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Dichotomous but stringent substrate selection by the dual-function Cdk7 complex revealed by chemical genetics

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Cdk7 performs two essential but distinct functions as a CDK-activating kinase (CAK) required for cell-cycle progression and as the RNA polymerase II (Pol II) CTD kinase of general transcription factor IIH. To investigate the substrate specificity underlying this dual function, we created an analog-sensitive (AS) Cdk7 able to use bulky ATP derivatives. Cdk7-AS–cyclin H–Mat1 phosphorylates ~10–15 endogenous polypeptides in nuclear extracts. We identify seven of these as known and previously unknown Cdk7 substrates that define two classes: proteins such as Pol II and transcription elongation factor Spt5, recognized efficiently only by the fully activated Cdk7 complex, through sequences surrounding the site of phosphorylation; and CDKs, targeted equivalently by all active forms of Cdk7, dependent on substrate motifs remote from the phosphoacceptor residue. Thus, Cdk7 accomplishes dual functions in cell-cycle control and transcription not through promiscuity but through distinct, stringent modes of substrate recognition.

The cyclin-dependent kinase-7 (Cdk7) complex was first identified biochemically as the metazoan CDK-activating kinase (CAK)[1–3]. The major form of the enzyme in cell extracts consists of the catalytic subunit Cdk7 together with cyclin H and the RING finger protein Mat1 in a heterotrimeric complex[4–8], which is also an intrinsic component of general transcription factor IIH (TFIIH)[9–11]. Although once controversial[12], the assignment of dual functions in cell-cycle control and transcription was validated by experiments that established Cdk7 (or an ortholog) as a Cdk1-activating kinase *in vivo* in *Drosophila melanogaster*[13], *Schizosaccharomyces pombe*[14–16] and *Caenorhabditis elegans*[17]. However, the incongruous ability of Cdk7 to phosphorylate such dissimilar substrates as the Pol II C-terminal domain (CTD) and CDK activation segments (T-loops) has not been explained and seems to require unusual substrate-selection strategies[18,19].

Cdk7 is a member of the CDK family, which belongs to a larger group of serine/threonine kinases with a preference for a proline in the +1 position relative to the phosphoacceptor residue. The heptad sequence repeated in the CTD, 1-YSPTSPS-7, within which Cdk7 preferentially phosphorylates Ser5 (refs. 9,20,21), conforms to this preference. In addition, Cdk7 has been reported to phosphorylate sites containing Ser-Pro or Thr-Pro dipeptides within a number of DNA-binding transcription factors, including the tumor suppressor p53 (ref. 22), retinoic acid receptor-α[23], E2F-1 (ref. 24) and Oct-1 (ref. 25). In contrast, the consensus among Cdk7 target sites in mammalian CDKs is TXXVVTL (in which the first threonine is phosphorylated and where X indicates lack of conservation). Cdk1, Cdk2 and Cdk6, all substrates of Cdk7 *in vitro*[4,26], lack a proline on the C-terminal side of the threonine residue phosphorylated by Cdk7.

One possible solution to the problem of substrate recognition is promiscuity. Cdk1, the prototypic cell-cycle CDK, phosphorylates hundreds of substrates containing the consensus Ser/Thr-Pro-X-Lys/Arg (where X is any residue) in whole-cell extracts of budding yeast[27], and in addition it phosphorylates some substrates with the abbreviated consensus Ser/Thr-Pro[28]. The ability of Cdk7 to phosphorylate sites both with and without proline residues would seem to suggest relaxed sequence recognition and perhaps a substrate range comparable to that of Cdk1. That would be counterintuitive, however, given that Cdk7 functions in cell-cycle control as an upstream activator of effector kinases such as Cdk1. The alternative hypothesis is that Cdk7 maintains two (or more) separate but nonetheless stringent modes of recognition. Possibly consistent with that idea, previous studies have shown differential effects on kinase activity toward different purified substrates upon addition of Mat1 to Cdk7–cyclin H to form a heterotrimeric complex[29,30] or upon Cdk7 T-loop phosphorylation[31].

To distinguish between promiscuity and dichotomy in substrate recognition, we needed a way to label the endogenous substrates of Cdk7 selectively in unfractionated extracts. We therefore engineered

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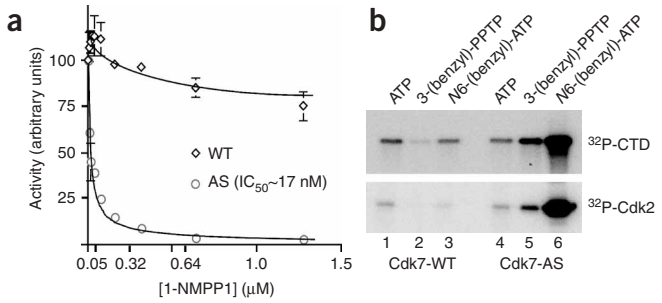


Figure 1 Generation and characterization of Cdk7-AS. (**a**) *In vitro* phosphorylation results showing that the AS mutation renders Cdk7 sensitive to the ATP analog 1-NMPP1. WT denotes wild-type Cdk7. (**b**) Cdk7-AS uses *N*6-(benzyl)-ATP with high efficiency. Phosphorylation of GST-CTD (top) or Cdk2 (bottom) using wild-type Cdk7 or Cdk7-AS with radiolabeled ATP (lanes 1 and 4), 3-(benzyl)-PPTP (lanes 2 and 5) or *N*6-(benzyl)-ATP (lanes 3 and 6) is shown.

an analog-sensitive mutant version of human Cdk7 (Cdk7-AS), following a strategy applicable to many eukaryotic protein kinases, in which a conserved bulky hydrophobic residue lining the ATP-binding pocket is mutated to a glycine residue to enable binding of nucleoside analogs with substituent groups appended to the purine ring[32]. This provides a way to label the substrates of a single kinase specifically in extracts, because the AS kinase is uniquely able to use the ATP analog[27].

