

# Cell Chemical Biology

## Bidirectional Allosteric Communication between the ATP-Binding Site and the Regulatory PIF Pocket in PDK1 Protein Kinase

### Highlights

- PS653 inhibits the binding of PIFtide by interaction with the ATP-binding site
- Adenosine binds at the ATP-binding site and enhances the binding of PIFtide
- Molecular dynamics simulations describe the conformational changes of PDK1
- Potent drugs to the ATP site of PDK1 have different effects on the PIF pocket

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### In Brief

Schulze et al. show that compounds binding to the ATP-binding site of a kinase can produce profound effects on a distant site, enhancing or inhibiting the interaction with allosteric regulators. The principle can be exploited in drug discovery and development.

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# Bidirectional Allosteric Communication between the ATP-Binding Site and the Regulatory PIF Pocket in PDK1 Protein Kinase

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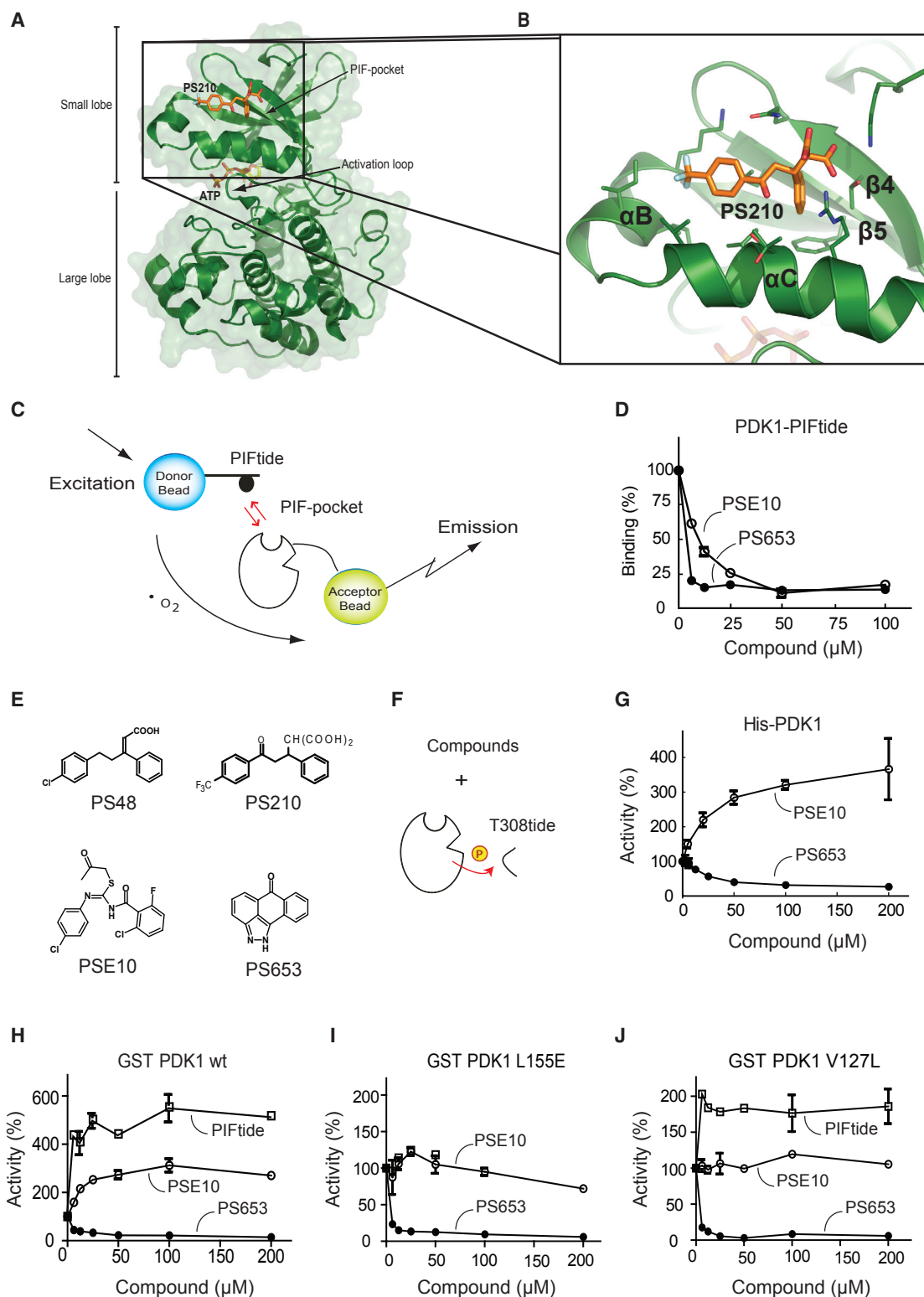
## SUMMARY

Allostery is a phenomenon observed in many proteins where binding of a macromolecular partner or a small-molecule ligand at one location leads to specific perturbations at a site not in direct contact with the region where the binding occurs. The list of proteins under allosteric regulation includes AGC protein kinases. AGC kinases have a conserved allosteric site, the phosphoinositide-dependent protein kinase 1 (PDK1)-interacting fragment (PIF) pocket, which regulates protein ATP-binding, activity, and interaction with substrates. In this study, we identify small molecules that bind to the ATP-binding site and affect the PIF pocket of AGC kinase family members, PDK1 and Aurora kinase. We describe the mechanistic details and show that although PDK1 and Aurora kinase inhibitors bind to the conserved ATP-binding site, they differentially modulate physiological interactions at the PIF-pocket site. Our work outlines a strategy for developing bidirectional small-molecule allosteric modulators of protein kinases and other signaling proteins.

## INTRODUCTION

Allostery is a fundamental and widespread mechanism of intramolecular signal transmission whereby local perturbations on a

protein affect the structure and dynamics of specific distal regions (Changeux, 2012; Goodey and Benkovic, 2008; Nussinov and Tsai, 2013). The term “allostery,” which was once restricted to oligomeric proteins, is now also used to describe the conformational changes that intramolecularly link two given distant sites on a protein (e.g., an orthosteric site and an allosteric site). The transmission of signals across long distances relies on dynamic coupling between different structural motifs and can be described as a shift in the population or dynamics of conformers (Bray and Duke, 2004). The formulations for the allosteric conformational changes have been studied over the last 50 years (Fenton, 2008; Monod et al., 1965; Nussinov and Tsai, 2014; Weber, 1972). Allostery thus mediates the responses of regulatory proteins to different stimuli along the transduction of cellular signals. Although the allosteric process is intrinsically bidirectional, cell signaling most often uses allostery unidirectionally to transduce a signal downstream in a signaling pathway (Kuriyan and Eisenberg, 2007). Protein kinases as well as other regulatory proteins that participate in signal transduction have evolved stringent switches (allosteric sites) that control their activation, i.e., modulate the conformation of the ATP-binding site (orthosteric site) in response to the appropriate upstream signals. In recent years there is growing interest in the development of allosteric drugs (Conn et al., 2014; Fang et al., 2013; Gray and Fabbro, 2014). However, the development of allosteric drugs is hampered by the poor knowledge of the molecular details of the allosteric process and how this can be rationally modulated at will with small compounds. Here, we describe the “reverse” allosteric regulation by small compounds on the phosphoinositide-dependent protein kinase 1 (PDK1), i.e., how binding of molecules to the ATP-binding site (orthosteric site) affects a



**Figure 1. The Kinase Domain, the PIF Pocket Binding to PIFtide, and the Identification of Small Molecules that Displace PIFtide from the PIF Pocket**

(A and B) Crystal structure of the catalytic domain of PDK1 in complex with ATP (yellow carbon atoms) binding at the active site and PS210 (orange carbon atoms) binding at the PIF-pocket allosteric site (PDB: 4AW1).

(C) Schematic representation of the AlphaScreen interaction assay employed for the screening of the library of small molecules.

(legend continued on next page)

regulatory allosteric site, reversing the direction in which the allosteric regulation between the two sites is observed in cell signaling.

The protein kinase domain is formed by two lobes with the ATP-binding site located in the cleft between the two (Figure 1A) (Zheng et al., 1993). It is currently proposed that dynamics is the underlying mechanism for allosteric regulation in protein kinases (Kornev and Taylor, 2015). In the prototype protein kinase A (PKA), the catalytic domain is constitutively active, and regulation of the enzymatic activity is provided by interaction with regulatory subunits (Taylor et al., 2012). In contrast, other members of the AGC group of protein kinases are not constitutively active and use a conserved hydrophobic pocket called the PDK1-interacting fragment (PIF) pocket as a key allosteric regulatory site. The PIF pocket was originally described in the PDK1 as a regulatory site that binds PIF, a polypeptide sequence derived from a PDK1 substrate (Biondi et al., 2000). The pocket is formed by the conserved  $\alpha$ C helix,  $\beta$  strands 4 and 5, and helix  $\alpha$ B (Figure 1B). The PIF sequence comprises an extended hydrophobic motif (HM) present in AGC kinases but has a phosphomimetic Asp residue instead of the Ser/Thr phosphorylation site. Phospho-HM polypeptides and PIFtide activate PDK1 and diverse AGC kinases (Biondi et al., 2000; Engel et al., 2006; Frodin et al., 2002; Yang et al., 2002) in vitro by interaction with the PIF pocket (Figures S1A and S1B). In PDK1, the PIF pocket plays an additional role in the specific recognition of a subgroup of its substrates. The HM of PDK1 substrates such as SGK, S6K, PKC, and RSK, but not protein kinase B (PKB)/Akt, require the docking of their HM to the PIF pocket of PDK1 to become efficiently phosphorylated (Bayascas, 2008; Biondi et al., 2001; Collins et al., 2003) (Figure S1C). We previously characterized the mechanism of activation of PDK1 upon binding of peptides or small molecules to the PIF pocket (Engel et al., 2006; Hindie et al., 2009; Sadowsky et al., 2011; Stockman et al., 2009; Wei et al., 2010) (Figures S1D and S1E). Crystal structures of PDK1 with the bound reversible activators PS48 (Hindie et al., 2009) and PS210 (Busschots et al., 2012) revealed structural aspects of the allosteric activation mechanism and the associated conformational changes. For instance, the crystal structure of PDK1 in complex with PS210 shows the complete closure of the ATP-binding site (Busschots et al., 2012).

The allosteric regulation of protein kinases mediated by the small lobe of the kinase domain is not restricted to AGC kinases. Aurora kinases are activated by the polypeptides of their interacting partners, which bind to a site equivalent to the PIF pocket (Bayliss et al., 2003). Furthermore, epidermal growth factor receptor kinases are activated by dimerization; an activating kinase interacts with a receiver kinase at a site equivalent to the PIF pocket (Zhang et al., 2006). Abl kinase is both allosterically inhibited and activated by intramolecular interaction with its SH2 domain (Wojcik et al., 2010), which, in the active state, docks at the top of the small lobe (Dolker et al., 2014; Nagar

et al., 2003). However, except for the case of AGC kinases, these regulatory features mediated by the small lobe have not been investigated using small compounds.

Despite the great interest in drug development of protein kinases over the last 20 years, the allosteric effects of small compounds binding to the ATP-binding site have remained mostly unexplored. In the present work, we investigate the bidirectional allosteric regulation of PDK1 using small compounds that bind to the PIF pocket and ATP-binding site. The results show how different small compounds that bind at the ATP-binding site produce allosteric effects on the PIF-pocket regulatory site, enhancing or inhibiting the binding to PIFtide. This work highlights how the “old” concept of allostery provides new exciting opportunities for drug development of protein kinases.

## RESULTS

### Identification of Small Compounds that Bind to the ATP-Binding Site and Allosterically Affect the Binding of PIFtide to the PIF Pocket of PDK1

PDK1 interacts with the HM-polypeptide PIFtide with high affinity, and this interaction increases the specific activity of PDK1 in vitro as measured by its increased ability to phosphorylate T308tide, a polypeptide derived from the activation loop of PKB/Akt (Biondi et al., 2000) (Figure S1A). The interaction between His-PDK1 and biotin-PIFtide can be measured by means of AlphaScreen technology (Figure 1C) (Busschots et al., 2012). Using this homogeneous assay, we screened a library of 14,400 small molecules (average molecular weight 320 Da) for their ability to affect the PDK1-PIFtide interaction. We identified small compounds that displaced the interaction, e.g., PSE10 and PS653 (Figures 1D and 1E). PSE10 (‘2-oxopropyl N-(4-chlorophenyl)-[(2-chloro-6-fluorobenzoyl)amino]methanimidothioate) has two ring systems joined by a linker and a side chain, resembling compound **1** (Engel et al., 2006), PS48 (Hindie et al., 2009) and PS210 (Busschots et al., 2012), which we previously characterized as activators that bind to the PIF pocket of PDK1. In contrast, PS653 (‘1,6-dihydrodibenzo[c,d,g]indazol-6-one) presents a small, planar, anthrone-derived structure. We tested the ability of the newly identified compounds to affect the intrinsic kinase activity of PDK1 (Figure 1F). PSE10 activated PDK1 but did not affect the activity of PDK1 with mutations at the PIF pocket (Figures 1F–1J). The crystal structure of PSE10 in complex with PDK1<sub>50–359</sub> confirmed that PSE10 bound to the PIF pocket (Figure S2 and Table S1) in a manner similar to the binding modes of PS48 and PS210. In contrast, PS653 inhibited the activity of different PDK1 constructs (Figures 1G, 1H, and S3). We previously showed that PDK1 Leu155Glu, which contains a mutation at the center of the PIF pocket, is not activated by PIFtide (Biondi et al., 2000) or by small compounds that bind to the PIF pocket (Engel et al., 2006). Similarly, other

(D) Effect of PSE10 and PS653 on the interaction between His-PDK1 50–360 and biotin-PIFtide.

(E) Structures of PSE10 and PS653 in comparison with previously described activators of PDK1, PS48, and PS210.

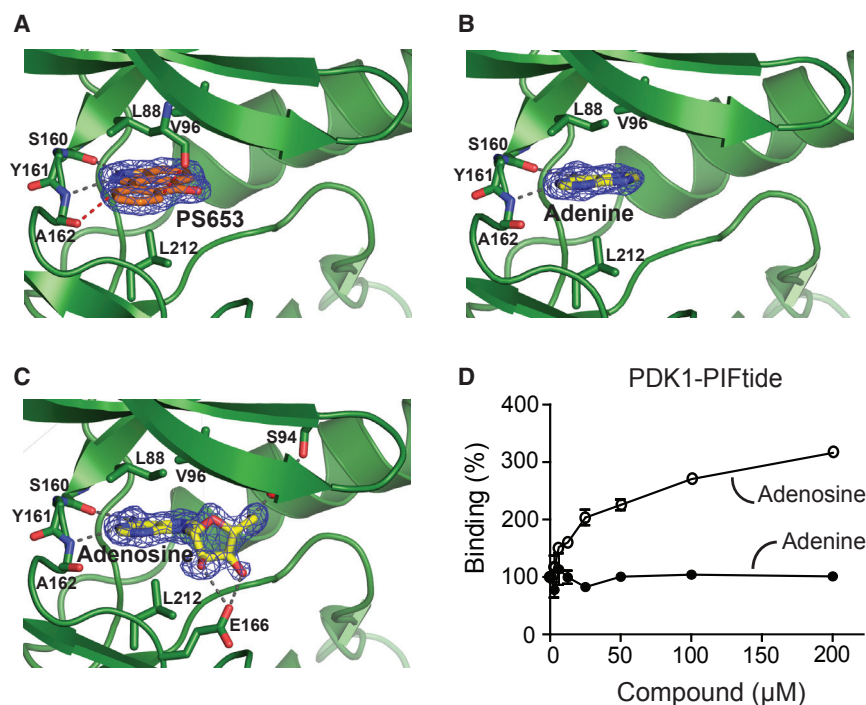
(F) Schematic representation of the PDK1 in vitro activity assay, using the polypeptide T308tide as substrate of PDK1.

