

Cyclin-dependent kinase control of the initiation-to-elongation switch of RNA polymerase II

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Promoter-proximal pausing by RNA polymerase II (Pol II) ensures gene-specific regulation and RNA quality control. Structural considerations suggested a requirement for initiation-factor eviction in elongation-factor engagement and pausing of transcription complexes. Here we show that selective inhibition of Cdk7—part of TFIIF—increases TFIIE retention, prevents DRB sensitivity-inducing factor (DSIF) recruitment and attenuates pausing in human cells. Pause release depends on Cdk9–cyclin T1 (P-TEFb); Cdk7 is also required for Cdk9-activating phosphorylation and Cdk9-dependent downstream events—Pol II C-terminal domain Ser2 phosphorylation and histone H2B ubiquitylation—in vivo. Cdk7 inhibition, moreover, impairs Pol II transcript 3′-end formation. Cdk7 thus acts through TFIIE and DSIF to establish, and through P-TEFb to relieve, barriers to elongation: incoherent feedforward that might create a window to recruit RNA-processing machinery. Therefore, cyclin-dependent kinases govern Pol II handoff from initiation to elongation factors and cotranscriptional RNA maturation.

In metazoans, Pol II frequently pauses in the promoter-proximal region, either stably to control expression of specific genes or transiently to promote recruitment of RNA-processing enzymes before elongation^{1,2}. Proteins implicated in pause establishment and release are targets for phosphorylation by multiple distinct cyclin-dependent kinases (CDKs) embedded within the transcription machinery, which implies coordination, by unknown mechanisms, of individual CDK functions. Cdk7, together with its regulatory partners cyclin H and Mat1, forms a subcomplex within the transcription initiation factor TFIIF³. We previously reported construction of a human colon carcinoma cell line in which both copies of wild-type *CDK7* are replaced by mutant alleles encoding an analog-sensitive mutant (Cdk7^{as}), which is inhibited by bulky adenine analogs that do not affect wild-type kinases⁴. Selective inhibition of Cdk7 in *CDK7^{as}* cells attenuated pausing by Pol II and led to reduced 3′-end cleavage of a β -globin reporter transcript and increased read-through transcription of the U2 small nuclear RNA (snRNA) gene⁵, consistent with a role for Cdk7 in the recruitment of factors involved in cotranscriptional RNA processing^{6–8}. Work in *Drosophila* has also implicated Cdk7 in the establishment of a paused polymerase at heat-shock loci⁹.

Pausing by Pol II depends on DSIF, a heterodimer of Spt4 and Spt5 that is conserved in all eukaryotes. DSIF is required in metazoans for recruitment of a negative elongation factor (NELF), which promotes stable pausing and is absent in yeast. Recent structural and biophysical studies indicate that the archaeal homologs of DSIF and the initiation factor TFIIE bind overlapping sites in the clamp region of RNA

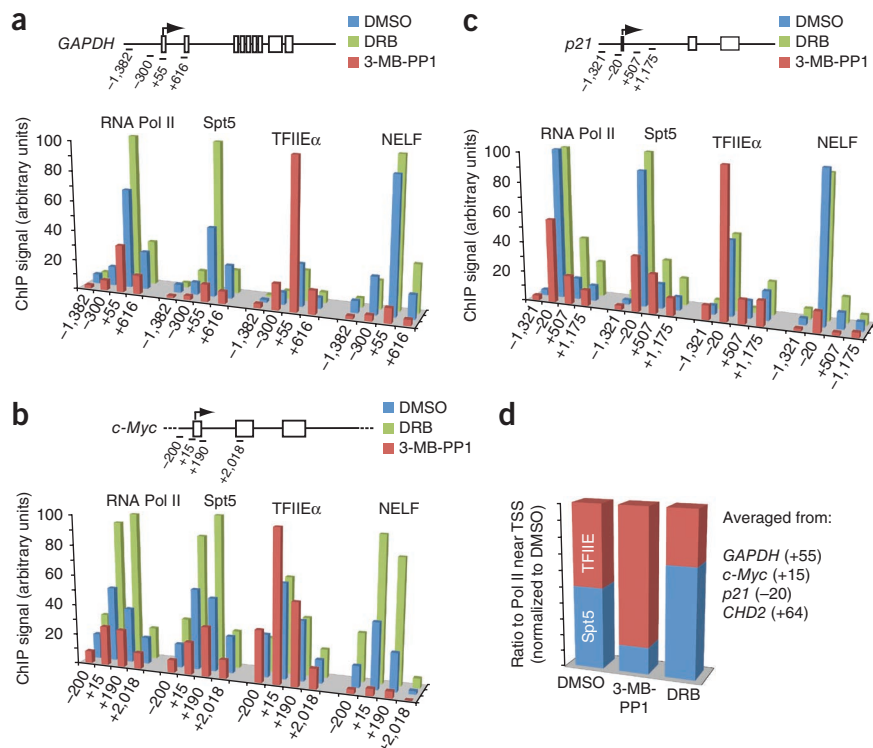
polymerase in a mutually exclusive fashion^{10–12}. These observations suggest that, in metazoans, TFIIE eviction would be a prerequisite for recruitment of DSIF and NELF to pause the transcription complex.

Overcoming the elongation block requires phosphorylation by Cdk9–cyclin T1, a complex also known as positive transcription elongation factor b (P-TEFb), to release NELF and convert DSIF into a processivity factor^{13–15}. In common with other CDKs, Cdk9 contains an activation loop (T loop) that must be phosphorylated for maximal activity; however, a kinase capable of activating Cdk9 in metazoans has not been identified. Phosphorylation at Thr186 of the T loop also facilitates binding to the inhibitory 7SK small nuclear ribonucleoprotein (snRNP)^{16–18} and thus has the potential to affect Cdk9 activity positively or negatively in different contexts. Identification of the kinase(s) upstream of Cdk9 therefore has important implications for the understanding of elongation control in mammalian cells. A candidate Cdk9-activating kinase is Cdk7 itself. As part of TFIIF, Cdk7 phosphorylates the C-terminal domain (CTD) of Rpb1 (the largest subunit of Pol II) and other proteins involved in transcription. The CTD consists of multiple heptad repeats (52 in human Rpb1) with the consensus sequence 1-YSPSPS-7; Cdk7 prefers to phosphorylate positions 5 and 7 (Ser5 and Ser7), whereas Cdk9 is generally thought to prefer Ser2 but can target Ser5 and Ser7 *in vitro*^{5,19}. Cdk7 also has an essential role in cell-cycle progression as a CDK-activating kinase (CAK)³. Previous attempts to detect activation of human Cdk9 by Cdk7 were unsuccessful, however, leading to suggestions that Cdk9 might (i) not require T-loop phosphorylation to achieve full activity²⁰, (ii) phosphorylate its own T loop in *cis*²¹ or (iii) have a CAK distinct from Cdk7 (ref. 17).

