

RESEARCH ARTICLE SUMMARY

STRUCTURAL BIOLOGY

p27 allosterically activates cyclin-dependent kinase 4 and antagonizes palbociclib inhibition

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INTRODUCTION: The cyclin D (CycD)-dependent kinases CDK4 and CDK6 (CDK4/6) phosphorylate the retinoblastoma (Rb) tumor suppressor protein to cause entry into the cell cycle in normal cells and in many cancers. Three small-molecule CDK4/6 inhibitors—palbociclib, ribociclib, and abemaciclib—are clinically approved in HER2-negative, ER-positive breast cancer in combination with antiestrogens. Although these drugs are already being used in clinical trials for diverse cancers, ongoing research is needed to better understand the mechanisms of inherent or acquired resistance to CDK4/6 inhibition.

RATIONALE: The intrinsically disordered proteins p21 and p27 are commonly known as CDK inhibitors, yet CDK4-CycD requires p21 or p27 (p21/p27) for assembly and activity in vivo. Moreover, all approved CDK4/6 inhibitors

were developed against purified CDK4/6-CycD complexes that lacked p21/p27. To date, there are no structures or kinetic data explaining whether and how p21/p27 activates CDK4-CycD and influences the response of chemical inhibitors. We therefore set out to structurally and functionally characterize the trimeric p21/p27-CDK4-CycD complexes, as well as to investigate the mechanism of clinically approved CDK4/6 inhibitors.

RESULTS: We present a crystal structure of the CDK4 holoenzyme, which reveals that p27 allosterically activates CDK4 to phosphorylate Rb by remodeling the adenosine triphosphate-binding site and by promoting release of the kinase activation segment. We find that tyrosine phosphorylation of p27 is required to activate CDK4 and that the lack of a key tyrosine in p21 makes it a poor ac-

tivator. Surprisingly, we also find that the purified p27-CDK4-CycD complex is refractory to inhibition by approved CDK4/6 inhibitors and that endogenous p27-, CDK4-, and CycD-associated activity is also insensitive. Instead, palbociclib primarily targets CDK4/6 monomer in breast cancer cells, and this association indirectly inhibits the downstream Rb-inactivating kinase CDK2.

CONCLUSION: The success of CDK4/6 inhibitors demonstrates the clinical importance of Rb inactivation in cancer. Although treatment

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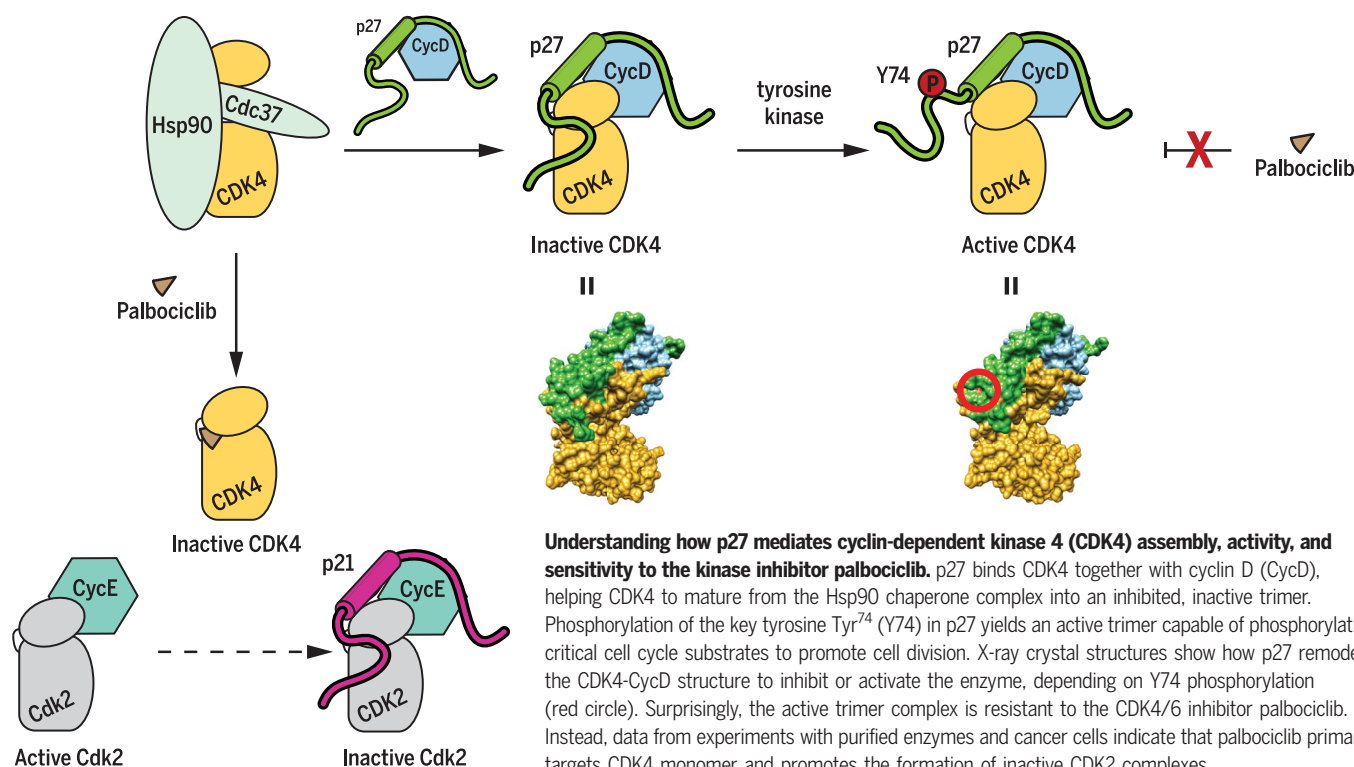
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with palbociclib, abemaciclib, or ribociclib leads to low concentrations of phosphorylated Rb, we conclude that these small molecules do not directly inhibit CDK4 kinase activity in the breast cancer cells we examined.

The mechanism that we propose, inhibition of complex assembly, parallels that of the endogenous CDK4/6 inhibitor protein p16. We conclude that mechanisms leading to CDK2 inhibition are critical for inducing cell cycle arrest and are likely critical determinants of whether cells are sensitive to the CDK4/6 inhibitors. ■

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p27 allosterically activates cyclin-dependent kinase 4 and antagonizes palbociclib inhibition

Keelan Z. Guiley^{1,2}, Jack W. Stevenson², Kevin Lou², Krister J. Barkovich², Vishnu Kumarasamy³, Tilini U. Wijeratne¹, Katharine L. Bunch¹, Sarvind Tripathi¹, Erik S. Knudsen³, Agnieszka K. Witkiewicz³, Kevan M. Shokat², Seth M. Rubin^{1*}

The p27 protein is a canonical negative regulator of cell proliferation and acts primarily by inhibiting cyclin-dependent kinases (CDKs). Under some circumstances, p27 is associated with active CDK4, but no mechanism for activation has been described. We found that p27, when phosphorylated by tyrosine kinases, allosterically activated CDK4 in complex with cyclin D1 (CDK4-CycD1). Structural and biochemical data revealed that binding of phosphorylated p27 (phosp27) to CDK4 altered the kinase adenosine triphosphate site to promote phosphorylation of the retinoblastoma tumor suppressor protein (Rb) and other substrates. Surprisingly, purified and endogenous phosp27-CDK4-CycD1 complexes were insensitive to the CDK4-targeting drug palbociclib. Palbociclib instead primarily targeted monomeric CDK4 and CDK6 (CDK4/6) in breast tumor cells. Our data characterize phosp27-CDK4-CycD1 as an active Rb kinase that is refractory to clinically relevant CDK4/6 inhibitors.

Cyclin-dependent kinases 4 and 6 (CDK4/6) drive cell proliferation by partnering with D-type cyclins (CycD) to phosphorylate the retinoblastoma protein (Rb). Rb is subsequently hyperphosphorylated and inactivated by CDK2 to trigger passage through the G₁ phase of the cell cycle (1–3). Disruption of this CDK4/6-Rb signaling pathway is ubiquitous in tumors and typically occurs through overexpression of CycD1 or loss of the CDK4/6-specific inhibitor p16^{INK4a}, both of which increase CDK4/6 activity, leading to uncontrolled proliferation (4–6). Specific CDK4/6-targeting adenosine triphosphate (ATP)-competitive drugs such as palbociclib are approved for estrogen receptor-positive breast cancer and are being tested in clinical trials for application in diverse cancer types (4, 5, 7, 8). As the use of CDK4/6 inhibitors as therapies increases, it becomes critical to understand their mechanism and factors that promote sensitivity or resistance.

CDK4/6 regulation is multilayered, reflecting the need to integrate diverse growth signals to control the cell cycle (3). Canonical CDKs require cyclin binding to properly structure their catalytic site (9). CDK4-CycD is unique in that CycD binding alone does not induce an active kinase structure (10, 11). CDK4-CycD also has relatively fewer characterized substrates and poorer catalytic activity compared to other CDKs (12–16). There are several cofactors that

interact with CDK4-CycD to modulate complex activity, assembly, and localization. The Rb family members (Rb, p107, and p130), which are the best-characterized substrates of CDK4, contain a specific activating interaction sequence (15, 17, 18). The Hsp90-Cdc37 chaperone complex stabilizes monomeric CDK4 by binding the unfolded N-lobe of the kinase (19, 20). The INK4 family (p19, p18, p16, and p15) inhibits CDK4/6 by obstructing cyclin binding and by pulling the activation segment (also called the T-loop) into an inactive conformation (21, 22). Activation segment phosphorylation by the CDK-activating kinase (CAK) stimulates CDK4 activity (23, 24).

CIP (p21) and KIP (p27 and p57) proteins are CDK2 inhibitors in vitro and in cells under conditions of growth arrest (25). They are intrinsically disordered proteins that fold onto a cyclin and then a CDK sequentially to form ternary complexes (26). Mice lacking p21 or p27 are susceptible to tumorigenesis (27, 28), which is consistent with the important roles of CIP and KIP proteins in negatively regulating the cell cycle through CDK2 inhibition. p27 degradation is critical for licensing entry into S phase, and p21 is a key effector of p53-activated senescence (25, 29). p27 directly inhibits CDK2-CycA by occluding a substrate-docking site and by inserting a small helix within the p27 CDK-inhibitory domain into the CDK2 ATP site (30).

p21 and p27 have a more complex role in regulating CDK4. Although they can inhibit CDK4 under some conditions, they are also necessary for CDK4 activity. Embryonic fibroblasts that lack both p21 and p27 fail to assemble active CDK4-CycD complexes (31). Much of p27 is found in a complex with CDK4-CycD

in proliferating cells, and active CDK4 complexes in cells contain both CycD and p27 (25, 32–36). High levels of p21 are inhibitory, whereas low levels induce assembly and nuclear localization of enzymatically active CDK4 complexes (37). The activity of CDK4 complexes requires phosphorylation of p27 by non-receptor tyrosine kinases (NRTKs) (34, 35, 38), including the breast tumor kinase Brk (also called PTK6). However, it is unclear whether and how p21 and p27 directly stimulate CDK4 catalytic activity, how this activation is mediated by p27 phosphorylation, and how p27 influences CDK4's sensitivity to chemical inhibitors such as palbociclib.

