MECHANISMS OF RECRUITMENT OF THE CTD PHOSPHATASE RTR1 TO RNA POLYMERASE II

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LIST OF ABBREVIATIONS

2D-PAGE	Two dimensional-polyacrylamide gel electrophoresis
CBP	Calmodulin binding peptide
ChIP	Chromatin Immunoprecipitation
CTD	C-terminal domain of yeast RNA polymerase II subunit Rpb1
CTDK-I	C-terminal domain kinase I
DALPC	Direct analysis of large protein complexes
ddH_2O	Distilled and de-ionized water
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
Fc	Fragment crystallizable region of IgG
FDR	False discovery rate(s)
IEF	Isoelectric focusing
IGEPAL	Octylphenoxypolyethoxyethanol
mRNA	Messenger RNA
MS	Mass spectrometry/mass spectrometer
MudPIT	Multidimensional protein identification technology
NSAF	Normalized spectral abundance factor(s)
ORF(s)	Open reading frame(s)
pI	Isoelectric point
RNAPII	Yeast RNA polymerase II complex
Rpb1-12	RNA polymerase II subunits 1-12
S2	Serine 2 of yeast RNA polymerase II C-terminal domain

S2-P	Phosphorylated serine 2 of yeast RNA polymerase II C-terminal domain
S5	Serine 5 of yeast RNA polymerase II C-terminal domain
S5-P	Phosphorylated serine 5 of yeast RNA polymerase II C-terminal domain
S7	Serine 7 of yeast RNA polymerase II C-terminal domain
S7-P	Phosphorylated serine 7 of yeast RNA polymerase II C-terminal domain
S. cerevisiae	Baker's yeast, *Saccharomyces cerevisiae*
SCX	Strong cation exchange
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
RP	Reversed phase chromatography
TAP	Tandem affinity purification
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
WT	Wild-type

INTRODUCTION

I. RNA Polymerase II-Dependent Transcription

There are three highly conserved RNA polymerases in eukaryotes that collectively are responsible for the transcription of all classes of cellular RNA. One of these enzymes, RNA polymerase II (RNAPII), is responsible for transcribing DNA to mRNA as well as most snRNAs and miRNAs. RNAPII is a large, 550 KDa complex that consists of 12 protein subunits known as Rpb1 through Rpb12 (Figure 1), several of which have conserved homologs in the eukaryotic polymerases RNAPI and RNAPIII (Cramer et al., 2008). In addition to its subunits, a wide variety of sequence specific and general transcription factors are required for RNAPII to bind to a promoter, form a competent initiation complex, and to modulate gene-specific messenger RNA (mRNA) transcript levels in response to environmental and developmental signals (Nikolov and Burley, 1997).

During RNA transcription in eukaryotes, nascent mRNA is processed at its 5' and 3'-ends and is often spliced, reviewed in (Kuehner et al., 2011). These processing steps are closely coupled to RNAPII transcription through a variety of mechanisms, one of which involves the recruitment of specific sets of RNA processing factors to specific modified forms of the C-terminal domain (CTD) of Rpb1, the largest subunit of RNAPII, reviewed in (Buratowski, 2009). The CTD of Rpb1 in yeast consists of 26-27 repeats of the sequence $Y_1S_2P_3T_4S_5P_6S_7$, which serves as a signaling platform that is unique to RNAPII (Corden et al., 1985). Specifically, serine-2 (S2), serine-5 (S5), and serine-7 (S7) of the CTD repeat are subject to phosphorylation by cellular kinases, and the pattern of serine

phosphorylation is specific for a given stage of transcription in the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) (Akhtar et al., 2009; Chapman et al., 2007; Kim et al., 2009; Komarnitsky et al., 2000). It is through this mechanism; the stage-specific phosphorylation of S2, S5, and S7, that information about the location of RNAPII relative to the target gene is encoded in the CTD and is used to recruit accessory proteins at the proper time during transcription.

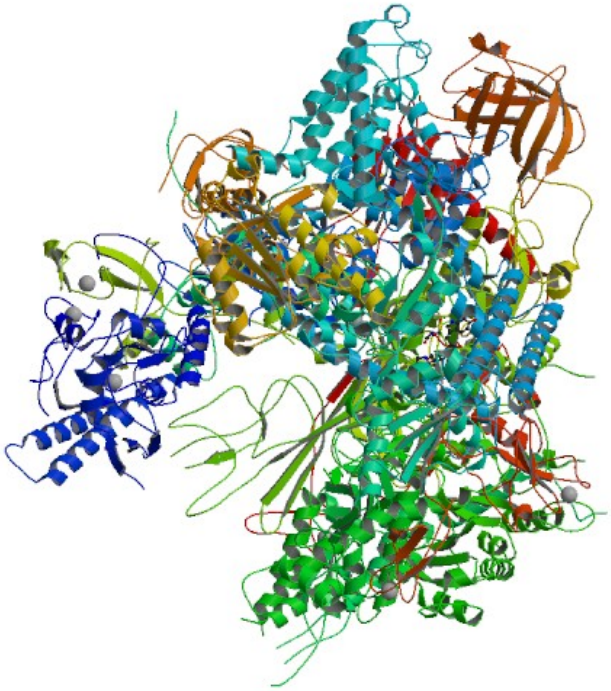

Figure 1. Ribbon structure of *Saccharomyces cerevisiae* RNA polymerase II.

RNAPII must exist in a hypophosphorylated state before a viable initiation complex can be formed at the promoter of a target gene (Laybourn and Dahmus, 1990; Lu et al., 1991). Immediately following the formation of the initiation complex, there is an increase in the level of serine-5 phosphorylation (S5-P), which is responsible for recruiting the 5'-capping machinery (Fabrega et al., 2003; Komarnitsky et al., 2000;

Schroeder et al., 2000). As transcription proceeds into elongation, there is a change in the CTD phosphorylation pattern that results in increases in S2-P (Cho et al., 2001). The phosphorylation of S2 is especially important as transcription nears termination because S2-P serves as a binding site for the termination and polyadenylation machinery to interact with the CTD (Kim et al., 2004; Licatalosi et al., 2002; Meinhart and Cramer, 2004).

II. Additional Proteins Involved in the Regulation of RNAPII Activity

In addition to the subunits of RNAPII, numerous other proteins are involved in the regulation of transcription. These factors respond to a wide range of cellular signals that relay information about the cell's environment (e.g. oxidative and heat stress), state of nutrition, and proliferation status back to the transcription machinery (Mager and De Kruijff, 1995). The role of transcription complexes such as Mediator, Elongator, and SAGA, for example, have been previously reviewed (Bhaumik, 2011; Casamassimi and Napoli, 2007; Creppe and Buschbeck, 2011); however, a detailed understanding of the kinases and phosphatases that are involved in the regulation of transcription through their role in mediating the phosphorylation state of the CTD is less clear.

Our current working model of transcription (Figure 2) shows RNAPII at the promoter of a target gene during transcription initiation. Successively, the Kin28 subunit of the general transcription factor TFIIH phosphorylates S5 of the CTD, which recruits the capping machinery and initiates mRNA processing as RNAPII is released from the promoter and moves into the coding region of the gene (Komarnitsky et al., 2000; Schroeder et al., 2000). In addition to phosphorylating S5, TFIIH has been shown to

phosphorylate S7 *in vitro* and was also proven to be necessary for S7-P *in vivo* (Akhtar et al., 2009; Kim et al., 2009). During transcription elongation, the Ctk1 subunit of the CTDK-I complex catalyzes the phosphorylation of CTD S2 (Cho et al., 2001), and it is this increase in S2-P that recruits specific members of the termination and polyadenylation machinery as RNAPII approaches the 3'-end of the target gene (Kim et al., 2009; Licatalosi et al., 2002; Meinhart and Cramer, 2004). Additionally, the cyclin-dependent kinase Bur1 was shown to phosphorylate CTD S2 near target gene promoters and was also shown to stimulate CTDK-I mediated S2-P during transcription elongation (Qiu et al., 2009).

During the transcription cycle, the transition from CTD S5-P to S2-P is affected by the aforementioned kinases as well as by the opposing action of CTD-phosphatases. Three CTD phosphatases have been characterized in yeast that remove S2, S5, and S7 phosphorylation (Bataille et al., 2012; Kobor et al., 1999; Meinhart and Cramer, 2004; Mosley et al., 2009; Zhang et al., 2012). The first characterized phosphatase, Fcp1, has been shown to dephosphorylate S2-P during the transcription cycle and may play a role in recycling RNAPII by converting it back to its hypophosphorylated state following transcription termination (Archambault et al., 1997; Aygun et al., 2008; Chambers et al., 1995; Cho et al., 2001; Kobor et al., 1999; Kong et al., 2005).

The phosphatase Ssu72 removes S5-P from the RNAPII CTD (Krishnamurthy et al., 2004) and has recently been characterized as a S7-P phosphatase (Kobor et al., 1999; Zhang et al., 2012). Ssu72 is enriched at the 3'-end of RNAPII target genes where it functions as a subunit of the cleavage and polyadenylation factor (CPF) complex (He et al., 2003) and is consequently also involved mRNA 3' end processing (He et al., 2003).

However, Ssu72 phosphatase activity has been shown to be independent of its role in dephosphorylating S5-P (Krishnamurthy et al., 2004). The involvement of Ssu72 in the later stages of transcription is supported by one, evidence that Ssu72 enrichment at the promoter is low if not absent as determined by Chromatin Immunoprecipitation (ChIP) experiments (Ansari and Hampsey, 2005; Nedea et al., 2003) and two, that Ssu72 temperature sensitive mutants show defects in transcription termination (Steinmetz and Brow, 2003). Furthermore, Ssu72 was recently shown to recognize the S5-P-cis-proline-6 isomer, and the cis/trans isomerization reaction catalyzed by the proline isomerase Ess1 was shown to facilitate the dephosphorylation of S5-P by Ssu72 (Werner-Allen et al., 2011; Xiang et al., 2010; Zhang et al., 2011). ChIP experiments have shown that Ess1, much like Ssu72, is recruited mainly to the transcription terminator of RNAPII target genes and not to the gene body (Krishnamurthy et al., 2009). Since the majority of S5-P is dephosphorylated during transcription elongation, these observations raise doubt the Ssu72 could be the only S5-P phosphatase (Figure 2).

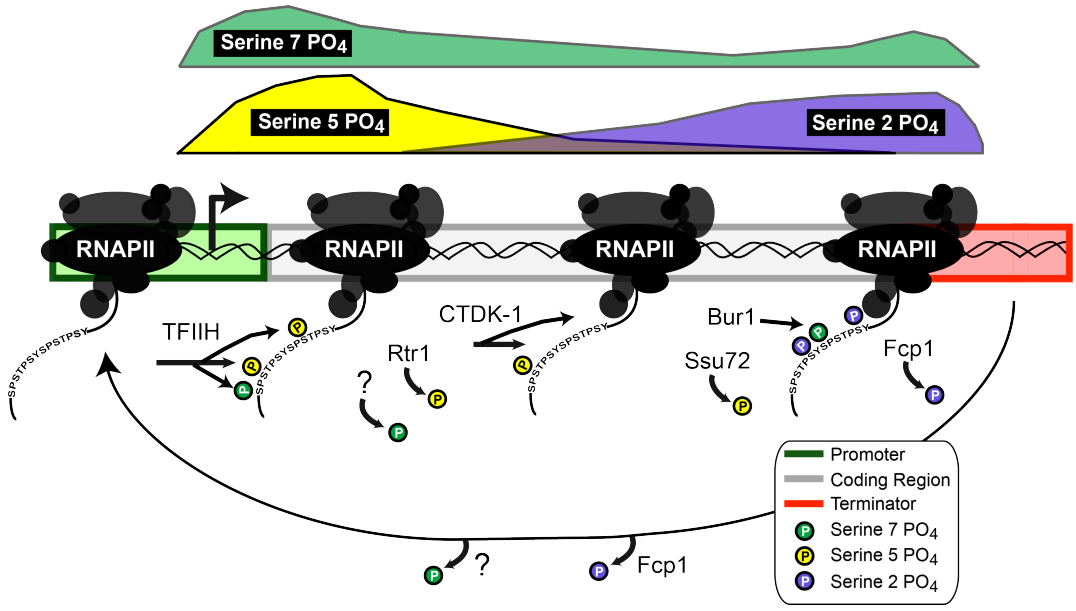

Figure 2. Model of RNAPII-dependent transcription in *Saccharomyces cerevisiae*. The CTD of RNAPII exists in a hypophosphorylated state prior to forming a competent initiation complex at a gene promoter. TFIIH increases S5-P and S7-P at the promoter following initiation, and as elongation proceeds, the phosphatases Rtr1 and Ssu72 decrease S5-P as Bur1 and CTDK-1 phosphorylate S2. As RNAPII approaches the terminator, Fcp1 removes S2-P, and Ssu72 attenuates S7-P to facilitate RNAPII recycling.

Fcp1 has been experimentally shown to dephosphorylate S2-P (Cho et al., 2001; Kong et al., 2005), and Ssu72 has been shown to dephosphorylate both S5-P and S7-P (Krishnamurthy et al., 2004; Zhang et al., 2012). However, neither phosphatase has been shown to regulate the transition from S5-P to S2-P during transcription elongation, where the largest drop in S5-P occurs. Another protein, Rtr1 (regulator of transcription 1) was found to bind to phosphorylated RNAPII and was required for the induction of *GAL* genes by galactose (Gibney et al., 2008). In 2009, using both *in vitro* and *in vivo* experiments, Mosley *et al.* demonstrated that Rtr1 is a CTD phosphatase that plays a crucial role in transcription elongation by regulating the transition of CTD S5-P to S2-P (Mosley et al., 2009). Rtr1 was found to co-purify with a transcription competent form of

RNAPII with peak levels of Rtr1 localizing to active RNAPII target genes between the peaks of S5-P and S2-P RNAPII (Mosley et al., 2009). In addition, *rtr1Δ* resulted in an accumulation of CTD S5-P in both whole cell extracts as well as across the open reading frames (ORFs) of two genes in yeast. *In vitro* experiments revealed that Rtr1 is able to dephosphorylate the CTD alone or in the context of an elongation complex (containing DNA, RNA, and RNAPII) (Mosley et al., 2009). Additionally, using both *in vitro* and *in vivo* methods, Egloff *et al.* recently identified that the human homolog of Rtr1 known as RPAP2 (RNAPII-associated polymerase 2) is a CTD phosphatase that dephosphorylates S5-P (Egloff et al., 2012). The authors demonstrated that RPAP2 was recruited to RNAPII via S7-P, which resulted in the recruitment of Integrator and the dephosphorylation of S5-P at a representative RNAPII target snRNA gene in humans.

The kinases and phosphatases that were introduced in this section and that have been shown to modify the RNAPII CTD during transcription are summarized in Table 1.

Table 1. Kinases and phosphatases responsible for modifying the RNA polymerase II CTD during transcription. The kinases responsible for phosphorylating the CTD are listed on the left side of the table and their opposing phosphatases are on the right.

RNAPII CTD Kinases*	Kinase Activity	CTD Site	Phosphatase Activity	RNAPII CTD Phosphatases*
TFIIK†				TFIIB
(Kin28)¥	**Cyclin dependent**	**Ser-5/Ser-7**	**TFIIB associated**	**(Ssu72)¥**
(Ccl1)	Kin28 cyclin			
(Tfb3)				
		Ser-5	**Phosphatase**	**Rtr1**
CTDK-I				TFIIF
(Ctk1)	**Kinase**	**Ser-2**		(Tfg1)
(Ctk2)	Regulatory subunit			(Tfg2)
(Ctk3)				(Taf14)
BUR1-BUR2		**Ser-2**	**Tfg1 associated**	**(Fcp1)**
(Bur1)	**Cyclin dependent**	**Ser-2**		
(Bur2)	Bur1 cyclin			

*Complex subunits or associated proteins are in parentheses

†TFIIK is a subcomplex of the general transcription factor TFIIH, which is comprised of Ssl2, Tfb1, Tfb2, and Ssl1subunits in addition to the TFIIK subunits

¥Dual CTD-serine specificity kinase/phosphatase

III. The Recruitment of Rtr1 Phosphatase to RNAPII

It was previously observed that RNAPII must exist in a hypophosphorylated state prior to forming a competent transcription initiation complex (Laybourn and Dahmus, 1990; Lu et al., 1991). Additionally, during transcription, specific kinases and phosphatases act on the CTD of RNAPII to regulate the phosphorylation state of S2, S5, and S7, which contributes to the regulation of RNAPII transcription as described. Specifically, the phosphatase Rtr1 was shown to regulate a key step important for transcription elongation and termination, likely through regulation of the transition of the phosphorylation state of the RNAPII-CTD from S5-P to S2-P during transcription

elongation (Mosley et al., 2009). Although the role that Rtr1 plays in regulating the aforementioned transition has been described, the mechanism involved in the recruitment of Rtr1 to RNAPII has not been elucidated in yeast. Consequently, the present work will focus on testing two key hypotheses. The first of these was that Rtr1 is recruited to the RNAPII complex during transcription elongation through an interaction with an unidentified protein(s), and that post-translation modification of Rtr1 or its interacting partner(s) may also play a role in its recruitment to RNAPII. Therefore, experiments were conducted to uncover protein-protein interactions and post-translational modifications involved in the recruitment of Rtr1 to RNAPII during transcription. In 2011, Fasolo *et al.* used protein arrays to demonstrate a direct interaction between two of the subunits of the CTDK-I complex (specifically Ctk1 and Ctk2) and Rtr1 (Fasolo et al., 2011). Therefore, the second hypothesis tested was that an interaction between Rtr1 and the CTDK-I complex is involved in the recruitment of Rtr1 to RNAPII. Furthermore, it was hypothesized that Rtr1 is a substrate of Ctk1, the kinase subunit of CTDK-I, and that Rtr1 phosphorylation is also involved in its recruitment to RNAPII. Alternatively, the requirement for CTDK-I may be associated with its kinase activity as a known modifier of S2 in the RNAPII CTD.

The first objective of the present work was to identify novel proteins that interact with Rtr1 and RNAPII that could potentially facilitate its recruitment to RNAPII during transcription. Specifically, we searched for proteins that interacted with Rtr1 alone, with RNAPII alone, and with the RNAPII-Rtr1 complex using affinity purifications followed by MudPIT analyses. Special attention was given to interactions with the PAF complex, which is important in transcription initiation and elongation, as well as to post-

translational modifications of the interacting partners, including RNAPII, which may play a role in recruiting Rtr1 to RNAPII.

The PAF complex is found in both yeast and higher eukaryotes, is comprised of five subunits (Paf1, Cdc73, Ctr9, Rtf1, and Leo1), and has been found to associate with RNAPII throughout the mRNA transcription cycle (Mueller and Jaehning, 2002; Mueller et al., 2004). Furthermore, consistent with its presence at the promoter and continuing through transcription termination, PAF has been demonstrated to associate with an impressive list of transcription factors that are involved in regulating initiation and elongation, reviewed in (Jaehning, 2010), chromatin remodeling and modification (Gerber and Shilatifard, 2003), and mRNA export/processing (Mueller et al., 2004). Following its initial recruitment to RNAPII, PAF remains associated with RNAPII throughout transcription and may serve as a scaffold for the recruitment of transcription factors during elongation (Gerber and Shilatifard, 2003; Penheiter et al., 2005). The interaction between Rtr1 and PAF subunits was previously observed (Mosley, A.L., unpublished data); therefore, we focused on confirming the interaction between the PAF complex and Rtr1 to determine if their interaction plays a role in Rtr1 recruitment.

The second objective in understanding the mechanism involved in the recruitment of Rtr1 to RNAPII during transcription involved confirming the interaction between CTDK-I and Rtr1 observed *in vitro* by Fasolo *et al.* in 2011. CTDK-I plays an important role in regulating the activity of RNAPII through its phosphorylation of S2 and S5 of the CTD (Jones et al., 2004). CTDK-I is comprised of catalytic (Ctk1) and regulatory (Ctk2) subunits, as well as a third subunit of unknown function (Ctk3) (Lee and Greenleaf, 1989). In 2004, Joes *et al.* reported the pattern of CTD phosphorylation by CTDK-I

(Jones et al., 2004); the authors used direct chemical analysis of *in vitro* experiments to determine that CTDK-I phosphorylates S5, that it phosphorylates S2 if S5 was previously phosphorylated, and that it shows increased affinity for CTD substrates that have been previously phosphorylated at either S2 or S5. However, *in vivo* studies on CTDK-I mutants have shown that the kinase complex is responsible for the majority of the S2-P in yeast, whereas S5-P is not affected *in vivo* (Cho et al., 2001). The focus of the present work in studying the role that CTDK-I may play in recruiting Rtr1 to RNAPII during transcription is two-fold. First, the RNAPII-CTD phosphorylation pattern, for which CTDK-I plays a role, has been shown to recruit factors to RNAPII during transcription (Komarnitsky et al., 2000). Additionally, studies on human RPAP2 have shown that it is capable of binding to a phosphorylated CTD at one site (S7-P) while targeting a different site for dephosphorylation (S5-P) (Egloff et al., 2012). Second, a direct interaction between Rtr1 and two separate subunits of CTDK-I (specifically Ctk1 and Ctk2) was previously observed, although no biological role for this interaction was determined (Fasolo et al., 2011). Additionally, the possibility that Rtr1 may serve as substrate for CTDK-I, and that the phosphorylation of Rtr1 by CTDK-I may play a role in the recruitment of Rtr1 to RNAPII, was investigated herein as well.