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Figure 1 Cdk7 inhibition prevents exchange of TFIIIE for DSIF and attenuates promoter-proximal pausing. (a–c) ChIP of Pol II, DSIF (Spt5), NELF and TFIIIE α on *GAPDH* (a), *c-Myc* (b) and *p21* (c) genes. DMSO, dimethylsulfoxide control. Treatment with 50 μ M DRB results in increased Spt5 and Pol II cross-linking over the TSS, whereas inhibition of Cdk7 in *CDK7^{as}* cells with 10 μ M 3-MB-PP1 results in reduced Pol II pausing and a loss of Spt5 and NELF recruitment, which is accompanied by an increase in TFIIIE cross-linking ($n = 2-4$, $P < 0.01$; **Supplementary Fig. 1**). (d) Change in the ratio of Spt5 and TFIIIE relative to Pol II after drug treatments. The data, averaged from four genes (*GAPDH*, *c-Myc*, *p21* and *CHD2*), were normalized to the ratios of Spt5 and TFIIIE to Pol II in the DMSO-treated condition, which were each set at 1.



In both budding and fission yeast, P-TEFb is activated by a single-subunit CAK that is not present in metazoans^{22,23}.

Here we sought to establish the functional relationship between Cdk9 and its potential activator Cdk7 during the transcription cycle. By chemical genetics, we uncover two seemingly antagonistic functions of Cdk7 in governing Pol II dynamics on transcribed genes in human cells. First, we show that Cdk7 activity is required for displacement of TFIIIE and recruitment of DSIF at the promoter-proximal pause site, which potentially explains how inhibition of Cdk7 *in vivo* leads to diminished Pol II pausing. Second, we show that Cdk7 activates Cdk9 *in vitro* and that Cdk7 activity is required for T-loop phosphorylation of Cdk9 on chromatin *in vivo*. Inhibition of Cdk7 leads to reduced phosphorylation of chromatin-associated Pol II on CTD Ser2 residues and a global reduction in mono-ubiquitylation of histone H2B at Lys123 (H2Bub1)—post-translational protein modifications that both depend on Cdk9 activity²⁴. Therefore, a unitary network of CDKs with a shared upstream activating kinase controls

critical transitions in both the cell-division cycle and the transcription cycle of Pol II. The opposing functions of Cdk7 in establishing and releasing the pause, moreover, suggest that the CDK network relies on incoherent feedforward to produce a transient slowing of transcript elongation and thus a kinetic window for recruitment of RNA-processing machinery.

RESULTS

Pol II handoff requires Cdk7 activity

Because Cdk7 was reported to phosphorylate both TFIIIE^{25,26} and the Spt5 subunit of DSIF²⁷, we sought to test its involvement in a putative

Figure 2 Cdk7 activates Cdk9^{as} *in vitro*. (a) Kinase assay of wild-type Cdk9 (Cdk9^{WT}) and analog-sensitive variant Cdk9^{as}, purified as a complex with cyclin T1 from insect cells co-infected with the corresponding baculoviruses. As shown, Cdk9^{as} prefers the bulky analog *N*⁶-(benzyl)-ATP over natural ATP, whereas wild-type Cdk9—cyclin T1 (purified identically) prefers natural ATP. (b) Assay of Cdk9^{as} kinase activity, following incubation with activating kinases. Samples are Cdk9^{as}–cyclin T1, reconstituted with separately purified, unphosphorylated subunits and incubated with either Cdk7 or Csk1 before measurement of activity toward GST-Spt5. (c) Phosphorylation with radiolabeled *N*⁶-(benzyl)-ATP of HeLa nuclear extract or purified fractions of DSIF and Pol II reveals activation of Cdk9^{as}–cyclin T1 by Cdk7. The labeling in nuclear extract demonstrates the CAK-dependent phosphorylation by Cdk9^{as} of a limited number of substrates, several of which remain to be identified. Asterisks denote autophosphorylation products that do not depend on addition of substrates to purified Cdk9^{as}–cyclin T1. (d) Activation of Cdk9^{as} by cell extracts. Cdk9^{as}–cyclin T1 was incubated with ATP and nuclear extract (HeLa NE)—mock- or Cdk7-immunodepleted (Δ Cdk7)—before testing its activity on a recombinant GST-Spt5 substrate (**Supplementary Fig. 3a**). Depletion of Cdk7 reduces the ability of the extract to activate Cdk9^{as}, indicating the removal of a Cdk9-activating kinase. (e) Activation of Cdk9^{as} by cell extracts. Treatment with 200 nM 3-MB-PP1 (**Supplementary Fig. 3b**) diminishes the Cdk9^{as}-activating kinase activity in *CDK7^{as}* but not wild-type HCT1116 extracts; activity is restored to the inhibited extract by the addition of purified, inhibitor-resistant, wild-type Cdk7 (Cdk7^{WT}).

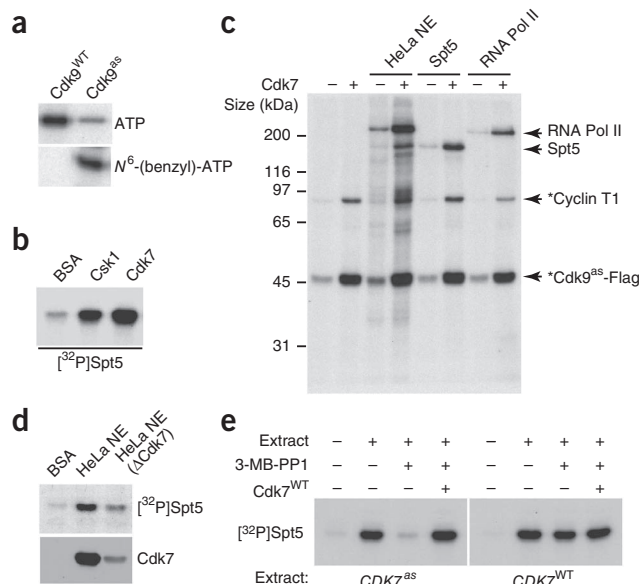
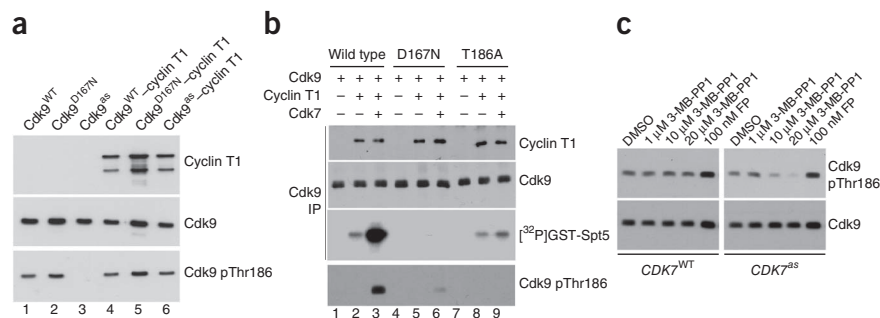


Figure 3 Cdk7 activates wild-type Cdk9 *in vitro* and *in vivo*. (a) Immunoblot of various preparations of recombinant Cdk9. When expressed as a monomer in insect cells, Cdk9^{as} lacks T-loop (Thr186) phosphorylation, in contrast to other Cdk9 variants tested (lanes 1–3). All variants, including Cdk9^{as}, become phosphorylated when coexpressed with cyclin T1 (lanes 4–6). (b) Activation assay of Cdk9 translated *in vitro*. Cdk7 phosphorylates wild-type Cdk9 on Thr186, resulting in increased activity toward a recombinant GST-Spt5 substrate.



(c) Immunoblots of total and Thr186-phosphorylated Cdk9. Bulk Cdk9 T-loop phosphorylation is reduced in a dose-dependent manner by 3-MB-PP1 treatment of *CDK7^{as}* but not wild-type HCT116 cells, whereas flavopiridol (FP) has no effect.