We find that Cdk7-AS is not promiscuous in its substrate selection, but instead shows exquisite specificity for a small number of polypeptides in crude extracts of human tissue culture cells. The new targets of Cdk7 we identified, together with the previously known substrates, define two structurally unrelated classes, requiring two distinct but equally efficient and highly selective mechanisms for recognition. Recognition of one class is directed by the amino acid residues surrounding the site of phosphorylation on the substrate and is dependent on both the presence of Mat1 and phosphorylation of the T-loop in Cdk7 complexes. Recognition of the other class, in contrast, depends on substrate features not found near the phosphorylation site and requires either Mat1 or phosphorylation of the T-loop for optimal efficiency.

RESULTS

An analog-sensitive Cdk7

To render Cdk7 capable of binding derivatized ATP analogs, we changed the conserved Phe91 to a glycine residue. We expressed the Cdk7-AS mutant protein, together with cyclin H, in insect cells infected with recombinant baculoviruses and purified the resulting dimeric complex (in which Cdk7 is bis-phosphorylated on its own T-loop) before reconstituting the fully active form of the complex by addition of purified Mat1 (ref. 31). Cdk7-AS was sensitive to inhibition by the nonhydrolyzable analog 1-NMPP1 (**Fig. 1a**), whereas wild-type (Cdk7-WT) complexes purified and assembled in the same way were insensitive.

Cdk7-AS selectively used two analogs, 3-(benzyl)-pyrazolopyrimidine triphosphate (PTTP) and *N*6-(benzyl)-ATP[33] (**Supplementary Fig. 1** online), to phosphorylate two model substrates: the Pol II CTD and Cdk2 (**Fig. 1b**). One analog, 3-(benzyl)-PPTP, was a poor substrate for Cdk7-WT but was preferred over natural ATP by Cdk7-AS (**Fig. 1b**, compare lanes 2 and 5). Cdk7-WT showed a preference for natural ATP (K_m ~90 μM) over the other analog, *N*6-(benzyl)-ATP (K_m ~500 μM) (**Supplementary Table 1** online). However, under conditions of substrate excess Cdk7-WT could use the unnatural analog to phosphorylate glutathione *S*-transferase (GST)-CTD, but not Cdk2, efficiently (**Fig. 1b**, compare lanes 1 and 3). Cdk7-AS, by contrast, preferred *N*6-(benzyl)-ATP (K_m ~1 μM) over ATP (K_m ~550 μM) (**Fig. 1b**, compare lanes 3 and 6; **Supplementary Table 1**). The basis for selectivity is an apparent K_m for the analog nearly 100-fold lower than that of Cdk7-WT for ATP. This preference does not seem to slow enzyme turnover; the apparent k_{cat} values for Cdk7-WT with ATP (~10 s^{-1}) and for Cdk7-AS with either ATP (~18 s^{-1}) or *N*6-(benzyl)-ATP (~23 s^{-1}) were of similar magnitude (**Supplementary Table 1**). Consistent with the minimal impairment of natural ATP use, *Drosophila* with a *cdk7-AS* allele as their sole source of the enzyme were viable, fertile and phenotypically wild type (S.L. and R.P.F., unpublished data). All subsequent selective labeling reactions with Cdk7-AS were carried out with the *N*6-(benzyl)-ATP analog.

Exquisite selectivity of Cdk7-AS in crude extracts

We next performed labeling reactions with the purified wild-type or Cdk7-AS complex added, at approximately physiological concentrations (see Methods), to human whole-cell (not shown) or nuclear extracts (**Fig. 2**). In the presence of 1 mM unlabeled ATP and an ATP-regenerating system, neither the added Cdk7-WT nor any endogenous kinase in the extract was capable of using the radioactive *N*6-(benzyl)-ATP to phosphorylate protein substrates detectably (**Fig. 2a**,**b** and **Supplementary Fig. 2** online). In contrast, Cdk7-AS labeled three major, discrete bands of ~200, ~110 and ~33 kDa (in addition to the Cdk7 autophosphorylation signal at ~45 kDa), which were resolved by one-dimensional denaturing gel electrophoresis (**Fig. 2a**, lane 8). On longer autoradiographic exposure, a less intensely labeled set of about seven additional endogenous substrates (and the ~36-kDa recombinant His-Mat1) became visible (**Fig. 2b**, lane 4, and **Supplementary Fig. 2**). The pattern of labeling was qualitatively similar in whole-cell extracts, except for decreased labeling of the ~110-kDa polypeptide (data not shown).

Because the electrophoretic mobilities of the ~200- and ~33-kDa labeled polypeptides corresponded to those of known Cdk7 substrates (Rpb1, the largest subunit of Pol II, and the cell-cycle CDKs, respectively) we performed immunoprecipitations with the appropriate antibodies after labeling reactions with Cdk7-AS and *N*6-(benzyl)-ATP. This analysis confirmed that the ~200-kDa labeled band is Pol II and that the ~33-kDa band is a mixture of Cdk1 and Cdk2 (**Fig. 2c**) as well as Cdk4 and Cdk5 (**Supplementary Fig. 3** online). Moreover, in crude extracts of asynchronous cells, the fully active Cdk7-AS complex phosphorylated Pol II and the ~33-kDa CDKs (considered as a single substrate) to virtually identical levels (**Fig. 2**).

Cdk7-AS complexes with distinct substrate preferences

We compared labeling by two different Cdk7-AS–containing complexes: the dimeric complex with cyclin H, assembled and phosphorylated on the T-loop *in vivo*; and the trimeric, phosphorylated complex, reconstituted *in vitro* by addition of Mat1 to the dimer (**Fig. 2d**). Although both complexes labeled the major ~110- and ~33-kDa substrates with about equal intensity, only the trimer efficiently phosphorylated Pol II (**Fig. 2d**, compare lanes 1 and 2). The appearance of signals at ~150, ~60 and ~38 kDa was also dependent on the presence of Mat1 in the Cdk7-AS complex.

