Using ancestral proteins to unravel the molecular mechanism and druggability of Aurora A kinase

Master’s Thesis

Presented to

The Faculty of the Graduate School of Arts and Sciences
Brandeis University
Department of Biochemistry
Dorothee Kern, Advisor

In Partial Fulfillment
of the Requirements for the Degree

Master of Science
in
Biochemistry

by
Yuejiao Zheng
May 2016
Copyright by
Yuejiao Zheng

© 2016
ABSTRACT

Using ancestral proteins to unravel the molecular mechanism and druggability of Aurora A kinase

A thesis presented to the Department of Biochemistry
Graduate School of Arts and Sciences
Brandeis University
Waltham, Massachusetts

By Yuejiao Zheng

Overexpression of Aurora A, a Ser/Thr kinase, underlies many human cancers. Aurora A’s activity is regulated by phosphorylation on the activation segment and/or binding of activator TPX2 in the PIF pocket of Aurora A. Despite more than a decade of studies on this important oncoprotein, the molecular mechanism of TPX2 binding and activation of Aurora A are still poorly understood. Particularly, pinpointing the allosteric network within Aurora A that is responsive to activation via TPX2, is as of yet, elusive.

We used Ancestral Sequence Reconstruction and a number of biophysical techniques to search for the necessary and sufficient residues on Aurora A needed to respond to the effect of TPX2. TPX2 is evolutionarily younger than Aurora A. Aurora A ancestors were reconstructed from eras pre-dating (Aur\textsubscript{ANC1} and Aur\textsubscript{ANC2}) and post-dating TPX2 (Aur\textsubscript{ANC3}, Aur\textsubscript{ANC4} and Aurora A\textsubscript{human}). The kinase activity of ancestral and modern Aurora species, binding affinity to TPX2 and activation by TPX2 was initially studied. Using site-directed mutagenesis, HPLC, ATP/NADH-coupled assays and ITC, we found that changes in Aurora sequences in the course of evolution serve to accommodate TPX2 binding and mediate allosteric activation. Moreover, a
network of 15 amino acid were shown to be necessary for eliciting the full allosteric activation response by TPX2.

In parallel, to determine whether the allosteric binding site of TPX2 (the PIF-pocket) could be used in drug design, we screened for monobodies that could elicit the opposite effect to TPX2 and thus inhibit Aurora A activity. This work led to the identification of several inhibitors and an activator of Aurora A kinase.

In conclusion, we show that a network of 15-residues is responsible for allosterically responding to TPX2 and that binding of other activators and inhibitors in the PIF-pocket (where TPX2 binds), favors either activation or inhibition of Aurora A kinase. Use of the PIF pocket as a drug-design hotspot could allow for synthesis of more specific, less toxic inhibitors of Aurora A kinase.
Table of Contents

Abstract..................................................................................................................................................iii
Table of Contents......................................................................................................................................v
List of Figures...........................................................................................................................................vii
List of Tables.............................................................................................................................................ix
Introduction................................................................................................................................................1
  Aurora and TPX2......................................................................................................................................1
  PIF binding pocket (PIF pocket)................................................................................................................3
  Ancestral Sequence Reconstruction.........................................................................................................4
  Monobodies...............................................................................................................................................4
Results and Discussions..........................................................................................................................6
  Ancestral Aurora Kinase Activity and TPX2 Activation..........................................................................6
  Monitoring TPX2 Binding and Activation in Ancestral Aurora using Site-Directed Mutagenesis........8
  Attempts at crystallization of Aur_{ANC4}, the youngest ancestral Aurora A species......................19
  Monobodies.............................................................................................................................................22
Conclusion.........................................................................................................................26
Material and Methods.......................................................................................................28
References.........................................................................................................................33
List of Figures

Figure 1. TPX2 activates Aurora A \textit{in vivo}................................................................. 2

Figure 2. The front and back view of TPX2 $^{1-45}$ bound to Aurora A$_{\text{modern}}$$^{122-403}$ in cartoon representation ................................................................. 3

Figure 3. Ancestral sequence reconstruction of Aurora A and TPX2................................................................. 6

Figure 4. Aurora A evolves to respond to TPX2 activation................................................................. 7

Figure 5. Sequence alignment of Aurora ancestors......................................................................................... 8

Figure 6. A structural comparison of wild type and Y199H Aurora A$_{\text{modern}}$................................................................. 9

Figure 7. TPX2 binds to Aurora A and activates Aurora A \textit{in vitro}. ................................................................. 10

Figure 8. ITC measurements for Y199H Aurora A$_{\text{modern}}$ and H199Y Aur$_{\text{ANC2}}$ ................................................................. 11

Figure 9. ATP/NADH-coupled assay for Y199H Aurora A$_{\text{modern}}$ and H199Y Aur$_{\text{ANC2}}$ ................................................................. 12

Figure 10. A structural comparison of wild type and H187N Aurora A$_{\text{modern}}$................................................................. 13

Figure 11. ITC measurements for H187N Aurora A$_{\text{modern}}$......................................................................................... 13

Figure 12. ATP/NADH-coupled assay for H187N Aurora A$_{\text{modern}}$ and N187H Aur$_{\text{ANC2}}$................................................................. 15

Figure 13. A structural comparison of wild type and E211H R232T Aurora A$_{\text{modern}}$................................................................. 16

Figure 14. HPLC assay for wild type and E211H R232T Aurora A$_{\text{modern}}$................................................................. 17

Figure 15. ATP/NADH-coupled assay for Aur$_{\text{ANC2}}$ and Aur$_{\text{ANC2}+15\text{aa}}$................................................................. 18

Figure 16. A structural comparison of wild type and Aur$_{\text{ANC2}+15\text{aa}}$. ................................................................. 19
Figure 17. Structural representation of Long Construct $\text{Aur}_{\text{ANC4}}^{122-403}$ ..................................................21

Figure 18. Preliminary screening for $\text{Aur}_{\text{ANC4}}^{122-403}$ crystal.................................................................21

Figure 19. Structural representation of T288V Avi-AurA-TPX2 chimera..............................................................22

Figure 20. HPLC assay for T288V Avi-AurA-TPX2 chimera..............................................................................23

Figure 21. ITC measurements for Aurora A$_{\text{modern}}$ bound to sGFP Mb2.........................................................24

Figure 22. HPLC assay for Mb1, Mb2, and sGFP Mb2..........................................................................................25

Figure 23. Scheme of the coupled assay..............................................................................................................31
List of Tables

Table 1. Testing Conditions for Aur\textsubscript{ANC4} Crystallization.............................................................................. 20
Introduction

Aurora A and TPX2

Aurora A, a serine/threonine kinase, has become an attractive target cancer therapeutics, given its important role in cell cycle regulation and thus cell proliferation (Aliagas-Martin et al. 2009; Bebbington et al. 2009; Cheok et al. 2010). During mitosis, Aurora A regulates centrosome separation and bipolar spindle assembly (Glover 2003; Giubettini et al. 2010). However, over-expression of Aurora A has been found in different mammalian tumor cell lines, where cells are show centrosomal abnormalities and chromosomal instability (Kallioniemi et al., 1994; Zhou et al., 1998; Jeng et al., 2004). Aurora A is known to regulate spindle formation by phosphorylating pole-area kinetochore to ensure correct chromosome alignment and kinetochore-microtubule connections (Ye et al. 2015).