In the present work, we studied the recruitment of Rtr1 to RNAPII in *S. cerevisiae* by investigating novel Rtr1-interacting proteins using affinity purification methods followed by MudPIT-LC/MS analysis. The first set of experiments was conducted in yeast that expressed a TAP-tagged version of the RNAPII subunit, Rbp3, and C-terminally His-FLAG-HA-tagged Rtr1 phosphatase (hence referred to as Rtr1-HFH). This was followed by a single purification experiment in yeast that expressed C-terminally V5- or FLAG-

tagged Rtr1. The second research objective was pursued using single and double affinity purification steps to isolate CTDK-I subunits and Rtr1, which were subsequently detected by western blot. Furthermore, the *in vivo* phosphorylation of Rtr1 by Ctk1 was investigated through the affinity purification of Rtr1-TAP from wild-type or CTDK-I mutant cells followed by MudPIT-LC/MS analysis.

IV. The Development of Multi-Dimensional Protein Identification Technology

The ability to identify global changes in gene expression has been feasible for more than a decade through the use of DNA microarrays to measure mRNA transcript levels (Boisvert et al., 2011; Lockhart and Winzeler, 2000). However, complex mechanisms involved in mRNA translation, and differences in protein stability, can lead to a discrepancy between mRNA transcript levels and protein concentrations (Boisvert et al., 2011). This discrepancy uncovered the need to directly measure proteins in complex samples, which is a prerequisite to predict the effect of gene expression on biological function (Hatzimanikatis and Lee, 1999).

Mass spectrometry (MS) is the method of choice for identifying and quantifying proteins in complex samples such as cell lysates. In order to generate molecules with better mass spectrometric properties, the intact proteins in a sample are typically digested with a protease such as trypsin. This step, although necessary, serves to further increase the complexity of the sample. Therefore, prior to proteolytic digestion and mass spectrometric analysis, the intact proteins in a sample must be resolved from each other.

Historically, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was the approach most widely used to achieve protein resolution (Hanash, 2000). Using this

approach, proteins are separated in the first dimension by their isoelectric point (pI) using isoelectric focusing (IEF) followed by a second dimension of separation by molecular weight using SDS-PAGE (Friedman et al., 2009). Following separation, each protein spot must be excised and extracted from the SDS-PAGE gel preceding proteolytic digestion and MS analysis. Major shortcomings to this approach include that it is tedious, time-consuming, and is insufficient for low abundance proteins, membrane bound proteins, or proteins with extreme pI values or molecular weight, reviewed in (Coombs, 2011).

The need to improve the throughput of direct protein analyses, as well as to measure low abundance proteins and those that cannot easily be resolved by 2D gels provided an impetus for the development of multidimensional liquid chromatographic approaches. In 1999, Link *et al.* reported their strategy for the direct analysis of large protein complexes (DALPC) that they implemented to identify individual proteins in large complexes within a cell (Link et al., 1999). Unlike 2D-PAGE, their approach could be placed online with a mass spectrometer, which greatly improved the efficiency of protein measurements. Following denaturation and proteolytic digestion of the proteins in a sample, the DALPC process separates the resulting complex mixture of proteolytic peptides using two-dimensional liquid chromatography. Peptides are separated in the first dimension by their isoelectric point using strong cation exchange (SCX), eluted from SCX using increasing concentrations of KCl, and further separated in the second dimension by their hydrophobicity using reverse phase (RP) chromatography. The chromatographically resolved peptides exit the RP directly into the tandem mass spectrometer for analysis. Following analysis, the resulting MS/MS spectra are submitted to a protein search

algorithm such as SEQUEST that correlates them with theoretical spectra generated from the protein sequences in a species-specific FASTA database (MacCoss et al., 2002; Moore et al., 2001). This process was subsequently adapted for the rapid analysis of large-scale proteomes, such as *Saccharomyces cerevisiae*, and was termed multidimensional protein identification technology (MudPIT) (Washburn et al., 2001). A major difference between MudPIT and DALPC is the use of ammonium acetate as the salt for elution of the peptides from SCX (Link et al., 1999; Washburn et al., 2001). MudPIT-LC/MS has been demonstrated to be highly reproducible for quantitatively measuring proteins complex components in *S. cerevisiae* as well as peptide mapping of post-translational modification (Black et al., 2008; Litovchick et al., 2011; McDonald and Yates, 2002; Mosley et al., 2011). Consequently, MudPIT-LC/MS was utilized as the primary analytical tool for the work presented here.

V. Introduction to Tandem Affinity Purification

The recent sequencing of the complete genomes of several species has provided the means to delve deeper into the biochemistry of these biological systems. The availability of genomic information facilitates determining the identity of encoded proteins using methods such as mass spectrometry. Additionally, using techniques such as mass spectrometry, the direct measurement of protein post-translational modifications, protein-protein interactions, and protein expression level is achievable.

Historically, studying protein-protein interactions at the level of an organism was accomplished by two-hybrid screening, reviewed in (Petschnigg et al., 2011). However, issues with selectivity and the need for orthogonal strategies to complement two-hybrid

experiments has led to the combination of protein purification schemes and mass spectrometry, which has allowed the rapid identification of interacting partners (Puig et al., 2001). Because of the wide range of physicochemical properties that can be taken advantage of to isolate a target protein, it is exceedingly difficult to develop a generic protein purification scheme. To address this shortcoming, *Puig et al.* published their Tandem Affinity Purification (TAP) method in 2001, which they targeted to the purification of native protein complexes (Puig et al., 2001). One shortcoming of Puig's methodology is difficulty in purifying insoluble membrane and chromatin-bound proteins; however, affinity-purification schemes have subsequently been utilized to study chromatin-associated (Lambert et al., 2009; Mosley et al., 2009) and membrane-bound proteins (Wisniewski, 2011).

The TAP method for the purification of protein complexes (Rigaut et al., 1999) involves the fusion of a TAP tag to the N- or C-terminus of the target protein. In yeast, this can be accomplished with standard molecular biology techniques and polymerase chain reaction (Knop et al., 1999); homologous recombination is efficient in yeast and can be used to introduce the TAP tag to the protein of interest through the direct incorporation of the C-terminal TAP tag sequence into the chromosomal locus of the protein-encoding gene of interest. The work presented here relies on the use of the C-terminal TAP tag, which incorporates two different epitopes that can be taken advantage of for purification (see Table 2). These include the ZZ binding domains of Protein A (*Staphylococcus aureus*), which bind strongly to the fragment crystallizable region (Fc) of IgG, and calmodulin binding peptide (CBP) (Chang, 2006). The two binding regions are separated by a Tobacco Etch Virus (TEV) protease cleavage site, which is used to

facilitate cleavage of the fusion protein N-terminal to the Protein A portion of the epitope tag resulting in elution of the protein of interest from the IgG resin. Following preparation of the yeast cell extract, the protein of interest and its interacting partners are isolated using a two-step purification process, which relies on the Protein A and CBP portions of the TAP tag. The resulting proteins are highly purified and are subjected to tandem MS analysis.

In addition to the TAP tag, other affinity tags can be used individually or in combination to study the interaction of proteins of interest and identify new interacting partners that may be required for their recruitment or function. The use of different protein tag combinations for tandem affinity purification was reviewed by Li in 2010 (Li, 2010), and a list of the tags used in the present work is presented in Table 2. It should also be mentioned that the methods presented here can be applied to mammalian system, and Burckstummer *et al.* presented their method of TAP purification to study interaction proteomics in mammalian cells (Burckstummer et al., 2006).

Table 2. Affinity tags used in the purification of Rtr1, RNAPII, CTDK-I, and their interacting partners. The table includes the tag epitopes and the reagents used to capture/elute them during the purification process.

Tag	Epitope	Affinity to	Elution
FLAG	DYKDDDDK	FLAG antibody-conjugated agarose resin	FLAG peptide, acid, on-resin digestion (trypsin)
6XHIS	HHHHHH	Ni(2+), Co(2+)	Imidazole
CBP	KRRWKKNFIAVSA-ANRFKKISSSGAL	Target of Calmodulin resin (+Ca dependent)	EGTA
V5	GKPIPNPLLGLDST	V5 antibody-conjugated agarose resin	V5 peptide, acid
Protein A	*Staphylococcus aureus* IgG-binding domains	IgG-conjugated sepharose resin	DTT, surfactant; TEV protease (when part of TAP tag)
HA	YPYDVPDYA	HA antibody-conjugated agarose resin	HA peptide, acid
HFH*	6XHis-3XFLAG-TEV-3XHA	See 6XHIS, FLAG, HA	See 6XHIS, FLAG, HA
TAP*	CBP-TEV-Protein A	See CBP, Protein A	See CBP, Protein A

*The HFH and TAP tags are prepared from combinations of the other listed tag formats.

MATERIALS AND METHODS

I. Materials and Reagents

Table 3. Materials and reagents used in the investigation of protein-protein interactions with Rtr1 phosphatase

Reagent	Source / Preparation
5% SDS-PAGE Stacking Gel	1.25 mL polyacrylamide (40%), 1.25 mL tris-HCl (1 M, pH 6.8), 0.1 mL ammonium persulfate (10%), 0.1 mL SDS (10%), 0.01 mL TEMED, 7.3 mL ddH_2O
10% SDS-PAGE Gel	7.5 mL polyacrylamide (40%), 7.5 mL Tris-HCl (1 M, pH 6.8), 0.3 mL ammonium persulfate (10%), 0.3 mL SDS (10%), 0.012 mL TEMED, 14.4 mL ddH_2O
12% SDS-PAGE Gel	8 mL polyacrylamide (40%), 7.5 mL Tris-HCl (1 M, pH 8.8), 0.2 mL ammonium persulfate (10%), 0.2 mL SDS (10%), 0.008 mL TEMED, 4.1 mL ddH_2O
AcTEV Protease	Invitrogen, catalog # 12575-023; 10,000 units/μL
Agar	Thermo Fisher catalog # BP1423-500
Anti-Calmodulin Binding Protein Antibody (α-CBP)	Thermo Fisher catalog # CAB1001, 0.5 mg/mL
Anti-FLAG-HRP Antibody	Sigma catalog # A8592-1MG
Anti-FLAG Resin	Sigma catalog # A2220-5ML, M2-Agarose
Anti-Rabbit IgG-HRP	GE Healthcare catalog # NA934V
Anti-V5 Agarose	Sigma catalog # A7345-1ML
Anti-V5 Antibody/Horse Radish Peroxidase (α-V5-HRP)	Invitrogen catalog # R96125
Blocking Buffer	5% Milk in Tris Buffered Saline
Bio-Rad Precision Plus Protein Standards	Bio-Rad catalog # 161-0374
Calmodulin Binding Buffer	10 mM Tris (pH 8), 1 mM magnesium acetate, 1 mM imidazole, 2 mM calcium chloride, 10% glycerol, 0.5 mM DTT, 1X yeast protease inhibitors
Calmodulin Elution Buffer	10 mM Tris (pH 8), 0.3 M sodium chloride, 1 mM magnesium acetate, 1 mM imidazole, 2 mM EGTA, 10% glycerol, 0.5 mM DTT, 1X yeast protease inhibitors
Calmodulin-sepharose resin	GE Healthcare catalog # 17-0529-01
Chloroacetamide (CAM)	Sigma catalog # 22790

Reagent	Source / Preparation
Developing Solution	2.5% sodium carbonate, 0.05% formaldehyde, 0.005% sodium thiosulfate in ddH_2O (w/v)
Digestion Buffer	95:5 ammonium bicarbonate (50 mM, pH 8.5)/methanol (v/v)
DNAse I	Sigma catalog # D4623
ECL Plus Reagent	GE Healthcare catalog # RPN2133, reagent A and B were mixed in a 40:1 ratio, respectively
EconoPrep Column	Bio-Rad catalog # 731-1550
Endoproteinase Lys-C	Promega catalog # V1071
Ethanol Wash	30:70 ethanol/ddH_2O (v/v)
Fixing Solution	30:10:60 ethanol/acetic acid/ddH_2O (v/v/v)
Heparin	Sigma catalog # H3393
IgG-Sepharose Resin	GE Healthcare catalog # 17-0969-01
Immun-Star WesternC Kit	Bio-Rad catalog # 170-5070
Laemmli Buffer	Bio-Rad catalog # 161-0737
Mini-ProteanTGX Any KD Protein Gel	Bio-Rad catalog # 456-9034
Peptone	BD catalog # 211820-2KG
Precision Plus Ladder	Bio-Rad catalog # 161-0374
Precision Plus Protein Dual Xtra Standards (protein ladder)	Bio-Rad catalog # 161-0377
Protease Inhibitor Cocktail	10,000X, Sigma catalog # P8215-5ML
Salmon Sperm DNA	Sigma catalog # D7656-1ML
Sensitizer Solution	0.02% sodium thiosulfate in ddH_2O (w/v)
Silver Nitrate Solution	0.1% silver nitrate, 0.02% formaldehyde in ddH_2O (w/v)
Stop Solution	0.5% glycine in ddH_2O (w/v)
TAP Lysis Buffer	40 mM hepes (pH 7.5), 10% glycerol, 350 mM NaCl, 0.1% tween-20, 0.5 mM DTT, 1X yeast protease inhibitors
TCEP	0.1 M, Pierce catalog # 77720
TEV Cleavage Buffer	10 mM tris (pH 8), 150 mM NaCl, 0.1% IGEPAL, 0.5 mM EDTA, 10% glycerol, 1 mM DTT, 1X yeast protease inhibitors
Transfer Buffer	5.8 g tris-HCl, 2.9 g glycine, 900 mL ddH_2O, 100 mL methanol, 3.75 µL SDS (10%)
Tris/Gly/SDS Run Buffer	Bio-Rad catalog # 161-0732
Tris Buffered Saline (TBS)	Sigma catalog # T5912
Tris-HCl Buffer	100 mM Tris-HCl in ddH2O (pH 8.5)
Trypsin Gold	Promega catalog # V-5280, 100 µg/mL following reconstitution with 50 mM acetic acid
Yeast Extract	Sigma catalog # Y1625-1KG
Yeast Protease Inhibitors	10,000X, Sigma catalog # P8215

Reagent	Source / Preparation
YPD Media	30 g yeast extract, 60 g peptone, 2.7 L distilled water, 300 mL of 20% dextrose (w/v); sterilize by autoclaving

II. Rpb3-TAP/Rtr1-HFH Purification Scheme

Yeast Cell Lysis

Yeast cells that express RNAPII with a TAP-tagged version of the Rpb3 subunit (Rpb3-TAP), and Rtr1 containing an HFH tag (Rtr1-HFH), were cultured in YPD media at 30 ºC and grown to an OD_{600} ~2. The cells were centrifuged, and the supernatant was removed, and the cells were washed with ddH2O. Following centrifugation to remove the cell wash, the cells were re-suspended in Tap Lysis Buffer (10:1 Lysis Buffer/Cells, v/v), which was subsequently transferred to a liquid nitrogen bath using a syringe with an 18-gauge hypodermic needle. The frozen cell pellet was pulverized with a mortar and pestle, and two parts cell pellet and one part powdered dry ice were transferred into a Waring blender for further homogenization. The frozen homogenate was transferred to a second vessel and was allowed to thaw in a water bath at ambient temperature. The cell homogenate was treated with DNase I (100 units) and heparin (300 μg) for 10 min (ambient temperature) and was clarified by centrifugation as previously described (Mosley et al., 2009; Mosley et al., 2011). Following centrifugation, the clarified supernatant was transferred to a clean beaker to be used for affinity purification.

Purification of Proteins that Interact with Rtr1-HFH

The first step of the purification targeted proteins that interact with Rtr1-HFH when it was not in complex with RNAPII (Figure 3). In this experiment, the supernatant from the yeast whole cell lysate was treated with 500 μL of IgG-sepharose to immunoprecipitate the RNAPII complex via the Protein A portion of the Rpb3-TAP subunit. Following

incubation on a stir plate at 4 ºC, the IgG-sepharose was separated from the yeast cell lysate supernatant by gravity flow through a 30 mL Bio-Rad Econoprep column. The IgG-sepharose resin was washed with 30 mL of TAP Lysis Buffer, and the column flow-through containing unbound Rtr1-HFH that was not associated was transferred to a clean beaker. To isolate Rtr1-HFH and any interacting proteins, the Bio-Rad column flow-through was further treated with 100 µL of anti-FLAG resin and incubated with stirring overnight at 4 ºC. Following incubation, the sample was transferred to a second Econoprep column, and the supernatant was allowed to drain through the column by gravity flow. The column containing the anti-FLAG resin was washed with 30 mL of TAP Lysis Buffer followed by 1 mL of 50 mM ammonium bicarbonate (pH 8.5) to remove any proteins that did not interact with Rtr1. The anti-FLAG resin was re-suspended with 500 µL of Digestion Buffer and 10 µL of 100 µg/mL Trypsin Gold. The re-suspended sample was transferred to a 1.5 mL centrifuge tube and was digested "on-resin" overnight (with mixing) at 37 ºC. The following day, the sample wash centrifuged to remove the anti-FLAG resin, and the supernatant was acidified with 10 µL of formic acid and was loaded to a MudPIT column for LC/MS analysis.

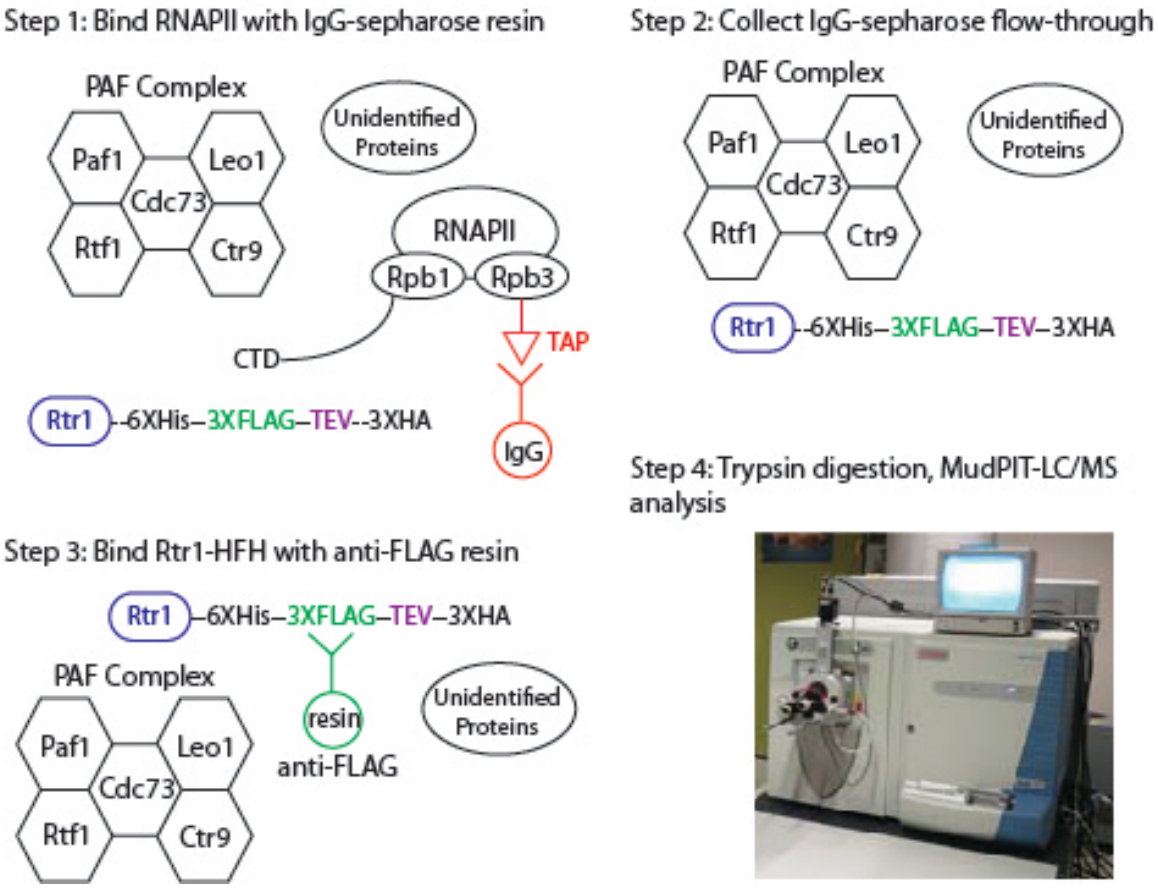

Figure 3. Purification scheme used to identify Rtr1-interacting proteins not in complex with RNAPII. In step 1, RNAPII is immunoprecipitated from yeast cell lysate with IgG-sepharose resin. The proteins that were not removed by the IgG-sepharose (step 2) and that interact with Rtr1-HFH were immunoprecipitated with anti-FLAG resin (step 3). Following "on-resin: digest with trypsin, the resulting peptides were analyzed by MudPIT-LC/MS (step 4).