Crystal structures of p21-CDK4-CycD1 and p27-CDK4-CycD1 complexes

To better understand p21 and p27 regulation of CDK4, we determined the crystal structures of p21-CDK4-CycD1 and p27-CDK4-CycD1 complexes at 3.2 Å and 2.3 Å resolution, respectively (Fig. 1 and tables S1 and S2). p21 and p27 similarly fold into a single helix that spans CDK4-CycD1. The structures demonstrate why both proteins function as assembly factors. p21 and p27 contain a subdomain 1 (D1), which docks into a hydrophobic cleft in CycD1, and a subdomain 2 (D2), which binds the N-lobe of CDK4 (Figs. 1 and 2). CDK4 and CycD1 are joined through the bridging helix (α 1), which provides a rigid constraint to define the relative orientation of the cyclin and kinase N-lobe domains (Fig. 1, A and B).

The trimer structures demonstrate two key mechanisms of inhibition. First, p21 and p27 use their RxLF motif in D1 to block access to a critical substrate-docking site on CycD (Fig. 2, A and B) (39, 40). Similar D1 domain-mediated interactions are also observed between p27 and CDK2-CycA (fig. S1, A and B). Second, the p21 and p27 D2 domains displace the first strand (β 1) of the CDK4 N-lobe β sheet (Fig. 2C). As a result, the glycine-rich loop (Gly-loop; residues 13 to 19 in CDK4) is dislodged from the CDK4 active site and the ATP-binding pocket is disrupted. A striking difference in how p27 binds CDK2 and CDK4 was observed in the interaction between p27 D2 and the kinase domain. Whereas p27 inserts a small 3_{10} helix into the CDK2 ATP site (30), a 3_{10} helix was not observed at the corresponding location in CDK4 (Fig. 2D and fig. S1C). Differences in the CDK4 hinge region from CDK2 suggest that 3_{10} -helix insertion of p27 into the CDK4 active site would be sterically hindered (fig. S1D).

p21 and p27 binding rotates the CDK4 N-lobe and releases the kinase activation segment

We compared the trimer structures to the known CDK4-CycD dimer structures (10, 11). Unexpectedly, p21 and p27 induced structural changes that better shape the active site for

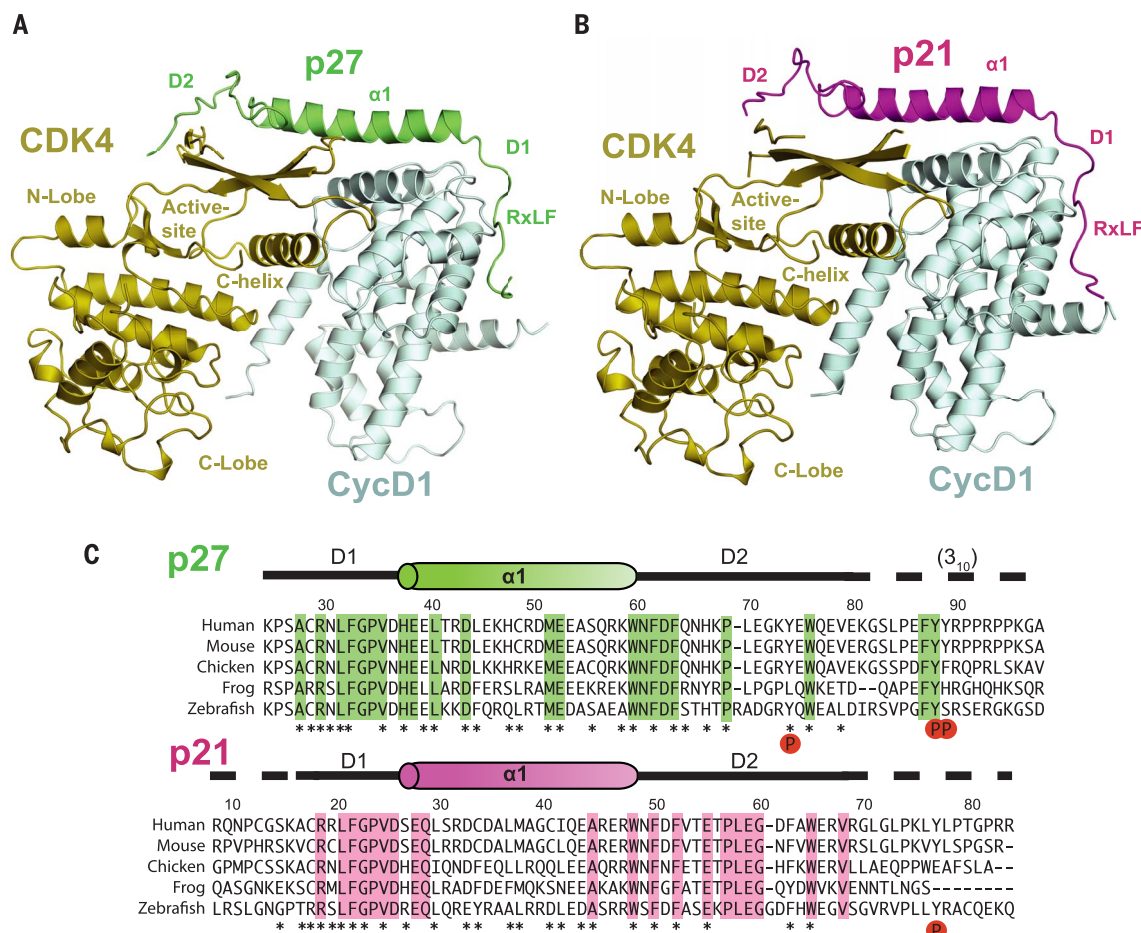
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Fig. 1. Structures of the p27-CDK4-CycD1 and p21-CDK4-CycD1 complexes. (A) Overall structure of p27-CDK4-CycD1. p27 (green) binds CycD1 (cyan) with its D1 domain and CDK4 (gold) with its D2 domain.

(B) Structure of p21-CDK4-CycD1. p21 (magenta) adopts a fold similar to that of p27, bridging CDK4 (gold) and CycD1 (cyan).

(C) Sequence alignment of p27 and p21. Asterisks represent residues directly interacting with CDK4 or CycD1. The known tyrosine phosphorylation sites are noted. Secondary structure observed in the crystal is indicated above the sequences. Dashed lines indicate sequences in the crystallized protein that are not visible in the electron density, including the C-terminal sequence in p27 that forms a 3_{10} helix when bound to CDK2 (in parentheses). Amino acid abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.



catalysis. p27 binding rotated the N-lobe of CDK4 relative to the C-lobe (Fig. 3, A to D). The strands in the N-lobe β sheet were shifted by ~ 4 to 6 Å such that the β -strand 2 ($\beta 2$) of the trimer complex replaced the β -strand 3 ($\beta 3$) position of the dimer, $\beta 3$ replaced $\beta 5$, and $\beta 5$ replaced $\beta 4$. An important consequence of this altered N-lobe conformation is that the activation segment was released from the CDK4 active site, because specific interactions between $\beta 3$ and $\beta 5$ and the activation segment helix were broken (Fig. 3B). The release represents an important step that allows substrate binding in the kinase active site (41). For CDK1 and CDK2, activation segment release occurs upon binding to a cognate cyclin, and phosphorylation of the activation segment by CAK further improves substrate binding (42). However, in the crystal structure of the CDK4-CycD1 dimer, the activation segment remains in a substrate-blocking conformation despite the introduction of a phosphomimetic (T172D) at the CAK phosphorylation site (Fig. 3B) (10). The release of the activation segment observed upon p27 binding (Fig. 3B) is a unique mechanism to

CDK4 and likely explains the requirement of p27 for CDK4 activation segment phosphorylation by CAK (24).

A second result of the β -sheet rearrangement was that the catalytic lysine (Lys³⁵) on $\beta 3$ was pulled into a position to accept the β - and γ -phosphates of ATP (Fig. 3, C and D). This position of Lys³⁵ is similar to that of the corresponding catalytic lysine (Lys³³) in active CDK2-CycA (fig. S1E) (9, 30). In contrast, the position of Lys³⁵ in the CDK4-CycD1 dimer is similar to its position in the inactive CDK2 monomer structure (10, 11). This conformational change in the N-lobe does not occur when p27 binds CDK2 and is therefore an allosteric activating mechanism of p27 specific to CDK4 (fig. S1, A and F) (30).

Although the p21 and p27 trimer complexes are primed for catalysis, in that Lys³⁵ is positioned to coordinate ATP and the activation segment is released, there are still aspects of the structure that indicate a requirement for additional activating mechanisms. The D2 domain must be displaced to permit formation of the ATP-binding G-loop. In addition, com-

parison of the CDK4 trimer structure with an active CDK2 structure indicates that the C helix (also known as the PSTAIRE helix) in CDK4 remains in an inactive position, with its critical catalytic glutamate pointing away from the active site (Figs. 2C and 3D). We propose that the structure determined here is an intermediate along a pathway to activation, and that substrate binding, ATP binding, or activation segment phosphorylation in conjunction with p27 binding may induce the required rotation of the C helix (fig. S2A).

