Quantitative Analysis of Dynamic Protein Interactions during Transcription Reveals a Role for Casein Kinase II in Polymerase-associated Factor (PAF) Complex Phosphorylation and Regulation of Histone H2B Monoubiquitylation

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Using affinity purification MS approaches, we have identified a novel role for casein kinase II (CKII) in the modification of the polymerase associated factor complex (PAF-C). Our data indicate that the facilitates chromatin transcription complex (FACT) interacts with CKII and may facilitate PAF complex phosphorylation. Posttranslational modification analysis of affinity-isolated PAF-C shows extensive CKII phosphorylation of all five subunits of PAF-C, although CKII subunits were not detected as interacting partners. Consistent with this, recombinant CKII or FACT-associated CKII isolated from cells can phosphorylate PAF-C in vitro, whereas no intrinsic kinase activity was detected in PAF-C samples. Significantly, PAF-C purifications combined with stable isotope labeling in cells (SILAC) quantitation for PAF-C phosphorylation from wild-type and CKII temperature-sensitive strains (cka1Δ cka2–8) showed that PAF-C phosphorylation at consensus CKII sites is significantly reduced in cka1Δ cka2–8 strains. Consistent with a role of CKII in FACT and PAF-C function, we show that decreased CKII function in vivo results in decreased levels of histone H2B lysine 123 monoubiquitylation, a modification dependent on FACT and PAF-C. Taken together, our results define a coordinated role of CKII and FACT in the regulation of RNA polymerase II transcription through chromatin via phosphorylation of PAF-C.

Transcription elongation by RNA polymerase II (RNAPII) is a coordinated process that is regulated to ensure the proper expression of protein-coding genes. Numerous protein complexes play a role in aiding RNAPII loading onto a target gene promoter through the formation of preinitiation complexes. Following initiation, RNAPII proceeds into productive transcript elongation, during which the enzyme must cope with a chromatin landscape that can have an inhibitory effect on RNAPII passage. The polymerase-associated factor complex (PAF-C) plays a central role in the regulation of RNAPII elongation and co-transcriptional histone methylation at histone H3 lysine residues 4 and 36 as well as monoubiquitylation of histone H2B at lysine 123 (H2B-K123ub1) (1–5). In the model organism Saccharomyces cerevisiae, PAF-C is composed of five subunits: Cdc73, Ctr9, Leo1, Paf1, and Rtf1 (6). The human Paf1 complex contains an additional subunit, Ski8, which has been shown to be important in 3′-5′ mRNA degradation (7). Human PAF-C has been shown to interact directly with RNAPII (8). Various studies in yeast have linked PAF-C function to the facilitates chromatin transcription (FACT) complex, a histone chaperone that facilitates removal of a H2A/H2B dimer during transcription and replacement of that dimer following RNAPII passage (9–11). The FACT complex is composed of Spt16 and Pob3. FACT makes contacts with the H2A/H2B dimer and has also been shown to interact with histones H3/H4, histone tails, and intact nucleosomes in some contexts (10, 12–15). Spt16 associates with all five subunits of yeast PAF-C as well as casein...

* This work was supported by National Institutes of Health Grants R01 GM099714 (to A. L. M.) and R01 GM110058 (to B. D. S.) and a biomedical research grant from the Indiana University School of Medicine. Summer support for part of the work on this project was provided by the DePauw University Student Faculty Summer Research Fund and Professional Development Fund (to L. G. B. and A. K. B.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

†† Supported by a postdoctoral fellowship awarded by University of North Carolina Lineberger Comprehensive Cancer Center Basic Mechanisms of Viral and Chemical Carcinogenesis Training Grant 5 T32 CA009156.

‡‡ Supported by the summer undergraduate program for prospective physician scientists at the Indiana University School of Medicine.

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kinase II (CKII), as determined by qualitative mass spectrometry analysis (16). Affinity purification experiments have shown that PAF-C interacts genetically and physically with conserved transcription elongation factors, including Spt6, 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole sensitivity-inducing factor, and FACT (16, 17). It has been proposed that Pafl mediates the interaction between FACT and RNAPII in yeast (18). In higher eukaryotes, human PAF-C has been shown to interact with the general transcription elongation factor Transcription factor SII, the superelongation complex, and the FACT complex, consistent with its known interactions in yeast (8).

