Supporting Information

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SI Materials and Methods

Plasmids and Yeast Strains. *Saccharomyces cerevisiae C160* (*RPO31*) and *C82* were separately cloned into the high copy number 2 micron vector pRS425 (2 μ, *LEU2*) (1) and both genes were driven by the yeast *ADH1* promoter. *C160* was 13Myc-tagged and *C82* was V5-tagged at their C termini, yielding pCP1 (*C160*-Myc, 2 μ, *LEU2*) and pYL3 (*C82*-V5, 2 μ, *LEU2*). The pRS425-based C160 and C82 plasmids were used to generate plasmids containing TAG-codon substitutions (generally referred to as amber plasmids) by oligonucleotide-directed phagemid mutagenesis. The amber plasmids were applied in the subsequent yeast transformation to provide strains for nonnatural amino acid *p*-benzoyl-*L*-phenylalanine (BPA) insertions into C160 and C82. For *C160* and *C82* mutagenesis studies, genes were cloned into vector pRS315 with a single HA epitope tag in the C-termini and driven by their native promoters, yielding pCP2 (*C160*-HA, *ars cen*, *LEU2*), pYL4 (*C82*-HA, *ars cen*, *LEU2*).

To conduct plasmid shuffle to generate strains bearing amber or mutant plasmids, yeast shuffle strains were all derived from BY4705 (2) with the chromosomal deletion of individual gene by the KanMX4 cassette to create the following strains: CPy1 (*MATα ade2::his3G his3Δ200 leu2Δ0 met15Δ0 lys2Δ0 trp1Δ63 ura3Δ0* [*C160::KanMX4*] *C160*-pRS316 (*URA3*$^+$)) and YLy2 (*MATα ade2::his3G his3Δ200 leu2Δ0 met15Δ0 lys2Δ0 trp1Δ63 ura3Δ0* [*C82::KanMX4*] *C82*-pRS316 (*URA3*$^+$)). The procedure to site-specifically insert BPA (Bachem) in yeast and preparation of yeast whole-cell extract was as described previously (3).

PIC Isolation and BPA Photo-Cross-Linking. Pol III PIC isolation with immobilized DNA templates from whole-cell extract and the subsequent BPA cross-linking procedures were as described previously (3). Briefly, 5′-biotinylated DNA fragments containing SUP4 tRNA and U6 snRNA gene sequences were used in the immobilized template assay. For a typical BPA photo-cross-linking experiment, 800 μg of whole-cell extract was incubated with 4 μg of DNA template immobilized on Streptavidin magnetic beads (Dynal). After washing three times with transcription buffer [20 mM Hepes (pH 7.9), 80 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, and 2% (vol/vol) glycerol] containing 0.01% Tween 20, reactions were divided into two fractions, one for UV irradiation (+UV) and the other as the control (−UV). UV irradiation was conducted with a Spectrolinker XL-1500 UV oven (Spectronics) with a total energy of 7,500 μJ·cm^{-2}. The isolated PICs were then resuspended in NuPAGE loading buffer (Invitrogen) for SDS/PAGE/Western blot analysis. Immunoblotting results were visualized by the LI-COR Bioscience Odyssey infrared imaging system.

In Vitro Transcription. PIC isolated by immobilized template assay as described above was resuspended in 10 μL of transcription buffer containing 200 ng α-amanitin, 4 units of RNase inhibitor (Roche), and 1 mM DTT. Arrested ternary complex was formed by a 15-min incubation in the presence of 250 μM ATP, 250 μM UTP, and 250 μM CTP and transcription was allowed to resume at 30 °C for 20 min by incubation with 25 μM GTP, 0.16 μM [α-^{32}P]GTP (3,000 Ci/mmol), and 0.3 mg/mL heparin to prevent reinitiation (single-round transcription) or with 25 μM GTP and 0.16 μM [α-^{32}P]GTP (3,000 Ci/mmol) (multiple-round transcription). Transcription reactions were stopped by adding 180 μL of 0.1 M sodium acetate, 10 mM EDTA, 0.5% SDS, and 200 μg/mL glycogen and extracted by phenol/chloroform. After ethanol precipitation, transcripts were analyzed on 8% (wt/vol) denaturing urea polyacrylamide gels and quantified by PhosphorImager analysis.

