Structure of TFIIK for phosphorylation of CTD of RNA polymerase II

Trevor van Eeuwen1,2, Tao Li3, Hee Jong Kim1,2,4, Jose J. Gorbea Colón1,2, Mitchell L. Parker5,6, Roland L. Dunbrack5, Benjamin A. García1,4, Kuang-Lei Tsai3, Kenji Murakami1*

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 TFIIH 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 TFIIFK 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 TFIIFK.

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 Y3S4P3T4S5P6S7 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 (Ser5) 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 TFIIFK 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 TFIIFK and Mediator (17), suggesting functional and physical interactions between Mediator and TFIIFK (18). Consistent with biochemical studies, recent cryo–electron microscopy (cryo-EM) structures of PIC-Mediator localized the position of TFIIFK in contact with the Mediator head module (19, 20) and suggested the path of CTD from pol II, although the Mediator head module, to TFIIFK (19). However, the structure of TFIIFK 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 TFIIFK to the rest of TFIIH (core TFIIH) (22).

Here, we have determined a structure of TFIIFK 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 TFIIFK into the previous cryo-EM map of PIC-Mediator, we now localize the active site of TFIIFK in PIC-Mediator and thus extend the previously proposed model of the CTD path on the Mediator head module to the active site of TFIIFK.

RESULTS

Cryo-EM structure determination of TFIIFK

Active TFIIFK, with a phosphorylated Thr162 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). TFIIFK was incubated with 10-fold molar excess CTD peptide and nonhydrolyzable analog of adenosine triphosphate (ATP) [adenosine diphosphate–aluminum fluoride–stabilized (ADP-AIF3)] 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). TFIIFK particles (~130,000) selected from these classifications were processed with 3D autoalignment, CTF refinement, and Bayesian polishing routines in Relion at a nominal resolution of 3.64 Å (fig. S1, L and K), 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

1Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA. 2Biochemistry and Molecular Biophysics Graduate Group, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA. 3Department of Biochemistry and Molecular Biology, McGovern Medical School, University of Texas Health Science Center at Houston, Houston, TX 77030, USA. 4Epigenetics Institute, Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA. 5Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111, USA. 6Molecular and Cell Biology and Genetics Program, Drexel University College of Medicine, Philadelphia, PA 19102, USA.

*Corresponding author. Email: kenjim@pennmedicine.upenn.edu (K.M.); kuang-lei.tsai@uth.tmc.edu (K.L.T.)
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 H₃₃ and H₄₃ 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$_{N3-4}$ 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 disuccinimidyldibutyrlic 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 (Cα-Cα distances less than 25 Å) (Fig. 2, A to C, and fig. S2B), which are in good agreement with previous studies using bis(sulfo)succinimidylsuberate/disuccinimidylsuberate 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 Thr162, 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 cleft between the two lobes of Kin28 (Fig. 3A and fig. S3A) in Map 2. Ccl1 contains two canonical cyclin boxes (fig. S3B), each consisting of five helices (H1 to H5 and H1′ to H5′) and four extra N-terminal α helices [H N1 (residues 48 to 54), H N2 (residues 62 to 73), H N3 (residues 77 to 82), and H N4 (residues 95 to 105)]. The first cyclin (N-terminal) box primarily interacts with Kin28, while the second (C-terminal) cyclin box and two N-terminal α helices (H N1 and H N2) interact with Tfb3 (Fig. 3A and fig. S3B).

The C-terminal 62 residues (residues 259 to 320) of Tfb3 were identified at the interface between the C-lobe of Kin28 and the second cyclin box of Ccl1 (i.e., on the opposite side to the active site of Kin28) (Fig. 3A and fig. S3C). These residues do not form a hydrophobic core of their own but rather snake along a groove between Kin28 and the second cyclin box of Ccl1. Of the C-terminal 62 residues, its N-terminal (residues 271 to 285) and C-terminal (residues 313 to 320) regions primarily interact with the second cyclin box, HN1 and HN2 of Ccl1, as described above, while the central region of Tfb3 reaches into the active site of Kin28, where a short 310 helix (residues 289 to 292) is in contact with the KHYT 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 active site of TFIIFK