(G) Effect of PSE10 and PS653 on the in vitro activity of His-PDK1<sub>50–556</sub>; the inhibition of other PDK1 constructs is presented in Figure S3.

(H–J) Effect of PSE10, PS653, and PIFtide on the activity of GST-PDK1<sub>1–556</sub> and the PIF-pocket mutants Leu155Glu and Leu127Val.

Data are presented as means  $\pm$  SEM. See also Figures S1 and S2.





**Figure 2. PS653, Adenine, and Adenosine Bind to the ATP-Binding Site but Produce Distinct Allosteric Effects at the PIF Pocket**

(A–C) Crystal structures of PDK1 in complex with PS653 (A), adenine (B), and adenosine (C). Only residues in direct contact with the compounds are shown as sticks. Hydrogen bonds are visualized as gray dotted lines. Close contacts of PS653 mentioned in the main text are indicated as red dotted lines.  $|2F_o - F_c|$  electron density of the compounds is shown in blue and contoured at  $1\sigma$ . (D) Effect of adenine and adenosine on the interaction between PDK1 and PIFtide. The interaction between His-PDK1<sub>50-359</sub> and biotin-PIFtide was measured using the AlphaScreen assay depicted in Figure 1C and the effect of adenine and adenosine quantified.

Data are presented as means  $\pm$  SEM. See also Figure 1C and Table S1.

mutations in the PIF pocket also partially abolished the activating effect of PIFtide and small compounds (Engel et al., 2006). In contrast to PSE10, PS653 still inhibited PDK1 (Leu155Glu) and PDK1 (Val127Leu), which have mutations at different key residues in the PIF pocket (Figures 1I and 1J). These results were not compatible with PS653 binding to the PIF pocket; instead, they suggested that the binding site of PS653 was allosterically coupled to the PIF pocket.

The high-resolution crystal structure of PDK1<sub>50-359</sub> in complex with PS653 (Figures 2A and S2C; Table S1) revealed that PS653 binds at the ATP-binding site, thus confirming that the displacement of PIFtide by PS653 is mediated by a reverse allosteric effect and not due to a direct competition for the PIF-binding pocket. Like the adenine moiety of ATP and most ATP-competitive inhibitors, PS653 forms two hydrogen bonds with the main chain atoms Ser160-CO and Ala162-N of the so-called linker region and is sandwiched between the hydrophobic side chains of Leu88, Val96, and Leu212. However, a major difference is that PS653 extends outside the usual ATP-binding cavity. Its outer ring is in close contact (3.25 and 3.0 Å, respectively) with the carbonyl groups of both Leu99 of the small lobe and Ala162 of the large lobe.

We then investigated whether adenine or adenosine, which bind to the same site as PS653 (Figures 2B and 2C; Table S1), could produce similar allosteric effects. Surprisingly, adenosine enhanced the binding of PIFtide to PDK1, providing evidence of cooperativity, whereas adenine did not produce any allosteric effect on the PIF pocket (Figure 2D). Interestingly, the enhancing effect was observed selectively with adenosine and not with adenine, AMP, ADP, ATP, the non-hydrolyzable ATP analog thio-ATP, or cAMP (Figure S3B). The high-resolution crystal structure of PDK1 in complex with adenosine showed that the ribose moiety forms hydrogen bonds with Glu166 of the large

lobe and an indirect hydrogen bond via a water molecule with Ser94 of the small lobe (Figure 2C). Thus, the presence of the sugar moiety between the lobes played a role in the allosteric enhancement of the binding of PIFtide to the PIF

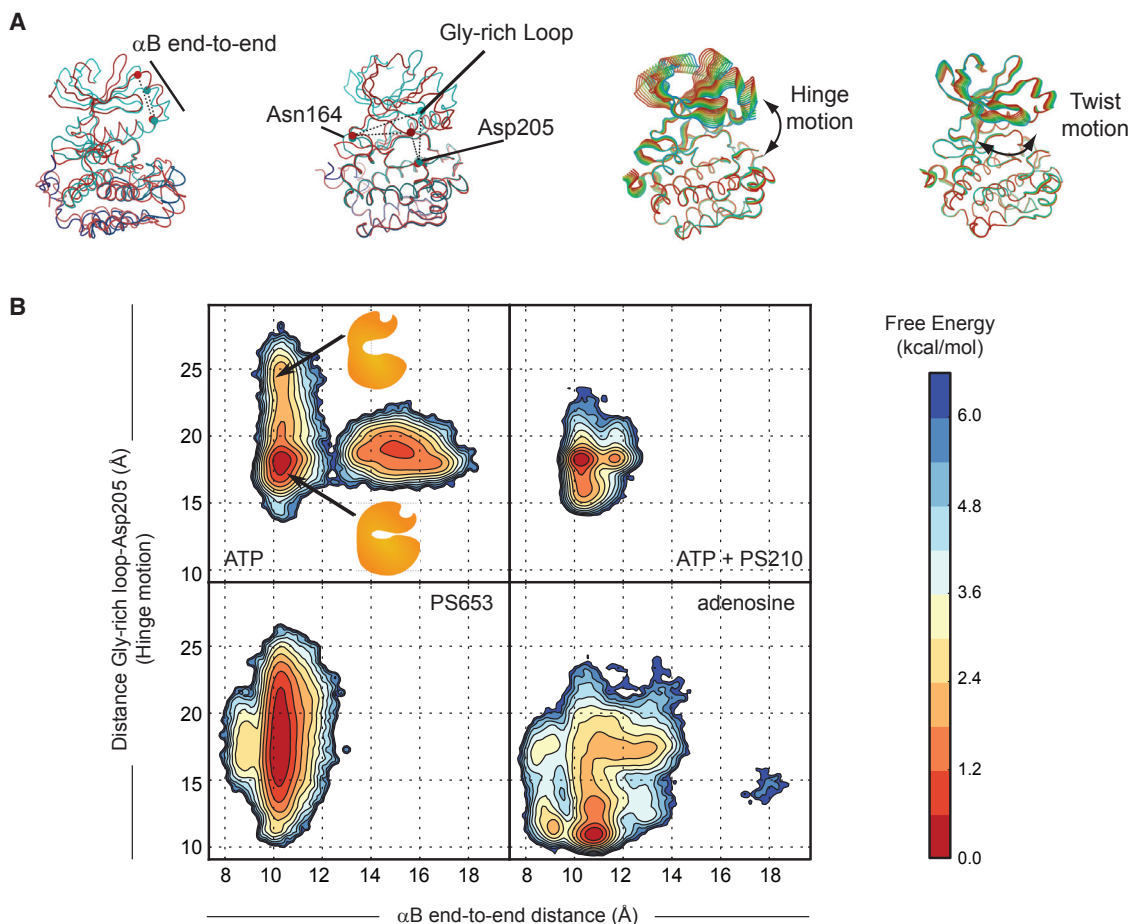
pocket. The differential effects caused by distinct compounds binding to the ATP-binding site showed that the resulting allosteric effects were highly selective.

The co-crystal structures revealed the precise binding mode of the allosteric and non-allosteric compounds under investigation. The crystal structures, on the other hand, did not reveal significant structural differences that could explain the observed allosteric effects.

### Molecular Dynamics Simulations Describe Changes to the Conformational Dynamics of PDK1 upon Ligand Binding

To obtain more detailed information on the allosteric mechanisms, we analyzed the changes in the dynamics of PDK1 upon the binding of the different effectors using atomistic molecular dynamic (MD) simulations. We first performed long MD simulations to observe the effect of the different ligands on the sub- $\mu\text{s}$  dynamics of PDK1. This is an unbiased approach to evaluate the whole dynamics of the protein. Interestingly, we identified an equilibrium between two populations of interconverting conformations, which differed in the stability of the small  $\alpha\text{B}$  helix and could be appropriately quantified by the distance between the  $C\alpha$  atoms of Lys115 and Lys123 (end-to-end distance), shown as yellow spheres in Figures S4A and S4B.

To fully sample the conformational changes that in protein kinases can take place on timescales ( $\mu\text{s}$  to  $\text{ms}$ ) not easily accessible to conventional MD (Saladino and Gervasio, 2012), and to reconstruct the conformational free energy landscape, we performed long multiple-replica parallel tempering (PT; Sugita and Okamoto, 1999) simulations in the well-tempered ensemble (WTE; Bonomi and Parrinello, 2010). Using this approach we clearly observed multiple transitions of PDK1 between the two conformations differing in the orientation and folding of helix



### Figure 3. Free Energy Calculation of PDK1 with Compounds

(A) Schematic representation of parameters measured. Free energy surfaces as projected along the identified  $\alpha$ B parameter (end-to-end distance) and two distances describing the opening and rotation of the small lobe with respect to the large lobe.

(B) Free energy calculation of PDK1 with ATP, PS210, PS653, and adenosine. PS653 clearly enhances the hinge motion and lobe rotation, while adenosine has the opposite effect, similar to that observed for PS210.

See also [Figures S4](#) and [S5](#).

$\alpha$ B, with PS210 suppressing the population of the molecules with a more disordered helix. In [Figure 3](#), we report the free energy projected along the variable describing the length of helix  $\alpha$ B helix (end-to-end distance) and the distance between the Gly-rich loop (residues 90–94) and Asp205, which describes the kinase hinge motion. It is clear that the two conformations observed in the previous MD of the kinase with ATP that are well resolved along the  $\alpha$ B end-to-end distance variable are not observed in the presence of PS210, the free energy surface (FES) of which has a single minimum with a well-ordered  $\alpha$ B helix ([Figure 3B](#)). The second minimum of the FES of PDK1 with ATP is also compatible with a lower degree of  $\alpha$ B integrity and with our observation that partial unfolding of the C-terminal end of the helix takes place. Although the FES along the Gly-rich loop-Asp205 distance (hinge motion) shows a clear main minimum for ATP and ATP + PS210, the morphology and position of the basins suggests that the binding of PS210 restricts the kinase lobe dynamics, enforcing a more closed catalytic domain ([Figure 3B](#)), in agreement with the crystal structure of the complex ([Busschots](#)

[et al., 2012](#)). Indeed, structures with a Gly-rich loop-Asp205 distance larger than 20 Å, appear to be relatively well populated in the presence of ATP, but are rarely populated upon addition of PS210. A highly conserved salt bridge between a Lys that positions the phosphate of ATP at the active site and a Glu from helix  $\alpha$ C (Lys111 and Glu130 in PDK1) is widely considered a hallmark of active structures of protein kinases. The MD simulations show that in the presence of ATP, the Lys111-Glu130 salt bridge is present in closed structures. In agreement with being an activator, PS210 stabilizes conformations with a closed hinge and well-formed  $\alpha$ B helix. The same conclusion can be drawn by observing the projection of the FES along the first two eigenvectors of the principal component analysis ([Figure S4D](#)). Different orientations of Tyr126 were observed in our new simulations for PDK1 with both ATP and PS210 ([Figures S4C](#) and [S5A](#)). Interestingly, the same alternative conformation of Tyr126, with the OH in close proximity to the terminal phosphates of ATP, was observed independently in a new crystal form of PDK1 obtained in complex with ATP and a phosphorylated HM polypeptide

**Table 1. Thermodynamic Parameters of the PDK1 Interaction with Low-Molecular-Weight Compounds Obtained by Isothermal Titration Calorimetry**

Compound	Temperature (K)	N	$K_a$ ( $M^{-1}$ )	$K_d$ ( $\mu M$ )	$\Delta H$ ( $kcal\ mol^{-1}$ )	$\Delta S$ ( $cal\ mol^{-1}\ deg^{-1}$ )	$T\Delta S$ ( $kcal\ mol^{-1}$ )
Adenosine	298	$1.2 \pm 0.2$	$(5 \times 10^4) \pm (1 \times 10^4)$	20	$-3.2 \pm 0.7$	10.7	3.2
PS653	310	$0.87 \pm 0.02$	$(8 \times 10^5) \pm (2 \times 10^5)$	1.25	$-1.20 \pm 0.06$	23.1	7.2

N is the stoichiometry of binding.

See also [Figure S7](#).

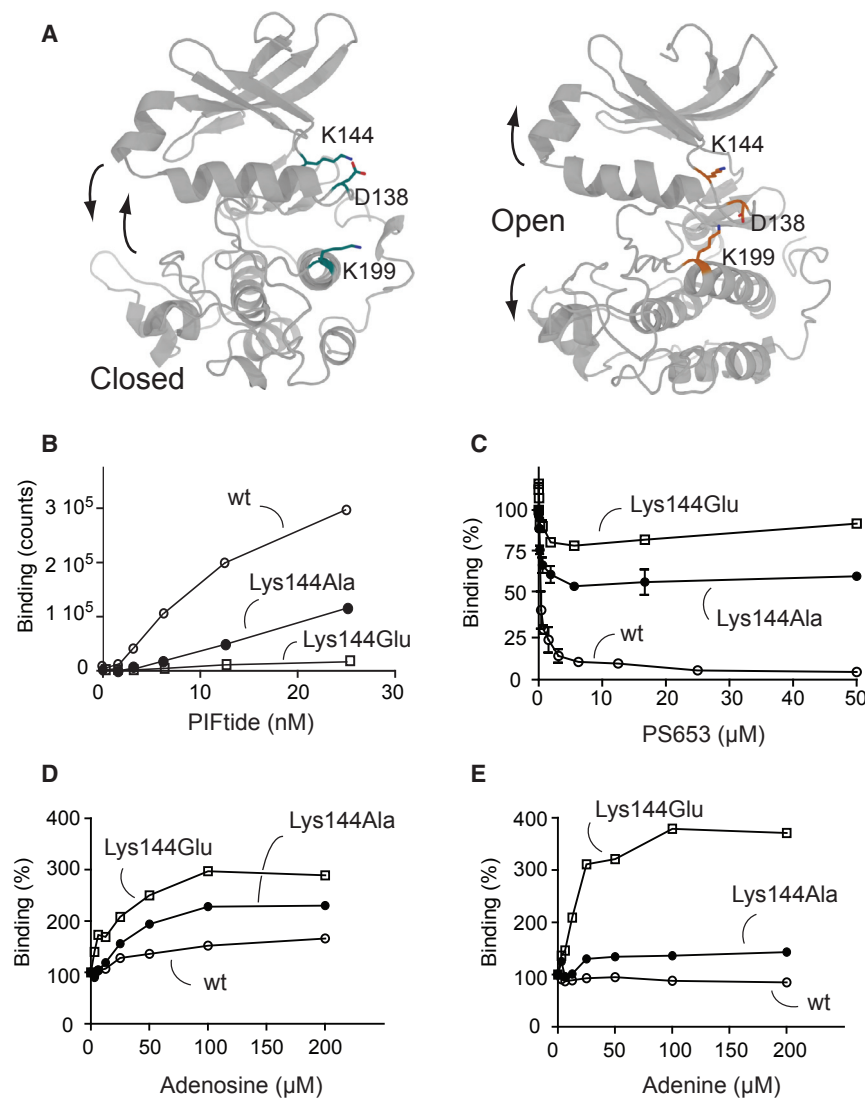
binding to the PIF pocket ([Figure S4C](#) and [Table S1](#)). In addition, this new crystal structure confirms the PIF pocket as the binding site of substrate HM polypeptides ([Figure S5B](#)). Overall, the PT-WTE simulations confirmed the differences in the behavior of ATP- and ATP-PS48/PS210-bound PDK1 previously observed by MD and allowed better characterization of the dynamic and structural shifts arising from the binding of the allosteric effector. The MD simulations were in excellent agreement with the experimental data, and predicted Tyr126 movements that were independently confirmed in successive crystallography work. It should be noted that the simulations in the presence of ATP revealed that, in the open conformation, the PIF pocket is always well formed and stable, while the closed structure presents also a destabilized  $\alpha B$  helix.