Pol III Immunoprecipitation. Yeast cell extracts containing the indicated mutations in C160 or C82 were used for immunoprecipitation. Generally, whole-cell extract (800 μg) containing Flag-tagged Pol III subunit was mixed with 50 μL of anti-Flag (M2; Sigma-Aldrich) agarose in extract dialysis buffer [20 mM Hepes (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, and 20% (vol/vol) glycerol] and incubated at 4 °C for 1 h. The immunoprecipitated Pol III was washed three times with 400 μL of extract dialysis buffer. The bound fraction was directly subjected to SDS/PAGE analysis without elution. Proteins were transferred to polyvinylidene fluoride membranes and probed with the indicated antibodies.

C82 Purification and FeBABE Conjugation. *C82* was cloned in the pET21a (Novagen) vector with a V5 tag followed by six histidines in the C terminus (pET21a-C82-V5-His_6, pCW8), and the plasmid was transformed into an *Escherichia coli* BL21 (DE3) strain (Novagen) that also contains the plasmid pG-KJE8 (Takara) for coexpression with GroEL/ES. The cells were induced by 0.4 mM isopropyl β-d-1-thiogalactopyranoside and 5 ng/mL tetracycline and grown at 13 °C overnight. Cells from a 10-L culture were harvested by centrifugation and resuspended in 60 mL of lysis buffer [20 mM Tris·Cl (pH 7.5), 150 mM NaCl, 10% (vol/vol) glycerol, and 30 mM imidazole]. After lysis by microfuildizer (Microfluidics), cell extract was clarified by centrifugation and was subsequently applied to affinity purification with Ni-Sepharose (GE Healthcare). C82 was eluted in elution buffer [20 mM Tris·Cl (pH 7.5), 150 mM NaCl, 10% (vol/vol) glycerol, and 250 mM imidazole] and the eluate was subsequently dialyzed against buffer A [20 mM Tris·Cl (pH 7.5), 50 mM KCl, and 10% (vol/vol) glycerol]. C82 was further purified by anion exchange chromatography (Mono Q 1 mL; GE Healthcare) with a linear gradient of 20 column volumes from 0 to 500 mM KCl in the buffer containing 20 mM Tris·Cl (pH 7.5) and 10% (vol/vol) glycerol. Pooled peak fractions containing C82 protein were concentrated and stored at −80 °C. All buffers were supplemented with 2 mM β-mecaptoethanol and phenylmethylsulfonyl fluoride (PMSF). For conjugation of the hydroxyl-radical–generating reagent FeBABE (*p*-bromoacetamido benzyl-EDTA, iron(III); Dojindo), all C82 protein variants were based on the noncysteine C82 with four endogenous cysteines changed to noncys residues as follows: Cys103Leu, Cys195Ala, Cys510Ala, and Cys568Ile. All of the expression and purification procedures of noncys and other single-cys variants were as described above.

C34 and C31 Purification. C34 and C31 were separately expressed as N-terminally $histidine_6$ SUMO-tagged fusion proteins in *E. coli* BL21 (DE3) RIL cells. Cells were induced by 0.4 mM isopropyl β-d-1-thiogalactopyranoside and grown at 25 °C for 6 h. For both C34 and C31, cells from 3 L of *E. coli* culture were harvested, lysed, and purified by Ni-Sepharose using the same buffers for C82 purification as described above. The proteins were dialyzed against buffer A to reduce the salt concentration. SUMO tag was subsequently removed by adding purified yeast Ulp1 (SUMO protease) to the concentration of 1.76 μg/mL for 1 h at room temperature. SUMO protease, SUMO tag, and uncleaved fusion proteins were removed by a second passage through Ni-Sepharose. C34 was further purified using a 5-mL HiTrap Heparin HP column (GE Healthcare) by a six-column volume linear gradient

from 100 to 600 mM KCl in the buffer containing 20 mM Tris·Cl (pH 7.5) and 10% (vol/vol) glycerol. C34 was eluted at ∼300 mM KCl. Similarly, C31 was purified over a HiTrap Heparin HP column using a 10-column volume linear gradient from 100 to 600 mM KCl and the proteins was eluted at 360 mM KCl. Eluted protein were concentrated and stored at −80 °C for further use.

