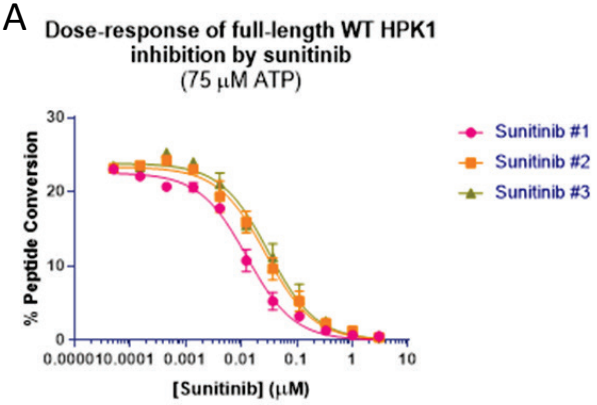
Supporting Information

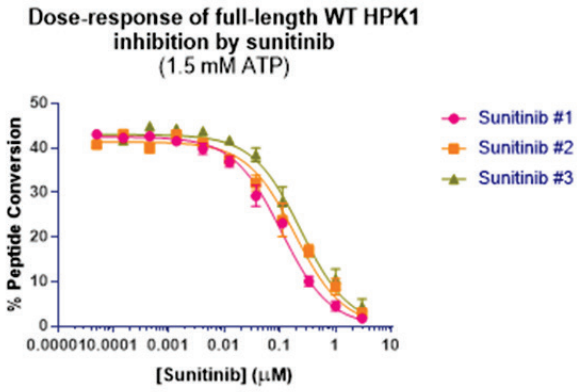
Table S1. Properties of dimer interfaces (PISA analysis). Values indicate contributions from both subunits.

HPK1-sunitinib complex	+0P	+2P	2PM
Atoms in the interface	431	122	380
Residues in the interface	122	36	98
Buried ASA (Å^2)	4506.6	1124.4	3703.1
Total ASA (Å^2)	28953.2	26710.6	30855.3
Solvation energy (kcal/mol)	-573.2	-487.4	-503.3
SE gain (kcal/mol)	-22.0	-9.4	-32.4
interface area (Å^2)	2253.3	562.2	1851.6
Delta G (kcal/mol)	-22	-9.4	-32.4
Binding energy (kcal/mol)	-32.2	-11.2	-36.7
Hydrogen bonds	13	4	3
Salt bridges	12	0	8

A

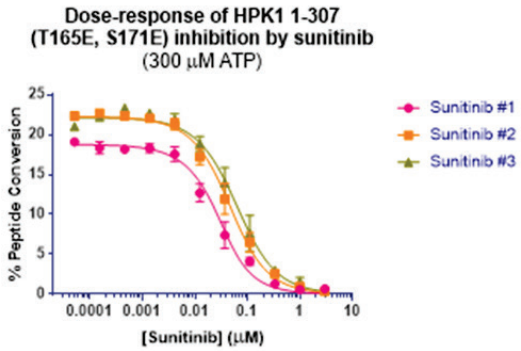


	E	Ki	S	Km
Sunitinib #1	0.001	0.0059	75	72
Sunitinib #2	0.001	0.0132	75	72
Sunitinib #3	0.001	0.0149	75	72

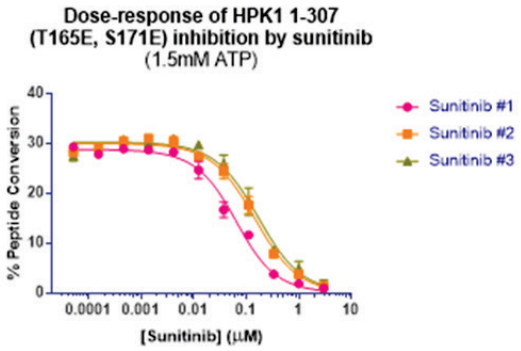


	E	Ki	S	Km
Sunitinib #1	0.001	0.0048	1500	72
Sunitinib #2	0.001	0.0084	1500	72
Sunitinib #3	0.001	0.0109	1500	72

