

Supplementary Information:

Distinct allosteric networks in CDK4 and CDK6 in the cell cycle and in drug resistance

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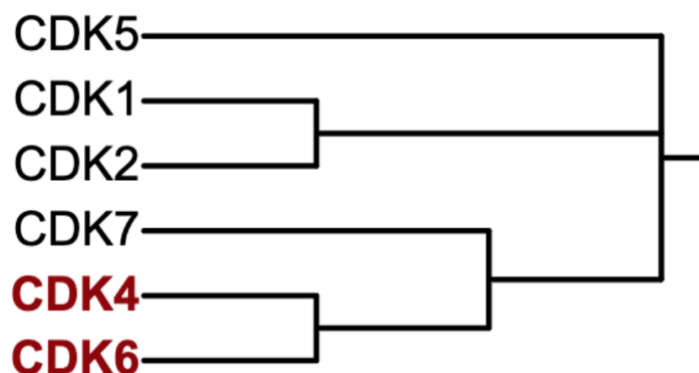


Figure S1. Phylogenetic tree of human cell cycle CDKs. It shows the evolutionary closeness of CDK4 and CDK6, compared to the rest of the cell cycle CDKs. The figure is visualized using [1].

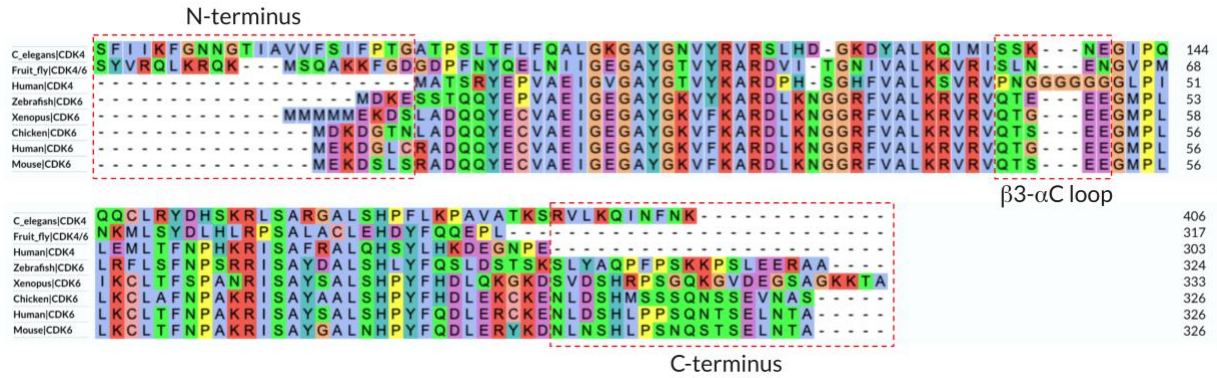


Figure S2. Multi-species sequence alignment comparing the N- and C-terminal region and β3-αC loop across CDK 4 and CDK6. The sequence divergence between human CDK4 and CDK6 is mostly conserved in other species as well. The alignment is colored by amino acid properties (red = positive charge, magenta= negative charge, blue = hydrophobic, green = polar, pink = cysteines, orange = glycines, yellow = prolines, cyan = aromatic) to highlight sequence conservation and variation. The key regions (N- and C-termini, and β3-αC loop) are highlighted in red boxes. Each row represents a CDK4 or CDK6 of species ortholog.

