Pol II CTD kinases Bur1 and Kin28 promote Spt5 CTR-independent recruitment of Paf1 complex

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Paf1 complex (Paf1C) is a transcription elongation factor whose recruitment is stimulated by Spt5 and the CDKs Kin28 and Bur1, which phosphorylate the Pol II C-terminal domain (CTD) on Serines 2, 5, and 7. Bur1 promotes Paf1C recruitment by phosphorylating C-terminal repeats (CTRs) in Spt5, and we show that Kin28 enhances Spt5 phosphorylation by promoting Bur1 recruitment. It was unclear, however, whether CTD phosphorylation by Kin28 or Bur1 also stimulates Paf1C recruitment. We find that Paf1C and its Cdc73 subunit bind diphosphorylated CTD repeats (pCTD) and phosphorylated Spt5 CTRs (pCTRs) *in vitro*, and that *cdc73* mutations eliminating both activities reduce Paf1C recruitment *in vivo*. Phosphomimetic (acidic) substitutions in the Spt5 CTR sustain high-level Paf1C recruitment in otherwise wild-type cells, but not following inactivation of Bur1 or Kin28. Furthermore, inactivating the pCTD/pCTR-interaction domain (PCID) in Cdc73 decreases Paf1C-dependent histone methylation in cells containing non-phosphorylatable Spt5 CTRs. These results identify an Spt5 pCTR-independent pathway of Paf1C recruitment requiring Kin28, Bur1, and the Cdc73 PCID. We propose that pCTD repeats and Spt5 pCTRs provide separate interaction surfaces that cooperate to ensure high-level Paf1C recruitment.

The EMBO Journal (2012) 31, 3494–3505. doi:10.1038/emboj.2012.188; Published online 13 July 2012

Subject Categories: chromatin & transcription

Keywords: CTD; Paf1C; Spt5; transcription elongation

Introduction

The Paf1 complex (Paf1C) is an important cofactor for elongating RNA Polymerase II (Pol II), comprised of five subunits: Paf1, Rtf1, Cdc73, Ctr9, and Leo1, which is recruited to the coding sequences (CDS) of actively transcribed genes (Kim *et al*, 2004; Sims *et al*, 2004; Mayer *et al*, 2010). Paf1C orchestrates the co-transcriptional ubiquitylation of histone H2B on Lys-123 and methylation of histone H3 on Lys-4, which requires H2B ubiquitylation, and also the methylation of H3 on Lys-36 and Lys-79 (Sims *et al*, 2004; Smith and Shilatifard, 2010). Paf1C also promotes correct

3' end formation by its role in recruiting termination factors (Penheiter *et al*, 2005; Sheldon *et al*, 2005; Nordick *et al*, 2008; Rozenblatt-Rosen *et al*, 2009; Kim and Levin, 2011), and enhances Pol II recruitment to promoters that depend on activator SBF (Swi4/Swi6) (Kim and Levin, 2011). Thus, efficient Paf1C recruitment is crucial for events occurring throughout the transcription process and spanning the entire CDS; however, the mechanism of Paf1C recruitment by elongating Pol II is not well understood.

Multiple factors have been implicated in Paf1C recruitment, including the FACT, Ccr4-Not, and Spt4/Spt5 (DSIF) complexes, and the cyclin-dependent kinases (Cdk) Kin28 (cyclin Ccl1) and Bur1 (cyclin Bur2) (Larabee *et al*, 2005; Wood *et al*, 2005; Pavri *et al*, 2006; Qiu *et al*, 2006; Mulder *et al*, 2007; Liu *et al*, 2009; Zhou *et al*, 2009). Kin28 phosphorylates the heptad repeats ($Y_1S_2P_3T_4S_5P_6S_7$) in the C-terminal domain (CTD) of Pol II subunit Rpb1 on Ser5 and Ser7 (Akhtar *et al*, 2009; Glover-Cutter *et al*, 2009; Kim *et al*, 2009), and this activity stimulates recruitment of mRNA processing factors, histone modifying enzymes and elongation factors near the promoter (Phatnani and Greenleaf, 2006; Buratowski, 2009; Govind *et al*, 2010). As inhibiting Kin28 does not impair Spt4 recruitment, it appears that Spt4/Spt5 acts directly in Paf1C recruitment (Qiu *et al*, 2006). Indeed, strong evidence was presented that Bur1 stimulates Paf1C recruitment by phosphorylating the C-terminal repeats (CTRs) of Spt5 (Liu *et al*, 2009; Zhou *et al*, 2009). Similar to the role of the phosphorylated Pol II CTD in cofactor recruitment, the hexad repeats in Spt5, with consensus sequence $S_1A_2W_3G_4G_5Q_6$, could provide a platform for Paf1C recruitment when phosphorylated by Bur1. However, it was reported that Paf1C does not bind directly to phosphorylated Spt4/Spt5 complex (Liu *et al*, 2009), making it unclear how Bur1 and Spt5 promote Paf1C recruitment. Moreover, the reduction in Paf1C recruitment evoked by inhibiting Bur1 exceeded that of Ala substitutions of the Ser₁ residue in all 15 hexad repeats of Spt5 (Liu *et al*, 2009), raising the possibility that Bur1 promotes Paf1C recruitment by an Spt5-independent pathway.

In fact, we and others have shown that Bur1 also phosphorylates Ser2 of the Pol II CTD (S2P) *in vivo*, augmenting the activity of the major, but non-essential, S2P kinase Ctk1 (Liu *et al*, 2009; Qiu *et al*, 2009). We found that Bur1 is recruited to the 5' end of the CDS at ARG1 in a manner stimulated by Kin28 and the pCTD-interaction domain (PCID) in the Bur1 C-terminus. Moreover, Bur1 contributed significantly to S2P formation near the ARG1 promoter and in the fraction of bulk elongating Pol II highly enriched in Ser5P (S5P), while Ctk1 was responsible for the majority of S2P at promoter-distal ARG1 sequences and in bulk Pol II (Qiu *et al*, 2009). An auxiliary role for Bur1 in S2P formation in the CDS was also detected by genome-wide measurements of S2P occupancy (Tietjen *et al*, 2010; Bataille *et al*, 2012). Interestingly, Tietjen *et al* (2010) further implicated Bur1 in S7P formation at sites distal from the promoter; although

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Received: 2 November 2011; accepted: 20 June 2012; published online: 13 July 2012

Bataille *et al* (2012) found that Bur1's contribution to S7P was most pronounced under conditions of Kin28 inhibition. Thus, it appears that Bur1 contributes to the formation of both Ser2P and Ser7P in elongating Pol II.

Considering our previous finding that Kin28 promotes Bur1 recruitment (Qiu *et al*, 2009), it was possible that the stimulatory role of Kin28 in Paf1C recruitment (Qiu *et al*, 2006) merely reflects the positive effect of Kin28 on Bur1 recruitment and attendant phosphorylation of Spt5 CTRs. We set out to test the alternative hypothesis that Kin28 acts more directly by phosphorylating the Pol II CTD on Ser5 and Ser7 to provide an alternative platform for Paf1C recruitment. In addition to phosphorylating the CTD directly, Kin28 could also cooperate with Bur1 to produce S2,S5- or S5,S7-diphosphorylated CTD repeats, and this collaboration with Bur1 in CTD phosphorylation, as well as Spt5 CTR phosphorylation by Bur1, would be enhanced by Kin28's stimulatory role in Bur1 recruitment. Our results indicate that Paf1C binds specifically to both types of diphosphorylated CTD repeats and to phosphorylated Spt5 CTRs (pCTRs) *in vitro*, and they provide strong evidence that Kin28 and Bur1 can promote Paf1C recruitment independently of Spt5 CTR phosphorylation. Accordingly, we envision a dual mechanism of Paf1C recruitment via diphosphorylated Pol II CTD repeats and Spt5 pCTRs, with both pathways stimulated by Kin28 and Bur1.