#### Molecular Mechanism of the Reverse Allosteric Effects of PS653 and Adenosine on the PIF Pocket

We next analyzed the molecular mechanism of the reverse allosteric effects of PS653 and adenosine on the PIF pocket. The free energy projections along the same variables used before are reported in [Figure 3B](#). PS653 produced a marked enhancement of the PDK1 hinge/twist motions ([Figures 3B](#) and [S4D](#)). This, in turn, dramatically increases the population of more open structures (distances larger than 17 Å in [Figure 3B](#)). In contrast, adenosine enriched the most closed conformation ([Figure 3B](#)), suppressing the hinge motion, similar to what was observed in the PS210 FES. While both PS210 and PS653 rigidify the PIF pocket, the most populated conformations are quite different, and the two lobes appear more twisted in the structure with PS653 ([Figures S6A](#) and [S6B](#)). The additional hydrogen bonds formed by the ribose moiety of adenosine ([Figure 2C](#)) could trigger the hinge motion and tip the balance of the conformational equilibrium toward being more closed. Moreover, the changes observed in the conformational free energy landscape suggest that the stabilization of more closed structures by adenosine enhances the allosteric coupling between the two lobes and affects the binding of PIFtide to the PIF pocket by increasing the flexibility of the  $\alpha B$  region. In contrast, PS653 has unfavorable close contacts in the crystal structure, and in the MD simulation induces the displacement of Leu88, hindering the full closure of the two lobes and breaking the catalytic spine ([Kornev et al., 2006](#)). We next compared the interaction of PDK1<sub>50-360</sub> with PS653 and adenosine using isothermal titration calorimetry. In both cases the results confirmed the existence of a single binding site for the compounds in solution, with dissociation constants in the low micromolar range ([Figure S7](#) and [Table 1](#)). Notably, the interaction of PDK1<sub>50-360</sub> with PS653 was less enthalpic and more entropy driven ( $-1.2\ kcal/mol$ ;  $\Delta H/\Delta G = 15\%$ ) than the interaction with adenosine ( $-3.2\ kcal/mol$ ;  $\Delta H/\Delta G = 50\%$ ), in agreement with the

above data showing that PS653 establishes less hydrogen bonds with residues in the enzyme active site, and that it promotes the hinge movement stabilizing the open conformation of the catalytic domain. Thus, the presence of PS653 tips the balance of the conformational equilibrium toward more open structures and in turn to a specific conformation of the PIF pocket that is less apt at binding PIFtide. This finding also suggests that the conformational flexibility of the  $\alpha B$  region might be required for proper recognition of PIFtide and HM polypeptides of other physiological substrates that dock in the PIF pocket. Overall, the results indicated that the reverse allosteric effects that enhance the binding of PIFtide are transduced through the two lobes when the hinge motion brings them together in the closed conformation, while the decrease in binding to PIFtide is due to an induced opening of the hinge that interrupts the allosteric network.

The computational results point toward a central role played by the hinge and twist motions and an allosteric communication network that extends from one lobe to the other. Numerous residues increase interactions in the closed conformation, including residues from the  $\alpha B$  and  $\alpha C$  helices, the DFG motif, and the Gly-rich loop, as well as residues from the large lobe ([Figure S6C](#)). In addition, the MD simulations identified a series of salt bridges that were formed or broken with the hinge motion, e.g., Glu130-Lys111, Asp138-Lys199, Asp138-Lys144, and Lys163-Glu215. Of these, the salt bridge formed by Lys144 and Asp138 was only observed in the closed form but not in the open form, where it is replaced by the Lys199-Asp138 interaction ([Figure 4A](#)). A mutagenesis study based on the simulations confirmed a major role for Lys144. Firstly, we found that the purified Lys144Glu and Lys144Ala mutant proteins had slightly lower specific activities (approximately 30% decreased) than the control GST-PDK1 protein. In spite of lower basal activity, the Lys144 mutants were normally activated by an excess of PIFtide (2  $\mu M$ ), reaching 93% and 89%, respectively, of the wild-type activity in the presence of PIFtide, indicating that they were active and overall well folded, suggesting that the mutation stabilized open-inactive forms of the kinase. Secondly, the Lys144 mutations importantly decreased the binding of PIFtide ([Figure 4B](#)). Together, we validated that Lys144 indeed played an important role in the hinge motion, stabilizing open conformations that have decreased ability to bind PIFtide. This is in remarkable agreement with MD simulations of the Lys144Glu mutant. Starting from a structure where the original Asp138-Lys144 salt bridge was formed, the Lys144Glu mutation quickly leads to the formation of the alternative Lys199-Asp138, observed only in the open structures of the wild-type. Thus, the mutation actively shifts the population toward more open structures that, as previously demonstrated, are generally less prone to bind PIFtide than the mutant itself. The mutated



**Figure 4. The Reverse Allosteric Effect Is Mediated by Lys144 and Is Differentially Affected by ATP-Competitive PDK1 Inhibitors**

(A) Asp138 can form salt bridges with either Lys144 or Lys199. These residues are shown as sticks in a cartoon representation of a PDK1 crystal structure (PDB: 3HRC). Asp138, Lys144, and Lys199 are shown.

(B–E) The ability of GST-PDK1<sub>1–359</sub> wt and Lys144 mutants to bind biotin-PIFtide was studied in the AlphaScreen interaction assay. (B) Mutation of Lys144 to Ala or Glu affects the binding to PIFtide. (C–E) Effect of PS653 on the interaction between GST-PDK1 proteins and biotin-PIFtide.

Data are presented as means ± SEM. See also Figure S6.

mechanism of allosteric coupling between the ATP-binding site and the PIF pocket.

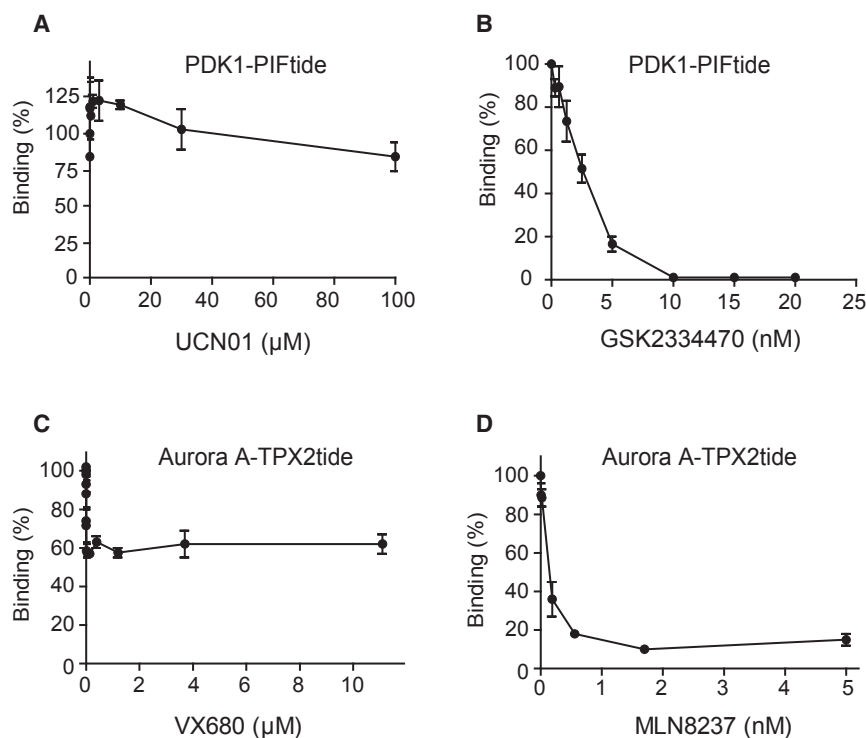
**Drugs in Development Binding at the ATP-Binding Site and Allosterically Affecting the PIF Pocket**

We further investigated whether our findings can explain observed selective in-cell inhibition of downstream signaling by some PDK1 inhibitors. UCN01 is the 7'OH derivative of staurosporine, which binds to the ATP-binding site in PDK1 (Komander et al., 2003; Takahashi et al., 1989) and inhibits the phosphorylation of substrates. The recently developed GSK2334470 is a very potent ATP-competitive inhibitor of PDK1 (Axten et al., 2010) that shows high selectivity for PDK1. Intriguingly, GSK2334470 very

Glu144 does not appear to form any interaction and remains exposed to the solvent for the remainder of the simulation, suggesting that the effect of the mutation is not due to new interactions, but to the severing of important interactions in the existing allosteric network. In spite of the much lower affinity for PIFtide, we could perform a suitable interaction assay upon increasing the concentration of biotin-PIFtide in the assays (Figures 4D and 4E). Notably, the PDK1 Lys144 mutants increased the binding of PIFtide in the presence of adenosine (Figure 4D). In contrast to the lack of reverse allosteric effects by adenine on wild-type PDK1, adenine induced very high reverse allosteric induction of the binding of PIFtide in the PDK1 Lys144Glu mutant (Figure 4E). As the increased population of open structures is generally a consequence of the higher interlobe dynamics (as seen for PS653), the effect seen in the Lys144Glu PDK1 mutant could be due to adenine shifting back the equilibrium toward more closed structures to maximize the interactions within the binding site, de facto restoring the open-closed equilibrium of wild-type PDK1. We conclude that Lys144 plays a key role in the regulated hinge movement, which is central to the

efficiently inhibits S6K phosphorylation in cells but is a relatively poor inhibitor of the phosphorylation of another PDK1 substrate, PKB/Akt (Najafov et al., 2011). The reason for this differential inhibition of PDK1 substrates has remained elusive (Rettenmaier et al., 2014). Early studies described that the PIF pocket of PDK1 is required for the phosphorylation of S6K but not PKB/Akt (Biondi et al., 2001), and this was confirmed in knockin cells and tissues expressing PDK1 (Leu155Glu) (Arencibia et al., 2013; Bayascas, 2008; Collins et al., 2003) as well as pharmacologically using compounds binding to the PIF pocket of PDK1, which block the phosphorylation of S6K but not PKB/Akt (Busschots et al., 2012; Rettenmaier et al., 2014). Given that S6K but not PKB/Akt requires a docking interaction of its HM to the PIF pocket of PDK1 for its efficient phosphorylation, we hypothesized that the differential effect of GSK2334470 on S6K and PKB/Akt could be related to the identified reverse allosteric regulation effect. We performed a set of experiments with both GSK2334470 and UCN01 and observed that only the GSK compound displaced the binding of PIFtide from PDK1 (low nanomolar median inhibitory concentration), acting similar to PS653,





**Figure 5. Negative Allosteric Modulation of the Binding to the PIF Pocket by Drugs under Development to the ATP-Binding Site of PDK1 and Aurora Kinase**

(A and B) Effect of UCN01 and GSK2334470 ATP-competitive inhibitors of PDK1 on the interaction of His-PDK1<sub>50-359</sub> and biotin-PIFtide.

(C and D) Effect of VX680 and MLN8237 ATP-competitive inhibitors of Aurora kinase on the interaction of His-Aurora A and biotin-TPX2tide. Data are presented as means  $\pm$  SEM.

while UCN01 did not affect the binding of PIFtide even at high  $\mu$ M concentrations (Figures 5A and 5B). The results suggest a model in which the reverse allosteric effect induced by GSK2334470 enhances the inhibition of S6K phosphorylation, which requires binding to the PIF pocket. In this manner, GSK2334470 could inhibit S6K through a dual mechanism, inhibiting the intrinsic activity of PDK1 and additionally disturbing the docking interaction by reverse allostery, whereas PKB/Akt phosphorylation would be affected by the inhibition of PDK1 intrinsic activity but not by the reverse allosteric effect.

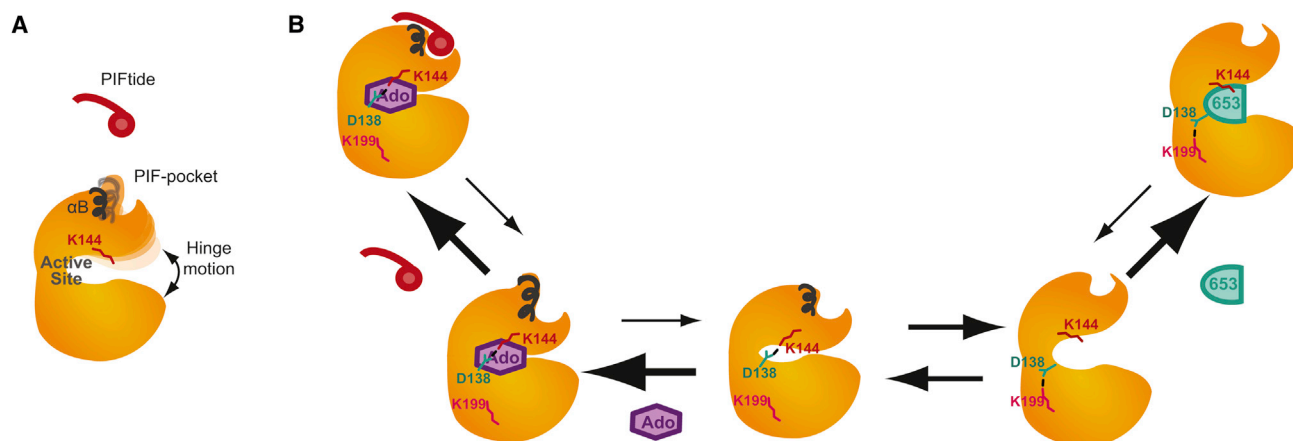
The above findings enable the development of substrate-selective inhibitors of kinases by targeting the ATP-binding site. In order to better illustrate possible uses of the described mechanism we tested the existence of such effects on the mitotic protein kinase Aurora A. The site equivalent to the PIF pocket in Aurora A binds to a protein partner, TPX2, a microtubule-associated protein which localizes the kinase in the centrosomes during mitosis. Aurora kinase also forms complexes with the oncogenes N- and C-Myc, thereby stabilizing Myc and supporting tumor growth. There is interest in the identification of drugs that bind to Aurora kinase, disturb the complex, and destabilize the Myc oncogene (Brockmann et al., 2013; Gustafson et al., 2014). We have thus set up Aurora A-TPX2tide interaction assays, similar to those developed to test the interaction between PDK1 and PIFtide. Two different ATP-competitive drugs that have entered clinical trials were then tested on the reverse allosteric effect in Aurora kinase. Interestingly, while VX680 virtually did not affect the interaction (at  $\mu$ M concentrations), MLN8237 potently displaced the interaction (at low nM concentrations) (Figures 5C and 5D). The finding on Aurora kinase indicates that different compounds binding to the ATP-binding site can produce very different reverse allosteric effects on TPX2, an

approach that can be exploited for the destabilization of N- and C-Myc as well as for the inactivation of Aurora kinase by delocalization.

compounds that take advantage of the bidirectional nature of allosteric networks, enhancing or inhibiting the binding to a downstream signaling partner and further define the molecular mechanism for the reverse allostery between the active site and the PIF pocket in PDK1 (Figure 6). This knowledge can be exploited for protein kinase drug discovery and drug development.

