UNCOVERING THE MECHANISM OF CHROMATIN ASSOCIATION OF THE PAF1 TRANSCRIPTION ELONGATION COMPLEX

by

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Paf1C co-localizes with Pol II and influences gene expression by regulating transcription initiation, elongation and termination. Some crucial functions of Paf1C include promoting co-transcriptional histone modifications and recruiting termination factors. The mechanism of chromatin recruitment of Paf1C was obscure. We identified the importance of a conserved region within the Rtf1 subunit of Paf1C, termed the ORF association region (OAR), in chromatin-tethering of Paf1C. I found that the interaction of Paf1C with the transcription elongation factor Spt5 was mediated by the Rtf1 OAR and the Spt5 C-terminal region (CTR). Binding assays established the direct nature of the Rtf1-Spt5 interaction and the sufficiency of the Rtf1 OAR and the Spt5 CTR for this interaction. ChIP assays demonstrated the ability of the OAR to mimic the chromatin association pattern of Paf1C, independent of Paf1C but dependent on the Spt5 CTR and the Bur1 kinase. This suggests that the targeting of the OAR tethers Paf1C to chromatin. Collectively, these results provide a molecular mechanism for coupling Paf1C with the transcription machinery.

Additionally, I found that substitution of OAR residues predicted to be important for the human Rtf1 OAR-Spt5 CTR interaction in the OAR-CTR co-crystal impaired the chromatin association of Paf1C supporting the relevance of the co-crystal interactions. Furthermore, I showed that strains that are doubly mutated in the OAR and the Cdc73 C-domain exhibited cumulative reduction in Paf1C chromatin occupancy. Consistently, I showed that cells lacking

both the OAR and the C-domain lose Paf1C-mediated histone modifications. This indicates that the Rtf1 OAR and the Cdc73 C-domain facilitate dual-attachment of Paf1C to chromatin.

My work has also provided better understanding of the function of the histone modification domain (HMD) of Rtf1. I found that overexpression of the HMD was essential for it to promote histone modifications. Additionally, I showed that the HMD is sufficient for the H2B K123 Ub, the mark upstream of the H3 K4 and H3 K79 methylation events, but the rest of Paf1C is required for the HMD to stimulate H3 K4 Me3 modification. Cumulatively, my findings provide additional insight into the regulation of histone modifications by the Rtf1 HMD.

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PREFACE

I am very thankful to my advisor Dr. Karen Arndt for helping me identify my weaknesses and work on them and evolve as an independent researcher. Her trust in me has boosted my self-confidence. I would like to thank her for being supportive during my tough times. Her excitement and enthusiasm for science has certainly been contagious. Her attitude of always asking me what I plan to do next after a failed experiment has helped me become resilient over time and think of alternative strategies to answer a scientific question immediately instead of being disappointed over a failed experiment. She has certainly made PhD a wonderful experience for me. I would also like to thank all my other Committee members- Dr. Joseph Martens (past member), Dr. Andrew VanDemark, Dr. Michael Tsang, Dr. Martin Schmidt and Dr. Anthony Schwacha for providing me excellent mentoring, insightful comments, very helpful advice and sharing reagents and equipments.

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Words cannot express how thankful I am to my family in India, especially my Mother, without whose support I could not never get where I am today. They have always encouraged and supported me to pursue what I wanted. Having raised me in a very protective environment, it was very tough for my family to let me go to the other end of the world, but they did believe in my decisions and me. My family kept me going when I was getting overwhelmed with all my problems staying independently for the first time ever in my life.

I have always been grateful to God for giving me the strength to face my problems and I consider him responsible for all the good things that have ever happened to me.

1.0 INTRODUCTION

The packaging of the eukaryotic genome into chromatin allows it to fit within the realm of the nucleus but makes the DNA inaccessible to DNA-templated processes such as transcription. A nucleosome, which is the smallest unit of chromatin, consists of a histone octamer containing two copies each of histone H2A, H2B , H3 and H4, around which is wrapped about 145-147 bp of DNA. Eukaryotes have evolved several accessory factors that facilitate the passage of Pol II through nucleosomes by sliding or evicting histones, incorporating histone variants or post-translationally modifying histones. These chromatin alterations make the DNA more accessible by altering the stability of nucleosomes or by facilitating the recruitment of downstream effector proteins. Additionally, they also coordinate the co-transcriptional processing of mRNA.

A subset of the multifunctional accessory factors categorized as transcription elongation factors associate with RNA polymerase II (Pol II), influence the rate of transcription or processivity of Pol II and regulate transcription not only at the elongation stage but also at the initiation and termination stages through various mechanisms. One such elongation factor that executes several crucial functions including regulating co-transcriptional histone modifications, nucleosome occupancy, phosphorylation of Pol II and RNA 3'-end formation is the Paf1 transcription elongation complex (Polymerase-associated factor 1 complex). This complex regulates the expression of many genes including those involved in development, antiviral response and maintenance of stem cell pluripotency. Since my dissertation research involves

uncovering the mechanism of recruitment of this crucial transcription elongation complex and better understanding its role in regulating co-transcriptional histone modifications, this chapter reviews the current knowledge of the mechanisms of transcriptional regulation by various regulatory factors.

1.1 CHROMATIN ALTERATIONS DURING TRANSCRIPTION

Regulation of gene expression allows cells to respond to environmental cues and properly execute their functions. Several complex molecular mechanisms involving a plethora of proteins coordinately regulate the expression of genes. Control of transcription is essential for regulation of gene expression. Transcription involves the synthesis of an RNA transcript using DNA as the template. To accommodate meters long genomic DNA within the eukaryotic nucleus, it has to be compacted into chromatin. However, chromatin acts as a hindrance to DNA-templated processes such as transcription. The smallest unit of chromatin is the nucleosome formed by the wrapping of 145-147 base pairs of DNA around a histone octamer that consists of two copies each of histone H2A, H2B, H3 and H4 [reviewed in (LUGER *et al.* 2012)]. Nucleosomes are stabilized by several hydrogen bonds and Van der Waals interactions between the histones and between the histones and DNA wrapped around them [reviewed in (LUGER *et al.* 2012)].

Chromatin is variable throughout the genome due to the presence of different post-translational histone modifications and histone variants. These variations and the actual sliding of the nucleosomes by chromatin remodelers allow transcription, DNA repair, DNA recombination and DNA replication machinery to overcome the chromatin barrier [reviewed in (CLAPIER and CAIRNS 2009; TALBERT and HENIKOFF 2010)]. They make the DNA more

accessible to these processes either by weakening the histone-DNA interaction, by serving as docking sites for other chromatin regulators that aid this process or by sliding or evicting the histones.

1.1.1 Histone chaperones and Histone variants

Nucleosome assembly is believed to occur in a stepwise fashion and involves the deposition of an H3-H4 tetramer on DNA followed by addition of two H2A-H2B dimers, while nucleosome disassembly likely occurs in the reverse order (BOHM *et al.* 2011). Histone chaperones facilitate the assembly, eviction, exchange or redeposition of histones by binding to them. They generally have specificity for either H3-H4 or H2A-H2B dimers or histone variants. Disassembly of nucleosomes during transcription is facilitated by histone chaperones Nap1 and FACT that facilitate the disassembly of H2A-H2B dimers ahead of Pol II and nucleosome reassembly upon Pol II passage through the template DNA (DEL ROSARIO and PEMBERTON 2008; SCHWABISH and STRUHL 2004; SCHWABISH and STRUHL 2006) [reviewed in (ELSASSER and D'ARCY 2012)] (discussed in section 1.3.5). Asf1 and Spt6 also maintain the chromatin integrity over the promoter and the coding regions of transcribed genes but they selectively bind histone H3-H4 [reviewed in (EITOKU *et al.* 2008)] (BORTVIN and WINSTON 1996; SCHWABISH and STRUHL 2006). Pol II has been shown to be able to travel through a histone hexamer that lacks just one H2A-H2B dimer (KIREEVA *et al.* 2002).

Histone variants are isoforms of the canonical histones and may differ from canonical histones in their biochemical characteristics or their positioning within the genome. The affinity of the histone chaperones for histone variants affects the incorporation of histone variants into nucleosomes. Histone variants alter the structure and stability of nucleosomes or their ability to

undergo compaction into higher order chromatin structure. Histone variant H2AX differs from the canonical histone H2A in its C-terminus where it can be phosphorylated at Ser139 by phosphoinositide kinase-like kinases in response to DNA damage (ROGAKOU *et al.* 1998) [reviewed in (SARMA and REINBERG 2005)]. This mark stabilizes or facilitates the association of DNA repair proteins with DNA at the sites of DNA damage. The centromeric histone variant (CenH3 in humans, chromosome segregation protein and Cse4 in yeast), found within the centromeric nucleosome instead of histone H3, is important for the association of the centromere with the kinetochore (WESTERMANN *et al.* 2003). Histone H2A.Z (Htz1 in yeast) is a variant of the H2A family. Nucleosomes containing H2A.Z acetylated at Lys14 are found at the promoters of active genes where they facilitate the recruitment of Pol II (TANABE *et al.* 2008; WAN *et al.* 2009). Acetylated H2A.Z at the transcription start sites (TSS) has been shown to be involved in the activation of oncogenes while deacetylated H2A.Z at the TSS is associated with the repression of tumor suppressor genes in cancer cells (VALDES-MORA *et al.* 2012).

1.1.2 Histone modifications

Histones can be subjected to several posttranslational modifications including acetylation, methylation, ubiquitination, phosphorylation and sumoylation. Monoubiquitination of histone H2B K123 (H2B K120 in mammals), catalyzed by the ubiquitin conjugating enzyme and ubiquitin ligase, Rad6 (HR6A and HR6B in humans) and Bre1 (Rnf20 in humans), is a pre-requisite for di- and tri-methylation of H3 K4 by Set1 methyltransferase (methylation of H3 K4 in humans is catalyzed by at least six methyltransferases including Set1A, Set1B, MLL/MLL1, MLL2, MLL3 and MLL4) as well as di- and tri-methylation of H3 K79 by Dot1 methyltransferase (human homolog Dot1L; Dot1 like) (BRIGGS *et al.* 2002; KIM *et al.* 2009;

KOKEN *et al.* 1991; NG *et al.* 2002b) [reviewed in (RUTHENBURG *et al.* 2007)]. Monoubiquitination of H2B K123 impacts downstream methylation of H3 K4 and H3 K79 by facilitating the recruitment of the proteosomal ATPases Rpt4 and Rpt6 and also by promoting the recruitment and activity of Set1 and Dot1 methyltransferases (EZHKOVA and TANSEY 2004; KIM *et al.* 2009; MCGINTY *et al.* 2008; OH *et al.* 2010; RACINE *et al.* 2012). H2B K123 Ub also stimulates the recruitment and activity of Set1 methyltransferase (KIM *et al.* 2009; RACINE *et al.* 2012). Paf1C also regulates these modifications by facilitating the targeting of Rad6, Bre1 and Set1 to chromatin (KIM *et al.* 2009; KIM and ROEDER 2009; KROGAN *et al.* 2003a; NG *et al.* 2003b; XIAO *et al.* 2005). Work from the Arndt laboratory also suggests that Paf1C may not just serve as a platform for recruiting these histone modifiers but also play an active role through physical association with the histones and hence could make them more accessible to these enzymes (PIRO *et al.* 2012). Consistent with this, Paf1C has also been shown to directly interact with histone H3 (CHU *et al.* 2013; MARAZZI *et al.* 2012).

Histone methylation is a relatively stable mark and hence has been proposed to create a transcriptional memory for cells (NG *et al.* 2003b). The MLL1 methyltransferase positively regulates the expression of genes encoding the homeobox family of transcription factors (*HOX* genes) that regulate development. Translocations involving the *MLL1* gene result in persistently high expression of the *HOXA9* gene and hence are the underlying cause of childhood leukemias [reviewed in (RUTHENBURG *et al.* 2007; TAN *et al.* 2010)]. Methylation of H3 K79 by Dot1 inhibits the promiscuous binding of the Sir proteins and hence indirectly facilitates the association of Sir proteins with specific loci where they can induce chromatin compaction (VAN LEEUWEN *et al.* 2002). The methylation of H3 K79 at the site of double stranded breaks leads to

recruitment of DNA repair protein 53BP1 (Rad9 in yeast) through association of the Tudor domain of 53BP1 with methylated H3 K79 [reviewed in (NGUYEN and ZHANG 2011)].

Interestingly, the H2B monoubiquitination mark was also recently shown to be essential for the methylation of a non-histone protein Dam1 (LATHAM *et al.* 2011). Independent of its role in methylation, H2B ubiquitination acts as repressive mark at the promoters of quiescent genes while it has an activating effect when present at active genes (BATTA *et al.* 2011). Rnf20 has also been observed to exert opposing effects on tumor suppressor and oncogenes. Rnf20 positively regulates expression of tumor suppressor genes while it negatively regulates expression of oncogenes (SHEMA *et al.* 2008). Based on the effects observed upon downregulation of Rnf20 such as increased cell migration and induction of tumorigenesis, Rnf20 has been implicated as a tumor suppressor (SHEMA *et al.* 2008). Consistent with this, cancer cells appear to downregulate the expression of Rnf20 by hypermethylating its promoter DNA (SHEMA *et al.* 2008). Further H2B K123 Ub has been shown to collaborate with the histone chaperone Spt16 to maintain nucleosome reassembly in the wake of Pol II (FLEMING *et al.* 2008). *In vitro* studies have also demonstrated an inhibitory effect of H2B K123 Ub on chromatin compaction (FIERZ *et al.* 2011).

The regulation of both the recruitment and activity of the enzymes that catalyze histone modifications (writers) and the enzymes that remove these marks (erasers) results in a specific genome-wide pattern of distribution of histone modifications. The downstream effects of these modifications are facilitated by the proteins that recognize these modified histones (readers). The C-terminal domain (CTD) of Pol II also facilitates the establishment of the pattern of histone modifications by serving as a platform for recruitment of the enzymes catalyzing these modifications. The CTD of Pol II contains several repeats of the consensus sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 (52 repeats in mammals and 26 in yeast) (ALLISON *et al.* 1985). The

differential phosphorylation of these repeats of Pol II CTD regulates the recruitment of Set1 and Set2 methyltransferases (Figure 1) (KROGAN *et al.* 2003a; NG *et al.* 2003b; XIAO *et al.* 2003) [reviewed in (ZHANG *et al.* 2012b)]. Set1 methyltransferase is recruited through association with Ser5 phosphorylated form of Pol II CTD and this form of Pol II is enriched over the 5' ends of genes (NG *et al.* 2003b) (Figure 1). The localization of the tri-methylated H3 K4 residues near the promoters of active genes results in the recruitment of the H3 K14 NuA3 acetyltransferase complex through association of the plant homeodomain (PHD) of the Yng1 subunit of the NuA3 complex with the histones trimethylated at H3 K4 (NG *et al.* 2003b; TAVERNA *et al.* 2006) (Figure 1). These acetylated histones near the transcription start site can then recruit chromatin remodeling complexes by binding to their bromodomains and hence increase the accessibility of promoter DNA. Additionally, trimethylated H3 K4 can also be directly recognized by the PHD domain within the nucleosome remodeling factors (NURF) (WYSOCKA *et al.* 2006). On the other hand, the di-methylated H3 K4 mark is enriched downstream of the tri-methylated H3 K4 mark [reviewed in (SMOLLE and WORKMAN 2013)]. This mark triggers the chromatin association of the HDAC (histone deacetylase complex) Set3 through association with its PHD finger domain (Figure 1). This complex deacetylates histones and prevents cryptic intragenic transcription that could occur upon the passage of transcribing Pol II (KIM and BURATOWSKI 2009). Histones di- and tri-methylated at H3 K36 by the Set2 enzyme are enriched in the middle and at the 3' ends of active genes [reviewed in (CRISUCCI and ARNDT 2011)]. This pattern of histones methylated at H3 K36 is established by targeted recruitment of Set2 through its association with the serine 2 phosphorylated form of the CTD of Pol II, a form of Pol II that predominates over the 3' region of genes (KROGAN *et al.* 2003a; XIAO *et al.* 2003) (Figure 1). The histones containing di- and tri-methylated H3 K36 in turn recruit the histone deacetylase complex Rpd3S through interaction

with the chromodomain of the Eaf3 subunit of Rpd3S (CARROZZA *et al.* 2005; KEOGH *et al.* 2005; LI *et al.* 2007; LI *et al.* 2009) (Figure 1). The hypoacetylation of the histones prevents spurious transcription from within these regions. Thus methylation of H3 K4, H3 K79 and H3 K36 are marks mainly associated with active genes [reviewed in (MARTIN and ZHANG 2005)].

Histone modifications can also facilitate gene silencing. Methylation of H3 K9, H3 K27 and H4 K20 are linked with transcriptional repression [reviewed in (MARTIN and ZHANG 2005)]. Methylation of H3 K9 by SUV39 (Suppressor Variegation 3-9 homolog) and H3 K27 by Polycomb Repressive Complex 2 (PRC2) promotes the chromatin association of the Heterochromatin Protein 1(HP1) and PRC1 through association with the chromodomain within these proteins (LACHNER *et al.* 2001) [reviewed in (MARGUERON and REINBERG 2011)].

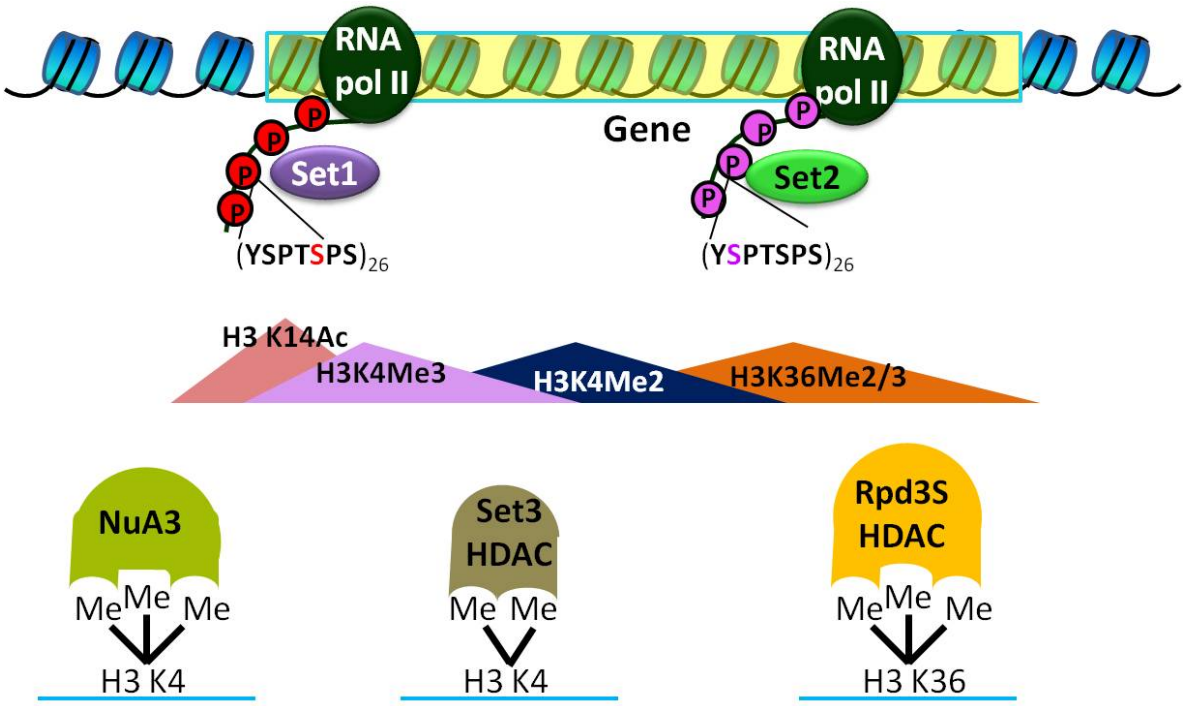


Figure 1. Chromatin alterations during Pol II transcription

H3 K4 methyltransferase Set1 is recruited by association with the Ser5 phosphorylated Pol II CTD. The tri-methylated form of H3 K4 is enriched near the promoter while its di-methylated form is enriched downstream of this modification [reviewed in (CRISUCCI and ARNDT 2011)]. Tri-methylated H3 K4 recruits NuA3 histone acetyltransferase complex which catalyzes the acetylation of H3 K14 in the promoter region while di-methylated H3 K4 recruits the HDAC Set3 via association with its PHD finger domain and facilitates the deacetylation of histones within this region [reviewed in (CRISUCCI and ARNDT 2011)]. H3 K36 methyltransferase Set2 is recruited by Ser2 phosphorylated Pol II CTD (KROGAN *et al.* 2003b). Histones with di- and tri-methylated H3 K36 in the middle of the gene and at the 3' regions facilitate the association of the Rpd3S HDAC thus maintaining these histones in the deacetylated state [reviewed in (SMOLLE and WORKMAN 2013)]. These histone modifications thus facilitate the passage of transcribing Pol II and maintain chromatin integrity in the wake of Pol II.

1.1.3 Nucleosome remodeling

Chromatin remodeling complexes alter accessibility of DNA by altering the local positioning of the nucleosomes. They harness the energy from ATP hydrolysis to slide, evict or alter the composition of the nucleosomes. Based on domains flanking the conserved ATPase domain of the remodelers, they can be classified into four different families including SWI-SNF (Switching defective, sucrose nonfermenting), ISWI (Imitation switch), INO80 (Inositol requiring 80) and CHD (Chromodomain helicase DNA binding) [reviewed in (CLAPIER and CAIRNS 2009)]. The domains flanking the ATPase domain such as the chromodomain and bromodomain target the remodeling complexes to specific regions in the genome by binding to methylated and acetylated nucleosomes, respectively. Acetylated lysines in the histones recruit chromatin remodelers such as Swi/Snf or RSC through association with their bromodomains [reviewed in (CLAPIER and CAIRNS 2009)]. Methylated lysines on the other hand, facilitate the recruitment of chromatin remodelers containing chromodomains such as Chd1 in humans or the PHD finger domain such as BPTF (Bromodomain PHD finger transcription factor) [reviewed in (PETTY and PILLUS 2013)]. Presence of both the chromodomain and the PHD finger within a chromatin remodeler such as the BPTF facilitates combinatorial recognition of histone marks i.e. BPTF binds histones that are acetylated at H4 K16 and di- and tri-methylated at H3 K4 using its bromodomain and chromodomain, respectively [reviewed in (PETTY and PILLUS 2013)].

In summary, the chromatin alterations mediated by histone chaperones, histone variants, chromatin remodelers and the post-translational modifications of histones together facilitate the transit of transcribing Pol II through the chromatin template and maintain the integrity of chromatin upon passage of Pol II.

1.2 STAGES OF TRANSCRIPTION

The production of a mature mRNA transcript involves mainly three stages each of which can be regulated to control gene expression.

1.2.1 Initiation

The transcription cycle begins with the sequential assembly of the pre-initiation factors including TFIID, TFIIA, TFIIB, TFIIE, TFIIF, TFIIH and Pol II over the promoter of a gene [reviewed in (ORPHANIDES *et al.* 1996)]. In an ATP-dependent process requiring TFIIE and TFIID the hydrogen-bonded DNA strands are separated near the transcription start site (GOODRICH and TJIAN 1994). RNA synthesis is then initiated at the transcription start site through the catalytic formation of phosphodiester bonds between the first two nucleotide triphosphates complementary to the template DNA strand. Pol II has to relinquish its contacts with the promoter DNA and the transcription initiation factors in order to productively synthesize RNA. The pre-initiation complex contains the hypophosphorylated form of Pol II. TFIIH, which includes a sub-complex of cyclin H (Ccl1 in yeast), cyclin dependent kinase 7 (CDK7; Kin28 in yeast) and MAT1 (Tfb3 in yeast), mediates the phosphorylation of the CTD of Pol II at Ser5 [reviewed in (EGLY and COIN 2011)]. This phosphorylation event marks the initiation of transcription.

1.2.2 Elongation

Earlier studies were focused on the regulation of transcription at the initiation stage but in the last few years, the importance of regulation at the elongation stage has also been recognized. The Pol II CTD is one of the key targets for the regulation of transcription at the elongation stage. All the residues of the conserved heptapeptide sequence of the Pol II CTD (Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7) other than the proline residues have been shown to be dynamically phosphorylated in a manner dependent on the stage of transcription [reviewed in (ZHANG *et al.* 2012b)]. The differentially phosphorylated CTD of Pol II serves as a binding platform for stage-specific recruitment of proteins that facilitate transcription by altering the chromatin template and regulating the co-transcriptional processing of nascent mRNA (See section 1.1.2 for examples of facilitation of transcription by altering the chromatin template). The Pol II CTD phosphorylated at Ser5 recruits the 5'-mRNA capping machinery and this 5'-capping of mRNA protects the nascent mRNA from RNAases and facilitates its cytoplasmic transport (FABREGA *et al.* 2003; MCCRACKEN *et al.* 1997).

After initiation of transcription, movement of Pol II may be paused by association of NELF (negative elongation factor) with the Pol II-associated transcription elongation factor DSIF [5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) sensitivity-inducing factor; human homologue of yeast Spt5]. Promoter proximal pausing has been shown to occur in metazoans and not in yeast since yeast lack a NELF homologue [reviewed in (GILMOUR 2009)]. Release of paused Pol II is facilitated by recruitment of P-TEFb (positive transcription elongation factor b), comprised of cyclin T and Cdk9, which phosphorylates DSIF at its C-terminal repeats (CTR), NELF and/or transcriptional activators such as c-myc (FUJINAGA *et al.* 2004; RAHL *et al.* 2010; WADA *et al.* 1998b; YAMADA *et al.* 2006; YAMAGUCHI *et al.* 1999). The

association of positive factors such as Paf1C and Human Immunodeficiency Virus Type 1 regulatory protein, Tat (transactivator of transcription), with DSIF has been suggested to mediate the transition to the elongation phase in biochemical assays measuring transcriptional activity (CHEN *et al.* 2009). Additionally, Mediator, the coregulator complex, which includes cyclin C and Cdk8, has also been shown to act as molecular switch for transition from initiation to elongation stage by releasing its contacts with the initiation factor TFIID and binding to elongation factors such as eleven nineteen lysine-rich in leukemia (ELL) and ELL-associated factors (TAKAHASHI *et al.* 2011). Release of paused Pol II provides a mechanism of rapid induction of gene expression.

1.2.3 Termination

As Pol II approaches the 3' ends of genes, the Ser2 phosphorylated form of Pol II is enriched. This form of Pol II serves as a binding platform for factors required for cleavage and polyadenylation of the nascent mRNA such as Pcf11 (Protein 1 of cleavage and polyadenylation factor I) and Rtt103 (regulator of Ty1 transposition) (LUNDE *et al.* 2010) [reviewed in (HSIN and MANLEY 2012)]. In mammals, the addition of the poly(A) tail is directed by two regions flanking the cleavage and polyadenylation site. These include the consensus sequences AAUAAA, which occurs 10-30 nucleotides upstream of the cleavage and the polyadenylation site and is a docking site for CPSF (cleavage polyadenylation specificity factor), and a U/GU rich region located 30 nucleotides downstream of the cleavage site, which binds CstF (cleavage stimulation factor) [reviewed in (KUEHNER *et al.* 2011; RICHARD and MANLEY 2009)]. The 73-KDa subunit of CPSF complex catalyzes the endonucleolytic cleavage after which the addition of the poly(A) tail is catalyzed by poly(A) polymerase (MANDEL *et al.* 2006). Binding of the poly(A) binding

protein to the poly(A) site protects the nascent mRNA from degradation (MINVIELLE-SEBASTIA *et al.* 1997). In yeast, cleavage and polyadenylation is regulated by CPF (cleavage and polyadenylation factor) and CFI and II (Cleavage factors I and II) which is comprised of factors that are homologous to the factors of the mammalian complexes, CPSF and CstF. Two models had been proposed to explain the mechanism of transcription termination. The allosteric model of termination suggests that the swapping of anti-termination proteins for termination proteins may elicit allosteric changes that trigger the release of Pol II (LOGAN *et al.* 1987). In accordance with this model, a major swapping of elongation factors for termination factors occurs at the poly(A) site (MAYER *et al.* 2010). The torpedo model proposes that the endonucleolytic cleavage provides an entry site for the Xrn2 exonuclease (Rat1 in yeast) that catalyzes the degradation of the RNA downstream of the cleavage site and hence promotes the release of the transcription elongation complex (CONNELLY and MANLEY 1988; KIM *et al.* 2004b). However, recent studies have suggested that transcription termination may involve a combination of the two mechanisms proposed by the two models for transcription termination [reviewed in (RICHARD and MANLEY 2009)]. Thus recognition of the poly(A) site is crucial for termination to occur. The action of Ser5 phosphatases such as Ssu72 and Rtr1 and the Ser2 phosphatase Fcp1 ensures the regeneration of the hypophosphorylated form of Pol II, which can engage in another round of transcription (KUEHNER *et al.* 2011).

In summary, all the stages of transcription are highly regulated by well-coordinated recruitment and exchange of accessory proteins that regulate the journey of Pol II along the chromatin template at different stages and orchestrate transcription with the co-transcriptional processing of mRNA (Figure 2).

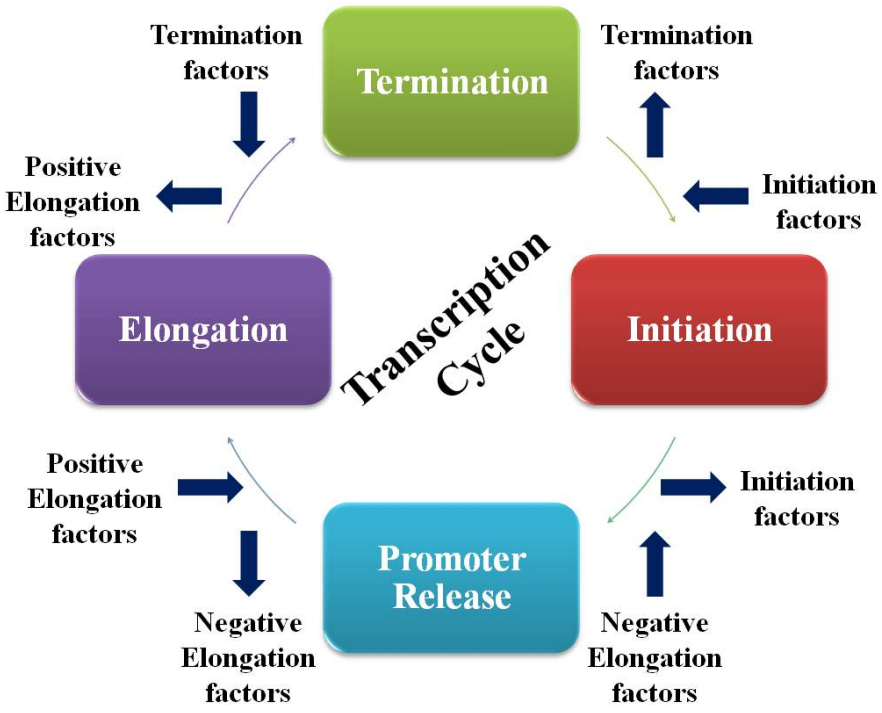


Figure 2. Transcription Cycle

The four main stages of transcription include initiation, promoter release, elongation and termination. Transition from one stage to the next involves exchange of factors mediating that stage with the factors regulating the next stage of transcription (GILMOUR 2009; MAYER *et al.* 2010).

1.3 ELONGATION FACTORS

In the last few years, a plethora of information has been obtained about the roles of several transcription elongation factors that aid the passage of Pol II through chromatin during the elongation stage of transcription. Additionally, elongation factors also regulate the initiation of transcription, co-transcriptional processing of mRNA, such as mRNA capping, splicing and mRNA 3' processing, as well the termination of transcription and mRNA turnover. Elongation factors can be divided into three main functional groups: ones that enhance the rate of elongation and inhibit transient pausing of Pol II such as Spt5; ones that facilitate the release of arrested Pol II such as TFIIS and Ccr4-Not complex; and ones that promote the passage of Pol II through the chromatin template such as P-TEFb, FACT, Spt6, Chd1 and Paf1C (Table 1).

Table 1. RNA polymerase II elongation factors

Elongation factor	**Important functions during transcription**
Spt5 (DSIF in humans)	• Stimulates processivity of Pol II (HARTZOG *et al.* 1998) • Associates with NELF to induce promoter-proximal pausing (YAMAGUCHI *et al.* 1999) • Facilitates recruitment of capping machinery, Paf1C and CFI [reviewed in (HARTZOG and FU 2013)]
TFIIS	• Reactivates arrested Pol II [reviewed in (FISH and KANE 2002)]
P-TEFb (Bur1 kinase in yeast)	• Phosphorylates the CTR of Spt5 (YAMADA *et al.* 2006) • Forms a transcriptionally active complex with Brd4 [reviewed in (ZHOU and YIK 2006)] • Forms a transcriptionally inactive complex termed 7SK snRNP complex [reviewed in (ZHOU and YIK 2006)] • Bur1 kinase phosphorylates Rad6 at serine120 and activates it (WOOD *et al.* 2005) • Bur1 kinase facilitates recruitment of Paf1C by phosphorylating the CTR of Spt5 and the CTD of Pol II (LIU *et al.* 2009; QIU *et al.* 2012; ZHOU *et al.* 2009) • P-TEFb was previously shown to phosphorylate Ser2 of Pol II CTD that has now been shown to be a function of Cdk12 (Ctk1 in yeast) [reviewed in (KOHOUTEK and BLAZEK 2012)]
FACT	• Serves as a histone chaperone [reviewed in (WINKLER and LUGER 2011)] • Facilitates chromatin disassembly to allow passage of Pol II and reassembly upon passage of Pol II and hence also prevents cryptic initiation of transcription from intragenic regions [reviewed in (WINKLER and LUGER 2011)]

Spt6	• Functions as a histone chaperone [reviewed in (DUINA 2011)] • Promotes passage of transcription machinery by disassembling nucleosomes and helps maintain chromatin integrity upon passage of Pol II [reviewed in (DUINA 2011)] • Facilitates the recruitment of histone H3 K36 methylase Setd2 (Set2 in yeast) and RNA export factor REF1 (Yra1 in yeast) and nuclear exosome factor Rrp6 (YOH *et al.* 2007; YOH *et al.* 2008)
Ccr4-Not complex	• Deadenylation activity of the complex regulates mRNA turnover [reviewed in (REESE 2013)] • Ubiquitination activity of the complex regulates stability of proteins such as the histone demethylase Jhd2 [reviewed in (REESE 2013)] • Facilitates the reactivation of transcription from paused Pol II (KRUK *et al.* 2011)
Chd1	• Maintains chromatin integrity and hence prevents cryptic initiation of transcription within gene bodies in collaboration with Isw1 chromatin remodeling complex by inhibiting trans-histone exchange (SMOLLE *et al.* 2012) • Increases histone exchange in the regions close to the promoter and hence facilitates chromatin remodeling over these regions (EHRENSBERGER and KORNBERG 2011; LIN *et al.* 2011; RADMAN-LIVAJA *et al.* 2012)
Paf1C	• Recruits Chd1 (SIMIC *et al.* 2003) • Promotes co-transcriptional histone modifications such as monoubiquitination of H2B K123, downstream methylation of H3 K4 and H3 K79 and tri-methylation of H3 K36 [reviewed in (CRISUCCI and ARNDT 2011)] • Represses inducible genes by maintaining nucleosome occupancy over their promoters (PRUNESKI *et al.* 2011) • Facilitates 3' RNA processing and transcription termination partly by promoting the Ser2 phosphorylation of Pol II CTD that serves as a platform for recruiting factors involved in these processes [reviewed in (CRISUCCI and ARNDT 2011)]

1.3.1 Spt5

Among all the proteins that regulate transcription elongation, the only one that is conserved across all three domains of life including bacteria, archaebacteria and eukaryotes is the transcription elongation factor Spt5 (PONTING 2002) [reviewed in (HARTZOG and FU 2013)]. Spt5 and its heterodimeric partner, Spt4, were discovered in a genetic screen directed towards uncovering factors involved in maintaining chromatin structure during transcription (WINSTON *et al.* 1984). A seminal study in yeast strengthened the evidence for the role of Spt5 as an elongation factor through identification of Pol II mutants, which reduce transcription rates, as the suppressors of the conditional mutants of Spt5 (HARTZOG *et al.* 1998). This study also demonstrated the existence of the Spt4-Spt5 complex, showed that Spt5 also interacted with Pol II *in vivo* and identified Spt5 as the homologue of the bacterial transcription elongation factor, NusG (HARTZOG *et al.* 1998). Around the same time, the human Spt5-Spt4 complex, termed DSIF, was also discovered based on its identification as a factor conferring sensitivity to the transcription elongation inhibitor DRB (5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole) (WADA *et al.* 1998a). These and other studies that revealed physical and genetic interactions of the Spt4-Spt5 complex with other elongation factors such as Spt6 and TFIIS and the impact of Spt5 on the rate of elongation and processivity of Pol II during transcription further established the identity of Spt5 as an elongation factor (HARTZOG *et al.* 1998; LINDSTROM and HARTZOG 2001; SWANSON and WINSTON 1992).

Eukaryotic Spt5 is a large protein containing several domains (Figure 3). The NGN (NusG N-terminal) domain facilitates the interaction between Spt5 and clamp helices of Pol II through mainly hydrophobic interactions. These interactions between Spt5 and Pol II allow the clamping of Pol II onto the underlying DNA and thus enhance the processivity of Pol II

(HIRTREITER *et al.* 2010; KLEIN *et al.* 2011; MARTINEZ-RUCOBO *et al.* 2011; PONTING 2002). Interestingly, the binding sites of the transcription initiation factor TFIIE and Spt5 on Pol II overlap and their affinities for Pol II depend on the factors associated with Pol II (GROHMANN *et al.* 2011). The presence of Pol II within the initiation complex favors the binding of TFIIE while Spt5 can displace TFIIE and bind Pol II within an elongation complex. Spt5 also interacts with its heterodimeric binding partner Spt4, a small zinc finger protein, through its NGN domain via a β-sheet interface and several hydrogen bonds and this association with Spt4 is important for the stability of Spt5 (DING *et al.* 2010; GUO *et al.* 2008; MALONE *et al.* 1993).

The KOW (Kyprides, Ouzounis and Woese) motifs found within Spt5 have the ability to bind nucleic acids and are also found within ribosomal proteins (CHARIER *et al.* 2004; STEINER *et al.* 2002). They resemble the Tudor domain and may facilitate the interaction of Spt5 with other protein known to interact with Spt5 such as Spt6, proteins of the capping machinery and Bur1-Bur2 kinase complex (LINDSTROM *et al.* 2003). The C-terminal region of Spt5 has multiple repeats of a peptide sequence that varies among eukaryotes. The CTR of *S. cerevisiae* has the consensus sequence ST/AWGGA/Q while the repeats in Spt5 of higher eukaryotes such as humans, mice and *Drosophila* are located upstream of the C-terminus and have the sequence GSR/QTP (SWANSON *et al.* 1991; YAMADA *et al.* 2006). The serine residue within the consensus sequence of the CTR of yeast Spt5 has been shown to be phosphorylated by the Bur1-Bur2 kinase (yeast homologue of P-TEFb) and this phosphorylation event was shown to facilitate the recruitment of Paf1C through a mechanism that was obscure (LIU *et al.* 2009; ZHOU *et al.* 2009). Additionally, the CTR of yeast Spt5 has also been shown to be important for the recruitment of the capping enzyme and the pre-mRNA cleavage factor 1 (MAYER *et al.* 2012; PEI *et al.* 2003). The phosphorylation of the threonine residue within the repeats in human Spt5 by P-TEFb

facilitates the release of paused Pol II (see section 1.2.2 for more discussion on paused Pol II) (YAMADA *et al.* 2006). Transcriptional activation upon phosphorylation has been shown to be facilitated by Paf1C and the Tat1 transcriptional activator protein in HIV (CHEN *et al.* 2009). Thus, the CTR of Spt5 like the CTD of Pol II could serve as platform for recruitment of proteins during stages of transcription to facilitate the movement of Pol II and co-transcriptional mRNA processing.