B



	E	Ki	S	Km
Sunitinib #1	0.030	0.0030	300	78
Sunitinib #2	0.030	0.0064	300	78
Sunitinib #3	0.030	0.0099	300	78



	E	Ki	S	Km
Sunitinib #1	0.030	0.0024	1500	78
Sunitinib #2	0.030	0.0064	1500	78
Sunitinib #3	0.030	0.0077	1500	78

Figure S1. Inhibition of full-length autophosphorylated HPK1 and phosphomimetic HPK1$^{\mathbf{2PM}}$ kinase domain (1-307, T165E, S171E) by sunitinib (synthesized by Pfizer, Inc, in-house) was measured by microfluidic mobility-shift assay using 3 μM phosphoacceptor peptide 5FAM-AKRRRLSSLRA-COOH (CPC Scientific, Sunnyvale, CA) and sunitinib (11-dose 3-fold serial dilutions, 2% DMSO final) in 50 mM MOPS, pH 7.8, buffer, 0.002% Tween-20, 1 mM DTT and 2.5 mM $MgCl_2$. Reactions were initiated by addition of 0-2000 μM ATP, following a 20-min preincubation. K_i values were calculated by fitting the initial velocities (peptide substrate % conversion) to the Morrison equation (1) for tight-binding competitive inhibition by non-linear regression using apparent ATP K_m values of 72 and 78 μM for full-length WT HPK1 and HPK1$^{\mathbf{2PM}}$, respectively. (A) Dose-response curves for activated full-length WT HPK1 at 75μM and 1.5 mM ATP. The concentration of HPK1 was 1 nM. (B) Dose response curves for HPK1$^{\mathbf{2PM}}$ at 300 μM and 1.5 mM ATP. The concentration of HPK1$^{\mathbf{2PM}}$ was 30 nM. The experiment was conducted by a mobility-shift assay as above.

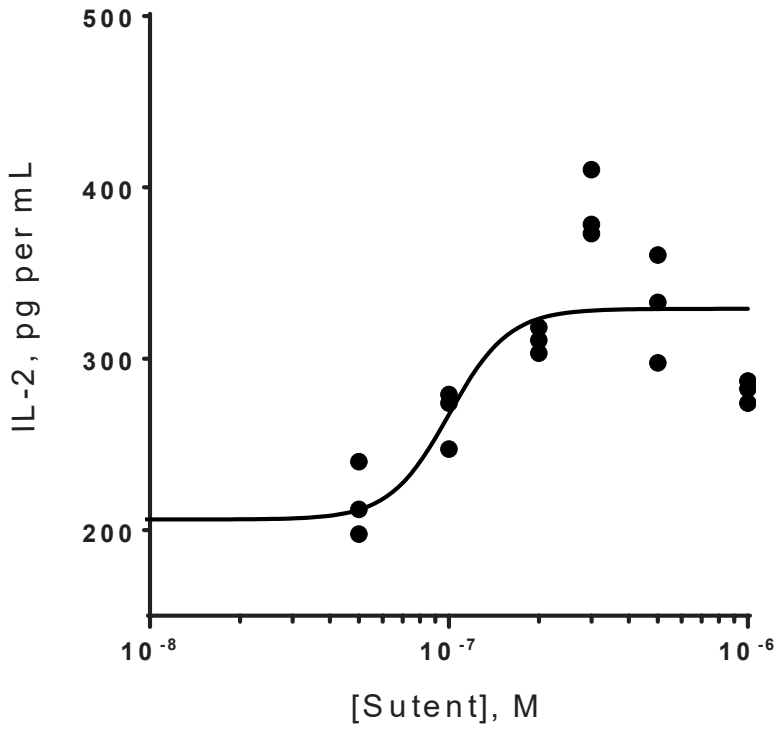


Figure S2. TCR X CD28-induced IL-2 production by Jurkat cells pre-treated with Sunitinib. Jurkat cells were pre-incubated for 30 minutes with either DMSO (control) or 0.05 0.1, 0.2, 0.3, 0.5 and 1.0 µM of sunitinib before stimulating for 24 hours with plate-bound anti-CD3e antibody in the presence of 5 µg per mL of soluble anti-CD28 mAb. The levels of IL-2 in supernatant were determined by ELISA kit (Invitrogen Carlsbad, CA).