We not only describe compounds that make full use of the bidirectional nature of allosteric communication by binding at the ATP-binding site and affecting the PIF pocket regulatory site, but we are also depicting the global molecular mechanism underlying the allosteric cross-regulation of the two sites. The hinge motion plays a pivotal role in allowing the allosteric communication. Orthosteric compounds (e.g., adenosine) that re-modulate the hinge motion and bring the lobes together promote the formation of an allosteric network connecting the ATP-binding site and the PIF pocket. Various residues pertaining to the catalytic and regulatory spines (C- and R-spines) are part of the network (Kornev et al., 2006), and two salt bridges alternatively formed by Lys144 act as a switching mechanism turning the communication on or off. Adenosine, by allosterically increasing the flexibility of  $\alpha$ B and  $\alpha$ C helices (Figure 4B), increases the ability of the PIF-pocket region to adapt to the binding PIFtide peptide. In stark contrast to adenosine, PS653 weakens the C-spine and favors an open-hinge conformation, breaking the allosteric communication network and locking the  $\alpha$ B and  $\alpha$ C helices lining the PIF pocket in a conformation less favorable to the binding of the PIFtide peptide. In this manner, PS653 disfavors the binding of the prototypical HM polypeptide that physiologically bind to the PIF pocket.

Our initial unbiased MD simulations clearly showed the existence of different conformations of PDK1, which fitted very well



**Figure 6. Schematic Representation of the Bidirectional Allosteric Modulation of AGC Kinases by Small Compounds**

(A) Representation of the hinge motion in equilibrium between open and closed structures of the protein kinase catalytic domain, showing the orthosteric ATP-binding site (active site), the HM polypeptide PIFtide, and the PIF pocket (allosteric site). The end-to-end distance variations of the  $\alpha$ B helix are represented by the length of helices. The key residue Lys144 is represented as a red stick.

(B) Representation of the reverse allosteric mechanism by which PS653 and adenosine (Ado) affect the conformation of the PIF pocket and the binding of PIFtide. PS653 binds to the ATP-binding site, stabilizes open conformations, and rigidifies the PIF pocket, resulting in the inhibition of the binding of PIFtide. Adenosine binds to the ATP-binding site, favors the dynamic closure of the structure and disorients the PIF pocket in the closed conformation, resulting in the cooperative binding of PIFtide. Lys144, Asp138, and Lys199 (shown as sticks) are represented in alternative salt bridge interactions formed in the closed and open conformations of PDK1.

to all previous knowledge on the biochemical and structural information on the activation of PDK1 by PIFtide and small compounds binding to the PIF pocket. With the current availability of computer resources it is not possible to extend such unbiased simulations up to the high  $\mu$ s to ms time scales. However, this unbiased result prompted us to explore the dynamics of PDK1 by focusing on particular areas of the protein, using PT-WTE, an effective enhanced-sampling algorithm. The detailed information from biochemical studies, structural work and MD simulations, and PT-WTE provided a clear mechanism of the bidirectional allosteric process in PDK1. We believe that a similar approach can be effectively used to investigate the mechanisms of allosteric regulation by occupancy of the site equivalent to the PIF pocket in other protein kinases.

The previous characterization that GSK2334470 is a much stronger inhibitor of S6K than of PKB/Akt could be explained by the fact that this compound does not produce a double inhibitory effect on PKB/Akt, because this kinase does not require the HM-docking interaction with the PIF pocket of PDK1 for its physiological phosphorylation. Thus, at concentrations where PDK1 is not fully inhibited by GSK2334470, its effects are similar to compounds binding to the PIF pocket, which selectively inhibit S6K without affecting PKB/Akt (Busschots et al., 2012; Rettenmaier et al., 2014). There is some precedence for the paradoxical effects observed by drugs binding at the ATP-binding site of protein kinases. A reverse allosteric effect is probably responsible for the nucleotide-pocket-induced activation of PKC $\epsilon$  (Cameron et al., 2009) and the allosteric RNase activation of IRE1 $\alpha$  (Wang et al., 2012). In addition, there is some evidence that a similar reverse allosteric modulation might be involved in the “paradoxical activation” of partially inhibited B-RAF dimers (Heidorn et al., 2010). Interestingly, the detailed characterization of the allosteric modulation by compounds binding to the ATP-binding site could

inspire the rational development of drugs with add-on characteristics; a rich example to use for discussion of the implications of this is that of Aurora kinase. Compounds that bind to the ATP-binding site and allosterically affect the interaction with TPX2 are expected to destabilize the oncogene Myc by destabilizing the Aurora-Myc complexes. In addition, since TPX2 provides proper localization to Aurora A, compounds that bind to the ATP-binding site and allosterically disrupt the interactions with TPX2 may render molecules functionally unable to phosphorylate their specific substrates, even after the dissociation of the compound. Similarly, it can be expected that compounds that produce desired double effects, i.e., blocking the active site and, in addition, disrupting or enhancing a given allosteric interaction may be desired. We must note that the current nomenclature to describe the mode of inhibition of kinases by small compounds (type I, type II, and type III inhibitors) does not appropriately describe the allosteric effects of the compounds. A more appropriate nomenclature, in use for allosteric compounds acting on receptors, requires the naming of the identity of the receptor, the binding site of the small molecule, the site which is being allosterically affected, and the kind of modulation. In one of the examples described here, GSK2334470 is a type I inhibitor but is also a negative allosteric modulator binding to the ATP-binding site and displacing the binding of PIFtide from the PIF pocket of PDK1.

Here, we also provide the crystal structure of PDK1 in complex with the phosphorylated HM of Akt/PKB. The equivalent docking interaction is necessary for the phosphorylation and activation of most PDK1 substrates, including S6K and SGK. However, the PIF-pocket docking interaction is not considered to be required for the phosphorylation of Akt/PKB (Biondi et al., 2001; Busschots et al., 2012; Collins et al., 2003; Rettenmaier et al., 2014). Our structure suggests that the docking interaction still

takes place for Akt/PKB, even if it is not the determinant of the phosphorylation of this kinase. During the writing of this article, the crystal structure of PDK1 in complex with a short version of PIFtide was published (Rettenmaier et al., 2014). From our results, it appears that PDK1 might require a limited increased flexibility of the PIF pocket to sample the appropriate conformation for PIFtide binding. According to this scenario, the disorder of the  $\alpha$ C helix previously observed in solution (Hindie et al., 2009) and the disorder of  $\alpha$ B helix predicted here by the MD simulations might provide a mechanism to facilitate the binding of different substrate HM polypeptides. This entropy-driven binding mechanism, also observed in ternary complexes of PKA (Masterson et al., 2011), may be an essential component of the ability of PDK1 to act as a “conformational sensor” (Biondi, 2004).

The initial crystal structures of PKA already depicted the versatility of the kinase domain revealing open, intermediate, and closed conformations of the ATP-binding site (Johnson et al., 2001). The hinge interlobe motion has subsequently been described by MD simulations of PKA (Masterson et al., 2011) and other kinases (Dolker et al., 2014). In PKA, it was revealed that the rate of hinge motion of the PKA catalytic domain ( $20 \text{ s}^{-1}$ ) correlated with the turnover of the enzyme (Kim et al., 2015; Srivastava et al., 2014). The turnover of PDK1 phosphorylation of different substrates, however, is approximately 1,000 times slower ( $0.6\text{--}1 \text{ min}^{-1}$ ) (Biondi et al., 2001), therefore indicating that most opening and closing motions of the PDK1 kinase domain are non-productive, not leading to the phosphorylation of substrates.

It is worth noting that a very important cellular molecule, such as adenosine, produces reverse allosteric effects on PDK1 in vitro. Adenosine is not only a metabolite from the synthesis of ATP, the substrate of PDK1, but is also a signaling molecule on its own (Borea et al., 2016). Our results suggest that an increase in adenosine could lead to the engagement of PDK1 in complexes with S6K, subtly regulating the identity of substrates of PDK1 to become phosphorylated. It is tempting to speculate that other protein kinases could also respond to the levels of ATP intermediates or other small physiological nucleotide molecules. This mechanism could indeed regulate cellular signaling by affecting the interactions of proteins with regulatory domains or regulatory subunits, formation of protein complexes, modulation of localization, or phosphorylation by upstream kinases, etc.

Previous data indicate that the full bidirectional allosteric regulation between the active site and the PIF pocket is functional in the regulation of AGC kinases, perhaps best exemplified in PRK2, where a pseudosubstrate inhibitory polypeptide, PLKtide binding to the active site, and PIFtide binding to the PIF pocket, are allosterically mutually excluded and contribute to the complex mechanism of regulation of PRKs (Bauer et al., 2012).

Although the mechanisms of allostery have been investigated for the last 50 years, it is still not possible to rationally design small molecules to modulate allosteric cellular switches at will. This work provides a deep understanding of the bidirectional allosteric coupling mechanism and suggests ways to exploit it. The use of MD simulations as described above enables the in silico testing of molecules before synthesis, and provides a technology to guide the design of variant compounds that produce a desired reverse allosteric effect. We expect that the results from this work will inspire the development of drugs with

reverse allosteric effects on other protein kinases and, more generally, on other signaling proteins.

## SIGNIFICANCE

**Allostery is a fundamental and widespread regulation mechanism by which proteins transfer information between remote sites and functional sites in response to different stimuli. It is of interest to modulate allosteric processes for treatment of diseases and for future design of regulated molecular machines. Our proposed PDK1 model provides information on the conformational transitions of the catalytic domain and depicts for the first time, in atomistic detail, the bidirectional allosteric communication between the ATP-binding site and the regulatory PIF pocket, where compounds binding to the ATP-binding site can allosterically enhance or inhibit the interaction with a cellular partner. More generally, drugs directed to the ATP-binding site that have equal ability to inhibit a given kinase may have very different effects on the formation of protein complexes and cell signaling, different on-target side effects, and, overall, different degrees of success in the treatment of patients. The PDK1/AGC kinase allosteric model system provides a rich example of the potential of small molecules to bidirectionally modulate the conformation of a protein kinase. By providing a validated computer simulation it will now be possible to use this tool to design allosteric drugs with a given allosteric effect. In turn, we expect that the perspective presented here will benefit drug development of members of the protein kinase family by providing a computer platform from which to rationally design compounds directed to the PIF pocket or ATP-binding site and produce desired secondary allosteric effects. Similarly, the above approach could also be applied more generally for drug discovery projects of other allosteric signaling proteins the functions of which could be modulated by molecules designed to produce desired conformational changes.**

## EXPERIMENTAL PROCEDURES

### Materials

The polypeptide substrate of protein kinase PDK1 was T308tide (KTFC GTPEYLAPEVRR; >75% purity). Other polypeptides used were PIFtide (REPRILSEEEQEMFRDFDYIADWS), biotin-PIFtide (biotin-REPRILSEEEQEMFRDFDYIADWS) and biotin-TPX2tide (biotin-MSQVKSSYSYDAPSDFINFSSL DDEGDTQNIDSWFEEKANLEN-NH<sub>2</sub>). Adenine and adenosine ( $\geq 99\%$ ) were from Sigma-Aldrich.

### Crystal Structures

To obtain the crystal structure of PDK1 in complex with PS653, PSE10, adenine, and adenosine, PDK1 was expressed, purified, concentrated, crystallized, and soaked with compounds as previously described (Hindie et al., 2009). The crystal structure of PDK1 in the new crystal packing was obtained in a screening for new crystallization conditions in the presence of HM polypeptides. PDK1<sub>50-359</sub> (Y288G,Q292A) was co-crystallized in the presence of the phosphorylated peptide KGAGGGGFQFS(P)YSA (underlined residues are conserved in the HM phosphorylation site of Akt/PKBs, SGKs, S6Ks, and PKCs).

### PDK1 Expression, Purification, and In Vitro Activity Test

His-tagged PDK1<sub>1-556</sub> and His-tagged PDK1<sub>50-359</sub> employed in activity assays and in the AlphaScreen interaction assays were expressed in insect cells and

purified through Ni-NTA and gel-filtration chromatography as previously described (Busschots et al., 2012; Engel et al., 2006). His-tagged human Aurora A<sub>1-403</sub> was recombinantly expressed in bacteria from pET28 plasmid and purified as described above for PDK1. The GST-fusion proteins were obtained from HEK293 cells after transient transfection of the corresponding pEBG2T plasmids and purified as previously described (Engel et al., 2006). The detailed conditions for the activity assay are described in the [Supplemental Information](#).

#### AlphaScreen Interaction-Displacement Assays

The AlphaScreen assay was performed according to the standard protocol from the manufacturer (PerkinElmer). The setup of the interaction-displacement assay was previously described (Busschots et al., 2012). In short, the assay was performed in a final volume of 25  $\mu$ L in white 384-well microtiter plates (Greiner Bio-One) with His-PDK1<sub>50-359</sub> (Tyr288Gly; Gln292Ala) (25 nM) and biotin-PIFtide (25 nM) in a buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM DTT, 0.01% (v/v) Tween 20, and 0.1% (w/v) BSA, followed by the addition of 5  $\mu$ L of beads (nickel chelate-coated acceptor beads and streptavidin-coated donor beads; 20  $\mu$ g/mL final concentrations). The setup for Aurora A-TPX2tide interaction assay was the same, except that His-Aurora A (5 nM) and biotin-TPX2tide (10 nM) were employed.

#### Molecular Dynamics

We performed MD simulations using the GROMACS package (Hess et al., 2008) and the Amber99SB\*-ILDN force field (Best and Hummer, 2009; Lindorff-Larsen et al., 2010). PT simulations were performed using PLUMED 2.1 (Tribello et al., 2014). We performed PT with five replicas in the 300–400 K range using the WTE (Bonomi and Parrinello, 2010).

#### Isothermal Titration Calorimetry

Calorimetric titrations were performed using the MicroCal iTC200 instrument (GE Healthcare) as previously described (Hindie et al., 2009) with the modifications indicated in the [Supplemental Experimental Procedures](#).

#### Small Molecules

Compounds **PS653** and **PSE10** were commercially available from Maybridge. The commercial compounds were further analyzed by <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, and ESI-MS (see [Supplemental Experimental Procedures](#)).