Unlike their eukaryotic homologues, archeal and bacterial Spt5 proteins (NusG: N-utilization substance G) are small and only have the NGN domain and one KOW domain [reviewed in (HARTZOG and FU 2013)]. NusG interacts with a ribosomal protein NusE and the termination factor Rho through its C-terminal domain in a mutually exclusive fashion (BURMANN *et al.* 2010). Hence, transcription termination can occur only at the end of operons once translation and transcription are uncoupled and the interaction between NusG and NusE is lost, facilitating the binding of Rho to NusG. The interaction of NusG with NusE also suppresses the backtracking of RNA polymerase and hence rate of translation affects the rate of transcription during bacterial co-transcriptional translation (PROSHKIN *et al.* 2010). Additionally, bacteria express a paralogue of Spt5 called RfaH that binds to RNA polymerase in a manner similar to that of NusG through the hydrophobic interaction of its NGN domain with the RNA polymerase clamp. However, the recruitment of RfaH also requires an 8 bp consensus site, GGCGGTAG, termed the ops (operon polarity suppressor) element (BAILEY *et al.* 1996; NIETO *et al.* 1996). This difference in the mechanisms of recruitment of RfaH and NusG prevents the interference from RfaH to the binding of NusG to RNA polymerase although both bind RNA polymerase in a similar fashion.

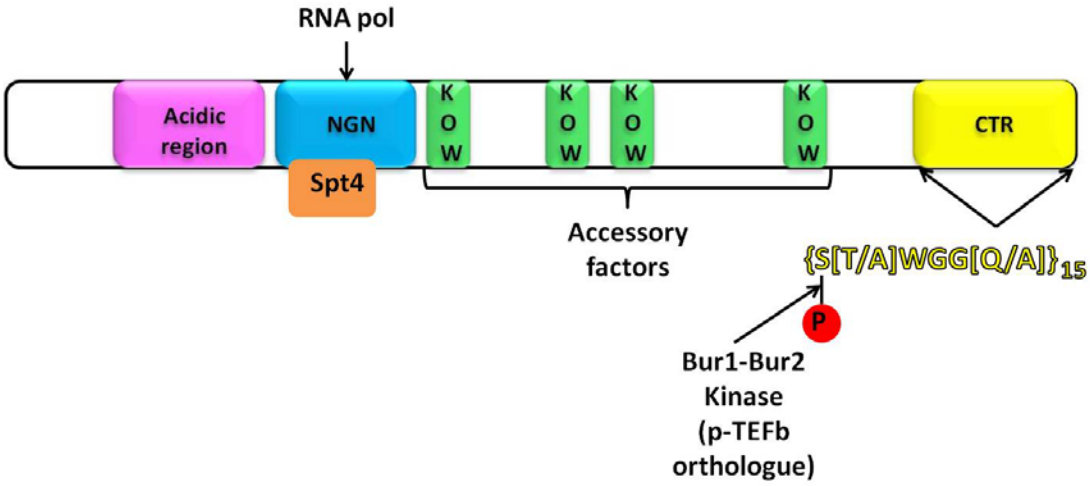


Figure 3. Schematic representation of *S. cerevisiae* Spt5

Spt5 is a large protein containing an N-terminal acidic region, followed by an NGN domain, which mediates its binding to Spt4 and Pol II, several KOW motifs and a C-terminal region containing several repeats of a short hexapeptide sequence (KYRPIDES *et al.* 1996; PONTING 2002; SWANSON *et al.* 1991).

1.3.2 TFIIS

The TFIIS elongation factor was discovered as a factor that was able to reactivate transcription from arrested RNA polymerase (SEKIMIZU *et al.* 1976; SEKIMIZU *et al.* 1979). The yeast homologue was discovered in a screen to identify genes that confer sensitivity to the base analog 6-Azauracil and was also termed *DST1* (DNA strand Transfer 1) since Dst1 mutants showed defective homologous recombination (CLARK *et al.* 1991) [reviewed in (UPTAIN *et al.* 1997)]. TFIIS helps reactivate arrested Pol II by stimulating the intrinsic RNA cleavage activity of arrested Pol II. Upon encountering a block during transcription, Pol II backtracks causing displacement of the 3' end of the growing RNA transcript from the catalytic site of Pol II. This leads to an arrest of Pol II. The stimulation of the intrinsic RNA cleavage activity of arrested Pol II by TFIIS leads to cleavage of the misaligned 3' end of the nascent transcript and creates new 3' end that is now properly aligned at the active site of Pol II so that it can then resume transcription [reviewed in (FISH and KANE 2002)]. TFIIS contains three domains, Domain I, II and III of which the conserved domains, domain II and III, are required to stimulate the cleavage of the nascent transcript and reactivate the arrested Pol II (GUO and PRICE 1993). Domain II and the linker region between domains II and III facilitate the binding of TFIIS to Pol II (AGARWAL *et al.* 1991; SHIMASAKI and KANE 2000). Prokaryotic homologues of TFIIS include GreA and GreB, which also facilitate the release of arrested RNA polymerase by stimulating the intrinsic RNA cleavage ability of RNA polymerase [review in (FISH and KANE 2002)].

1.3.3 P-TEFb

P-TEFb is comprised of the kinase Cdk9 and its regulatory cyclin T1 (PENG *et al.* 1998). Treatment of HeLa cells with flavopiridol, an inhibitor of P-TEFb, indicated its requirement for the expression of a large number of genes (CHAO and PRICE 2001). It is also essential for transcription elongation of the HIV genome in HIV-1 infected cells (WEI *et al.* 1998). Previous studies had identified Ser2 of the Pol II CTD, Thr4 of the CTR of DSIF and the RD subunit of NELF as the substrates of P-TEFb (FUJINAGA *et al.* 2004; RAHL *et al.* 2010; WADA *et al.* 1998b; YAMADA *et al.* 2006; YAMAGUCHI *et al.* 1999). However, recent studies have attributed the function of phosphorylation of the Ser2 of Pol II CTD to the kinase Cdk12 (Ctk1 in yeast) and its associated cyclin K (BARTKOWIAK *et al.* 2010; BLAZEK *et al.* 2011; KOHOUTEK and BLAZEK 2012) [reviewed in (KOHOUTEK and BLAZEK 2012)].

Half the cellular pool of P-TEFb exists in a transcriptionally active complex with Brd4 (bromodomain containing protein 4) protein. Brd4 interacts with cyclin T1 of P-TEFb through its tandem bromodomains, that it also utilizes to associate with acetylated histones and hence Brd4 couples P-TEFb with genes primed for transcriptional activation (BISGROVE *et al.* 2007; JANG *et al.* 2005; YANG *et al.* 2005). However, P-TEFb is also recruited by HIV-1 to stimulate transcription of the viral genes via a Brd4-independent mechanism. The viral regulatory protein, Tat, associates with P-TEFb and facilitates its incorporation into a ternary complex containing the TAR element (Trans-acting response RNA element), a stem-loop structure formed by a nascent viral transcript at the site of stalled Pol II, P-TEFb and Tat protein [reviewed in (ZHOU and YIK 2006)]. Upon recruitment of P-TEFb, viral transcription elongation is stimulated.

In addition to its transcriptionally active complex, the rest of P-TEFb is sequestered in a transcriptionally inactive complex, the 7SK snRNP complex, from which it can be released in response to certain signals. The 7SK snRNP complex consists of 7SK, a Pol III transcribed snRNA (short nuclear RNA), HEXIM1 (hexamethylene bisacetamide inducible1), BCDIN3 (Bicoid interacting 3) and Pip7S (P-TEFb interaction protein for 7SK stability)/LARP7 (L-related protein 7) (HE *et al.* 2008; JERONIMO *et al.* 2007; KRUEGER *et al.* 2008) [reviewed in (ZHOU and YIK 2006)]. HEXIM1 inhibits P-TEFb by binding to it and 7SK acts as a scaffold for this interaction of P-TEFb with HEXIM1 (YIK *et al.* 2003). BCDIN3 is a methylphosphate capping enzyme that protects 7SK from 5'-3' exonucleases by capping it (JERONIMO *et al.* 2007). LARP7, on the other hand, protects 7SK from 3'-5' exonucleases by binding to its 3'-UUU-OH sequence (HE *et al.* 2008).

The yeast homologue of the Cdk9 kinase of P-TEFb complex is the Bur1 kinase that phosphorylates Ser2 of Pol II CTD during early stages of transcription elongation and the CTR of Spt5 (LEE and GREENLEAF 1991; LIU *et al.* 2009; PATTURAJAN *et al.* 1999; QIU *et al.* 2012; ZHOU *et al.* 2009). The Bur1-Bur2 complex is recruited to genes through association with the Ser5-phosphorylated Pol II CTD (QIU *et al.* 2009). Bur1-Bur2 in turn phosphorylates Ser2 of the Pol II CTD in the promoter-proximal regions, and upon recruitment of Ctk1, Ctk1 takes over the function of the phosphorylating Ser2 of the Pol II CTD (QIU *et al.* 2009). Furthermore, the Bur1-Bur2 complex phosphorylates the CTR of Spt5 (discussed in section 1.1.3). Additionally, Bur1 kinase phosphorylates Ser120 on Rad6, which activates the catalytic activity of Rad6 towards the monoubiquitination of H2B K123 (WOOD *et al.* 2005). Because Bur1 kinase facilitates the recruitment of Paf1C and activates Rad6, Bur1-Bur2 mutants have reduced levels of monoubiquitinated H2B K123 and methylated H3 K4, H3 K79 and H3 K36 (CHU *et al.* 2007;

WOOD *et al.* 2005). On the other hand, monoubiquitination of H2B K123 impedes the recruitment of Ctk1. Only upon deubiquitination of H2B K123 by the deubiquitinating enzyme Ubp8, can Ctk1 be recruited to genes (WYCE *et al.* 2007). Ctk1-catalyzed Ser2 phosphorylation of the Pol II CTD stimulates the recruitment of H3 K36 methyltransferase Set2 and 3'-RNA processing factors (AHN *et al.* 2004). Ctk1 is required for maintaining the boundary of H3 K4 Me3 and H3 K4 Me2 across genes. Absence of Ctk1 results in spreading of H3 K4 Me3 and H3 K4 Me2 towards the 3' ends of genes (XIAO *et al.* 2007). This regulation of the distribution of di- and tri-methylated H3 K4 by Ctk1 is independent of its role in the recruitment of Set2 methyltransferase (XIAO *et al.* 2007).

1.3.4 FACT

Genes encoding the two major subunits of the yeast FACT (Facilitates chromatin transactions) complex, Spt16 and Pob3, were discovered in screens to identify mutants that alter transcription at genes having a transposon insertion within their promoters, cause cell proliferation defects and genetically interact with transcriptional activators, all suggesting a role for the FACT complex in transcription (LYCAN *et al.* 1994; PRENDERGAST *et al.* 1990; WINSTON *et al.* 1984). The human FACT complex was discovered as an activity stimulating transcription elongation on a nucleosomal template in an *in vitro* transcription assay (ORPHANIDES *et al.* 1998). The association between Spt16 and Pob3 was discovered in the search for complexes involved in DNA replication highlighting the importance of this histone chaperone in DNA-templated processes other than transcription (WITTMEYER and FORMOSA 1997). The function of the human homologue of *S. cerevisiae* Pob3, SSRP1 (Structure specific recognition protein 1), has been split between two yeast proteins, Pob3 and Nhp6. SSRP1 has an additional DNA binding domain

compared to Pob3, a domain also found in high-mobility group (HMG) proteins such as yeast Nhp6, which associates with yeast Spt16. However, the interaction between Spt16 and Nhp6 is not stable indicating that the DNA-binding function of Nhp6 may also be executed by other proteins that associate with yeast Spt16 (FORMOSA *et al.* 2001). In addition to the experiments leading to the discovery of FACT in human and yeast, its physical and functional interactions with other elongation factors such as Paf1C and its association with Pol II at actively transcribed genes, further underscored the role of FACT as a transcription elongation factor (COSTA and ARNDT 2000; SAUNDERS *et al.* 2003; SQUAZZO *et al.* 2002).

FACT is a histone chaperone that can associate with both H3-H4 dimers and H2A-H2B dimers. Hence, it can also promote nucleosome assembly during DNA replication by facilitating the incorporation of H3-H4 tetramers or H2A-H2B dimers (BELOTSERKOVSKAYA *et al.* 2003; ORPHANIDES *et al.* 1998; ORPHANIDES *et al.* 1999). Due to its histone chaperoning ability, FACT can aid the passage of Pol II through chromatin during transcription by reorganizing the nucleosomes via displacement of single H2A-H2B dimer and reassemble the nucleosomes upon the passage of Pol II (BELOTSERKOVSKAYA *et al.* 2003; ORPHANIDES *et al.* 1999).

Spt16 has an N-terminal domain (NTD), a dimerization domain (D) via which it associates with Pob3, a middle (M) and a C-terminal domain (C). Pob3 protein also contains an N-terminal domain (N) through which it associates with Spt16, a middle (M) and an acidic C-terminal (C) domain. The Spt16-NTD, which bears structural similarity to aminopeptidases and the Pob3-M domain, which has the double pleckstrin homology motif, have the ability to bind the N-terminal histone tails (VANDEMARK *et al.* 2006; VANDEMARK *et al.* 2008). Additionally, the Pob3 M-domain also facilitates the association of FACT with replication protein A (RPA), which is involved in other DNA-templated processes such as replication and repair

(VANDEMARK *et al.* 2006). The Spt16 C-domain binds histones and makes the nucleosomal DNA more accessible while the D and M domains facilitate nucleosome reassembly and hence maintain chromatin integrity upon the passage of Pol II (HAINER *et al.* 2012; WINKLER *et al.* 2011). A recent comprehensive study has suggested that the hydrophobic interactions of the M domain of Spt16 with the H2A-H2B heterodimer are crucial for the histone chaperoning function of Spt16, although the other regions of Spt16 are also involved in the binding of Spt16 to the histones (HONDELE *et al.* 2013). This study also implicated the dimerization domain of Spt16 in the interaction of Spt16 with the DNA replication machinery (HONDELE *et al.* 2013).

1.3.5 Spt6

Spt6 was discovered along with Spt5, Spt4 and Spt16 in genetic screens to identify suppressors of the transcriptional defects conferred by transposon insertions (FASSLER and WINSTON 1988; WINSTON *et al.* 1984). The physical and functional interactions of Spt16 with other transcription elongation factors such as Spt5 and TFIIS and its effects on the transcription of genes such as the histone genes and on the transcriptional activity of human estrogen receptor provided further evidence for its role in transcription (BANIAHMAD *et al.* 1995; CLARK-ADAMS and WINSTON 1987; COMPAGNONE-POST and OSLEY 1996; DENIS and MALVAR 1990; HARTZOG *et al.* 1998; SWANSON and WINSTON 1992). Early studies also provided evidence that Spt6 can directly associate with histones (with higher affinity for histone H3) and facilitate the assembly of nucleosomes *in vitro*, thus uncovering its function as a histone chaperone (BORTVIN and WINSTON 1996). Subsequent experiments identified its role in preventing spurious initiation of transcription from intragenic regions (CHEUNG *et al.* 2008; KAPLAN *et al.* 2003). Spt6 also maintains chromatin integrity over highly transcribed genes by facilitating nucleosome

reassembly in the wake of Pol II (IVANOVSKA *et al.* 2011). Spt6 negatively regulates the expression of some genes by maintaining nucleosome occupancy over promoter DNA and blocks the access of the promoter DNA to transcriptional activators (ADKINS and TYLER 2006; HAINER *et al.* 2011).

That Spt6 may have additional functions independent of its role as a histone chaperone, was shown by the lack of a remarkable overlap between Spt6 targets identified by transcriptome analysis and targets requiring Spt6 for maintenance of their chromatin integrity (IVANOVSKA *et al.* 2011). Roles of Spt6 in transcription that may be independent of its histone chaperone activity were uncovered after the identification of the physical interactions of Spt6 with Ser2 phosphorylated Pol II CTD and Iws1 (interacts with Spt6) (KROGAN *et al.* 2002; YOH *et al.* 2007). Spt6 interacts with the Ser2 phosphorylated form of Pol II CTD through its SH2 domain (Src homology 2). The binding of Isw1 to the CTD-bound Spt6 facilitates the recruitment of Setd2 (SET domain 2; human homologue of Set2), human RNA export factor REF1 (homologue of yeast Yra1; Yeast RNA annealing protein 1) and human Rrp6 (ribosomal RNA processing), which is involved in RNA processing and mRNA surveillance (YOH *et al.* 2007; YOH *et al.* 2008). Spt6 has been shown to be important for several developmental and other signaling pathways in higher eukaryotes (ARDEHALI *et al.* 2009; BANIAHMAD *et al.* 1995; KEEGAN *et al.* 2002; SHEN *et al.* 2009).

1.3.6 Ccr4-Not complex

Ccr4 (Carbon catabolite repression 4) was named after its discovery as a gene required for the expression of glucose-repressed genes (DENIS 1984). While the Not1 and Not2 components were identified as genes required for proper cell cycle division (REED 1980; SHUSTER and BYERS

1989). These genes were termed the NOT genes (negative on TATA) due to their negative effect on expression from unconventional TATA promoters (COLLART and STRUHL 1994). The presence of the Not proteins in the Ccr complex was uncovered in a proteomics analysis of the Ccr complex (LIU *et al.* 1998). These and other studies led to the identification of the nine core subunits of the Ccr4-Not complex: Not1-5, Ccr4, Caf1 (Ccr4-associated factor 1), Caf40 and Caf130 (BAI *et al.* 1999; CHEN *et al.* 2001; LIU *et al.* 1998). Subsequent studies led to the identification of several functions of this complex performed over the entire lifetime of mRNA from its synthesis to its degradation. Identification of the functional and physical interaction between the Ccr4-Not complex and the TFIID transcription initiation factor and the localization of Ccr4-Not over promoter regions suggested a role for this complex in transcription initiation (BADARINARAYANA *et al.* 2000; DELUEN *et al.* 2002). The sensitivity of the mutants of the Ccr4-Not complex to drugs that induce greater dependency on transcription elongation factors such as 6-Azauracil and Mycophenolic acid (MPA), genetic interactions of this complex with transcription elongation factors such as TFIIS and Spt16, the occupancy of this complex over transcribed genes, its physical interaction with Pol II, its reduced GLAM (gene length accumulation of mRNA) ratio and its ability to restore transcription from stalled Pol II, emphasized the role of the Ccr4-Not complex as a transcription elongation factor (DENIS *et al.* 2001; GAILLARD *et al.* 2009; KRUK *et al.* 2011).

Identification of the cytoplasmic deadenylation activity of the Ccr4-Not complex established the role of this complex in the regulation of mRNA turnover (TUCKER *et al.* 2001). The deadenylation activity is dependent on the Ccr4 subunit and Caf1 subunit. The Ccr4 subunit belongs to the EEP (Exonuclease/Endonuclease/Phosphatase) superfamily and has the DNaseI-like domain that confers upon it 3'-5' exonuclease activity on substrates such as poly(A) RNA

and single stranded DNA (CHEN *et al.* 2002; MARCHLER-BAUER *et al.* 2013; TUCKER *et al.* 2001). The deadenylase activity is also contributed by the Caf1 subunit, which is a member of the DEDD family (has catalytically important and conserved D and E residues in the exonuclease domain) and has the RNaseD-like domain, in higher eukaryotes but not in yeast, where it only tethers the Ccr4 subunit with the rest of the complex (MARCHLER-BAUER *et al.* 2013; TEMME *et al.* 2010; TUCKER *et al.* 2001).

The Ccr4-Not complex also exhibits ubiquitination activity. This activity is contributed by the Not4 subunit, an E3 ubiquitin ligase that has a highly conserved RING (Really Interesting New Gene) finger domain (ALBERT *et al.* 2002). Not4 has been shown to function with two E2 ubiquitin conjugases, Ubc4 and Ubc5, to regulate the levels of proteins such as the histone demethylase Jhd2 (JumonjiC domain-containing Histone Demethylase), DNA polymerase alpha, nascent polypeptide associated complex, cyclin C and the transcription factor Yap1 [reviewed in (REESE 2013)].

Thus, the Ccr4-Not complex is a multifunctional complex that influences gene expression through several mechanisms.

1.3.7 Chd1

Chd, represents a family of chromatin remodeling complexes that possess tandem chromodomains at their N-terminus, a central helicase domain and a C-terminal DNA binding domain (WOODAGE *et al.* 1997). They are conserved from yeast to humans. The 6-Azauracil resistance of mutants of this chromatin remodeling factor had suggested a negative role for this complex in transcription elongation (WOODAGE *et al.* 1997). Subsequent studies uncovered the physical and functional interactions of Chd1 with transcription elongation factors such as Spt16,

Spt5 and Paf1C and the localization of Chd1 over actively transcribed genes, highlighting the role of Chd1 in transcription elongation (SIMIC *et al.* 2003).

Chd1 has been shown to function with another chromatin remodeler Isw1 (Imitation Switch subfamily) to inhibit trans-histone exchange and hence maintain chromatin integrity over gene bodies and prevent spurious intragenic transcription (GKIKOPOULOS *et al.* 2011; LEE *et al.* 2012; QUAN and HARTZOG 2010; SMOLLE *et al.* 2012). This function of Chd1 has been attributed to its ability to reduce histone turnover over the 3' end of genes by maintaining the histones in the deacetylated state (QUAN and HARTZOG 2010; RADMAN-LIVAJA *et al.* 2012). However, Chd1 increases histone exchange near 5' ends of genes (RADMAN-LIVAJA *et al.* 2012). Consistent with these results, Chd1 has been shown to facilitate chromatin remodeling over the promoters in an activator-dependent manner upon recruitment by the Mediator coactivator complex (EHRENSBERGER and KORNBERG 2011; LIN *et al.* 2011).

The requirement for the chromodomains of Chd1 for its recruitment to chromatin differs between organisms. The chromodomains of human Chd1 may mediate its recruitment to chromatin through association with di- and tri-methylated H3 K4 (SIMS *et al.* 2005). In yeast, however, it remains obscure if the binding of the chromodomain to methylated H3 K4 targets Chd1 to chromatin (PRAY-GRANT *et al.* 2005; SIMS *et al.* 2005). Yeast Chd1 has been shown to be recruited to chromatin through interaction with the Rtf1 subunit of Paf1C (SIMIC *et al.* 2003).

In mice, Chd1 has been shown to be important for maintaining stem cell pluripotency and reduced levels of Chd1 causes an increase in the heterochromatin levels (GASPAR-MAIA *et al.* 2009). Furthermore, human Chd1 has been shown to function as tumor suppressor in prostrate cancer by positively regulating transcription mediated by androgen receptor including transcription of tumor suppressor genes (BURKHARDT *et al.* 2013).

1.3.8 Paf1C

Yeast Paf1C is composed of five subunits including Paf1, Ctr9 (Cln Three Requiring), Cdc73 (Cell Division Cycle), Leo1 (Left Open reading frame) and Rtf1 (Restores TBP Function). Human Paf1C is associated with an additional subunit, Ski8 (ZHU *et al.* 2005). Paf1 and Cdc73 subunits were discovered during a search for accessory proteins associated with Pol II (WADE *et al.* 1996). The Rtf1 subunit was discovered in a screen to identify suppressors of a TBP mutant with altered specificity for DNA (STOLINSKI *et al.* 1997). Ctr9 was discovered in a screen to uncover the transcriptional regulators of G1 cyclin (KOCH *et al.* 1999). The role of Leo1 and Cdc73 in transcription was uncovered after the discovery of their association with the other subunits of Paf1C. The proteomic analysis of the affinity purified subunits led to identification of all the components of Paf1C (KOCH *et al.* 1999; KROGAN *et al.* 2002; MUELLER and JAEHNING 2002; SQUAZZO *et al.* 2002). The phenotypes exhibited by the members of Paf1C such as the Spt$^-$ phenotype, sensitivity to drugs such as 6-AU and MPA, their effects on expression of some genes as determined by microarray analysis, their genetic and physical interactions with other elongation factors such as Spt5, Spt16 and TFIIS, their localization over actively transcribed genes, their effect on the GLAM (gene length accumulation of mRNA) ratio and their impact on co-transcriptional histone modifications underscored the identity of Paf1C as a transcription elongation complex (ADELMAN *et al.* 2006; GAILLARD *et al.* 2009; RONDON *et al.* 2004; SQUAZZO *et al.* 2002; STOLINSKI *et al.* 1997) [reviewed in (CRISUCCI and ARNDT 2011)]. The stimulatory effect of Paf1C alone and its synergistic effect with TFIIS on transcription over chromatin templates in transcription assays with biochemically defined factors suggested that Paf1C also has an intrinsic ability to promote transcription and it can also synergistically

stimulate transcription through association with other elongation factors such as TFIIS (KIM *et al.* 2010).

Paf1C is a multifunctional complex that regulates transcription through several different mechanisms. One of the better-studied roles of Paf1C is the regulation of co-transcriptional histone modifications (Figure 4). Paf1C mediates the monoubiquitination of lysine 123 of histone H2B, which is catalyzed by the ubiquitin-conjugating enzyme Rad6 and the ubiquitin ligase Bre1, by facilitating the chromatin recruitment of Rad6 possibly by binding to Bre1 (KIM and ROEDER 2009; NG *et al.* 2003a; WOOD *et al.* 2003; XIAO *et al.* 2005). Recent studies from our laboratory also suggest that the Rtf1 subunit of Paf1C may facilitate this histone modification through interaction with nucleosomes (PIRO *et al.* 2012). Monoubiquitinated H2B K123 collaborates with the histone chaperone FACT to facilitate the reassembly of nucleosomes in the wake of Pol II (FLEMING *et al.* 2008; PAVRI *et al.* 2006). The effect of this mark is context-dependent. While it acts as an activating mark within the body of genes, at the promoter it acts an inhibitory mark by facilitating nucleosome formation (BATTA *et al.* 2011). This mark also serves as a pre-requisite for the di- and tri-methylation of H3 K79 and H3 K4 residues, which is catalyzed by the Dot1 and Set1 enzymes, respectively (DOVER *et al.* 2002; KROGAN *et al.* 2003a; NG *et al.* 2003b; SUN and ALLIS 2002; WOOD *et al.* 2003). Additionally, Paf1C also promotes H3 K4 methylation by mediating the recruitment of the Set1 complex (KROGAN *et al.* 2003a). H3 K4 Me2 and H3 K4 Me3 modifications facilitate the recruitment of histone acetyltransferases and histone deacetylases (discussed in section 1.1.2). By regulating the methylation of the H3 K4 residue, Paf1C facilitates the activation of genes and prevents the initiation of cryptic transcription from intragenic regions (CHU *et al.* 2007; KIM and BURATOWSKI 2009; LIN *et al.* 2011; STOLINSKI *et al.* 1997) (also discussed in section 1.1.2). H3 K79 methylation on the other

hand inhibits the binding of proteins involved in transcriptional silencing at active loci and restricts them to the specific silenced loci (NG *et al.* 2002a; VAN LEEUWEN *et al.* 2002). Thus, Paf1C is also important for telomeric silencing. Paf1C also facilitates the maintenance of chromatin integrity by promoting tri-methylation of H3 K36, which recruits the HDAC that maintains the histones in a deacetylated state and prevents spurious initiation of transcription from intragenic regions (discussed in section 1.1.2). This effect of Paf1C on Set2 recruitment is likely due to the impact of Paf1C on the phosphorylation of Ser2 of the Pol II CTD (NORDICK *et al.* 2008; PENHEITER *et al.* 2005).

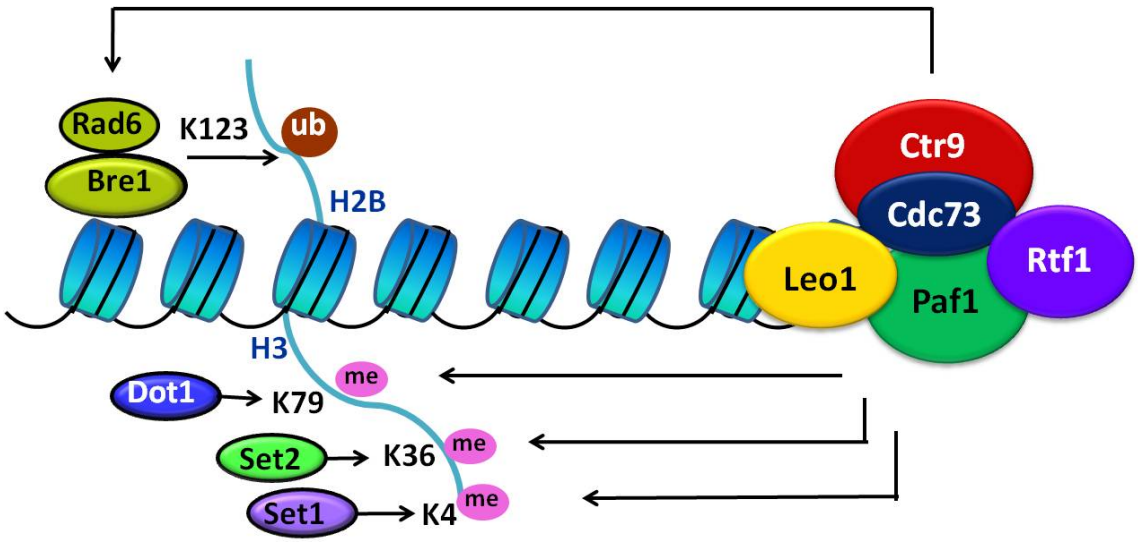


Figure 4. Regulation of histone modifications by Paf1C

Paf1C is required for the monoubiquitination of H2B K123, which is catalyzed by the Rad6 and Bre1 proteins [reviewed in (CRISUCCI and ARNDT 2011)]. This mark is involved in a histone cross-talk and facilitates methylation of H3 K79 and H3 K4 methylation marks, which are catalyzed by methyltransferase Set1 and Dot1 [reviewed in (CRISUCCI and ARNDT 2011)]. Additionally, Paf1C is also required for tri-methylation of H3 K36 by the Set2 methyltransferase [reviewed in (CRISUCCI and ARNDT 2011)].

Through regulation of the Ser2 phosphorylation of the Pol II CTD, Paf1C also regulates transcription termination (Figure 5). The Ser2 phosphorylation of the Pol II CTD serves as a binding platform for recruitment of termination factors involved in cleavage and polyadenylation of nascent mRNA such as Pcf11 (MUELLER *et al.* 2004; NORDICK *et al.* 2008). Hence, loss of Paf1C subunits causes shortening of the poly(A) tails of mRNA, which destabilizes those mRNAs (MUELLER *et al.* 2004). Depletion of the components of Paf1C also results in utilization of alternative poly(A) sites at some genes sensitizing those transcripts to nonsense mediated mRNA decay, an mRNA surveillance mechanism that eliminates aberrant mRNAs with premature stop codons or abnormally long 3' UTRs (untranslated regions) (MUHLRAD and PARKER 1999; PELTZ *et al.* 1993; PENHEITER *et al.* 2005). Not only does Paf1C indirectly influence the recruitment of termination factors through regulation of Ser2 phosphorylation of the Pol II CTD but it also directly facilitates the association of the termination factor Cft1 with the Ser5 phosphorylated Pol II CTD (NORDICK *et al.* 2008). Additionally, the Ski8 subunit that associates with human Paf1 mediates 3'-5' mRNA degradation and hence mRNA surveillance co-transcriptionally by associating with actively transcribed genes through Paf1C (ZHU *et al.* 2005).

Studies from the Arndt laboratory and the Buratowski laboratory have uncovered the importance of Paf1C for proper termination of snoRNAs, a class of non-coding, non-polyadenylated RNAs that facilitate post-translational modifications of ribosomal RNAs (rRNA), transfer RNAs (tRNAs) and small nuclear RNAs (snRNA) [reviewed in (MATERA *et al.* 2007)]. Paf1C prevents the read-through transcription of snoRNAs by facilitating the recruitment of Nrd1 and Nab3, which regulate snoRNA termination through the Nrd1-Nab3-Sen1 pathway (SHELDON *et al.* 2005; STEINMETZ *et al.* 2001). Furthermore, Paf1C also facilitates the

termination of snoRNA transcripts through regulation of histone modifications such as monoubiquitination of H2B K123 and methylation of H3 K4 and H3 K36 and additional unknown mechanisms (TERZI *et al.* 2011; TOMSON *et al.* 2012; TOMSON *et al.* 2011).

Paf1C not only regulates transcription elongation and termination but also transcription initiation. Support for the role of Paf1C in transcription initiation mainly comes from studies in higher eukaryotes. Paf1C facilitates the expression of genes required for maintaining stem cell pluripotency such as Oct4 and Nanog, and the transcriptional targets of the Notch signaling pathway by maintaining the chromatin over promoters in a transcriptionally active state through promotion of tri-methylation of H3 K4 over the promoter regions (AKANUMA *et al.* 2007; BRAY *et al.* 2005; DING *et al.* 2009; TENNEY *et al.* 2006). Furthermore, Paf1C is recruited by the Gli (Glioma associated oncogene family zinc finger 1) transcription factors through a direct physical association with Cdc73 to activate transcription through the Hedgehog signaling pathway (MOSIMANN *et al.* 2009). Similarly, Paf1C is also deployed by the transcription factor beta-catenin through a direct interaction with the Cdc73 subunit to activate transcription downstream of the Wnt signaling pathway (MOSIMANN *et al.* 2006). Thus, Paf1C regulates several developmentally important signaling pathways and hence is essential for normal development of multicellular organisms. Additionally, Paf1C has also been shown to repress transcription of genes by maintaining nucleosome occupancy over the promoter region (Figure 5) (PRUNESKI *et al.* 2011).

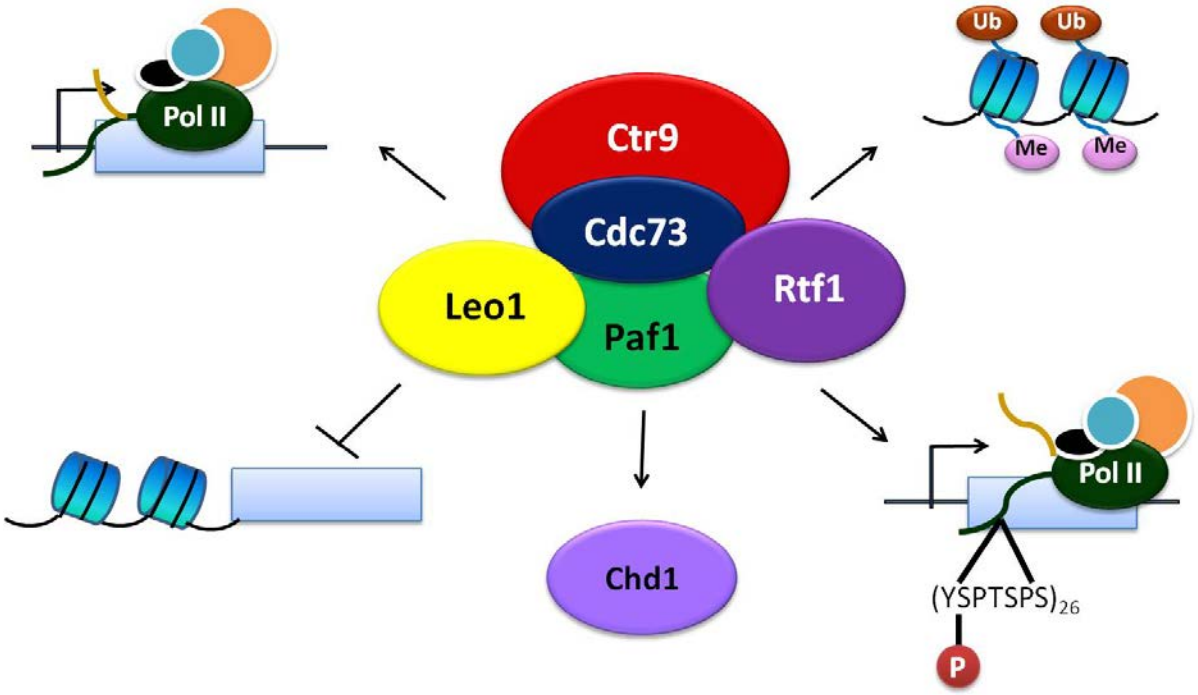


Figure 5. Functions of Paf1C

Paf1C performs several functions within the cell such as facilitating the passage of Pol II through chromatin through association with other elongation factors, maintaining histone occupancy over the promoter region to repress genes, mediating the recruitment of chromatin remodeling factor Chd1, promoting the phosphorylation of Ser2 of Pol II CTD and stimulating co-transcriptional histone modifications [reviewed in (CRISUCCI and ARNDT 2011)]. This figure has been adapted from published material (CRISUCCI and ARNDT 2011).

Misregulation of Paf1C can result in severe pathological conditions (Figure 6). Paf1C mediates immunological response through regulation of the interleukin 6 (IL-6) signaling pathway by stabilizing the interaction of the transcription factor Stat3 with the promoters of IL-6 responsive genes (YOUN *et al.* 2007). Influenza virus A H3N2 subtype suppresses the Paf1C-mediated antiviral immune response by deploying its viral non-structural protein 1, a histone mimic, to block the activity of Paf1C by binding to it (MARAZZI *et al.* 2012). Mutations within genes encoding components of Paf1C have been identified as drivers of cancer. Germline or somatic mutations that cause frameshift, missense or nonsense mutations within the human Cdc73 protein have been identified as the underlying cause of hyperthyroidism jaw tumor syndrome (CARPTEN *et al.* 2002; HOWELL *et al.* 2003; SHATTUCK *et al.* 2003). These mutations have been shown to affect the association of Cdc73 with the rest of Paf1C or with a histone methyltransferase complex (ROZENBLATT-ROSEN *et al.* 2005). These reports and functional studies in human cell lines which showed that overexpression of Cdc73 can inhibit cell proliferation by silencing the cyclin D1 gene suggest the role of Cdc73 as a tumor suppressor (WOODARD *et al.* 2005). However, amplifications of the locus harboring this gene have also been observed in pancreatic cancer, breast cancer, brain cancer and head cancer [reviewed in (CHAUDHARY *et al.* 2007)]. Furthermore, amplification of the locus encoding *PAF1* gene and the corresponding upregulation of the Paf1 subunit have been observed in cancers such as pancreatic cancer. Since overexpression of Paf1 has also been shown to induce the transformed phenotype in cells, the amplification of Paf1 may likely be a driver mutation in these cancers [reviewed in (CHAUDHARY *et al.* 2007)]. The ability of Paf1C to act as an oncogene may be attributed to its ability to positively regulate the transcription of oncogenes such as the targets of the Wnt or the Notch signaling pathway.

In-frame translocations within the gene encoding the human histone H3 K4 methyltransferase, MLL (Mixed Lineage Leukemia), with the region encoding one or more of over fifty different nuclear transcription activators such as ENL, AF4 or AF9 or cytoplasmic proteins like AF6 that dimerize and acquire the ability to activate transcription, results in oncogenic fusion proteins [reviewed in (TAN *et al.* 2010)]. These fusion proteins contain the N-terminal portion of MLL, which includes the AT hook motifs and the CXXC zinc-finger motif that facilitates the targeting of the fusion protein to chromatin and are the underlying cause of myeloid, lymphoid and mixed lineage leukemias [reviewed in (TAN *et al.* 2010)]. The oncogenic ability of cells bearing these genetic lesions is likely due to the persistent activation of homeobox transcription factor (HOX) genes, which are involved embryonic development and hematopoetic differentiation mainly *HOXA9* [reviewed in (HESS 2004)]. Normally, *HOXA9* is briefly expressed in the progenitor hematopoetic cells and downregulated during differentiation. Paf1C is important for the transcriptional activation by the oncogenic fusion protein since it facilitates the recruitment of the fusion protein to *HOXA9* gene (MILNE *et al.* 2010). Many of the proteins that fuse with MLL because of the genetic translocations have also been shown to be a part of the Super elongation complex (SEC) that positively regulates the expression of *HOX* genes. This suggests that the SEC could also be facilitating the overexpression of the *HOX* genes by the MLL fusion proteins [reviewed in (SMITH *et al.* 2011)].

Together, these studies suggest that misregulation of Paf1C can result in several diseases, including cancers.