TFIIE-DSIF switch. We compared the effects of selective Cdk7 inhibition, by treatment of *CDK7^{as}* cells with the bulky adenine analog 3-MB-PP1, to those of a Cdk9-selective inhibitor, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB). We chose DRB because, unlike the more potent Cdk9 inhibitor flavopiridol, it had little or no Cdk7-inhibiting activity *in vitro* and produced more consistent effects on Pol II chromatin immunoprecipitation (ChIP) signals *in vivo* (data not shown). DRB increased promoter-proximal Pol II and Spt5 occupancy on *c-Myc* (*MYC*) and *GAPDH* (Fig. 1a,b), presumably by preventing phosphorylation of Spt5 and release of NELF^{28,29}. In contrast, 3-MB-PP1 decreased cross-linking of Pol II and, to a greater extent, Spt5 on *c-Myc*, *GAPDH* and *p21* (*CDKN1A*) in *CDK7^{as}* cells. (Protein cross-linking to the *p21* promoter is relatively insensitive to DRB, consistent with the ability of DRB to induce *p21* transcription³⁰.) Impaired DSIF recruitment after Cdk7 inhibition is likely to explain the near absence of NELF at the transcription start site (TSS) of the three genes we tested (Fig. 1a–c and Supplementary Fig. 1c), consistent with previous results⁵. Decreased DSIF occupancy after Cdk7 inhibition was accompanied by reciprocally increased TFIIE (Fig. 1a–d; Supplementary Figs. 1a,b and 2). The relationship between Spt5-to-Pol II and TFIIE-to-Pol II ratios varied little among the tested genes (Supplementary Fig. 2), suggesting a generalized anticorrelation between Pol II-associated DSIF and TFIIE. This is consistent with a competition between the two factors for the Pol II clamp, by analogy with their archaeal homologs. Moreover, the activity of TFIH-associated Cdk7 appears to control disengagement of TFIIE from, and recruitment of DSIF and NELF to, the transcription complex.

Cdk7 activates Cdk9 *in vitro*

Our results thus far indicate that Cdk7 activity is required to establish a promoter-proximal pause by Pol II, in apparent opposition to P-TEFb, which relieves the block to elongation imposed by DSIF and NELF. To identify potential positive regulators of Cdk9 activity in mammalian cell extracts, we replaced the ‘gatekeeper’ residue Phe103 of human Cdk9 with glycine to generate an analog-sensitive variant (Cdk9^{as}) capable of accepting bulky adenine analogs in its active site³¹. Cdk9^{as}-cyclin T1 complexes purified from insect cells were able to use N⁶-(benzyl)-ATP selectively to phosphorylate a fragment of Spt5 *in vitro* (Fig. 2a). We reconstituted human P-TEFb with Cdk9^{as} and cyclin T1 purified from insect cells and bacteria, respectively; preincubation of this complex with unlabeled ATP and either Cdk7 or the fission-yeast CAK Csk1 stimulated its activity toward Spt5 in a subsequent reaction with N⁶-(benzyl)-[γ -³²P]ATP (Fig. 2b). Similarly, preincubation with Cdk7 increased activity of Cdk9^{as} toward endogenous HeLa-cell Rpb1 and Spt5, either in a nuclear extract or after partial purification of Pol II and DSIF (Fig. 2c).

In the unfractionated extract, Cdk9^{as} labeled several additional unidentified proteins; many of these signals were also enhanced by pretreatment with Cdk7.

It was previously suggested that HeLa cells contained a Cdk9-activating kinase distinct from Cdk7 (ref. 17). We therefore sought to measure the relative contribution of Cdk7 to the Cdk9 activation we observed in cell extracts (Supplementary Fig. 3a). We incubated a Cdk9^{as} complex with ATP and bovine serum albumin (BSA) or HeLa nuclear extract, which was either mock treated or immunodepleted of Cdk7. After a 30-min incubation, we added Spt5 and N⁶-(benzyl)-[γ -³²P]ATP and allowed the reaction to proceed for 5 min; labeling of the exogenous substrate specifically measures activity of Cdk9^{as}, which is uniquely able to use the bulky ATP analog. Incubation with nuclear extract increased activity of Cdk9^{as}-cyclin T1 relative to that of a BSA-treated complex, whereas prior depletion of Cdk7 diminished the ability of the extract to stimulate Cdk9^{as}, which suggested that the activation was due to Cdk7 (Fig. 2d). To exclude the possibility that immunoprecipitation removed another Cdk7-associated protein capable of stimulating Cdk9, we measured activation in extracts of *CDK7^{as}* HCT116 cells, in which Cdk7 activity can be specifically inhibited by 3-MB-PP1 (Supplementary Fig. 3b). Because Cdk9^{as} is also sensitive to 3-MB-PP1 (data not shown), we immunoprecipitated it after incubation in the extract and tested Spt5 kinase activity in a second drug-free reaction. The addition of 200 nM 3-MB-PP1 during the preincubation nearly abolished the ability of *CDK7^{as}*, but not wild-type, extracts to activate Cdk9^{as} (Fig. 2e). Moreover, Cdk9^{as}-activating capacity could be restored in *CDK7^{as}* cell extracts containing 3-MB-PP1 by the addition of wild-type Cdk7, which is impervious to the drug. We conclude that Cdk7 is the major—and possibly sole—enzyme able to activate Cdk9^{as} in human cell extracts.

We next tried to reconstitute activation of wild-type Cdk9 by Cdk7. Insect cell-derived, wild-type Cdk9 assembled with cyclin T1 from bacteria (as described above for Cdk9^{as}) was active and could not be further stimulated by Cdk7 or Csk1 (data not shown). This suggested that wild-type Cdk9 might be phosphorylated on the T loop when expressed as a monomer in insect cells, in contrast to Cdk9^{as} (and wild-type Cdk1, Cdk2 or Cdk7). We analyzed Cdk9 variants expressed with baculoviruses by immunoblotting with an antibody specific for the isoform phosphorylated on Thr186. Although both wild-type and analog-sensitive forms were phosphorylated when coexpressed with cyclin T1, the wild-type but not the analog-sensitive Cdk9 was phosphorylated when expressed as a monomer (Fig. 3a). This explained why we were only able to activate the latter with purified CAKs (and, perhaps, why previous efforts to identify a CAK for Cdk9 were unsuccessful). Previously, we observed a similar monomer-specific defect in T-loop phosphorylation of Cdk2 containing

the analogous gatekeeper mutation in human cells³². The catalytically inactive Cdk9^{D167N} mutant was phosphorylated when expressed alone or in combination with cyclin T1 (Fig. 3a), which argues against autoactivation *in cis*.

To exclude the possibility that activation by Cdk7 was a unique property of the analog-sensitive mutant, we needed a source of wild-type Cdk9 not already phosphorylated at Thr186. Toward this end, we programmed rabbit reticulocyte lysates to synthesize Cdk9, which we analyzed for binding to purified cyclin T1, T-loop phosphorylation state and activity toward Spt5 (Fig. 3b). Cdk9 translated *in vitro* was not phosphorylated at Thr186, even after a 60-min incubation in the presence of cyclin T1 and ATP, which indicated that it was incapable of autoactivation even when bound to cyclin. That binding was productive because it generated basal activity of Cdk9 toward Spt5. The addition of active Cdk7 to the translation reaction resulted in phosphorylation of Thr186 and further activation of Cdk9. T-loop phosphorylation of Cdk9^{D167N} was also dependent on Cdk7 but was lower than that of wild-type Cdk9, possibly owing to a conformational defect of the mutant protein translated *in vitro*. Cdk7 was unable to stimulate the basal activity of Cdk9^{T186A}, indicating that activation of wild-type Cdk9 occurred through a canonical T-loop phosphorylation mechanism.

