Structure of TFIIK for phosphorylation of CTD of RNA polymerase II

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During transcription initiation, the general transcription factor TFIIH marks RNA polymerase II by phosphorylating Ser5 of the carboxyl-terminal domain (CTD) of Rpb1, which is followed by extensive modifications coupled to transcription elongation, mRNA processing, and histone dynamics. We have determined a 3.5-Å resolution cryo–electron microscopy (cryo-EM) structure of the TFIH kinase module (TFIIK in yeast), which is composed of Kin28, Ccl1, and Tfb3, yeast homologs of CDK7, cyclin H, and MAT1, respectively. The carboxyl-terminal region of Tfb3 was lying at the edge of catalytic cleft of Kin28, where a conserved Tfb3 helix served to stabilize the activation loop in its active conformation. By combining the structure of TFIK with the previous cryo-EM structure of the preinitiation complex, we extend the previously proposed model of the CTD path to the active site of TFIK.

INTRODUCTION

The C-terminal domain (CTD) of the largest subunit (Rpb1) of RNA polymerase II (pol II) is composed of 26 and 52 repeats of the consensus sequence of Y$_5$S$_7$P$_3$T$_4$S$_5$P$_8$S$_7$ in yeast and human, respectively, and is subjected to extensive posttranslational modifications during transcription, which serves as a platform for binding of transcription factors, mRNA processing factors, and histone modifiers (1–3).

CTD modifications begin with phosphorylation on the fifth residue of the consensus repeat (Ser$^5$) during transcription initiation by TFIIH, the 10-subunit general transcription factor (4–9). Whereas this CTD phosphorylation depends on the three-subunit kinase module (called TFIK in yeast), composed of Kin28, Ccl1, and Tfb3, yeast homologs of cyclin-dependent kinase 7 (CDK7), cyclin H, and MAT1, respectively (10–13), CTD phosphorylation levels are substantially enhanced in the preinitiation complex (PIC) containing pol II and all five general transcription factors (8, 14), and, to a greater extent, in PIC-Mediator (15, 16). The enhanced CTD phosphorylation can be reconstituted in vitro when a CTD peptide is combined with TFIK and Mediator (17), suggesting functional and physical interactions between Mediator and TFIK (18). Consistent with biochemical studies, recent cryo–electron microscopy (cryo-EM) structures of PIC-Mediator localized the position of TFIK in contact with the Mediator head module (19, 20) and suggested the path of CTD from pol II, although the Mediator head module, to TFIK (19). However, the structure of TFIK remained to be determined, due to its local mobility.

In contrast to CDKs for the cell cycle, such as CDK2, CDKs for transcription, such as CDK7 in TFIIH, CDK8 in Mediator, and CDK9 in positive transcription elongation factor b (P-TEFb), have subunit(s) or cofactor(s) that activate respective cyclin kinases during distinct steps of transcription (21). In the case of CDK7 (Kin28 in yeast), previous biochemical studies suggest that Tfb3 binds a Kin28-Ccl1 dimer and activates the kinase activity through its C-terminal region, while the N-terminal region of Tfb3, containing the Ring domain, serves to tether TFIK to the rest of TFIIH (core TFIIH) (22).

Here, we have determined a structure of TFIK using cryo-EM and chemical cross-linking and mass spectrometry (XL-MS). The C-terminal 62 residues of Tfb3 were identifiable, lying along the interface between Kin28 and Ccl1, stabilizing the activation loop (T-loop) in its catalytically active form. By docking the structure of TFIK into the previous cryo-EM map of PIC-Mediator, we now localize the active site of TFIK in PIC-Mediator and thus extend the previously proposed model of the CTD path on the Mediator head module to the active site of TFIK.

RESULTS

Cryo-EM structure determination of TFIK

Active TFIK, with a phosphorylated Thr$^{162}$ in the activation loop, was isolated from yeast through a TAP tag on the Tfb3 subunit, which is capable of pol II CTD phosphorylation, as previously published (fig. S1, A and B) (23). TFIK was incubated with 10-fold molar excess CTD peptide and nonhydrolyzable analog of adenosine triphosphate (ATP) [adenosine diphosphate–aluminum fluoride–stabilized (ADP-AIF$_3$)] and was vitrified by plunge freezing. We imaged ~3 million particles, with a Titan Krios equipped with a K3 direct electron detector (fig. S1C). Reference-free two-dimensional (2D) class averaging with cryoSPARC (24) yielded a set of homogeneous classes, with clearly visible secondary structures (fig. S1D). Approximately 1 million images selected through the 2D class averaging were subjected to ab initio calculation of initial maps and following iterative 3D classifications with Relion (25). TFIK particles (~130,000) selected from these classifications were processed with 3D autorefinement, CTF refinement, and Bayesian polishing routines in Relion at a nominal resolution of 3.64 Å (fig. S1, I, K, and L), referred to as Map 2 (table S1). While Map 2 showed the well-defined features of the cyclin kinase ascribable to Kin28-Ccl1 and flanking density attributable to the C-terminal region of Tfb3 (fig. 1, A and B), another run of 3D classification was performed using a mask excluding flexible