Results

Paf1C can bind directly to phosphorylated Rpb1 CTD repeats

Considering that Paf1C recruitment is stimulated by both Kin28 and Bur1 (Larabee *et al*, 2005; Qiu *et al*, 2006; Liu *et al*, 2009), and the evidence cited above that these Cdk

phosphorylate the Pol II CTD on Ser5 and Ser7 (Kin28) or Ser2 and Ser7 (Bur1), we investigated whether Paf1C can bind directly to Pol II CTD repeats in a manner stimulated by S2P, S5P, or S7P. To this end, Paf1C was purified from a *CDC73-TAP* strain through the first step of tandem affinity purification, that is, prior to cleavage of the calmodulin binding domain (CBD) (Puig *et al*, 2001; see Supplementary Figure S1A), and tested for binding to biotinylated peptides comprised of three CTD repeats, either unphosphorylated, monophosphorylated at Ser2, Ser5, or Ser7, or diphosphorylated at either Ser2,Ser5 or Ser5,Ser7, in all three repeats. (Note that S2P,S7P-diphosphorylated peptides were not successfully synthesized.) Cdc73-CBD and co-purifying Rtf1 show no binding to unphosphorylated peptides, little or no binding to S2P and S7P peptides, and relatively weak binding to S5P peptides (Figure 1A). Remarkably, the purified Paf1C binds much more strongly to both S2P,S5P and S5P,S7P peptides, with consistently slightly greater binding for the S2P,S5P peptides (Figure 1A, lanes 1–25 and data not shown). As expected if Cdc73-CBD and Rtf1 bind as subunits of the same complex, the extent of depletion of Cdc73-CBD in the supernatant (S) fraction using S2P,S5P peptides relative to the 'mock' no-peptide reaction was comparable to that observed for Rtf1 (Figure 1A, cf. lane 14 versus 10 and 25 versus 17). (The depletion of signal in the supernatant does not equal the increase in signal in the bound fraction (B) because a larger proportion of the latter was examined.) These findings suggest that native Paf1C interacts with CTD repeats in a manner stimulated moderately by S5P and strongly by S2,S5 or S5,S7 diphosphorylation.

To confirm that enhanced binding of Paf1C to the phosphorylated peptides is dependent on the phosphate groups, the

Figure 1 Paf1C binds to phosphorylated CTD peptides. **(A, B)** Biotinylated CTD peptides (1.5 µg) phosphorylated on Ser2 (S2P), Ser5 (S5P), Ser7 (S7P), Ser2 and Ser5 (S2P S5P), Ser5 and Ser7 (S5P S7P), unphosphorylated (CTD) were adsorbed to streptavidin-coated magnetic beads. Native Paf1C purified from a *CDC73-TAP* strain was incubated with beads alone (Mock) or beads bearing peptides at 4°C. 1/40 input (Inp), 1/40 supernatants (S) and bound (B) proteins were subjected to Western analysis with anti-TAP and anti-Rtf1 antibodies. For lanes 24–29, immobilized (S2P S5P) peptides were treated with CIP in the presence or absence of PhosSTOP prior to incubation with purified Paf1C. **(B)** As in **(A)** except CTD peptides with Ser2 and Ser5 substituted by Asp were also used. Lanes 1–3 and 4–7 derive from different portions of the same blot as described in Supplementary Figure S10A. **(C, D)** As in **(A, B)** except using the indicated purified recombinant GST fusion proteins (500 ng) in place of Paf1C and anti-GST antibodies in Western analysis. Also, 1/10 input (Inp) and supernatants (S) (1/20 input and supernatant in **(D)**) for GST-Ctr9(594–855) were examined. Figure source data can be found with the Supplementary data.

S2P,S5P peptides were pre-treated with calf intestine phosphatase (CIP), in the absence or presence of phosphatase inhibitor PhosSTOP, before incubation with purified Paf1C. Indeed, specific binding of Cdc73-CBD and Rtf1 to S2P,S5P peptides was abolished by CIP treatment and fully recovered by inclusion of PhosSTOP (Figure 1A, lanes 24, 26, and 28). Similar results were observed for the S5P peptides (Supplementary Figure S2A). Second, we tested purified Paf1C for binding to phosphomimetic peptides containing Asp2 and Asp5 in all three repeats, and observed no detectable binding (Figure 1B). Accordingly, Paf1C binding to phosphorylated CTD repeats likely involves specific recognition of the phosphate dianion in a manner inefficiently mimicked by monoanionic carboxylate groups. The results in Figure 1A and B support the possibility that Paf1C is recruited by Pol II CTD repeats diphosphorylated on Ser5/Ser2 or Ser5/Ser7.

Cdc73, Ctr9, and Rtf1 each contains a putative PCID

In an effort to determine which Paf1C subunits can mediate binding to phosphorylated CTD peptides, GST fusions to each subunit were purified from *E. coli* and used in binding assays. As the full-length GST-Ctr9 fusion was not well expressed, we analysed fusions containing different segments of Ctr9. Interestingly, GST-Rtf1 binds only to the S2P,S5P peptides. By contrast, the GST fusion containing residues 594–855 of Ctr9 binds weakly to S2P and S5P, but tightly to both kinds of diphosphorylated peptides, with a slight preference for S2P,S5P over S5P,S7P (Figure 1C; Supplementary Figure S2B). The GST-Cdc73 fusion binds to all three peptides harbouring monophosphorylated serines but, similar to the above findings on other recombinant subunits and purified Paf1C, it binds much better to the S2P,S5P- and S5P,S7P-diphosphorylated peptides. Based on the fractions of input bound, and the ratios of signals in the B versus S fractions, recombinant Cdc73 binds more tightly than does Ctr9 or Rtf1 to all peptides to which the latter two subunits can bind (Figure 1C; Supplementary Figure S2B). The fusions to Paf1, Leo1, and three other segments of Ctr9 (1–491, 414–800, and 701–1077) showed no binding to any CTD peptides (Figure 1C and data not shown) using comparable levels of fusion proteins in the binding reactions (Supplementary Figure S1B). As shown above for purified Paf1C, dephosphorylating the S2P,S5P peptides greatly reduced binding by GST-Cdc73, GST-Ctr9(594–855), and GST-Rtf1 in a manner restored with PhosSTOP (Supplementary Figure S2C). Furthermore, GST-Rtf1 and GST-Ctr9(594–855) showed little or no binding to the S2D,S5D phosphomimetic peptides (Figure 1D), and GST-Cdc73 showed reduced binding to the S5D and S2D,S5D peptides compared to the cognate mono- and diphosphorylated peptides (Supplementary Figure S2D).

Together, these findings suggest that Cdc73, Rtf1, and Ctr9 each contains a PCID that binds more tightly to one or both kinds of diphosphorylated CTD repeats than to monophosphorylated repeats and that also prefers dianionic phosphate groups over monoanionic acidic residues. These results can account for the higher affinity of native Paf1C for diphosphorylated versus monophosphorylated repeats (Figure 1A), and they support our hypothesis that Bur1 stimulates Paf1C recruitment, in part, by phosphorylating Ser2 or Ser7 in CTD repeats already phosphorylated on Ser5 by Kin28.