CKII is an abundant and constitutively active serine kinase that phosphorylates many targets in yeast and mammalian cells (19, 20). CKII contributes to the pathology of many human cancers (21–23). Multiple complexes containing CKII have been identified, including the transcription elongation factor FACT (16, 24). In mammalian cells, FACT co-purifies with CKII in a complex that phosphorylates p53 on serine 392 in response to DNA damage (24). In addition, deletion mutants of CKII exhibit defective transcription of specific cell-cycle genes, which results in a delay in entrance into S phase (25).

Quantitative analysis of dynamic protein interactions remains a significant challenge for proteomics because transient interaction partners are obtained at substoichiometric levels relative to bait proteins (reviewed in Ref. 26). Here we focused on the transient interactome of FACT and PAF-C and found that they are interaction partners with CKII. The use of hierarchical clustering and normalized spectra abundance factor (NSAF) values from multiple baits readily identify reciprocal interactions between FACT/CKII and PAF-C/RNAPII. In-depth mass spectrometry analysis using MudPIT of biological replicate purifications of the FACT complex (Spt16-TAP) isolated under low-salt conditions followed by significance analysis of interactome (SAINT) resulted in the identification of statistically significant interactions between FACT, CKII, PAF-C, and histones. Additionally, we showed that all five subunits of PAF-C are targeted for phosphorylation by CKII in vivo. Although PAF-C is subjected to extensive phosphorylation by CKII, reciprocal interactions between CKII and PAF-C were not observed. However, reciprocal interactions were detected between CKII and FACT, suggesting that the FACT complex may facilitate CKII modification of PAF-C. In support of this idea, we showed that CKII copurified with Spt16-TAP readily phosphorylates PAF-C in vitro, whereas no detectable kinase activity copurified with PAF-C through Ctr9-FLAG. In addition, we purified PAF-C from WT and CKII-defective cells using a SILAC approach and definitively show that CKII activity is required for phosphorylation of multiple residues across the subunits of PAF-C. Finally, and consistent with a role for CKII in PAF-C and FACT function, we show that temperature-sensitive mutants of CKII display reduced levels of histone H2B-K123ub1.

Experimental Procedures

Yeast Strains and Growth Conditions—All yeast strains are listed in supplemental Table S1. Gene deletions were performed using gene replacement (27). All expression plasmids contained the endogenous gene promoter. Mutagenesis of plasmids was performed with the QuikChange Lightning Multi site-directed mutagenesis kit (Agilent Technologies) with the primers and plasmids described in supplemental Table S2. Plasmids were transformed into deletion strains using standard methods (28). Endogenous Paf1 and Ctr9 were C-terminally tagged with the 3×-FLAG epitope using the p3FLAG plasmid (29). All strains were confirmed by Western blotting and PCR. TAP-tagged cells used for standard purifications were grown to an A600 of 2.0–2.5 in yeast extract, peptone, dextrose medium and pelleted by centrifugation. For stable isotope labeling in cells (SILAC) experiments, preconditions of Ctr9-FLAG WT and cka1Δ cka2–8 were grown in yeast nitrogen base 2% glucose without arginine or lysine overnight. The cka2–8 allele is a temperature-sensitive mutant with defective function at 25 °C and 37 °C (30–32). The preconditions were used to inoculate 3-liter cultures of Ctr9-FLAG WT cells in yeast nitrogen base 2% glucose containing [13C6,15N2]lysine and [13C6,15N2]arginine, referred to as heavy medium. The Ctr9-FLAG mutant cka1Δ cka2–8 was grown in YNB 2% glucose containing standard 13C6,14N-containing lysine and arginine, referred to as light medium. For the SILAC experiment, cells were grown to an A600 = 1.5–2.0 at 30 °C and then subjected to a 2-h heat shock at 37 °C.

