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A water-mediated allosteric network governs activation of Aurora kinase A

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Abstract

The catalytic activity of many protein kinases is controlled by conformational changes of a conserved Asp-Phe-Gly (DFG) motif. We used an infrared probe to track the DFG motif of the mitotic kinase Aurora A (AurA) and found that allosteric activation by the spindle-associated protein Tpx2 involves an equilibrium shift towards the active DFG-In state. Förster resonance energy transfer experiments show that the activation loop undergoes a nanometer-scale movement that is tightly coupled to the DFG equilibrium. Tpx2 further activates AurA by stabilizing a water-mediated allosteric network that links the C-helix to the active site through an unusual polar residue in the regulatory spine. The polar spine residue and water network of AurA are essential for phosphorylation-driven activation, but an alternative form of the water network found in related kinases can support Tpx2-driven activation, suggesting that variations in the water-mediated hydrogen bond network mediate regulatory diversification in protein kinases.

Introduction

Protein kinases orchestrate signaling pathways that control cellular functions ranging from metabolism to cell division¹. Kinase activity is tightly controlled in the cell, and the disruption of kinase regulation mechanisms is widely associated with cancer^{2–4}. Crystal structures have shown that kinase domains are regulated by switching between active and inactive states, involving large-scale conformational rearrangements of essential catalytic

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Author contributions

N.M.L. conceived and designed the project. S.C. performed the kinase activity assays and thermal shift assays, and S.C. and N.M.L. performed the infrared spectroscopy experiments. E.R. performed the fluorescence experiments. J.D.C. and J.B. conceived and performed the molecular dynamics simulations and analysis. N.M.L. and J.D.C. wrote the manuscript.

Competing financial interests

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elements⁵. Conformational switching is triggered by the binding of allosteric regulators or by phosphorylation, but how these trigger events are coupled to changes in the conformational equilibria and dynamics of the kinase to tightly control catalytic activity remains poorly understood. In this paper we address this question for the mitotic kinase Aurora A (AurA) by using a site-specific infrared probe to monitor the conformation of the catalytic Asp-Phe-Gly (DFG) motif, and Förster resonance energy transfer (FRET) to track the regulatory activation loop during allosteric activation.

AurA is a central player in mitosis, controlling mitotic entry, centrosome maturation and bipolar spindle assembly⁶. These disparate functions are mediated by two cellular pools of AurA, which are differentially localized to the centrosome and the mitotic spindle, and are regulated by distinct biochemical mechanisms. Centrosomal AurA is activated, like many protein kinases, by phosphorylation on a conserved site in the regulatory activation loop (T288)^{7,8}, and subsequently controls mitotic entry and centrosome maturation^{9,10}. Conversely, the pool of AurA that is recruited to the mitotic spindle and controls bipolar spindle assembly is activated by a unique mechanism involving binding to the spindle-associated protein Tpx2^{11,12}.

Activation of AurA by Tpx2 is independent of phosphorylation, and, in normal cells, spindle-associated AurA is maintained in the unphosphorylated state through the action of the protein phosphatase PP6^{13,14}. Inactivating mutations in PP6, observed in ~10% of melanoma, lead to hyperactivation of spindle-associated AurA and genomic instability^{15–17}. The binding of Tpx2 to unphosphorylated AurA results in a robust ~50-fold increase in kinase activity⁷, but the structural and dynamic changes that accompany Tpx2 binding and mediate allosteric activation remain unclear.

Regulation in many protein kinases is mediated by conformational changes of the DFG motif and the adjacent activation loop⁵. In the activated state, referred to as DFG-In, the DFG aspartate is oriented into the active site to coordinate Mg:ATP. Catalytic activity of the DFG-In state is further promoted by the formation of a hydrophobic “regulatory spine” involving the DFG phenylalanine and additional amino acids located on the C-helix and the N- and C-terminal lobes of the kinase, locking these structural elements together^{18,19}. In autoinhibited protein kinases the DFG motif often adopts inactive conformations, referred to as DFG-Out states, in which the DFG aspartate is repositioned to point out of the active site^{20,21}.

X-ray structures show AurA adopts the DFG-Out state when bound to a variety of kinase inhibitors^{22,23}, and the DFG-In state when bound to Tpx2²⁴. However, structures also show that AurA can adopt the DFG-In state when bound to nucleotide alone^{24–26}, and it remains unclear whether the DFG-In/Out transition occurs during activation. The only clear change upon Tpx2 binding is an adjustment of the C-terminal half of the activation loop that promotes peptide substrate binding²⁴. However, these data are difficult to reconcile with enzyme kinetics measurements, which show that Tpx2 binding is accompanied by a decrease in the K_m value for ATP, and an increase in the catalytic rate constant k_{cat} , pointing to allosteric communication between Tpx2 and the kinase active site^{7,27}. Tpx2 alters the

affinity of AurA for ATP-competitive inhibitors²⁸, suggesting this communication may be clinically relevant and targetable.

Here we resolve the confounding structural and kinetic data on AurA by showing that the kinase exists in a conformational equilibrium between DFG-In and DFG-Out states, and that Tpx2 causes a population shift towards the DFG-In state. Furthermore, Tpx2 promotes activity by stabilizing a polar analog of the regulatory spine that couples the C-helix to the DFG motif through a water-mediated hydrogen bond network. Reconstituting an alternative hydrogen bond network from a related kinase into AurA restores activation by Tpx2 but not by phosphorylation, suggesting that variations in the water network contribute to altered allosteric wiring in different kinases.

Results

Tpx2 triggers a shift towards the DFG-In state

The formation of a hydrophobic regulatory spine is a central feature of activated protein kinases^{18,19}. In AurA, however, the regulatory spine residue located on the C-helix is a polar glutamine (Q185), as opposed to the hydrophobic residues normally found at this position in protein kinases. The significance of this highly unusual feature of AurA is unknown. X-ray structures show that the Q185 sidechain points into a cavity in the active site where it can form hydrogen bonds with structured water molecules²⁴. The water binding sites, referred to as W1 and W2, are formed by the backbone amides of the aspartate and phenylalanine residues of the DFG motif, respectively, and are a characteristic feature of the DFG-In state in many protein kinases²⁹ (Figure 1a).