The dependence of Pol II labeling on assembly of Cdk7-AS into a trimeric complex seemed consistent with a proposed role for Mat1 as a specificity factor capable of stimulating the CTD kinase activity

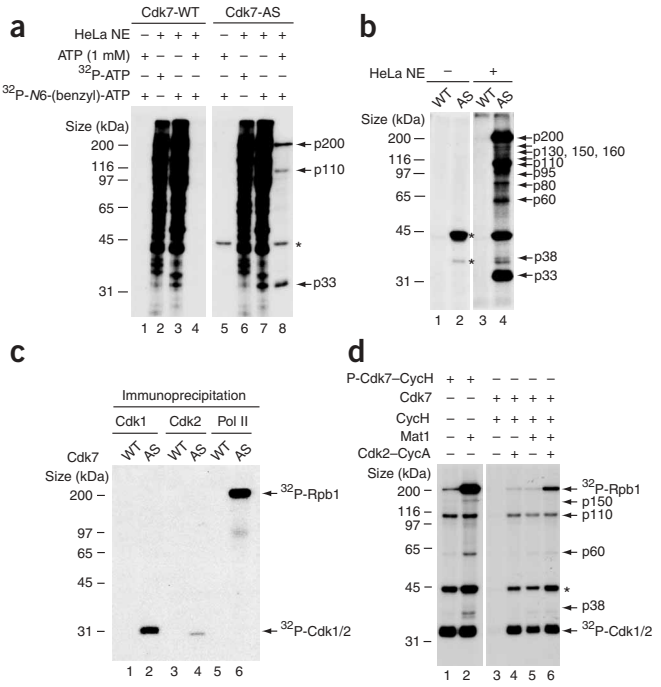


Figure 2 Cdk7-AS is exquisitely selective in crude extracts. (**a**) Incubation of wild-type Cdk7 (Cdk7-WT) or Cdk7-AS in HeLa nuclear extract (NE) with either [γ-^{32}P]ATP or [γ-^{32}P]*N*6-(benzyl)-ATP, in the absence of unlabeled ATP, results in nonspecific labeling (lanes 2, 3, 6 and 7). Labeling by [γ-^{32}P]*N*6-(benzyl)-ATP is completely abolished by the inclusion of 1 mM unlabeled ATP and an ATP-regenerating system in reactions containing Cdk7-WT (lane 4). Under the same conditions, a specific pattern of phosphorylation emerges in the presence of Cdk7-AS (lane 8). (**b**) Phosphorylation by Cdk7-AS–cyclin H–Mat1 of endogenous proteins in HeLa nuclear extract. Extract was omitted from reactions to reveal autophosphorylation of recombinant ~45-kDa Cdk7-AS and ~36-kDa His-Mat1 (lane 2, asterisk). The dried gels were exposed for longer periods than were those in **a**, to reveal additional substrates. Arrows indicate bands specifically labeled by Cdk7. (**c**) Results of immunoprecipitation of Cdk1, Cdk2 and RNA Pol II from extracts labeled with wild-type (lanes 1, 3, 5) or AS (lanes 2, 4, 6) Cdk7. (**d**) T-loop phosphorylation enhances activity of the Cdk7–cyclin H–Mat1 trimer in a substrate-specific manner. Labeling of endogenous proteins in HeLa nuclear extracts was carried out with [γ-^{32}P]*N*6-(benzyl)-ATP and the fully phosphorylated Cdk7-AS–cyclin H dimer (lane 1) or the phosphorylated Cdk7-AS–cyclin H–Mat1 trimer generated by adding pure Mat1 to the dimer (lane 2). CAK complexes were also assembled from individual subunits, including unphosphorylated Cdk7-AS (lanes 3–6). Inactive mixtures of Cdk7-AS and cyclin H (lane 3) can be activated through phosphorylation of the Cdk7 T-loop by Cdk2–cyclin A (lane 4) or through the addition of Mat1 (lane 5). Each pathway results in a similar, low level of activity toward Pol II. The phosphorylation of Cdk7 within the Mat1-containing complex results in an increased activity toward Pol II and other substrates (lanes 2 and 6). Cdk1/2 denotes the Cdk1–Cdk2 doublet; p200, for example, denotes a labeled polypeptide of ~200 kDa.

of Cdk7 (ref. 29,30). As was the case with purified recombinant CTD substrates, however[31], it is actually Cdk7 T-loop phosphorylation within the context of the trimeric complex that enhances the enzymatic activity of Cdk7-AS toward endogenous Rpb1. To demonstrate this, we reconstituted Cdk7-AS complexes *in vitro* from individual, monomeric subunits and tested their ability to phosphorylate endogenous Pol II and CDKs in nuclear extracts in the presence of *N*6-(benzyl)-ATP (**Fig. 2d**). Simple mixing of purified Cdk7-AS and cyclin H monomers did not generate detectable kinase activity (lane 3), presumably reflecting the lack of T-loop phosphorylation in the preparation of purified Cdk7 monomer[7]. Pretreatment with Cdk2–cyclin A (lane 4) or preincubation with Mat1 (lane 5) before the labeling reaction with the analog generated Cdk7-AS complexes that were active toward the CDK doublet and the ~110-kDa polypeptide, but only weakly active toward Pol II. The reconstituted trimer was slightly but reproducibly less efficient than the Cdk7–cyclin H dimer at phosphorylating endogenous Cdk1 and Cdk2 in the nuclear extract (compare lanes 4 and 5). We have previously observed a similar effect with purified enzymes and substrates, which might be due to an approximately two-fold higher apparent K_m for CDK–cyclin complexes[31]. The addition of Mat1 and pretreatment with active Cdk2–cyclin A together restored the ability to phosphorylate Pol II (lane 6), albeit not to the same level as could be achieved by addition of Mat1 to Cdk7-AS–cyclin H complexes phosphorylated and assembled *in vivo* (compare lanes 2 and 6).

The stimulation of kinase activity toward Rpb1 and other unidentified substrates did not come at the expense of activity toward CDKs (**Fig. 2d**, compare lanes 4 and 6), consistent with our previous enzymological characterization of the different Cdk7-containing complexes[31]. Notably, whereas most of the additional substrates we detected with Cdk7-AS were sensitive to T-loop phosphorylation, the ~110-kDa band was not, suggesting that it might also be a CDK (see below).