The presence of PCIDs in Cdc73 and Rtf1 is consistent with the known requirements for these two subunits in Paf1C

recruitment *in vivo* (Mueller *et al*, 2004; Nordick *et al*, 2008). Given the strong evidence that Rtf1 acts directly in Paf1C recruitment (Warner *et al*, 2007), we examined whether native Rtf1 can interact with pCTD peptides independently of the other two Paf1C subunits harbouring PCIDs by purifying Rtf1-CBD from an *RTF1-TAP cdc73Δ ctr9Δ* strain. As Rtf1 association with Paf1C requires Cdc73 (Nordick *et al*, 2008), the Rtf1-CBD preparation should also be devoid of Paf1 and Leo1. As shown in Supplementary Figure S2E, Rtf1-CBD bound only to the S2P,S5P peptides, and the proportion of input recovered in the bound fraction was considerably smaller than that seen for intact Paf1C (cf. Supplementary Figure S2E, lanes 15–16 versus Figure 1A, lanes 13–14). Thus, while native Rtf1 can interact with S2P,S5P peptides independently of Cdc73 and Ctr9, it appears that Cdc73 or Ctr9 contributes to the relatively stronger pCTD-binding activity of intact Paf1C and also to its interaction with S5P,S7P peptides. Based on the relative affinities of recombinant Cdc73 and Ctr9 for S2P,S5P peptides (Figure 1C), it is likely that Cdc73 makes the greater contribution to Paf1C interaction with this diphosphorylated species of CTD heptad.

Evidence that Paf1C binds directly to phosphorylated Spt5 CTRs

There is strong evidence that Bur1 promotes Paf1C recruitment by phosphorylating Ser₁ in the 15 CTRs of Spt5 (Liu *et al*, 2009; Zhou *et al*, 2009); however, evidence was lacking for direct interaction of Paf1C with phosphorylated Spt5 (Liu *et al*, 2009). To determine whether Paf1C can interact with Ser1-phosphorylated CTRs, we conducted peptide binding assays with purified Paf1C and biotinylated peptides consisting of four repeats of the Spt5 consensus sequence S₁A₂W₃G₄G₅Q₆, synthesized with Ser1 unphosphorylated (CTR), phosphorylated (pCTR), or substituted with aspartic acid (CTR_D).

The Paf1C purified from the *CDC73-TAP* strain described above displayed strong, phosphorylation-dependent binding to the Spt5 CTR peptides, binding at high levels to pCTR but not to CTR peptides, and binding in a manner abolished by CIP treatment of pCTR and recovered fully with PhosSTOP (Figure 2A). Moreover, purified Paf1C displayed reduced binding to CTR_D versus pCTR peptides (Figure 2A). Similar to our results with Pol II CTD peptides, bacterially expressed GST-Cdc73, GST-Rtf1, and GST-Ctr9(594–855) all bind to pCTR peptides in a manner greatly diminished by CIP treatment and recovered with PhosSTOP (Figure 2B), and show no binding to unphosphorylated CTR peptides (Figure 2C). By contrast, GST-Paf1 and GST-Leo1 do not bind to either pCTR or CTR peptides (Figure 2C). Based on the proportion of input recovered with the pCTR peptides, GST-Cdc73 and GST-Rtf1 bind more tightly than GST-Ctr9(594–855) to pCTR peptides (Figure 2B and C). Consistent with this, Rtf1-CBD purified from *cdc73Δ ctr9Δ* cells also displays strong binding to pCTR peptides (Supplementary Figure S2F).

Summarizing the binding data for the recombinant subunits in Figure 1C and Supplementary Figure S2B and C, Cdc73 binds tightly to phosphorylated CTR peptides and to both kinds of diphosphorylated CTD peptides, Rtf1 binds strongly to pCTR but only moderately to S2P,S5P peptides, and Ctr9 binds relatively weakly to both pCTR and both kinds of diphosphorylated CTD peptides. Unlike our findings

Figure 2 Paf1C binds to phosphorylated Spt5 C-terminal repeats (CTR) peptides. (**A–D**) Peptide binding assays were conducted as described in Figure 1 using biotinylated CTR peptides (1.5 µg) with Ser1 unphosphorylated (CTR), Ser1 phosphorylated (pCTR), or Ser1 substituted with Asp (CTR_D) or Glu (CTR_E), and purified native Paf1C or recombinant GST fusion proteins, except using 1/5 or 1/10 input and supernatants of GST-Rtf1 and GST-Ctr9(594–855), respectively. Lanes 10–18 in (**A**) derive from the same experiment that produced Figure 1B, and the input and Mock lanes from the latter are reproduced here as lanes 10–12. Figure source data can be found with the Supplementary data.

for purified Paf1C, recombinant GST-Cdc73, GST-Rtf1, and GST-Ctr9(594–855) fragments bound to the phosphomimetic Spt5 CTR_D peptides to nearly the same extent as to pCTR peptides (Figure 2D). While this last discrepancy remains to be explained, the results in Figure 2 provide strong evidence that Paf1C can interact specifically with phosphorylated Spt5 CTRs.

The Cdc73 PCID promotes Paf1C recruitment in vivo

Having found that recombinant Cdc73 displays the strongest affinity for both kinds of diphosphorylated CTD repeats, we sought to identify the PCID in Cdc73 and assess its importance in Paf1C recruitment. To this end, we tested a panel of GST-Cdc73 deletion variants for binding to the S5P-CTD peptides *in vitro*. As shown in Figure 3A, a C-terminal fragment of residues 201–393 was the smallest Cdc73 segment that bound S5P-CTD peptides indistinguishably from full-length Cdc73 (Figure 3A), and it showed the same relative affinities for different CTD peptides observed for full-length Cdc73, namely S2P,S5P>S5P>S2P>unphosphorylated peptides (cf. Figure 3B, top row (WT) with Figure 1C, GST-Cdc73). These results suggested that the functional PCID spans a relatively large region (of 193 residues) in the Cdc73 C-terminus.

A multiple sequence alignment of Cdc73 from different yeasts and fungi revealed blocks of conserved residues throughout the C-terminal segment (Supplementary Figure S3), including a possible WW motif—a PCID containing two conserved Trp residues (Meinhart *et al*, 2005)—and other strings of conserved residues with no obvious similarity to previously established PCIDs (Li *et al*, 2005; Meinhart *et al*, 2005; Phatnani and Greenleaf, 2006; Vojnic *et al*, 2006; Lunde *et al*, 2010; Xiang *et al*, 2010; Ghosh *et al*, 2011).

We introduced mutations into the GST-Cdc73(201–393) construct to generate Ala substitutions in the conserved Trp residues (W357A and W380A), or in 6–7 consecutive residues in three other conserved blocks, and tested the mutant proteins for peptide binding. As shown in Figure 3B and C, all of the mutations except W357A reduced or abolished binding of the fusion protein to pCTR peptides. Interestingly, all substitutions besides W357A also greatly impaired binding to the Spt5 pCTR peptides (Figure 3D), consistent with the possibility that the same structural elements in the Cdc73 C-terminus responsible for binding to pCTR repeats mediate binding to the Spt5 pCTRs. However, it is possible that these substitutions disrupt folding of the Cdc73 C-terminal region and impair binding to pCTR and/or pCTR peptides by an indirect mechanism.

After introducing a subset of these PCID mutations into chromosomal *CDC73* (along with a C-terminal *myc* tag), we discovered by Western analysis of yeast WCEs that they moderately reduce abundance of Cdc73–myc protein. However, for three of the mutants we could mitigate these reductions by introducing a low-copy plasmid harbouring the same mutant allele: W380A; W357AW380A; and 304-309Ala (Supplementary Figure S4A). The resulting strains also display normal levels of Paf1C, as indicated by the fact that nearly WT amounts of Rtf1 (Figure 4A) and HA-tagged Paf1 (Supplementary Figure S4B) co-immunoprecipitated with each *cdc73*–myc protein. Accordingly, we proceeded to evaluate the effects of the *cdc73* mutations on Paf1C recruitment by chromatin immunoprecipitation (ChIP) analysis of the myc-tagged Cdc73 subunits.

