

1 **A dynamic mechanism for allosteric activation of Aurora kinase A by activation loop**  
2 **phosphorylation**

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23 **Abstract**

24 Many eukaryotic protein kinases are activated by phosphorylation on a specific conserved  
25 residue in the regulatory activation loop, a post-translational modification thought to stabilize the  
26 active DFG-In state of the catalytic domain. Here we use a battery of spectroscopic methods

27 that track different catalytic elements of the kinase domain to show that the ~100-fold activation  
28 of the mitotic kinase Aurora A (AurA) by phosphorylation occurs without a population shift to the  
29 DFG-In state, and that the activation loop of the activated kinase remains highly dynamic.  
30 Instead, molecular dynamics simulations and electron paramagnetic resonance experiments  
31 show that phosphorylation profoundly alters the structure and dynamics of the DFG-In  
32 subpopulation, leading to activation of the kinase. Kinetics experiments tracking structural  
33 transitions during nucleotide binding suggest that a substantial DFG-Out subpopulation is an  
34 important feature of activated AurA that evolved to optimize the kinetics of substrate binding and  
35 product release.

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38 **Introduction**

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40 Protein phosphorylation is a central feature of cellular signal transduction, and stringent  
41 regulatory control of the participating protein kinases is critical for the integrity of these  
42 pathways. Kinase activity is typically regulated by finely-tuned allosteric mechanisms that  
43 reversibly switch the kinase domain between active and inactive conformational states<sup>1</sup>.  
44 Disruption of these mechanisms, leading to constitutive kinase activity, is a major cause of  
45 cancer, and small molecules that inhibit specific disease-associated kinases constitute an  
46 important class of modern cancer drugs<sup>2</sup>.

47 Phosphorylation on a specific site in the activation loop of the kinase domain is the most  
48 widely conserved regulation mechanism in protein kinases<sup>3</sup>. X-ray structures suggest that ionic  
49 interactions between the phosphate moiety and a pocket of basic residues lock the activation  
50 loop into a conserved active conformation<sup>4-6</sup>. In this conformation, the catalytic asp-phe-gly  
51 (DFG) motif at the N-terminal end of the activation loop adopts an active “DFG-In” conformation,  
52 in which the aspartate residue of the DFG motif points into the active site to coordinate Mg-ATP.

53 In the absence of phosphorylation protein kinases often adopt autoinhibited states, in which  
54 activity is blocked by conformational rearrangements of the activation loop and DFG motif. An  
55 important mode of autoinhibition involves a flip of the aspartate residue of the DFG motif out of  
56 the active site, preventing magnesium coordination<sup>7-9</sup>. Many protein kinases have been  
57 observed to adopt these “DFG-Out” states, and some have been targeted with small-molecule  
58 inhibitors that preferentially bind to the DFG-Out conformation<sup>10</sup>. In addition to phosphorylation,  
59 kinase conformation is typically also modulated by the binding of accessory proteins that further  
60 tune the activity level of the enzyme.

61 The serine/threonine kinase Aurora A (AurA) is an essential mitotic protein that controls  
62 many cellular processes including mitotic spindle assembly, centrosome maturation, and mitotic  
63 entry<sup>11-15</sup>. These functions of AurA are driven by two distinct activation mechanisms of the  
64 kinase operating in different spatiotemporal contexts. At the centrosome, AurA is activated by  
65 autophosphorylation on the activation loop residue T288. In contrast, at the mitotic spindle, AurA  
66 is activated by the spindle-associated protein Tpx2<sup>16</sup>, and this pool of the kinase is kept in the  
67 unphosphorylated state by the continual action of the phosphatase PP6<sup>17,18</sup>.

68 Extensive *in vitro* studies have shown that Tpx2 and phosphorylation act independently  
69 to increase AurA kinase activity by up to several hundred-fold<sup>19,20</sup>. Binding of Tpx2 to  
70 unphosphorylated AurA triggers a population shift from a DFG-Out to the DFG-In state in  
71 solution<sup>21</sup>. Crystal structures of phosphorylated AurA bound to Tpx2 show that the T288  
72 phosphothreonine residue forms extensive ionic interactions unique to the DFG-In state,  
73 suggesting that both factors simply stabilize the same active conformation<sup>22-24</sup>. In this paper, we  
74 show that phosphorylation on T288 in fact activates AurA through a completely different  
75 mechanism than Tpx2. Using three complementary spectroscopic methods we show that  
76 phosphorylation does not trigger a switch to the DFG-In state, and that the phosphorylated  
77 activation loop of AurA continually samples both active and inactive conformational states.  
78 Instead, phosphorylation acts by enhancing the catalytic activity of the subpopulation of

79 molecules adopting the DFG-In state. Stopped flow kinetics experiments point to the DFG-Out  
80 state being important for nucleotide dissociation, and suggest that the transition between DFG-  
81 Out and DFG-In states may be a central feature of the catalytic cycle of the enzyme.

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84 **Results and Discussion**

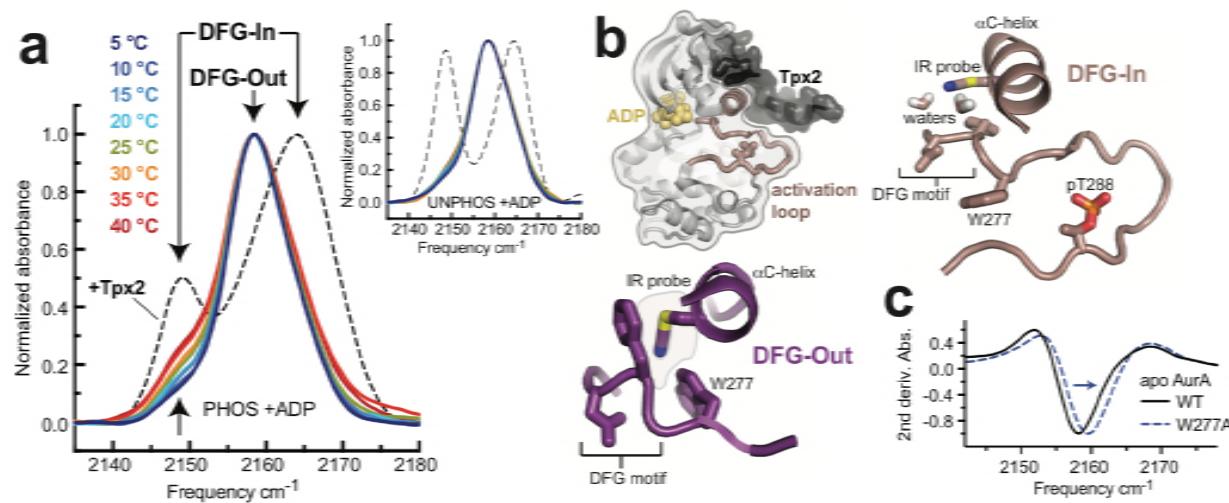
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86 **The DFG motif of phosphorylated AurA is predominantly in the DFG-Out state.**

87 We set out to explain how phosphorylation of AurA on T288 leads to a ~100-fold  
88 increase in catalytic activity (Supplementary Figure S1)<sup>19,20</sup>. We previously used an infrared (IR)  
89 probe that tracks the DFG motif of AurA to show that Tpx2 binding triggers a conformational  
90 change from the DFG-Out to the DFG-In state<sup>21</sup>. In this method, a cysteine residue is introduced  
91 at position Q185 at the back of the active site of AurA, and chemical labeling is used to  
92 introduce a nitrile infrared probe at this position<sup>25</sup>. To test whether phosphorylation of AurA also  
93 causes a conformational shift of the DFG motif, we prepared samples of AurA Q185C  
94 phosphorylated on T288. Homogeneous phosphorylation and nitrile labeling were verified by  
95 western blotting and mass spectrometry (Supplementary Figure S2 and S3).

96 IR spectra of labeled phosphorylated AurA bound to ADP showed predominantly a  
97 single absorbance band centered at 2158 cm<sup>-1</sup> (Figure 1a). We previously assigned this peak in  
98 spectra of unphosphorylated AurA to the DFG-Out form of the kinase, in which the nitrile probe  
99 is buried in a hydrophobic pocket (Figure 1b)<sup>21</sup>. Addition of saturating amounts of Tpx2 peptide  
100 (residues 1-43 of human Tpx2) to the IR samples caused a dramatic change in the spectra in  
101 which the central peak at 2158 cm<sup>-1</sup> largely disappears, and two new peaks appear at 2149 cm<sup>-1</sup>  
102 and 2164 cm<sup>-1</sup> (Figure 1a). These changes are indicative of a shift to the DFG-In state, in which  
103 water molecules coordinated to the DFG motif form hydrogen bonds to the probe, causing

104 pronounced spectral shifts (Figure 1b)<sup>21</sup>. To confirm that the peak at 2158 cm<sup>-1</sup> arises from the  
105 DFG-Out state, we mutated residue W277, which is positioned directly against the IR probe in  
106 the DFG-Out state, but is displaced away from it in the DFG-in state, to alanine (Figure 1b,c). IR  
107 spectra of the W277A mutant showed a clear spectral shift of the 2158 cm<sup>-1</sup> peak (Figure 1c),  
108 consistent with this peak arising from the DFG-Out state.



109 **Figure 1. The DFG motif of phosphorylated AurA remains predominantly in the DFG-Out**  
110 **state. a)** IR spectra of nitrile-labeled and phosphorylated AurA bound to ADP, measured at the  
111 indicated temperatures (colored curves). The spectrum for the same sample bound to Tpx2 is  
112 shown for comparison (dashed black line, measured at 20°C). Arrows indicate peaks assigned  
113 to the DFG-In and DFG-Out states. The inset shows the same experiments performed with  
114 unphosphorylated AurA. Single representative spectra are shown, normalized to peak maxima.  
115 **(b)** Overview of the structure of AurA in the active conformation bound to ADP (yellow) and  
116 Tpx2 (black), with enlarged views of the DFG-In (right, PDB ID: 1OL5) and DFG-Out (bottom,  
117 PDB ID: 5L8K) states with the nitrile probe (Q185CN) modeled into the structures. **(c)** Second  
118 derivatives of IR spectra of apo WT and W277A AurA, showing the spectral shift of the 2158 cm<sup>-1</sup>  
119 peak (arrow).  
120

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122 Experiments performed over a range of temperatures highlighted the presence of a  
123 DFG-In subpopulation in the phosphorylated samples bound to nucleotide, apparent as small  
124 shoulders on either side of the main 2158 cm<sup>-1</sup> peak that increase in amplitude at higher

125 temperature (Figure 1a). A similar DFG-In subpopulation was also detected in  
126 unphosphorylated AurA bound to ADP<sup>21</sup>, although the temperature dependence is absent in the  
127 unphosphorylated protein (Figure 1a, inset). These surprising results show that, at physiological  
128 temperatures, phosphorylation does not significantly change the DFG-In/Out equilibrium and the  
129 DFG-In subpopulation remains low. Tpx2 binding appears to be required to elicit a shift to the  
130 DFG-In state.

