Transcription of genes encoding small structured RNAs such as transfer RNAs, spliceosomal U6 small nuclear RNA and ribosomal 5S RNA is carried out by RNA polymerase III (Pol III), the largest yet structurally least characterized eukaryotic RNA polymerase. Here we present the cryo–electron microscopy structures of the *Saccharomyces cerevisiae* Pol III elongating complex at 3.9 Å resolution and the apo Pol III enzyme in two different conformations at 4.6 and 4.7 Å resolution, respectively, which allow the building of a 17–subunit atomic model of Pol III. The reconstructions reveal the precise orientation of the C82–C34–C31 heterotrimer in close proximity to the stalk. The C53–C37 heterodimer resides involved in transcription termination close to the non–template DNA strand. In the apo Pol III structures, the stalk adopts different orientations coupled with closed and open conformations of the clamp. Our results provide novel insights into Pol III–specific transcription and the adaptation of Pol III towards its small transcriptional targets.

### Molecular structures of unbound and transcribing RNA polymerase III

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In eukaryotes, transcription of structured, small RNAs such as tRNAs, spliceosomal U6 small nuclear RNA (snRNA), ribosomal 5S RNA and 7 SL RNA is mediated by RNA polymerase III (Pol III)1. Transcriptional activity of Pol III is elevated in cancer cells, and the tumour suppressors p53 and Rb and the proto-oncogene Myc directly regulate Pol III transcription2. In the last decade, detailed structural studies of Pol I and Pol II contributed to elucidate basic mechanisms of DNA-dependent RNA transcription3–5. However, structural insight into Pol III-mediated transcription has been limited so far as the highest resolution Pol III structures only extend to 10 Å for apo Pol III6 and to 16.5 Å and 19 Å for the Pol III elongation complex7,8. Here, we present the first atomic structures of apo Pol III and elongating Pol III obtained by single-particle electron cryo-microscopy (cryo-EM). Our reconstructions consolidate biochemical data, allow the detailed comparison with the Pol I and Pol II enzymes, and provide additional insight into Pol III–specific transcription.

### Overall architecture of Pol III

*Saccharomyces cerevisiae* Pol III was purified as previously described9 yielding pure, homogenous and transcriptionally active enzyme (Fig. 1). To shed light onto the Pol III architecture and to further investigate Pol III-mediated transcription, we acquired cryo-EM images of Pol III bound to an assembled transcriptionally active bubble (elongating Pol III, Fig. 1c) and of native unbound Pol III (apo Pol III) on an FEI Titan Krios equipped with a Falcon II direct electron detector (Methods, Extended Data Fig. 1). The final map of elongating Pol III was determined using maximum-likelihood based 3D classification in RELION20 at an average resolution of 3.9 Å (Extended Data Fig. 1), but extends beyond 3.5 Å in the Pol III core (Extended Data Figs 1c, 2 and 3). This electron microscopy density was used to build and refine an atomic model of the complete 17-subunit structure of elongating Pol III (Fig. 1a, Extended Data Fig. 4 and Extended Data Table 1). Representative densities (Fig. 1b, Extended Data Fig. 2) and the relatively isotropic resolution (Extended Data Fig. 1c, Extended Data Table 2) demonstrate that the quality of the cryo-EM density map is comparable to electron density maps of Pol I and Pol II obtained by X-ray crystallography at nominally higher resolutions (Extended Data Fig. 3). The overall architecture of
the Pol III core is conserved with respect to Pol I and Pol II (Fig. 1d). However, the clamp head part of subunit C160 is enlarged compared to its Pol I and Pol II counterparts (Extended Data Fig. 5). In addition, C160 contains an extended foot that, unlike Pol I and Pol II, forms a large interface with the shared subunit ABC14.5. Furthermore, a carboxy-terminal extension of C160, unique to Pol III, protrudes from the core and together with the C160 N terminus contacts the stalk. Similarly, the second largest subunit C128 shows an overall conserved fold, but contains an extended protrusion that increases the depth of the DNA-binding cleft in comparison to Pol I and Pol II (Extended Data Fig. 5). The Pol III cryo-EM structure also includes the Pol III-specific C82–C34–C31 heterotrimer and the C53–C37...
heterodimer, both showing several unexpected features as discussed later.

The apo Pol III data set yielded two major 3D classes showing distinct conformations at 4.6 Å and 4.7 Å resolution (Extended Data Fig. 1b, c). One reconstruction is very similar to the elongating Pol III (root mean square deviation (r.m.s.d.) = 0.43 Å), whereas the second reconstruction shows an altered orientation of the stalk, the heterotrimer and a more open cleft resulting in a larger difference with elongating Pol III (r.m.s.d. = 2.73 Å). The two apo Pol III conformations presumably result from ‘closed’ and ‘open’ states of the clamp domain as discussed below (Extended Data Fig. 6a–c).