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Fig. 1. Cryo-EM structure of TFIIK. (A) Left: Cryo-EM map of the core yeast TFIIK at 3.5 Å shows clear density for each subunit: Kin28 (blue), Ccl1 (cyan), and Tfb3 (orange) with density colored by subunits indicated. Right: Structural model of TFIIK with subunits colored as indicated. Activation loop, ADP-AlF₃, and phosphorylated Thr₁₆₂ annotated. (B) Cryo-EM map and fit structural model of yeast TFIIK at 3.64 Å resolution, including H₃₁₃ and H₃₁₄ helices of Ccl1. (C to E) EM density with side chains of Kin28 (C), Ccl1 (D), and Tfb3 (E). (F) Schematic diagram of domains of TFIIK subunits. The Ring domain and helical domain of Tfb3 are not resolved in the EM map.
H$_{3-4}$–4 helices of Ccl1 (fig. S1H) to further improve map quality for model building (Fig. 1A). In this 3.5-Å-resolution map of the core TFIIK, referred to as Map 1 (table S1 and fig. S1, E to G), many side chains were clearly visible (Fig. 1, C to E), allowing us to build an atomic model, aided by homology models of Kin28 and Ccl1 constructed from the published crystal structures of CDK7 (26) and cyclin H (27). The Kin28/Ccl1/Tfb3 model was iteratively refined against the cryo-EM map using real-space refinement in Phenix and Coot with good refinement statistics (table S1). The resulting model contained Kin28 (296 of 306 residues), Ccl1 (287 of 393 residues), and the C-terminal 62 residues of Tfb3 (residues 259 to 320) (Fig. 1, A and B to F). The other regions were not built because of missing or poor densities. The density corresponding to ADP-AlF$_3$ was observed in the ATP-binding site of Kin28, whereas there was no density attributable to the CTD peptide in the substrate catalytic site (Fig. 1, A and B).

**Cross-linking and mass spectrometry of TFIIK**

The structure of TFIIK determined by cryo-EM was validated by chemical XL-MS (Fig. 2A). Endogenously purified holo-TFIIH (23) containing core-TFIIH subunits (Ssl2, Rad3, Tfb1, Tfb2, Ssl1, Tfb4, and Tfb5) and TFIIK was reacted with MS-cleavable cross-linker disuccinimidyl dibutyric urea (DSBU) (28), and the cross-linked peptides were acquired by MS and analyzed by the search engine, MeroX (29). To obtain cross-links of high confidence, identifications with a false discovery rate (FDR) of 1% or lower were retained. We obtained a total of 635 cross-links, comprising 564 within core TFIIH, 43 within TFIIK, and 28 between core TFIIH and TFIIK. Of the 43 cross-links identified in TFIIK, 12 cross-links could be directly compared with corresponding residues in the model (the other 31 were on flexible loops or on the N-terminal portion of Tfb3 and not modeled), and all cross-links were consistent with our model (Ca-Ca distances less than 25 Å) (Fig. 2, A to C, and fig. S2B), which are in good agreement with previous studies using bis(sulfosuccinimidyl)suberate/disuccinimidyl suberate with similar spacer arm length (30–32).