Specific phosphorylation sites exclusive to Cdk7

We were unable to immunoprecipitate suspected Cdk7 substrates Cdk6 (ref. 26), p53 (ref. 22) or E2F-1 (ref. 24) that had been labeled by Cdk7-AS in HeLa whole-cell or nuclear extracts (data not shown), and labeling of Cdk4 and Cdk5 was relatively inefficient (**Supplementary Fig. 3**). Different Cdk7 substrates might be labeled with varying efficiencies in extracts for a number of reasons (see Discussion). We explored one mechanism that could potentially prevent detection of a true Cdk7 substrate: the prior saturation of all potential sites for phosphorylation. Incubating nuclear extract in the presence of unlabeled ATP and an ATP-regenerating system before adding the radiolabeled *N*6-(benzyl)-ATP and Cdk7-AS caused the time-dependent diminution of labeling of both the ~33-kDa and ~110-kDa polypeptides but not the ~200-kDa band (**Fig. 3**). Immunodepletion of endogenous Cdk7 from the extract before incubation (**Fig. 3a**) prevented the bleaching of the AS kinase signals (**Fig. 3b**). We immunoprecipitated Cdk1 from the reactions to show that its specific labeling by Cdk7-AS could be abolished by extract preincubation (**Fig. 3c**); reversal of the bleaching effect by prior depletion of Cdk7 indicated that Cdk7 was the only kinase in the extract capable of phosphorylating the relevant site (Thr161 of the Cdk1 T-loop).

Identification of novel Cdk7 substrates

To identify additional substrates of Cdk7, we fractionated the HeLa nuclear extracts according to the separation scheme outlined in **Figure 4a** and performed labeling of chromatographic fractions *in vitro* with the AS kinase complex (**Fig. 4b**). We were unable to identify the major ~110-kDa substrate (p110) by mass spectrometry of polypeptides excised from a stained SDS-PAGE gel (**Fig. 4c**); the phosphorylation signal and a band immunoreactive with antibodies to human Cdk11 comigrated, however, through the three steps of the fractionation (**Fig. 4d** and data not shown). We also immunoprecipitated p110 labeled by Cdk7-AS in nuclear extract with an antibody to Cdk11 (**Fig. 4e**).

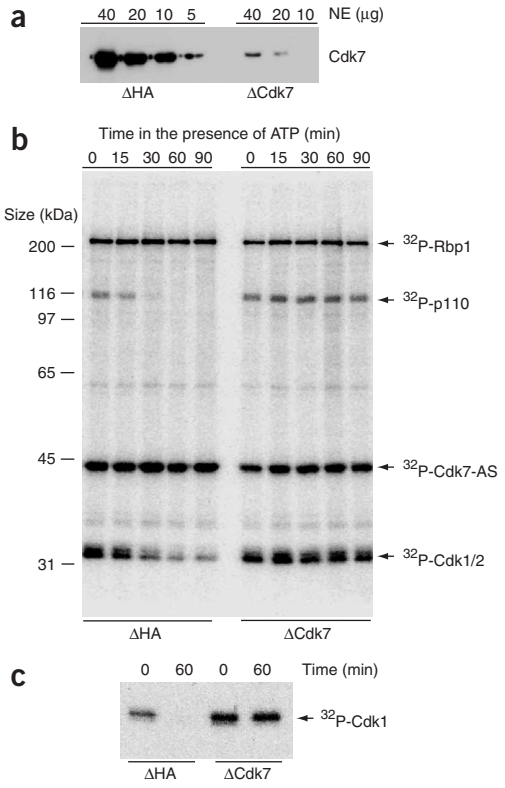


Figure 3 Identification of sites uniquely phosphorylated by Cdk7 on nuclear-extract proteins. (**a**) Anti-Cdk7 immunoblots of nuclear extract after immunodepletion with anti-Cdk7 or mock depletion with anti-HA, revealing ~90% depletion of endogenous Cdk7. (**b**) The mock- or Cdk7-depleted extracts (ΔHA or ΔCdk7, respectively) were incubated with unlabeled ATP for the indicated amount of time before labeling. Labeling of the ~33-kDa CDK doublet (^{32}P-Cdk1/2) and the ~110-kDa unidentified substrate (^{32}P-p110) was prevented by prior incubation of the extract with ATP. This bleaching was prevented by prior immunodepletion of endogenous Cdk7. (**c**) Results of immunoprecipitation of Cdk1 from the mock- or Cdk7-depleted extract after the labeling reaction. As indicated, the extracts were incubated for 0 or 60 min in the presence of unlabeled ATP before labeling.

During fractionation of the nuclear extract, the lightly labeled ~150-kDa polypeptide (**Fig. 2b,d** and **Supplementary Fig. 4** online) became progressively more prominent (**Fig. 4b**), presumably because of its enrichment and the simultaneous removal of competing substrates such as Pol II. In the final step of purification, Superdex 200 gel-exclusion chromatography, the phosphorylation signal at ~150 kDa (**Fig. 4b**) migrated with a major polypeptide (**Fig. 4c**), which was identified by mass spectrometry as the transcription elongation factor Spt5. We confirmed the identity of the ~150-kDa polypeptide labeled by Cdk7-AS in the unfractionated nuclear extract by immunoprecipitation with an antibody to Spt5 (**Fig. 4e**).

To confirm Cdk11 as an authentic Cdk7 substrate and to map the site of Cdk7-dependent phosphorylation, we tested the ability of Cdk7-AS to phosphorylate the recombinant 58-kDa isoform of Cdk11, which arises *in vivo* from translation initiation at an internal ribosome entry site (IRES)[34]. **Figure 5a** shows an alignment of the T-loops of Cdk2, Cdk7 and Cdk11. The Cdk11 sequence contains, in addition to the putative activating threonine residue, Thr219, a perfect Cdk1–Cdk2 consensus phosphorylation site at Ser213. We expressed the 58-kDa isoform of Cdk11 (wild-type, S213A or T219A) in rabbit reticulocyte lysates; all three forms were expressed at equivalent levels, as indicated by [^{35}S]methionine incorporation (**Fig. 5b**). Notably, in the control lysate not expressing human protein, Cdk7-AS did not label any endogenous proteins (**Fig. 5b**). In lysates programmed to express wild-type Cdk11 (lane 3) or the S213A mutant (lane 4), but not the T219A mutant (lane 5), Cdk7-AS labeled a single band at the expected size of ~58 kDa. We conclude that Cdk7 phosphorylates Cdk11, dependent on the conserved Thr219 residue in the Cdk11 T-loop, and is therefore likely to be a genuine Cdk11-activating kinase. Efficient phosphorylation of recombinant Cdk11, like that of endogenous p110 in nuclear extracts (**Fig. 2d**), did not depend on the presence of Mat1 (data not shown).