We reasoned that a spectroscopic probe introduced at the Q185 position of AurA might sense conformational changes of the DFG motif that accompany kinase activation through their effects on the bound water molecules. We used cysteine labeling in the context of a functional cysteine-light (“Cys-lite”) version of AurA to introduce a thiocyanate infrared (IR) probe³⁰ into the active site at the Q185 position (Figure 1b, Supplementary Results, Supplementary Figures 1, 2). The CN stretch vibration of the thiocyanate group undergoes predictable frequency shifts in response to changes in local electrostatics and hydrogen bonding, making it a useful probe of local polarity^{31,32}. We refer to the nitrile-labeled AurA as Q185CN.

We measured infrared absorbance spectra of AurA Q185CN in the apo form, and in the presence of saturating concentrations of either ADP or Tpx2 alone, or both ADP and Tpx2. While in the apo sample a single nitrile absorbance peak was observed, the addition of the ligands caused profound spectral changes in which the single peak splits into multiple absorbance bands (Figure 1c). Peak fitting revealed that the spectra were comprised of three absorbance bands: two outlying peaks, and a central peak that matches the peak position of the apo sample (Figure 1d). The addition of the two ligands leads to a concerted increase in the magnitude of the two outlying peaks, with a corresponding decrease in the magnitude of the central peak, pointing to an equilibrium that is shifted by the ligands.

The following peak assignments are based on solvatochromism studies (Figure 1e) and deuterium isotope effects (Supplementary Figure 3) and are discussed in more detail in the Online Methods. The blue- and red-shifted peaks promoted by the ligands arise from the DFG-In state, in which the Q185CN probe forms hydrogen bonds to the W1 and W2 water molecules coordinated to the DFG motif (Figure 1f), and appear as separate peaks in slow exchange due to the picosecond vibrational timescale. The large separation between these peaks ($>15\text{ cm}^{-1}$), compared to spectral shifts seen in aprotic solvents (Figure 1e), is due to two different hydrogen bond geometries that cause shifts of opposite sign. A linear hydrogen bond with W1 gives rise to the blue-shifted peak, whereas a bent (π) hydrogen bond with W2 gives rise to the red-shifted peak, consistent with previous work on nitrile probes^{33,34}. This situation arises because of the unique geometry of the DFG-In state in which two water binding sites are positioned in close proximity to the probe.

The position of the central absorbance band, which dominates the spectra of the apo protein, is consistent with the loss of the hydrogen bonds to W1 and W2, suggesting a rearrangement of the DFG motif to an inactive DFG-Out state. The existence of a two-state conformational equilibrium is supported by the concerted changes of the intensities of the red- and blue-shifted peaks (DFG-In), and the reciprocal changes in the central peak (DFG-Out), observed in spectra measured at different temperatures (Figure 1g).

A measure of the equilibrium constant for the DFG-In/Out equilibrium can be obtained from the integrals of the two absorbance bands assigned to the DFG-In state, and the single absorbance band assigned to the DFG-Out state (Figure 1d), within the approximation that the extinction coefficient of the probe is the same in all three states (see Online Methods). This analysis suggests a surprisingly balanced DFG equilibrium, with the DFG-In state significantly populated in the apo enzyme, and increased ~3-fold by Tpx2.

Tpx2 triggers a large-scale shift of the activation loop

To further investigate the conformational shift promoted by Tpx2, we used Förster resonance energy transfer (FRET) to track the position of the activation loop. Two fluorescent dyes, a donor and an acceptor, were incorporated by cysteine labeling, one on the tip of the activation loop (S284C), and one on a distal surface of the kinase domain (L225). This labeling scheme is expected to yield higher FRET efficiencies for inactive DFG-Out states than for the DFG-In state (Figure 2a). The kinase activity of labeled samples was ~30% of the unlabeled protein (Supplementary Figure 4). Titrations of Tpx2 and nucleotide caused pronounced increases in the relative fluorescence intensity of the donor dye (Figure 2a), consistent with a shift to a more active state, and revealed that the binding of ADP and Tpx2 is cooperative, with the affinities of both ligands increased in the presence of the other (Figure 2b).

X-ray structures show AurA adopting a variety of DFG-Out states, with the activation loop displaced across the active site cleft from its position in the DFG-In state by as little as 1 or by up to 4 nanometers^{22,23,35,36}. For each biochemical state of AurA we measured the distance between the dyes from the degree of donor quenching in the presence of acceptor (see Online Methods). The distance values derived from these FRET experiments represent ensemble averages, and in the case of a conformational equilibrium provide a qualitative

measure of the balance of the equilibrium. The data show that ADP and Tpx2 increase the apparent separation of the dyes by 3 and 5 angstroms, respectively, and 8 ± 1 angstrom for both ligands together (Figure 2c). The larger response to both ligands together is consistent with a shifting equilibrium, and provides a lower bound on the scale of the movement between the underlying structural states. These results are most consistent with apo AurA adopting a DFG-Out conformation observed in structures of the protein bound to an inhibitory nanobody³⁵ and the inhibitor VX-680³⁷, in which the tip of the activation loop moves ~ 1 nanometer from its position in the active state. In this DFG-Out state the N-terminal β -strand of the activation loop is shifted in register but remains clamped to the catalytic loop (Figure 2d), restricting movement of the loop compared to other DFG-Out states. A similar DFG-Out conformation has been observed in the tyrosine kinase Abl³⁸.

Although the FRET and IR experiments probe different structural elements of the protein, they are both consistent with Tpx2 promoting a transition from an autoinhibited DFG-Out state to an active DFG-In state. To further test whether the two methods probe the same conformational equilibrium, we compared the IR and FRET data obtained at different temperatures. The temperature dependence of the DFG equilibrium was determined by fitting variable-temperature IR spectra. The resulting Van 't Hoff plot (Figure 2e) shows that the equilibrium in the presence of Tpx2 exhibits significant temperature dependence, with the DFG-In state favored at lower temperature. Variable-temperature FRET experiments were performed for all four biochemical states, and the extracted distances plotted as a function of inverse temperature to facilitate comparison with the IR data (Figure 2f). A striking correspondence is apparent between the two sets of data, both in terms of the relative effects of ADP and Tpx2, as well as the temperature dependence.

These experiments provide a highly consistent view of AurA allostery in which the DFG motif and activation loop are tightly coupled and exist in a delicately balanced conformational equilibrium that is shifted towards the active DFG-In state in a graded fashion by nucleotide and Tpx2. This graded response appears too small to fully explain the 50-fold activation of AurA, suggesting that Tpx2 may also enhance the activity of the DFG-In state.