TPX2, Aurora A’s specific binding protein, stabilizes Aurora A by forming a TPX2-Aurora A complex to prevent Aurora A from proteasome-dependent degradation (Giubettini et al. 2010) TPX2 also localizes Aurora A to the spindle microtubules during mitosis, which is a necessary step in Aurora A’s regulation of spindle formation (Carmena, Earnshaw 2003; Kufer et al. 2002)(Figure 1). It is previously reported that autophosphorylation of Aurora A’s T288 residue is correlated with increased catalytic activity (Littlepage et al., 2002). By directly measuring kinase activity using HPLC-based assays, Zorba et al proposed two activation
mechanisms for Aurora A: autophosphorylation at T288 and allosteric activation by TPX2 (Zorba et al. 2014). Autophosphorylation increases the catalytic activity of TPX2-independent, centrosome-associated dephosphorylated Aurora A by 100 fold. Comparably, TPX2-dependent, spindle-associated dephosphorylated Aurora A has a 50-fold increase in activity due to TPX2 binding (Zorba et al. 2014). TPX2 protects T288 by favoring movement of T288 to a solvent-inaccessible position that cannot be reached by phosphatases (Bayliss et al. 2003). Additionally, TPX2 activates by binding to Aurora A and inducing an active conformation of the dephosphorylated, lowly-active form of the kinase (Zorba et al. 2014).

Figure 1. TPX2 activates Aurora A \textit{in vivo}. Gradient of Ran-GTP facilitates TPX2’s release from Importin. TPX2 binds to Aurora A and activates it by interfering with protein phosphatase 1 γ (PP1γ). TPX2 then targets Aurora A to spindle microtubules and enables Aurora A to phosphorylate substrate such as pole-area kinetochore. Copied from Carmena, Earnshaw et al., 2003. (Carmena, Earnshaw et al., 2003)
PIF Binding Pocket (PIF pocket)

PDK1 interacting fragment (PIF) is a flexible segment of the protein kinase C-related kinase-2 (PRK2) that interacts with the kinase domain of PDK1 (Balendran et al. 1999). PIF specifically bind to a hydrophobic region in the N-lobe of Pdk1, also known as the “PIF-binding pocket” (Biondi 2002). Further studies showed that the PIF-binding pocket of PDK1 was the anchoring site of two more kinases in the AGC family, S6K1 and SGK1 (Biondi 2001). PIF-binding pockets, ubiquitous in AGC kinase family, are critical to kinase activity regulation and have become important docking sites for designing inhibitors that are specific for AGC kinases (Arencibia et al. 2013). Closely related to AGC kinases, Aurora A also has a PIF pocket (PIF pocket) analogous to that of AGC kinases with typical elements of β5 sheet, αB and αC helices (Biondi 2002; Bayliss et al. 2003). Aurora’s PIF pocket is found to be the binding site of TPX2, making this area a key interface for allosterically regulating Aurora A’s by its physiologically binding partner, TPX2 (Zorba et al. 2014) (Figure 2, PDB file: 1OL5). However, efforts to exploit Aurora A’s PIF pocket in drug design, are currently only rudimentary.

![Figure 2. The front and back view of TPX2 bound to Aurora A in cartoon representation (PDB 1OL5, Bayliss et al., 2003). Aurora A is in pink and TPX2 in purple. The dotted line shows missing electron density in TPX2, connecting the αhelix and the PIF-pocket-bound segment. Orange circles locate binding hotspot tyrosine 199.](image-url)
Ancestral Sequence Reconstruction

It is known that Aurora A is prevalent in most eukaryotes while TPX2 has been found only in animals and plants, which suggests that TPX2 appears later in the evolution timeframe than Aurora A (Goshima, Li 2011). To study the molecular characteristics of allosteric activation of Aurora A by TPX2, the approach that we used was to study the sequence diversity of Aurora A along the evolutionary lineage, in particular, the changes in sequence space that Aurora A incurred and how they affect binding to, and activation by, TPX2. To generate the sequences of Aurora A ancestors, ancestral sequences reconstruction techniques were used. In 1963, Linus Pauling and Emile Zuckerkandl proposed a strategy that can be used to resurrect ancestral sequences of a target protein by aligning sequences of related proteins found in contemporary species, later known as ancestral sequences reconstruction (Pauling et al. 1963). Designed based on Pauling and Zuckerkandl’s concept, Bali-Phy is a software that estimates phylogenies using Markov chain Monte Carlo and posterior samples (Suchard and Redelings, 2006). Christopher Wilson and Adelajda Zorba used Bali-Phy to construct the lineage of Aurora A, followed by PAML phylogeny optimization. Four Aurora A ancestors, two of them predating (Aur_{ANC1} and Aur_{ANC2}) and two of them postdating (Aur_{ANC3} and Aur_{ANC4}) the existence of TPX2, were resurrected.

Monobodies

Monobodies are antibody-like, small binding proteins modified from fibronectin type III domain (FN3) (Koide et al. 2012). Combinatorial libraries of FN3 are created by introducing mutations to antibody-equivalent complementarity determining regions (CDRs) as well as alternative surfaces (Koide et al. 2012). Monobodies have been used as specific binding
regulators to disrupt protein-protein interactions. When used as allosteric regulators for kinases, monobodies can either activate or inhibit the activity of the targeted kinase. Monobody HA4 activates Bcr-Abl by binding to Bcr-Abl’s SH2 domain, 1000 times tighter than phosphopeptides that mimic Abl’s natural cellular activators (Wojcik et al. 2010). In contrast, by binding to the same Bcr-Abl’s SH2 domain as HA4, monobody HA4-7c12 inhibits Bcr-Abl’s activity (Wojcik et al. 2010). Inspired by the therapeutic design of HA4 and HA4-7c12 based on the characteristics of protein-protein interfaces, we wanted to investigate if there are monobodies that regulate Aurora A’s kinase activity by binding to its PIF pocket with similar or better potency than Aurora A’s natural binding protein, TPX2. After studying the in vitro effects of monobodies binding to Aurora A, we then wanted to characterize the in vivo outcomes of Aurora A – inhibitory monobody interactions. In mammalian cells, super-negatively charged proteins are aggregation-resistant and can be loaded onto cationic, non-viral macromolecule delivery vehicles (Thompson et al. 2012). Therefore, in preparation for in vivo experiments, super-negatively charged, GFP tagged monobodies were constructed and purified.
Results and Discussion

Ancestral Aurora A Kinase Activity and TPX2 activation

To identify sites in Aurora A that are critical for allosteric regulation by TPX2, Christopher Wilson and Adelajda Zorba used Ancient Sequence Reconstruction to resurrect sequences of four ancestors of human Aurora A: Aur\textsubscript{ANC1}, Aur\textsubscript{ANC2}, Aur\textsubscript{ANC3}, and Aur\textsubscript{ANC4} (Figure 3).