Affinity Purifications—Tandem affinity purifications (TAPs) were performed as essentially described by Puig et al. (33) and Mosley et al. (34–36). Control purifications were performed from untagged parental strains (BY4741 for the TAP strains). Cell pellets were resuspended in lysis buffer (40 mM Hapes-KOH (pH 7.5), 10% glycerol, 100 or 350 mM NaCl (depending upon purification), 0.1% Tween 20, 0.5 mM DTT, 2 mM Na orthovanadate, and fresh yeast protease inhibitor mixture) and lysed using glass beads in a bead beater. The resulting lysate was treated with DNaseI and heparin sulfate at room temperature for 10 min to release any chromatin-bound proteins. The lysate was cleared by centrifugation and incubated with 500 μl of IgG- Sepharose (GE Healthcare) beads overnight at 4 °C. The slurry was washed to remove nonspecific binding proteins, resuspended in tobacco etch virus (TEV) protease cleavage buffer, and incubated with 100 units of AcTEV™ (Invitrogen) protease overnight at 4 °C. A disposable Poly-prep chromatography column was used to separate the beads from the cleaved protein by gravity flow. The beads, retained on the column, were washed with Calmodulin binding buffer. The protein flow-through was incubated with Calmodulin-Sepharose beads for 3 h at 4 °C and eluted from the beads with Calmodulin elution buffer containing 2 mM EGTA. FLAG tag affinity purifications were performed as described above with the following changes. After clarification of the lysate by centrifugation, the lysate was incubated with 500 μl of anti-FLAG M2 antibody resin (Sigma) overnight at 4 °C. The next day, the lysate/resin slurry was passed through a disposable Poly-prep chromatography column to capture the FLAG resin. The resin was then washed extensively with lysis buffer prior to 3× FLAG peptide elution. The elutions were performed by incubation of the resin with 250 μl of a 1 mg/ml solution of 3× FLAG peptide resuspended in lysis buffer. Following elution, the resin was washed with an additional 250 μl of lysis buffer that was also collected with the eluate. This process was repeated three to four times. Affinity
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purification elutions were TCA-precipitated overnight at 4 °C, followed by overnight digestion with endoproteinase Lys-C and then another overnight digestion with Trypsin Gold (Promega) at 37 °C. All digestions were quenched with formic acid to a final concentration of ~5%.

MudPIT Mass Spectrometry and Database Searching—Peptide mixtures were analyzed by MudPIT mass spectrometry as described previously (37). Each sample was pressure-loaded onto a 100-μm fused-silica nanospray column pulled to an ~5-μm tip using a P-2000 laser puller. The microcapillary columns contained two C18 reverse phases (Aqua, Phenomenex) separated by strong cation exchange resin (Luna, Phenomenex). Each MudPIT column was placed in line with the LTQ Velos ion trap or LTQ Velos Orbitrap mass spectrometer, and a spray voltage of 2.0 kV was applied to the nanocolumn. The automated MudPIT cycles consisted of four to ten 120-min steps with increasing concentrations of ammonium acetate. Four-step MudPIT consisted of 8-μl injections of 50, 100, 200, and 300 mM ammonium acetate, respectively, followed by a 20-min wash with buffer A (5% acetonitrile and 0.1% formic acid) and then a 90-min organic gradient of 5–80% buffer B (80% acetonitrile and 0.1% formic acid) to facilitate peptide elution from the reverse-phase resin. Four MudPIT steps were used for the SILAC-labeled Ctr9-FLAG purifications. Ten-step MudPIT consisted of 8-μl injections of 25, 50, 75, 100, 150, 200, 250, 300, and two 350 mM ammonium acetate steps, followed by a buffer A wash and buffer B gradient as described above. Ten-step MudPIT was performed for all other AP-MS samples. Each full scan (at a resolution of 60,000) in the Orbitrap) was followed by 10–15 MS/MS scans using data-dependent acquisition in the ion trap, where the most intense precursor ions were individually fragmented by collision-induced dissociation (collision energy = 35).

