Supplemental Data

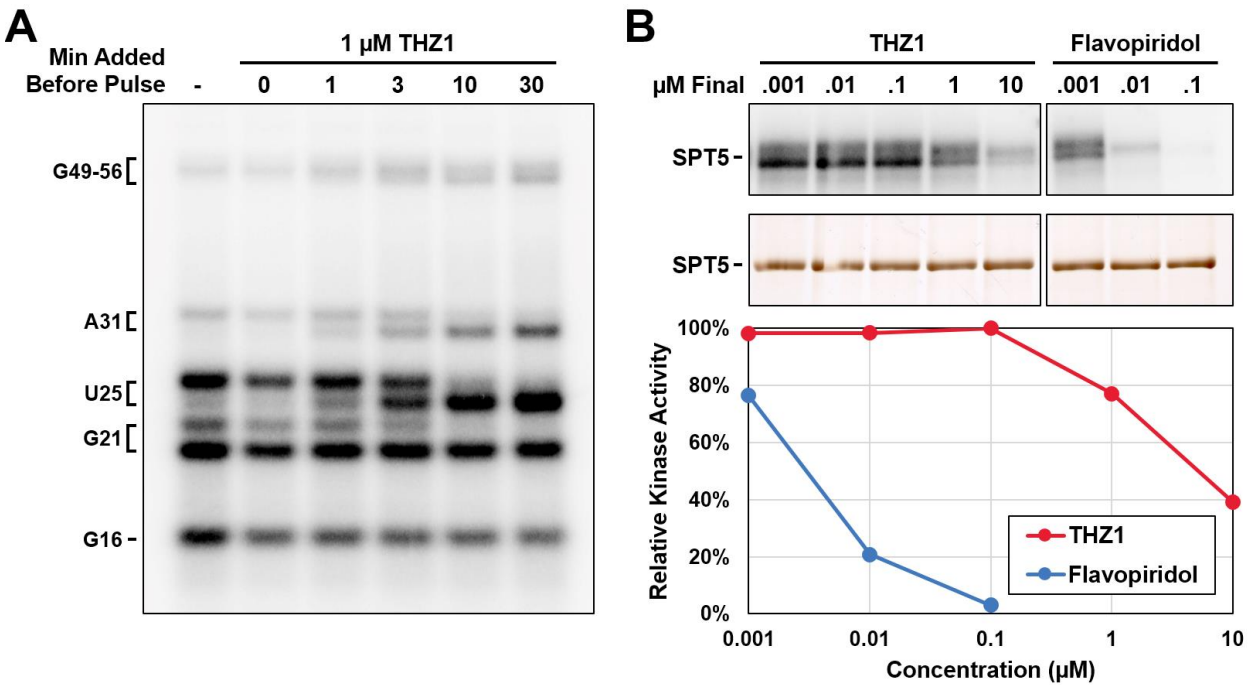

Figure S1: Effects of THZ1 on transcription and P-TEFb kinase activity, related to Figure 1.

(A) HeLa nuclear extract and template DNA were preincubated for 30 min and pulsed for 30 sec with limiting α-^{32}P-CTP. At the indicated times, THZ1 was introduced to give a final concentration of 1 μM during the pulse. Brackets indicate the positions of capped and uncapped transcripts that end in the indicated base and position. 9% Urea-PAGE.

(B) Kinase assay. 1 pmole DSIF and 0.04 pmole P-TEFb were incubated for 10 min with THZ1 or Flavopiridol and 10 min with 30 μM cold ATP with 2.5 μCi γ-^{32}P-ATP. Final concentrations of THZ1 and Flavopiridol are indicated. Label incorporation into the Spt5 subunit of DSIF was quantified and plotted in the lower panel. Note that in vitro transcription reactions instead use 500 μM ATP and non-covalent THZ1 activity will be reduced 17-fold. 9% SDS-PAGE followed by phosphorimaging and silver staining.