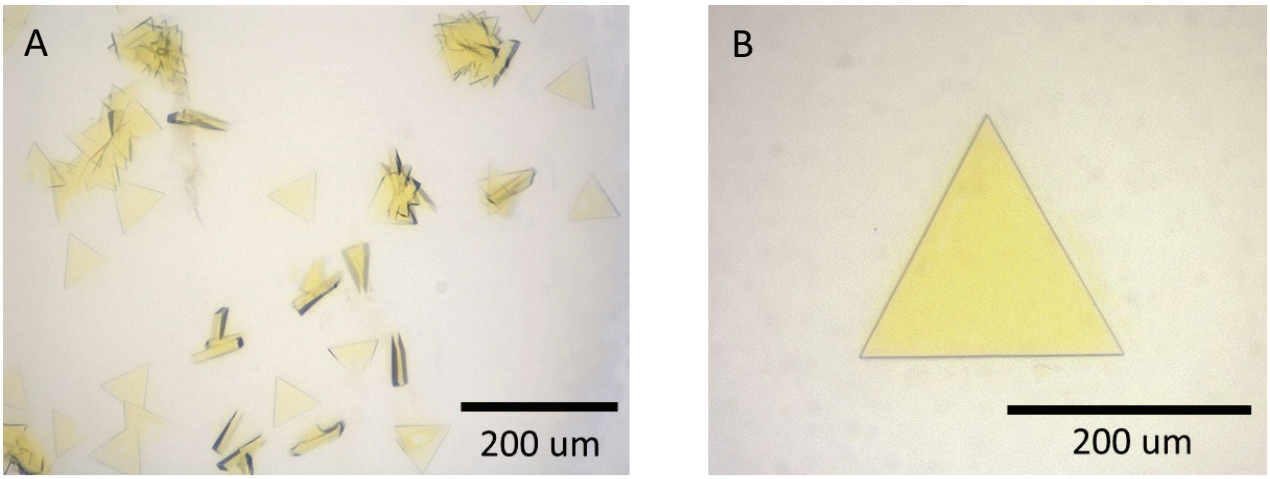


Figure S3. Crystals of WT HPK1^{+0P}-sunitinib. (A) A shower of small (~50 um) yellow crystals was observed in the initial crystal hit. (B) Optimized crystals were of similar morphology but much larger (200 um or larger in the greatest dimension).