#### ACCESSION NUMBERS

The coordinates of the structures have been deposited in the PDB under accession codes PDB: 5LVL (PS653), 5LVM (adenine), 5LVN (adenosine), 5LVO (PSE10), and 5VLP (HM-peptide).

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.chembiol.2016.06.017>.

#### AUTHOR CONTRIBUTIONS

J.O.S. obtained and analyzed most of the crystallography data. V.H. solved initial PDK1 crystals. G.S. and F.L.G. designed the computational analysis. G.S. did the MD simulations. K.B. set up the screening assay and M.N.L. did biophysical assays. D.O. provided medicinal chemistry support. Screening and biochemical characterizations were performed by E.S., K.B., A.K.H., and S.N. under the supervision of R.M.B. S.Z. provided advice. P.M.A. supervised V.H. and analyzed data. R.M.B. supervised the whole research project. The manuscript was written by R.M.B. and F.L.G., supported by J.O.S., G.S., and P.M.A.

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#### REFERENCES

- Arencibia, J.M., Pastor-Flores, D., Bauer, A.F., Schulze, J.O., and Biondi, R.M. (2013). AGC protein kinases: from structural mechanism of regulation to allosteric drug development for the treatment of human diseases. *Biochim. Biophys. Acta* 1834, 1302–1321.
- Axten, J.M., Blackledge, C.W., Brady, G.P., Feng, Y., Grant, S.W., Medina, J.R., Milller, W.H., and Romeril, S.P. (2010). Preparation of 6-(4-pyrimidinyl)-1H-indazole derivatives as PDK1 inhibitors. Patent Application Number, PCT/US2009/064891, WO 2010059658.
- Bauer, A.F., Sonzogni, S., Meyer, L., Zeuzem, S., Piiper, A., Biondi, R.M., and Neimanis, S. (2012). Regulation of protein kinase C-related protein kinase 2 (PRK2) by an intermolecular PRK2-PRK2 interaction mediated by its N-terminal domain. *J. Biol. Chem.* 287, 20590–20602.
- Bayascas, J.R. (2008). Dissecting the role of the 3-phosphoinositide-dependent protein kinase-1 (PDK1) signalling pathways. *Cell Cycle* 7, 2978–2982.
- Bayliss, R., Sardon, T., Vernos, I., and Conti, E. (2003). Structural basis of Aurora-A activation by TPX2 at the mitotic spindle. *Mol. Cell* 12, 851–862.
- Best, R.B., and Hummer, G. (2009). Optimized molecular dynamics force fields applied to the helix-coil transition of polypeptides. *J. Phys. Chem. B* 113, 9004–9015.
- Biondi, R.M. (2004). Phosphoinositide-dependent protein kinase 1, a sensor of protein conformation. *Trends Biochem. Sci.* 29, 136–142.
- Biondi, R.M., Cheung, P.C., Casamayor, A., Deak, M., Currie, R.A., and Alessi, D.R. (2000). Identification of a pocket in the PDK1 kinase domain that interacts with PIF and the C-terminal residues of PKA. *EMBO J.* 19, 979–988.
- Biondi, R.M., Kieloch, A., Currie, R.A., Deak, M., and Alessi, D.R. (2001). The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. *EMBO J.* 20, 4380–4390.
- Bonomi, M., and Parrinello, M. (2010). Enhanced sampling in the well-tempered ensemble. *Phys. Rev. Lett.* 104, 190601.
- Borea, P.A., Gessi, S., Merighi, S., and Varani, K. (2016). Adenosine as a multi-signalling guardian angel in human diseases: when, where and how does it exert its protective effects? *Trends Pharmacol. Sci.* 37, 419–434.
- Bray, D., and Duke, T. (2004). Conformational spread: the propagation of allosteric states in large multiprotein complexes. *Annu. Rev. Biophys. Biomol. Struct.* 33, 53–73.
- Brockmann, M., Poon, E., Berry, T., Carstensen, A., Deubzer, H.E., Rycak, L., Jamin, Y., Thway, K., Robinson, S.P., Roels, F., et al. (2013). Small molecule inhibitors of aurora-A induce proteasomal degradation of N-Myc in childhood neuroblastoma. *Cancer Cell* 30, 357–358.
- Busschots, K., Lopez-Garcia, L.A., Lammi, C., Stroba, A., Zeuzem, S., Piiper, A., Alzari, P.M., Neimanis, S., Arencibia, J.M., Engel, M., et al. (2012). Substrate-selective inhibition of protein kinase PDK1 by small compounds that bind to the PIF-pocket allosteric docking site. *Chem. Biol.* 19, 1152–1163.



- Cameron, A.J., Escribano, C., Saurin, A.T., Kostecky, B., and Parker, P.J. (2009). PKC maturation is promoted by nucleotide pocket occupation independently of intrinsic kinase activity. *Nat. Struct. Mol. Biol.* **16**, 624–630.
- Changeux, J.P. (2012). Allosteric and the Monod-Wyman-Changeux model after 50 years. *Annu. Rev. Biophys.* **41**, 103–133.
- Collins, B.J., Deak, M., Arthur, J.S., Armit, L.J., and Alessi, D.R. (2003). In vivo role of the PIF-binding docking site of PDK1 defined by knock-in mutation. *EMBO J.* **22**, 4202–4211.
- Conn, P.J., Lindsley, C.W., Meiler, J., and Niswender, C.M. (2014). Opportunities and challenges in the discovery of allosteric modulators of GPCRs for treating CNS disorders. *Nat. Rev. Drug Discov.* **13**, 692–708.
- Dolker, N., Gorna, M.W., Sutto, L., Torralba, A.S., Superti-Furga, G., and Gervasio, F.L. (2014). The SH2 domain regulates c-Abl kinase activation by a cyclin-like mechanism and remodulation of the hinge motion. *PLoS Comput. Biol.* **10**, e1003863.
- Engel, M., Hindie, V., Lopez-Garcia, L.A., Stroba, A., Schaeffer, F., Adrian, I., Imig, J., Idrissova, L., Nastainczyk, W., Zeuzem, S., et al. (2006). Allosteric activation of the protein kinase PDK1 with low molecular weight compounds. *EMBO J.* **25**, 5469–5480.
- Fang, Z., Grutter, C., and Rauh, D. (2013). Strategies for the selective regulation of kinases with allosteric modulators: exploiting exclusive structural features. *ACS Chem. Biol.* **8**, 58–70.
- Fenton, A.W. (2008). Allosteric: an illustrated definition for the 'second secret of life'. *Trends Biochem. Sci.* **33**, 420–425.
- Frodin, M., Antal, T.L., Dummler, B.A., Jensen, C.J., Deak, M., Gammeltoft, S., and Biondi, R.M. (2002). A phosphoserine/threonine-binding pocket in AGC kinases and PDK1 mediates activation by hydrophobic motif phosphorylation. *EMBO J.* **21**, 5396–5407.
- Goodey, N.M., and Benkovic, S.J. (2008). Allosteric regulation and catalysis emerge via a common route. *Nat. Chem. Biol.* **4**, 474–482.
- Gray, N.S., and Fabbro, D. (2014). Discovery of allosteric BCR-ABL inhibitors from phenotypic screen to clinical candidate. *Methods Enzymol.* **548**, 173–188.
- Gustafson, W.C., Meyerowitz, J.G., Nekritz, E.A., Chen, J., Benes, C., Charron, E., Simonds, E.F., Seeger, R., Matthay, K.K., Hertz, N.T., et al. (2014). Drugging MYCN through an allosteric transition in Aurora kinase A. *Cancer Cell* **26**, 414–427.
- Heidorn, S.J., Milagre, C., Whittaker, S., Noury, A., Niculescu-Duvas, I., Dhomen, N., Hussain, J., Reis-Filho, J.S., Springer, C.J., Pritchard, C., et al. (2010). Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. *Cell* **140**, 209–221.
- Herbert, C., Schieborr, U., Saxena, K., Juraszek, J., De Smet, F., Alcouffe, C., Bianciotto, M., Saladino, G., Sibrac, D., Kudlinzki, D., et al. (2013). Molecular mechanism of SSR128129E, an extracellularly acting, small-molecule, allosteric inhibitor of FGF receptor signaling. *Cancer Cell* **23**, 489–501.
- Hess, B., Kutzner, C., van der Spoel, D., and Lindahl, E. (2008). GROMACS 4: Algorithms for highly efficient, load-balanced, and scalable molecular simulation. *J. Chem. Theory Comput.* **4**, 435–447.
- Hindie, V., Stroba, A., Zhang, H., Lopez-Garcia, L.A., Idrissova, L., Zeuzem, S., Hirschberg, D., Schaeffer, F., Jorgensen, T.J.D., Engel, M., et al. (2009). Structure and allosteric effects of low molecular weight activators on the protein kinase PDK1. *Nat. Chem. Biol.* **5**, 758–764.
- Johnson, D.A., Akamine, P., Radzio-Andzelm, E., Madhusudan, M., and Taylor, S.S. (2001). Dynamics of cAMP-dependent protein kinase. *Chem. Rev.* **101**, 2243–2270.
- Kim, J., Masterson, L.R., Cembran, A., Verardi, R., Shi, L., Gao, J., Taylor, S.S., and Veglia, G. (2015). Dysfunctional conformational dynamics of protein kinase A induced by a lethal mutant of phospholamban hinder phosphorylation. *Proc. Natl. Acad. Sci. USA* **112**, 3716–3721.
- Komander, D., Kular, G.S., Bain, J., Elliott, M., Alessi, D.R., and Van Aalten, D.M. (2003). Structural basis for UCN-01 (7-hydroxystaurosporine) specificity and PDK1 (3-phosphoinositide-dependent protein kinase-1) inhibition. *Biochem. J.* **375**, 255–262.
- Kornev, A.P., and Taylor, S.S. (2015). Dynamics-driven allostery in protein kinases. *Trends Biochem. Sci.* **40**, 628–647.
- Kornev, A.P., Haste, N.M., Taylor, S.S., and Eyck, L.F. (2006). Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. *Proc. Natl. Acad. Sci. USA* **103**, 17783–17788.
- Kuriyan, J., and Eisenberg, D. (2007). The origin of protein interactions and allostery in colocalization. *Nature* **450**, 983–990.
- Lindorff-Larsen, K., Piana, S., Palmo, K., Maragakis, P., Klepeis, J.L., Dror, R.O., and Shaw, D.E. (2010). Improved side-chain torsion potentials for the Amber ff99SB protein force field. *Proteins* **78**, 1950–1958.
- Masterson, L.R., Cheng, C., Yu, T., Tonelli, M., Kornev, A., Taylor, S.S., and Veglia, G. (2011). Dynamics connect substrate recognition to catalysis in protein kinase A. *Nat. Chem. Biol.* **6**, 821–828.
- Monod, J., Wyman, J., and Changeux, J.P. (1965). On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.* **12**, 88–118.
- Nagar, B., Hantschel, O., Young, M.A., Scheffzek, K., Veach, D., Bornmann, W., Clarkson, B., Superti-Furga, G., and Kuriyan, J. (2003). Structural basis for the autoinhibition of c-Abl tyrosine kinase. *Cell* **112**, 859–871.
- Najafav, A., Sommer, E.M., Axten, J.M., Deyoung, M.P., and Alessi, D.R. (2011). Characterization of GSK2334470, a novel and highly specific inhibitor of PDK1. *Biochem. J.* **433**, 357–369.
- Nussinov, R., and Tsai, C.J. (2013). Allosteric in disease and in drug discovery. *Cell* **153**, 293–305.
- Nussinov, R., and Tsai, C.J. (2014). Unraveling structural mechanisms of allosteric drug action. *Trends Pharmacol. Sci.* **35**, 256–264.
- Rettenmaier, T.J., Sadowsky, J.D., Thomsen, N.D., Chen, S.C., Doak, A.K., Arkin, M.R., and Wells, J.A. (2014). A small-molecule mimic of a peptide docking motif inhibits the protein kinase PDK1. *Proc. Natl. Acad. Sci. USA* **111**, 18590–18595.
- Sadowsky, J.D., Burlingame, M.A., Wolan, D.W., McClendon, C.L., Jacobson, M.P., and Wells, J.A. (2011). Turning a protein kinase on or off from a single allosteric site via disulfide trapping. *Proc. Natl. Acad. Sci. USA* **108**, 6056–6061.
- Saladino, G., and Gervasio, F.L. (2012). New insights in protein kinase conformational dynamics. *Curr. Top. Med. Chem.* **12**, 1889–1895.
- Srivastava, A.K., McDonald, L.R., Cembran, A., Kim, J., Masterson, L.R., McClendon, C.L., Taylor, S.S., and Veglia, G. (2014). Synchronous opening and closing motions are essential for cAMP-dependent protein kinase A signaling. *Structure* **22**, 1735–1743.
- Stockman, B.J., Kothe, M., Kohls, D., Weibley, L., Connolly, B.J., Sheils, A.L., Cao, Q., Cheng, A.C., Yang, L., Kamath, A.V., et al. (2009). Identification of allosteric PIF-pocket ligands for PDK1 using NMR-based fragment screening and 1H-15N TROSY experiments. *Chem. Biol. Drug Des.* **73**, 179–188.
- Sugita, Y., and Okamoto, Y. (1999). Replica-exchange molecular dynamics method for protein folding. *Chem. Phys. Lett.* **314**, 141–151.
- Takahashi, I., Asano, K., Kawamoto, I., Tamaoki, T., and Nakano, H. (1989). UCN-01 and UCN-02, new selective inhibitors of protein kinase C. I. Screening, producing organism and fermentation. *J. Antibiot. (Tokyo)* **42**, 564–570.
- Taylor, S.S., Ilouz, R., Zhang, P., and Kornev, A.P. (2012). Assembly of allosteric macromolecular switches: lessons from PKA. *Nat. Rev. Mol. Cell Biol.* **13**, 646–658.
- Tribello, G.A., Bonomi, M., Branduardi, D., Camilloni, C., and Bussi, G. (2014). PLUMED 2: new feathers for an old bird. *Computer Phys. Commun.* **185**, 604–613.
- Wang, L., Perera, B.G., Hari, S.B., Bhatarai, B., Backes, B.J., Seeliger, M.A., Schurer, S.C., Oakes, S.A., Papa, F.R., and Maly, D.J. (2012). Divergent allosteric control of the IRE1 $\alpha$  endoribonuclease using kinase inhibitors. *Nat. Chem. Biol.* **8**, 982–989.
- Weber, G. (1972). Ligand binding and internal equilibria in proteins. *Biochemistry* **11**, 864–878.
- Wei, L.Y., Gao, X.Q., Warne, R., Hao, X.S., Bussiere, D., Gu, X.J., Uno, T., and Liu, Y. (2010). Design and synthesis of benzazepin-2-one analogs as allosteric binders targeting the PIF pocket of PDK1. *Bioorg. Med. Chem. Lett.* **20**, 3897–3902.

Wojcik, J., Hantschel, O., Grebien, F., Kaube, I., Bennett, K.L., Barkinge, J., Jones, R.B., Koide, A., Superti-Furga, G., and Koide, S. (2010). A potent and highly specific FN3 monobody inhibitor of the Abl SH2 domain. *Nat. Struct. Mol. Biol.* 17, 519–527.