Database searching of the RAW files was first done with SEQUEST HT (version 1.4.1.14) in Proteome Discoverer (1.4.0.288) using trypsin as the enzyme restriction and the following parameters: two missed cleavages for trypsin, a precursor mass tolerance of 1.4 Da for ion trap data and 10 ppm for Orbitrap data, a fragment mass tolerance of 1.0 daltons, a Δ CN value of [mteq]0.15, and a fixed modification of +57 Da on cysteine residues and variable modifications of +16 Da on methionine and +80 daltons on serine, tyrosine, and threonine. The SILAC experiments were searched as above but included dynamic modifications for [13C6,15N2]lysine and [13C6,15N4]arginine. A custom S. cerevisiae FASTA database was used for a database search that contained 6631 protein sequences, including the entire yeast proteome from Uniprot (downloaded on February 27, 2014), and ~150 common contaminant proteins, including proteolytic enzymes, human keratins, and common laboratory contaminants. Additionally, we included the peptide sequence for the TAP tag used for isolation of all protein complexes studied. The PSM counts for the TAP tag were manually added to the PSM count of the bait (i.e. TAP-tagged) subunit for subsequent quantitative analysis. The msf files from Proteome Discoverer were imported into Scaffold 4, and the peptides from Scaffold were used for subsequent quantitative and post-translational modification analysis for SAINT. The data obtained for the SILAC experiments were analyzed by the quantitation module within Proteome Discoverer 1.4 (Thermo) to calculate the total peak area for each precursor ion and the relative ratio of heavy to light precursor ions. For posttranslational modification analysis of Cdc73-TAP, spectra were analyzed with X!Tandem with the addition of protein N-terminal acetylation (+42 Da) as a dynamic search option. Each protein is required to have at least two peptides to be considered identified. Additionally, site-specific modifications are reported from manual interpretation of spectra to confirm the fragment ion coverage of the specific phosphorylation site. Peptides used for phosphorylation mapping were also required to have ≤150 ppm from the LTQ Velos ion trap or ≤10 ppm from the LTQ Velos Orbitrap. Peptide spectrum matches (PSMs) used for protein interaction analysis were identified at a peptide false discovery rate of less than or equal to 1% as calculated by Scaffold. Hierarchical clustering analysis was performed as described previously (35, 36). The peptide and protein identifications for the Spt16-TAP (n = 4), and Cdc73-TAP (n = 3) purifications are available upon request. Additionally all RAW data files, Scaffold data files, and peak list files have been deposited into the MassIVE data repository under the title “FACT, PAF-C, and CKII.”

Label-free Quantitative Proteomics Approaches for Interaction Analysis—The total number of PSMs passing the criteria listed above were used for relative quantitation using the following approaches. Two empirical -fold change scores were calculated (38). The first, referred to as FC-A, calculates the -fold enrichment of affinity purifications over control purifications using the average mean of the PSMs per protein across replicates. The second score, referred to as FC-B, calculates the -fold change over control using the geometric mean of replicates. SAINT probability scores were also calculated for Spt16-TAP replicates as described previously using the Contaminant Repository for Affinity Purification (CRAPome) web site as detailed in multiple publications (37–42). Finally, we also normalized the total spectral abundance for proteins of interest using NSAF calculations as described previously (35, 36, 43, 44).

In Vitro Kinase Assays—For kinase reactions, ~300 ng of low-salt (100 mM) purified Spt16-TAP-associated proteins and/or Ctr9-FLAG-associated proteins were incubated alone, in combination, or with recombinant CKII (Millipore) for 2 h at 30 °C in kinase buffer (40 mM HEPES (pH 7.5), 10 mM MgCl2, 5 mM dithiothreitol, and 10 μCi of [γ-32P]ATP (6000 Ci/mmol, PerkinElmer Life Sciences)). Kinase reactions were stopped by the addition of 2× SDS-PAGE loading buffer and boiling at 100 °C for 10 min. Reactions were then separated by SDS-PAGE on a 10–20% precast gel (Bio-Rad). The gels were dried under a vacuum prior to exposure to a PhosphorImager screen for ≥1 h prior to scanning on a Fuji scanner.

Immunoblotting—Yeast cells of the indicated genotypes along with their wild-type counterparts were grown in YPD either at permissive or restrictive temperatures. Overnight-saturated cultures were back-diluted to an A600 of 0.2 and allowed to grow until they reached an A600 of 1. Five A600 equivalents of cells were lysed by bead beating using SUMEB lysis buffer containing 1% SDS, 8 M urea, 10 mM MOPS (pH 6.8), 10 mM EDTA, and 0.01% bromphenol blue. Lysates were separated by SDS-PAGE and probed using anti-HA (UNC Antibody Core Facility,
Results