Purification of Proteins that Interact with RNAPII

The second step of the purification targeted proteins that interact with RNAPII when it was not in complex with Rtr1-HFH (Figure 4). In this experiment, the supernatant from the yeast whole cell lysis was treated with 500 µL of IgG-sepharose to immunoprecipitate the RNAPII complex via the Protein A portion of the Rpb3-TAP subunit. Following incubation on a stir plate at 4 ºC, the IgG-sepharose was separated from the yeast cell lysate supernatant with a 30 mL Bio-Rad Econoprep column. The

IgG-sepharose resin was washed with 30 mL of TAP Lysis Buffer and 3 mL of TEV Cleavage Buffer to remove non-interacting proteins. Next, the washed IgG-sepharose resin containing immunoprecipitated RNAPII was re-suspended with 1 mL of TEV Cleavage Buffer and transferred to a 1.5 mL centrifuge tube. The sample was treated with 10 μL of AcTEV protease to elute RNAPII and its interacting partners from the IgG-sepharose resin (incubated overnight at 4 ºC with agitation).

The following day, the sample was transferred to a 30 mL Econoprep column and was allowed to drain into a 500 mL beaker by gravity flow. The column was subsequently washed with 30 mL of TAP Lysis Buffer, and the column flow-through (including the wash) was treated with 100 μL of anti-FLAG resin to remove any Rtr1 that was associated with RNAPII (incubated with stirring overnight at 4 ºC).

Following incubation, the sample was transferred to a second Econoprep column, and the eluate was allowed to drain through the column by gravity flow. The column containing the anti-FLAG resin was washed with 15 mL of Calmodulin Binding Buffer to remove RNAPII and interacting proteins that did not interact with Rtr1. The column flow-through was collected in a 15 mL centrifuge tube. Following the addition of 3 μL of 1 M calcium chloride to facilitate the CBP-calmodulin interaction, 200 μL of calmodulin-sepharose resin (pre-washed with Calmodulin Binding Buffer) was added to the sample to immunoprecipitate RNAPII and interacting proteins via the calmodulin-binding portion of the Rpb3-TAP tag. The sample was incubated overnight at 4 ºC on a rotating wheel.

The resin containing immunoprecipitated RNAPII and interacting proteins was transferred to a 20 mL Econoprep column to drain by gravity flow, and the remaining

resin wash washed with 30 mL of Calmodulin binding Buffer. Next, RNAPII and interacting proteins were eluted from the calmodulin resin with five 1 mL aliquots of Calmodulin Elution Buffer containing EGTA to chelate the calcium chloride; the individual elutions, numbered E1-E5, were collected in 1.5 mL centrifuge tubes. (Each elution was incubated at ambient temperature for five minutes prior to be collected by gravity flow.)

The individual calmodulin elutions (E1-E5) were analyzed by SDS-PAGE with silver staining by first preparing a 10% SDS-PAGE gel with a 5% stacking gel. Aliquots (8 μL) of 4X Loading Dye were added to 24 μL aliquots of each of the calmodulin elutions, and the samples were denatured at 100 ºC for 5 min. Following denaturation, the entire volume of each aliquot was loaded to the SDS-PAGE gel along with 2.5 μL of a 1:10 dilution of the Precision Plus Protein Standards. The gel was run in Tris/Gly/SDS buffer at 180 V for 50 min. Following PAGE, the lanes and stacker was cutoff the SDS-PAGE gel, and the gel was placed in 100 mL of Fixing Solution overnight at ambient temperature. The following day, the Fixing Solution was discarded, and 100 mL of Ethanol Wash were added; the gel was incubated at room temperature for 10 min. This procedure (discarding the previous wash, adding the next wash, and incubating at ambient temperature for 10 min) was repeated for a water wash, the Sensitizer Solution, a water wash, Silver Nitrate Solution, a water wash, the Developing Solution, respectively. Following addition of the developing solution, the progress of the silver staining was monitored until the proper level of staining was achieved (2-10 min), and washing the gel with Stop Solution for 10 min at ambient temperature quenched the development. The

Stop Solution was removed, the gel was rinsed with ddH2O, and the gel was imaged with a flatbed digital scanner.

Prior to LC/MS analysis, calmodulin elution E2 was prepared using a trichloroacetic acid (TCA) precipitation protocol preceding proteolytic digestion. Briefly, 100 µL of sample E2 was transferred to a 1.5 mL centrifuge tube, and 200 µL of 4 ºC Tris-HCl were added to the sample. Next, 100 µL of 100% TCA were added to the sample followed by vortex mixing; the sample was incubated overnight at 4 ºC. The following day, the sample was centrifuged for 30 min at 14,000 RPM and 4 ºC (full speed), and the supernatant was discarded. Acetone (500 µL at 4 ºC) was added to the pellet followed by vortex mixing, and the sample was centrifuged for 10 min at full speed. The sample was washed with acetone a second time as previously described, the acetone was removed, and the tube was uncapped until the remaining acetone had evaporated. The sample was reconstituted with 30 µL of 8 M urea in Tris-HCl, and 1.5 µL of TCEP (5 mM final concentration) were added; the sample was incubated at ambient temperature for 30 min. Following sample reduction, the sample was alkylated by the addition of 0.6 µL of CAM (final concentration 10 mM); the sample was incubated in the dark for 30 min. Next, the sample was digested with 0.3 µL of endoproteinase Lys-C overnight at 37 ºC. The following day, the urea was diluted by the addition of 90 µL of Tris-HCL and 0.6 µL of calcium chloride (final concentration 2 mM), and the sample was digested with Trypsin Gold (0.5 µg) overnight at 37 ºC preceding analysis by MudPIT-LC/MS.

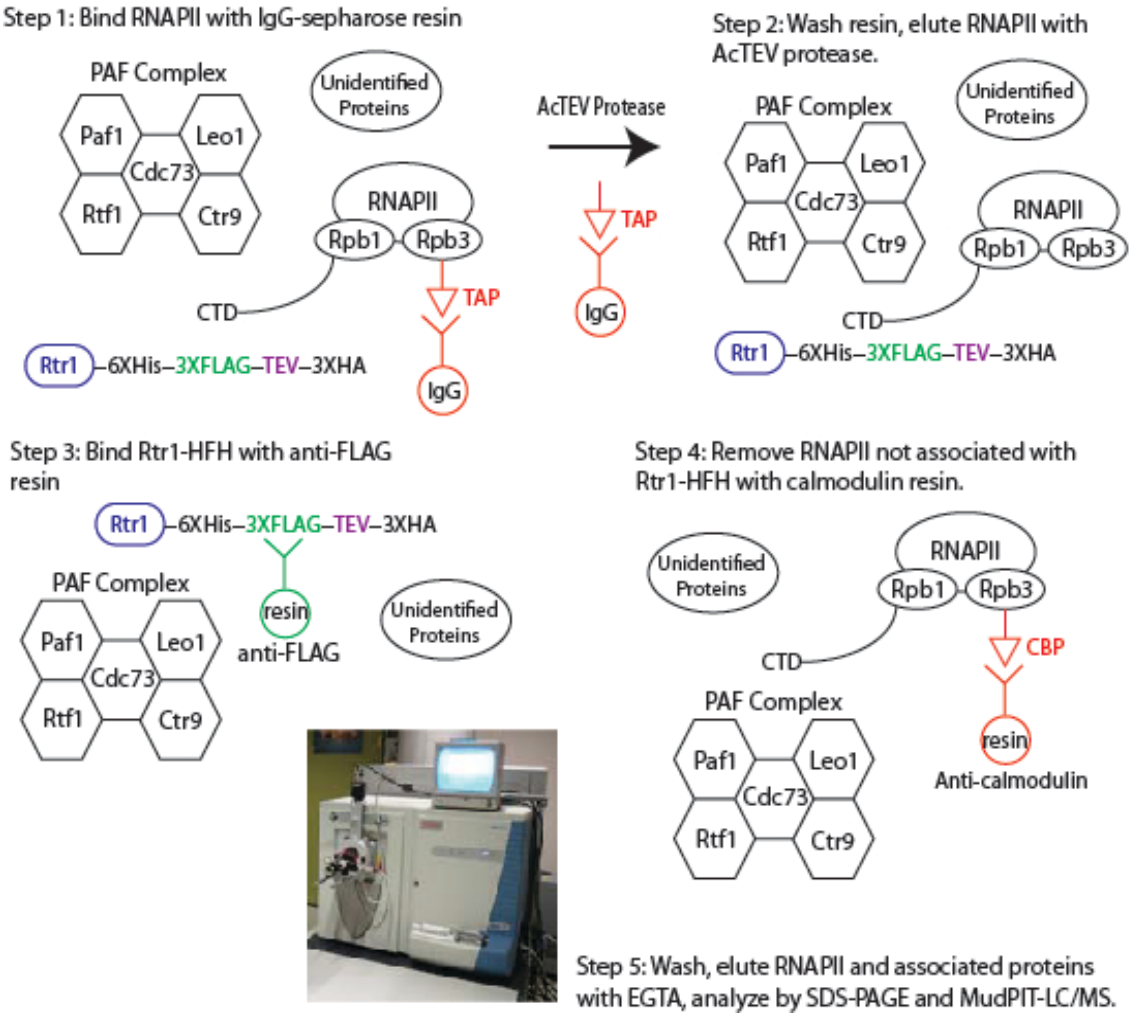

Figure 4. Purification scheme used to identify RNAPII-interacting proteins not in complex with Rtr1. In step 1, RNAPII is immunoprecipitated from yeast cell lysate with IgG-sepharose resin, unassociated proteins are washed away, and RNAPII was eluted from IgG-sepharose with AcTEV protease (step 2). In step 3, Rtr1-HFH and associated proteins were removed through immunoprecipitation with anti-FLAG resin, and the remaining RNAPII and associated proteins were immunoprecipitated with calmodulin resin (step 4). In step 5, RNAPII and associated proteins are eluted from calmodulin resin with EGTA and analyzed by SDS-PAGE and MudPIT-LC/MS.

Purification of Proteins that Interact with the RNAPII-Rtr1 Complex

The third step of the purification targeted proteins that interact with the Rtr1-RNAPII complex (Figure 5). The yeast cell lysate was prepared in the same manner as was presented in the above section, "Purification of Proteins that Interact with RNAPII,"

including the immunoprecipitation with IgG-sepharose, the elution of RNAPII and interacting proteins from IgG-sepharose with AcTEV protease, and the immunoprecipitation of proteins in the IgG-sepharose eluate with anti-FLAG resin. However, at this point in the purification, the two procedures diverged. On the day following the immunoprecipitation of the protein that interacted with the Rtr1-RNAPII complex, The EconoPrep column containing the anti-FLAG resin was washed with 30 mL of TAP Lysis Buffer, which was allowed to drain by gravity flow into a 500 mL beaker. The anti-FLAG resin collected in the column was subsequently washed with 1 mL of 50 mM ammonium bicarbonate, and the resin was re-suspended with 500 µL of Digestion Buffer. The re-suspended resin was transferred to a clean, 1.5 mL centrifuge tube, the sample was centrifuged, and 300 µL of the supernatant was removed and discarded. An aliquot of Trypsin Gold (10 µL of 100 µg/mL) was added to the remaining supernatant/anti-FLAG resin, and the sample was digested "on-bead" overnight at 37 ºC. Following incubation, the sample was centrifuged, and the supernatant containing the digested proteins was transferred to a clean 1.5 mL centrifuge tube prior and stored at -80 ºC prior to MudPIT-LC/MS analysis.

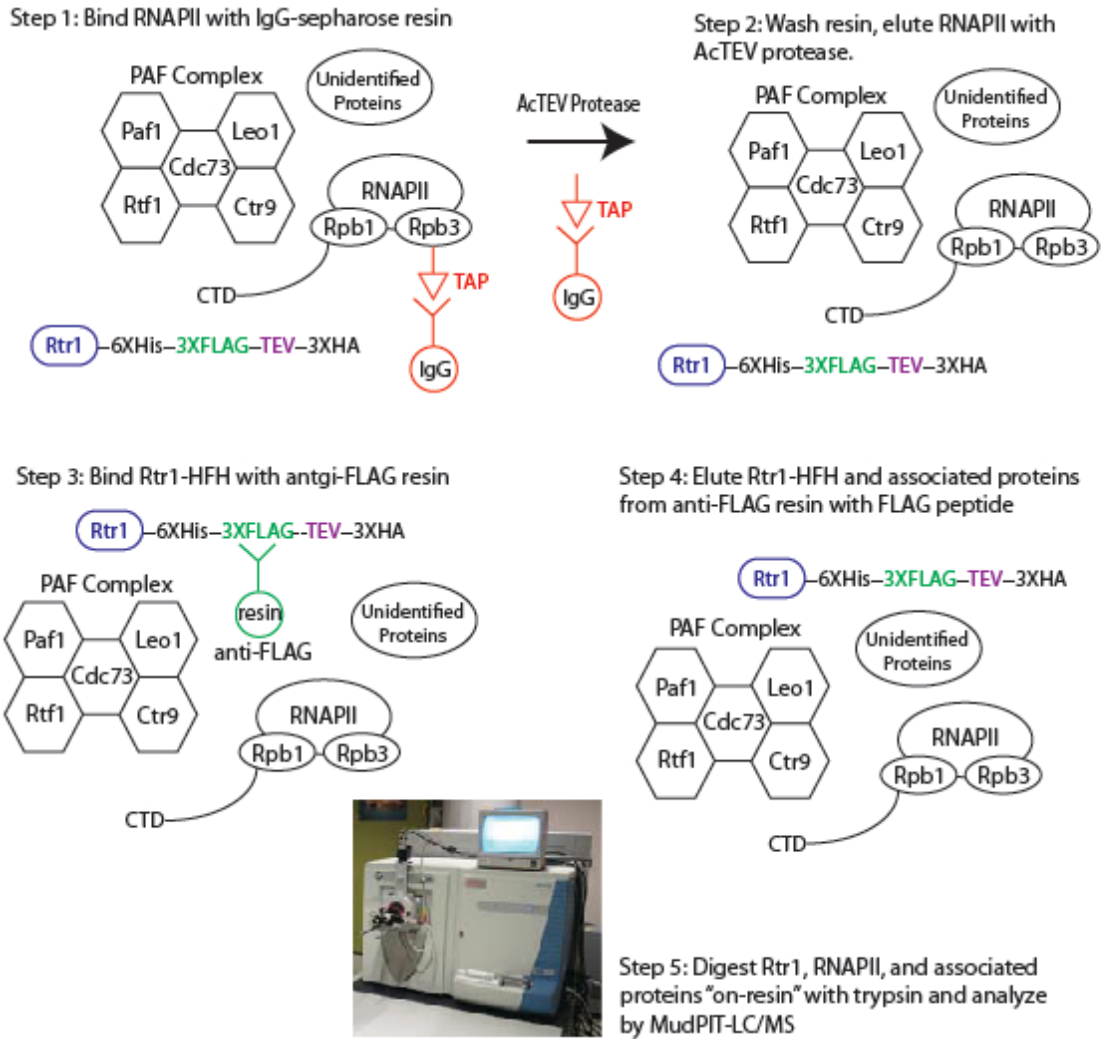

Figure 5. Purification scheme used to identify proteins that interact with the RNAPII-Rtr1 complex. In step 1, RNAPII is immunoprecipitated from yeast cell lysate with IgG-sepharose resin, unassociated proteins are washed away, and RNAPII is eluted from IgG-sepharose with AcTEV protease (step 2). In step 3, Rtr1-HFH and associated proteins are removed with anti-FLAG resin. In step 4, RNAPII, Rtr1, and associated proteins are eluted from anti-FLAG resin with FLAG peptide, and analyzed by MudPIT-LC/MS following digestion with trypsin.

III. Rtr1-1XFLAG Purification Scheme

Yeast Cell Lysis

Yeast cells that express a 1X-FLAG tagged version of the phosphatase Rtr1 (Rrt1-1XFLAG) were cultured in YPD media at 30 ºC to an OD_{600} of ~4. The cells were

centrifuged, the supernatant was removed, and the cells were washed with ddH2O. Following centrifugation to remove the cell wash, the cells were re-suspended in TAP Lysis Buffer and were processed in the same fashion as was described in Section II above (Rpb3-TAP/Rtr1-HFH Purification Scheme, Yeast Cell Lysis).

Purification of Proteins that Interact with Rtr1-1XFLAG

This purification scheme targeted proteins that interact with Rtr1-1XFLAG (Figure 6). In this experiment, an aliquot (200 µL) of anti-FLAG resin was washed with 1 mL of TAP Lysis Buffer and was added to the supernatant from the yeast cell lysate. The sample was incubated for approximately 24 hours at 4 ºC with stirring, and the anti-FLAG resin containing Rtr1-1XFLAG and any interacting proteins was recovered by passing the sample over a 30 mL EconoPrep column. The sample was allowed to drain into a 500 mL beaker by gravity, and the anti-FLAG resin remaining in the column was washed with 30 mL of TAP Lysis Buffer followed by 1 mL of 50 mM ammonium bicarbonate (pH 8.5) to remove any proteins that did not interact with Rtr1. The anti-FLAG resin was re-suspended with 500 µL of Digest Buffer and 10 µL of 100 µg/mL Trypsin Gold. The re-suspended sample was transferred to a 1.5 mL centrifuge tube and was digested "on-resin" overnight (with mixing) at 37 ºC. The following day, the sample was centrifuged to remove the anti-FLAG resin, and the supernatant was acidified with 10 µL of formic acid and was loaded to a MudPIT column for LC/MS analysis.

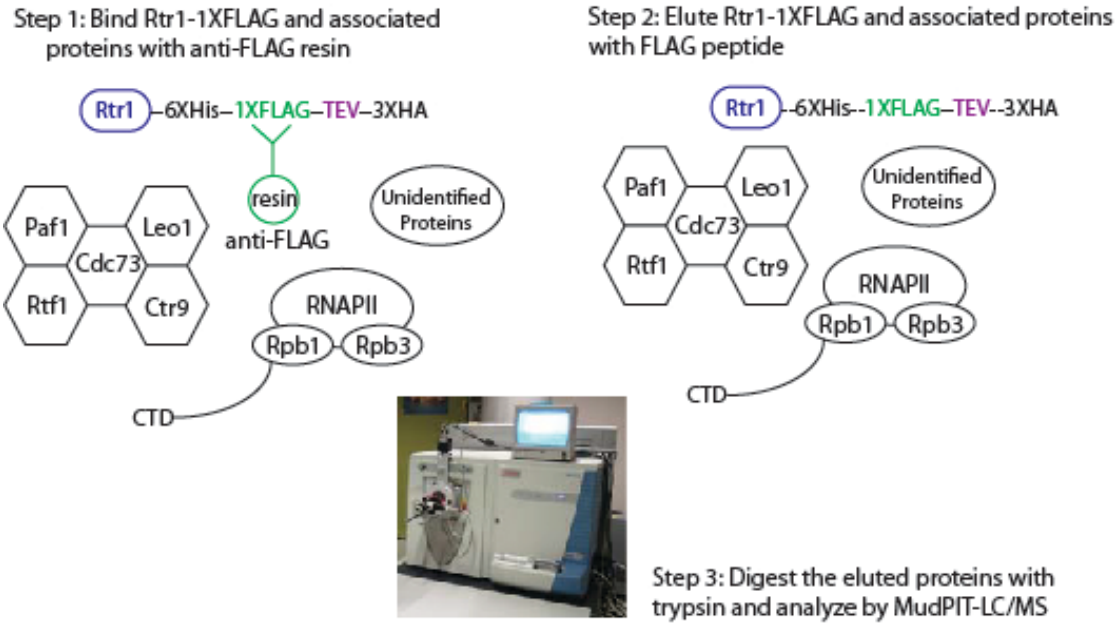

Figure 6. Purification scheme used to identify proteins that interact with Rtr1. In step 1, Rtr1-1XFLAG is immunoprecipitated with anti-FLAG resin, unassociated proteins are washed away, and Rtr1 and associated proteins are eluted from anti-FLAG resin with FLAG peptide (step 2). In step 3: the eluted proteins are precipitated with TCA, digested with trypsin, and analyzed by MudPIT-LC/MS.