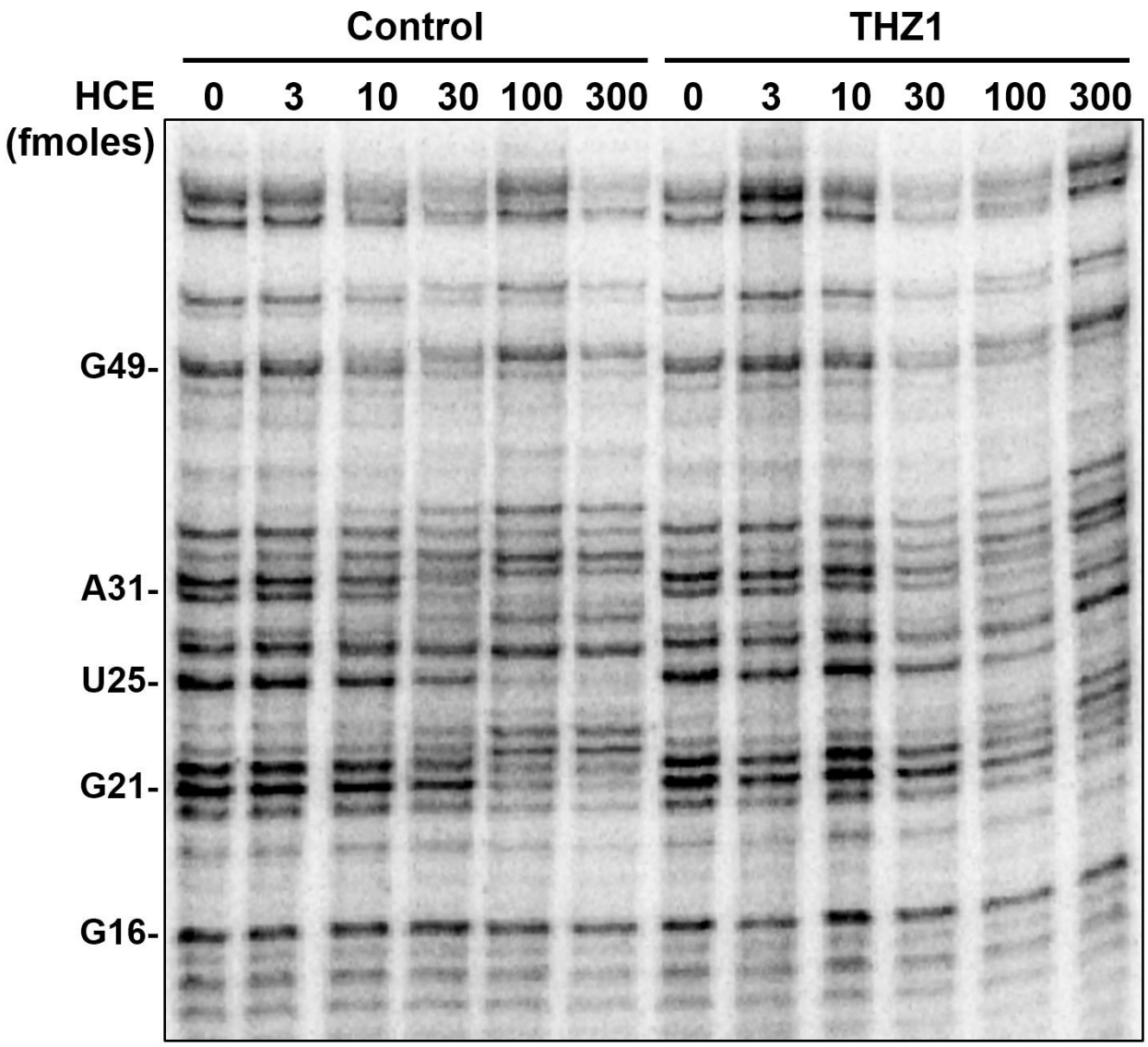

Figure S2: Effect of THZ1 in a defined transcription system, related to Figure 3.

Defined transcription reactions containing only TFIIB, TBP, TFIIE, TFIIF, TFIIH, and Pol II were preincubated for 60 min with CMV template DNA ± 1.5 μM THZ1 and pulsed for 45 sec with indicated amounts of HCE. 12.5% Urea-PAGE.