Tyrosine phosphorylation of p27 but not p21 activates CDK4 trimer complexes

To quantify the kinase activity of p21- and p27-CDK4-CycD1, we assayed the activity of recombinant CDK4 complexes (Fig. 3, E to G). We used a phosphorylation site mimetic in the CDK4 activation segment (CDK4 T172E) because there was heterogeneity in phosphorylation of the Thr¹⁷² site in our purified protein (fig. S2, B and C). We tested the CDK4-CycD1 dimer (called K4D1/- because it lacks p21 or p27), the trimer with p21 or p27 (K4D1/p21 or

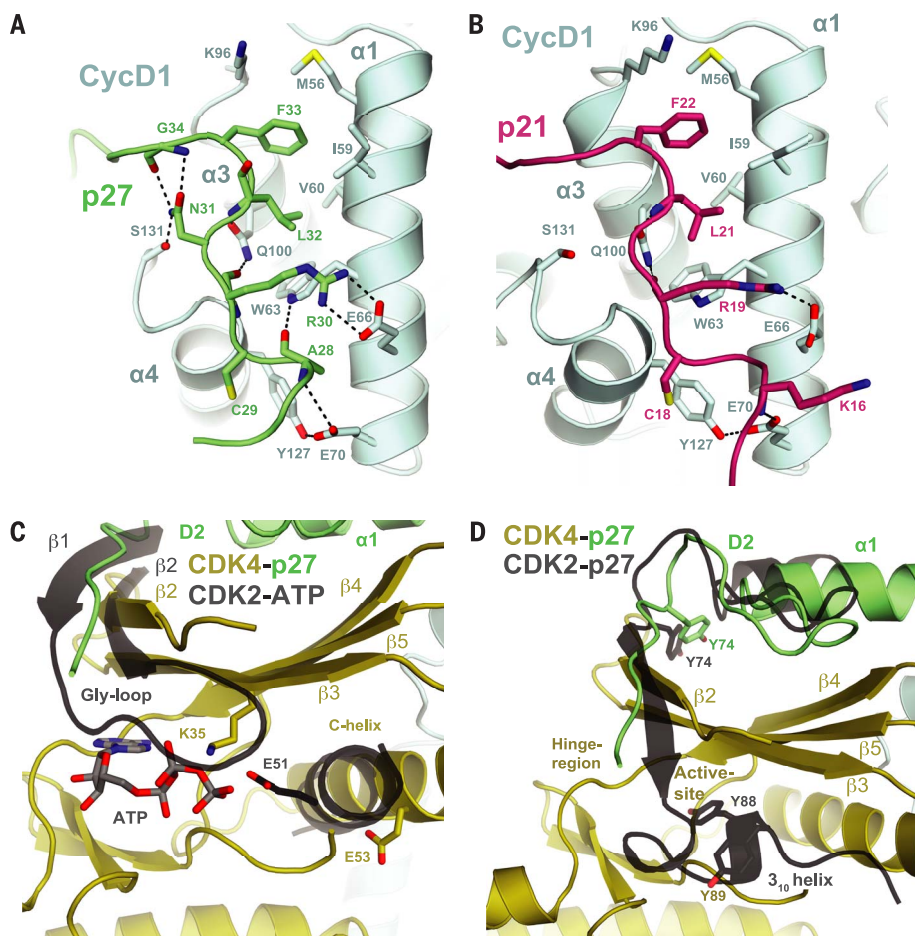


Fig. 2. p27 and p21 inhibit substrate binding and catalytic activity. (A) Association between the p27 RxF motif (green) and the MVRIL cleft in CycD1 (cyan) competes for substrate docking. (B) The p21 RxF (magenta) bound to CycD1 (cyan). (C) Binding of the D2 region in p27 (green) displaces the β 1 strand in the CDK4 N-lobe (gold), disrupting the ATP-binding site. The structure shown in gray for comparison, including the ATP, is from CDK2 in the active CDK2-CycA dimer. The CDK4 β 1 strand and following Gly loop are not visible in the p27-CDK4-CycD1 trimer structure, indicating they are disordered. The C helix remains in an inactive conformation in the CDK4-p27 structure. (D) Comparison of binding of p27 to CDK2 (p27 in gray) and to CDK4 (p27 in green, CDK4 in gold). The 3_{10} helix in p27 that binds the CDK2 ATP site is not visible and likely remains disordered upon CDK4 binding. Tyrosine phosphorylation sites are shown.

K4D1/p27), and the trimer with tyrosine kinase (Brk/PTK6)-phosphorylated p21 and p27 (K4D1/phosp21 or K4D1/phosp27) (Fig. 3E and fig. S3). The dimer demonstrated strong activity toward the purified Rb C-terminal domain (residues 771 to 928, Rb⁷⁷¹⁻⁹²⁸), whereas the trimer with unphosphorylated p21 or unphosphorylated p27 demonstrated poor activity. Consistent with observations in cell culture (34, 35), tyrosine phosphorylation of p27 restored its recombinant trimer enzyme activity. The tyrosine kinase-phosphorylated p21 had only slight additional activity, indicating that p21 trimer complexes are mostly inhibited despite tyrosine phosphorylation.

We focused on the strong activity of the phosphorylated p27 trimer and used steady-state kinetic experiments to quantify enzyme

processing of ATP (Fig. 3, F and G, and fig. S4). We tested CDK4 activity of the dimer and the phosp27-trimer complex toward Rb⁷⁷¹⁻⁹²⁸, Rb⁷⁷¹⁻⁸⁷⁴ (residues 771 to 784; this shorter Rb fragment lacks a CDK4 docking sequence) (15, 17, 18), Cdc6¹⁻¹¹⁹ (residues 1 to 119, a CDK4 substrate that contains an RxF docking sequence) (13), and p107⁹⁴⁹⁻¹⁰⁶⁸ (residues 949 to 1068, which lacks any known docking sequence). In the case of all four substrates, the K_M of ATP for the K4D1/phosp27 trimer was reduced relative to that of K4D1/- and was more similar to the K_M of CDK2 and that of most serine/threonine kinases (16). This decrease in K_M , which we also observed for CDK6-CycD1 but not for CDK2-CycA (fig. S5), is consistent with our structural results that p27 binding induces an N-lobe conformation in

CDK4 that supports ATP coordination. We note that although we observed activity from reconstituted phosp27-CDK6-CycD1 trimers in vitro, it is unclear whether these complexes exist in cycling cells (43).

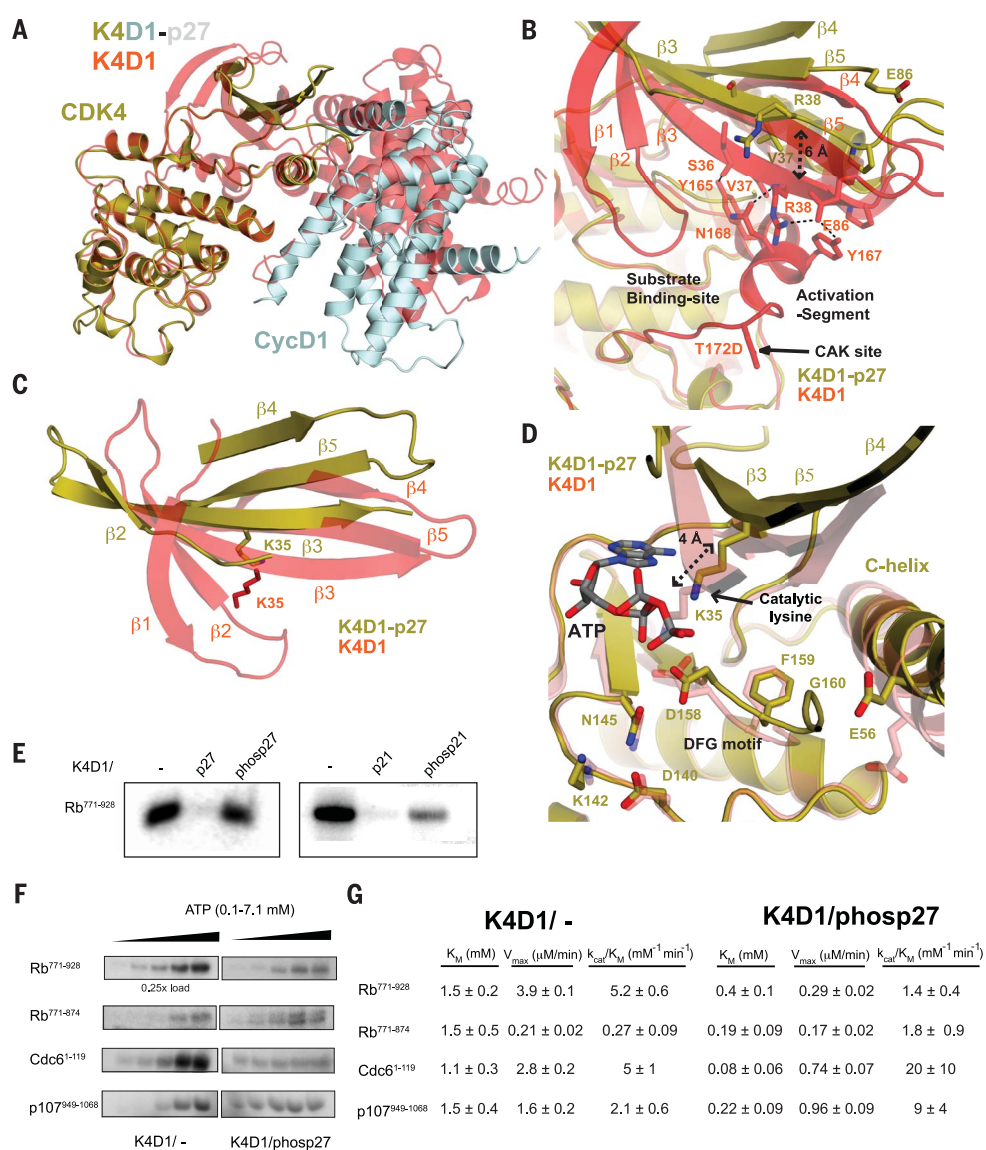
In the activity assays, phosp27 also had an inhibitory effect that is specific for substrates containing a CDK docking sequence. When assaying the K4D1/- dimer, the ATP-dependent maximum reaction rate V_{max} and the Rb substrate k_{cat}/K_M both decreased upon deletion of the docking sequence in Rb (compare Rb⁷⁷¹⁻⁹²⁸ to Rb⁷⁷¹⁻⁸⁷⁴), supporting the importance of docking in dimer phosphorylation of Rb (Fig. 3G and fig. S4B) (17, 18). The ATP V_{max} decreased upon addition of phosp27 when assaying Rb⁷⁷¹⁻⁹²⁸ (factor of 13 decrease) and Cdc6¹⁻¹¹⁹ (factor of 4 decrease), which both contain docking sequences (Fig. 3G). In contrast, the ATP V_{max} only modestly decreased for p107⁹⁴⁹⁻¹⁰⁶⁸ and for Rb⁷⁷¹⁻⁸⁷⁴, which lack docking sequences. The Rb⁷⁷¹⁻⁹²⁸ substrate k_{cat}/K_M , but not the Rb⁷⁷¹⁻⁸⁷⁴ substrate k_{cat}/K_M , decreased upon addition of p27 (fig. S4B), and the kinetics of the trimer activity toward both Rb substrates was similar (Fig. 3G). These observations indicate that even in the active complex, p27 inhibits CDK4 activity toward some substrates by occluding the docking site in CycD (Fig. 2A).