During the transcription cycle, RNAPII interacts with numerous accessory proteins to facilitate transcription initiation, elongation through chromatin, co-transcriptional RNA processing and transcription termination. Unlike RNA polymerase I and III, which have “built-in” elongation factor activities, all RNAPII-associated elongation factors interact in a dynamic fashion (35, 45). One approach to study transient interaction partners using AP-MS is to perform reciprocal purifications of low-level prey proteins to confirm the interaction with the original bait protein of interest (Fig. 1). Although this approach is common, the coordinated analysis of these purifications (35, 36). A representative Spt16-TAP LS purification was used for hierarchical clustering analysis (Fig. 1, A, left panel, and B, second column). A high-salt (350 mM NaCl) purification was also performed for Spt16-TAP for comparison (Fig. 1, first column). In addition, we performed MudPIT analysis of Ckb2-TAP (a subunit of casein kinase II) and Cdc73-TAP (a subunit of PAF-C) and included datasets published previously for Spt4-TAP, Tfg1-TAP (a subunit of TFIIF), and Rpb11-TAP for comparison (36) (Fig. 1). For each dataset, NSAF values were calculated and analyzed by hierarchical clustering.

As shown in Fig. 1B, hierarchical clustering readily separates the known protein complexes into groups based on the AP-MS data (see dendrogram on the left and compare with protein complex labels on the right). Using these data, the Spt4/Spt5 heterodimer is readily identified as a complex based on the high abundance of the complex in Spt4-TAP samples. Overall, the cluster analysis identified two main sets of elongation factors. The first set includes TFIIF, Spt4/Spt5, and PAF-C as interaction partners that readily co-purify with RNAPII (Fig. 1, B, first column). In addition, we performed MudPIT analysis of Ckb2-TAP (a subunit of casein kinase II) and Cdc73-TAP (a subunit of PAF-C) and included datasets published previously for Spt4-TAP, Tfg1-TAP (a subunit of TFIIF), and Rpb11-TAP for comparison (36) (Fig. 1). For each dataset, NSAF values were calculated and analyzed by hierarchical clustering.

SAINT calculates interaction probabilities for proteins that are isolated through affinity purification approaches (referred
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for serine, threonine, or tyrosine phosphorylation on Spt16-TAP and Cdc73-TAP purifications. Using this approach, we found that all five subunits of PAF-C are putative CKII targets, suggesting that PAF-C and CKII are not only direct interacting partners but that the interaction rates are likely too rapid to capture high levels of CKII subunits in PAF-C purifications (supplemental Table S5, Fig. 3). Two of the phosphorylation sites, Ser422 in Pf1 and Ser538 in Leo1, consist of a SPD/E sequence, which suggests that these sites could be targets of cyclin-dependent kinases or CKII based on their consensus motifs (49). Three of the PAF-C subunits (Ctr9, Pf1, and Rtf1) are putatively modified by CKII at multiple sites (Figs. 3, A, C, and D, with representative phosphosite mapping spectra provided in supplemental Fig. S1). Serine 132 in Leo1, a putative CKII consensus site, is modified in over 80% of the PSMs identified that contain that amino acid.

To define the consensus motifs for the PAF-C amino acids modified by CKII, we performed sequence enrichment analysis using Seq2Logo (50) (Fig. 4B). The major consensus sequence (n = 10 sites) for PAF-C phosphorylation sites is SDX[D/E][D/E] (Fig. 3 and supplemental Table S4). These data, in light of our protein interaction analysis, suggest that FACT may facilitate CKII recruitment to PAF-C for its subsequent phosphorylation (18). Although we performed full phosphorylation analyses of the FACT subunits Spt16 and Pob3, we did not identify any phosphorylated peptides in either protein. However, the C-terminal domain sequence of Spt16 was not detected in our analyses, suggesting that alternate protease digestions may be needed to fully analyze potential modification sites in FACT.