131

132 **Tpx2 binding shifts the phosphorylated activation loop to a more active conformation.**

133 We used intramolecular FRET to track movements of the activation loop of AurA with  
134 and without phosphorylation on T288. Donor (D) and acceptor (A) Alexa fluorophores were  
135 incorporated on the activation loop (S284C) and  $\alpha$ D helix (L225C) as previously described<sup>21</sup>.  
136 These labeling positions were chosen to track the movement of the activation loop across the  
137 active site as the kinase switches from the DFG-Out to the DFG-In state, with the dyes  
138 predicted to be further apart in the DFG-In state (Figure 2a). Phosphorylation of the protein on  
139 T288 was confirmed by tryptic mass spectrometry (Supplementary Figure S4). The labeled  
140 phosphorylated kinase exhibits robust catalytic activity in the absence of Tpx2, and is further  
141 activated only modestly by the addition of Tpx2 (3-4 fold), a characteristic feature of WT AurA  
142 phosphorylated on T288 (Supplementary Figure S5)<sup>19,20</sup>.

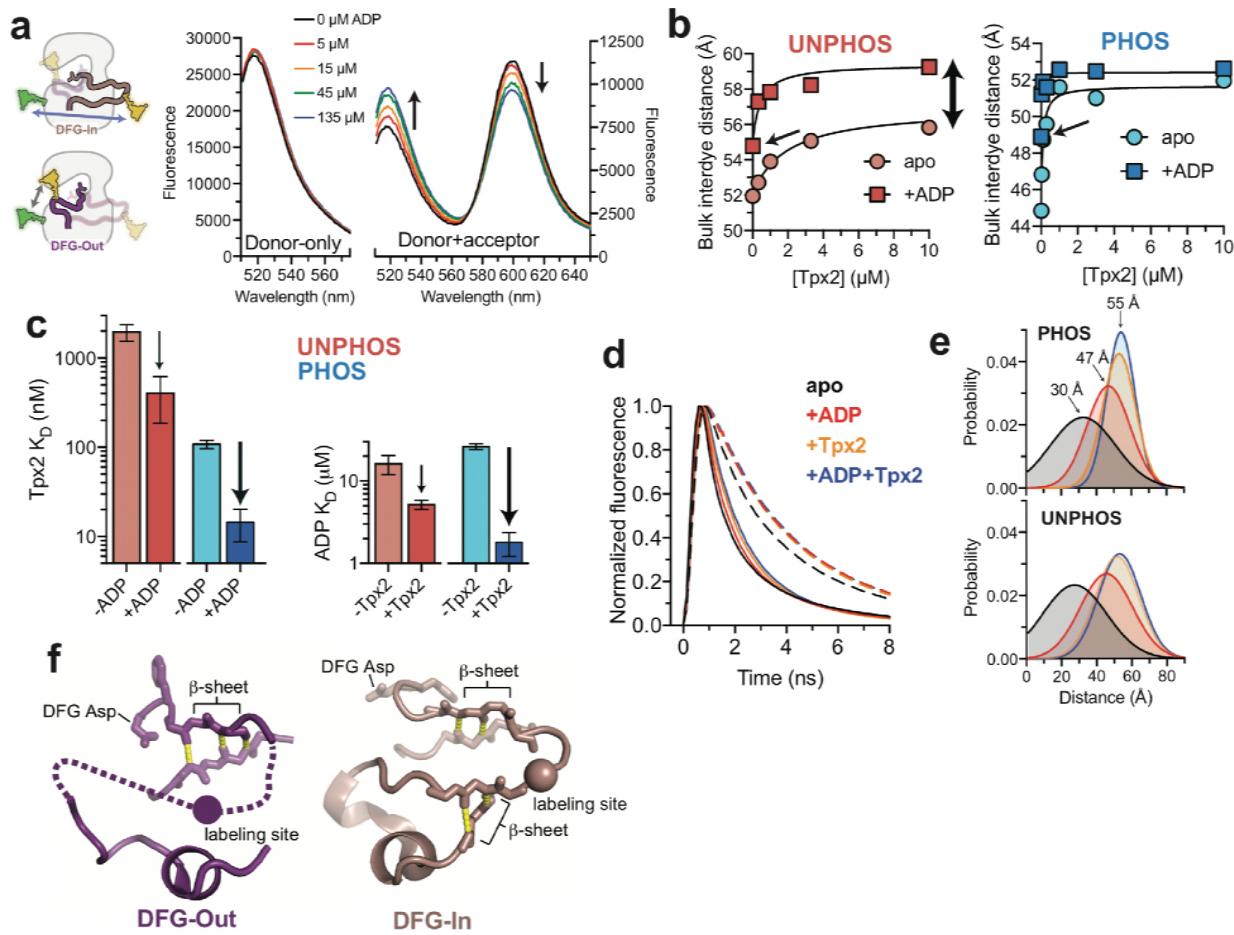
143 Steady-state fluorescence emission spectra were measured for D- and D+A-labeled  
144 samples in ligand titration experiments. The addition of either nucleotide or Tpx2 resulted in  
145 enhanced fluorescence emission from the donor dye and reduced emission from the acceptor,  
146 consistent with a decrease in FRET efficiency (Figure 2a). This indicates that upon binding  
147 nucleotide and Tpx2, phosphorylated AurA undergoes a conformational change to a more active  
148 conformation in which the activation loop is extended and the dyes are farther apart. The scale  
149 of the conformational change was estimated by calculating ensemble-averaged inter-dye  
150 distances from the bulk FRET efficiencies (see Methods). The maximal increase in distance is

151 observed when the kinase is saturated with both nucleotide and Tpx2, and is on the order of ~1  
152 nanometer for both phosphorylated and unphosphorylated AurA (Figure 2b, Supplementary  
153 Figure S6)<sup>21</sup>. This suggests that the activation loop undergoes a similar structural change in  
154 response to ligand binding regardless of its phosphorylation state. However, we noted several  
155 differences between the unphosphorylated and phosphorylated enzymes in how they respond to  
156 Tpx2 binding. Firstly, the affinity of the phosphorylated kinase for Tpx2, determined from the  
157 titration data, is ~20-fold higher than for the unphosphorylated kinase (Figure 2c). Secondly,  
158 phosphorylation enhances the cooperativity between nucleotide and Tpx2 binding observed in  
159 the unphosphorylated enzyme (Figure 2c, black arrows). Thirdly, Tpx2 binding alone is sufficient  
160 to produce a maximal conformational shift in the phosphorylated kinase, whereas, even at  
161 saturating concentrations, Tpx2 is insufficient to achieve this for the unphosphorylated kinase,  
162 and ADP and Tpx2 must both be present (double-headed arrow in Figure 2b, Supplementary  
163 Figure S6). These results suggest that Tpx2 and phosphorylation have synergistic effects on  
164 AurA and work together to fully stabilize the kinase in the active DFG-In state.

165 In contrast to the differing responses to Tpx2, AurA responds similarly to nucleotide  
166 binding regardless of the phosphorylation state of the enzyme. Specifically, the magnitude of the  
167 conformational change induced by nucleotide binding, as inferred from the increase in inter-dye  
168 distance, is similar in both cases and approximately half the maximal change (arrows in Figure  
169 2b), and the nucleotide affinity is not increased by phosphorylation in the absence of Tpx2  
170 (Figure 2c, right panel). Similar results were obtained with the non-hydrolyzable ATP analog  
171 AMPPNP (Supplementary Figure S6). These data suggest that the binding of nucleotide to the  
172 active site of phosphorylated AurA is only weakly coupled to the conformation of the activation  
173 loop, but that they become tightly coupled when Tpx2 is present.

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176

177 **Figure 2. The phosphorylated activation loop remains flexible and shifts to a more active**  
 178 **conformation upon Tpx2 binding.** (a) (left) Schematics showing the labeling scheme used  
 179 and (right) emission spectra of donor-only (D, left) and donor + acceptor (D+A, right) labeled  
 180 phosphorylated AurA samples in the presence of different concentrations of ADP. Single  
 181 representative experiments. (b) Ensemble-averaged distances between donor and acceptor  
 182 dyes, calculated from bulk FRET measured for unphosphorylated (left) and phosphorylated  
 183 (right) AurA with varying concentrations of Tpx2 in the presence and absence of saturating  
 184 ADP. Thin arrows highlight the intermediate distances observed with saturating ADP alone, and  
 185 the double-headed arrow shows the incomplete shift observed with saturating Tpx2 alone for  
 186 the unphosphorylated sample. Single representative experiments are shown. (c) Binding  
 187 constants of Tpx2 (left) and ADP (right) for phosphorylated (blue) and unphosphorylated (red)  
 188 AurA in the presence and absence of the other ligand. Arrows highlight cooperativity between  
 189 ADP and Tpx2. Data represent mean values  $\pm$  s.d.;  $n = 3$ . (d) Time-resolved fluorescence  
 190 waveforms for D-only (dashed lines) and D+A (solid lines) phosphorylated AurA in the presence  
 191 and absence of 100  $\mu$ M Tpx2 and 200  $\mu$ M ADP. Data are for a single representative

192 experiment, normalized to the fluorescence peak. (e) Comparison of single-Gaussian distance  
193 distribution fits to fluorescence lifetime data obtained with phosphorylated (top) and  
194 unphosphorylated AurA (bottom). Same coloring as d. (f) Structures of the DFG-Out (left) and  
195 DFG-In (right) states of AurA, highlighting the  $\beta$ -sheet hydrogen bonds constraining the N- and  
196 C-terminal segments of the activation loop. The S284C labeling site is shown as a sphere.

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198

199 **The phosphorylated activation loop adopts a range of conformations but becomes highly**  
200 **ordered upon Tpx2 binding.**

201 The steady-state FRET measurements provide ensemble-averaged measures of  
202 distance. To gain insight into the distribution of conformations present in AurA and how it is  
203 altered by ligand binding, we performed time-resolved (TR) FRET experiments to quantify  
204 energy transfer through its effect on the fluorescence lifetime of the donor dye. TR fluorescence  
205 decays were recorded for phosphorylated and unphosphorylated AurA samples in the presence  
206 and absence of saturating ADP and Tpx2 using time-correlated single-photon counting  
207 (TCSPC) (Figure 2d). These data were then fit to a structural model consisting of a Gaussian  
208 distribution of inter-fluorophore distances for each condition<sup>26-28</sup> (Figures 2e). The fraction of the  
209 D+A samples lacking acceptor dye was explicitly accounted for in the TR-FRET fitting, yielding  
210 more reliable distances than the values estimated by steady-state FRET.