Tpx2 stabilizes an allosteric network in the active site

We wondered whether the unique polar spine residue of AurA (Q185) forms hydrogen bonding interactions that are important for catalytic activation. Since the interactions between the Q185CN probe and the W1 and W2 water molecules are not representative of the wildtype enzyme, we turned to molecular dynamics simulations to investigate how the native Q185 residue interacts with W1 and W2. Simulations were initiated from the x-ray structure of WT AurA bound to ADP and Tpx2 (pdb code 1OL5)²⁴, with Tpx2 removed from the starting structure in half the simulations to provide a model for the protein in the absence of Tpx2. For each initial structure (with and without Tpx2), a total of 250 independent simulations up to 500 nanoseconds in length, totaling over 100 microseconds of aggregate simulation time each, were run in explicit solvent using the distributed computing platform Folding@home. Simulations of the Q185L and Q185M mutants were prepared

similarly, with over 15 microseconds of aggregate simulation time collected for each mutant with and without Tpx2.

Inspection of the WT trajectories revealed that a specific hydrogen bonding geometry predominated in the active site cavity, with W1 and W2, and a third water molecule we refer to as W3, forming a ring of hydrogen bonds linking the catalytically essential Glu-Lys salt bridge³⁹ (E181-K162) and the alpha phosphate of the nucleotide (representative snapshot shown in Figure 3a). The Q185 sidechain forms hydrogen bonds with both the W1 and W2 water molecules, linking the water-mediated hydrogen bond network to the C-helix. The positions of the water molecules in the simulations are consistent with x-ray data on AurA and other kinases (see Figure 1a).

To assess the stability of the hydrogen bond network we calculated autocorrelation functions $C(t)$ for sites W1 and W2 in the presence and absence of Tpx2, measuring the probability of finding the *same* water molecule in the site both at some initial time t_0 and time t later (see Online Methods). Surprisingly, the autocorrelation functions exhibited a slow decay component on a timescale longer than 25 nanoseconds, indicating that subpopulations of waters with extremely long residence times occupy these sites some fraction of the time (Figure 3b). Both the populations and the residence times of this long-lived subpopulation were significantly increased in the presence of Tpx2. Simulations performed on Q185L and Q185M mutants showed much shorter correlation times for the long-lived subpopulations, indicating that the Q185 sidechain is important for stabilization (Figure 3c). Interestingly, the presence of Tpx2 also resulted in much lower RMSD values for the C-helix, and these lower values were associated with increased populations of long-lived water molecules (Figure 3d). Stabilization of the C-helix by Tpx2 was greatly reduced in the Q185L mutant (Figure 3d). Together these results suggest that the polar Q185 residue and the associated water-mediated hydrogen bonds form an allosteric network that couples Tpx2 and the C-helix to the active site.

The water network is essential for allosteric activation

To assess whether the polar properties of the Q185 residue are important for activation of AurA, we prepared variants of AurA that possess a canonical hydrophobic regulatory spine by replacing Q185 with the hydrophobic leucine and methionine residues found at this position in most other protein kinases. We found that the Q185 mutations severely compromise the kinase activity of unphosphorylated AurA in the presence of Tpx2, decreasing it by up to 100-fold (Figure 4a). To test whether Q185 is also important for activation of AurA by phosphorylation, we made the same mutations in the WT construct of AurA, which is homogeneously phosphorylated on T288 in the activation loop when purified from *E. coli*. Phosphorylation of the mutants was confirmed by mass spectrometry and western blotting (Supplementary Figure 6). While WT AurA is robustly activated by phosphorylation alone, the Q185 mutations strongly reduced the kinase activity of phosphorylated AurA in the absence of Tpx2 (Figure 4b). All mutants were well folded and possessed similar melting temperatures to WT AurA, as assessed by differential scanning fluorimetry (Supplementary Figure 7).

We also measured the kinase activity of the phosphorylated Q185 mutants in the presence of Tpx2. Strikingly, the addition of Tpx2 largely rescued the activity of the phosphorylated mutants (Figure 4c). We observed the same dramatic rescue effect with the Q185CN protein used for the IR experiments. Like the Q185L and Q185M mutants, the Q185CN protein exhibited low activity in both unphosphorylated and phosphorylated forms (Figure 4d,e), but in the presence of Tpx2 the phosphorylated form was robustly active (Figure 4e).

These results show that the Q185 mutants are not fundamentally defective, but require the combined actions of Tpx2 and phosphorylation for significant kinase activity, whereas WT AurA requires only one or the other. As these activation pathways are thought to be mutually exclusive in normal cells, where the spindle-associated pool of AurA is not phosphorylated and phosphorylated centrosomal AurA does not encounter Tpx2^{13,15}, Q185 may be essential for both cellular pools of AurA. However, in melanoma cells with mutated PP6, the spindle pool of AurA becomes phosphorylated on T288. Our data show that in this hyperactivated state, with the activation loop and C-helix clamped together by both Tpx2 and phosphorylation, the additional coupling provided by the Q185 sidechain is no longer necessary.

It is remarkable that the Q185M and Q185L mutations have such pronounced effects considering that three quarters of protein kinases have a leucine or methionine at this position in the regulatory spine, and that mutations that enhance the hydrophobic character of the spine increase catalytic activity in other kinases^{40,41}. Our experimental results strongly support the conclusion from the MD simulations that, in AurA, the polar glutamine and its hydrogen bonds with the W1 and W2 water molecules form an important allosteric network that couples the C-helix to the active site. This network appears to function independently from the conformational shift of the DFG motif and activation loop, as the IR data obtained with the Q185CN variant and the FRET data obtained with a native Q185 residue show very similar responses to Tpx2 (see Figure 2e,f).

A PKA-like allosteric network supports function in AurA

The Q185 residue is not conserved outside the Aurora kinase family, raising the question of whether the W1 and W2 water molecules play any functional role in other kinase families. X-ray structures of the related AGC family kinase PKA show that the structure of the cavity containing W1 and W2, and the positions of the waters, are different from AurA⁴². Firstly, a leucine-to-valine substitution in the cavity (L194 in AurA, V104 in PKA) results in shifted position of the nucleotide in PKA (Figure 5a). Secondly, although PKA has a leucine at the position equivalent to Q185, a polar threonine residue at a second cavity position (A273 in AurA, T183 in PKA) instead forms a hydrogen bond to one of the water molecules. These differences enforce an alternative pentagonal water network with 2 water molecules (W1 and W2), instead of the hexagonal 3-water structure in AurA (W1, W2, and W3). Note that similar polygonal hydrogen bond structures are common in liquid water and at protein interfaces^{43,44}.

