The Paf1 Complex Broadly Impacts the Transcriptome of *Saccharomyces cerevisiae*

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ABSTRACT The Polymerase Associated Factor 1 complex (Paf1C) is a multifunctional regulator of eukaryotic gene expression important for the coordination of transcription with chromatin modification and post-transcriptional processes. In this study, we investigated the extent to which the functions of Paf1C combine to regulate the *Saccharomyces cerevisiae* transcriptome. While previous studies focused on the roles of Paf1C in controlling mRNA levels, here, we took advantage of a genetic background that enriches for unstable transcripts, and demonstrate that deletion of *PAF1* affects all classes of Pol II transcripts including multiple classes of noncoding RNAs (ncRNAs). By conducting a *de novo* differential expression analysis independent of gene annotations, we found that *Paf1* positively and negatively regulates antisense transcription at multiple loci. Comparisons with nascent transcript data revealed that many, but not all, changes in RNA levels detected by our analysis are due to changes in transcription instead of post-transcriptional events. To investigate the mechanisms by which *Paf1* regulates protein-coding genes, we focused on genes involved in iron and phosphate homeostasis, which were differentially affected by *PAF1* deletion. Our results indicate that *Paf1* stimulates phosphate gene expression through a mechanism that is independent of any individual Paf1C-dependent histone modification. In contrast, the inhibition of iron gene expression by *Paf1* correlates with a defect in H3 K36 trimethylation. Finally, we showed that one iron regulon gene, *FET4*, is coordinately controlled by *Paf1* and transcription of upstream noncoding DNA. Together, these data identify roles for Paf1C in controlling both coding and noncoding regions of the yeast genome.

KEYWORDS Paf1 complex; RNA polymerase II; histone modifications; chromatin; noncoding RNA

IN the context of chromatin, accurate and controlled transcription by RNA polymerase II (Pol II) requires the functions of many regulatory factors. One highly conserved regulatory factor is Paf1C, which in yeast is composed of *Paf1*, *Ctr9*, *Leo1*, *Rtf1*, and *Cdc73* (Jaehning 2010; Crisucci and Arndt 2011; Tomson and Arndt 2013). Paf1C associates with Pol II during transcription elongation and regulates both transcriptional and post-transcriptional processes, including the cotranscriptional deposition of histone modifications and the nuclear export of RNAs (Tomson and Arndt

2013; Fischl *et al.* 2017; Van Oss *et al.* 2017). Histone modifications dependent on Paf1C include H3 lysine 4 di- and trimethylation (H3 K4me_{2/3}), H3 K79me_{2/3}, and H2B K123 mono-ubiquitylation (ub) in *Saccharomyces cerevisiae* (H2B K120 in humans). Paf1C facilitates the deposition of H2B K123ub via its *Rtf1* subunit (Piro *et al.* 2012; Van Oss *et al.* 2016), which interacts directly with the ubiquitin-conjugating enzyme *Rad6* through its histone modification domain (Van Oss *et al.* 2016). H2BK123ub is a prerequisite to H3 K4me_{2/3} and H3 K79me_{2/3}, modifications catalyzed by the histone methyltransferases *Set1* and *Dot1*, respectively (Dover *et al.* 2002; Sun and Allis 2002). Paf1C also promotes the deposition of H3 K36me₃ by the *Set2* histone methyltransferase (Krogan *et al.* 2003b; Chu *et al.* 2007). Consistent with the binding of Paf1C to the Pol II elongation machinery (Qiu *et al.* 2006, 2012; Amrich *et al.* 2012; Wier *et al.* 2013; Xu *et al.* 2017; Vos *et al.* 2018), the histone modifications

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dependent on Paf1C are found at regions of active transcription (Smolle and Workman 2013).

The absence of specific histone modifications in Paf1C mutants is associated with transcriptional defects. These defects include the transcriptional read-through of terminators found at the 3' ends of Pol II-transcribed small nucleolar RNA (snoRNA) genes (Sheldon *et al.* 2005; Terzi *et al.* 2011; Tomson *et al.* 2011, 2013). In addition to promoting a histone modification state that facilitates transcription termination, Paf1C physically associates with proteins implicated in transcription termination and RNA 3'-end formation (Nordick *et al.* 2008; Rozenblatt-Rosen *et al.* 2009). The importance of Paf1C in regulating snoRNA termination supports a functional interaction with the *Nrd1-Nab3-Sen1* (NNS) transcription termination pathway (Arndt and Reines 2015; Porrua and Libri 2015), which is responsible for the termination of snoRNAs and other noncoding transcripts including cryptic unstable transcripts (CUTs) in yeast (Schulz *et al.* 2013). Many of these same noncoding transcripts are rapidly degraded by the nuclear exosome through a process mediated by the *Trf4/Trf5-Air1/Air2-Mtr4* polyadenylation complex (TRAMP) (Schmid and Jensen 2008). For example, loss of *Trf4*, the polyA polymerase subunit of the TRAMP complex that adds short polyA tails to transcripts destined for degradation or processing by the nuclear exosome, has been shown to stabilize CUTs and snoRNAs in *S. cerevisiae* (LaCava *et al.* 2005; Vaňáčová *et al.* 2005; Wyers *et al.* 2005; Thiebaut *et al.* 2006; Nishimura *et al.* 2009; Xu *et al.* 2009).

Despite growing understanding of the molecular functions of Paf1C, few studies have probed how these functions lead to a transcriptional outcome. Moreover, little is known about the roles of Paf1C in controlling noncoding transcription. To begin to address these questions, we sought to comprehensively investigate the importance of Paf1C in modulating the *S. cerevisiae* transcriptome, taking advantage of a genetic background that allows enhanced detection of unstable transcripts. To this end, we used strand-specific whole-genome tiling arrays to measure steady state RNA levels in *PAF1* and *pafl1Δ* strains that contain or lack the TRAMP subunit gene *TRF4*. We found that deletion of *PAF1* affects all classes of Pol II transcripts including both stable and unstable noncoding RNAs (ncRNAs) and antisense transcripts. Comparisons with published NET-seq experiments, which detect Pol II-engaged, nascent transcripts (Harlen and Churchman 2017), indicate that most, but not all, changes in steady state transcript abundance in the *pafl1Δ* background can be attributed to altered transcription. Analysis of subsets of protein-coding genes suggests that *Paf1* represses the transcription of some genes through facilitating H3 K36me3 and stimulates the transcription of other genes independently of any single Paf1C-dependent histone modification. Finally, we report a regulatory mechanism governing the *FET4* locus, which incorporates both CUT transcription and *Paf1*. Together, these data support a role for Paf1C in multiple regulatory mechanisms that collectively and broadly impact the Pol II transcriptome.

Materials and Methods

Yeast strains and culturing methods

All *S. cerevisiae* strains used in this study are listed in Table 1 and are isogenic to the FY2 strain, which is a *GAL2⁺* derivative of S288C (Winston *et al.* 1995). Deletion of specific loci was achieved by one-step gene disruption (Lundblad *et al.* 2001), and confirmed by PCR. Genetic crosses were conducted as described (Rose *et al.* 1991). Cells were grown to log phase at 30° in rich media (YPD) supplemented with 400 μM tryptophan and harvested by centrifugation. Cell pellets were washed once with sterile water, flash frozen, and stored at –80° prior to RNA isolation for RT-qPCR and Northern blotting experiments.

RNA isolation

RNA was extracted by the hot phenol extraction method (Collart and Oliviero 1993). Briefly, frozen cells were suspended in 400 μl of TES extraction buffer (10 mM Tris-Cl pH 7.5, 10 mM EDTA, 0.5% SDS) and 400 μl of acid phenol, followed by incubation at 65° for 1 hr. The aqueous phase was collected and re-extracted using acid phenol and then chloroform. Extracted RNA was combined with 40 μl of 3 M sodium acetate and 1 ml of 100% ethanol, mixed, and placed at –80° for at least 1 hr. Precipitated RNA was collected by centrifugation and suspended in RNase-free water before quantification and quality check by agarose gel electrophoresis.