211 The distance distributions measured for the phosphorylated and unphosphorylated  
212 kinase are strikingly similar (Figure 2e). In both cases, a broad distribution centered at  $\sim$ 30  
213 angstroms is observed for apo AurA, indicating that the activation loop is highly flexible under  
214 these conditions. This is consistent with adoption of the DFG-Out state, in which the C-terminal  
215 half of the activation loop lacks contacts with the rest of the kinase domain, and is typically  
216 disordered in x-ray structures<sup>29-31</sup> (Figure 2f). The addition of both ADP and Tpx2 together yields  
217 the longest distances ( $\sim$ 55 angstroms) with the narrowest distributions, indicative of a well-  
218 defined structure consistent with the DFG-In state, in which the segment of the loop containing

219 the labeling site is anchored to the C-terminal lobe of the kinase on both sides by backbone  
220 hydrogen bonds<sup>22</sup> (Figure 2f). For the +Tpx2 samples, the presence of phosphorylation resulted  
221 in additional narrowing of the distributions, suggesting that phosphorylation further restricts the  
222 movement of the loop within the DFG-In state. In the presence of ADP alone the observed  
223 distance distributions are intermediate in both distance and width between the other samples,  
224 consistent with both unphosphorylated and phosphorylated AurA remaining in an equilibrium  
225 between DFG-Out and DFG-In states (Figure 2e).

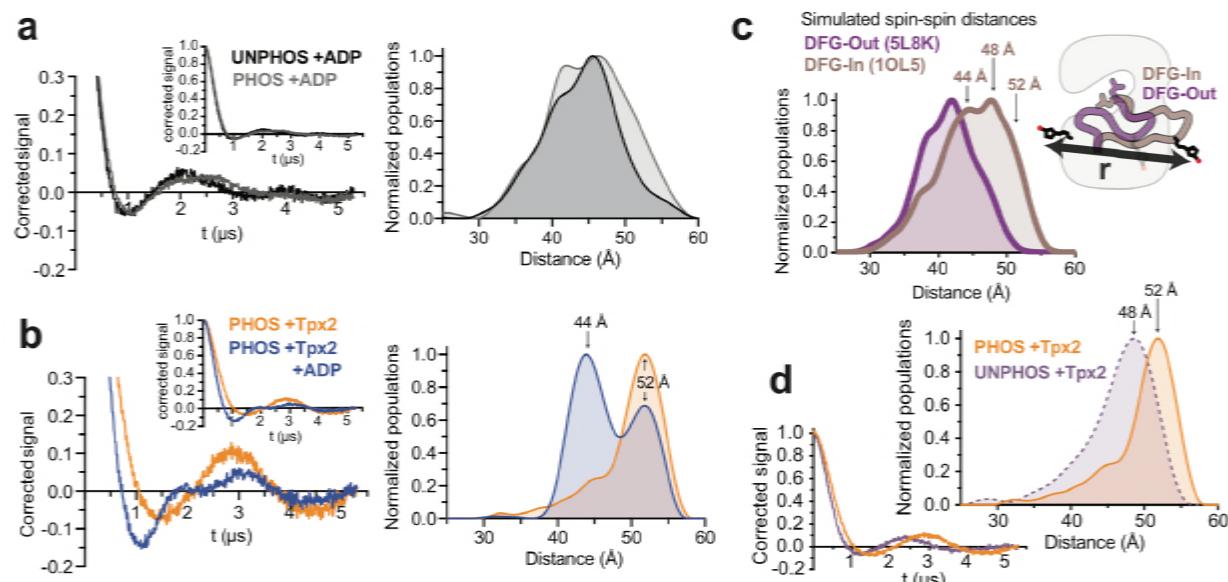
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227 **DEER experiments confirm that phosphorylated AurA requires Tpx2 to switch to the  
228 active state**

229 To independently confirm that the activation loop of phosphorylated AurA samples  
230 multiple conformational states, we used double electron-electron resonance (DEER) EPR  
231 spectroscopy<sup>32</sup>. DEER experiments probe the distance-dependent dipole-dipole interactions of  
232 unpaired electron spins, providing information about the distribution of spin-spin distances  
233 present in the sample. Two MTSL spin labels were incorporated into AurA at the same positions  
234 used for FRET experiments (L225C and S284C; labeling and phosphorylation were confirmed  
235 by mass spectrometry (Supplementary Figure S7)), and samples were flash frozen in the  
236 presence of saturating concentrations of ADP and/or Tpx2 for DEER experiments.

237 Background-corrected dipolar evolution data (DEER spectra) acquired for the  
238 unphosphorylated and phosphorylated samples lacking Tpx2 were very similar, with the DEER  
239 signal decaying rapidly, consistent with phosphorylation failing to trigger a shift towards the  
240 DFG-In state, and the activation loop adopting multiple conformations (Figure 3a). The addition  
241 of Tpx2 to the phosphorylated samples resulted in striking changes (Figure 3b), with  
242 pronounced oscillations apparent in the DEER spectra that persist beyond 5 microseconds,  
243 indicating a high degree of structural order in the activation loop. Consistent with this, spin-spin  
244 distance distributions determined from these data by Tikhonov regularization<sup>33</sup> are similar and

245 broad for the samples bound to nucleotide alone (Figure 3a), but display two sharp peaks at 44  
246 and 52 angstroms for the phosphorylated samples containing Tpx2 (Figure 3b). The 52-  
247 angstrom distance is considerably longer than the distances observed in the samples lacking  
248 Tpx2, consistent with the activation loop adopting the extended conformation characteristic of  
249 the active DFG-In state (see Figure 2f). Although the shorter 44-angstrom distance could in  
250 principle arise from a fraction of the sample occupying the DFG-Out state, we consider this  
251 unlikely. Firstly, the sharp nature of the 44- and 52-angstrom peaks is indicative of the high  
252 degree of structural order expected for the DFG-In state, but not the dynamic DFG-Out state, as  
253 discussed above (see Figure 2f). Secondly, the IR and FRET results indicate that Tpx2 shifts  
254 the phosphorylated kinase mostly to the DFG-In state.



255  
256 **Figure 3. DEER spectroscopy confirms that the phosphorylated kinase requires Tpx2 to**  
257 **switch fully to the active state. (a-b)** Enlarged view of the background-corrected DEER  
258 spectra are shown on the left with the full spectra shown as insets. The corresponding  
259 population densities obtained by Tikhonov regularization are shown on the right. All figures  
260 show data from a single representative experiment. **(a)** Comparison of unphosphorylated (black)  
261 and phosphorylated (gray) AurA in the presence of ADP. **(b)** Comparison of phosphorylated  
262 AurA bound to Tpx2 alone (orange), and both Tpx2 and ADP (blue). The 44- and 52-angstrom  
263 peaks in the distance distribution are highlighted. **(c)** Spin-spin distance distributions obtained

264 by molecular dynamics simulations initiated from x-ray structures of AurA in either the DFG-Out  
265 inactive state (purple) or the DFG-In state with both Tpx2 and phosphorylation, representing the  
266 fully active conformation (pink). The inset shows a schematic of the labeled kinase. (d) DEER  
267 spectra and distance distributions comparing unphosphorylated (purple) and phosphorylated  
268 AurA (orange) bound to Tpx2.

269

270 To support the DEER experiments we performed molecular dynamics simulations of  
271 MTSL-labeled AurA in either the DFG-Out state (PDB ID: 5L8K) or the DFG-In state (PDB ID:  
272 1OL5), totaling 75-110 microseconds of aggregate simulation data for each state. As expected,  
273 simulated spin-spin distances were considerably longer for the DFG-In state than the DFG-Out  
274 state (Figure 3c). In simulations of the most active state (the phosphorylated kinase in the DFG-  
275 In state bound to Tpx2), different spin label rotamers give rise to a range of distances, with  
276 peaks in the distribution apparent around ~44, ~48 and ~52 angstroms (Figure 3c). The 44- and  
277 52-angstrom spin-spin distances are consistent with the DEER data, although it is not clear why  
278 the relative contribution of the 52-angstrom distance decreases upon addition of ADP (Figure  
279 3b). Presumably the rotamer states corresponding to the 48-angstrom distance seen in the  
280 simulations are sparsely populated at the low temperature of the DEER experiment.

281 DEER spectra were also measured for AurA bound to Tpx2 but lacking phosphorylation  
282 (Figure 3d), and the corresponding distance distributions showed a sharp peak at a longer  
283 distance than in the corresponding sample without Tpx2 (see Figure 3a), consistent with a  
284 switch to the DFG-In state. Interestingly, this distance was ~4 angstroms shorter than the 52-  
285 angstrom peak measured for the phosphorylated sample in the presence of Tpx2 (Figure 3d),  
286 suggesting that phosphorylation alters the structure of the DFG-In state.

287 Taken together, the IR, FRET, and EPR data conclusively show that phosphorylation on  
288 T288 alone is not sufficient to shift AurA into the DFG-In state. Instead, the phosphorylated  
289 activation loop samples a range of different conformations spanning the DFG-In and DFG-Out  
290 states, and phosphorylation must drive catalytic activation of AurA by other mechanisms,

291 perhaps by altering the structure and/or dynamics of the DFG-In subpopulation to populate  
292 catalytically competent geometries.

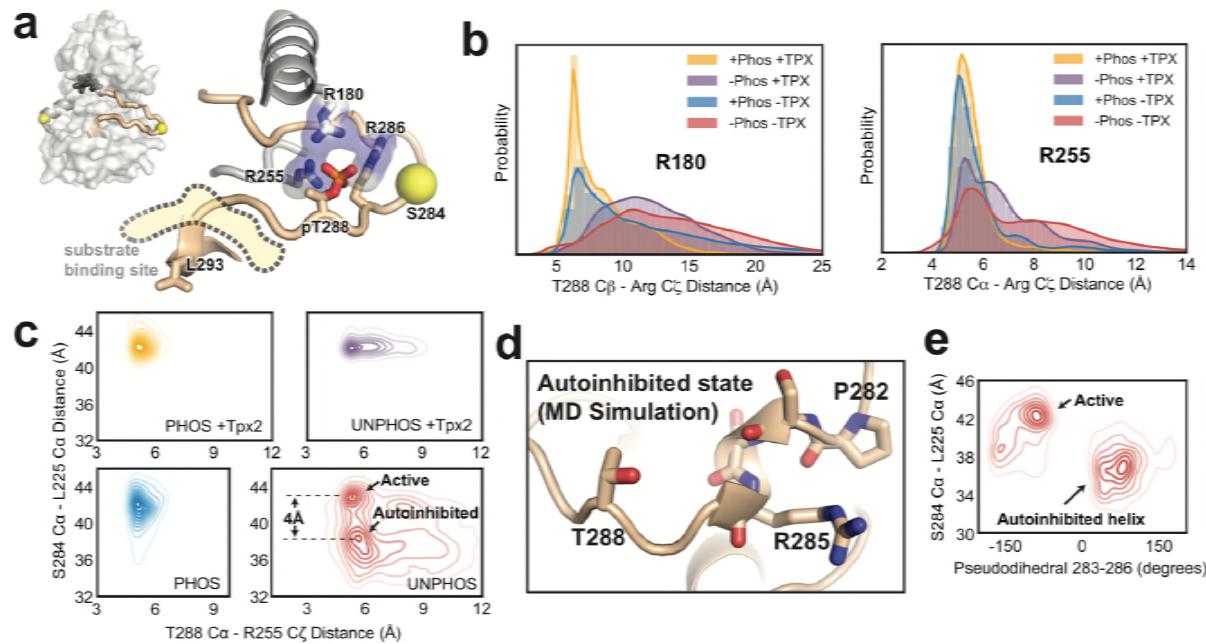
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294 **Phosphorylation promotes a single functional conformation in the DFG-In state**

295 To provide insight into how phosphorylation alters dynamics in the DFG-In state, we  
296 performed molecular dynamics simulations of the wild-type kinase. Simulations were initiated  
297 from the x-ray structure of active DFG-In AurA bound to ADP and Tpx2 (PDB ID: 1OL5)<sup>22</sup>, and  
298 were run in the presence and absence of Tpx2, with and without phosphorylation on T288. For  
299 each of these four biochemical states, 250 trajectories up to 500 nanoseconds in length were  
300 obtained on the distributed computing platform Folding@home, for a total of over 100  
301 microseconds of aggregate simulation time for each biochemical state. Analysis of the DFG  
302 conformation revealed that the simulations remained predominantly in the DFG-In state (Figure  
303 S13), suggesting that the simulation time was insufficient to capture the slow conformational  
304 change to the DFG-Out state. The simulations can thus be regarded as probing the  
305 conformational dynamics of the DFG-In kinase.