We showed previously that the occupancy of myc-tagged Paf1 in *ARG1* chromatin is increased on induction of transcriptional activator Gcn4 (by starvation for isoleucine and

Figure 3 Phospho-CTD interaction domain (PCID) of Cdc73 is located in its C-terminal region. (A) GST fusions to full-length (F.L.) Cdc73 or the Cdc73 truncations shown schematically on the right were purified and used in peptide binding assays with the CTD peptide S5P as described in Figure 1. The results are summarized in the schematic. (B–D) Peptide binding assays conducted as in Figure 1 using the indicated CTD peptides (B, C) or Spt5 pCTR peptides (D) and either WT or the indicated mutant versions of GST-Cdc73(201–393). Lanes 1–3 and 4–5 of the W357A panel derive from different sections of the same blot as described in Supplementary Figure S10B. Figure source data can be found with the Supplementary data.

Figure 4 Substitutions in the Cdc73 PCID eliminating binding to pCTD peptides reduce Cdc73-myc occupancies at ARG1. (A) Untagged WT strain BY4741 and the following *CDC73-myc₁₃* strains were cultured in synthetic complete medium lacking leucine (SC-Leu) to log phase (A_{600} of ~ 0.6): HQY1147 (*CDC73-myc₁₃*); HQY1468 (*cdc73-W380A-myc₁₃* harbouring low-copy plasmid pHQ1862 with *cdc73-W380A-myc₁₃*, henceforth abbreviated as -W380A*); HQY1469 (*cdc73-W357A, W380A-myc₁₃* harbouring pHQ1861, abbreviated as -W357A, W380A*); and HQY1471 (*cdc73-6Ala(304-309)-myc₁₃* harbouring pHQ1952, abbreviated as -304-309Ala*). WCEs were prepared and immunoprecipitated with anti-myc antibodies and subjected to Western analysis with anti-myc and anti-Rtf1 antibodies. The last six lanes derive from a separate blot as described in Supplementary Figure S10C. (B–D) ChIP analysis of Cdc73-myc binding at ARG1. The *CDC73-myc₁₃* strains described in (A) and *CDC73-myc₁₃* gcn4 Δ strain HQY1148 were cultured in SC lacking Ile, Val, and Leu (SC-Ilv-Leu) and treated with sulfometuron (SM) for 30 min to induce Gcn4. ChIP analysis was conducted using anti-myc antibodies for Cdc73-myc (B, C) or anti-Rpb3 antibodies for Rpb3 (D). DNA extracted from immunoprecipitates (IP) and input chromatin (Input) samples was subjected to PCR in the presence of [³³P]-dATP with the appropriate primers to amplify radiolabelled ARG1 5'ORF or 3'ORF sequences, or sequences from a chromosome V intergenic region (ChrV) examined as a control. PCR products were resolved by PAGE and visualized by autoradiography, with representative results shown for two biological replicates in adjacent lanes of (B), and quantified with a phosphorimager. The resulting values were expressed as ratios of IP to Input signals and normalized to the corresponding ratio for WT *CDC73-myc* cells. Error bars correspond to standard errors of the mean derived from four or more independent cultures and two independent immunoprecipitations for each culture. (See Materials and methods in Supplementary data for further details on quantification of ChIP data.) Figure source data can be found with the Supplementary data.

valine) to levels ~6-fold above background in *GCN4* cells but remains at background levels in *gcn4Δ* cells (Qiu *et al*, 2006). As shown in Figure 4B, Cdc73-myc occupancy in the *ARG1* CDS is similarly induced by Gcn4, showing much higher levels in *GCN4* (WT) versus *gcn4Δ* cells (lanes 11–12 versus 9–10). Importantly, the *W380* and *304-309Ala* mutations significantly reduced Cdc73-myc occupancy under inducing conditions (Figure 4B and C). An even stronger reduction was observed for the *W357A*, *W380A* double mutant, although the *W357A* single mutation had little or no effect on its own. The reductions in Cdc73 occupancy occurred without significant decreases in Pol II (Rpb3) occupancy at *ARG1* (Figure 4D). These results suggest that the Cdc73 PCID is crucial for efficient Paf1C recruitment at induced *ARG1*.

We also measured Cdc73-myc occupancies at the *GAL1* gene under conditions of galactose induction, and at the constitutively expressed genes *PMA1* and *ADH1*. As shown in Supplementary Figure S5A–F, the *W380A*, *W357A*, *W380A* and *304-309Ala* mutations all produced significant reductions in Cdc73-myc occupancies, but not Pol II (Rpb3) occupancies, in the coding sequences of these three genes, consistent with a general requirement for the Cdc73 PCID in Paf1C recruitment.

Bur1 promotes Paf1C recruitment by Spt5 pCTR dependent and independent pathways

The results above indicate that the Cdc73 PCID is required for robust Paf1C recruitment *in vivo*, but do not reveal whether it mediates interaction with Spt5 pCTRs, Pol II pCTD repeats, or both, to promote Paf1C recruitment. It was shown previously that substituting Ser1 with Ala in all 15 CTRs of Spt5 (*spt5-S1-15A*) reduced Paf1-myc occupancy at the 5' ends of *ARG1* and *PYK1* *in vivo* (Liu *et al*, 2009), consistent with the idea that Spt5 phosphorylation by Bur1 promotes Paf1C recruitment. To provide additional evidence supporting this interpretation, we examined whether the phosphomimetic Asp and Glu substitutions in the Spt5 CTRs would have less effect than Ala substitutions on Paf1C recruitment to *ARG1*. Indeed, Paf1-myc recruitment was reduced by a factor of 3–4 in the non-phosphorylatable *spt5-S1-15A* mutant, but only by 20–30% in the phosphomimetic mutants *spt5-S1-15D* and *spt5-S1-15E*, at the 5' and 3' ends of the *ARG1* CDS (Figure 5A and B). Thus, even though the Spt5 CTR_D and CTR_E peptides were inferior to the pCTR peptides in binding Paf1C *in vitro* (Figure 2), the phosphomimetic Spt5 substitutions can sustain a substantial level of Paf1C recruitment *in vivo*, supporting the conclusion that phosphorylation of Ser1 in the Spt5 CTRs by Bur1 stimulates Paf1C recruitment (Liu *et al*, 2009). We speculate that phosphomimetic Spt5 CTRs are highly effective in Paf1C recruitment *in vivo* because they contain an immutable negative charge, whereas the phosphorylation level of the WT CTRs is likely modulated by protein phosphatases.