**Overall structure of TFIIK**

In TFIIK, Kin28 [root mean square deviation (RMSD) of ~5.7 Å with CDK7 [Protein Data Bank (PDB): 1UA2] across 278 residue pairs],
and Ccl1 [RMSD of ~5.0 Å with cyclin H (PDB: 1KXU) across 224 residue pairs] form a canonical CDK-cyclin complex (Fig. 3A, and fig. S3, A and B) (33–35). Kin28 was phosphorylated at Thr316, stabilizing the activation loop in a conformation characteristic of active CDKs (Fig. 3, B and C). The X-DFG motif at the N terminus of the activation loop was in a “BLAminus” conformation, consistent with an active kinase (36). ADP-AlF3 was identified in the catalytic site with an active kinase (36). ADP-AlF3 was identified in the catalytic site at position +0 (Fig. 4 and fig. S4C). Similar to Cdk2, L150 and N164–T167 of Kin28 were positioned to make a suitable pocket for proline (Pro4) at position +1, while T167, the hydrophobic stem of R168, and W169 formed a hydrophobic pocket to accept proline (Pro4) at position −2. M49 of the STAIR helix also packed against tyrosine (Tyr4) at position +3. By contrast, the side chains of Thr4, Ser7, and Ser2 at positions −1, +2, and +4, respectively, were free of contacts. This binding mode is in good agreement with a previous mutational study (39) showing requirement for Tyr4, Pro4, and Pro6 but not Ser2, Thr4, and Ser7 for Ser5 phosphorylation. When the CTD modeling was repeated by positioning Ser3, Ser7, or Thr4 in the catalytic site (fig. S4, D to F), hydrophobic residues, Pro3, Pro6, and Tyr4, were mostly free of contacts. For example, when Ser2 is at the active site, the residues in the hydrophobic sites occupied by the Pro3, Pro6, and Tyr4 in the Ser2 substrate peptide are replaced with Ser3, Pro3, and Ser7, respectively. Only Pro3 makes hydrophobic contacts with the kinase domain. When Thr4 is the substrate, there is no proline at position +1, and the Pro3, Pro6, and Tyr4 side chains are pointed away from the kinase domain. When Ser7 is the substrate, the side chain of Tyr1 is at the +1 position and points away from the hydrophobic site that binds the +1 Pro. Calculations with Rosetta of the ΔΔG of binding for the four peptides were not ordered as we expected. S5 was higher (less favorable) than S2, S7, and T4, although the distribution of the top 10 models overlapped significantly (fig. S4B) so we could not discriminate between potential Kin28 substrates (Ser3, Thr4, Ser7, and Ser7) from this modeling alone.

The active site of TFIIK

Activation loop phosphorylation and Tfb3 binding are both likely to be key determinants of TFIIK activation but are independent mechanisms: The salt bridges between phosphorylated Thr162 (pT162) and three Arginine residues (Arg31, Arg278, and Arg152) of Kin28 stabilize the flattened, active conformation of the activation loop on the HYTF motif of Kin28, and the following amphipathic helix (termed as Tfb3 activation helix, residues 299 to 309) is in contact with the activation loop (Fig. 3, A and D). Notably, this region containing two α helices is the most conserved part of Tfb3/MAT1 (Fig. 3E, top, and fig. S3C).

The structure of TFIIK in the PIC with Mediator

Previous cryo-EM structures of the PIC-Mediator localized TFIIK, but the structure of TFIIK was not determined because of its local mobility relative to the rest of the structure (19, 20). The structure of TFIIK was fitted into the corresponding density (20) in good agreement with XLs in the PIC (Fig. 5, A and B, and fig. S5C), except HN3 (residues 77 to 82) and HN4 (residues 94 to 111) of Ccl1, which are yeast specific and the most mobile part in the structure of TFIIK based on local resolution calculations (fig. S11). The N- and C-lobes of Kin28 are in contact with the middle module’s hook and head module’s neck of Mediator (40–42), respectively (Fig. 5B), forming a tunnel that may direct the CTD (see below). The point of contact with the Mediator neck is the region of CDK7/Kin28 that differs the most from CDK2 (26), comprising αD-αE loop (Kin28 residues 95 to 105), and the Pro-rich C-terminal region (Kin28 residues 293 to 303). On the back side of TFIIK, Tfb3 was facing toward the ARCH anchor domains of Tfb3 (Fig. 5C). In between, Tfb3 linker region (resides 145 to 268) is likely disordered, but its localization was supported by previous XL-MS (Fig. 5, A and C) (19, 20).