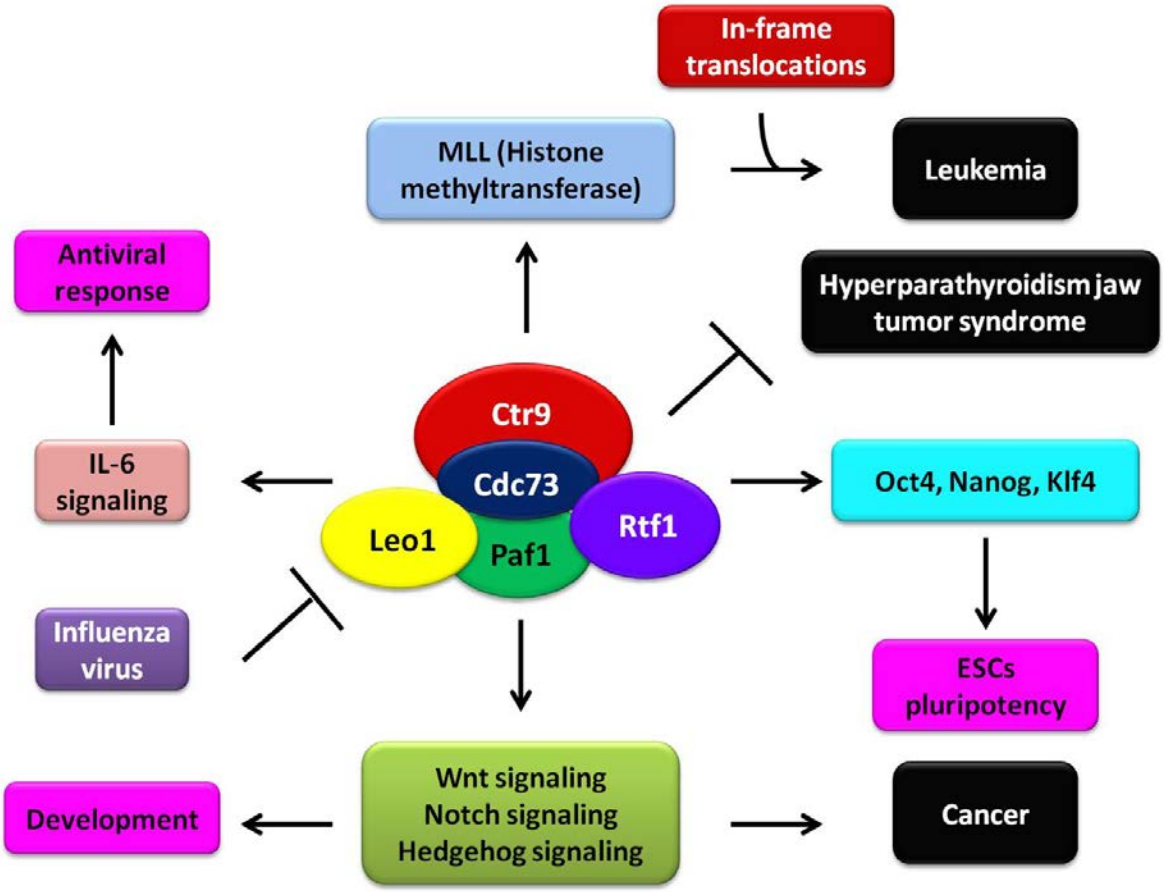

In-frame translocations
MLL (Histone methyltransferase)
Leukemia
Antiviral response
Hyperparathyroidism jaw tumor syndrome
Ctr9
Cdc73
Leo1
Paf1
Rtf1
IL-6 signaling
Oct4, Nanog, Klf4
Influenza virus
ESCs pluripotency
Wnt signaling
Notch signaling
Hedgehog signaling
Development
Cancer

Figure 6. Health significance of Paf1C

Paf1C facilitates the expression of downstream targets of Notch signaling, Wnt signaling, Hedgehog signaling and hence is essential for normal development [reviewed in (TOMSON and ARNDT 2013)]. Overexpression of the components of Paf1C can lead to cancer presumably due to its role in signaling pathways such as Notch and Wnt (AKANUMA *et al.* 2007; TENNEY *et al.* 2006). Paf1C mediates antiviral response by facilitating transcriptional activation downstream of the IL-6 signaling pathway (YOUN *et al.* 2007). Additionally, influenza virus uses its non-structural protein 1 to bind to Paf1C and suppress Paf1C-mediated antiviral response by the host (MARAZZI *et al.* 2012). Furthermore, it regulates embryonic stem cell (ESC) pluripotency by facilitating the expression of genes required for maintaining a pluripotent state, such as Oct4, Nanog and Klf4 (DING *et al.* 2009). Also, mutations within the Cdc73 subunit of Paf1C are the cause of hyperparathyroidism jaw tumor syndrome (CARPTEN *et al.* 2002). The chromosomal rearrangements within the MLL gene results in the production of oncogenic fusion proteins that require Paf1C for their oncogenic activity [reviewed in (TAN *et al.* 2010)]. This figure has been adapted from published material (TOMSON and ARNDT 2013).

1.4 THESIS AIMS

Paf1C is a highly conserved transcription elongation complex composed of five members in yeast including Paf1, Ctr9, Leo1, Rtf1 and Cdc73. Although termed an elongation factor, Paf1C facilitates initiation and termination of transcription as well. Paf1C is involved in several important functions such as regulating co-transcriptional histone modifications, RNA 3'-end formation and phosphorylation of the Pol II CTD. Paf1C regulates expression of many important genes including those involved in development, antiviral response and maintenance of stem cell pluripotency. Misregulation of Paf1C has been associated with developmental defects, cancers and other diseases.

However, prior to my work the mechanism of recruitment of this important complex was obscure. Structure-function analysis of Rtf1 had uncovered small internal deletions within the central region of Rtf1 that diminished the association of Paf1C with actively transcribed genes. My goal was to determine how this region, termed the ORF association region (OAR) based on its ability to facilitate the chromatin association of Paf1C to actively transcribed genes, mediated the recruitment of Paf1C. To gain insight into the mechanism of recruitment of Paf1C, I sought to find answers to many different questions such as what are the boundaries of the functional ORF association region of Rtf1 (OAR) and what are the functional and the structural consequences of the lack of this OAR? Which protein-protein interactions of Paf1C are mediated by the OAR? Are the protein interactions mediated by the OAR direct or indirect? Is the OAR just necessary or also sufficient to mediate these interactions? Are these protein interactions modulated by post-translational modifications? Is the OAR sufficient for chromatin association? Is the chromatin association by the OAR dependent on the other members of Paf1C? Is the chromatin association and dissociation of the OAR regulated in the same manner as that of entire

of Paf1C? What are the functionally important residues of the OAR? Does the Paf1C associate with chromatin and execute its functions through a dual attachment mediated by the OAR of Rtf1 and the C-domain of Cdc73, which has been previously implicated in the recruitment of Paf1C?

I performed affinity purification of an OAR deletion derivative of Rtf1 and sent it for mass spectrometry analysis to our collaborator, Dr. Richard Gardner, at Fred Hutchinson Cancer Research Centre, to identify the protein interactions that were lost in this deletion derivative. This subtractive proteomics approach revealed that the interaction of Paf1C with the universally conserved transcription elongation factor Spt5 was mediated by the OAR. I validated the mass spectrometry data through co-immunoprecipitation assays and gel-filtration chromatography analysis. I further determined if this interaction was direct by performing *in vitro* binding assays with bacterially expressed, purified recombinant derivatives of Rtf1 and Spt5, which also led to the identification of the minimal regions of Rtf1 and Spt5 mediating the interaction of Paf1C with Spt5. Pull-down assays revealed that the post-translational modification of Spt5 was important for this interaction. Next, by performing several chromatin immunoprecipitation (ChIP) assays to determine the chromatin occupancy of OAR in the context of a wild-type strain and many mutant strains, I identified that the OAR can associate and dissociate from chromatin in the same manner as that of the entire Paf1C without obligatory assistance from any other components of Paf1C. I also showed that Spt5 and the Bur1-Bur2 kinase complex regulate the chromatin association and dissociation of the entire Paf1C by targeting the OAR of Rtf1. Furthermore, I also identified the functionally important residues within the OAR mainly by performing growth assays and ChIP assays.

I also determined the reason for the low levels of Rtf1-mediated (H3 K4 Me3, H3 K4 Me3 and H3 K79 Me2/3) and high levels of Rtf1-independent histone modifications (H3 K36 Me3) promoted by Paf1C in the cells lacking the OAR, although the chromatin occupancy of the entire Paf1C was dramatically low in these cells. My results suggest that extremely low levels of Paf1C, undetectable by ChIP, may be associating with the actively transcribed genes in the absence of the OAR, through the C-domain of Cdc73. These low levels of Paf1C associated with the chromatin through the C-domain of Cdc73 in the absence of the OAR are likely responsible for the low levels of Rtf1-dependent histone modifications (H3 K4 Me3, H3 K4 Me2 and H3 K79 Me2/3) and high-level of Rtf1-independent histone modification (H3 K36 Me3) regulated by Paf1C, in cells lacking the OAR. Only cells lacking both the OAR of Rtf1 and the C-domain of Cdc73 show an almost complete loss of all the Paf1C-mediated histone modifications by immunoblotting analysis.

In addition to identifying the mechanism of chromatin association of Paf1C, my work on the histone modification domain (HMD) of Rtf1 has provided better insight into how this small region within Rtf1 (Rtf1 residues 63-152) mediates histone modifications. I determined that the HMD could execute its function of promoting histone modifications such as monoubiquitination of H2B K123, di-methylation of H3 K4 and di- and tri-methylation of H3 K79 without assistance from other components of Paf1C but it requires aid from other members of Paf1C for the H3 K4 Me3 mark. My work also revealed the dependency of the functional activity of the HMD on the protein levels of the HMD. Consistent with this, I also found that fusing the HMD to a Gal-4 DNA binding domain (GBD) caused a reduction in the levels of HMD and correspondingly reduced the levels of HMD-mediated histone modifications. Furthermore, I have also obtained a

preliminary result that suggests that the HMD may mediate the histone modification function through interaction with the ubiquitin ligase, Bre1.

Collectively, my work has provided a mechanistic insight into the chromatin association and dissociation of Paf1C and a better understanding of how Paf1C promotes co-transcriptional histone modifications.

2.0 THE RECRUITMENT OF PAF1C OCCURS THROUGH A DIRECT INTERACTION BETWEEN THE OAR OF RTF1 AND THE CTR OF SPT5

Most of the work presented in this Chapter is adapted from published material (MAYEKAR *et al.* 2013) and is reprinted with some changes.

2.1 INTRODUCTION

Packaging of the eukaryotic genome into chromatin hinders the movement of Pol II during transcription. Hence, eukaryotes have evolved numerous initiation and elongation factors that orchestrate the recruitment and movement of Pol II across active genes. Paf1C is one such conserved elongation factor. In *S. cerevisiae*, Paf1C consists of five subunits: Paf1, Ctr9, Cdc73, Leo1 and Rtf1 (MUELLER and JAEHNING 2002; SHI *et al.* 1997; SHI *et al.* 1996; SQUAZZO *et al.* 2002) [reviewed in (CRISUCCI and ARNDT 2011; TOMSON and ARNDT 2013)]. Paf1C regulates transcription and chromatin structure through several mechanisms. The most well studied functions of Paf1C are its roles in regulating co-transcriptional histone modifications. Through a mechanism that requires the histone modification domain of Rtf1, Paf1C is required for efficient monoubiquitination of histone H2B K123 by the ubiquitin-conjugating enzyme Rad6 and the

ubiquitin-protein ligase Bre1 (NG *et al.* 2003a; PIRO *et al.* 2012; WARNER *et al.* 2007; WOOD *et al.* 2003; XIAO *et al.* 2005). This mark is a pre-requisite for di- and tri-methylation of H3 K4 and K79 by the Set1 and Dot1 histone methyltransferases, respectively (DOVER *et al.* 2002; KROGAN *et al.* 2003a; NG *et al.* 2003a; NG *et al.* 2003b; SUN and ALLIS 2002). Paf1C also facilitates H3 K36 tri-methylation on the bodies of active genes (CHU *et al.* 2007). Additional functions of Paf1C include the Rtf1-mediated recruitment of the Chd1 chromatin remodeling protein (SIMIC *et al.* 2003), the stimulation of serine 2 phosphorylation on the C-terminal domain (CTD) of Pol II (MUELLER *et al.* 2004; NORDICK *et al.* 2008), the maintenance of nucleosome occupancy on highly expressed regions of the genome (PRUNESKI *et al.* 2011), and the recruitment of factors involved in transcription termination and RNA processing (MUELLER *et al.* 2004; NORDICK *et al.* 2008; TOMSON *et al.* 2012). Either individually or collectively, the multiple functions of Paf1C can influence the rate of transcription elongation (KIM *et al.* 2010; RONDON *et al.* 2004; TOUS *et al.* 2011). In addition to its roles in elongation, Paf1C has also been shown to affect the initiation and termination stages of the transcription cycle (DING *et al.* 2009; MOSIMANN *et al.* 2006; MOSIMANN *et al.* 2009; MUELLER *et al.* 2004; TOUS *et al.* 2011).

Members of Paf1C were first discovered in a search for proteins that associate with Pol II (WADE *et al.* 1996). Consistent with its physical association with Pol II, Paf1C is enriched on the bodies of actively transcribed genes at levels that correlate with gene expression (MAYER *et al.* 2010). Previous studies have implicated several proteins in the recruitment of yeast Paf1C to active chromatin, including the transcription elongation factors Spt16-Pob3/FACT, the Ccr4-Not complex, and Spt4-Spt5/DSIF (MULDER *et al.* 2007; PAVRI *et al.* 2006; QIU *et al.* 2012; QIU *et al.* 2006). Conflicting results have been obtained regarding the role of the Ccr4-Not complex in Paf1C recruitment. Deletion of the Not4 subunit of the Ccr4-Not complex causes a reduction in

the levels of H3 K4 Me3 modification, but has no impact on H3 K4 Me2, H3 K4Me, H3 K79 Me2, H3 K79Me3 and H2B Ub levels (LARIBEE *et al.* 2007; MULDER *et al.* 2007). While the Timmers groups attributed this reduction in H3 K4 Me3 levels to the reduced occupancy of Paf1C in the absence of Not4, the Strahl group observed no impact of the loss of Not4 on the recruitment of Paf1C. They identified the association between Ccr4-Not complex and the proteasome as the underlying cause of this reduction (LARIBEE *et al.* 2007; MULDER *et al.* 2007).

FACT was implicated in the recruitment of Paf1C based on the recruitment of FACT prior to Paf1C to an inducible promoter and a physical interaction between FACT and Paf1C (PAVRI *et al.* 2006). That the phosphorylation of the CTR of Spt5 facilitates the recruitment of Paf1C to chromatin was suggested, due to reduction in the chromatin occupancy of Paf1C in mutant cells lacking the Spt4 subunit of the Spt4-Spt5 complex or the CTR of Spt5 or mutants having non-phosphorylatable Spt5 CTR (LIU *et al.* 2009; QIU *et al.* 2006; ZHOU *et al.* 2009). However, the mechanistic understanding of how the phosphorylated CTR of Spt5 facilitates the recruitment of Paf1C *in vivo* is lacking. The Bur1-Bur2 protein kinase has also been shown to stimulate the recruitment of Paf1C to Pol II through the phosphorylation of the C-terminal repeats (CTRs) of Spt5 and through a pathway independent of this function (LIU *et al.* 2009; QIU *et al.* 2012; QIU *et al.* 2006; ZHOU *et al.* 2009). In addition, the Kin28 protein kinase was shown to promote the recruitment of Paf1C through phosphorylation of the CTD of Pol II and by facilitating the chromatin association of Bur1-Bur2 complex (QIU *et al.* 2012; QIU *et al.* 2009).

With respect to members of Paf1C, loss of the Rtf1, Cdc73 or Leo1 subunits reduces the occupancy of Paf1C on chromatin (DERMODY and BURATOWSKI 2010; MUELLER *et al.* 2004; WARNER *et al.* 2007). *In vitro*, recombinant Cdc73, Rtf1, and Ctr9 can bind to peptides corresponding to the Pol II CTD phosphorylated on serine 2 and 5 and to peptides corresponding to the phosphorylated Spt5 CTR (AMRICH *et al.* 2012; PHATNANI *et al.* 2004; QIU *et al.* 2012; SHI *et al.* 1997). For Cdc73, this peptide binding activity maps to a domain that adopts a Ras-like fold and is important for Paf1C recruitment *in vivo* (AMRICH *et al.* 2012; PHATNANI *et al.* 2004; QIU *et al.* 2012; SHI *et al.* 1997). The ability of Leo1 to facilitate chromatin association of Paf1C correlates with its ability to bind RNA (DERMODY and BURATOWSKI 2010). The mechanism of chromatin association of Paf1C through the Rtf1 subunit remains obscure.

Through genetic deletions, we previously identified a highly conserved region within Rtf1 that is important for the chromatin association of Paf1C and termed this region the ORF association region (OAR) of Rtf1 (WARNER *et al.* 2007). The Rtf1 OAR contains a Plus3 motif, highlighted by the presence of three conserved, positively charged amino acids (DE JONG *et al.* 2008). An NMR study of the human Rtf1 Plus3 domain demonstrated that this domain is structurally similar to Tudor domains, which can recognize the methylation marks on histones and participate in protein-protein interactions, and the PAZ domains found in Dicer and Argonaute proteins (DE JONG *et al.* 2008; LINGEL *et al.* 2004; LU and WANG 2013; PEK *et al.* 2012). In this study, we sought to identify the mechanism of recruitment of Paf1C to active chromatin through this highly conserved region of Rtf1. Using a subtractive proteomics approach, we discovered that the OAR of Rtf1 mediates the interaction of Paf1C with Spt5. Using purified recombinant proteins, I obtained evidence for a direct interaction between the Rtf1 OAR and Spt5, independent of the other members of Paf1C. I also showed that both the

deletion of the Spt5 CTR and mutation of the Bur1-Bur2 complex impaired the recruitment of full-length Rtf1 and the OAR alone, suggesting that the OAR is a crucial target for recruitment of Paf1C by the Spt5 CTR and Bur1-Bur2 kinase complex.

2.2 MATERIALS AND METHODS

2.2.1 Yeast strains and growth

S. cerevisiae strains used in this study (Table 2) are isogenic to FY2, a *GAL2*$^{+}$ derivative of S288C (WINSTON *et al.* 1995). Yeast transformations and matings were performed as previously described (AUSUBEL *et al.* 1988; ROSE *et al.* 1991). Rich (YPD), synthetic complete (SC), and minimal (SD) media were made as previously described (ROSE *et al.* 1991). 6-azauracil (6-AU) was added to SC-URA medium at a final concentration of 50 μg/mL. For serial dilution growth assays, yeast strains were grown overnight to saturation in the appropriate media and washed twice with sterile water. Using sterile water, cells were 10-fold serially diluted from a starting concentration of 1 x 10^{8} cells/mL. 2.5 μL of each dilution was then spotted onto the indicated media and incubated at 30°C for the specified number of days.

2.2.2 Plasmid construction

Some of the plasmids used in this study are described in Table 3. Mutations encoding amino acid substitutions in the OAR were constructed by site-directed mutagenesis using the QuikChange mutagenesis kit (Agilent) and pLS21-5 (STOLINSKI *et al.* 1997) as the template.

pMM25 encoding GST-OAR was created by subcloning the coding sequence for amino acids 235-373 of Rtf1, which was PCR-amplified from pLS21-5 with primers that introduce EcoRI and BamHI sites at the 5' and 3' ends of the PCR product and ligated to EcoRI/BamHI-digested pGEX-3X (SMITH and JOHNSON 1988). pAP21 expresses GST-Rtf1-His_6. pAP21 was generated by insertion of the coding sequence for the His_6 tag at the 3' end of the *RTF1* ORF in plasmid pJS4 to create a version of Rtf1 that has an N-terminal GST tag and a C-terminal His_6 tag (WARNER *et al.* 2007). pMM26, which expresses GST-Rtf1ΔOAR-His_6, was constructed by deleting the sequence coding for Rtf1 amino acids 230-390 from pAP21 using a QuikChange mutagenesis kit (Agilent). pGH25, pGH100, and pGH258 were gifts from Dr. Grant Hartzog and express an MBP-Spt5 CTR fusion protein (Spt5 residues 807-1063), MBP, and Flag-Spt5-His_6 plus Spt4, respectively. The plasmid expressing His_6-OAR (Rtf1 residues 235-373) was a gift from Dr. Andrew VanDemark.

2.2.3 Immunoblot analyses

Ten ml log phase cultures of yeast cells grown to an OD_{600} of approximately 0.8 were used to make trichloroacetic acid (TCA) extracts. The cell pellets were washed with 20% (w/v) TCA and then resuspended in 200 µl of 20% (w/v) TCA. They were lysed after addition of 250 µl acid-washed glass beads by vigorous vortexing (setting 10) twice for 1 min and then gentle vortexing (setting 3) for 15 minutes. The lysate was transferred to a 1.5 ml tube and spun in a microfuge at 8000 rpm for 10 minutes. The pellet was then gently washed (by inversion) with 0.5 M Tris, pH 7.4 and then resuspended in 100 µl of 0.5 M Tris, pH 7.4 and 150 µl of 3X SDS PAGE loading buffer. The resuspended pellet was boiled for 5 minutes, cooled to room temperature and then

spun in a microfuge at 8000 rpm for 10 minutes. The supernatant was boiled for 5 minutes and then loaded on SDS-polyacrylamide gels.

Proteins were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose or PVDF membranes and probed with primary antibodies against either Rtf1 (SQUAZZO *et al.* 2002), the HA epitope (1:2500 dilution, Roche), total histone H3 (1:30,000 dilution, (PIRO *et al.* 2012)), H3 K4 Me3 (1:2000 dilution, Active Motif 39159), H3 K4 Me2 (1:2000 dilution, Upstate 07-030), H3 K79 Me2/3 (1:2000 dilution, Abcam ab2621), Paf1 (1:1000 dilution, gift from Dr. Judith Jaehning), glucose-6-phosphate dehydrogenase (1:30,000 dilution, Sigma A9521), tobacco etch virus (TEV) protease-cleaved TAP tag (1:2500; Thermo Scientific CAB 1001) or an antibody that detects the uncleaved TAP tag (peroxidase-anti-peroxidase; 1:2000 dilution; Sigma P1291). After incubation with the primary antibodies, membranes were probed with sheep anti-mouse or donkey anti-rabbit secondary antibodies (1:5000 dilution, GE Healthcare) and visualized using enhanced chemiluminescence substrate (Perkin Elmer).

2.2.4 Co-immunoprecipitation (Co-IP) assays

Yeast transformants, grown in selective medium to a density of 1-2 x 10^7 cells/mL, were lysed by bead-beating in a lysis buffer containing 20 mM HEPES, pH 7.4, 100 mM sodium acetate, 2 mM magnesium acetate, 10 mM EDTA, 0.1% Tween-20, 10% glycerol, 1 mM DTT and protease inhibitors (Halt protease inhibitor cocktail, Thermo Scientific). 500-1000 µg of the clarified extract was incubated with anti-Spt5 antisera (1:1000 dilution, gift from Dr. Grant Hartzog) for 2 hrs at 4^{o}C, followed by incubation with Protein A-conjugated agarose (GE Healthcare) for 1 hr at 4^{o}C. The agarose beads were then washed with lysis buffer containing 400 mM sodium acetate and the immunoprecipitated proteins were subjected to immunoblot

analysis using anti-Spt5 and anti-HA (1:2500, Roche) antibodies to detect the immunoprecipitated Spt5 and HA-Rtf1 proteins, respectively.

2.2.5 Chromatin immunoprecipitation (ChIP) assays

The indicated strains were grown to a density of 1-2 x 10^7 cells/mL. Cells were cross-linked with formaldehyde, quenched with glycine, harvested, and lysed (PIRO *et al.* 2012). Soluble chromatin was prepared by sonication (PIRO *et al.* 2012) and then incubated with agarose-conjugated α-Myc antibody (Santa Cruz Biotechnology; 9E10, sc-40AC), agarose-conjugated α-HA antibody (Santa Cruz Biotechnology; sc-7392AC), α-Spt5 antibody (gift from Dr. Grant Hartzog), α-Spt16 antibody (gift from Dr. Tim Formosa) or α-Rpb3 antibody (Neoclone) overnight at 4ºC. This was followed by 2 hours incubation at 4°C with Protein A-conjugated agarose (GE Healthcare) for α-Spt5 and α-Spt16 antibodies or Protein G-conjugated agarose (GE Healthcare) for α-Rpb3 antibody. Input and immunoprecipitated DNA were purified and analyzed by quantitative real-time PCR using Maxima SYBR Green/ROX qPCR master mix (Fermentas) and primers (relative to ATG = +1) to the 5' coding region of *PYK1* (+253 to +346), 3' coding region of *PYK1* (+1127 to +1270), region beyond the poly(A) site of *PYK1* (+1803 to +1938), 5' coding region of *PMA1* (+214 to +319), 3' coding region of *PMA1* (+2107 to +2194), region beyond the poly(A) site of *PMA1* (+3373 to +3475) or a telomeric region of chromosome VI (coordinates: 269495 to 269598). Graphs represent the average values for three biological replicates and the error bars indicate the standard error of the mean (SEM) for those values.

2.2.6 Purification of TAP-tagged proteins

Transformants of *rtf1*Δ strains (KY1258 or KY619) expressing plasmid-encoded Rtf1 derivatives were grown to log phase (3–4 x 10^7 cells/mL). Whole cell extracts were made by bead beating in a lysis buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl and 0.1% NP-40. Ten mgs of the clarified extracts were then subjected to one step-affinity purification by incubation with rabbit IgG (Sigma I5006) conjugated magnetic beads (Invitrogen, M-270 Epoxy) for 1 hr and 15 minutes at 4°C. They were then washed three times with the lysis buffer. The bound proteins were eluted by cleavage with TEV protease (Invitrogen, 12575-015) for 3 hours at 15°C in a buffer containing 100 mM Tris, pH 8.0, 150 mM NaCl, 0.1% NP-40, and 0.5 mM EDTA, concentrated by TCA precipitation and then run on SDS-polyacrylamide gels. For the identification of proteins that co-purified with the Rtf1 derivatives, samples were run approximately 1 cm into the gel, excised, and analyzed by tandem mass spectrometry in an Orbitrap mass spectrometer (Fred Hutchinson Cancer Research Centre Proteomics Facility). The identified peptides were validated as described previously (AMRICH *et al.* 2012). The average number of peptides identified from a minimum of three independent purifications is shown.

2.2.7 Expression and purification of recombinant proteins

Expression of FLAG-Spt5-His_6 together with Spt4 in *E. coli* codonplus-RIL cells transformed with plasmid pGH258 was induced by growth in LB medium containing 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 0.3 mM zinc chloride overnight at 20°C. Cells were harvested and lysed using a homogenizer in a buffer containing 25 mM Tris, pH 8.0, 500 mM NaCl, 10% glycerol and 5 mM imidazole. Spt5 was purified from the clarified lysate by nickel affinity

chromatography (Qiagen). It was then subjected to a second round of purification by heparin affinity chromatography in a buffer containing 25 mM Tris, pH 6.5, 100 mM NaCl and 8% glycerol. OAR-His_6 expression was induced in *E.coli* codonplus-RIPL cells in ZY autoinduction medium (STUDIER 2005) for ~24 hrs at 37°C and purified following the same procedure as that used for the purification of Spt5. Expression of GST in *E.coli* codonplus-RIL cells and GST-Rtf1-His_6 and GST-Rtf1ΔOAR-His_6 in *E.coli* codonplus-RIPL cells was induced in LB medium containing 0.1 mM IPTG at 37°C for 3 hrs. As described for the purification of Spt5, GST-Rtf1-His_6 and GST- Rtf1ΔOAR-His_6 were purified by nickel affinity chromatography in a buffer containing 25 mM Tris, pH 8.0, 250 mM NaCl, 10% glycerol and 5 mM imidazole and then subjected to a second round of purification with glutathione sepharose resin (GE Healthcare) in a buffer containing 25 mM Tris, pH 7.4, 150 mM NaCl and 10% glycerol. Expression of GST and GST-OAR was induced using the procedure described for GST-Rtf1-His_6 and GST-Rtf1ΔOAR-His_6, and the proteins were purified from the clarified extract by affinity purification with glutathione sepharose resin (GE Healthcare) in a buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 10% glycerol and 5 mM EDTA. The GST-bound proteins were used in the binding assay. MBP-tagged proteins were expressed in *E.coli* codonplus-RIPL cells by growth in LB medium containing 0.1 mM IPTG and 0.3 mM zinc chloride for 4 hrs at 37°C. The pellets were lysed in a homogenizer in a buffer containing 25 mM Tris-Cl, pH 7.4, 250 mM NaCl and 10% glycerol, and the proteins were isolated by affinity purification of the clarified lysate with amylose resin (New England Biolabs).

2.2.8 *In vitro* protein interaction assays with purified proteins

Four micrograms of purified recombinant FLAG-Spt5-His_6/Spt4 was added to glutathione sepharose-bound, GST-tagged proteins in a binding buffer containing 25 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 5mM EDTA, 2% NP-40 and 1 mM DTT and the mixtures were incubated for 1 hr at 4^{o}C with gentle agitation. The glutathione sepharose beads were washed six times in binding buffer containing 500 mM NaCl and the bound proteins were eluted from the beads by boiling for 5 min in 4X SDS sample buffer. Samples were then loaded onto 12% SDS-polyacrylamide gels. Spt5 and the GST-tagged proteins were detected by immunoblot analysis with antibodies against Spt5 and GST (1:1000, Molecular Probes A5800). For the binding assay with MBP-tagged proteins, 4 µg of purified recombinant OAR-His_6 was incubated with 4 µg of MBP or MBP-CTR and magnetic nickel beads (Qiagen) for 1 hr at 4^{o}C in a binding buffer containing 25 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol and 20 mM imidazole. After incubation, the beads were washed three times with buffer containing 25 mM Tris, pH 7.4, 250 mM NaCl, 10% glycerol and 25 mM imidazole. Bound proteins were eluted from the beads with a buffer containing 500 mM imidazole and run on a 15% SDS-polyacrylamide gel. MBP-tagged proteins and OAR-His_6 were detected by immunoblotting with anti-MBP (1:10,000 dilution; New England Biolabs E8030S) and anti-His_6 (1:500 dilution; GE Healthcare, 24-4710-01) antibodies.

2.2.9 Gel filtration chromatography

Transformants of an *rtf1*Δ strain (KY2529) expressing plasmid-encoded full-length Rtf1 or Rtf1 lacking the OAR were grown to log phase (3–4 x 10^7 cells/mL). Whole cell extracts were made

using the homogenizer in the gel filtration buffer (30 mM HEPES-KOH pH 7.4, 200 mM potassium acetate, 1 mM magnesium acetate, 1 mM EGTA, 0.05% Tween-20, 10% glycerol). The extract was then subjected to sonication with a Misonix 3000 sonicator (Farmingdale, NY) three times for 20 seconds at power 2 and twice for 20 seconds at power 4 with icing for at least 1 minute in between each sonication. The extracts were clarified by ultracentrifugation at 1,00,000 g, quantified using Bradford assay and ~16 mg of the clarified extract was run through a Sephacryl S-500 column previously equilibrated with the gel filtration buffer. Three ml fractions were collected, concentrated by TCA precipitation and then run on 8% and 10% SDS-polyacrylamide gels. Rtf1 and Paf1 proteins were detected by immunoblotting with anti-Rtf1 (SQUAZZO *et al.* 2002) and anti-Paf1 antiserum (gift from Dr. Judith Jaehning) and Ctr9-Myc_6, Cdc73-TAP and Leo1-HA_3 were detected using anti-Myc (1:1000; Santa Cruz Biotechnology; 9E10), antibody against the uncleaved TAP tag (peroxidase anti-peroxidase), and anti-HA (1:2500, Roche) antibodies.

2.2.10 GST pull-down assays using yeast extracts

GST pull-down assays were performed to test the interaction between recombinant GST-OAR protein and Spt5 derivatives, provided in the form of yeast extracts. Log phase cultures (2 liters at OD_{600}= 0.6-1.0) of *E.coli* codonplus-RIL cells containing plasmid encoding GST (pGEX-3X) and *E.coli* codonplus-RIPL cells containing plasmid encoding GST-OAR (pMM25) were induced with 0.1 mM IPTG for 2 hr 45 min, lysed in phosphate-buffered saline (PBS) buffer containing 1 mM EDTA and protease inhibitors using a homogenizer. Clarified lysates were incubated with 1 ml of BSA-blocked, 50% glutathione sepharose resin (GE Healthcare) for 1 hour at 4°C to purify GST and GST-OAR. The purified glutathione-bound proteins were

incubated with 2 mg of clarified yeast extracts, prepared by homogenization, of strains KA181, KA183, and KA185 for 1.5 hr in binding buffer (150 mM NaCl, 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 5 mM EDTA, 10% glycerol and protease inhibitors). The glutathione beads containing the GST-tagged proteins and their interactors bound to them were then washed with binding buffer to remove the non-specifically bound proteins. SDS loading buffer was then added to the washed beads. Samples were boiled for 5 min at 100°C and resolved on 8% and 15% SDS-polyacrylamide gels to analyze the presence of Spt5 and GST-tagged proteins by immunoblotting, respectively.

Table 2. *Saccharomyces cerevisiae* strains used for the study in this Chapter

Strain	Genotype
KY619	*MAT*a *rtf1Δ102::ARG4 arg4-12 his4-912∂ leu2Δ1 lys2-173R2 trp1Δ63*
KY995	*MATα rtf1Δ::URA3 CTR9-6XMYC::LEU2 his3Δ200 leu2 Δ(0 or 1) ura3(Δ0 or 52) trp1 Δ63*
KY1220	*MATα HA$_3$-PAF1 his3Δ200 leu2Δ1 ura3Δ0*
KY1258	*MAT*a *rtf1Δ::URA3 RAD6-13xMYC::KanMX leu2Δ1 ura3-52 trp1Δ63*
KY1758	*MATα rtf1Δ101::LEU2 his4-912∂ lys2-128∂ leu2Δ1 trp1Δ63 arg4-12*
KY1813	*MATα paf1Δ::KanMX rtf1Δ::LEU2 his4-912∂ leu2Δ1 trp1Δ63 ura3-52*
KY2124	*MAT*a *rtf1Δ::KanMX4 hta1-htb1Δ::LEU2 hta2-htb2Δ::KanMX his3Δ200 lys2-128∂ leu2Δ1 ura3-52 trp1Δ63 pJH23 WT=[HTA1-HTB1/HIS3/CEN/ARS/Amp^R]*
KY2125	*MAT*a *rtf1Δ::KanMX4 hta1-htb1Δ::LEU2 hta2-htb2Δ::KanMX his3Δ200 lys2-128∂ leu2Δ1 ura3-52 trp1Δ63 pJH23 FL=[HTA1-FLAG-HTB1/HIS3/CEN/ARS/Amp^R]*
KY2195	*MATα rtf1Δ101::LEU2 cdc73Δ::KanMX4 his4-912∂ leu2Δ1 trp1Δ63*
KY2410	*MAT*a *rtf1Δ101::LEU2 HA$_3$-PAF1 his3Δ200 leu2Δ1 ura3(Δ0 or 52)*
KY2413	*MAT*a *his3Δ200 leu2Δ1 ura3(Δ0 or 52) trp1Δ63*
KY2414	*MAT*a *rtf1Δ101::LEU2 HA$_3$-PAF1 leu2Δ1 ura3(Δ0 or 52) trp1Δ63*
KY2529	*MAT*a *rtf1Δ::KanMX LEO1-3XHA::HIS3 CTR9-6XMYC::LEU2 CDC73-TAP::TRP1 SPT5-FLAG his3Δ200 leu2Δ (0/1) ura3 Δ0 trp1Δ63*
KA150*	*MATα rtf1Δ101::LEU2 spt5Δ5::NAT^R his3Δ200 lys2-128∂ leu2Δ1 ura3Δ0 trp1Δ63 arg4-12*
KA181	*MATα spt5 Δ::HIS3 rtf1Δ::KanMX his3 Δ(1/200) leu2 Δ(0/1) trp1Δ63 pHQ1494 [LEU2 SPT5-3XHA]*
KA183	*MATα spt5 Δ::HIS3 rtf1Δ::KanMX his3 Δ(1/200) leu2 Δ(0/1) ura3 Δ0 trp1Δ63 met15Δ0 pHQ1494 [LEU2 spt5-S1-15A-3XHA]*
KA185	*MAT*a *spt5 Δ::HIS3 rtf1Δ::KanMX his3 Δ(1/200) leu2 Δ(0/1) pHQ1494 [LEU2 spt5-S1-15D-3XHA]*
AY777[¥]	*MATα bur2-1 rtf1Δ:: KanMX4 his4-912∂ lys2-128∂ suc2Δuas(-1900/-390) ura3-52 trp1Δ63*

*Provided by Dr. Grant Hartzog

¥ Provided by Dr. Gregory Prelich

Table 3. Plasmids used for the study in this Chapter

Plasmid	Construction	Origin	Promoter	Protein
pMM01	pLS20 derivative (SQUAZZO *et al.* 2002), created using site-directed mutagenesis	*CEN/ARS*	*RTF1* promoter	HA_3-Rtf1ΔOAR (lacks residues 230-390 of Rtf1)
pMM03	pPC59 derivative (SQUAZZO *et al.* 2002), created using site-directed mutagenesis	*CEN/ARS*	*RTF1* promoter	Rtf1ΔOAR-TAP (lacks residues 230-390 of Rtf1)
pMM35	pAP37 derivative (PIRO *et al.* 2012); PCR-amplified fragment encoding the OAR and containing NdeI and PstI restriction sites was subcloned into NdeI and PstI digested pAP37	2 micron	*ADH1* promoter	NLS-Myc-OAR (residues 230-390 of Rtf1)
pMM36	pMM35 derivative, the Myc tag was removed and an EcoRI site was introduced by site-drected mutagenesis to create a vector for introducing a PCR-amplified HA_3 tag sequence from pLS21-5 having EcoRI and NcoI overhangs	2 micron	*ADH1* promoter	NLS-HA_3-OAR (residues 230-390 of Rtf1)
pMM41	pLS20 derivative; a PCR-amplified NLS-HA_3-OAR encoding fragment with NdeI and AflII overhangs was subcloned into pLS21-5 digested with NdeI and AflII to replace the *RTF1* gene in pLS21-5	*CEN/ARS*	*RTF1* promoter	NLS-HA_3-OAR (residues 230-390 of Rtf1)

pMM43	pMM40 derivative (PIRO *et al.* 2012); NdeI fragment of pLS21-5 encoding the HA_3 tag was subcloned into NdeI-digested pMM40	*CEN/ARS*	*ADH1* promoter	HA_3-Rtf1
pMM44	pMM41 derivative; the *RTF1* promoter was replaced with the *ADH1* promoter, which was PCR-amplified from pGBKT7 (Clontech) using primers that introduce SalI and NdeI sites at the 5' and 3' ends of the PCR product	*CEN/ARS*	*ADH1* promoter	NLS-HA_3-OAR (residues 230-390 of Rtf1)
pMM47	pMM01 derivative; NdeI restriction fragment encoding the HA_3 tag was deleted	*CEN/ARS*	*RTF1* promoter	Rtf1ΔOAR (lacks residues 230-390 of Rtf1)
pMM61	pRS316 derivative; XhoI-SacI fragment from pLS20 including the *RTF1* promoter and the *RTF1* (untagged) coding region was ligated to XhoI-SacI digested pRS316	*CEN/ARS*	*RTF1* promoter	Rtf1
pMM62	pRS316 derivative; XhoI-SacI fragment from pMM47 including the *RTF1* promoter and the *rtf1ΔOAR* coding region was ligated to XhoI-SacI digested pRS316	*CEN/ARS*	*RTF1* promoter	Rtf1ΔOAR (lacks residues 230-390 of Rtf1)

2.3 RESULTS

2.3.1 The OAR of Rtf1 is important for the chromatin association and transcriptional functions of Paf1C

Full association of Paf1C with active ORFs requires the Cdc73, Leo1 and Rtf1 subunits of the complex. In this study, we sought to explore the mechanism by which Rtf1 facilitates the recruitment of Paf1C to chromatin. In a previous functional analysis of internal deletion derivatives of Rtf1, we identified a region within Rtf1, termed the ORF association region (OAR), which is critical for the chromatin association of Paf1C (WARNER *et al.* 2007). Together, the consecutive deletions that defined the OAR in this earlier study removed residues 201-395 of Rtf1. To better map the boundaries of the OAR, we examined the predicted secondary structure of this region as well as the degree of amino acid conservation (Figure 7A). Based on this analysis, I constructed a single complete OAR deletion mutation that removes residues 230-390 of Rtf1. Confirming that removal of these residues did not affect the stability of the protein, levels of the new deletion derivative, Rtf1ΔOAR, and full-length Rtf1 were similar, as determined by immunoblotting for the triple HA tag present on these proteins (Figure 7B).