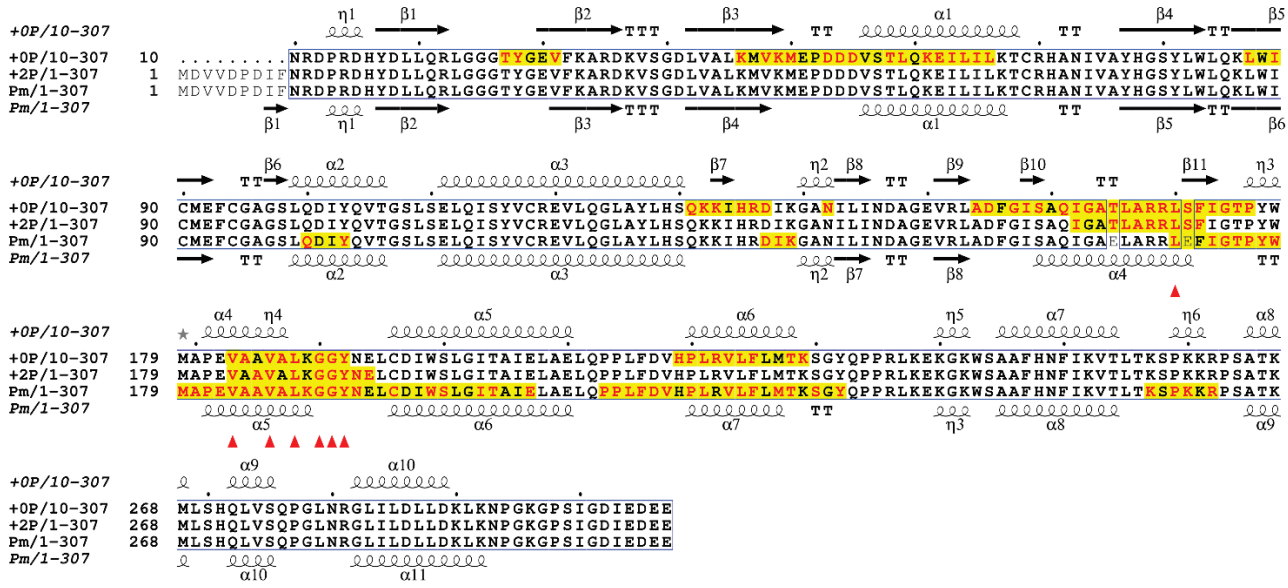
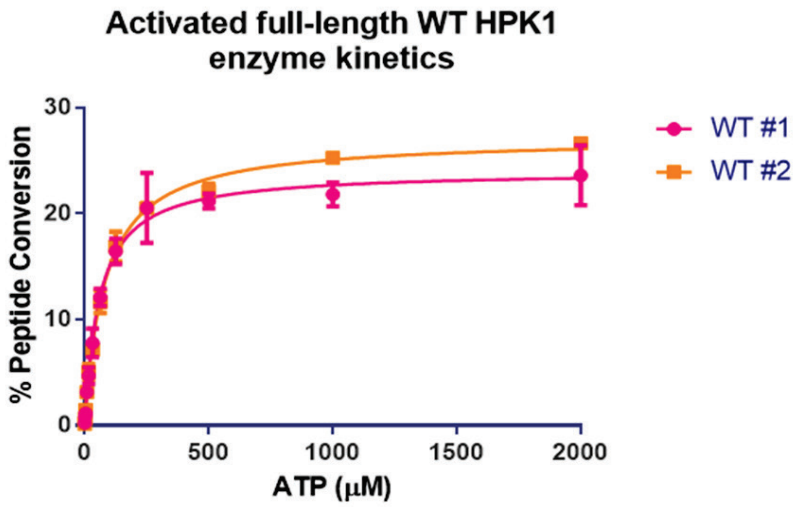


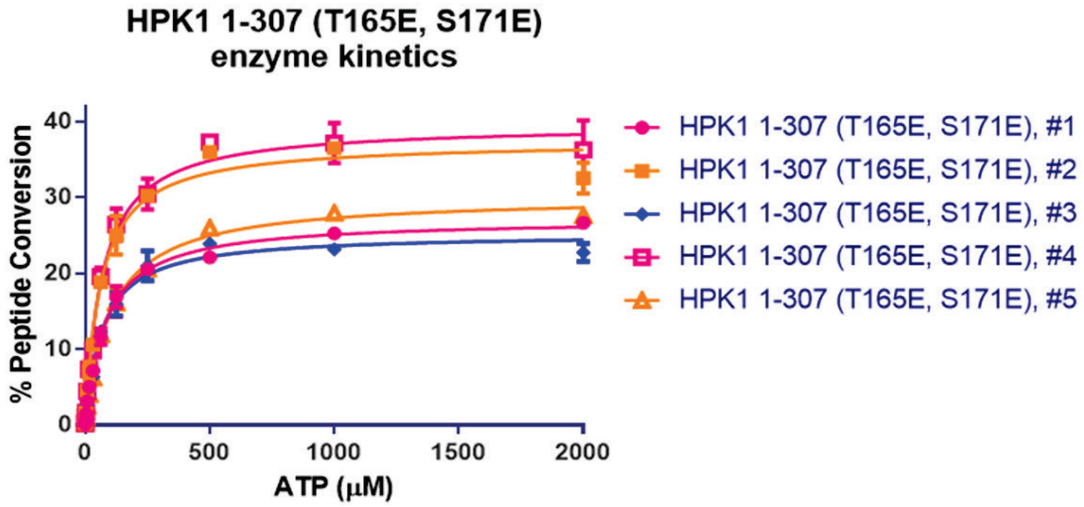
Figure S4. Structure based sequence alignment showing location of interface residues for the HPK1^{+0P}, HPK1^{+2P} and HPK1^{2PM} head-to-head dimers. Red characters indicate interface residues with yellow highlight showing regions with gaps of ≤ 2 non-interfacing residues. Red arrows indicate interface residues shared between all three structures. Secondary structure elements are indicated for HPK1^{+0P} and HPK1^{2PM} at the top and bottom of the sequence alignment respectively.