Cdk7 activity is required for Cdk9 activation on chromatin

Next, we asked whether Cdk7 activity was required for Cdk9 activation *in vivo*. Treatment of *CDK7^{as}* but not wild-type HCT116 cells with 3-MB-PP1 resulted in dose-dependent loss of Cdk9 Thr186 phosphorylation in whole-cell extracts (Fig. 3c), which suggested that Cdk7 is responsible for most or all Cdk9-activating phosphorylation *in vivo*. In contrast, there was no loss of Cdk9 T-loop phosphorylation upon treatment with 100 or 500 nM flavopiridol, a potent inhibitor of Cdk9 (Fig. 3c and data not shown), which is, again, inconsistent with autoactivation.

We next analyzed Cdk9 occupancy and T-loop phosphorylation state on transcribed genes, in the presence or absence of Cdk7 activity, by ChIP. The P-TEFb signal measured with an antibody against total Cdk9 (that is, not specific for the phosphorylated isoform) was concentrated in the 5' regions of the *c-Myc*, *GAPDH* and *p21* genes; 3-MB-PP1 treatment had little or no effect on its distribution in either wild-type or *CDK7^{as}* cells (Fig. 4a,b; Supplementary Figs. 4 and 5). Therefore, human P-TEFb does not depend on TFIIH-associated kinase activity for recruitment to the elongation complex, as do its orthologs in budding and fission yeast^{33,34}. Moreover, that recruitment can occur independently of DSIF engagement (Fig. 1a–c).

We then looked at the spatial pattern of Cdk9 T-loop phosphorylation. In mock-treated cells, the distribution of Thr186-phosphorylated Cdk9 was different from that of total Cdk9, increasing toward the 3' end of the *c-Myc* and *GAPDH* genes, both in absolute levels and relative to total Cdk9 (Fig. 4a,b; Supplementary Figs. 4 and 5). A similar enrichment of phospho-Cdk9 occurred at the 3' end of the *p21* gene induced by doxorubicin (Supplementary Fig. 5). Cdk9 Thr186 phosphorylation was reduced when we treated *CDK7^{as}* cells with 3-MB-PP1; the reduction was apparent at all positions on all three genes and was most prominent at the 3' ends, where the signal intensities were highest in untreated cells (Fig. 4a,b and Supplementary Fig. 5). The ChIP profile of phospho-Cdk9 was not affected by 3-MB-PP1 treatment in wild-type cells (Supplementary Fig. 4), which indicated that loss of T-loop phosphorylation was a specific consequence of Cdk7 inhibition. We conclude that Cdk7 activity is required for activation of Cdk9 on transcribed chromatin and therefore might function both to establish and relieve pausing by Pol II.

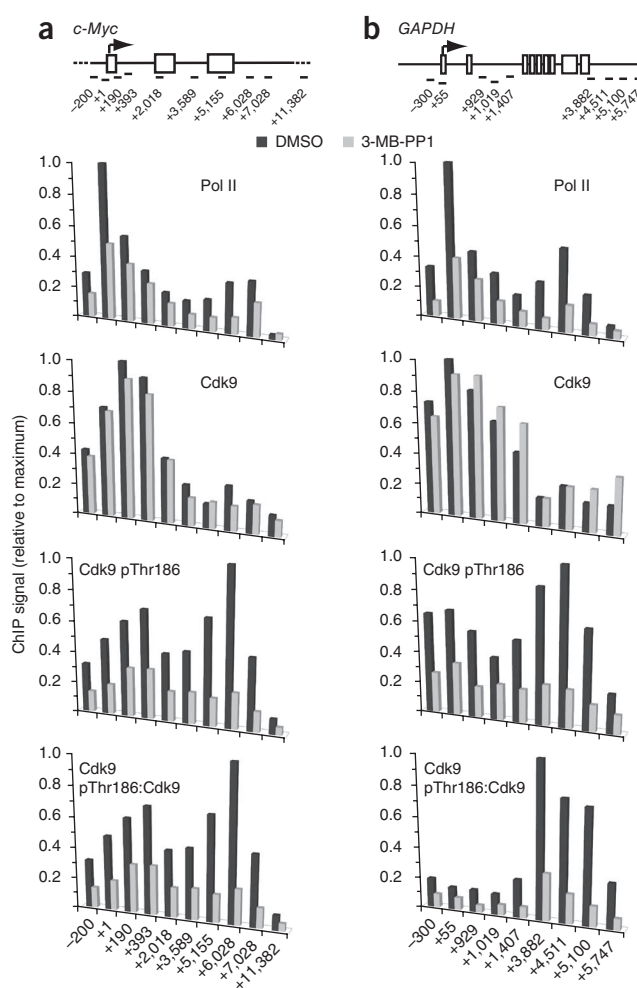


Figure 4 Cdk7 activity is required for Cdk9 Thr186 phosphorylation on chromatin *in vivo*. (a) ChIP of Pol II, total Cdk9 and Cdk9 pThr186 at different positions along the *c-Myc* gene. The Thr186-phosphorylated form of Cdk9 is enriched toward the 3' end of the gene and is diminished by addition of 3-MB-PP1 (10 μ M) to *CDK7^{as}* cells. (b) Same as in a, for the *GAPDH* gene. Cdk9 pThr186:Cdk9 is a normalized ratio with the maximum set equal to 1.0.

Cdk7 inhibition impairs CTD Ser2 phosphorylation

Diminished T-loop phosphorylation of chromatin-associated Cdk9 predicts lowered specific activity of P-TEFb and reduced phosphorylation of its downstream targets. To test this prediction, we performed ChIP analysis on *c-Myc* and *GAPDH* with antibodies specific for Pol II isoforms phosphorylated at CTD positions Ser2 or Ser5 (pSer2 and pSer5) (Fig. 5a,b). Inhibition of Cdk7 with 10 μ M 3-MB-PP1 caused reductions in ChIP signal intensities with both pSer2- and pSer5-specific antibodies but also reduced total Pol II occupancy—measured with an antibody specific for the Rpb1 amino terminus—throughout the bodies of both genes. Nonetheless, there were significant reductions in ratios of pSer2 to total Pol II in the 3' regions of both *c-Myc* and *GAPDH*, where pSer2 (and Cdk9 pThr186) levels normally peak. These were similar in magnitude and degree of statistical significance to reductions in ratios of pSer5 to total Pol II nearer to the 5' ends of genes, where pSer5 signals are highest. Therefore, selective inhibition of Cdk7 caused diminished Rpb1 CTD phosphorylation on a site directly modified by Cdk7 and on one targeted more efficiently by Cdk9.