Northern blot analysis

For Northern blot analyses, 10–20 μg of total RNA were separated on a gel containing 2% agarose, 6.5% formaldehyde, and 1× MOPS for 500 V hr and then transferred to a Nytran supercharge nylon transfer membrane (#10416296; Schleicher & Schuell BioScience, Dassel, Germany) prior to hybridization with radiolabeled DNA probes. DNA probes were generated by PCR corresponding to the following genomic regions relative to the +1 nucleotide of the annotated coding sequence for the *FET4* gene: *CUT* 793/794 (–479 to –114) and *FET4* (+261 to +651). Detection of *SCR1* (–181 to +284) served as a loading control. Oligonucleotides used to generate these probes are listed in Supplemental Material, Table S1. Probes were made using [α^{32} P]-dATP (single labeling) or [α^{32} P]-dATP and [α^{32} P]-dTTP (double labeling). Signals were quantified using a Typhoon FLA 7000 phosphorimager (GE, Boston, MA) and normalized to the *SCR1* internal loading control using ImageJ software.

Reverse transcription quantitative polymerase chain reaction

A total of 10 μg of RNA from each sample to be used in reverse transcription quantitative polymerase chain reaction (RT-qPCR) was treated with TURBO DNase (AM1907; Ambion, Thermo Fisher, Waltham, MA) following the manufacturer's instructions. To ensure that there was no DNA contamination after the DNase treatment, 1 μl of DNase-treated RNA was

Table 1 Yeast strains used in this study

Strain ^a	Genotype
KY292 (FY118 ^b)	<i>MATa his4-912Δ lys2-128Δ leu2Δ1 ura3-52 trp1Δ63</i>
KY307 (FY838 ^b)	<i>MATα his3Δ200 lys2Δ202 leu2Δ1 ura3-52</i>
KY457	<i>MATa rtf1Δ::URA3 leu2Δ1 ura3-52 trp1Δ63</i>
KY508 (FY737 ^b)	<i>MATa his3Δ200 lys2-128Δ leu2Δ1 ura3-52 snf2Δ::HIS3</i>
KY583 (GHY280 ^c)	<i>MATa his3Δ200 lys2-128Δ leu2Δ1 ura3-52 trp1Δ63 chd1Δ::HIS3</i>
KY632	<i>MATα his3Δ200 lys2-128Δ leu2Δ1 ura3-52 chd1Δ::URA3</i>
KY802	<i>MATa his3Δ200 lys2-173R2 ura3Δ(0 or 52) paf1Δ::URA3</i>
KY804	<i>MATα his3Δ200 leu2Δ2(0 or 1) ura3Δ(0 or -52) paf1Δ::URA3</i>
KY884	<i>MATa his3Δ200 lys2-173R2 leu2Δ1 ura3-52 trp1Δ63 isw2Δ::HIS3</i>
KY901	<i>MATα his3Δ200 lys2-128Δ leu2Δ1 ura3-52 trp1Δ63 isw1Δ::HIS3</i>
KY907	<i>MATa his3Δ200 lys2-128Δ leu2Δ1 ura3-52 set1Δ::HIS3</i>
KY914	<i>MATα his3Δ200 lys2-173R2 leu2Δ1 ura3-52 set2Δ::HIS3</i>
KY934	<i>MATα his3Δ200 leu2Δ1 trp1Δ63 dot1Δ::HIS3</i>
KY938	<i>MATα his3Δ200 leu2Δ1 trp1Δ63 set1Δ::HIS3</i>
KY972	<i>MATα his3Δ200 lys2-128Δ leu2Δ1 ura3-52 swr1Δ::KANMX</i>
KY1021	<i>MATa his4-912Δ lys2-128Δ leu2Δ1 trp1Δ63</i>
KY1235	<i>MATa his3Δ200 lys2-173R2 ura3-52 rco1Δ::HIS3MX6</i>
KY1250	<i>MATa his3Δ200 leu2Δ1 ura3-52 set2Δ::HIS3 paf1Δ::KANMX</i>
KY1683	<i>MATα his3Δ200 lys2-128Δ leu2Δ1 trp1Δ63</i>
KY1702	<i>MATa leu2Δ0 ura3Δ0 paf1Δ::KANMX</i>
KY1952	<i>MATα his4-912Δ lys2-128Δ trp1Δ63 leu2Δ1 bre1Δ::KANMX</i>
KY2012	<i>MATa leu2Δ0 ura3Δ0 trf4Δ::CLONAT</i>
KY2016	<i>MATa leu2Δ0 ura3Δ0 trf4Δ::CLONAT paf1Δ::KANMX</i>
KY2027	<i>MATα ura3-52 (hta2-htb2)Δ::KANMX</i>
KY2045	<i>MATα his3Δ200 leu2Δ1 trp1Δ63 rad6Δ::KANMX</i>
KY2167	<i>MATα ura3Δ0 HTA1-htb1-K123R (hta2-htb2)Δ::KANMX</i>
KY2239	<i>MATα his4-912Δ lys2-128Δ trp1Δ63 ctr9Δ::KANMX</i>
KY2241	<i>MATa his4-912Δ lys2-128Δ trp1Δ63 cdc73Δ::KANMX</i>
KY2243	<i>MATα his4-912Δ lys2-128Δ leu2Δ1 trp1Δ63 rtf1Δ::KANMX</i>
KY2244	<i>MATa his4-912Δ lys2-128Δ ura3-52 trp1Δ63 leo1Δ::URA3</i>
KY2271	<i>MATα his4-912Δ lys2-128Δ leu2Δ1 trp1Δ63 paf1Δ::KANMX</i>
KY2276	<i>MATa leu2Δ0 ura3Δ0</i>
KY2845	<i>MATa leu2Δ0 ura3Δ0 trf4Δ::CLONAT paf1Δ::KANMX FET4::HIS3 TTS at -400</i>
KY2846	<i>MATa leu2Δ0 ura3Δ0 paf1Δ::KANMX FET4::HIS3 TTS at -400</i>
KY2851	<i>MATa his3Δ200 leu2Δ0 ura3Δ0 trf4Δ::CLONAT FET4::HIS3 TTS at -400</i>
KY3460	<i>MATa his3Δ200 lys2-128Δ leu2Δ1 ura3-52 trp1Δ63 arp8Δ::HIS3</i>
KY3461	<i>MATa his3Δ200 his4-912Δ leu2Δ1 lys2-173R2 ura3-52 ino80::HIS3</i>
KY3462	<i>MATa his3Δ200 leu2Δ1 ura3-52 trp1Δ63 paf1Δ::URA3</i>
KY3463	<i>MATa his3Δ200 lys2-128Δ leu2Δ1 ura3-52 isw1Δ::HIS3</i>
KY3464	<i>MATa his3Δ200 leu2Δ1 ura3-52 ade8 paf1Δ::URA3</i>
KY3465	<i>MATa his3Δ200 leu2Δ1 ura3-52 ade8 arp8Δ::HIS3</i>
KY3466	<i>MATa his3Δ200 leu2Δ0 ura3Δ0 FET4::HIS3 TTS at -400</i>

^a All strains derived from S288C.

^b FY strains were provided by Fred Winston.

^c GHY strains were provided by Grant Hartzog.

subjected to 40 cycles of PCR and analyzed by agarose gel electrophoresis. All samples used in RT-qPCR showed no PCR product after 40 cycles. Reverse transcription reactions were performed on 1 μg of DNase-treated RNA using the RETRO-Script Reverse Transcription Kit (AM1710; Ambion, Thermo Fisher) following the manufacturer's instructions.

RT-qPCR experiments were performed in technical duplicate, and all strains were tested in at least biological triplicate. Reactions were prepared in a volume of 20 μl using Maxima SYBR Green/ROX qPCR Master Mix (2×) (#K0221; Thermo Fisher) following the manufacturer's instructions. Each 20 μl reaction was then divided into two, 10-μl reactions, which were analyzed on a StepOne Real-Time PCR System (Thermo Fisher) beginning with a hold at 95° for 10 min

followed by 40 cycles of 95° for 15 sec, and 58° for 1 min, and finally terminating with the generation of a melt curve. Efficiencies were determined for all primer sets by measuring C_t values across a series of six 10-fold dilutions starting with 250 ng/μl and ending with 2.5 pg/μl. RT-qPCR data were analyzed using the mathematical formula developed by Pfaffl (2001) and normalized to *SCR1* levels. RT-qPCR primers and their efficiencies are listed in Table S1.

Affymetrix tiling array analysis

All RNA samples used in tiling array analysis were prepared using established methods (Juneau *et al.* 2007; Perocchi *et al.* 2007), and quality was assessed by agarose gel electrophoresis. RNA samples (100 μg total) were DNase treated

(#EN0521; Fermentas, Waltham, MA) and purified using an RNeasy kit (#74104; Qiagen, Hilden, Germany). RNA was reverse transcribed into cDNA in the presence of 6 ng/ μ l actinomycin D to prevent antisense artifacts (Perocchi *et al.* 2007), fragmented, and labeled with a 3' biotin tag. Affymetrix custom tiling arrays (A-AFFY-116, Affymetrix Custom Array, *S. cerevisiae* Tiling Steinmetz, GEO Platform ID: GPL4563) were used to quantify gene expression (David *et al.* 2006; Huber *et al.* 2006; van Bakel *et al.* 2013). Arrays were processed and scanned using published methods (van Bakel *et al.* 2013).