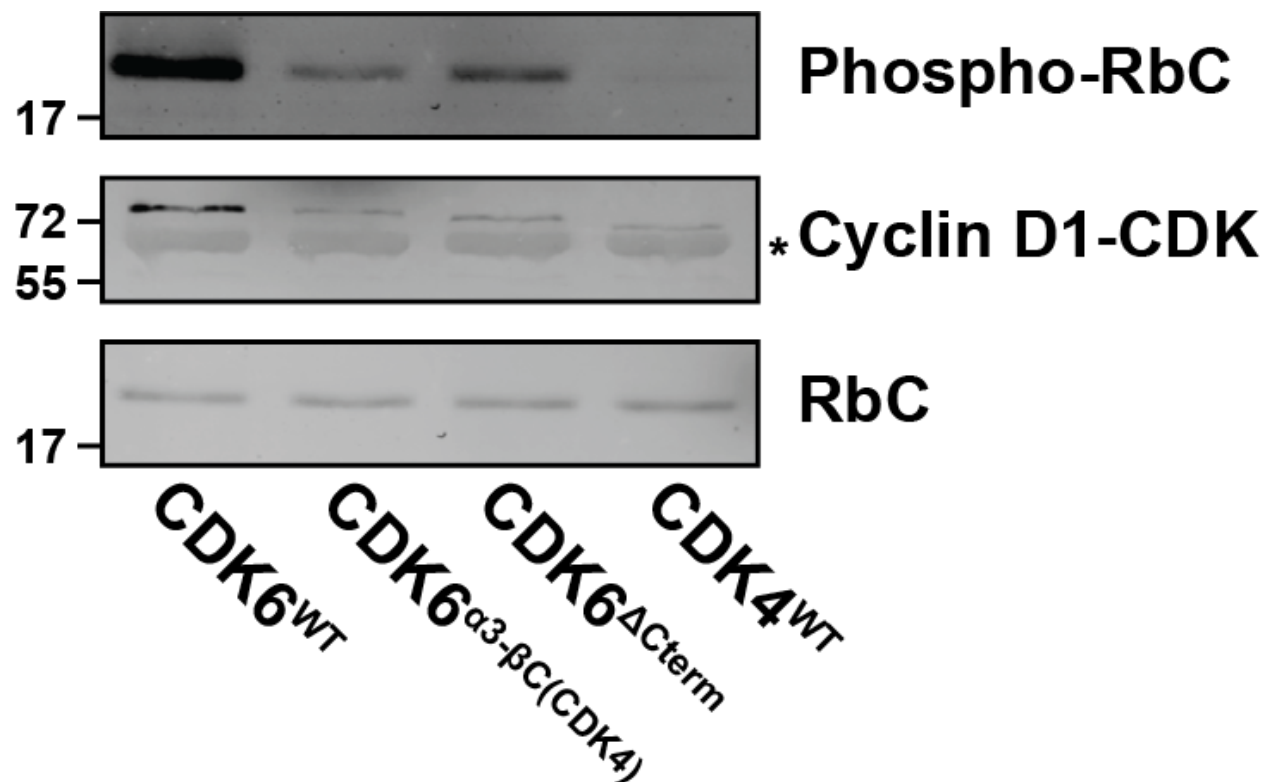


Figure S3: Representative immunoblot of RbC phosphorylation by Cyclin D1-CDK4/6 complexes. *In vitro* phosphorylation of RbC by CDK6 (WT, $\beta 3$ - α C(CDK4), and Δ Cterm) and CDK4 (WT). Shown is phosphorylated RbC (detected by anti-thiophosphate ester antibodies), cyclin D1-CDK fusions (detected by anti-FLAG antibodies), and RbC detected by total protein stain.

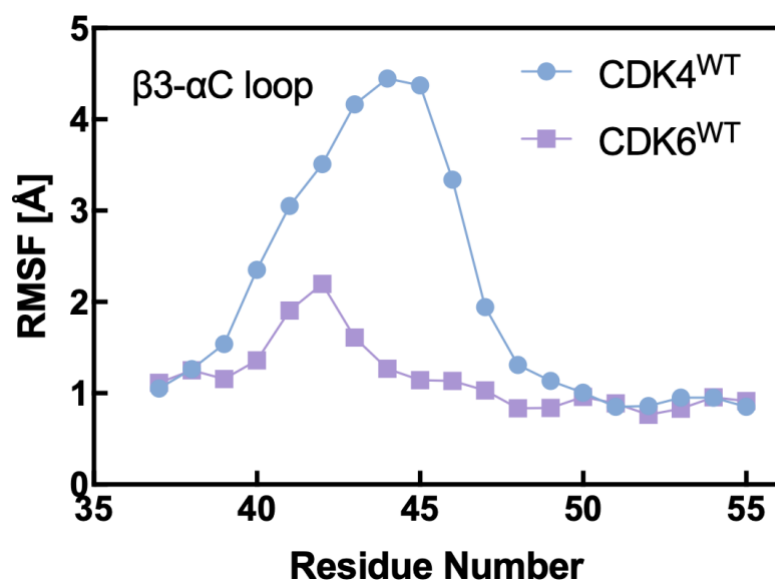


Figure S4. Root mean square fluctuations (RMSFs) of $\beta 3$ - αC loop in CDK4^{WT} and CDK6^{WT}. The $\beta 3$ - αC loop in CDK4^{WT} has significantly more fluctuation than that in CDK6^{WT}. Residue number for CDK6 is subtracted by 8 to match the $\beta 3$ - αC loop residues in CDK4.