IV. CTDK1-TAP/Rtr1-V5 Purification Scheme

Yeast Cell Lysis

Two different yeast strains that express a TAP-tagged version of either of the CTDK-I subunits Ctk1 or Ctk2, and a V5-tagged version of Rtr1 were cultured in YPD media at 30 ºC to an OD_{600} of approximately four. The cells were centrifuged, the supernatant was removed, and the cells were washed with ddH2O. Following centrifugation to remove the cell wash, the cells were re-suspended in TAP Lysis Buffer and were processed in the same fashion as was described in Section II above (Rpb3-TAP/Rtr1-HFH Purification Scheme, Yeast Cell Lysis).

CTDK-I and Rtr1 Single Purification Scheme

The objective of this purification scheme was to confirm the *in vivo* interaction between Rtr1-V5 and CTDK-I (via Ctk1-TAP or Ctk2-TAP), see Figure 7. The present experiment utilized a single purification step in which an aliquot (200 µL) of pre-washed IgG-sepharose resin was added to the supernatant from the yeast cell lysates (Ctk1-TAP/Rtr1-V5 or Ctk2-TAP/Rtr1-V5 strains); separate 100 µL aliquots of the yeast lysates were set aside to serve as the input controls. The samples were incubated for approximately 24 hours at 4 ºC with stirring, and the IgG-sepharose resin containing Ctk1/2-TAP and Rtr1-V5, if present, was recovered by passing the samples over 30 mL EconoPrep columns. The samples were allowed to drain into 500 mL beakers by gravity flow, and the IgG-sepharose resin remaining on the columns was washed with 30 mL of TAP Lysis Buffer to remove any non-interacting proteins. The IgG-sepharose resin from each sample was re-suspended with 200 µL of TAP Lysis Buffer, transferred to clean microcentrifuge tubes, and stored at -80 ºC.

IgG-sepharose treated and input control samples were removed from the freezer and were allowed to thaw in a water bath at ambient temperature. An aliquot of 4X Loading Dye was added to the samples, which we subsequently boiled for 10 min to elute the proteins from the resin. The samples were centrifuged at 14,000 RPM at 4 ºC for 10 min, and the supernatants were transferred to clean microcentrifuge tubes. The input control samples (10 µL) and the experimental samples (30 µL, Ctk1-TAP/Rtr1-V5 and Ctk2-TAP/Rtr1-V5) were loaded in duplicate to a 12% SDS-PAGE gel, which was run in Tris/Gly/SDS Run Buffer at 200 V for 50 min. Following the SDS-PAGE analysis, the gel was blotted to nitrocellulose overnight at 30 V in Transfer Buffer.

Following the Western transfer, the nitrocellulose membrane was placed in Blocking Buffer for approximately 2 hours and cut in half; each of the halves contained a full complement of samples: Ctk1-TAP/Rtr1-V5 input and experimental samples, and Ctk2-TAP/Rtr1-V5 input and experimental samples. The left half of the nitrocellulose membrane was probed by incubating it overnight at 4 ºC in 1:1000 diluted (in Block Buffer) anti-calmodulin binding protein antibody (α-CBP). Likewise, the right half of the membrane was probed with 1:1000 diluted anti-V5 antibody directly conjugated to horseradish peroxidase (α-V5-HRP). The next day, the membranes were removed from the primary antibody solutions. The sample probed with α-V5-HRP was washed three times with TBS (10 min per wash) and developed with ECL Plus reagent. The sample probed with α-CBP was washed three times with TBS (10 min per wash) and was placed in 1:1000 diluted (in Block Buffer) secondary antibody (anti-rabbit IgG-HRP) for two hours at ambient temperature. Following incubation, the membrane was removed from the secondary antibody solution, washed as above, and developed with ECL Plus reagent. Both membranes were imaged using a Fuji Scanner (FLA 5000) at 473 nm using the LPB filter setting.

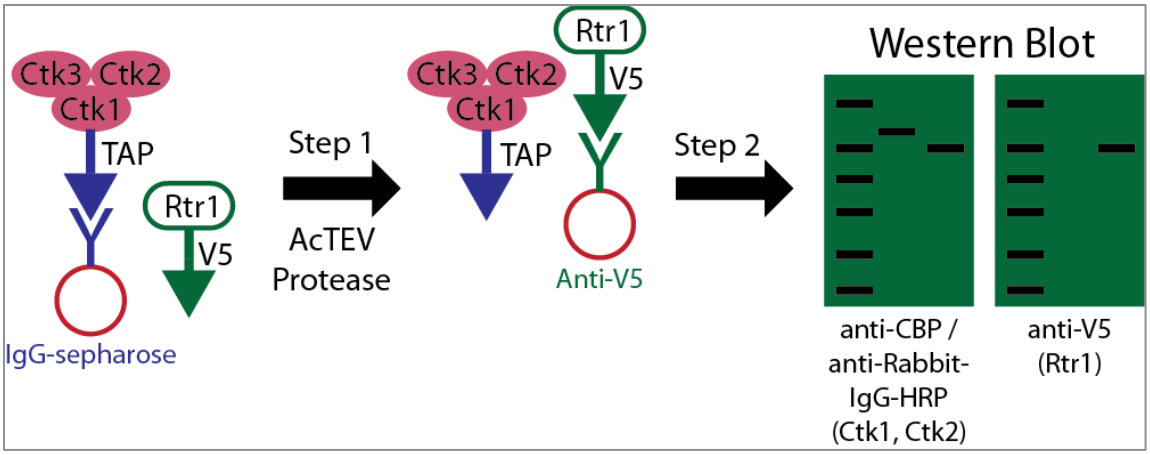

Figure 7. Rtr1-V5/CTDK-I-TAP single and double purification schemes are shown. In the single purification scheme, CTDK-I and associated proteins are immunoprecipitated from yeast cell lysate via the Ctk1-TAP (or Ctk2-TAP) subunit using IgG-sepharose. CTDK-I and associated proteins are eluted from IgG-sepharose using TEV protease (step 1), and CTDK-I and Rtr1 are detected by Western blot. In the double purification scheme CTDK-I and Rtr1 (if present) are immunoprecipitated via the Rtr1-V5 tag (using anti-V5 resin) prior to detection by Western blot.

CTDK-I and Rtr1 Double Purification Scheme

The objective of this purification scheme was to confirm the *in vivo* interaction between Rtr1-V5 and CTDK-I (via Ctk1-TAP or Ctk2-TAP), see Figure 7. The present experiment utilized a two purification steps. In the first step, an aliquot (250 µL) of pre-washed IgG-sepharose was added to the supernatant from the yeast cell lysates (Ctk1-TAP/Rtr1-V5 or Ctk2-TAP/Rtr1-V5 strains); separate 100 µL aliquots of the yeast lysates were set aside to serve as the input controls. The samples were incubated for approximately 24 hours at 4 ºC with stirring, and the IgG-sepharose resin containing Ctk1/2-TAP and Rtr1-V5, if present, was recovered by passing the samples over 30 mL EconoPrep columns. The samples were allowed to drain into 500 mL beakers by gravity flow, and the IgG-sepharose resin remaining on the columns was washed with 30 mL of TAP Lysis Buffer to remove any non-interacting proteins. Next, the IgG-sepharose was

washed a second time with 3 mL of TEV Cleavage Buffer, was re-suspended with 1 mL of TEV Cleavage Buffer, and was transferred to clean microcentrifuge tubes. Afterwards, CTDK-I and Rtr1 (if present) were eluted from the IgG-sepharose resin following the addition of 10 µL of AcTEV protease, which was incubated overnight at 4 ºC. The following day, the samples were passed through a 10 mL EconoPrep column, and the flow-through was collected into a 15 mL centrifuge tube. The column was washed with 9 mL of TAP Lysis Buffer, which was added to the same tubes. The eluted proteins were incubated with 100 µL of pre-washed anti-V5 agarose overnight at 4 ºC on a rotating wheel.

The next day, the anti-V5 agarose containing Rtr1-V5 and CTDK-I, if present, was recovered by passing the samples over 30 mL EconoPrep columns. The samples were allowed to drain into 500 mL beakers by gravity flow, and the agarose resin remaining on the columns was washed with 30 mL of TAP Lysis Buffer to remove any non-interacting proteins. Next, the resin from each sample was re-suspended with 200 µL of TAP Lysis Buffer, transferred to clean microcentrifuge tubes, and stored at -80 ºC. On the day of analysis, the samples were analyzed by Western blot in the same fashion as was described in the above section (*Purification of CTDK-I and Rtr1, Single Purification*).

V. MudPIT-LC/MS Analysis

Samples were prepared for MudPIT-LC/MS analysis either by TCA precipitation or by direct digestion with trypsin as described in the individual protein purification sections above. Following digestion with trypsin, the samples were quenched by the addition of 1% formic acid prior to being loaded on a two dimensional MudPIT capillary column as

previously described (Florens and Washburn, 2006). Each of the samples from the aforementioned protein purifications was subjected to a 10-step MudPIT analysis as previously described with specific updates and modifications as indicated below (Wolters et al., 2001). Following each of the 10 steps, the chromatographically resolved peptides were eluted into a ThermoFisher LTQ Velos for mass spectrometric (MS) analysis; the LC/MS system was equipped with a Proxeon nano-liquid chromatograph and an in-house modified nano-electrospray source designed specifically for MudPIT-LC/MS. Following MS analysis, the tandem mass spectra were submitted to the SEQUEST algorithm for protein identification using Proteome Discoverer software (Thermo). Spectra were search against a FASTA database containing 5819 Saccharomyces cerevisiae protein sequences (downloaded from NCBI build from 04/26/11) and 281 common contaminant proteins including human keratins, immunoglobins, and proteolytic enzymes. Reversed versions of all non-redundant peptides were included in the database as a means of estimating false discovery rates (FDR). FDRs were kept at 2% or lower for all the data analysis performed in this work. To account for reduction and carbamidomethylation (alkylation, CAM) of cysteine-containing peptides, a static modification of +57 Da was added to all SEQUEST searches when TCEP reduction and CAM treatment were performed prior to the digestion procedure. Additionally, a dynamic addition of +16 Da was included to identify oxidized methionine residues when present.

RESULTS

I. Rpb3-TAP/Rtr1-HFH Purification

Rtr1 phosphatase was shown to regulate a key step in the transition of the RNAPII-CTD phosphorylation state from S5-P to S2-P, which is important for transcription elongation and termination in eukaryotes (Mosley et al., 2009). However, the mechanism involved in the recruitment of Rtr1 to RNAPII during transcription is unclear. Consequently, three TAP purification experiments were performed in *S. cerevisiae* that expresses various combinations of tagged versions of a RNAPII subunit and Rtr1: Rpb3-TAP and Rtr1-HFH. The first purification experiment targeted proteins that interacted with Rtr1 when it was not in complex with RNAPII. In the second experiment, proteins were identified that interacted with RNAPII when it was not in complex with Rtr1, and the third experiment uncovered proteins that associated with the RNAPII-Rtr1 complex.

The proteins that were identified in the aforementioned experiments were included in the results tables below if at least 2 unique peptides and 100 Peptide Spectral Matches (PSMs) were detected following MudPIT-LC/MS analysis. Table 4 presents a list of transcription-relevant proteins in *S. cerevisiae*, and proteins that were detected from at least one unique peptide that were present in Table 4 were included in the results tables as well. Conversely, proteins that were identified by Krogan *et al.* as common contaminants in *S. cerevisiae* TAP affinity purifications were removed from the Rpb3-TAP/Rtr1-HFH results tables presented below (Krogan et al., 2006). In this work, Krogan and coworkers used MALDI and LC/MS to identify contaminant proteins that represented more than 3% of 4,562 proteins that were isolated from 2,357 tandem affinity purifications in *S.*

cerevisiae providing a much larger number of purifications to identify common TAP contaminants than the studies we performed here.

Table 4. Transcription-relevant proteins. The descriptions of the protein functions were obtained from the Saccharomyces Genome Database (www.yeastgenome.org).

Gene	Protein	Description
YDL140C	Rpb1 (Rpo21)	RNAPII subunit (largest), phosphorylation of Rpb1-C-terminal domain (CTD) regulates its association with transcription factors
YOR151C	Rpb2	RNAPII subunit, second largest
YIL021W	Rpb3	RNAPII subunit, third largest
YJL140W	Rpb4	RNAPII subunit forms a dissociable complex with Rpb7; involved in 3'-end processing factors, translation initiation, export on mRNA under stress
YBR154C	Rpb5	Subunit common to RNA polymerase I, II, and III; binds DNA
YPR187W	Rpb6 (Rpo26)	Subunit common to RNA polymerase I, II, and III
YDR404C	Rpb7	See Rpb4
YOR224C	Rpb8	Subunit common to RNA polymerase I, II, and III
YGL070C	Rpb9	RNAPII subunit, binds DNA
YOR210W	Rpb10	Subunit common to RNA polymerase I, II, and III
YOL005C	Rpb11	RNAPII subunit
YHR143W-A	Rpb12 (Rpc10)	Subunit common to RNA polymerase I, II, and III
YBR279W	Paf1	Subunit of PAF complex involved in transcription elongation
YLR418C	Cdc73	Subunit of PAF complex involved in transcription elongation
YOR123C	Leo1	Subunit of PAF complex involved in transcription elongation
YGL244W	Rtf1	Subunit of PAF complex involved in transcription elongation
YOL145C	Ctr9	Subunit of PAF complex involved in transcription elongation
YKL139W	Ctk1	Catalytic subunit of the C-terminal Repeat Domain Kinase I (CTDK-I), CTDK-I phosphorylates the CTD of RNAPII to regulate transcription and mRNA 3'-end processing
YJL006C	Ctk2	Regulatory subunit of the C-terminal Repeat Domain Kinase I (CTDK-I), CTDK-I phosphorylates the CTD of RNAPII to regulate transcription and mRNA 3'-end processing

Gene	Protein	Description
YML112W	Ctk3	Subunit (function unknown) of the C-terminal Repeat Domain Kinase I (CTDK-I), CTDK-I phosphorylates the CTD of RNAPII to regulate transcription and mRNA 3'-end processing
YJR072C	Npa3	Member of the GTPase family (has ATPase activity); involved in transport of RNAPII to the nucleus; involved in cohesion of sister chromatids
YLR243W	NA	An essential gene, but of unknown function
YGR063C	Spt4	Forms a complex with Spt5 that is involved in regulating RNA polymerase I and II transcription and processing of pre-mRNA
YML010W	Spt5	See Spt4; has a CTD that is a substrate for phosphorylation
YGR116W	Spt6	Protein involved in transcription, the maintenance of chromatin, and RNA processing
YGL207W	Spt16	With Pob3, forms the heterodimeric FACT complex; modifies nucleosomes to provide RNA/DNA polymerases access to DNA
YML069W	Pob3	See Spt16
YPR086W	Sua7	Transcription factor TFIIB, a factor required for transcription initiation
YPR133C	Spn1	Protein involved in RNAPII transcription
YDR172W	Sup35	eRF3, a translation termination factor eRF3 involved in mRNA deadenylation and decay
YBR143C	Sup45	eRF1, a polypeptide release factor involved in translation termination
YDL084W	Sub2	Subunit of the TREX complex that is required for mRNA export to nucleus; in the DEAD-box RNA helicase superfamily, involved in spliceosome assembly
YGL105W	Arc1	Protein that delivers tRNA to methionyl- and glutamyl-tRNA synthetases
YER164W	Chd1	A protein involved in chromatin remodeling that regulates transcription; a component of the SAGA and SLIK complexes
YMR235C	Rna1	GTPase activating protein involved in nuclear transport
YDL074C	Bre1	E3 ubiquitin ligase required for transcription, among other functions
YHR168W	Mtg2	A putative GTPase
YGR186W	Tfg1 (Ssu71)	Largest subunit of TFIIF involved in RNAPII transcription initiation and elongation
YGR005C	Tfg2	Subunit of TFIIF involved in RNAPII transcription initiation and elongation
YPL129W	Tfg3 (Taf14)	Subunit of several complexes: TFIID, TFIIF, INO80, SWI/SNF, NuA3; involved in RNAPII transcription initiation and modification of chromatin
YGL043W	Dst1	TFIIS, general transcription elongation factor

Gene	Protein	Description
YPR093C	Asr1	A ubiquitin ligase that modifies RNAPII, which contributes to its regulation
YJL168C	Set2	A histone methyltransferase that is involved in transcription elongation
YDL115C	Iwr1	A RNAPII transport factor

Proteins Identified that Interact with Rtr1

The results of the Rpb3-TAP/Rtr1-HFH TAP purification that targeted proteins that interacted with Rtr1 when it was not in complex with RNAPII are presented in Table 5. In all, 66 proteins were detected that interacted with Rtr1 through the detection of at least two unique peptides that were present with at least 100 PSMs; all transcription-relevant proteins that were identified by at least one unique peptide were also included in Table 5. Of the 66 proteins identified, 28 proteins were members of the transcription-relevant list. This included all 12 subunits of RNAPII, which should not be present following the IgG-sepharose purification step (see Discussion).

Table 5. Proteins identified that interacted with Rtr1-HFH when it was not in complex with Rpb3-TAP (RNAPII) following tandem affinity purification from *S. cerevisiae* and MudPIT-LC/MS analysis.