Figure 3. Ancestral sequence reconstruction of Aurora A and TPX2. Lineages of Aurora kinase and TPX2 show that Aurora precedes TPX2 in the evolution timeframe (Christopher Wilson, Adelajda Zorba)
I investigated \textit{in vitro} kinase activity of four ancient Aurora As and Aurora A \textsubscript{modern} by conducting HPLC assays, with and without the presence of TPX2 (Figure 4). TPX \textsubscript{21-45} was chosen to carry out activity assays because this fragment is sufficient to activate Aurora A (Bayliss et al. 2003). We showed that all ancestral proteins are active on their own: dephosphorylated ancestral Aurora kinases are lowly active and phosphorylation drastically increases kinase activity, as expected (data not shown). We thus concluded that our Ancestral Sequence Reconstruction gave rise to viable, active kinases and is robust.

Additionally, Aurora As post-dating the appearance of TPX2, namely, Aurora A \textsubscript{ANC3}, Aurora A \textsubscript{ANC4}, and Aurora A \textsubscript{modern}, in addition to phosphorylation, they can also be activated by TPX2. In contrast, Aurora kinases pre-dating TPX2, cannot be activated by this binding partner. It is noteworthy that younger Aurora As demonstrate more folds of activation by TPX2. In all five sets of HPLC assays, I used T288V Aurora ancestors to prevent autophosphorylation in the experimental condition of 10 \textmu M enzyme.
Monitoring TPX2 Binding and Activation in Ancestral Aurora using Site-Directed Mutagenesis

In the evolutionary timeframe, TPX2 appears after the existence of Aur\textsubscript{ANC2} and before that of Aur\textsubscript{ANC3}. Sequence alignment of Aurora A ancestors shows 25 residue differences between Aur\textsubscript{ANC2} and Aur\textsubscript{ANC3} (Figure 5).
Figure 5. Sequence alignment of Aur\textsubscript{ANC1}, Aur\textsubscript{ANC2} and Aur\textsubscript{ANC3}. (Adelajda Zorba)

One of the 15 residues, Y199, is a binding hotspot in Aurora A\textsubscript{modern}'s PIF pocket and is known to interact with Y8, Y10, P13 of TPX2’s conserved segment \textsuperscript{8}YSYDAPS\textsuperscript{14} (Bayliss et al. 2003) (Figure 6, PDB file: 1OL5). Interestingly, in Aur\textsubscript{ANC1} and Aur\textsubscript{ANC2}, a histidine is at position 199, which is outside the hydrogen-bonding range with the aforementioned, potential interacting residues in TPX2. We thus expect that mutating Aurora A\textsubscript{modern} Y199 into histidine can disrupt Aurora A\textsubscript{modern}'s binding to TPX2.
Y199H Aurora A<sub>modern</sub> was successfully expressed in E. coli (BL21) and purified using the AKTA FPLC system. ITC data confirm the assumption that the Y199H mutation will cause weakened Aurora A – TPX2 interaction, with a TPX2 binding $K_d$ of 97.1 ± 6.7μM for Y199H Aurora A<sub>modern</sub> (Figure 8a), indicating a 20-fold decrease in TPX2 binding affinity when compared to $K_d$ of 5.4 ± 0.2μM for Aurora A<sub>modern</sub> (Figure 7a).
Figure 7. TPX2 binds to Aurora A and activates Aurora A in vitro. (a) Isothermal titration calorimetry (ITC) measurement conducted with 80μM Aurora A modern and 940μM TPX2\(^{1-45}\) shows a tight binding of \(K_d = 5.4 \pm 0.2\) μM. (b) TPX2 increases initial rate of dephosphorylated Aurora A modern to \(1.13\ s^{-1}\), a 15.9-fold increase compared to Aurora alone \((0.07s^{-1})\). Reactions were conducted in the presence of 1 μM Aurora A modern, 50 μM TPX2\(^{1-45}\), 5mM ATP, and 1mM Kemptide in assay buffer (20mM TrisHCl, 200mM NaCl, 20mM MgCl\(_2\), 3% (vol/vol) glycerol, 1mM TCEP, pH 7.50) at 25°C. Phosphorylated peptide production was monitored by reverse phase-high performance liquid chromatography (RP-HPLC). (c) TPX2 increases overall catalytic efficiency of Aurora A modern by increasing \(k_{cat}\) and decreasing \(K_M\) for Lat2 peptide. ATP/NADH-coupled assay was used to monitor Lats2 phosphorylation. Reactions were carried in the presence of 1μM dephosphorylated Aurora or 0.25μM dephosphorylated Aurora with 500μM TPX2 and saturated with 5mM ATP in assay buffer (20mM TrisHCl, 200mM NaCl, 20mM MgCl\(_2\), 10% (v/v) glycerol, 1mM TCEP, pH 7.50) at 25°C.
Reverse mutation of H199Y was introduced to Aur\textsubscript{ANC2} with a $K_d$ of $135.8 \pm 6.3 \mu M$ (Figure 8b). Comparing to a $K_d$ of $160.6 \pm 22.2 \mu M$ for Aur\textsubscript{ANC2} (measured by Adelajda Zorba, data not shown in figures), the H199Y Aur\textsubscript{ANC2} mutant slightly favors TPX2 binding but it’s far from achieving the tighter binding ranges of Aurora A\textsubscript{modern} – TPX2.

In NADH/ATP assays, the turnover of mutants are characterized by Michaelis-Menten kinetics. Results show that the Y199H mutation in Aurora A\textsubscript{modern} decreases not only the initial TPX2 activation rate at low substrate concentration, but also the overall fold increase in TPX2 activation (Figure 9a). However, the reverse mutant H199Y Aur\textsubscript{ANC2} demonstrates no change in initial rate or fold increase in TPX2 activation (Figure 9b). ITC and ATP/NADH-coupled assay results indicate that H199Y mutation cannot fully restore TPX2 binding in an Aurora ancestor.
that predates the existence of TPX2 and has not developed a TPX2-responding system. Despite slight establishment of TPX2 binding, the H199Y mutation does not allow Aur\textsubscript{ANC2} to respond to allosteric activation by TPX2. The results encouraged me to further look into other sites that are responsible for the interaction as well as activation by TPX2.