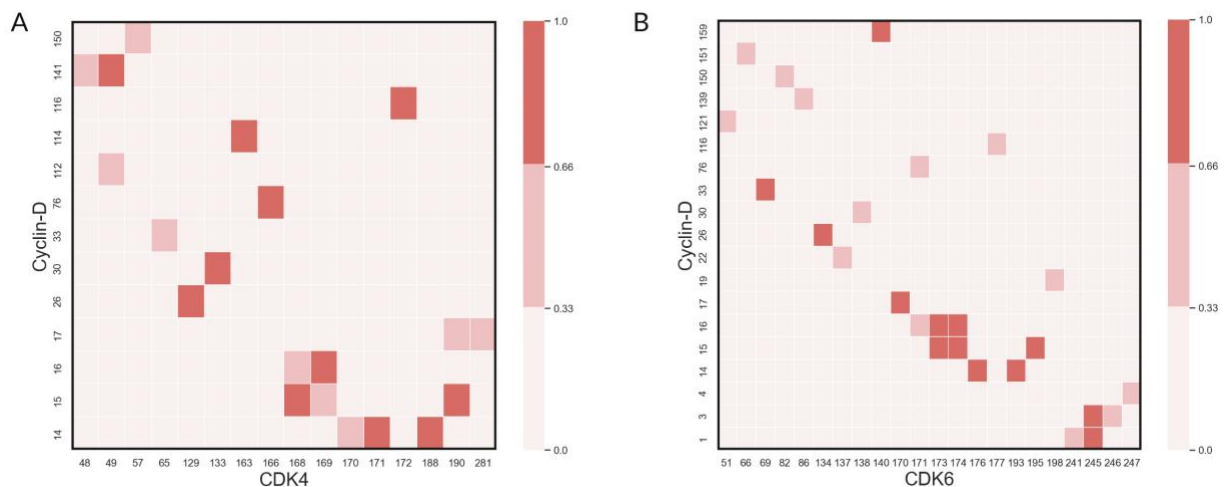


Figure S5. Contacting residue map between CDK and cyclin for (A) cyclin-D/CDK4, and (B) cyclin-D/CDK6 complexes. The x-axis represents residue numbers from CDK4/6, while the y-axis shows cyclin-D residues. The color intensity indicates contact frequency between residue pairs during the simulation, with darker red representing higher frequency contacts (scale from 0.0 to 1.0). These maps show more extensive contacting residues between CDK6 and cyclin-D, resulting in its stronger binding affinity.

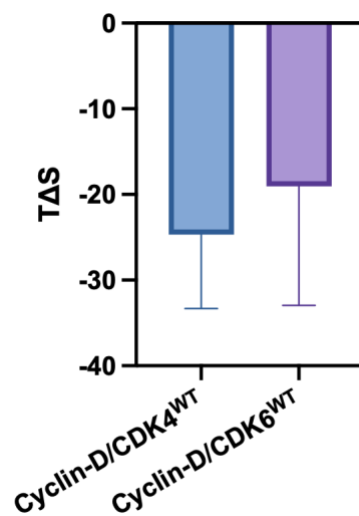


Figure S6. Entropic contribution to the binding free energy between cyclin-D and either CDK4^{WT} or CDK6^{WT}. The higher degree of entropic contribution of CDK6 complex decreases its binding free energy with cyclin-D.

