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The cryoelectron microscopy structure of the human CDK-activating kinase

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The human CDK-activating kinase (CAK), a complex composed of cyclin-dependent kinase (CDK) 7, cyclin H, and MAT1, is a critical regulator of transcription initiation and the cell cycle. It acts by phosphorylating the C-terminal heptapeptide repeat domain of the RNA polymerase II (Pol II) subunit RPB1, which is an important regulatory event in transcription initiation by Pol II, and it phosphorylates the regulatory T-loop of CDKs that control cell cycle progression. Here, we have determined the three-dimensional (3D) structure of the catalytic module of human CAK, revealing the structural basis of its assembly and providing insight into CAK activation in this context. The unique third component of the complex, MAT1, substantially extends the interaction interface between CDK7 and cyclin H, explaining its role as a CAK assembly factor, and it forms interactions with the CDK7 T-loop, which may contribute to enhancing CAK activity. We have also determined the structure of the CAK in complex with the covalently bound inhibitor THZ1 in order to provide insight into the binding of inhibitors at the CAK7 active site and to aid in the rational design of therapeutic compounds.

cryoelectron microscopy | cyclin-dependent kinase | transcription | cell cycle | phosphorylation

Transcription initiation by eukaryotic RNA polymerase II (Pol II) depends on the formation of a preinitiation complex (PIC) on promoter DNA, which includes general transcription factors, Pol II, and Mediator (1). Like many other cellular processes, transcription and the assembly of the PIC are regulated by phosphorylation events. During PIC assembly, Mediator interacts with the C-terminal heptapeptide repeat domain of RPB1, the largest subunit of Pol II (2), also referred to as the Pol II-CTD. Phosphorylation of this region by the cyclin-dependent kinase (CDK) 7 subunit of transcription initiation factor IHH (TFIHH) disrupts its interactions with Mediator, allowing initiating Pol II to break free from the PIC and clear the promoter (3). Pol II-CTD phosphorylation and dephosphorylation are thus key events that regulate transcription of eukaryotic protein-coding genes and have been implicated in the formation of phase-separated transcriptional subcompartments (4, 5). This process is not only important for normal transcription, but also contributes to aberrantly elevated transcription levels in cancer cells. Consequently, transcription-related CDKs have been identified as promising drug targets (6), and inhibition of CDK7 by compounds such as THZ1 or SY-1365 has been shown to selectively kill cancer cells that require elevated levels of transcription (7, 8).

In addition to its conserved role in transcription, human CDK7 phosphorylates CDKs that control the cell cycle, a function that is not conserved in yeast. The activity of cell cycle CDKs is generally controlled by association with partner cyclins (9). Cyclin binding provides the bulk of CDK stimulation (10) by inducing conformational changes in the CDK that lead to extension of a regulatory loop near the active site, known as the T-loop, and to the proper arrangement of the active site for phosphoryl transfer (11). However, in addition to cyclin binding, full activation of cell cycle CDKs requires phosphorylation of the T-loop (9, 12). In animal cells, these activating phosphorylations are carried out by CDK7 (13, 14), itself a cyclin-dependent kinase whose activity depends on cyclin H (14).

In human and other metazoan cells, regulation of transcription initiation by phosphorylation of the Pol II-CTD and phosphorylation of cell cycle CDKs thus depend on a single kinase, CDK7, which functions in a complex termed the CDK-activating kinase (CAK) (14). In addition to CDK7 and cyclin H, human CAK comprises a third subunit, MAT1 (15–18). CDK7 and cyclin H form a canonical CDK-cyclin pair, while MAT1 serves as a CAK assembly factor (15–17), increases CAK activity toward target CDKs (16, 19), and tethers the CAK to the core of TFIHH when this 10-subunit complex carries out its function in transcription initiation (19–21). When TFIHH functions in DNA repair, MAT1 is dislodged from its binding sites on the TFIHH core complex, releasing the 3-subunit CAK module (22, 23). The functional unit of the human CAK complex is thus the CDK7-cyclin H-MAT1 trimer (16, 18).