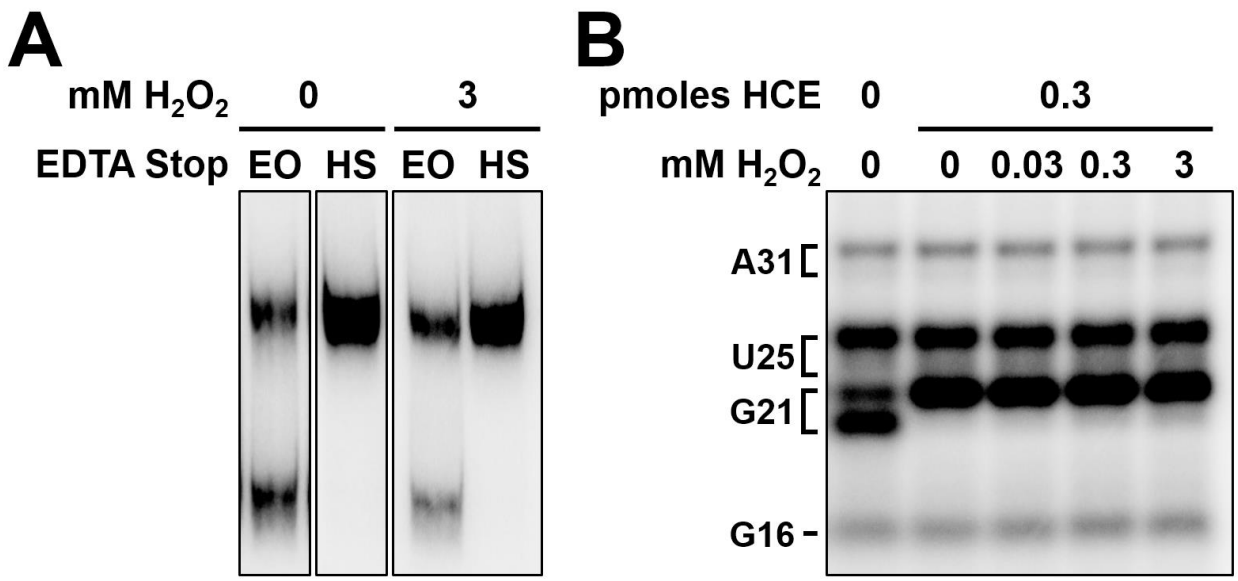

Figure S3: Effects of H_2O_2 on capping enzyme, phosphatase, and CTD phosphorylation, related to Figure 4.

(A) Migration of elongation complexes was analyzed as in Figure 2E, except preincubations were performed in the presence of indicated amounts of H_2O_2. Figure 2E and Figure S3A were run on the same gel and the 0 mM H_2O_2 lanes are shared. EO: EDTA only. HS: high salt.

(B) HCE was preincubated for at least 5 min with increasing amounts of H_2O_2 before addback to high salt washed elongation complexes as in Figure 3B. Figure 3B and Figure S3B were run on the same gel and the first lane is shared. 9% Urea-PAGE.