Hydroxyl Radical Cleavage with C82-FeBABE Conjugate. In a typical FeBABE hydroxyl radical protein cleavage experiment, 400 μg of yeast whole-cell extract containing N-terminally HA_6-tagged C160 and the C82 deletion-mutant Δ(50-52) was incubated with 0.72 μg of C31, 0.94 μg of C34, and 2 μg of C82-FeBABE conjugate (130 nM) in a 200-μL reaction before PIC isolation with immobilized template assay. Pol III PICs were isolated with immobilized DNA template containing SUP4 tDNA gene. After washing to remove nonspecifically bound proteins, FeBABE cleavage assay was conducted as previously described (3). The C160 cleavage sites were determined by calculating the mobility shifts of the cleaved peptide fragments compared with the in vitro-translated N-terminally HA_6-tagged C160 peptide ladders. Detailed procedures were as previously described (3).

Pol III Preparation and Cross-Linking-MS Analysis. Pol III was purified as described (4), except for the final size-exclusion chromatography step. Purified Pol III (95 μL containing 100 μg of protein) was mixed with 25 mM DSS (Creative Molecules) dissolved in DMF to a final concentration of 1.2 mM and incubated for 30 min at 30 °C. The reaction was stopped by the addition of 1 M NH_4HCO_3 to a final concentration of 100 mM and incubation was followed for 15 min at 30 °C. To increase the cross-linking yield, Pol III was also cross-linked with DSS at a final concentration of 3.5 mM. To remove potential polymerase oligomers, which might have emerged during cross-linking, a final size-exclusion step was added to the cross-linking protocol. Size-exclusion chromatography after cross-linking was performed in a Superose 6 10/300 GL column with 40 mM Hepes (pH 7.8), 120 mM KCl, 100 μM $MgCl_2$, 10 μM $ZnCl_2$, 100 mM NH_4HCO_3, 10% (vol/vol) glycerol, and 5 mM DTT. Mass spectrometric analysis and database searching was carried out as described (5).

1. Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene* 110(1):119–122.
2. Brachmann CB, et al. (1998) Designer deletion strains derived from Saccharomyces cerevisiae S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 14(2):115–132.
3. Wu CC, Lin YC, Chen HT (2011) The TFIIF-like Rpc37/53 dimer lies at the center of a protein network to connect TFIIIC, Bdp1, and the RNA polymerase III active center. *Mol Cell Biol* 31(13):2715–2728.
4. Vannini A, et al. (2010) Molecular basis of RNA polymerase III transcription repression by Maf1. *Cell* 143(1):59–70.
5. Jennebach S, Herzog F, Aebersold R, Cramer P (2012) Crosslinking-MS analysis reveals RNA polymerase I domain architecture and basis of rRNA cleavage. *Nucleic Acids Res* 40 (12):5591–5601.

