden, H. Gohlke, R. Luo et al., The Amber biomolecular simulation programs. *J Comput Chem* **26**, 1668-1688 (2005).

75. P. Akamine, Madhusudan, J. Wu, N. H. Xuong, L. F. Ten Eyck et al., Dynamic features of cAMP-dependent protein kinase revealed by apoenzyme crystal structure. *J Mol Biol* **327**, 159-171 (2003).

76. J. A. Maier, C. Martinez, K. Kasavajhala, L. Wickstrom, K. E. Hauser et al., ff14SB: Improving the Accuracy of Protein Side Chain and Backbone Parameters from ff99SB. *J Chem Theory Comput* **11**, 3696-3713 (2015).

77. M. H. M. Olsson, C. R. Søndergaard, M. Rostkowski, J. H. Jensen. PROPKA3: Consistent Treatment of Internal and Surface Residues in Empirical p K_a Predictions. *Journal of Chemical Theory and Computation*, **7**, 525–537 (2011).

78. C. R. Søndergaard, M. H. M. Olsson, M. Rostkowski, J. H. Jensen, Improved Treatment of Ligands and Coupling Effects in Empirical Calculation and Rationalization of p K_a Values. *Journal of Chemical Theory and Computation*, **7**, 2284–2295 (2011).

79. K. L. Meagher, L. T. Redman, H. A. Carlson. Development of polyphosphate parameters for use with the AMBER force field. *Journal of Computational Chemistry*, **24**, 1016–1025 (2003).

80. O. Allnér, L. Nilsson, A. Villa. Magnesium Ion–Water Coordination and Exchange in Biomolecular Simulations. *Journal of Chemical Theory and Computation*, **8**, 1493–1502 (2012).

81. N. Homeyer, A. H. Horn, H. Lanig, H. Sticht, AMBER force-field parameters for phosphorylated amino acids in different protonation states: phosphoserine, phosphothreonine, phosphotyrosine, and phosphohistidine. *J Mol Model* **12**, 281-289 (2006).

82. D. J. Price, C. L. Brooks, 3rd, A modified TIP3P water potential for simulation with Ewald summation. *J Chem Phys* **121**, 10096-10103 (2004).

83. R. J. Loncharich, B. R. Brooks, R. W. Pastor, Langevin dynamics of peptides: the frictional dependence of isomerization rates of N-acetylalanyl-N'-methylamide. *Biopolymers* **32**, 523-535 (1992).

84. R. W. Pastor, B. R. Brooks, A. Szabo, An Analysis of the Accuracy of Langevin and Molecular-Dynamics Algorithms. *Mol Phys* **65**, 1409-1419 (1988).

85. H. J. C. Berendsen, J. P. M. Postma, W. F. Vangunsteren, A. Dinola, J. R. Haak, Molecular-Dynamics with Coupling to an External Bath. *Journal of Chemical Physics* **81**, 3684-3690 (1984).

86. T. Darden, D. York, L. Pedersen, Particle Mesh Ewald - an N.Log(N) Method for Ewald Sums in Large Systems. *Journal of Chemical Physics* **98**, 10089-10092 (1993).

87. M. A. Balsera, W. Wriggers, Y. Oono, K. Schulten, Principal component analysis and long time protein dynamics. *J Phys Chem-Us* **100**, 2567-2572 (1996).

88. I. Bahar, A. R. Atilgan, M. C. Demirel, B. Erman, Vibrational dynamics of folded proteins: Significance of slow and fast motions in relation to function and stability. *Physical Review Letters* **80**, 2733-2736 (1998).

89. G. G. Maisuradze, A. Liwo, H. A. Scheraga, Principal component analysis for protein folding dynamics. *J Mol Biol* **385**, 312-329 (2009).

90. H. Frauenfelder, S. G. Sligar, P. G. Wolynes, The Energy Landscapes and Motions of Proteins. *Science* **254**, 1598-1603 (1991).

91. G. G. Maisuradze, A. Liwo, H. A. Scheraga, Relation between Free Energy Landscapes of Proteins and Dynamics. *Journal of Chemical Theory and Computation* **6**, 583-595 (2010).

