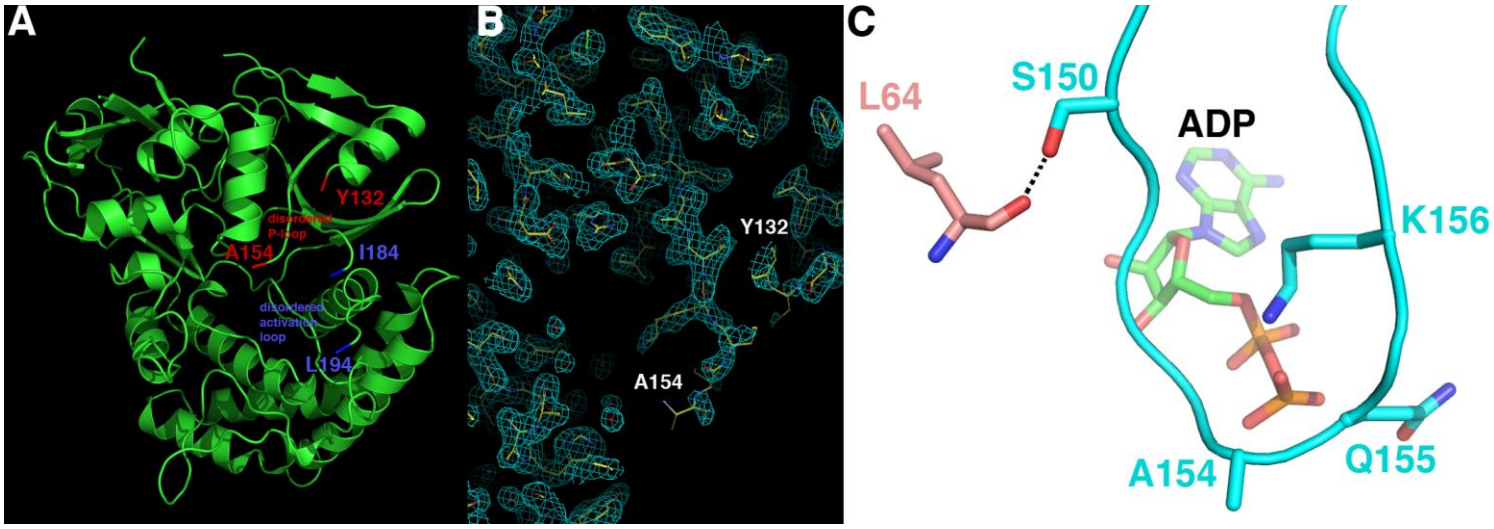

Figure S1. Structure of HipA(S150A). (A) Ribbon diagram of the HipA(S150A) structure. Shown in red and labelled are the first and last visible residues of the P-loop motif, which is disordered in the structure. Shown in blue and labelled are the residues that connect the flexible activation loop/region. (**B**) $2F_o$-F_c composite omit map of the HipA(S150A) structure around the disordered P-loop, contoured at 1 σ. (**C**) Close up of the ADP-binding P-loop motif of the HipA(D309Q)-ADP structure. Ribbon diagram of the P-loop motif showing selected side chains that contact the ADP phosphate and ribose moiety. Also shown is the key hydrogen bond between the Ser150 side chain and carbonyl oxygen of Leu64 that is present when the protein adopts the in-state conformation.