A narrow cleft encloses DNA

In both apo Pol III data sets and the elongating Pol III structures we observe a characteristic narrower cleft in comparison to Pol I and Pol II (Extended Data Fig. 6d). The DNA duplex is embedded into the Pol III cleft and extends from downstream base pair (bp) +14 towards the active site until the upstream bp −9 (Fig. 2a). The DNA duplex is anchored between the jaw and lobes domains and the oppositely positioned extended clamp head. In addition, the WH2 and WH3 domains of C82 (see below) lie in close proximity and further stabilize downstream DNA (Fig. 2b). Subunit ABC27 completes this enclosure by inserting a proline-containing loop into the minor groove between bps +11 to +14, thus threading the DNA duplex towards the active centre. In the crystal structure of elongating Pol II bound to a second DNA–RNA hybrid that comprises upstream and downstream DNA duplexes (53-mer DNA oligonucleotides), the proline-containing loop also protrudes into the minor groove of downstream DNA, while upstream DNA interacts with a hairpin-loop/wedge in Pol II subunit Rbp2 (residues 862–874) also present in Pol III subunit C128 (residues 794–806) as well as in Pol I. Global recognition of upstream and downstream DNA therefore appears to be conserved among the three eukaryotic RNA polymerases.

The template DNA strand unwinds at bp +2 and a characteristic A-type DNA–RNA hybrid forms at positions −1 to −9 (Fig. 2a). Additional density in the RNA exit channel likely corresponds to the emerging and thus more flexible single-stranded RNA (Fig. 2c). No clear density for upstream DNA is visible, indicating flexibility of the emerging duplex. Another noticeable feature is the strong density of the downstream DNA double strand in comparison to the much weaker density for the DNA–RNA duplex (Fig. 2a). This is in contrast to the Pol II elongation complex, where both densities are of equal quality. Remarkably, the DNA–RNA duplex is tightly associated with the Pol II elongation complex between the wall and fork loop 1/rudder, whereas in Pol III the rudder and fork loop 1 reach towards the protrusion, thereby reducing the association of the DNA–RNA hybrid with the Pol III active centre (Fig. 2d). While the DNA–RNA hybrid appears to be only loosely associated with the active site, we observe a tight enclosure of the downstream DNA duplex at the entrance of the DNA-binding cleft.

Pol III heterotrimer protrudes into the cleft

Although the crystal structure of the human C82 orthologue hRPC62 (ref. 32) is available, its integration into the C82–C34–C31 heterotrimer and the precise orientation of the heterotrimer within the complete Pol III enzyme could not be clarified by previous cryo-EM reconstructions owing to their limited resolution. In addition, the functional roles of the seven winged-helix (WH) domains present in subunits C82 and C34 and often found in transcription factors as DNA-binding or protein–protein interaction modules are still poorly understood. Our cryo-EM structure shows how the heterotrimer packs onto the clamp head by forming a large, hydrophobic interface through various WH domains (C82-WH1/WH4 and C34-WH3; Fig. 3). In this case the WH domains serve as protein–protein interacting domains, although they
might still contact DNA during open complex formation in the transcription initiation process. The coiled-coil domain of C82 protrudes towards the stalk and the remaining C82-WH2 and WH3 are facing away from the core. Notably, C82-WH2 and C82-WH3 align with the clamp head to reach around bp +15 towards downstream DNA. Furthermore, an additional long ‘cleft loop’ extending from C82-WH4 passes through a canyon in the clamp head into the DNA-binding cleft close to DNA bp +7 (Extended Data Fig. 7a). Subunit C34 comprises three WH domains that span from C82 towards the protrusion crossing the DNA-binding cleft\cite{16}. TFIIIE\(\alpha/\beta\) also possesses three WH domains and, in the cryo-EM structure of the human Pol II pre-initiation complex, crosses the DNA-binding cleft\cite{14}. The two N-terminal C34 WH domains are not visible in the Pol III cryo-EM density like the A49 tandem WH domain in the Pol I crystal structure\cite{24,25}, while the third WH domain tightly associates with C82 and is located at the periphery of the heterotrimer. The C34 C-terminal region following the third WH domain passes the C82-WH4 and contacts the C82 coiled-coil domain (Fig. 3). The third subunit of the heterotrimer, namely C31, was predicted to be largely unstructured and associated between C82 and the stalk, as shown by crosslinking analysis\cite{16}. However, we were able to build a mainly helical element of C31 (residues 42–69), demonstrating that C31 extends along the surface from the C34 C terminus towards C82-WH4 over the C82 coiled-coil domain to reach the stalk, where it becomes disordered. Additional density close to the interface of C82-WH1 and WH2 and between the heterotrimer and the stalk is most likely to correspond to C-terminal stretches of C31, but no sequence could be unambiguously assigned to it (Extended Data Fig. 7b). Nevertheless, the topology of C31 and the extended interaction interface with at least three WH domains, the C82 coiled-coil domain and the stalk confirm the previously reported role of C31 in connecting the heterotrimer to the Pol III core and stalk\cite{17,35,36}.

**Transcription termination by Pol III heterodimer**

Previous electron microscopy and crosslinking studies of Pol III and the C53–C37 subcomplex possessed a conserved dimerization module at the lobe of Pol III\cite{12,13,21,26} similar to TFIIIF and A49–A34.5 in Pol II\cite{27} and Pol I\cite{24,25}, respectively. On the other hand, extensions of C53 and C37 crosslink close to the active site, the stalk and the heterotrimer\cite{13,16}. Our structure better characterizes the interaction network of the C53–C37 heterodimer with other Pol III subunits. Notably, C37 shows an extended contact surface with C11 and the cleft when compared to TFIIIF\(\alpha\) and A49 (Fig. 4a), consistent with its proposed role in C11 association with the core\cite{3}.