Bayliss et al reported that in TPX2, a conserved residue W\textsubscript{34} packed against the αC-helix which is a crucial element of Aurora A’s catalytic core. Specifically, TPX2’s W\textsubscript{34} forms a π-π interaction with Aurora A\textsubscript{modern}’s H187 (Figure 10, PDB file: 1OL5). However, this π-π interaction would be absent if TPX2 were to bind to Aur\textsubscript{ANC2}. Instead of a histidine, Aur\textsubscript{ANC2} has an asparagine at 187 which forms a plane perpendicular to the plane of TPX2’s W\textsubscript{34}. 

Figure 9. ATP/NADH-coupled assay for Y199H Aurora A\textsubscript{modern} and H199Y Aur\textsubscript{ANC2}. (a) Y199H decreases fold of activation by TPX2 from 10.6 fold to 4.3 fold. (b) H199Y Aur\textsubscript{ANC2} does not increase activation by TPX2. ATP/NADH-coupled assay was used to monitor Lats2 phosphorylation. Reactions were carried in the presence of 1μM dephosphorylated Aurora or 0.25μM dephosphorylated Aurora with 500μM TPX2 and saturated with 5mM ATP in assay buffer (20mM TrisHCl, 200mM NaCl, 20mM MgCl\textsubscript{2}, 10% (v/v) glycerol, 1mM TCEP, pH 7.50) at 25 °C.
Similar to the Y199H and H199Y mutation pair, I introduced H187N to Aurora A\textsubscript{modern} and N187H to the previous mutant, H199Y Aur\textsubscript{ANC2}, of which the binding affinity to TPX2 was slightly improved. TPX2 binding affinity for H187N Aurora A\textsubscript{modern} is comparable to the wild type, suggesting that the absence of π-π interaction does not affect binding (Figure 11).

Figure 10. A structural comparison of wild type and H187N Aurora A\textsubscript{modern}. Residue 187 is a histidine in Aurora ancestors postdating the existence of TPX2 (Aur\textsubscript{ANC3}, Aur\textsubscript{ANC4}, and Aurora A\textsubscript{modern}), but an asparagine in Aurora ancestors predating the existence of TPX2 (Aur\textsubscript{ANC1} and Aur\textsubscript{ANC2}). In the wild type, H187 interacts with TPX2’s W34 through π stacking; in the mutant, N187 lacks a ring structure to interact with W34.

Figure 11. ITC measurements conducted with 35μM H187N Aurora A\textsubscript{modern} and 248μM TPX2\textsubscript{1-45} show undisturbed binding of K\textsubscript{d}=9.9±0.5μM, within the same magnitude with wild type Aurora A\textsubscript{modern}’s affinity to TPX2\textsubscript{1-45} (K\textsubscript{d}=5.4±0.2μM).
H187N Aurora A\textsubscript{modern} can still be activated by TPX2, but the fold increase at substrate concentration of 1mM is smaller (8.7 fold) compared to the effect of TPX2 to the wild type (10.7 fold). Along the same line, the $K_M$ of Lats2 is 9.3 mM for the H187N mutant, which is slightly higher than the $K_M$ for Aurora A\textsubscript{modern} on its own (8.4 mM). ITC and activity data manifest that the $\pi-\pi$ interaction between Aurora A\textsubscript{modern}'s H187 and TPX2’s W34 does not directly affect binding to TPX2; nevertheless, this interaction is essential for TPX2 activation. Activity assay results of the reverse double mutant N187H/H199Y Aur\textsubscript{ANC2} also align with this proposal, which shows that at 1mM Lats2 concentration, the initial activation by TPX2 is 1.8 fold, a higher fold increase compared to H199Y Aur\textsubscript{ANC2}'s response to TPX2 activation (1.2 fold) (Figure 12). However, the reverse mutation only results in a minimal decrease in $K_M$ and even a 14% decrease in $k_{cat}$. The results suggest that H187 is an important site for TPX2 activation in Aurora A\textsubscript{modern}, however, only by mutating the respective residue in Aur\textsubscript{ANC2} cannot restore the full activation observed in Aurora A\textsubscript{modern}. Therefore, we continued our screening for more residues responsible for TPX2 activation, within as well as remote from the PIF pocket.

Figure 12. ATP/NADH-coupled assay for H187N Aurora A\textsubscript{modern} and N187H Aur\textsubscript{ANC2}. H187N leads to minimal decrease in fold of activation by TPX2 from 10.6 fold (wild type) to 8.7 fold. N187H Aur\textsubscript{ANC2} does not increase activation by TPX2. ATP/NADH-coupled assay was used to monitor Lats2 phosphorylation. Reactions were carried in the presence of 1$\mu$M dephosphorylated Aurora or 0.25$\mu$M dephosphorylated Aurora with 500$\mu$M TPX2 and saturated with 5mM ATP in assay buffer (20mM TrisHCl, 200mM NaCl, 20mM MgCl\textsubscript{2}, 10% (v/v) glycerol, 1mM TCEP, pH 7.50) at 25 °C.
With the help of our collaborator, computational biologist Dina Mirijinian, two residues in Aurora A\textsubscript{modern}, E221 and R232, were identified as potential regulatory residues for TPX2 activation. Although E221 and R232 are far from the TPX2 binding pocket, they are involved in forming a salt bridge based on the structure of Aurora A\textsubscript{modern} (Figure 13, PDB file: 1OL5).

Figure 13. A structural comparison of wild type and E211H R232T Aurora A\textsubscript{modern}. Residue 211 and 232 are occupied by glutamate and arginine, which forms a salt bridge, in Aurora ancestors postdating the existence of TPX2 (Aur\textsubscript{ANC3}, Aur\textsubscript{ANC4}, and Aurora A\textsubscript{modern}). These two residues are histidine and threonine, respectively, in Aurora ancestors predating the existence of TPX2 (Aur\textsubscript{ANC1} and Aur\textsubscript{ANC2}).