A

β7   Catalytic Machinery   αE

M²³¹   L²²⁷   L²²⁴   W²²¹   D²²⁰   K²¹⁷

I²²⁸   A²²³   αF

A²³²   E²³⁰   A²¹⁸

A²³³   Y²²⁹   V²²⁶   W²²²   V²¹⁹

Tethering Sites   Tethering Sites

αH-αI

B

αA   PKI   P-2 Arg

YRD

D220   E230   αG

αE   αF

αI   αH

C

```
          220              230
PKA    KAVDWWALGVLIYEMAA
BRAF   FQSDVYAFGIVLYELMT
LRRK2  QQADVYSFGLLLYDILT
PKCβ   KSVDWWAFGVLLYEMLA
PKCα   KSVDWWAYGVLLYEMLA
SRC    IKSDVWSFGILLTELTT
```
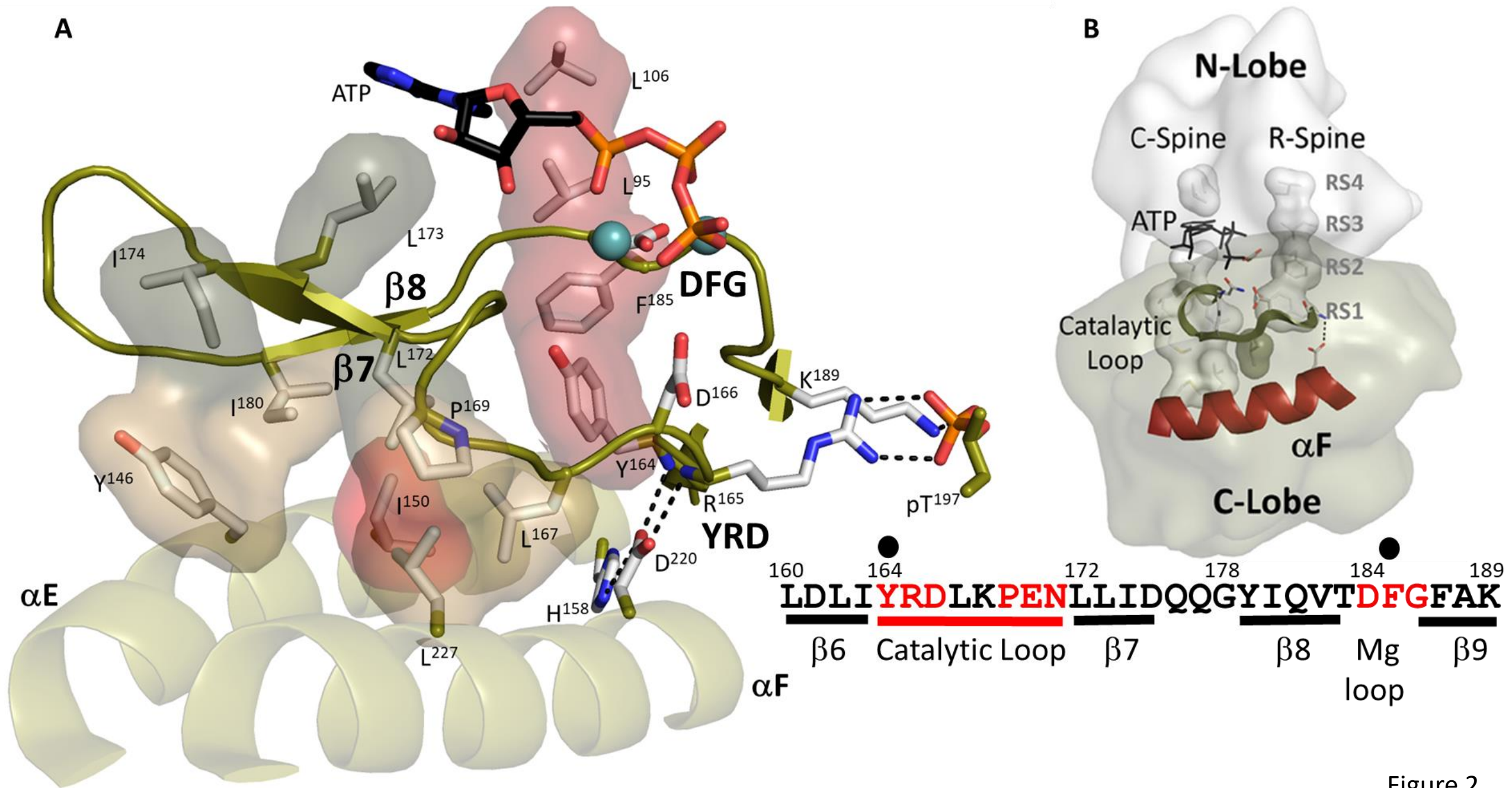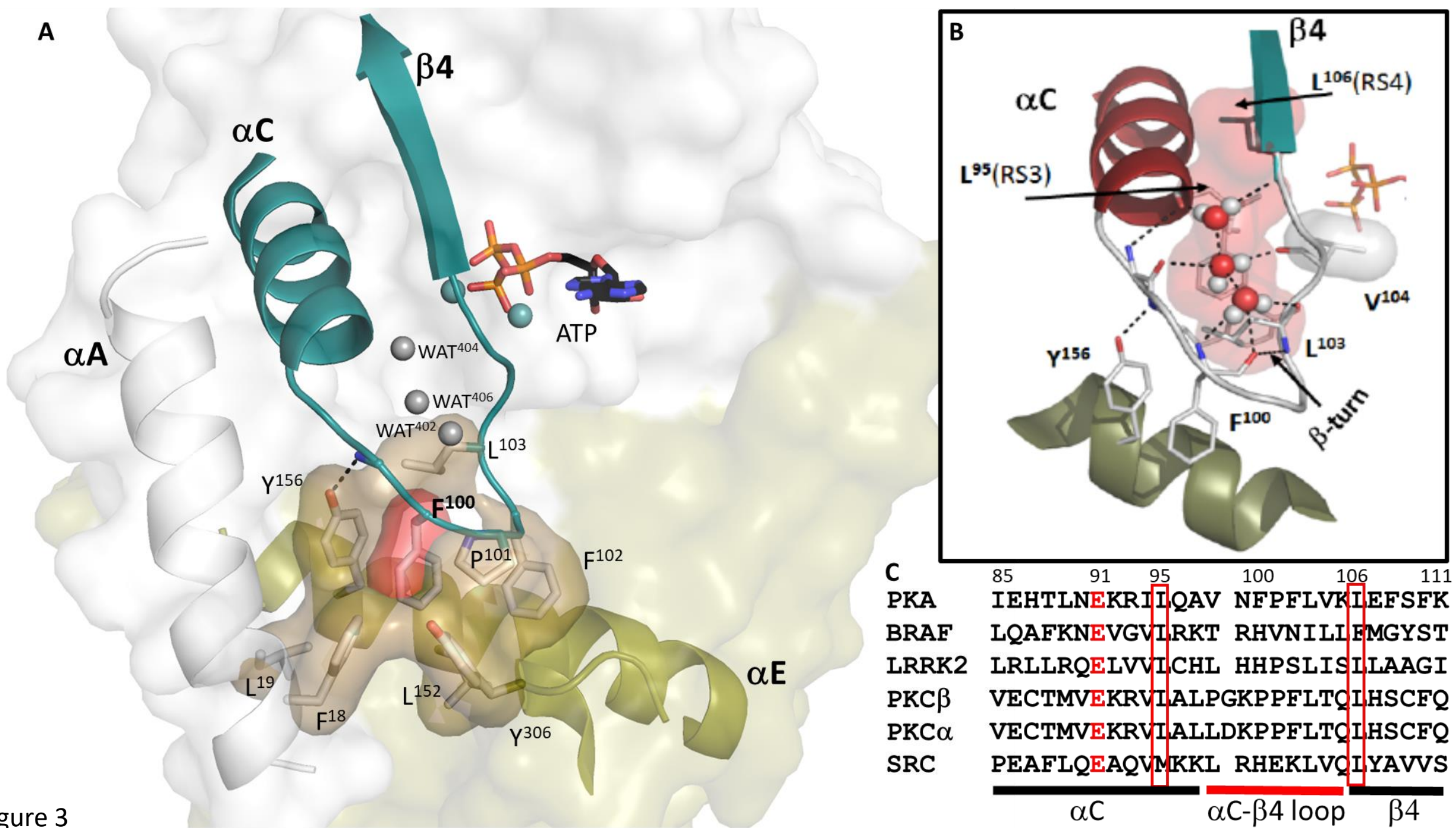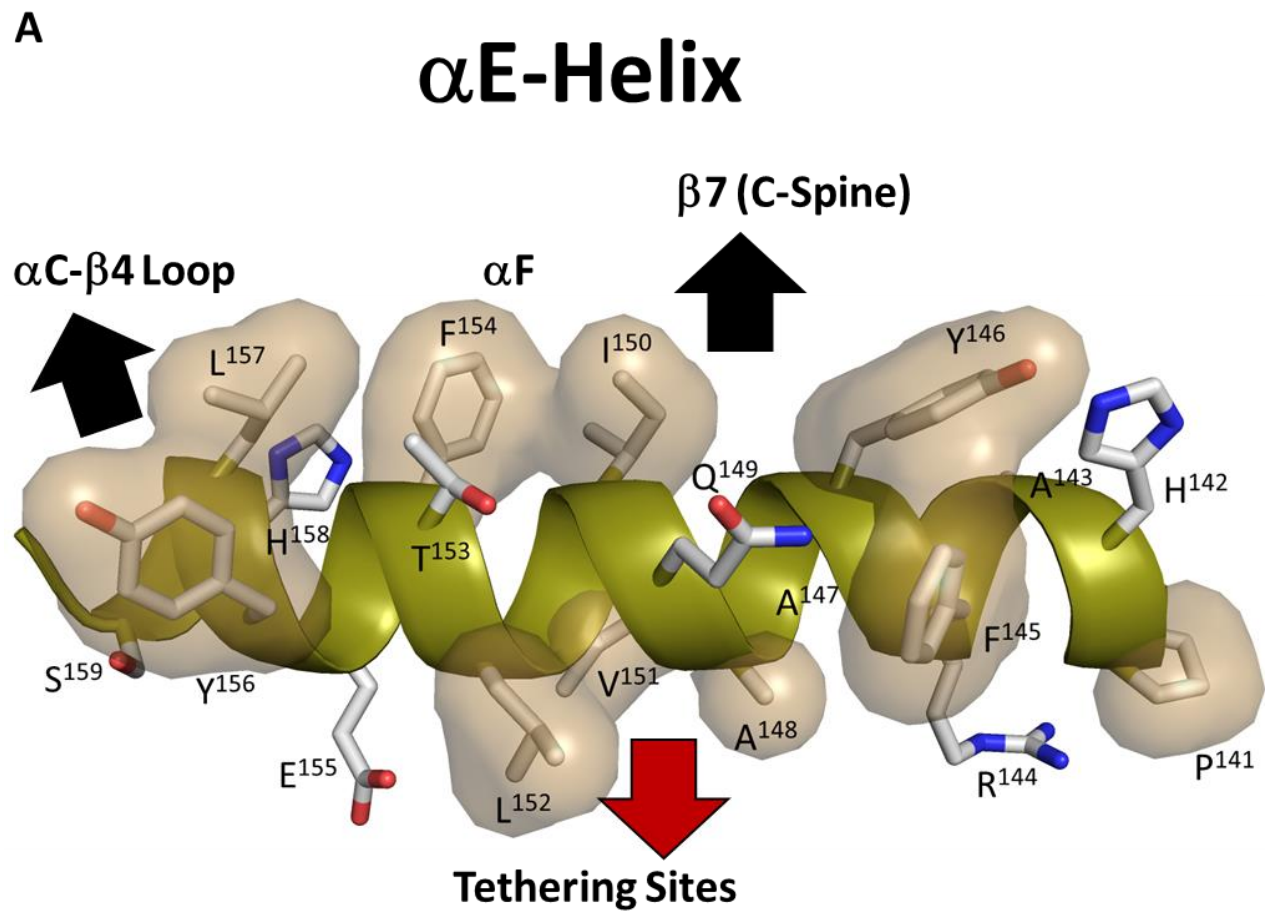
Figure 1