While X-ray crystal structures of CDK7 and of cyclin H have been determined (24–26), structural information on the entire three-subunit CAK, which is critical for a mechanistic understanding of the role of MAT1 in CAK assembly and the regulation of its activity, has remained elusive. Here, we present cryoelectron microscopy (cryo-EM) structures of human CAK

Significance

Control of gene expression and the cell cycle is critical for appropriate cell growth and timely cell division. Failure of the mechanisms regulating these processes can result in proliferative diseases. A molecular complex termed the CDK-activating kinase (CAK) impinges on both of these regulatory networks in human cells and is thus a possible drug target for treatment of cancer. Here, we use cryoelectron microscopy to describe the detailed molecular structure of the human CAK, revealing its architecture and the interactions between its regulatory elements. Additionally, we have obtained the structure of the CAK in complex with a small-molecule inhibitor.

Author contributions: B.J.G. and E.N. designed research; B.J.G., J.M.P.-B., K.L., and A.T.I. performed research; A.T.I. and D.B.T. contributed new reagents/analytic tools; B.J.G. analyzed data; B.J.G. and E.N. wrote the paper; and E.N. supervised research.

The authors declare no competing interest.

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bound to either ATPγS or the inhibitor THZ1, thereby revealing the architecture of the human CAK and the structural basis for its inhibition by covalent inhibitors. Our structure of the CAK also completes the high-resolution structural analysis of human TFIIH and shows how MAT1 interlinks CDK7, XPB, and XPD, the three major functional centers in TFIIH.

Results and Discussion

Structure Determination of the Human CAK. To determine the structure of the human CAK, we recombinantly coexpressed CDK7, cyclin H, and MAT1 in insect cells, purified the resulting complex by affinity and size-exclusion chromatography, and collected cryo-EM data of frozen-hydrated CAK bound to the nucleotide analog ATPγS (SI Appendix, Fig. S1) using a 200-kV instrument (27). Three-dimensional (3D) reconstruction resulted in a structure at 2.8-Å resolution (Fig. 1A and B and SI Appendix, Fig. S2A–C), which allowed unambiguous docking of the high-resolution crystal structures of CDK7 and cyclin H (24–26). We rebuilt the docked models according to the cryo-EM map, which showed clear density for amino acid side chains and the bound nucleotide (Fig. 1C and SI Appendix, Fig. S2D and E). After assignment and rebuilding of the CDK7-cyclin H dimer, the remaining density allowed building of residues 244 to 308 of MAT1, consistent with biochemical data showing that a C-terminal fragment of MAT1 (residues 229 to 309) is sufficient for binding to CDK7-cyclin H and to enhance kinase activity of CDK7 (19). The reconstructed volume thus corresponds to a mass of ~84 kDa, which is among the smallest asymmetric particles for which better than 3-Å resolution have been obtained using cryo-EM.

Complete Structural Description of Human TFIIH. The CAK subcomplex is an important functional unit of TFIIH and critical for its activity as a general transcription factor. Previous cryo-EM studies of human and yeast TFIIH have revealed the structure of the TFIIH core complex and residues 1 to 210 of MAT1 (28–32), but were lacking the remainder of the CAK module due to disorder (28, 29) or low local resolution (30, 31). Our structure of human CAK completes the structure of human TFIIH (Fig. 1D–F). The combined structures show how MAT1 simultaneously interacts with both the TFIIH core complex and the CDK7-cyclin H module. Distinct and independently folding structural modules, the RING domain and helical regions near the N terminus, and the CAK anchor near the C terminus of MAT1, engage with the TFIIH core complex and the CDK-cyclin module, respectively. These regions are connected by a flexible tether of ~35 residues that are not resolved in any of the structures reported (Fig. 1D–F). The flexible nature of the tether and the lack of observed interactions of the CDK7-cyclin H module with other regions of MAT1 in the full-length CAK complex (Fig. L4) explain why the CDK7-cyclin H module and parts of MAT1 bound to it were not resolved in previous structures of free TFIIH (28, 29). This structural flexibility may support the ability of the CAK module to occupy distinct positions in the Pol II-PIC depending on the presence of mediator (30, 31, 33). Our findings also underscore the role of MAT1 as
a key regulator of TFIIF that physically links the DNA translocase XPD, the helicase XPD, and the kinase CDK7 (Fig. 1E), the three ATP-consuming, functional centers of the complex. MAT1 forms interactions with regulatory elements on both CDK7 (see below) and XPD (28, 32, 34), highlighting its central regulatory role.