I mutated these two residues to H221 and T232, residues found in Aur\textsubscript{ANC1} and Aur\textsubscript{ANC2}. The Activity assays showed that the activity of the double mutant E221H/R232T Aurora A\textsubscript{modern} can still be increased by TPX2 by 11.5 fold (Figure 14). This is a minimal decrease in activation, compared with the activity of TPX2 activation on Aurora A\textsubscript{modern} (15.9 fold), when substrate phosphorylation is monitored on Kemptide. Therefore, the data indicate that the two residues, when mutated to their pre-TPX2 era Aurora kinase counterparts, brought about less activation by TPX2, as expected, however, they were not completely irresponsive to TPX2 as was the case with Aur\textsubscript{ANC1} and Aur\textsubscript{ANC2}. 
Having explored residues within and outside the PIF pocket, we conclude that a single-site mutagenesis can slightly improve TPX2 binding in Aur\textsubscript{ANC2}, an ancestor that predates the appearance of TPX2. Additionally, we observed an interesting contradiction where some single/double-site mutageneses can partially decrease TPX2 activation in Aurora A\textsubscript{modern}, but the reverse mutations in Aur\textsubscript{ANC2} cannot help the ancestor feel the activation by TPX2. Inspired by this paradox, we postulated that along the evolutionary lineage of Aurora A, a group of residues might be responsible for Aurora As’ ability to respond to TPX2, as opposed to distinct single or double residue substitutions. Recall that a difference of 25 residues marked the transition from Aur\textsubscript{ANC2} to Aur\textsubscript{ANC3}. Of the 25 residues, we selected 15 residues that we hypothesized were needed in order to respond to the presence of TPX2. These 15 residues fulfill the following two requirements: 1) conserved among Aur\textsubscript{ANC3}, Aur\textsubscript{ANC4}, and Aurora A\textsubscript{modern}; 2) conserved between Aurora A\textsubscript{modern} and Aurora B. Aurora B is another member of the Aurora kinase family that

![HPLC assay for wild type and E211H R232T Aurora A\textsubscript{modern}](image)

Figure 14. HPLC assay for wild type and E211H R232T Aurora A\textsubscript{modern}. Mutations in E221 and R232 lead to minimal decrease in TPX2 activation compared to TPX2 activation in Aurora A\textsubscript{modern}; thus, these two residues alone are not sufficient to facilitate TPX2 activation. Reactions were conducted in the presence of 1 μM protein, with or without 400μM TPX2\textsubscript{1−45}, 5mM ATP, and 1mM Kemptide in assay buffer (20mM TrisHCl, 200 mM NaCl, 20mM MgCl\textsubscript{2}, 3% (vol/vol) glycerol, 1mM TCEP, pH 7.50) at 25°C. Phosphorylated peptide production was monitored by reverse phase-high performance liquid chromatography (RP-HPLC).
shares 70% sequence identity (in its kinase domain alone) with Aurora A. Aurora B is responsive to allosteric activation by its own binding partner, INCEP. We thus postulated that an allosteric network must be present in Aurora B as it is present in Aurora A (Carmena, Earnshaw 2003). Sequence alignment between Aurora A\textsubscript{modern} and Aurora B enables us to discard residues that are redundant in the developmental process of an allosteric network. Adelajda Zorba designed a construct of Aur\textsubscript{ANC2}+15aa and used ITC experiments to show that this construct processes a TPX2 binding affinity ($K_d=4.2 \pm 3.7\mu M$, data not shown) comparable to Aurora A\textsubscript{modern} ($K_d=5.4 \pm 0.2\mu M$) (Figure 7).

I then conducted ATP/NADH-coupled Assays for the Aur\textsubscript{ANC2}+15aa construct, with and without the existence of TPX2 (Figure 15). Assays show that this construct can feel the effect of TPX2 much better than the previous site-directed mutants, with a significant $K_M$ tightening effect and an increase in the initial phosphorylation rate at low substrate concentrations.

![Graph 1](image1)

**Figure 15.** Group mutation establishes TPX2 activation in Aur\textsubscript{ANC2}, an ancestor that originally does not respond to TPX2. ATP/NADH-coupled assay was used to monitor Lats2 phosphorylation. Reactions were carried in the presence of 1μM dephosphorylated Aurora or 0.25μM dephosphorylated Aurora with 500μM TPX2 and saturated with 5mM ATP in assay buffer (20mM TrisHCl, 200mM NaCl, 20mM MgCl\textsubscript{2}, 10% (v/v) glycerol, 1mM TCEP, pH 7.50) at 25 °C.
Therefore, this 15-residue group is the smallest network we have found so far to re-establish the binding as well as activation by TPX2, in an ancestral Aurora A that is not responsive to TPX2 by nature. Based on the structure of dephosphorylated Aurora A modern (Figure 16, PDB file: 1OL5), the 15 residues locate across the span of the entire kinase, with the majority of them in α-helices.

![Figure 16](image)

Figure 16. A structural comparison of wild type and Aur\(\text{ANC2+15aa}\). Aurora A \(\text{modern}\)’s structure is used as a reference to locate the 15 residues in Aur\(\text{ANC2+15aa}\). We assume Aur\(\text{ANC2}\) adopts a similar structure as Aurora A modern.

**Attempts at crystallization of Aur\(\text{ANC4, the youngest}\) ancestral Aurora A species**

As confirmed by the previous mutagenesis results, computational biology is a powerful tool in determining important residues in allosteric activation. However, an X-ray structure of the ancient Aurora kinase would ultimately be the best way to discover residues important in
allosteric activation. The goal of determining the X-Ray structure of ancestral Aurora A kinases in the presence and absence of TPX2 is to obtain a snapshot of the structural determinants of the interaction between ancestral Aurora A species and TPX2. I worked on obtaining the structure of Aur\textsubscript{ANC4}\textsuperscript{133-388} ancestral Aurora A in an effort to ultimately determine specific and highly potent druggable site in Aurora A kinase. I chose Aur\textsubscript{ANC4}\textsuperscript{133-388} to start with because it is the youngest ancestral Aurora A and presumably it can feel the activation by TPX2 best, among all the ancestral Aurora A species. I used an E.coli-based expression system in the presence of phosphatase \lambda PP to produce dephosphorylated Aur\textsubscript{ANC4}\textsuperscript{133-388}. I tested 5 conditions (CZ1-5) (Table 1) using the hanging drop and sitting drop methods, as is shown in table 1.