Structural mechanism of p27-CDK4-CycD activation by tyrosine phosphorylation

We solved the structure of a phosp27-CDK4-CycD1 complex to determine how tyrosine phosphorylation relieves p27 inhibition (table S1). Tyr⁷⁴ was in a position similar to that in the unphosphorylated trimer, but there was clear electron density for the phosphate (Fig. 4, A and B, and fig. S6). Tyr⁷⁴ is part of an aromatic cluster in p27 D2 that binds the CDK4 N-lobe. The structural data predict that Tyr⁷⁴ phosphorylation would weaken this association by destabilizing the hydrophobic interface between Tyr⁷⁴ and the N-lobe of CDK4 (Fig. 4, A and B). In p21, a phenylalanine (Phe⁶²) is in the position of Tyr⁷⁴ in p27 such that the p21 D2 domain would remain bound without the addition of a phosphate (Figs. 1C and 4C).

In a structure we solved of the trimer containing p27 with phosphomimetics (table S1), temperature B-factors were ~10 to 20 Å² higher for side chains in the aromatic cluster in D2 and the electron density was weaker around Trp⁶⁰ and Glu⁷⁴ (fig. S6). The higher B-factors and weaker density are consistent with lower D2 occupancy and higher disorder. Deletion of the D2 domain from p27 or mutation of the tyrosines to glutamate phosphomimetics generated a trimer complex that phosphorylated Rb⁷⁷¹⁻⁹²⁸ (Fig. 4D and fig. S7). Solution studies with p27 binding to CDK2-CycA demonstrate that phosphorylation of Tyr⁷⁴ results in loss of interactions between the D2 domain of p27

Fig. 3. p27 induces structural changes that promote ATP coordination and processing. (A to D) Structural alignment of CDK4-CycD1 with p27 (gold-cyan) and without p27 (red, PDB code 2W96) reveals movement of both the CDK4 N-lobe and CycD1 domains relative to the CDK4 C-lobe. **(E)** Phosphorylation of purified Rb⁷⁷¹⁻⁹²⁸ with [³²P]ATP and the indicated dimer (K4D1/-) or trimer complex. **(F and G)** Steady-state kinase assays measuring effects of ATP concentration on initial reaction rate, as measured by incorporation of [³²P]ATP. Reactions include CDK4 dimer (K4D1/-) or active trimer (K4D1/phosp27) and the indicated substrate.



and the N-lobe of CycA (44). Together these results support a model in which phosphorylation or phosphomimetics weaken the affinity of the D2 domain for the CDK4 N-lobe, allowing for the formation of the Gly loop and activation of the kinase. The activation segment was not homogeneously phosphorylated in our structure (fig. S2B), so we cannot rule out the possibility that activation segment phosphorylation and the ultimate repositioning of the C helix also play a role in removing the D2 domain from the N-lobe.

Tyr⁸⁸ and Tyr⁸⁹ were disordered in all the p27-CDK4-CycD1 crystal structures, and the CDK4 trimer assembled with p27 containing a phosphomimetic at Tyr⁷⁴ had similar (although slightly less) activity to that of the enzyme assembled with phosphomimetics at all the tyrosines (Fig. 4D). Thus, Tyr⁷⁴ phosphorylation appeared to be nearly sufficient for p27 activation of CDK4, although the data in-

dicate that there may have been some contribution from Tyr⁸⁸/Tyr⁸⁹ phosphomimetics in the p27 triple glutamate mutant (Fig. 4D). This mechanism for turning off p27 inhibition is different from the mechanism in CDK2, in which phosphorylation of p27 Tyr⁸⁸ and Tyr⁸⁹ is necessary for ejection of a p27 3₁₀ helix from the catalytic site (fig. S1C) (45). These phosphorylation sites in p27 and the corresponding site in p21 (Tyr⁷⁷) are located in a sequence that remains disordered upon CDK4 binding (Fig. 1C), which is consistent with their weaker role in the mechanism of CDK4 activation. The more important role of the Tyr⁷⁴ phosphorylation site also explains the observed poor activity of Brk-phosphorylated p21 complexes (Fig. 3E), as p21 lacks a tyrosine at the equivalent position of Tyr⁷⁴ in p27 (Figs. 1C and 4C). We conclude that although p21, like p27, primes CDK4 for catalysis by releasing the activation segment, p21 tyrosine phosphorylation does

not relieve D2 inhibition, and the complex remains mostly inhibited.

p27-CDK4-CycD1 complexes are insensitive to CDK4/6-specific inhibitors

The structural changes in the kinase N-lobe that are induced by p27 binding indicate that palbociclib-type inhibitors should have poor potency toward active CDK4 trimer complexes (Fig. 5A). The “gatekeeper” residue Phe⁹³ in β 5 and also Ala³³ in β 3 are rotated away from making contacts with the C5-methyl and C6-acetyl groups in the pyridopyrimidine scaffold of palbociclib (46, 47). We tested the effects of palbociclib, ribociclib, and abemaciclib on the activity of the reconstituted CDK4 enzyme complexes (Fig. 5B and fig. S8A). All three compounds inhibited Rb⁷⁷¹⁻⁹²⁸ phosphorylation by CDK4-CycD1 dimer (K4D1/-), as expected; however, the active phosp27-CDK4-CycD1 trimer (K4D1/phosp27) was relatively insensitive

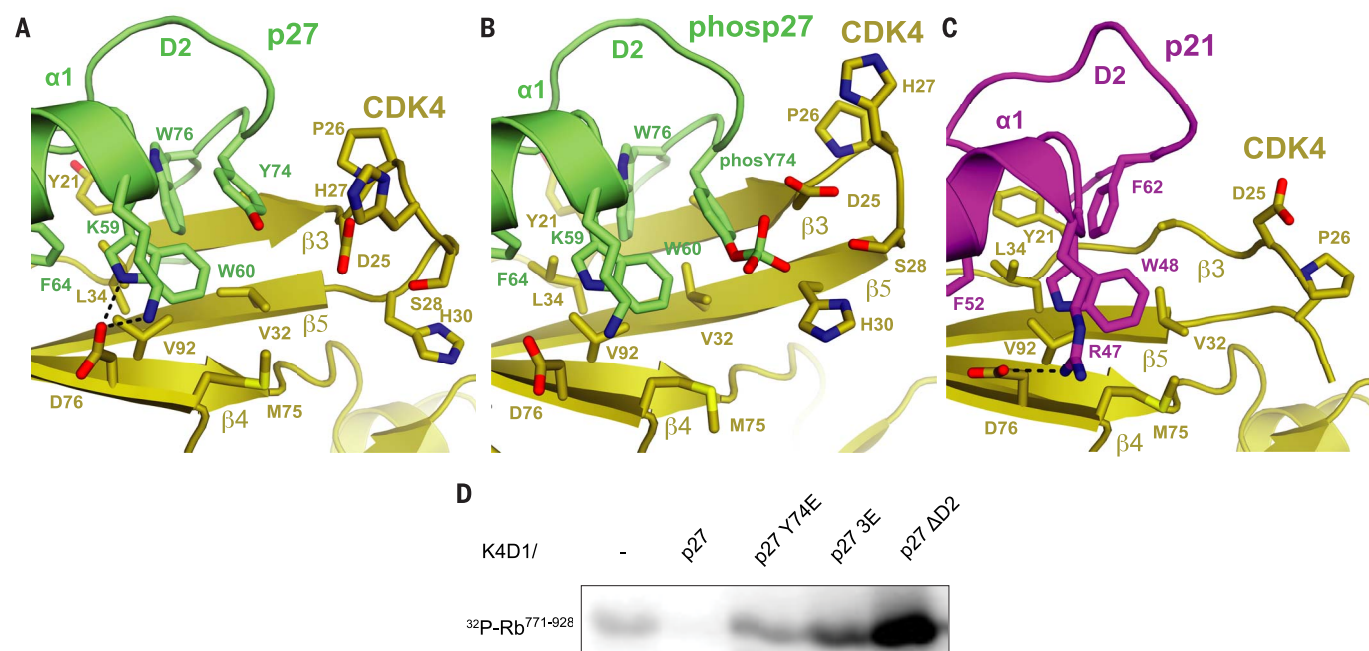


Fig. 4. Tyr⁷⁴ phosphorylation disrupts the p27 D2-CDK4 interface. (A to C) Comparison of CDK4-CycD1 structures with unphosphorylated p27 (A), phosphorylated p27 (B), and p21 (C). (D) [³²P]ATP phosphorylation of Rb⁷⁷¹⁻⁹²⁸ using K4D1 dimer or trimer enzymes assembled with the indicated p27 kinase inhibitory domain construct (residues 25 to 93). 3E contains three glutamate phosphomimetics at Tyr⁷⁴, Tyr⁸⁸, and Tyr⁸⁹. ΔD2 contains p27 residues 25 to 60, and therefore lacks the D2 CDK4-binding domain.

to the drugs, with apparent inhibition constants of ~10 μM or higher. We measured the binding affinity of palbociclib for CDK4 complexes by isothermal titration calorimetry (ITC). The drug bound only CDK4 monomer and K4D1/- dimer tightly. Consistent with our structural and kinetic data, we detected no affinity for K4D1/phosp27 trimer (Fig. 5C and fig. S8B). We also measured binding of p27 to CDK4 monomer and found no affinity if the enzyme was first saturated with palbociclib (Fig. 5C and fig. S8C). These data demonstrate that binding of p27 and of palbociclib to CDK4 are mutually exclusive.

Palbociclib does not inhibit endogenous CDK4 activity

We tested whether the activity of endogenous cellular CDK4 complexes is inhibited by palbociclib. We immunoprecipitated p27 complexes from MCF7, MDA-MB-231, and T98G cells. MCF7 and MDA-MB-231 cells are Rb-positive and palbociclib-sensitive breast cancer cells that differ in estrogen receptor (ER) status (46). T98G cells are Rb (+) and ER (-) glioma cells that are relatively less sensitive to palbociclib (see below). The immunoprecipitates phosphorylated purified Rb⁷⁷¹⁻⁹²⁸ in the [³²P]ATP assay; however, the activity was insensitive to palbociclib that was added to the kinase reactions (Fig. 5D). We considered that this palbociclib-insensitive activity might be from CDK2. Although cellular CDK2

complexes with p27 are thought to be inactive (32), we found that reconstituted K2A/phosp27 complexes phosphorylated Rb (fig. S5C). However, the p27 immunoprecipitate activity was also not inhibited by the CDK2 inhibitor dinaciclib, and therefore the immunoprecipitate likely contained a CDK4 activity and not a CDK2 activity (fig. S9).

To test the entire cellular pool of CDK4 activity, we immunoprecipitated CDK4 from the same cell lines using an antiserum raised against the CDK4 C terminus, which contains a sequence distinct from CDK2 (48). Palbociclib also poorly inhibited the immunoprecipitated CDK4 kinase activity (Fig. 5E), which is consistent with previous observations that CDK4 activity arises from trimer complexes (25, 31-37) and our observation that the trimer is insensitive to the drug (Fig. 5B).