A



	Vmax	Km
WT #1	24	60
WT #2	27	84

B



	Vmax	Km
HPK1 1-307 (T165E, S171E), #1	27	84
HPK1 1-307 (T165E, S171E), #2	38	62
HPK1 1-307 (T165E, S171E), #3	25	68
HPK1 1-307 (T165E, S171E), #4	40	68
HPK1 1-307 (T165E, S171E), #5	30	105

Figure S5. Enzyme kinetics of HPK1 constructs. (A) Activated full-length WT HPK1 at an enzyme concentration of 1 nM. (B) HPK1^{2PM} (1-307, S165E, S171E) phosphomimetic double mutant kinase domain at an enzyme concentration of 30 nM. The experiment was conducted using 0-2000 μM ATP and 3 μM phosphoacceptor peptide, 5FAM-AKRRRLSSLRA-COOH by a mobility-shift assay.

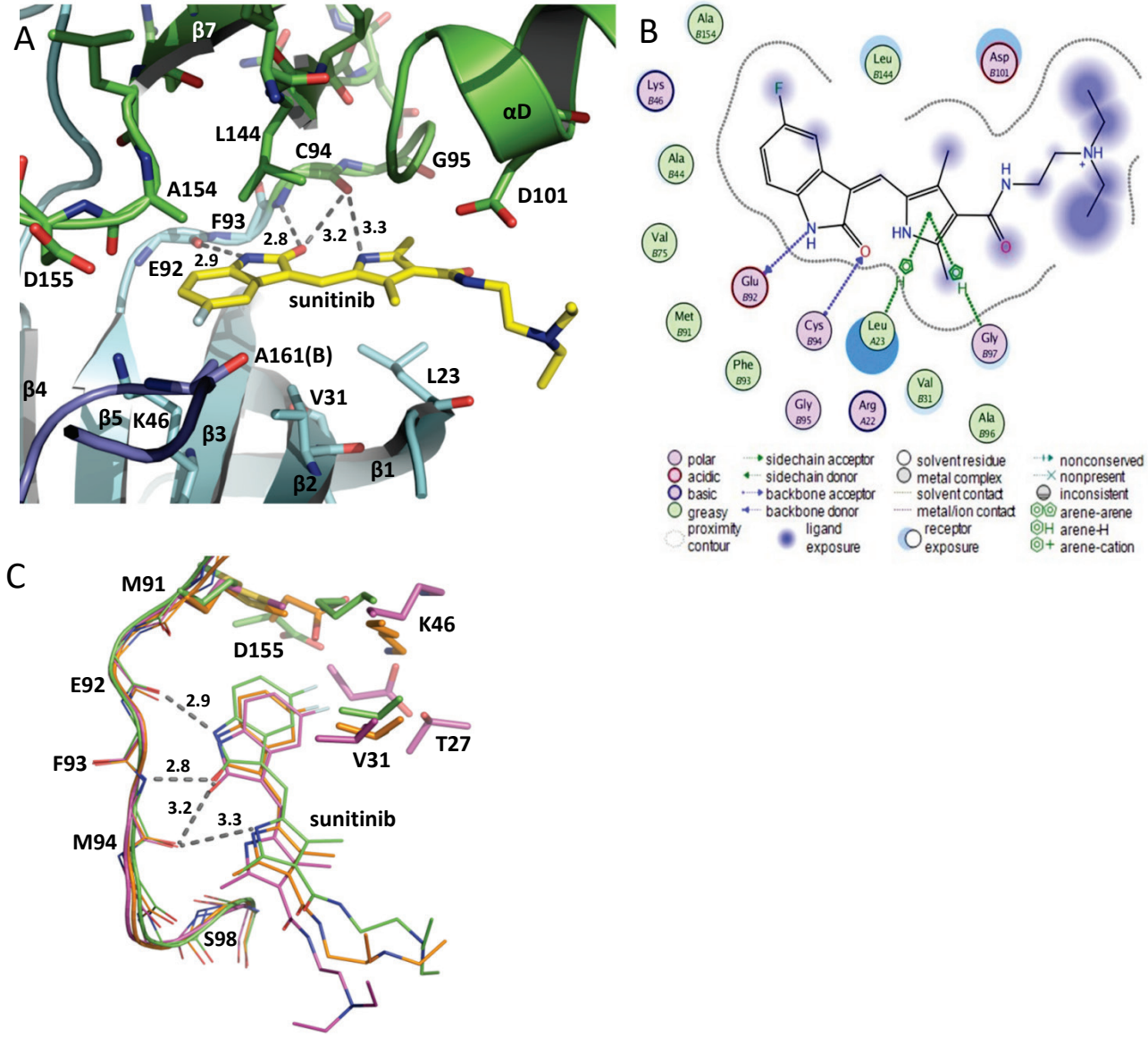


Figure S6. HPK1-sunitinib interactions. (A) Sunitinib bound to HPK1^{+0P} KD, and (B) a schematic of the protein-ligand interactions. (C) Superposition of +0P (green), +2P (magenta), and 2PM (orange) HPK1-sunitinib complexes showing the structurally conserved hinge residues M91–S98, and the highly variable positions of other key active site residues. T27 of the GL is ordered in the +2P complex only. The catalytic lysine (K46), and DFG aspartic acid (D155) do not interact with the ligand and adopt significantly different conformations. Distance (Å) indicated for the HPK1^{+0P}-sunitinib complex.