306 The T288 phosphorylation site lies in the C-terminal segment of the activation loop,  
307 which forms an integral part of the binding site for peptide substrates (Figure 4a). In the crystal  
308 structure used to initiate the simulations, this segment of the loop appears to be stabilized by  
309 interactions between the pT288-phosphate moiety and three arginine residues: R180 from the  
310  $\alpha$ C helix, R286 from the activation loop, and the highly conserved R255 from the catalytic loop  
311 “HRD motif” (Figure 4a)<sup>22</sup>. To probe the integrity of these interactions in the simulations, and to  
312 investigate loop dynamics in their absence, we examined the distribution of distances between  
313 the C $\zeta$  atoms of either R180 or R255 and the C $\beta$  or C $\alpha$  atoms of T288 following equilibration  
314 within the DFG-In state (Figure 4b). For the simulations performed in the presence of  
315 phosphorylation, both the T288-R255 and T288-R180 distances are tightly clustered around 5-6  
316 angstroms, confirming that the phosphate moiety is coordinated by both arginine residues

317 throughout the majority of the trajectories (Figure 4b). In contrast, the distributions are  
318 considerably broader in the absence of phosphorylation, confirming that this segment of the  
319 activation loop remains dynamic.



320  
321 **Figure 4. Molecular dynamics simulations of AurA in the DFG-In state show**  
322 **phosphorylation promotes a specific configuration of the activation loop. (a)** Structure of  
323 active phosphorylated AurA bound to Tpx2 and ADP (PDB ID: 1OL5) showing the interactions  
324 between pT288 and the basic arginine pocket. The S284 and L225 C $\alpha$  atoms are shown as  
325 spheres. **(b)** Distributions of the T288 C $\alpha$  - R180 C $\zeta$  (left) and T288 C $\alpha$  - R255 C $\zeta$  (right)  
326 distances determined from MD simulations of AurA performed in the indicated biochemical  
327 states. Note that ADP was present in all simulations. **(c)** Contour plots showing the L225 C $\alpha$  -  
328 S284 C $\alpha$  distances plotted against the T288 C $\alpha$  - R255 C $\zeta$  distances for all four biochemical  
329 conditions. The active and autoinhibited states observed for the unphosphorylated kinase in the  
330 absence of Tpx2 (red), and the shift in the L225-S284 distance between them, are indicated. **(d)**  
331 Simulation snapshot showing the helical turn in the activation loop and the position of the T288  
332 sidechain at the C-terminal end of the helix. **(e)** The L225 - S284 distance is plotted against the  
333 dihedral angle defined by the C $\alpha$  atoms of residues 283-286 (pseudodihedral). The helical  
334 conformation in the autoinhibited state is indicated.  
335

336 We also tracked the distance between the L225 and S284 Ca atoms (the sites used for  
337 incorporating spectroscopic probes) to capture movements of the tip of the activation loop  
338 containing S284 away from the active conformation. Plotting the L225-S284 distance versus the  
339 R255-T288 distance provides additional insight into the relative effects of Tpx2 and  
340 phosphorylation (Figure 4c). Phosphorylated AurA is locked into a single conformation with a  
341 long L225-S284 distance (42 Å) and short R255-T288 distance, indicative of a stable active  
342 state. Interestingly, phosphorylation alone is nearly as effective at constraining the loop in this  
343 state as phosphorylation and Tpx2 together (Figure 4c, left panels). In contrast, the simulations  
344 of unphosphorylated AurA bound to Tpx2 show a broader distribution of distances, indicating  
345 that Tpx2 is less effective than phosphorylation at stabilizing the activation loop. This may  
346 explain why Tpx2 activates AurA to a lesser extent than phosphorylation.

347

#### 348 **Phosphorylation may disrupt an autoinhibitory DFG-In state**

349 The simulations of unphosphorylated AurA without Tpx2 show a much greater degree of  
350 conformational heterogeneity than the simulations of the other three biochemical states. The N-  
351 terminal lobe of the kinase is particularly heterogeneous, and local unfolding occurs within the  
352 αC-helix in many of the trajectories, as seen previously in simulations of the epidermal growth  
353 factor receptor<sup>34</sup> and in x-ray structures of the related AGC-family kinase Akt<sup>35</sup>. Although the  
354 activation loop moves substantially away from the active conformation, giving rise to shorter  
355 L225-S284 distances, the loop is not in fact disordered. Instead, two discrete subpopulations  
356 are visualized in the simulations: one subpopulation corresponding to the active-like state, and  
357 another with a much shorter L225-S284 distance (38 Å, see Figure 4c), representing a stable  
358 DFG-In state in which the activation loop is not in a catalytically-competent conformation.  
359 Manual inspection of the trajectories revealed that in this subpopulation the tip of the activation  
360 loop folds into a short helical turn spanning residues P282-R286, with the P282 proline residue  
361 serving as the N-terminal capping residue in most of the trajectories<sup>36</sup> (Figure 4d). Calculating

362 the pseudodihedral angle for the C $\alpha$  atoms of S283-R286 across all trajectories confirmed that  
363 the inactive subpopulation possesses well-defined helical pseudodihedral values of 50-75°  
364 (Figure 4e). Although this conformation has not been observed in x-ray structures of AurA, the  
365 formation of short helices in the activation loop is a common feature of the inactive states of  
366 other protein kinases<sup>37-40</sup>.

367 An interesting feature of the autoinhibited DFG-In state observed in the simulations is  
368 that the T288 residue, which immediately follows the helical segment in the protein sequence, is  
369 positioned close to the C-terminal end of the helix in almost all of the trajectories (Figure 4d),  
370 with the sidechain hydroxyl forming hydrogen bonds to the backbone carbonyls of residues  
371 R285 and R286 in many of the simulation snapshots. We reasoned that upon phosphorylation  
372 of T288, the proximity of the phosphate group to the negatively-charged end of the helix dipole<sup>41</sup>  
373 would destabilize this autoinhibited state, promoting the refolding of the activation loop to the  
374 active conformation. The existence of such an autoinhibition mechanism may explain why  
375 unphosphorylated AurA exhibits very low catalytic activity in the absence of Tpx2 despite a  
376 substantial DFG-In subpopulation<sup>21</sup>.

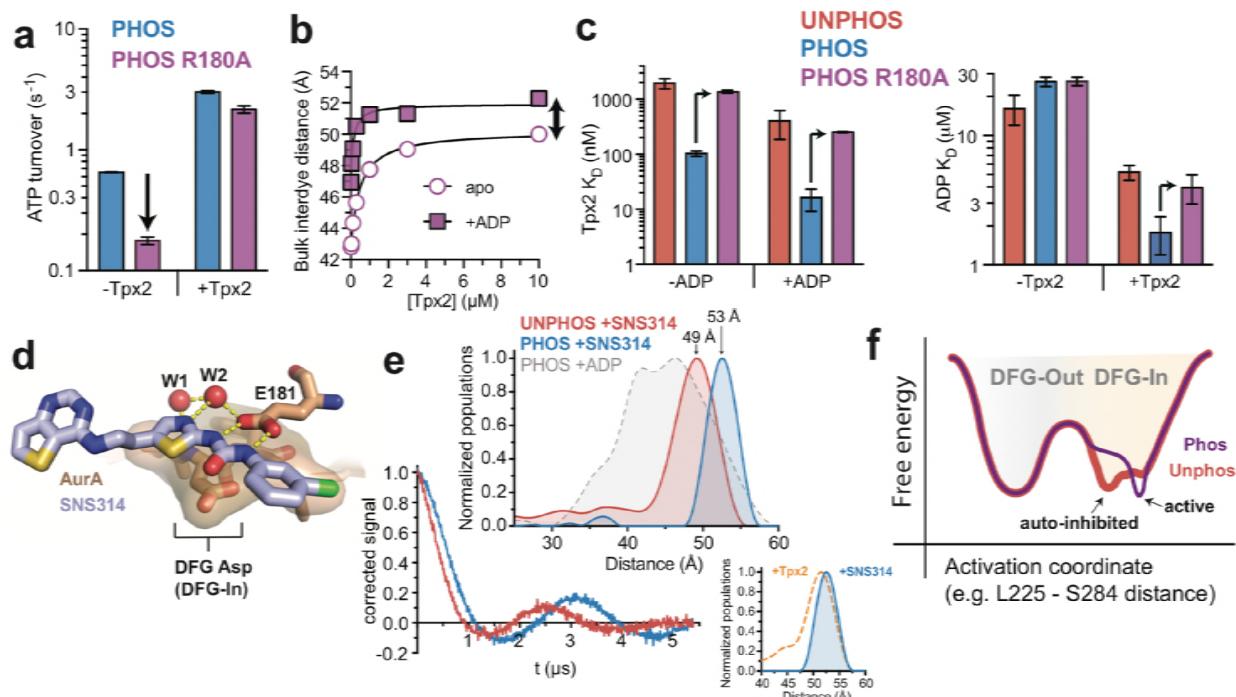
377 We wondered why the helical activation loop conformation has not been observed in x-  
378 ray structures of AurA. In fact, the activation loop adopts the active conformation in only a small  
379 subset of AurA structures determined in the presence of Tpx2<sup>22-24</sup> or other protein factors that  
380 stabilize the active state<sup>42</sup>. Instead, almost all of the structures of AurA in the DFG-In state (76  
381 structures out of 138 total structures of AurA in the PDB) were determined in the same  
382 hexagonal crystal form in which the kinase adopts an inactive conformation with the activation  
383 loop misaligned and the peptide binding site disassembled. Upon examination of the crystal  
384 lattice we noticed that this conformational state of the activation loop appears to be induced by a  
385 crystal contact between the peptide binding site and a neighboring molecule in the lattice  
386 (Supplementary Figure S11). This apparent crystallographic artifact may have prevented

387 previous observation of the helical autoinhibited DFG-In state visualized in our simulations,  
388 which model the kinase in solution rather than in the crystallographic context. In conclusion, the  
389 MD simulations, which represent over a millisecond of simulation data, suggest that  
390 phosphorylation has profound effects on the activation loop of AurA in the DFG-In state,  
391 disrupting an autoinhibited state and promoting an alternative conformation primed for catalytic  
392 function.