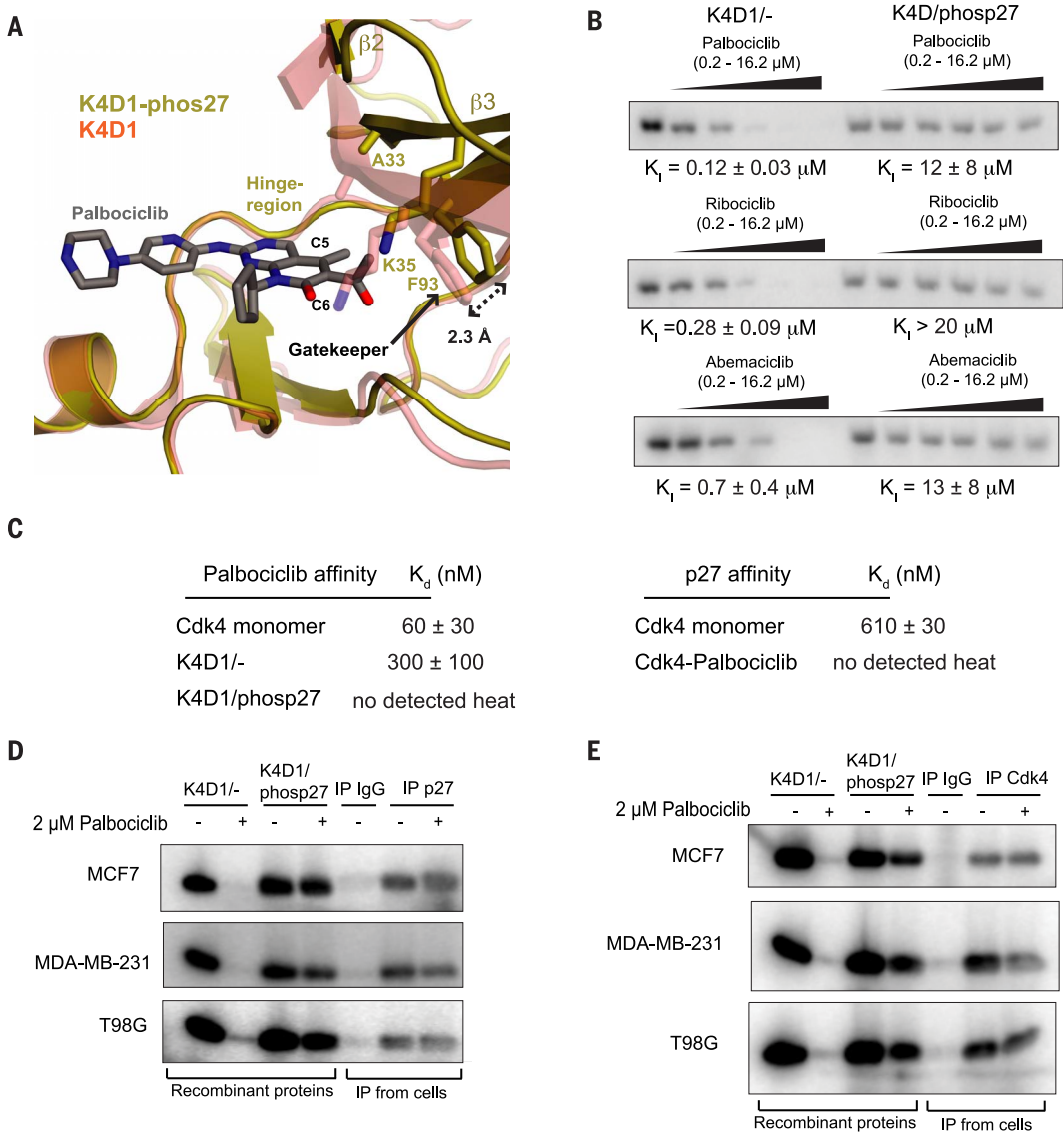
An ATP-site occupancy probe targets CDK4 monomers in cells

To examine the association of an inhibitor with endogenous CDK4 complexes, we treated MCF7 and MDA-MB-231 cells with the covalent ATP-site occupancy probe XO44 (49). XO44 labeled both CDK4 and CDK2 in these cells and could be detected by a gel shift upon coupling to an analysis probe (Fig. 6A and fig. S10, A and B). Although XO44 labeled most CDK2 and the mitotic kinase Aurora B (fig. S10A), it labeled only ~40 to 60% of CDK4 even at high concentrations, indicating that a resistant pop-

ulation of the protein exists. CDK4 labeling in cells was inhibited by palbociclib (Fig. 6B and fig. S10C); this finding shows that XO44 and palbociclib bind the ATP site competitively and that the population of CDK4 that is targeted by XO44 is also targeted by palbociclib-type inhibitors. XO44 labeled recombinant CDK4 efficiently in dimer complexes but not in unphosphorylated or phosphorylated trimer complexes (Fig. 6C).

We treated cells with XO44 and palbociclib and fractionated the lysates with size-exclusion chromatography (Fig. 6D and fig. S10D). We observed CDK4 in high-molecular size fractions comigrating with Hsp90 and Cdc37 (e.g., fractions 5 and 6), in mid-size fractions with CycD1 and p27 (fractions 9 and 10), and in low-size fractions that likely contain monomers (fractions 15 and 16) (34, 36). Consistent with ATP-competitive probes sequestering obligate Hsp90-client kinases from the chaperone system (20, 50), we observed a reduction in the abundance of large-sized, putatively Hsp90/Cdc37-bound CDK4 in extracts from XO44- and palbociclib-treated cells. We also detected greater quantities of CDK4 in small-sized monomer fractions upon treatment with either compound, and only the CDK4 population observed in these fractions was labeled with XO44 (Fig. 6D, fractions 15 and 16, XO-44 + click). Considering that CDK4 inhibitors do not associate with CDK4 bound by INK proteins (51), we propose that the tested inhibitors primarily

Fig. 5. Palbociclib does not bind and poorly inhibits purified and endogenous CDK4 trimer complexes. (A) K4D1 dimer (PDB ID 2W96) and the phosph27-K4D1 trimer structures were aligned with palbociclib-bound CDK6 (PDB ID 2EUF, not shown) to model the position and interactions of the drug when bound to CDK4. (B) [³²P]ATP phosphorylation of Rb⁷⁷¹⁻⁹²⁸ using CDK4-CycD1 dimer (K4D1/-) and phosph27-CDK4-CycD1 trimer (K4D1/phosp27) enzymes in the absence (leftmost lane in each titration) and presence of increasing inhibitor concentrations (see fig. S8A for quantification). Each drug is dosed from 0.2 μM to 16.2 μM in factor of 3 increments. (C) ITC affinities for palbociclib (left) or p27 (right) titrated into the indicated enzyme. (D) The indicated cell lysates were immunoprecipitated with control or with p27 antibody, and the activity of the immunoprecipitate was used to phosphorylate Rb⁷⁷¹⁻⁹²⁸ with [³²P]ATP in the absence or presence of palbociclib. Reactions with the indicated recombinant dimer (K4D1/-) or trimer (K4D1/phosp27) enzymes are shown for comparison in the first four lanes. (E) As in (D), except lysates were precipitated with antiserum raised against a CDK4 C-terminal peptide.



bind CDK4 monomers in cells and increase the abundance of the inactive monomer population. We synthesized a palbociclib analog linked to biotin, and we used it to precipitate CDK4/6 from cell extracts to determine which proteins exist in the complexes targeted by palbociclib-type inhibitors (Fig. 7A). Consistent with our activity and ITC binding assays, the palbociclib-biotin precipitated recombinant CDK4-CycD1 dimer but not recombinant p27-CDK4-CycD1 trimer (Fig. 7B). Strikingly, in breast cancer cell lysates, the palbociclib-biotin strongly precipitated CDK4 and CDK6 but did not precipitate any detectable p21 or p27 (Fig. 7, C and D). This observation further demonstrates that the drug does not bind trimer complexes with p21 and p27 in these cells. A faint band reproducibly appeared in the CycD1 blot, indicating that there may be some small population of dimer complexes targeted by the drug. How-

ever, we conclude that monomer CDK4 and monomer CDK6 are the predominant targets of palbociclib in these cells. **Palbociclib-induced cell cycle arrest coincides with loss of CDK2 but not CDK4 activity** We treated cells with palbociclib for 48 hours, which, similar to previous descriptions, led to strong arrest of the breast cancer cells and to weaker arrest of T98G cells, as determined by 5-ethynyl-2-deoxyuridine (EdU) incorporation and propidium iodide staining (52, 53) (Fig. 8A and fig. S11). Endogenous CDK4 and CycD1 complexes retained kinase activity despite the cells arresting with drug treatment (Fig. 8B and fig. S12A). This similar activity in palbociclib-treated cells was observed despite increased levels of CycD1 (Fig. 8C and fig. S12B) (52). In contrast, CDK4 complexes immunoprecipitated from serum-starved NIH/3T3 cells

lack Rb-directed kinase activity (fig. S12C), and the lack of activity coincides with reduced CycD1 levels. In the breast cancer cells tested, we did not observe palbociclib inhibiting CDK4- or CycD1-associated kinase activity, which is consistent with the lack of susceptible dimer complexes that could be detected in their lysates (Fig. 6D and Fig. 7, C and D). Unlike CDK4 complexes, CDK2 complexes that were immunoprecipitated from the breast cancer cells had diminished activity after 48 hours of palbociclib treatment (Fig. 8B). CDK2 inhibition at this drug concentration is likely indirect (5, 46), resulting at least in part from lower levels of CycA (Fig. 8C) (52). We also observed evidence for an increase in p21 but not p27 in CDK2-CycE complexes (Fig. 8, C and D, and fig. S12, D and E). In reciprocal coimmunoprecipitation experiments, additional p21-CycE association was detected after