Yang, J., Cron, P., Thompson, V., Good, V.M., Hess, D., Hemmings, B.A., and Barford, D. (2002). Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation. *Mol. Cell* 9, 1227–1240.

Zhang, X., Gureasko, J., Shen, K., Cole, P.A., and Kuriyan, J. (2006). An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell* 125, 1137–1149.

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

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## **Supplemental Information**

### **Bidirectional Allosteric Communication between the ATP-Binding Site and the Regulatory PIF Pocket in PDK1 Protein Kinase**

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## Supplemental Information

Bidirectional allosteric communication between the ATP-binding site and the regulatory PIF-pocket in PDK1 protein kinase

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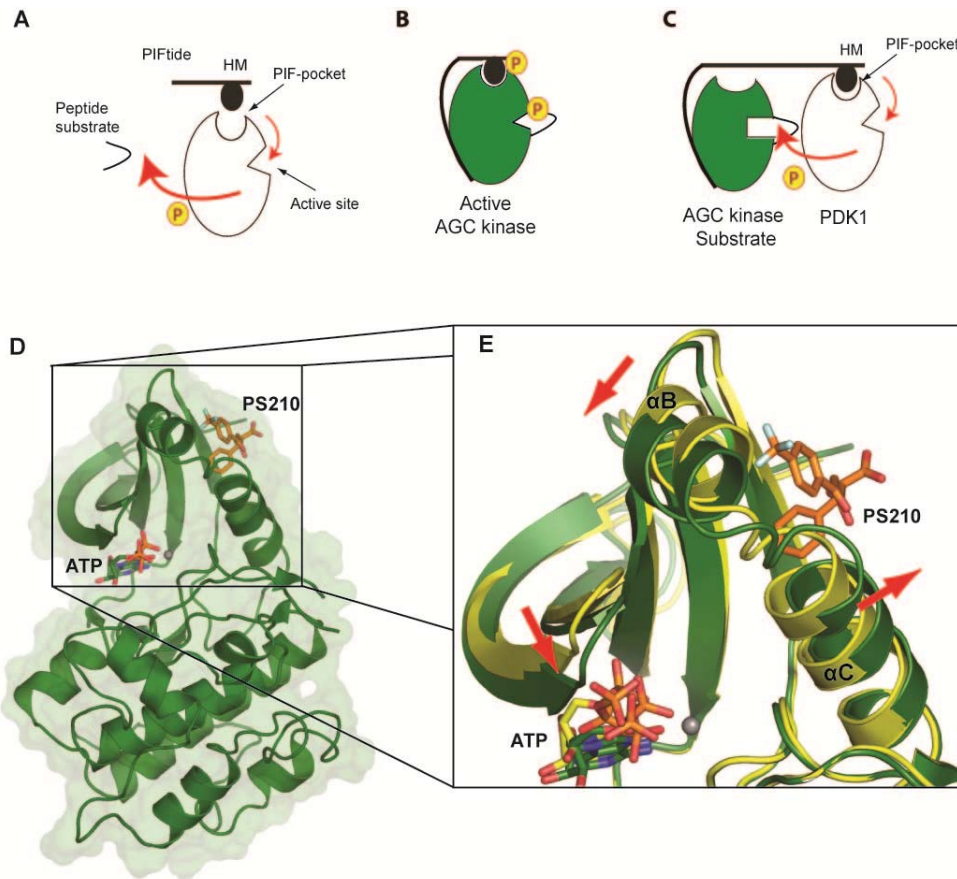
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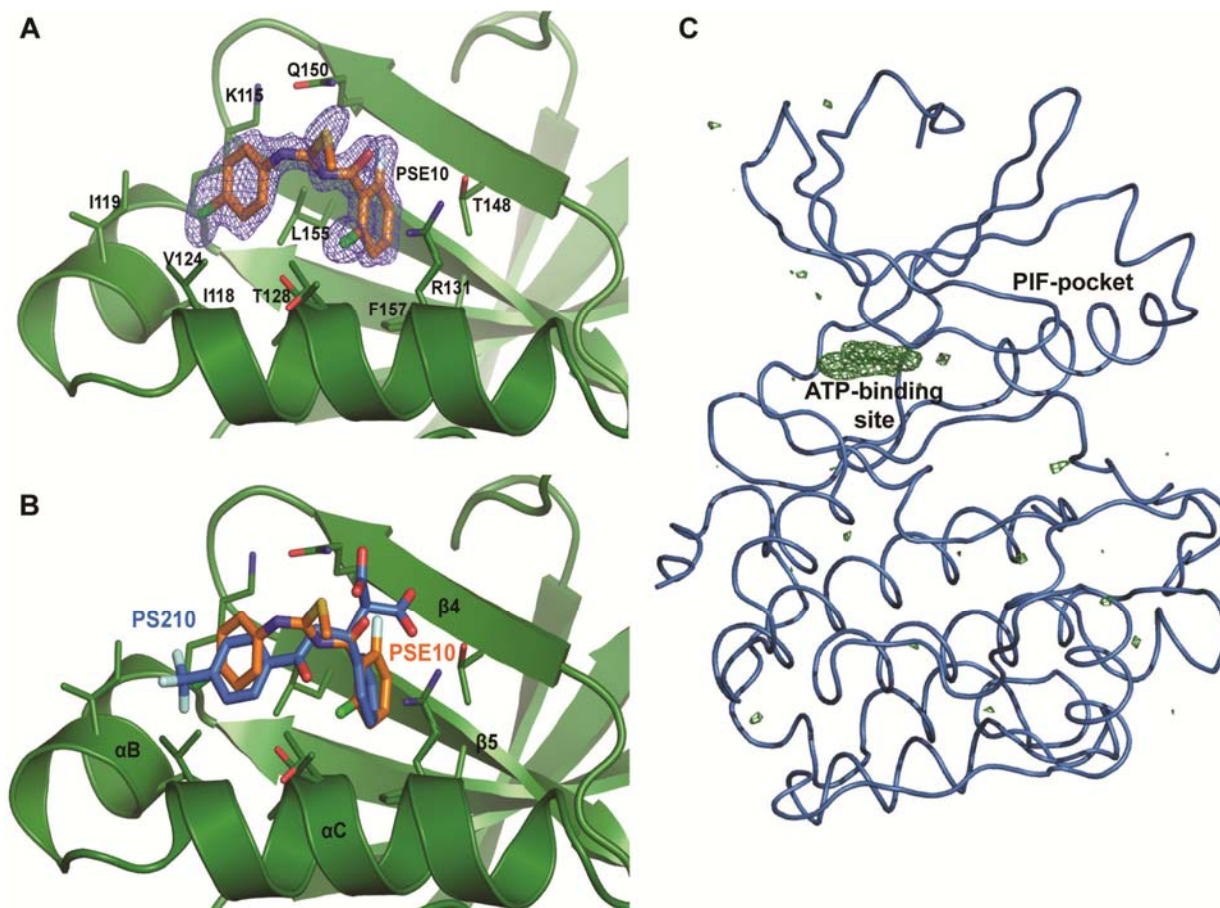
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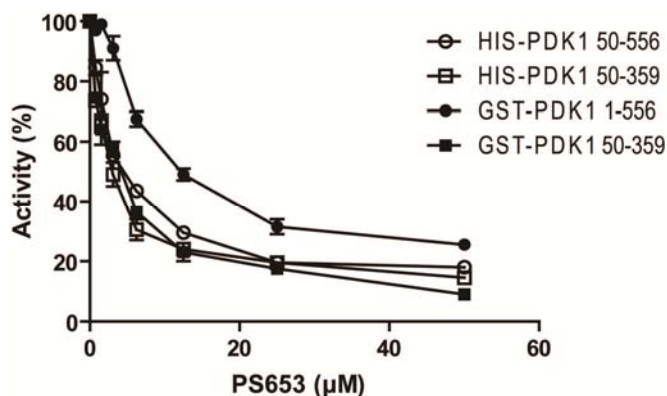


**Figure S1. Related to Figure 1.** Role of the PIF-pocket in the activation of AGC kinases and in the docking interaction of PDK1 with substrates. **(A)** Scheme of the catalytic domain of an AGC kinase that is activated *in trans* by a polypeptide comprising a hydrophobic motif (HM), such as PIFtide. The scheme represents the PDK1 *in vitro* activity assay used in this work (Biondi, et al., 2000). The mechanism of activation of by the binding of HM to the PIF-pocket has also been described in members of multiple families of kinases within the AGC kinase group, such as SGK, S6K, RSK, MSK, PKC (Biondi, et al., 2001; Frodin, et al., 2002) and Akt/PKB (Biondi, et al., 2001; Frodin, et al., 2002; Yang, et al., 2002). **(B)** Scheme representing a prototype AGC kinase. The HM, which is located C-terminal to the catalytic domain, binds intramolecularly to the PIF-pocket and acts in concert with the activation loop to stabilize the active conformation after HM and activation loop phosphorylation. **(C)** The PIF-pocket of PDK1 is the docking site for the specific docking interaction with substrates. PDK1 is a conformational sensor that only interacts with inactive protein kinases, which are recognized by the exposure of the HM. The docking interaction between the HM sequence and the PIF-pocket provides selectivity for the phosphorylation of substrates and also activates PDK1. The docking interaction is required for the phosphorylation of most substrates of PDK1 studied (e.g. S6K, SGK, PKCs, PRKs), but not for the phosphorylation of Akt/PKB, as verified *in vitro* (Biondi, et al., 2001) and in cells in culture (Bayascas, 2008; Collins, et al., 2003). Upon activation loop phosphorylation, the substrate acquires the active conformation as shown in (B). While PKA is an AGC kinase, its catalytic domain has evolved to be regulated by the interacting subunit and not by the PIF-pocket. In PKA, the C-terminal HM is constitutively docked into the PIF-pocket and does not play a regulatory role as described for other members of the AGC kinase group. **(D)** Allosteric activation of PDK1 by small compounds binding to the PIF-pocket. The binding of small compounds to the PIF-pocket produces local changes at the binding site and also allosteric effects at the activation loop and the Gly-rich loop, as verified in crystallography and deuterium exchange experiments (Hindie, et al., 2009). The allosteric effect on the ATP-binding site was confirmed using a fluorescent ATP probe, whose fluorescence varied in the presence of activating compounds that bind to the PIF-pocket (Hindie, et al., 2009). The allosteric effect on the ATP-binding site was most notably observed in the crystal structure of PDK1 in complex with PS210. The binding of PS210 induces the complete closure of the ATP-binding site, similar to the most closed-active structure of PKA (Zheng, et al., 1993).

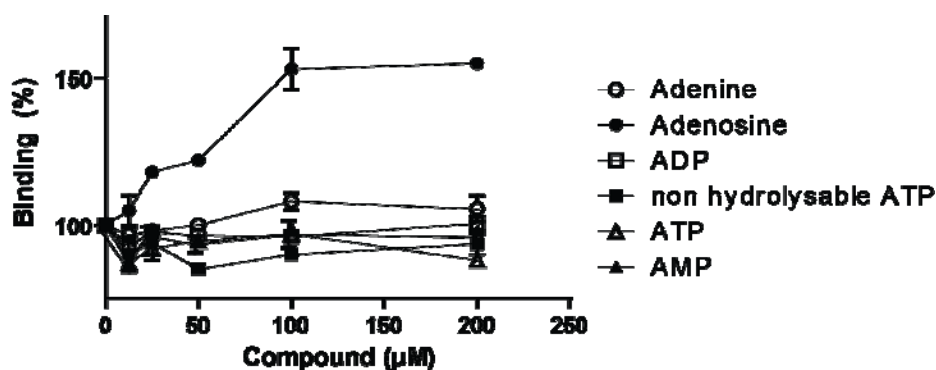


**Figure S2. Related to Figure 1, Figure 2 and Table S1.** Crystal structure of PDK1 in complex with PSE10 in comparison to PS210. **(A)** PDK1 (green) is shown in cartoon representation with residues in direct contact with allosteric compound PSE10 (orange carbon atoms) shown as sticks.  $|2F_o - F_c|$  electron density of the compound is shown in blue and contoured at  $1\sigma$ . **(B)** Superposition with the crystal structure of PS210 (blue carbon atoms) bound to PDK1 (PDB ID 4AW1). The binding mode of both compounds is very similar. The two ring systems align well and occupy the same subpockets of the PIF-pocket. **(C)** PS653 binds only to the ATP-binding site.  $|F_o - F_c|$  electron density of the whole structure is shown in green and contoured at  $5\sigma$ . This “positive difference density” represents only electron density that is not explained well by the structural model. For comparison, PS653 was omitted from the structure before calculation. No electron density of a second PS653 molecule can be observed, in particular not in the PIF-pocket. The remaining difference density belongs only to lowly occupied alternative water molecules and side chains.