There are some notable features of the tunnel formed by Mediator and TFIIK (Fig. 6). The tunnel lies in the path of the CTD where it emerges from the “CTD channel” (19) formed by the Mediator head and middle modules. The active site of TFIIK lies on the inner wall of the tunnel, so that CTD phosphorylation may be processive as it threads through the tunnel (5, 19). The ~25-residue segment of CTD bound to the Mediator head in the CTD channel (19, 43) is oriented for the delivery to the active site of TFIIK. When a seven-residue CTD (SPTPSYS) is modeled on Tfb3 (Fig. 6, right), its
Fig. 3. Activation mechanism of TFIIK. (A) Structural model of TFIIK activation. Kin28 (blue), Ccl1 (cyan), Tfb3 (orange), and the activation loop (red) are colored. (B) EM density of the activation loop with important activating residues. The phosphate group on Thr162 (pT162) is apparent as in other activated CDKs (30). (C) Electrostatic potential map of activation loop and surrounding residues shows a conserved basic patch surrounding pT162, suggesting a similar activation mechanism conserved through CDKs. (D) Activation of the Kin28 activation loop by the Tfb3 activation helix and the Kin28 KHYT motif. The Tfb3 activation helix makes direct contact with the activation loop via hydrogen bonding interaction between Tfb3 R304 and Kin28 backbone carbonyl and hydrophobic interaction between Tfb3 F296 and Kin28 P157. Tfb3 also binds the Kin28 KHYT motif (residues 180 to 183) by a hydrogen bonding networking including Tfb3 F291–Kin28 H181 and Tfb3 Y300–Kin28 T183. The KHYI motif also helps stabilize the activated activation loop by hydrogen bonding interactions with Y182 and R128 and hydrophobic interaction between Y182 and L161. (E) Sequence alignment of Tfb3 activation loop (top) and Kin28 activation loop (bottom). Tfb3 activation helix is highly conserved from yeast to human. Kin28 activation loop is highly conserved though A156 is replaced with a serine/threonine in other eukaryotes for CAK regulation. (F) Structures of inactive human CDK7 (PDB: 1UA2) (pink, left), active yeast Kin28 (this study) (blue, middle), and active CDK2 (PDB: 1FIN) (purple, right). Inactive CDK7 has activation loop (red) covering the active site, while activated Kin28 and CDK2 moved the activation loop, which are stabilized by the Tfb3 activation helix and the CycA αN helix, respectively. The CycA αN helix is absent in Ccl1/cyclin H.
Our in silico analysis suggests that residues Pro \(3\), Pro \(6\), CTD \(\pm 0\) and \(\pm 1\), and \(\pm 3\), respectively, in the substrate binding site (Fig. 5), which is in good agreement with previous mutational study (39). Such hydrophobic interactions were not possible when the CTD modeling was repeated by positioning Ser\(^2\) at position +0 (fig. S4), which may explain its substrate specificity of Ser\(^5\) over Ser\(^2\).

Previous biochemical studies demonstrated that CTD phosphorylation levels are substantially enhanced in the presence of Mediator (15–17). In addition to the activation loop, Mediator contacts the \(\alpha E\) and \(\alpha I\) helices of Kin28/CDK7 as well as the Pro-rich C-terminal region, which is a long, structurally variable insertion in CMGC family kinases between the \(\alpha G\) and \(\alpha H\) helices (36). The Pro-rich region provides an additional recognition site and thus confers diverse substrate selection (26). In the mitogen-activated protein kinase p38 (44), the equivalent region, called the docking site, binds docking site recognition sequences and brings neighboring phosphorylatable regions closer to the catalytic site. In a similar manner, the Pro-rich region of Kin28/CDK7 serves as a docking site, which binds Mediator and thereby aligns the CTD path for delivery to its active site (Fig. 6). Moreover, the tunnel formed between the middle module’s hook and middle module’s neck of Mediator sterically confines the CTD path and thus further enhances the chance for CTD to access TFIIK. It may even facilitate processive CTD phosphorylation (5, 19) as it threads through the tunnel.

DISCUSSION
There is an extended family of CDKs in eukaryotes (21): While ancestral CDK family members function to regulate the cell cycle, some CDKs, such as CDK7, CDK8, and CDK9, have evolved to function in other cellular processes, most notably in transcription regulation. We have determined the structure of TFIIK, composed of Kin28 (the yeast ortholog of CDK7), Ccl1, and Tfb3, which is responsible for the CTD Ser\(^5\) phosphorylation during transcription initiation. In the structure, Kin28 and Ccl1 form a canonical CDK-cyclin complex. Tfb3 was identified on the back side of TFIIK, stabilizing the active form of the activation loop at the edge of the catalytic cleft and facilitating access of a substrate to the active site. When TFIIK is superimposed onto the CDK2–cyclin A complex, the Tfb3 activation helix is approximately in the position and orientation of the N-terminal helix (\(\alpha N\)) of cyclin A (Fig. 3F and fig. S3, D and E). In some ancestral cell cycle CDKs, such as CDK2, the N-terminal helix of cyclin (e.g., cyclin A) directly stabilizes the activation loop (33). In contrast, in TFIIK, the equivalent N-terminal \(\alpha\) helices (\(H_{N1}\) and \(H_{N2}\)) of Ccl1/cyclin H interact with Tfb3/MAT1, which, in turn, stabilizes the activation loop (Fig. 3F and fig. S3, D and E). Thus, the activation loop stabilization through Tfb3/MAT1 may have been acquired during evolution of the transcriptional lineage of cyclins.