<table>
<thead>
<tr>
<th>CZ_1</th>
<th>pH=5.5</th>
<th>CZ_2</th>
<th>pH=7.5</th>
<th>CZ_3</th>
<th>pH=5.5</th>
<th>CZ_4</th>
<th>pH=6.5</th>
<th>CZ_5</th>
<th>pH=7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M</td>
<td>MES</td>
<td>0.1M</td>
<td>MES</td>
<td>0.1M</td>
<td>MES</td>
<td>0.1M</td>
<td>MES</td>
<td>0.1M</td>
<td>MES</td>
</tr>
<tr>
<td>0.2M</td>
<td>Ammonium Sulfate</td>
<td>0.2M</td>
<td>Ammonium Sulfate</td>
<td>0.2M</td>
<td>Ammonium Sulfate</td>
<td>0.2M</td>
<td>Ammonium Sulfate</td>
<td>0.2M</td>
<td>Ammonium Sulfate</td>
</tr>
<tr>
<td>25%(w/v)</td>
<td>PEG5000 MME</td>
<td>25%(w/v)</td>
<td>PEG5000 MME</td>
<td>30%(w/v)</td>
<td>PEG5000 MME</td>
<td>30%(w/v)</td>
<td>PEG5000 MME</td>
<td>30%(w/v)</td>
<td>PEG5000 MME</td>
</tr>
<tr>
<td>CZ_6</td>
<td>pH=5.5</td>
<td>CZ_7</td>
<td>pH=7.5</td>
<td>CZ_8</td>
<td>pH=6.5</td>
<td>CZ_9</td>
<td>pH=5.5</td>
<td>CZ_10</td>
<td>pH=7.5</td>
</tr>
<tr>
<td>0.1M</td>
<td>MES</td>
<td>0.1M</td>
<td>MES</td>
<td>0.1M</td>
<td>MES</td>
<td>0.1M</td>
<td>MES</td>
<td>0.1M</td>
<td>MES</td>
</tr>
<tr>
<td>0.2M</td>
<td>Ammonium Sulfate</td>
<td>0.2M</td>
<td>Ammonium Sulfate</td>
<td>0.2M</td>
<td>Ammonium Sulfate</td>
<td>0.2M</td>
<td>Ammonium Sulfate</td>
<td>0.2M</td>
<td>Ammonium Sulfate</td>
</tr>
<tr>
<td>30%(w/v)</td>
<td>PEG8000 MME</td>
<td>30%(w/v)</td>
<td>PEG8000 MME</td>
<td>25%(w/v)</td>
<td>PEG8000 MME</td>
<td>25%(w/v)</td>
<td>PEG8000 MME</td>
<td>25%(w/v)</td>
<td>PEG8000 MME</td>
</tr>
<tr>
<td>4% (v/v)</td>
<td>1,3-propanediol</td>
<td>4% (v/v)</td>
<td>1,3-propanediol</td>
<td>4% (v/v)</td>
<td>1,3-propanediol</td>
<td>4% (v/v)</td>
<td>1,3-propanediol</td>
<td>4% (v/v)</td>
<td>1,3-propanediol</td>
</tr>
</tbody>
</table>

Table 1. Testing Conditions for Aur\textsubscript{ANC4} Crystallization.

However, no crystals were seeded in the end. We proposed that it was because the Aur\textsubscript{ANC4}\textsuperscript{133-388} sequence I used was a fragment of the common sequence, Aur\textsubscript{ANC4}\textsuperscript{122-403}. We speculated that the shorter Aur\textsubscript{ANC4}\textsuperscript{133-388} was not enough for crystal anchoring because it is lacks the flanking glycine-rich sequences that are important for forming secondary turn structures before the beta sheet structure at the N-terminus and after the alpha helix at the C-terminus (Figure 17).
Thus, I designed a longer construct, Aur\textsubscript{ANC4}^{122-403} by adding residue 122-132 to the N-terminal and residue 389-403 to the C-terminal. Same crystallization methods were used for Aur\textsubscript{ANC4}^{122-403}, under testing conditions CZ1-10 (Table 1). Condition CZ 2 showed crystal-like compound on Day 0 (Figure 18), but no crystals formed in the end. Therefore, more conditions need to be tested in future experiments.

Figure 17. Structural representation of Long Construct Aur\textsubscript{ANC4}^{122-403}. Labeled in red are sequences added to the N-terminal and C-terminal of Aur\textsubscript{ANC4}^{133-388}.

Figure 18. Preliminary screening for Aur\textsubscript{ANC4}^{122-403} crystal. Condition CZ2 on Day 0 showed possible crystal formation, but no crystal was formed in the end.
Monobodies

Guided by Aurora A’s activation mechanisms involving the PIF pocket and TPX2 allosteric network, we wanted to advance the study of allosteric interaction into the field of drug design. Monobodies are well-known for their high target affinity and specificity, however, they have not been extensively used as binding proteins for serine/threonine kinases. In order to screen for monobodies that bind to the PIF pocket, I constructed T288V Avi-AurA-TPX2 chimera, a recombinant fusion protein created by joining Aurora A\textsubscript{modern} and TPX2\textsuperscript{1-45} with a flexible 33-residue linker (Figure 19, PDB file 1OL5). The Avi-tag allowed for biotinylation of Aurora A which was necessary in anchoring Aurora A to streptavidin beads and thus selecting monobodies of interest.

![Figure 19. Structural representation of T288V Avi-AurA-TPX2 chimera. Aurora in pink, TPX2 in purple, flexible 33-residue linker in green. Orange circle shows binding hotspot Y199.](image)

Figure 20 shows the results from HPLC activity assays, which indicate that the chimeric protein is more active than T288V Aurora A\textsubscript{modern}. The results imply that the Aurora A
component of the T288V Avi-AurA-TPX2 chimera can be activated by the TPX2 component.
Due to the length limit of the linker, the TPX2 component might not be able to position itself well to exert a full activation of 15.9 fold on Aurora A_{modern}.

Later, through a phage display library of fibronectin type III (FN3), our collaborators, Akiko Koide and Shohei Koide, selected monobodies that bind to Avi-AurA but not to Avi-AurA-TPX2 chimera. The positive selection through Avi-AurA binding screens monobodies that binding to AurA unspecifically, while the negative selection through Avi-AurA-TPX2 chimera binding enables us to identify and discard monobodies that do not bind to the PIF pocket, as the PIF pocket is closed off in the fusion protein by its TPX2 component. More negative selections were conducted using Y199H (characterized above) and Y199K (H and K disrupt TPX2 binding.

Figure 20. HPLC assay for T288V Avi-AurA-TPX2 chimera. T288V Avi-AurA-TPX2 chimera has a higher activity than TV Aurora A_{modern} while a lower than the TPX2-activated TV Aurora A_{modern}. Reactions were conducted in the presence of 1 μM protein, with or without 500μM TPX2^{1-45}, 5mM ATP, and 1mM Kemptide in assay buffer (20mM TrisHCl, 200mM NaCl, 20mM MgCl\textsubscript{2}, 3% (vol/vol) glycerol, 1mM TCEP, pH 7.50) at 25°C. Phosphorylated peptide production was monitored by reverse phase-high performance liquid chromatography (RP-HPLC).
at diff degrees) mutations, aimed at screening for monobodies that binds to the PIF pocket utilizing the same allosteric network as TPX2.

Two monobodies were selected for further studies, Mb1 and Mb2, of which the binding network were identified as highly similar to TPX2’s. In preparation for in vivo experiments, we also constructed sGFP Mb1 and sGFP Mb2. With the fluorescent GFP tag, we can better visualize the monobodies under microscopes. Additionally, the super-negatively charged group optimizes monobodies’ entry into mammalian cells through cationic-transporter-facilitated endocytosis (Thompson et al. 2012).