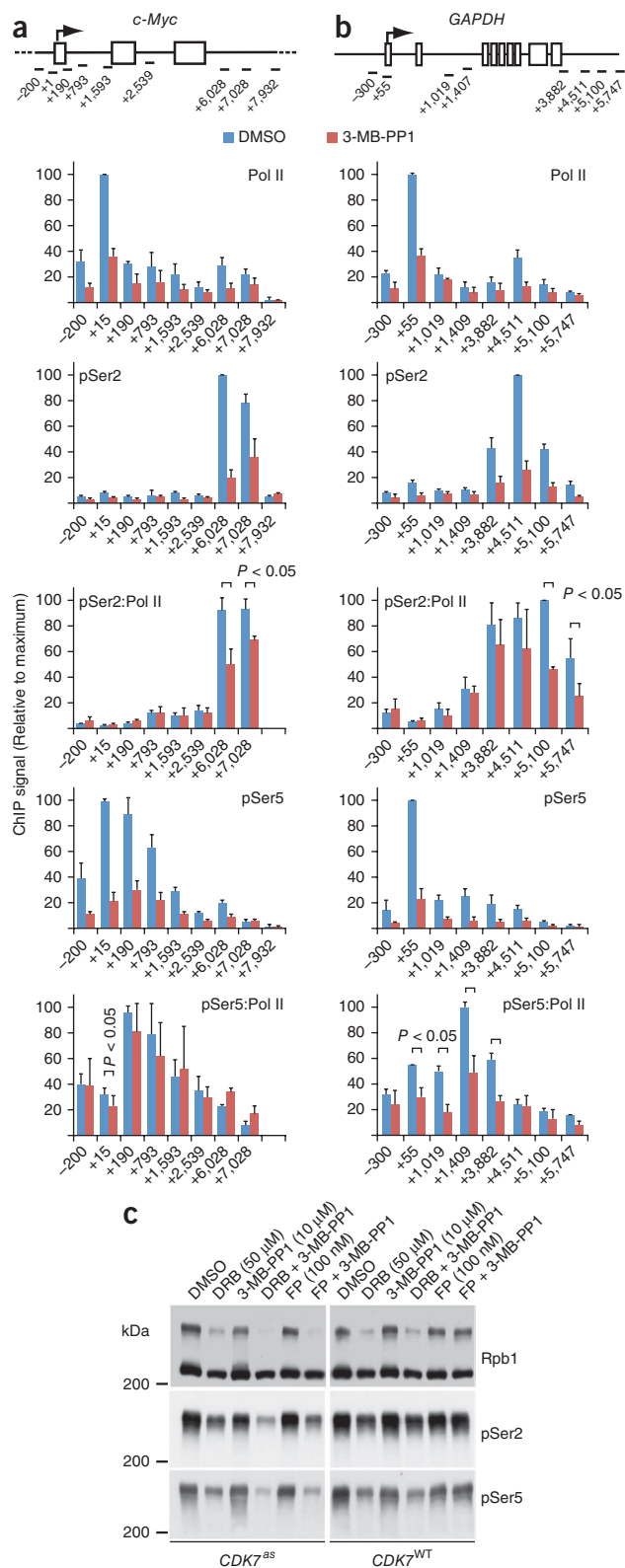
Figure 5 Cdk7 promotes Rpb1-Ser2 phosphorylation in human cells. (a,b) ChIP analysis of Pol II on *c-Myc* (a) and *GAPDH* (b) genes, showing the effect of Cdk7 inhibition on the relative levels of CTD phosphorylation at Ser2 and Ser5. When expressed as a ratio to total Pol II, Ser5 phosphorylation (pSer5) is reduced at the 5' ends of both genes. Levels of Ser2 phosphorylation (pSer2) are reduced relative to total Pol II at the 3' ends of both genes, where Cdk9 pThr186 and pSer2 signals are highest in mock-treated cells ($n = 4$, error bars, s.d.; P values determined by Student's t -test). (c) Western blot analysis showing that simultaneous inhibition of Cdk7 and Cdk9 has synergistic effects on bulk Pol II phosphorylation. Single-kinase inhibition of Cdk7^{as} with 10 μ M 3-MB-PP1 or of Cdk9 with 50 μ M DRB or 100 nM flavopiridol had modest or no effects on levels of pSer2, pSer5 or the hyperphosphorylated II₀ form of Rpb1, whereas combined Cdk7 and Cdk9 inhibition reduced all three signals.

Our previous analysis of CTD phosphorylation in *CDK7^{as}* human cells treated with allele-specific inhibitors suggested that other kinases could maintain near-normal levels of bulk Ser5 phosphorylation when Cdk7 activity was reduced⁴; ChIP analysis at selected loci in the same cells indicated that Cdk7 and Cdk9 had redundant functions in generating pSer5 (ref. 5). Consistent with that interpretation, we observed synergistic effects on bulk pSer5 levels when we combined Cdk7 inhibition (10 μ M 3-MB-PP1 in *CDK7^{as}* cells) with doses of flavopiridol or DRB that had modest effects on pSer5 by themselves (Fig. 5c). Cdk7 inhibition also synergized with the more Cdk9-selective drugs to decrease bulk levels of pSer2, and of the slowest-migrating, hyperphosphorylated II₀ form of Rpb1. There was no additive effect of 3-MB-PP1 with flavopiridol or DRB in wild-type cells, which indicated that increased sensitivity to Cdk9-selective drugs was a specific effect of Cdk7 inhibition. The data are consistent with diminished T-loop phosphorylation leading to decreased specific activity of Cdk9 and a consequent lowering of the pSer2-inhibitory threshold for both DRB and flavopiridol.

Cdk7 promotes H2Bub1 and histone mRNA 3'-end formation

A post-translational protein modification that depends indirectly on Cdk9 activity is H2Bub1, which is implicated in Pol II transcript elongation and 3'-end formation^{24,35}. Treatment with moderately selective Cdk9 inhibitors, or reduction of Cdk9 levels by RNA interference (RNAi), was shown to diminish H2Bub1 levels in human cell extracts; this did not appear to be a general consequence of deregulated Pol II pausing or elongation, because RNAi-dependent knockdown of Spt5 or a NELF subunit did not lower global H2Bub1 levels²⁴. As expected, flavopiridol treatment reduced H2Bub1 in both wild-type and mutant HCT116 cells (Fig. 6a); *CDK7^{as}* cells were slightly more sensitive than wild-type cells, perhaps because the *CDK7^{as}* allele is hypomorphic⁴. Treatment with 3-MB-PP1 caused a dose-dependent loss of H2Bub1 in *CDK7^{as}* but not wild-type HCT116 cells, consistent with a requirement for Cdk7 activity upstream of Cdk9 in promoting H2Bub1.