Role of MAT1 in CAK Architecture and Stability. Overall, the arrangement of CDK7 and cyclin H resembles that of other CDK-cyclin complexes (reviewed in ref. 35). Interactions are mostly confined to the N-terminal domains of both CDK7 and cyclin H (Fig. 2A and SI Appendix, Fig. S3A), burying approximately 1,100 Å² of accessible surface area. Previous analysis revealed that both the extent of the interaction surface and the geometry of CDK-cyclin complexes show marked variability (36). We find that the C-terminal lobe of CDK7 and the C-terminal cyclin fold of cyclin H are rotated away from each other compared to the structure of CDK2-cyclin A (11), resulting in a geometry that is intermediate between that of CDK2-cyclin A and that described for the transcriptional CDK9-cyclin T complex (P-TEFb) (37) (Fig. 2B and SI Appendix, Fig. S3 B and C). In the CAK, the C-terminal CAK-anchor region of MAT1 (residues 255 to 309) fills the space vacated by this rotation (Fig. 2A and B). Most of this region of MAT1 is sandwiched between the C-terminal lobe of CDK7 and the C-terminal cyclin fold of cyclin H, thereby bridging the two subunits (Fig. 2C and D). Indeed, MAT1 buries more surface area with CDK7 and cyclin H (>1,200 Å² each) than CDK7 and cyclin H bury with each other. This rationalizes biochemical data that established MAT1 as a CAK assembly factor that enhances the stability of the CDK7-cyclin H interaction (15, 16), but requires revision of a previously proposed architecture of the CAK based on low-resolution cryo-EM data, which placed the MAT1 components such that the space between CDK7 and cyclin H was left unoccupied (33).

The most prominent and well-ordered part of the MAT1 density is its C-terminal α-helix (Fig. 1C, residues 288 to 300), which spans the distance between the C-terminal lobe of CDK7 and the C-terminal cyclin fold of cyclin H and interacts with both proteins. Comparison of the CAK structure to that of CDK2 with cyclins A, B, and E (11, 12, 38, 39) shows that the C-terminal α-helix of MAT1 occupies a similar position as an extended α-helix at the N terminus of the cyclin folds in cyclin A.
B, and E that contributes to the enlarged interaction surface in CDK2-cyclin complexes (SI Appendix, Fig. S3D). Both cyclin H and cyclin T within P-TEFb (37), which is more closely related to cyclin H than are the cell cycle cyclins (40), lack this extended α-helix at the N terminus of the cyclin folds, leaving room for other interactions at this site. However, it is unclear whether any associated factors bind at this site in P-TEFb or other transcriptional CDKs, and thus the cooption of this interaction site by an associated protein may be unique to CAK.

The yeast equivalent of the human CDK7-cyclin H-MAT1 complex is called TFIIK and is formed by the proteins Kin28, Ccl1, and Tfb3 (41, 42). Analysis of our structure shows that the MAT1 residues that form contacts to cyclin H and CDK7 are mostly conserved in Tfb3 (SI Appendix, Figs. S4 and S5), suggesting that the molecular contacts between MAT1/Tfb3 and the CDK-cyclin pair are likely equivalent, and confirming a conserved architecture between CAK and TFIIK, and likely across all eukaryotes.