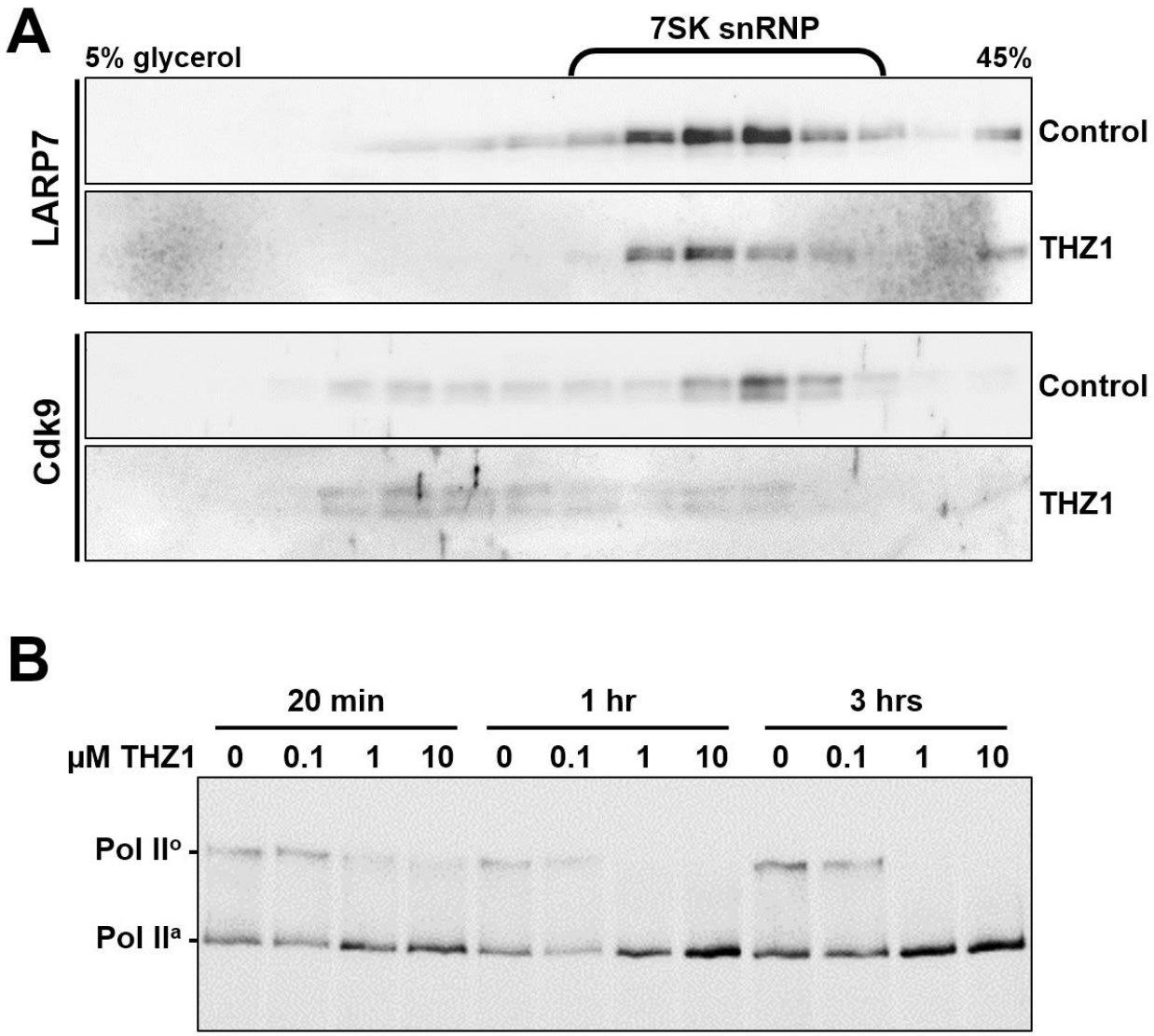

Figure S4: Effect of THZ1 on 7SK snRNP stability and CTD phosphorylation in cells, related to Figure 6.

(A) HeLa cells were optionally treated for 1 hr with 200 nM THZ1, harvested, and lysed in a buffer containing 10 mM KCl, 150 mM NaCl, and 0.5% Triton X-100. Supernatants were sedimented on a 5-45% glycerol gradient as described previously (Krueger et al., 2010). Western blots of fractions were probed with antibodies to LARP7 and Cdk9 as indicated. 9% SDS-PAGE.

(B) HeLa cells were treated with the indicated concentrations of THZ1 for the indicated times and lysed in 2% SDS. Western blots of cell lysates were probed using a Pol II large subunit antibody with a non-CTD epitope (sc-899). Pol II^{a}: hypo-phosphorylated CTD. Pol II^{o}: hyper-phosphorylated CTD. 9% SDS-PAGE.