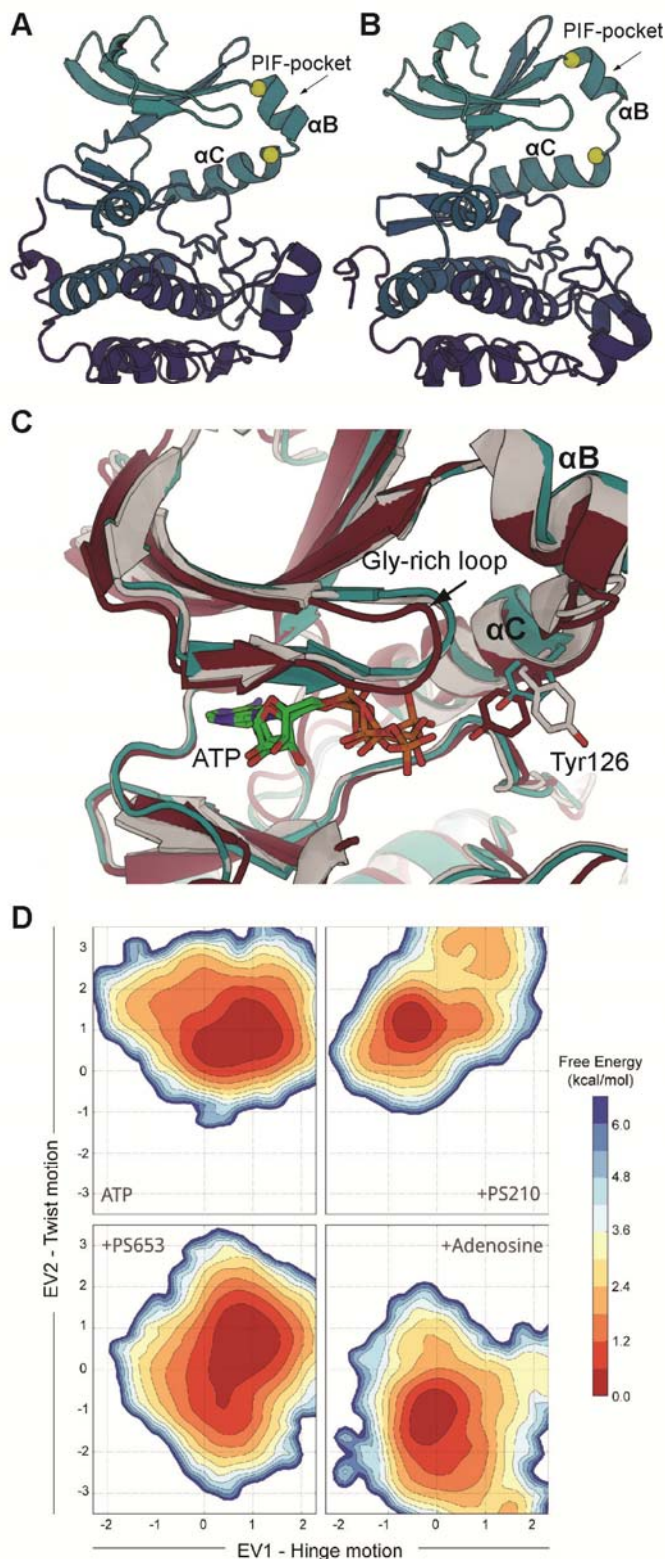
A



B

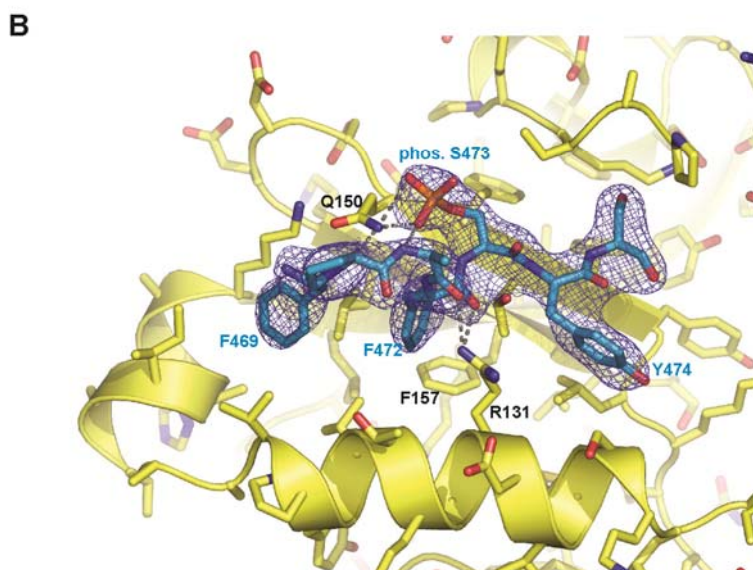
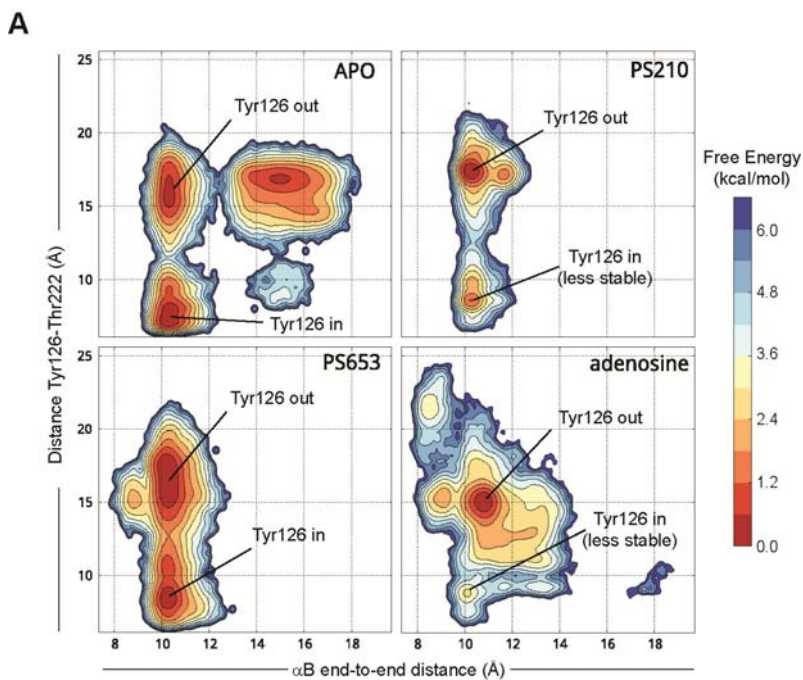


**Figure S3. Related to Figures 1 and 2.** Effect of small compounds that bind at the ATP-binding site on the allosteric modulation of the interaction with PIFtide. **A.** Effect of PS653 negative allosteric modulator on the *in vitro* activity of PDK1. PS653 inhibits the activity of His-PDK1, GST-PDK1, His-PDK1<sub>50-359</sub> and GST-PDK1<sub>1-359</sub>. PDK1 comprises a catalytic domain located N-terminally and a C-terminal PH domain. PS653 inhibits the activity of different constructs of PDK1 comprising or lacking the first 49 aminoacids or the C-terminal region comprising the PH domain (His-PDK1 1-556, IC<sub>50</sub>= 3,3 µM, IC= 1,5-7,3 µM; GST-PDK1 1-556, IC<sub>50</sub>= 7,9 µM, IC= 6,3-9,9 µM; His-PDK1<sub>50-359</sub> [Tyr288Gly; Gln292Ala], IC<sub>50</sub>= 3,3, IC= 2,3-4,4 µM and GST-PDK1<sub>1-359</sub> [Tyr288Gly; Gln292Ala], IC<sub>50</sub>= 5,1 µM; CI= 3,6-7,2 µM). CI, 95% confidence interval. **B.** Adenosine is a positive allosteric modulator that binds to the ATP-binding site of PDK1 and enhances the interaction with PIFtide. The interaction was measured using the alphascreen assay described in Figure 1. Adenine, AMP, ADP, ATP or the non-hydrolyzable ATP analog (up to 200 µM), do not affect the interaction of PDK1 with PIFtide.

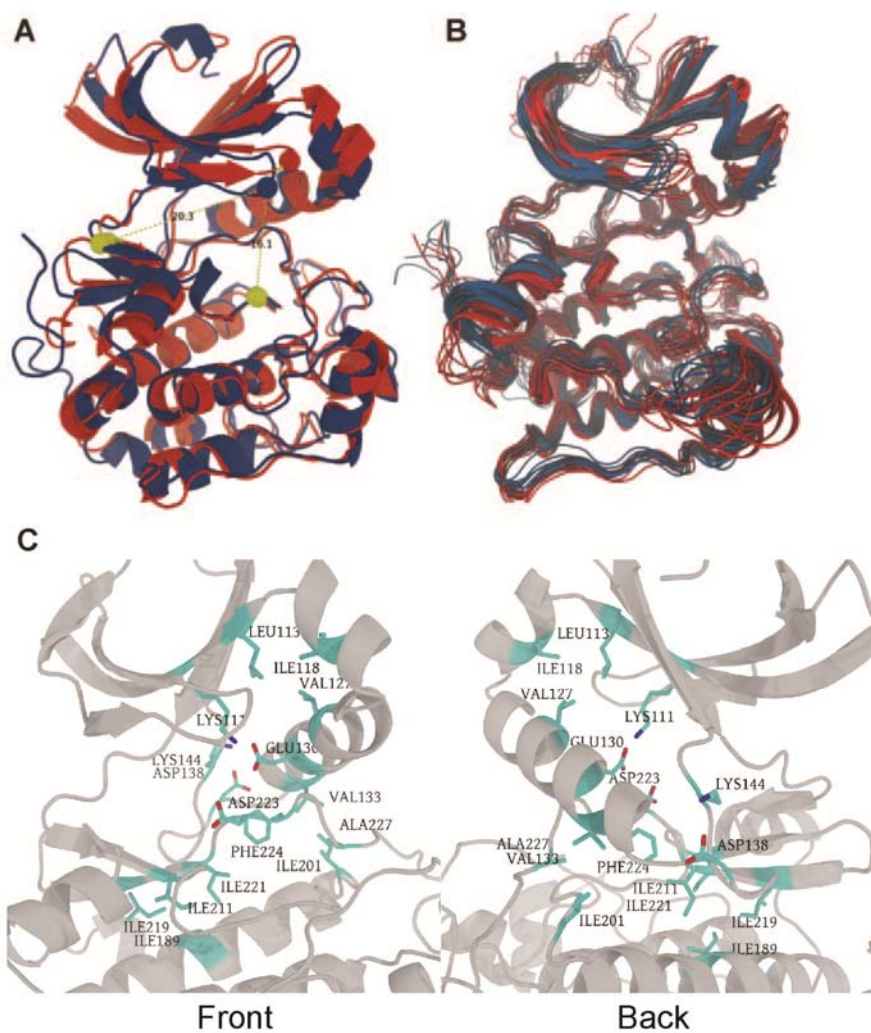


**Figure S4. Related to Figure 3.** Structure of PDK1 from the 400ns MD simulation. (A-B) Two different conformations are observed (a-b) differing by the orientation of helix  $\alpha B$ . The residues used to compute the end-to-end distance (later in the main text) are highlighted as yellow spheres. (C) Different orientations of Tyr126 from the simulation (gray, main orientation; cyan, alternative orientation) and from the new crystal (purple). (D) Projection of the free energy along the first two PCA vectors obtained from the unbiased MD of PDK1 with ATP. When PS210 is bound, the accessible conformations span a narrower range of values along both the first eigenvector, which describes the *hinge motion*, and the second, which depicts the rotation (“twist”) of the N-lobe with respect to the C-lobe. Interestingly, the twist movement coincides with the opening of the ATP-binding site, and its suppression upon PS210 binding also leads to tighter binding. This is in perfect agreement with the crystal structure of PDK1 in complex with PS210, which shows a rotation of the hinge resulting in the most closed structure of PDK1 (Busschots, et al., 2012).

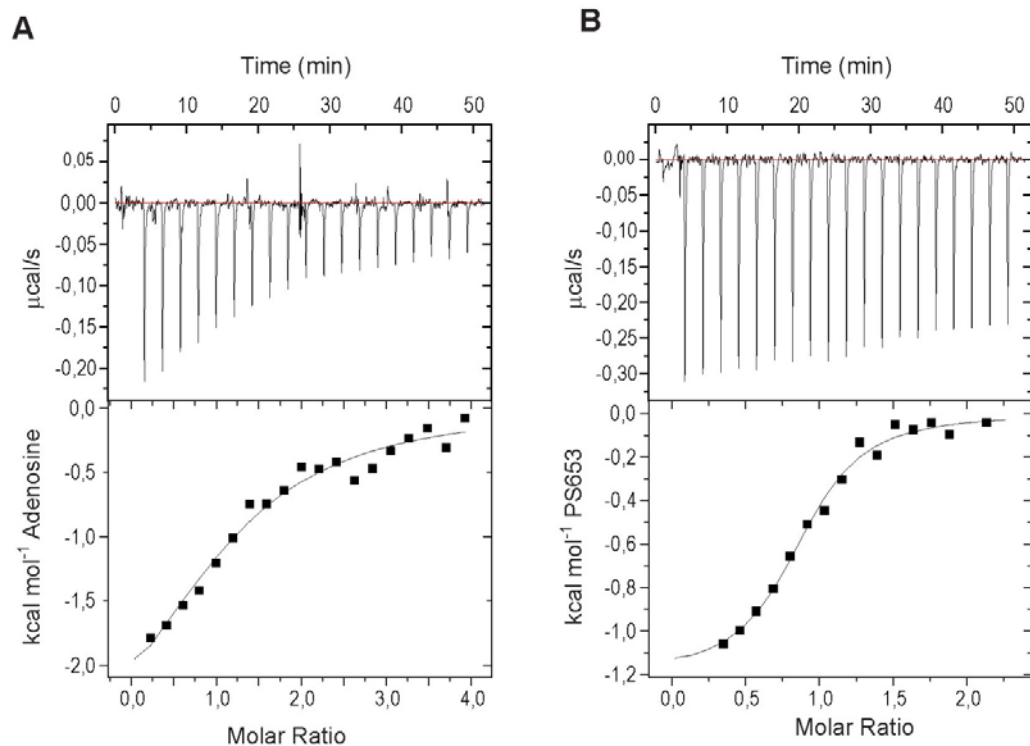




**Figure S5. Related to Figure 3 and Table 1.** PDK1 crystal in complex with phosphorylated HM polypeptide and MD simulations reveal new conformation of Tyr126. (A) Molecular dynamics of Tyr126 in the presence of ATP, PS210, PS653 and adenosine. Free energy projected along the  $\alpha$ B end-to-end distance as in Figure 3 and figure S6 and along the distance Tyr126-Thr222, which discriminates the different orientations of Tyr126. The “alternative” conformation with the OH-group pointing towards ATP (Tyr126 “in”) is less stable with PS210. (B) Crystal structure of PDK1 in complex with a phosphorylated HM polypeptide and ATP. The figure shows a zoom of the PIF-pocket with PDK1 (yellow) in cartoon representation and side chains as sticks. The HM polypeptide is derived from PKB/Akt and the residue numbers correspond to PKB $\alpha$ /Akt1. The peptide is depicted in light blue; its  $|2F_o - F_c|$  electron density is shown in dark blue and contoured at  $1\sigma$ . The crystal structure contains four molecules in the asymmetric unit. Residues 469-475 are well resolved in all four molecules. The structure depicts the docking interaction between the HM of substrates of PDK1 and the PIF-pocket of PDK1. This docking interaction is required for the phosphorylation of a number of substrates of PDK1, i.e. S6K and SGK, but is not required for efficient phosphorylation and activation of PKB/Akt (Biondi, et al., 2001; Busschots, et al., 2012; Collins, et al., 2003; Rettenmaier, et al., 2014). Our structure suggests that the docking interaction still takes place for Akt/PKB, even if it is not the determinant of the phosphorylation of Akt/PKB by PDK1. While writing the current manuscript, the crystal structure of PDK1 in complex with a short version of PIFtide was published (Rettenmaier, et al., 2014).



**Figure S6. Related to Figure 3 and Figure 4.** PT-MetaD simulations with PS210 (red) and PS653. (A,B) Most populated structures from the PT-MetaD simulations with PS210 (red) and PS653 (blue). While both ligands stabilize the PIF pocket, different conformations are observed, with PDK1 lobes being more twisted with PS653. (C) Residues that have increased interactions in the close conformation of PDK1.



**Figure S7. Related to Table 1.** Characterization of adenosine and PS653 interaction with PDK1<sub>50-359</sub> by ITC. **(A)** Interaction of PDK1<sub>50-359</sub> with adenosine. **(B)** Interaction of PDK1<sub>50-359</sub> with PS653. The panels show the raw heat signal for successive injections of compound into a PDK1<sub>50-359</sub> solution. Bottom panels show the integrated heats of injections corrected for heats of dilution for compounds. The solid lines corresponding to the best fit of the data were calculated using Origin<sup>TM</sup> software. Thermodynamic parameter values are given in Table 1.

**Table S1. Related to Figure 2.** Crystallography: Data collection and refinement statistics. See also Figure 2; Figures S2 and S5.