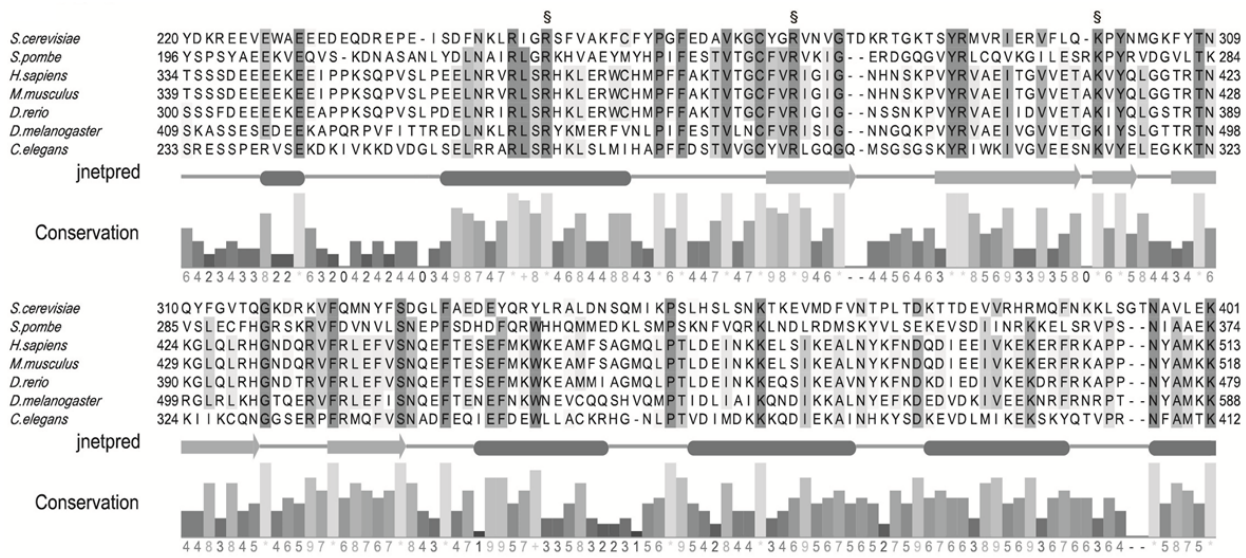
Using this new deletion derivative, I assessed the impact of the OAR on chromatin association of Rtf1 at the 5' and 3' regions of two actively transcribed genes, *PYK1* and *PMA1*, by performing chromatin immunoprecipitation (ChIP) assays. I observed that deletion of the OAR severely reduced the ability of Rtf1 to associate with these ORFs (Figure 7C and 7D), indicating that residues 230-390 comprise the functional OAR of Rtf1. To test if the absence of the OAR, like a complete deletion of *RTF1* (MUELLER *et al.* 2004), diminished the association of

Paf1C with chromatin, I performed ChIP analysis of the Paf1 subunit, using strains that express HA_3-tagged Paf1 and untagged full-length Rtf1, untagged Rtf1ΔOAR, or no form of Rtf1 (Figure 7E). The absence of the OAR reduced the association of Paf1 with active ORFs to levels similar to those caused by complete deletion of *RTF1*. Therefore, consistent with our previous results (WARNER *et al.* 2007), the OAR of Rtf1 is important for tethering not only the Rtf1 subunit but also other Paf1C subunits to actively transcribed chromatin.

Given the importance of the OAR for chromatin association of Paf1C, I next tested if a full deletion of the OAR caused mutant phenotypes indicative of transcriptional defects such as the suppressor of Ty phenotype (Spt^- phenotype) and 6-AU sensitivity (COSTA and ARNDT 2000). To assess the Spt^- phenotype, I introduced plasmids expressing full-length Rtf1, Rtf1ΔOAR or no form of Rtf1 into an *rtf1*Δ *his4-912δ* strain. Suppression of the transcriptional effects of the Ty δ-element insertion mutation within the *HIS4* promoter of the *his4-912δ* allele is indicated by growth on medium lacking histidine (WINSTON 1992). Whereas the pattern of transcription initiation at *his4-912δ* in *RTF1* cells leads to an extended, nonfunctional *HIS4* transcript and a His^- phenotype, deletion of *RTF1* restores transcription initiation at the normal start site of the *HIS4* gene and growth on medium lacking histidine (STOLINSKI *et al.* 1997) (Figure 7F). Similar to the effect of deleting *RTF1* entirely, absence of the OAR caused a strong Spt^- phenotype (Figure 7F). Sensitivity to the uracil analogue 6-AU, which depletes certain nucleotide pools, is frequently used as an indicator of a defect in transcription elongation (EXINGER and LACROUTE 1992). At the 6-AU concentrations used in my assay, wild-type cells grew normally, but cells lacking Rtf1 or just the OAR of Rtf1 grew poorly (Figure 7F). Thus, removal of the OAR of Rtf1 caused strong transcription-related phenotypes that are similar to those caused by a complete loss of the protein (COSTA and ARNDT 2000).

An important function of Rtf1 is in facilitating the modification of histones during transcription elongation. To test the importance of the OAR in promoting H2B K123 mono-ubiquitination and downstream histone methylation events, I performed immunoblotting analyses on extracts prepared from *RTF1*, *rtf1*Δ*OAR*, and *rtf1*Δ strains. Absence of the OAR caused a dramatic reduction in the global levels of H3 K4 Me3 and H3 K79 Me2/3 and also led to diminished levels of H3 K4 Me2 (Figure 7B). (Note that the antibody used for the analysis of H3 K79 methylation detects both the di-methyl and tri-methyl modification states). Consistent with the reduction in H3 K4 and K79 methylation, absence of the OAR also led to greatly reduced levels of H2B K123 ubiquitination as revealed by immunoblotting analysis of *RTF1* and *rtf1*Δ*OAR* strains that express FLAG-tagged H2B as the only source of H2B (Figure 7G). These results indicate that the OAR-mediated chromatin association of Paf1C is important for full levels of transcription-coupled histone modifications in agreement with our previous observations (WARNER *et al.* 2007).

A.


S.cerevisiae
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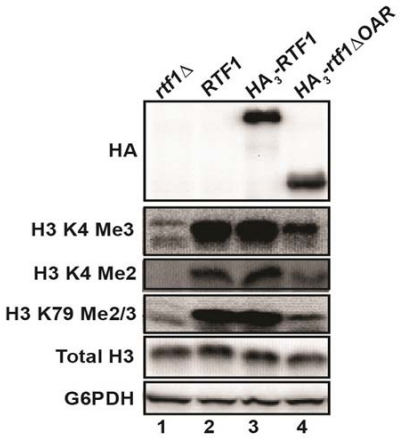
H.sapiens
M.musculus
D.rerio
D.melanogaster
C.elegans
jnetpred
Conservation

B.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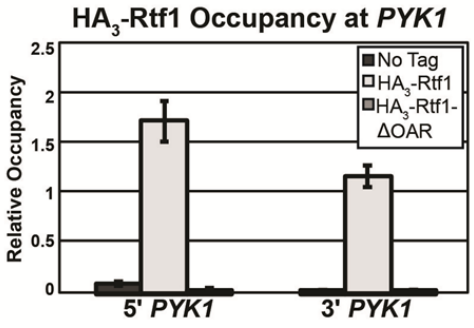
HA3-RTF1
HA3-rtf1ΔOAR
HA
H3 K4 Me3
H3 K4 Me2
H3 K79 Me2/3
Total H3
G6PDH
1
2
3
4

C.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1
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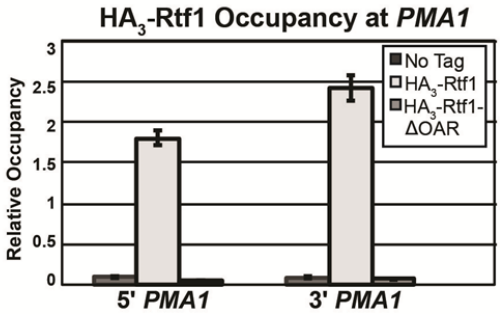
HA3-Rtf1-ΔOAR
5' PYK1
3' PYK1

D.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Relative Occupancy
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1.5
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0
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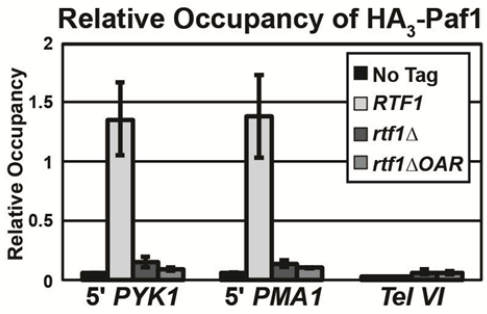
HA3-Rtf1-ΔOAR
5' PMA1
3' PMA1

E.


Relative Occupancy of HA3-Paf1
Relative Occupancy
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1
0.5
0
No Tag
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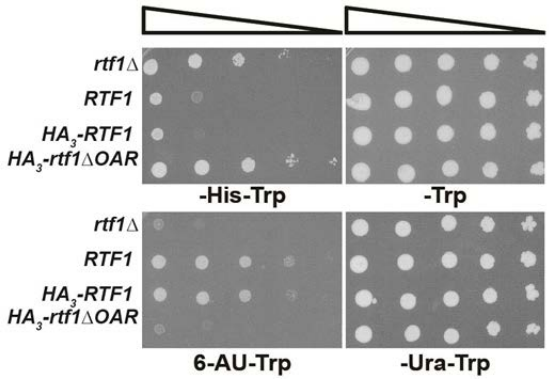
rtf1ΔOAR
5' PYK1
5' PMA1
Tel VI

F.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HA3-rtf1ΔOAR
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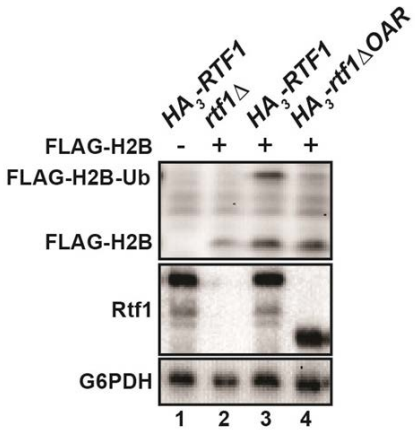
G.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rtf1Δ
HA3-RTF1
HA3-rtf1ΔOAR
FLAG-H2B - + + +
FLAG-H2B-Ub
FLAG-H2B
Rtf1
G6PDH
1 2 3 4

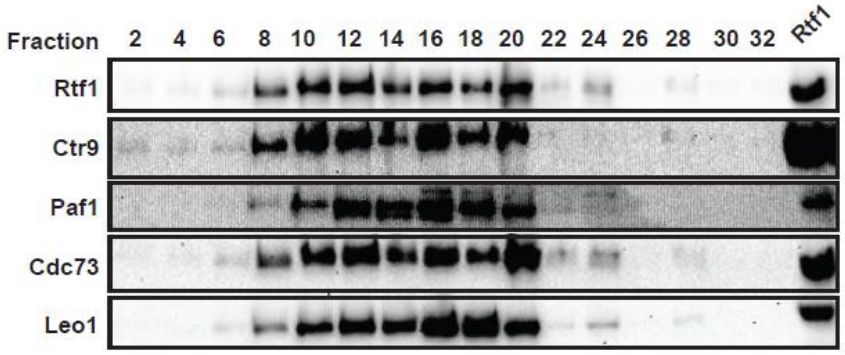
Figure 7. OAR is a highly conserved and a functionally important region of Rtf1

(A) Multiple sequence alignment of Rtf1 homologues developed using Clustal X 2.0 (Larkin et al. 2007) and secondary structure prediction developed using jNET (Cole et al. 2008). The coloration indicates the percentage of identity among the residues of Rtf1 homologues. Darker shades of grey represent a higher percentage of identity. Tubes and arrows denote α helices and β sheets, respectively. "§" indicates the Plus3 residues (S. cerevisiae R251, R273 and K299). (B) Immunoblotting analyses were performed using indicated antibodies on transformants of an rtf1Δ (KY619) strain containing plasmids expressing HA3-Rtf1 (pLS21-5), HA3-Rtf1ΔOAR (pMM01) and Rtf1 (pLS20). An rtf1Δ strain (KY619) transformed with empty vector (pRS314) was used as the negative control. G6PDH and total H3 levels serve as loading controls. (C and D) ChIP analysis was performed on strains used in (B) with anti-HA beads to determine the localization of HA_3-tagged Rtf1 proteins at *PYK1* (C) and *PMA1* (D). (E) ChIP analysis was performed on the strains KY2413, KY1220, KY2410 strains and the KY2414 strain transformed with pMM47 using anti-HA beads to determine the occupancy of HA_3-Paf1 over the indicated regions. For C-E, average values of three biological replicates are shown with the error bars representing the SEM. (F) Tenfold serial dilutions, ranging from 10^8 cells/ml to 10^4 cells/ml, of an *rtf1*Δ strain (KY619) transformed with an empty vector (pRS314) and the strains used for ChIP analysis in (B) were spotted on SD-His-Trp, SC-Ura-Trp media containing 50 μg/mL 6-AU, and appropriate control media and incubated for 4 days at 30°C. (G) Immunoblotting analysis was performed using the indicated antibodies on transformants of an *rtf1Δ* strain expressing FLAG-tagged H2B (KY2125) and either Rtf1 (pLS21-5) or Rtf1ΔOAR (pMM01) from plasmids. An *rtf1Δ* strain expressing untagged H2B (KY2124) and Rtf1 from plasmid pLS21-5 served as a negative control for the FLAG antibody. An *rtf1Δ* strain expressing FLAG-tagged H2B (KY2125) and containing the empty vector pRS314 served as a negative control for H2B K123 ubiquitination. G6PDH served as the loading control.

2.3.2 The OAR of Rtf1 does not affect the integrity of the Paf1 complex

We considered the possibility that the reduced functionality of the Rtf1 derivative lacking the OAR may be to the lack of integrity of Paf1C in the absence of the OAR. To test this possibility, I performed gel filtration chromatography analysis on yeast extracts prepared from wild-type strains and strains lacking the OAR of Rtf1 and analyzed several fractions by immunoblotting to determine the presence of all the members of Paf1C. Immunoblotting analysis of these strains expressing tagged versions of Ctr9, Cdc73 and Leo1 subunits showed that all the members of the Paf1C co-elute in similar fractions in both strains (mainly fractions 8-20) (Figure 8A and B) and hence suggests that the transcriptional defects exhibited by the OAR deletion may not be due to a general loss of Paf1C integrity in this mutant.

A.



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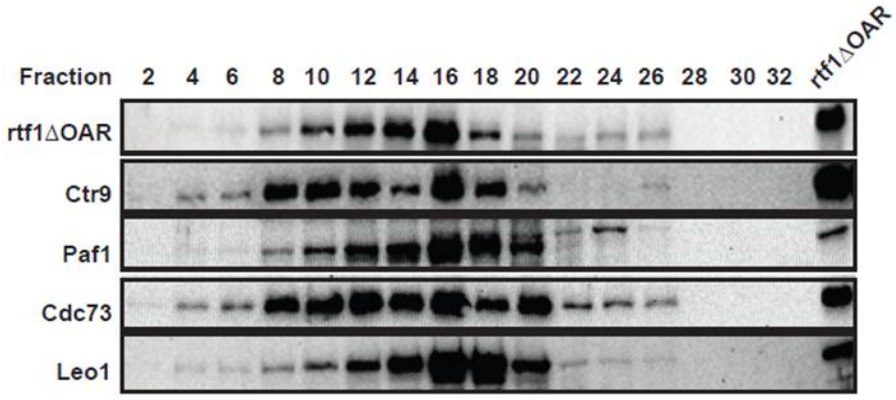


Figure 8. Members of the Paf1 complex co-elute in similar fractions in strains expressing Rtf1 and Rtf1ΔOAR

(A and B) Extracts of an *rtf1*Δ strain (KY2529) containing plasmid expressing Rtf1 (pMM61) (A) or Rtf1ΔOAR (pMM62) (B) were fractionated on a an S500 column and the indicated fractions of the eluate were subjected to immunoblotting using antibodies against Rtf1, Myc-tag, Paf1, TAP-tag and HA-tag to detect Rtf1, Ctr9, Paf1, Cdc73 and Leo1 proteins respectively.

2.3.3 The OAR is required for the interaction of Rtf1 with Spt5

We hypothesized that interactions of Paf1C mediated by the OAR could be involved in tethering Paf1C to chromatin. To uncover these interactions, I performed one-step affinity purification of TAP-tagged full-length Rtf1 and a TAP-tagged Rtf1ΔOAR derivative, lacking amino acids 230-390, from yeast extracts (see section 2.2.6). This one-step affinity purification approach allowed me to isolate the Rtf1 proteins under native conditions, thereby preserving potentially weak or transient interactions. In parallel, untagged Rtf1 was subjected to the same purification scheme to identify non-specific associations. Of the proteins identified by mass spectrometry analysis of our purified samples, the essential transcription elongation factor Spt5 was unique in its ability to interact with full-length Rtf1 but not with Rtf1 lacking the OAR (Figure 9A). Immunoblotting analysis indicated that the loss of this interaction was not due to reduced expression or general instability of the Rtf1ΔOAR-TAP protein relative to the full-length Rtf1-TAP protein or to reduced Spt5 levels in cells lacking the OAR (Figure 9B; Figure 9E, lanes 1 and 2). Furthermore, the mass spectrometry results showed that the Rtf1ΔOAR protein retained its interactions with the four other Paf1C subunits, Paf1, Ctr9, Cdc73 and Leo1 (Figure 9A) consistent with my assessment of Paf1C composition by gel filtration chromatography (see section 2.2.9) that revealed similar co-elution profiles for all five Paf1C subunits in fractions obtained from *RTF1* and *rtf1ΔOAR* extracts. Together, these observations suggest that the OAR does not play a major role in governing overall Paf1C integrity.

To validate the mass spectrometry results, I performed immunoblotting analysis of the affinity-purified Rtf1 derivatives. This analysis showed that both full-length Rtf1 and Rtf1 lacking the OAR interacted with Paf1 (Figure 9C) in accordance with the mass spectrometry and gel filtration data and our identification of a Paf1-interacting region at the C-terminus of Rtf1,

which is retained in the Rtf1ΔOAR protein (WARNER *et al.* 2007). Interestingly, our mass spectrometry data showed that the Rtf1ΔOAR-TAP protein, like full-length Rtf1-TAP, interacted with Spt16, a result confirmed by immunoblotting with antibodies against Spt16 (Figure 9C). Since deletion of the OAR greatly impaired the interaction between Rtf1 and chromatin, we asked if chromatin occupancy of Spt16 was reduced in the *rtf1ΔOAR* strain. Surprisingly, my ChIP analysis revealed that the Spt16 levels over actively transcribed regions were not greatly altered in cells lacking the OAR (Figure 9D). These results suggest that Paf1C interacts with some factors involved in transcription even when it is not tightly associated with chromatin. They also indicate that the chromatin occupancy of Spt16 is not strongly dependent on the chromatin occupancy of Paf1C and that the interaction between Spt16 and Rtf1ΔOAR is insufficient to recruit the latter to chromatin.

To uncover the mechanism of recruitment of Paf1C to chromatin through the Rtf1 OAR, I focused on the interaction between Rtf1 and Spt5. Both mass spectrometry analysis and immunoblotting analysis of the affinity-purified proteins demonstrated that deletion of the OAR disrupted the interaction between Rtf1 and Spt5 (Figure 9A and 9C). Reciprocally, immunoprecipitation of Spt5 resulted in the co-immunoprecipitation of full-length Rtf1 but not Rtf1 lacking the OAR (Figure 9E, lanes 5 and 6). Based on reports demonstrating that the CTR of Spt5 is important for the chromatin association of Paf1C (LIU *et al.* 2009; ZHOU *et al.* 2009) and that recombinant Paf1C subunits can bind to CTR peptides *in vitro* (QIU *et al.* 2012), we hypothesized that the CTR of Spt5 could mediate the interaction of Spt5 with Rtf1. Using an antibody against an internal region of Spt5, I immunoprecipitated Spt5 or a Spt5 mutant protein lacking the CTR from yeast extracts and asked if HA-tagged full-length Rtf1 could co-immunoprecipitate with either Spt5 protein. Interestingly, full-length Rtf1 co-

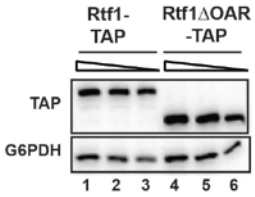
immunoprecipitated with full-length Spt5 but not with Spt5 lacking the CTR (Figure 9E, lanes 5 and 7). To determine if removal of the Spt5 CTR affected the co-transcriptional histone modification functions of Rtf1, I performed immunoblotting analysis of the *spt5*Δ*CTR* strain used in our studies. In agreement with previous results (LIU *et al.* 2009; ZHOU *et al.* 2009), the absence of the Spt5 CTR caused a reduction in H3 K4 Me3 and H3 K79 Me2/3 levels (Figure 9F). Together, these results demonstrate that the interaction of Rtf1 with Spt5 requires the OAR of Rtf1 and the CTR of Spt5, and this interaction is important for the function of Rtf1.

Our protein interaction studies revealed a requirement for the Rtf1 OAR in mediating an interaction between Paf1C and Spt5. We were, therefore, interested in determining if the OAR was important for the chromatin association of Spt5. Analysis of Spt5 occupancy at the constitutively active genes *PYK1* and *PMA1* by ChIP in strains expressing full-length Rtf1 or Rtf1ΔOAR showed that the levels of Spt5 associated with these genes were not greatly impacted by the lack of the OAR (Figure 9G). This finding indicates that Spt5 acts upstream of Rtf1 in promoting Paf1C recruitment.

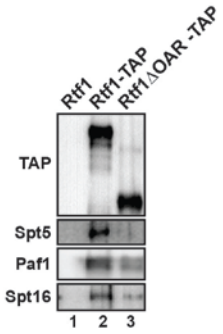
A.

Proteins	Average number of peptides		
	Rtf1	Rtf1-TAP	Rtf1Δ OAR-TAP
Rtf1	0	33	14
Spt5	0	13	0
Paf1	1	33	25
Ctr9	1	54	25
Cdc73	0	21	13
Leo1	0	24	17
Spt16	0	12	14

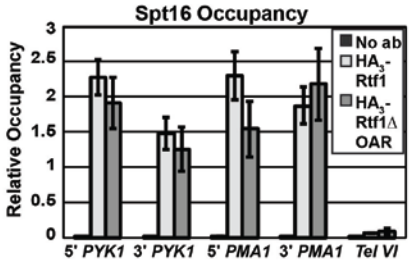
B.



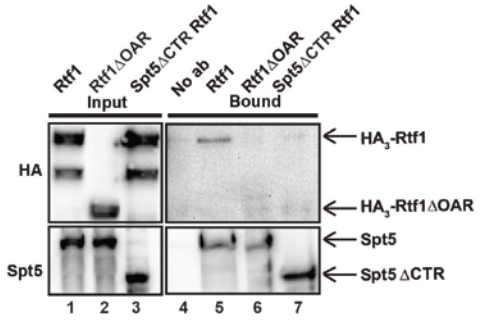
C.



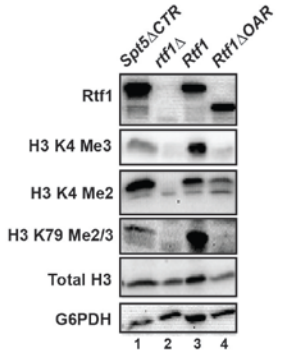
D.



E.



F.



G.

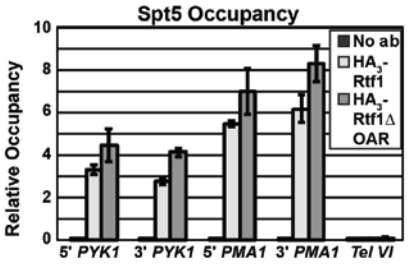


Figure 9. The OAR is important for the interaction of Rtf1 with Spt5

(A) Extracts of an *rtf1*Δ strain (KY1258) bearing plasmids expressing Rtf1 (pLS20), Rtf1-TAP (pPC59) and Rtf1ΔOAR-TAP (pMM03) were subjected to one-step affinity purification using IgG-conjugated magnetic beads, and mass spectrometry analysis was performed on the isolated proteins. The average number of peptides identified for each protein (minimum of three trials) is listed. A subset of the results is shown. (B) Immunoblotting analysis was performed to determine the levels of Rtf1 in transformants of an *rtf1*Δ strain (KY619) expressing Rtf1-TAP (pPC59) or Rtf1ΔOAR-TAP (pMM03) by loading 9 µl, 7 µl and 5 µl of each of the indicated extracts. G6PDH served as the loading control. (C) Immunoblotting analysis of the affinity-purified Rtf1 proteins from extracts of transformants of an *rtf1*Δ strain (KY619) containing the plasmids described in (A). (D) ChIP analysis was performed using anti-Spt16 antibody to determine the occupancy of Spt16 over 5' and 3' regions of *PYK1* and *PMA1* and a telomeric region of chromosome VI. Chromatin from transformants of an *rtf1*Δ strain (KY619) expressing HA_3-Rtf1 (pLS21-5) or HA_3-Rtf1ΔOAR (pMM01) was used. As a negative control, ChIP analysis was performed with chromatin from HA_3-Rtf1 (pLS21-5) expressing transformants without the addition of antibody (no ab). (E) Spt5 was immunoprecipitated from extracts of transformants of an *rtf1*Δ strain (KY619) transformed with a plasmid expressing HA_3-Rtf1 (pLS21-5) or HA_3-Rtf1ΔOAR (pMM01) and a transformant of an *rtf1*Δ *spt5*Δ*CTR* strain (KA150) expressing HA_3-Rtf1 (pLS21-5). Extracts of transformants expressing HA_3-Rtf1 (pLS21-5) subjected to similar analysis without the addition of anti-Spt5 antibody served as the negative control (no ab). Immunoblotting with anti-HA and anti-Spt5 antibodies was performed to detect Rtf1 and Spt5, respectively. The results are representative of those from three separate experiments. (F) Immunoblotting analyses were performed using indicated antibodies on transformants of an *rtf1*Δ (KY619) strain containing plasmids expressing HA_3-Rtf1 (pLS21-5) or HA_3-Rtf1ΔOAR (pMM01) and transformants of an *rtf1*Δ *spt5ΔCTR* (KA150) strain containing plasmid expressing Rtf1 (pLS21-5). An *rtf1*Δ strain (KY619) transformed with empty vector (pRS314) was used as the negative control. G6PDH and total H3 levels serve as loading controls. (G) ChIP analysis of the occupancy of Spt5 over 5' and 3' regions of *PYK1* and *PMA1* and over a telomeric region of chromosome VI was performed with chromatin prepared from strains described in (D).

2.3.4 The OAR of Rtf1 interacts directly with the CTR of Spt5

Our data are consistent with the possibility that a direct physical interaction between the Spt4-Spt5 complex and the Rtf1 OAR mediates the coupling of Paf1C to Pol II. To determine whether the interaction between Rtf1 and Spt4-Spt5 is direct or indirect, I performed *in vitro* binding assays with bacterially expressed Spt4-Spt5 complex and Rtf1 derivatives. Like in yeast cells (WARNER *et al.* 2007), I have found bacterially expressed Rtf1 to be susceptible to proteolytic breakdown. To maximize recovery of recombinant, intact wild-type Rtf1 and Rtf1ΔOAR, we designed expression constructs that encode doubly tagged forms of these proteins. The N-terminal GST tag and C-terminal His_6 tag were exploited in a two-step affinity purification strategy (see section 2.2.8) to enrich for the intact Rtf1 proteins. Subsequently, the GST tagged-Rtf1 proteins were bound to glutathione sepharose and mixed with purified recombinant Spt4-Spt5 to test for binding. We found that full-length Rtf1 interacted with Spt4-Spt5 *in vitro* (Figure 10A, lane 2) and removal of the OAR from Rtf1 diminished this interaction (Figure 10A, lane 3). Together, my *in vitro* and *in vivo* experiments indicate that the OAR is important for the direct interaction of Rtf1 with Spt4-Spt5. To determine if the OAR is sufficient for the interaction with Spt4-Spt5, I performed an *in vitro* binding assay using a purified recombinant GST-OAR protein (amino acids 235 to 373 of Rtf1) and recombinant Spt4-Spt5. Interestingly, the OAR alone strongly interacted with Spt4-Spt5 (Figure 10A, lane 4). These results show that the Rtf1 OAR is both necessary and sufficient for a direct interaction between Rtf1 and Spt4-Spt5.

My co-immunoprecipitation experiments revealed a requirement for the CTR of Spt5 in the *in vivo* association of Spt5 with Rtf1 (Figure 9E, lane 7). We therefore hypothesized that the

CTR of Spt5 could be facilitating the recruitment of Paf1C by directly interacting with the OAR of Rtf1. To test this idea, I performed an *in vitro* binding assay to examine the binding of the isolated OAR to the Spt5 CTR. I purified His_6-tagged OAR and MBP-tagged Spt5 CTR proteins from bacterial expression strains, mixed the purified proteins, and used affinity chromatography to pull down the His_6-OAR protein. I then performed immunoblotting analysis to determine if the MBP-CTR protein or MBP alone was retained on the beads after multiple washes. This analysis revealed that, relative to MBP alone, significantly higher amounts of MBP-CTR were bound to His_6-OAR (Figure 10B, lanes 1 and 3), providing support for a direct interaction between the OAR of Rtf1 and the CTR of Spt5.

To address the importance of CTR phosphorylation in regulating the physical interaction between Spt5 and the OAR, I performed a GST pull-down assay using GST-OAR and extracts prepared from yeast cells that express wild-type Spt5 or mutant derivatives of Spt5 in which the phosphorylated serine residues are substituted to alanine residues, as a non-phosphorylatable version (Spt5-S1-15A), or to aspartic acids, as a phosphomimetic version (Spt5-S1-15D) (QIU *et al.* 2012). In accordance with the results I obtained with recombinant proteins, the GST-OAR protein, but not GST alone, interacted with wild-type Spt5 provided in the yeast extract (Figure 10C. lanes 1 and 2). A similar level of association was observed between the GST-OAR protein and the Spt5-S1-15D phosphomimetic derivative (Figure 10C, lane 4). In contrast, the interaction between the GST-OAR protein and Spt5 was diminished by substitution of the Bur1-phosphorylated serine residues (LIU *et al.* 2009) with alanine residues (Figure 10C, lane 3). These results are consistent with the conclusion that phosphorylation of the Spt5 CTR stimulates its interaction with the Rtf1 OAR.

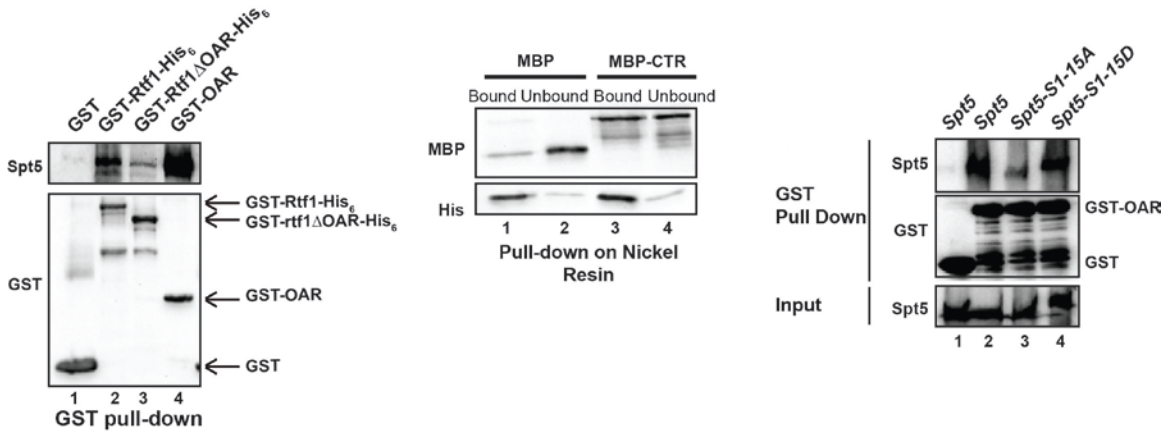


Figure 10. The OAR of Rtf1 interacts directly with the CTR of Spt5

(A) Recombinant GST (pGEX-3X), GST-Rtf1-His_6 (pAP21), GST-Rtf1ΔOAR-His_6 (pMM26) and GST-OAR (pMM25) proteins, bound to glutathione sepharose beads, were incubated with the same amount of purified recombinant Flag-Spt5-His_6/Spt4 (pGH258). Beads were washed and bound samples were analyzed by immunoblotting for Spt5 (upper panel) or GST (lower panel). The latter serves as a control for the amount of GST-tagged derivatives used in the assay. The results are representative of three experiments. (B) *In vitro* binding assay of the OAR of Rtf1 with the CTR of Spt5 was performed by incubating equal amounts of recombinant purified His_6-OAR with MBP alone or MBP-CTR. The OAR was pulled down using magnetic nickel beads. The amount of MBP or MBP-CTR and OAR was determined by immunoblotting with anti-MBP (upper panel) and anti-His_6 (lower panel). Results are representative of three experiments. (C) Extracts of *rtf1*Δ strains expressing wild-type Spt5 (KA181), Spt5 with a non-phosphorylatable CTR (KA183) or Spt5 with a phosphomimetic version of the CTR (KA185) were used for GST pull-down assays with bacterially purified GST-OAR (pMM25) bound to glutathione beads. A reaction using GST bound to glutathione beads and extracts of an *rtf1*Δ strain expressing wild-type Spt5 (KA181) served as a negative control. Results are representative of two experiments.

2.3.5 The OAR is sufficient for chromatin association and mimics the chromatin association pattern of Rtf1

Since deletion of the Rtf1 OAR reduced the occupancy of Paf1C on chromatin, we hypothesized that the OAR alone might be able to associate with active chromatin. To test this hypothesis, I constructed a plasmid that expresses the OAR (amino acids 230 to 390) as a fusion protein with a nuclear localization sequence (NLS) and a triple HA tag and transformed this plasmid into an *rtf1*Δ strain. ChIP was performed to analyze the chromatin association levels of NLS-HA_3-OAR and full-length HA_3-Rtf1 at *PMA1* and *PYK1*. Interestingly, even in the absence of the remainder of the Rtf1 protein, the OAR associated with the *PYK1* and *PMA1* genes and not with an untranscribed telomeric region (Figure 11A). The levels of association were however lower than those for full-length Rtf1, which is likely due to decreased cellular levels of the NLS-HA_3-OAR protein compared to full-length HA_3-Rtf1 (Figure 11B). Since the OAR lacks the C-terminal region of Rtf1 required for the interaction with other members of Paf1C, its association with chromatin would not be expected to be facilitated by other members of the complex (WARNER *et al.* 2007). To test this idea, I assessed the chromatin occupancy of Rtf1 and the OAR in the absence of Cdc73 or Paf1. Consistent with previous observations that Cdc73 is important for Paf1C recruitment (AMRICH *et al.* 2012; MUELLER *et al.* 2004; QIU *et al.* 2012) and that Paf1 is important for Rtf1 stability (MUELLER *et al.* 2004), Rtf1 showed lower chromatin occupancy in the absence of Cdc73 or Paf1 subunits (Figure 11C and D). This reduced chromatin occupancy correlated with reduced levels of Rtf1 in both the *cdc73Δ* and *paf1Δ* strains (Figure 11B, lanes 2, 4 and 6). Surprisingly, chromatin occupancy of the OAR alone was increased in the absence of *CDC73* (Figure 11C and D). Immunoblotting analysis showed that levels of the OAR protein, unlike full-length Rtf1, were unaltered in the *cdc73Δ* and *paf1Δ*

strains compared to the *PAF1 CDC73* control strain (Figure 11B, lanes 3, 5 and 7). The higher chromatin levels of the OAR in a *cdc73Δ* strain may reflect a competition between the OAR and Cdc73 for a common binding partner on chromatin, consistent with observations that both can interact with the Spt5 CTR (Figure 10) (QIU *et al.* 2012).

Although the OAR is sufficient to bind chromatin, we were interested in knowing whether its occupancy pattern mimicked that of full-length Rtf1. Therefore, I used ChIP analysis to ask if the OAR alone, like Rtf1 and other subunits of Paf1C (KIM *et al.* 2004a; MAYER *et al.* 2010), dissociated from chromatin near the poly(A) site of *PYK1* and *PMA1*. To better visualize the decrease in Rtf1 association, I normalized occupancy levels to those at the 5' ends of the genes. Interestingly, I observed that the OAR occupancy levels dropped significantly beyond the poly(A) site, similar to the pattern of Rtf1 occupancy (Figure 12A-C). Reduction in the levels of the OAR beyond the poly(A) site could be explained by reduced chromatin levels of Pol II or Spt5 in cells expressing the OAR alone as compared to cells expressing full-length Rtf1. However, ChIP analysis revealed that levels of Spt5 and Pol II across *PYK1* and *PMA1* were similar in both strains (Figure 12A and D-G). These results indicate that the lower levels of the OAR beyond the poly(A) site are not due to lower levels of Spt5 or Pol II at these sites specifically in OAR-expressing cells but are due to the dissociation of the OAR, like full-length Rtf1, from chromatin near the poly(A) site. Together, our data show that the isolated OAR is capable of associating with chromatin independently of the other members of Paf1C in a pattern similar to that of full-length Rtf1.

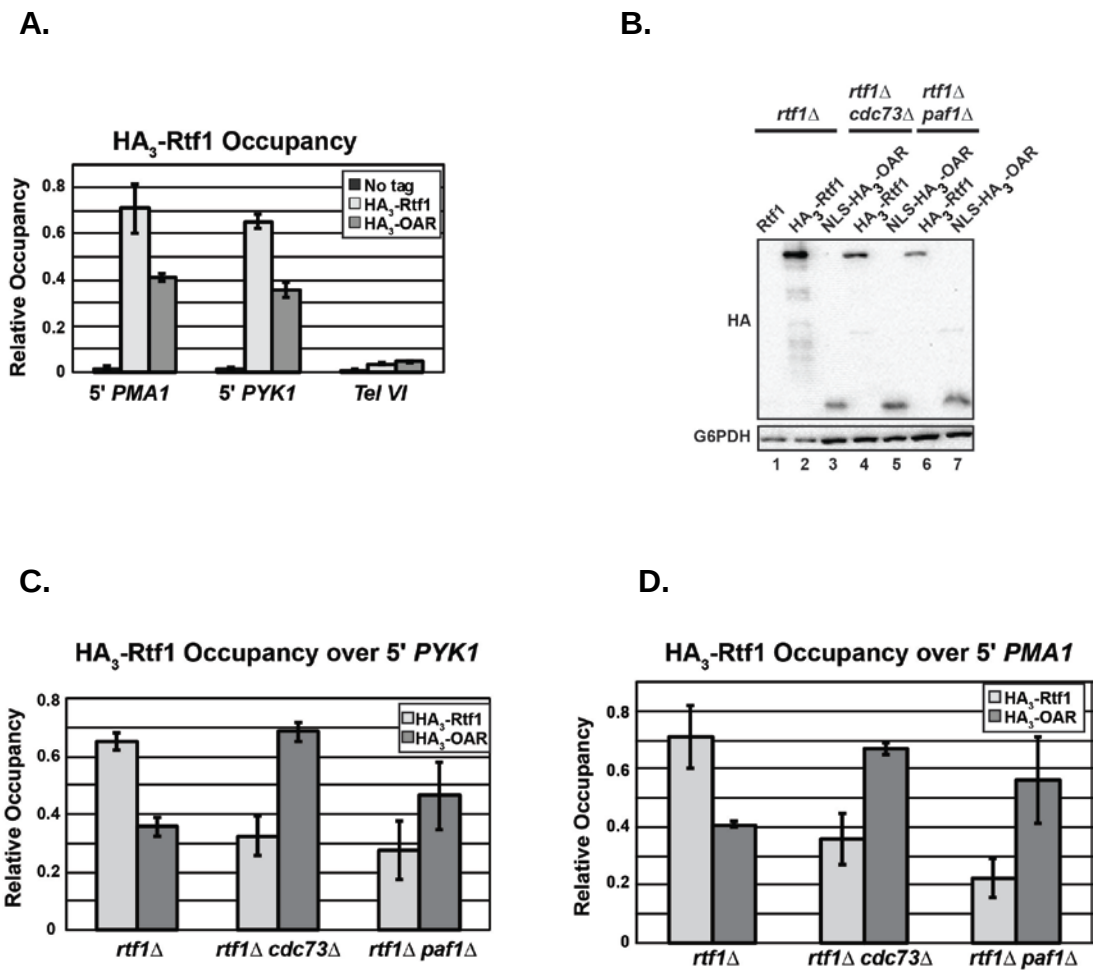


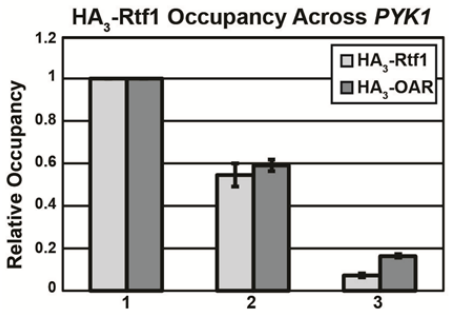
Figure 11. The OAR is sufficient for chromatin association

(A) ChIP analyses of transformants of an *rtf1Δ* strain (KY619) expressing NLS-HA_3-OAR (pMM44), HA_3-Rtf1 (pMM43) and untagged Rtf1 (pMM40) were performed using anti-HA beads to determine the occupancy of HA-tagged Rtf1 derivatives over 5' regions of *PYK1* and *PMA1* and a telomeric region of chromosome VI. (B) Immunoblotting analysis of transformants of *rtf1Δ* (KY619), *rtf1Δ cdc73Δ* (KY2195) and *rtf1Δ paf1Δ* (KY1813) strains expressing NLS-HA_3-OAR (pMM44) or HA_3-Rtf1 (pMM43) was performed using antibody against the HA tag. The anti-G6PDH immunoblot served as a loading control. (C and D) ChIP analyses were performed on strains used in (B) to determine the chromatin occupancy of NLS-HA_3-OAR (pMM44) and HA_3-Rtf1 (pMM43) over 5' regions of *PYK1* (C) and *PMA1* (D). For (A), (C) and (D), graphs represent the average of three biological replicates with error bars indicating the SEM.