Cdk7 therefore functions upstream of NELF, P-TEFb and H2Bub1—all of which are implicated in 3'-end processing of histone mRNAs^{24,36,37}. Histone mRNAs are not normally polyadenylated but rather are recognized by the stem-loop binding protein (SLBP) and cleaved by the U7 snRNP. When the primary pathway of 3'-end formation is impaired, for example by NELF knockdown or Cdk9 inhibition, read-through transcription yields polyadenylated transcripts from cryptic downstream processing sites. We tested a role for Cdk7 in this pathway by PCR-based quantification of RNA transcripts of the *HIST1H2BD* locus. Concomitant with the reduction in H2Bub1, treatment with 3-MB-PP1 caused a decrease in total, and an increase in polyadenylated, histone H2B mRNA in *CDK7^{as}* but



not wild-type cells (Fig. 6b). Therefore, inhibition of Cdk7 mimics the effects on histone gene expression of NELF knockdown³⁷ or direct inactivation of Cdk9 (ref. 24). We previously showed that Cdk7 activity is required for correct 3'-end processing of an snRNA and a polyadenylated mRNA⁵. The effect on a third processing pathway required for histone mRNA maturation suggests pervasive

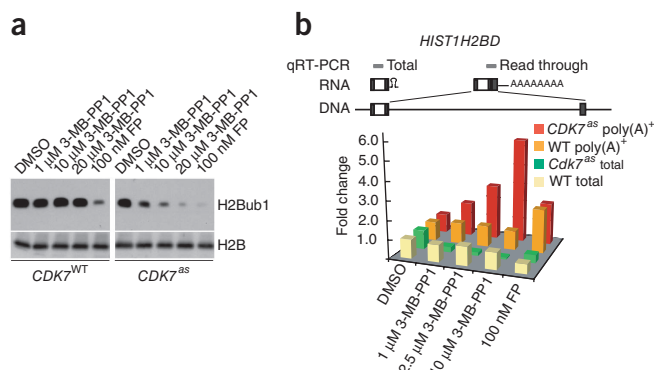


Figure 6 Cdk7 activity is required for normal histone mRNA 3'-end formation. **(a)** Immunoblot of total (H2B) and ubiquitylated (H2Bub1) histone H2B. Flavopiridol treatment of wild-type (WT) or *CDK7^{as}* HCT116 cells or selective inhibition of Cdk7 by 3-MB-PP1 in *CDK7^{as}* cells leads to decreased H2Bub1. **(b)** Quantitative reverse-transcription PCR (qRT-PCR) measurement of histone H2B RNA. Increased read-through transcription of the *HIST1H2BD* gene, detected by accumulation of an aberrant polyadenylated transcript.

derangement of 3'-end formation of Pol II transcripts when Cdk7 is inactive, perhaps due to ineffective pausing or pause release.

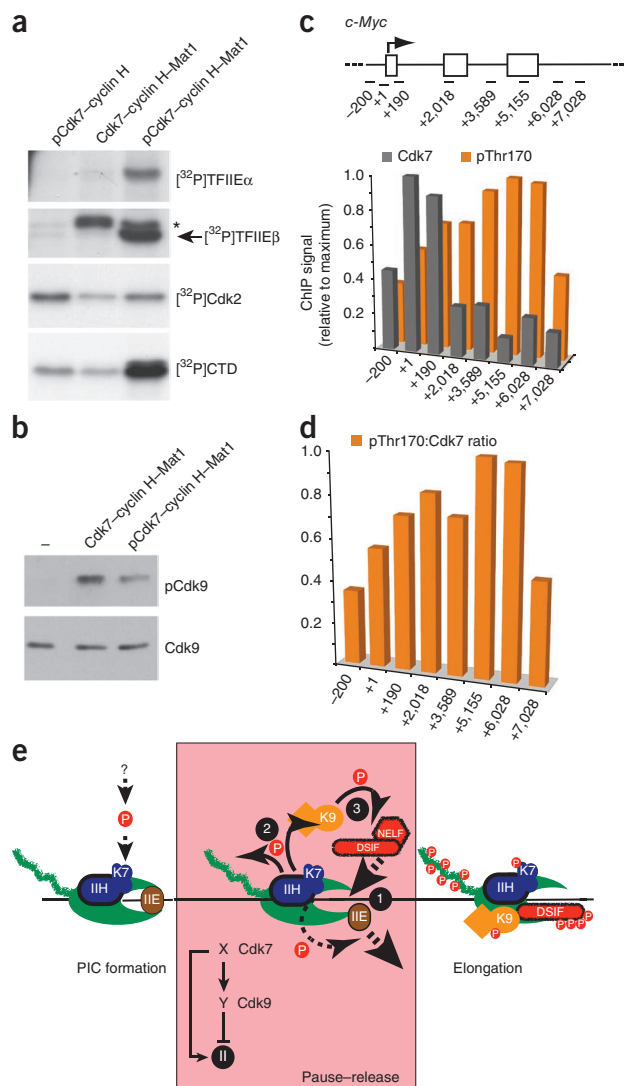
Fully activated Cdk7 phosphorylates TFIIEβ

To identify the possible mechanism(s) by which Cdk7 activity might facilitate the handoff of Pol II from TFIIE to DSIF, we tested the ability of purified Cdk7 complexes to phosphorylate TFIIE purified from bacteria. Cdk7 phosphorylated both subunits of holo-TFIIE but labeled the TFIIEβ subunit more heavily (Fig. 7a), whereas TFIIEH isolated from mammalian cells was reported to phosphorylate only TFIIEα^{25,26}. We showed previously that CAK and CTD kinase functions of Cdk7 complexes could be differentially regulated by subunit composition or T-loop phosphorylation^{26,38}. Phosphorylation of Cdk2–cyclin A complexes by Cdk7–cyclin H was relatively slow ($k_{cat} \sim 0.02 \text{ s}^{-1}$) and depended on Cdk7 T-loop phosphorylation or presence of the accessory subunit Mat1 but not both. In contrast, the basal rate of Pol II CTD phosphorylation by the Cdk7–cyclin H–Mat1 trimer was about ten times faster and was further accelerated ~20-fold when the Cdk7 T loop was phosphorylated³⁸. Activation of Cdk9–cyclin T1 by trimeric Cdk7 was also insensitive to Cdk7 phosphorylation (Fig. 7b). In contrast, phosphorylation of TFIIE, like that of Pol II, Spt5 and other non-CDK substrates of Cdk7, depended on both Mat1 and Cdk7 T-loop phosphorylation (Fig. 7a).

Figure 7 Cdk7 T-loop phosphorylation stimulates TFIIE phosphorylation and is spatially regulated on chromatin. **(a)** Activity assay of Cdk7. Efficient phosphorylation of TFIIEα and TFIIEβ by Cdk7 depends on both T-loop phosphorylation of Cdk7 and the presence of Mat1, as in the case of the Pol II CTD. Arrow points to labeled TFIIEβ; asterisk denotes Mat1 phosphorylated by associated Cdk7^{as}. **(b)** Cdk9 phosphorylation by Cdk7. Cdk9, like Cdk2, is phosphorylated with similar efficiency by all isoforms of Cdk7. T-loop phosphorylation of Cdk9^{as} was detected by immunoblotting with anti-pThr186. **(c)** ChIP analysis of Cdk7. Total Cdk7 signals are enriched in the promoter-proximal region, whereas T-loop phosphorylation increases from the 5' to the 3' end of the *c-Myc* gene. **(d)** The ratio of T-loop-phosphorylated to total Cdk7 increases along the length of *c-Myc*. **(e)** Proposed model of CDK-dependent, promoter-proximal pause establishment and release. (1) After recruitment of TFIIE and TFIIEH (Cdk7), Cdk7 activity allows TFIIE to be dislodged by DSIF from the Pol II clamp. The involvement of TFIIE phosphorylation by Cdk7 (thin dashed arrow) in this switch remains hypothetical, and other phosphorylations catalyzed by Cdk7 could be implicated. Binding of DSIF and NELF blocks elongation (pause). (2) Cdk9 is activated by Cdk7 and (3) phosphorylates DSIF and NELF to trigger processive elongation (release). Inset, Cdk7 sits atop a feedforward loop; its catalytic activity is required to initiate Pol II pausing through the TFIIE-DSIF switch and to release the pause through activation of Cdk9.