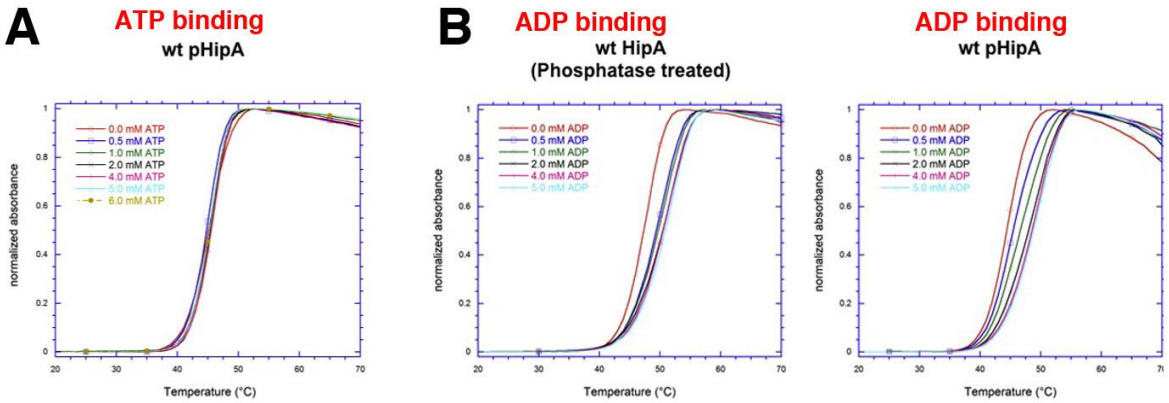

Figure S2. The affect of S150 phosphorylation on ATP and ADP binding to HipA. (A) Thermal stability of pHipA as a function of increasing ATP concentration. ATP does not increase pHipA stability up to 6.0 mM demonstrating that HipA does not bind ATP at physiologically relevant concentrations. (**B**) (Left) Thermal stability of dephosphorylated HipA as a function of increasing ADP concentration. The concentration range is indicated within the plot. (Right) Thermal stability of pHipA as a function of increasing ADP concentration. Note that the pHipA is not fully saturated until 4.0 mM ADP is added whereas phosphatase treated pHipA is saturated around less than 0.5 mM ADP.