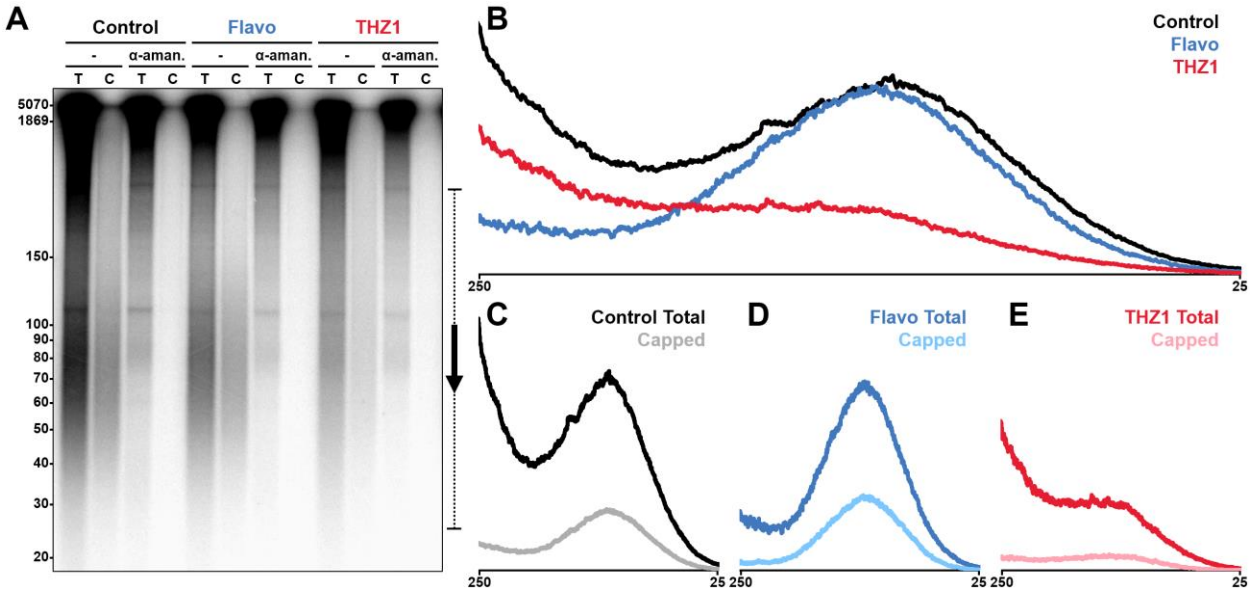

Figure S5: THZ1 inhibits proper mRNA capping and Pol II elongation in cells, related to Figure 6.

(A) Cap status determination of transcripts generated by nuclear run-ons performed with 500 μM ATP/UTP/GTP, 2 μM cold CTP, and 0.33 μM α-^{32}P-CTP in the absence or presence of 4 μg/ml α-amanitin using nuclei from HeLa S3 cells treated 1 hr with DMSO only (Control; black), 1 μM Flavopiridol (blue), or 1 μM THZ1 (red). 6% Urea-PAGE.

(B) Profiles from the indicated region (bracket) of total Pol II transcripts generated by first normalizing each sample pair to cold nuclear RNAs visible in the ethidium bromide stained gel to account for variations in nuclear material and then taking the difference between run-ons performed with or without α-amanitin.

(C-E) Profiles generated as in (B) of total (dark) or capped (light) Pol II transcripts from control (C), Flavopiridol (D), or THZ1 (E) nuclear run-ons. (B-E) have the same vertical axis heights.

Supplemental Experimental Procedures

In vitro transcription

The use of soluble and paramagnetic bead-immobilized CMV templates and HeLa nuclear extract (HNE) to study transcription in vitro was described previously (Adamson et al., 2003; Cheng and Price, 2007, 2009). All steps were performed at room temperature (RT). Template DNA from -800 to +175 (Figure 2, S3A) or +508 (Figures 1, 3-5, S1A, S3B) was incubated for 30 min with 1 µl/rxn HNE in the presence of 60 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES pH 7.6, 1 mM DTT, and 0.5 U/µl SUPERase-In (Ambion AM2696) or 1 U/µl RNaseOUT (Invitrogen 10777-019). 1 mM THZ1 (Kwiatkowski et al., 2014) and Flavopiridol (Chao et al., 2000) stocks in DMSO were diluted to 10 µM in H_2O immediately before use. In Figure 4, 1 mM DTT was replaced with 3 mM H_2O_2 as indicated (2 mM final during the chase). Initiation was accomplished with a 30 or 45 sec pulse containing 60 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES pH 7.6, 0.21 µM α-^{32}P-CTP (PerkinElmer BLU008H001MC), and 500 µM ATP/UTP/GTP (limiting CTP) or 500 µM ATP/GTP 0.1 µM UTP (limiting UTP/CTP). Elongation complexes were either stopped by addition of EDTA to 20 mM or chased for 3 min with 500 µM CTP. Before the chase, immobilized complexes were optionally isolated with high salt wash (1.6 M KCl, 20 mM HEPES pH 7.6, 1 mM DTT, and 0.02% Tween20) to remove associated factors or low salt wash (60 mM KCl, 20 mM HEPES pH 7.6, 1 mM DTT, 0.2 mg/ml BSA (New England BioLabs B9000), and 0.02% Tween20) to allow retention of associated factors. Labeled transcripts were extracted with phenol, precipitated with 95% ethanol and 500 mM $NH_4C_2H_3O_2$, separated on denaturing RNA gels (6 M urea, 1X TBE, and 6 or 9% 37.5:1 acrylamide:bis-acrylamide), scanned using a Fujifilm Typhoon FLA-7000 phosphorimager, and analyzed using Fujifilm MultiGauge v3 software.