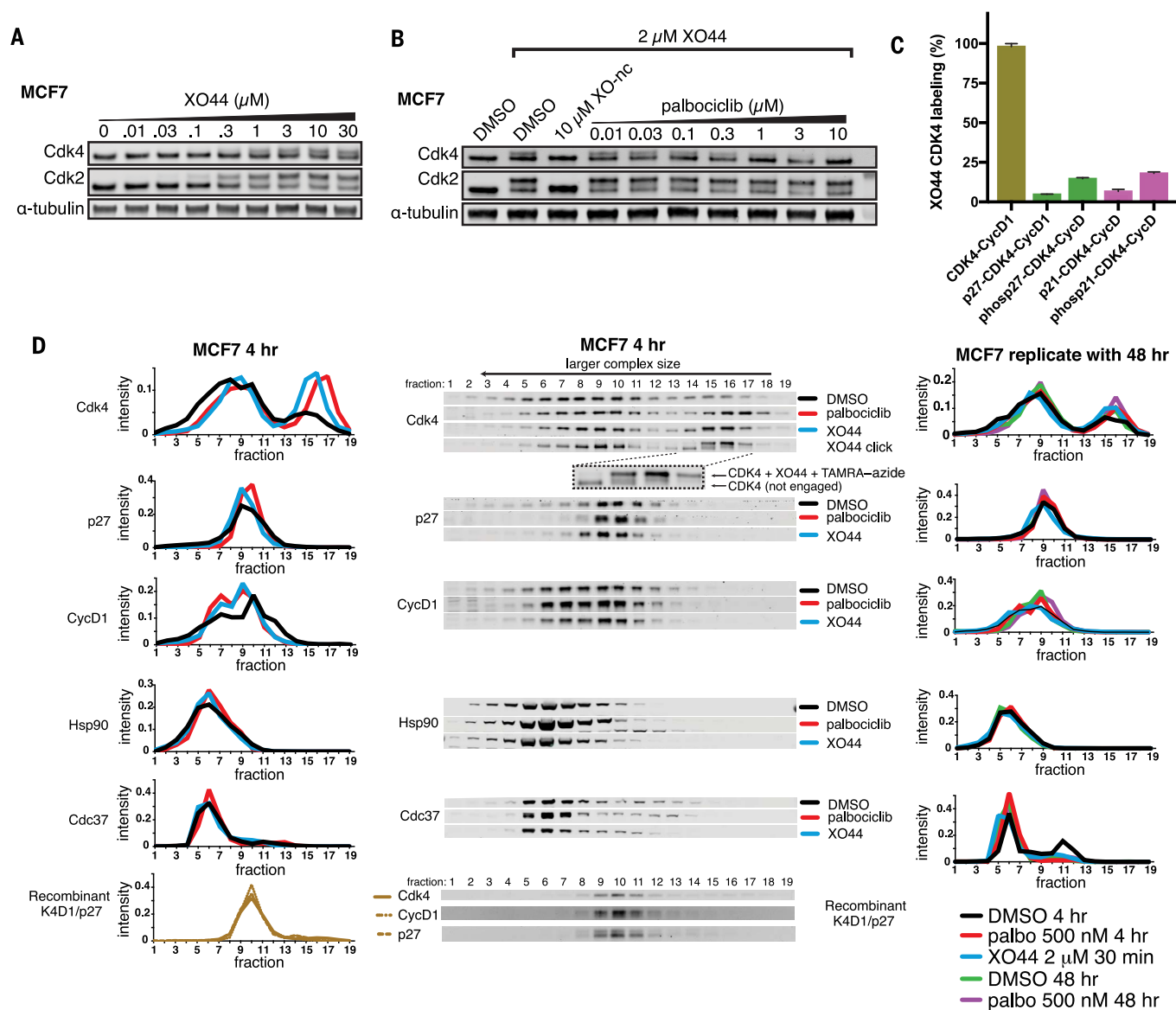


Fig. 6. The ATP-site occupancy probe XO44 labels monomer CDK4 in MCF7 cells. (A) Labeling of endogenous CDK4 and CDK2 by the promiscuous covalent ATP-site probe XO44. MCF7 cells were treated with DMSO vehicle or XO44 at the indicated concentrations for 30 min. Lysates were subjected to click reaction with TAMRA-azide to visualize XO44 labeling of proteins by gel mobility shift. (B) Palbociclib competes with XO44 for CDK4 binding but not CDK2 binding. Experiment performed as in (A), but cells were pretreated for 60 min with DMSO, a nonclickable analog of XO44 (XO-nc), or increasing concentrations of palbociclib. (C) XO44 efficiently labels purified recombinant CDK4-CycD1 dimer but not trimer complexes with p21 or p27, as determined by

electrospray ionization mass spectrometry. Protein complexes were treated with DMSO or XO44. Average percent labeling was determined for three replicates; error bars denote SD. (D) Asynchronous MCF7 cells were treated with DMSO, XO44 (2 μ M, 30 min) or palbociclib (500 nM, 4 hours), and lysates were fractionated using Superdex200 size-exclusion chromatography. XO44 labeling was monitored by gel mobility shift after click reaction with TAMRA-azide ("XO44 click"). Normalized quantifications of band signals from the Western blots using the indicated antibody are shown. A benchmark chromatography experiment using the recombinant protein complex is displayed at the bottom of the panel. See fig. S10 for data using MDA-MB-231 cells.

48 hours. We could not detect p27 in the CycE1 IP with or without palbociclib treatment, so we cannot determine whether p27 also plays a role in down-regulating the CDK2-CycE activity. We conclude that palbociclib treatment indirectly leads to CDK2 inhibition and that this CDK2 inhibition, and not CDK4 inhibition, correlates with cell cycle arrest.

Discussion

CIP/KIP proteins were first characterized as CDK inhibitors, in particular as potent inhibitors of CDK2-CycA/E (25, 54–58). In contrast, other evidence implicated noninhibitory roles for p21 and p27 in mediating CDK4-CycD function, including complex assembly and nuclear localization (31, 37). Moreover, the observations

that active CDK4-CycD tolerated the presence of p27 and that Rb-directed kinase activity in cells contained p27 led to models that CDK4-CycD may titrate inhibitory p27 away from CDK2 (25, 33, 37, 59). Our data demonstrate that p27 is not merely a noninhibitor, but in fact allosterically activates CDK4-CycD, remodeling the kinase to increase the catalytic

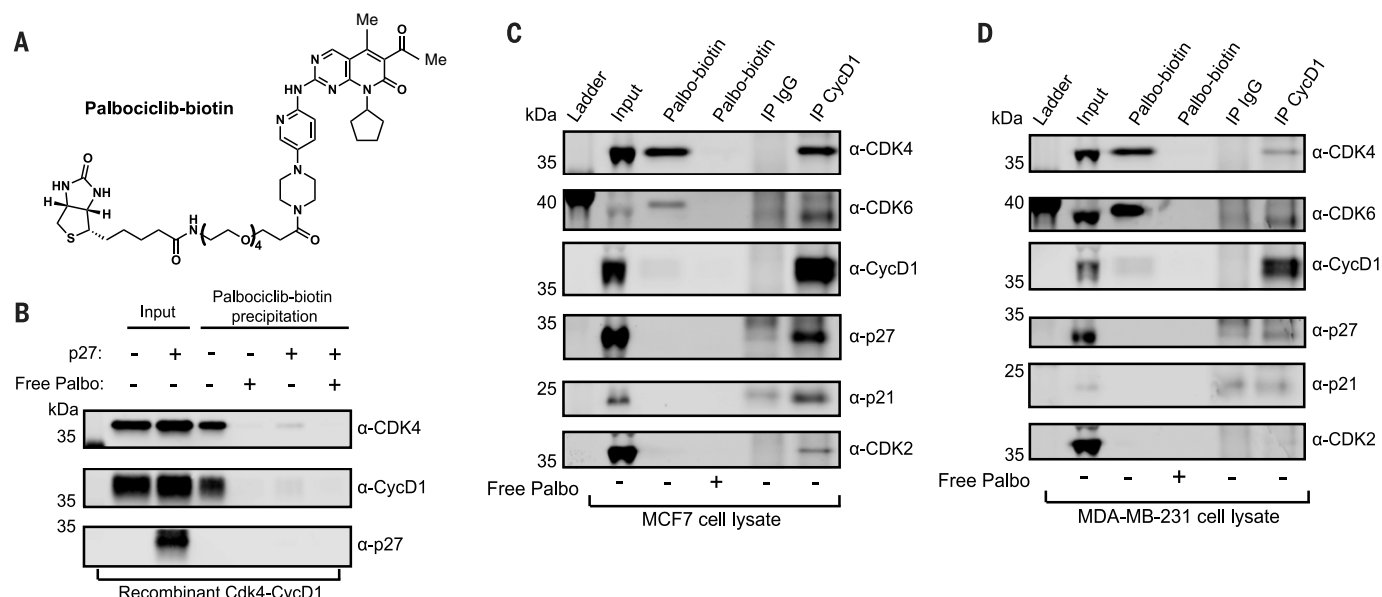


Fig. 7. Palbociclib directly targets CDK4/6 monomer in cell lysate. (A) Chemical structure of palbociclib-biotin synthesized here. (B) Palbociclib-biotin precipitated purified recombinant CDK4-CycD1 dimer, but not p27-CDK4-CycD1 trimer. (C and D) MCF7 or MDA-MB-231 cell lysates were used in a precipitation reaction with palbociclib-biotin. A CycD1 antibody was also used to precipitate CDK4/6 from cell lysates to compare the total pool of CycD1 complexes to palbociclib-bound complexes.

efficiency of ATP processing. Our structural data demonstrate that this effect is specific to CDK4 (versus CDK2) and is primarily induced by Tyr⁷⁴ phosphorylation in p27. Although p21 also acts as an assembly factor for active CDK4-CycD1 (31), p21 contains a phenylalanine at a position equivalent to that of Tyr⁷⁴ (Figs. 1C and 4C), and p21 trimer complexes cannot be activated by D2 displacement as in p27. As a result, phosphorylated p21 trimer complexes have relatively less activity than phosphorylated p27 complexes.

The catalytic efficiency of the phosp27-CDK4-CycD1 trimer was greater than the catalytic efficiency of the dimer for most of the substrates we investigated (Fig. 3G). The requirement of p27 for efficient phosphorylation may explain why fewer cell cycle substrates have been identified for CDK4/6 than for (e.g.) CDK2, and why it has been thought that Rb is a preferred CDK4/6 substrate (12, 14, 15, 17, 18). Additional CDK4/6 substrates have been identified more recently (13, 60, 61), and it will be important to explore the role of p27 in their regulation by CDK4/6 phosphorylation. Our observation that the CDK4-CycD1 dimer catalytic efficiency was dependent on the last ~50 amino acids of Rb is consistent with reports of a CDK4 docking site in the Rb C-terminal domain (17, 18). Docking allows Rb to more tightly associate with the dimer and be phosphorylated even when ATP affinity is so low. In contrast, we observed that the trimer activity did not depend on a substrate docking sequence, which suggests that a role of p27 activation may be to broaden CDK4 substrate specificity. Our data also implicate a

competitive association between Rb and p27 for CDK4-CycD and indicate that p27 still functions to temper Rb-directed CDK4 activity even in the context of active trimer complexes.

CDK4/6-specific inhibitors were developed through optimizing potency and selectivity toward CDK4-CycD dimer, despite evidence that the physiological complex contains CIP or KIP family inhibitors such as p27 (25, 31–37, 46). Our data demonstrate that active recombinant CDK4 complexes containing p27 are not sensitive to these inhibitors and that palbociclib does not associate with active endogenous p27 trimer complexes. Moreover, we did not detect inhibition of CDK4 activity in drug-arrested breast cancer cells, and we did not robustly detect CDK4-CycD1 dimer complexes in cell lysates. Still, we recognize that dimer inhibition may occur in some contexts and that some small population of dimer may be targeted even in the breast cancer cells tested here. In light of the differences between dimer and trimer response to CDK4/6 palbociclib-type inhibitors *in vitro*, it will be important to explore how the balance of dimer and trimer complexes may influence sensitivity to drugs in cells or *in vivo*.