A

ATP
L¹⁰⁶
L⁹⁵
L¹⁷³
I¹⁷⁴
β8
DFG
F¹⁸⁵
β7
L¹⁷²
I¹⁸⁰
P¹⁶⁹
D¹⁶⁶
K¹⁸⁹
Y¹⁴⁶
I¹⁵⁰
L¹⁶⁷
Y¹⁶⁴
R¹⁶⁵
YRD
pT¹⁹⁷
H¹⁵⁸
D²²⁰
αE
L²²⁷
αF

B

N-Lobe
C-Spine   R-Spine
RS4
RS3
ATP   RS2
RS1
Catalytic
Loop
αF
C-Lobe

```
160        164              172        178        184   189
LDLI YRD LKPEN LLIDQQGY IQVT DFG FAK
     β6  Catalytic Loop  β7        β8      Mg    β9
                                          loop
```

Figure 2

Figure 3

A

αE-Helix

αC-β4 Loop

β7 (C-Spine)

αF

L¹⁵⁷   F¹⁵⁴   I¹⁵⁰   Y¹⁴⁶

H¹⁵⁸   T¹⁵³   Q¹⁴⁹   A¹⁴³   H¹⁴²

S¹⁵⁹   Y¹⁵⁶   V¹⁵¹   A¹⁴⁷   F¹⁴⁵

E¹⁵⁵   L¹⁵²   A¹⁴⁸   R¹⁴⁴   P¹⁴¹

Tethering Sites

B

PKA WT (1ATP)
PKA Apo (1J3H)
PKA E230Q (1SYK)
PKA R194A (4DFY)
Inactive SRC (2SRC)
Active SRC (1YI6)
Fl LRRK2 (7LHW)
BRAF (4MNF)