To test whether a PKA-like water network could be reconstituted in AurA, we mutated the cavity residues in our Cys-lite construct to their counterparts in PKA, and purified this Q185L L194V A273T triple mutant in both phosphorylated and unphosphorylated forms.

Kinase assays performed with the unphosphorylated triple mutant showed that the additional mutations rescued the response to Tpx2, restoring it to a similar level as the unphosphorylated Cys-lite construct (Figure 5b). This dramatic result shows that a canonical hydrophobic regulatory spine can in fact support Tpx2-driven activation of AurA, but only in the context of the alternative water network of PKA.

In striking contrast, we found that the phosphorylated triple mutant lacked activity in the absence of Tpx2, but that activity was restored by Tpx2 (Figure 5c), much like the phosphorylated Q185L mutant (Figure 4c). It appears that the unusual Q185 residue and water network of AurA are essential for Tpx2-independent activation by phosphorylation. Presumably by tightly coupling the C-helix to the active site through the hydrogen bonds of the water network, Q185 makes further stabilization of the C-helix by Tpx2 largely redundant (Figure 5d). Interestingly, in PKA and most AGC kinases, a hydrophobic motif in the C-terminal tail docks onto the C-helix in manner that closely resembles the Tpx2:AurA interaction. Unlike AurA, this interaction is essential for PKA activity⁴⁵, presumably because the C-helix lacks the link to the water network provided by Q185 in AurA (Figure 5e).

The ability of the PKA-like water network to support activation of AurA by Tpx2 suggests a conserved role for the water network in kinase function. A multiple sequence alignment of the AGC kinases⁴⁶ showed that nearly half of them have identical cavity residues to PKA, suggesting they possess a similar water network. However, there is another group of AGC kinases in which the polar residue linked to the water network is instead located at a third cavity position (104 in PKA numbering) (Figure 5f). The x-ray structure of MRCK, a representative member of this group, shows that this leads to a more AurA-like hexagonal water network (Figure 5f)⁴⁷. Apparently there are several classes of water network in different kinases, distinguished by the position of the polar amino acid that participates in the hydrogen bonding. Considering our results with AurA, these variations may serve to differentially couple key structural elements of the kinase to mediate diverse regulatory functions in different kinase subfamilies.

Discussion

It is widely appreciated that conformational changes of the DFG motif are a central feature of allosteric regulation in protein kinases^{20,48}. X-ray structures give the impression that these structural changes function as a binary switch, with allosteric regulators trapping the kinase in the DFG-In or DFG-Out state. Here, we obtain a more nuanced view of allosteric activation by combining spectroscopic methods that track the DFG motif and activation loop with molecular dynamics simulations and biochemical assays probing a novel allosteric network in the active site. Our data reveal a delicate conformational balance between active and inactive states in AurA, in which allosteric activation is mediated only in part by an equilibrium shift towards the active state, and enhancement of the catalytic activity of the DFG-In state is an equally important feature of the activation mechanism.

The surprisingly balanced nature of the DFG equilibrium raises the intriguing possibility that some function of the kinase is dependent upon the equilibrium remaining readily

reversible⁴⁹, and that the two-part activation mechanism described here evolved to provide robust activation while satisfying this constraint. For instance, the observation that the equilibrium constant is close to unity for the activated AurA:Tp α 2 complex could point to a role for the DFG transition in the catalytic cycle, such as in nucleotide release⁵⁰.

The second activation step in AurA involves enhancing the catalytic activity of the DFG-In state by stabilizing a hydrogen bonding network interconnecting the C-helix, DFG motif and catalytic Glu-Lys salt bridge. In other protein kinases, assembly of these same structural elements is driven by hydrophobic interactions between regulatory spine residues^{18,19,40}. Interestingly, mutations that disrupt the hydrophobic character of the spine block activity in PKA just as mutations of Q185 do in AurA, and these effects can be similarly rescued by activation loop phosphorylation in PKA⁴⁰. We therefore envision that Q185 and the associated water network of AurA represent a polar analog of the regulatory spines of other protein kinases, performing a related function.

The spines and water networks of AurA and PKA are not interchangeable, however, as the latter cannot support activation of AurA by phosphorylation. Our results suggest that the unusual polar spine and water network of AurA may have evolved to substitute for the hydrophobic motif of AGC kinases by more tightly linking the C-helix to the DFG motif and other active site residues. The regulatory spine and water network may coevolve in protein kinases to tune the extent of allosteric coupling between the active site and diverse regulatory inputs.

Online Methods

Expression and Purification of AurA Constructs

Aurora A kinase domain constructs (human Aurora A, residues 122–403 containing an N-terminal hexahistidine tag) were expressed in BL21-DE3-RIL cells (Agilent) at 18°C overnight. Cell pellets were resuspended in lysis buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 10% glycerol, 20 mM imidazole) and lysed using an Emulsiflex C3 (Avestin). Lysates were centrifuged at 20,000 rpm for 1 hour, and loaded onto Ni NTA columns (GE), washed with lysis buffer, and eluted with elution buffer (20 mM HEPES, pH 7.5, 200 mM NaCl, 10% glycerol, 500 mM imidazole). Samples were desalted into desalting buffer (300 mM NaCl, 10% glycerol, 20 mM HEPES, pH 7.5). Samples were further purified by cation exchange chromatography after diluting 5-fold into buffer SP A (100 mM NaCl, 10% glycerol, 20 mM HEPES, pH 7.5) using a HiTrap SP cation exchange column (GE) and eluted with a 20 column volume gradient from 0% to 100% Buffer B (1 M NaCl, 10% glycerol, 20 mM HEPES, pH 7.5). Cation exchange chromatography was also used to separate phosphorylation states when necessary. Lambda protein phosphatase (New England Biolabs) was used to dephosphorylate AurA constructs when necessary.

The nitrile labeled Q185CN mutant was prepared using a cysteine-light (Cys-lite) construct of AurA in which all native DTNB-labile cysteine residues were mutated (C290S/A, C393S, C247A). Note that the C290S mutation results in the loss of autophosphorylation in *E. coli* due to the disruption of dimerization⁷, whereas C290A does not, allowing Cys-lite constructs to be conveniently prepared in either phosphorylated or unphosphorylated form.

The kinase activity of the phosphorylated and unphosphorylated forms of the Cys-lite construct are similar to their WT AurA counterparts (Supplementary Figure 1).