In summary, our data establish that MAT1 forms extensive, conserved interfaces with both CDK7 and cyclin H, explaining its role as a CAK assembly factor.

Conformation and Interactions of the CDK7 T-Loop in the Human CAK.

In our structure, which has clear density for ATPγS in the CDK7 active site (SI Appendix, Fig. S2D), the regulatory T-loop of CDK7 is found in an extended, active conformation, pointing away from the nucleotide-binding site and toward cyclin H (Fig. 3A and B and SI Appendix, Fig. S6 A–D). This conformation contrasts with the structure of isolated CDK7, in which the T-loop occludes the substrate-binding site (Fig. 3B and SI Appendix, Fig. S6C). Furthermore, the presence of cyclin H pushes the C-helix (CDK7 residues 57 to 68) into position to promote assembly of the CDK7 active site (Fig. 3B), analogous to the conformational change of the C-helix described for CDK2-cyclin A complex (12). Accordingly, our purified CAK was able to phosphorylate a synthetic (YSPTSPS)KKKK peptide, mimicking the RPB1 CTD, on Ser5 of the YSPTSPS heptapeptide repeat (SI Appendix, Fig. S6 E and F). Notably, the tip of the CDK7 T-loop closely approaches the C-terminal α-helix of MAT1 (Fig. 2D), suggesting that interactions with MAT1 promote the extended conformation of the CDK7 T-loop within the CAK complex (Fig. 3A and C), likely contributing to the reported CAK activation by MAT1 (19).

Like its CDK substrates, CDK7 itself is the target of regulatory phosphorylation. Specifically, its T-loop harbors residues S164 and T170 (Fig. 3C), both of which are phosphorylation sites (16, 43, 44). In our cryo-EM maps, S164 and to a lesser degree T170 showed enlarged densities that may arise from the presence of substoichiometric levels of the phosphorylated species (Fig. 3D). These map features are consistent with mass spectrometric analysis, indicating the presence of some phosphorylated molecules in our CAK samples (SI Appendix, Fig. S7). The presence of both phosphorylated and unphosphorylated CDK7 in otherwise homogeneously stable CAK is in agreement with previous reports of MAT1-mediated assembly of CAK and with data showing that full, active complex can exist in the absence of T-loop phosphorylation (16).