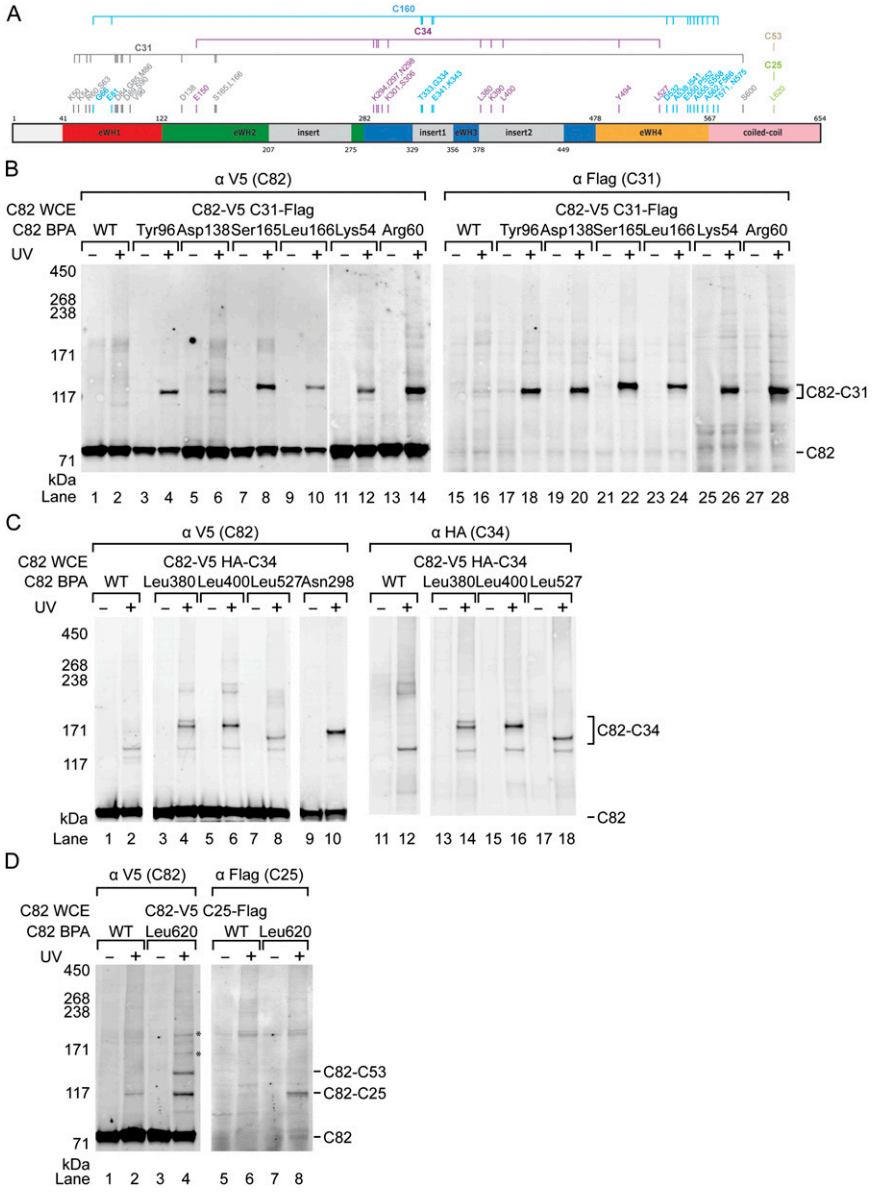

Fig. S1. C82 BPA photo-cross-linking (related to Fig. 2). (*A*) Schematic representation of BPA incorporation in C82 and cross-linked polypeptides. Domains homologous to hRPC62 are shown in red (eWH1), green (eWH2), blue (eWH3), orange (eWH4), and salmon (coiled-coil). The insertions in the eWH2 and eWH3 are in gray. Numbers labeled above the schematic bar indicate BPA-substituted residues that gave rise to cross-links. Color coding of each residue corresponds to the identity of cross-linked polypeptide as indicated above the horizontal connecting lines. (*B*) Representative C82–C31 cross-links in the eWH1 and eWH2. C82 and the cross-links were detected by anti-V5 antibody, and C31 in the indicated fusion bands was confirmed by second immunostaining to reveal Flag-epitope-tagged C31 (*Right*). (*C*) Representative C82–C34 cross-linking. (*D*) Confirmation of a C82–C25 cross-link. The Western blot shows that C82 Leu620-BPA cross-links with C53 and C25 simultaneously. Similar to the confirmation of above C82 cross-linked polypeptides, the Flag-epitope tagging method was applied to confirm both cross-linked proteins (*Right*). Asterisks indicate unidentified cross-links.