393

394 **Experimental evidence for ordering of the activation loop in the DFG-In state by**  
395 **phosphorylation**

396 A key insight from the MD simulations is that phosphorylation alone is sufficient to  
397 promote the active configuration of the activation loop, and that coordination of the  
398 phosphothreonine by the R180 and R255 residues is likely important for this. To test the role of  
399 these interactions, we mutated the R180 residue to an alanine in the context of our  
400 phosphorylated FRET construct (phosphorylation was confirmed by mass spectrometry, see  
401 Supplementary Figure S8). The R180A mutant possessed 4-fold lower activity in the absence of  
402 Tpx2, whereas the activity in the presence of Tpx2 was only modestly affected (Figure 5a),  
403 indicating that the pT288-arginine interactions are particularly important for activation of AurA by  
404 phosphorylation alone. Steady-state FRET experiments on the R180A mutant showed broadly  
405 similar ligand-induced conformational shifts as observed in the absence of the mutation, but the  
406 response to Tpx2 alone was somewhat smaller and more similar to unphosphorylated AurA<sup>21</sup>  
407 (Figure 5b and Supplementary Figure S6). Consistent with this observation, the binding affinities  
408 for Tpx2 and ADP are reduced by the R180A mutation, and are similar to those observed for  
409 unphosphorylated AurA (Figure 5c). This indicates that the pT288-arginine interactions are  
410 necessary for the synergy between phosphorylation and Tpx2 observed with the wildtype  
411 enzyme.



412  
413 **Figure 5. Experimental support for ordering of the activation loop in the DFG-In state**  
414 **mediated by phosphorylation.** (a) Kinase activity (shown as ATP turnover per second) for  
415 phosphorylated WT (blue) and phosphorylated R180A (purple) AurA unlabeled FRET constructs  
416 in the presence and absence of 10 μM Tpx2. The decrease in the activity in the absence of  
417 Tpx2 due to the R180A mutation is highlighted by the arrow. Data represent mean values ± s.d.;  
418 n = 3. (b) Ensemble-averaged distances between donor and acceptor dyes, calculated from  
419 bulk FRET, for phosphorylated R180A AurA with varying concentrations of Tpx2 in the presence  
420 and absence of saturating ADP. The double-headed arrow highlights the incomplete shift  
421 observed with saturating Tpx2 in the absence of ADP. Single representative experiments are  
422 shown. (c) Binding constants for Tpx2 (left) and ADP (right) for phosphorylated (blue),  
423 phosphorylated R180A (purple) and unphosphorylated (red) unlabeled AurA FRET constructs in  
424 the presence and absence of saturating concentrations of the other ligand. Arrows highlight the  
425 effects of the R180A mutation. Data represent mean values ± s.d.; n = 3. (d) X-ray structure of  
426 SNS-314 bound to AurA highlighting interactions with the DFG motif, structured water molecules  
427 and the catalytic glutamate (E181) that promote the DFG-In state. (e) DEER spectra (bottom)  
428 and distance distributions (top) measured for unphosphorylated and phosphorylated AurA  
429 bound to SNS-314. The distribution measured for phosphorylated AurA bound to ADP is shown  
430 in the top panel for comparison. The inset shows a comparison of the distributions obtained for  
431 the phosphorylated kinase bound to either SNS-314 (blue) or Tpx2 (orange), highlighting their

432 similarity. (f) Hypothesized energy landscape for AurA, highlighting the effect of phosphorylation  
433 on the DFG-In state.

434

435 The catalytic defect of the R180A mutant suggests that the pT288-phosphate moiety  
436 does dock into the arginine pocket even when Tpx2 is absent, as observed in the MD  
437 simulations. We hypothesized that our FRET and EPR experiments did not detect this in the  
438 form of a conformational change in the activation loop (see for instance Figure 3a) because  
439 under these conditions the substantial DFG-Out subpopulation masks the structural changes  
440 occurring in the DFG-In subpopulation. To test this, we used the ATP-competitive AurA inhibitor  
441 SNS-314, which preferentially binds to the DFG-In state of AurA<sup>43</sup> (Figure 5d), to induce a  
442 homogeneous population of DFG-In kinase. DEER spectra measured on unphosphorylated and  
443 phosphorylated AurA bound to SNS-314 were strikingly different from one another, and the  
444 Tikhonov distributions confirmed that phosphorylation causes a pronounced shift of ~4  
445 angstroms to longer distance (Figure 5e). Both distance distributions are very narrow, consistent  
446 with the activation loop adopting a well-defined structure in the absence of phosphorylation that  
447 differs from that of the active state. The increase in spin-spin distance upon phosphorylation is  
448 similar in magnitude to the change in the L225-S284 C $\alpha$  distance between the autoinhibited and  
449 active DFG-In states in the MD simulations, and may correspond to this conformational change  
450 (Figure 4c). Importantly, the distance measured for phosphorylated AurA bound to SNS-314 is  
451 nearly identical to that observed for the phosphorylated kinase bound to Tpx2 (Figure 5e inset),  
452 the most catalytically active form of the enzyme that x-ray structures show to be in the fully  
453 active state.

454 These results confirm that phosphorylation is both necessary and sufficient to fully  
455 constrain the activation loop in the active conformation for the fraction of the kinase adopting the  
456 DFG-In state. We conclude that while phosphorylated AurA samples both the DFG-Out and  
457 DFG-In states, the structure and dynamics of the DFG-In subpopulation are profoundly altered

458 by phosphorylation, leading to catalytic activation of the kinase. The autoinhibited DFG-In state  
459 identified in the MD simulations provides an explanation for how phosphorylation can promote  
460 activity without triggering a shift in the DFG equilibrium (Figure 5f). In this model,  
461 phosphorylation acts as much by destabilizing the autoinhibited DFG-In state as by stabilizing  
462 the active DFG-In state, leading to a population shift within the DFG-In state without much effect  
463 on the relative populations of the DFG-In and DFG-Out states.

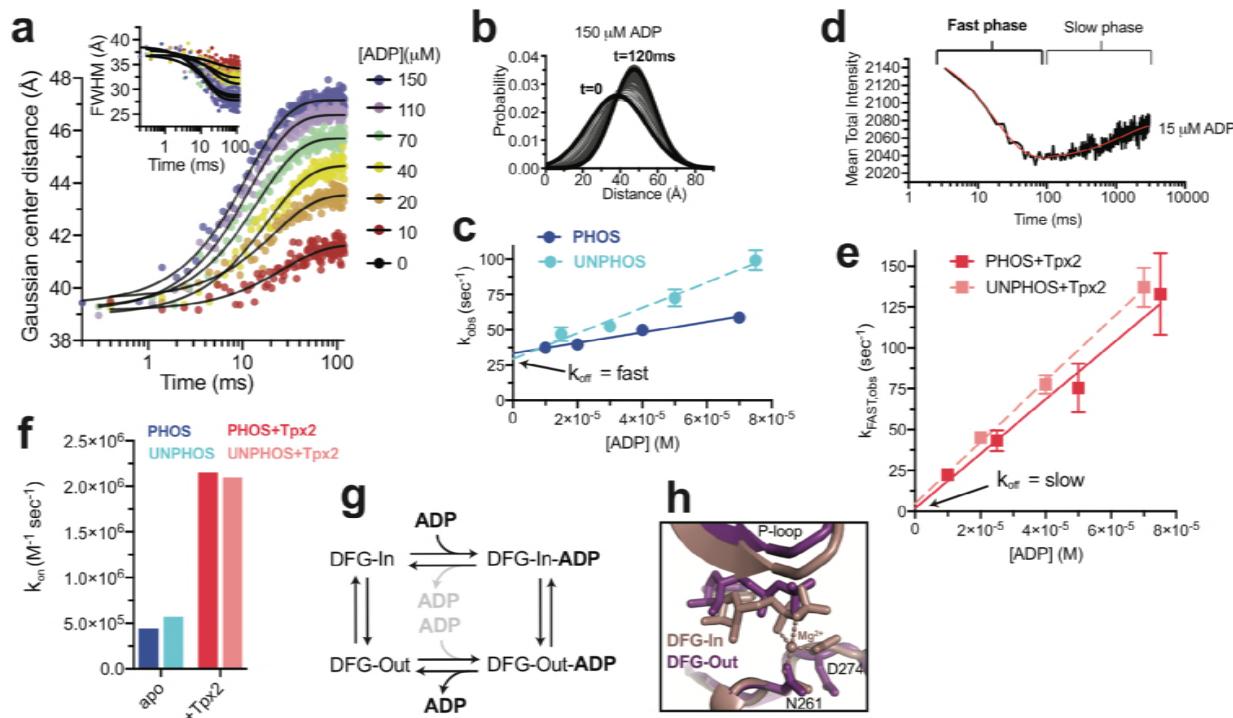
464

465 **A speculative model for a role of the DFG flip in nucleotide release during catalytic  
466 turnover of AurA**

467 We wondered whether maintaining a significant DFG-Out population might be important  
468 for the physiological function of AurA. In the closely-related kinase PKA catalytic turnover is  
469 rate-limited by product dissociation<sup>44-46</sup>, and it has been previously suggested for other kinases  
470 that the DFG flip may be coupled to nucleotide binding and release<sup>47,48</sup>. We therefore performed  
471 rapid mixing experiments to measure the rate of ADP binding to fluorescently labeled AurA,  
472 using a transient time-resolved fluorescence instrument that can track structural changes by  
473 time-resolved FRET with millisecond time resolution<sup>49</sup>.

474 For both unphosphorylated and phosphorylated samples lacking Tpx2 a single kinetic  
475 phase was observed in the mixing experiments (Figure 6a). Fitting of the time-resolved  
476 waveforms for the phosphorylated sample yielded FRET distances that evolved from ~37  
477 angstroms at early time points to ~47 angstroms at the end of the mixing experiment,  
478 demonstrating that the experiment monitors the shift towards the DFG-In state triggered by  
479 nucleotide binding (Figure 6a,b). A linear dependence of the apparent association rate constant,  
480  $k_{obs}$ , on ADP concentration was measured for both samples (Figure 6c), consistent with the  
481 kinetics reporting directly on nucleotide binding. In the presence of Tpx2 the kinetic behavior  
482 changed dramatically, with both the unphosphorylated and phosphorylated samples exhibiting

483 two well-separated kinetic phases (Figure 6d, Figure S9), which were analyzed separately as  
 484 discussed in more detail in the Supporting Information. The observed rate constants for the fast  
 485 phase,  $k_{\text{FAST,obs}}$ , were linearly dependent on ADP concentration (Figure 6e), consistent with ADP  
 486 binding, whereas the slow phase was not strongly dependent on ADP concentration (Figure  
 487 S10), suggesting a structural change.