Our data implicate an additional mechanism toward cell cycle arrest in which palbociclib associates with CDK4 monomer instead of binding and inhibiting CDK4 dimer or trimer activity. One outcome is that the population of CDK4 monomer increases, and it is possible that this CDK4-palbociclib complex is recognized and induces a response, such as the decrease in CycA abundance (52) or an increase

of p21 in CDK2-CycE complexes. Both of these outcomes correlate with our observed lower CDK2 activity. A reported CDK4/6 degrader drug is not as effective as palbociclib in inhibiting Rb phosphorylation (62), and, a recent study finds that cells expressing a catalytically inactive CDK4 are arrested when treated with palbociclib (63). These results perhaps reflect the fact that palbociclib-induced cell cycle arrest does not require inhibition of CDK4 activity but alternatively results from indirect inhibition of CDK2. This indirect role of CDK4 in modulating CDK2 activity is consistent with observations that CDK4-CycD expression can activate CDK2 through sequestration of p27 and without the requirement of CDK4 catalytic activity (25, 58).

We note the similarity of this mechanism of palbociclib inhibition to that of the p16-INK family of CDK4 protein inhibitors, which sequester inactive monomer CDK4 (Fig. 8E). The ability of palbociclib to mimic p16 may in part explain why cells lacking p16 are often most sensitive to palbociclib-type inhibitors (52, 53, 64, 65). We suggest that, similar to the downstream effects of p16, accumulation of CDK4 monomer upon palbociclib treatment indirectly lowers CDK2 activity, potentially through the shuttling of p21 to CDK2 complexes (25, 36, 66, 67). In cycling cells, this drug-induced reorganization of CDK complexes may occur when p21 is re-synthesized after its degradation before S phase and/or when CycD1 is low in S phase and would lead to cell cycle arrest (68). The targeting of an inactive kinase, rather than inhibition of assembled kinase activity, may apply to

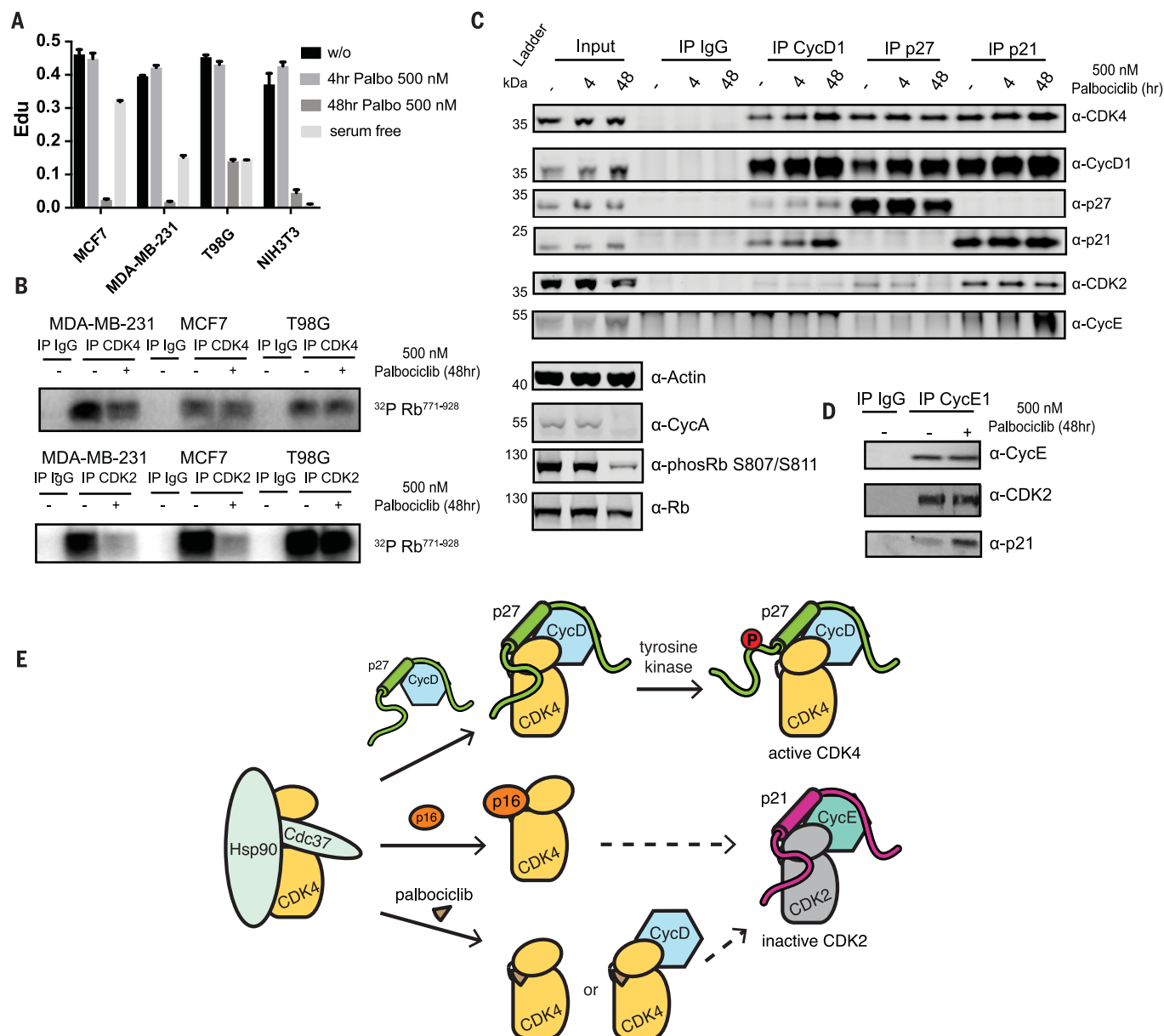


Fig. 8. Palbociclib indirectly leads to down-regulation of CDK2 through p21.

(A) Cell cycle profiling of the cell lines used in this study upon treatment with 500 nM palbociclib for 4 or 48 hours or without drug treatment. Cells were assayed for EdU incorporation as a marker of S phase at the different time points after drug treatment. The fraction of cells showing EdU staining is reported. EdU incorporation in cells that were serum-starved (serum free) for 48 hours is also shown for comparison. (B) Cells were treated with palbociclib as indicated for 48 hours, and lysates were immunoprecipitated with CDK4 antiserum or a CDK2 antibody.

Activity of the immunoprecipitated complexes was assayed as in Fig. 5, D and E.

(C) CycD1-, p27-, and p21-associated complexes were immunoprecipitated from MCF7 cells treated with palbociclib for the indicated time, and proteins were detected by Western blot. (D) CycE1 was immunoprecipitated from MCF7 cells treated with palbociclib for 48 hours. (E) Model for trimer assembly and CDK4 inhibition by palbociclib. Like p16 family CDK4 inhibitors, palbociclib binds monomeric CDK4 and indirectly leads to inactive CDK2 complexes. Although not observed in the breast cancer cells studied here, there may be some contexts in which palbociclib also targets CDK4 dimers.

other kinase therapeutic targets as well (69), and we note that Gleevec (imatinib) similarly inhibits an inactive conformation of the Abl kinase (70).

Materials and methods

Recombinant protein expression

Full-length human CDK4, CDK6, cycD1, p21, p27, and Brk were expressed and purified from Sf9 cells. CDK4, CDK6, p21, p27, and Brk were

expressed as GST fusion proteins. CycD1 was coexpressed with other components untagged. Lysates were first purified by GS4B affinity chromatography. Proteins were then eluted from the resin and subject to Source 15Q (GE Healthcare) anion exchange chromatography. The elution fraction was then subjected to TEV protease cleavage overnight in 25 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT, 0.5 mM EDTA. The protein was then passed over GS4B

affinity resin again to remove free GST, concentrated, and stored in a buffer containing 20 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT, and 20% glycerol. CDK2-CycA was expressed in *E. coli* and purified as described (71).

Phosphorylation of p21 and p27

Recombinant p21 and p27 were expressed as a GST fusion in *E. coli* and purified as described above. Purified proteins were treated with 10%

GST-Brk (by mass) in a buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 1 mM DTT, 10 mM MgCl₂, and 1 mM ATP and incubated at 4°C for 24 hours. The phosphorylated p21 or p27 was purified by passing through GS4B affinity resin and eluted from a Superdex 75 column (GE Healthcare) in a buffer containing 25 mM Tris pH 8.0, 100 mM NaCl, and 1 mM DTT. The extent of phosphorylation was confirmed using electrospray mass spectrometry on a SCIEX X500 QTOF spectrometer.

Crystallization, data collection, structure determination, and model refinement

For crystallization, human CDK4 residues 1-303 (Δ G45-G47, G43E, G44E), truncated cycD1 (16-267), p27 kinase-inhibitory domain (p27^{KID}; 25-93), and the p21 kinase-inhibitory domain (p21^{KID}; 14-81) were prepared as described above. The CDK4 contains mutations in a glycine-rich sequence that mimic CDK6 and were required for crystallization (10, 17). The CDK4 complexes were prepared for crystallization by elution from a Superdex 200 column (GE Healthcare) in a buffer containing 10 mM Tris pH 8.0, 100 mM NaCl, and 1 mM DTT. The p27-CDK4-CycD1, p27(3E)-CDK4-CycD1, and p21-CDK4-CycD1 complexes were crystallized from a 10 mg/ml solution by microbatch method under Al's oil at 22°C. Rods formed after 3 days in 100 mM Tris, 10% PEG 8000, and 200 mM MgCl₂, pH 7.0. Crystals were cryo-protected in reservoir solution supplemented with 25% glycerol and cryo-cooled in liquid nitrogen.

The phospho27-CDK4-CycD1 complex was prepared for crystallization by mixing 3:1 molar ratio of phosphorylated p27 and CDK4-CycD1 dimer followed by elution from a Superdex 200 column in a buffer containing 10 mM Tris, 100 mM NaCl, and 1 mM DTT, pH 8.0. The complex was crystallized from a 10 mg/ml solution by sitting drop vapor diffusion method at 22°C. Rods formed after 3 days in 100 mM Tris, 17% PEG 3350, 100 mM CaCl₂, and 10 mM MgCl₂, pH 7.0. Crystals were cryo-cooled in the reservoir solution supplemented with 25% glycerol.