Nearly all CDKs phosphorylate their substrates at Ser (or Thr) residues with a strong sequence preference for Ser-Pro at positions \(\pm 0\) and \(\pm 1\) and weaker preferences for amino acids at other positions (38). We therefore asked how Kin28 (TFIIK) can distinguish between Ser\(^5\) and Ser\(^2\), both of which are followed by a proline in the CTD (39). Our in silico analysis suggests that residues Pro\(^3\), Pro\(^6\), and Tyr\(^1\) formed stable hydrophobic interactions at positions \(-2\), \(-1\), and \(+3\), respectively, in the substrate binding site (Fig. 5), which is in good agreement with previous mutational study (39). Such hydrophobic interactions were not possible when the CTD modeling was repeated by positioning Ser\(^2\) at position +0 (fig. S4), which may explain its substrate specificity of Ser\(^5\) over Ser\(^2\).

MATERIALS AND METHODS
Protein purification
TFIIK and holo-TFIIF were purified from yeast as previously described (23) with minor modifications. In short, yeast containing TAP tags on TFIIF subunits Tfb4 and Ssl2 was grown in 100 liters of YPAD (yeast extract, peptone, adenine, glucose) medium to an optical density (OD) of 10.0. Whole cell lysate was prepared by bead beating in buffer A [50 mM Heps (pH 7.6), 1 mM EDTA, 5% glycerol, 400 mM potassium acetate, 2-mercaptoethanol, and protease inhibitors]. Following the addition of 100 mM ammonium sulfate and 0.1% polyethyleneimine (PEI), lysed cells were stirred for 1 hour and centrifuged, and then the cleared lysate was loaded onto an immunoglobulin
G (IgG) column. The column was washed with 5 to 10 column volumes of buffer 300 [50 mM Hepes (pH 7.6), 1 mM EDTA, 5% glycerol, 300 mM potassium acetate, 2 mM dithiothreitol (DTT), and protease inhibitors] and then resuspended in buffer 300 and allowed to settle. IgG beads were washed by batch with another 10 column volumes of buffer 300. TFIIF was treated with tobacco etch virus in buffer 300, eluted from the IgG column, and then resuspended in buffer 300 and allowed to settle. TFIIF was eluted by salt gradient of concentration from 300 mM to 1.2 M potassium acetate. Fractions containing different TFIIF subunits were separated and concentrated separately.

**Cryo-EM sample preparation and data collection**

To prepare cryo-EM grids, purified TFIIF (final concentration, 0.08 mg/ml) was incubated with 10-fold molar excess CTD peptide (three-repeat CTD peptide) and 2.5 mM ADP-AlF₃ for 30 min in buffer 100 [20 mM Hepes (pH 7.5), 100 mM potassium acetate, and 2 mM DTT]. The sample (2 μl) was then applied to glow-discharged (1 min; easiGlow, Pelco) R1.2/1.3 200-mesh or R2/2 300-mesh QUANTIFOIL holey carbon grids (Electron Microscopy Sciences). The grids were subsequently blotted for 2 s using Whatman grade 41 filter paper (Sigma-Aldrich) and flash-frozen in liquid ethane with
a Leica EM CPC manual plunger (Leica Microsystems). EM grids were prepared in batches, and the freezing conditions were optimized by screening on a FEI TF20 microscope operating at 200 kV and equipped with a FEI Falcon III direct electron detection camera at the Electron Microscopy Research Lab (University of Pennsylvania).

Cryo-EM specimens were imaged at the Beckman Center for Cryo-Electron Microscopy (University of Pennsylvania) using a FEI Titan Krios G3i transmission electron microscope operating at 300 kV, equipped with a K3 direct electron detector (Gatan) and a Bioquantum energy quantum filter (Gatan) at a nominal magnification of ×105,000 in super-resolution mode (pixel size of 0.415 Å) at a defocus range between 0.8 and 2.8 μm. A total of 4620 images was collected over the course of 2 days. The exposure time was 2.24 s, divided into 35 frames, at a nominal dose of 45 electrons/Å².