Accession	Description*	Coverage	# PSMs	# Peptides	# AAS	MW
gi6323278	Yef3p	30.65	898	27	1044	115.9
gi6320061	Rpb1; TXN	35.14	736	47	1733	191.5
gi6319612	Mec1p	36.91	646	62	2368	273.2
gi6322084	Pfk26p	44.01	624	28	827	93.4
gi6320987	Rtr1; TXN	44.69	564	12	226	26.2
gi6321631	Tdh3p	50.30	433	11	332	35.7
gi6325300	Rpl43ap	29.35	368	3	92	10.1
gi6323057	Rpl15ap	24.02	323	4	204	24.4
gi6322468	Tdh2p	46.39	280	10	332	35.8
gi6325167	Rps6ap	28.81	276	7	236	27.0
gi6321315	Rps2p	40.16	258	7	254	27.4
gi6324725	Rpb2; TXN	31.21	252	27	1224	138.7

Accession	Description*	Coverage	# PSMs	# Peptides	# AAS	MW
gi6322409	Tdh1p	22.29	240	5	332	35.7
gi6321291	Rpl9ap	39.27	236	6	191	21.6
gi14318555	Rpl2ap	36.61	226	7	254	27.4
gi6319282	Fun12p	32.63	222	23	1002	112.2
gi6324298	Hhf2p	42.72	205	4	103	11.4
gi6320949	Rps8bp	39.50	205	6	200	22.5
gi6324262	Rpl9bp	39.27	197	6	191	21.6
gi6322168	Rpb3; TXN	47.17	193	9	318	35.3
gi6323654	Clu1p	35.00	188	29	1277	145.1
gi6320707	Lcd1p	26.64	181	14	747	86.4
gi6322313	Rpa34p	36.05	176	6	233	26.9
gi6325036	Rpl1ap	24.88	173	6	217	24.5
gi6320963	Rpl23bp	16.79	172	2	137	14.5
gi6323567	Rpl6ap	27.84	160	4	176	19.9
gi9755341	Rps16ap	48.95	151	7	143	15.8
gi6320065	Rpl35ap	33.33	144	6	120	13.9
gi6323226	Nop56p	42.46	142	14	504	56.8
gi6321968	Eno2p	50.34	142	13	437	46.9
gi6322847	Rpl14ap	31.16	136	4	138	15.2
gi6324260	Rpl16bp	28.28	133	7	198	22.2
gi37362658	Ssz1p	43.49	131	14	538	58.2
gi86558907	Rhr2p	41.20	125	7	250	27.9
gi6321754	Rpl8ap	38.67	124	10	256	28.1
gi6320190	Nop1p	30.28	119	8	327	34.4
gi6319666	Rps9bp	34.87	117	9	195	22.3
gi6322271	Rps22ap	42.31	113	5	130	14.6
gi6324886	Nop58p	35.23	110	12	511	56.9
gi6321693	Eno1p	41.88	109	11	437	46.8
gi37362649	Gus1p	21.89	104	13	708	80.8
gi6321203	Ade5,7p	37.16	103	18	802	86.0
gi6319630	Rpb5; TXN	33.02	88	5	215	25.1
gi6324569	Rpb11; TXN	42.50	37	4	120	13.6
gi6321333	Arc1p; TXN	26.86	33	7	376	42.1
gi6325445	Rpb6; TXN	14.19	28	1	155	17.9
gi6324798	Rpb8; TXN	21.23	27	2	146	16.5
gi6321231	Spt16p; TXN	16.33	25	12	1035	118.6
gi6324784	Rpb10; TXN	52.86	13	2	70	8.3
gi330443717	Ctr9; TXN	4.55	11	3	1077	124.6
gi6321193	Rtf1; TXN	2.69	11	2	558	65.8

Accession	Description*	Coverage	# PSMs	# Peptides	# AAS	MW
gi6320119	Sub2p; TXN	6.05	9	2	446	50.3
gi6322321	Rpb4; TXN	23.98	9	4	221	25.4
gi6321012	Chd1p; TXN	5.86	8	6	1468	168.1
gi6322532	Npa3p; TXN	9.35	7	3	385	43.2
gi6321368	Rpb9; TXN	8.20	7	1	122	14.3
gi6323632	Spt5; TXN	2.73	3	3	1063	115.6
gi6321625	Tfg1; TXN	1.63	3	1	735	82.1
gi6319619	Sup45p; TXN	6.18	3	2	437	49.0
gi6321937	Rpb12; TXN	28.57	3	1	70	7.7
gi6320612	Rpb7; TXN	9.36	2	1	171	19.0
gi6320377	Sup35p; TXN	1.61	1	1	685	76.5
gi6319756	Paf1; TXN	2.92	1	1	445	51.8
gi6325390	Spn1p; TXN	3.66	1	1	410	46.1
gi6325128	Taf14; TXN	6.56	1	1	244	27.4
gi6323272	YLR243W; TXN	4.78	1	1	272	30.6

*All transcription-relevant proteins that were detected were included (TXN in description)
Coverage: The total percentage of amino acid sequence identified by matching tryptic peptides
PSMs: The number of peptide spectral matches (proteins with <100 PSMs were excluded unless they were classified as transcription related -TXN)
Peptides: The number of unique peptides that were detected (proteins with <2 were excluded unless they were classified as transcription related -TXN)
AAS: The number of amino acids in the intact protein
MW: The protein molecular weight (kilodaltons)

Proteins Identified that Interact with RNAPII

RNAPII and associated proteins were eluted from the calmodulin resin, the last step of the TAP purification that targets RNAPII, by five aliquots of Calmodulin Elution Buffer containing EGTA. These calmodulin elutions were analyzed by SDS-PAGE with silver staining to evaluate the yield of the purification as determined by the presence and intensity of the RNAPII subunits (Figure 8). Elution two and three were found to provide the best recovery of RNAPII subunits, and all twelve subunits were present including

phosphorylated Rpb1 as determined by comparison to previously obtained silver-stained gels for RNAPII purifications (Mosley et al., 2011).

The results of the Rpb3-TAP/Rtr1-HFH TAP purification that targeted proteins that interacted with RNAPII when it was not in complex with Rtr1 are presented in Table 6. A total of 14 proteins that satisfied the aforementioned criteria were detected by MudPIT-LC/MS. Among the list was a single protein, Dbp2, that is not a member of the transcription-relevant list (see Table 4) and was identified by more than two unique peptides having greater than 100 PSMs. Additionally, eight of the twelve RNAPII subunits were detected by MudPIT-LC/MS (Rpb6, Rpb9, Rpb10, and Rpb12 were absent), as well as four additional transcription-relevant proteins.

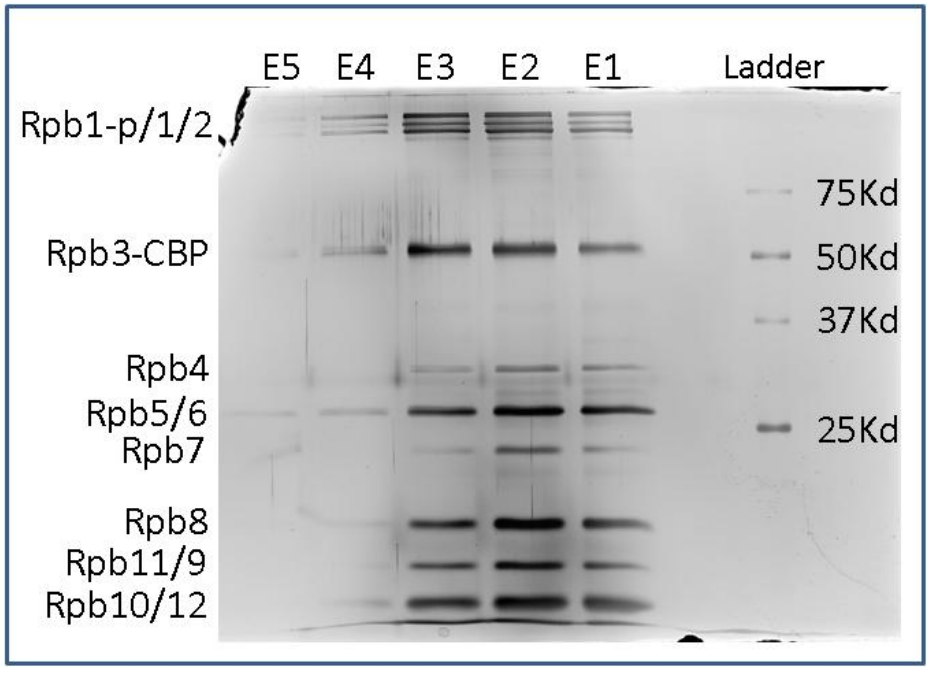

Figure 8. SDS-PAGE analysis with silver staining showing RNAPII subunits. The RNAPII subunits were present in the Rpb3-TAP/Rtr1-HFH purification scheme targeting proteins that interact with RNAPII in the absence of Rtr1.

Table 6. Proteins identified that interacted with Rpb3-TAP (RNAPII) when not in complex in Rtr1

Accession	Description*	Coverage	# PSMs	# Peptides	# AAs	MW
gi6320061	Rpb1p; TXN	20.95	3153	32	1733	191.5
gi6324725	Rpb2p; TXN	20.18	1647	22	1224	138.7
gi6322321	Rpb4p; TXN	22.62	647	4	221	25.4
gi6322168	Rpb3p; TXN	8.18	515	2	318	35.3
gi6324569	Rpb11p; TXN	31.67	464	4	120	13.6
gi6324798	Rpb8p; TXN	21.23	457	2	146	16.5
gi6319630	Rpb5p; TXN	12.09	292	2	215	25.1
gi6324217	Dbp2p	18.86	122	9	546	61.0
gi6323632	Spt5p; TXN	10.82	88	9	1063	115.6
gi6320612	Rpb7p; TXN	7.60	44	1	171	19.0
gi6325390	Spn1p; TXN	14.15	14	4	410	46.1
gi6321231	Spt16p; TXN	1.35	3	1	1035	118.6
gi6321333	Arc1p; TXN	3.46	1	1	376	42.1
gi6321552	Spt6p; TXN	1.10	1	1	1451	168.2

*All transcription-relevant proteins that were detected were included (TXN in description)
Coverage: The total percentage of amino acid sequence identified by matching tryptic peptides
PSMs: The number of peptide spectral matches (proteins with <100 PSMs were excluded unless they were classified as transcription related -TXN)
Peptides: The number of unique peptides that were detected (proteins with <2 were excluded unless they were classified as transcription related -TXN)
AAS: The number of amino acids in the intact protein
MW: The protein molecular weight (kilodaltons)

Proteins Identified that Interact with the RNAPII-Rtr1 Complex

The results of the Rpb3-TAP/Rtr1-HFH TAP purification that targeted proteins that interacted with the RNAPII-Rtr1 complex are presented in Table 7. A total of 26 proteins were identified that satisfied the criteria of having at least 2 unique peptides detected that had at least a total of 100 PSMs. An additional 6 proteins were included in Table 7 that did not meet the inclusion criteria but were members of the transcription-relevant list presented in Table 4. In all, 8 of the RNAPII subunits were detected; Rpb4, Rpb6, and Rpb7 were not detected. Furthermore, 9 of the proteins that were found to interact with the RNAPII-Rtr1 complex were not members of the transcription-relevant list.

Table 7. Proteins identified that interacted with the RNAPII-Rtr1 complex

Accession	Description*	Coverage	# PSMs	# Peptides	# AAs	MW
gi6320061	Rpb1; TXN	24.75	801	32	1733	191.5
gi6324725	Rpb2; TXN	28.68	550	27	1224	138.7
gi6323278	Yef3p	26.15	532	19	1044	115.9
gi6320987	Rtr1; TXN	45.13	270	8	226	26.2
gi6322168	Rpb3; TXN	43.40	238	7	318	35.3
gi6324298	Hhf2p	50.49	213	5	103	11.4
gi6324886	Nop58p	27.40	178	10	511	56.9
gi6325167	Rps6ap	23.31	177	4	236	27.0
gi37362649	Gus1p	26.98	138	14	708	80.8
gi6319559	Rpl19ap	29.10	135	5	189	21.7
gi6320190	Nop1p	27.83	117	8	327	34.4
gi6323654	Clu1p	17.85	104	16	1277	145.1
gi6319378	Rpl32p	23.85	100	3	130	14.8
gi6324569	Rpb11; TXN	42.50	71	4	120	13.6
gi6319630	Rpb5; TXN	28.84	68	4	215	25.1
gi6324798	Rpb8; TXN	21.23	49	2	146	16.5
gi6324784	Rpb10; TXN	52.86	36	2	70	8.3
gi6321333	Arc1p; TXN	11.97	35	4	376	42.1
gi6321231	Spt16p; TXN	5.12	17	4	1035	118.6
gi6321937	Rpb12; TXN	28.57	11	1	70	7.7
gi6322532	Npa3p; TXN	3.64	9	1	385	43.2
gi6323632	Spt5; TXN	2.54	2	2	1063	115.6
gi6320129	Bre1p; TXN	1.29	2	1	700	80.6
gi6321368	Rpb9; TXN	17.21	1	1	122	14.3
gi6323571	Pob3p; TXN	1.63	1	1	552	63.0
gi6323272	YLR243W; TXN	4.78	1	1	272	30.6

*All transcription-relevant proteins that were detected were included (TXN in description)
Coverage: The total percentage of amino acid sequence identified by a matching tryptic peptide
PSMs: The number of peptide spectral matches (proteins with <100 PSMs were excluded unless they were classified as transcription related -TXN)
Peptides: The number of unique peptides that were detected (proteins with <2 were excluded unless they were classified as transcription related -TXN)
AAS: The number of amino acids in the intact protein
MW: The protein molecular weight (kilodaltons)

II. Rtr1-1XFLAG Purification

Proteins Identified that Interact with Rtr1

The next experiment that was designed to identify proteins that interacted with Rtr1 that might plan a role in the recruitment of Rtr1 to RNAPII was performed in *S. cerevisiae* that expressed a 1XFLAG-tagged version of Rtr1 (Rtr1-1XFLAG, see Table 2). This experiment relied on a single affinity purification step with anti-FLAG resin to co-immunoprecipitate Rtr1 and its interacting partners from *S. cerevisiae* lysate followed by their detection by MudPIT-LC/MS. In the same fashion as the Rpb3-TAP/Rtr1-HFH experiments, proteins were only included in the results table (Table 8) if they were detected by the presence of at least 2 unique peptides and at least 100 PSMs. Furthermore, all transcription-relevant proteins (Table 4) were included in the results table, and any contaminant proteins were excluded.

The results of the Rtr1-1XFLAG single purification are presented in Table 8, which includes 2 proteins, in addition to Rtr1, that met the inclusion criteria, and an additional 9 proteins that were included because they were members of Table 4. Among the detected proteins were RNAPII subunits (Rpb1, Rpb2, Rpb5, Rpb6, Rpb10) as well as proteins that were not part of the list of transcription-relevant proteins: Mec1, Hyp2.

Table 8. Proteins identified that interacted with Rtr1

Accession	Description*	Coverage	# PSMs	# Peptides	# AAs	MW
gi6319612	Mec1p	13.51	145	20	2368	273.2
gi6320801	Hyp2p	19.11	124	2	157	17.1
gi6320987	Rtr1; TXN	56.19	117	11	226	26.2
gi6320061	Rpb1; TXN	6.35	20	8	1733	191.5
gi6324725	Rpb2; TXN	4.58	13	3	1224	138.7
gi6325445	Rpb6; TXN	14.19	3	1	155	17.9
gi6321193	Rtf1; TXN	6.81	2	2	558	65.8
gi6324784	Rpb10; TXN	37.14	2	1	70	8.3
gi6319630	Rpb5; TXN	7.91	1	1	215	25.1
gi6320377	Sup35p; TXN	1.90	1	1	685	76.5
gi6321368	Rpb9; TXN	20.49	1	1	122	14.3
gi330443587	Mtg2p; TXN	6.37	1	1	518	57.8

*All transcription-relevant proteins that were detected were included (TXN in description)
Coverage: The total percentage of amino acid sequence identified by matching tryptic peptides
PSMs: The number of peptide spectral matches (proteins with <100 PSMs were excluded unless they were classified as transcription related -TXN)
Peptides: The number of unique peptides that were detected (proteins with <2 were excluded unless they were classified as transcription related -TXN)
AAS: The number of amino acids in the intact protein
MW: The protein molecular weight (kilodaltons)

III. Scaffold Data Analysis

The computer program Scaffold™ (hence referred to as Scaffold) is a tool used by proteomics researchers to integrate protein data generated following a search using a combination of three common MS-based proteomics search algorithms (SEQUEST, X!tandem, Mascot) (NRPP, 2008). Upon integrating MS results from proteins identified in different experiments, Scaffold generates peptide and protein identification probabilities and recalculates false discovery rates based on the Peptide Prophet algorithm (Keller et al., 2002). Furthermore, as in the work presented here, Scaffold can align hundreds of proteins identified in single and double purifications schemes that originated from both experimental and mock preparations.

In order to increase the confidence that the proteins detected in the RNAPII-Rtr1 double purification, and the Rtr1 single purification, experiments were associated with

Rtr1 and/or RNAPII, all of the MudPIT-LC/MS data from both experimental and mock preparations were subjected to a Scaffold analysis. This included data from experimental and mock samples from Rtr1-TAP (Mosley Lab, Indiana University), Rtr1-1XFLAG, and Rpb3-TAP/Rtr1-HFH purification schemes.

After performing the Scaffold analysis, normalized spectral abundance factors (NSAF) were calculated using Equation I and Microsoft Excel as previously described (Zhang et al., 2010; Zybailov et al., 2006); PSM is the number of spectrum-peptide matches for a given protein, Length is the length of the protein in amino acids, k represents all proteins identified in the purification, and N is the total number of proteins identified. PSM is an indication of protein abundance as measured using mass spectrometry; however, larger proteins tend to generate more proteolytic fragments upon digestion. Therefore, NSAF values are a way to normalize PSM values obtained from a protein by considering its length and then putting all PSM/Length values for all proteins detected in a sample on a scale from 0-1(hence normalizing the values).

Equation I $$NSAFi = \frac{\left(\frac{PSM}{Length}\right)i}{\sum_{k=1}^{N}(PSM/Length)k}$$

To be included in the results table (Table 9), proteins had to identified by MudPIT-LC/MS with at least 20 total peptide counts, and a protein had to be enriched by at least 2-fold over the relevant mock preparation (i.e. $NSAF_{exp}/NSAF_{mock} > 2$).

The results of the Scaffold analysis are presented in Table 9. The first column of data presents all proteins that were detected across all of the different purification schemes as Rtr1-interacting proteins. All twelve of the RNAPII subunits were detected with the exception of Rpb4, Rpb6, Rpb7, and Rpb9. Thirteen additional proteins were also detected that included transcription-relevant proteins (Table 4) Npa3 and two subunits of

the PAF complex (Cdc73, Ctr9). In the second column, proteins that were enriched greater than 2-fold in the RNAPII-Rtr1 double purifications are listed. This column should be enriched in proteins that are required for Rtr1 recruitment to RNAPII. As with the first column, the same set of RNAPII subunits were detected along with 8 additional proteins, all of which were common to the first column. Npa3 was the only protein detected in the double purifications, other than RNAPII subunits), that was a member of the transcription-relevant proteins listed in Table 4. Likewise, column three lists the proteins that were greater than 2-fold enriched in the Rtr1-TAP and Rtr1-1XFLAG single purification experiments specifically. In all, 8 proteins were detected including three PAF complex subunits (Cdc73, Ctr9, Leo1). Four proteins listed in Table 9 (Rpb8, Rpb12, Spn1, Rpa49) were not detected in the mock purification experiments.

Table 9. Proteins that co-purified with Rtr1 following tandem affinity purification; single and double affinity purifications that were enriched 2-fold over protein from mock purifications

Proteins Identified[#] (all purification schemes)	Proteins Enriched ≥2-fold (Rtr1-RNAPII double purifications)	Proteins Enriched ≥2-fold (Rtr1 single purifications)
Rpb1*	Rpb1*	
Rpb10*	Rpb10*	
Rpb11*	Rpb11*	
Rpb12*†	Rpb12*†	
Rpb2*	Rpb2*	
Rpb3*	Rpb3*	
Rpb5*	Rpb5*	
Rpb8*†	Rpb8*†	
Hhf2	Hhf2	
Npa3	Npa3	
Prp22	Prp22	
Rad9	Rad9	
Rpa34	Rpa34	
Rpc53	Rpc53	
Rpt6	Rpt6	
Ssn2	Ssn2	
Cdc73		Cdc73
Ctr9		Ctr9
Gal11		Gal11
Hsc82		Hsc82
Nut1		Nut1
		Spn1†
		Rpa49†
		Leo1

#Proteins identified with <20 total peptide counts were rejected
*RNAPII subunits
†Not detected in MOCK purifications

IV. Post-translational Modification of Rtr1

To help clarify if post-translational modification of Rtr1 plays a role in its recruitment to RNAPII, MudPIT-LC/MS spectra obtained from the Rtr1-TAP single purification were searched using the SEQUEST. The data obtained from Rtr1-TAP was analyzed for the presence of the following PTMs: phosphorylation of Ser, Thr, and Tyr, which will result in a mass shift of 80 daltons per phosphorylation site on the peptide. The PTM

search result is presented in Figure 9, which indicates the presence of a phosphorylation site at Rtr1 Ser_{217}. The MS/MS spectrum of the phosphorylated tryptic peptide IVENDNPSILGDFTR (Figure 9, pane D) shows an extensive and diagnostic neutral loss of 98 da (phosphoric acid) from the parent $[M+2H]^{+2}$ ion.