Infrared Spectroscopy

The purified Q185C mutant was labeled with a nitrile probe using a 1.5:1 molar ratio of DTNB (Ellman's reagent) followed by 50 mM KCN. Samples were run over cation exchange to remove unreacted DTNB and KCN. Homogeneous incorporation of single nitrile labels was confirmed by mass spectrometry (Supplementary Figure 2). Samples were prepared by concentrating labeled protein (~80 μ M) with and without excess Tpx2 (~160 μ M, approximate 100-fold above the reported K_D -value⁷), ADP (4 mM), and $MgCl_2$ (8 mM) in FTIR buffer (20 mM HEPES, pH 7.5, 300mM NaCl, 20% glycerol). Samples concentrated to ~1 mM were loaded into a calcium fluoride sample cell with an 80 μ m pathlength (Biotools), and infrared absorbance spectra were recorded on a Vertex 70 FTIR spectrometer (Bruker) equipped with a liquid nitrogen cooled indium antimonide detector, using 2 cm^{-1} spectral resolution, a zero-filling factor of 4, and 256 interferometer scans. Spectra were background-subtracted using the buffer flow-through from sample concentration and baselined using the polynomial method in the Opus software (Bruker). The height of the absorbance peaks for the apo and +ADP samples were ~1 mAU (1×10^{-3} absorbance units), and the +Tpx2 and +ADP+Tpx2 samples ~0.5 mAU. Measurements were taken in triplicate of the APO, ADP-bound, Tpx2-bound, and ADP+Tpx2-bound forms of the protein and averaged without smoothing. Peak positions of baselined spectra were verified using the 2nd-derivatives of the absorption spectra calculated prior to baselining. The 2nd-derivative spectra shown in Figure 1g had a smoothing function applied in the Opus software (17 smoothing points). Variable temperature experiments were performed using a TempCon-2X (Biotools) at 5 degree intervals from 5 °C to 40 °C. Samples were left to equilibrate for 10 minutes after each temperature change before acquiring spectra.

Spectral fitting of the Q185CN+ADP/+Tpx2 data was performed in the OPUS software using three Gaussian lineshapes with unconstrained peak positions, widths and maxima. The peak positions derived from these unconstrained fits matched the minima in the second derivatives of the absorbance. The peak positions of these basis functions (2148.7 cm^{-1} , 2158 cm^{-1} , and 2164.3 cm^{-1}) were subsequently constrained when fitting all other spectra. Representative fits and residuals are shown in Supplementary Figure 8; the RMS values for the residuals were in the range of 6–12 μ AU units (1–2% of the peak heights).

Deuterium isotope effects were measured by preparing samples in buffer (20 mM HEPES, pH7.5, 300 mM NaCl) prepared in D_2O (Sigma Aldrich). Protein samples were concentrated in the presence of a two-fold excess of Tpx2 (residues 1–43) to 100 μ L, followed by twice diluting the sample to 15 mL in D_2O buffer and concentrating to 1 mL. Deuterated glycerol (Sigma Aldrich) was added to a final concentration of 20% and the sample concentrated for infrared absorbance experiments.

Infrared Peak Assignments and Calculation of Equilibrium Constants

The red and blue-shifted peaks observed in spectra of the protein bound to Tpx2 are separated by >15 cm^{-1} . This shift is too large to arise from non-hydrogen bonding effects, as

demonstrated by IR measurements on ethylthiocyanate in aprotic solvents of widely varying polarity (see Figure 1e), indicating that the peaks arise from changes in the hydrogen bonding state of the Q185CN probe. To test whether the probe forms hydrogen bonds with water we performed IR experiments using samples prepared in deuterated buffer. Control experiments on the model compound ethyl thiocyanate in water showed the nitrile peak in D₂O is ~0.5 cm⁻¹ blue-shifted compared to the value in H₂O (Supplementary Figure 3a). In deuterium exchange experiments using the AurA Q185CN protein bound to ADP and Tpx2, we found that the isotope effect for the blue-shifted peak could not be reliably measured due to the confounding shoulder arising from the central peak, but we did observe a 0.5 cm⁻¹ isotope shift for the more isolated red-shifted peak. Strikingly, this shift is in the opposite direction to the shift observed for ethyl thiocyanate in solution, i.e. D₂O induced a *red-shift* of the peak instead of a blue-shift (Supplementary Figure 3b).

This puzzling result can be rationalized by considering previous *ab initio* calculations of acetonitrile-water clusters, which predicted that the nitrile stretch frequency can vary by >20 cm⁻¹ depending on the hydrogen bond angle, with a linear geometry causing a blue shift, and a bent geometry a red shift³⁴. This pointed to a model in which the linear hydrogen bond arises from an interaction between the probe and the W1 water molecule, and the bent hydrogen bond arises from an interaction with W2 (see Figure 1f). Based on the *ab initio* calculations the ~15 cm⁻¹ separation of the peaks is consistent with the bent hydrogen bond having an angle of ~100°, in good agreement with this structural model.

The integrated areas of the red- and blue-shifted peaks assigned to the DFG-In state, and the central peak assigned to the DFG-Out state, were calculated in OPUS and used to determine the populations of the DFG-In and DFG-Out states and the equilibrium constant for the DFG-In/Out transition. This analysis assumes that the oscillator strength of the nitrile vibration is the same for all three peaks. Although the oscillator strengths of the underlying states are unknown, we can place an upper bound on the differences based on a consideration of nitrile-containing compounds in solvents. The nitrile oscillator strength can increase by up to a factor of two on going from a non-hydrogen bonded to a hydrogen bonded state⁵¹. If the central band corresponds to a free nitrile, the blue-shifted peak could have an extinction coefficient up to twice that of the central band. This would lead to an error in the DFG-In population of less than 20%, and would be associated with consistent over- or underestimation of the equilibrium constants without altering the trends across different biochemical states.

Kinase Assays

Kinase activities of purified proteins were measured in triplicate using a coupled kinase assay (DiscoverX). Reactions were carried out in the ADP Quest Assay buffer (15 mM HEPES, pH 7.4, 20 mM NaCl, 1 mM EGTA, 0.02% TWEEN20, 10 mM MgCl₂, and 0.1 mg/mL bovine-γ-globulins). Assays were performed using 2.5 μM, 30 nM, 10 nM, or 2 nM protein depending on the activity of each kinase mutant, 10 μM Tpx2 (residues 1–43, Selleckchem), and 1 mM kemptide peptide substrate (Anaspec). Reactions were initiated by adding 50 μM ATP to each well of a 96-well black microplate (Corning). Samples were incubated at 30 °C in a fluorescence plate reader (Tecan Infinite M1000 PRO) for 30

minutes before starting reactions. Samples were excited at 500 nm and fluorescence emission recorded at 590 nm every 20 seconds for 240 cycles. Activity measurements were determined from the initial slopes of the fluorescence measurements as a function of time, determined by linear regression. Kinase activity was determined as the difference in activity between the samples with and without peptide substrate.