488

489 **Figure 6. Tpx2 enhances nucleotide binding kinetics and slows nucleotide release.** (a)  
 490 Gaussia FRET distances (main graph) and corresponding full-width half maximum values  
 491 (inset) determined by transient time-resolved FRET for phosphorylated AurA mixed with  
 492 indicated concentrations of ADP. Black lines are single exponential fits. Each trace represents a  
 493 single experiment. (b) The distance distributions derived from the lifetime data are shown for a  
 494 single injection experiment for time points between 0 and 120 milliseconds. (c) Apparent rate  
 495 constants  $k_{\text{obs}}$  determined from single exponential fits to the fluorescence data, plotted as a  
 496 function of [ADP] for phosphorylated (dark blue) and unphosphorylated (light blue) samples.  
 497 Data represent single [ADP] series experiments, and error bars represent the estimated errors  
 498 from linear regression. The arrow highlights the measured off rates of  $\sim 30\text{ s}^{-1}$  for both samples.  
 499 (d) Fluorescence transient for unphosphorylated AurA bound to Tpx2 and mixed with  $15\text{ }\mu\text{M}$   
 500 ADP. The fast and slow phases are indicated. (e) Rate constants  $k_{\text{fast,obs}}$  determined from the

501 fast phase are plotted as a function of [ADP]. (f) Elementary rate constants  $k_{ON}$  for ADP  
502 association in the presence and absence of 20  $\mu$ M Tpx2. Data represent the average of two  
503 independent experiments. (g) Kinetic scheme for ADP binding to AurA, emphasizing the  
504 proposed model in which ADP binds preferentially to the DFG-In state and dissociates from the  
505 DFG-Out state. (h) Comparison of x-ray structures of AurA bound to ADP in the DFG-In (pink,  
506 PDB ID: 1OL5) and DFG-Out (purple, PDB ID: 5L8K) states, with the magnesium coordination  
507 indicated.

508

509 We propose the following interpretation of these results (see Supporting Information for  
510 further discussion). The DFG-In and DFG-Out states are in equilibrium, and nucleotide binds  
511 preferentially to the DFG-In subpopulation. In the absence of Tpx2, the DFG flip is fast  
512 compared to binding resulting in a single kinetic phase in which the relatively slow on rate  $k_{ON}$   
513 ( $\sim 4 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ) reflects binding to the small DFG-In subpopulation. In the presence of Tpx2, the  
514 same kinetic scheme applies, but the DFG flip slows dramatically to  $\sim 2 \text{ s}^{-1}$  (the slow phase in  
515 Figure 6d) so that the DFG-In and DFG-Out states now interconvert slowly compared to  
516 nucleotide binding. The on and off rates determined from the fast phase (Figure 6e) therefore  
517 reflect nucleotide binding and dissociation from the kinetically isolated DFG-In subpopulation.  
518 The  $\sim 5$ -fold increase in the on rate  $k_{ON}$  observed with Tpx2 ( $\sim 2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ , Figure 6f) is  
519 consistent with the much larger DFG-In subpopulation under those conditions. The off rate  $k_{OFF}$   
520 (the intercept in Figure 6e) is difficult to measure precisely due to the steep concentration  
521 dependence, but is clearly much slower than in the absence of Tpx2 (compare arrows in Figure  
522 6c and e). This indicates that nucleotide dissociation from the DFG-In state is very slow, and  
523 suggests that the DFG-Out state is responsible for the fast dissociation observed in the absence  
524 of Tpx2 ( $30 \text{ s}^{-1}$ ), and may even be necessary for efficient nucleotide dissociation (Figure 6g).  
525 This hypothesis is consistent with x-ray structures of DFG-Out AurA bound to nucleotides<sup>19,50</sup>,  
526 which show that magnesium coordination is lost and the nucleotide is partly dissociated from the  
527 C-terminal lobe of the kinase (Figure 6h).

528 In our model, the slow phase observed in the presence of Tpx2 arises from the DFG-Out  
529 subpopulation binding nucleotide and converting to the DFG-In state. FRET analysis of the  
530 lifetime data for the slow phase suggested a small shift of ~1-2 angstroms to longer distance,  
531 consistent with a small subpopulation undergoing the DFG flip (see Supporting Information and  
532 Figure S10), but the limited size of this change prevents us from ruling out other possible  
533 explanations, and our model therefore remains speculative. Interestingly, the observed  
534 timescale of this structural change ( $\sim 2 \text{ s}^{-1}$ ) is similar to the maximum catalytic turnover rate  
535 observed for Tpx2-bound AurA ( $\sim 3 \text{ sec}^{-1}$ , Figure 5a), raising the possibility that the DFG flip is a  
536 rate limiting step for catalytic turnover under these conditions. We propose that the DFG-Out  
537 state represents an intermediate in the catalytic cycle, and that an important function of the DFG  
538 flip in AurA may be to promote efficient nucleotide exchange.

539

540

## 541 **Discussion**

542 The majority of eukaryotic protein kinases are activated by phosphorylation on the  
543 activation loop<sup>3</sup>. X-ray structures have suggested that the functional role of phosphorylation is to  
544 trap the kinase in the active DFG-In state and rigidify the flexible activation loop in a specific  
545 configuration that promotes catalysis and substrate binding<sup>4-6</sup>. Our results show that  
546 phosphorylation can drive catalytic activation of a protein kinase without restraining the  
547 activation loop in the DFG-In state, providing a contrasting and highly dynamic view of an  
548 activated kinase in which major conformational changes of catalytic elements may occur  
549 continuously during the catalytic cycle. A recent single-molecule fluorescence study also  
550 reported that phosphorylated AurA dynamically transitions between multiple structural states<sup>51</sup>.

551 We previously reported that the binding of Tpx2 to unphosphorylated AurA causes a  
552 population shift towards the DFG-In state, in striking contrast with the phosphorylation-mediated  
553 activation mechanism described here<sup>21</sup>. Our simulation and EPR data also reveal differences in

554 how phosphorylation and Tpx2 affect the DFG-In subpopulation, with Tpx2 failing to fully  
555 constrain the C-terminal segment of the activation loop in the active conformation, unlike  
556 phosphorylation. Thus it appears that, when acting alone, phosphorylation and Tpx2 activate  
557 AurA through distinct pathways, with phosphorylation promoting a specific configuration of the  
558 activation loop for the DFG-In subpopulation, and Tpx2 instead triggering a DFG flip as well as  
559 stabilizing the kinase  $\alpha$ C-helix and an associated water-mediated hydrogen bond network<sup>21</sup>.  
560 When AurA is simultaneously activated by both Tpx2 and phosphorylation, the two regulatory  
561 inputs synergize, with the kinase switching fully to the DFG-In state and the activation loop  
562 becoming highly ordered. This synergy likely accounts for the observation that Tpx2 and  
563 phosphorylation together can overcome the deleterious effects of destabilizing mutations in the  
564 regulatory spine of AurA<sup>21</sup>. Interestingly, this doubly-activated form of AurA is not thought to  
565 occur in normal cells, but is prominent in about 10% of melanoma patients, where mutational  
566 inactivation of the PP6 phosphatase leads to accumulation of phosphorylated AurA on the  
567 mitotic spindle, and results in genomic instability<sup>52,53</sup>. The synergistic action of phosphorylation  
568 and Tpx2 on the conformational dynamics of AurA may provide unique opportunities for the  
569 development of inhibitors that selectively target this pathological form of the kinase in  
570 melanoma<sup>54</sup>.

571        Although activation of AurA by phosphorylation and Tpx2 are normally mutually  
572 exclusive, similar activation steps must occur in concert in the related AGC-family kinases,  
573 which require both phosphorylation and the docking of their C-terminal hydrophobic motifs (HM)  
574 to the  $\alpha$ C-helix, which resembles the Tpx2 interaction. The suppressed dynamics of doubly-  
575 activated AurA may therefore be representative of canonically-activated AGC kinases, and the  
576 highly dynamic nature of phosphorylated AurA in the absence of Tpx2 may be a unique property  
577 of the Aurora kinases that reflects the loss of the HM and the emergence of the dual activation  
578 mechanisms in this lineage<sup>21</sup>. These dynamics may facilitate further regulation of AurA by

579 additional cellular factors, allowing for graded levels of catalytic activity. For instance,  
580 phosphorylated AurA is known to interact with Cep-195<sup>55</sup>, Bora<sup>14,15</sup>, and Ajuba<sup>56</sup> at the  
581 centrosome, and these interactions can further regulate AurA activity towards specific  
582 substrates.

583 The DFG flip has long been considered one of the key regulatory mechanisms used by  
584 nature to control the catalytic activity of protein kinases<sup>7,8</sup>. Although AurA does adopt the DFG-  
585 Out state, our results show that activation of the enzyme by phosphorylation is not mediated by  
586 a DFG flip, but rather by tuning the catalytic activity of the DFG-In subpopulation. This suggests  
587 that evolution has moderated the ionic interactions between the phosphothreonine residue and  
588 the basic residues it interacts with to ensure a substantial DFG-Out subpopulation. Based on  
589 our kinetic studies, we speculate that this may be due to the importance of the DFG-Out state  
590 for promoting nucleotide release during the catalytic cycle. If this model is correct, the surprising  
591 result reported here that phosphorylation is not tightly coupled to the DFG equilibrium may  
592 simply be the consequence of evolutionary selective pressure to optimize the kinetics of  
593 substrate binding and product release for efficient catalytic turnover. It remains to be seen  
594 whether a large DFG-Out subpopulation is a unique feature of AurA, or a more general property  
595 of activated protein kinases.

596

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603

## 604 **Author contributions**

605 E.F.R. and N.M.L conceived and designed experiments; E.F.R. prepared fluorescently labeled  
606 samples and performed steady-state fluorescence experiments; E.F.R. and J.M.M. performed  
607 time-resolved fluorescence experiments and analyzed the data; E.F.R. and E.L. prepared  
608 MTSI-labeled samples and A.T. performed EPR experiments and analyzed the data; D.D.T.  
609 helped conceive the EPR experiments and supervised analysis of the time-resolved FRET and  
610 EPR data; S.C. prepared nitrile-labeled IR samples and performed IR experiments; J.D.C. and  
611 N.M.L. conceived and designed molecular dynamics simulations; J.M.B. performed the MD  
612 simulations of WT AurA, S.K.A. performed the simulations of spin-labeled AurA, and S.K.A. and  
613 S.M.H. analyzed MD simulation data; E.F.R. and N.M.L. wrote the manuscript.

614

## 615 **Materials and Methods**

616

### 617 **Expression and purification of AurA constructs**

618 Aurora A kinase domain constructs were expressed and prepared as previously  
619 described<sup>21</sup>. Site-directed mutagenesis using the QuikChange Lightning kit (Agilent) was used  
620 to incorporate mutations R180A and W277A. Homogeneous phosphorylation of our Cys-lite  
621 constructs was achieved using a C290A variant which autophosphorylates efficiently on T288  
622 when expressed in E.coli<sup>57</sup>. Phosphorylation was verified by mass spectrometry and activity  
623 assays (see Supplemental Information).