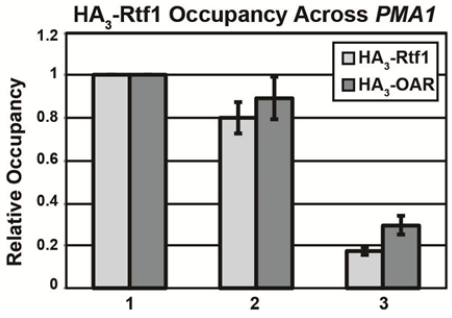
A.


1
253
346
2
1127
1270
3
1803
1938
PYK1
TATA
1(ATG)
1503(STOP)
1642
1
214
319
2
2107
2194
3
3373
3475
PMA1
TATA
1(ATG)
2757(STOP)
2823
3277

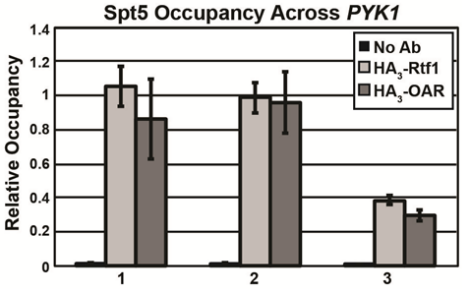
B.


HA3-Rtf1 Occupancy Across PYK1
Relative Occupancy
HA3-Rtf1
HA3-OAR
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3

C.


HA3-Rtf1 Occupancy Across PMA1
Relative Occupancy
HA3-Rtf1
HA3-OAR
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3

D.


Spt5 Occupancy Across PYK1
Relative Occupancy
No Ab
HA3-Rtf1
HA3-OAR
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3

E.


Spt5 Occupancy Across PMA1
Relative Occupancy
No Ab
HA3-Rtf1
HA3-OAR
3
2.5
2
1.5
1
0.5
0
1
2
3

F. **G.**

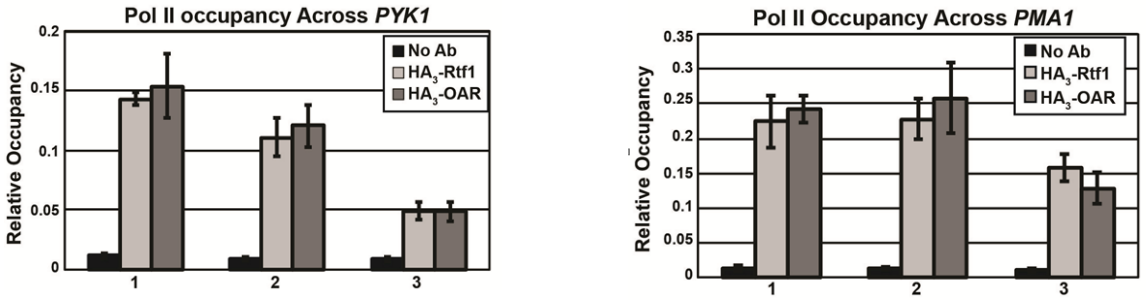


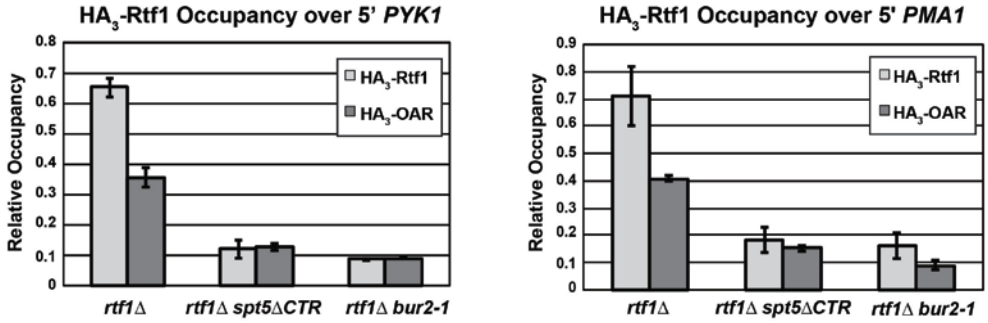
Figure 12. The OAR mimics the chromatin association pattern of Rtf1

Occupancy of Rtf1 derivatives (B and C), Spt5 (D and E) and Pol II (F and G) at *PYK1* and *PMA1* was measured by ChIP. Locations of PCR products are shown in A. Transformants of an *rtf1*Δ strain (KY619) expressing either HA_3-Rtf1 (pMM43) or NLS-HA_3-OAR (pMM44) were used for ChIP assays with anti-HA beads (B and C), Spt5 antisera (D and E) or Rpb3 antibody (F and G). Occupancy levels of HA_3-Rtf1 and NLS-HA_3-OAR were normalized to their occupancy levels at the 5' regions of the genes, which were set to 1 (panels B and C). A no antibody reaction (No Ab) served as a negative control for the non-specific association of Spt5 and Pol II with the beads (panels D-G). All graphs depict the average of three biological replicates with SEM.

2.3.6 OAR occupancy on chromatin is dependent on the CTR of Spt5 and Bur1-Bur2 kinase complex

Our findings so far show that the OAR is necessary for chromatin association of Paf1C, that the OAR can interact directly with the CTR of Spt5 *in vitro*, and that the isolated OAR behaves similarly to Paf1C in its ability to interact with chromatin both in terms of the localization pattern and the preference for active genes. I also found that the phosphorylation of the CTR of Spt5 enhances the interaction of the OAR with Spt5. Also, previous studies have shown that the recruitment of Paf1C is dependent on the CTR of Spt5 and on the Bur1-Bur2 protein kinase, which targets the CTR of Spt5 and the CTD of Pol II (LARIBEE *et al.* 2005; LIU *et al.* 2009; QIU *et al.* 2009; QIU *et al.* 2006; WOOD *et al.* 2005; ZHOU *et al.* 2009). Based on all these findings, we hypothesized that the association of the isolated OAR with chromatin would be dependent on the CTR of Spt5 and Bur2. To test this hypothesis, I analyzed the chromatin occupancy of the NLS-HA_3-OAR protein and full-length HA_3-Rtf1 in *spt5ΔCTR* and *bur2-1* strains. Strikingly, just like for full-length Rtf1, the levels of chromatin association of the OAR at *PYK1* and *PMA1* were significantly reduced in the *spt5ΔCTR* and *bur2-1* strains (Figure 13A and B). Immunoblotting analysis showed that the OAR protein levels were slightly reduced in the *spt5ΔCTR* and *bur2-1* strains but not to the same degree as the reduction in the ChIP signals (Figure 13C, lanes 3, 5 and 7). Together, these results suggest that the OAR is responsive to the same regulatory factors as Paf1C and underscore the central role of this domain in the chromatin association of Paf1C.

A. **B.**



C.

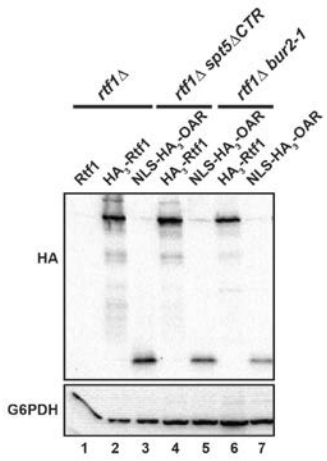


Figure 13. OAR recruitment is reduced in strains lacking the Spt5 CTR or mutated in BUR2

(A and B) ChIP analyses of the isolated OAR and full-length Rtf1 in transformants of *rtf1Δ* (KY619), *rtf1Δ spt5ΔCTR* (KA150), and *rtf1Δ bur2-1* (AY777) strains expressing NLS-HA_3-OAR (pMM44) or HA_3-Rtf1 (pMM43) over 5' regions of *PYK1* (A) and *PMA1* (B) using anti-HA beads. The averaged results from three biological replicates with SEM are shown. (C) Immunoblot analysis of HA_3-Rtf1 and NLS-HA_3-OAR levels of strains used for ChIP analysis in A and B was performed using antibody against the HA tag. G6PDH levels serve as the loading control.

2.4 DISCUSSION

Both the initial purification of Paf1 as an Pol II-associated factor (SHI *et al.* 1996) and the subsequent discovery that the subunits of Paf1C co-localize with Pol II on open reading frames (KROGAN *et al.* 2002; POKHOLOK *et al.* 2002) argue that the physical coupling of Paf1C to Pol II is likely to be important for directing the functions of Paf1C to active genes. Our previous deletion analysis identified a region of Rtf1, the OAR, as being important for the chromatin association of Paf1C (WARNER *et al.* 2007); however, the nature of the interaction between the OAR and the Pol II elongation machinery was unknown. In this study, we investigated the mechanism of recruitment of Paf1C through the Rtf1 OAR. Affinity purification of Rtf1 proteins containing or lacking the OAR revealed that the OAR was critical for the physical association of Spt5 with Paf1C, and my co-immunoprecipitation analysis demonstrated that the interaction between Rtf1 and Spt5 required the CTR of Spt5. My *in vitro* binding assays performed with purified recombinant versions of Rtf1 and Spt4-Spt5 provided evidence for a direct interaction between these elongation factors. In addition, these experiments suggested that the OAR of Rtf1 and the CTR of Spt5 mediate the direct physical interaction between Rtf1 and Spt5. My GST-pull down assay with the OAR also suggested that the phosphorylation of the Spt5 CTR facilitated better interaction between the OAR and the Spt5 CTR. Using ChIP studies, I found that the OAR can occupy chromatin independently of other Paf1C components and exhibit a localization pattern similar to that of full-length Rtf1. Moreover, chromatin occupancy of the isolated OAR was significantly reduced in strains lacking the Spt5 CTR or the Bur2 cyclin component of the Bur1-Bur2 protein kinase, which phosphorylates the Spt5 CTR as well as the CTD of Pol II (LIU *et al.* 2009; QIU *et al.* 2009; ZHOU *et al.* 2009). Taken together, our results

suggest that the OAR of Rtf1 plays a prominent role in mediating the recruitment of Paf1C to elongating Pol II through an interaction with the CTR of Spt5.

In organisms ranging from bacteria to humans, Spt5 and its homologues have fundamental roles in regulating transcription elongation. In eukaryotes, Spt5 has been shown to promote Pol II pausing and processivity (WERNER 2012). Spt5 consists of several domains including an acidic N-terminal domain, a NusG N-terminal domain (NGN), multiple KOW (Kyprides, Ouzounis, Woese) domains and the CTR (PONTING 2002). There have been several structural studies on the interaction between Spt5 and Spt4 (GUO *et al.* 2008; MARTINEZ-RUCOBO *et al.* 2011). The crystal structure of the archaeal homologues of a fusion protein of Spt4 and the NGN domain of Spt5 revealed that Spt4 interacts with the NGN domain of Spt5 (GUO *et al.* 2008). Both the NGN and the KOW motifs of Spt5 are involved in the interaction of Spt5 with Pol II (HIRTREITER *et al.* 2010; VIKTOROVSKAYA *et al.* 2011). Curiously, sequence homology between the KOW motifs of Spt5 and the Plus3 domain of human Rtf1 has been noted, although the significance of this similarity is unclear (DE JONG *et al.* 2008). In this work we provide evidence that the Rtf1 OAR is an interacting partner for the CTR of Spt5; however, our results do not rule out the possibility that other regions within Spt5 or Rtf1 may enhance their interaction.

The mechanisms that direct the recruitment of Paf1C to RNA polymerase II are likely to be important for targeting Paf1C functions to active genes, and previous studies have implicated several different transcription elongation factors as well as the Pol II CTD in this process (MULDER *et al.* 2007; PAVRI *et al.* 2006; QIU *et al.* 2012; QIU *et al.* 2006). Particularly relevant to our results, mutations that alter the Spt5 CTR or its kinase, Bur1-Bur2, were previously shown to impair the recruitment of Paf1C to chromatin (LARIBEE *et al.* 2005; LIU *et al.* 2009; QIU *et al.*

2012; WOOD *et al.* 2005; ZHOU *et al.* 2009). Interestingly, chromatin association levels of Paf1 in cells expressing a mutant form of Spt5 that could not be phosphorylated on the CTR were higher than those in cells expressing Spt5 lacking the CTR (LIU *et al.* 2009). This observation suggests that, while the phosphorylation of the Spt5 CTR by Bur1-Bur2 enhances the affinity of the OAR for the CTR, other aspects of the CTR sequence and structure are also important for this interaction. Consistent with this idea, our *in vitro* binding assays revealed an interaction between recombinant OAR and CTR proteins, even though the latter is unlikely to be properly phosphorylated in *E. coli*.

Our observation that the chromatin association pattern of the OAR was similar to that of full-length Rtf1 indicates that features of Paf1C that control its pattern of chromatin association and dissociation are contained within the OAR. Interestingly, other components of Paf1C, including the Cdc73 C-domain, which interacts with the Pol II CTD in a phospho-specific manner, and Leo1, which binds to RNA, are also important for ensuring full levels of Paf1C recruitment (AMRICH *et al.* 2012; DERMODY and BURATOWSKI 2010; QIU *et al.* 2012). Together, these results argue for the existence of more than one attachment point between Paf1C and transcribing Pol II. Unlike its Spt5 and Pol II interaction partners which continue on to the transcription termination site, Paf1C dissociates from coding regions near the poly(A) site where 3'-end processing factors are recruited to the Pol II machinery (KIM *et al.* 2004a; MAYER *et al.* 2010). The dissociation of Paf1C at this site may be governed by changes in the phosphorylation patterns of the Pol II CTD, the Spt5 CTR, or both proteins. In addition, the association of Leo1 with RNA may contribute to the release of Paf1C near the RNA cleavage site. Interestingly, a recent study showed that the recruitment of the 3'-end RNA processing factor, RNA cleavage factor I, requires the CTR of Spt5 (MAYER *et al.* 2012). Together with our discovery of a direct

physical interaction between the Rtf1 OAR and the Spt5 CTR, this observation, as well as the previous identification of numerous RNA processing factors in complex with Spt5 (LINDSTROM *et al.* 2003), raises the possibility that the Spt5 CTR, like the Pol II CTD, acts as a platform for the recruitment and exchange of proteins that coordinate the synthesis and processing of Pol II transcripts.

3.0 SURFACE-EXPOSED, CONSERVED RESIDUES WITHIN THE OAR OF RTF1 ARE FUNCTIONALLY IMPORTANT

Some of the work presented in this Chapter is adapted from published material (MAYEKAR *et al.* 2013; WIER *et al.* 2013) and is reprinted with changes. The crystal structures shown in this Chapter represent the work of Adam Wier, Dr. Annie Heroux and Dr. Andrew VanDemark.

3.1 INTRODUCTION

Transcription is a highly regulated process that involves a functional crosstalk between Pol II and several proteins that directly or indirectly facilitate the transit of Pol II through chromatin and couple the synthesis of RNA with the co-transcriptional processing of RNA. Paf1C is one such transcriptional regulator that has roles at various stages of transcription from initiation to termination including the activation of transcription upon recruitment by other transcription factors, regulation of co-transcriptional histone modifications and 3' processing of newly synthesized RNA (MOSIMANN *et al.* 2006; MOSIMANN *et al.* 2009) [reviewed in (CRISUCCI and ARNDT 2011; TOMSON and ARNDT 2013)]. Since most the functions of Paf1C are co-transcriptional the coupling of Paf1C with the transcription machinery is expected to be

indispensable for the proper execution of these functions. In the previous Chapter, I provided a molecular mechanism for coupling Paf1C to the transcription machinery through the OAR of Rtf1. Comparison of amino acid sequences of homologues of Rtf1 from yeast to humans had revealed that the OAR is the most highly conserved region of Rtf1 (Figure 7A). The presence of highly conserved residues on the surface suggested that they could be mediating functionally-important interactions of Paf1C with a binding partner(s) that tethers Paf1C to actively transcribed genes.

My work described in Chapter 2 indicated that the OAR mediated the targeting of Paf1C to active chromatin through association with the phosphorylated CTR of Spt5. However, the OAR was still recruited to active chromatin, although at much lower levels, in the absence of the CTR of Spt5 (Figure 13A and B). This suggests that the OAR may also be involved in additional interactions required for the chromatin recruitment of Paf1C that were not identified via our subtractive proteomics approach (discussed in section 2.3.3). Furthermore, in the absence of OAR, we can still observe low levels of histone modifications, which suggest that weak association of Paf1C (undetectable by ChIP) with active chromatin may still occur in cells lacking the OAR through an OAR-independent mechanism of recruitment. Furthermore, there may be accessory factors that may act in a synergistic fashion with the OAR to tether Paf1C to chromatin. These accessory factors mediating the recruitment of Paf1C by OAR-dependent and OAR-independent mechanisms could be uncovered in a high-copy-number suppressor screen to identify proteins that when overexpressed can suppress the transcriptional defects (Spt^- phenotype) exhibited by a hypofunctional OAR.

To uncover functionally-important residues within the OAR that when mutated confer transcriptional defects without affecting the stability of Rtf1, I took a mutational-analysis

approach. I found that OAR mutants having amino acid substitutions for the conserved, surface-localized residues of the OAR exhibited Spt$^-$ phenotype, suggesting that they are transcriptionally defective. Furthermore, I saw an exacerbation of the Spt$^-$ phenotype when more than one residue in the OAR was mutated. Additionally, my immunoblotting, co-immunoprecipitation and ChIP analysis attributed the transcriptional defect in these mutants to reduced association of Paf1C with Spt5 that resulted in reduced chromatin association of Paf1C.

Adam Wier, Dr. Annie Heroux and Dr. Andrew VanDemark obtained a crystal structure for the human OAR/Plus3 with a phosphorylated CTR of Spt5 and hence proved that the interaction that I discovered in yeast is conserved in humans as well. His work provided a better understanding of the structural basis of the OAR-CTR interaction. To determine if the interactions observed in the crystal structure were physiologically relevant, I performed ChIP analysis with the mutants predicted to affect the OAR-CTR interaction. My results show that the chromatin occupancy of Rtf1 along with the rest of Paf1C is significantly diminished in cells expressing these mutants thus suggesting that the interactions between the OAR of Rtf1 and the CTR of Spt5 observed in the OAR-CTR co-crystal are important for tethering Paf1C to chromatin.

The Cdc73 subunit of Paf1C has also been implicated in the chromatin attachment of Paf1C (MUELLER *et al.* 2004). The chromatin association function of Cdc73 has been attributed to a C-terminal domain of Cdc73 (C-domain) (AMRICH *et al.* 2012; QIU *et al.* 2012). My ChIP and western blot analysis suggest that the chromatin association of Paf1C is mediated by a dual attachment via the OAR of Rtf1 and the C-domain of Cdc73.

3.2 MATERIALS AND METHODS

3.2.1 Yeast growth assays

Yeast cells (Table 4) were grown overnight in the appropriate medium. They were then washed twice with sterile water and 10-fold serial dilutions of the washed cells were made using sterile water. 2.5 µl of each dilution was then spotted onto the indicated medium and incubated at 30°C or 37°C for the number of days specified.

3.2.2 Plasmids

Plasmids pLS20 and pLS21-5 (STOLINSKI *et al.* 1997) express untagged and HA-tagged full-length Rtf1, respectively, under the control of the *RTF1* promoter on *TRP1*-marked *CEN/ARS* plasmids. The *TRP1*-marked *CEN/ARS* plasmids encoding the indicated HA_3-tagged mutant derivatives of Rtf1 were made using the Quikchange site-directed mutagenesis kit (Agilent) and pLS21-5 as the template. The *URA3*-marked plasmids expressing untagged mutant versions of Rtf1 were made by first removing the HA_3-tag from plasmids that express HA_3-Rtf1 by NdeI digestion and then ligating the XhoI-SacI fragment from these plasmids, containing the *RTF1* promoter and coding region, to pRS316 digested with XhoI-SacI. pMM61 and pMM62 expresses untagged full-length Rtf1 and Rtf1 lacking the OAR, respectively, under the control of the *RTF1* promoter on a *URA3*-marked CEN/ARS plasmids. pMM65 was generated using a QuickChange site-directed mutagenesis kit to introduce the R251A and Y327A substitutions in the Rtf1 protein. pCD3, pWR4 and pCD8 express untagged Cdc73, HA-tagged Cdc73 and HA-tagged Cdc73 lacking the C-domain (AMRICH *et al.* 2012).

3.2.3 Western Blot Analysis

Trichloroacetic acid extracts were made from log phase yeast cultures (OD_{600} 0.8-1.2) (Table 4) lysed by bead beating as described in Chapter 2 (section 2.2.3). These yeast extract were subjected to western blot analysis as described previously (MAYEKAR *et al.* 2013) using antibodies against the HA epitope (1:2500 dilution, Roche), Rtf1 (1:3000 dilution) (SQUAZZO *et al.* 2002), Histone H3 K4 Me3 (1:2000, Active motif 39159), H3 K4 Me2 (1:2000, Upstate 07-030), H3 K79 Me2/3 (1:2000, Abcam ab2621), H3 K36 Me3 (1:1000, Abcam ab9050), total histone H3 (1:30,000) (PIRO *et al.* 2012) or, as a loading control, glucose-6-phosphate dehydrogenase (1:30,000 dilution, Sigma A9521).

3.2.4 Chromatin Immunoprecipitation

Chromatin was prepared from log phase cultures of the indicated yeast strains (Table 4) grown to a density of 1-2 $x10^7$ cells/ml. Cells were treated with formaldehyde, quenched with glycine, harvested, and lysed as previously described (MAYEKAR *et al.* 2013). Chromatin was fragmented by sonication and incubated overnight at 4°C with agarose-conjugated α-HA antibody (Santa Cruz Biotechnology; sc-7392AC). Quantitative real-time PCR using Maxima SYBR Green/ROX qPCR master mix (Fermentas) and primers to the 5' region of *PYK1* (+253 to +346/ATG=+1), 3' region of *PYK1* (+1127 to +1270/ATG=+1), 5' region of *PMA1* (+214 to +319/ATG=+1), 3' region of *PMA1* (+2107 to +2194/ATG=+1) or telomeric region of chromosome VI (coordinates: 269495 to 269598) were used to determine the amount of *PYK1*, *PMA1* or telomeric DNA associated with the HA-tagged proteins. The y-axis of the graphs depicts the average values of

the efficiency of the primer set$^{(Ct\ of\ input)-(Ct\ of\ IP)}$ for three biological replicates and the error bars show the standard error of the mean (SEM).

3.2.5 Indirect immunofluorescence

Indirect immunofluorescence was performed as described previously (GUERRIERO *et al.* 2013). Briefly, transformants of an *rtf1Δ* strain (KY619) (Table 4) transformed with plasmid expressing HA_3-Rtf1 (pLS21-5), HA_3-Rtf1ΔOAR (pMM01), HA_3-Rtf1-R251, R273E, K299E or untagged Rtf1 (pLS20; served as the negative control) were grown to mid-log phase (OD_{600} ~0.5), fixed with 4% formaldehyde for one hour and treated with zymolyase 20T at 37°C to generate spheroplasts. Spheroplasts were adhered to poly-lysine treated slides and then permeabilized by treatment with methanol and acetone. After permeabilization, they were blocked with 0.5% BSA, 0.5% ovalbumin and 0.6% fish gelatin at 37°C and then incubated overnight with mouse anti-HA antibody (1:250, Roche) overnight at room temperature followed by 2 hour incubation with fluorophore-conjugated anti-mouse antibody (1:500; Alex 647, Molecular Probes) and DAPI (1:250). Prolong Antifade Gold (Invitrogen) was used as the mounting medium to image the slide under Olympus FV1000 (100X oil immersion lens).

Table 4. *Saccharomyces cerevisiae* strains used for the study in this Chapter

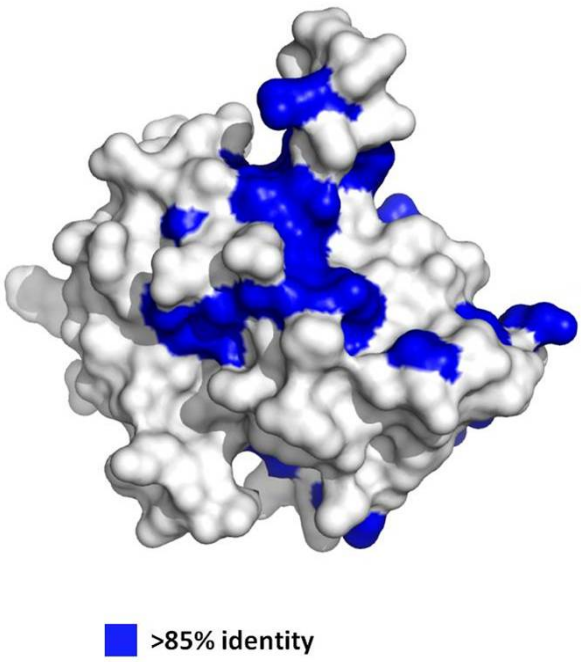
Strain	Genotype
KY619	*MAT*a *rtf1Δ102::ARG4 arg4-12 his4-912∂ leu2Δ1 lys2-173R2 trp1Δ63*
KY2417	*MAT*α *rtf1Δ::LEU2 cdc73Δ::KanMx his3Δ200 leu2Δ1 ura3(Δ0 or 52) trp1Δ63*

3.3 RESULTS

3.3.1 Mutational analysis approach to identify the functionally important residues of the OAR

Upon obtaining the crystal structure for the human OAR (WIER *et al.* 2013) we were interested in identifying the region of the OAR important for its function. One feature of functionally important residues of proteins is their high degree of conservation. Hence, we mapped the residues showing greater than 85% identity among nine homologues of Rtf1 from humans to yeast onto this structure (Figure 14A). These conserved residues of the OAR also included the three invariant basic residues within this region (R366, R388 and K414 in human Rtf1 and R251, R273 and K299 in *S. cerevisiae* Rtf1) that confer upon OAR its alternative name, the Plus3 region. Strikingly, most of the conserved residues were clustered together on the surface indicating that this region may be involved in mediating functionally important protein-protein interactions (Figure 14A and B).

A.



B.

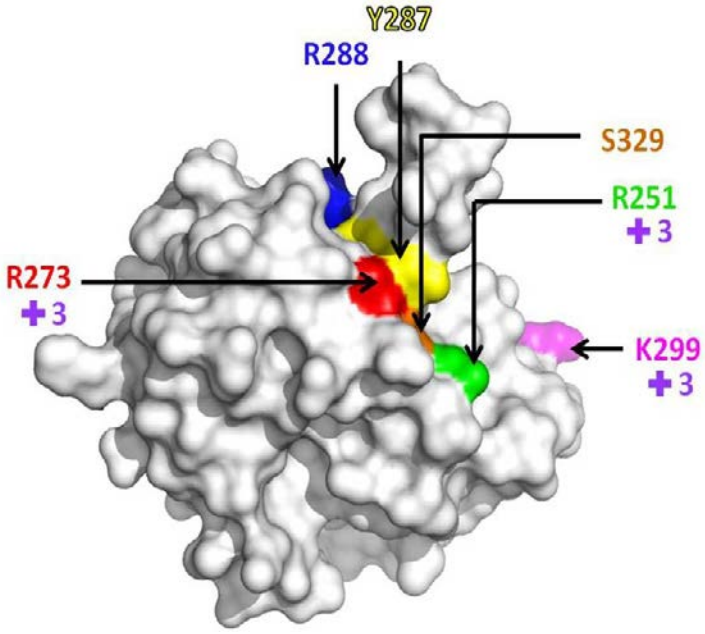


Figure 14. Crystal structure of the human Plus3 domain

(A) The residues showing greater than 85% identity among the homologues of Rtf1 were mapped onto the surface view of the crystal structure of human OAR and colored blue using Pymol (WIER *et al*. 2013). (B) The colored residues represent the corresponding yeast residues that I mutated as a part of my mutational analysis to identify the functionally important residues of the OAR of Rtf1. The Plus3 sign indicates the three highly conserved positively charged residues corresponding to the *S. cerevisiae* residues R251, R273 and K299 that confer upon the Rtf1 OAR its alternative name, Plus3.

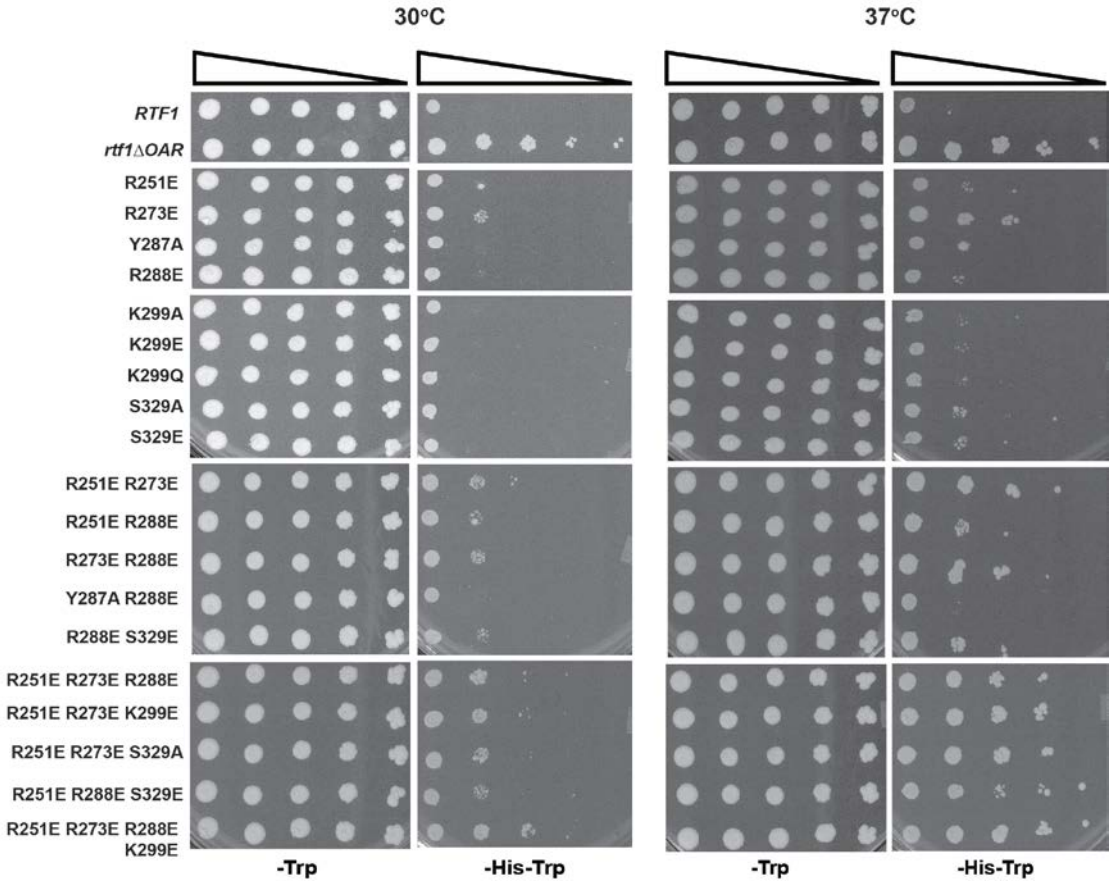
To test their biological significance, I mutated some of these conserved residues, either singly or in combination, to glutamic acid or alanine residues and then tested the ability of the resulting Rtf1 mutant proteins to cause Spt$^-$ phenotype. Most of the single-residue charge-swap substitutions did not suppress the *his4-91δ* allele at 30°C (Figure 15A). R273E was the only single residue substitution that caused the Spt$^-$ phenotype at 30°C, albeit weakly (Figure 15A). However, mutation of multiple conserved residues enhanced the Spt$^-$ phenotype (Figure 15A). Immunoblotting analysis indicated that the mutant Rtf1 protein levels were comparable to wild-type Rtf1 levels at 30°C (Figure 15B and C), suggesting that the enhanced mutant phenotypes in the double and triple mutants were not a consequence of reduced protein levels.

In addition to examining the Spt$^-$ phenotype at 30°C, I also tested this phenotype at 37°C to determine if the higher temperature exacerbated the Spt$^-$ phenotype of the mutants. Of the three single amino acid substitutions, the R273E substitution caused the strongest Spt$^-$ phenotype at 37°C (Figure 15A). The multiple-residue mutants also showed stronger Spt$^-$ phenotypes at 37°C (Figure 15A). Most multiple-residue mutants mimicked *rtf1ΔOAR* at 37°C although this may be partly due to reduced stability of the mutant proteins at 37°C (Figure 15D and E). We considered the possibility that the mutant phenotypes of strains expressing Rtf1 mutant proteins were due to loss of Rtf1 from the nucleus. However, my indirect immunofluorescence assays combined with confocal microscopy revealed that the Rtf1ΔOAR and Rtf1-R251E, R273E, K299E proteins, like wild type Rtf1, were localized to the nucleus (Figure 15F).

To test whether the surface-localized, highly conserved residues are important for the Spt5-mediated recruitment of Paf1C to chromatin, I determined the impact of the R273E single-residue substitution and R251E R273E K299E (Plus3 mutant) triple-residue substitution on the interaction of Rtf1 with Spt5 and assessed their chromatin association levels using ChIP analysis.

Even when the cells were grown at 30°C, these Rtf1 mutant proteins showed impaired interaction with Spt5 and exhibited significant chromatin association defects (Figure 16A and B). In these conditions, the levels of the mutant proteins were similar to wild-type Rtf1 (Figure 15B-C). Impairment of the ability of Rtf1 to associate with chromatin by deletion of the OAR significantly reduced the levels of Rtf1-dependent histone modifications (Figure 7B). Likewise, substitution of the three Plus3 residues lowered global levels of H3 K79 Me2/3 and H3 K4 Me3 (Figure 16C, lane 5). Quantification of the immunoblots revealed a very modest reduction in the levels of H3 K79 Me2/3 in the Rtf1-R273E mutant strain but no effect on H3 K4 Me2 or H3 K4 Me3 levels (Figure 16C, lane 4). Taken together, my results show that the surface-localized conserved residues of the OAR such as the Plus3 residues are important for the chromatin association of Rtf1 and full levels of Rtf1-mediated histone modifications.

A.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37°C
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rtf1ΔOAR
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R273E
Y287A
R288E
K299A
K299E
K299Q
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S329E
R251E R273E
R251E R288E
R273E R288E
Y287A R288E
R288E S329E
R251E R273E R288E
R251E R273E K299E
R251E R273E S329A
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R251E R273E R288E K299E
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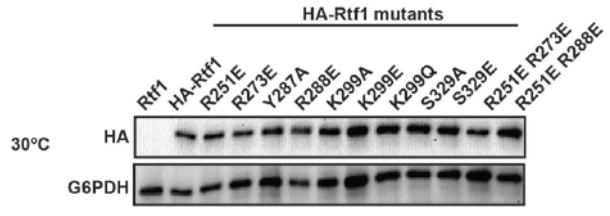
-His-Trp
-Trp
-His-Trp

B.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HA-Rtf1
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R273E
Y287A
R288E
K299A
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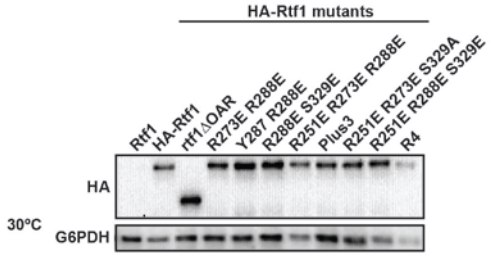
R251E R273E
R251E R288E
30°C
HA
G6PDH

C.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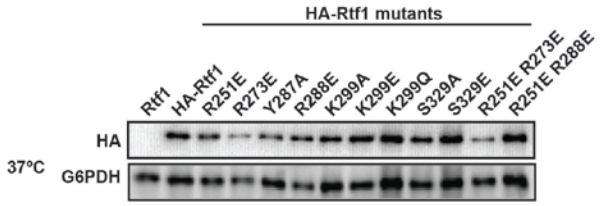
HA-Rtf1
rtf1ΔOAR
R273E R288E
Y287 R288E
R288E S329E
R251E R273E R288E
Plus3
R251E R273E S329A
R251E R288E S329E
R4
HA
30°C
G6PDH

D.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Rtf1
HA-Rtf1
R251E
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Y287A
R288E
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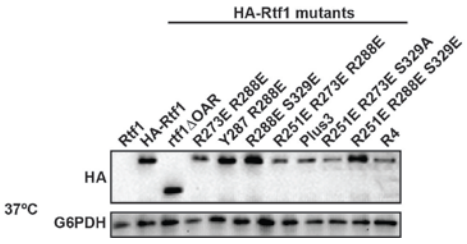
HA
37°C
G6PDH

E.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Rtf1
HA-Rtf1
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R273E R288E
Y287 R288E
R288E S329E
R251E R273E R288E
Plus3
R251E R273E S329A
R251E R288E S329E
R4
HA
37°C
G6PDH

F.