Differential Scanning Fluorimetry

Melting temperatures were determined using the Thermal Melt program on the StepOnePlus Real Time PCR System (Applied Biosystems) on the fast setting. The temperature ramp was from 25 °C to 95 °C, over 45 minutes. Each reaction mixture consisted of protein melt buffer (40mM HK₂PO₄, pH 7.6), 5.4 μM protein, 5× Sypro Orange (Life Technologies), with or without ADP (4 mM) and MgCl₂ (8 mM) and was performed in triplicate in a MicroAmp Fast 96-well Reaction Plate (Applied Biosystems). Melting temperatures were determined from the minima in the first derivatives of the fluorescence curves.

Förster resonance energy transfer experiments

Samples of AurA for intramolecular FRET experiments were prepared by double cysteine labeling. Two cysteine mutations were incorporated into the cysteine-lite construct (C290S C393S) of AurA by site directed mutagenesis, S284C on the activation loop, and L225C on the kinase D-helix. The first labeling step was performed with stoichiometric amounts of Alexa 488 maleimide donor dye (ThermoFisher), and singly-labeled protein was purified by ion exchange chromatography prior to labeling with an excess of Alexa 568 maleimide acceptor dye. A portion of the sample labeled only with Alexa 488 was used for donor-only control experiments. This labeling procedure resulted in homogeneous singly- and doubly-labeled samples as measured by mass spectrometry. Labeled samples retained 30–40% of the kinase activity of the unlabeled Cys-lite protein (Supplementary Figure 4).

Ligand titration FRET experiments were carried out in a Fluoromax 4 Spectrofluorometer (Horiba) at room temperature. Assays were performed in 15 mM HEPES, pH 7.4, 20 mM NaCl, 1 mM EGTA, 0.02% TWEEN20, 10 mM MgCl₂, and 0.1 mg/mL bovine-γ-globulins, at AurA concentrations of 20–50 nM.

For the temperature dependence studies, fluorescence spectra of samples containing 200–300 nM protein, 140 μM ADP, and/or 100 μM Tpx2 were measured on a Cary eclipse fluorimeter with peltier-based temperature controller. The buffer was the same as listed above, minus bovine-γ-globulins. The binding affinity of AurA for Tpx2 is highly temperature dependent (consistent with previous ITC data⁷), with K_D values determined by fluorescence of 0.3 μM at 10 °C, 1.5 μM at 22 °C, and 12 μM at 35 °C (Supplementary Figure 5). The variable temperature FRET experiments were therefore performed using 100 μM Tpx2 to ensure >90% saturation even at the high temperatures.

FRET efficiency values were calculated using the values of the donor fluorescence intensities measured for the donor and acceptor-labeled samples (F_{DA}) and the donor intensities measured for the donor-only control samples (F_D), according to the equation

$E = 1 - \frac{F_{DA}}{F_D}$. Distances were determined using the Förster inverse 6th-power law

$E = \frac{1}{1 + (r/R_0)^6}$, where r is the distance between the dyes, and R_0 is the Förster distance for the dye pair, using the established value of 62 angstroms for Alexa 488 and Alexa 568 (Thermo Fisher). Fluorescence anisotropy measurements confirmed that the anisotropy values of both dyes were below 0.2 in all samples under all measurement conditions (Supplementary Figure 9). Under these conditions the error in the inferred distances due to deviations of the orientation factor κ^2 from the isotropic value of 2/3 is expected to be less than ~25%⁵².

Western Blotting

Purified Aurora (50 ng) was run on a 4–20% Novex WedgeWell Tris-Glycine gel (Life Technologies). Protein was transferred to a 0.45 μ m pore size pure nitrocellulose membrane (VWR International) running at 10 V for 1 hour using a Mini Blot Module (Life Technologies). The membrane was blocked for 1 hour at room temperature in 5% BSA (Fisher Scientific) dissolved in PBST. The membrane was incubated with a 1:10,000 dilution of phospho-AurA (Thr288) Rabbit AB primary antibody (Cell Signaling Technology) for 1 hour at room temperature. The membrane was washed in PBST and then blocked in 5% BSA in PBST for one hour. The membrane was incubated with a 1:10,000 dilution of peroxidase-conjugated AffiniPure Donkey Anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc) for 45 minutes at room temperature. The membrane was washed with PBST. Supersignal Chemiluminescent Substrate (ThermoScientific) was added to the membrane and the membrane was visualized on a myECL imager (ThermoScientific).

Determination of the amino acids found at the regulatory spine and water cavity positions in human kinases

We used the multiple sequence alignments derived from the sequencing of the human genome⁴⁶ and freely available on the Kinase Phylogeny website (<http://kinase.com/web/current/human/phylogeny/>) to determine the residues found at the regulatory spine position equivalent to Q185 across all human kinases, and the residues found at the L194 and A273 cavity positions across the 63 AGC kinases. For the Q185 position the calculated residue frequencies are 59% Leu, 28% Met, 3.6% His, 2.3% Thr, 1.8% Val, 1.4% Gln, 1.6% Ser, 1.6% Cys, <1% Asn.

Protein Databank Search

We used the PDBeMotif server to search the Protein Databank for crystal structures of protein kinases determined in the DFG-In state bound to nucleotides. DFG constraints for the search were derived from the backbone torsion angles of the DFG motif in the x-ray structure of AurA bound to ADP and Tpx2 (1OL5). A total of 65 x-ray structures were identified bound to ADP (28), AMPPNP (18) and ATP (19). X-ray structures lacking bound magnesium ions in the active site were excluded from the analysis. The pdb codes of the structures shown in Figure 1a are 1OL5 (AurA), 1PHK, 1JJK and 3A99 (Phosphorylase Kinase, DAPK, and PIM1, CAMK family), 1L3R, 1O6K and 4UAK (PKA, AKT and

MRCK, AGC family), 3QHW and 1PYX (Cdk2 and GSK3b, CMGC family), 1DAW (CK2), 3DLS (PASK, CAMKL family), 4JDI (PAK4, STE family), 1IR3 (IRK, TK family), 3G2F (BMPR2, TKL family).