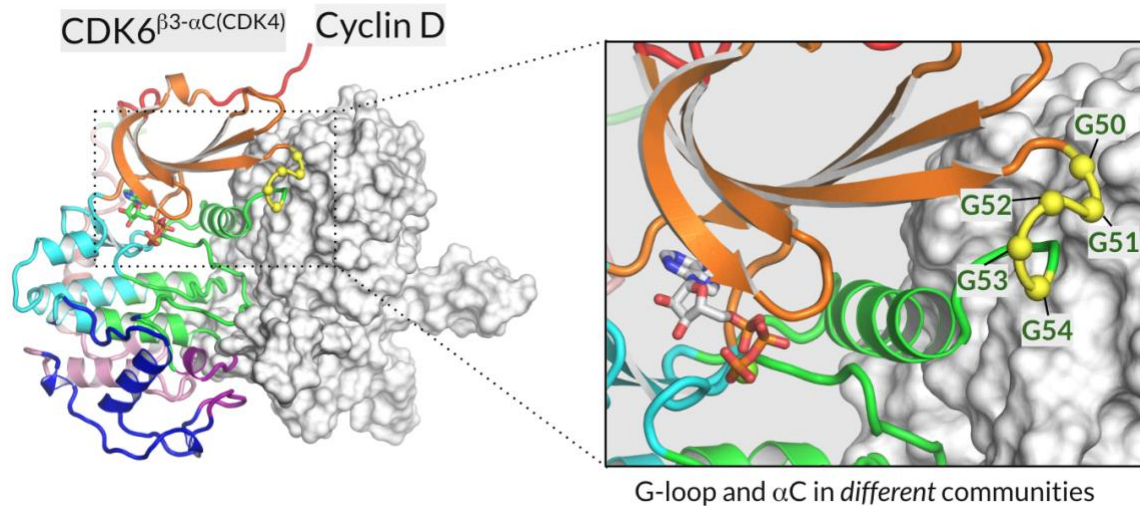


Figure S7. Community analysis of CDK6^{WT} and CDK6^{β3-αC(CDK4)}. It shows the impact of the longer CDK4 β3-αC loop can disrupt community organization breaking the coupling between β-strands in C-lobe and αC-helix in CDK6^{WT} (**Fig. 3**). CDK6 is shown in cartoon representation with different colors representing distinct dynamic communities identified through community network analysis. Cyclin-D is displayed in surface representation (gray), highlighting the protein-protein interface. In the zoomed-in view of the G-loop and αC-helix, the G-loop residues (G50-G54) are highlighted in yellow against the cyclin-D surface (gray). The separation of the G-loop and αC-helix into different dynamic communities (shown by distinct colors: orange and green) demonstrates how incorporating the CDK4 β3-αC loop disrupts the normal coupling between these regulatory elements.

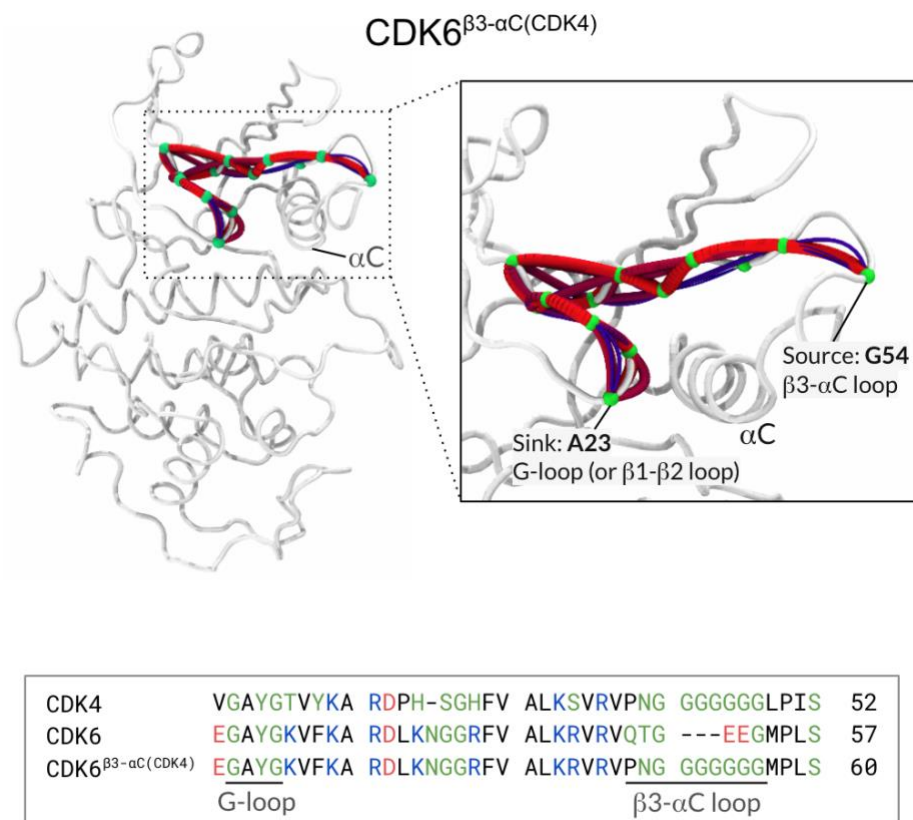


Figure S8. Impact of β3-αC loop substitution on allosteric communication in CDK6^{β3-αC(CDK4)}. Structural visualization of allosteric pathways in CDK6^{β3-αC(CDK4)}. CDK6^{β3-αC(CDK4)} is mutant of CDK6 with β3-αC loop swapped for that from CDK4. The zoomed-in view of the allosteric pathways between source residue A29 (in β3-αC loop) and G54 in the G-loop. It exhibits similar allosteric pathways compared to that of CDK4 in **Fig. 4**. Swapping β3-αC weaken the allosteric network between the β3-αC and G-loop, resulting in more dynamic G-loop (β1-β2 loop).