Our cryo-EM structure also rationalizes the role of the C53–C37 heterodimer in Pol III transcription termination that only requires a stretch of 5–7 thymines in the non-template DNA strand for efficient transcription termination\cite{18}. Subunit C37 extends towards the DNA-binding cleft where it positions a flexible loop (residues 197–224) that has been shown to contact C34 and the Pol III-specific TFIIIB subunit Bdp1\cite{13,26} before folding back into a helix (residues 230–240; Extended Data Fig. 8a). Deleting the five residues (R226, L227, T228, G229, S230) leading into this helix produces a terminator read-through phenotype in *S. cerevisiae*\cite{3}, and in *Schizosaccharomyces pombe* the corresponding region was identified as a hotspot for terminator read-through mutations\cite{40}. The same residues have also been cross-linked to C128 (ref. 13) and in the cryo-EM structure are packed onto a section of the C128 lobe that when deleted also results in a termination read-through phenotype\cite{41}. In addition, these five residues are in close proximity to the non-template DNA strand that is flexible in the Pol III structure, but was only identified in both apo Pol III structures.
where it occupies a cleft between Rpb5 and the C160 funnel as observed at a low density threshold (Extended Data Fig. 7c). The position of the C11 C-terminal domain is far away from the position of the corresponding A12.2 C-terminal domain, suggesting that the C11 C-terminal domain is mobile and only temporally recruited to the catalytic centre (in analogy to TFIIS in Pol II). A long linker (residues 37–61) that connects both domains presumably accounts for the required mobility (Fig. 4b). C11 mutations at the extended interface between C37 and C11 N-terminal domain induce terminator read-through transcripts, (Fig. 4b). C11 mutations at the extended interface between C37 and C11 N-terminal domain induce terminator read-through transcripts, reflecting the involvement of the flexibly linked N- and C-terminal domains of C11 in two termination-related, yet distinct activities.

**Pol III stalk relays conformational changes**

The Pol III stalk subunits C25 and C17 are homologous to Pol II Rpb7–Rpb4 and Pol I A43–A14 (refs 7, 36). Consistent with previous low-resolution electron microscopy studies12,21,26, the Pol III cryo-EM structure confirms that the closed HDRC domain fold observed in the *S. pombe* C25–C17 heterodimer45 (PDB 3ayh) describes the conformation of the stalk on active Pol III most appropriately. Furthermore, we see a tight interaction network of the Pol III stalk and the C160 N- and C-terminal extensions, the latter contacting and positioning the C17 HDRC domain on C25 (Extended Data Fig. 8c). This leads to a tight anchoring of the stalk to the core, which is additionally strengthened by the Pol III-specific helix of C25 that extends from the stalk and contacts the clamp.

Remarkably, the open and closed clamp conformations of apo Pol III (Extended Data Fig. 6b) demonstrate two structurally distinct conformations of the stalk, the clamp head and the heterotrimer (Fig. 5). A flexible clamp has been also reported in the bacterial RNA polymerase, where it was shown that the clamp is predominantly open in the unbound conformation, then closes during initiation and elongation46. Furthermore, the archaeal RNA polymerase and Pol II contain a flexible clamp and in both systems the status of the clamp is associated with the stalk30,34,47. In contrast to archaeal RNA polymerase and Pol II, in Pol III the clamp movement is less pronounced, resulting in a narrower Pol III cleft compared to other RNA polymerases even in the open clamp conformation (Extended Data Fig. 6c, d). Here, we show both clamp conformations in Pol III visualized from the same sample and speculate that a moving stalk in Pol III can mediate the observed conformational changes. The open clamp conformation opens the cleft, the clamp head and the heterotrimer. Further, in Pol III the clamp movement is less pronounced, resulting in a narrower Pol III cleft compared to other RNA polymerases even in the open clamp conformation.
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Electron microscopy. For 5 min. The samples were analysed on a denaturing polyacrylamide gel (17% EM-buffer with additional 10 mM MgCl2 at 28 °C for 20 min. The reaction was used as template. An excess of transcription bubble was incubated with Pol III in Pol III complexes were then diluted 5 fold for grid freezing. To form the Pol III elongation complex, the buffer was exchanged to EM-buffer and 5 mM MgCl2; were added. Pol III was then diluted to 1 mg mL−1 and incubated with 5× excess of transcription bubble for 1 h at 7 °C. The elongating Pol III complexes were then diluted 5 fold for grid freezing.

RNA extension assay. Labelling of the RNA and annealing was performed as previously described except that the full transcription bubble (see above) was used as template. An excess of transcription bubble was incubated with Pol III in EM-buffer for 1 h at 4 °C. The RNA elongation was initiated by addition of NTPs used as template. An excess of transcription bubble was incubated 4 °C. Finally, 5 mM MgCl2 and 10 mM DTT were added to the annealed transcription bubble.