Molecular Dynamics Simulations

System Preparation

Modeling: WT AurA in complex with ADP was simulated with and without Tpx2. All simulations were started from the x-ray structure of WT AurA bound to Tpx2 and ADP in the presence of three magnesium ions (1OL5) in order to prevent biases from different starting configurations. From the crystal structure, PDBFixer version 1.2 was used to model in Tpx2 residues 23–29 (unresolved in 1OL5), add hydrogens belonging to standard protein residue protonation states corresponding to pH 7.4, and remove phosphorylation from threonine residues 287 and 288⁵³. Crystallographic waters were retained to prevent nonphysical collapse of hydrophilic pockets during minimization. For mutant simulations, point mutations were introduced by PDBFixer at this point. The chain containing Tpx2 was then removed for simulations without Tpx2 and retained for simulations with Tpx2. Sulfate ions present in the crystal structure were manually removed. The crystallographic ADP residue (containing only heavy atoms) was extracted from the structure and converted to a protonated Tripos mol2 file using OpenEye toolkit OEChem v2015.June⁵⁴. The protein structure was then loaded to an OpenMM version 7.0.1 *Modeller* object, and the protonated ADP was reintroduced through conversion from mol2 to OpenMM format via MDTraj 1.4.2^{53,55}.

Parameterization: An OpenMM *ForceField* was instantiated using AMBER99SBildn force field parameters⁵⁶ for the protein and TIP3P water model, along with ADP parameters generated by Carlson and accessed from the Amber Parameter Database(<http://sites.pharmacy.manchester.ac.uk/bryce/amber>)⁵⁷.

Minimization: Local energy minimization was performed in three separate steps in order to gradually introduce bond constraints. An OpenMM *System* was instantiated with no constraints on bonds or angles for the first minimization, which took place in vacuum (with crystallographic waters) with no constraints on bonds or angles. After this minimization, a new *System* was instantiated with constraints on the lengths of all bonds involving a hydrogen atom, and minimization was repeated. The structure and positions of all atoms were then put into a new OpenMM *Modeller* object, where TIP3P waters with NaCl ions to achieve an effective salt concentration of 300 mM was added to a cubic box extending 11.0 Å beyond the outermost protein atoms. Another *System* with constrained hydrogen-involved bonds was created from the solvated structure and minimized.

Equilibration: To minimally relax the structure before deploying simulations to Folding@Home, 5000 steps of Langevin dynamics were run using a Langevin integrator using at a time step of 2.0 fs, temperature of 300.0 K, and collision rate of 5.0 ps⁻¹. Nonbonded forces were modeled using the particle-mesh Ewald (PME) method with a cutoff distance of 9.0 Å. All other settings remained at default values.

Production simulation: The resulting system, integrator, and state data from minimal equilibration were serialized to XML format for simulation on Folding@Home using a simulation core based on OpenMM 6.3⁵³. This entire process was repeated 5 times each for the WT simulations with and without Tpx2, and once for each of the mutants considered above, to set up individual Folding@home RUNs, with each RUN representing a distinct initial configuration. For each of the RUNs, 50 CLONES with different initial random velocities and random seeds were simulated on Folding@home, where each clone ran for a maximum of 500 ns (250 million Langevin dynamics steps with all-atom output frames saved every 125,000 steps), generating over 100 μ s of aggregate simulation data for each of the WT conditions (with and without Tpx2) and over 10 μ s of aggregate simulation data for each of the mutant conditions.

Analysis: All analysis regarding formation of hydrogen bonds and salt bridges was performed using MDTraj 1.6. For the purposes of defining waters occupying the W1 and W2 sites, distances were computed between protein residues or atoms of interest and neighboring waters, using distance cutoffs between heavy atoms and no angle requirement. The W1 site was defined as any waters within 3.5 Å of D274 N, and W2 as any water within 4.1 Å of F275 N OR within 3.5 Å of both E181 and Q185 (any sidechain atom of both residues simultaneously). Trajectories were visually analyzed to qualitatively characterize the quantitative results using both Visual Molecular Dynamics (VMD) 1.9.2 and PyMOL 1.7⁵⁸. Autocorrelation functions for water molecules occupying the conserved sites were calculated using the `timeseries` module from the `pymbar` package^{59,60}, adapted to compute the autocorrelation of individual waters that remain within the water site definition. In order to report on how long the same water molecule resided within the site without exchanging to bulk solvent, correlation function computation utilized a scalar product $s(t_1, t_2)$ that assumed the value of unity if the *same* water molecule occupied the water site at both times t_1 and t_2 , and zero otherwise. The initial 50 ns of simulation data were discarded to equilibration prior to autocorrelation analysis. The resulting autocorrelation function $C(t)$ quantifies the probability that the same water is found within the site definition both at some initial time t_0 and a time t later, such that $C(0)$ is equal to the equilibrium probability of finding at least one water matching the water site criteria, and $C(t)$ approaches zero with large t due to the improbability of finding the same water in the site a very long time later. The population and autocorrelation time of the long-lived subpopulation was extracted by fitting the tail of the correlation function [15,75] ns with a single exponential using the `scipy.optimize.curve_fit` function, with the amplitude of the exponential representing the population and the timescale representing the autocorrelation time of these long-lived waters. 95% confidence intervals were determined by the bootstrap procedure, using 500 bootstrap replicates in which independent trajectories were drawn with replacement and fit quantities were computed over the ensemble of bootstrap replicates.

RMSD histograms: The alpha-carbon (CA) RMSD to the α C helix of 1OL5 (residues 175–188) was computed after aligning the trajectory frame using all AurA alpha carbons. 50 histogram bins spanning the range [0,15] Å RMSD were used. For histograms separated by long-lived and short-lived waters, long-lived waters were identified as those frames

containing at least one water matching the site definition for which at least one water was *still* located in the site 20 ns later.

Code availability: In house scripts used in data processing will be made freely available.