Spt5 is a component of the DRB sensitivity–inducing factor (DSIF)[35], which imposes a block to transcript elongation by Pol II that can be relieved by the action of positive transcription elongation factor b (P-TEFb), the complex of Cdk9 and cyclin T[36]. Spt5 contains two C-terminal repeat (CTR) regions with limited sequence similarity to the Pol II CTD[37]; Cdk9 phosphorylates Spt5 predominantly within CTR1, which is required for both positive and negative effects of Spt5 on elongation *in vitro*[37].

We compared phosphorylation site-specificity of Cdk7-AS and Cdk9 toward bacterially expressed protein fusions of GST with CTR1 or CTR2 (**Fig. 6a**). Whereas Cdk9 had a ~14-fold preference for sites within GST-CTR1 (**Fig. 6b**), consistent with previous reports[37], Cdk7-AS phosphorylated GST-CTR1 and GST-CTR2 equally well (**Fig. 6b**). Efficient phosphorylation of either CTR1 or CTR2 by Cdk7-AS was dependent on addition of Mat1 to the phosphorylated dimer (**Fig. 6b**, compare lanes 5 and 6 with lanes 8 and 9). Labeling by Cdk7-AS of the full-length Spt5 in nuclear extracts

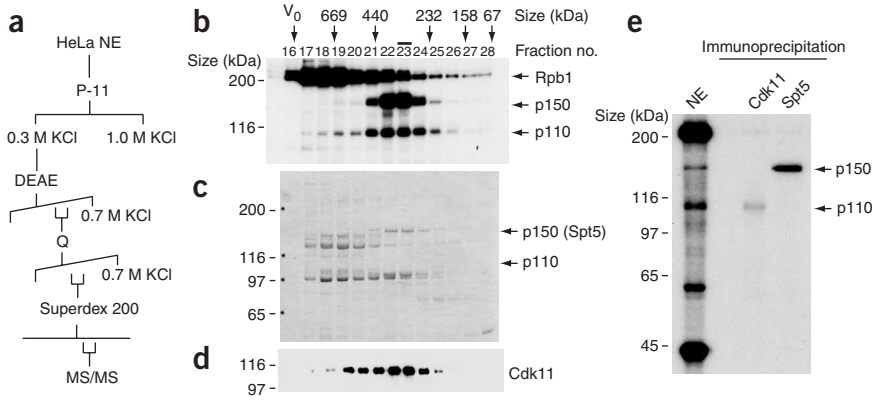


Figure 4 Purification and identification of Cdk7 substrates in nuclear extract (NE). (**a**) Chromatographic scheme to purify the unknown substrates. (**b**) Results of phosphorylation of fractions from a Superdex 200 size-exclusion column after sequential DEAE and Q-Sepharose chromatographies (**Supplementary Fig. 4**). (**c**) The polypeptides, visualized by staining with Coomassie blue. The ~150-kDa labeled band, p150, was unambiguously identified as Spt5 by mass spectrometry. (**d**) Anti-Cdk11 immunoblot of fractions from Superdex 200 chromatography, showing the cofractionation of Cdk11 with the Cdk7-AS substrate p110. (**e**) Confirmation that Spt5 and Cdk11 are substrates by immunoprecipitation from labeled nuclear extract.

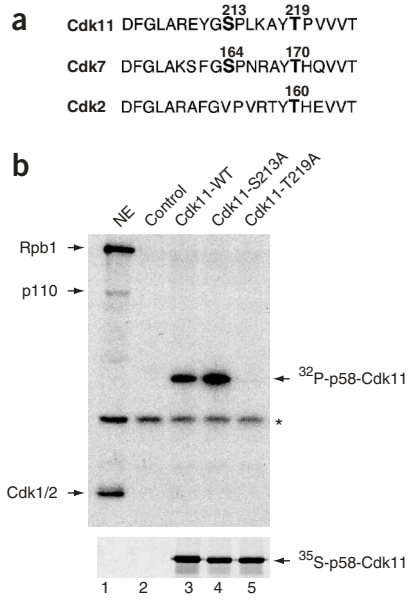
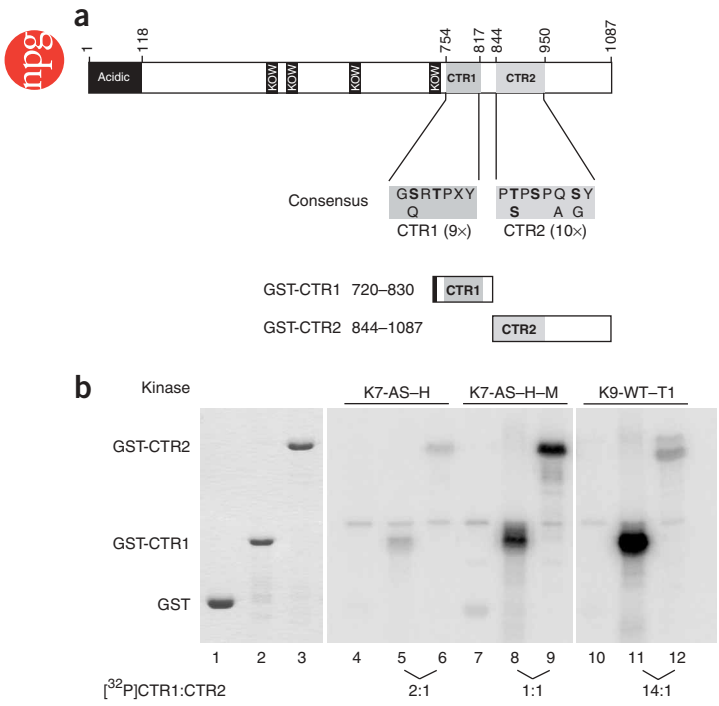


Figure 5 Cdk7 is a potential Cdk11-activating kinase. (**a**) Alignment of the T-loop sequences of Cdk2, Cdk7 and Cdk11. Both Cdk7 and Cdk11 T-loops have a similarly positioned serine residue in a perfect consensus CDK phosphorylation motif, in addition to the conserved, activating threonine. (**b**) Autoradiographic analysis of reticulocyte lysates programmed with cDNAs encoding the wild-type (WT), S213A or T219A p58 isoform of Cdk11 and either phosphorylated with Cdk7-AS and [γ-^{32}P]*N*6-(benzyl)-ATP (top) or labeled with [^{35}S]methionine (bottom). NE denotes nuclear extract. Control denotes mock transcription/translation in which reticulocyte lysate was not programmed with any cDNA.

was also Mat1 dependent (**Fig. 2d**). Thus, the requirements for recognition of Spt5 by Cdk7 are similar to those for Pol II and distinct from those for Cdk1, Cdk2 and Cdk11.