Generation of annotation files for the tilingArray R package

Following the guidelines provided with the *davidTiling* Bioconductor package (David *et al.* 2006), tiling array probes were mapped to the *S. cerevisiae* genome (S288C version = R64-2-1) (Cherry *et al.* 2012; Engel *et al.* 2014). Briefly, the probe FASTA file was extracted from the array design file and used as input for MUMmer3.23 (Kurtz *et al.* 2004), along with the chromosome FASTA files for the S288C genome. Both the output of MUMmer3.23 and the S288C genome annotations were read into R (R Development Core Team 2016), and a slightly modified version of the *makeProbeAnno.R* script was used to generate an up-to-date probe annotation file for use with the *tilingArray* package (Huber *et al.* 2006). All R packages used in this study can be found at www.bioconductor.org (Gentleman *et al.* 2004), and R scripts can be found at https://github.com/mae92/Paf1C-Transcriptome-Analysis-Code/tree/master/R_Code.

Variance stabilizing normalization

CEL files for 12 tiling arrays were read into R as a single expression set using the *readCel2eSet()* function of the *tilingArray* package. The probe intensities for all 12 arrays were log2 transformed and normalized (variance stabilized normalization R code available at: https://github.com/mae92/Paf1C-Transcriptome-Analysis-Code/tree/master/R_Code) to minimize batch effects using the *vsr* package (Huber *et al.* 2002). This normalization method, as opposed to spike-in methods, assumes that the expression of most genomic regions will be equivalent between experiments, and therefore, the extent of expression changes overall in the *paf1 Δ* strains may be underestimated.

Mapping probe intensities to probe positions across the S. cerevisiae genome

The expression set containing the normalized log2 transformed probe intensities was used as input for the *segChrom()* function of the *tilingArray* package, and the locations of probes across the genome were extracted for use in downstream analysis using basic R commands (R code can be found at: https://github.com/mae92/Paf1C-Transcriptome-Analysis-Code/tree/master/R_Code). Probe locations were averaged for triplicate samples and these averaged values were used to generate BedGraph files, which were converted

into bigWig files for visualization in the Integrative Genomics Viewer (IGV) from the Broad Institute (Thorvaldsdottir *et al.* 2013).

Annotation-guided differential expression analysis

Normalized log2 transformed probe intensity values were extracted from the *tilingArray* output. Using a custom file (available at: https://github.com/mae92/Paf1C-Transcriptome-Analysis-Code/blob/master/Transcript_Annotations/combined.fix.csv) containing transcript annotations (listed in Table S2) from the Saccharomyces Genome Database (SGD) and recent studies of novel ncRNA transcripts (Cherry *et al.* 1998; Xu *et al.* 2009; Yassour *et al.* 2010; van Dijk *et al.* 2011; Schulz *et al.* 2013; Venkatesh *et al.* 2016), we calculated the average log2 intensity values for probes spanning each annotation. This process was carried out using an in-house Python script (available at: https://github.com/mae92/Paf1C-Transcriptome-Analysis-Code/tree/master/Python_Code) that calculates the average intensity of all probes occupying a given annotation found in the annotation file. Average log2 intensity values for all transcripts in each replicate and strain background were loaded into the *limma* package, where a linear model was used to determine statistical significance. Log2 fold change, and *P* values for all transcripts tested, were extracted from the output for each strain comparison. These *P* values were adjusted using Benjamini and Hochberg's false discovery rate (FDR) (Benjamini and Hochberg 1995) method using *top.table* command in *limma* (R code for *limma* analysis can be found at: https://github.com/mae92/Paf1C-Transcriptome-Analysis-Code/tree/master/R_Code) (Ritchie *et al.* 2015). Significantly differentially expressed genes (adjusted *P* value <0.05) that were present in both comparisons (*paf1 Δ* vs. WT and *paf1 Δ trf4 Δ* vs. *trf4 Δ*) were loaded into SGD's YeastMine database (Balakrishnan *et al.* 2012), where additional annotation and gene ontology information could be extracted. Gene ontology results are shown in Table 2. Plots of differential expression data were produced in R.

De novo differential expression analysis

BedGraph files were created containing normalized log2 probe intensity values (averaged for the three biological replicate arrays) mapped to the yeast genome. Differentially expressed transcripts identified by the *de novo* differential expression analysis were defined by a six-step process (Figure S2). (1) Average log2 (probe intensity) was calculated for three biological replicates. (2) The data were smoothed by averaging across a sliding window of 20 tiling array probes (roughly 160 bp). (3) The log2 fold change (experimental vs. control) was calculated across the entire genome. (4) All regions where the absolute value of the observed fold change was >1 (log2 fold change of 0) were identified. (5) Regions of the genome with an absolute change of 1.5-fold (log2 fold change of 0.58) were identified, and any of these regions <80 bp long (a length comparable to the shortest snoRNA) were excluded. (6) The two lists of regions from

Table 2 Gene ontology results for genes that showed increased or decreased expression (1.5-fold) in both *paf1Δ* to WT and *paf1Δ trf4Δ* to *trf4Δ* comparisons

Increased		
	<i>P</i> value	<i>n</i>
Biological process		
Siderophore transport [GO:0015891]	3.26E−04	5
Iron chelate transport [GO:0015688]	5.91E−04	5
Iron coordination entity transport [GO:1901678]	5.33E−03	5
Glycerol transport [GO:0015793]	2.29E−02	4
Iron ion homeostasis [GO:0055072]	3.25E−02	7
Cellular component		
Integral component of plasma membrane [GO:0005887]	2.77E−03	10
Intrinsic component of plasma membrane [GO:0031226]	7.38E−03	10
Extracellular region [GO:0005576]	2.00E−02	9
Plasma membrane part [GO:0044459]	2.22E−02	11
Decreased		
	<i>P</i> value	<i>n</i>
Biological process		
Small molecule metabolic process [GO:0044281]	1.95E−09	50
Small molecule biosynthetic process [GO:0044283]	6.07E−08	30
Single-organism biosynthetic process [GO:0044711]	8.96E−07	42
Oxoacid metabolic process [GO:0043436]	1.32E−06	33
Organic acid metabolic process [GO:0006082]	1.39E−06	33
Organic acid biosynthetic process [GO:0016053]	9.25E−06	21
Carboxylic acid biosynthetic process [GO:0046394]	9.25E−06	21
Single-organism metabolic process [GO:0044710]	6.55E−05	61
Branched-chain amino acid biosynthetic process [GO:0009082]	9.27E−05	7
Polyphosphate metabolic process [GO:0006797]	7.04E−04	6
Carboxylic acid metabolic process [GO:0019752]	2.44E−03	27
Cellular amino acid biosynthetic process [GO:0008652]	4.44E−03	14
Branched-chain amino acid metabolic process [GO:0009081]	7.73E−03	7
Alpha-amino acid biosynthetic process [GO:1901607]	1.30E−02	13
Organic hydroxy compound metabolic process [GO:1901615]	4.57E−02	13
Cellular component		
Vacuolar transporter chaperone complex [GO:0033254]	1.08E−03	4

steps 4 and 5 were intersected to yield a list of extended differentially expressed regions where some portion of the transcript had an absolute fold change of 1.5-fold or greater. The transcripts defined by this method were then treated as their own list of transcript annotations for use in the comparisons described herein. This was done using a combination of AWK (Aho *et al.* 1979) and the BedTools suite (Quinlan and Hall 2010; Quinlan 2014). The shell script used to define differentially expressed transcripts can be found at https://github.com/mae92/Paf1C-Transcriptome-Analysis-Code/tree/master/Shell_Code and R code used to generate the input BedGraph files can be found at https://github.com/mae92/Paf1C-Transcriptome-Analysis-Code/tree/master/R_Code.