CDK T-loop phosphorylation: a basis for substrate ordering?

Because T-loop phosphorylation of Cdk7 could affect its substrate specificity and, thereby, the order in which it phosphorylates different proteins during the transcription cycle, we asked whether this modification—which appeared to be constant in the soluble fraction of Cdk7 extracted from human cells at different points in the cell cycle^{38,39}—was evenly or unevenly distributed on chromatin. Although the peak of Cdk7 cross-linking occurred at or near the TSS, ChIP analysis with a phospho-specific antibody (Supplementary Fig. 6) revealed increased Cdk7 pThr170, relative to total Cdk7, as we sampled further downstream on multiple genes (Fig. 7c,d and data not shown). This suggests that Cdk7, like Cdk9 (and Rpb1 and Spt5), is recruited to chromatin preferentially in unphosphorylated form, whereas the Cdk7 that co-localizes with elongating Pol II is



more likely to be phosphorylated. That could be because of spatially regulated Cdk7 T-loop phosphorylation, preferential retention of the phosphorylated isoform in the elongation complex or both. In any case, the dynamic nature of Cdk7 T-loop phosphorylation on chromatin suggests a means to regulate events in transcription such as the TFIIE-DSIF handoff. On the other hand, Cdk9 activation is likely to be a slow step not subject to acceleration by Cdk7 modification. Therefore, the T-loop phosphorylation status of chromatin-associated Cdk7, determined by still-unidentified kinases and phosphatases, has the potential to regulate the duration of promoter-proximal pausing by Pol II.

DISCUSSION

We have shown here that Cdk7 can act to establish the promoter-proximal pause, through its control of the TFIIE-DSIF switch, and to release Pol II from the pause, through its ability to activate Cdk9. Therefore, within the context of the Pol II transcription machinery, Cdk7 is both an effector CDK, which phosphorylates Pol II and other proteins directly involved in transcription, and an upstream regulator of Cdk9 (and, possibly, other transcriptional CDKs). The identification of Cdk7 as a Cdk9-activating kinase in human cells resolves a long-standing puzzle and suggests that mammals might have only a single CAK for all CDKs that depend on T-loop phosphorylation, regardless of their function. In contrast, transcriptional CDKs in fungi are activated by a non-cyclin-dependent, single-subunit CAK, even in fission yeast, where the Cdk7 ortholog is a physiologic activator of cell-cycle CDKs^{22,40,41}.

To drive the cell cycle, CDKs work in sequences defined by the timed expression of different cyclins and by checkpoints that ensure dependence of later events on completion of earlier ones⁴². Although transcription by Pol II likewise involves multiple CDKs, mechanisms enforcing their order of action have been slow to emerge. Recent work in budding and fission yeast revealed one basis for sequential CDK function: recruitment of P-TEFb depends on activity of the TFIH-associated kinase^{33,34}. In fission yeast, moreover, Ser7 phosphorylation of the Pol II CTD by the Cdk7 ortholog Mcs6 can promote subsequent phosphorylation by Cdk9 (refs. 34,43). Although the latter mechanism—CTD ‘priming’ by an early-acting CDK—appears to be conserved in human cells⁴⁴, data presented here indicate that the former is unlikely to operate in metazoans; inhibition of Cdk7 in human cells did not diminish recruitment of Cdk9 to chromatin.

Instead, we uncovered a third way in which distinct CDK functions can be ordered in the Pol II transcription cycle—direct activation of one CDK by another—that is likely to be unique to metazoans. In human cells, P-TEFb is activated by Cdk7, as are CDKs involved in cell division^{44,45}. Therefore, cell proliferation and gene expression are controlled by CDK cascades with a common upstream activating kinase. During cell-cycle progression, different CDK-cyclin complexes are active at different times and phosphorylate substrates in a temporal order determined by properties of both enzyme (distinct recognition motifs on different cyclins) and substrate (relative affinity for given CDK-cyclin pairs)⁴⁶. The individual subunits of TFIH and P-TEFb are expressed constitutively and apparently are recruited to chromatin *en bloc*. Our ChIP analyses of Cdk7 and Cdk9 suggest that a degree of temporal regulation might instead be achieved by T-loop phosphorylation during the transcription cycle: the ratios of phosphorylated to unphosphorylated isoforms at different positions on transcribed chromatin predict that the specific activity of both CDKs would increase along genes in a 5′-to-3′ direction. Consistent with this idea, a readout of chromatin-associated CDK activity—Ser2 phosphorylation of the Rpb1 CTD—also increases toward the 3′ end

and is diminished when Cdk7 is inhibited. The decrease in the ratio of pSer2 to total Pol II was modest relative to the drop in the ratio of Cdk9 pThr186 to total Cdk9, perhaps because reduced Pol II density on transcribed chromatin lowered the effective concentration of the Cdk9 substrate (Rpb1 CTD) simultaneously with the reduction in specific activity of the CTD-modifying enzyme (Cdk9).

Cdk7 activity is required at two distinct execution points in the G1 and G2 phases of the cell cycle⁴. Cdk7 also influences the Pol II cycle at multiple points, promoting pausing by recruitment of DSIF and NELF to chromatin and activating a downstream CDK to overcome the pause. Inefficient recruitment of DSIF and NELF would lower the threshold of P-TEFb activity required to overcome pausing and thus might explain why pausing is attenuated by inhibition of Cdk7 even while Cdk9 is also indirectly inhibited. Moreover, that inhibition is not complete: wild-type Cdk9 had measurable activity in the absence of detectable Thr186 phosphorylation, and a Cdk9^{T186A} mutant also had basal activity toward Spt5 *in vitro* (Fig. 3b), which might suffice to support elongation when Cdk7 is inhibited. Although elongation is permitted under these circumstances, Pol II density in coding regions is generally diminished, steady-state levels of many transcripts are decreased and RNA processing is disrupted (ref. 5, this report and S.L., R.A. and R.P.F., unpublished observations). Antagonistic downstream effects elicited by the same kinase suggest incoherent feedforward (Fig. 7e)—a network motif that produces biphasic response kinetics in cellular signaling pathways⁴⁷. In this case, the proposed delay between recruitment of elongation factors and activation of Cdk9 could ensure a transient pause to promote processive transcription and proper loading of mRNA-processing machinery.

Our results raise the possibility that CDKs also act in similar fashion to enforce the stable pausing of Pol II in promoter-proximal regions of stringently regulated genes, together with known pause factors such as NELF^{1,2}. Like NELF depletion^{2,37}, selective inhibition of Cdk7 caused decreased Pol II cross-linking to chromatin at multiple genes and deranged mRNA 3′-end formation (ref. 5 and this report). The model of elongation control that we have proposed (Fig. 7e) appears ‘hardwired’ to produce a transient pause. The relative rates of the individual steps might be subject to differential regulation, however, which could shorten or lengthen the pause as needed. For example, T-loop phosphorylation of Cdk7—which appears to be a postrecruitment event at the genes we have analyzed—could accelerate phosphorylation of Pol II, Spt5 and TFIIE without affecting the rate of Cdk9 activation and thereby increase pause duration. An intrinsically slow rate of Cdk9 T-loop phosphorylation might be further retarded, in contrast, by negative regulatory factors or features of the chromatin landscape at specific genes, to generate a more durable pause. The chemical-genetic approach uncovered a CDK cascade at the core of the Pol II transcription machinery. It could now illuminate the regulatory capabilities of that module and allow the identification of additional targets of both Cdk7 and Cdk9 within the transcription machinery.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Supplementary information is available in the [online version of the paper](#).