Pol III was purified endogenously from Saccharomyces cerevisiae as previously described. For apo Pol III, the buffer was exchanged to EM-buffer (15 mM Tris pH 7.5, 150 mM (NH4)2SO4, TempRecommended). Template-free cDNA templates were heated to 95 °C and cooled to room temperature allowing formation of the 11-nucleotide mismatch double-stranded DNA. In a second step, RNA was added, the mixture heated to 45 °C, and slowly cooled to 4 °C. Finally, 5 mM MgCl2 and 10 mM DTT were added to the annealed transcription bubble.

The procedure deblurs images by particle-based motion correction and minimizes processing routine (including the 'particle polishing' step) using all seven frames. The resulting frame stacks and total exposure images obtained two reconstructions, class 1 ('closed clamp' apo Pol III) with a resolution of 4.6 Å and class 2 (open clamp) apo Pol III1 at 4.7 Å. For the elongating Pol III, class 1 showed improved alignment and yielded a map with increased resolvability compared to the 75,751 particle reconstruction, whereas class 2 showed no apparent conformational difference to class 1, but showed reduced resolvability, corresponding to less well aligned particles. Class 1 was subsequently refined to a resolution of 3.9 Å according to FSC0.143 criterion. In order to estimate resolution, we compensated mask effects by performing the high-resolution noise subtraction procedure within RELION (Extended Data Fig. 1c). The resulting maps were sharpened by using an automatically calculated B-factor of 136 Å² and 140 Å² for class 1 and 2 of apo Pol III and −100 Å² for elongating Pol III, respectively.

Local resolution was assessed using the tool RESMAP (Extended Data Fig. 1c). The elongating Pol III map shows local resolution beyond 3.5 Å in the core and generally a uniform distribution of resolution between 3.5–4.6 Å for all subunits except the C34 WH3 (Extended Data Fig. 1c, Extended Data Table 2). Both apo Pol III maps show stronger resolution differences between the core and peripheral subunits due to mobility of the heterotrimer and the stalk.

Model building and localization of subunits in the elongating Pol III density. The building of initial models into the experimental density map was guided by homology models of Pol III subunits and crystal structures of archaeal RNA polymerase (PDB 4abc), Pol I (PDB 4c3i), Pol II (PDB 1wcm) as well as available crystal structures for the stalk (PDB 2ckz, 3ayh), the C82 homologue hRPC62 (PDB 2xub) and the TFIIF heterodimer (PDB 1f3u). All homology models were built using MfoldExplorer59, HHsearch57 and Modeller58. Homology models of subunits C160 and C128 were built based on the Pol II structure, C11 based on the A12.2 subunit of Pol I, C82 on hRPC62 (PDB 2xub) and C34 WH3 domain based on an unrelated WH domain of highest sequence similarity (PDB 1lfd). The homology model of the stalk (C17–C25 dimer) was built based on the structures of C17–C25 dimer from O. nova1 (PDB 2ckz) and the HRDC (helicase and RNaseD C-terminal) domain of C17 taking the domain orientations from S. pombe C17–C25 structure (PDB 3ayh) that improved the fit with the electron microscopy map.

Manual model building and local real-space refinement was performed in COOT60. For initial model building, the homology models were used to assemble a 15-subunit Pol III model consisting of all subunits except C34 and C31 which together with the above-mentioned crystal structures was placed into the elongating Pol III map by rigid body fitting. The fitted structures of individual subunits were used to aid de novo model building and re-building of reference models. Additionally, Xlink Analyzer60 was used to display available lysine-lysine crosslinks and photo-crosslinks46 on the Pol III model, which allowed us to assign several peripheral densities to subunits C31, C34, C37 and C53. Our current models of apo and elongating Pol III account for most features observed in the experimental electron microscopy density maps, except for a few short stretches of density where unambiguous assignment to a subunit was not possible and two strong density peaks observed in both apo Pol III reconstructions and located close to the active site. We speculate that these densities might arise from variations in sample preparation and correspond to bound nucleotides and metal ions, although one of them could also result from a conformational switch of the rudder. There are four distinct Rhp11 binding sites located in the Pol III core subunits C160 (2), C128 (1), C11 (1), ABC103 (1) and ABC106 (1). We accounted for metal binding at these sites where the experimental density sufficiently supported the placement of metal ions.