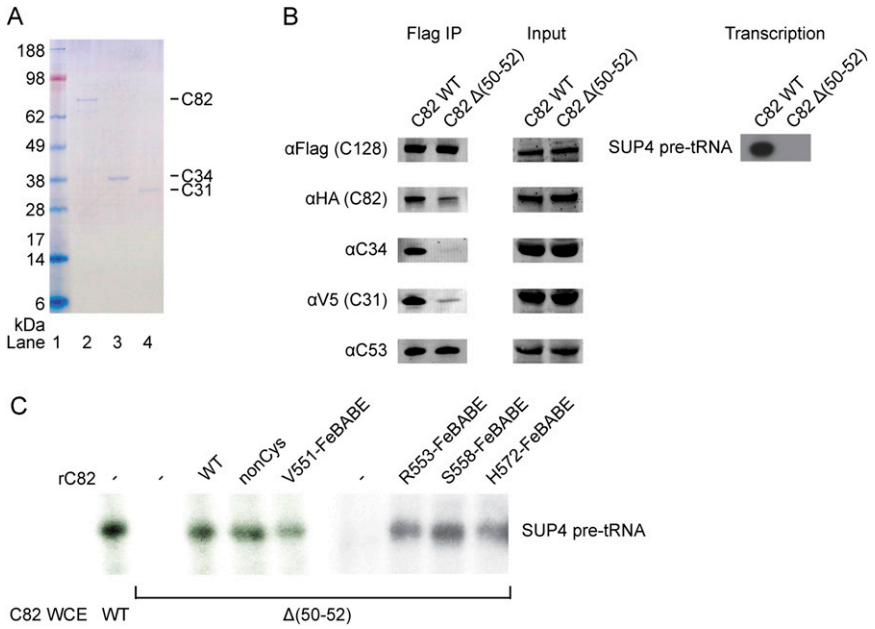

Fig. S2. Functional analysis of the recombinant C82/34/31 complex and C82 FeBABE conjugation (related to Fig. 3). (*A*) SDS/PAGE analysis of purified recombinant C82, C34 and C31. (*B*) Association of the C82/34/31 subcomplex with the Pol III core. The left panel demonstrates Western-blot analysis of Pol III components from coimmunoprecipitation assays using whole-cell extracts from yeast strains containing C82 WT or the internal deletion mutant Δ(50-52). Pol III was immunoprecipitated through C128-Flag (*Left*). The autoradiogram in the right panel shows in vitro transcription activity of WT and the mutant (SUP4 pre-tRNA). (*C*) Transcriptional activity analysis for C82-FeBABE derivatives. Whole-cell extracts (WCE) containing C82 WT or Δ(50-52) mutant are indicated below. The indicated C82-FeBABE derivative was added in the C82 Δ(50-52) WCE along with recombinant C34 and C31 to restore transcription activity compared with the wild-type WCE.