We next performed a series of experiments to confirm that the subunits of PAF-C are bona fide substrates of CKII using both in vitro and in vivo approaches. Using [γ32P]ATP, we first performed in vitro kinase assays using LS Spt16-TAP-purified FACT and Ctr9-FLAG-purified PAF-C as substrates in the presence or absence or recombinant CKII. As shown in Fig. 4A, lane 2, proteins corresponding to the molecular weight of both subunits of the FACT complex can be phosphorylated in vitro in reactions containing recombinant CKII (Fig. 4A, Spt16 and Pob3). Recombinant CKII is also autophosphorylated in this experiment, as visualized in Fig. 4A, lane 5, which only contains recombinant CKII and ATP. Interestingly, phosphorylated Spt16 and Pob3 bands were also visualized in reactions lacking recombinant CKII, suggesting that CKII (or potentially some other co-purifying kinase) can also phosphorylate FACT in vitro (Fig. 4A). Importantly, we found that PAF-C was not phosphorylated in the absence of recombinant CKII, suggesting that CKII is not a stable interacting partner of PAF-C (Fig. 4A, lane 3). These data are also in agreement with our AP-MS studies (Figs. 1 and 2). In contrast, four bands corresponding to the molecular weights of PAF-C subunits are readily phosphorylated in the presence of recombinant CKII (Fig. 4A, lane 4). Notably, we did find that four of the five PAF-C subunits were phosphorylated in our in vitro reaction following incubation with Spt16-TAP and ATP (Fig. 4B, lane 3). These data clearly show that the FACT-CKII complex will readily phosphorylate PAF-C in vitro.

FIGURE 2. Identification of significant Spt16-TAP interaction partners. Shown are stringent fold change scores versus SAINT interaction probabilities for FACT, CKII, and PAF-C complex members from analysis of Spt16-TAP samples (n = 4). The specific subunit names and their interaction scores are listed in supplemental Table S3. A legend including a color code for the subunits of FACT, CKII, PAF-C, and nucleosomes is shown at the bottom right.
To test whether CKII is responsible for phosphorylation of PAF-C using an in vivo approach, we performed quantitative proteomics analysis of Ctr9-FLAG-isolated PAF-C complexes from WT and CKII temperature-sensitive strains (cka1Δ cka2–8) using SILAC-based quantitation (Fig. 5). The experimental scheme used for these studies is shown in Fig. 5A. Both
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WT and CKII temperature-sensitive strains were grown to an A600 = 1.5 and then heat shocked at 37 °C for 2 h prior to mixing of the cell pellets, lysis, and PAF-C purification. The full set of significantly changed peptides identified in the Ctr9-FLAG SILAC dataset is included in supplemental Table S5. As shown in supplemental Table S5, we readily identified peptides for 10 subunits of PAF-C for which the levels of the phosphorylated peptides decreased, whereas the unmodified peptides increased in cka1Δ cka2–8 strains relative to the levels of total histone H2B. Additionally, the levels of histone H2B-K123ub1 were decreased in cka1Δ cka2–8 strains relative to the levels of total histone H2B. Regardless, these findings suggest that reduced H2B-K123ub1 levels can occur without significant effects on H3K4 methylation (56). Taken together, these data show that CKII is an important regulator of histone H2B-K123ub1, likely through its regulation of PAF-C and FACT. Additional analysis of phosphosite mutants of the PAF-C subunits Paf1, Ctr9, and Cdc73 did not individually reveal defects in histone modification levels at H2B-K123ub1, histoneH3lysine4trimethylation, or histoneH3lysine36trimethylation (Fig. 6A). No changes in histone H3K4 methylation levels were observed, which is in line with studies that show that reduced H2B-K123ub1 levels can occur without significant defects on H3K4 methylation (56).

Discussion

In this study, we have performed an in-depth proteomics and genetic analysis of two transiently associated RNAPII elongation factors that are recruited to target genes during active gene transcription. Although PAF-C subunits were not identified as significant interaction partners of RNAPII when using Rpb3-TAP as bait in previous studies, purification of PAF-C through Cdc73-TAP revealed that RNAPII is a major interaction partner of PAF-C (Fig. 1). However, purifications of FACT through Spt16-TAP under low-salt conditions revealed a significant interaction between FACT and PAF-C (supplemental Table S3 and Fig. 2). These data suggest that PAF-C may be involved in FACT recruitment to RNAPII, as has been hypothesized previously, because PAF-C readily interacts with RNAPII in affinity purifications (18). FACT readily co-purifies with CKII in low-
salt purifications. Although Cdc73-TAP samples did not contain significant levels of FACT or CKII subunit peptides, we found that four of five subunits of PAF-C are phosphorylated by CKII in vitro and in vivo (Figs. 3–5). In addition, we found that Spt16-interacting CKII can readily phosphorylate purified PAF-C in vitro (Fig. 4B). Together, these findings implicate the FACT complex in phosphorylation of PAF-C by CKII. The human FACT complex has been proposed previously to play a similar role in the regulation of p53 phosphorylation by CKII (24).