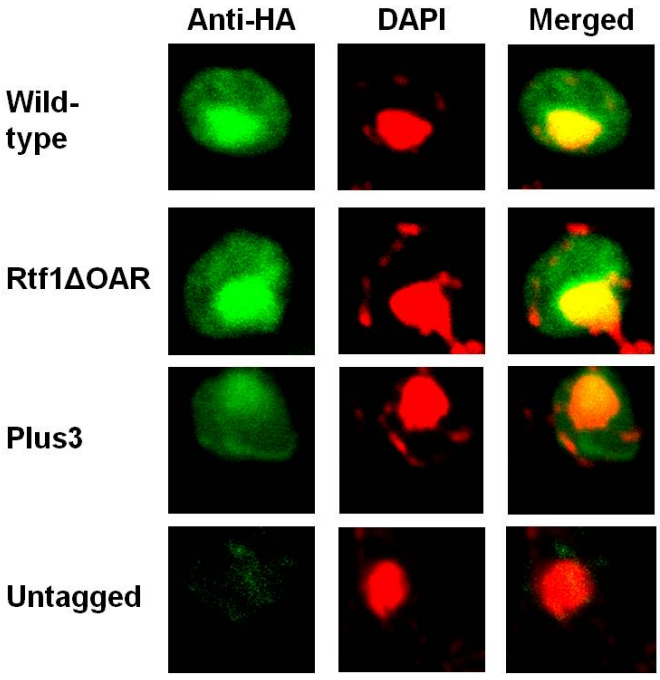
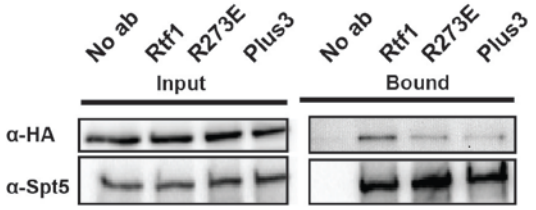

Anti-HA
DAPI
Merged
Wild-type
Rtf1ΔOAR
Plus3
Untagged

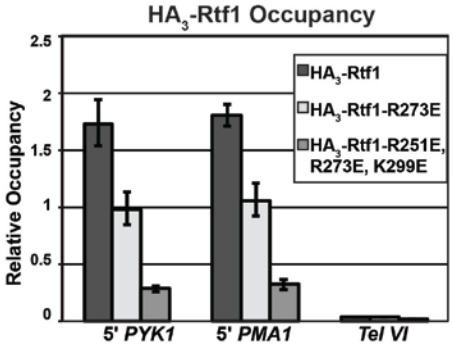
Figure 15. Mutations in the surface-localized conserved residues of the OAR of Rtf1 cause transcriptional defects

(A) An *rtf1Δ* strain (KY619) was transformed with derivatives of HA_3-Rtf1 (pLS21-5) having the indicated amino acid substitutions in the OAR. Tenfold serial dilutions of these mutants, ranging from 10^8 cells/ml to 10^4 cells/ml, were spotted on SD-His-Trp plates to assess their Spt^- phenotype and on SC-Trp as a growth control and incubated for four days at 30°C or 37°C. (B to E) Immunoblotting analysis was performed on strains used in A grown at either 30°C (B and C) or 37°C (D and E), with anti-HA antibody to determine Rtf1 protein levels. Extracts of an *rtf1Δ* strains (KY619) expressing untagged Rtf1 (pLS20) grown at 30°C or 37°C served a negative control for anti-HA western analysis. G6PDH levels were measured as a loading control. The R251, R273, R288 and K299 residues have been mutated to glutamic acid residue in the R4 mutant. (F) Indirect immunofluorescence confocal microscopy was used to determine the cellular localization of the indicated OAR mutants. Nucleus was stained using DAPI while the HA-tagged OAR mutants were visualized using antibody against the HA-tag and a fluorophore-conjugated anti-mouse secondary.

A.


No ab
Rtf1
R273E
Plus3
No ab
Rtf1
R273E
Plus3
Input
Bound
α-HA
α-Spt5

B.


HA3-Rtf1 Occupancy
Relative Occupancy
2.5
2
1.5
1
0.5
0
HA3-Rtf1
HA3-Rtf1-R273E
HA3-Rtf1-R251E, R273E, K299E
5' PYK1
5' PMA1
Tel VI

C.

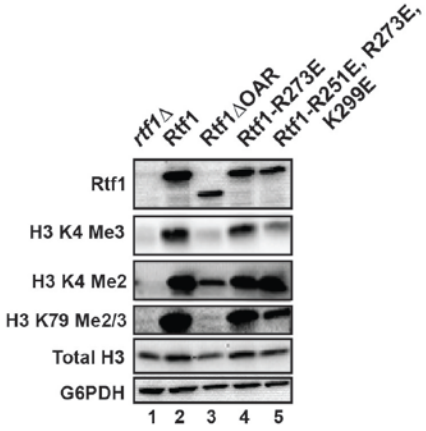

rtf1Δ
Rtf1
Rtf1ΔOAR
Rtf1-R273E
Rtf1-R251E, R273E, K299E
Rtf1
H3 K4 Me3
H3 K4 Me2
H3 K79 Me2/3
Total H3
G6PDH
1 2 3 4 5

Figure 16. The Plus3 residues of the OAR are important for the chromatin association of Rtf1 and Rtf1-dependent co-transcriptional histone modifications

(A) Spt5 was immunoprecipitated using an antibody against it from extracts of an *rtf1*Δ (KY619) strain expressing HA_3-Rtf1 (pLS21-5) or its specified mutant derivatives. Spt5 and Rtf1 were detected by immunoblotting with anti-Spt5 and anti-HA antibodies. An immunoprecipitation performed in parallel without the addition of anti-Spt5 antibody to the extracts of a strain expressing HA_3-Rtf1 (pLS21-5) served as a negative control. The results are representative of two separate experiments. (B) ChIP analyses of transformants of an *rtf1Δ* strain (KY619) bearing plasmids expressing HA_3-Rtf1 (pLS21-5) or the indicated mutant proteins, which were expressed from pLS21-5 derivatives, were performed using anti-HA beads to determine the occupancy of the Rtf1 proteins over 5' regions of *PYK1* and *PMA1* and a telomeric region of chromosome VI. Average of three biological replicates is shown with the error bars depicting the SEM. (C) Immunoblotting analyses were performed using indicated antibodies on transformants of an *rtf1*Δ (KY619) strain containing plasmids expressing HA_3-Rtf1 (pLS21-5), HA_3-Rtf1ΔOAR (pMM01) or the indicated mutant proteins, which were expressed from derivatives of pLS21-5. An *rtf1*Δ strain (KY619) transformed with empty vector (pRS314) was used as the negative control. G6PDH and total H3 levels serve as loading controls.

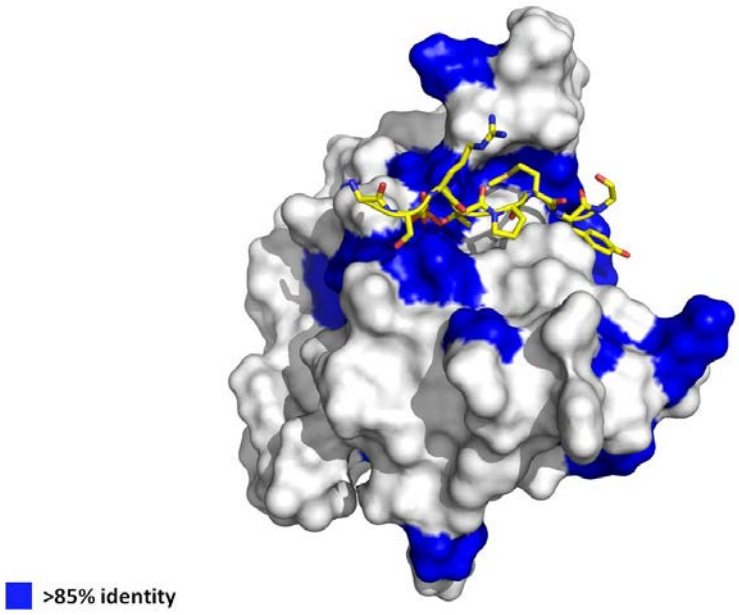
3.3.2 Structural basis for the interaction of the OAR /Plus3 region of Rtf1 with the CTR of Spt5

To determine the structural basis for the interaction of the OAR of Rtf1 and the Spt5 CTR and the importance of the phosphorylation of the CTR in this interaction, Adam Wier, Dr.Annie Heroux and Dr. Andrew VanDemark determined the structure of the human OAR-pCTR complex (Figure 17A). This crystal structure provides evidence that the OAR-CTR interaction that I discovered in *S. cerevisiae* is conserved in humans as well. The crystal structure shows that the Rtf1 OAR and the Spt5 pCTR peptide are held together partly via hydrogen bonds between surface localized conserved residues within the OAR including two of the Plus3 residues (Human Rtf1 residues R366, R388, Y400, S443, Q445) and the phosphate group of the phosphorylated threonine residue (pT784) within the CTR (Figure 17B). This co-crystal structure is consistent with my phenotypic analysis data, which suggested that *S. cerevisiae* Rtf1 residues R251 (a Plus3 residue), R273 (a Plus3 residue), Y287 and S329 corresponding to human Rtf1 residues R366, R388, Y400 and S443, respectively, are involved in functionally important multivalent interactions with its binding partner (Figure 15A, 16A and 16B).

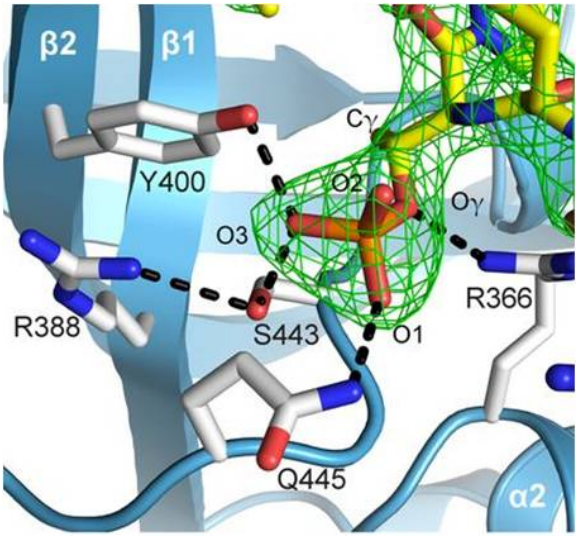
The other interactions tethering the Rtf1 OAR to the Spt5 pCTR peptide are the Van der Waals interactions between the hydrophobic residues of the human OAR/Plus3 (human Rtf1 residues N393, Y400, F437 and F441) and the Spt5-CTR (Figure 17C). This interaction involves two residues within the consensus human CTR sequence, G-S-R/Q-T-P (pT784 and P785) and two residues within the linker region (M786 and Y787) of the human Spt5 CTR (YAMADA *et al.* 2006). The length of this linker region is conserved in humans (two residues) (YAMADA *et al.* 2006). Although the residues within the linker region may vary, the binding pocket containing

the M786 is large enough to also accommodate other hydrophobic residues such as His or Leu, while the second binding pocket including the consensus residue Y787 can also fit Gln and His, the other residues found at this position in some of the linker regions of the CTR (WIER *et al.* 2013). Together, Adam Wier, Dr. Annie Heroux and Dr. VanDemark's work suggests that the association of the human OAR with the human CTR is held together by two sets of interactions- hydrophilic and hydrophobic interactions between the OAR and the phosphorylated CTR.

A.



B.



C.

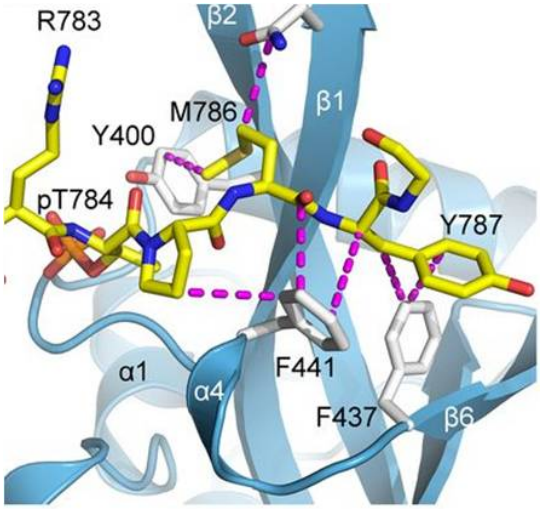


Figure 17. Rtf1 OAR and Spt5 CTR interaction is mediated by two sets of interactions

(A) The co-crystal of the human OAR with a peptide of Spt5 CTR. Shown in blue are the residues that are greater than 85% identical among the homologues of Rtf1. (B) The black dotted lines represent the hydrogen bonds mediating the interaction of the human OAR residues (white) Y400 (yeast Y287), R388 (yeast R273, one of the three plus3 residues), S443 (yeast S329), Q445 (not a conserved residue) and R366 (yeast R251, one of the three plus3 residues) with the phosphothreonine residue of the peptide of human Spt5 CTR (yellow). (C) The Van der Waals interactions between the human OAR residues (white) N393 (yeast T278), Y400 (yeast Y287), F441 (yeast Y327) and F437 (yeast F323) and the Spt5 CTR peptide (yellow) are depicted using pink dotted lines.

3.3.3 Residues of the OAR of Rtf1 mediating the human OAR-CTR interaction are also important for proper chromatin association in yeast

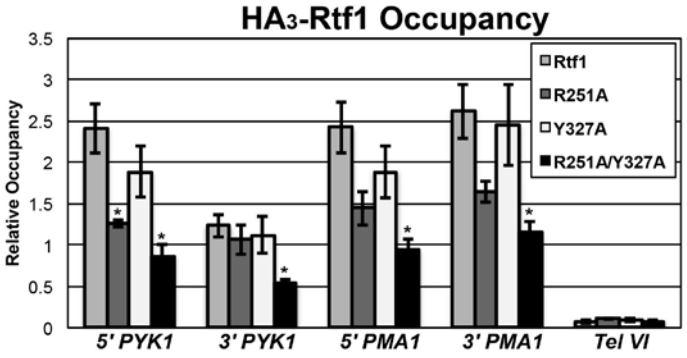
The structural and biochemical studies performed by Adam Wier, Dr. Annie Heroux and Dr. VanDemark identified amino acids R366 and F441 of human Rtf1 as being important for the Plus3-pCTR interaction *in vitro* due to their involvement in the hydrophilic and hydrophobic interactions holding the Plus3 domain and the CTR together. To test if these residues are important *in vivo* for the recruitment of Rtf1 to chromatin, I performed chromatin immunoprecipitation assays in *S. cerevisiae*. Individually, alanine substitutions for *S. cerevisiae* Rtf1 residues R251 and Y327, which correspond to human Rtf1 residues R366 and F441, respectively, caused a modest decrease in Rtf1 occupancy on two active genes, *PYK1* and *PMA1* (Figure 18A). Interestingly, when both the R251A and Y327A substitutions were incorporated into Rtf1, it caused a greater reduction in the chromatin occupancy of Rtf1. Consistent with the localization of the corresponding human Rtf1 residues on the surface of the OAR/Plus3 (Figure 17A), individual and simultaneous substitution of R251 and Y327 did not affect the stability of yeast Rtf1 (Figure 18B). These results indicate that the hydrophilic and hydrophobic interactions that mediate the OAR/Plus3-pCTR association in the co-crystal are indeed physiologically relevant and are important for the chromatin recruitment of Rtf1.

Previous studies have also implicated the carboxy-terminal domain of Cdc73 (Cdc73 C-domain) for recruitment of Paf1C to chromatin (AMRICH *et al.* 2012; QIU *et al.* 2012) in addition to the OAR of Rtf1, raising the possibility that the Cdc73 C-domain and the Rtf1 Plus3-Spt5 CTR interaction surface may function collaboratively to tether Paf1C to the transcription machinery. To test this possibility, I constructed yeast strains that express Cdc73 mutant protein lacking the C-domain or Rtf1 mutant protein having alanine substitutions at the R251 and Y327

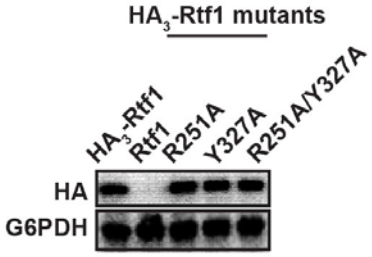
residues or strains expressing these mutant versions of Cdc73 and Rtf1 proteins. Chromatin occupancy of Cdc73 determined through the presence of the HA-tag on the full-length Cdc73 and Cdc73 lacking the C-domain, showed that simultaneous removal of the Cdc73 C-domain and the Spt5 CTR interacting residues within the OAR/Plus3 domain led to a greater defect in Paf1C chromatin localization than either single mutant condition (Figure 18C). Western analysis demonstrated that the mutations did not affect the stability of Cdc73 protein (Figure 18D). My ChIP analysis thus suggests that the Rtf1 OAR and the Cdc73 C-domain collaborate to tether Paf1C to active genes.

In addition to testing the contribution of both the OAR of Rtf1 and the C-domain of Cdc73 for chromatin association, I also tested the impact of deleting both the Rtf1 OAR and the Cdc73 C-domain on all the Paf1C-regulated histone modifications. Lack of the C-domain of Cdc73 alone caused only a modest reduction in Paf1C-mediated histone modifications, while lack of the OAR alone had a large impact on the H3 K4 Me3 and H3 K79 Me2/3 levels, a small effect on H3 K4 Me2 and no impact on H3 K36 Me3 levels. However, my immunoblotting analysis suggested that the deletion of both almost completely abolished all the histone modifications mediated by Paf1C (Figure 18E). Thus, my ChIP and western blot analyses further support the importance of the OAR/Plus3-Spt5 CTR interface in Paf1C recruitment and indicate that the association of Paf1C with the transcription machinery is facilitated by at least two attachments mediated by the OAR of Rtf1 and the C-domain of Cdc73.

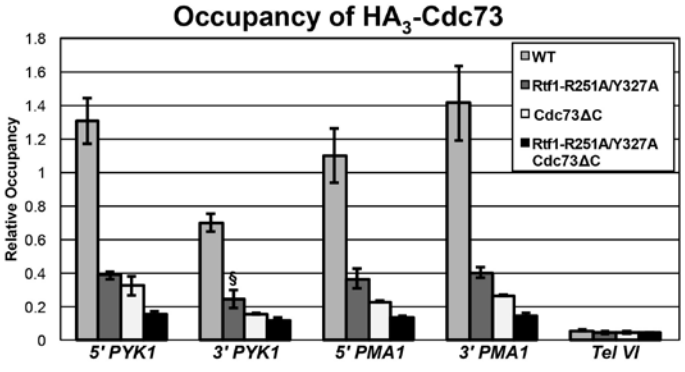
A.


HA3-Rtf1 Occupancy
Relative Occupancy
3.5
3
2.5
2
1.5
1
0.5
0
Rtf1
R251A
Y327A
R251A/Y327A
5' PYK1
3' PYK1
5' PMA1
3' PMA1
Tel VI

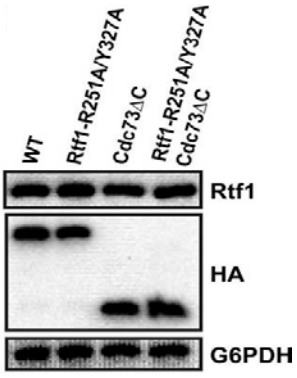
B.


HA3-Rtf1 mutants
HA3-Rtf1
Rtf1
R251A
Y327A
R251A/Y327A
HA
G6PDH

C.


Occupancy of HA3-Cdc73
Relative Occupancy
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
Rtf1-R251A/Y327A
Cdc73ΔC
Rtf1-R251A/Y327A Cdc73ΔC
5' PYK1
3' PYK1
5' PMA1
3' PMA1
Tel VI

D.


WT
Rtf1-R251A/Y327A
Cdc73ΔC
Rtf1-R251A/Y327A Cdc73ΔC
Rtf1
HA
G6PDH

E.

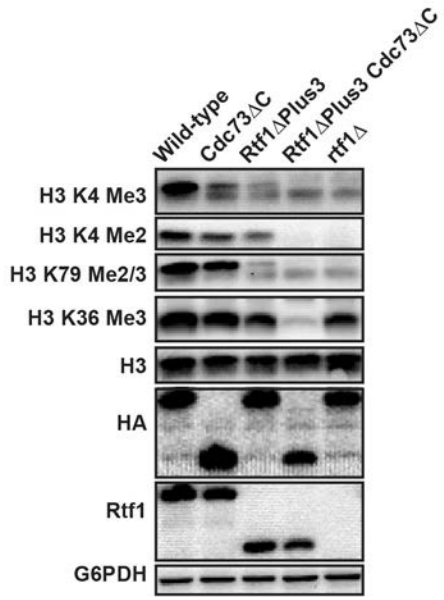


Figure 18. The OAR of Rtf1 and the C-domain of Cdc73 collaborate to mediate the chromatin association of Paf1C and hence facilitate Paf1C-mediated histone modifications

(A) ChIP analysis was performed on transformants of an *rtf1*Δ strain (KY619) containing plasmids expressing the indicated HA-tagged derivatives of Rtf1. Error bars represent the SEM from three biological replicates, and asterisks denote p-values of <0.05. (B) Western blot analysis of extracts prepared from yeast transformants used in (A) using α-HA antibody to determine the levels of Rtf1 protein. G6PDH levels serve as the loading control. (C) ChIP analysis of HA_3-Cdc73 was performed on transformants of an *rtf1*Δ*cdc73*Δ (KY2417) strain carrying plasmid expressing full-length HA-tagged Cdc73 (pWR4) or Cdc73 lacking the C-domain (pCD8) and plasmid encoding full-length Rtf1 (pMM61) or R251A Y327A mutant of Rtf1. The error bars indicate the SEM. The chromatin occupancy of all the single mutants is significantly different (p>0.05) compared to wild-type and Rtf1-R251A, Y327A Cdc73ΔC mutant at 5' *PYK1*, 3' *PYK1*, 5' *PMA1* and 3' *PMA1* except where indicated (§). (D) Immunoblotting analysis of the extracts of strains used in (C), using α-Rtf1 and α-HA to determine the Rtf1 and Cdc73 protein levels. G6PDH levels were used as the loading control. (E) Western blot analysis was performed on transformants of an *rtf1*Δ *cdc73*Δ (KY2417) strain containing plasmid expressing full-length HA-tagged Cdc73 (pWR4) or Cdc73 lacking the C-domain (pCD8) and plasmid encoding full-length Rtf1 (pMM61) or Rtf1 lacking the OAR (pMM62) or empty vector (pRS316) using the indicated antibodies.

3.4 DISCUSSION

Sequence alignments indicate that the OAR is the most highly conserved region of Rtf1 across eukaryotes (WARNER *et al.* 2007). This conservation suggests that the OAR makes key contribution(s) to the function of Paf1C, which in higher eukaryotes has crucial roles in preventing cancer, promoting stem cell pluripotency, and ensuring proper cellular differentiation and organismal development [reviewed in (TOMSON and ARNDT 2013)]. In this study, we sought to identify the residues of the OAR that are important for its function. My work described in the previous chapter, provided a mechanism for the chromatin association of Paf1C through the OAR of Rtf1 where I found that an interaction of the OAR of Rtf1 with the phosphorylated CTR of Spt5 tethers Paf1C to actively transcribed genes. Performing a genetic screen for suppressors of a hypofunctional OAR could lead to the identification of additional regulators of chromatin association of Paf1C. Thus uncovering the functionally important residues within the OAR could lead to the identification of an OAR mutant that might be a useful for genetic screens in the future.

To identify the functionally important residues, I took a mutational analysis approach. We anticipated that conserved residues on the surface would play a functionally important role. Upon mutating conserved residues on the surface of the OAR to oppositely charged residues or alanine residues, I observed that these substitutions caused Spt$^-$ phenotype, indicative of transcriptional defects (Figure 14A and B and 15A). My co-immunoprecipitation analysis indicated that the interaction of these mutant Rtf1 proteins with Spt5 was impaired (Figure 16A). My ChIP analysis showed that the strength of the Spt$^-$ phenotype caused by the substitutions, which is indicative of transcriptional defects correlated with the degree of defect in chromatin-association, suggesting that the defect in chromatin association was the cause of the Spt$^-$

phenotype (Figure 16B). The single-residue OAR/Plus3 mutant (Rtf1-R273E) which caused a weak Spt$^-$ phenotype, caused a small defect in the chromatin association of Paf1C, while the triple-residue OAR/Plus3 mutant which caused a strong Spt$^-$ phenotype, caused a greater defect in the ability of Paf1C to associate with actively transcribed genes. My immunoblotting analysis with these mutants suggested that a large chromatin association defect causes only a small defect in the levels of H3 K4 Me3 and H3 K79 Me2/3 and no defect in H3 K4 Me2 levels and a two-fold defect in chromatin association is not enough to influence the level of histone modifications (Figure 16C). These results suggest that low levels of chromatin-associated Paf1C are sufficient for the regulation of histone modifications by Paf1C.

The molecular details about the interactions involved in the association of the Rtf1 OAR/Plus3 with the Spt5 CTR were provided by determining the structure of the OAR/Plus3-CTR co-crystals (Figure 17A). This co-crystal structure supports that the interaction between the OAR and the phosphorylated CTR that I uncovered, is also conserved in humans. The co-crystal structure revealed that the OAR of Rtf1 and the CTR of Spt5 are held together by two sets of interactions. A set of hydrophilic interactions were mediated by the phosphorylated residue within the CTR and the conserved surface-localized polar residues of the OAR, that I had also identified as being important for preventing transcriptional defects through mutational analysis (Figure 17B). These hydrophilic interactions with the phosphorylated residues also provided an explanation for the impaired interaction of the OAR with mutant Spt5 having a non-phosphorylatable CTR (Spt5-S1-15A mutant) (Figure 10C). Additionally, a set of Van der Waals interactions are mediated by the non-polar residues within the CTR and the aromatic residues within the OAR (Figure 17C). When I mutated residues of *S. cerevisiae* Rtf1 (R251 and Y327) corresponding to residues of the human OAR (R366 and F441) involved in the hydrophilic

and/or Van der Waals interactions, I observed a reduction in the chromatin occupancy of Paf1C (Figure 18A), thus suggesting that the interactions observed in the OAR-CTR crystal structure are indeed functionally important.

In a screen for conditional *rtf1* mutations, we previously identified two point mutations that alter residues within the OAR and confer Spt$^-$ phenotype at 37°C (SHELDON *et al.* 2005). The crystal structure of the human Rtf1 Plus3 domain indicated that the corresponding human residues are buried within the protein and substitution of these residues is likely to destabilize the core of the structure. In contrast, the Plus3 residues that I mutated here are surface-exposed. Interestingly, *in vitro* studies performed with the human Plus3 domain suggested that this domain has the ability to bind single stranded DNA (DE JONG *et al.* 2008), suggesting models in which this domain interacts with the Pol II transcription bubble. Our data strongly suggest that the *S. cerevisiae* Rtf1 OAR interacts with the phosphorylated CTR of Spt5. However, we have not ruled out the possibility that the OAR could mediate recruitment of Paf1C through additional mechanisms such as through interactions with single-stranded DNA.

Previous studies had indicated that the C-domain of the Cdc73 subunit of Paf1C was also important for the chromatin association of Paf1C (AMRICH *et al.* 2012; QIU *et al.* 2012). However, deletion of C-domain alone did not greatly affect the histone modification function of Paf1C (AMRICH *et al.* 2012). This result is consistent with my observations that the OAR mutants that cause significant chromatin association defects have much less bearing on the levels of histone modifications. Although I could not detect any chromatin association of Paf1C lacking the OAR by ChIP analysis, I could still observe low levels of Rtf1-mediated histone modification and the levels of an Rtf1-independent, Paf1-mediated histone modification (H3 K36 Me3) was unaffected by the lack of OAR. I reasoned that these low levels of histone modifications were a

result of very low levels (undetectable by my ChIP analysis) of chromatin-associated Paf1C and the small reduction in chromatin association levels caused by the C-domain were not enough to impact the regulation of histone modifications by Paf1C. Hence, I hypothesized that removing two attachment points of Paf1C, the OAR of Rtf1 and the C-domain of Cdc73 would abolish all Paf1C-mediated histone modifications. My immunoblotting analysis is consistent with this hypothesis (Figure 18E). My ChIP analysis with mutations in both the OAR and the C-domain also suggested that the OAR and C-domain collaborate to facilitate the chromatin association of Paf1C (Figure 18C).

Collectively my results described in this Chapter, provide *in vivo* evidence to support the OAR-CTR co-crystal structure and a dual-attachment hypothesis in which Paf1C is tethered to actively transcribed genes through the OAR of Rtf1 and C-domain of Cdc73. My data indicate that the OAR is a major contributor to chromatin recruitment of Paf1C. The removal of both the Rtf1 OAR and the Cdc73 C-domain can almost completely detach Paf1C from chromatin as indicated by the almost complete loss of all Paf1C-mediated histone modifications in the immunoblotting analysis.

4.0 THE HMD OF RTF1 IS SUFFICIENT TO REGULATE CO-TRANSCRIPTIONAL HISTONE MODIFICATIONS

Some of the figures in this Chapter have been taken from published material and reprinted with some changes (PIRO *et al.* 2012). I have performed all the experiments for the figures shown in this Chapter. The work of others required for understanding the rationale behind those experiments has been summarized without including the corresponding figures and citing the paper wherever necessary.

4.1 INTRODUCTION

Eukaryotic transcription occurs within restrictive chromatin that can be dynamically modified during transcription. One of the major mechanisms through which chromatin can be altered is through the post-translational modification of histones. These modifications can either create docking sites for proteins that recognize these marks and hence facilitate their recruitment or alter the strength of DNA-histone interactions enhancing or diminishing the accessibility of DNA. Acetylated and methylated histones can target proteins containing bromodomains or chromodomains, respectively, to chromatin [reviewed in (SMITH and SHILATIFARD 2010)]. The recognition by these chromatin-binding proteins is highly specific and dependent on the number of groups of each modification that are covalently conjugated to histones and the residue

modified. Proteins with closely related recognition domains can have different specificities and hence different outcomes. For example, the tri-methylation of H3 K4 residues serves as a docking site for the PHD domain of the NuA3 histone acetyltransferase complex (TAVERNA *et al.* 2006). Since this mark predominates near the 5' end of genes it helps maintain histones in an acetylated state in the regions close to the promoter. Since this modification also weakens the electrostatic interaction between the histone and DNA, it makes the DNA more accessible to the transcriptional activators and transcription machinery (TAVERNA *et al.* 2006). The H3 K4 Me2 mark, on the other hand, is recognized by the PHD domain within the histone deacetylase complex known as the Set3 complex (KIM and BURATOWSKI 2009). Since this mark is enriched downstream of the H3 K4 Me3 modification, it maintains the histones in that region in the deacetylated state and prevents spurious initiation of transcription from intragenic regions in the wake of Pol II (KIM and BURATOWSKI 2009). Histones can also be subjected to additional modifications such as phosphorylation, ubiquitination and sumoylation.

The chromatin alterations that influence the transit of Pol II through a chromatin template are regulated by transcription elongation factors coupled to the transcription machinery. One such elongation factor is the Paf1 transcription elongation complex. The Paf1 complex regulates tri-methylation of H3 K36 by facilitating the recruitment of H3 K36 methyltransferase Set2 (CHU *et al.* 2007). Additionally, the Rtf1 subunit of Paf1C is required for the monoubiquitination of H2B K123 (WOOD *et al.* 2003). This mark is a pre-requisite for downstream H3 K4 di- and tri-methylation and H3 K79 di- and tri-methylation (DOVER *et al.* 2002; KROGAN *et al.* 2003a; KROGAN *et al.* 2003b; NG *et al.* 2003a; NG *et al.* 2003b; WOOD *et al.* 2003). The Paf1 complex can facilitate these modifications by facilitating the recruitment of the ubiquitin-conjugating

enzyme Rad6, the ubiquitin ligase Bre1 and the H3 K4 methyltransferase Set1 (KIM and ROEDER 2009; KROGAN *et al.* 2003a; NG *et al.* 2003b; WOOD *et al.* 2003; XIAO *et al.* 2005).

Functional analysis using sequential internal deletion derivatives of Rtf1 resulted in the identification of a region within Rtf1 that is necessary for facilitating these Rtf1-mediated histone modifications (WARNER *et al.* 2007). We hypothesized that this region within Rtf1, which we termed the histone-modification domain (HMD), may play a direct role in facilitating these co-transcriptional histone modifications. To test this hypothesis, we determined if the HMD could regulate these modifications on its own with/without being tethered to a chromatin-targeting domain, the Gal4 DNA binding domain (GBD), and attempted to uncover the interactions mediated by the HMD. We found that the HMD on its own was sufficient to perform Rtf1-mediated histone modifications even without being tethered to the GBD. However, the lack of association of the HMD with the rest of Paf1C may lead to the mistargeting of these marks to transcriptionally inactive loci. The HMD can facilitate Rtf1-dependent co-transcriptional histone modifications through interaction with chromatin (PIRO *et al.* 2012) and possibly Bre1. Our findings suggest that Paf1C may play an active role in facilitating co-transcriptional histone modifications.

4.2 MATERIALS AND METHODS

4.2.1 Yeast strains and growth

Yeast strains (Table 5) are isogenic to FY2 (WINSTON *et al.* 1995) and were generated through transformation or tetrad analysis (ROSE *et al.* 1990). Rich (YPD) and synthetic complete (SC) media were prepared as previously described (ROSE *et al.* 1990).

4.2.2 Plasmids

Plasmids pGBKT7 and pGBT9 are commercially available plasmids. For the rest of the plasmids, the second and the third letters in the names of the plasmids indicate the initials of the person who constructed them. Specifically, I constructed the "pMM" plasmids. "pMW" plasmids were made by Dr. Marcie Warner and "pAP" plasmids were made by Anthony Piro. The "pLS" and "pPC" plasmids were made by Lori Stolinski and Dr. Patrick Costa (SQUAZZO *et al.* 2002; STOLINSKI *et al.* 1997). Plasmid pRG147 is a gift from Dr. Richard Gardner.

pMW8 and pAP44 are derivatives of pGBKT7 (Clontech). pMW8 expresses an amino-terminal fusion of GBD-Myc to amino acids 63-152 of *S. cerevisiae* Rtf1 (GBD-Myc-HMD). To construct pMW8, PCR was performed to amplify base pairs 187-456 of *RTF1* using plasmid pLS21-5 (STOLINSKI *et al.* 1997) as a template and primers that introduce NdeI and EcoRI sites at the 5' and 3' ends of the product, respectively. The resulting PCR product was cloned into the polylinker of pGBKT7. pAP44 is analogous to pMW8, expresses the full-length Rtf1 protein as a fusion to GBD-Myc (GBD-Myc-Rtf1), and was constructed in the same manner. pLS28

(SIMIC *et al.* 2003) is a derivative of pGBT9 (Clontech) and expresses an amino-terminal fusion of the GBD to the full-length Rtf1 protein (GBD-Rtf1).

pAP37 expresses an amino-terminal fusion of the Large T antigen nuclear localization signal (NLS) to the c-Myc epitope. To construct pAP37, oligonucleotides corresponding to the sense and antisense strands of the NLS were hybridized, phosphorylated using T4 polynucleotide kinase, and ligated to MluI/SpeI digested pAP36. pAP39 expresses an amino-terminal fusion of NLS-Myc to Rtf1 residues 63-152 (NLS-Myc-HMD), and was derived from pMW8 in the same step-wise-manner as pAP37 was derived from pGBKT7. pAP45 expresses the c-Myc epitope fused to the full-length Rtf1 protein (Myc-Rtf1), and was constructed by amplifying the *RTF1* coding sequence from pLS21-5, as for pAP44, and cloning it into pAP36 using the *Nde*I and *Eco*RI sites present within the polylinker.

pMM38 is a derivative of the *CEN/ARS* plasmid pLS20 (STOLINSKI *et al.* 1997) and directs the expression of the NLS-Myc-HMD (Rtf1 residues 63-152) under the control of the *RTF1* promoter. A fragment containing the NLS-Myc-HMD coding sequence was amplified by PCR from template pAP39 using primers that introduce *Nco*I and *Eco*RI restriction sites at the 5' and the 3' ends of the product, respectively. After digestion of the PCR product with *Nco*I and *Eco*RI, the ends were made blunt by treatment with Klenow and then ligated to pLS20, which had been digested with *Nde*I and *Afl*II to remove the *RTF1* coding region and blunt-ended with Klenow. pMM39 encodes NLS-Myc-HMD under the control of the *ADH1* promoter and is also a derivative of pLS20 (STOLINSKI *et al.* 1997). A DNA fragment containing the *ADH1 promoter-NLS-Myc-HMD* sequence was amplified from pAP39 using primers that introduce *Sal*I and A*fl*II restriction sites at the 5' and the 3' ends of the product, respectively. The PCR product was then ligated to pLS20 that had been digested with *Sal*I and *Afl*II to remove the *RTF1* coding

region and 800 bp of 5’ sequence. pMM40 encodes full-length Rtf1 under the control of the *ADH1* promoter. The *ADH1* promoter sequence was PCR-amplified from pGBKT7 with primers that introduce *Sal*I and *Nde*I restriction sites at the 5' and the 3' ends of the product, respectively. The PCR product was then ligated to pLS20, which had been digested with *Sal*I and *Nde*I to remove 800 bp upstream of the *RTF1* coding region. All final plasmids were confirmed by DNA sequencing.

pPC59 encodes C-terminally TAP-tagged full length Rtf1 (SQUAZZO *et al.* 2002). pMW11 encoding TAP-tagged derivative of Rtf1, lacking the region encoding PID (Paf1C interaction domain, residues 491-558), was created by introducing the TAP tag at the last amino acid of region 11 (residue 490) by homologous recombination. Plasmid pMW13 encodes Rtf1Δ3-TAP (lacks Rtf1 residues 62-109) and it was obtained by site-directed mutagenesis using pPC59 as the template. Plasmid pMW14 encodes Rtf1Δ3ΔPID-TAP (lacks Rtf1 residues 62-109 and residues 491-558) and was made using site-directed mutagenesis of plasmid pMW11. Plasmid pRG147 is a *URA3*-marked 2 micron plasmid expressing HA_3-tagged ubiquitin (gift of Dr. Richard Gardner).

4.2.3 Immunoblotting Analysis

TCA extracts were made and western blot analysis was performed as described in Chapter 2 (section 2.2.3). Nitrocellulose or PVDF membranes containing proteins transferred from the SDS-polyacrylamide gels were probed with anti-H3 K4 Me3 (1:2,000; Active Motif 39159), anti-H3 K4 Me2 (1:2,000; Millipore 07-030;), anti-H3 K79 Me2/3 (1:1,000; Abcam ab2621), anti-H3 (TOMSON *et al.* 2011), anti-G6PDH (1:30,000; A9521 Sigma), anti-Gal4 (DBD) (1:000; sc-577), anti-FLAG (1:1000;), primary antibodies, followed by incubation with donkey anti-

rabbit horseradish peroxidase-conjugated secondary antibody (1:5,000, GE Healthcare) or sheep anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5000; GE Healthcare).

4.2.4 Chromatin Immunoprecipitation

ChIP analyses described in this Chapter were performed as described in Chapter 2 (section 2.2.5). Immunoprecipitation was performed using Rtf1 antiserum (SQUAZZO *et al.* 2002) followed by incubation by protein A-conjugated agarose (GE Healthcare). Quantitative real-time PCR was performed on immunoprecipitated DNA using Maxima SYBR Green/ROX qPCR mix (Fermentas) in an Applied Biosystems 7300 Real Time PCR machine, and data analysis was performed using 7300 System SDS Software. The association of immunoprecipitated protein was examined at the 5' end of *PYK1* (+253 to +346; ATG = +1), *GAL7* UAS (-238 to -128; ATG = +1) or the telomere on the right arm of chromosome VI (coordinates: 269495/269598). Immunoprecipitated chromatin from three independent transformants was analyzed. Graphs represent $(\text{primer set efficiency})^{(Ct\ Input-\ Ct\ IP)}$ and error bars represent the standard error of the mean for the three biological replicates.

A sequential ChIP assay was used to measure chromatin-associated levels of H2B K123 ubiquitination (CHANDRASEKHARAN *et al.* 2011). Chromatin was prepared from transformants of yeast strain KY2085, containing plasmids pAP37, pAP39, or pAP45 and pRG147. Strain KY2086, which expresses FLAG-tagged H2B K123R, served as a control to demonstrate that the ubiquitination signal was dependent on K123. Chromatin was subjected to 20 cycles of sonication using a Misonix 3000 sonicator. The first two cycles were performed at an output setting 2, and the remaining cycles were performed at an output setting 4. Each cycle consisted of 20 seconds of sonication followed by a 1 min rest. The solubilized chromatin was recovered

by centrifugation. FLAG-tagged H2B was immunoprecipitated from 2.5 mg solubilized chromatin using anti-FLAG antibody (Sigma) and protein G beads and then eluted using 3XFLAG peptide (Sigma) as described (CHANDRASEKHARAN *et al.* 2011). 50 µl of the eluate were set aside as input and 400 µl were incubated with anti-HA conjugated agarose (sc-7392AC) overnight at 4^{o}C with end-over-end mixing. Beads were washed twice with FA140 buffer (CHANDRASEKHARAN *et al.* 2011), once with buffer containing 10 mM Tris-HCl, pH 8, 0.25 M LiCl, 1 mM EDTA, 0.5% NP-40 and 0.5% sodium deoxycholate and once with buffer containing 10 mM Tris-HCl, pH 8 and 1 mM EDTA. The immunoprecipitated chromatin was eluted from the beads, pronase-treated and reverse-crosslinked as described (KURAS and STRUHL 1999). The immunoprecipitated DNA from two independent chromatin preparations was used in quantitative real-time PCR reactions performed with SYBR Green (Fermentas). Mean values are shown with error bars representing the range of the measurements.