624

### 625 **IR spectroscopy**

626 Phosphorylated Q185C AurA protein samples (human AurA residues 122-403 containing  
627 an N-terminal hexahistidine tag) were prepared using a cysteine-light form of the kinase in  
628 which all endogenous solvent-accessible cysteines were removed by mutagenesis (Q185C,  
629 C247A, C290A, C393S). After Nickel-affinity purification, repeated rounds of cation exchange  
630 chromatography were used to isolate the homogeneous singly phosphorylated species, with

631 enrichment of the phosphorylated species tracked during purification by western blotting and  
632 activity assays (Supplementary Figure S2). Nitrile labeling of the purified protein was performed  
633 using a 1.5:1 molar ratio of DTNB (Ellman's reagent), followed by 50 mM KCN, and excess  
634 labeling reagents were removed using a fast desalting column (GE Healthcare). Incorporation of  
635 a single nitrile label was confirmed by whole-protein mass spectrometry (Supplementary Figure  
636 S3). Samples for IR spectroscopy were prepared by concentrating labeled protein (50-100  $\mu$ M)  
637 in the presence or absence of 4 mM ADP and 8 mM MgCl<sub>2</sub>, and/or excess Tpx2 peptide (~150  
638  $\mu$ M, residues 1-43 of human Tpx2, Selleckchem) in FTIR buffer (20 mM Hepes, pH 7.5, 300 mM  
639 NaCl, 20% glycerol). Samples were concentrated to ~1 mM and loaded into a calcium fluoride  
640 sample cell mounted in a temperature-controlled housing (Biotools) for IR experiments. IR  
641 spectra were recorded on a Vertex 70 FTIR spectrometer (Bruker) equipped with a liquid  
642 nitrogen cooled indium antimonide detector with 2  $\text{cm}^{-1}$  spectral resolution. Spectra were  
643 averaged across 256 scans, background subtracted using absorbance spectra of the buffer  
644 flow-through from sample concentration, and baselined using the polynomial method in the  
645 OPUS software (Bruker).

646

#### 647 **Kinase assays**

648 Kinase activity was measured using the ADP Quest coupled kinase assay (DiscoverX) in  
649 a fluorescence plate reader (Tecan Infinite M1000 PRO) as described previously<sup>21</sup>. Assays were  
650 performed using 2, 5, 10, 100, or 200 nM kinase (depending on the protein variant), 1 mM  
651 kemptide peptide substrate (Anaspec), 10  $\mu$ M Tpx2 residues 1-43 (Selleckchem), and 50  $\mu$ M  
652 ATP (Sigma Aldrich). Activity was determined using the initial fluorescence intensity slopes as a  
653 function of time (determined by linear regression) for ADP concentrations 1-10  $\mu$ M. Background  
654 ATPase activity was determined for samples with no peptide substrate added, and was  
655 subtracted from the activity in the presence of kemptide. We then determined the average

656 fluorescence over the time of the assay for known ADP concentrations in the dynamic range of  
657 the assay to construct a calibration curve, and used this to convert the background-corrected  
658 activity to ATP turnover numbers. Activities given are the average of three experiments, where  
659 error bars are the standard deviation of the replicates.

660

### 661 **Fluorescence and Förster resonance energy transfer (FRET) experiments**

662 For FRET experiments, the variant AurA C290S/A C393S L225C S284C was expressed,  
663 purified and labeled with donor (Alexa 488, Invitrogen) and acceptor (Alexa 568) using cysteine-  
664 maleimide chemistry, as previously described<sup>21</sup>. Labeled samples were validated using activity  
665 assays and mass spectrometry and retained close to full kinase activity (Supplementary Figures  
666 S3 and S4).

667

#### 668 *Steady-state FRET*

669 Ligand titration FRET experiments were performed in a Fluoromax 4 Spectrofluorometer  
670 (Horiba) at 22 °C. Assays were performed in 15 mM HEPES pH 7.5, 20 mM NaCl, 1 mM EGTA,  
671 0.02% Tween-20, 10 mM MgCl<sub>2</sub>, 1% DMSO and 0.1 mg/mL bovine-γ-globulins at AurA  
672 concentrations of 5-50 nM. Bulk FRET efficiency and inter-fluorophore distance were calculated  
673 from the ratios of the donor fluorescence in the presence and absence of acceptor, assuming a  
674 value of 62 angstroms for the Förster radius, as previously described<sup>21</sup>. The steady-state  
675 anisotropy was below 0.2 for all samples and did not change appreciably between biochemical  
676 conditions (Supplementary Figure S12).

677 Dissociation constants K<sub>D</sub> were determined using the spectra obtained with the D+A-  
678 labeled sample. The ratio F<sub>D</sub>/F<sub>A</sub> (where F<sub>D</sub> is the donor fluorescence maximum and F<sub>A</sub> is the  
679 acceptor fluorescence maximum), a highly sensitive measure of ligand binding, was fit as a  
680 function of ligand concentration. For calculation of the K<sub>D</sub> for Tpx2 with saturating ADP bound,  
681 K<sub>D</sub> is near the concentration of fluorescent protein. Ligand depletion was accounted for by fitting

682 the raw data to determine the plateau  $F_D/F_A$  value (representing ligand saturation), calculating  
683 the percent saturation for each total ligand concentration, and then back calculating the  
684 concentration of free ligand.  $F_D/F_A$  was then re-fit as a function of free ligand concentration.

685

686 *Time-resolved (TR) FRET*

687 The instrument used to collect time-resolved fluorescence at equilibrium has been  
688 previously described<sup>27</sup>. Data were detected by time-correlated single photon counting. The  
689 instrument response function was obtained with the emission polarization set at vertical, while  
690 fluorescence data were collected with the emission polarization set at 54.7°, with a GFP band  
691 pass filter in place (Semrock).

692 Experimental buffer contained 15 mM HEPES pH 7.5, 20 mM NaCl, 1 mM EGTA, 0.02%  
693 Tween-20, 10 mM MgCl<sub>2</sub>. Experiments were performed at 100-200 nM unphosphorylated or  
694 phosphorylated FRET-labeled AurA, in the presence and absence of 100 μM Tpx2 and 200 μM  
695 ADP; one phosphorylated AurA experiment also contained 1 mM DTT. For both  
696 unphosphorylated and phosphorylated AurA, two independent experiments were performed and  
697 analyzed.

698 Data fitting was performed as previously described<sup>26</sup>. Briefly, time-resolved fluorescence  
699 waveforms were fit using custom software designed for analysis of time-resolved  
700 fluorescence<sup>49</sup>. The instrument response function and the model of the fluorescence decay were  
701 convolved to fit the measured time-resolved fluorescence waveform. Donor-only fluorescence  
702 waveforms were modeled using a multiexponential decay function, which accounts for the  
703 intrinsic lifetimes of Alexa 488, and two exponentials were required to fit the Alexa 488  
704 fluorescence decay. Donor + acceptor (D+A) waveforms were modeled from the amplitudes and  
705 lifetimes present in the matched donor-only sample and modified so that a distance-dependent  
706 resonance energy transfer term, corresponding to a Gaussian distribution of inter-probe  
707 distances, describes the decrease in fluorescence lifetime relative to the donor-only control. The

708 mean distance and full-width half maximum of the Gaussian functions were fit individually for  
709 each D+A and D-O pairing, while the parameters that described general conditions of the  
710 experiment common among all samples, such as the fraction of a given D+A sample containing  
711 D-only protein, were globally linked.

712

#### 713 *Stopped-flow fluorescence kinetics*

714 The transient time-resolved fluorometer used has been previously described<sup>28,49</sup> and  
715 uses a *Biologic USA* SFM/20 single-mix stopped-flow instrument coupled to a transient time-  
716 resolved fluorescence spectrophotometer based on direct waveform recording technology. The  
717 flow rate was 8 mL/sec, the instrument dead time is >2 ms, and 3-5 waveforms were averaged  
718 every 1 ms. At each ADP concentration, 12-20 successive replicate mixing experiments were  
719 performed and averaged together, and these averaged waveforms were fit to extract time-  
720 resolved FRET distance information and kinetic constants.

721 For experiments, 20-50 nM phosphorylated or unphosphorylated FRET-labeled AurA  
722 was loaded into syringe A, 10-300  $\mu$ M ADP was loaded into syringe B, and samples were  
723 rapidly mixed. For measurements of ADP binding in the presence of Tpx2, 20  $\mu$ M Tpx2 was  
724 added to the buffer in both syringes A and B. Stopped-flow experiments were performed at 25  
725 °C in buffer containing 20 mM HEPES pH 7.4, 200 mM NaCl, 10% glycerol, 10 mM MgCl<sub>2</sub>, 0.1  
726 mg/mL bovine- $\gamma$ -globulins, and 1 mM DTT. The kinetic constants plotted are the average  
727 obtained in two experiments.

728 For details of the kinetic fitting, see Supplementary Information.

729

#### 730 **EPR experiments**

731 DEER samples were prepared in the Cys-lite mutant construct AurA C290S/A C393S  
732 C247A L225C S284C, purified as described above. AurA was labeled with MTSL (Santa Cruz  
733 Biotechnology), purified by cation exchange chromatography, and concentrated. Labeling was

734 verified using mass spectrometry, and MTSI-labeled samples retained close to full activity of  
735 unlabeled AurA in the presence and absence of Tpx2 (Supplementary Figure S7). The protein  
736 was then buffer exchanged into the experimental buffer, which was 20 mM HEPES pH 7.5, 300  
737 mM NaCl, 10% deuterated glycerol, 2% v/v H<sub>2</sub>O in D<sub>2</sub>O. For DEER experiments, samples  
738 containing 30-60  $\mu$ M MTSI-labeled AurA were prepared in the presence and absence of 100-  
739 200  $\mu$ M Tpx2 and 300  $\mu$ M ADP (8 mM MgCl<sub>2</sub> was added to samples containing ADP). Final  
740 samples varied in v/v H<sub>2</sub>O concentration from 2-14%; however, no significant differences were  
741 observed in Tikhonov distributions derived from experiments performed in protonated and  
742 deuterated buffers. Samples were flash-frozen in an isopropanol dry ice bath followed by liquid  
743 nitrogen. Data shown are from one of two replicate experiments.

744 DEER spectra were detected at 65 °K using an Elexsys E580 Q-Band spectrometer  
745 (Bruker Biospin) equipped with an ER 5107D2 resonator (Bruker Biospin) using the standard 4-  
746 pulse pulse sequence with  $\pi/2$  and  $\pi$  pulses (including ELDOR) set to 16 and 32 ns  
747 respectively. The pump frequency was set to the central resonance position of the nitroxide  
748 echo-detected field swept spectrum while the observe position was set 24G up-field to avoid  
749 excitation bandwidth overlap<sup>27</sup>.