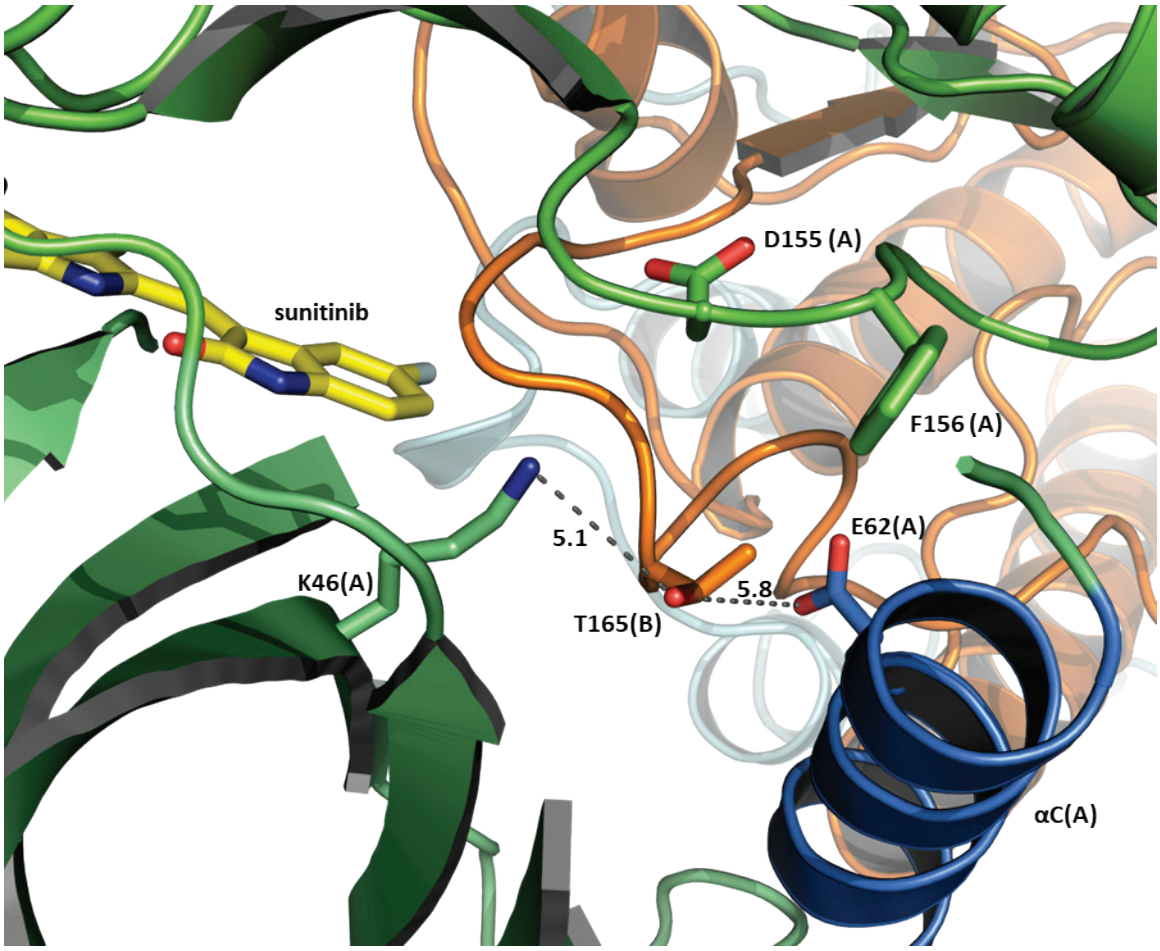


Figure S7. Location of T165 autophosphorylation site in the active site of HPK1^{+0P} dimer. Chain A: N-lobe (cyan cartoon), αC-helix (blue cartoon), C-lobe (green cartoon), and chain B, orange cartoon. The Chain B AL is inserted between the catalytic lysine (K46) and the conserved αC glutamate (E62) at the Chain A active site. Numbers and dashed lines indicated distance (Å) between the T165(B) OG1 atom and the K46(A) NZ and E62(A) OE2 atoms.