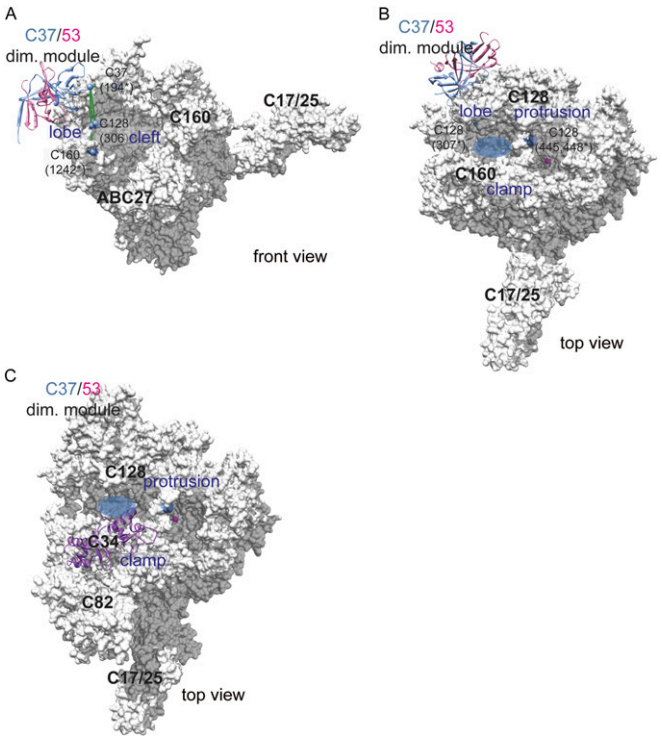

Fig. S3. Localization of the C37/53 subcomplex within Pol III and the model of C34 WH domains (related to Fig. 5). (*A*) Model of the C37/53 dimerization module in Pol III. The model of C37/53 dimerization module (C37, cyan ribbon; C53, pink ribbon) on the lobe domain of C128 is based on previous EM analyses and biochemical probing (1–4). Cross-linked C37/C160 intersubunit lysine pairs (connected with green lines) that further constrain the docking of the dimerization module are highlighted in light blue. (*B*) Localization of C37 C-terminal region within the active site cleft. C128 residues that cross-link to C37 C terminus are highlighted in light blue and labeled (C128 Lys445). Cross-linked lysines in C128 that do not have predictable structures in the Pol III core model, the nearest residues are colored and labeled with an asterisk (C128 Lys307* and Lys448*). The blue semitransparent oval in the active site cleft depicts the possible position of the C37 C-terminal region. (*C*) Colocalization of C34 WH domains and C37 C-terminal region in the active site cleft. As in *B*, the C37 C-terminal region is represented by a blue semitransparent oval. C34 WH domains (purple ribbon) are positioned above the cleft based on biochemical probing analyses in this study. C82 and Pol III core are depicted as white molecular surface. C128 Lys445 and Lys448 (colored in light blue) on the fork loop 2 cross-linked to the C37 C-terminal region.

1. Wu CC, Lin YC, Chen HT (2011) The TFIIF-like Rpc37/53 dimer lies at the center of a protein network to connect TFIIIC, Bdp1, and the RNA polymerase III active center. *Mol Cell Biol* 31 (13):2715–2728.
2. Vannini A, et al. (2010) Molecular basis of RNA polymerase III transcription repression by Maf1. *Cell* 143(1):59–70.
3. Fernández-Tornero C, et al. (2010) Conformational flexibility of RNA polymerase III during transcriptional elongation. *EMBO J* 29(22):3762–3772.
4. Jennebach S (2011) RNA polymerase I domain architecture and basis of rRNA cleavage. PhD dissertation (Ludwig-Maximilians-Universität München, Munich).