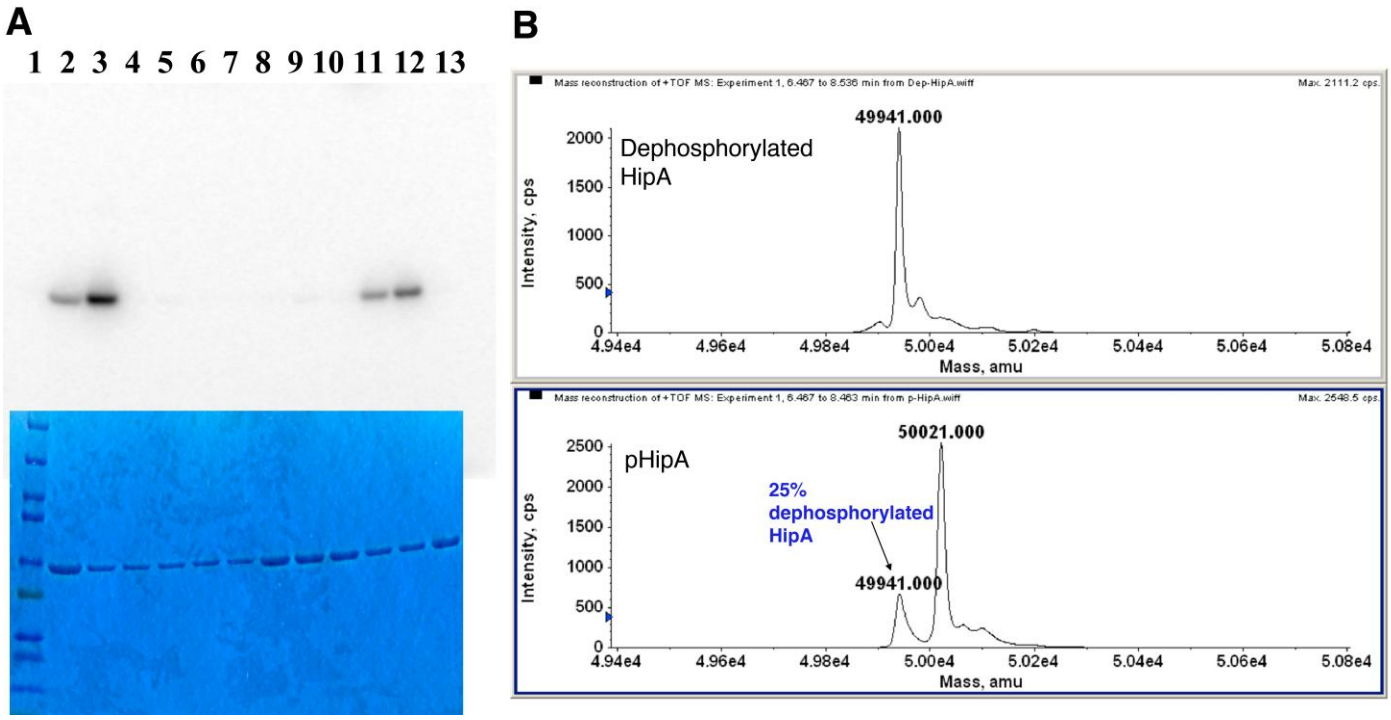

Figure S3. Autophosphorylation assays of HipA proteins. (A) Autophosphorylation assays of wt HipA (λ phosphatase-treated), HipA(S150A), HipA(D309Q) and pHipA. Two time points were taken at 1 min and 60 min for each sample. Each assay was performed with 1 μL of HipA protein (~1 mg/mL) added to 19 μL of the reaction solution, which contained 20 mM Hepes pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1.0 mM DTT, 50 μM ATP, [γ-^{32}P]ATP (10 μCi). See Extended Experimental Procedures for more details. HipA(S150A) and pHipA phosphorylation assays were done with and without 1 μL of HipA(D309Q) at 1 mg/mL. The top panel shows the autophosphorylation assays (autoradiogram) and the bottom panel showed the same gel stained with Coomassie brilliant blue (load control). The lanes are follows: (1) Molecular weight marker. (2) Dephosphorylated HipA, 1 min (3) Dephosphorylated HipA, 60 min (4) HipA(D309Q), 1 min (5) HipA(D309Q), 60 min (6) HipA(S150A), 1 min (7) HipA(S150A), 60 min (8) HipA(S150A) + HipA(D309Q), 1 min (9) HipA(S150A) + HipA(D309Q), 60 min, (10) HipA(S150A) + HipA(D309Q) + 3 mM ATP, 60 min, (11) pHipA, 1 min (note as shown in Figure S3B this "pHipA" sample is ~25% dephosphorylated, which can and does autophosphorylate), (12) pHipA, again containing ~25% dephosphorylated ATP, 60 min, (13) HipA(S150A) + HipA(D309Q) + 3 mM ATP, 60 min. **(B)** Mass spectrometry analysis of the lambda phosphatase-treated HipA and pHipA proteins used in the autophosphorylation assays shown in Figure S3A. Purified wild type HipA is typically 65-90% phosphorylated on residue Ser150 (Correia et al., 2006). This analysis clearly shows that the pHipA protein used in this experiment was only ~75% phosphorylated, leaving ~25% active kinase. This explains the kinase activity shown in panel A, lanes 11 and 12.

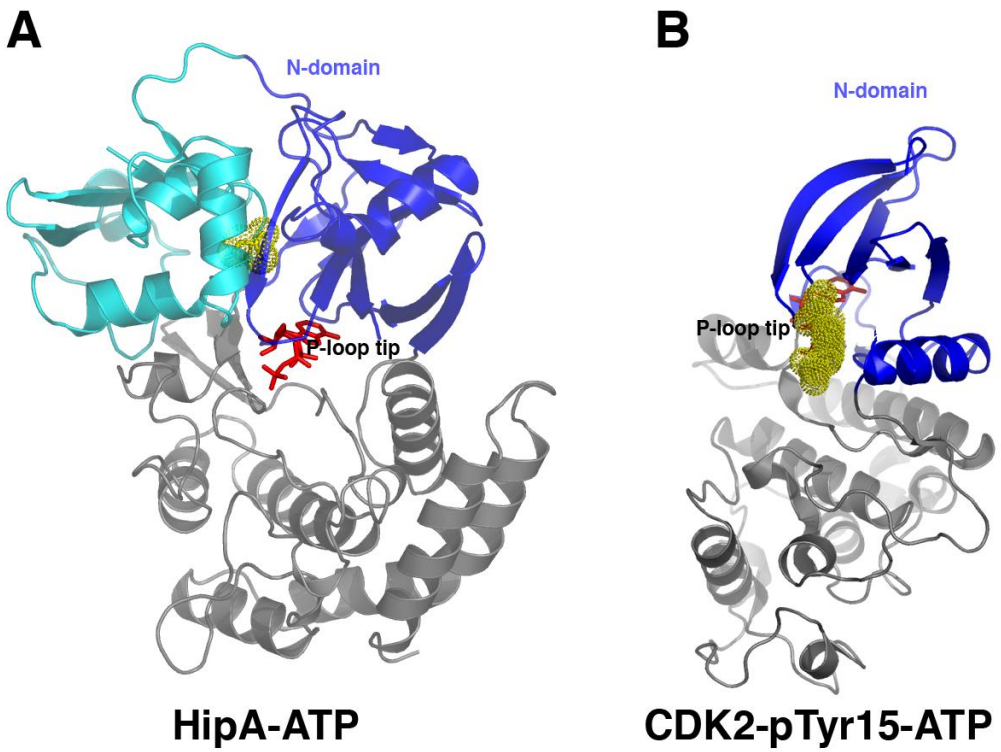

Figure S4. Phosphorylation of HipA P-loop motif residue Ser150 but not CDK2 P-loop residue Tyr15 requires a large conformational change. (A) The HipA(D309Q)-ATP structure showing ATP (red sticks) and residue Ser150 (yellow dotted surface within the N-domain). The HipA N-domain is larger than those of other protein kinases and contains an extra subdomain (colored cyan). This extra subdomain helps bury the Ser150 residue. **(B)** Structure of the CDK2-ATP structure in which Tyr15 has been phosphorylated by Wee1 kinase. The N-domain of CDK2 corresponds to N-subdomain 2 of HipA and is colored blue. ATP is shown as red sticks and the entire pTyr15 residue is shown as a yellow dotted surface. Notably, the Tyr15 residue of CDK2 is located on the tip of the P-loop, which in this structure is fully exposed. Hence, phosphorylation of CDK2 residue Tyr15 requires no conformational change.