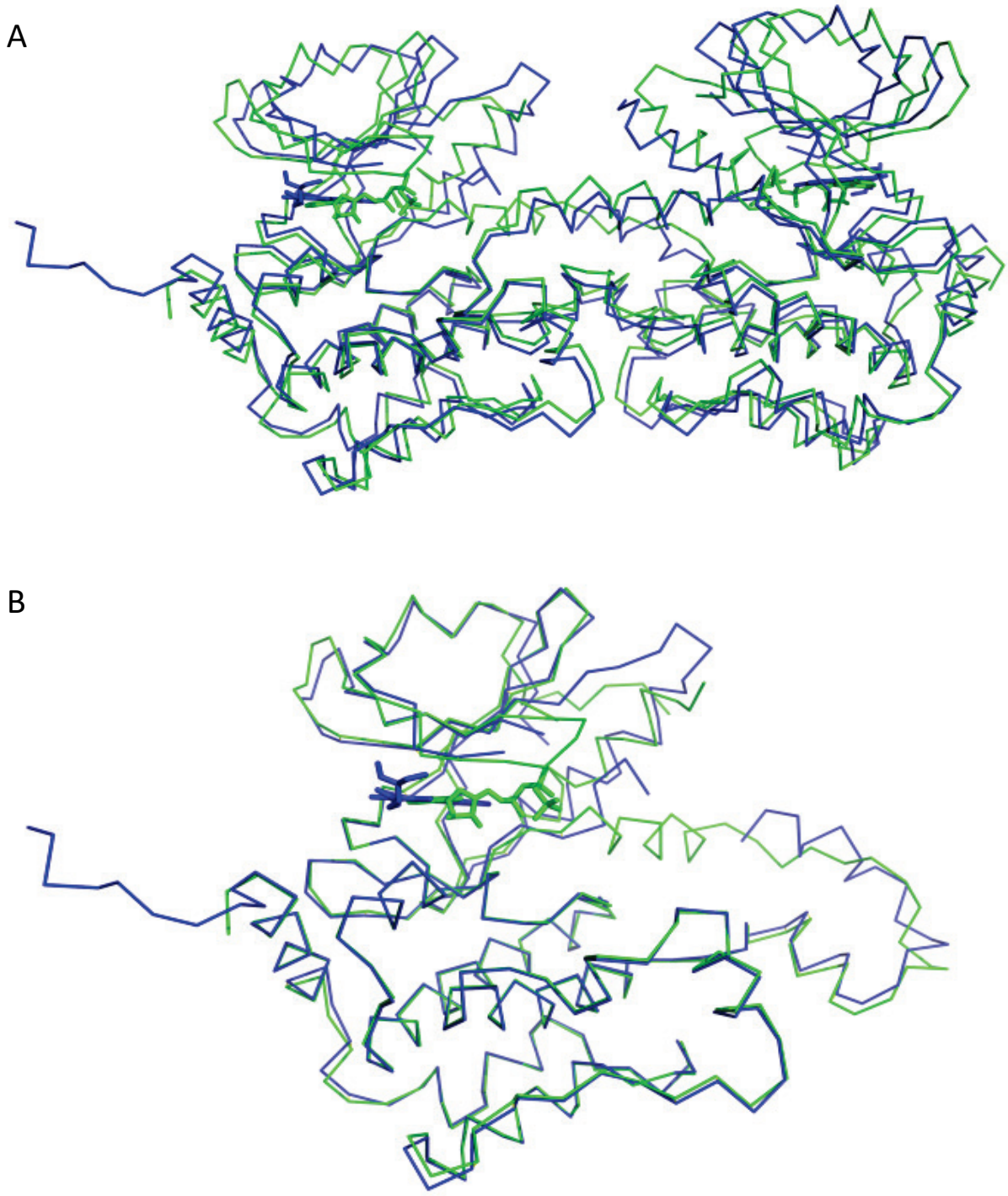


Figure S8. Comparison of domain-swapped HPK1 structures from this study and at the PDB. (A) Domain-swapped dimer of HPK1^{2PM}-sunitinib (blue ribbon) superimposed onto domain swapped dimer of HPK1 in complex with AMPPNP published by Wu et al. (green ribbon, PDB ID code 6CQF) (2). RMSD = 1.564 Å for 482 Cα atoms (B) Chain A of HPK1^{2PM}-sunitinib superimposed onto chain A of HPK1- AMPPNP). RMSD = 0.429 Å for 202 Cα atoms.

References

1. Morrison, J. F. (1969) Kinetics of the reversible inhibition of enzyme-catalysed reactions by tight-binding inhibitors. *Biochimica et Biophysica Acta (BBA) - Enzymology* **185**, 269-286

2. Wu, P., Sneeringer, C. J., Pitts, K. E., Day, E. S., Chan, B. K., Wei, B., Lehoux, I., Mortara, K., Li, H., Wu, J., Franke, Y., Moffat, J. G., Grogan, J. L., Heffron, T. P., and Wang, W. (2019) Hematopoietic Progenitor Kinase-1 Structure in a Domain-Swapped Dimer. *Structure* **27**, 125-133.e124