4.2.5 Purification of TAP-tagged proteins

One step-affinity purification and mass spectrometry analysis was performed as described in Chapter 2 (section 2.2.6). However, for the targeted approach to detect the interaction of the HMD with Bre1 protein, the bead-bound proteins were not eluted using TEV protease. Instead, the washed beads were boiled for 5 minutes after resuspension in SDS loading buffer. They were then resolved on SDS polyacrylamide gels, transferred to nitrocellulose membranes and probed with anti-FLAG (1:1000; Sigma) and anti-TAP tag (1:2500; Thermo Scientific CAB 1001) primary antibodies and sheep anti-mouse and donkey anti-rabbit secondary antibodies (1:5000 dilution, GE Healthcare), respectively. They were then visualized using enhanced chemiluminescence substrate (Perkin Elmer).

Table 5. *Saccharomyces cerevisiae* strains used for the study in this Chapter

Strain	Genotype
KY619	*MAT***a** *rtf1Δ102::ARG4 arg4-12 his4-912∂ leu2Δ1 lys2-173R2 trp1Δ63*
KY1021	*MAT***a** *his4-912∂ leu2Δ1 lys2-128∂ trp1Δ63*
KY1258	*MAT***a** *rtf1Δ::URA3 RAD6-13xMYC::KanMX leu2Δ1 ura3-52 trp1Δ63*
KY1876	*MAT***a** *leu2Δ1 ura3-52 trp1Δ63 arg4-12 gal4Δ::LEU2 rtf1Δ::NatMx HSV-SET1 BRE1-FLAG RAD6-13xMYC::KanMX*
KY2084	*MATα rtf1Δ::KanMX4 hta1-htb1Δ::LEU2 hta2-htb2Δ::KanMX ubp8Δ::NatMX his3Δ200 leu2Δ1 lys2-128∂ trp1Δ63 ura3-52 pJH23WT =[HTA1-HTB1/HIS3/CEN/ARS/Amp^R]*
KY2085	*MATα rtf1Δ::KanMX4 hta1-htb1Δ::LEU2 hta2-htb2Δ::KanMX ubp8Δ::NatMX his3Δ200 leu2Δ1 lys2-128∂ trp1Δ63 ura3-52 pJH23FL =[HTA1-FLAG-HTB1/HIS3/CEN/ARS/Amp^R]*
KY2086	*MATα hta1-htb1Δ::LEU2 hta2-htb2Δ::KanMX ubp8Δ::NatMX his3Δ200 leu2Δ1 lys2-128∂ trp1Δ63 ura3-52 pCD2 =[HTA1-FLAG-HTB1-K123R/HIS3/CEN/ARS/Amp^R]*
KY2123	*MAT***a** *rtf1Δ::KanMX4 gal4Δ::LEU2 leu2Δ1 lys2-128∂ trp1Δ63 ura3-52*
KY2124	*MAT***a** *rtf1Δ::KanMX4 hta1-htb1Δ::LEU2 hta2-htb2Δ::KanMX his3Δ200 leu2Δ1 lys2-128∂ trp1Δ63 ura3-52 pJH23WT =[HTA1-HTB1/HIS3/CEN/ARS/Amp^R]*
KY2125	*MAT***a** *rtf1Δ::KanMX4 hta1-htb1Δ::LEU2 hta2-htb2Δ::KanMX his3Δ200 leu2Δ1 lys2-128∂ trp1Δ63 ura3-52 pJH23FL =[HTA1-FLAG-HTB1/HIS3/CEN/ARS/Amp^R]*
KY2195	*MATα rtf1Δ101::LEU2 cdc73Δ::KanMX4 his4-912∂ leu2Δ1 trp1Δ63*
KY2232	*MAT***a** *rtf1Δ::KanMX paf1Δ::URA3 hta1-htb1Δ::LEU2 hta2-htb2Δ::KanMX ubp8Δ::NatMX his3Δ200 leu2Δ1 lys2-128∂ trp1Δ63 ura3-52 pJH23FL =[HTA1-FLAG-HTB1/HIS3/CEN/ARS/Amp^R]*
KY2234	*MATα rtf1Δ::KanMX ctr9Δ::URA3 hta1-htb1Δ::LEU2 hta2-htb2Δ::KanMX ubp8Δ::NatMX his3Δ200 leu2Δ1 lys2-128∂ trp1Δ63 ura3-52 pJH23FL =[HTA1-FLAG-HTB1/HIS3/CEN/ARS/Amp^R]*

GHY1144*	*MAT***a** *rtf1Δ101::LEU2 leo1Δ::URA3 his4-912∂ leu2Δ1 lys2-128∂ trp1Δ63 ura3-52*

*provided by Dr. Grant Hartzog

4.3 RESULTS

4.3.1 The Rtf1 HMD is sufficient to promote Rtf1-dependent histone modifications

Previous studies involving functional analysis of sequential internal deletion derivatives of Rtf1 led to the identification of a region within Rtf1 (Rtf1 residues 62-152) that when deleted leads to the loss of Rtf1-dependent histone modifications including H2B K123 Ub, H3 K4 Me3 and H3 K79 Me2 and hence this region was termed the histone modification domain (HMD) (WARNER *et al.* 2007). This finding laid the foundation for future work on the HMD in the Arndt laboratory that was aimed at exploring the mechanism by which the HMD facilitates histone modifications. This functional analysis of Rtf1 had also led to the identification of a central region within Rtf1 that was important for the chromatin association of Paf1C (WARNER *et al.* 2007). Based on these results, it was hypothesized that if the HMD was expressed in conjunction with a chromatin-targeting domain such as the Gal4 DNA binding domain (GBD), which can bind to the *GAL7* upstream activating sequence (UAS), it could facilitate histone modifications at that region (*GAL7* UAS).

ChIP analysis and immunoblotting analysis performed with the GBD-Myc-HMD expressed under the *ADH1* promoter suggested that the Rtf1 HMD (Rtf1 residues 63-152) can indeed bind to the *GAL7* UAS and facilitate Rtf1-dependent co-transcriptional histone modifications including H3 K4 Me3 and H3 K79 Me2 at this locus (PIRO *et al.* 2012). Interestingly, ChIP analysis also suggested that the fusion protein of the HMD with the GBD also facilitated these modifications at other transcriptionally active loci not normally bound by

the GBD (PIRO *et al.* 2012). The appearance of Rtf1-mediated histone modifications at the *PYK1* gene suggested that the GBD-HMD may have the ability to bind this locus. Since the GBD does not specifically bind to the *PYK1* gene, it was anticipated that the overexpression of either the GBD or the HMD could be leading to association of the GBD-HMD protein with the locus. To confirm that the GBD-HMD could bind to loci other than the *GAL7* UAS, I performed ChIP analysis on the GBD-HMD protein. As expected, the GBD-HMD protein showed high levels of occupancy at the *GAL7* UAS. However, it also showed binding to the *PYK1* gene and a telomeric region although at much lower levels compared to that at the *GAL7* UAS (Figure 19A).

To test the possibility that the binding of the GBD-HMD fusion protein to loci other than the *GAL7* UAS was mediated by the HMD, the region encoding the HMD was fused to a nuclear localization signal (NLS) to direct the HMD to the nucleus. Immunoblotting and ChIP analyses were then performed with the NLS-Myc-HMD protein. Just like the GBD-HMD (Figure 19A), the NLS-Myc-HMD protein bound to the active genes and facilitated H3 K4 Me3 and H3 K79 Me2/3 at these loci (PIRO *et al.* 2012). Additionally, it also bound non-transcribed loci and facilitated low level of H3 K79 Me2/3 at a non-transcribed locus (PIRO *et al.* 2012). Thus, the HMD may have the ability to bind to chromatin in a non-specific manner, which subsequently may lead to the targeting of a co-transcriptional histone modification at a non-transcribed locus. Consistent with the ChIP analysis, my western blot analysis also suggests that the NLS-Myc-HMD protein also facilitates H3 K4 Me3, H3 K4 Me2 and H3 K79 Me2/3 at global levels (Figure 19B).

We wanted to determine if the HMD was facilitating these methylation marks through the known pathway for H3 K4 Me2/3 and H3 K79 Me2/3, which requires the H2B K123 Ub mark as a pre-requisite (DOVER *et al.* 2002; NG *et al.* 2002b). Hence, I performed ChIP analysis and

western blot analyses to determine the levels of H2B K123 Ub mark at an active gene and the global levels of H2B K123 Ub in cells expressing NLS-Myc-HMD protein. I performed sequential ChIP analysis using cells expressing FLAG-tagged H2B and overexpressing HA-tagged ubiquitin on plasmids to determine the levels of H2B K123 Ub at the *PYK1* gene, since we lacked an antibody specific for the H2B K123 Ub mark and a good antibody against ubiquitin. The presence of FLAG-tagged H2B allowed me to immunoprecipitate H2B from crosslinked chromatin of these cells using an antibody against the FLAG tag. I then immunoprecipitated ubiquitinated H2B from this immunoprecipitated fraction using an antibody against the HA tag of the overexpressed ubiquitin. Strains expressing FLAG-tagged H2B K123 that cannot be ubiquitinated at lysine 123 (H2B K123R) and overexpressing HA-tagged ubiquitin served as a negative control in this sequential ChIP analysis since these cells lack the H2B K123 Ub mark. Consistent with the ability of the NLS-Myc-HMD protein to facilitate methylation of H3 K4 and H3 K79, my sequential ChIP analysis also indicated that the NLS-Myc-HMD protein can promote monoubiquitination of histone H2B at lysine 123 at an active gene (Figure 19C).

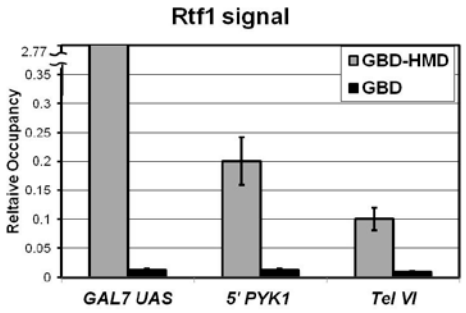
To confirm the rescue of the H2B K123 Ub mark by the NLS-Myc-HMD protein at the global level, I performed western blot analysis with an antibody against the FLAG tag in strains expressing FLAG-tagged histone H2B to visualize both the unmodified H2B as well as monoubiquitinated H2B. Since monoubiquitinated H2B runs slower than the unmodified H2B on a 15% SDS-PAGE gel it can be differentiated from unmodified H2B. An *rtf1*Δ strain containing the vector alone serves as a negative control for H2B K123 Ub in this western analysis since *rtf1*Δ strains are incapable of monoubiquitinating H2B K123 [reviewed in (CRISUCCI and ARNDT 2011)] and hence the extracts of these strains lack the lower mobility band corresponding to the ubiquitinated form of H2B K123 (Figure 19D, lane 2). A strain expressing untagged H2B served

as negative control for the FLAG western analysis since they lack the bands corresponding to both the unmodified H2B and H2B that is monoubiquitinated at lysine 123 (Figure 19D, lane 1). In accordance with my ChIP analysis, my western blot analysis also suggested that the HMD can facilitate H2B K123 Ub at a global level (Figure 19D, lane 3).

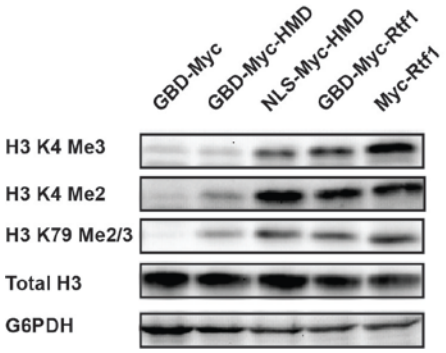
However, I found that global levels of H3 K4 Me3, H3 K4 Me2 and H3 K79 Me2/3 in cells expressing NLS-Myc-HMD protein were higher than those of cells expressing a fusion protein of GBD and HMD indicating that fusion to the GBD was impairing the function of the HMD (Figure 19B, lanes 2 and 3). However, fusing the GBD to full-length Rtf1 did not greatly affect the levels of histone modifications (Figure 19B, lanes 4 and 5). This functional impairment of the HMD could be a result of diminished protein levels of the GBD-HMD fusion protein or reduced activity of the GBD-HMD protein or both. To test the effect of protein levels, I performed immunoblot analysis. This analysis indicated that both the GBD-HMD and the GBD-Rtf1 fusion proteins were expressed at much lower levels than the NLS-Myc-HMD and Myc-Rtf1 proteins (Figure 19E). This suggests that reduction of total protein levels by fusion of the GBD to the HMD greatly affects the ability of the HMD to promote the co-transcriptional modification of histones while full-length Rtf1 even when expressed at lower levels by fusion to the GBD continues to function as well as Rtf1 alone. However, we cannot rule out the possibility that the lower signal for the GBD-HMD and the GBD-Rtf1 proteins with anti-Rtf1 antibody could be an impact of reduced accessibility of the epitope. Thus, although our western blot analysis that the functional impairment of the GBD-HMD was due to diminished protein levels, we cannot rule out the possibility that fusion to the GBD could have affected the ability of the HMD to facilitate the addition of the ubiquitin and methyl groups to histones.

Since the GBD-HMD was observed to be hypofunctional and the NLS-Myc-HMD protein was found to be capable of associating with chromatin and modifying histones, the rest of the studies aimed at uncovering the mechanism of regulation of histone modifications by the HMD were pursued using the construct expressing the NLS-Myc-HMD fusion protein.

A.


Rtf1 signal
GBD-HMD
GBD
Reltaive Occupancy
2.77
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
GAL7 UAS
5' PYK1
Tel VI

B.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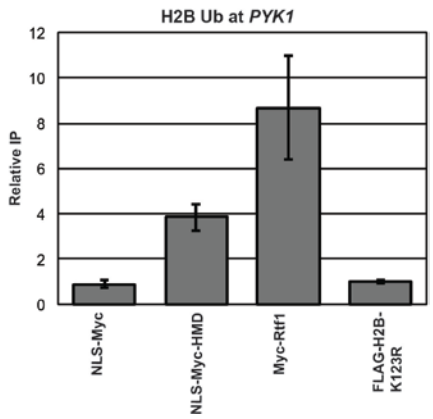
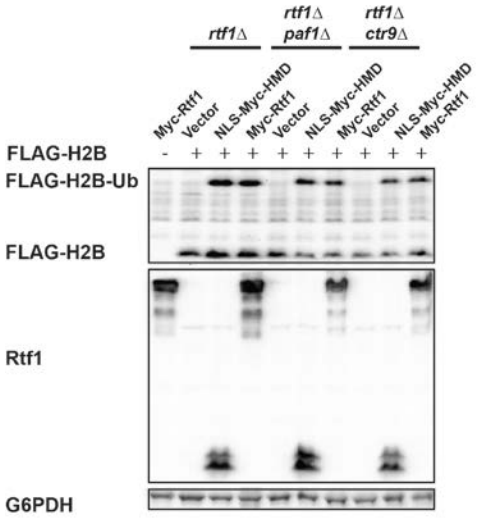
GBD-Myc-HMD
NLS-Myc-HMD
GBD-Myc-Rtf1
Myc-Rtf1
H3 K4 Me3
H3 K4 Me2
H3 K79 Me2/3
Total H3
G6PDH

C.


H2B Ub at PYK1
Relative IP
12
10
8
6
4
2
0
NLS-Myc
NLS-Myc-HMD
Myc-Rtf1
FLAG-H2B-K123R

D.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rtf1Δ paf1Δ
rtf1Δ ctr9Δ
Myc-Rtf1
Vector
NLS-Myc-HMD
Myc-Rtf1
Vector
NLS-Myc-HMD
Myc-Rtf1
Vector
NLS-Myc-HMD
Myc-Rtf1
FLAG-H2B
- + + + + + + + + +
FLAG-H2B-Ub
FLAG-H2B
Rtf1
G6PDH

E.

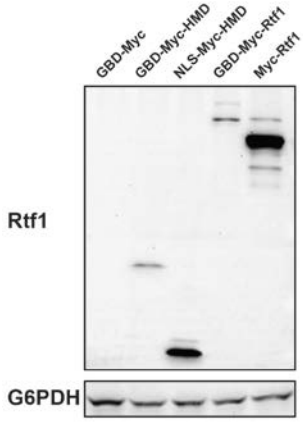

GBD-Myc
GBD-Myc-HMD
NLS-Myc-HMD
GBD-Myc-Rtf1
Myc-Rtf1
Rtf1
G6PDH

Figure 19. The HMD is sufficient to promote H3 K4 and K79 methylation and H2B K123 monoubiquitination

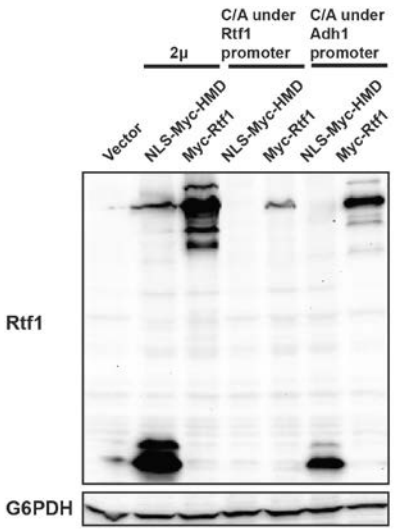
(A) ChIP analysis of Rtf1 levels at the *GAL7 UAS*, 5' region of *PYK1* or at telomeric region in *rtf1*Δ *gal4*Δ (KY2123) transformants expressing GBD-Myc (pGBKT7) or GBD-Myc-HMD (pMW8). (B) Immunoblot analysis of histone modifications in *rtf1*Δ *gal4*Δ (KY2123) transformants expressing GBD-Myc (pGBKT7), GBD-Myc-HMD (pMW8), NLS-Myc-HMD (pAP39), GBD-Myc-Rtf1 (pAP44) and Myc-Rtf1 (pAP45). Immunoblot analysis of H3 served as a control for total amount of histones and G6PDH levels as a control for the amount of extract loaded. (C) Sequential ChIP analysis of FLAG-H2B K123 Ub at the 5' region of *PYK1*. An *rtf1*Δ strain (KY2085), containing a *FLAG-HTB1* expression plasmid as the sole source of H2B, was co-transformed with a 2-micron plasmid expressing HA_3-ubiquitin (pRG147) and a 2-micron plasmid expressing NLS-Myc (pAP37), NLS-Myc-HMD (pAP39) or Myc-Rtf1 (pAP45). A strain (KY2086) containing FLAG-H2B-K123R as the sole source of H2B and transformed with HA_3-ubiquitin (pRG147) provides a specificity control for the H2B K123ub mark. Mean values from two biological replicates are shown with error bars representing the range of the data. (D) Anti-FLAG immunoblot analysis of *rtf1Δ* strains expressing untagged H2B (KY2084) or *rtf1Δ* strains (KY2085), *rtf1Δ paf1Δ* strains (KY2232) or *rtf1Δ ctr9Δ* strains (KY2234) expressing FLAG-H2B and, NLS-Myc vector (pAP37), NLS-Myc-HMD (pAP39) or Myc-Rtf1 (pAP45). (E) Immunoblot analysis of the Rtf1/HMD levels was performed using extracts of strains described in (B).

4.3.2 The over-expression of the HMD is required for the full-functionality of the HMD

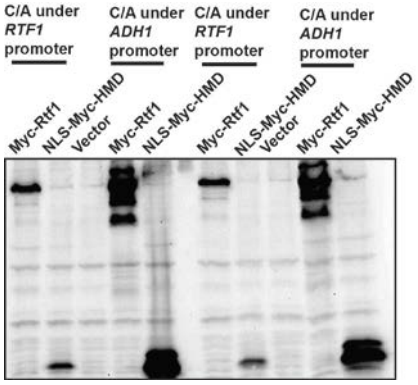
Our immunoblotting and ChIP analyses with the GBD-HMD fusion protein suggested that the functionality of the HMD might be dependent on the levels of the HMD protein. To test the dependency of the functionality of the HMD on the levels of the HMD expressed, I made plasmid constructs expressing the NLS-Myc-HMD protein on a low-copy-number plasmid (*CEN/ARS* origin) or a high-copy-number plasmid (2µ origin of replication) and under the control of the endogenous *RTF1* promoter or a strong promoter (*ADH1*). I performed immunoblotting analysis with transformants of cells expressing varying levels of the NLS-Myc-HMD protein and full-length Rtf1 protein to determine the impact of the HMD protein levels on the histone modifications. When similar amounts of extracts were loaded on SDS-polyacrylamide gels for western blot analysis, the HMD and Rtf1 signals for the high-copy and low-copy plasmids with the *ADH1* promoter were saturated. Hence, I stripped this blot partially by a short incubation with stripping buffer and redeveloped it, which allowed a comparison of the HMD expression levels under a strong promoter on high and low copy plasmids in the linear range. However, this partial stripping protocol led to complete stripping of the relatively weak Rtf1 signal for the HMD expressed under its endogenous promoter on a low copy plasmid (Figure 20A). Hence, I separately analyzed the expression of the HMD and Rtf1 on *CEN/ARS* plasmids (biological duplicates) (Figure 20B). As expected, the HMD expressed on a high-copy-number plasmid under a strong promoter showed highest level of expression, followed by the HMD expressed on a low copy number plasmid under a strong promoter (Figure 20A). The HMD expressed on a low-copy-number plasmid under the endogenous *RTF1* promoter was

expressed at the lowest levels (Figure 20A and B). Consistent with the dependency of the full-functionality of the HMD on the total levels of the HMD protein, I found the highest levels of H3 K4 Me3, H3 K4 Me2 and H3 K79 Me2/3 in cells expressing the HMD on a high-copy-number plasmid under a strong promoter followed by a low-copy-number plasmid under a strong promoter. The cells expressing the HMD on a low-copy-number plasmid under the endogenous *RTF1* promoter did not show any detectable levels of these Rtf1-mediated histone modifications (Figure 20C).

A.


2μ
C/A under Rtf1 promoter
C/A under Adh1 promoter
Vector
NLS-Myc-HMD
Myc-Rtf1
NLS-Myc-HMD
Myc-Rtf1
NLS-Myc-HMD
Myc-Rtf1
Rtf1
G6PDH

B.


C/A under RTF1 promoter
C/A under ADH1 promoter
C/A under RTF1 promoter
C/A under ADH1 promoter
Myc-Rtf1
NLS-Myc-HMD
Vector
Myc-Rtf1
NLS-Myc-HMD
Myc-Rtf1
NLS-Myc-HMD
Vector
Myc-Rtf1
NLS-Myc-HMD

C.

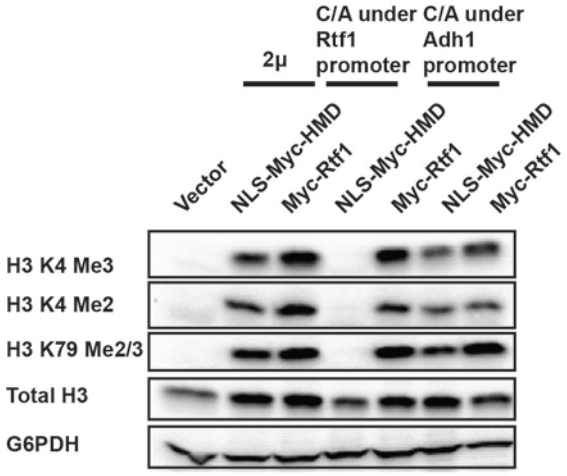

2μ
C/A under Rtf1 promoter
C/A under Adh1 promoter
Vector
NLS-Myc-HMD
Myc-Rtf1
NLS-Myc-HMD
Myc-Rtf1
NLS-Myc-HMD
Myc-Rtf1
H3 K4 Me3
H3 K4 Me2
H3 K79 Me2/3
Total H3
G6PDH

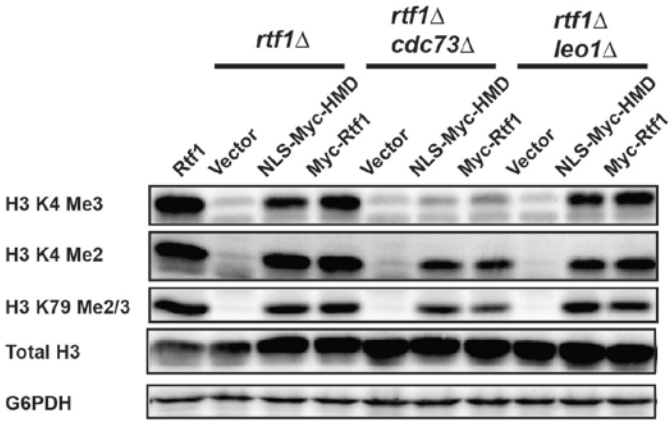
Figure 20. Overexpression of the HMD is essential for the HMD protein to promote Rtf1-dependent histone modifications

(A-C) Immunoblot analysis was performed using polyclonal Rtf1 antiserum to measure the levels of Rtf1 proteins (A and B) and histone modifications (C) in *rtf1*Δ *gal4*Δ strains (KY2123) transformed with either empty vector (pAP37) or 2-micron (*ADH1* promoter) plasmids encoding NLS-Myc-HMD (pAP39) or Myc-Rtf1 (pAP45) or *rtf1*Δ strains (KY619) transformed with *CEN/ARS* (*RTF1* promoter) plasmids that express NLS-Myc-HMD (pMM38) or Rtf1 (pLS20) or *rtf1*Δ strains (KY619) transformed with *CEN/ARS* (*ADH1* promoter) plasmids that express NLS-Myc-HMD (pAP39) or Rtf1 (pMM40). G6PDH and H3 levels serve as loading controls. Figure (B) shows a comparison of Rtf1 and HMD expression driven by the *ADH1* and the *RTF1* promoters in two different transformants for the indicated *CEN/ARS* plasmids.

4.3.3 The HMD can facilitate most of the Rtf1-mediated histone modifications in the absence of other members of Paf1C

Although the HMD lacks the domain of Rtf1 indispensable for the interaction of Rtf1 with the rest of Paf1C, it is possible that it could still be aided by the rest of Paf1C in promoting the histone modifications. To test this possibility, I performed immunoblotting analysis with cells lacking the Cdc73 or the Leo1 subunit of Paf1C and expressing the HMD and determined the levels of the Rtf1-mediated histone modifications in these cells. My immunoblotting analysis indicated that lack of Leo1 had no effect on the levels of the histone modifications tested and the total HMD levels. The absence of Cdc73 had no effect on the levels of H3 K79 Me2/3, a small effect on H3 K4 Me2 levels and a bigger impact on the H3 K4 Me3 levels with no apparent effect on the levels of the HMD expressed (Figure 21A and B). The absence of Paf1 and Ctr9 subunits were also shown to have similar effect as Cdc73 on the H3 K4 Me3, H3 K4 Me2 and H3 K79 Me2/3 levels and the proteins levels of the HMD (PIRO *et al.* 2012). However, the absence of Paf1 and Ctr9 subunits did not greatly affect the ratio of global levels of modified H2B (H2B K123 Ub) to unmodified H2B facilitated by the NLS-Myc-HMD protein (Figure 19D; lanes 3, 4, 6, 7, 9 and 10). This suggests that other members of Paf1C may facilitate the higher extent of methylation on the histone H3 K4 residue through an unknown mechanism.

A.



B.

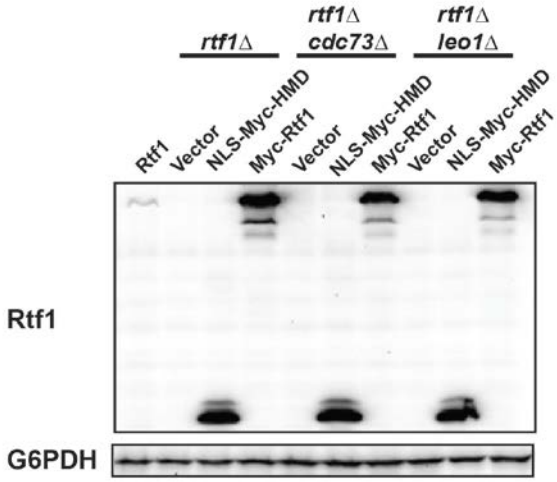


Figure 21. The HMD functions independently of other Paf1C members

(A and B) Immunoblot analysis of H3 K4 and K79 methylation (A) or Rtf1 protein levels (B) in *rtf1*Δ (KY2123), *rtf1*Δ *cdc73*Δ (KY2195) or *rtf1*Δ *leo1*Δ (GHY1144) strains expressing NLS-Myc-HMD (pAP39), Myc-Rtf1 (pAP45) or transformed with vector (pAP37) or a wild-type strain (KY1021).

4.3.4 The HMD physically associates with Bre1

Uncovering the protein-protein interactions mediated by the HMD using a targeted approach and an unbiased proteomics approach could provide us better insight into the mechanism through which the HMD facilitates histone modifications. To do this, we decided to take two complementary approaches. As part of the subtractive proteomics approach, I performed one-step affinity purification with extracts of strains expressing a TAP-tagged derivative of Rtf1 that lacked a part of the HMD, the removal of which was previously shown by our laboratory to abolish the Rtf1-dependent histone modifications (WARNER *et al.* 2007). Additionally, I also performed one-step affinity purification with a TAP-tagged derivative that lacked the domain of Rtf1 important for the interaction of Rtf1 with the other members of Paf1C which we termed the PID (Paf1C interaction domain) (WARNER *et al.* 2007) alone and in combination with partial deletion of the HMD. Identifying the interactions lost in these TAP-tagged deletion derivatives of Rtf1 could allow the identification of the interactions that were collaboratively maintained by the HMD and the other members of Paf1C in order to facilitate Rtf1-dependent histone modifications. I also performed one-step affinity purification in parallel with full-length TAP-tagged and untagged Rtf1 to identify all the interactions mediated by Rtf1 and the non-specific interactions occurring through the non-specific binding of the proteins in the clarified yeast extracts to the IgG-conjugated magnetic beads. Consistent with our previous observations (WARNER *et al.* 2007), the mass spectrometry data showed that the interaction of Rtf1 with the rest of the members of Paf1C was lost in the affinity-purified derivatives of TAP-tagged Rtf1 that lacked the PID alone and PID in combination with part of the HMD (Figure 22A). However, we could not identify any interactions that were consistently lost in the affinity-purified derivatives of TAP-tagged Rtf1 that lacked part of the HMD nor did we detect any interactions

that were retained in the PID deletion derivative but lost in the double deletion derivative lacking the PID and part of the HMD (Figure 22A).

As a complementary approach, I also performed one-step affinity purification of TAP-tagged HMD, TAP-tagged Rtf1, untagged Rtf1 and untagged HMD and attempted to identify the specific interactions of Rtf1 that were mediated by the HMD using immunoblotting analysis. The strains that I used in this analysis expressed FLAG-tagged Bre1 from the endogenous locus to allow detection of a possible interaction between the HMD and Bre1 protein by immunoblotting with an antibody against the FLAG tag. Since the HMD is required for the monoubiquitination of H2B K123 catalyzed by the ubiquitin-conjugating enzyme Rad6 and ubiquitin-ligating enzyme Bre1 and Bre1 is required for targeting Rad6 to H2B K123 (KIM and ROEDER 2009), we anticipated that the HMD could be facilitating the monoubiquitination of H2B K123 through an interaction with Bre1.

When I performed immunoblotting analysis on the one-step affinity purified Rtf1-TAP, NLS-HMD-TAP and untagged Rtf1 and NLS-HMD, I detected a band corresponding to FLAG-Bre1 in the bound fractions of Rtf1-TAP and HMD-TAP but not in the bound fractions of untagged Rtf1 and HMD proteins (Figure 22B). This suggests that the HMD may be interacting with Bre1 protein and hence facilitating the monoubiquitination of H2B K123. However, this experiment was performed only once and hence needs to be repeated to confirm the result. Also, Bre1 was not identified as an interactor in the mass spectrometry analysis of the Rtf1-TAP protein. This may be due to association of small amounts of Bre1 with the HMD protein that could lead to the masking of the mass spectrometry signal for Bre1 peptides by the peptides of other major interactors present in higher amounts in the affinity purified Rtf1-TAP.

A.

Proteins	Rtf1	Rtf1-TAP	Rtf1Δ3-TAP	Rtf1ΔPID-TAP	Rtf1Δ3ΔPID-TAP
CTR9	1	54	43	5	1
PAF1	1	34	35	0	0
RTF1	1	33	21	37	28
PAB1	3	25	16	21	19
LEO1	0	24	21	1	0
CDC73	0	22	19	1	0
IMD3	2	17	15	16	20
VMA2	2	16	12	8	8
TY1B-DR1	0	14	6	0	4
SPT5	0	13	8	10	11
TY1B-DR5	0	13	0	1	10
SPT16	0	12	9	5	9
RRP5	0	12	5	13	14
DED1	1	11	10	17	12
URA2	0	11	3	2	1
RPL4A	3	10	5	8	9
CPA2	0	9	2	1	0
ATP2	2	8	5	3	4
PFK1	1	8	3	3	2
KAP123	1	8	6	3	3
HTB2	2	7	6	8	9
SSBR1	1	7	5	6	5
TY1B-ER1	0	7	13	8	10
CKA2	0	7	5	12	8
KEM1	2	7	3	5	5

B.

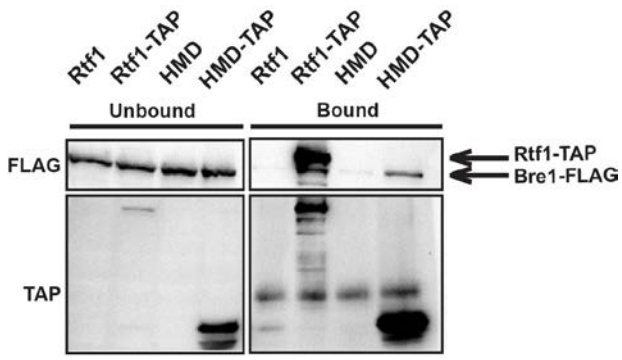


Figure 22. Identifying the HMD-mediated interactions of Rtf1

(A) Extracts of *rtf1*Δ strains (KY1258) bearing plasmids expressing Rtf1 (pLS20), Rtf1-TAP (pPC59), Rtf1Δ3-TAP (pMW13), Rtf1ΔPID-TAP (pMW11) and Rtf1Δ3ΔPID-TAP (pMW14) were subjected to one-step affinity purification using IgG-conjugated magnetic beads, and mass spectrometry analysis was performed on the isolated proteins. The average number of peptides identified for each protein in four trials is listed. The first 25 hits sorted by the results for Rtf1-TAP are shown after elimination of the interactors that showed less than three-fold enrichment for Rtf1-TAP over untagged full-length Rtf1. (B) Immunoblotting analysis was performed with antibody against the FLAG tag to determine if one step affinity purified HMD (expressed from pAP46) from an *rtf1Δ* strain expressing FLAG-tagged Bre1 (KY1876) could interact with Bre1. One-step affinity purified Rtf1-TAP (expressed from pPC59) served as control to show that an interaction observed with the HMD alone was not an artifact arising from overexpression of the isolated HMD. Untagged Rtf1 (expressed from pLS20) and HMD (expressed from pAP39) served as negative controls for the affinity purification. Immunoblotting with antibody against the TAP tag served as a control to show the successful pull-down of the TAP-tagged proteins. Rtf1 protein has a seven-peptide sequence similar to that of FLAG epitope (Rtf1 residues 196-202) that leads to detection of Rtf1 by an antibody against the FLAG tag.

4.4 DISCUSSION

Paf1C regulates the expression of several genes within the cell such as those required for maintaining stem cell pluripotency, genes involved in eliciting an antiviral immune response and the downstream target genes of crucial signaling pathways including Notch, Hedgehog and Wnt signaling pathways [reviewed in (TOMSON and ARNDT 2013)]. One of the major mechanism through which Paf1C affects gene expression is through its role in promoting co-transcriptional histone modifications. Our laboratory previously identified the region (HMD) necessary for Paf1C mediated histone modifications including H2B K123 Ub, H3 K4 Me3, H3 K4 Me2 and H3 K79 Me2 (WARNER *et al.* 2007). This study laid the foundation for future studies in our lab directed towards uncovering the mechanism of regulation of histone modifications by the HMD of Rtf1.

The work described in this Chapter mainly represents my contribution to a recently published study from the Arndt laboratory which showed that the HMD is not only necessary but also sufficient to mediate most of the Rtf1-dependent histone modifications and it does so by binding to chromatin (PIRO *et al.* 2012). I showed that the HMD when fused to the GBD not only binds to *GAL7* UAS but also another actively transcribed locus not normally bound by GBD and also a transcriptionally inactive locus (a telomeric region) although at lower levels compared to its occupancy at the *GAL7* UAS (Figure 19A). This and additional ChIP analyses performed with the HMD alone, which showed that the HMD binds to non-transcribed loci and leads to the targeting of a co-transcriptional histone modification (H3 K79 M2/3) at the telomeric region (PIRO *et al.* 2012), suggest that if the HMD is expressed on its own in the absence of the rest of the Rtf1 protein, it may not be targeted to specific areas of the genome. My work described in Chapter 2 showed that the region of Rtf1 previously implicated in the chromatin association of

the entire Paf1C, the OAR of Rtf1, can however mimic the chromatin association pattern of the entire Paf1C (Figure11 and 12). Thus, the OAR of Rtf1 is critical for the proper chromatin targeting of Paf1C and dictates the localization pattern of the HMD, which also appears to have some intrinsic chromatin-association activity.

Additionally, I also showed that the HMD does not require aid from the other members of Paf1C to monoubiquitinate lysine 123 of histone H2B (Figure 20D) and also to methylate H3 K79 and H3 K4 (Figure 21) (PIRO *et al.* 2012). However, it does need some assistance from the rest of Paf1C for mainly H3 K4 Me3 and to a small extent for full levels of H3 K4 Me2 (Figure 21). The effect of the absence of the other members of Paf1C on the functionality of the HMD seems to recapitulate the effect of the absence of the Cps40 and Cps60 subunits of COMPASS (Complex of proteins associated with Set1) on these modifications (SCHNEIDER *et al.* 2005). The absence of these subunits affects the extent of methylation by COMPASS without affecting the association of Set1. The rest of Paf1C may be required for the chromatin association of Cps40 or Cps60 subunits, which could be tested using ChIP analyses. The other mechanism by which the rest of Paf1C could influence the higher states of H3 K4 methylation could be by facilitating the longer residence of the methyltransferase Set1 on genes, which may be essential for the addition of multiple methyl groups. This could be tested by looking at the rate at which Set1 dissociates from a long gene *YLR454* (an 8 kb long gene) under the *GAL1* promoter upon shifting the cells from an activating galactose-containing medium to a repressing glucose-containing medium in cells expressing the HMD and lacking or containing the rest of Paf1C. Another mechanism by which the absence of the rest of Paf1C could result in reduced H3 K4 tri-methylation could be that the other members of Paf1C may protect the methylation groups from the action of the H3 K4 demethylase. This could be tested by determining if the chromatin bound or global levels of

Jhd2 protein, the demethylase for the methylation at the H3 K4 residue (HUANG *et al.* 2010), are increased in cells lacking the Paf1, Ctr9 or Cdc73 subunits of Paf1C using ChIP and immunoblotting analyses, since the absence of these subunits greatly reduced the effect of the HMD on H3 K4 Me3 levels (Figure 21A) (PIRO *et al.* 2012).

Furthermore, I showed that the ability of the HMD to facilitate histone modifications correlates with the levels of HMD expression (Figure 20). The HMD when expressed at high levels on a high-copy-number plasmid under a strong promoter (*ADH1*) showed complete rescue of all the Rtf1-mediated modifications, but when it was expressed on a low-copy-number plasmid under endogenous *RTF1* promoter, which is weaker compared to *ADH1* promoter, it did not show any detectable rescue of histone modifications (Figure 20). Consistent with this, I also observed that fusion of the HMD to the GBD diminished the levels of total protein and correspondingly resulted in a reduction in the global levels of histone modifications (Figure 19B and E). This suggests that the HMD has to be present at high levels to facilitate Paf1C-mediated functions on its own. This may be due to weaker or transient association of the HMD that is stabilized in the presence of the rest of Rtf1. A much higher amount of HMD may be required to stabilize the association of the HMD with chromatin when the rest of Rtf1 is lacking. That overexpression of the HMD does not override the need for the components of the canonical H2B ubiquitination pathway, mainly Rad6 and Bre1, suggests that overexpression of the HMD does not lead to histone modifications by an alternative mechanism (PIRO *et al.* 2012).