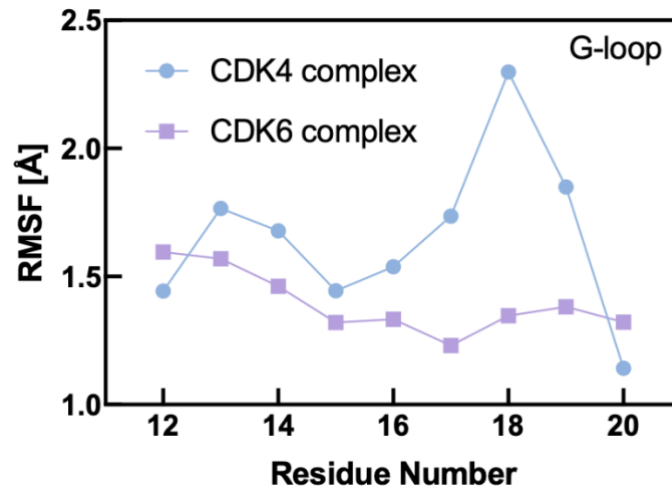


Figure S9. Root mean square fluctuations (RMSFs) of G-loop in CDK4 and CDK6. The G-loop of CDK4 has significantly more fluctuation than that in CDK6. The residue number for CDK6 is subtracted by 7 to match the G-loop residues in CDK4.