αC   β4   ATP   L¹⁰³   P¹⁰¹   F¹⁰²   Y¹⁵⁶   F¹⁰⁰   L¹⁹   L¹⁵²   F¹⁸   Y³⁰⁶   αE

Figure 4

C

```
        140       146           156     160
PKA     EPHARFYAAQIVLTFEYLHSL
BRAF    MIKLIDIARQTAQGMDYLHAK
LRRK2   RTLQHRIALHVADGLRYLHSA
PKCβ    EPHAVEYAAEIAIGLFFLQSK
PKCα    EPQAVEYAAEISIGLFFLHKR
SRC     LPQLVDMAAQIASGMAYVERM
```
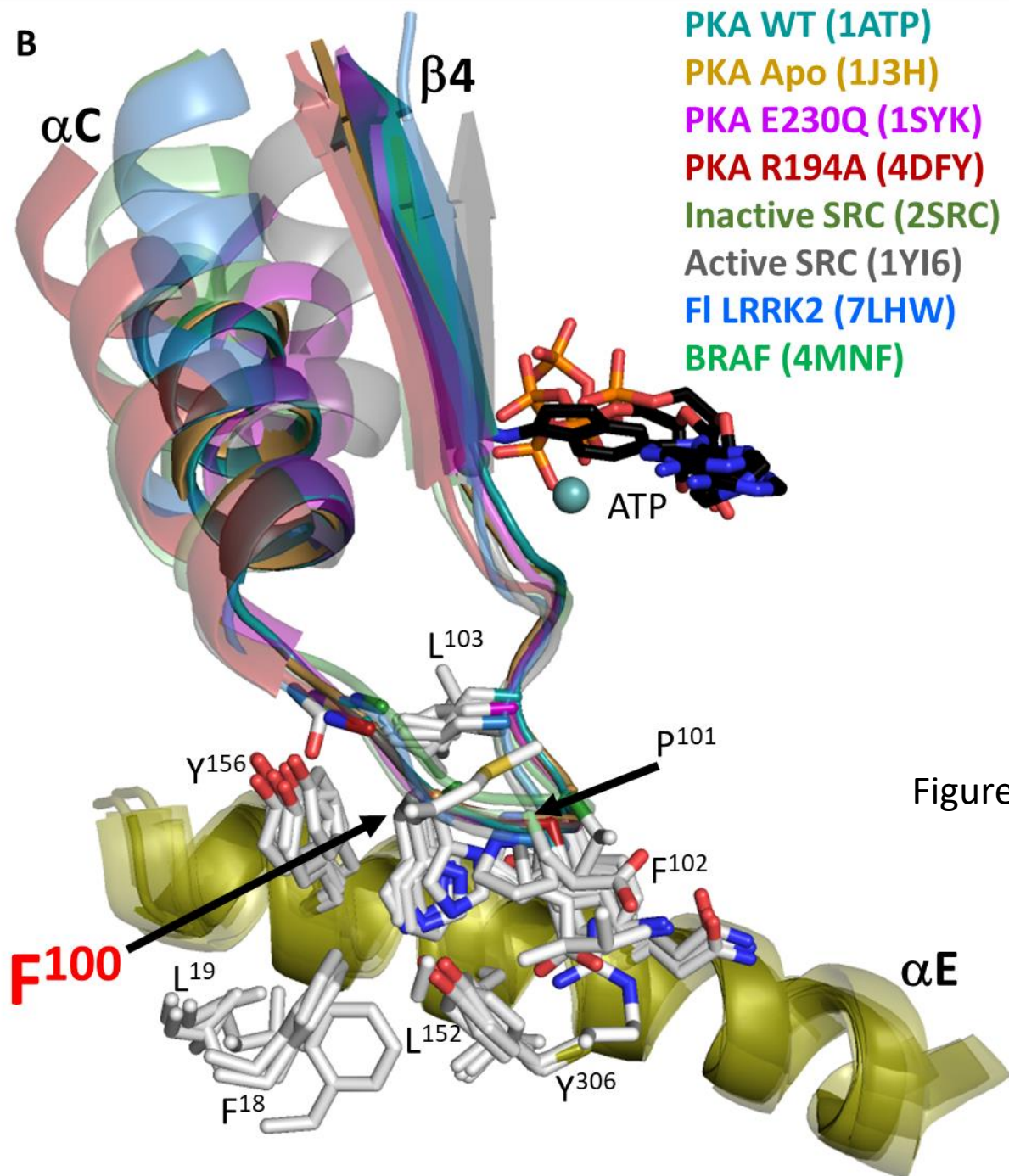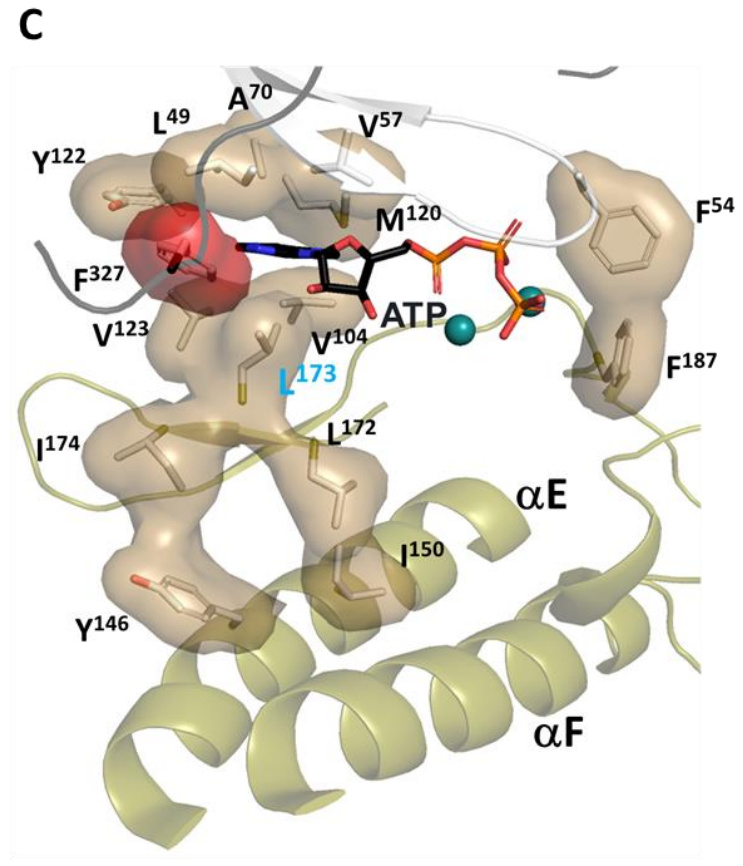