To identify the position of the C34 WH3 domain, the homology model was fitted into the full density map at 5.5 Å using a systematic global search with the ‘Fit In Map’ tool of UCSF Chimera61. The fitting was performed using 100,000 random initialplacements. These fits were clustered (leading to 30,000 unique fits) and scored by the normalized cross-correlation coefficient with the density map. The statistical significance of the scores was assessed as previously described62. In order to fit specific WH domains of C34 WH3 into a stretch of density unassigned by any other subunit next to subunit C82. The other significant fits corresponded to regions occupied by WH domains of C82 thus validating the ability of the systematic fitting procedure to locate WH domains in the experimental density. Although the WH1 and WH2 domains of C34 also fitted to this unassigned region, the positioning of WH3 domain was validated by cross-links to C82 and Consurf63 that revealed a conserved surface of the WH3 domain towards C82 in contrast to C34 WH2 and WH1.
resolution observed for different parts of the model, we calculated the local resolution distribution using RESMAP. We then computed a local mean resolution for each subunit by averaging all RESMAP voxels contained in a low-pass filtered volume generated from the model coordinates of this subunit (Extended Data Table 2). Segments comprising all voxels including and extending 3.5 Å outwards of the model coordinates were then carved from the elongating Pol III density map. A uniform isotropic B-factor of 50 Å² was assigned to all model atoms at the start of the refinement. The individual map segments and the corresponding coordinate models were centred in a cubic box of P1 symmetry with a cell dimension of 260.16 Å (240 × 240 × 240 pixels) to allow uniform grid sampling of model and experimental maps at the experimental pixel size. Coordinate refinement was performed by geometry-restrained real-space refinement based on gradient-driven minimization of a combined map and restraint target as implemented in ccxtb/PHENIX up to the computed local mean resolution of each subunit. Grid searches were employed for automated identification of optimal refinement weights to balance the relative contribution of geometry and experimental restraints. Additional restraints were applied between hydrogen-bonded atoms in secondary structure elements. For the DNA–RNA of the transcription bubble, an initial model was obtained from the structure of a Pol II–Mediator complex (pdb 4v1n) and restraints were generated with the LibG program distributed with CCP4/REFMAC (Fei Long, unpublished). The geometries of Zn²⁺ binding sites in C160, C128, C11 and ABC10(3) and ABC10(6) were restrained to reference values according to Harding,66,67.

Each round of model optimization was evaluated by computing the real-space cross-correlation (RSCC) between experimental map and a map calculated from the model coordinates. To this end, model maps were generated by calculating B-factor-weighted structure factors from the model coordinates (using electron atomic form factors)68 and computing the inverse Fourier transform. We refined individual isotropic atomic displacement parameters (ADPs) by optimizing the real-space correlation between model and experimental map. ADPs were obtained by fitting to the computed RSCC profile. The resulting B-factor distributions correlate well with local resolution estimates, with lowest B-factors in the Pol III core and higher B-factors in the peripheral subunits of the stalk (C25–C17) and the C53–C37 and C82–C34–C31 subcomplexes (Extended Data Fig. 4).

Automated real-space refinement cycles were alternated with manual model building in COOT59. Manual model building was aided by experimental maps filtered at lower resolution and experimental maps that were re-sharpened by applying the structure factor amplitudes computed from the current model69,70, which leads to decreased noise levels and increased visibility of structural features in some regions of the map, in particular in the peripheral Pol III subunits. Taking into account the refined B-factor distribution in the model, this map manipulation enhances structural features due to better correction of the Fourier amplitude fall-off resulting from experimental factors, but does not introduce model bias since experimental phases are used.

Following initial refinement of all individual subunits against the corresponding map segments, the entire elongating 17-subunit Pol III complex and the DNA–RNA transcription bubble was assembled and subjected to additional refinement against the full map reconstruction filtered at 3.9 Å resolution to account for inter-subunit interactions. The DNA–RNA model was not further refined at this stage. Since refinement target weights are optimized based on overall map resolution, restraint weighting can be suboptimal in regions of the map with a resolution lower than the average map resolution. To compensate for this effect in the full map refinement, we restrained peripheral subunits by an additional harmonic potential applied to the Cα positions, which was scaled relative to the average local resolution in this map region calculated as described above.

The Fourier shell correlation between model map and half-set 3D reconstructions was used to assess the possibility of overfitting66. Briefly, the atoms in the model were randomly displaced by up to a maximum of 0.5 Å, followed by five cycles of real-space refinement against one of the half maps (work map) using the same protocol as described above. We then computed the FSC between the resulting model and the work map (FSCwork) as well as the cross-validated FSC between the refined model and the other half map not used in refinement (FSCcv). The close agreement between both curves for the elongating Pol III model indicates that no overfitting took place (Extended Data Fig. 4). We attribute the minor discrepancies in particular at low-resolution between FSCwork/FSCcv and the FSC between two independent half sets (Extended Data Fig. 1) to the incomplete model and solvent effects.67,71 The very good correlation in the high-resolution region beyond 6 Å provides support that our model accounts well for the defined density features observed in the experimental map. The quality of the final model was validated using MOLPROBITY72 and was found to range in the top percentiles for the corresponding resolution range.

Model building and refinement of apo Pol III (closed/open clamp conformation). The initial model for apo Pol III in the closed and open clamp conformations was obtained from the model coordinates of the individual subunits refined against the higher resolution map of elongating Pol III. The 10-subunit core, the C35–C37 heterodimer, the C82–C34–C31 heterotrimer and the stalk (C25–C17) were placed by rigid body fitting into the respective density maps. The C-terminal TFFS-like domain of C11, not visible in the elongating Pol III structure, was added to complete the model. The complete model was then refined against the respective maps filtered at 4.6/4.7 Å resolution. Sharpened maps for both closed and open clamp conformations of apo Pol III were used as the refinement target. Restraints on secondary structure and metal binding geometries were implemented as described for elongating Pol III. Automatic real-space refinement was performed as described above, with additional harmonic restraints on Cα positions weighted by the average local resolution to account for resolution anisotropy observed in both maps. Manual adjustments to the model were done in COOT; model assessment and validation were performed as described above. A summary of the refinement statistics for all three models can be found in Extended Data Table 1.