The phosphomimetic substitutions should render the contribution of Spt5 CTRs to Paf1C recruitment independent of Bur1. Hence, if Bur1 stimulates Paf1C recruitment solely by phosphorylating Spt5, then inhibiting an analogue-sensitive Bur1 variant (encoded by *bur1-as*) should not affect Paf1C recruitment in the *spt5* phosphomimetic mutants. Consistent with previous results (Qiu *et al*, 2006; Liu *et al*, 2009), treatment of the *bur1-as* strain with the inhibitory ATP analogue 3MB-PP1 dramatically reduced Gcn4-dependent

Paf1-myc occupancy at *ARG1* (Supplementary Figure S6A, *bur1-as*, +MB versus –MB). Neither inhibition of *bur1-as* with 3MB-PP1 nor the *spt5-S1-15A* mutation reduces Pol II (Rpb3) occupancy at *ARG1* (Supplementary Figure S6B). Accordingly, the ratio of Paf1-myc to Rpb3 occupancies was strongly reduced by inhibition of *bur1-as* (Figure 5C, *bur1-as*, +MB versus –MB). The Paf1-myc:Rpb3 ratio was also reduced in the *spt5-S1-15A bur1-as* double mutant in the absence of *bur1-as* inhibition by 3MB-PP1, but not to the extent observed in the *bur1-as* single mutant treated with 3MB-PP1 (Figure 5C, *bur1-as spt5-S1-15A*, –MB versus *bur1-as*, +MB). Importantly, analogue treatment of all three *bur1-as spt5* double mutants evoked reductions in the Paf1-myc:Rpb3 ratios at *ARG1* compared to the levels seen without 3MB-PP1 (Figure 5C and Supplementary Figure S6A and B, *bur1-as spt5* double mutants, +MB versus –MB). These results indicate that Bur1 contributes to Paf1C recruitment at *ARG1* by a mechanism independent of Ser1 phosphorylation of the Spt5 CTRs. ChIP analysis of Paf1-myc recruitment at the *GAL1*, *PMA1*, and *ADH1* genes in the same mutants gave qualitatively similar findings, with inhibition of *bur1-as* evoking significant reductions in Paf1-myc occupancies in both the *bur1-as spt5-S1-15D* and *bur1-as spt5-S1-15E* double mutants (Supplementary Figure S7A–C). These findings support the possibility that diphosphorylated CTD repeats produced by Bur1 and Kin28 act together with the Spt5 pCTRs to promote Paf1C recruitment.

Paf1C recruitment also requires Kin28 kinase activity in phosphomimetic spt5 mutants

We showed previously that robust Paf1 recruitment is dependent on Kin28 (Qiu *et al*, 2006), and that Kin28 promotes Bur1 recruitment to promoter-proximal CDS (Qiu *et al*, 2009). Hence, we predicted that Kin28 should promote Paf1C recruitment, at least in part, by enhancing Bur1 recruitment and attendant phosphorylation of Spt5 CTRs. Supporting this prediction, inhibiting the analogue-sensitive variant of Kin28 (*kin28-as*) with NA-PP1 strongly reduces phosphorylation of the Spt5 CTRs, as judged by Western analysis of bulk Spt5 with phosphospecific antibodies (Liu *et al*, 2009; Figure 6A, lanes 3–4). Strikingly, phosphorylated Spt5 was eliminated on removal of the PCID in the C-terminus of Bur1 by the *bur1-ΔC* mutation (Figure 6A, lanes 5–6). Given that *bur1-ΔC* reduces Bur1 binding to S5P CTD repeats *in vitro* and decreases Bur1 recruitment to *ARG1* *in vivo* (Qiu *et al*, 2009), these findings strongly support the idea that Kin28 promotes Spt5 CTR phosphorylation by enhancing co-transcriptional recruitment of Bur1.

We asked next whether the contribution of Kin28 to recruitment of Paf1C is limited to its role in stimulating Spt5 phosphorylation by Bur1. If so, then analogue inhibition of *kin28-as* should not reduce Paf1C recruitment in strains harbouring the Spt5 variants with non-phosphorylatable CTRs. Consistent with our previous results (Qiu *et al*, 2006), ChIP analysis showed that treatment of *kin28-as* cells with ATP analogue NA-PP1 lowers the occupancy of the S5P form of Rpb1 at the promoter and 5'ORF regions of *ARG1* (Figure 6B, *kin28-as*). Furthermore, inhibition of *kin28-as* by NA-PP1 evokes reduced Paf1-myc:Rpb3 ratios in the *ARG1* coding sequences (Figure 6C, *kin28-as*). Importantly, inhibition of *kin28-as* also reduced Paf1 recruitment in cells harbouring the phosphomimetic variants *spt5-S1-15D*

Figure 5 Inactivating *bur1-as* reduces Paf1-myc occupancy at *ARG1* in *spt5* phosphomimetic and non-phosphorylatable CTR mutants. (A, B) Phosphomimetic substitutions in Spt5 CTRs sustain Paf1 recruitment at *ARG1*. *PAF1-myc₁₃* strains with the indicated WT or mutant *SPT5-HA₃* alleles (HQY1379, HQY1414, HQY1441, and HQY1447) were subjected to ChIP analysis with anti-myc antibodies as described in Figure 4. Representative results are shown in (A), with results from independent cultures displayed in adjacent lanes, and the quantification of results from four or more biological replicates is summarized in (B) as percentages of the values measured in the WT *SPT5* strain. (C) Inactivating *bur1-as* reduces Paf1-myc occupancy at *ARG1* in phosphomimetic *spt5* mutants. *PAF1-myc₁₃ bur1-as* strains with the indicated *SPT5-HA₃* alleles (HQY1379, HQY1416, HQY1444, Y1449, and HQY1419) were cultured in SC-Ilv to *A*₆₀₀ of ~0.4 and half of each culture was treated with 6 μM 3MB-PP1 (+3 MB) for 20 min while the other half was left untreated (–3 MB); and all cultures were then treated with sulfometuron (SM) for 30 min to induce Gcn4. ChIP analysis was conducted using anti-myc or anti-Rpb3 antibodies. Paf1-myc occupancies at *ARG1* were calculated as described in Figure 4, normalized for the corresponding Rpb3 levels and the resulting Paf1-myc:Rpb3 ratios are presented in (C) relative to those observed for the WT *SPT5-HA₃* strain. The Paf1-myc and Rpb3 occupancies used to calculate the ratios in (C) are presented in Supplementary Figure S6.

and *spt5-S1-15E*, and also in the strain containing the non-phosphorylatable mutant *spt5-S1-15A* (Figure 6C). ChIP analysis of Spt5 in the same strains insured that inhibition of kin28-as by NA-PP1 does not unexpectedly reduce the occupancies of WT or mutant forms of Spt5 at *ARG1* (Figure 6D). Similar results were obtained for the induced *GAL1* gene, at least for the 5' end of the CDS, where inhibition of kin28-as reduced the Paf1:Rpb3 ratio in cells harbouring the phosphomimetic *spt5* mutations (Supplementary Figure S8C). Although Paf1 occupancies were likewise reduced at the 3' end of *GAL1* on kin28-as inhibition (Supplementary Figure S8A), this result is complicated by the similar reductions in Rpb3 occupancies that occur at the *GAL1* 3' end (Supplementary Figure S8B). Nevertheless, the bulk of the data in Figure 6 and Supplementary Figure S8 indicate that cells harbouring Spt5 variants that cannot be phosphorylated by Bur1 are still dependent on Kin28 activity for efficient Paf1C recruitment. This implies that Kin28 promotes Paf1C recruitment by a mechanism distinct from its stimulation of Spt5 phosphorylation by Bur1, consistent with the notion that CTD repeats diphosphorylated by Kin28 and Bur1 provide an alternative platform for Paf1C recruitment.

The fact that the *kin28-as* mutation alone does not evoke a more dramatic reduction in Paf1C recruitment (Figure 6C) might be explained by noting that inhibition of kin28-as alone reduces, but does not abolish, Spt5 CTR phosphorylation (Figure 6A), and previous results indicating that Bur1 and Srb10 contribute to Ser5P and that Bur1 promotes Ser7P

formation *in vivo* (Liu *et al*, 2004; Kanin *et al*, 2007; Tietjen *et al*, 2010; Bataille *et al*, 2012). Thus, the residual Spt5 CTR and Pol II CTD phosphorylation occurring in analogue-inhibited *SPT5 kin28-as* cells could be sufficient for appreciable Paf1C recruitment by both partially phosphorylated scaffolds.