EC-EMSA

EC-EMSAs were described previously (Cheng and Price, 2008; Guo et al., 2014). All steps except antibody incubation were performed at RT. Elongation complexes were formed as described above using a 30 sec limiting CTP (Figure 2A) or limiting UTP/CTP (Figure 2B-E) pulse. In Figure 2A, complexes were stopped by addition of 0.5 M EDTA to a final concentration of 20 mM, isolated by three high salt and two low salt washes, restriction digested to remove the magnetic beads with 10 U SacI-HF (New England BioLabs R3156) in low salt wash containing 7.5 mM $MgCl_2$ for 15 min, and incubated with antibody for 30 min at 4° (Pol II: sc-899; CTD: 8WG16; Ser7p: 4E12-1-1; Ser5p: 3E8-2-7; Ser2p: 3E10-1-1). In Figure 2B, complexes were stopped by addition of high salt wash containing 20 mM EDTA. In Figure 2C, complexes were

stopped by addition of 0.5 M EDTA to a final concentration of 20 mM, incubated for 3 min, isolated, incubated for 10 min in kinase buffer (60 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES pH 7.6, 1 mM DTT, 8 µM single-stranded DNA (sequencing primer) to limit TTF2 driven termination, and 1 mM ATP) with or without 2.3 µg recombinant P-TEFb (Cheng and Price, 2007), low salt washed, and restriction digested. In Figure 2D, complexes were stopped by 1) addition of 0.5 M EDTA to a final concentration of 20 mM, 2) one wash and resuspension in low salt wash containing 20 mM EDTA, or 3) addition of high salt wash containing 20 mM EDTA. These complexes were then incubated for 3, 10, or 30 min prior to isolation and restriction digestion. In Figure 2E, complexes were stopped by 1) addition of high salt wash containing 20 mM EDTA, or 2) addition of 0.5 M EDTA to a final concentration of 20 mM. These complexes were incubated for 30 min prior to isolation and restriction digestion. Mobilities of elongation complexes containing labeled transcripts were determined by native gel electrophoresis (0.5X Tris-glycine and 4% 37.5:1 acrylamide:bis-acrylamide) and phosphorimaging.

Capping enzyme add-back

Recombinant human capping enzyme (HCE) was purified as described previously (Moteki and Price, 2002). The following steps were performed at RT. Serial dilutions of HCE in low salt wash were used for Figure 3B-D. In Figure 3A-C, high or low salt isolated elongation complexes were incubated for 1 min (Figure 3A) or 3 min (Figure 3B-C) with equal volumes of reaction buffers containing 60 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES pH 7.6, 1 mM DTT, 0.2 mg/ml BSA, 500 µM GTP, and indicated amounts of HCE. Capping was stopped with a solution of 1% sarkosyl, 100 mM NaCl, 50 mM Tris pH 7.6, and 20 mM EDTA immediately before phenol extraction.

Transcript cap status determination

Cap status determination using recombinant human cap methyltransferase (HCM) was described previously (Moteki and Price, 2002). Prior to use, anti-2,2,7-trimethylguanosine agarose beads (Calbiochem NA02A) which recognize m^7G-capped RNAs were blocked for 30 min at RT in blocking buffer (25 mM Tris pH 7.6, 5 mM $Mg(C_2H_3O_2)_2$, 25 mM $KC_2H_3O_2$, 25 mM NaCl, 1 mM DTT, 0.02% Tween20, 0.05 mg/ml Torula yeast RNA (Sigma R6625), 0.2 mg/ml BSA, and 0.02 U/µl SUPERase-In), washed three times with washing buffer (25 mM Tris pH 7.6, 5 mM $Mg(C_2H_3O_2)_2$, 75 mM $KC_2H_3O_2$, 75 mM NaCl, 1 mM DTT, 0.1% Tween20, and 0.02 U/µl SUPERase-In), rinsed three times with binding buffer (blocking buffer without BSA), and resuspended in binding buffer as a 20% slurry. For Figure 6, 0.5 ml of anti-2,2,7-trimethylguanosine ascites fluid (Calbiochem CS214155) was diluted 1:10 in 35 mM KCl

HGKEDP (25 mM HEPES pH 7.6, 15% glycerol, 35 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.1% isopropanol-saturated PMSF) containing 0.5% Triton X-100 and fractionated with an 80 ml gradient (50 mM to 1 M KCl HGKEDP) over a Mono Q HR 10/10 column. Pure IgG eluted around 100 mM KCl and antibody-containing fractions were pooled, bound to Protein G Sepharose Fast Flow beads (Sigma P3296) pre-blocked with blocking buffer lacking Torula yeast RNA, and washed once with RIPA buffer (20 mM Tris pH 7.6, 150 mM $KC_2H_3O_2$, 1% Triton X-100, 0.2% sodium deoxycholate, 0.1% SDS, and 1 mM EDTA), once with LiCl buffer (20 mM Tris pH 7.6, 400 mM LiCl, 0.02% Tween20, and 1 mM EDTA), and three times with binding buffer lacking Torula yeast RNA.