Distinct modes of substrate recognition by Cdk7

Because of the apparent dichotomy in its protein substrates, we examined the ability of Cdk7, in either dimeric or trimeric form, to phosphorylate peptides corresponding to its target sequences in Rpb1 and Cdk2. Cdk7 phosphorylated a peptide consisting of two tandem CTD heptad repeats (**Fig. 7a**); the trimeric complex was more active than the dimer toward the peptide substrate, as was the case with a GST-CTD fusion or with the full-length Rpb1 (**Fig. 2d**). In contrast, neither Cdk7 complex was able to phosphorylate a peptide derived from the T-loop of Cdk2 (**Fig. 7a**). However, Csk1, the monomeric CAK from *S. pombe*[15], was able to phosphorylate the same Cdk2 T-loop peptide. It therefore seems that the CTD of RNA Pol II contains all the information required for recognition by Cdk7, whereas the larger protein context is required for recognition of the Cdk2 T-loop. Notably, trimeric Cdk7 forms a stable complex *in vitro* with Cdk2 but not with a CTD substrate (**Fig. 7b**). (Interaction between the dimeric Cdk7–cyclin H and Cdk2–cyclin A complexes has been detected previously[38].) Taken together, our results suggest that Cdk7 has two distinct modes of protein substrate recognition (**Fig. 7c**): one for CDKs, which relies primarily on features distant from the actual site of phosphorylation; and one that depends on the sequence surrounding the phosphoacceptor residues in substrates such as Rpb1, Spt5 and others.



DISCUSSION

We have taken a chemical genetic approach[39], specifically engineering a protein kinase to accept a bulky variant of ATP as a phosphate donor, to define the substrate preferences of Cdk7 in the context of the entire, soluble intracellular proteome. Cdk7-AS shows exquisite selectivity in crude extracts for the same targets that the wild-type enzyme has been shown to phosphorylate by classical enzymological methods with purified substrate proteins. Our results reveal, moreover, a dichotomy in the proteins phosphorylated by Cdk7. For example, the additional substrates we identified, Cdk11 and Spt5, are structurally related to known Cdk7 substrates (Cdks and the Pol II CTD, respectively) but not to each other.

The mechanisms by which Cdk7 recognizes Pol II and Spt5 are likely to be similar. We base this prediction on (i) the similarly repetitive sequences of the CTD and CTRs[37]; (ii) the preponderance of Ser-Pro and Thr-Pro dimers within both substrates; and (iii) the dependence of Rpb1 and Spt5 phosphorylation on both the subunit composition and T-loop phosphorylation of the Cdk7 complex. We have reported previously that Thr170 phosphorylation of Cdk7 in the trimeric complex boosts the enzyme's turnover of model CTD substrates, whereas the activity toward Cdk2 is relatively insensitive to T-loop modification[31]. The endogenous proteins

Figure 6 Cdk7 phosphorylates recombinant Spt5. (**a**) Schematic representation of Spt5 and GST-fusion constructs expressing either the CTR1 or CTR2 repeats. Four regions of homology to KOW motifs in prokaryotic NusG are also indicated[37]. (**b**) Left, SDS-PAGE analysis showing purified GST (lane 1), GST-CTR1 (lane 2) and GST-CTR2 (lane 3) stained with Coomassie blue. Middle and left, autoradiograph showing GST (lanes 4, 7 and 10), GST-CTR1 (lanes 5, 8 and 11) and GST-CTR2 (lanes 6, 9 and 12) phosphorylated by Cdk7-AS–cyclin H dimer (K7-AS–H, lanes 4–6), Cdk7-AS–cyclin H–Mat1 trimer (K7-AS–H–M, lanes 7–9) or Cdk9–cyclin T1 (K9-WT–T1, lanes 10–12). The ratio of CTR1 phosphorylation to CTR2 phosphorylation ([^{32}P]CTR1:CTR2) by each kinase is indicated at bottom. In direct comparisons, Cdk9 has about ten-fold higher activity than Cdk7 toward CTR1-containing substrates (data not shown).