Analysis of published datasets

Next generation sequencing datasets from previous studies (Churchman and Weissman 2011; Van Oss *et al.* 2016; Harlen and Churchman 2017) were obtained in BedGraph format directly from the authors or FASTQ format from NCBI SRA database and converted into BigWig format for use with DeepTools (Ramírez *et al.* 2014, 2016). Files received in BedGraph format were converted using the University of California Santa Cruz (UCSC) Genome Browser (Kent *et al.* 1976)

utility *BedGraphToBigWig*. Files downloaded from the SRA database in FASTQ format were mapped to the *S. cerevisiae* genome (S288C version = R64-2-1) (Cherry *et al.* 2012; Engel *et al.* 2014) using HISAT2 (Kim *et al.* 2015), and converted to BAM format using Samtools (Li *et al.* 2009). BAM files were converted to Wig format using the bam2wig utility (found at <https://github.com/MikeAxtell/bam2wig>), and converted to BigWig format using the UCSC utility *WigToBigWig*. Heatmaps were plotted using *computeMatrix* and *plotHeatmap* tools in the deepTools package by summing the tag counts using 50-bp bins.

Statistical analysis

At least three biological replicates were performed for every assay shown in this manuscript including tiling arrays. Each biological replicate is a pure yeast culture derived from a single colony initiated from a single cell of a given strain. Tiling array data analyzed using the *limma* package were subjected to the standard *limma* workflow, which utilizes linear modeling and an empirical Bayes method to determine differentially expressed genes from as little as three biological replicates. The *limma* *p* values were adjusted for multiple comparisons using Benjamini and Hochberg's FDR (Benjamini and Hochberg 1995). All RT-qPCR and northern blot *P* values were

generated using an unpaired, two-sided, Student's *t*-test, assuming equal variance carried out between the mutant strain and the wild-type strain.

Data availability

Strains are available upon request. Tiling array data (raw CEL files, BedGraph files, annotation-guided differential expression results, and files containing annotations for differentially expressed transcripts defined by our *de novo* analysis in BED6 file format) have been deposited in the Gene Expression Omnibus database under accession number GSE122704. Code used for analysis of tiling array data has been uploaded to the following GitHub repository (<https://github.com/mae92/Paf1C-Transcriptome-Analysis-Code>). Tables S1–S6 and Figures S1–S7 are available via FigShare. Supplemental material available at FigShare: <https://doi.org/10.25386/genetics.7796837>.

Results

Deletion of PAF1 affects coding and noncoding transcripts genome-wide

To investigate the impact of Paf1C on the *S. cerevisiae* transcriptome, we used high-resolution whole-genome tiling arrays to measure steady state RNA levels in *S. cerevisiae* strains deleted for the *PAF1* gene, which encodes a core member of Paf1C important for complex integrity (Mueller *et al.* 2004; Deng *et al.* 2018). Additionally, to assess the Paf1-dependency of unstable ncRNAs in these experiments, we deleted *TRF4* in both *PAF1* and *paf1Δ* strains. We chose to delete *TRF4* instead of the nuclear exosome subunit gene, *RRP6*, because in our previous survey of CUTs upstream of protein-coding genes, we observed enhanced CUT levels at four out of six loci in the *trf4Δ* background relative to the *rrp6Δ* background (Raupach *et al.* 2016).

When compared to a *trf4Δ* strain, the *paf1Δ trf4Δ* double mutant revealed wide-ranging effects on all Pol II transcript classes examined: mRNAs, small nuclear RNAs (snRNAs), snoRNAs, CUTs, stable unannotated transcripts (SUTs; Xu *et al.* 2009), *Xrn1*-dependent unstable transcripts (XUTs; van Dijk *et al.* 2011), *Nrd1*-unterminated transcripts (NUTs; Schulz *et al.* 2013), and *Set2*-repressed antisense transcripts (SRATs; Venkatesh *et al.* 2016) (Figure 1, A–G and Table S3). In general, levels of snRNAs, snoRNAs, and SRATs increased in the *paf1Δ trf4Δ* double mutant relative to the *trf4Δ* single mutant (Figure 1, D and G and Table S3) indicating that, in wild-type cells, Paf1 suppresses their transcription or destabilizes the transcripts. In the case of SRATs, increased transcript levels are consistent with a requirement for Paf1 in facilitating H3 K36me3—a modification that negatively regulates transcription (Churchman and Weissman 2011; Kim *et al.* 2016; Venkatesh *et al.* 2016) by activating the Rpd3S histone deacetylase complex and by inhibiting histone exchange (Carrozza *et al.* 2005; Joshi and Struhl 2005; Keogh *et al.* 2005; Govind *et al.* 2010; Venkatesh *et al.* 2012). Levels of many CUTs, SUTs, XUTs, and NUTs decreased upon deletion of *PAF1* (Figure 1, B, C, E, and F and Table S3). For

NUTs and CUTs, these changes in transcript abundance suggest that Paf1 impacts NNS-dependent termination beyond the snoRNA genes. At protein-coding genes, Paf1 positively and negatively affects mRNA levels in a locus-specific manner (Figure 1A and Table S3), in agreement with previous studies (Shi *et al.* 1996; Porter *et al.* 2005; Cao *et al.* 2015; Chen *et al.* 2015; Yu *et al.* 2015; Yang *et al.* 2016; Fischl *et al.* 2017).

For many snoRNA genes, we detected an increase in RNA levels downstream of the annotated gene in the *paf1Δ* and *paf1Δ trf4Δ* strains relative to wild type and *trf4Δ* strains, respectively (Figure 1H and Figure S1). This observation is consistent with previous studies showing Paf1 is required for efficient termination at many snoRNA genes (Sheldon *et al.* 2005; Tomson *et al.* 2013). Note that the log2 fold change values calculated for any particular snoRNA gene and its downstream region do not always agree. In many cases, RNA levels mapping to the gene body do not change even when downstream changes are observed, suggesting that read-through transcription is occurring at these loci (Figure 1H). Interestingly, through k-means clustering analysis, we also identified snoRNA genes that are relatively unaffected by deletion of *PAF1* (Figure S1). Further studies will be needed to elucidate the mechanistic distinctions between the Paf1-dependent and Paf1-independent snoRNA genes.

De novo differential expression analysis reveals effects of Paf1 on antisense transcripts

As an independent analysis and to facilitate detection of unannotated transcripts, we performed a *de novo* differential expression analysis of our tiling array data. Here, we relied on the data to reveal the boundaries of differentially expressed transcripts instead of using predetermined annotations. Strains lacking *PAF1* were compared to control strains on a probe-by-probe basis. Genomic regions with a 1.5-fold or greater difference in expression between *paf1Δ* and *PAF1* strains were selected as differentially expressed and extended until the expression difference was no longer observed (see Figure S2 and *Materials and Methods* for a detailed description of this analysis).

Confirming the accuracy of the *de novo* analysis, we found that nearly all the mRNAs identified as differentially expressed in the *paf1Δ trf4Δ* strain by our annotation-guided analysis (585 mRNAs; 1.5-fold or greater expression change relative to *trf4Δ* strain) were also detected by the *de novo* analysis (Figure S3A). We note that, compared to the annotation-guided analysis, a larger number of differentially expressed transcripts that overlap mRNAs on the sense strand were detected in the *de novo* analysis. This observation is not due to technical differences, but rather a consequence of multiple *de novo* transcripts overlapping with a single annotated mRNA and the increased sensitivity of the analysis. Additionally, we found that the length distribution of all transcripts identified in the *de novo* analysis was similar to that reported in SGD for mRNAs (Figure S3B), confirming that we were not calling exceedingly long or short transcripts. Further, separation of the *de novo* analysis data by transcript class

Figure 1 Deletion of *PAF1* affects all Pol II transcript classes. (A–G) Volcano plots graphing statistical significance (y-axis) against expression change (x-axis) between *paf1Δ trf4Δ* and *trf4Δ* strains (KY2016 and KY2012, respectively) for the indicated Pol II transcript classes. In (G), snRNAs and snoRNAs are shown in red and black, respectively. Each point represents an individual transcript. Tiling array probe intensities were averaged over annotated regions using a custom Python script and an average log2 fold change and *P* value were calculated using the *limma* R package. The horizontal line indicates an FDR adjusted *P* value of 0.05 and the vertical lines indicate a 1.5-fold change in expression (log2 fold change of 0.58). Counts and percentages of differentially expressed transcripts shown here are listed in Table S3. (H) Heatmap of log2 fold change in expression between *paf1Δ* and WT strains (KY1702 and KY2276, respectively) for the 29 most affected snoRNA genes. The snoRNA gene bodies and regions 0–50, 50–100, and 100–150 bp downstream of their annotated 3′ ends are plotted and sorted by the 0–50 bp region.

revealed effects of *paf1Δ* on ncRNAs and coding RNAs similar to those observed in the annotation-guided analysis (compare Figure 2A to Figure 1, A–G and Table S4). In the *paf1Δ trf4Δ* strain, for example, SRATs and snoRNAs were predominantly upregulated, and other ncRNAs were predominantly downregulated. When viewed as a whole, the *de novo* analysis detected far more differentially expressed transcripts in the *paf1Δ trf4Δ* strain (relative to the *trf4Δ* control strain) than in the *paf1Δ* strain (relative to the *PAF1* control strain) (Figure S3C). Therefore, a functional TRAMP complex, which promotes processing and degradation of unstable transcripts, obscures many of the transcriptional effects of deleting *PAF1*.