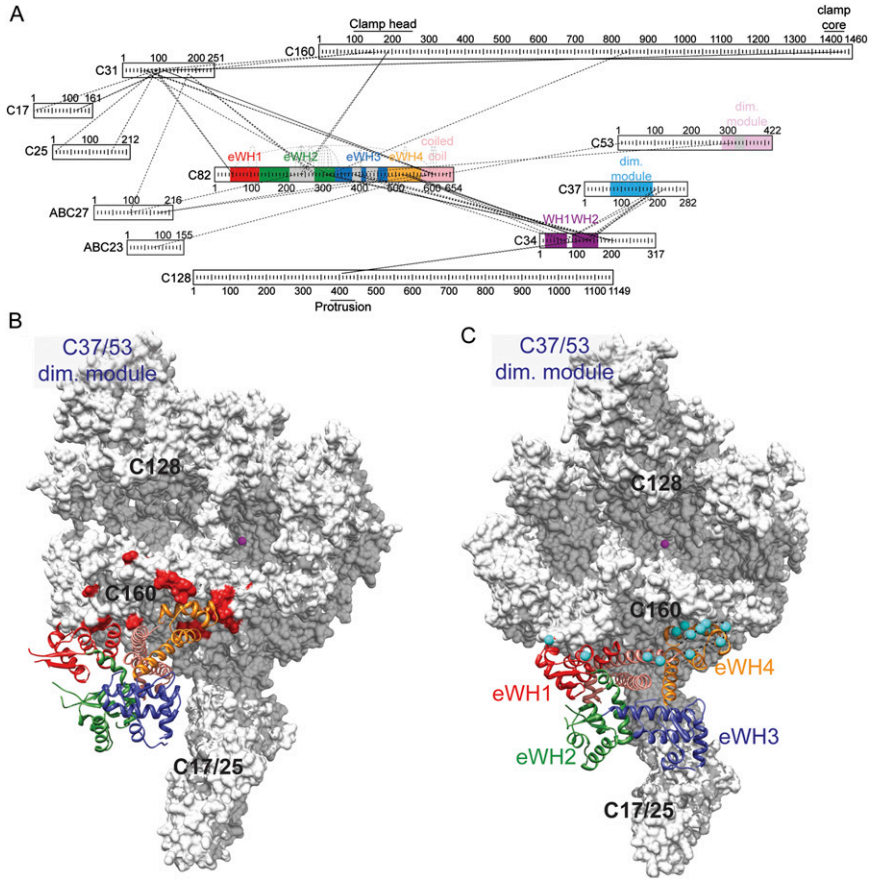

Fig. S4. MS analysis of lysine–lysine cross-linking of Pol III and the model of C82 on the Pol III core (related to Figs. 3 and 5). (*A*) Schematic representation of the Pol III subunits and lysine–lysine cross-linking map for the C82/34/31 subcomplex. C82 structural domains are colored as in Fig. 2. The WH domains in C34 are shown in purple, and the dimerization domains in C37/53 are shown in light blue and pink, respectively. Black dashed lines and gray dashed arcs indicate intersubunit and intrasubunit cross-links, respectively. Cross-validation of C82-Pol III core model by the C160–C82 cross-linking pattern of C160 BPA substitution in the clamp (*B*) and that of C82 BPA substitution in the eWH1, eWH4, and coiled-coil (*C*). The Pol III core is shown in white and C160 residues showing C82 cross-links are colored red and mapped on the Pol III core model. C82 residues yielding C160 cross-links are shown as cyan spheres.

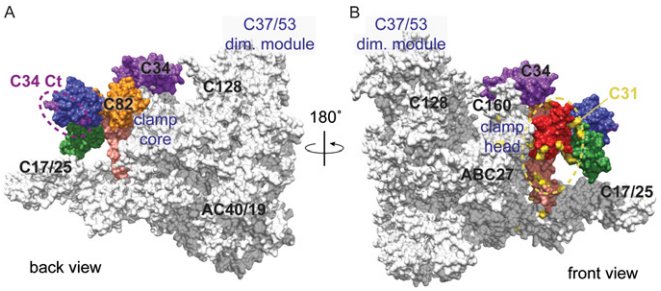

Fig. S5. Possible positions for C34 C-terminal region and C31 (related to Fig. 5). (*A*) C82 and C34 WH domains are shown as molecular surfaces on C160 clamp and are colored as above. Lysine residues on C82 eWH3 (in blue) giving rise to cross-linking pairs with C34 C terminus are shown in purple. The purple dashed oval depicts the possible location for C34 C terminus. (*B*) Possible position for C31 in Pol III. Residues in C160, C82 and C17/25 showing lysine–lysine cross-links with C31 are colored yellow. The yellow dashed oval indicates a possible C31 location between C17/25 stalk and ABC27.