Image processing and 3D reconstruction
The cryo-EM data were processed, and maps were calculated with using a combination of software including cryoSPARC v2.12.4 (24) and Relion 3.0.8 (25). The TFIIK dataset was motion-corrected with MotionCorr2 (45) and then imported into cryoSPARC for CTF correction with CTFFIND4 (46). Blob-based picking with cryoSPARC was used to produce a small subset of particles for the generation of 2D references. A total of 3,288,475 particles were picked by template-based picking, and two rounds of reference-free 2D classification were performed to remove particles that lacked clear features (fig. S1D), resulting in a subset of 938,135 particles. This subset was then transferred to Relion 3.0.8 for initial model generation by stochastic gradient descent. The initial model was consistent with 2D class averages and could accommodate a cyclin kinase pair (fig. S1H).

This initial model was then used as a reference for multiple rounds of 3D classification (fig. S1I). Last, two classes consisting of 129,955 particles were selected and combined in 3D autorefinement yielding a map of 4.2 Å. The data were further processed by iterating rounds of Beamtilt estimation and Bayesian polishing, yielding a final map of 3.64-Å resolution (fig. S1G). 3D variability analysis was conducted in cryoSPARC showing motion and heterogeneity in the HN3 and HN4 helices (fig. S1M).

CryoEF (47) was used to evaluate the orientation distribution and anisotropic effects using a particle size of 100 Å (fig. S1K). As determined by cryoEF, Map 2 (EOD = 0.69) showed slight anisotropy. To decrease anisotropy and improve the map quality, a further run of 3D classification was performed masking out the flexible HN3 and HN4 helices (fig. S1H). A good class of 81,446 particles was obtained, and subsequent refinement in Relion, as outlined above, yielded a final map of 3.5-Å resolution (fig. S1G). Repeated cryoEF analysis using these particles showed an improved orientation distribution (fig. S1F), and the EOD was increased to 0.71. Maps were post-processed independently with deepEMhancer (48) and Relion and both were deposited. The local resolution of the maps was determined using Fourier shell correlation (cutoff of 0.5) with blocres (49) (fig. S1, E and I).

Model building and refinement
To build the atomic model of Kin28/Ccl1/Tfb3, we started by rigid-body fitting crystal structures of human CDK7 (PDB: 1UA2) (26) and cyclin H (PDB: 1KXU) (50) into the cryo-EM map using UCSF Chimera (51), which showed an apparent continuous density corresponding to the Tfb3 subunit (fig. S3F). Because of slight sequence variations in Kin28 and Ccl1 between yeast and human, sequence alignments, secondary structure predictions, and homology modeling were used to facilitate the model building. In Kin28, a phosphate group was added to the side chain of Thr162 due to phosphorylation, and the ADP-AlF₃ was placed into the density at the ATP-binding site. There was no observable density for the CTD peptide. The density map, corresponding to the Tfb3 subunit, was of sufficient quality for ab initio model building. Residues (259 to 320) in the
C-terminal region of the Tfb3 were modeled into the density map. The remaining portion of Tfb3 was missing or disordered in the density map. The model building and adjustments were done using Coot (fig. S3, G and H) (52). Refinement of the Kin28/Ccl1/Tfb3 model against the cryo-EM map was carried out using the real space refinement in Phenix (53). In the final model, amino acids for Kin28 (26 to 31, 42 to 43, and 304 to 306) and Ccl1 (1 to 46, 288 to 325, and 371 to 393) were not built because of missing or poor densities. The final model statistics are shown in table S1.

Cross-linking mass spectrometry sample preparation

One hundred fifty micrograms of purified holo-TFIIF at a concentration of 1 mg/ml in buffer 300 [20 mM Hepes (pH 7.6), 300 mM potassium acetate, 5% glycerol, and 2 mM DTT] was mixed with 6 mM DSBU (Thermo Fisher Scientific) and incubated on ice for 2 hours. The reaction was quenched by adding 50 mM ammonium bicarbonate, and the reaction was further stopped by trichloroacetic acid (TCA) precipitation. Cross-linked proteins were precipitated with 20% (w/v) TCA (Sigma-Aldrich) on ice for 90 min. Proteins were pelleted by centrifugation at 21,000 g for 15 min and washed with 10% TCA in 0.1 M tris-HCl and then with acetone (Thermo Fisher Scientific). The solvent was discarded, the pellet was air-dried and then stored at −80°C for analysis by MS.