Unstable ncRNAs are often found near mRNA loci in tandem or antisense orientations (Neil *et al.* 2009; Xu *et al.* 2009; van Dijk *et al.* 2011; Schulz *et al.* 2013; Castelnovo *et al.* 2014). Murray *et al.* (2015) demonstrated that regions of high antisense transcription are deficient in H2BK123ub, H3K4me3, H3K79me3, and H3K36me3, while regions of low antisense transcription are enriched for H3K79me2. Levels of all these histone modifications are affected by *PAF1* deletion. Therefore, deletion of *PAF1* and loss of Paf1C-dependent histone modifications may generate a chromatin landscape that promotes antisense transcription at some loci and represses it

at others. Interestingly, in the *de novo* analysis, we observed enrichment of many transcripts oriented antisense to mRNA loci in the *paf1Δ trf4Δ* strain, relative to the *trf4Δ* strain, and found that *Paf1* both positively and negatively regulates antisense transcript levels in *S. cerevisiae* (Figure 2B and Table S5). Deeper analysis revealed that many of the antisense transcripts detected by the *de novo* analysis overlapped with previously annotated noncoding transcripts (Figure 2C), consistent with earlier studies showing that many noncoding transcripts are oriented antisense to genes (Xu *et al.* 2009; van Dijk *et al.* 2011; Schulz *et al.* 2013; Venkatesh *et al.* 2016). This suggests that a large portion of the ncRNA differential expression profile observed in the *paf1Δ trf4Δ* strain results from antisense transcription.

To investigate the antisense transcriptional landscape further, we plotted sense and antisense transcript levels relative to the transcription start site (TSS) and transcription end site (TES) of protein-coding genes at which we detected an absolute change of ≥ 1.5 -fold in antisense transcription overlapping the gene (Figure S4, A and B). For both the antisense-downregulated class and the antisense-upregulated class, k-means clustering analysis revealed five clusters differing in the patterns and levels of antisense transcription relative to sense transcription. (Note that when these clusters are

Figure 2 Paf1 positively and negatively regulates antisense transcription. (A) Horizontally stacked bar graphs showing the percentage of each transcript class (listed in Table S2) found to overlap with a differentially expressed transcript identified in *paf1Δ* or *paf1Δ trf4Δ* strains by *de novo* analysis (counts and percentages listed in Table S4). (B) Vertically stacked bar graph plotting percentage of transcripts, identified in the *de novo* analysis, that overlap with mRNA coding regions on the sense or anti-sense strand. These data are also presented in Table S5. (C) Bar graph

broken down by overlap with various ncRNA classes, no one cluster is dominated by an individual ncRNA class (Figure S4, C and D and Table S6). A small number of protein-coding genes show an apparent anti-correlation between sense and antisense transcription in the *paf1Δ trf4Δ* mutant (cluster 1 in Figure S4A and cluster 5 in Figure S4B). However, for most genes experiencing a 1.5-fold or greater increase in antisense transcription, a clear relationship between antisense and sense transcript levels was not detected. This result agrees with previous work on antisense transcription (Murray *et al.* 2015). Further, a plot of sense and antisense transcript levels for all protein-coding genes suggests that antisense transcription is not governing the changes we detect in sense transcription for most genes (Figure S4E).

Paf1 regulates transcript abundance at the transcriptional level

Paf1C has been shown to regulate both transcriptional and post-transcriptional processes at protein-coding genes (Porter *et al.* 2005; Van Oss *et al.* 2016; Fischl *et al.* 2017). To determine if changes in RNA levels detected by our tiling array analysis occurred at the transcriptional level, we compared our results to published NET-seq data (Harlen and Churchman 2017). Tiling array data comparing *paf1Δ* and *PAF1* strains or *paf1Δ trf4Δ* and *trf4Δ* strains were used to generate heatmaps for comparison to *paf1Δ* NET-seq data (Figure 3A). Overall, we observed similarity between the *paf1Δ trf4Δ* tiling array data and the *paf1Δ* NET-seq data, indicating that Paf1 is regulating the abundance of many transcripts, including unstable ncRNAs and mRNAs, through an effect on transcription (Figure 3, A and B). However, our analysis also indicates, that at some genes, Paf1 is likely playing a post-transcriptional role. For example, for the majority of Paf1-stimulated protein-coding genes (73%), a decrease in steady state RNA levels in *paf1Δ* cells was reflected in reduced nascent transcript levels (Figure 3B). In contrast, a smaller fraction of Paf1-repressed genes (52%) showed a corresponding increase in NET-seq signal in the *paf1Δ* background (Figure 3B). Therefore, both positive and negative effects of Paf1 occur at the transcriptional level at many loci, but for protein-coding genes repressed by Paf1, a larger fraction appear to be post-transcriptionally regulated.

One possible difference between Paf1-stimulated and Paf1-repressed genes is related to their level of expression in wild-type cells. To investigate this possibility, we used ChIP-exo data from Van Oss *et al.* (2016) to analyze Pol II occupancy (Rpb3 subunit) at protein-coding genes with absolute expression changes of 1.5-fold or greater in a *paf1Δ* background as measured by our tiling array analysis. The Rpb3 ChIP-exo data indicate that, in general, Paf1-stimulated genes are more highly transcribed than Paf1-repressed genes

summarizing the overlap between differentially expressed antisense transcripts detected by the *de novo* analysis and previously annotated non-coding RNAs (see sums in Table S6 for counts).

Figure 3 Paf1 regulates many of its target loci at the transcriptional level. (A) Heatmaps plotting log2 fold-change in transcript levels detected by tiling array for *paf1Δ* vs. WT (KY1702 vs. KY2276) and *paf1Δ trf4Δ* vs. *trf4Δ* (KY2016 vs. KY2012) as well as a *paf1Δ* vs. WT NET-seq comparative analysis (Harlen and Churchman 2017). Previously annotated coding and noncoding transcripts were scaled so that each row in the heatmap represents a single transcript from transcription start site (TSS) to transcription end site (TES). (B) Pie charts showing the direction of change in

(Figure S5, A and B). Similarly, Paf1 occupancy is higher at Paf1-stimulated genes compared to Paf1-repressed genes, consistent with the known association of Paf1C with Pol II. In agreement with the Rpb3 ChIP-exo data, analysis of transcript abundance in wild-type strains further demonstrated that Paf1 generally plays a positive role at highly active genes (Figure S5, A–C). Since defects in Paf1C cause a disruption in telomeric silencing (Krogan *et al.* 2003a; Ng *et al.* 2003a), we also analyzed the chromosomal locations of Paf1-regulated genes. Our analysis revealed broad chromosomal distribution of Paf1-stimulated and Paf1-repressed genes, in both *TRF4* and *trf4Δ* backgrounds, with no obvious bias toward telomeres (Figure S6, A–C).

Paf1 stimulates the expression of phosphate homeostasis genes through a mechanism independent of its effects on individual histone modifications

Gene ontology analysis (Ashburner *et al.* 2000) revealed an enrichment in phosphate homeostasis genes among the genes where expression decreased upon deletion of *PAF1* in both the *TRF4* and *trf4Δ* backgrounds (Table 2). Given the wealth of information on the importance of chromatin structure in regulating genes in the phosphate regulon (Korber and Barbaric 2014), we explored the mechanism of Paf1 involvement at these genes. Our tiling array data show that many but not all genes activated by the Pho4 transcription factor (Zhou and O'Shea 2011) are downregulated in the absence of Paf1 (Figure 4A), indicating that the effects of Paf1 are unlikely to be due to a loss of Pho4 function. Consistent with this, *PHO4* transcript levels are not strongly affected in the *paf1Δ* strain (Figure 4A).