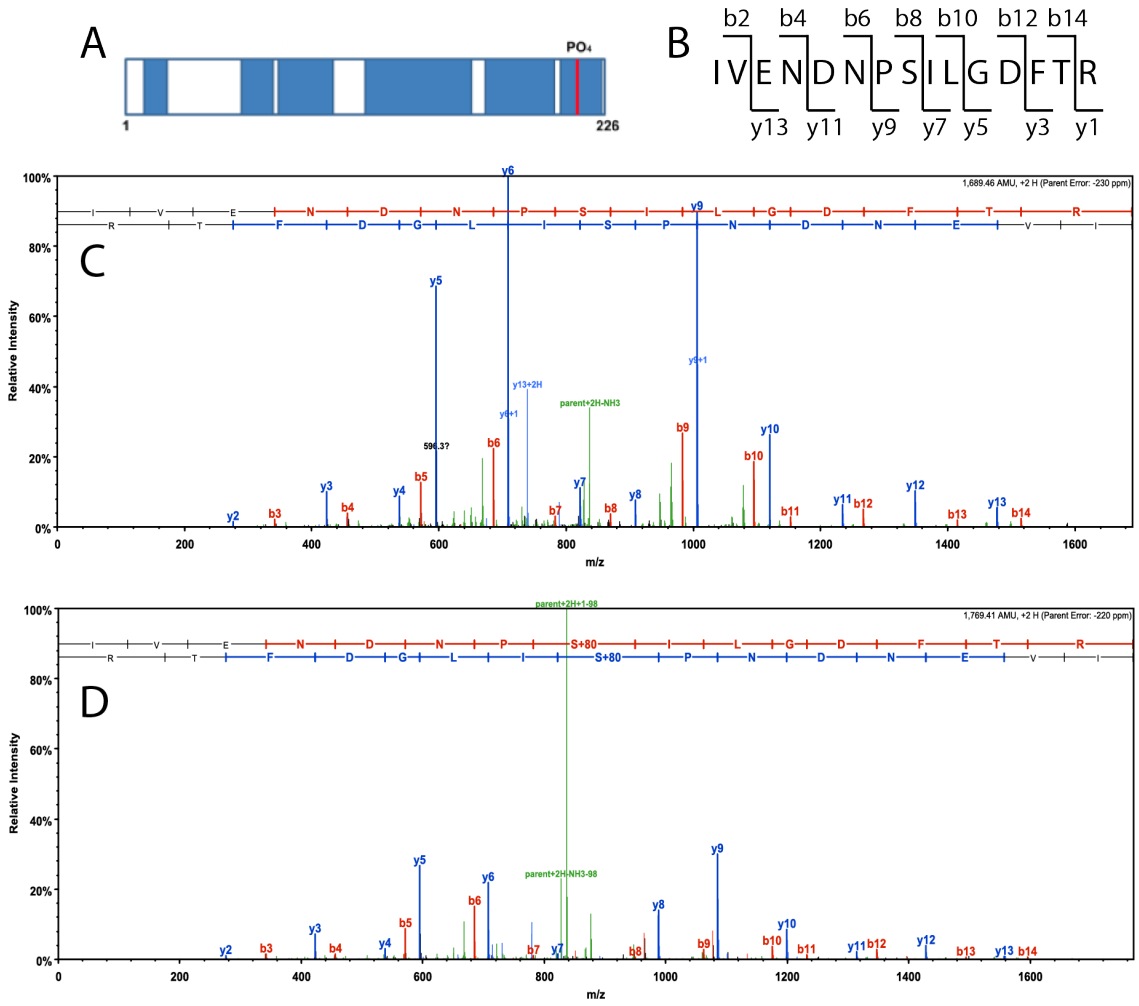

Figure 9. LC/MS identification of Rtr1 phosphorylation site at Ser_{217} (Berna et al., 2011). (A) Schematic representation of *Saccharomyces cerevisiae* yeast Rtr1 with sequence coverage indicated in blue and the position of the phosphorylation site in red. (B) Sequence of tryptic peptide containing the site of phosphorylation with every other y- and b-ion indicated. (C) MS/MS spectrum obtained from the unmodified Rtr1 tryptic peptide IVENDNPSILGDFTR. (D) MS/MS spectrum of phosphorylated (Ser_{217}) Rtr1 tryptic peptide indicating the signature neutral loss of 98 daltons from the parent $[M+2H]^{+2}$ ion.

V. CTDK1-TAP/Rtr1-V5 Purification

Fasolo *et al.* reported the interaction between the CTDK-I complex and Rtr1 in 2011 (Fasolo et al., 2011). Because of the involvement in CTDK-I in the regulation of RNAPII transcription, and its role in recruiting factors to RNAPII, it was hypothesized

that CTDK-I plays a role in the recruitment of Rtr1 to RNAPII during transcription. Consequently, single and double affinity purifications of *S. cerevisiae* that express TAP-tagged versions of CTDK-I subunits (Ctk1, Ctk2), and V5-tagged Rtr1, were performed to confirm the interaction between Rtr1 and CTDK-I. Following the purification schemes as described in the methods section, the presence or absence of specific CTDK-I subunits and Rtr1 were analyzed by Western blot.

Interaction of CTDK-I and Rtr1, Single Purification

The results of the IgG-sepharose single purification that targeted Ctk1-TAP or Ctk2-TAP, and Rtr1 if it was found to interact with CTDK-I, are presented in Figure 10. Ctk1 was identified in the Ctk1-Input sample, but an interaction between Ctk1 and Rtr1 was not observed. Rtr1 was not identified in the Ctk2-Input sample (result not shown), which invalidated this portion of the experiment and indicated that the Ctk2-TAP strain was incorrect.

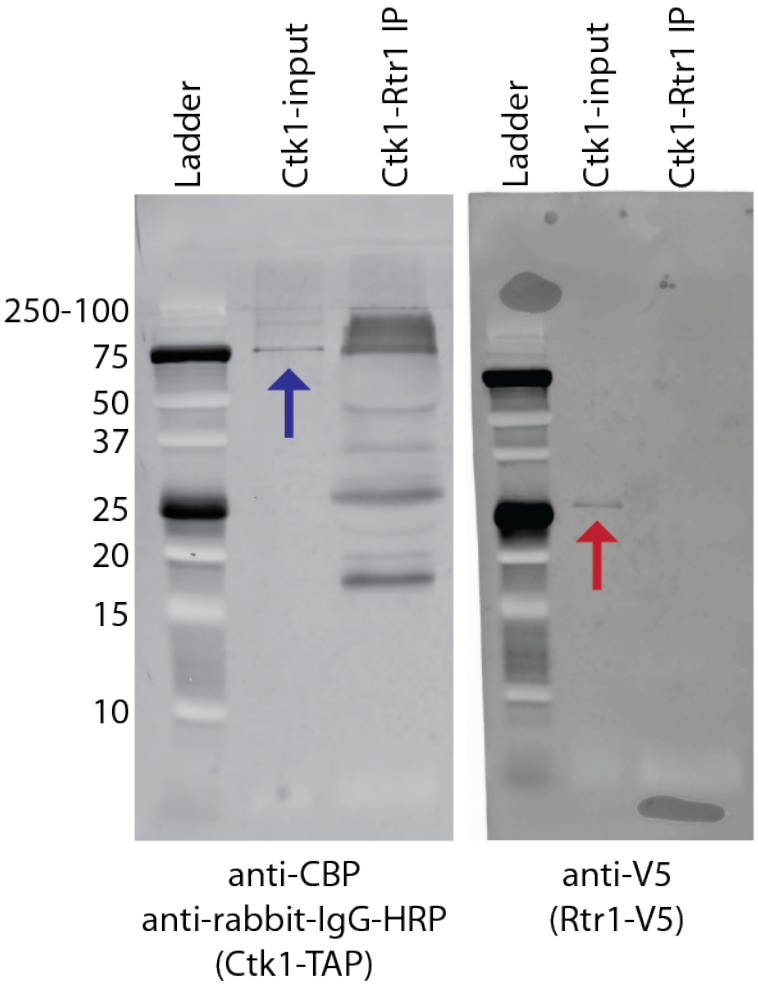

Figure 10. Western blot analysis of the CTDK-I subunit Ctk1 and Rtr1 following a single affinity purification targeting Rtr1-V5 and interacting proteins. The blue arrow indicates the position of Ctk1-TAP in the Ctk1 input sample, and the red arrow indicates the position of Rtr1-V5 in the Ctk1 input sample. The percentage of the input samples and IP samples loaded to the protein gel were 0.05% and 25%, respectively.

Interaction of CTDK-I and Rtr1, Double Purification

The results of the IgG-sepharose and anti-FLAG double purification that targeted Ctk1-TAP, Ctk2-TAP, and Rtr1 are presented in Figure 11. Both Ctk1 and Rtr1 were identified in the Ctk1-Input sample, but an interaction between Ctk1 and Rtr1 was not observed indicating that Rtr1 and Ctk1 do not interact at significant levels *in vivo*. Additionally, Ctk2 was identified in the Ctk2-Input, but Rtr1 was not detected in the

Ctk2-Input, so the interaction between CTDK-I and Rtr1 could not be confirmed from this part of the experiment.

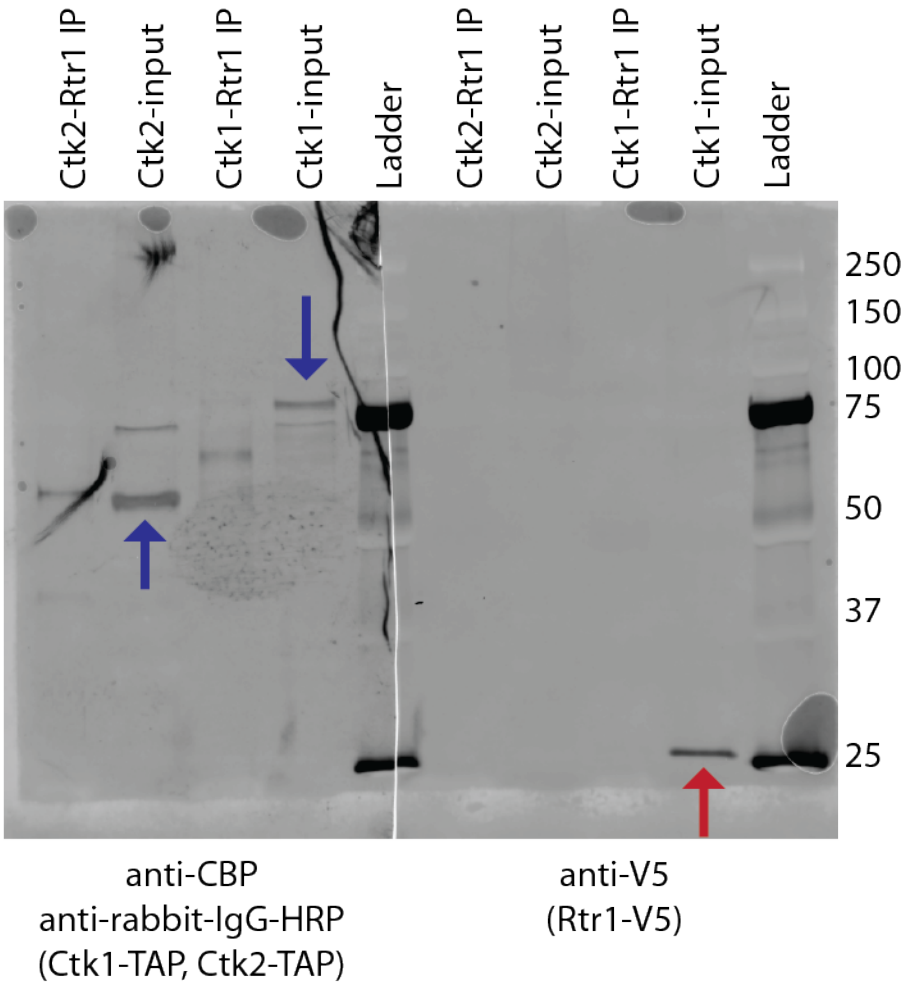

Figure 11. Western blot analysis of CTDK-I subunits (Ctk1 and Ctk2) and Rtr1 following a double affinity purification targeting Ctk1-TAP, Ctk2-TAP, and Rtr1-V5. The blue arrows indicate the position of Ctk2-TAP in the Ctk2 input sample and Ctk1-TAP in the Ctk1 input sample. The red arrow indicates the position of Rtr1-V5 in the Ctk1 input sample. The percentage of the input samples and IP samples loaded to the protein gel were 0.05% and 25%, respectively.

Effect of ctk1Δ on Rtr1 Interactions

Although the *in vivo* interaction between Rtr1 and CTDK-I was not confirmed in the work presented here, there interaction was previously reported using an *in vitro* approach to maximize kinase-interacting partner interactions (Fasolo et al., 2011). Therefore, the effect of *ctk1Δ* on Rtr1 interactions with RNAPII and transcription factors, and on the phosphorylation of Rtr1 was studied. *S. cerevisiae* that expressed Rtr1-TAP in a *CTK1* deletion genetic background (*ctk1Δ*) were purified using a TAP purification scheme. In the first part of the experiment, protein-protein interactions between Rtr1, RNAPII subunits, and transcription factors known to associate with Rtr1 Ser_{217} peptides was dramatically reduced in the absence of Ctk1 (Figure 12). Likewise, following post-translational modification searches for phosphorylation, the number of phosphorylated Rtr1 peptides was dramatically reduced in Rtr1-TAP *ctk1Δ* versus wild-type Rtr1-TAP (Figure 13).

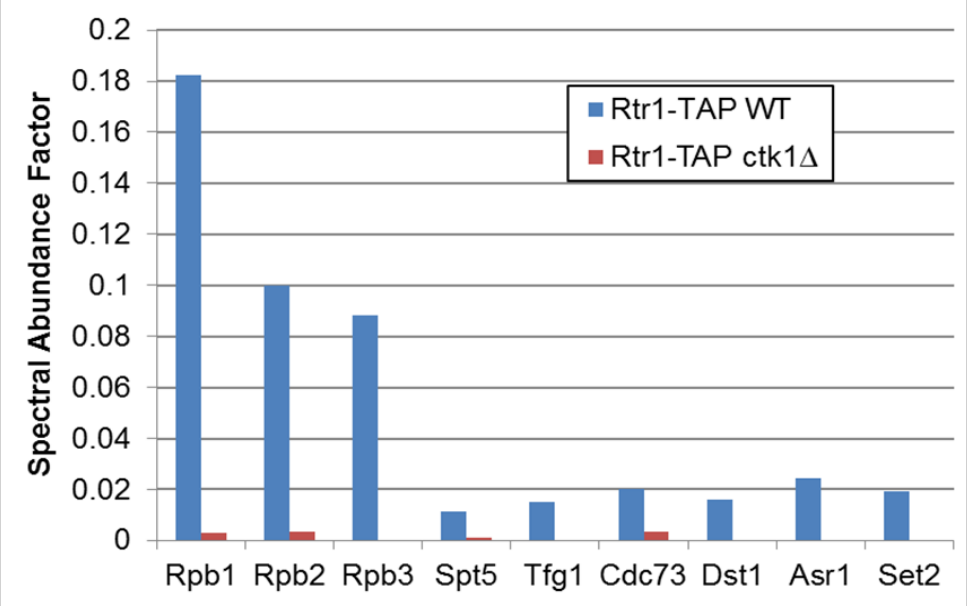

Figure 12. Effect of *ctk1Δ* on Rtr1 interactions with RNAPII and transcription factors (Berna et al., 2011). The spectral abundance factors of RNAPII subunits and transcription factors known to associate with Rtr1 were reduced in *ctk1Δ* versus wild-type *S. cerevisiae* as determined by LC/MS.

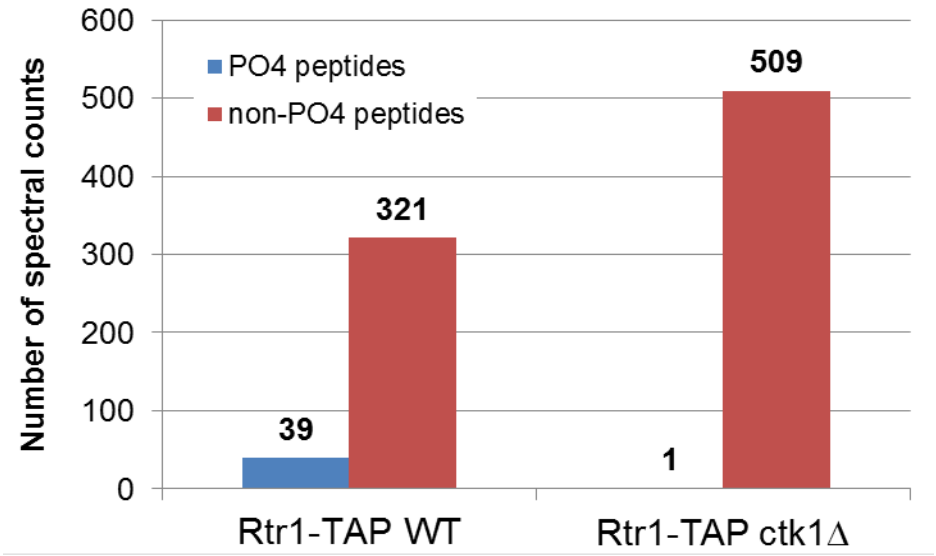

Figure 13. Effect of *ctk1Δ* on Rtr1 serine-217 phosphorylation (Berna et al., 2011). The presence of Rtr1 serine-217 phosphorylation was reduced in *ctk1Δ* versus wild-type *S. cerevisiae* as determined by LC/MS.

DISCUSSION

I. Rpb3-TAP/Rtr1-HFH Purification Scheme

In an effort to identify novel proteins responsible for recruiting Rtr1 phosphatase to RNAPII during transcription, we devised a tandem affinity purification scheme using a yeast strain that expressed Rtr1-HFH and a TAP-tagged version of the RNAPII subunit, Rpb3. The purification was designed to target proteins that interact with Rtr1 in the presence and absence RNAPII, as well as proteins that interact with RNAPII in the absence of Rtr1 (see Figure 14).

To isolate proteins that interact with Rtr1 alone, IgG-sepharose was used to remove RNAPII, through the interaction with the Protein-A portion of the Rpb3 TAP tag, from the *S. cerevisiae* extract. Next, Rtr1 and interacting proteins were isolated using anti-FLAG resin to target the 3XFLAG portion of the Rtr1-HFH tag. Following the affinity purification, the proteins were digested with trypsin and were identified by MudPIT-LC/MS. The results of the analysis are presented in Table 5, and it was noted that the purification was contaminated by all twelve of the RNAPII subunits. It was initially hypothesized that there was insufficient IgG-sepharose to remove all of the RNAPII from the sample; however, the same results was achieved upon repeating the purification with a 2-fold increase in the volume of IgG-sepharose resin used. Consequently, a second hypothesis was proposed that the *S. cerevisiae* used in the purification expressed both wild-type and TAP-tagged RNAPII (via the Rpb3 subunit). A PCR experiment was designed using primers that would generate products that could be used to differentiate Rpb3-TAP from wild-type Rpb3 following agarose gel electrophoresis. However,

following PCR analysis of genomic DNA isolated from the *S. cerevisiae*, no PCR products were obtained. The experiment was repeated with the same result and was therefore inconclusive (data not shown). Although the preparation was contaminated by RNAPII subunits, the data obtained were still useful to identify proteins that interact with the RNAPII-Rtr1 complex.

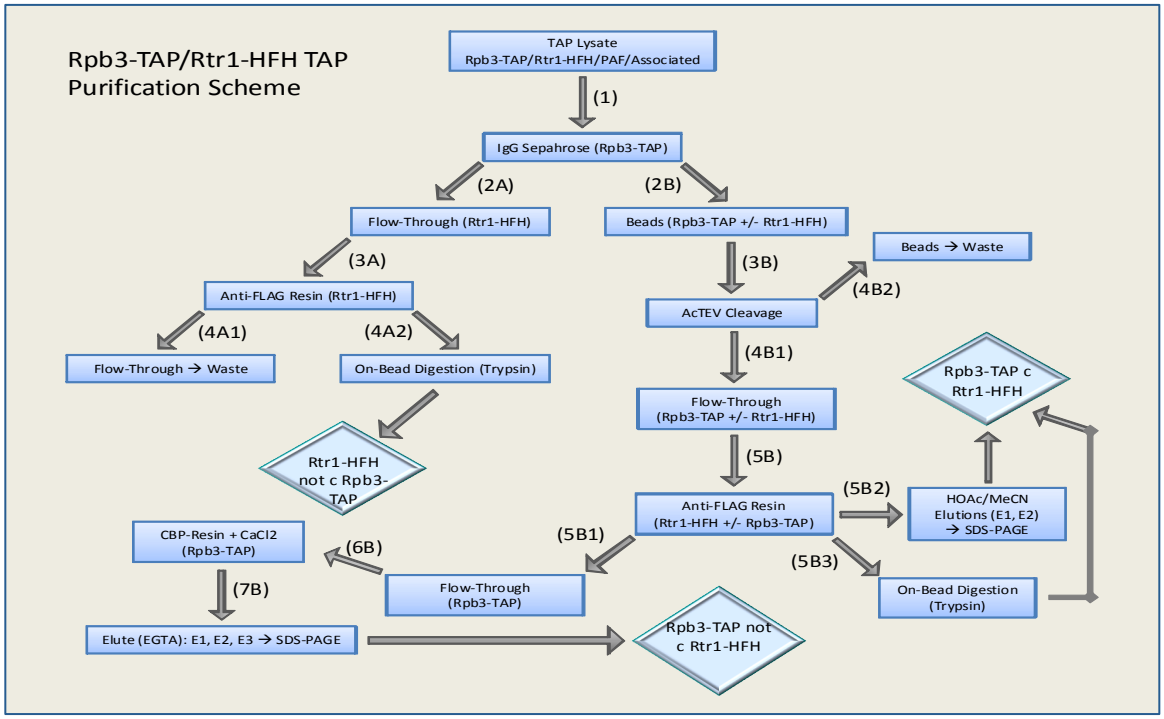

Figure 14. Overview of the Rpb3-TAP/Rtr1-HFH purification scheme. The purification was broken down into three sections that targeted proteins that interacted with: proteins that interacted with Rtr1 alone (4A2), proteins that interacted with RNAPII alone (7B), and proteins that interacted with the RNAPII-Rtr1 complex (5B2, 5B3).