![Fig. 3. Extended T-loop of CDK7. (A) The T-loop in the CAK-ATPγS complex is extended and points toward MAT1 and cyclin H. (B) In the crystal structure of free, inactive CDK7 (light gray), the T-loop (cyan) is folded across the active site and the C-helix is dislodged from the active site (PDB ID 1UA2) (26). Our structure is superposed (CDK7, gray; T-loop, teal; C-helix, dark red). Large conformational changes between the inactive and active states are indicated with arrows. (C) Positively charged residues near the CDK7 phosphorylation sites (S164, T170; Oγ shown as red spheres) are shown as sticks. (D) Structure of the CAK near S164; cryo-EM density shown in gray (combined surface and mesh) suggests that S164 is partially phosphorylated in our preparation of CAK. Positively charged residues surrounding the phosphate group are labeled.](https://www.pnas.org/cgi/doi/10.1073/pnas.2009627117)
Interestingly, S164 within the CDK7 T-loop is found in proximity to three arginine side chains (Fig. 3 C and D), each one from all three CAK subunits (CDK7 R167, MAT1 R295, and cyclin H R165). This patch of arginines is reminiscent of a positively charged pocket that accommodates the phosphate group of phosphorylated T160 in CDK2, an arrangement that was first visualized in the structure of phosphorylated CDK2 in complex with cyclin A (12), and which is also found in CDK7 (CDK7 residues R61, R136, and K160; Fig. 3C). The human CAK thus harbors two such positively charged pockets, the first of them formed by CDK7 alone and conserved across CDKs, the second specific to the CAK and formed by all three subunits. Phosphate binding in the latter pocket might influence complex assembly and/or the conformation of the T-loop. Indeed, we found that a phosphate group modeled into our density closely approaches cyclin H R165, suggesting formation of an intermolecular salt bridge, and the phosphate may form additional interactions with CDK7 R167 and N166 (Fig. 3D). These potential additional intermolecular interactions following phosphorylation of S164 might promote CAK stability or an extended conformation of the T-loop, both of which would likely contribute to regulation of CAK activity toward certain substrates. This idea is consistent with previous findings showing that T-loop phosphorylation can stabilize CDK7-cyclin H association in the absence of MAT1, and that an S164A mutant shows lower kinase activity toward CDK2 (16). Additionally, an S164A T170A double mutant shows reduced CAK assembly in Drosophila (45) and an equivalent mutant in Xenopus laevis CDK7 (S170A) shows impaired activation in response to cyclin H binding (43). Our structural results, which suggest formation of intermolecular contacts involving the phosphorylated T-loop, can now rationalize these biochemical data. However, our structure does not provide a clear mechanism for the inhibition of CTD-kinase activity by S164 phosphorylation (46), and it cannot easily explain why T170 phosphorylation was found to contribute more strongly to CAK assembly than S164 phosphorylation (16, 43), given that T170 is far removed from any structural elements of cyclin H or MAT1 (Fig. 3C). This interplay between T-loop phosphorylation and complex stability was also observed in the yeast Kin28-Ccl1-Tfb3 complex (42), even though yeast Kin28 lacks a secondary phosphorylation site equivalent to human S164. It is possible that T170 phosphorylation has an indirect effect by affecting CDK7 conformation, or that it stabilizes an extended T-loop conformation that enables formation of interactions involving the tip of the T-loop.

Taken together, our data suggest that T-loop contacts with MAT1 are a specific feature of the CAK, contribute to an extended state of the T-loop, and promote an active CDK7 conformation upon formation of the trimeric CAK. T-loop phosphorylation may enable further regulation of the activity of the CAK toward specific substrates or depending on the cell cycle stage (45, 46).

**Structural Mechanism of Irreversible Inhibition of CDK7 by THZ1.** Due to its activity in transcription and cell cycle regulation, the human CAK plays an important role in regulating cell growth and cell division. Consequently, inhibition of the CAK has been found to be a promising strategy for treatment of proliferative diseases (6, 7). To obtain insight into the covalent binding of the cysteine-reactive anticancer compound THZ1 (7) to CDK7 within the CAK, we have determined the 3.3-Å resolution structure of THZ1 bound to a CDK7-cyclin H-MAT1Δ219 complex (CAK-MAT1Δ219), in which the N-terminal region of MAT1, including its RING domain, has been removed (Fig. 4A and SI Appendix, Figs. S1A and S8). Consistent with mass spectrometric analysis that shows essentially complete occupancy of our complex with THZ1 (SI Appendix, Fig. S7), we observe well-defined density for the small-molecule inhibitor in the active site of CDK7 (Fig. 4B). THZ1 occupies the mostly hydrophobic binding pocket that otherwise accommodates the base and ribose of bound ATP (Fig. 4 C–E). Less defined density extends toward the comparably poorly ordered C-terminal region of CDK7, which is only visible until residue C312. In inhibitor-bound CDK7, the nucleotide-binding pocket is slightly contracted at the site where the phosphate groups would be located in the nucleotide-bound state (Fig. 4F), likely because there are no corresponding groups in the inhibitor occupying this position.

Our observations are in overall agreement with the proposed mode of binding of THZ1 based on computational modeling (7). Our density is best defined for the aromatic groups of THZ1 that are buried inside CDK7 and suggests that the THZ1 indole ring is rotated such that it points toward the open side of the nucleotide-binding pocket, rather than more deeply into it (Fig. 4 B and C). This aspect of the conformation of THZ1 more closely resembles a recent computational model for the binding of SY-1365 to CDK7 (8) and the structure of the related compound THZ531 bound to CDK12-cyclin K (47), as compared to the originally proposed model for binding of THZ1 (7).