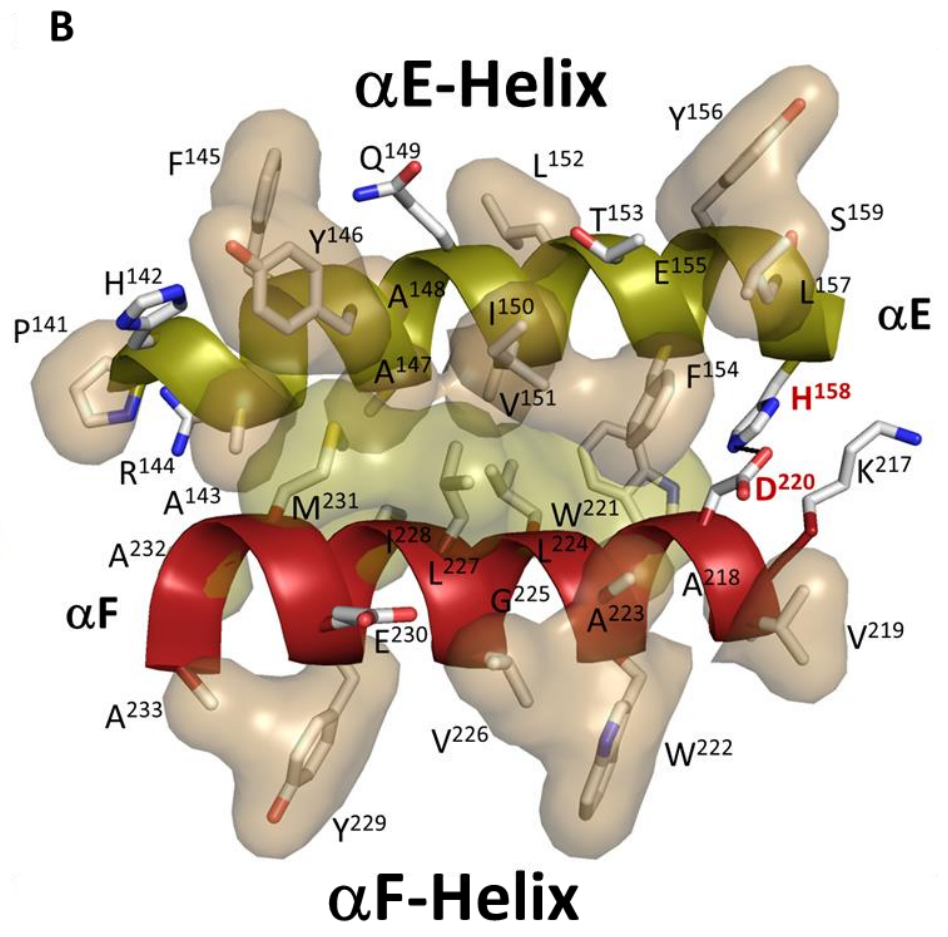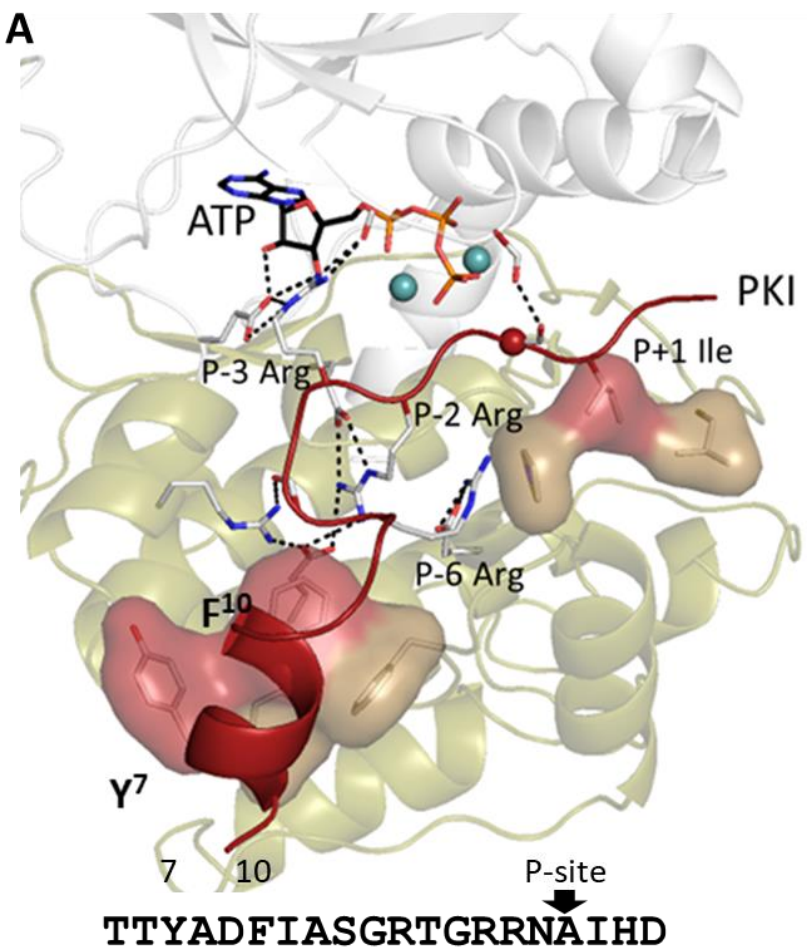
Figure 5
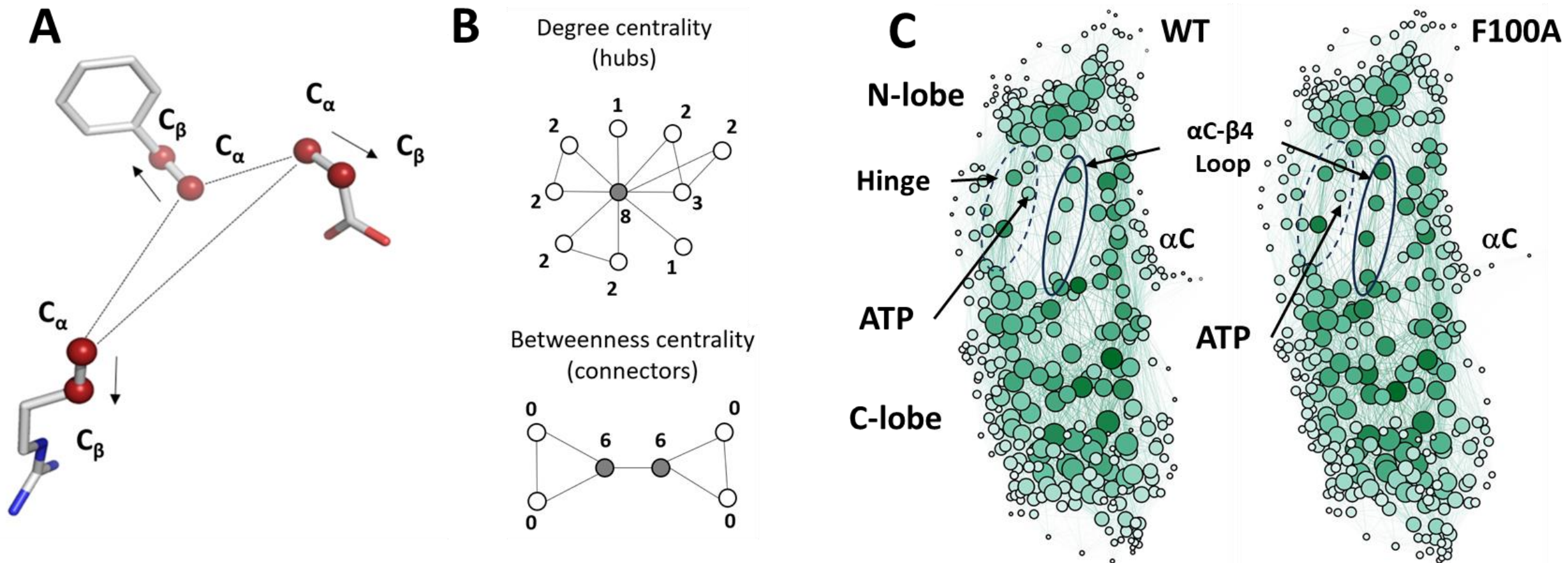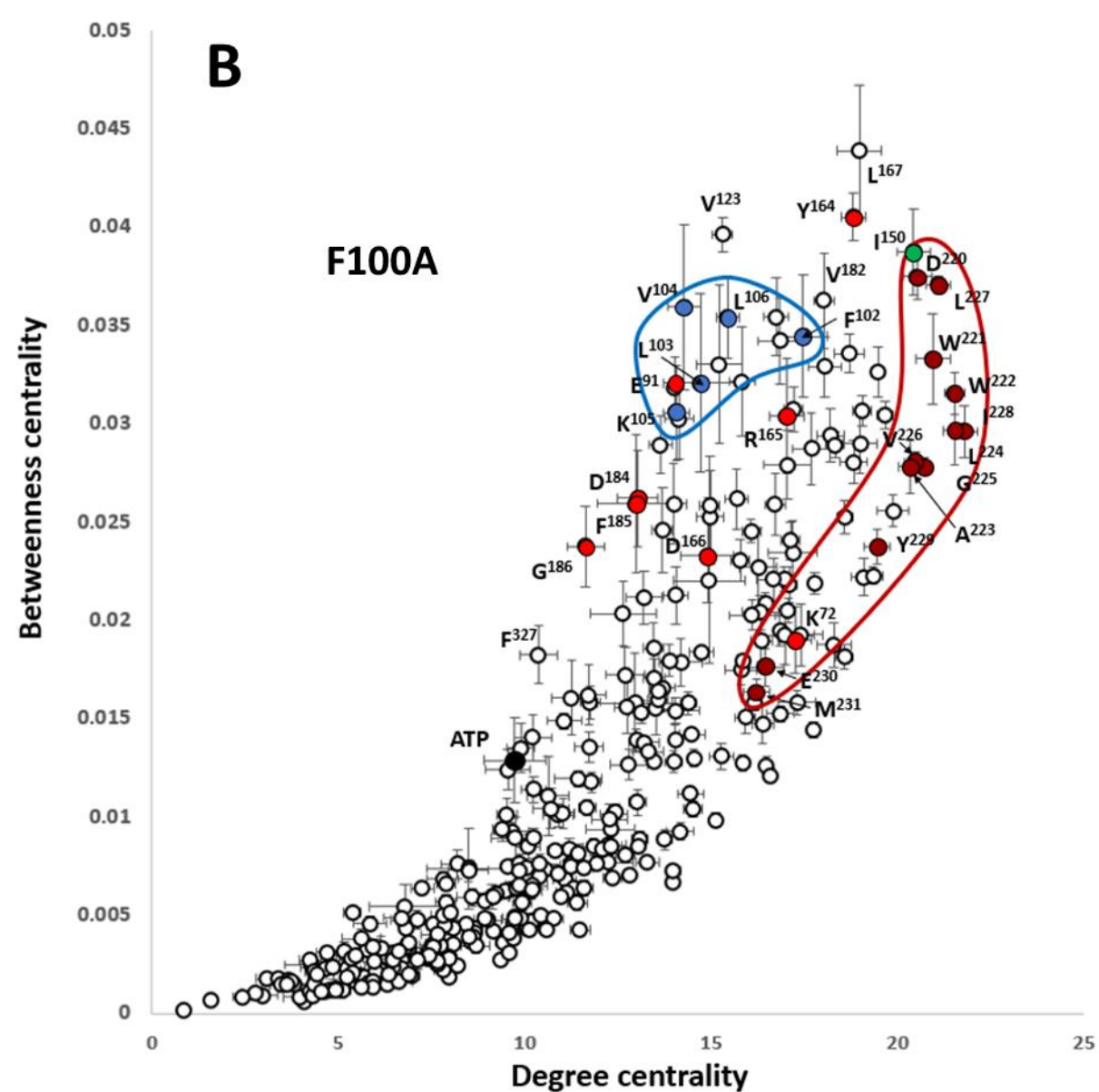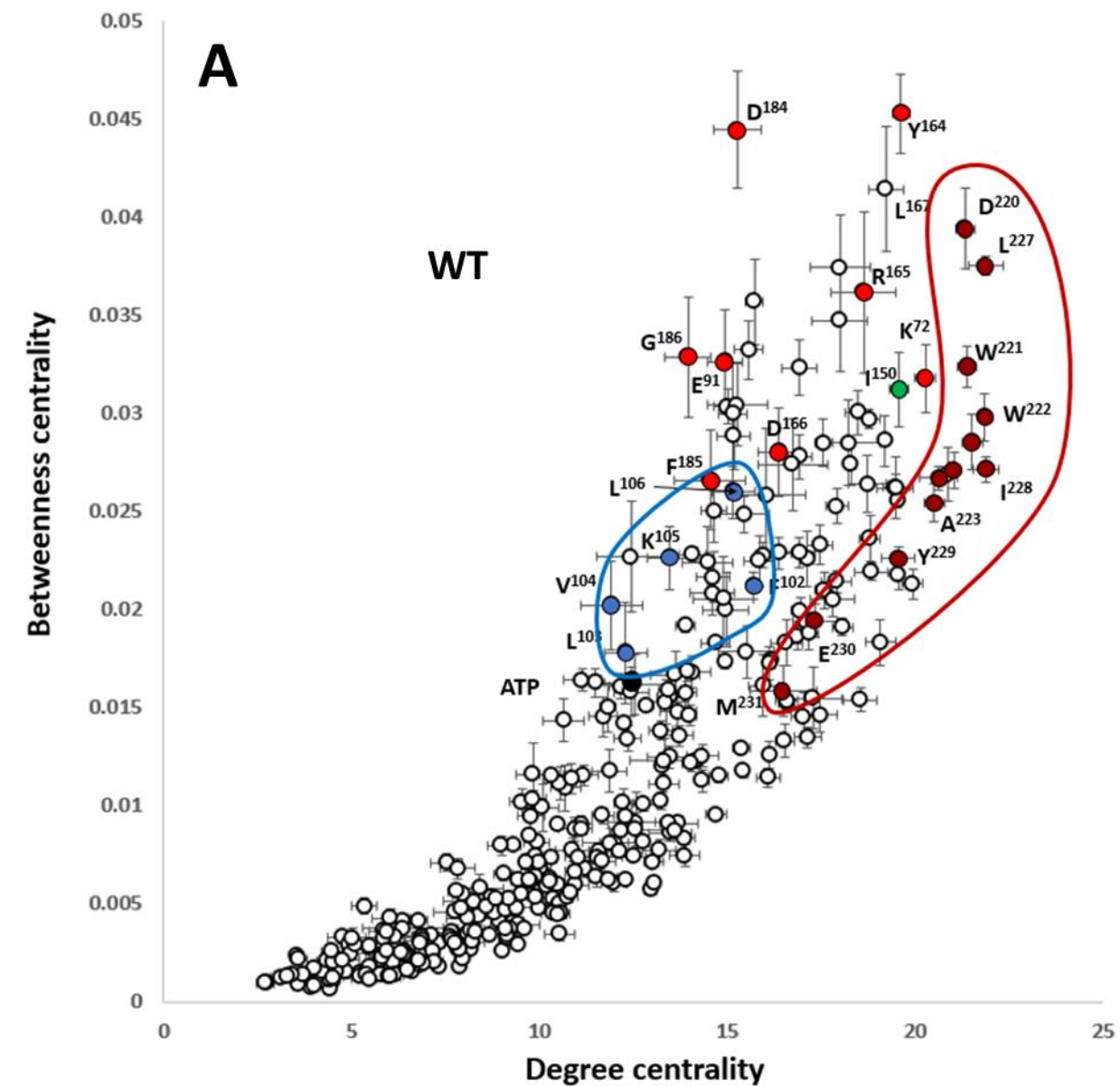
Figure 6