To assess the contribution of individual Paf1C members to the expression of phosphate homeostasis genes, we performed RT-qPCR analysis of RNA isolated from *paf1Δ*, *ctr9Δ*, *cdc73Δ*, *rtf1Δ*, and *leo1Δ* strains. The RT-qPCR results generally agreed with our tiling array results. RNA levels for *PHO5*, *PHO81*, and *PHO84* were significantly decreased in the absence of any single Paf1C subunit with deletions of *PAF1* and *CTR9* causing the greatest effects (Figure 4B). Occupancy of Paf1 on *PHO5*, *PHO81*, and *PHO84*, as demonstrated by ChIP-exo analysis (VanOss *et al.* 2016) argues for a direct role of Paf1 in regulating these genes (Figure S5D).

Given the prominent role of Paf1C in promoting transcription-coupled histone modifications, we asked if loss of these modifications could explain the gene expression changes we observed in the Paf1C mutant strains. To this end, we performed RT-qPCR assays on RNA prepared from strains lacking the H2Bub enzymes Rad6 and Bre1, the H3 K4 methyltransferase Set1, the H3 K36 methyltransferase Set2, or the H3

NET-seq data (Harlen and Churchman 2017) for mRNAs that increased or decreased expression by at least 1.5-fold in the *paf1Δ* vs. WT comparison as measured by tiling array. Direction of change in NET-seq was determined by summing the reads in the first 500 bp of protein-coding genes in both WT and *paf1Δ* NET-seq datasets and calculating a fold-change (1.5-fold cutoff).

Figure 4 Paf1 positively regulates many phosphate homeostasis genes. (A) Heatmap of expression differences observed in a *paf1*Δ strain (KY1702) relative to a WT strain (KY2276) at Pho4-responsive genes (Zhou and O'Shea 2011). (B–D) RT-qPCR analysis of phosphate gene expression in strains lacking (B) individual Paf1C subunits (KY1021, KY2271, KY2239, KY2243, KY2241, and KY2244), (C) histone modification enzymes (KY1683, KY2045, KY1952, KY938, KY914, KY934) or (D) H2B K123. In (D), RNA levels in the H2B K123R mutant (KY2167) were

K79 methyltransferase *Dot1* (Figure 4C). RNA levels for *PHO5*, *PHO81*, and *PHO84* decreased in the *rad6*Δ and *bre1*Δ strains, which, like an *rtf1*Δ strain (Van Oss *et al.* 2016), are severely deficient in H2Bub. However, deletion of *PAF1* and *CTR9* had a greater impact on the transcription of these genes than deletion of either *BRE1*, which encodes the ubiquitin ligase for H2B K123, or *RTF1*, which encodes the primary Paf1C determinant of H2Bub (Figure 4, B and C). The larger effect of *rad6*Δ compared to *bre1*Δ suggests that, as a ubiquitin conjugating enzyme, *Rad6* may play roles in *PHO* gene regulation beyond catalyzing H2Bub. In agreement with this, we observed only a slight decrease in *PHO5*, *PHO81*, and *PHO84* RNA levels in a H2B K123R mutant compared to the control strain (Figure 4D). Other than a slight, but statistically significant, upregulation of *PHO5* and *PHO81* in the *dot1*Δ strain, loss of individual H3 methyltransferases did not alter transcription of the *PHO* genes (Figure 4C). Therefore, the loss of individual Paf1C-mediated histone modifications does not explain the strong reduction in phosphate homeostasis gene expression observed in *paf1*Δ and *ctr9*Δ mutants.

The absence of a clear effect of Paf1C-dependent histone modifications on *PHO* gene regulation prompted us to investigate other connections between *Paf1* and chromatin. Previous work by Batta *et al.* (2011) showed reduced nucleosome occupancy within coding regions in a *paf1*Δ strain, and the importance of nucleosome occupancy changes for *PHO* gene expression have been well documented (Barbaric *et al.* 2007; Korber and Barbaric 2014). Therefore, we investigated genetic interactions between *Paf1* and chromatin remodeling factors. Genetic crosses were performed between strains lacking *Paf1* and strains mutated for the following chromatin remodeling factors: *Chd1*, *Isw1*, *Isw2*, *Swi/Snf*, *Ino80*, and *Swr1* (Figure 4E). We observed synthetic lethality or severe synthetic growth defects in double mutants containing *paf1*Δ, and a deletion of *SWR1*, *ISW1*, *SNF2*, or *ARP8*, which encodes a subunit of the *Ino80*

compared to the appropriate WT control strain (KY2027). Relative expression ratio is calculated using primer efficiency, normalization to the RNA polymerase III transcript *SCR1* and a comparison to a WT strain (Pfaffl 2001). Error bars represent SEM and all statistically significant results are reported as asterisks ($0.01 < P < 0.05 = *$, $0.001 < P < 0.01 = **$, $0 < P < 0.001 = ***$). All *P* values were derived from a Student's *t*-test between the mutant strain and WT. (E) Cumulative data from crosses between a *paf1*Δ strain and strains deleted for chromatin remodeling factors. Following tetrad analysis of the following crosses, growth defects of double mutants were determined: *chd1*Δ *paf1*Δ = KY583 × KY804; *isw1*Δ *paf1*Δ = KY3464 × KY901; *isw2*Δ *paf1*Δ = KY884 × KY804; *snf2*Δ *paf1*Δ = KY508 × KY804; *arp8*Δ *paf1*Δ = KY3460 × KY804; *swr1*Δ *paf1*Δ = KY3462 × KY972. (F) Northern blot analysis of *SPL2* and *VTC3* RNA levels. Strains used in this analysis were KY292, KY802, KY457, KY508, KY3465, KY3461, KY884, KY3463, KY972, and KY632. *SCR1* serves as a loading control. (G) Genome browser view showing antisense transcription at the *PHO84* locus. The browser view shows smoothed differential expression tracks ($\log_2(-\text{paf1}\Delta \text{trf4}\Delta/\text{trf4}\Delta)$), 160 bp sliding window) with both SGD and *de novo* transcript annotations. Plus (+) and minus (−) symbols refer to DNA strand. The *PHO84* gene is oriented right to left.

complex. While the molecular basis for these genetic interactions is unclear, it is likely that Paf1C and chromatin remodeling factors regulate the expression of a shared group of genes. To test this idea for genes in the *Pho4* regulon, we focused on two genes, *SPL2* and *VTC3*, known to be stimulated by *Ino80* (Ohdate *et al.* 2003). Northern analysis revealed greatly reduced *VTC3* and *SPL2* expression in cells lacking *PAF1*, *SNF2*, *ARP8*, or *INO80* (Figure 4F). Although deletion of *SWR1* or *ISW1* did not affect *SPL2* or *VTC3* mRNA levels, it is possible that other Paf1-dependent genes are regulated by these remodeling factors. Taken together, these data suggest that Paf1C and chromatin remodeling factors work in parallel to maintain gene expression levels required for cell viability and phosphate homeostasis.

A well-studied example of locus-specific antisense control of transcription occurs at the *PHO84* gene in yeast (Castelnuovo *et al.* 2013). At this locus, accumulation of an antisense transcript in an *rrp6Δ* strain leads to repression of the sense transcript through a mechanism dependent on particular histone modifications (Castelnuovo *et al.* 2014). In light of the changes in antisense RNAs detected in the *paf1Δ* background (Figure 2B and Figure S4), we examined our *de novo* differential expression data for evidence of Paf1-regulated antisense transcription at *PHO84* (Figure 4G). When comparing the *paf1Δ trf4Δ* mutant to the *trf4Δ* control strain, we observed increased antisense and decreased sense transcript levels across the *PHO84* gene. Interestingly, *PHO84* fell into one of the two clusters of genes for which an anticorrelation between sense and antisense transcription was observed in the *paf1Δ trf4Δ* mutant (Figure S4B, cluster 5). These data suggest that, at the *PHO84* gene, deletion of *PAF1* elevates antisense transcription and coordinately decreases sense transcription.

Paf1 represses iron homeostasis gene expression in part through its role as a facilitator of H3 K36me3

Gene ontology analysis of genes that increased expression in *paf1Δ* strains revealed enrichment for genes in various iron-related processes (Table 2). As with the phosphate genes, an additional motivation for choosing iron homeostasis genes for follow-up experiments was the extent to which they have been characterized in the literature (Yamaguchi-Iwai *et al.* 2002; Rutherford and Bird 2004; Courel *et al.* 2005; Kaplan and Kaplan 2009; Cyert and Philpott 2013). Our tiling array analysis of genes that are normally activated by the *Aft1* and *Aft2* transcription factors in iron-limiting conditions (Cyert and Philpott 2013) revealed that many but not all of these genes are repressed by Paf1 in iron-replete media (Figure 5A).