In addition to CDK7, THZ1 can irreversibly inhibit CDK12 and CDK13 because of the high sequence conservation of their active site pockets and because similarly to CDK7, CDK12 and CDK13 also have cysteine residues located in proximity to the active site that can be targeted for covalent modification (7, 47). The structure of CDK12-cyclin K bound to the related cysteine-reactive compound THZ531 (47) shows that the aromatic ring systems of THZ1 and THZ531 inserted into the kinase active sites exhibit very similar conformations, in agreement with the sequence similarity of the binding pockets in CDK7 and CDK12 (SI Appendix, Fig. S9 A and B). In contrast, the arms of the inhibitors spanning the distance to the covalently modified cysteines assume dramatically different conformations, both between CAK-THZ1 and CDK12-cyclin K-THZ531 as well as between the two copies of the molecule present in the asymmetric unit of the latter structure (SI Appendix, Fig. S9C).

In summary, our structure is in overall agreement with a previous computational model for the interaction of THZ1 with CDK7 at the ATP-binding pocket and, together with structural data on the related CDK12-cyclin K-THZ531 complex, informs on the interactions of different regions of the inhibitor with its target. Additionally, our structure now refines our understanding of the conformation of the inhibitor inside the CDK7 nucleotide-binding pocket.

**Conclusion**

The cryo-EM structure of the 84-kDa CDK-cyclin module of the human CAK reveals the molecular architecture of this trimeric CDK assembly, how the unique third subunit MAT1 contributes to the stability of the complex, and how molecular interactions within the CAK promote the extended, activated conformation of the T-loop of CDK7. We also determined the structure of the small-molecule inhibitor THZ1 at the active site of CAK. Our study shows that cryo-EM is suitable for structure determination of a small, asymmetric complex at better than 3-Å resolution, opening the way for structure-guided design and improvement of therapeutic compounds that inhibit the activity of the human CAK and other important targets of similar size.

**Methods**

**Cloning and Expression of CAK and CAK-MAT1Δ219.** Codon-optimized genes for MAT1, cyclin H, and CDK7 (sequences were synthesized by GenScript) were amplified by PCR using primers with suitable overhangs and cloned into 438-series vectors harboring expression cassettes with baculoviral promoters and terminators (48) using the in-fusion variant (49) of SLIC cloning (50). The expression cassettes encoding His6-tagged MAT1, Strep-tagged CDK7, and tag-free cyclin H (SI Appendix, Fig. S1A) were then combined into expression constructs containing MAT1-cyclin H-CDK7 using Pmel (Msd) and Swal (SmiI) restriction digestis (FastDigest enzymes, Thermo Fisher Scientific) and subsequent in-fusion reactions. The construct for expression of

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MAT1Δ219-cyclin H-CDK7 (CAK-MAT1Δ219) was assembled using the same approach but using cloning primers that remove the N-terminal 219 residues of MAT1 and allow creation of an N-terminal MBP-His6 fusion construct. A construct harboring a Pol II-CTD peptide mimic fused to the CDK7 N terminus was constructed by linearization of the plasmid encoding CDK7 by PCR, followed by insertion of a gene block (synthesized by IDT DNA) encoding the peptide sequence and a linker by an in-fusion reaction. The peptide could not be visualized in any of the subsequent cryo-EM maps (see below).

The vectors encoding the expression constructs were transformed into EMBacY cells (51) for preparation of bacmids by standard methods. Bacmid DNA was prepared by isopropanol precipitation and transfected into Sf9 (Spodoptera frugiperda) insect cells using FuGENE HD transfection reagent (Promega). After two rounds of virus amplification in Sf9 cells, 1-L cultures of High5 (Trichoplusia ni) insect cells were infected with 10 mL supernatant from the previous virus amplification cultures. The cells were harvested by centrifugation after 72 h of incubation and frozen in liquid N2 for later use.