Data were collected at the Advanced Light Source, Lawrence Berkeley National Laboratory at beamline 8.3.1. Diffraction spots were integrated using MOSFLM (72), and data were merged and scaled using Scala in the CCP4 software package (73, 74). The structure of the p27-CDK4-CycD1 trimer was first determined by molecular replacement using Phaser and the CDK4-CycD1 dimer as a search model (PDB ID 2W9F) (75). A model for p27 was then built into the extra electron density with Coot (76), and the model was refined with Phenix (77). Models for the CDK4-CycD1 trimer complexes containing phospho27, p27(3E), or p21 were then built and refined starting from the unphosphorylated p27 structure.

Western blots and antibodies

Details on the antibodies used and the procedures for Western blotting can be found in the supplementary materials.

Kinase assays

Recombinant CDK4 (0.5 μ M), CDK2 (0.5 μ M), or CDK6 (1 μ M) complexes were mixed with substrate (30 μ M) in a kinase buffer containing 25 mM Tris pH 8.0, 200 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 250 μ M ATP (or as indicated), and 100 μ Ci of [³²P] γ -ATP. Substrate was diluted into the reaction buffer, and the reaction was initiated by addition of ATP. Reactions were quenched after 30 min by addition of SDS-PAGE loading buffer. Independent time-course experiments confirmed that phosphate addition is still linear with time beyond 45 min using our experimental conditions. SDS-PAGE gels were imaged with a Typhoon scanner and bands quantified using the ImageJ software package. For each assay, three replicates were performed. We note that our measured K_i values for the inhibitors on K4D1/- (Fig. 5B and fig. S8A) are consistent with our ITC-measured K_d value for palbociclib (Fig. 5C) but are higher than IC_{50} values previously reported (4, 7, 46). We believe the difference is due to the higher ATP concentration in our assay, which we chose to be closer to the ATP K_M for K4D1/-.

For the activity assays using immunoprecipitation of endogenous CDK4, CDK2, or p27 complexes, 1 mg of whole-cell extract was immunoprecipitated with 2 μ g of anti-p27 antibody (SCBT DCS-72), 2 μ g of anti-CDK2 antibody (SCBT D-12), or 20 μ l of CDK4 antiserum to C-terminal peptide (gift of C. Sherr, St. Jude Children's Research Hospital) (48) and 25 μ l of protein A/G PLUS beads (SCBT) for 2 hours at 4°C. The beads were then washed 3 times with 50 mM Tris pH 7, 150 mM NaCl, 1 mM DTT, and 5% glycerol. The final wash was performed in kinase buffer and the reaction was performed with the recombinant substrate while the enzyme complexes were on the beads.

For the activity assays using immunoprecipitated CycD1 complexes, MCF7 cells were lysed with lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 10 mM DTT, 10% glycerol, 1 \times Halt protease inhibitor (Thermo Fisher), and 1 mM PMSF. 5 μ g of anti-CycD1 (Invitrogen; MA5-12707) was incubated with 300 μ g of the lysate overnight at 4°C. Normal mouse IgG1 (Cell Signaling Technology, 5415) was used as a control. Protein G-agarose beads were added to each of the IP samples and incubated up to 4 hours at 4°C. Protein immunocomplexes were washed 3 times with the lysis buffer and 2 times with kinase reaction buffer (50 mM HEPES-KOH pH 7.5, 20 mM MgCl₂, 1 mM DTT). Kinase reactions were carried out in 100 μ l of kinase buffer in the presence of 2 mM ATP and 0.5 μ g

of recombinant Rb C-terminal domain as substrate by gently shaking at room temperature up to 1 hour. The resulting phosphorylated Rb protein was detected by Western blotting using anti-p-Rb (S780) antibody (Cell Signaling Technology, 9307L).

Activity assays with immunoprecipitated CycE1 complexes were performed similarly, but the lysis buffer contained 50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Tween-20, 1 \times Halt protease inhibitor, and 1 mM PMSF. Immunoprecipitation was performed with an anti-CycE1 antibody (Santa Cruz Biotechnology; SC-377100) as described for CycD1. The kinase reactions were performed in 100 μ l of kinase buffer (40 mM Tris-HCl pH 8.0, 20 mM MgCl₂, 0.1 mg/ml BSA, 50 μ M DTT) in the presence of 2 mM ATP and 0.5 μ g of Rb protein as substrate by gently shaking at room temperature up to 1 hour. The resulting phosphorylated Rb protein was detected by Western blotting using anti-p-Rb (S807/S811) antibody (Cell Signaling Technology, 8516S).

XO44 labeling assays

XO44 labeling was carried out as described (49) with some modifications. Live adherent cells were labeled by incubation with 2 μ M XO44 at 37°C for 30 min. Cells grown in 15-cm dish format to be analyzed by gel filtration were harvested as described below. Cells grown in 6-well plate format (seeded at 500,000 cells per well and allowed to recover for ~20 hours) to be analyzed by Western blot were then washed twice with cold PBS, perturbed and lysed in place with 80 μ l of lysis buffer containing 100 mM HEPES pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 \times cOmplete EDTA-free protease inhibitor cocktail and 1 \times PhosSTOP phosphatase inhibitor cocktail (both Roche). Lysates were clarified by centrifuging at 20,000g for 20 min, then normalized to 2.5 mg/ml protein concentration using the Pierce Rapid Gold BCA assay (Thermo). 20.75 μ l of lysate was treated with 4.25 μ l of a master mix to give final concentrations of 1% SDS, 50 μ M TAMRA-azide (in DMSO), 1 mM TCEP, 100 μ M TBTA (in 1:4 DMSO:t-butyl alcohol) and 1 mM CuSO₄. The resulting mixture was incubated at room temperature for 1 hour before being quenched with 5 μ l of 6 \times SDS loading buffer and analyzed by Western blot as described above. The TAMRA dye was used to add mass to XO44-labeled CDK4, so the XO44 modification is observable as a gel shift by using SDS-PAGE.

Gel filtration assay

Gel filtration assays on crude cell lysates were performed as described (36) with modifications. Cells were seeded on 15-cm dishes at 10 million cells per dish and allowed to recover for ~20 hours before treatment with compounds as indicated and harvesting by washing with

PBS followed by trypsinizing, pelleting and two further washes with cold PBS. Pellets of ~25 million cells were lysed in buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol, 1 mM EDTA plus cOmplete EDTA-free protease inhibitor cocktail (Roche) on ice for 20 min. Lysates were then clarified by centrifuging at 18,000g for 2 × 15 min. In each case, 500 µl (5 mg) of lysate was incubated with DNase I (NEB, 1 unit/mg lysate) in 1× DNase I reaction buffer (NEB) for 20 min on ice and briefly clarified again by centrifuging for 5 min. Samples were then loaded on a Superdex200 Increase 10/300 GL column pre-equilibrated in 50 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, 10% glycerol. The column was eluted for 1.25 column volumes at 0.35 ml/min and 0.5-ml fractions were collected starting at 5 ml. Fractions 10 to 26 were analyzed by Western blot as described above. Samples of fractions from XO44-treated cells were used in click reactions as described above. To benchmark purified protein complexes, 0.8 µg of p27-CDK4-CycD1 trimer was diluted in 500 µl of HEPES buffer for loading. Gel filtration and Western blot analysis were performed as described above.

LC-MS analysis of CDK4 covalent labeling

Recombinant purified protein complexes (1 µM) were suspended in a buffer containing 20 mM HEPES, 150 mM NaCl, and 1 mM MgCl₂, pH 7.5, and treated with XO44 (10 µM) or DMSO (1% (v/v) DMSO final). After 30 min at room temperature, reactions were stopped by the addition of 10% (v/v) formic acid to a final concentration of 2% (v/v) formic acid. The extent of covalent labeling was assessed by LC-MS (Waters Xevo G2-XS QToF, ACQUITY UPLC Protein BEH C4 Column, 300 Å, 17 µm, 2.1 mm × 50 mm). Deconvolution of multiply charged ions was performed using Waters MassLynx software (version 4.1).

Palbociclib-biotin

Details of the synthesis and characterization of palbo-biotin can be found in the supplementary materials. For palbo-biotin precipitations, 100 µM palbo-biotin was first incubated with streptactin beads in PBS for 30 min at room temperature. The beads were then washed 3× with PBS and resuspended in 1 mg of cell extract in 50 mM HEPES pH 7.0, 150 mM NaCl, 0.1% Tween-20, 1 mM DTT, 5% glycerol, 1× cOmplete EDTA-free protease inhibitor cocktail and incubated at 4°C for 2 hours. Complexes bound to streptactin beads were eluted using 2× SDS buffer and subjected to immunoblot analysis. For recombinant protein, the same protocol was followed except 2 µg of recombinant protein was added to the beads in IP buffer with 1% BSA. 10 µM excess free palbociclib was added to the extract or purified proteins where indicated.

Isothermal titration calorimetry

Equilibrium dissociation constants were obtained using a Micro Cal VP-ITC at 15°C. For drug binding experiments, 40 µM purified CDK4-CycD1, phospho27(p27^{KID}; 25-93)-CDK4-CycD1, and GST-CDK4 were dialyzed overnight in a buffer containing 25 mM Tris pH 8.0, 200 mM NaCl, and 5 mM BME. Proteins were titrated with 0.5 mM palbociclib dissolved in the same buffer. For measurement of p27 (p27^{KID}; 25-93) binding to GST-CDK4, proteins were eluted in separate runs from a Superdex 200 column equilibrated in a buffer containing 25 mM Tris pH 8.0, 200 mM NaCl, and 5 mM BME. 250 µM p27 was titrated into 40 µM GST-CDK4 in the absence and presence of 50 µM palbociclib.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S12

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p27 allosterically activates cyclin-dependent kinase 4 and antagonizes palbociclib inhibition

Keelan Z. Guiley, Jack W. Stevenson, Kevin Lou, Krister J. Barkovich, Vishnu Kumarasamy, Tilini U. Wijeratne, Katharine L. Bunch, Sarvind Tripathi, Erik S. Knudsen, Agnieszka K. Witkiewicz, Kevan M. Shokat and Seth M. Rubin

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Revised view of anticancer drug mechanism

A crystal structure of the active form of cyclin-dependent kinase 4 (CDK4) provides insight into regulation of the cell cycle and the mechanism of action of a drug used for breast cancer therapy. The protein p27 has been thought to act as a CDK inhibitor. Guiley *et al.* performed a structural analysis of active complexes of CDK4 with cyclin D1 (CycD1) and p27 (see the Perspective by Sherr). The results showed that p27 actually remodels the active site of CDK4 to allow full activation when p27 is phosphorylated on tyrosine (phosp27). Furthermore, they found that the breast cancer drug palbociclib, a CDK4 inhibitor, doesn't actually interact with active phosp27-CDK4-CycD1 trimers. Instead, it appears that the drug, which shows promise in the clinic, binds to inactive CDK4 monomers and prevents interaction with p27.

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