Radiolabeled transcripts were phenol extracted, ethanol precipitated, and resuspended at 37° in 10 µl/rxn 25 mM Tris pH 7.6 with 0.5 U/µl SUPERase-In. Half of each sample was set aside for analysis of total transcripts. The other half was incubated for 15 min at 37° with an equal volume of 25 mM Tris pH 7.6, 10 mM $Mg(C_2H_3O_2)_2$, 200 µM SAM, 1 mM DTT, 0.2 mg/ml BSA, and 30 ng/µl HCM to methylate existing RNA caps. After methylation, 50 µl/rxn 20% antibody bead slurry was added to each sample and incubated with rotation for 2-4 hr at RT. Samples were then washed three times with 70 µl washing buffer; unbound supernatants were saved and pooled. Total, bound, and unbound fractions were brought to equal volumes in washing buffer, spiked with glycogen, and phenol extracted, precipitated, and analyzed as described above.

Pausing factor add-back

Recombinant DSIF (Renner et al., 2001), NELF (Renner et al., 2001), and Gdown1 (Cheng et al., 2012) were purified as described previously. The following steps were performed at RT and all factors were diluted 10-fold in low salt wash immediately before use. High or low salt isolated complexes were incubated for 5 min prior to chase with equal volumes of reaction buffers containing 60 mM KCl, 20 mM HEPES pH 7.6, 1 mM DTT, 0.2 mg/ml BSA, 0.5 U/µl SUPERase-In, and 1) no additional factors, 2) 0.3 pmoles DSIF and 0.6 pmoles NELF, 3) 0.6 pmoles NELF, or 4) 1 pmole Gdown1. All add-back reactions were stopped with a solution of 1% sarkosyl, 100 mM NaCl, 50 mM Tris pH 7.6, and 20 mM EDTA immediately before phenol extraction.

Nuclear run-on

Nuclei isolation conditions were adapted from (Chao and Price, 2001). For Figure 6, HeLa cells were grown to 5×10^5 cells/ml in spinner flasks at 37° and 5% CO_2 in SMEM (Gibco 11380-037) supplemented with 10% FBS (Gibco 26140-079) and 2 mM L-Glutamine (Gibco 25030-081).

One hour before harvesting, 10 ml of media was removed, spiked with 1:1,000 final volume of DMSO optionally containing 1 mM of Flavopiridol or THZ1 (1 µM final), and re-added to cells. After compound incubation, suspension cells were pelleted at 500 × g for 10 min and media was decanted. Cell pellets were resuspended in 15 ml ice cold lysis buffer (10 mM Tris pH 7.6, 320 mM sucrose, 0.5% Triton X-100, 2 mM $Mg(C_2H_3O_2)_2$, 3 mM $CaCl_2$, 1 mM DTT, 0.004 U/µl SUPERase-In, and 0.1% isopropanol-saturated PMSF) and Dounce homogenized. Cell lysis was monitored by phase-contrast microscopy. Once lysed, cells were pelleted at 1,200 × g for 5 min and thoroughly resuspended in a mixture of 1 ml lysis buffer and 2 ml sucrose cushion (10 mM Tris pH 7.6, 1.9 M sucrose, 5 mM $Mg(C_2H_3O_2)_2$, 1 mM DTT, 0.004 U/µl SUPERase-In, and 0.1% isopropanol-saturated PMSF). This homogenate was carefully layered over a 2 ml sucrose cushion and spun at 30,000 × g for 45 min. Nuclei were resuspended in 1 ml storage buffer (10 mM Tris pH 7.6, 25% glycerol, 5 mM $Mg(C_2H_3O_2)_2$, and 5 mM DTT), pelleted at 1,200 × g for 5 min, and resuspended with storage buffer to about 5×10^7 nuclei/ml before storage in aliquots at -80°.