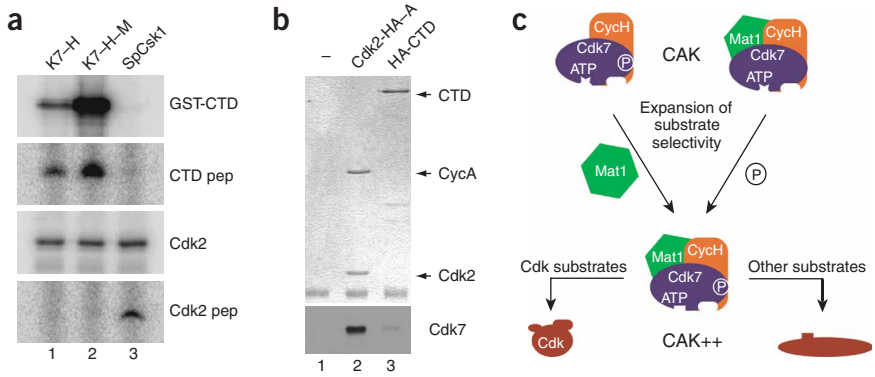


Figure 7 Alternative modes of substrate recognition. (**a**) Cdk7 efficiently phosphorylates a CTD peptide but not a T-loop peptide. Autoradiograph showing that phosphorylation of a peptide (pep) containing two tandem CTD heptad repeats by the dimeric (K7–H) or trimeric (K7–H–M) forms of Cdk7 roughly parallels labeling of a GST-CTD fusion protein. The monomeric CAK from fission yeast, Csk1 (SpCsk1) was active toward both full-length Cdk2 (in complex with cyclin A) and the Cdk2 peptide (but not GST-CTD or the CTD peptide). By contrast, neither Cdk7 complex, both of which phosphorylated full-length Cdk2, had detectable activity toward the peptide. (**b**) Co-immunoprecipitation results showing that Cdk7 associated with HA-tagged Cdk2–cyclin A but not with HA-tagged CTD after incubation of either substrate with Cdk7–cyclin H–Mat1 and ATP. (**c**) Schematic showing alternative modes of substrate recognition by Cdk7. Full activation of Cdk7 requires T-loop phosphorylation and binding to Mat1. This results in an expansion of substrate specificity, leading to the enhanced phosphorylation of substrates such as RNA Pol II and Spt5, which seem to be recognized through determinants surrounding the phosphorylation site, possibly by direct interaction with the kinase active site. In contrast, all three active forms of Cdk7 depicted efficiently recognize CDK substrates, probably by docking at a site distinct from the target T-loop.

labeled in crude extracts by Cdk7-AS can also be classified as dependent on (Pol II and Spt5) or independent of (Cdk1, Cdk2 and Cdk11) Mat1 binding and T-loop phosphorylation.

We suggest, on the basis of these differences, that Cdk7 adopts two distinct modes of substrate recognition to phosphorylate dissimilar target proteins, and that this flexibility is crucial for the enzyme to accomplish its dual functions. One mode is dependent on phosphorylation of the Cdk7 T-loop, whereas the other, through which Cdk7 phosphorylates other CDKs, is not. The CAK function of Cdk7 is therefore naturally buffered against changes in T-loop phosphorylation state that could potentially modulate the transcriptional function, and it might be regulated instead by the availability of substrates, by the activity of opposing T-loop phosphatases[40,41], by subcellular localization of Cdk7 itself[42] or by some combination of these. Also, in contrast to recognition of Pol II and Spt5, Cdk7 stably binds its CDK substrates and phosphorylates them essentially independently of the sequence surrounding the phosphoacceptor residue. This was suggested previously by the results of swapping T-loop sequences between CDKs that are natural Cdk7 substrates and those that are not[19].

Cdk11 is apparently recognized by the second mode, as expected because it is a CDK. Examination of its sequence, however, raises two paradoxes. First, in having a CDK consensus phosphorylation site six residues upstream of the putative activating threonine, the Cdk11 T-loop most closely resembles that of Cdk7 itself, but Cdk7 is incapable of T-loop autophosphorylation. Second, when the T-loop of Cdk7 is transplanted into Cdk2, both the CDK consensus site at Ser164 and the activating Thr170 become targets for phosphorylation by Cdk7 (ref. 19). In contrast, Cdk7-AS is selective for Thr219 of the Cdk11 T-loop and does not seem to phosphorylate the CDK consensus site Ser213. These preferences can also be explained if recognition and phosphorylation-site selection by Cdk7 are based on structural features of CDKs at some remove from the phosphoacceptor residue.

The two substrate-recognition mechanisms of Cdk7 operate with remarkably similar efficiency: in nuclear extracts, we measured a Pol II/Cdk1 + Cdk2 labeling ratio of 0.96. We measured the concentrations in our labeling reactions of Pol II, Cdk1 and cyclin B1 (upon which the phosphorylation of Cdk1 depends[4]) at ~4, ~22 and ~2.8 nM, respectively. There is a caveat: although we can estimate total concentrations of each individual substrate protein, we cannot know what fraction is available for phosphorylation by Cdk7 *in vitro*. Prior phosphorylation by endogenous Cdk7 can reduce labeling by Cdk7-AS, and the labeling efficiencies of different substrates might also reflect their relative accessibilities, the presence of phosphatases or both (S.L., J.B. and R.P.F., unpublished observations). It is nonetheless notable that the cell-cycle CDKs and Pol II are labeled by Cdk7-AS to nearly identical levels in asynchronous extracts, given the approximate parity in apparent values of k_{cat}/K_m measured for Cdk2–cyclin A and the CTD[31].

The AS kinase strategy has previously facilitated the identification of novel kinase substrates[27,43,44]. Here, it has led to the identification of two Cdk7 substrates, Cdk11 and Spt5, but perhaps more importantly, it has provided an insight into how Cdk7 carries out its two essential functions. Recognition of divergent primary structures does not hinge on relaxation of specificity, but rather on the maintenance of two stringent but distinct substrate-targeting mechanisms (**Fig. 7c**). The ability to phosphorylate short CTD peptides and the strong preference for Ser5 over Ser2 within the CTD heptad repeats[9,20,21,45,46] suggest precise docking of these substrates within the Cdk7 active site. Phosphorylation of CDKs, in contrast, relies on protein-protein interactions that bring enzyme and substrate together in a stable complex so as to ensure accurate phosphorylation of the T-loop, largely irrespective of the T-loop sequence. In support of that model, the apparent K_m for Cdk2 is an order of magnitude lower than that for CTD[31].

Several (about six) substrates detectably labeled by Cdk7-AS in nuclear extracts remain to be identified by additional fractionation or candidate-protein approaches. In addition, the AS kinase strategy will allow us to probe for differences in Cdk7 substrate specificity in different mammalian cell types or under different physiological conditions, for example at different points in the cell cycle. Finally, the identification of two new, *bona fide* Cdk7 substrates has expanded the known contours of an elaborate signaling network linking cell division, transcription and mRNA processing. Cdk11 is an essential kinase with suspected roles in mitosis, transcription and precursor-mRNA splicing[47,48]. Spt5 is implicated in the mechanisms that couple transcription elongation to mRNA capping[49–51]. We can now investigate possible roles for Cdk7-dependent phosphorylation in controlling, and perhaps coordinating, these processes.