The Cdc73 CID is required for robust histone H3 methylation in the absence of Ser1 phosphorylation of Spt5 CTRs

Finally, we reasoned that if the Cdc73 PCID mediates Paf1C binding to both the Spt5 pCTRs and Pol II pCTD, then the *cdc73* PCID substitutions that impair binding to phosphorylated CTD repeats should impair Paf1C recruitment even in cells where the Spt5 CTRs are rendered non-phosphorylatable by S1A substitutions. To test this prediction, we first exploited the fact that elimination of Cdc73 in *cdc73Δ* cells confers strong 6-azauracil (6-AU) sensitivity, a phenotype conferred by various mutants with defects in Pol II elongation, owing to reduced nucleotide pools evoked by 6-AU (Riles *et al*, 2004). Importantly, we found that all three double mutants harbouring the *spt5-S1-15A* allele combined with one of the three *cdc73* PCID mutations display slow-growth phenotypes on complete medium and also much greater sensitivity to 6-AU than observed in the corresponding *spt5-S1-15A* or *cdc73* single mutants (Figure 7A). Employing a second *in-vivo* assay for Paf1C function, we measured the levels of Lys4 and Lys36 trimethylation in bulk histone H3 in the same

Figure 6 Paf1 recruitment in *spt5* phosphomimetic mutants requires Kin28 activity. (A) Inactivating *kin28-as*, or eliminating the Bur1 PCID, reduces phosphorylation of Spt5 CTRs *in vivo*. WCEs were prepared by the denaturing method of TCA precipitation from strains BY4741 (WT) and HQY1455 (*kin28-as*) cultured in SC-Ilv medium to A_{600} of ~0.35, untreated (–) or treated (+) with 48 μ M NA-PP1 for 42 min, and from HQY1223 (WT) and HQY1280 (*bur1-CA*) untreated with NA-PP1, and subjected to Western analysis with antibodies against phosphorylated Spt5 (Spt5-P) or total Spt5. (B–D) Inactivating *kin28-as* reduces S5P and decreases the Paf1-myc:Rpb3 ratio in phosphomimetic *spt5* mutants without diminishing Spt5 occupancies at ARG1. Paf1-myc₁₃₇₉ strains (WT SPT5-HA₃), HQY1414 (*spt5-S1-15A*-HA₃), HQY1441 (*spt5-S1-15D*-HA₃), HQY1447 (*spt5-S1-15E*-HA₃), HQY1465 (*kin28-as* SPT5-HA₃), HQY1575 (*kin28-as spt5-S1-15A*-HA₃), HQY1466 (*kin28-as spt5-S1-15D*-HA₃), and HQY1467 (*kin28-as spt5-S1-15E*-HA₃) were cultured in SC-Ilv and treated with NA-PP1 at 12 μ M for 12 min and then with sulfometuron for 30 min. ChIP analysis was conducted as described in Figures 4 and 5 using H14 antibodies against S5P (B), anti-myc antibodies and anti-Rpb3 antibodies (C), and HA antibodies against Spt5-HA (D). Figure source data can be found with the Supplementary data.

set of strains. As expected (Larabee *et al*, 2005; Chu *et al*, 2007), these two modifications were greatly reduced in *cdc73* Δ cells (Figure 7B, lane 9 versus 1). The *spt5-S1-15A* and *cdc73* PCID mutations produced slight to moderate reductions in the modifications (Figure 7B, lanes 1–5), with the strongest effects observed for the W357A,W380A* mutant (lane 3). Importantly, however, both H3-K4me₃ and H3-K36me₃ were nearly undetectable in all three *cdc73 spt5-S1-15A* double mutants (Figure 7B, lanes 6–8).

The findings that the *cdc73* PCID mutations exacerbate the growth phenotypes (Figure 7A) and impair H3 methylation (Figure 7B) in *spt5-S1-15A* cells, where the Spt5 pCTRs cannot contribute to Paf1C recruitment, strongly supports our hypothesis that the Cdc73 PCID mediates an important interaction of Paf1C with Pol II pCTD repeats in addition to Spt5 pCTRs. This model can also explain why the -W357A,W380A* single mutation confers 6AU sensitivity and reduces H3-K36me₃ abundance while the *spt5-S1-15A* mutation provokes neither phenotype, as the -W357A,W380A* mutation is expected to reduce Paf1C recruitment by both the pCTD and pCTR, whereas *spt5-S1-15A* impairs only the pCTR-dependent mechanism. Presumably, the *spt5-S1-15A*, -W357A,W380A* double mutation reduces Paf1C occupancy below the level conferred by -W357A,W380A* alone because *spt5-S1-15A* also decreases the ability of the Rtf1 and Ctr9 subunits to support Paf1C recruitment via the pCTR. In addition, although W357A,W380A* reduces Cdc73 occupancy at ARG1 to the background level seen in *gcn4* Δ cells (Figure 4B), it clearly does not abolish Cdc73 occupancy

at the other genes we tested (Supplementary Figure S5), and it may reduce Cdc73 levels at ARG1 only to the basal levels observed in *gcn4* Δ cells. Hence, the *spt5-S1-15A* mutation likely also eliminates the residual contribution of the W357A,W380A* variant of the Cdc73 PCID to Paf1C recruitment by the pCTR.

Discussion

Paf1C is recruited directly by phosphorylated Spt5 CTRs in a manner stimulated by Kin28

Previous work established that phosphorylation of the Spt5 CTRs by Bur1 is a key pathway for stimulating Paf1C recruitment by elongating Pol II (Liu *et al*, 2009; Zhou *et al*, 2009), but it was unclear whether the Spt5 pCTRs interact directly with Paf1C. We observed here that purified Paf1C and recombinant forms of three Paf1C subunits, Cdc73, Rtf1, and Ctr9, bind specifically to Ser1-phosphorylated Spt5 CTR peptides *in vitro*. The pCTR-binding activity of Cdc73 was mapped to its C-terminus and we demonstrated that substitution mutations in this PCID that inactivate pCTR binding *in vitro* reduce Paf1C recruitment to ARG1, GAL1, ADH1, and PMA1 CDS *in vivo*. These findings provide the first evidence for direct binding of Paf1C to the Bur1-phosphorylated form of the Spt5 CTRs.

We showed previously that Paf1C recruitment is also enhanced by Kin28 (Qiu *et al*, 2006), but the underlying mechanism was unknown. Consistent with the fact that Kin28 promotes Bur1 recruitment near the promoter via the

Figure 7 The Cdc73 PCID promotes Paf1C function independently of phosphorylated Spt5 CTRs, and a model for Paf1C recruitment via the Spt5-pCTR and Pol II pCTD. **(A)** *cdc73* PCID mutations confer synthetic sensitivity to 6-AU and slow growth in combination with *spt5-S1-15A*. Strains HQY1468 (-W380A), HQY1469 (-W357AW380A), HQY1471 (-304-309Ala), HQY1509 (-W380A *spt5-S1-15A*), HQY1510 (-W357AW380A *spt5-S1-15A*), and HQY1507 (-304-309Ala *spt5-S1-15A*) were transformed with a low-copy *URA3* plasmid containing the cognate chromosomal *cdc73* allele present in that strain (designated as *cdc73** alleles, as described in Figure 4), whereas strains HQY1147 (WT), HQY1508 (*spt5-S1-15A*), and 5326 (*cdc73Δ*) were transformed with an empty *URA3* vector. The transformants were grown to saturation and serial 10-fold dilutions were spotted on SC-Ura and SC-Ura + 6AU (with 6-azauracil at 100 μg/ml) and incubated at 30°C for 3 or 4 days, respectively. **(B)** WCEs prepared from strains in **(A)** were subjected to Western analysis with antibodies against total H3, the indicated methylated forms of H3, or Gcd6 (as loading control). Non-specific bands are marked by an asterisk. **(C)** Model for dual pathway of Paf1C recruitment via the pCTR and pCTD. **(Upper panel)** Kin28 phosphorylates Ser5 and Ser7 in the CTD and the S5P stimulates Bur1/Bur2 recruitment to the CTD via the Bur1 PCID. Spt5/Spt4 associates with Pol II via the 'clamp domain' of Pol II (Martinez-Rucobo *et al*, 2011), independently of CTD phosphorylation. Recruited Bur1/Bur2 phosphorylates Ser2 (and possibly Ser7) in CTD repeats harbouring S5P to produce diphosphorylated repeats, and also phosphorylates the Spt5 CTRs. **(Lower panel)** Paf1C recruitment occurs through cooperation between interactions of the PCIDs in Cdc73, Rtf1 and Ctr9 with diphosphorylated CTD repeats and the Spt5 pCTR. Figure source data can be found with the Supplementary data.