To further investigate this subset of genes, we performed RT-qPCR analysis on RNA isolated from strains lacking individual Paf1C members (Figure 5B). A reproducible increase in expression was observed for *ARN1*, *FIT2*, *FIT3*, and *SIT1* in *paf1Δ* and *ctr9Δ* strains. Occupancy of Paf1 on these genes suggests that Paf1 is playing a direct role in mediating their repression (Figure S5D). With the exception of *SIT1*, deletion

of *CDC73* also led to derepression of these genes. In contrast, whereas Paf1, Ctr9, and Cdc73 repress the transcription of *ARN1*, *FIT2*, and *FIT3*, *Leo1* appears to play a stimulatory role at these genes, while *Rtf1* has little effect. Together, these data demonstrate that individual Paf1C subunits differentially regulate iron-responsive genes.

The Set2 histone methyltransferase catalyzes H3 K36me3—a modification that is dependent on Paf1 and Ctr9 (Krogan *et al.* 2003b; Chu *et al.* 2007). This epigenetic mark leads to the activation of the Rpd3S histone deacetylase complex and inhibition of histone exchange, generating a repressed chromatin state (Carrozza *et al.* 2005; Joshi and Struhl 2005; Keogh *et al.* 2005; Govind *et al.* 2010; Churchman and Weissman 2011; Venkatesh *et al.* 2012, 2016; Kim *et al.* 2016). In the *set2Δ* strain, RNA levels for *FIT3* and *SIT1* increased to those observed in the *paf1Δ* strain, suggesting that Paf1 represses these genes through stimulating H3 K36me3 (Figure 5C). This conclusion is supported by our observation that a *set2Δ paf1Δ* double mutant recapitulates the increased expression of *FIT3* and *SIT1* observed in the *set2Δ* and *paf1Δ* single mutants (Figure S7). Further, NET-seq data (Churchman and Weissman 2011; Harlen and Churchman 2017) indicate that the increase in steady state mRNA levels for *FIT3* and *SIT1* in *paf1Δ*, *set2Δ*, and *rcol1Δ* strains is associated with an increase in transcription (Figure 5D).

For two other genes, *ARN1* and *FIT2*, the level of derepression observed in the *paf1Δ* strain was greater than that observed in the *set2Δ* strain, despite evidence from NET-seq data (Figure 5D; Churchman and Weissman 2011; Harlen and Churchman 2017) that loss of Set2 strongly increases transcription of these genes. Similarly, with the exception of *FIT3*, steady state mRNA levels in a strain lacking the Rpd3S subunit *Rco1* did not reflect the increase in transcription detected by NET-seq (Figure 5, C and D). One likely explanation for the difference between the steady-state mRNA measurements (Figure 5C) and the nascent transcript data (Figure 5D) is that mRNA levels for the iron-responsive genes are post-transcriptionally regulated, possibly through a degradation pathway that involves Paf1. This conclusion is in line with observations made through our *de novo* analysis (Figure 3B), which indicated a post-transcriptional role for Paf1 at genes where it negatively regulates mRNA levels, and with previous descriptions of RNA degradation pathways that target mRNAs in the iron regulon (Lee *et al.* 2005; Puig *et al.* 2005).

In addition to the Set2/Rpd3S pathway, we tested other histone modifiers for a role in iron gene repression by examining *ARN1*, *FIT2*, *FIT3*, and *SIT1* expression in *bre1Δ*, *rad6Δ*, *set1Δ*, and *dot1Δ* strains by RT-qPCR (Figure 5E). With the exception of the *dot1Δ* mutation, which elevated *ARN1* and *SIT1* transcript levels, none of these mutations led to a significant derepression of the iron genes. The prominent role of Set2 in mediating *FIT3* repression is further supported by the enrichment of H3 K36me3 on this gene (Weiner *et al.* 2015) and the derepression of *FIT3* RNA levels in *set2Δ* and H3

Figure 5 Paf1 represses iron homeostasis genes. (A) Heatmap of expression differences observed in a *paf1* Δ strain (KY1702) relative to a WT strain (KY2276) at Aft1 and Aft2 responsive genes involved in maintenance of iron homeostasis (Cyert and Philpott 2013). (B and C) RT-qPCR analysis of the indicated genes in strains lacking (B) individual Paf1C subunits (KY1021, KY2271, KY2239, KY2243, KY2241, and KY2244) or (C) genes in the Set2/Rpd3S pathway (KY307, KY914, KY1702, and KY1235). Calculation of the relative expression ratio and statistical testing

K36A mutants (Venkatesh *et al.* 2016) (Figure 5F). Taken together, these results suggest that Paf1 represses expression of genes in the iron regulon by inhibiting transcription, most likely by facilitating histone marks such as H3 K36me3, and by influencing RNA stability.

FET4 is differentially regulated by Paf1 and upstream CUT transcription

To investigate the interplay between Paf1 and noncoding DNA transcription on a protein-coding gene in the iron regulon, we focused on the *FET4* gene, which encodes a low affinity iron transporter in *S. cerevisiae* (Dix *et al.* 1994). Two CUTs have been annotated upstream of the *FET4* coding region (Xu *et al.* 2009; Raupach *et al.* 2016) (Figure 6A). We hypothesized that CUT 794/793 transcription regulates *FET4* transcription possibly in a PAF1-dependent manner. To test this, we generated strains containing a transcription termination sequence (TTS) upstream of the *FET4* gene positioned to stop transcription of both upstream CUTs in wild type, *paf1* Δ , *trf4* Δ , and *paf1* Δ *trf4* Δ backgrounds.

Northern analysis showed that deletion of PAF1 reduced *FET4* transcript levels (Figure 6B top blot, cf. lanes 1 and 2 and lanes 3 and 4; Figure 6C) and CUT794/793 levels (Figure 6B middle blot, compare lanes 3 and 4; Figure 6D). When the TTS was inserted upstream of *FET4* (+TTS), CUT levels decreased and *FET4* transcript levels increased in all conditions tested, suggesting that transcription of the upstream CUT inhibits expression of the coding region (Figure 6, B–D). This is reminiscent of the inhibitory effect of non-coding transcription upstream of the well-studied *SER3* gene (Martens *et al.* 2004). Interestingly, even when CUT transcription was blocked, *FET4* transcript levels were reduced in the *paf1* Δ background. These data suggest that CUT transcription upstream of the *FET4* promoter negatively regulates transcription of the *FET4* gene and that Paf1 has a dual role in regulating *FET4* by stimulating expression of both the ORF and the inhibitory CUTs 794/793 (Figure 6E).

Discussion

The many roles of Paf1C and the pleiotropic phenotypes conferred by deletion of individual Paf1C subunits (Betz *et al.* 2002) suggest that this conserved transcription elongation complex regulates the expression of many genetic loci. While previous studies focused on the regulation of mRNAs (Shi *et al.* 1996; Penheiter *et al.* 2005; Porter *et al.* 2005;

were performed as in Figure 4. (D) Heatmaps of expression differences between mutant yeast strains and their respective WT strains in tiling array (this study) and NET-seq (Churchman and Weissman 2011; Harlen and Churchman 2017) datasets. (E) RT-qPCR results for iron homeostasis genes in strains lacking enzymes that catalyze Paf1C-associated histone modifications (KY1683, KY2045, KY1952, KY938, and KY934). (F) Genome browser view of the *FIT3* locus showing H3 K36A and *set2* Δ RNA-seq data from Venkatesh *et al.* (2016) and H3 K36me3 occupancy data from Weiner *et al.* (2015).

Figure 6 The *FET4* locus is regulated by Paf1 and transcription of ncDNA upstream of the coding region. (A) Diagram of the *FET4* locus and the position of a transcription termination sequence (*HIS3* TTS) inserted 400 bp upstream of the *FET4* start codon to block *CUT 794/793* transcription. (B) Northern analysis of *FET4* mRNA, *CUT 794/793* and *SCR1* RNA (loading control) from WT, *paf1Δ*, *trf4Δ*, and *paf1Δ trf4Δ* strains without the inserted TTS (KY2276, KY1702, KY2012, KY2016) or with the TTS (KY3466, KY2846, KY2851, KY2845). (C and D) Quantification of

Batta *et al.* 2011; van Bakel *et al.* 2013; Cao *et al.* 2015; Chen *et al.* 2015; Yang *et al.* 2016; Fischl *et al.* 2017; Harlen and Churchman 2017), here we sought to identify the full cohort of *Paf1*-regulated transcripts, both coding and noncoding, by exploiting a genetic background deficient in the TRAMP/exosome-dependent RNA degradation pathway and by performing *de novo* transcript identification analyses. Our high-resolution tiling array experiments revealed differential expression of transcripts in all Pol II transcribed RNA classes in strains deleted for *PAF1*. A comparison of our *paf1Δ trf4Δ* tiling array data with published *paf1Δ* NET-seq (Harlen and Churchman 2017) data demonstrated that *Paf1* regulates many coding and ncRNAs at the transcriptional level, and that the presence of a functional TRAMP complex obscures many of these transcriptional effects.