	PS653	PSE10 + ATP	adenine	adenosine	phos. PBK-HM peptide + ATP
<b>Data collection</b>					
Space group	C2	C2	C2	C2	P2 <sub>1</sub>
Cell dimensions <i>a, b, c</i> (Å)	148.2, 44.4, 47.3	148.2, 44.4, 47.9	148.0, 44.4, 47.8	147.4, 44.3, 47.7	47.8, 168.5, 94.9
$\alpha, \beta, \gamma$ (°)	90, 100.3, 90	90, 101.8, 90	90, 102.1, 90	90, 101.4, 90	90, 93.0, 90
Resolution (Å)	73-1.4 (1.5- 1.4)*	73-1.09 (1.19- 1.09)	44-1.27 (1.34- 1.27)	43-1.38 (1.46- 1.38)	95-2.5 (2.6-2.5)
$R_{\text{sym}}$	5.1 (68.3) *	5.1 (68.3)	4.9 (70.7)	3.4 (55.3)	10.4 (70.8)
$I / \sigma I$	14.7 (2.0) *	15.0 (2.0)	17.8 (2.4)	17.6 (2.0)	12.5 (2.2)
Completeness (%)	99.8 (99.8) *	99.7 (99.7)	99.6 (98.1)	99.0 (98.1)	99.7 (97.8)
Redundancy	4.0 (4.0) *	4.8 (3.0)	6.6 (6.2)	3.0 (3.0)	4.2 (3.8)
<b>Refinement</b>					
Resolution (Å)	1.4	1.09	1.27	1.38	2.5
No. reflections	59735	126722	80421	61796	51591
$R_{\text{work}} / R_{\text{free}}$	15.3 / 17.8	14.0 / 16.1	13.0 / 15.7	13.3 / 16.6	19.5 / 23.5
No. atoms					
Protein	2504	2371	2437	2375	9209
Ligand/ion	41	66	17	27	124
Water	267	361	310	256	324
<i>B</i> -factors					
Protein	21.1	14.4	22.0	21.0	50.7
Ligand/ion	27.5	26.3	23.3	28.0	60.2
Water	31.3	26.8	32.6	32.5	41.5
R.m.s deviations					
Bond lengths (Å)	0.013	0.016	0.012	0.008	0.008
Bond angles (°)	1.6	1.6	1.5	1.3	1.2

\*Values in parentheses are for highest-resolution shell.



## Supplemental Experimental Procedures

### Supplemental Materials

Complete protease inhibitor cocktail tablets were from Roche. Protein concentration was determined using Coomassie Plus from Perbio. Protein concentration was performed using Vivaspin concentrators. Ni-NTA and Glutathione sepharose resins were from GE Healthcare. Human embryonic kidney (HEK) 293 cells (ATCC collection) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco). Mammalian tissue culture materials were from Greiner. Insect cell expression system and all the insect cell related material were from Invitrogen. Site-directed mutagenesis was performed using a QuikChange strategy (Stratagene). DNA constructs used for transient transfection were purified from bacteria using a Qiagen plasmid Mega kit according to the manufacturer's protocol. DNA sequences were verified by automatic DNA sequencing (Applied Biosystems 3100 Genetic Analyzer).

### PDK1 expression, purification and in vitro kinase assay

PDK1<sub>50-359</sub> employed in the crystallography work (PDK1<sub>50-359</sub> [Tyr288Gly; Gln292Ala]) was in addition subjected to TEV protease cleavage and re-chromatography through Ni-NTA resin before gel filtration and concentration, as described (Hindie, et al., 2009). *In vitro* PDK1 activity tests were performed using T308tide as a substrate for PDK1 essentially as previously described (Biondi, et al., 2000; Engel, et al., 2006). In brief, the PDK1 activity assay was performed at room temperature (22°C) in a 20 µl mix containing 50 mM Tris pH 7.5, 0.05 mg/ml BSA, 0.1% β-mercaptoethanol, 10 mM MgCl<sub>2</sub>, 100 µM [ $\gamma$ <sup>32</sup>P]ATP (5-50 cpm/pmol), 0.003% Brij, 150-500 ng PDK1, and T308tide 300 µM, or 600 µM for GST-PDK1 assays). The assay was performed in a 96 well format and 4 µl aliquots spotted on p81 phosphocellulose papers (Whatmann) using ep motion 5070 (Eppendorf), washed in 0.01% phosphoric acid, dried, and then exposed and analyzed using PhosphorImager technology (FLA-9000 Starion, Fujifilm). The basal specific activities and the activity in the presence of excess of PIFtide (2 µM) of GST-PDK1 purified constructs were: GST-PDK1<sub>50-359</sub> [Y288G; Q292A] (wt), 0,93 U/mg, plus PIFtide 5,6 U/mg; GST-PDK1<sub>50-359</sub> [Tyr288Gly; Gln292Ala; Lys144Ala] (Lys144Ala), 0,65 U/mg, plus PIFtide 4,99 U/mg; GST-PDK1<sub>50-359</sub> [Tyr288Gly; Gln292Ala; Lys144Glu] (Lys144Glu), 0,65 U/mg, plus PIFtide 5,2 U/mg. Activity measurements were performed in duplicates or triplicates with less than 10 % difference between replicates. Activity measurements were repeated at least twice with different batches of purified proteins.

### Crystal structures

We soak PDK1 crystals with the different compounds over night. However, crystals soaked at the same time with both PS653 and PS210 were completely dissolved after only 3 h. When soaked for 1 h, the crystal structure contained only PS210 (data not shown). Crystals of PDK1 in complex with the phosphorylated HM polypeptide was obtained by hanging drop vapor diffusion at 20 °C. 1.5 µl of protein solution (20 mg/ml, 4.3 mM ATP) was added to 1.5 µl of reservoir solution (1.65 M ammonium sulfate, 100 mM sodium sulfate pH 5.7 and 10 mM DTT). Plate-like crystals grew to a size of 800 µm x 300 µm x 50 µm within two to three weeks.

X-ray diffraction data were collected at beamlines BL14.1 (BESSY II, HZB, Berlin) and PXIII (Swiss Light Source, Villigen). The structure of PDK1 (PDB code 3HRC(Hindie, et al., 2009)) served as a model for molecular replacement. Data were processed using the XDS program package, Phaser, PHENIX and Coot as previously performed (Busschots, et al., 2012). The following Ramachandran plot values (preferred regions/allowed regions/outliers) were obtained using Coot: PS653, 96.3/3.3/0.4; PSE10+ATP, 96.6/3.0/0.4; adenine, 98.0/2.0/0; adenosine, 96.5/3.2/0.4; HM-peptide, 94.7/5.3/0. Molecular graphic figures were prepared using PyMOL (Schrödinger).

### Screening of a library of small compounds using alphascreen technology

The effects of the 14400 diverse set of small compounds (Maybridge HitFinder library; average MW 320) were tested at a concentration of 50 µM. His-PDK1 and Biotin-PIFtide in the presence of DMSO or the different compounds from the library were incubated in the dark for 90 min at room temperature and the emission of light from the acceptor beads was measured in the EnVision reader (Perkin Elmer). The identified hits were compared with those identified in an equivalent screening using His-Aurora kinase and biotin-TPX2. The hits that specifically affected the PDK1-PIFtide screening were further investigated for validation. PS653 (Maybridge code RH00237) and PSE10 (Maybridge code RF02011) are representatives of different validated hits identified that

displaced the PDK1-PIFtide interaction. The interaction between GST-PDK1<sub>50-359</sub> [Tyr288Gly; Gln292Ala] (wt) and biotin-PIFtide was measured in the same assay but using anti-GST and streptavidin-coated donor beads. The conditions for all alphascreen assays were identified in a cross-titration experiment by varying the concentrations of PDK1 and biotin-PIFtide. Conditions were chosen to have 50000-100000 alphascreen counts and be in a linear range. All GST-PDK1 assays contained 1.6 nM PDK1; the interaction with PDK1 wt was measured in the presence of 6.25 nM biotin-PIFtide; 25 nM and 100 nM biotin-PIFtide were used to measure the effect of compounds on GST-PDK1<sub>50-359</sub> [Tyr288Gly; Gln292Ala; Lys144Ala] (Lys144Ala) and PDK1<sub>50-359</sub> [Tyr288Gly; Gln292Ala; Lys144Glu] (Lys144Glu), respectively. When the assay was performed with GST-PDK1 constructs, the displacement of biotin-PIFtide by PS653 reached 50%. Except for the original screening, the assays were performed at least twice, in duplicates and with proteins from at least two different purifications. Representative experiments are shown.

### Details of molecular dynamics simulations

The system was minimized with 10000 steps of conjugated gradient and equilibrated in the NPT ensemble for 10 ns. A production run of 400 ns was then performed in the NVT ensemble, with a time step of 2 fs. Neighbor searching was performed every 5 steps. The PME algorithm was used for electrostatic interactions with a cut-off of 1.2 nm, while a single cut-off of 1.2 nm was used for Van der Waals interactions. Temperature coupling was done with the V-rescale algorithm (Bussi, et al., 2007).

### Isothermal titration calorimetry

Titration were performed by a 0.4  $\mu$ l injection followed by 19 successive injections (2  $\mu$ l) of each compound (adenosine 500  $\mu$ M, PS653 250  $\mu$ M) into a 202  $\mu$ l reaction cell containing PDK1<sub>50-359</sub> (26.5  $\mu$ M and 23  $\mu$ M, respectively). For the titrations of PDK1 with adenosine, the protein and the compound were prepared in 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM DTT and 1% v/v DMSO. For the titration of PDK1 with PS653 the protein and the compound were prepared in 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM DTT and 5% v/v DMSO. Errors on the thermodynamic parameter values in Table 1 are non linear least square fitting errors of the experimental binding isotherms using the Levenberg-Markardt iteration method (Freire et al., 2009). The titration of PDK1 with adenosine was performed at 25°C, and the titration of PDK1 with PS653 was made at 37°C. Raw calorimetric data were corrected for heats of dilution. Binding stoichiometries, enthalpy values and  $K_d$  values were determined by fitting corrected data to a model with one type of sites using Origin7 software (MicroCal Inc.).

### Small molecules PSE10 and PS653

All <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectra were measured on AMX 400 (400MHz) spectrometers. Chemical shifts ( $\delta$ ) are reported in parts per million (ppm). For peak multiplicity are used: s, singlet; d, doublet; t, triplet; m, multiplet. J values are given in Hz. ESI mass spectra were recorded on a Fisons VG Plattform II spectrometer.

**PSE10** ('2-oxopropyl N-(4-chlorophenyl)- [(2-chloro-6-fluorobenzoyl)amino] methanimidothioate; Maybridge RF02011). C<sub>17</sub>H<sub>13</sub>Cl<sub>2</sub>FN<sub>2</sub>O<sub>2</sub>S [399.26 g/mol]. <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) [ $\delta$  ppm]: 7.52 (m, 2H), 7.38 (m, 1H), 7.31 (m, 3H), 7.19 (m, 1H), 7.03 (s, 1H), 3.64 (m, 1H), 3.41 (m, 1H), 1.40 (s, 3H). <sup>13</sup>C-NMR (100 MHz, DMSO-d<sub>6</sub>) [ $\delta$  ppm]: 173.08, 172.36, 159.64, 157.17, 136.21, 132.82, 131.28, 130.93, 130.61, 130.52, 130.37, 130.30, 128.79, 128.29, 125.40, 125.36, 114.72, 114.50, 91.78, 25.83. ESI-MS (M+H<sup>+</sup>): 399.00.

**PS653** ('1,6-dihydrodibenzo[cd, g]indazol-6-one; Maybridge RH00237). C<sub>14</sub>H<sub>8</sub>N<sub>2</sub>O [220.23 g/mol]. <sup>1</sup>H-NMR (400 MHz, DMSO-d<sub>6</sub>) [ $\delta$  ppm]: 13.70 (bs, 1H, NH), 8.32 (d, J= 8.06Hz, 1H), 8.23 (d, J= 8.06Hz, 1H), 8.02 (d, J= 8.22Hz, 1H), 7.96 (d, J= 7.15Hz, 1H), 7.82 (t, J= 7.45Hz, 1H), 7.74 (m, 1H), 7.61 (t, J= 7.45 Hz, 1H). <sup>13</sup>C-NMR (100 MHz, DMSO-d<sub>6</sub>) [ $\delta$  ppm]: 182.79, 139.13, 138.28, 133.66, 132.62, 131.64, 128.57, 128.52, 128.36, 125.17, 122.41, 122.10, 120.18, 117.02. ESI-MS (M+H<sup>+</sup>): 221.07.

## References

- Bayascas, J.R. (2008). Dissecting the role of the 3-phosphoinositide-dependent protein kinase-1 (PDK1) signalling pathways. *Cell Cycle* 7, 2978-2982.
- Biondi, R.M., Cheung, P.C., Casamayor, A., Deak, M., Currie, R.A., and Alessi, D.R. (2000). Identification of a pocket in the PDK1 kinase domain that interacts with PIF and the C-terminal residues of PKA. *Embo J* 19, 979-988.
- Biondi, R.M., Kieloch, A., Currie, R.A., Deak, M., and Alessi, D.R. (2001). The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. *Embo J* 20, 4380-4390.
- Busschots, K., Lopez-Garcia, L.A., Lammi, C., Stroba, A., Zeuzem, S., Piiper, A., Alzari, P.M., Neimanis, S., Arencibia, J.M., Engel, M., et al. (2012). Substrate-Selective Inhibition of Protein Kinase PDK1 by Small Compounds that Bind to the PIF-Pocket Allosteric Docking Site. *Chem Biol* 19, 1152-1163.
- Bussi, G., Donadio, D., and Parrinello, M. (2007). Canonical sampling through velocity rescaling. *J Chem Phys* 126, 014101.
- Collins, B.J., Deak, M., Arthur, J.S., Armit, L.J., and Alessi, D.R. (2003). In vivo role of the PIF-binding docking site of PDK1 defined by knock-in mutation. *Embo J* 22, 4202-4211.
- Engel, M., Hindie, V., Lopez-Garcia, L.A., Stroba, A., Schaeffer, F., Adrian, I., Imig, J., Idrissova, L., Nastainczyk, W., Zeuzem, S., et al. (2006). Allosteric activation of the protein kinase PDK1 with low molecular weight compounds. *Embo J* 25, 5469-5480.
- Frodin, M., Antal, T.L., Dummler, B.A., Jensen, C.J., Deak, M., Gammeltoft, S., and Biondi, R.M. (2002). A phosphoserine/threonine-binding pocket in AGC kinases and PDK1 mediates activation by hydrophobic motif phosphorylation. *Embo J* 21, 5396-5407.
- Hindie, V., Stroba, A., Zhang, H., Lopez-Garcia, L.A., Idrissova, L., Zeuzem, S., Hirschberg, D., Schaeffer, F., Jorgensen, T.J.D., Engel, M., et al. (2009). Structure and allosteric effects of low molecular weight activators on the protein kinase PDK1. *Nat. Chem. Biol.* 5, 758-764.
- Rettenmaier, T.J., Sadowsky, J.D., Thomsen, N.D., Chen, S.C., Doak, A.K., Arkin, M.R., and Wells, J.A. (2014). A small-molecule mimic of a peptide docking motif inhibits the protein kinase PDK1. *Proc Natl Acad Sci U S A* 111, 18590-18595.
- Yang, J., Cron, P., Thompson, V., Good, V.M., Hess, D., Hemmings, B.A., and Barford, D. (2002). Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation. *Mol Cell* 9, 1227-1240.
- Zheng, J., Knighton, D.R., ten Eyck, L.F., Karlsson, R., Xuong, N., Taylor, S.S., and Sowadski, J.M. (1993). Crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MgATP and peptide inhibitor. *Biochemistry* 32, 2154-2161.