Eighty-nine proteins, including Rtr1 and nine of the twelve RNAPII subunits, were reported in Table 5 that were found to interact with the RNAPII-Rtr1 complex; the focus of this branch of the purification was changed due to the aforementioned contamination by RNAPII subunits. Some of the notable proteins that were detected were PAF complex

subunits (Ctr9, Rtf1, and Paf1), the ATPase Npa3, the essential protein YLR243W, the FACT complex subunit Spt16; and Chd1, which is involved in chromatin remodeling and transcription regulation. All of the proteins detected in this experiment were included in a Scaffold analysis to determine their level of enrichment relative to mock preparations. Additionally, the results of the Scaffold analysis identified which of the proteins present in Table 5 were shown to consistently interact with RNAPII-Rtr1 across the different purification schemes reported herein.

In the second arm of the Rpb3-TAP/Rtr1-HFH purification scheme, proteins that interacted with RNAPII alone were targeted. RNAPII and interacting proteins were isolated from the cell lysate using IgG-sepharose, and Rtr1 was removed from this fraction using anti-FLAG resin. RNAPII and its interacting partners were further purified using calmodulin resin to target the calmodulin binding protein portion of the Rpb3-TAP tag. Following immunoprecipitation, the RNAPII subunits in the sample were analyzed by SDS-PAGE with silver staining (Figure 8), and a second aliquot of the affinity-purified sample was digested with trypsin and analyzed by MudPIT-LC/MS (Table 6). Rtr1 was not detected in this purification scheme, which served as a negative control by verifying its removal using anti-FLAG resin.

It was concluded from the SDS-PAGE analysis of the RNAPII subunits that the purification scheme had good yield; all twelve of the RNAPII subunits were detected. In addition to RNAPII subunits, nine other proteins were included in the results presented in Table 6 including Spt5, which has a CTD similar to Rpb1 that is a substrate for phosphorylation. The finding that Spt5 associates with RNAPII at high levels corroborates the same observation that was made by Mosley *et al.* in 2011 (Mosley et al.,

2011). Therein, the authors used label free quantitative proteomics to study RNAPII complexes, and they reported that Spt5 was one of the most abundant proteins found to associate with RNAPII. In addition, the transcription factors Spt6 and Spn1 were detected, as was the FACT subunit Spt16, and Arc1, which is involved in tRNA delivery to tRNA synthetases. In addition to the proteins presented in Table 6, all of the proteins detected in this branch of the purification scheme were included in the Scaffold analysis to determine their level of enrichment relative to mock preparations.

The final targets of the Rpb3-TAP/Rtr1-HFH purification were proteins that interacted with the RNAPII-Rtr1 complex. In this purification, RNAPII-Rtr1 was isolated from yeast lysate first by using IgG-sepharose to target Rpb3-TAP followed by a second immunoprecipitation using anti-FLAG resin to target Rtr1-HFH.

In addition to nine of the twelve RNAPII subunits and Rtr1, thirty-six proteins that were detected were included in the results presented in Table 7. In effect, this experiment was a repeat of the experiment that was intended to identify proteins that interacted with Rtr1 alone; however, that experiment was contaminated by the presence of RNAPII subunits (Table 5). Consequently, many of the same proteins were detected in this experiment as well, including YLR243W, Npa3, Spt16, and Arc1. Discounting Rtr1 and RNAPII subunits, seven transcriptionally relevant proteins were detected. As with the other Rpb3-TAP/Rtr1-HFH purification schemes, all of the proteins that were detected were included in the Scaffold analysis.

II. Rtr1-1XFLAG Purification Scheme

A single affinity purification scheme was performed in *S. cerevisiae* that expressed Rtr1-1XFLAG. By substituting the relatively simple anti-FLAG purification for the more complex tandem affinity purification, a wider net was cast in an effort to identify a larger range of proteins that may play a role in the recruitment of Rtr1 to RNAPII during transcription (see Figure 6). By reducing the number of purification steps, the Rtr1-FLAG single purification has the potential for increased background relative to the TAP purification scheme; however, Scaffold analysis was used to help identify non-specific Rtr1-interacting partners by measuring their level of enrichment relative to mock preparations.

The results of the Rtr1-1XFLAG purification are presented in Table 8. In addition to Rtr1 and six of the twelve RNAPII subunits, Mec1, Hyp2, Ssa1, and Ssa2 were detected with greater than 100 PSMs, and with the exception of Hyp2, greater than eight unique peptides. However, it should be noted that Hyp2 is relatively small (157 amino acid residues), which may help to explain the limited number of unique peptides that were detected. The proteins Sup35, Mtg2, and the PAF complex subunit Rtf1 were also detected, but with poor yield ($\leq$2 PSMs and Unique Peptides). As with the Rpb3-TAP/Rtr1-HFH tandem affinity purification, all of the proteins detected in this experiment were included in the Scaffold analysis discussed in the following section.

III. Scaffold Data Analysis

Scaffold Analysis Reveals 21 Proteins that Interact with RNAPII or Rtr1

As previously discussed, all of the proteins that were detected in the MudPIT-LC/MS analyses of Rtr1-TAP and Rtr1-1XFLAG single affinity purifications, and Rpb3-

TAP/Rtr1-HFH double purifications, were analyzed using the program Scaffold. Scaffold analysis allowed the side-by-side comparison of tandem mass spectrometry results from proteins that were detected in the experimental affinity-based preparations to determine their level of enrichment versus mock preparations (i.e. from strains that do not contain affinity-tagged proteins). The level of protein enrichment was determined by dividing NSAF values (Equation 1) from proteins enriched in experimental preparations by those from their respective mock preparations. Twenty-one proteins that were detected with at least twenty total peptide counts were presented in the Scaffold results table (see Table 9). Additionally, proteins that were enriched by greater than or equal to 2-fold versus their respective mock preparations were distinguished in Table 9 by the type of affinity purification from which they were recovered (i.e. Rtr1-RNAPII double purifications or Rtr1 single purifications).

The first column in Table 9 lists the proteins that were identified in all of the aforementioned purification schemes. This included, as expected, the RNAPII subunits Rpb1-3, Rpb5, Rpb8 and Rpb10-12. Rpb4, Rpb6, Rpb7, and Rpb8 were not detected (i.e. they were present with less than 20 total peptide counts). However, this can be explained by the fact that Rpb4, Rpb7, and Rpb9 were found to have decreased association with Rtr1; and Rpb6, Rpb8 are difficult to detect by mass spectrometry (Mosley et al., 2012; Mosley et al., 2011). The same RNAPII subunits listed herein were found to be greater than 2-fold enriched in the Rtr1-RNAPII double purifications as well. In fact, the results of the different purification schemes indicate that the most significant Rtr1 interacting partners identified were RNAPII subunits. In the Rbp3-TAP/Rtr1-HFH double purifications, Rpb1, Rpb2, Rpb3, and Rpb4 were part of the top six most abundant

proteins found to interact with Rtr1 (Tables 5 and 7). Similarly, Rpb1, Rpb2, and Rpb6 were among the top 5 most abundant proteins found to interact with Rtr1 in the Rtr1-1XFLAG single purification experiment (Table 8). Figure 15 shows the level of Rtr1 enrichment, relative to the RNAPII subunits Rpb1 and Rpb2, achieved using Rtr1-TAP and Rtr1-FLAG single purifications; and Rpb3-TAP/Rtr1-HFH double purification schemes.

Proteins that were Greater than 2-Fold Enriched in Rtr1-RNAPII Double Purifications

Sixteen proteins identified in the Scaffold analysis were found to be greater than 2-fold enriched in the Rtr1-RNAPII double purification schemes relative to their respective mock preparations. These included ten proteins that were RNA polymerase subunits, which suggests that RNAPII subunits may play the most important role in recruiting Rtr1 during transcription. As mentioned before, eight of these proteins were RNAPII subunits. Other RNA polymerase subunits were also detected. Rpc53, also known as Rpc4, is an RNA polymerase III subunit (Kassavetis et al., 2010), and Rpa34 is a RNA polymerase I subunit (Albert et al., 2011). It is unlikely that RNA polymerase I and III subunits are involved in recruiting Rtr1 to RNAPII.

The protein Histone H4 (Hhf1, Hhf2) was greater than 2-fold enriched in the Rtr1-RNAPII double purifications and is described in the Saccharomyces Genome Database (SGD, www.yeastgenome.org) as being required for the assembly of chromatin. The human homolog is encoded by the gene KCNJ11 and is known as Hist2H4B protein. As a histone core protein, it is reasonable that Hhf2 would be found to associate with RNAPII as they both interact with DNA. The interaction with DNA and Rtr1 is likely indirect via RNAPII, so it is possible that Rtr1 might not interact directly with Histone

H4. This is supported by the fact that Histone H4 was not enriched in the Rtr1single purification schemes.

Npa3 was greater than 2-fold enriched in the Rtr1-RNAPII double purifications but was absent in the Rtr1single purifications. This protein is a cytoplasmic protein that binds to RNAPII in a GTP-dependent fashion and was found to participate in its transport to the nucleus (Staresincic et al., 2011). Staresincic *et al.* reported that depletion of Npa3 in yeast resulted in the build-up of RNAPII in the cytoplasm and a corresponding decrease in the transcription of numerous genes as measured by clonal sequencing and RT-PCR (Staresincic et al., 2011); compared to wild-type Npa3, a temperature sensitive strain resulted in at least a 2-fold change in expression of 1392 genes, with 1294 of these being down-regulated. XAB1, the human homolog of Npa3, was found to interact with human RNAPII in a similar fashion (Staresincic et al., 2011). It is possible that the role that Npa3 plays in importing RNAPII into the nucleus might also signal the recruitment of Rtr1. Interestingly, the human homolog of Rtr1, RPAP2, was found to translocate between the nucleus and the cytoplasm (Boulon et al., 2010), and it was proposed that RPAP2 might serve to keep RNAPII in a hypophosphorylated state to prevent the recruitment of RNA processing factors and the resulting, premature processing of RNA (Corden, 2011).

The DEAH-box RNA helicase Prp22 is an ATPase that catalyzes the release of mRNA from the spliceosome during pre-mRNA splicing (Schwer, 2008). Prp22 was enriched in the Rtr1-RNAPII double affinity purification but not in the Rtr1 single purification schemes. The fact that Prp22 was not found to directly interact with Rtr1, and that it functions primarily to release mRNA from the spliceosome as opposed to affecting

its elongation, makes it unlikely that Prp22 is involved in the recruitment of Rtr1 to RNAPII during transcription elongation.

Checkpoints proteins are involved in regulating cell cycle checkpoints, which are signaling pathways that delay progression through the cell cycle in response to DNA damage or defective chromosomal replication (Labib and De Piccoli, 2011). The checkpoint protein Rad9 was enriched in the Rtr1-RNAPII double purifications. Rad9 is specifically involved in DNA damage repair and forms a ternary complex with the proteins Dpb11 and Mec1 (Pfander and Diffley, 2011). Based on the role of Rad9, it is not a primary candidate as a protein involved in recruiting Rtr1 to RNAPII.

Rpt6 was also found to be enriched in the present experiment and is one of six ATPases that are components of the regulatory particle of the 26S proteasome, which in eukaryotic cells degrades ubiquitinated proteins (Lee et al., 2012). In 2002, Ferdous *et al.* reported that the 19S regulatory particle of the proteasome plays an important, nonproteolytic role in the efficient initiation of transcription by human RNAPII (Ferdous et al., 2002). Furthermore, the authors reported that this function was general to all eukaryotes. The human homolog of Rpt6 is known as p45 and serves a similar function in the human 26S proteasome (Satoh et al., 2001).

The final protein found to be enriched in this purification scheme was Ssn2 (Srb9/Med13). Ssn2 protein was found to be identical to Srb9, a component of the mediator complex that interacts with RNAPII to mediate transcription activation and repression in *S. cerevisiae* (Song et al., 1996). More recently, Wery *et al.* reported that Srb9 (now Med13) associates with TFIIS, a transcriptional elongation factor, via its N-terminal domain (Wery et al., 2004). Furthermore, The role that Med13 plays in the

mediator complex is conserved in humans, and the human homolog of Med13 has the same designation (Belakavadi and Fondell, 2010). Unfortunately, Svejstrup *et al.* reported that Mediator was unable to bind to hyperphosphorylated RNAPII, and that as a result, Mediator was found to dissociate from RNAPII during transcription elongation (Svejstrup et al., 1997). Consequently, Mediator subunits are unlikely to be involved in recruiting Rtr1 to RNAPII during transcription elongation. Two additional Mediator subunits, Gal11 (Med15) and Nut1 (Med5) were similarly enriched in the Rtr1 single purification schemes.

Proteins that were Greater than 2-Fold Enriched in Rtr1 Single Purifications

Eight proteins were enriched in the Rtr1 single purification schemes relative to their respective mock preparations. Among them were three of the five subunits of the PAF complex, Cdc73, Ctr9, and Leo1. Mueller and Jaehning previously reported that PAF subunits were found to associate with RNAPII throughout the cycle of mRNA transcription (Mueller and Jaehning, 2002; Mueller et al., 2004); however, although PAF subunits were present, they were not found to be greater than 2-fold enriched in the Rtr1-RNAPII double purification schemes. As previously described, PAF associates with an impressive list of transcription factors and thereby plays a role in regulating RNAPII transcription (Krogan et al., 2002; Mueller and Jaehning, 2002; Squazzo et al., 2002). PAF may also serve as a scaffold for the recruitment of transcription factors during transcription elongation (Gerber and Shilatifard, 2003; Penheiter et al., 2005), which makes PAF a candidate involved in the recruitment of Rtr1 to the RNAPII complex.

Eukaryotic cells are capable of inducing the expression of heat shock genes as a protective mechanism to counter the effects of elevated temperatures. One such gene,

Hsc82, encodes a protein that was enriched in the current purification scheme (Hsp90). *S. cerevisiae* have two genes that encode isoforms of Hsp90, namely Hsc82 and Hsp82; the former is constitutively expressed and the latter is heat-inducible (Morano et al., 1999). It is unknown if and how Hsc82 association with Rtr1 is involved in the role that Rtr1 plays in regulating transcription elongation.

Spn1 (Iws1, human homolog) plays an essential and highly conserved role (from yeast to humans) in regulating RNAPII-mediated gene expression (Pujari et al., 2010). In addition to interacting with RNAPII throughout the entire reading frame of a number of constitutively expressed genes, Spn1 interacts with other factors such as the TATA-binding protein (TBP) and the general transcription TFIIS (Krogan et al., 2002; Pujari et al., 2010). Spn1 is thought to play an important role in forming an active transcription elongation complex through its interactions with RNAPII and other transcriptionally relevant factors such as Spt4, Spt5, Spt6, and TFIIS (Diebold et al., 2010; Pujari et al., 2010). Consequently, Spn1 is considered a candidate for follow-up experiments to further investigate its potential role in recruiting Rtr1 to RNAPII during transcription elongation.

The final protein found to be enriched in the Rtr1 single purification schemes was Rpa49, which is the RNA polymerase I subunit A49. Even though it is possible that Rpa49 could form a complex with RNAPII, perhaps at low levels, Rpa49 is not considered to be a primary candidate involved in recruiting Rtr1 to RNAPII during mRNA transcription.

A list of the proteins detected in the present work that are potentially involved in the recruitment of Rtr1 to RNAPII during transcription is presented in Table 10.

Table 10. Top candidate Rtr1-interacting proteins detected by MudPIT-LC/MS following single and double affinity purifications that are potentially involved in recruiting Rtr1 to RNAPII during transcription.

Rtr1 Interacting Proteins	**Cellular Function***	**Significance to Rtr1 recruitment to RNAPII**	**Human Homolog**
RNAPII subunits: Rpb1-3, Rpb5, Rpb8, Rpb10-12	mRNA, snRNA, miRNA transcription	1. Most consistently enriched Rtr1 interacting proteins 2. Rtr1 human homolog, Rpap2, is recruited to human RNAPII by S7-P 3. Recruitment may be mediated by CTD phosphorylation	RNAPII (subunits highly conserved)
Npa3	Cytoplasmic GTPase involved in RNAPII export to the nucleus	1. Npa3 and Rtr1 co-locate with RNAPII in both the cytoplasm and the nucleus 2. Npa3 and Rtr1 interact in human and yeast	XAB1
Rpt6	One of six core ATPases associated with the 26S proteasome	The 19S regulatory subunit of the proteasome plays an important, non-proteolytic role in efficient transcription initiation	p45
PAF complex subunits: Cdc73, Ctr9, Leo1	Regulation of RNAPII transcription	1. Associates with RNAPII throughout the transcription cycle 2. Known to serve as a scaffold for recruiting numerous transcription factors	hPaf1 complex
Hsc82	Heat shock protein	1. Hsc82 homolog found to interact with human Npa3 and Rtr1 2. Hsc82 is NOT a common contaminant although it is a heat shock protein	Hsp90
Spn1	Essential (yet unknown) role in regulating RNAPII gene expression	1. Involved in forming an active transcription elongation complex 2. Interacts with RNAPII throughout the entire coding region of numerous genes 3. Spn1 and Rtr1 share Spt5 as an interacting partner	Iws1

*Saccharomyces Genome Database (www.yeastgenome.org)

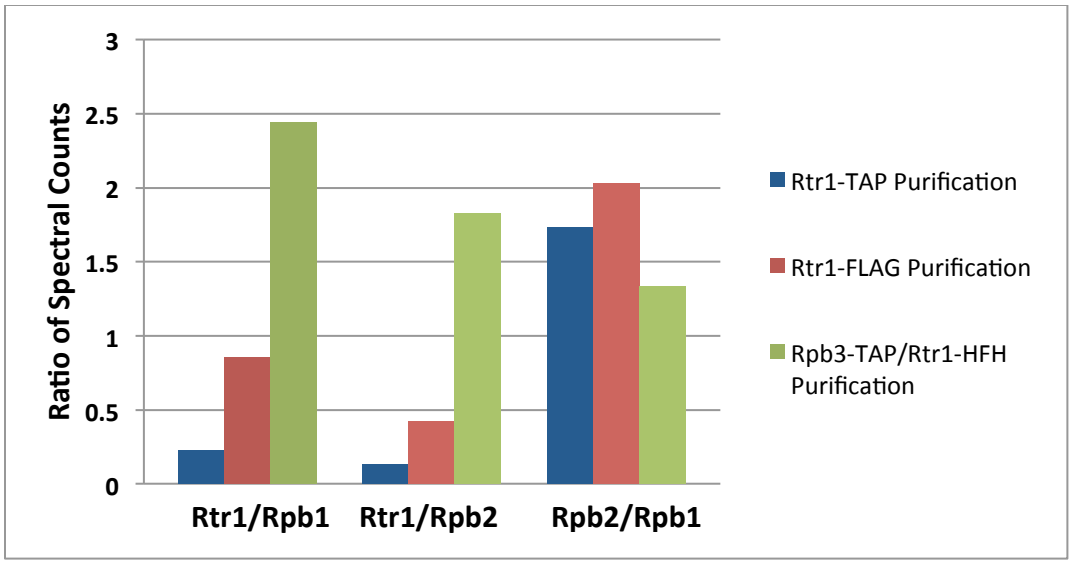

Figure 15. The level of Rtr1 enrichment relative to RNAPII subunits Rpb1 and Rpb2. The level of enrichment was determined by dividing the LC/MS spectral counts of Rtr1 by either Rpb1 or Rbp2 (Rpb2 enrichment relative to Rpb1 is presented as a relative comparator). The level of enrichment achieved using three different purifications schemes is presented: Rtr1-TAP and Rtr1-FLAG single purifications; and a Rpb3-TAP/Rtr1-HFH double purification.