METHODS

Generation of Cdk7-AS complexes. Wild-type and Cdk7-AS–containing complexes were purified as described previously[31,52]. To test the effect of T-loop

phosphorylation on activity of Cdk7 toward endogenous substrates, CAK complexes were reconstituted from individual components in the presence or absence of active Cdk2–cyclin A. Complexes were formed by first incubating ~1 μg each of Cdk7 and cyclin H at 23 °C for 30 min in a total volume of 25 μl 25 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$, 1 mM ATP and 1 mM DTT; then adding 5 μl buffer, or buffer plus 50 ng active Cdk2–cyclin A, and incubating for an additional 30 min at 23 °C; and finally further adding 5 μl buffer, or buffer plus 1 μg Mat1. An 8 μl aliquot (~200 ng) of each enzyme mix was then withdrawn to label HeLa nuclear extract. For further details regarding expression and purification of proteins, see **Supplementary Methods** online.

Immunological methods. We quantified Cdk7 in HeLa nuclear extract by comparison with purified Cdk7 on quantitative anti-Cdk7 immunoblots. The signal was acquired with a Fujifilm LAS-3000 imaging system and analyzed with ImageGauge software (Fujifilm). Endogenous Cdk7 was estimated at ~0.2% of total nuclear extract protein (data not shown). We typically added 100–200 ng of Cdk7-AS to labeling reactions containing 100 μg HeLa nuclear extract. Antibodies raised against Cdk1 (C19), Cdk2 (M2), Cdk11 (C17), Spt5 (H-300) and Rpb1 (N20) were obtained from Santa Cruz Biotechnology, anti-Cdk4 (35.1) from Biomol, anti-Cdk5 (DC17) from Upstate Biotechnology, anti–RNA polymerase II (8WG16) and anti-hemagglutinin (HA.11) from Covance and anti-Cdk7 (MO1.1) from Sigma.

Generation of ATP analogs. [γ-^{32}P]*N*6-(benzyl)-ATP was produced enzymatically by nucleoside diphosphate kinase (NDPK) as previously described[33]. Briefly, ~4 mg of His-tagged NDPK was bound to a 100-μl $CoCl_2$ iminodiacetic acid column. [γ-^{32}P]ATP (2 mCi at 6,000 mCi mmol^{-1}; Amersham Biosciences) was passed over the column, followed by several washes. *N*6-(benzyl)-ADP was then passed over the column and the eluate containing [γ-^{32}P]*N*6-(benzyl)-ATP collected with several washes of 10 mM HEPES (pH 7.4), 150 mM NaCl and 5 mM $MgCl_2$, resulting in a recovery of ~90% of the input radioactivity and a final concentration of 1.0–2.5 μCi μl^{-1}. Benzyl-PPTP was produced as previously described[33].

Protein phosphorylation in extracts. Nuclear extracts were typically prepared from 100 l of HeLa cells harvested at a density of 5 × 10^5 cells ml^{-1}. Nuclear extracts were prepared and dialyzed essentially as previously described[53], with modifications[54]. For labeling, ~100 μg of protein was incubated in 60 μl of solution containing 25 mM HEPES (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 80 mM sodium-β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4 and an ATP-regenerating system (1 mM ATP, 40 mM creatine phosphate, 0.2 mg ml^{-1} creatine phosphokinase) with 100 ng Cdk7-AS–cyclin H–Mat1 complex and 5 μCi [γ-^{32}P]*N*6-(benzyl)-ATP. We found that thorough mixing of the ATP regeneration system into the reaction before the addition of [γ-^{32}P]*N*6-(benzyl)-ATP reduced nonspecific background. Reactions were incubated at 23 °C for 10–15 min and stopped by the addition of 1 volume 2× SDS-PAGE sample buffer for direct analysis or by dilution in 25 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% (v/v) Triton X-100 and 20 mM EDTA for subsequent immunoprecipitation. Labeled bands were visualized by SDS-PAGE and exposure to film or Phosphorimager screen. Phosphorylation attained a steady state after 15 min, with no increase in specific labeling or background upon further incubation of up to 2 h. The phosphorylation by Cdk7-AS of certain substrates could be prevented by first assembling reactions as described above, omitting Cdk7-AS and [γ-^{32}P]*N*6-(benzyl)-ATP and allowing phosphorylation by endogenous kinases. For those experiments, a master mix consisting of 300 μg HeLa nuclear-extract protein in a final volume of 135 μl was incubated at 23 °C for various times before 17 μl was transferred to a fresh tube, where 1 μl Cdk7-AS (~100 ng μl^{-1}) and 2 μl [γ-^{32}P]*N*6-(benzyl)-ATP (~2 μCi μl^{-1}) were added. After an additional 15-min incubation at 23 °C, the reactions were stopped with 20 μl 2× SDS sample buffer. For further details regarding kinetic measurements and protein and peptide phosphorylation, see **Supplementary Methods**.

Interaction between Cdk7 and Cdk2. Cdk2-HA–cyclin A (2 μg Cdk2) or HA-GST-CTD (5 μg) were incubated with Cdk7–cyclin H–Mat1 (200 ng Cdk7) in 40 μl 25 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$ and 500 μM ATP for 10 min at 23 °C. The reaction was diluted by the addition of 400 μl 10 mM HEPES (pH 7.4), 150 mM NaCl and 0.1% (v/v) Triton X-100 and immunoprecipitated with anti-HA covalently coupled to protein G–Sepharose for 1 h at 4 °C. The immunoprecipitates were washed three times with 10 mM HEPES (pH 7.4), 150 mM NaCl and 0.1% (v/v) Triton and twice with 10 mM HEPES (pH 7.4) and 150 mM NaCl. Cdk2 and CTD were visualized by Coomassie blue staining, and associated Cdk7 was detected by an immunoblot.

***In vitro* transcription and translation.** Complementary DNAs encoding wild-type and phosphorylation site–mutant Cdk11 (58-kDa isoform) were transcribed and translated *in vitro* with the TNT Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's instructions. The programmed lysates (5 μl) were added directly to phosphorylation reactions with Cdk7-AS and [γ-^{32}P]*N*6-(benzyl)-ATP, performed as described above for nuclear extract. Separate translation reactions were carried out in the presence of [^{35}S]methionine in parallel to monitor the translation efficiency of each of the Cdk11 cDNAs.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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