My affinity purification and immunoblotting with TAP-tagged HMD suggests that Bre1 may interact with the HMD (Figure 22B). Work by Anthony Piro also suggested that the HMD can interact with nucleosomes using a nucleosome pull-down assay and immunoblotting, and mutations within the HMD that greatly reduce the levels of Rtf1-mediated histone modifications

also correspondingly impair the ability of the HMD to interact with nucleosomes (PIRO *et al.* 2012; TOMSON *et al.* 2011). These results suggest that interaction of the HMD with both the enzyme (Bre1) and the substrate (histones) may regulate the monoubiquitination of lysine 123 at histone H2B. The impact of the HMD on the chromatin association of Bre1 can be tested by determining the levels of Bre1 on chromatin in cells expressing HMD and HMD mutants that nearly abolish the Rtf1-mediated histone modifications.

Cumulatively, my work described in this Chapter has provided better insights into the mechanism of regulation of histone modifications by the HMD and has raised more interesting questions that can be addressed in the future.

5.0 CONCLUSIONS AND FUTURE DIRECTIONS

5.1 THE OAR OF RTF1 FACILITATES THE RECRUITMENT OF PAF1C THROUGH INTERACTION WITH THE CTR OF SPT5

Regulation of transcription is essential for proper gene expression. Chromatin obstructs the binding and the movement of the transcription machinery. Hence, eukaryotes have evolved numerous accessory factors that associate with the transcriptional machinery to facilitate its passage through chromatin. One such factor is the conserved Paf1C that regulates transcription through several mechanisms including promoting co-transcriptional histone modifications, recruiting chromatin remodelers, maintaining nucleosome stability during transcription and regulating 3'-end formation of RNA. Paf1C positively regulates the expression of several genes including those involved in maintaining stem cell pluripotency, antiviral response and cell cycle progression. Misregulation of Paf1C can cause developmental defects and diseases such as cancer. However, the mechanism of recruitment of this important complex was unclear when I began my studies. The Spt4-Spt5 complex, the Bur1-Bur2 kinase complex, the Ccr4-Not complex and Spt16 were previously implicated in the recruitment of Paf1C to actively transcribed genes [reviewed in (JAEHNING 2010)], but a detailed molecular mechanism for the recruitment of Paf1C was lacking. My work provided a molecular mechanism for tethering Paf1C to the transcription machinery, supported by both studies *in vivo* and *in vitro*.

Previous work in our laboratory had indicated that a central region within Rtf1, termed the ORF association region or the OAR, is important for the chromatin occupancy of Paf1C. OAR is the most conserved region of Rtf1 suggesting its involvement in a crucial functional role. Hence, we decided to uncover the details of the mechanism involved in the functioning of the OAR. After mapping the boundaries of the functional OAR by analysis of the predicted secondary structure and degree of conservation, I took a subtractive proteomics approach and discovered that the OAR of Rtf1 mediates the interaction of Paf1C with the evolutionarily conserved transcription elongation factor Spt5 (Figure 7 and 9). Since Spt5 can directly interact with Pol II and has other regions that can facilitate its interactions with other elongation factors, it seemed to be a perfect candidate for coupling Paf1C to the transcription machinery. Using co-immunoprecipitation analysis, I further demonstrated that the CTR of Spt5 is important for the OAR-mediated interaction between Spt5 and Paf1C (Figure 9). After I successfully identified the regions of both Paf1C and Spt5 that facilitate the interaction of Paf1C with Spt5 and hence allow the targeting of Paf1C to chromatin under physiologically relevant conditions, I then performed *in vitro* binding assays with purified recombinant derivatives of Rtf1 and Spt5 and showed that the Rtf1 OAR and the Spt5 CTR are necessary and sufficient for the interaction of Rtf1 with Spt5 (Figure 10).

To test if the OAR is also functionally sufficient to associate with chromatin in a regulated manner, I performed ChIP analyses and showed that that OAR alone associates with actively transcribed genes in a pattern similar to that of Paf1C (dissociating from the chromatin at the poly(A) site), does not require assistance from other subunits of Paf1C to accomplish its function and associates with chromatin in a manner dependent on the Spt5 CTR and the Bur1-

Bur2 kinase complex suggesting that the interaction of the OAR with phosphorylated Spt5 CTR is functionally important for the OAR (Figure 10-13).

Our collaborators, Adam Wier, Dr. Annie Heroux and Dr. Andrew VanDemark, obtained the crystal structure of both the human OAR/Plus3 alone and the human OAR/Plus3 in a complex with the phosphorylated human Spt5 CTR. The OAR-CTR crystal structure showed that the interaction that I discovered in yeast is conserved in humans as well (Figure 17). Most of the residues that I found to be functionally important using Spt$^-$ phenotypic analysis, a phenotype caused by transcriptional defects, and ChIP analysis were at the OAR-CTR interface mediating the OAR-CTR interaction, thus providing a structural validation of my mutational analysis (Figure 15-17). The OAR-CTR co-crystal is held by two interactions, a set of electrostatic interactions between the phosphate groups of the phosphorylated Spt5 CTR and the polar residues of the OAR and a set of Van Der Waals interactions between the aromatic residues of the OAR and the CTR (Figure 17). My ChIP analysis with the OAR mutant (Rtf1-R251A, Y327A) that impaired both the interactions suggested that the contacts observed in the context of the OAR-CTR crystal were indeed facilitating the chromatin association of Paf1C in the *S. cerevisiae* cells (Figure 17 and 18).

Low but measurable chromatin occupancy of the OAR alone in a strain expressing Spt5 lacking the CTR suggests the involvement of additional factors that could be mediating the recruitment of Paf1C through the OAR (Figure 13). Since these factors were not uncovered by our subtractive proteomics approach, we could perform affinity purification and mass spectrometry analysis with OAR-TAP to enrich for important interactions of the OAR that facilitate the chromatin association of Paf1C.

As an alternative approach to identify novel direct or indirect regulators of the chromatin association of Paf1C we can perform a high-copy suppressor screen to identify proteins that when overexpressed can suppress the Spt$^-$ phenotype caused by the impaired association of Paf1C in an OAR mutant such as the Plus3 mutant (Rtf1-R251E R273E K299E). Paf1C dissociates from the chromatin at the poly(A) site while Spt5 continues further with Pol II. Why is it important for Paf1C to dissociate from chromatin at the poly(A) site? To address this question, we could construct a fusion protein of Spt5 with Rtf1 and first determine if the fusion protein is functional. This can be done by performing growth assays to determine to test if the cells expressing the Spt5-Rtf1 fusion protein exhibit phenotypes indicative of transcriptional defects such as Spt$^-$ phenotype and sensitivity to the nucleotide analog 6-Azauracil. Immunoblotting analysis can also be performed to determine the levels of Paf1C-mediated co-transcriptional histone modifications in these cells. If the fusion protein is functional, we could use the cells expressing the fusion protein to determine the consequence of lack of dissociation of Paf1C from Spt5 at the poly(A) site. The presence of Rtf1 will also lead to the association of the rest of Paf1C with Spt5 through the PID (Paf1C interaction domain) of Rtf1.

ChIP analysis can then be performed to determine if Paf1C associates with chromatin beyond the poly(A) site in these cells. The cells expressing this fusion protein can then be subjected to several analyses to determine the biological outcome of tethering Paf1C beyond the poly(A) site such as determining if these cells have termination defects. Since the CTR of Spt5 can bind both elongation factors (Paf1C) and termination factors (CFI complex) the chromatin association of which is enriched at the poly(A) site, it is possible that these cells will exhibit termination defects due to impaired recruitment of termination factors (MAYEKAR *et al.* 2013; MAYER *et al.* 2012). If they do exhibit termination defects, ChIP analysis can be performed to

determine if the chromatin association of the termination factors known to bind to the CTR of Spt5 (members of CFI complex) is impaired in these strains.

Which event at the poly(A) site leads to an exchange of Paf1C for termination factors? Since we know that affinity of Paf1C for the CTR of Spt5 is greatly reduced by the lack of phosphorylation of the Spt5 CTR (Figure 10C) (WIER *et al.* 2013), we can predict that the dephosphorylation of Spt5 at the poly(A) site could be facilitating the exchange of termination factors for an elongation factor. Terminations factors such as Rna14, Rna15 and Hrp1, which are all subunits of the yeast cleavage factor I complex important for cleavage and polyadenylation of mRNA, were shown to bind the CTR of Spt5 in GST-pull down assay (GROSS and MOORE 2001; MAYER *et al.* 2012) [reviewed in (RICHARD and MANLEY 2009)]. To uncover if phosphorylation of the Spt5 CTR alters its affinity for the termination factors, GST pull-down assays could be performed with GST-tagged termination factors using extracts of cells expressing wild-type Spt5, phosphomimetic Spt5 and non-phosphorylatable Spt5 to determine if the termination factors exhibit higher affinity for non-phosphorylatable Spt5. If the termination factors do exhibit higher affinity for the non-phosphorylatable Spt5 compared to phosphomimetic Spt5, it should then be determined if they can directly bind the CTR of Spt5 in an *in vitro* binding assay with purified recombinant proteins. If the termination factors directly bind to the CTR of Spt5 then fluorescence anisotropy assays can be performed with fluorescently labeled phosphorylated CTR and non-phosphorylated CTR to determine their affinity for phosphorylated and non-phosphorylated CTR. If their affinity for the non-phosphorylated Spt5 CTR is higher than that for the phosphorylated one, it will support our hypothesis that the dephosphorylation of the Spt5 CTR at the poly(A) site triggers the exchange of an elongation factor for a termination factor

similar to the Pol II CTD, the phosphorylation and dephosphorylation of the Spt5 CTR could facilitate the association of specific factors during specific stages of transcription.

To identify other elongation factors that are recruited by the phosphorylated Spt5 CTR, quantitative mass spectrometry analysis could be performed with affinity purified TAP-tagged non-phosphorylatable Spt5 and wild-type Spt5. This could lead to the identification of the physical interactions of Spt5 that are impaired by the lack of phosphorylation of the Spt5 CTR. On the other hand, mass spectrometry analysis of affinity purified TAP-tagged phosphomimetic Spt5 and wild-type Spt5 could lead to the identification of the physical interactions of Spt5 that are impaired by the lack of dephosphorylation of Spt5 CTR. If our hypothesis that the dephosphorylation of the Spt5 CTR at the poly(A) site facilitates the exchange of an elongation factor for a termination factor is true, then a quantitative proteomic analysis could uncover the termination factors that preferably associate with non-phosphorylatable Spt5 CTR. ChIP analysis can then be performed to determine if the association of these termination factors with chromatin beyond the poly(A) site is diminished in cells lacking the CTR of Spt5.

5.2 DUAL ATTACHMENT OF PAF1C TO CHROMATIN IS MEDIATED BY THE OAR OF RTF1 AND THE C-DOMAIN OF CDC73

Our previous analysis had suggested that the C-domain of Cdc73 also contributes to the chromatin association of Paf1C (AMRICH *et al.* 2012). My ChIP analysis with the OAR and C-domain mutants indicated a greater reduction in the chromatin association of Paf1C in a double mutant strain, in which the both the Rtf1 OAR and Cdc73 C-domain were mutated, than either of the single mutants thus supporting our hypothesis that the chromatin attachment of Paf1C is

facilitated through a dual attachment mediated by the OAR of Rtf1 and the C-domain of Cdc73 (Figure 17). This ChIP analysis also suggested that the reduction in the chromatin occupancy caused by the lack of the C-domain is comparable to the reduction caused by the OAR point mutants, while complete deletion of the OAR has a much greater impact on the chromatin occupancy of Paf1C (Figure 7 and 17). This also provides an explanation for the lack of influence of the deletion of the C-domain of Cdc73 alone on the co-transcriptional histone modifications mediated by Paf1C (AMRICH *et al.* 2012) and suggests that even low levels of chromatin association of Paf1C are enough for Paf1C to exert its impact on co-transcriptional histone modifications.

We were curious about the residual histone modifications that seem to persist in cells expressing Rtf1 derivative lacking the OAR even though the chromatin occupancy of Paf1C is severely reduced in this strain (Figure 7). I anticipated that the C-domain of Cdc73 would be responsible for the low levels of histone modifications in the OAR deletion derivative. This hypothesis is supported by my analysis of Paf1C-mediated histone modifications in the strains lacking both the Rtf1 OAR and the Cdc73 C-domain, which showed an almost complete loss of all Paf1C-mediated histone modifications in the cells lacking these two domains (Figure 18).

The global levels of Ser2 phosphorylated Pol II CTD also could be measured in cells lacking both the Rtf1 OAR and the Cdc73 C-domain by immunoblotting analysis. Just as the histone modifications, we expect the Ser2 phosphorylation of the Pol II CTD, which is most dramatically impacted by the deletion of Paf1 or Ctr9 subunits alone, would be greatly reduced by the removal of both the OAR of Rtf1 and C-domain of Cdc73 even in the presence of Ctr9 and Paf1 subunits due to the inability of Paf1 and Ctr9 to independently associate with chromatin (NORDICK *et al.* 2008). Individual deletions of *PAF1* or *CTR9* cause stronger phenotypes than the

deletion of *CDC73* or *RTF1*. This is at least partly due to the reduced levels of other subunits of Paf1C in the absence of the Paf1 or Ctr9 subunits (MUELLER *et al.* 2004). Also, some functions of Paf1C such as the regulation of H3 K36 Me3 levels are impacted mainly by the Paf1 and Ctr9 subunits (CHU *et al.* 2007). To uncover the functions of Paf1 and Ctr9 subunits alone that require their chromatin association, we can express fusion proteins of OAR with Paf1 or Ctr9 in strains lacking the other subunits of Paf1C. A quick test to determine if fusion to the OAR allows the Paf1 and Ctr9 to perform some functions in the absence of the other members of Paf1C would be to compare the growth rate of cells expressing Paf1 or Ctr9 subunit alone or Paf1-OAR or Ctr9-OAR fusion protein alone in the absence of other subunits of Paf1C. If expressing the fusion protein improves the growth rate of these cells it will indicate that at least some of the functions of Paf1C could be rescued by targeting the Paf1 or Ctr9 subunit alone to chromatin by fusion to the OAR in the absence of other members of Paf1C. Identifying the physical interactions of the one-step affinity purified TAP-tagged OAR-Paf1 and OAR-Ctr9 fusion proteins using mass spectrometry analysis could provide insight into Paf1- or Ctr9-specific functional interactions of Paf1C and hence facilitate a mechanistic understanding of these functions.

The Hinnebusch laboratory showed that the Cdc73 subunit of Paf1C can bind *in vitro* to both the phosphorylated CTR peptide of Spt5 and a Pol II CTD peptide phosphorylated at Ser2 and Ser5 (QIU *et al.* 2012). However, this could be an artifact of an *in vitro* binding assay with purified recombinant proteins uncovering interactions that may not be physiologically relevant. However, my co-immunoprecipitation assay and affinity purification, which uncover physiologically relevant interactions, indicate that the OAR is important for the interaction of Paf1C with Spt5 and my GST pull-down assay suggests that the Rtf1 OAR interacts with the phosphorylated CTR of Spt5 even in the presence of other yeast proteins. However, I did see an

increase in the chromatin association of the isolated OAR in cells lacking the Cdc73 subunit of Paf1C (Figure 11C and D). This suggests that Cdc73 may interfere with the binding of the OAR to chromatin. It would be interesting to test if the OAR and Cdc73 compete for the phosphorylated CTR or if OAR induces an allosteric change in the phosphorylated CTR that facilitates the binding of Cdc73 to the Spt5 CTR. Whether Cdc73 competes with the OAR for binding the phosphorylated Spt5 CTR can be determined by comparing the affinity of the OAR alone and the OAR in the presence of increasing concentration of Cdc73 using native gel electrophoresis and western blot analysis. To uncover if the OAR facilitates the binding of Cdc73 to the phosphorylated Spt5 CTR, the amount of Cdc73 binding to the phosphorylated CTR can be determined in the presence of increasing concentration of the OAR using native gel electrophoresis and immunoblotting analysis.

5.3 IS THE OAR IMPORTANT FOR PROPER CHROMATIN TARGETING OF THE HISTONE MODIFICATION DOMAIN (HMD) OF RTF1?

Our studies with the HMD suggested that when overexpressed, the HMD alone can bind to chromatin and facilitate Rtf1-mediated histone modifications of Paf1C (PIRO *et al.* 2012). However, the lack of association of this HMD with the rest of Paf1C may lead to the non-specific binding of the HMD to transcriptionally inactive loci such as the telomeric regions and the appearance of the Rtf1-mediated histone modification at these regions (PIRO *et al.* 2012). The OAR alone, however specifically binds to chromatin in the same pattern as that of Paf1C (Figure 11). This suggests that the OAR facilitates the targeting of the HMD to the proper loci. To confirm this, we can express the OAR as a fusion protein with the HMD and determine if this

fusion protein can target Rtf1-dependent histone modifications to chromatin in the same manner as that of Paf1C and prevent the targeting of these modifications to transcriptionally inactive loci.

5.4 DOES THE HMD FACILITATE THE RECRUITMENT OF BRE1?

My one-step affinity purification and immunoblotting analysis with TAP-HMD suggests that HMD associates with Bre1 *in vivo*. If this result is reproducible, *in vitro* binding assays could be performed with purified recombinant proteins to determine if the interaction between the HMD and Bre1 protein is direct. Also, the occupancy of Rad6 and Bre1 over the ORFs of actively transcribed genes has been previously shown to be dependent on Paf1C (XIAO *et al.* 2005). To uncover if the interaction of Bre1 with the HMD facilitates the recruitment of Rad6 and Bre1 to chromatin, ChIP analysis can be performed to determine the occupancy of the ubiquitin conjugase and ubiquitin ligase, Rad6 and Bre1, in strains expressing the NLS-Myc-HMD protein.

Collectively, my work has provided a molecular mechanism for association of Paf1C with the transcription machinery and has improved our understanding of how a small region within Paf1C facilitates co-transcriptional histone modifications. Given the importance of Paf1C in the expression of downstream targets of important signaling pathways such as the Notch signaling and Hedgehog signaling and expression of genes required for mounting an antiviral response, genes important for proper transitions through the cell cycle and genes essential for maintaining stem cell pluripotency it was very important to understand how Paf1C gets to chromatin to execute all these essential functions [reviewed in (TOMSON and ARNDT 2013)].

While filling voids in the field of transcriptional regulation, my work has also raised interesting questions that could be addressed in the future to gain better insights into regulation of spatio-temporal recruitment of factors facilitating transcription.

APPENDIX A

A.1 PHOSPHOMIMETIC SPT5 IMPAIRS THE DISSOCIATION OF SPT5 AT THE POLY(A) SITE

Transcription of mRNAs commences with the assembly of the transcription initiations factors along with Pol II at the promoter. Once transcription is initiated, Pol II releases its contacts with the initiation factors and exchanges them for elongation factors. This exchange of elongation factors for initiation factors is facilitated by the CTD of Pol II. The CTD of Pol II consists of multiple repeats of the sequence $Y_1S_2P_3T_4S_5P_6S_7$, most residues of which are dynamically phosphorylated during transcription [reviewed in (HSIN and MANLEY 2012)]. The sequence is highly conserved across species although the number of repeats varies between species [reviewed in (HSIN and MANLEY 2012)]. The hypophosphorylated form of the CTD predominates during the initiation stage of transcription. The phosphorylation of Ser5 by Kin28/Cdk7 of Pol II CTD triggers the exchange of initiation factors for elongation factors [reviewed in (BURATOWSKI 2009; HSIN and MANLEY 2012)]. The localization of Kin28/Cdk7 phosphorylated Ser7 Pol II is similar to that of Ser5 phosphorylated Pol II (AKHTAR *et al.* 2009; CHAPMAN *et al.* 2007). This mark has been shown to be important for the recruitment of Rpap2 protein, which is required for the proper expression of snRNA genes (EGLOFF *et al.* 2012). During the later stages of transcription the Ser2 phosphorylated form of CTD catalyzed by

Ctk1/Cdk12 is enriched and this facilitates the recruitment of termination factors [reviewed in (BURATOWSKI 2009; HSIN and MANLEY 2012)]. Some elongation factors, such as Spt5 and Spt6, continue along with Pol II beyond the poly(A) site while other elongations factors, including Paf1C and Bur1-Bur2 kinase complex, dissociate at or near the poly(A) site (MAYER *et al.* 2010). In turn, 3' RNA processing factors are recruited by the transcribing machinery at the poly(A) site (MAYER *et al.* 2010). The mechanism underlying the exchange of elongation factors for the termination factors at the poly(A) site remains obscure.

Of the plethora of accessory factors that associate with Pol II and facilitate transcription, Spt5 is the only factor that has been conserved through evolution and is found in all the three kingdoms of life. The bacterial Spt5/NusG and archeal Spt5 have the NusG domain and the KOW motif [reviewed in (HARTZOG and FU 2013)]. The eukaryotic Spt5 is a much larger protein having multiple C-terminal repeats (CTR) in addition to KOW motifs and the NusG domain [reviewed in (HARTZOG and FU 2013)]. Although the actual sequence of the CTR is not conserved among eukaryotes, the nature of the residues in this region is conserved (WIER *et al.* 2013). The serine/threonine within this repeat is a substrate for phosphorylation by P-TEFb in higher eukaryotes and by Bur1-Bur2 kinase in yeast. The CTR of Spt5 just like the CTD of Pol II could serve as a platform for recruitment of regulatory factors during transcription. In accordance with this hypothesis, the CTR of Spt5 has been shown to bind the capping enzymes and termination factors (MAYER *et al.* 2012; PEI and SHUMAN 2002). Additionally, my work described in Chapter 2 indicated that Paf1C is recruited to chromatin through the binding of the Rtf1 OAR to the CTR of Spt5.

Both elongation factors and termination factors can be recruited through association with the CTR of Spt5. Paf1C binds to the phosphorylated CTR of Spt5 and dissociates at the poly(A)

site where termination factors such as the members of cleavage factor I complex are recruited through association with the CTR of Spt5 (MAYEKAR *et al.* 2013; MAYER *et al.* 2012). What alters the affinity of the Spt5 CTR for Paf1C at the poly(A) site that makes it relinquish its contacts with Paf1C and in turn associate with termination factors? My work in Chapter 2 (Figure 10C) and work from the VanDemark laboratory (WIER *et al.* 2013) suggests that dephosphorylation of the CTR dramatically diminishes the affinity of the Spt5 CTR for the Rtf1 OAR. Hence, we hypothesized that just like the Pol II CTD, the phosphorylation and dephosphorylation of the Spt5 CTR could act as a molecular switch for swapping termination factors for elongation factors at the poly(A) site.

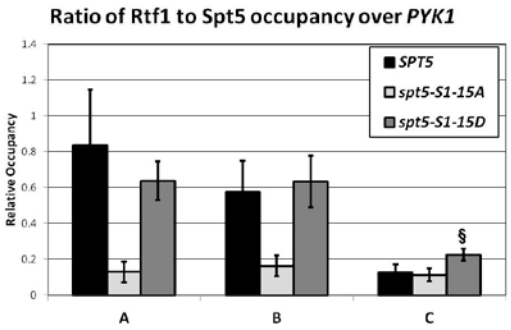
To test this hypothesis, I determined the chromatin occupancy pattern of Rtf1 relative to Spt5 using ChIP analysis in strains expressing wild-type Spt5, a phosphomimetic mutant of Spt5 in which all the phosphorylatable serine residues in the CTR have been mutated to aspartic acid, a phosphomimetic residue, (Spt5-S1-15D) and in strains expressing a mutant of Spt5 in which all the phosphorylatable serine residues in the CTR have been mutated to alanine making them non-phosphorylatable (Spt5-S1-15A). The occupancy of Rtf1 relative to that of Spt5 is much lower in cells expressing than wild-type Spt5 than cells expressing non-phosphorylatable Spt5 (Spt5-S1-15A) on actively transcribed genes in the regions before the poly(A) site (Figure 23A-C). This result is consistent with my results described in Chapter 2, which indicated that the affinity of the OAR of Rtf1 for Spt5 having non-phosphorylatable CTR is dramatically lower than that for wild-type or the phosphomimetic Spt5 derivative (Figure 10C). Beyond the poly(A) site, where Paf1C dissociates from chromatin, the levels of Rtf1 relative to Spt5 are similar in the wild-type cells and the non-phosphorylatable Spt5 mutant cells (Figure 23A-C). This result is in accordance with our hypothesis which predicts that the unphosphorylated form of Spt5

predominates beyond the poly(A) site. Excitingly, I found that the occupancy of Rtf1 relative to Spt5 is significantly increased beyond the poly(A) site in the strains expressing the phosphomimetic Spt5 derivative, while remaining comparable to that of wild-type at regions of actively transcribed genes 5’ to the poly(A) site (Figure 23). These results suggest that the presence of phosphomimetic Spt5 beyond the poly(A) site impairs the detachment of Paf1C from chromatin at the poly(A) site. Thus my results imply that the dephosphorylation of the CTR of Spt5 at the poly(A) site may lead to the dissociation of Paf1C from chromatin.

A.


A
253 346
B
1127 1270
C
1803 1938
PYK1
TATA 1(ATG)
1503(ST0P) 1642
A
214 319
B
2107 2194
C
3373 3475
PMA1
TATA
1(ATG)
2757(ST0P)
2823
3277

B.


Ratio of Rtf1 to Spt5 occupancy over PYK1
Relative Occupancy
SPT5
spt5-S1-15A
spt5-S1-15D
§
A
B
C

C.

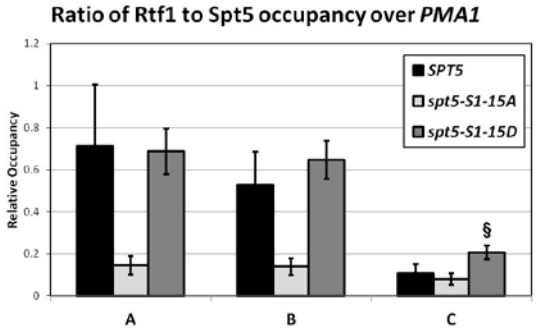

Ratio of Rtf1 to Spt5 occupancy over PMA1
Relative Occupancy
SPT5
spt5-S1-15A
spt5-S1-15D
§
A
B
C

Figure 23. Ratio of Rtf1 to Spt5 increases beyond the poly(A)

(A) The bars labeled A, B and C indicate the regions of *PYK1* and *PMA1* genes amplified in the real time PCR reactions to determine the occupancy of Rtf1 and Spt5 across these genes. (B and C) ChIP analysis was performed to determine the occupancy of Rtf1 and Spt5 in strains (Table 6) expressing wild-type Spt5 (HQY1379), Spt5 having a phosphomimetic CTR (HQY1441) and Spt5 having non-phosphorylatable CTR (HQY1414). ChIP analysis was performed as described in Chapter 2 (section 2.2.5). Briefly, crosslinked chromatin was obtained from the yeast strains and Rtf1 or Spt5 was immunoprecipitated by overnight incubation of the chromatin at 4°C with Rtf1 antiserum or antibody against Spt5 followed by incubation at 4°C with protein A beads for 2 hours. The immunoprecipitated chromatin was treated with proteinase K and the crosslinks were reversed by incubation overnight at 65°C. The amount of chromatin associated with the immunoprecipitated protein was then measured using real time PCR and primers sets specific to the indicated loci. The values shown above represent the average of the ratio of Rtf1 occupancy to Spt5 occupancy for each biological replicate. Error bars represent the standard error of the mean. The symbol "§" indicates that the relative occupancy of Rtf1 in the phosphomimetic mutant is significantly different compared to that of wild-type (p-value < 0.05) beyond the poly(A) site at both *PYK1* and *PMA1* genes while it was similar to that of wild-type at the other loci tested.

A.2 IDENTIFYING THE PHOSPHATASE FOR THE CTR OF SPT5

To begin to identify the phosphatase for the CTR of Spt5, I assessed the levels of phosphorylated Spt5 in selected phosphatase mutants using immunoblotting analysis. Our selection of phosphatase mutants to be tested was guided by Dr. Nathan Clark (University of Pittsburgh School of Medicine). He performed evolutionary rate covariance analysis with the proteins involved in the recruitment of Paf1C (all members of Paf1C, Spt5, Bur1-Bur2 kinase complex and Pol II) and all the phosphatases in yeast to identify the phosphatases that have co-evolved with the proteins involved in the recruitment of Paf1C (indicated by the p-values for the evolutionary rate covariance) (CLARK *et al.* 2012). This analysis is based on the assumption that proteins involved in similar functions co-evolve (CLARK *et al.* 2012). Fcp1, Ssu72, Ptp1 and Oca1 seemed to co-evolve with Spt5 and the subunits of Paf1C in this analysis (Figure 24A). Oca1 is a putative tyrosine phosphatase required for the cell cycle arrest induced upon oxidative damage of DNA (ALIC *et al.* 2001). Ptp1 is also a tyrosine phosphatase that dephosphorylates Fpr3 protein, an immunophilin, and it localizes in the cytoplasm (GUAN *et al.* 1991; WILSON *et al.* 1995). Since both Ptp1 and Oca1 are tyrosine phosphatases, they may not be the phosphatases dephosphorylating the phosphorylated serine residues of the Spt5 CTR. However, to confirm this we need to determine the levels of phosphorylated Spt5 in Oca1 and Ptp1 deletion mutants using immunoblotting analysis. To begin, we decided to focus on phosphatases previously known to have roles in transcription (Fcp1 and Ssu72) or known to interact with Spt5 (PP4).

Since Fcp1 and Ssu72 have well-characterized roles in transcription, they seemed to be the strongest candidates for the Spt5 CTR phosphatase. Fcp1 is the phosphatase for the

phosphorylated Ser2 of the Pol II CTD and Ssu72 dephosphorylates Ser5 and Ser7 of the Pol II CTD (CHO *et al.* 2001; KRISHNAMURTHY *et al.* 2004; ZHANG *et al.* 2012a). Additionally, Ssu72 also facilitates gene looping through interaction with TFIIB and it plays a role in RNA termination and 3'end processing through association with the APT complex (NEDEA *et al.* 2003; SINGH and HAMPSEY 2007). In addition, Ssu72 is enriched near the poly(A) site, making it a good candidate for the Spt5 CTR phosphatase since we anticipate that this dephosphorylation event may occur near the poly(A) site (DICHTL *et al.* 2002; NEDEA *et al.* 2003; SINGH and HAMPSEY 2007). The Fcp1 mutant that I tested was previously shown by an undergraduate researcher in our laboratory to be synthetically lethal with Rtf1. The functional implications of this mutant are however unknown. The Ssu72 mutant that I tested was previously shown by the Brow laboratory to impact the Nrd1-dependent and poly(A)-dependent termination of transcription (STEINMETZ and BROW 2003). However, the location of the residue mutated is not close to the catalytic site and its effect on the enzymatic activity of Ssu72 is unknown.

I also made a Psy2 null strain to test using this analysis, since proteomic studies with Psy2, a regulatory subunit of the phosphatase PP4 complex, have uncovered Spt5 as an interactor (GINGRAS *et al.* 2005; O'NEILL *et al.* 2007). Additionally, I also included mutants of other phosphatases previously shown to have roles in transcription such as Rtr1, Glc7 and Sit4. Rtr1 dephosphorylates Ser5 of the Pol II CTD and facilitates the transition of the Pol II CTD from its Ser5 phosphorylated form to its Ser2 phosphorylated form (MOSLEY *et al.* 2009). Glc7 is a serine/threonine phosphatase known to act on a variety of substrates and its targeting to specific substrates is achieved through association with regulatory subunits. For example, association with the Reg1 subunit targets Glc7 to the Snf1 protein (TU and CARLSON 1995). Interestingly, binding to the Ref2 subunit facilitates the association of Glc7 with cleavage and poly-

adenylation factor containing complex and hence Glc7 associated with Ref2 protein and seemed to be a good candidate for Spt5 CTR phosphatase since this form of Glc7 is enriched at the poly(A) site, our predicted site for Spt5 CTR dephosphorylation (NEDEA *et al.* 2003). A *REF2* null strain would have been a better strain that I could have used in my analysis, but we first decided to test the strains previously used in our laboratory (*glc7-T152K* and *reg1Δ*). Glc7-T152K is known to affect the association of Glc7 with Reg1 regulatory subunit (TU and CARLSON 1995). However, this mutant grows slower than a *reg1Δ* strain suggesting that it may have additional effects on the functioning of Glc7. We also included Sit4 in our analysis since Sit4 mutants exhibit Spt$^-$ phenotype indicative of transcriptional defects (SHIRRA *et al.* 2005). Sit4, also like Glc7, is the catalytic subunit found in many complexes and its association with specific regulatory subunits targets it to specific substrates (LUKE *et al.* 1996). I also used a *bur2Δ* strain as negative control in my western blot analysis. This mutant has a defective Bur1-Bur2 kinase complex and hence it diminishes the levels Spt5 phosphorylated at the CTR.

My immunoblotting analysis suggests that the Fcp1 point mutant that I tested causes a reduction and not an increase in the levels of phosphorylated Spt5 suggesting that Fcp1 impacts the CTR of Spt5 indirectly and not by directly dephosphorylating its CTR (Figure 24B). On the other hand, the temperature sensitive Ssu72 point mutation does not lower the levels of phosphorylated Spt5 after 1 hour incubation at the restrictive temperature (Figure 24C). However, the absence of increased levels of phosphorylated Spt5 in these mutants does not rule out the possibility that Fcp1 or Ssu72 could dephosphorylate the Spt5 CTR. Immunoblotting analysis should be performed with other hypofunctional mutants of Fcp1 or Ssu72 to confirm the effect of Fcp1 and Ssu72 on the levels of phosphorylated Spt5.

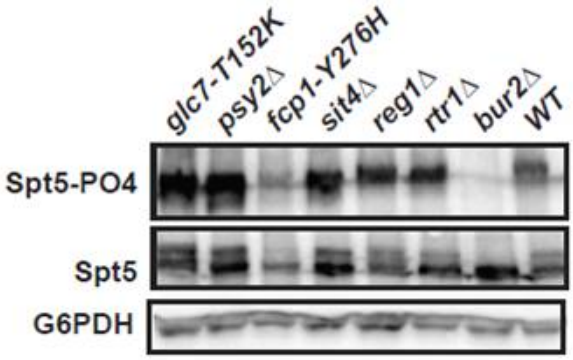
Interestingly, this analysis showed that Glc7 and Psy2 mutants may have increased levels of phosphorylated Spt5 (Figure 24B). However, this experiment has been performed only once. This experiment needs to be repeated with biological triplicates to make it possible to determine if the ratio of phosphorylated to total Spt5 in these mutants is statistically different from that of wild-type strains. Furthermore, since the *reg1Δ* strain did not show elevated levels of phosphorylated Spt5 it suggests that the *glc7-T152K* mutant may affect the level of phosphorylated Spt5 independently of Reg1 (Figure 24B).

Thus, Glc7 and Pph3 (catalytic subunit of PP4 complex) seem to be promising candidates as the phosphatases of the Spt5 CTR and the mutants of these phosphatases along with others need to be further tested by immunoblotting analysis to identify the phosphatase of Spt5 CTR that when mutated increases the levels of phosphorylated Spt5 and may hence induce terminations defects.

A.

SPT5	BUR1	BUR2	KIN28	RPO21	PAF1	LEO1	RTF1	CDC73	CTR9	
0.034	0.14	0.1358	0.4003	0.3146	0.186	0.108	0.346	0.067	0.013	**PPT1**
0.035	0.155	0.037	0.1607	0.1907	0.043	0.132	0.138	0.095	0.426	**GLC7**
0.052	0.053	0.0547	0.4123	0.2831	0.108	0.307	0.222	0.0574	0.073	**PPG1**
0.031	0.255	0.0401	0.0626	0.1844	0.103	0.367	0.058	0.1141	0.076	**PPH3**
0.139	0.422	0.0797	0.0381	0.0087	0.009	0.064	0.134	0.2704	0.541	**SIT4**
0.163	0.014	0.0337	0.4421	0.6011	0.395	0.477	0.378	0.3766	0.229	**PTC1**
0.013	0.062	0.097	0.3773	0.2135	0.133	0.166	0.274	0.0547	0.077	**PTC3**
0.012	0.009	0.0033	0.0243	0.101	0.125	0.205	0.019	0.1507	0.308	**PTC4**
0.122	0.12	0.0169	0.1646	0.0956	0.002	0.061	0.127	0.005	0.198	**PTC6**
0.301	0.416	0.0834	0.1844	0.2801	0.085	0.014	0.274	0.1296	0.168	**CDC14**
0.159	0.084	0.0525	0.038	0.253	0.604	0.559	0.037	0.2552	0.545	**YVH1**
0.01	0.003	0.0086	0.1463	0.0395	0.161	0.126	0.029	0.1908	0.358	**OCA1**
0.002	0.106	0.0924	0.0796	0.0101	0.124	0.22	0.001	0.0319	0.024	**SSU72**
0.082	0.022	0.0373	0.0524	0.0461	0.057	0.014	0.007	0.0242	0.085	**PTP1**
0.12	0.039	0.2619	0.0679	0.1348	0.162	0.6	0.087	0.2811	0.458	**PTP2**
0.012	0.047	0.0676	0.1235	0.0108	0.011	0.018	0.097	0.0194	0.107	**FCP1**
0.093	0.204	0.0261	0.0109	0.1219	0.091	0.063	0.131	0.0725	0.112	**RTR1**

B.



C.

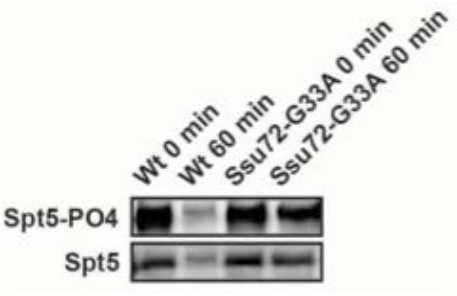


Figure 24. Screen for the phosphatase of the Spt5 CTR

(A) Covariance analysis was performed by Dr. Nathan Clarke to determine the p-values for the correlation of the covariance rates of the members of Paf1C and the proteins involved in recruitment of Paf1C including Bur1-Bur2 kinase complex, Spt5 and Pol II and the yeast phosphatases (CLARK *et al.* 2012). The degree of redness of the boxes correlates with the p-values with dark red indicating very low p-values (>0.05) and very light red indicating a p-value of less than 0.1. (B and C) Immunoblotting analysis was performed on TCA extracts of the indicated phosphatase mutants (prepared as described in Chapter 2, section 2.2.3) using antibody against the phosphorylated CTR of Spt5 (Gift from Dr. Steve Hahn). Immunoblotting analysis performed with antibody against Spt5 that recognizes both the phosphorylated and unphosphorylated forms of Spt5 served as a control for total Spt5 levels. G6PDH levels served as a control for the total amount of extract.

Table 6. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype
KY1200	*MATα his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY1351	*MATα bur2Δ::URA3 his3Δ200 lys2-128∂ ura3 leu2 CTR9-6xMYC::LEU2*
KY2094	*MATα psy2Δ::KanMX his3Δ200 lys2-128∂ leu2Δ1 ura3-52 ade8*
PY501	*MAT***a** *sit4Δ::HIS3 his3Δ200 leu2Δ1 ura3-52*
PY1048	*MAT***a** *reg1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
SRY55	*MATα fcp1-Y276H MATα lys2-128∂ leu2Δ1 ura3-52 trp1Δ63 ade8*
¥	*MATα rtr1Δ::KanMx his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*
OKA45ξ	*MATa glc7-T152K his3Δ200 lys2-801 ura3-52 trp1 Δ1*
OKA65£	*MATα cup1Δ::URA3 his3 trp1 lys2 ade2 leu2*
OKA257£	*MATα ssu72-G33A cup1Δ::URA3 his3 trp1 lys2 ade2 leu2*
HQY1379§	*MAT***a** *spt5Δ::HIS3 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 PAF1-13xMYC::KanMX4 pHQ1494 [LEU2 SPT5-3XHA]*
HQY1414§	*MATa spt5Δ::HIS3 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 PAF1-13xMYC::KanMX4 pHQ1876 [LEU2 spt5-S1-15A-3XHA]*
HQY1441§	*MAT***a** *spt5Δ::HIS3 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 PAF1-13xMYC::KanMX4 pHQ1894 [LEU2 spt5-S1-15D-3XHA]*

¥ Strain from the yeast deletion collection

ξ Provided by Dr. Marian Carlson

£ Provided by Dr. David Brow

§ Provided by Dr. Alan Hinnebusch

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