Cross-linked proteins were resuspended in 50 μl of resuspension buffer (2.5% SDS and 50 mM triethylammonium bicarbonate final concentrations) and reduced with final 10 mM DTT (US Biological) for 30 min at 30°C, followed by alkylation with final 50 mM iodoacetamide (Sigma-Aldrich) for 30 min at 30°C. The proteins were processed using an S-Trap column according to the protocol recommended by the supplier (Protifi, C02-mini) and digested with trypsin (Thermo Fisher Scientific) in 1:10 (w/w) enzyme/protein ratio for 1 hour at 47°C. Peptides eluted from this column were vacuum-dried and resuspended with the peptide fractionation-elution buffer [70% (v/v) liquid chromatography–MS (LC-MS) grade water (Thermo Fisher Scientific), 30% (v/v) acetonitrile (Thermo Fisher Scientific), and 0.1% (v/v) trifluoroacetic acid (TFA; Thermo Fisher Scientific)]. Peptides were first fractionated using AKTA Pure 25 with Superdex 30 Increase 3.2/300 (GE Life Sciences) at a flow rate of 30 μl min⁻¹ in the elution buffer, and 100-μl fractions were collected. On the basis of the elution profile, fractions containing enriched cross-linked peptides of higher molecular masses were vacuum-dried and resuspended with LC-MS grade water containing 0.1% (v/v) TFA for MS analysis. One-half of each fraction was analyzed by a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific) coupled to a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific) equipped with an in-house–made 15-cm-long fused silica capillary column (75 μm inner diameter), packed with reversed-phase ReproSil-Pur C18-AQ 2.4-μm resin (Dr. Maisch GmbH, Ammerbuch, Germany) column. Elution was performed using a gradient from 5 to 45% B (90 min), followed by 90% B (5 min), and reequilibration from 90 to 5% B (5 min) with a flow rate of 400 nl/min (mobile phase A: water with 0.1% formic acid; mobile phase B: 80% acetonitrile with 0.1% formic acid). Data were acquired in data-dependent tandem MS (MS/MS) mode. Full-scan MS settings were as follows: mass range, 300 to 1800 (mass/charge ratio); resolution, 120,000; MS1 AGC target 1E6; MS1 Maximum IT, 200 ms. MS/MS settings were as follows: resolution, 30,000; AGC target 2E5; MS2 Maximum IT, 300 ms; fragmentation was enhanced by higher-energy collisional dissociation with stepped collision energy of 25, 27, 30; loop count, top 12; isolation window, 1.5 m/z; fixed first mass, 130; MS2 Minimum AGC target, 800; charge exclusion: unassigned, 1, 2, 3, 8 and > 8; peptide match, off; exclude isotope, on; dynamic exclusion, 45 s. Raw files were converted to mgf format with TurboRawToMGF 2.0.8 (54).

Cross-linked peptide search

Search engine MeroX 2.0.1.4 (29) was used to identify and validate cross-linked peptides. MeroX was run in RISEUP mode, with default cross-linker mass and fragmentation parameters for DSBU: precursor mass range, 1000 to 10,000 Da; minimum precursor charge, 4; precursor and fragment ion precisions, 5.0 and 10.0 ppm, respectively; maximum number of missed cleavages, 3; carbamidomethylation of cysteine and oxidation of methionine, as fixed and variable modifications, respectively; results were filtered for score (>10) and FDR (<1%). Visualization of the cross-links on the TFIIF structure used Chimera with the Xlink Analyzer plug-in (55).

Modeling of CTD on TFIIF

The crystal structure of CDK2–cyclin A bound to a substrate peptide (PKTPKKA, the underlined phosphorylatable Thr defined as position +0 in the catalytic site) (PDB: 3QHR) (56) was aligned with TFIIF. Then, the side chains of the seven-residue segment of the CTD (sequence PTSPSYS) with S5 at the active site were replaced with the most common rotamer of each residue given the backbone conformation of the peptide according to the backbone-dependent rotamer library (57). The peptide was modified on the N and C terminus by adding acetyl and N-methyl amide groups, respectively, to mimic an extended peptide sequence. The structure of the Kin28/peptide complex was refined with 50 independent trials of the FastRelax algorithm in Rosetta (58). The ΔΔG of binding was estimated by performing the same refinement procedure on the Kin28 kinase domain alone and the peptide separated from the kinase domain and calculating the difference in Rosetta energy: ΔΔG = E(complex) − E(kinase alone) − E(peptide alone). The same procedure was repeated for the S2 (sequence SYSPSYTP), T4 (SPSYSPY), and S7 (SPSYSPY) phosphorylation sites of the CTD. The five lowest scoring (ΔΔG) reﬁned models for each peptide sequence in complex with Kin28 was selected for molecular visualization.