Activation loop phosphorylation and Tfb3 binding are both likely to be key determinants of TFIIFK activation but are independent mechanisms: The salt bridges between phosphorylated Thr162 (pT162) and three Arginine residues (Arg53, Arg728, and Arg152) of Kin28 stabilize the flattened, active conformation of the activation loop on the KHYT motif of Kin28 as in other active CDKs (Fig. 3, B and C), while distinct residues (Ala156 and Pro157) located at the tip of the activation loop pack against the hydrophobic face of the Tfb3 activation helix (Tyr309, Ala301, Arg302, and Val305) and of the flanking short 310 helix (Phe306) (Fig. 3D). In other eukaryotes, Ala156 of the activation loop is replaced with Ser (or Thr) (Fig. 3E, bottom), and its phosphorylation marks negative regulation of transcription during mitosis (37). This hydrophobic interaction is stabilized by surrounding hydrogen bonds between the Kin28 KHYT motif (His181 and Thr185) and Tfb3 (Phe291 and Tyr309) (Fig. 3D). Also, a hydrogen bond between the main chain of the activation loop and Arg301 of Tfb3 most likely contributes to Tfb3 binding (Fig. 3D).

To locate the substrate binding site of TFIIFK, 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) (38) was aligned with the coordinates of TFIIFK (RMSD of ~3.0 Å between CDK2–cyclin A and Kin28-Ccl1). The substrate peptide PKTPKKA could not discriminate between potential Kin28 substrates (Ser2, Thr4, Ser5, Ser7, Tyr1, Ser2) and Thr5, 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 (Ser6, Thr4, Ser5, and Ser7) from this modeling alone.

The structure of TFIIFK in the PIC with Mediator

Previous cryo-EM structures of the PIC-Mediator localized TFIIFK, but the structure of TFIIFK was not determined because of its local mobility relative to the rest of the structure (19, 20). The structure of TFIIFK 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 86) and HN4 (residues 94 to 111) of Ccl1, which are yeast specific and the most mobile part in the structure of TFIIFK 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 TFIIFK, Tfb3 was facing toward the ARCH anchor domains of Tfb3 (Fig. 5C). In between, Tfb3 linker region (residues 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 TFIIFK (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 TFIIFK 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 TFIIFK. When a seven-residue CTD (SPTSPSY) is modeled on TFIIFK (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 KHYT 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 , between Ser5 and Ser 2 , both of which are followed by a proline in the 38 +0 and +1 and weaker preferences for amino acids at other positions (+2, +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 αE and αI 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 αG and αH 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 TFIIF. It may even facilitate processive CTD phosphorylation (5, 19) as it threads through the tunnel.

MATERIALS AND METHODS
Protein purification
TFIIF and holo-TFIH were purified from yeast as previously described (23) with minor modifications. In short, yeast containing TAP tags on TFIH 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. TFIIH was washed by batch with another 10 column volumes of buffer 300. TFIIH was treated with tobacco etch virus in buffer 300, eluted from the IgG column, and loaded onto a UnoQ column (Bio-Rad). TFIIH was eluted by salt gradient of concentration from 300 mM to 1.2 M potassium acetate. Fractions containing different TFIIH subunits were separated and concentrated separately.

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

To prepare cryo-EM grids, purified TFIIK (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 liquid ethane.
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) and 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 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 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 (EO2D = 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 EO2D 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/CCLTfb3 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-TFIH 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 21,000g 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 for 1 hour at 4°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 2.5% SDS and 50 mM triethylammonium bicarbonate as the buffer (2.5% SDS and 50 mM triethylammonium bicarbonate final concentration). 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 4°C. Peptides eluted from this column were vacuum-dried with 10% TCA in 0.1 M tris-HCl and then with acetone (Thermo Fisher Scientific). The sample was 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 4°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 of the elution buffer, and 100–1000 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 enforced 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 TFIK structure used Chimera with the Xlink Analyzer plug-in (55).

**Modeling of CTD on TFIK**

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 TFIK. 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 SYSTPS), T4 (SPYSPS), and S7 (SPYSPY) 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 TFIK 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 TFIK in Mediator-PIC**

Integrative modeling (59) of TFIK 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 TFIK 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

Supplementary 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.

REFERENCES AND NOTES


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Structure of TFIIK for phosphorylation of CTD of RNA polymerase II

Trevor van Eeuwen, Tao Li, Hee Jong Kim, Jose J. Gorbea Colón, Mitchell I. Parker, Roland L. Dunbrack, Benjamin A. Garcia, Kuang-Lei Tsai and Kenji Murakami

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