ITC data show that Mb2’s affinity to Aurora A\textsubscript{modern} is in micromolar range ($K_d=9.9 \pm 0.3 \, \mu M$), similar to TPX2’s affinity of $5.4 \pm 0.2 \, \mu M$ (Figure 21).

![Aurora A\textsubscript{modern} vs. sGFP Mb2](image)

Figure 21. ITC measurements for Aurora A\textsubscript{modern} bound to sGFP Mb2. ITC measurements conducted with 34\,\mu M Aurora A\textsubscript{modern} and 236\,\mu M sGFP Mb2 show binding of $K_d=9.9\pm0.3\,\mu M$, comparable with 80\,\mu M wild type Aurora A\textsubscript{modern}’s affinity to 940\,\mu M TPX2\textsubscript{1−45} ($K_d=5.4\pm0.2\,\mu M$).
By analyzing T288V Aurora A<sub>modern</sub>‘s activity HPLC assays, with and without the existence of Mb1 and Mb2, I found that Mb1 is an activating regulator while Mb2 is an inhibiting regulator (Figure 22). HPLC activity assays demonstrate similar activity for Mb2 and sGFP Mb2, confirming that the sGFP tag does not alter Mb2’s effect on Aurora A<sub>modern</sub>’s kinase activity.

Overall, the monobody screening strategy enables us to search for tight-binding, high-specificity, small regulators that potentially inhibit Aurora A’s aberrant activity in cancer cells.

Figure 22. HPLC assay for Mb1, Mb2, and sGFP Mb2. Mb1 increases initial rate of T288V Aurora A<sub>modern</sub> by 7 fold while Mb2 decreases the rate to 1/5 or 1/30, with or without the presence of a sGFP tag. Reactions were conducted in the presence of 1 μM protein, with or without 40 μM Monobodies, 5 mM ATP, and 1 mM Kemptide in assay buffer (20 mM TrisHCl, 200 mM NaCl, 20 mM MgCl₂, 3% (vol/vol) glycerol, 1 mM TCEP, pH 7.50) at 25°C. Phosphorylated peptide production was monitored by reverse phase-high performance liquid chromatography (RP-HPLC).
Conclusion

Allostery is a process where changes in residues tens of angstrom away from the active site lead to a propagated response in the active site. Protein allostery is an important mechanism adopted by many organisms to regulate signal transduction and enzyme catalysis processes. Given that Aurora-TPX2 allostery exists in certain species yet not the others, we speculate that evolution would be the key to dissect this allosteric activation mechanism. It is true that protein evolution, where the amino acid sequence undergoes incremental changes, may also be driven by stability and protein folding, other than function-driven (Dokholyan et al., 2003). In the case of Aurora and TPX2, however, the fact that TPX2 brings about a 15.9 fold increase in Aurora A modern’s activity suggests that the development of TPX2 responsiveness through evolution is indeed function-driven and thus can provide us insights to unravel the allostery between TPX2 and Aurora kinase. Therefore, we used ASR to resurrect Aurora ancestors and tested ancestors’ responsiveness to TPX2 by conducting enzymatic assays, with and without the presence of TPX2. As we expected, appearance of TPX2, concurrent with the Aurora’s transition from AurANC2 to AurANC3, marks the establishment of responsiveness to TPX2.

We thus hypothesized that residue differences between AurANC2 and AurANC3 lead to a development of TPX2-activation mechanism in Aurora. To begin with, we noticed individual residues, previously reported to be critical in TPX2-Aurora interaction, are substituted by other residues in older Aurora ancestors that predate the appearance of TPX2. Based on the crystal
structure of TPX2 bound to Aurora A, younger Aurora species are able to interact with TPX2 by hydrogen-bonding or aromatic stacking these two residues, while older Aurora ancestors lack these characteristic interactions. However, ATP/NADH-coupled assays show that these individual residues are not sufficient to respond to TPX2 activation. As allostery implies a propagated effect across the entire enzyme, we postulate that a network of residues may be responsible for the relay of allosteric effect. Therefore, we re-examined the sequence alignment of Aur\textsubscript{ANC2} and Aur\textsubscript{ANC3}, which shows 25 residue differences. We narrowed down the network by highlighting 15 residues that are conserved among all modern Aurora species. Through coupled assays and ITC measurements, we show that the 15 residues are responsible for TPX2 allosteric activation.

These results paint a nuanced picture of allosteric activation in which key structural motifs in Aurora develop over time to converge on a specific structural mechanism capable of transferring maximum activation upon TPX2 binding. Given that crystal structures can only offer a static view of Aurora-TPX2 interaction, computational simulation of Aurora ancestors’ dynamics upon TPX2 binding can be an interesting future direction to pursue.
Material and Methods

Protein expression and Purification

Aurora A T288V plasmid was used as template to construct mutations through Poly Chain Reaction (PCR). Primers were ordered from IDT. The DNA fragment was digested with DpnI and subsequently co-transformed with Ser/Thr/Tyr phosphatase lambda (λPP) into DH5α E. coli cells. The recombinant plasmid was then transformed to BL21 (DE3) E. coli cells for protein expression at 37°C. Cells were induced with 0.6mM IPTG after OD reached 0.8. After 21°C overnight (16-18 hours) growth, cells were centrifuged at 5000 rpm for 15 min, resuspended in 50mM TrisHCl (pH 8.0), 300 mM NaCl, 40mM imidazole, 20mM MgCl₂, 10% (vol/vol) glycerol, and sonicated in the presence of EDTA-free protease inhibitor cocktail and DNAse for 4 min (20s on, 20s off, 3.0 V). After centrifuging for 45 min at 12000 rpm, lysate was collected then filtered using a 0.22μm filter and passed through a NiNTA column. The protein was eluted with 50mM TrisHCl (pH 8.0), 300mM NaCl, 500mM imidazole, 20mM MgCl₂, 10% (vol/vol) glycerol. Protein fractions were collected and TEV-cleaved overnight at 4°C in 10 kDa dialysis cassettes that were exchanged against 20mM TrisHCl (pH 7.0), 200mM NaCl, 20mM MgCl₂, 1mM TCEP, 10% (vol/vol) glycerol. Cleaved Aurora A was passed through a second nickel column to remove uncleaved fragments and His6-TEV-protease, followed by purification through a 26/60 S200 size exclusion column. (Zorba, Buosi et al., 2014)
TPX2\textsuperscript{1-45}, his6-tagged, GB1-tagged and thrombin cleavable, was transformed in BL21(DE3) cells and plated on Kan LB plates. Cells were induced at an OD=0.6-0.8 with 0.6mM IPTG. Cells grew for 3 hours at 37 °C. Cells were centrifuged, resuspended in binding buffer (50mM TrisHCl, 300mM NaCl, 20mM MgCl\textsubscript{2}, pH 8.0), sonicated in the presence of EDTA-free protease inhibitor cocktail and DNAse for 4 min (20s on, 20s off, 3.0 V). After centrifuging for 45 min at 12000 rpm, lysate was collected and then filtered using a 0.22μm filtering unit and passed through a NiNTA column. Bound protein was thrombin cleaved overnight in a dialysis cassette in gel filtration buffer (20mM TrisHCl, 200mM NaCl, 20mM MgCl\textsubscript{2}, 5mM TCEP, pH 7.50), passed through tandem NiNTA-benzamidine columns and finally through an S200 gel filtration column (Zorba, Buosi et al., 2014).