Figure preparation. Graphs and figures were prepared using Xmgrace, PyMol73 and UCSF Chimera.58
Extended Data Figure 1 | Pol III processing pipeline, Fourier-shell correlation curves and local resolution assessment. 

**a** Exemplary micrograph of elongating Pol III. All micrographs were low-pass filtered for particle picking. 

**b** General processing pipeline. The orange boxes display micrograph number and particles for elongating Pol III (left) and apo Pol III (right). The middle panel shows the general workflow that was followed for both data sets. For elongating Pol III (bottom left), a local classification step yielded one class with 49,543 particles (purple) that was subsequently refined and post-processed. For apo Pol III (bottom right), local classification diverged into two classes (purple) with 68,818 particles and 52,423 particles that were subsequently refined and post-processed. 

**c** Fourier-shell correlation (FSC) and local resolution assessment with RESMAP⁵⁵. All FSC calculations were performed with two independent half maps using RELION’s masking procedure⁵⁵. The resolution for the elongating Pol III cryo-EM map (top panel) is 3.9 Å according to the FSC 0.143 criterion, indicated by the black dashed line. The two apo Pol III cryo-EM reconstructions have a resolution of 4.6 Å (closed clamp Pol III, middle panel) and 4.7 Å (open clamp Pol III, bottom panel) according to the FSC 0.143 criterion. Local resolution is displayed on the post-processed full maps (first image column on the right) and a cross-section representation (second image column on the right). In both apo Pol III reconstructions, the peripheral subcomplexes show a strong decay in resolution compared to the core. In the elongating Pol III reconstruction, the resolution is more uniformly distributed, indicating stabilization of peripheral subunits.
Extended Data Figure 2 | Representative sections of the cryo-EM density for elongating Pol III. 

**a**, Cross-section of elongating Pol III in ribbon and stick representation, embedded in the experimental density at 3.9 Å, displayed in dark blue. **b**, Section displaying the core subunits C160 (grey) and C128 (wheat) shown in stick and ribbon representation. The experimental density of the core (dark blue) is well defined and has been filtered at 3.5 Å resolution for display. **c**, Section of stalk subunits C25 (blue) and C17 (pink). The estimated local resolution in this part is lower compared to the core (Extended Data Table 2). In **d** and **e**, the cryo-EM density is shown at 3.9 Å resolution. **d**, Section showing subunits C53 (blue) and C37 (purple). **e**, Close-up view of C82-WH1 (brown), C82-WH2 (green) and C31 (yellow) interface.
Extended Data Figure 3 | Comparison of electron microscopy densities with X-ray electron densities for shared subunits ABC23 (Rpb6) and ABC14.5 (Rpb8). Top left shows Pol III in front view, a stretch in ABC23 (cyan) and ABC14.5 (green) is coloured. The red boxes indicate the regions that are enlarged in the neighbouring panels. Corresponding density is displayed in tungsten. Models of Pol II and Pol I at nominally higher resolution are available, but for better comparison models in a similar resolution range are shown. For the $2F_o - F_c$ electron density maps obtained by X-ray crystallography a threshold of 1$\sigma$ was used for display. The top right shows three close-up views of the shared subunit ABC23 from elongating Pol III, Pol II (PDB 1wcm) and Pol I (PDB 4c3j). The bottom panels show 6 strands of shared subunit ABC14.5. Front view of the $\beta$-sheet and orthogonal views of individual strands in elongating Pol III, Pol II (PDB 1wcm) and Pol I (PDB 4c3j).
Extended Data Figure 4 | Model validation and temperature factor distribution of atomic models. a–c, FSC curves calculated between the refined atomic model and the half map used in refinement (FSC\textsubscript{work}) are shown in blue, those calculated between the refined atomic model and the second half map not used for refinement (FSC\textsubscript{test}) in red. Vertical lines mark the regular FSC 0.143 cutoff and the resolution target used in refinement as shown. Close agreement between FSC\textsubscript{work} and FSC\textsubscript{test} and the absence of a sharp drop beyond the refinement target resolution indicate that no overfitting took place. The respective FSC between the refined atomic model and the map obtained from 3D reconstruction using the entire data set (FSC\textsubscript{ref}) is also shown (black). d, e, Atomic B-factor distributions mapped on ribbon representations of elongating and apo Pol III. The overall distribution and relative differences between core and peripheral subunits for the different models correlate well with the distribution of local resolution (Extended Data Fig. 1).
Extended Data Figure 5 | Pol III-specific features of subunits C160 and C128 and comparison to the homologous Pol II and Pol I subunits.