Previous studies have addressed the challenge of transient interaction partners by overexpression of bait proteins (specifically kinases) to increase the relative abundance of significant protein-protein interactions (41). However, this approach could result in a large number of false positive interactions as a result of overexpression effects on the biological system in question. Additionally, single affinity purifications have been suggested as an approach to capture dynamic interaction partners. Unfortunately, single affinity purifications also have significantly higher interactions with cellular contaminants that, in our experience, are not removed by algorithms such as SAINT (37). Dynamic or transient interaction partners present even more of a challenge to existing statistical programs than small proteins (discussed above). This study suggests that inclusion of a large number of replicates in combination with follow-up reciprocal purifications and functional studies is the most robust approach for characterization of dynamic interactions such as those with kinases like CKII. However, it must also be acknowledged that the low detection frequency of transient interaction partners is a significant challenge for data-dependent acquisition-based approaches and their related statistical interpretation. For instance, confirmation of PAF-C modific-

![Diagram](image_url)
CKII Phosphorylates PAF-C

A

B

FIGURE 6. CKII regulates histone H2B-K123ub1 levels. A, Western blotting analysis of histone H2B-K123ub1, total histone H2B, and histone H3 lysine 4 trimethylation levels from whole cell extracts from cka1Δ cka2–8 strains grown under permissive (30 °C) and restrictive (25 °C and 37 °C) temperatures. The antibody used for each panel is indicated at the right, and the temperature used for cell growth is given at the bottom. B, Western blotting analysis of whole cell lysates for histone H2B-K123ub1, G6PDH, H2B, and HA of PAF1 or CTR9 deletion strains rescued with PAF1–3HA or CTR9–3HA expression vectors as indicated. Left panel, Vector; PrP513–3HA-SSN6; PAF1–WT, PAF1–3HA; S/T→E; A, PAF1–3HA S147A, T385A, T422A, S426A; S/T→E, PAF1–3HA S147E, T385E, T422E, S426E. Right panel, Vector, PrP513–3HA-SSN6; CTR9–WT, CTR9–3HA; S>T→A, CTR9–3HA S977A, S1015A, S1017A, S1046A, S1054A, S1056A; S>T→E, CTR9–3HA S977E, S1015E, S1017E, S1046E, S1054E, S1056E.

 tion by CKII in vitro and in vivo should result in the functional annotation of PAF-C and CKII as having both a transient protein-protein interaction and an enzyme-substrate relationship.

Our data show that CKII is a novel upstream regulator of H2B-K123ub1. We hypothesized that CKII phosphorylation of PAF-C could be required for FACT and/or PAF-C-dependent control of H2B-K123ub1. We have clearly shown that CKII regulates PAF-C phosphorylation and H2B-K123ub1. However, our initial genetic studies on PAF-C phosphorylation have not shown that PAF-C phosphorylation is required for H2B-K123ub1. There are many possible reasons for this. First, it is possible that CKII phosphorylation needs to be disrupted on multiple PAF-C subunits to fully recapitulate the cka1Δ cka2–8 phenotype. Additionally, it is possible that phosphorylation sites are present across these subunits that were not detected in our mass spectrometry analysis because of low peptide detectability and/or overdigestion with trypsin. Our findings that Pafl phosphorylation increases at threonine 127 in cka1Δ cka2–8 strains could suggest that other kinases can compensate for the loss of CKII activity. Finally, it is also plausible that the regulation of H2B-K123ub1 by CKII occurs in a PAF-C-independent mechanism.

Author Contributions—L. G. B., R. D., J. L. K., G. O. H., E. D. A., A. K. B., and A. L. M. generated the strains, conducted the experiments, and analyzed the results. L. G. B., G. O. H., and A. L. M. performed the mass spectrometry runs and data analyses. L. G. B. and A. L. M. wrote most of the paper with significant contributions from R. D., J. L. K., and B. D. S. L. G. B., R. D., J. L. K., B. D. S., and A. L. M. conceived the idea for the project.

Acknowledgments—We thank Whitney Smith-Kinnaman for technical contributions and project support, Nicole Novarese for strain generation, and members of the Mosley laboratory for comments on the manuscript. We also thank the Dr. David Stillman, Judith Jaehning, and Georjana Barnes laboratories for the spt16–11, PAF1 deletions and casein kinase II mutants, respectively.

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