Data availability

The full molecular dynamics simulation data will be made publically available on an online repository such as GitHub.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Tpx2 induces a population shift towards the DFG-in state

(a) Crystal structures of protein kinases, aligned on the DFG motif, showing conserved water molecules (see Online Methods for pdb codes). The Q185 residue of AurA is shown. (b) Labeling scheme used to incorporate an infrared (IR) probe into AurA. (c) IR spectra of Q185CN in the apo form (black line and gray shading) in the presence of ADP (dashed pink line), Tpx2 (dashed blue line), and both ligands (solid blue line). Single representative spectra, normalized to the peak maxima. (d) Each panel shows the results of fitting IR spectra measured for an individual biochemical state at 25 °C. Thick lines are the experimental data colored as in panel c, thin dashed lines are numerical fits, and shaded peaks are individual fit components. Populations are derived from integrating the central gray peak (DFG-Out) and the outlying blue peaks (DFG-In). Single representative spectra and fitting results are shown. (e) The outlying peaks from the AurA spectra are compared with IR spectra of ethyl thiocyanate dissolved in aprotic solvents of varying polarity (DMSO, dimethyl sulfoxide, DMF, dimethylformamide, DCM, dichloromethane). (f) Models for the probe-water hydrogen bonds giving rise to the red-shifted (left) and blue-shifted (right) peaks in the Q185CN spectra. (g) Second derivatives of IR spectra of Q185CN bound to Tpx2 measured at temperatures from 5 to 40 °C. Arrows indicate features in the 2nd derivatives corresponding to peaks in the absorbance spectra. Their temperature-dependent amplitudes are shown in the inset.

Figure 2. Tpx2 promotes a nanometer-scale shift of the activation loop

(a) Left: schematics of AurA labeled with donor and acceptor dyes, showing the activation loop and the DFG aspartate for DFG-Out (gray) and DFG-In (blue) states. Right: emission spectra of donor-only (D-only) and donor + acceptor (D+A) samples in the presence of different concentrations of Tpx2. (b) Left panels: normalized donor peak intensities (donor/acceptor) are shown for titrations of Tpx2 and ADP performed with and without the other ligand. Right panels: binding constants for ADP and Tpx2, determined from fitting the data in the left panels. Mean values $\pm$ s.d.; $n=3$. (c) Ensemble-averaged distances between the donor and acceptor dyes measured by FRET are shown for Tpx2 titrations performed with and without ADP (see Online Methods). A single representative experiment is shown, except for the titration end points for which values represent the mean; $n=2$. (d) Structural model for the conformational change detected by FRET, based on x-ray structures of AurA bound to ADP and Tpx2 (blue, pdb code 1OL5) and to an inhibitory nanobody (gray, pdb code 5L8K). (e) Van 't Hoff plots of the DFG equilibrium for each biochemical state. Each data point is derived from the ratios of the DFG-In and DFG-Out populations given by the numerical fits of a single absorbance spectrum. (f) FRET-based distances for each biochemical state are plotted as a function of inverse temperature. Data represent mean values $\pm$ s.d.; $n=3$.

Figure 3. The Q185 spine residue participates in a water-mediated allosteric network

(a) Representative snapshot from one of the molecular dynamics simulations of WT AurA bound to ADP and Tpx2. Water molecules are shown as sticks, and their hydrogen bonding interactions with surrounding residues as dashed lines. (b) Autocorrelation functions calculated for W1 and W2 water site occupancies across all MD simulations of WT AurA. Black lines are fits to the exponential tails quantifying long-lived subpopulations, with populations and residence times for the long-lived populations shown in the legend with 95% confidence intervals (subscripts and superscripts). (c) Long-lived water lifetimes (top) and populations (bottom) for the W1 and W2 water sites in simulations of WT AurA and the Q185M and Q185L mutants, in the presence of Tpx2. Vertical black lines denote 95% confidence intervals. (d) Left panel: C α RMSD distributions for the C-helix from simulations of WT AurA (colored lines and shading) and Q185L (black lines) with and without Tpx2. Right panels: for the WT simulations performed in the presence of Tpx2, the probability distributions were recalculated for simulation frames with or without long-lived waters (>20 ns). The results are shown for W1 (top panel) and W2 (bottom panel) sites.

Figure 4. The Q185 residue is essential for allosteric activation of AurA

(a) Kinase assays of unphosphorylated Cys-lite AurA and the corresponding Q185M and Q185L mutants measured at 30 nM enzyme concentration with and without Tpx2. (b) Kinase assays of WT phosphorylated AurA and phosphorylated Q185 mutants measured at 2 nM enzyme concentration in the absence of Tpx2. (c) WT phosphorylated AurA and phosphorylated Q185 mutants measured at 2 nM enzyme concentration in the same experiment shown in panel b but in the presence of Tpx2. The activities in the absence of Tpx2 are shown for comparison with fold activation by Tpx2 indicated by arrows. **a-c** show mean values $\pm$ s.d.; $n=3$. (d) Top panel: Kinase activity of unphosphorylated Cys-lite Q185M, Q185L and Q185CN AurA measured at 1 μ M enzyme concentration. The values are the mean $\pm$ s.d. of the maximal activity derived from fitting three independent Tpx2 titrations to a single binding site model. Bottom panel: Normalized kinase activity of the mutants plotted as a function of Tpx2 concentration. Mean normalized values $\pm$ s.d.; $n=3$. (e) Kinase activity assays of the Cys-lite and Q185CN forms of AurA prepared in phosphorylated form measured at 30 nM enzyme concentration in the presence and absence of Tpx2. Fold activation by Tpx2 is indicated by arrows. Mean values $\pm$ s.d.; $n=3$.

Figure 5. A PKA-like water network supports activation of AurA by Tpx2, but not by phosphorylation

(a) Comparison of the active sites of AurA and PKA (pdb code 1L3R). The geometries of the solvent networks are highlighted by shaded lines, and polar residues that participate in the network are shown. The AurA model is from the molecular dynamics snapshot shown in Figure 3a. (b) Kinase assay showing the activity of the unphosphorylated Q185L L194V A273T triple mutant compared to the other Q185L mutants and the Cys-lite construct. (c) Kinase assays of the phosphorylated triple mutant and the phosphorylated Cys-lite kinase. The left panel shows the activities in the absence of Tpx2, the right panel shows the activities in the presence of Tpx2, with the increase due to Tpx2 indicated by the arrows. **b-c** show mean values $\pm$ s.d.; $n=3$. (d) The structure of phosphorylated AurA bound to ADP and Tpx2 (pdb code 1OL5) with Q185 and the water network highlighted (DFG motif sidechains not shown). The regulatory spine is in gray, and Tpx2 is magenta. (e) The structure of phosphorylated PKA (pdb code 1L3R) with the equivalent structural elements highlighted as in panel d. (f) Structural comparison of the active sites of the AGC kinases PKA and the MRCK β (myotonic dystrophy-related Cdc42-binding kinase)(pdb codes 1L3R and 4UAK). Residues participating in the water-mediated hydrogen bond networks are shaded and numbered using the PKA numbering. The inset shows the frequencies of particular amino acid combinations at these two positions in AGC kinases.