IV. Post-translational Modification of Rtr1

One of the objectives of the present work was to investigate if Rtr1 post-translational modification is involved in its recruitment to RNAPII during transcription elongation. Consequently, tandem MS spectra obtained from Rtr1 single purification schemes were searched using the SEQUEST algorithm for potential PTMs including: phosphorylation (Ser, Thr, or Tyr), oxidation (Met, His), ubiquitination (Lys), methylation (Arg, Lys), and Sumoylation (Lys), which are some of the major PTMs involved in protein-protein interactions. The results of the MS/MS analysis and SEQUEST search suggest that Rtr1 is in fact phosphorylated at Ser_{217} (Figure 9). The phosphoserine was identified from an MS/MS spectrum obtained from the analysis of the Rtr1 tryptic peptide IVENDNPSILGDFTR, which indicated a single neutral loss of 98 da from the parent

$[M+2H]^{2+}$ ion. The neutral loss of H_3PO_4 (*m/z* 97.977) is diagnostic for the phosphorylation of Ser and Thr residues as it is typically a chemically labile bond in collision-induced dissociation (CID) than are peptide bonds (Lehmann et al., 2007).

The phosphorylation of Rtr1 is of particular interest in the present work as it has been found to directly associate with a kinase involved transcription regulation, CTDK-I (Fasolo et al., 2011). Consequently, we have hypothesized that CTDK-I is responsible for the phosphorylation of Rtr1, and that this Rtr1 PTM is involved in its recruitment to RNAPII during transcription elongation. Further experiments, of course, are required to support or reject this hypothesis.

V. CTDK1-TAP/Rtr1-V5 Purification Scheme

The second objective of the work presented here was to confirm the observation by Fasolo *et al.* in 2011 that Rtr1 interacts directly with CTDK-I, a regulatory kinase involved in RNAPII transcription (Fasolo et al., 2011). To this end, single and double purification schemes were performed in *S. cerevisiae* that express V5-tagged Rtr1 and a TAP-tagged version of either Ctk1 or Ctk2, which are CTDK-I subunits. The resulting samples were analyzed by Western blot, and the results of the single and double purifications schemes are presented in Figures 10 and 11, respectively. For an overview of the single and double purification schemes, see Figure 7.

The results of the Rtr1-V5/Ctk1-TAP and Rtr1-V5/Ctk2-TAP single purification experiments did not confirm the *in vivo* interaction between Rtr1 and CTDK-I. Ctk1 and Rtr1 were detected by Western blot in the input sample, which served as a positive control. In addition, Ctk1 was detected in the experimental sample; however, Rtr1 was

not detected (see Figure 10). This clearly indicates that either Rtr1 does not interact with CTDK-I, or more probably, that the interaction between Rtr1 and CTDK-I is temporally brief and was consequently below the limit of detection of this approach. Rtr1 was not detected in the Ctk2 input (results not shown), and consequently this portion of the experiment was not used for further analysis. One possible explanation was that the *S. cerevisiae* used in this experiment (i.e. Ctk2-TAP strain) did not express a V5-tagged version of Rtr1, but further investigation is required to support this hypothesis.

In an attempt to amplify the level of Rtr1, a double purification was performed that relied first on the immunoprecipitation of CTDK-I (via Ctk1-TAP or Ctk2-TAP) with IgG-sepharose followed by a second immunoprecipitation of CTDK-I/Rtr1 with anti-V5 resin (targeting the Rtr1 V5 tag). The results of the double purification were identical to those of the single purification in that both Ctk1 and Rtr1 were detected in the Ctk1 input sample; however, although Ctk2 was detected in the Ctk2 input sample, Rtr1 was absent (see Figure 11).

The *in vivo* interaction between Rtr1 and CTDK-I could not be confirmed in either the single or double purification schemes. However, the following section presents compelling evidence that CTDK-I regulates the function of Rtr1 *in vivo*, although it could not be determined if this regulation was direct or indirect. Additionally, confirmation of the *in vitro* interaction between CTDK-I and Rtr1 is currently being investigated.

VI. Effect of *ctk1Δ* on Rtr1 Interactions

As discussed above, direct evidence of the interaction between CTDK-I and Rtr1 could not be provided using Rtr1-V5/CTDK-I-TAP single and double purification schemes preceding analysis by Western blot. However, in an attempt to provide evidence

that CTDK-I regulates Rtr1, the interactions between Rtr1, RNAPII subunits, and transcription-relevant factors were studied in *S. cerevisiae* that lacked the catalytic subunits of CTDK-I (*ctk1Δ)*. Furthermore, the effect of *ctk1Δ* on the level of Rtr1 serine 217 phosphorylation was studied.

Rtr1-TAP single purification schemes in wild-type and *ctk1Δ S. cerevisiae* were performed preceding MudPIT-LC/MS analysis (in collaboration with Jerry Hunter). Following immunoprecipitation of Rtr1-TAP with IgG-sepharose resin, the interaction of Rtr1 with transcription-relevant proteins in wild-type *S. cerevisiae* were compared to those in *ctk1Δ* yeast. The list of transcription-relevant proteins included RNAPII subunits (Rpb1, Rpb2, Rpb3), transcription initiation and elongation factors (Spt5, Tfg1, Dst1), the PAF complex subunits Cdc73, the ubiquitin ligase Asr1, which is involved RNAPII regulation (www.yeastgenome.org), and the histone methyltransferase Set2. The results of the MudPIT-LC/MS analysis are presented in Figure 12, and it is evident that *ctk1Δ* has a significant, negative effect on the interaction of Rtr1 with RNAPII subunits and transcription-relevant proteins. For example, the interaction between the largest RNAPII subunit, Rpb1, and Rtr1 was decreased by more than 20-fold in the *ctk1Δ* yeast strain relative to wild-type. This trend was observed in the remaining transcription-relevant proteins as well, whose interactions with Rtr1 were significantly reduced in the *ctk1Δ* yeast. This finding supports the importance of CTDK-I in the regulation of Rtr1 function and suggests that Ctk1 acts upstream of the Rtr1-RNAPII interaction.

The same trend was observed for the presence of phosphorylated Rtr1 tryptic peptides in the absence of CTK1. In this experiment, Rtr1 phosphorylated peptides were significantly reduced in *ctk1Δ S. cerevisiae* versus wild-type following purification of

Rtr1-TAP (see Figure 13). The level of non-phosphorylated Rtr1 peptides was used to normalize the results across the different experiments. The level of phosphorylated peptides to non-phosphorylated peptides was decreased by more than 50-fold in *ctk1* yeast versus wild-type. These results, in addition to the finding that Rtr1 is phosphorylated at Ser_{217} (see Figure 9), suggest that Rtr1 is a potential CTDK-I substrate.

CONCLUSIONS

In 2009, Mosley and coworkers described the role of Rtr1 phosphatase in regulating a key step in transcription elongation, namely the transition of the RNAPII CTD phosphorylation state from S5-P to S2-P (Mosley, 2009). However, the mechanism responsible for recruiting Rtr1 to RNAPII during mRNA transcription has not been uncovered. Consequently, the focus of the work presented here was to elucidate protein-protein interactions and post-translation modifications involving Rtr1 that may be involved in its recruitment to RNAPII during transcription.

The first of two key hypotheses tested was that Rtr1 is recruited to the RNAPII complex during transcription elongation through an interaction with an unidentified protein(s), and that post-translational modification of Rtr1 or its interacting partner(s) may also play a role in Rtr1 recruitment. Additionally, special attention was given to the PAF complex as it was previously found to interact with Rtr1 (Mosley, unpublished data) and has been demonstrated to associate with numerous transcription factors that are involved in regulating initiation and elongation (Krogan et al., 2002; Mueller and Jaehning, 2002; Squazzo et al., 2002). Numerous proteins, including PAF complex subunits, were found to interact with Rtr1 following single and double affinity co-purification schemes from *S. cerevisiae*; protein identification was by MudPIT-LC/MS (see Tables 5-8).

In order to increase the confidence that the proteins detected in the affinity purification schemes interact with Rtr1 and RNAPII, they were subjected to data analysis using the computer program Scaffold. The results of the Scaffold data analysis are

presented in Table 9, and several proteins were enriched in experimental sample preparations (relative to mock purifications which were used as negative controls) that could play a role in recruiting Rtr1 to RNAPII. In addition to RNAPII subunits, seven proteins of particular interest were greater than 2-fold enriched in either Rtr1-RNAPII double purification schemes or Rtr1 single purification schemes. These included three PAF complex subunits (Cdc73, Ctr9, and Leo1), the heat shock protein Hsc82, the GTPase Npa3 (human homolog, XAB1), the ATPase Rpt6 (human homolog, p45); and Spn1 (human homolog Iws1), which is involved regulating RNAPII-mediated gene expression. Although it was not confirmed that these proteins recruit Rtr1 to RNAPII during transcription, they serve as candidates for further investigation. For example, the recruitment of Rtr1 to RNAPII during transcription elongation could be studied using individual yeast strains that have deleted the putative proteins thought to be involved in the recruitment of Rtr1.

To understand if post-translational modification of Rtr1 is involved in its recruitment during transcription elongation, a PTM search was performed using tandem mass spectra obtained from and Rtr1-TAP affinity purification. The results of the PTM search (Figure 9) indicate that Rtr1 was indeed phosphorylated at Ser_{217} (S217-P). Follow-up experiments are necessary to study the dependence of S217-P on Rtr1 recruitment to RNAPII. One possible set of experiments would involve mutating Rtr1-S217 to a neutral amino acid residue (S217A), to an acidic amino acid residue that could mimic a phospho-group (S217D), and to a neutral but structurally similar amino acid residue (S217N). These Rtr1 mutants could be used to study the impact of Rtr1-S217-P on its interaction with RNAPII as well as the importance of S217-P on transcription elongation.

Fasolo and co-workers reported that the CTDK-I kinase interacts with Rtr1 in *S. cerevisiae* (Fasolo et al., 2011). CTDK-I plays an important role in regulating RNAPII transcription via phosphorylation of CTD S2 and S5. Consequently, the second hypothesis that was tested in the present work was that CTDK-I recruits Rtr1 to RNAPII during transcription, and that the recruitment of Rtr1 involves its phosphorylation by CTDK-I. Unfortunately, the *in vivo* interaction between CTDK-I and Rtr1 could not be confirmed following single and double affinity purification schemes followed by Western blot analysis (see Figures 10 and 11). However, indirect evidence of a functional relationship between CTDK-I and Rtr1 was obtained while studying the interactions between Rtr1, RNAPII subunits, and transcription factors in WT and *ctk1Δ* yeast. Interactions between RNAPII subunits and relevant transcription factors were significantly reduced in *ctk1Δ* (Figure 12), which indicates that CTDK-I regulates Rtr1 function in some way. Likewise, the negative effect of *ctk1Δ* in *S. cerevisiae* on the presence of phosphorylated Rtr1 suggests that Rtr1 may be a substrate for CTDK-I kinase activity (Figure 13). Additional experiments are required to verify these studies, which could involve studying the *in vitro* interaction between purified Rtr1 and CTDK-I subunits (Ctk1 and Ctk2) as well as demonstrating that Rtr1 is a substrate for phosphorylation by the catalytic subunit Ctk1 (e.g. at Rtr1 Ser_{217}).

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CURRICULUM VITAE

Michael J. Berna Sr.

EDUCATION

Indiana University, Indianapolis, IN

- M.S. in Biochemistry and Molecular Biology (2012)
  (Thesis: Mechanisms of recruitment of the CTD phosphatase Rtr1 to RNA Polymerase II)

- Graduate Certificate in Biotechnology (2010)

Eastern Michigan University, Ypsilanti, MI

- B.S. in Biochemistry (1990)

PROFESSIONAL EXPERIENCE

Eli Lilly and Company, Dept. of Drug Disposition, Indianapolis, IN

Key Accomplishments:

- **Protein Mass Spectrometry.** Designed a qualitative/quantitative strategy for simultaneously measuring the *in vivo* PK/PD and proteolytic stability of multifunctional antibodies and their ligands. This strategy relies on the use of new high resolution/accurate mass orbitrap mass spectrometers.
- **Biomarkers.** Developed and implemented a mass spectrometry (MS)-based biomarker facility. Established and published a strong record of implementing immunoaffinity techniques and mass spectrometry to support low abundance biomarker verification, especially in the area of drug-induced toxicity.
- **Innovation.** Established the use of microwave-assisted proteolysis and immunoprecipitation in ELISA format to support biologic drug development. These techniques, in combination with nanoUPLC have reduced the gap between immunoassays and mass spectrometric assay sensitivity while dramatically improving analyte specificity.
- **Supervision, Mentoring, Collaboration.** Proven record of supervising and training post-doctoral fellows, associate and technician scientists in small/large molecule quantitation, structural characterization, and PK/PD assessment. Developed strong sourcing relationships with external contract organizations to support rules-based activities.

Promotion History:

- Research Scientist (2009-present), Associate Senior Biochemist (2004), Assistant Senior Bioanalytical Chemist (2000), Bioanalytical Chemist (1997), Associate Bioanalytical Chemist (1994)

Sterling Winthrop Inc., Dept. of Drug Metabolism/PK, Collegeville, PA (presently Sanofi-Aventis)

Key Accomplishments:

- **Innovation.** Co-designed and published an automated system to develop 2D-HPLC assays to support in PK/PD assessment and drug discovery.

Promotion History:

- Research Scientist (1993-94), Associate Research Scientist (1992)

Prior Experience

- Clinical/Forensic Toxicologist, South Bend Medical Foundation, South Bend, IN (1991-92)
- Associate Research Scientist, Gelman Sciences, Inc., Ann Arbor, MI (1988-91)

AFFILIATIONS

- American Society for Mass Spectrometry (ASMS)
- Golden Key International Honour Society

HONORS AND AWARDS

- Quality Advocate Award, Eli Lilly and Company (2004, 2003, 1999)
- John L. Emmerson Recognition Award, Eli Lilly and Company (2002)
- President's Recognition Award, Eli Lilly and Company (1997)

SCIENTIFIC REFEREE

- Journal of Molecular & Cellular Proteomics
- Journal of Chromatography (A & B)
- Analytica Chimica Acta
- Analytical Chemistry
- Rapid Communications in Mass Spectrometry

PATENTS

- Well Plate Seal, U.S. Patent D464,734

BOOK CHAPTERS

- B. L. Ackermann, **M. J. Berna**, J. A. Eckstein, L. W. Ott, A. K. Chaudhary, *Annual Reviews in Analytical Chemistry*, Volume 1, Annual Reviews, 2008.

- B. L. Ackermann, **M. J. Berna**, and A. T. Murphy, Advances in High-Throughput Quantitative Drug Discovery Bioanalysis, *Integrated Strategies for Drug Discovery Using Mass Spectrometry*, John Wiley & Sons, June 2005.

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- **M. J. Berna**, B. L. Ackermann, and A. T. Murphy, High-Throughput Chromatographic Approaches to LC/MS/MS Bioanalysis to Support Drug Discovery and Development, *Analytica Chim Acta* 2004; 509(1): 1-9.
- B. L. Ackermann, A. T. Murphy and **M. J. Berna**, The Resurgence of Column Switching Techniques to Facilitate Rapid LC/MS/MS Based Bioanalysis in Drug Discovery, *American Pharmaceutical Review* 2002; 5(1): 54-63.
- B. L. Ackermann, **M. J. Berna**, A. T. Murphy, Recent Advances in the Use of LC/MS/MS for Quantitative High-Throughput Bioanalytical Support of Drug Discovery, *Current Topics in Medicinal Chemistry* 2002; 2: 53-66.

PUBLICATIONS

- S. K. Engle, P. F. Solter, K. M. Credille, C. M. Bull, S. Adams, **M. J. Berna**, A. E. Schultze, R. C. Rothstein, M. D. Cockman, M. L. Pritt, H. Liu, Y. Lu, A. Y. Chiang, D. E. Watson, Detection of Left Ventricular Hypertrophy in Rats Administered a Peroxisome Proliferator-Activated Receptor {alpha}/{gamma} Dual Agonist using Natriuretic Peptides and Imaging, *Toxicol Sci* 2010; 114(2): 183-92.
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- Y. Zhen, **M. J. Berna**, M. L. Pritt, Z. Jin, D. E. Watson, B. L. Ackermann, J. E. Hale, Quantification of Heart Fatty Acid Binding Protein as a Biomarker for Drug-Induced Cardiac and Musculoskeletal Necroses, *Proteomics – Clinical Applications* 2007; 1(7): 661-671.
- **M. J. Berna**, Y. Zhen, D. E. Watson, J. E. Hale, B. L. Ackermann, Strategic Use of Immunoprecipitation and LC/MS/MS for Trace-Level Protein Quantitation: Myosin Light Chain 1, a Biomarker of Cardiac Necrosis, *Anal Chem* 2007; 79(11): 4199-205.
- **M. J. Berna** and B. L. Ackermann, Quantitation of Serine Enantiomers in Rat Brain Microdialysate Using Marfey's Reagent and LC/MS/MS, *J Chromatogr B* 2007; 846: 359-363.

- T. Oe, B. L. Ackermann, K. Inoue, **M. J. Berna**, C. O. Garner, V. Gelfanova, R. A. Dean, E. R. Siemers, D. M. Holtzman, M. R. Farlow, I. A. Blair, Quantitative Analysis of Amyloid beta Peptides in Cerebrospinal Fluid of Alzheimer's Disease Patients by Immunoaffinity Purification and Stable Isotope Dilution Liquid-Chromatography-Negative Electrospray Ionization/Tandem Mass Spectrometry, *Rapid Commun Mass Spectrom* 2006; 20(24): 3723-35.
- **M. J. Berna**, C. Schmalz, K. Duffin, P. Mitchell, M. G. Chambers, B. L. Ackermann, Online Immunoaffinity LC/MS/MS Determination of a Type II Collagen Peptide Biomarker in Rat Urine: Investigation of the Impact of CID Fluctuation on Peptide Quantitation, *Anal Biochem* 2006; 356(2): 235-43.
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- **M. J. Berna**, A. T. Murphy, B. Wilken, and B. L. Ackermann, Collection, Storage and Filtration of In-vivo Study Samples Using 96-Well Filter Plates to Facilitate Automated Sample Preparation and LC/MS/MS Analysis, *Anal Chem* 2002; 74(5): 1197-201.
- **M. J. Berna**, B. L. Ackermann, K. Ruterbories, S. Glass, Determination of Olanzapine in Human Blood by Liquid Chromatography-Tandem Mass Spectrometry, *J. Chromatogr B Biomed Sci Appl* 2002; 767(1): 163-8.
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- B. L. Ackermann and **M. J. Berna**, The use of Diagnostic Markers in Translational Medicine: expediting protein biomarker verification using LC/MS/MS, Land O' Lakes Conference on Drug Metabolism/Applied Pharmacokinetics, Merrimac, WI, September 8-12 (2008).
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- B. L. Ackermann and **M. J. Berna**, Evaluation of 96-Well Filter Plate Technology for Collecting, Storing, and Manipulating Plasma Samples for Drug Discovery Bioanalysis; North American Bioanalytical Forum (NABF), Kansas City, MO, September 29-October 2 (2002).
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- T. Oe, B. L. Ackermann, **M. J. Berna**, C. O. Garner, K. Inoue, I. A. Blair, Using LC-ESI-MS/MS with Stable Isotope Dilution to Track the Recovery of Amyloid-beta Peptides During CSF Collection and Processing, The 55th ASMS Conference on Mass Spectrometry, Indianapolis, IN, June 3-7 (2007).
- E. Zhen, **M. J. Berna**, Z. Jin, D. E. Watson, B. L. Ackermann, J. Hale, Development of a Mass Spectrometry-Based Multiplex Assay for Cardiac Biomarkers, The 55th ASMS Conference on Mass Spectrometry, Indianapolis, IN, June 3-7 (2007).
- P. G. Mitchell, M. G. Chambers, M. A. Carozza, C. Lin, H. W. Cole, J. L. Oskins, M. Schreiweis, N. H. Kulkarni, **M. J. Berna**, C. A. Schmalz, B. L. Ackermann, K. L. Duffin, J. E. Hale, J. E. Onyia, Pharmacodynamic Modulation of Bone-Derived and Cartilage-Derived Biomarkers in the Ovariectomized Rat Model, Osteoarthritis Research Society International Congress, Prague, Czech Republic, December 7-10 (2006).
- Y. Zhen, **M. J. Berna**, Z. Jin, D. E. Watson, B. L. Ackermann, J. E. Hale, Quantification of Heart Fatty Acid Binding Protein as a Biomarker for Drug-Induced Cardiac and Musculoskeletal Necroses, The 54th ASMS Conference on Mass Spectrometry, Seattle, WA, May 29-June 1 (2006).
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- T. Gillespie, R. Barbuch, **M. J. Berna**, R. Bowsher, C. Jensen, LC/MS/MS Identification and Profiling of Pergolide Mesylate Metabolites in Human, Rat, and Dog Liver Slices, The 46th ASMS Conference on Mass Spectrometry and Allied Topics, Orlando, FL (1998).