Figure 7
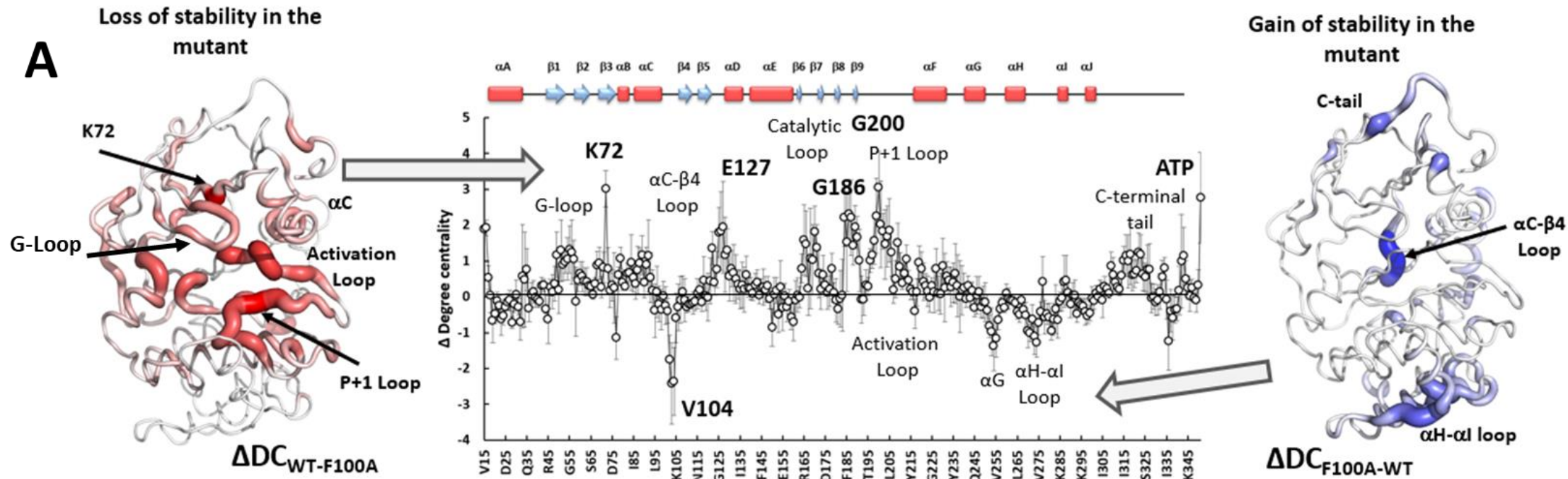
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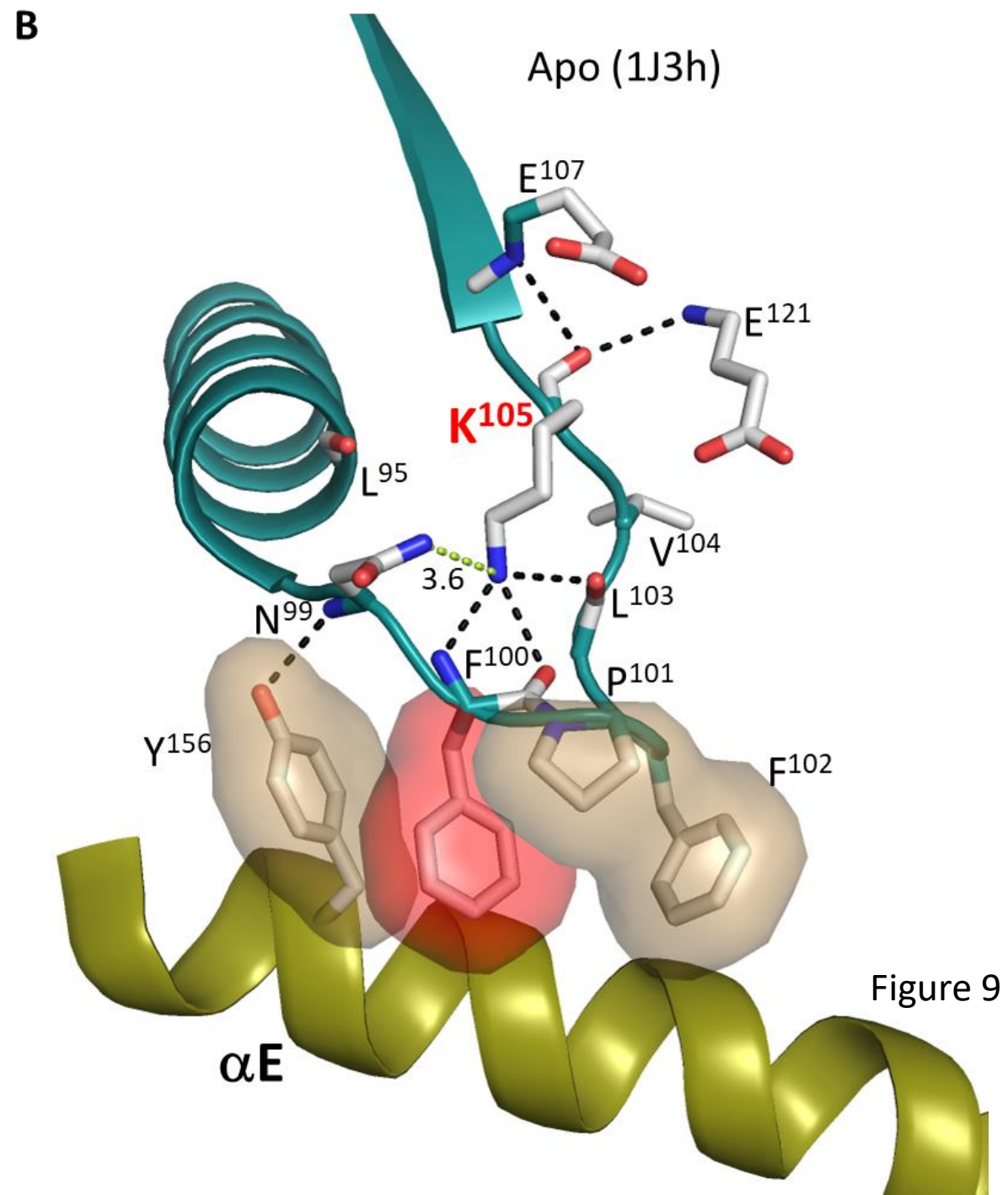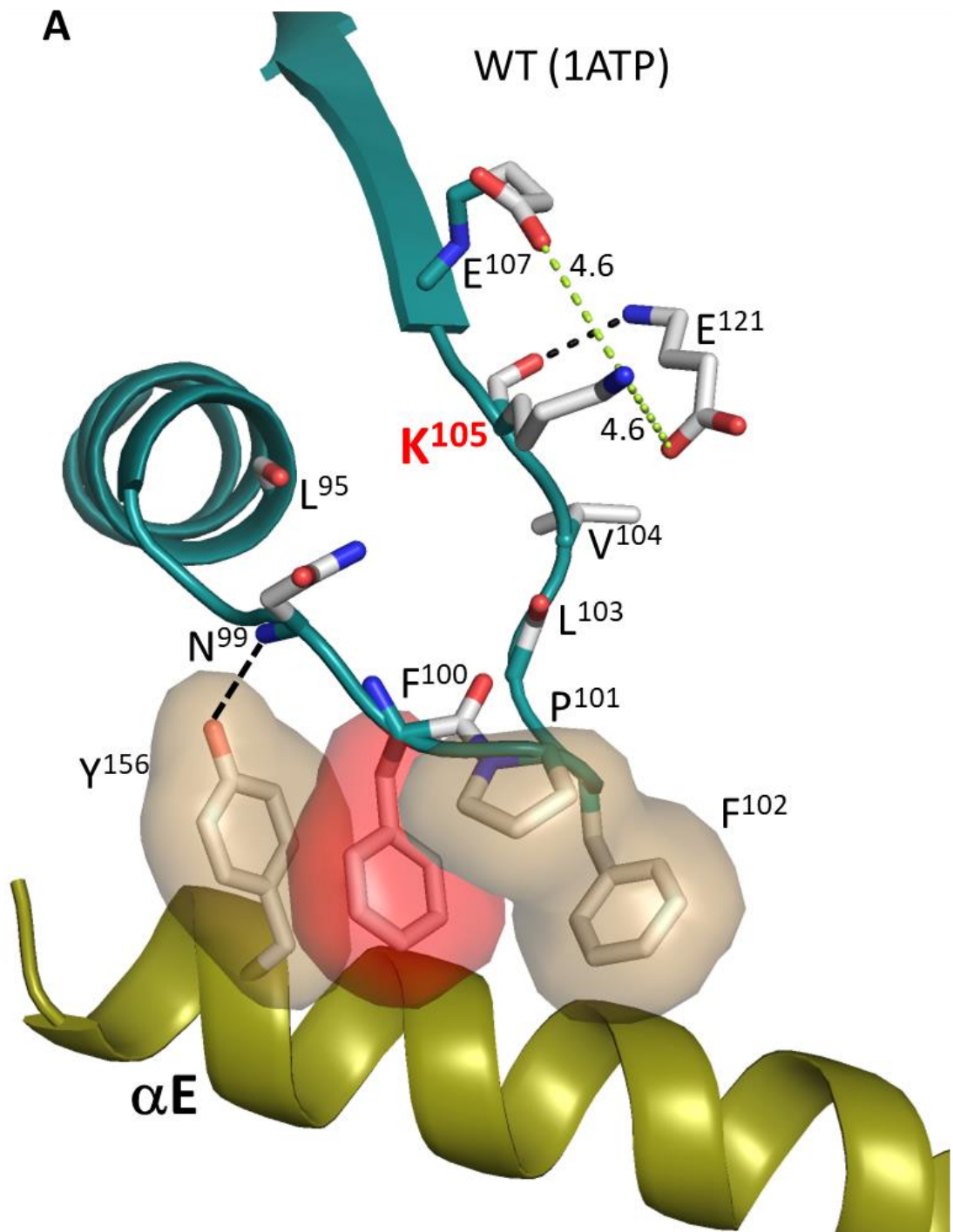
Figure 9

Figure 10

Figure 11

Figure 12