PCID in the Bur1 C-terminus (Qiu *et al*, 2009), we showed here that inactivating kin28-as, or deleting the Bur1 PCID, reduces phosphorylation of the Spt5 CTRs *in vivo*. This finding implies that Kin28 promotes Paf1C recruitment, at least partly, by stimulating Bur1 recruitment and attendant Ser1 phosphorylation of the Spt5 CTRs.

Evidence that Paf1C is recruited by diphosphorylated CTD repeats generated by Kin28 and Bur1 in addition to Spt5 pCTRs

We obtained evidence that Kin28, and possibly Bur1, also promote Paf1C recruitment more directly through Paf1C binding to the phosphorylated Pol II CTD. This idea was stimulated by the fact that Kin28, a component of TFIIF recruited to promoters (Mayer *et al* 2010), phosphorylates the CTD on Ser5 and Ser7 (Akhtar *et al*, 2009; Glover-Cutter *et al*, 2009; Kim *et al*, 2009) at promoter-proximal sites genomewide (Kim *et al*, 2010; Mayer *et al*, 2010; Tietjen *et al*, 2010; Bataille *et al*, 2012). Furthermore, because Kin28 promotes Bur1 recruitment (Qiu *et al*, 2009), and Bur1 also contributes to CTD phosphorylation on Ser2 and Ser7 (Liu *et al*, 2009; Qiu *et al*, 2009; Tietjen *et al*, 2010; Bataille *et al*, 2012), Kin28-stimulated Bur1 recruitment should augment formation of S5P,S7P-CTD repeats at promoter-distal sites and also produce S2P,S5P-CTD repeats near the promoter by phosphorylating Ser7 and Ser2,

respectively, in repeats already phosphorylated on Ser5 by Kin28. Importantly, we found that purified Paf1C can interact directly with pCTD peptides *in vitro* with a strong preference for S2P,S5P- and S5P,S7P-CTD repeats, and the same three Paf1C subunits that interact with Spt5 pCTR peptides also interact tightly with one or both of diphosphorylated CTD peptides. Moreover, point mutations in the Cdc73 C-terminus that reduce Paf1C recruitment *in vivo* abolish Cdc73 binding to both diphosphorylated CTD and pCTR peptides. Thus, Paf1C could be recruited by the Pol II pCTD in addition to the Spt5 pCTRs.

Strong support for this hypothesis came from examining the effects of inhibiting bur1-as or kin28-as in cells harbouring phosphomimetic Spt5 CTRs, where Ser1 in all 15 CTR repeats is substituted with Asp or Glu. These substitutions sustain efficient Paf1C recruitment in otherwise WT cells, whereas Spt5 substitutions with non-phosphorylatable Ala do not, supporting the model that phosphorylated Spt5 CTRs are crucial for Paf1C recruitment. Although Bur1 cannot regulate Ser1 phosphorylation in the Spt5 CTRs of these mutants, inhibiting bur1-as still reduced Paf1C recruitment in these strains, thus indicating that another Bur1 substrate is important for Paf1C recruitment. There is evidence that Bur1 phosphorylates Rad6 (Wood *et al*, 2005); however, we showed previously that eliminating Rad6 does not affect Paf1C recruitment at *ARG1* (Qiu *et al*, 2006). Hence, we

favour the model that the Spt5 pCTR-independent pathway of Paf1C recruitment involves Bur1's other known substrates, Ser2 and Ser7 of the Pol II CTD, and proceeds via direct binding of Paf1C to S2,S5- and S5,S7-diphosphorylated CTD repeats. This model can also account for the fact that inhibiting kin28-as in the mutants harbouring phosphomimetic Spt5 CTRs also reduces Paf1C recruitment, as expected from the reduction in S2,S5- and S5,S7-diphosphorylated CTD repeats produced by diminished Kin28 activity. Accordingly, we propose that Kin28 enables direct binding of PCIDs in Paf1C subunits to diphosphorylated CTD repeats in addition to stimulating Spt5 CTR phosphorylation by Bur1. Finally, the fact that the *cdc73* mutations that impair Cdc73 binding to pCTD peptides confer synthetic 6-AU sensitivity and strong reductions in Paf1C-dependent H3 methylation when combined with non-phosphorylatable *spt5-S1-15A* further supports the notion that the Cdc73 PCID mediates Paf1C binding to the pCTD in addition to the Spt5 pCTRs.

A model for dual pathways of Paf1C recruitment via Pol II pCTD and Spt5 pCTRs

Considering all of our findings and those described previously (Liu *et al*, 2009; Qiu *et al*, 2009; Zhou *et al*, 2009), we propose that Ser5 CTD phosphorylation by Kin28 at the promoter stimulates Bur1 recruitment with attendant phosphorylation of the Spt5 CTRs, and that Kin28 and Bur1 also collaborate to produce S2,S5-diphosphorylated CTD repeats at the promoter and 5' end of the CDS and S5,S7-diphosphorylated repeats further downstream in the CDS. The resulting pCTRs and pCTD repeats cooperate to enable robust recruitment of Paf1C via the multiple PCIDs in Paf1C subunits Cdc73, Rtf1, and Ctf9 (Figure 7C). Leo1 interaction with the nascent transcript likely also promotes Paf1C recruitment (Dermody and Buratowski, 2010), reminiscent of the finding that co-transcriptional recruitment of yeast Yra1 depends on an RNA recognition motif (RRM) that binds both RNA and the pCTD via distinct residues (Mackellar and Greenleaf, 2011). Additional experiments will be required to determine whether Paf1C binds with thermodynamic cooperativity to the pCTD and pCTRs in the Pol II elongation complex. However, recent structural analysis of the Pol II:Spt5/Spt4 complex is consistent with this idea in suggesting that the CTRs and CTD extend for comparable distances from the same side of Pol II (Martinez-Rucobo *et al*, 2011) and, thus, could interact simultaneously with different Paf1C subunits (Figure 7C).

Our proposal that Ser2 CTD phosphorylation promotes Paf1C recruitment might seem at odds with the previous finding that eliminating Ctk1, the major Ser2 CTD kinase, does not reduce Paf1C recruitment at *ARG1* (Qiu *et al*, 2006). However, we showed previously that Ctk1 and Bur1 make similar contributions to S2P in bulk elongating Pol II containing S5P, and in promoter-proximal Rpb1 at *ARG1* (Qiu *et al*, 2009). Thus, in *ctk1Δ* cells, the residual S2P generated by Bur1 in promoter-proximal Rpb1 could be sufficient for robust Paf1C recruitment by the pCTD early in the elongation cycle in combination with the Bur1-dependent Spt5 pCTR recruitment pathway. By contrast, eliminating Bur1 activity affects both pathways, abrogating Paf1C recruitment via Spt5 pCTRs in addition to reducing S2P levels in the CTD. It is also possible that the known

recruitment of Bur1 by CTD repeats phosphorylated on Ser5 by Kin28 (Qiu *et al*, 2009) makes Bur1 more efficient than Ctk1 in generating the S2,S5-diphosphorylated repeats that would constitute the Paf1C binding site near the promoter. Tethering of Bur1 to S5P repeats could also enhance S2P formation in clusters of CTD repeats and thereby facilitate binding of the different Paf1C subunits harbouring PCIDs to adjacent S2P,S5P-CTD repeats (Lunde *et al*, 2010). Finally, it is possible that the role of Bur1 in replacing Ser7P at promoter distal sites, which is apparently not shared by Ctk1 (Tietjen *et al*, 2010), is more important than its contribution to Ser2 CTD phosphorylation in Paf1C recruitment.