For Figure S5, nuclei isolation was performed as in Figure 6 with the following changes. HeLa S3 cells were grown to 80% confluency in T75 flasks in 30 ml DMEM (Gibco 11965-092) supplemented with 10% FBS. After compound incubation, adherent cells were aspirated, rinsed with 20 ml ice cold PBS, incubated for 5 min on ice with 20 ml swelling buffer (10 mM Tris pH 7.6, 2 mM $Mg(C_2H_3O_2)_2$, and 3 mM $CaCl_2$), scraped, and pelleted at 500 × g for 5 min.

For Figure 6, 2.5×10^6 nuclei were diluted to 50 µl in storage buffer lacking glycerol. Two 20 µl aliquots were incubated for 3 min at 30° with an equal volume of reaction buffer containing 10 mM Tris pH 7.6, 1% sarkosyl, 300 mM $KC_2H_3O_2$, 5 mM $Mg(C_2H_3O_2)_2$, 5 mM DTT, 0.5 U/µl SUPERase-In, 0.33 µM α-^{32}P-CTP, and optionally 4 µg/ml α-amanitin. Reactions were stopped by addition of EDTA to 20 mM and labeled transcripts were extracted with Trizol LS (Ambion 10296-028), precipitated with 95% ethanol and 500 mM $NH_4C_2H_3O_2$, and assayed for capping as described above.

Figure S5 nuclear run-on nucleotide conditions were adapted from published studies (Core et al., 2012; Core et al., 2008). 5×10^5 DMSO, Flavopiridol, or THZ1 HeLa S3 nuclei were first diluted to 25 µl in storage buffer, and then to 50 µl with 10 mM Tris pH 7.6, 5 mM $Mg(C_2H_3O_2)_2$, 5 mM DTT, and 0.25 U/µl SUPERase-In. Each sample was equally divided and incubated for 4 min at 30° with an equal volume of reaction buffer containing 10 mM Tris pH 7.6, 1% sarkosyl, 300 mM $KC_2H_3O_2$, 5 mM $Mg(C_2H_3O_2)_2$, 5 mM DTT, 0.5 U/µl SUPERase-In, 500 µM ATP/UTP/GTP, 2 µM cold CTP, 0.33 µM α-^{32}P-CTP, and optionally 4 µg/ml α-amanitin. Reactions were stopped, precipitated, and assayed for capping as described above.

Kinase assay

Kinase assay conditions were adapted from (Marshall et al., 1996). 10 mM THZ1 and Flavopiridol were serial diluted in 1% DMSO and preincubated for 10 min in 15 µl reactions containing 1 pmole DSIF, 0.04 pmoles P-TEFb, 25 mM HEPES pH 7.2, 50 mM KCl, 6.67 mM $MgCl_2$, 1.33 mM DTT, 0.067 mg/ml BSA, and 0.02% Triton X-100. After 10 min, reactions were completed to 20 µl and incubated for 10 min with the following buffer conditions: 25 mM HEPES pH 7.2, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.05 mg/ml BSA, 0.02% Triton X-100, 30 µM cold ATP, and 2.5 µCi γ-^{32}P-ATP. Reactions were stopped by addition of 5 µl 5X protein loading buffer (20% Ficoll, 10% SDS, 50 mM EDTA, 50 mM DTT, and 50 mM Tris pH 7.6), denatured at 95° for 5 min, separated by 9% SDS-PAGE electrophoresis (1X Tris-glycine with 0.1% SDS, 9% 37.5:1 acrylamide:bis-acrylamide resolving gel, and 4% 19:1 acrylamide:bis-acrylamide stacking gel), and analyzed by silver staining and phosphorimaging.

Defined in vitro transcription

TBP, TFIIB, TFIIE, TFIIF and Pol II were purified as described in (Mullen Davis et al., 2014). TFIIH was purified from HeLa nuclear extract using successive elutions from phosphocellulose and DE52, a modification of the method described in (Luse et al., 2011). Preinitiation complexes were assembled for 60 min at 30° as described by (Mullen Davis et al., 2014), except that soluble CMV template was used at 50 ng per 10 µl reaction. Initiation was accomplished with a 45 sec pulse containing 0.71 µM α-^{32}P-CTP, 3 µM UTP, and 500 µM ATP/GTP. Indicated amounts of HCE diluted in a buffer containing 25 mM HEPES pH 7.6, 15% glycerol, 185 mM KCl, and 0.1 mM EDTA were added at the start of the pulse.

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