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AUTHOR CONTRIBUTIONS

S.L., D.L.B. and R.P.F. designed the research and interpreted data. S.L., R.A., K.G.-C. and M.S. performed experiments and analyzed data. J.J.A., C.Z. and K.M.S. provided reagents. S.L. and R.P.F. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Antibodies. We obtained antibodies to Cdk9 (sc-8338), cyclin T1 (sc-10750), RNA Pol II (sc-899 and sc-9001), Spt5 (sc-28678), TFIIIE α (sc-237) and NELF-A (sc-23599) from Santa Cruz Biotechnology; RNA Pol II phosphorylated at Ser2 (A300-654A) or Ser5 (A300-655A) from Bethyl Laboratories; histone H2B (07-371) and H2BUb1 (05-1312) from Millipore; Cdk9-T186P (2549), used for immunoblots only, from Cell Signaling Research; and Cdk7 (MO1.1) from Sigma. The anti-phospho-Cdk9 (Thr186), and anti-phospho-Cdk7 (Thr170), used in ChIP and immunoblot experiments, were custom produced by 21st Century Biochemicals by using the peptide sequences SQPNRY(pT)NRV and SPNRAY(pT)HQVVTRW, respectively. All antibodies were used at 1:500 to 1:1,000 dilutions for immunoblotting, or 2–5 μ g per chromatin immunoprecipitation.

Cell culture and drug treatments. HCT116 cells were grown in McCoy's medium (Cellgro) supplemented with 10% FBS (Atlas Biologicals, CO) and 1% penicillin-streptomycin (Cellgro). Cells were treated at ~70–80% confluence by replacing the growth medium with fresh medium containing either DMSO, 10 μ M 3-MB-PP1, 50 μ M DRB (EMD cat. # 287819) or 100 nM flavopiridol (Sigma cat. # F3055), dissolved in DMSO.

Chromatin immunoprecipitation (ChIP). Primers used for PCR amplification in ChIP and RNA-quantification experiments are listed in **Supplementary Table 1**. Experiments were carried out essentially as previously described³⁰. Cells were grown in 15-cm dishes and treated with drugs for 4 or 8 h before fixation in 1% paraformaldehyde (Fisher BP531) in PBS for 15 min. The reaction was quenched with 100 mM glycine for 5 min. The plates were washed 3 \times in cold PBS and harvested in 1 ml each RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 50 mM NaF, Complete protease inhibitor (Roche)), then flash frozen in liquid nitrogen. Sonication was carried out in a volume of 2 ml with a Fisher Scientific sonic dismembrator 550 fitted with a micro tip, at 25% power in 20-s pulses for a total of 6 min. Lysates were clarified by centrifugation at 20,000g for 15 min. Lysate from one 15-cm dish was used for 3–5 immunoprecipitations, performed overnight with protein A or protein G beads, pre-blocked with bovine serum albumin and salmon sperm DNA, with 2–5 μ g of antibody. Nonimmune rabbit IgG was used as negative control. Quantitative PCR was carried out by using a Stratagene MX3005P instrument with SYBR green mixes from various manufacturers.

Protein purification and kinase assays. Cdk9^{as} was produced in Sf9 insect cells as a C-terminal His₆-Flag-tagged protein as previously described for Cdk7 (ref. 27). To generate cyclin T1, the coding sequence was cloned into the pET-28 vector (Novagen) and the protein expressed in bacteria as a C-terminal histidine-tagged protein and purified on Nickel-NTA (Qiagen). The Cdk9^{as}-cyclin T1 complex was reconstituted by incubation of equimolar amounts of each purified protein on ice for 30 min in 25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM DTT before activation reactions. The activated, Thr186-phosphorylated, Cdk9^{as}-cyclin T1 complex could be purified from Sf9 cells after co-infection. Csk1 tagged at the C terminus with Flag and His₆ was expressed in Sf9 cells and purified by affinity chromatography on nickel-NTA. A PCR-amplified fragment of Spt5-coding sequence corresponding to amino acids 720–830 (CTR1) was cloned into pGEX-4T1

(Amersham), expressed as a GST fusion protein in bacteria and purified on glutathione-Sepharose according to the supplier's instructions. For Cdk9^{as} activation assays, Cdk9^{as} purified as a monomer from Sf9 cells was combined with bacterially produced cyclin T1 and incubated with a source of activating kinase—either purified or in cell extracts—in the presence of MgCl₂ and ATP, followed by the phosphorylation of recombinant GST-Spt5 in the presence of N⁶-(benzyl)-[γ -³²P]ATP or N⁶-(2-phenethyl)-[γ -³²P]ATP. Cdk7-cyclin H dimers were generated by co-infection in insect cells; this results in binary complexes in which Cdk7 is phosphorylated at Thr170 (pCdk7-cyclin H), whereas coexpression of Cdk7 with cyclin H and Mat1 yields a trimeric unphosphorylated complex (Cdk7-cyclin H-Mat1). The phosphorylated trimeric complex was generated by adding purified His-Mat1 to the Cdk7-cyclin H dimer (pCdk7-cyclin H-Mat1)³⁸.

Generation of ATP analogs. Radiolabeled ATP analogs were produced enzymatically as previously described⁴⁸. Briefly, ~4 mg of His-tagged nucleoside diphosphate kinase (NDPK) was bound to a 100- μ l CoCl₂-iminodiacetic acid column. [γ -³²P]ATP (2 mCi at 6,000 mCi/mmol, Amersham Biosciences) was passed over the column, followed by several washes. N⁶-(benzyl)- or N⁶-(2-phenethyl)-ADP was then passed over the column, and the eluate containing N⁶-(benzyl)- or N⁶-(2-phenethyl)-[γ -³²P]ATP was collected with several washes of 10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, resulting in a recovery of ~90% of the input radioactivity and a final concentration of 1.0–2.5 μ Ci/ μ l.

Protein phosphorylation in extracts. Nuclear extracts were prepared and dialyzed essentially as previously described⁴⁹ and modified⁵⁰. For labeling, ~100 μ g of protein was incubated in 60 μ l of 25 mM HEPES, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 80 mM Na- β -glycerophosphate, 50 mM NaF, 1 mM Na₃VO₄, containing an ATP-regenerating system (1 mM ATP, 40 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase) with 100 ng Cdk9^{as}-cyclinT1 complex and 5 μ Ci N⁶-(benzyl)-[γ -³²P]ATP. Reactions were incubated at 23 °C for 10–15 min and stopped by the addition of 1 volume 2 \times SDS-PAGE sample buffer for direct analysis, or by dilution in 25 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 20 mM EDTA for subsequent immunoprecipitation. Labeled bands were visualized by SDS-PAGE and exposure to film or Phosphorimager screen.

In vitro transcription and translation. cDNAs encoding wild-type and mutant forms of Cdk9 were transcribed and translated *in vitro* with the TNT-Quick Coupled Transcription/Translation System (Promega), according to the manufacturer's instructions.

Histone gene expression analysis. For analysis of read-through transcription of the *HIST1H2BD* gene, total RNA was isolated after 12-h drug treatments and reverse transcribed by using olido(dT) to quantify read-through transcripts and with random hexamer primers to quantify total RNA as previously described²⁴.

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