Although we consider the scheme in Figure 7C to be the simplest interpretation of the data, we cannot exclude the possibility that Bur1 has another unknown substrate in the elongation complex, not recognized by Ctk1, that enhances Paf1C recruitment, and that the Spt5 pCTR-independent role of Kin28 in Paf1C recruitment is limited to promoting Bur1 recruitment and attendant phosphorylation of this unknown Bur1 substrate rather than the formation of diphosphorylated CTD repeats to which Paf1C can bind. This explanation would require that the ability of Paf1C subunits to bind diphosphorylated CTD repeats *in vitro* is completely incidental to their physiologically relevant interactions with Spt5 pCTRs. However, this alternative model seems inconsistent with the synthetic growth defects and reductions in Paf1C-dependent histone H3 methylation events we observed in the *cdc73 spt5-S1-15A* double mutants, which are more readily explained by simultaneous impairment of Paf1C binding to both pCTD and pCTR repeats.

The significance of pCTD and pCTR interactions with multiple Paf1C subunits

The results of our peptide binding assays indicated that purified Paf1C binds with comparable affinities to S2P,S5P- and S5P,S7P pCTD and monophosphorylated pCTR repeats. These interactions are quite specific for the phosphorylated ligands, as purified Paf1C does not interact stably with phosphomimetic S2D,S5D peptides and also binds less tightly to CTR_D versus pCTR peptides. Thus, tight interactions of Paf1C with both pCTD and pCTR repeats seem to require dianionic $-PO_3^-$ groups, and presumably their greater potential for forming ionic interactions or H bonds, compared to carboxylate groups of Asp or Glu side chains. In this view, the weaker binding seen for the CTR_D peptide presumably reflects its ability to make only a subset of the interactions afforded by the Ser1- PO_3^- group with the PCID binding pocket. This specificity makes it unlikely that the pCTD- and pCTR-binding activities of Paf1C merely reflect an RNA binding activity of the complex that would involve interactions with monoanionic phosphate groups in the backbone of the nascent transcript. Indeed, the Leo1 and Rtf1 subunits were shown to bind RNA *in vitro* (Dermody and Buratowski, 2010), yet we found that Leo1 does not interact with pCTD or pCTR peptides, and that Rtf1 is exquisitely specific in its binding to S2P,S5P versus S5P,S7P pCTD peptides. Thus, the ability to bind RNA does not automatically endow a protein with the ability to bind different kinds of diphosphorylated pCTD repeats or pCTR repeats. Indeed, Mackellar and Greenleaf (2011) showed that the RNA binding and S2P,S5P-CTD binding activities of Yra1 require distinct residues in the protein.

The recombinant Cdc73 and Ctr9 proteins most closely resemble the purified Paf1C complex in pCTD-binding specificity, but Cdc73 binds much more tightly than does the Ctr9 C-terminal segment to pCTD peptides. Hence, Cdc73 probably makes the greatest contribution to pCTD binding by Paf1C. By contrast, recombinant Rtf1 displays the strongest binding to pCTR peptides and could make the greater contribution to pCTR binding by Paf1C. As noted above, both Cdc73 and Rtf1 have been implicated in Paf1C recruitment *in vivo*, and while this manuscript was under revision, VanDemark, Arndt, and colleagues reported that truncation of nearly the entire Cdc73 PCID (as defined here) impaired Paf1C recruitment without affecting Paf1C integrity (Amrich *et al*, 2012), in accordance with our findings. They also found that removing the Cdc73 PCID reduced the H3-K4me₃ level only in cells lacking Rtf1, which is consistent with our findings that *cdc73* PCID substitutions reduce H3-K4me₃ only in combination with *spt5-S1-S15A*, which eliminates contacts between Paf1C subunits (likely Rtf1) and the Spt5 pCTR. Interestingly, the crystal structure of this Cdc73 segment reveals a Ras-like domain lacking key elements required for GTP binding but containing several clusters of conserved surface-exposed residues that could comprise one or more binding sites for CTD or CTR repeats (Amrich *et al*, 2012). Certain of our clustered Ala substitutions that eliminate GST-Cdc73 binding to both pCTD- and pCTR peptides affect a subset of these conserved surface residues; however, it is possible that they alter the folding of the PCID in a way that disrupts overlapping, or even separate, pCTD- and pCTR-binding surfaces. Assuming that a single surface is responsible for both binding activities of Cdc73, a hypothetical scenario can be proposed to account for this promiscuity based on known principles of pCTD interaction derived from structural analyses of PCID-pCTD complexes (see Supplementary Figure S9). Obviously, further work is required to determine whether Cdc73, Rtf1, and Ctr9 interact with only pCTD or pCTR repeats when present as subunits of intact Paf1C; and in the event that recognition of both ligands occurs, whether single or multiple binding pockets are involved.

In conclusion, there is a large body of evidence indicating that the phosphorylated CTD provides a scaffold for recruitment of mRNA processing and histone modification enzymes, and of factors that regulate elongation or termination by Pol II (Phatnani and Greenleaf, 2006; Govind *et al*, 2010). Our results point to cooperation between the

pCTD and Spt5 pCTR pathways for efficient Paf1C recruitment and Paf1C-dependent H3 methylation events. It is tempting to speculate that other factors recruited by the pCTD might also take advantage of the phosphorylated scaffold presented by the Spt5 pCTRs to enhance their recruitment by elongating Pol II.

Materials and methods

Yeast strains and plasmids employed are listed in Supplementary Tables S1 and S2, respectively, and are described in Supplementary data. Synthetic biotin-conjugated peptides purchased from AnaSpec are listed in Supplementary Table S4. Peptide binding assays and ChIP experiments were conducted as described in Supplementary data. Briefly, for ChIP assays, chromatin was prepared from formaldehyde-crosslinked cells, immunoprecipitated with the appropriate antibodies, and the DNA was extracted and subjected to PCR amplification in the presence of [³³P]-dATP using primers listed in Supplementary Table S3. PCR conditions were optimized for each primer set to ensure that the amounts of amplified ³³P-labelled products are proportional to amounts of input DNA over the range of concentrations of the relevant sequences in total or immunoprecipitated chromatin.

Co-immunoprecipitation assays using WCEs, and Western analysis of WCEs prepared under denaturing conditions using trichloroacetic acid, were conducted as described in Supplementary Data, as was the purification of GST fusions from bacteria and native Paf1C from yeast.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Tom Dever for excellent suggestions and advice; Fred Winston and Steven Hahn for plasmids; and Karen Arndt, Grant Hartzog, and Steven Hahn for antibodies. We also appreciate Kevan Shokat's kind gift of 3MB-PP1 at the outset of this study. We thank Andy VanDemark and Karen Arndt for critical reading of the manuscript and for communicating results prior to publication. This research was supported by the Intramural Research Program of the National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH).

Author contributions: HQ conceived and executed experiments, analysed the data and assisted in writing the manuscript. CU and NG assisted in executing experiments. AGH conceived experiments, analysed the data, and assisted in writing the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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