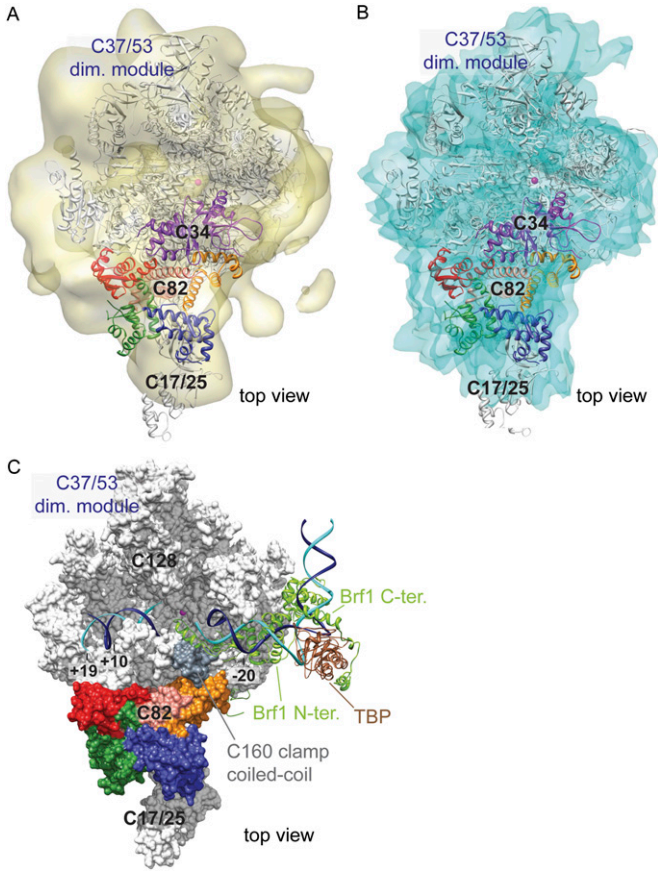

Fig. S6. Fitting of the Pol III model into the cryo-EM structure (related to Figs. 5 and 6). The Pol III model is depicted as the ribbon model of Cα trace: Pol III core in white and C34 WH domains and C82 in the same colors as the color scheme above. The cryo-EM structures of Pol III-DNA-RNA complex are shown as semitransparent envelops in khaki (EMD-1754) (*A*) and cyan (EMD-1804) (*B*) (1, 2). (*C*) An additional view of Pol III open promoter complex. Nucleotide positions at −20, +10, and +19 on the template DNA strand are labeled. C34 WH domains are omitted to show the coiled-coil motif in C160 clamp (gray).

1. Vannini A, et al. (2010) Molecular basis of RNA polymerase III transcription repression by Maf1. *Cell* 143(1):59–70.
2. Fernández-Tornero C, et al. (2010) Conformational flexibility of RNA polymerase III during transcriptional elongation. *EMBO J* 29(22):3762–3772.

Table S1. Summary of C160 clamp BPA cross-links (related to Fig. 1)

Table S1

Positions of BPA substitution in C160 are indicated, and the growth of each substitution is denoted as + or −, indicating a viable strain or lethal phenotype after BPA insertion, respectively. The indicated cross-linked proteins were identified as described in Materials and Methods.

Table S2. Summary of C82 BPA cross-links (related to Fig. 2)

Table S2

Positions of BPA substitution in C82 are indicated and the growth of each substitution is shown in + or –, indicating a viable strain on BPA-containing plate or a lethal phenotype after BPA incorporation, respectively.

Table S3. Summary of C82-FeBABE cleavages (related to Fig. 3)

Table S3

The residues tethered with FeBABE and the corresponding cleavage sites in C160 are listed.

Table S4. Summary of cross-linked peptide pairs in Pol III (related to Figs. 3 and 5)

Table S4

Inter- and intrasubunit cross-links were identified according to the previously described workflow using the search engine xQuest (1). ΔAA, number of amino acids separating the linked lysines; Distance, cross-link distance measured in PDB structure in Å; Error, mass deviation from the monoisotopic precursor mass in ppm; Id, amino acid sequences of peptides indicating the relative position of the linked lysines; Id-Score, xQuest identification score; M_r, molecular mass of cross-link precursor; MS2, minimum number of cross-link ions per peptide and cross-link; M/z, mass to charge ratio of cross-link precursor; No., cross-link identifier; Pos1, Pos2, absolute amino acid position of linked lysines; Protein1, Protein2, UniProt entry of cross-linked Pol III subunits; XLType, indicates inter- or intrasubunit linkages; z, charge of cross-link precursor.

1. Jennebach S, Herzog F, Aebersold R, Cramer P (2012) Crosslinking-MS analysis reveals RNA polymerase I domain architecture and basis of rRNA cleavage. *Nucleic Acids Res* 40(12): 5591–5601.