Monobodies were transformed in BL21 (DE3) cells and plated on Kan LB plates. Cells were induced at an OD=0.6-0.8 with 0.6mM IPTG. Cells were induced for 18°C overnight (16-18 hours). Cells were centrifuged, resuspended in binding buffer (50mM TrisHCl, 300mM NaCl, 20mM MgCl\textsubscript{2}, pH 8.0), sonicated in the presence of EDTA-free protease inhibitor cocktail and DNAse for 4 min (20s on, 20s off, 3.0 V). After centrifuging for 45 min at 12000 rpm, lysate and pellet were separately collected. Round I lysate was loaded on to NiNTA column after filtered with a 0.22μm filtering unit. Pellet was resuspended in GuHCl buffer (20mM TrisHCl, 6M GuHCl, pH 8.0), followed by centrifuge at 12000 rpm for 45 min. Round II lysate was collected and passed through a 0.22μm filtering unit. Round II lysate was loaded onto the NiNTA column previously loaded with round I lysate and washed with binding buffer. The column was then washed with 10X column volume of the GuHCl buffer, triton X buffer (binding buffer+ 0.1% Triton X), β-cyclodextrin buffer (binding buffer+ 5mM β-cyclodextrin) and binding buffer. Protein was then eluted with 100% eluting buffer (50mM TrisHCl, 500mM imidazole, 300mM...
NaCl, 20mM MgCl₂, pH 8.0). Protein fractions were collected and passed through a 26/60 S200 size exclusion column (Zorba et al., 2016).

**In-vitro kinase activity assay**

**HPLC**

Aurora A, kemptide, PEP, Pk, in the absence or presence of 50 μM TPX2₁-₄₅, were mixed in kinase buffer (20mM TrisHCl, 200mM NaCl, 3% [vol/vol] glycerol, 20mM MgCl₂, 1mM TCEP, pH 7.50). The assay was initiated by 5mM ATP. Then 5μl time points were collected, resuspended in 10μl 6% (vol/vol) trichloroacetic acid (in water) to quench the reaction, and neutralized with 100μl 100mM KH₂PO₄, pH 8.0 to provide the appropriate pH for nucleotide separation. The mixture was then passed through a 0.22μm SpinX column to remove protein precipitation. Reverse phase-high performance liquid chromatography (RP-HPLC) was used to separate phosphorylated from non-phosphorylated species. The running buffer was 0.1% TFA (vol/vol) in water, while the elution buffer was 100% acetonitrile. In cases where TPX2 was added, full saturation of Aurora A by TPX 2 1-45 was reached ([TPX2]>5 μM (Zorba, Buosi et al., 2014).

**ATP/NADH-coupled Assay**

Figure 23 shows the scheme of the ATP/NADH-coupled assay. Oxidation of NADH is monitored at A340 for 20mins using SOFTmaxPRO software on a 96-well microplate reader (SpectraMax). Assays were performed in a white, clear-bottom 96-well plate (Corning #3994) in a final reaction volume of 100μl per well.
Assay buffer (20mM TrisHCl, 200mM NaCl, 20mM MgCl2, 5mM TCEP, 10% glycerol, pH 7.5) was used, with 6.6mM phosphoenolpyruvate (PEP), 725μM NADH, 370nM pyruvate kinase (PK), 350nM lactate dehydrogenase (LDH), and 0.6mg/mL BSA. The rate of peptide phosphorylation was calculated from the equation:

$$k_{obs}(s^{-1}) = -\frac{\frac{dA_{340}}{dt} \left(\frac{OD}{s}\right)}{K_{path} \left(\frac{OD}{\mu M}\right) \times [Aurora] (\mu M)}$$

where $K_{path}$ is the molar absorption coefficient for NADH. For a 100μl well volume, the change in optical path length was experimentally determined to be $K_{path}=3800$ OD/M. Appropriate controls (ensuring PEP/NADH/PK/LDH are not limiting factors) were carried out before data was gathered (Zorba et al., 2016).

![Figure 23. Scheme of the coupled assay.](image)

Isothermal Titration Calorimetry
Nano ITC Low Volume (TA instruments) was used to conduct ITC measurements. 1μL injectant (TPX2 or Mb) was added every 180s, with a constant stirring at 350 rpm and at 25 °C. Data were analyzed via the NanoAnalyze software using the independent fit model. Prior to ITC titration, both protein and injectant were dialyzed in ITC buffer (20 mM TrisHCl, 200 mM NaCl, 20mM MgCl$_2$, 10% glycerol, 1 mM TCEP, pH 7.50) at 4°C and filtered using SpinX filter columns (Corning).

**Crystallographic methods**

Crystals of dephosphorylated ancient Aur$_{ANC4}^{133-388}$, in complex with AMPPCP, were grown at 18°C via the hanging drop method and the sitting drop method. 1:1, 2:1 and 3:1 ratios of protein mixture to mother liquor were obtained. The protein, peptide, and nucleotide were originally stored in 20mM TrisHCl (pH 7.0), 200mM NaCl, 20mM MgCl$_2$, 5mM TCEP, 10% (vol/vol) glycerol. Crystallization was optimized by repeating the process with a longer construct Aur$_{ANC4}^{122-403}$. 


References

Aliagas-Martin, Ignacio; Burdick, Dan; Corson, Laura; Dotson, Jennafer; Drummond, Jason; Fields, Carter et al. (2009): A Class of 2,4-Bisanilinopyrimidine Aurora A Inhibitors with Unusually High Selectivity against Aurora B †. In J. Med. Chem. 52 (10), pp. 3300–3307. DOI: 10.1021/jm9000314.


Balendran, Anudharan; Casamayor, Antonio; Deak, Maria; Paterson, Andrew; Gaffney, Piers; Currie, Richard et al. (1999): PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. In Current Biology 9 (8), pp. 393–404. DOI: 10.1016/S0960-9822(99)80186-9.


Zorba, Adelajda; Wilson, Christopher; Villali, Janice; Kern, Nadja; Zheng, Yuejiao and Kern, Dorothee. (2016): Ancient Origins of Allosteric Activation exposed through the evolution
of a Ser/Thr kinase. Unpublished manuscript. Department of biochemistry, Brandeis University, Waltham, MA.