Our study revealed both positive and negative roles of *Paf1* in regulating ncRNA levels. For many transcripts in the CUT, NUT, XUT, and SUT classes, *Paf1* stimulates their expression. For two other important classes of ncRNAs, snoRNAs and SRATs, *Paf1* functions primarily as a negative regulator. The elevation in SRAT expression was not unexpected given the importance of *Paf1* for H3 K36me3 (Krogan *et al.* 2003b; Chu *et al.* 2007)—a mark important for the maintenance of a repressive chromatin environment. In our previous work, we showed that *Paf1* is important for snoRNA termination (Sheldon *et al.* 2005; Tomson *et al.* 2011, 2013). We recapitulate those results here, and identify additional snoRNA loci that exhibit transcription termination defects in the absence of *Paf1* (Figure 1H). These results, together with our finding that *Paf1* impacts the transcription of many CUTs and NUTs, extends the functional connections between *Paf1* and the machinery that terminates and processes these ncRNAs, including the NNS machinery and the nuclear exosome. Similar to a *paf1Δ* strain, snoRNA 3' ends are extended in *rrp6* and *nrd1* mutants, which lack subunits of the nuclear exosome and NNS, respectively (Schulz *et al.* 2013; Fox *et al.* 2015). In contrast, while NUTs and CUTs are elevated in *nrd1* and *rrp6* mutants, levels of many of these unstable ncRNAs are decreased in strains deleted for *PAF1*. The reduced levels of these RNAs in *paf1Δ* strains are likely due, at least in part, to the stimulatory effect *Paf1* has on their transcription (Figure 3).

By performing a *de novo* differential expression analysis of our tiling array data, we uncovered effects of *Paf1* on anti-sense transcription (Figure 2B). Interestingly, many of the histone modifications promoted by Paf1C are reduced in regions experiencing higher levels of antisense transcription, but still others are present at high levels in these same regions (Murray *et al.* 2015). The loss of Paf1C-promoted histone modifications may therefore contribute to changes in

northern blot results for *FET4* and *CUT 794/793* normalized to *SCR1*. Error bars represent SEM and all statistically significant results are reported as asterisks that represent *P* values from Student's *t*-test as in Figure 4. (E) Diagram of the observed effects of *PAF1* and *CUT794/793* at the *FET4* locus.

antisense transcription (Castelnuovo *et al.* 2014; Murray *et al.* 2015). Indeed, we found instances of both increased and decreased antisense transcription in our *paf1Δ* strains. Although some global anticorrelation exists between sense and divergent antisense transcription initiating from nucleosome depleted regions (Xu *et al.* 2009; Churchman and Weissman 2011), antisense transcription does not universally correlate or anticorrelate with sense transcription (Murray *et al.* 2015). Our results agree with this observation (Figure S4E), but also point to a small subset of genes where sense and antisense transcription appear to be anticorrelated when *PAF1* is deleted (Figure S4A cluster 1 and S4B cluster 5).

One gene that fits into this category is *PHO84* (Figure S4B cluster 5; Figure 4G). Our data suggest a role for *Paf1* in preventing antisense transcription at *PHO84* independently of its functional connections with the TRAMP/exosome pathway, as we detect higher antisense and lower sense transcript levels in the *paf1Δ trf4Δ* strain relative to the *trf4Δ* strain (Figure 4G). The ability to detect changes in antisense transcription was enhanced by the absence of *Trf4*. This observation agrees with studies on *PHO84* and other genes, which showed elevated antisense transcription in the absence of *Rrp6* (Castelnuovo *et al.* 2013). With respect to *PHO84*, the mechanism by which *Paf1* facilitates sense and represses antisense transcription remains undefined. Although previous studies showed that *set1Δ* strongly upregulates *PHO84* sense transcription in the presence of *RRP6* (Castelnuovo *et al.* 2014) and that *Paf1C* is required for *Set1*-dependent H3 K4 methylation (Krogan *et al.* 2003a; Ng *et al.* 2003b), our strand-specific tiling array data showed that *paf1Δ* strongly downregulates *PHO84* sense transcription (Figure 4A). Similarly, our results do not ascribe the stimulatory effect of *Paf1* on *PHO5* and *PHO81* to any single *Paf1C*-dependent histone modification or an obvious change in antisense transcription; however, it remains possible that the individual modifications function redundantly in promoting the expression of these genes.

At many genes that are normally induced in iron-limiting conditions, *Paf1* plays a repressive role under iron-replete conditions. For the four strongly upregulated genes examined, deletion of *PAF1* increased steady state RNA levels as well as nascent transcript levels, arguing that *Paf1* is controlling the transcription of these genes. The increase in transcription in the *paf1Δ* strain correlates with a decrease in *Set2* function, as shown by NET-seq data (Churchman and Weissman 2011; Harlen and Churchman 2017). Indeed, in our tiling array experiments, we detected a global increase in SRAT transcription in a *paf1Δ* strain (Figure 1D and Figure 2A). Interestingly, when comparing steady state RNA levels and nascent transcript levels, we noted an apparent post-transcriptional effect of *Paf1* (Figure 3B and Figure 5, C and D). With respect to the iron metabolism genes, we saw a strong overlap between *Paf1*-repressed mRNAs and *Rnt1*-repressed mRNAs (Lee *et al.* 2005).

Rnt1 is a double-stranded RNA endonuclease that cleaves RNAs with a particular stem-loop structure (Chanfreau *et al.* 2000), and, in iron replete conditions, executes an RNA degradation pathway for mRNAs that encode iron uptake proteins (Lee *et al.* 2005). While other explanations are possible, the overlap between *Paf1*- and *Rnt1*-repressed mRNAs suggests that the post-transcriptional role of *Paf1* at iron regulon genes may involve a functional interaction with *Rnt1*. A recent study showed that the rate of transcription elongation can influence the folding and processing of histone pre-mRNAs (Saldi *et al.* 2018), raising the possibility that deletion of *PAF1* might alter the rate of elongation in a way that affects the folding of substrates for *Rnt1* or another RNA processing factor. Together, our results suggest that through stimulating *Set2*-mediated H3 K36 methylation, *Paf1* represses genes in the iron regulon, but has an additional role in reducing the stability of these mRNAs.

Numerous examples of protein-coding gene regulation by ncDNA transcription or ncRNAs have been observed (Castelnuovo and Stutz 2015). Adding to this body of evidence, we investigated the regulatory mechanisms governing expression of *FET4*, which encodes a low affinity iron transporter. Our analysis indicates that *FET4* is regulated by the expression of upstream CUTs and by *Paf1*. Insertion of a transcription termination sequence upstream of *FET4* decreased *CUT794/793* levels and increased *FET4* transcription. Deletion of *PAF1* reduced both *CUT794/793* and *FET4* transcript levels. Together with these targeted experiments, our tiling array results on the *paf1Δ* strain also revealed a stimulatory effect of *Paf1* on *FET4* mRNA levels. However, we note that our tiling array analysis of the *paf1Δ trf4Δ* strain indicated that, in some circumstances, *Paf1* can repress *FET4* expression. Previous studies have shown that genetic background and growth conditions can influence the levels of *FET4* mRNA and the noncoding RNAs adjacent to or overlapping *FET4* (*CUT794/793* and the *SUT322* antisense ncRNA) (Xu *et al.* 2009). Since our tiling array and northern blotting experiments used RNA from yeast grown on separate days, it is possible that slight differences in media may be responsible for differences in expression dynamics at the *FET4* locus, highlighting the intricacies of the regulatory system operating at this gene. Collectively, our results add to the interesting list of telomere-proximal metal-responsive genes under the control of noncoding transcription (Toesca *et al.* 2011).

The complexity of the transcription process and its regulation by chromatin provides numerous opportunities for multifunctional transcription factors, like *Paf1C*, to regulate gene expression. Our study reveals genome-wide effects of *Paf1* on both coding and noncoding RNAs and provides mechanistic explanations for its diverse effects on specific classes of protein-coding genes. An understanding of the locus-specific effects of *Paf1C* will be an important step in elucidating the numerous connections of this complex to gene expression changes that cause human disease (Tomson and Arndt 2013; Karmakar *et al.* 2018).

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