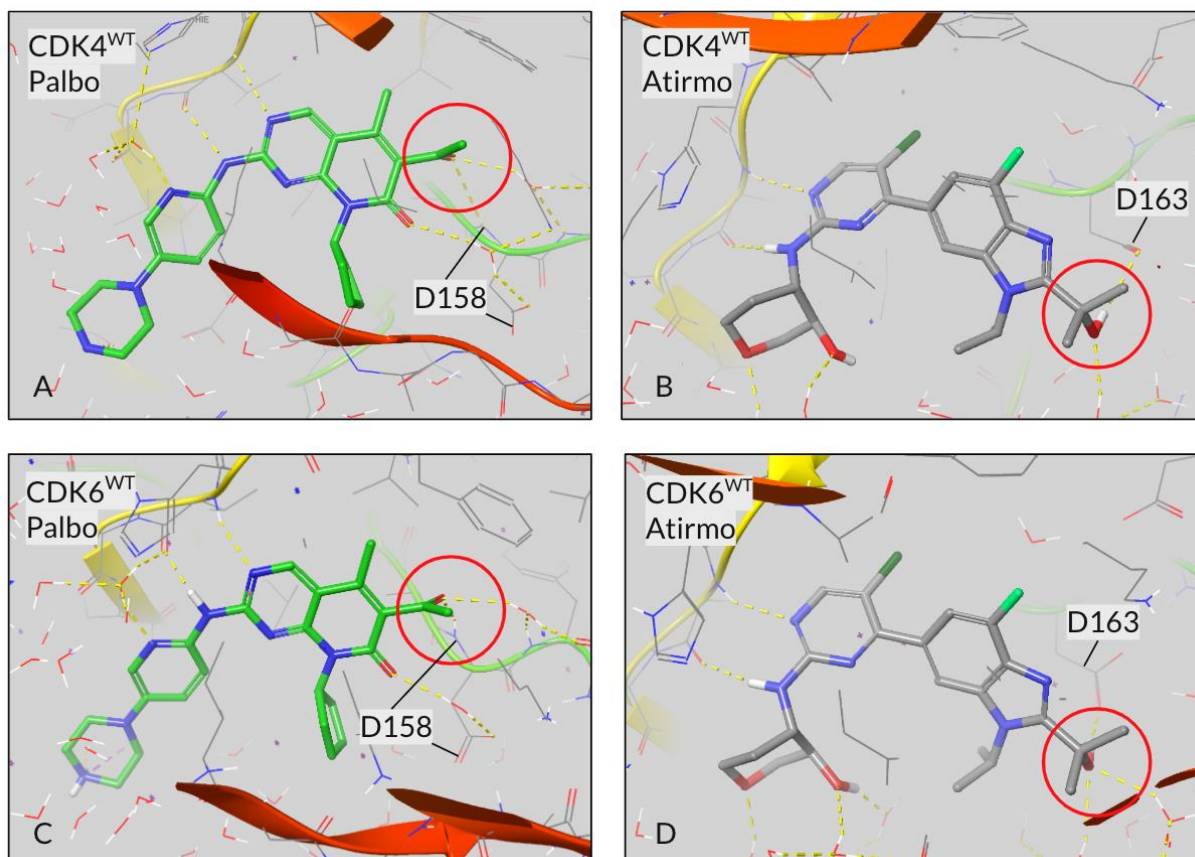


Figure S10. Comparison of binding modes of palbociclib (PD 0332991) and atirmociclib (PF-07220060) in CDK4 and CDK6 active sites. Both inhibitors interaction with the aspartic acid (D158 in CDK4, D163 in CDK6) in the DFG motif. (A) CDK4-palbociclib. (B) CDK4-atirmociclib. (C) CDK6-palbociclib. (D) CDK6-atirmociclib. Red circles highlight the carboxylic acid in palbociclib and isopropyl alcohol in atirmociclib. The additional methyl group in isopropyl alcohol of atirmociclib is responsible for the selectivity of CDK4 due to the flexible G-loop right above this allowing for this bulkier group, in contrast to the less flexible G-loop in CDK6.

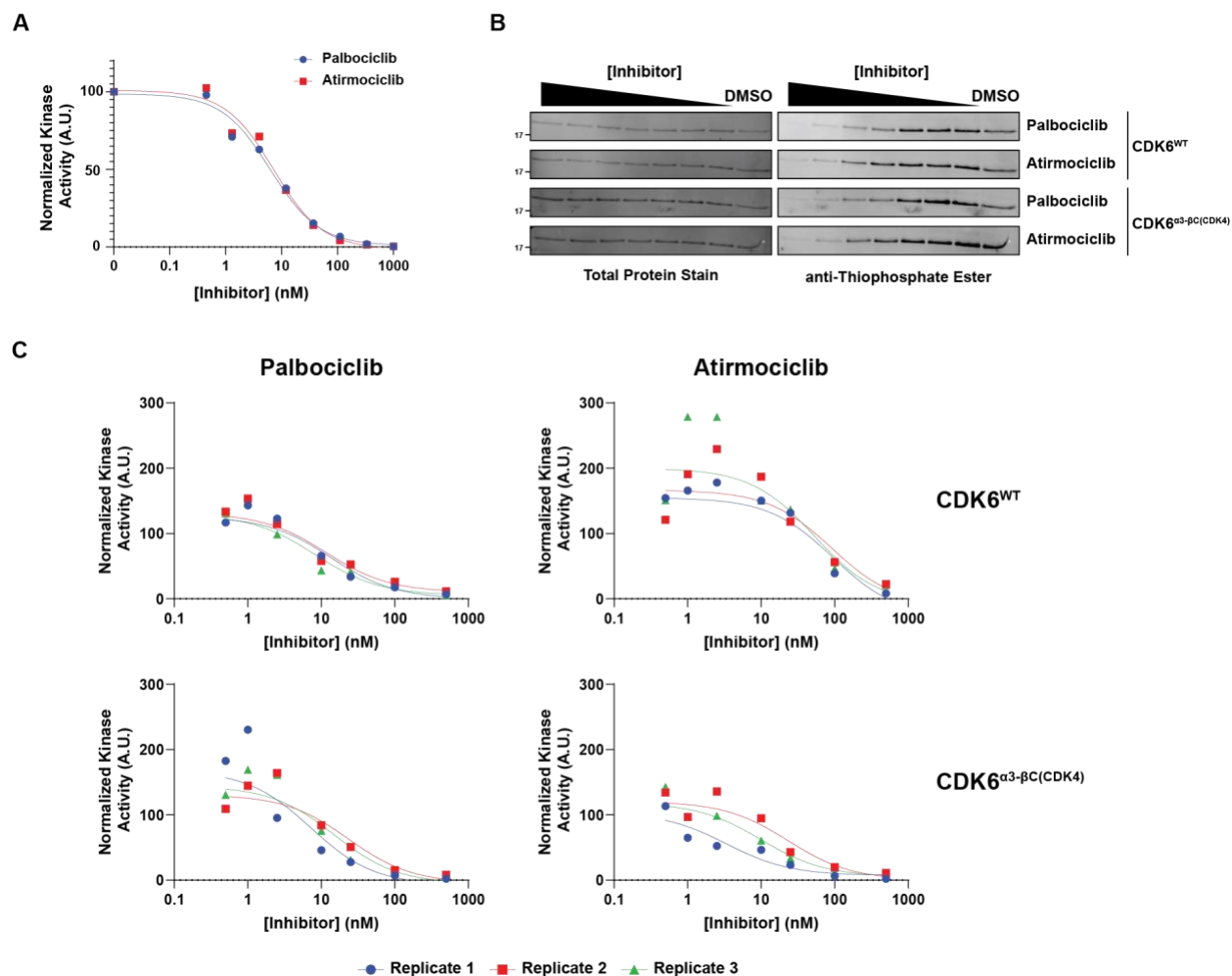


Figure S11. *In vitro* CDK4/6 inhibition by palbociclib and atirmociclib. (A) Palbociclib and atirmociclib mediated inhibition of CDK4 kinase activity toward RbC. (B) Representative immunoblots of palbociclib and atirmociclib mediated inhibition of CDK6^{WT} and CDK6^{β3-αC(CDK4)} kinase activity toward RbC. (C) Individual inhibition curves used to calculate IC₅₀ values displayed in Fig. 4I.