a, Top (left) and front (right) view of Pol III, with subunits C160 and C128 displayed in ribbon representation and the other subunits in surface representation (grey). Coloured stretches highlight characteristic features denoted in b. b, Bar diagram shows the domain organization of Pol III C160. Arrows and corresponding numbers below the bar diagram indicate insertions and deletions of five or more residues in Pol III relative to Pol II subunit Rpb1 as indicated by structure-based alignment. Coloured regions are also shown in Pol III subunit C160 (lower panel, left) and in a. Lower panel middle and right show Pol II Rpb1 and Pol I A190 subunits, respectively. c, Same as in b for Pol III subunit C128. Insertions and deletions compared to Pol II subunit Rpb2 are displayed in the box diagram.
Extended Data Figure 6 | Open and closed clamp conformation in Pol III compared to other RNA polymerases. a, Top view of aligned elongating Pol III and apo Pol III (left panel, closed clamp; middle panel, open clamp) and both closed clamp and open clamp apo Pol III conformations (right panel). r.m.s.d. values (core-heterodimer:all) for elongating Pol III – apo Pol III (closed clamp) (0.43 Å, 4,813 Cα atoms aligned), elongating Pol III – apo Pol III (open clamp) (0.71 Å, 4,795 Cα atoms aligned) and both apo Pol III open and closed clamp (0.71 Å, 4,829 Cα atoms aligned) demonstrate the similarity between closed clamp apo Pol III and elongating Pol III conformations. b, Schematic representation of Pol III in top view showing the conformational changes of clamp head, heterotrimer and stalk. The closed clamp conformation (elongating Pol III and closed clamp apo Pol III) is displayed in red, the open clamp conformation (open clamp apo Pol III) in green. The DNA–RNA duplex is shown in blue, the core and heterodimer in grey. c, Front view on open and closed clamp conformations in other RNA polymerases. The closed clamp state (green) and open clamp state (red) is indicated for archaeal polymerase (left panel, PDB 4ayb and 4qiw), for Pol II (middle panel, PDB 1wcm and 1twf) and for Pol III (right panel). Green and red angles describe the cleft opening in the closed and open clamp conformations. Black arrows and corresponding values indicate the relative distance of the subunits between the two conformations. d, Front view of apo Pol III closed clamp (left panel), apo Pol III closed clamp vs apo Pol II (middle panel, Pol II (PDB 1wcm) in red) and apo Pol III closed clamp vs apo Pol I (right panel, Pol I (PDB 4c3i) in blue). The cleft opening is indicated by a dashed line and the Cα-Cα distance across the cleft (black for Pol III, red for Pol II and blue for Pol I). However, some of the observed differences in cleft width between Pol I, Pol II and Pol III might also reflect differences between conditions of cryo-EM and crystal structures as well as different packing contacts in the crystals.
Extended Data Figure 7 | Pol III-specific subunits C82, C31 and C11.

a, Left panel: overall surface representation of Pol III with the C82–C34–C31 heterotrimer in ribbon representation. Right panels: two enlarged and orthogonal views of the region marked with a dotted black square. In subunit C82 WH4 inserts in the DNA binding cleft passing through a canyon in the clamp head. WH2 and WH3 extensions reach over the clamp head and are positioned in close proximity to downstream DNA.

b, Ribbon model of Pol III fitted into the electron microscopy density of the open clamp apo Pol III filtered at 6 Å resolution. For C31, additional density is visible in the cavity between the stalk and the heterotrimer, as shown in the top right panel. The described densities are also present in the closed clamp apo Pol III and the elongating Pol III reconstructions. No attempts were made to fit atomic models into these densities.

c, Electron microscopy density of the C11 TFIIS-like domain at 6 Å resolution as observed in the open clamp apo Pol III reconstruction. The left panel shows a side view of Pol III, the middle and right panels show close-ups at two different density thresholds.
Extended Data Figure 8 | C53–C37 heterodimer and stalk subunits C25–C17. a, Visualization of the photo-crosslinks between C53–C37 heterodimer and subunit C128. Pol III is shown in surface, C53–C37 and C11 in ribbon representation. In addition, the C128 lobe is shown in cartoon representation (small inset). Purple spheres on the lobe indicate residues that photo-crosslink to C37 (ref. 13), beige spheres on C37 indicate residues that photo-crosslink to C128 (ref. 13). The dashed line marks the tentative path of the non-template DNA strand. The experimental photo-crosslinks fit well to the cryo-EM structure. The C37 loop is disordered between Glu196 and Asn225, although photo-crosslinks indicate that this region is in close proximity to the lobe and the non-template DNA strand. b, Bottom view of Pol III in surface representation, with C53–C37 and C11 shown in ribbon representation. The black dotted square indicates the enlarged area in the centre of the image (small inset). The red density (shown at 4.5 Å) was not of sufficient quality to build an atomic model. However, photo-crosslinks from C37 and C128 to C53 (blue spheres on C37 ribbon and C128 surface mark crosslink positions) indicate that C53 N-terminal residues are located in this region. c, Stalk anchoring with C160 extensions. Top view (left panel) and bottom view (right panel) of the stalk subunits C17 (magenta), C25 (blue) and the C160 extensions (grey). Electron microscopy density corresponding to the C160 N- and C-terminal extensions is shown in tungsten blue. Individual entities and subunits are labelled.
Extended Data Table 1 | Refinement statistics