**Purification of CAK.** CAK complexes were purified using Nickel affinity, Strep-Tactin affinity, and size exclusion chromatography. Cells were resuspended into lysis buffer (250 mM KCl, 40 mM Hepes-KOH pH 7.9, 5 mM MgCl2, 5 mM β-mercaptoethanol, 10 mM imidazole, and 10% [vol/vol] glycerol supplemented with protease inhibitors and DNase I) and homogenized by sonication. The lysate was cleared using a JA-20 rotor (18,000 rpm for 30 min at 4 °C) and incubated with Ni-NTA superflow resin (Qiagen) for 30 min. The beads were washed in purification buffer (250 mM KCl, 25 mM Hepes-KOH pH 7.9, 5 mM MgCl2, 5 mM β-mercaptoethanol, and 10% [vol/vol] glycerol)
supplemented with 25 mM imidazole and eluted in purification buffer with 300 mM imidazole. The eluate was incubated with Strep-Tactin superflow plus resin (Qiagen) for 30 min, washed with 10 bed volumes of purification buffer, and eluted with purification buffer supplemented with 10 mM desthiobiotin (Sigma-Aldrich). The eluted fractions were concentrated by ultrafiltration using 30-kDa molecular weight cutoff Centricon centrifugal filter units (EMD Millipore) and applied onto a Superdex 200 10/300 GL column (GE Healthcare) for size exclusion chromatography (SI Appendix, Fig. S18). Purified fractions (SI Appendix, Fig. S1C) were pooled, concentrated to 2 mg/mL, and frozen in liquid N2 for storage at −80 °C.

Purification of CAK-MAT1Δ219 and THZ1 Complex Formation. For studies involving THZ1 binding to CAK, the CAK-MAT1Δ219 construct was used because this THZ1 induced precipitation of full-length CAK at various stages during the preparation of the complex, possibly because the zinc-coordinating cysteines in the MAT1 RING domain can engage in unwanted reactions with THZ1. CAK-MAT1Δ219 was purified as described above, with modifications to the protocol to enable binding of THZ1. Specifically, the MBP-His tag on MAT1 and the Streptag on CDK7 were cleaved using TEV (tobacco etch virus) protease (2 h at room temperature). The resulting protein was incubated with nickel beads to remove the cleaved MBP-tag and TEV protease and subsequently subjected to gel filtration to remove β-mercaptoethanol. This step was followed by reactions of the thiol-reactive acrylamide group in THZ1 with the thiol on β-mercaptoethanol. After formation of the CAK-MAT1Δ219-THZ1 complex by incubation of 0.2 mg/mL CAK-MAT1Δ219 (approximately 2 μM) with 5 μM THZ1 (EMD Millipore) for 1.5 h at room temperature, the complex was concentrated and subjected to a second gel filtration step, again without β-mercaptoethanol, to remove excess THZ1 and residual DMSO (dimethyl sulfoxide), in which THZ1 had been solubilized. The complex was then concentrated, flash frozen, and stored at −80 °C.

Cryo-EM Specimen Preparation. Cryo-EM specimens were prepared on Talos Arctica cryo-EM operated at 200-kV acceleration voltage according to a conventional defocus-based cryo-EM approach (27). Electron micrographs were taken using a Tecnai T12 (FEI) at 120 kV (amorphous carbon support grid). A total of 100 micrographs were selected using fast subsets in RELION 3.1 (53) and the resulting particles were divided using the Laplacian-of-Gaussian (LoG) algorithm in RELION 3.1 (53) and converted to a map using University of California San Francisco (UCSF) Chimera (56). The cryo-EM map refined from these LoG-picked particles subsequently served as the autopicking template and initial reference for processing of all other datasets.