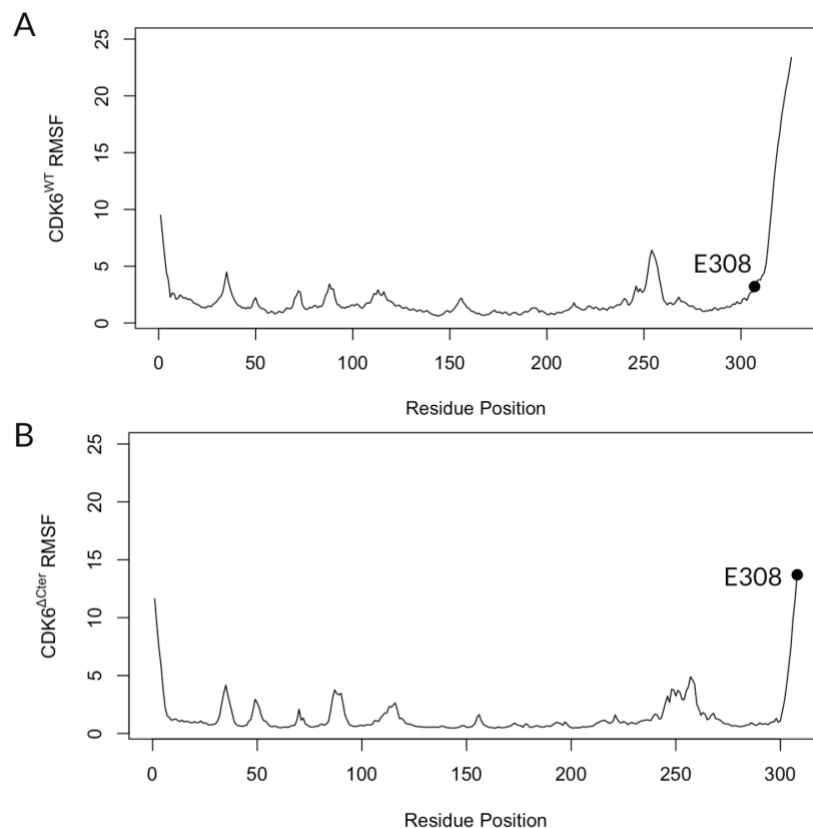


Figure S12. Root mean square fluctuations (RMSFs) of CDK6^{WT} and CDK6^{ΔCterm}. E308, the last residue in CDK6^{ΔCterm} has significantly more fluctuation than the same residue in CDK6^{WT}. The truncation of C-terminus results in an enhanced fluctuation in the last a few residues in the C-lobe, which leads to the disruption of the intrinsic dynamics of the kinase. For CDK6^{WT}, the longer C-terminus shielded E308 from significant fluctuation.