750 Data were analyzed using custom software written in Mathematica which was based  
751 heavily on DeerAnalysis 2017<sup>58</sup>. The raw spectra were phase and background corrected  
752 assuming a homogeneous background model to produce the DEER waveform. Distance  
753 distributions were determined using Tikhonov regularization, with an optimal smoothing  
754 parameter chosen using a combination of the I-curve and leave one out cross validation  
755 (LOOCV) techniques<sup>59</sup>. After choice of smoothing parameter, a range of background fits were  
756 performed to identify stable populations in the distance distributions, with highly unstable, long  
757 distance populations being largely attributable to errors in the background fit and model  
758 choice<sup>60</sup>.

759

760 **Molecular dynamics simulations**

761 *System Preparation*

762 *Modeling WT unphosphorylated AurA.* WT AurA in complex with ADP was simulated  
763 with and without Tpx2. All simulations were started from the x-ray structure of WT AurA bound  
764 to Tpx2 and ADP in the presence of three magnesium ions (PDB ID: 1OL5<sup>22</sup>). From the crystal  
765 structure, PDBFixer (<https://github.com/pandegroup/pdbfixer>) version 1.2 was used to model in  
766 Tpx2 residues 23-29 (unresolved in 1OL5), add hydrogens belonging to standard dominant  
767 protein residue protonation states at pH 7.4, and remove phosphorylation from threonine  
768 residues 287 and 288<sup>61</sup>. Crystallographic waters were retained to prevent nonphysical collapse  
769 of hydrophilic pockets during minimization. The chain containing Tpx2 was then removed for  
770 simulations without Tpx2 (-Tpx2) and retained for simulations with Tpx2 (+Tpx2). Sulfate ions  
771 present in the crystal structure were manually removed. The crystallographic ADP (containing  
772 only heavy atoms) was extracted from the structure and converted to a protonated Tripos mol2  
773 file using OpenEye toolkit OEChem v2015.June<sup>62,63</sup>. The protein structure was then loaded as  
774 an OpenMM version 7.0.1 Modeller object, and the protonated ADP was reintroduced through  
775 conversion from mol2 to OpenMM format via MDTraj 1.4.2<sup>61,64</sup>.

776 *Modeling WT phosphorylated AurA.* Simulations of phosphorylated WT AurA in complex  
777 with ADP were prepared as above, but the phosphothreonines (denoted TPO in the PDB file) at  
778 positions 287 and 288 were left in place and parameterized using the Sticht T1P AMBER  
779 parameters<sup>65</sup> retrieved from the AMBER parameter database<sup>66</sup>. The AMBER phosphothreonine  
780 parameter file was converted to OpenMM fxml using a python script that has been made  
781 publically available (<https://github.com/choderalab/AurA-materials>), and subsequently converted  
782 into a hydrogen specification file for OpenMM's Modeller by hand. The PDB file generated by  
783 PDBFixer was loaded into an OpenMM Modeller object where the hydrogens and bonds were  
784 added to the TPO residues using a Forcefield object instantiated with the AMBER99Bildn  
785 parameters as well as the custom TPO parameters described above. All crystallographic

786 waters, ADP, sulfate ions and magnesium ions were handled as with the unphosphorylated WT  
787 AurA simulations.

788 *Parameterization the WT AMBER simulations.* An OpenMM ForceField was instantiated  
789 using AMBER99SBildn force field parameters<sup>67</sup> for the protein and TIP3P water model, along  
790 with ADP parameters generated by Carlson and accessed from the Amber Parameter  
791 Database<sup>65-67</sup>. The phosphorylated simulations also used custom phosphothreonine parameters  
792 described above<sup>65,66</sup>.

793 *Minimization and equilibration for the WT AMBER simulations.* Local energy  
794 minimization was performed in three separate steps in order to gradually introduce bond  
795 constraints. An OpenMM System was instantiated with no constraints on bonds or angles for the  
796 first minimization, which took place in vacuum (with crystallographic waters) with no constraints  
797 on bonds or angles. After this minimization, a new System was instantiated with constraints on  
798 the lengths of all bonds involving a hydrogen atom, and minimization was repeated. The  
799 structure and positions of all atoms were then put into a new OpenMM Modeller object, where  
800 TIP3P waters were added to a cubic box extending 11 Å beyond the outermost protein atoms,  
801 along with neutralizing counterions and sufficient excess NaCl to achieve an effective salt  
802 concentration of 300 mM. Another System with constrained bonds to hydrogen was created  
803 from the solvated structure and minimized. To minimally relax the structure before deploying  
804 simulations to Folding@Home, 5000 steps of Langevin dynamics were run using a Langevin  
805 integrator with a time step of 2.0 fs, temperature of 300.0 K, and collision rate of 5.0 ps<sup>-1</sup>.  
806 Nonbonded forces were modeled using the particle-mesh Ewald (PME) method with default  
807 parameters with a cutoff distance of 9.0 Å. All other settings remained at default values, except  
808 double precision was used throughout the minimization-and-equilibration process.

809 *Production simulation for WT amber simulations.* The resulting system, integrator, and  
810 state data from minimal equilibrations were serialized to XML format for simulation on  
811 Folding@Home using a simulation core based on OpenMM 6.3<sup>61,68</sup> for both the phosphorylated

812 and unphosphorylated systems. This entire process was repeated 5 times each phosphorylation  
813 simulations with and without Tpx2 to set up individual Folding@home RUNs, with each RUN  
814 representing a distinct initial configuration generated by the minimization-and-equilibration  
815 procedure. For each of the RUNs, 50 CLONEs with different initial random velocities and  
816 random seeds were simulated on Folding@home, where each clone ran for a maximum of 500  
817 ns (250 million Langevin dynamics steps of 2 fs timestep with all-atom output frames saved  
818 every 125,000 steps using single precision and a Monte Carlo Barostat with pressure of 1 atm,  
819 temperature of 300 Kelvin, and barostat update interval of 50 steps), generating over 100  $\mu$ s of  
820 aggregate simulation data for each of the WT conditions (with and without Tpx2).

821 *Modeling Spin Probe-labeled AurA.* Because AMBER parameters for spin probes were  
822 not widely available, we used the equivalently modern CHARMM36 generation of forcefields for  
823 MTSI-labeled AurA, simulated in complex with ADP and with and without both TPX2 and  
824 phosphorylation, for a total of four possible combinations per starting structure. Simulations  
825 were started from two different starting configurations: DFG-in (PDB ID: 1OL5) and DFG-out  
826 (5L8K<sup>50</sup>). For the crystal structure of 1OL5, Schrödinger's PrepWizard<sup>69</sup> (release 2016-4) was  
827 used to model in Tpx2 residues 23-29 (unresolved in 1OL5), and add in hydrogens at pH 7.4 for  
828 both protein residues and ADP. The protonation state of ADP was assigned the lowest energy  
829 state using Epik at pH 7.4 $\pm$ 2. Hydrogen bonding was optimized using PROPKA at pH 7.4 $\pm$ 2.  
830 The entire structure was minimized using OPLS3 and an RMSD convergence cutoff of 0.3 $\text{\AA}$ .  
831 Because TPX2 is not present in the 5L8K structure, the coordinates and crystal waters of Tpx2  
832 in 1OL5 after preparation were transferred to the unprepared 5L8K after aligning the kinase  
833 domain to 1OL5 and deleting the vNAR domain (chain B) from 5L8K. 5L8K was then prepared  
834 using the same protocol as above, removing any organic solvent molecules but retaining all  
835 crystal waters. All three structures were run through CHARMM-GUI Solvator tool<sup>70</sup>  
836 (<http://www.charmm-gui.org/?doc=input/solvator>). In the first stage of this tool, all crystal waters  
837 and magnesiums in the structures were retained, while sulfates were deleted. Tpx2 was

838 deleted at this stage for the non-Tpx2 conditions. Phosphorylation was either built in or deleted  
839 for the threonine at residue 288 in the second stage of the Solvator tool. Also in this stage,  
840 residues 225 and 284 were mutated to cysteines and the MTSI spin label (named CYR1) was  
841 added to those residues. C290 was mutated to serine in the unphosphorylated conditions and  
842 alanine in the phosphorylated conditions, to match the DEER experimental conditions. After the  
843 PDB file was generated, a rectangular solvent box was generated using 10Å edge distance fit to  
844 the protein size, with 300 mM NaCl placed using the Monte-Carlo method.

845 *Parameterization the MTSI labeled CHARMM simulations.* An OpenMM ForceField was  
846 instantiated using CHARMM36<sup>71</sup> force field parameters for the protein and water model, along  
847 with ADP, TPO, and CYR1 parameter files output by CHARMM-GUI.

848 *Minimization and equilibration for the MTSI labeled CHARMM simulations.* A local  
849 energy minimization was performed with no constraints on bonds or angles, which took place in  
850 the solvated water box output by CHARMM-GUI and loaded into an OpenMM object. After  
851 minimization, 5000 steps of NVT dynamics were run using a Langevin integrator with a time  
852 step of 1.0 fs, temperature of 50K and a collision rate of 90.0 ps<sup>-1</sup>. Nonbonded forces were  
853 modeled using the particle-mesh Ewald (PME) method with a cutoff distance of 9.0 Å. All other  
854 settings remained at default values, except mixed precision was used throughout. After this, a  
855 second equilibration was run using 500000 steps of NPT dynamics using a Langevin integrator  
856 with a temperature of 300 Kelvin, collision rate of 90 ps<sup>-1</sup>, and timestep of 2.0 fs. A Monte Carlo  
857 Barostat was used with pressure of 1 atm and barostat update interval of 50 steps. To minimally  
858 relax this structure, 500000 steps of Langevin dynamics were run using a Langevin integrator  
859 with a 2 fs time step, 300 K temperature, and a collision rate of 5 ps<sup>-1</sup>.

860 *Production simulation for MTSI labeled CHARMM simulations.* The resulting system,  
861 integrator, and state data from the minimization and equilibration were serialized to XML format  
862 for simulation on Folding@Home using a simulation core based on OpenMM 6.3. This was done  
863 for all 8 conditions, where each combination of starting structure, phosphorylation status and

864 Tpx2 status was set up as a RUN. For each of the RUNs, 100 CLONEs with different initial  
865 random velocities and random seeds were simulated on Folding@home, where each clone ran  
866 for a maximum of 3  $\mu$ s (1.5 billion Langevin dynamics steps with all-atom output frames saved  
867 every 250,000 steps using mixed precision and a Monte Carlo Barostat with pressure of 1 atm,  
868 300 Kelvin, and barostat frequency of 50). In aggregate, each of the 12 configurations totaled  
869 between 75-110  $\mu$ s per starting configuration.

870 *Data Analysis.* Distances and torsions were computed using the compute\_distances and  
871 compute\_dihedrals functions in MDTraj v 1.8.0. For the WT Amber simulations, the first 100 ns  
872 of each CLONE were discarded. The first 250 ns were discarded for the CHARMM MTSL  
873 labeled AurA simulations to allow sufficient relaxation following the introduction of spin probes.  
874 Distance probability plots were generated using Seaborn v0.8.1  
875 (<https://doi.org/10.5281/zenodo.883859>) distplot using the norm\_hist parameter. The distance  
876 and dihedral contour plots were generated using the kdeplot function in Seaborn v0.8.1. All  
877 analysis scripts have been made publically available<sup>9</sup>.

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