From a typical micrograph with high CAK concentration, 1,000 to 1,500 particles could be picked. To speed up calculations, given the computational cost of processing several million particle picks per dataset, particles were initially extracted at 2.744 Å/pixel in 64-pixel boxes for initial 2D and 3D classification. Initial 2D classifications were performed using fast subsets in RELION 3.1 (53) and the resulting classes were generally selected, excluding mostly false positives or ice contamination. The subsequent low-resolution refinement reached near-Nyquist frequency resolution, and 3D classification without alignment (which applies to all 3D classifications in this work) from this refinement allowed selection of a pool of high-quality particles that immediately refined to better than 4 Å resolution upon reextraction of the particles at 1.28 Å/pixel (SI Appendix, Figs. S2A). These particles were then either directly subjected to Bayesian polishing (57) in RELION 3.1 (dataset 2) or subclassified and polished (dataset 1). In subclassifications, class selection was guided mostly by inspection of the slices of the 3D volumes, and classes with the crispest features were retained. During polishing, the box size was expanded from 144 pixels to 216 pixels to account for signal delocalization by the CTF (58). In our case, the resolution gain from this procedure was minimal, possibly because the high-resolution signal is contributed mostly by very low defocus particles, which are less affected by signal delocalization. However, more particles were included in equivalent 3D classification samples than when using smaller boxes, which appeared to benefit the map quality, and thus the larger box sizes were retained. After a 2D classification to remove particles impacted by detector defects and other artifacts revealed by polishing and box size expansion, these datasets refined to resolution of 3.1 to 3.2 Å. One more round of 3D classification and optimized CTF refinement (53, 59) yielded subsets of 67,890 and 101,016 particles, which were used for final automated refinement (Figs. S1A, S8). In the final refinement step, the map was further refined to 2.8 Å resolution, which was chosen to be 1.372 Å/pixel, and the corresponding enlarged box size after particle polishing was 192 pixels. The resolution of the map immediately after polishing (from 310,052 particles) was estimated at 3.2 Å, which is nominally better than the 3.3 Å estimated for the final map from 31,198 particles, obtained after two more 3D classification steps (SI Appendix, Fig. S8A). However, the latter map exhibited far fewer side chain density, better overall connectivity, and a lower B-factor (approximately −60 Å² vs. −90 Å² as automatically determined by the RELION postprocessing function) and was therefore used for interpretation, model building, and refinement.

Model Building, Refinement, and Validation. Before model building, the 3D volumes obtained from cryo-EM map refinement were reboxed to 150 pixels (CAK reconstruction) or 134 pixels (CAK-THZ1) without changing the pixel size. The atomic model of the CAK complex was assembled by fitting the high-resolution structures of CDK7 and cyclin H (24–26) into the cryo-EM map using UCSF Chimera (56), followed by rebuilding in COOT (63). Both crystal structures showed a good initial fit to the cryo-EM map and needed only minor rebuilding, with the exception of the CDK7 T-loop, which occupies the inactive conformation in the crystal structure, and CDK7 residues 104 to 111, which were found to be register shifted by one residue. After rebuilding according to the density, the stretch of remaining unassigned density was identified and assigned to MAT1 based on agreement of the density with secondary structure prediction and the pattern of large side chains near the C terminus of MAT1.
The model was then iteratively rebuilt in COOT and refined using PHENIX ELBOW (66), and the CAK-THZ1 structure was initially refined using the higher-resolution CAK-ATP5 structure to provide reference restraints, before five final macrocycles of real-space refinement were carried out without reference restraints.

Activity assay and Western blotting. Activity assays were performed in 25 mM Hepes-KOH pH 7.7, 150 mM KCl, 5 mM MgCl2, 5 mM β-mercaptoethanol, 5% glycerol. The 100-μL reactions containing 500 nM CAK, 500 μM (Y567PS)KΔKX-biotin substrate peptide (synthesized by Genescript), and 2 mM ATP were incubated at 30 °C for 30 min.

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