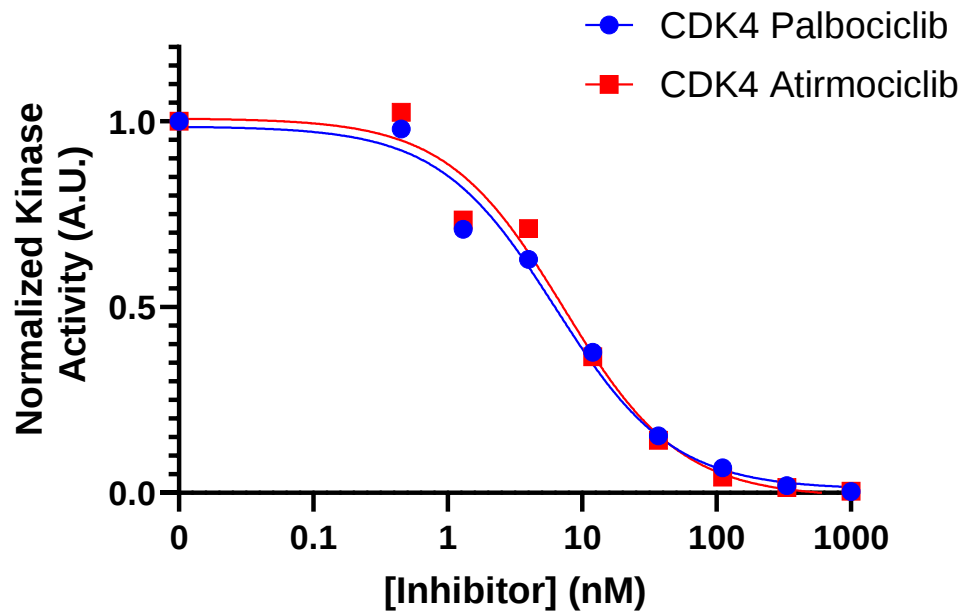


Figure S13. Dose-response curves showing the effect of CDK4/6 inhibitors, palbociclib (PD 0332991) and atirmociclib (PF-07220060), on the phosphorylation of the retinoblastoma protein (pRbC) in CDK4 complexes. The x-axis is the concentration of the inhibitors on a logarithmic scale, and the y-axis shows normalized pRb phosphorylation levels. Each curve represents a different condition: CDK4 with palbociclib (blue circles), CDK4 with atirmociclib (red squares).

Table S1. Summary of the simulations of CDK4 and CDK6 complexes. The mutations of CDKs are shown in Table S2.

System	Label	Source PDB	Components	α C	A-loop	DFG	Simulation time (μ s)	Number of replicas
Cyclin-D1/CDK4 (pT172)	CDK4 ^{WT}	7SJ3, 2W9Z	Cyclin-D1, CDK4 (pT172), ATP, Mg ²⁺	in	extended	in	2	3
Cyclin-D1/CDK6 (pT177)	CDK6 ^{WT}	1XO2, 2W9Z	Cyclin-D1, CDK6 (pT177), ATP, Mg ²⁺	in	extended	in	2	3
Cyclin-D1/CDK6 ^{ΔCterm} (pT177)	CDK6 ^{ΔCterm}	1XO2, 2W9Z	Cyclin-D1, CDK6 (pT177), ATP, Mg ²⁺	in	extended	in	2	3
Cyclin-D1/CDK4 ^{β3-αC(CDK6)} (pT172)	CDK4 ^{β3-αC(CDK6)}	7SJ3, 2W9Z	Cyclin-D1, CDK4 ^{β3-αC(CDK6)} (pT172), ATP, Mg ²⁺	in	extended	in	2	3
Cyclin-D1/CDK6 ^{β3-αC(CDK4)} (pT177)	CDK6 ^{β3-αC(CDK4)}	1XO2, 2W9Z	Cyclin-D1, CDK6 ^{β3-αC(CDK4)} (pT177), ATP, Mg ²⁺	in	extended	in	2	3

Table S2. CDK4 and CDK6 and mutant sequences. Altered sequences are underlined; delete sequence is denoted as red dash.

Name	Sequence	Ending residue ID
CDK4 ^{WT}	-----MAT <u>S</u> RYEPVAEIG VGAYGT <u>VYKA</u> RDPH-SGHFV ALKSVRVPNG GGGGGGLPIS	52
CDK6 ^{WT}	MEKDGLCRAD QQYECVAEIG EGAYGKV <u>FKA</u> RDLKNGGRFV ALKRVRVQTG ---EEGMPLS	57
CDK6 ^{β3-αC(CDK4)}	MEKDGLCRAD QQYECVAEIG EGAYGKV <u>FKA</u> RDLKNGGRFV ALKRVRV <u>PNG</u> GGGGGGMPLS	60
CDK4 ^{β3-αC(CDK6)}	-----MAT <u>S</u> RYEPVAEIG VGAYGT <u>VYKA</u> RDPH-SGHFV ALKSVRV <u>QTG</u> ---EEGLPIS	57
CDK6 ^{ΔCterm}	SALSHPYFQD LERCKE-----	308

Table S3: Yeast strains used in this study.

Strain	Protein Expressed	Mutation
pBTB_003-1	pRS425-pGAL1-3XFLAG-CCND1-Linker-CDK4	Wild Type

pBTB_004-1	pRS425-pGAL1-3XFLAG-CCND1-Linker-CDK6	Wild Type
MK_1149	pRS425-pGAL1-3XFLAG-CCND1-Linker-CDK6	β 3- α C(CDK4)
yBT73	pRS425-pLEXA-3XFLAG-CCND1-Linker-Cdk6	Δ Cterm

References

1. Letunic, I. and P. Bork, *Interactive Tree of Life (iTOL) v6: recent updates to the phylogenetic tree display and annotation tool*. Nucleic Acids Res, 2024. **52**(W1): p. W78-W82.