|                           | Elongating Pol III | Apo Pol III (closed clamp) | Apo Pol III (open clamp) |
|---------------------------|--------------------|-----------------------------|---------------------------|
| **Model composition**     |                    |                             |                           |
| No. of chains             | 17+3               | 17                          | 17                        |
| Non-hydrogen atoms        | 39276              | 38677                       | 38427                     |
| Protein residues          | 4839               | 4882                        | 4845                      |
| Nucleic acid              | 47                 | --                          | --                        |
| Ligand (Zn$^{2+}$)        | 6                  | 6                           | 6                         |
| **Refinement**            |                    |                             |                           |
| PDB ID                    | 5fj8               | 5fj9                        | 5fja                      |
| Resolution (Å)            | 260.2-3.9          | 260.2-4.6                   | 260.2-4.7                 |
| Map sharpening B-factor (Å$^2$) | -100            | -136                        | -140                      |
| Average B-factor (Å$^2$)  |                    |                             |                           |
| **Protein**               | 64.9               | 161.8                       | 182.0                     |
| **Nucleic acid**          | 76.3               | --                          | --                        |
| **Ligand (Zn$^{2+}$)**    | 58.6               | 62.4                        | 107.9                     |
| Molprobity score          | 2.6                | 2.58                        | 2.48                      |
| Clashscore (all atoms)    | 14.5               | 13.41                       | 13.84                     |
| Rotamer outliers (%)      | 2.00               | 2.08                        | 1.5                       |
| Ramachandran statistics   |                    |                             |                           |
| Favored (%)               | 82.46              | 82.52                       | 82.58                     |
| Disallowed (%)            | 1.15               | 1.23                        | 1.03                      |
| RMS (bonds, Å)            | 0.0032             | 0.0032                      | 0.0030                    |
| RMS (angles, °)           | 1.03               | 1.01                        | 0.89                      |

**Nucleic Acid**

Correct sugar puckers (%) | 91.5 | -- | -- |
Good backbone conform. (%) | 68.7 | -- | -- |
Extended Data Table 2 | Model statistics for elongating Pol III

| Subunit | Protein | Chain ID | Mw (kDa) | No. of residues | Residues built | Chain breaks | All-atom clashscore | Molprobity score | Average B-factor (Å²) | Local resolution (Å) |
|---------|---------|----------|----------|-----------------|-----------------|--------------|---------------------|-----------------|---------------------|---------------------|
| Core    | RPC1    | C160     | A        | 162.3           | 1460            | 1422 (97.4%) | 3                   | 10.42           | 2.24                | 56.35               | 3.8                  |
|         | RPC2    | C128     | B        | 129.5           | 1149            | 1115 (87.0%) | --                  | 12.61           | 2.40                | 60.10               | 3.8                  |
|         | RPC40   | AC40     | C        | 37.7            | 335             | 335 (100%)   | --                  | 10.60           | 2.40                | 60.42               | 3.8                  |
|         | RBP8    | ABC27    | E        | 25.1            | 215             | 215 (100%)   | --                  | 16.07           | 2.81                | 67.97               | 4.1                  |
|         | RBP6    | ABC23    | F        | 17.9            | 155             | 83 (53.5%)   | --                  | 3.67            | 2.34                | 57.97               | 3.6                  |
|         | RBP8    | ABC14.5  | H        | 16.5            | 148             | 140 (95.9%)  | 1                   | 9.05            | 2.15                | 62.64               | 4.2                  |
|         | RPC11   | C11      | I        | 12.5            | 110             | 49 (39.1%)   | --                  | 16.00           | 2.73                | 73.40               | 4.3                  |
|         | RPC10   | ABC10β   | J        | 8.3             | 70              | 68 (97%)     | --                  | 5.40            | 2.39                | 56.79               | 3.6                  |
|         | RPC19   | AC19     | K        | 16.1            | 142             | 101 (71.1%)  | --                  | 8.85            | 2.41                | 59.38               | 3.7                  |
|         | RPC10   | ABC10ct  | L        | 7.2             | 70              | 46 (65.7%)   | --                  | 12.13           | 2.65                | 67.80               | 4.2                  |
| Stalk   | RPC9    | C17      | D        | 18.6            | 161             | 119 (73.9%)  | 2                   | 15.82           | 2.51                | 73.39               | 4.4                  |
|         | RPC8    | C25      | G        | 24.3            | 219             | 191 (90.1%)  | 2                   | 16.86           | 2.67                | 70.03               | 4.3                  |
| Heterotrimer | RPC3 | C32      | O        | 74.0            | 654             | 539 (82.4%)  | 2                   | 15.99           | 2.86                | 73.08               | 4.5                  |
|         | RPC8    | C34      | P        | 36.1            | 317             | 89 (28.1%)   | 2                   | 26.41           | 3.54                | 75.84               | 5.3                  |
|         | RPC7    | C31      | Q        | 27.7            | 251             | 63 (25.1%)   | 2                   | 10.13           | 2.13                | 76.61               | 4.6                  |
| Heterodimer | RPC5 | C37      | M        | 32.1            | 282             | 184 (58.2%)  | 1                   | 11.72           | 2.59                | 74.18               | 4.3                  |
|         | RPC4    | C33      | N        | 46.7            | 422             | 110 (26.1%)  | 1                   | 12.10           | 2.98                | 71.51               | 4.2                  |
| RNA     | RNA     | R        |          | 18              |                | 9 (50.0%)     | --                  |                 |                     | 75.00               | 3.9                  |
| DNA Non-Template | S |          |          | 38              |                | 15 (50.0%)  | --                  |                 |                     | 75.91               | 3.9                  |
| DNA Template | T   |          |          | 38              |                | 23 (60.5%)  | --                  |                 |                     | 78.03               | 3.9                  |