Kinase assay

Pol II (4 pmol) was treated with 3 pmol of TFIIF in 20 mM Hepes (pH 7.6), 2.5 mM magnesium acetate, 100 mM potassium acetate, 5 mM DTT, 5% glycerol, and 5 mM ATP for 1 hour at room temperature. Reactions were stopped by adding EDTA. Phosphorylated and unphosphorylated pol II were analyzed by running a 6% SDS–polyacrylamide gel electrophoresis gel for 2 hours at 120 V (fig. S1B).

Integrative modeling of TFIIF in Mediator-PIC

Integrative modeling (59) of TFIIF on core Mediator-PIC was performed on the basis of a previously described approach (19), with minor modifications, using a Cryo-EM map for the core Mediator-bound transcription PIC at 5.8-Å resolution (EMDB-3850) (20) and two cross-link datasets (19). The TFIIF trimer and the core Mediator-PIC were treated as two rigid bodies, modeled at a residue level where possible and represented by flexible coarse-grained beads encompassing 5 to 40 amino acids elsewhere, as specified in a model topology file. A scoring function considering satisfaction of the EM volume, cross-linking dataset pairwise distance restraints, sequence connectivity, and nonoverlapping volumes was used in two separate simulations,
producing 320,000 models from 160 initial configurations each. From the top-scoring 500 models of each simulation (fig. S5A), a single structural cluster was determined, positioning the TFIIK structure at an overall sampling precision of~9 Å (fig. S5, B and C).

SUPPLEMENTARY MATERIALS

Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/15/eabd4420/DC1

View/request a protocol for this paper from Bio-protocol.

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Acknowledgments: We would like to acknowledge the use of instruments at the Electron Microscopy Resource Lab and at the Beckman Center for Cryo-Electron Microscopy at the University of Pennsylvania Perelman School of Medicine. We also thank D. Johnson-McDaniel for assistance with Krios microscope operation. Funding: This research was supported by NIH grants R01-GM123233 to K.M, and CA196539 and AG031862 to B.A.G. and the Cancer Prevention Research Institute of Texas, grant number 13127, to CPRIT Scholar in Cancer Research, K.-L.T.; NIH training grants T32-GM08275 to T.e.V and T32-GM071339 to H.J.K. and the National Science Foundation Graduate Research Fellowship UDPGE-1845298 to J.J.G.C.; NIH grant R35 GM122517 to R.L.D. and P30 CA006927 to the Fox Chase Cancer Center (in support of the Molecular Modeling Facility at Fox Chase). Computational resources were supported by NIH Project Grant S10OD023592. Author contributions: T.v.E., K.-L.T., and K.M. designed the experiments. T.e.V prepared cryo-EM samples and analyzed the data. H.J.K. and B.A.G. performed XL-MS. J.J.G.C., K.-L.T., and T.L. built models. R.L.D. and M.I.P. performed the molecular modeling of Kin28/substrate interactions. T.e.V, K.-L.T., and K.M. wrote the paper and prepared the figures for publication. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors. The cryo-EM density maps were deposited in the Electron Microscopy Data Bank (EMDB-23036, 22191). The atomic coordinates were deposited in the Protein Data Bank (accession codes: 7KUE and 6XII). The cross-linking data were deposited in the PRIDE repository and available at PXD021211. IMP files and Rosetta models are available at the Murakami Lab GitHub repository: https://github.com/cryomurakami/Structure_of_TFIIK_for_phosphorylation_of_CTD_of_RNA_polymerase_II.

Submitted 21 June 2020
Accepted 5 February 2021
Published 7 April 2021
10.1126/sciadv.abad4420

Citation: T. van Eeuwen, T. Li, H. J. Kim, J. J. Gorbea Colón, M. I. Parker, R. L. Dunbrack, B. A. Garcia, K.-L. Tsai, K. Murakami, Structure of TFIIK for phosphorylation of CTD of RNA polymerase II. *Sci. Adv.* **7**, eabad4420 (2021).
