CHARACTERIZATION OF PROTEIN-PROTEIN INTERACTION DOMAINS WITHIN THE *S. CEREVISIAE* NUA3 HISTONE ACETYLTRANSFERASE COMPLEX

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Abstract

Eukaryotic DNA is coiled around octamers of core histone proteins (H2A, H2B, H3, and H4), forming nucleosomes- the basic unit of chromatin. Nucleosome organization is coordinated in part by chromatin-associated protein complexes that modulate histone post-translational modifications (PTMs). NuA3, a conserved *S. cerevisiae* histone acetyltransferase (HAT) complex, engages trimethylation on lysine 4 of histone H3 (H3K4me3) through the plant homeodomain (PHD) finger in the Yng1 subunit. Subsequently, NuA3 acetylates H3K14 through the HAT domain of Sas3, leading to transcription initiation at NuA3-regulated genes. In addition to Yng1, NuA3 contains other potential protein-protein interaction domains including the proline-tryptophan-tryptophan-proline (PWWP) domain protein, Pdp3, and the extra-terminal (ET) domain protein, Taf14, which have undefined functions.

Here we performed co-immunoprecipitation combined with mass-spectrometry, biochemical binding assays, and genetic analyses to classify the NuA3 HAT complex into two functionally distinct forms: NuA3a and NuA3b. While NuA3a uses the PHD finger of Yng1 to bind H3K4me3 at the 5'-ends of open reading frames (ORFs), NuA3b uses the PWWP domain of the unique subunit, Pdp3, to bind H3K36me3 at gene bodies. We find that deletion of *PDP3* decreases NuA3-regulated transcription and results in growth defects when combined with transcription elongation mutants, suggesting NuA3b acts as a positive elongation

factor. Therefore, we ascribe a new function to H3K36me3 in yeast- in addition to repressing intergenic transcription, H3K36me3 also positively regulates transcription elongation via HAT complex recruitment into ORFs.

Additionally, we performed mass-spectrometry and biochemical binding assays to further characterize ET domains as mediators of protein-protein interactions. Specifically, the ET domain of Taf14 engages residues 105-125 of Yng1 and this interaction is critical for NuA3-directed HAT activity. Interestingly, the *H. sapiens* ET domain proteins AF9 and BRD4 also engage residues 105-125 of Yng1. Therefore, this *S. cerevisiae* Yng1 sequence may contain a conserved ET domain recognition motif and provide a novel mechanism to inhibit pathological ET domain interactions.

Dissertation Referees

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Chapter 1: General Introduction

1.1 Epigenetics and Chromatin

Although each cell in the human body contains the same DNA, different cell types express unique gene signatures that can be passed on through cell division[1]. This phenomenon, termed epigenetics, is defined as "the study of heritable changes that occur without a change in the DNA sequence"[2-4]. Epigenetic mechanisms are propagated in part through DNA methylation, modification of histone proteins, and non-coding RNAs, which collectively modulate gene expression[2,3,5,6]. Disruption of these mechanisms leads to aberrant gene expression patterns and epigenetic diseases including cancer and mental retardation syndromes[7,8]. Because of the high prevalence and poor prognosis of such diseases, understanding epigenetic pathways at a molecular level is critical for therapeutic development.

In eukaryotic cells, DNA is condensed within the nucleus in the form of chromatin. Chromatin is comprised of repetitive segments of DNA, histones, and non-histone proteins[9,10]. The basic unit of chromatin is the nucleosome, which contains approximately 146 base pairs of DNA coiled around octamers of conserved core histone proteins H2A, H2B, H3, and H4[11,12] **(Figure 1A)**. Nucleosomes are assembled with linker histone H1 into dynamic higher-order chromatin structures, which act as barriers to multiple cellular processes including DNA replication, DNA repair, and transcription[13,14,15,16]. Therefore, nucleosome organization provides a

Figure 1

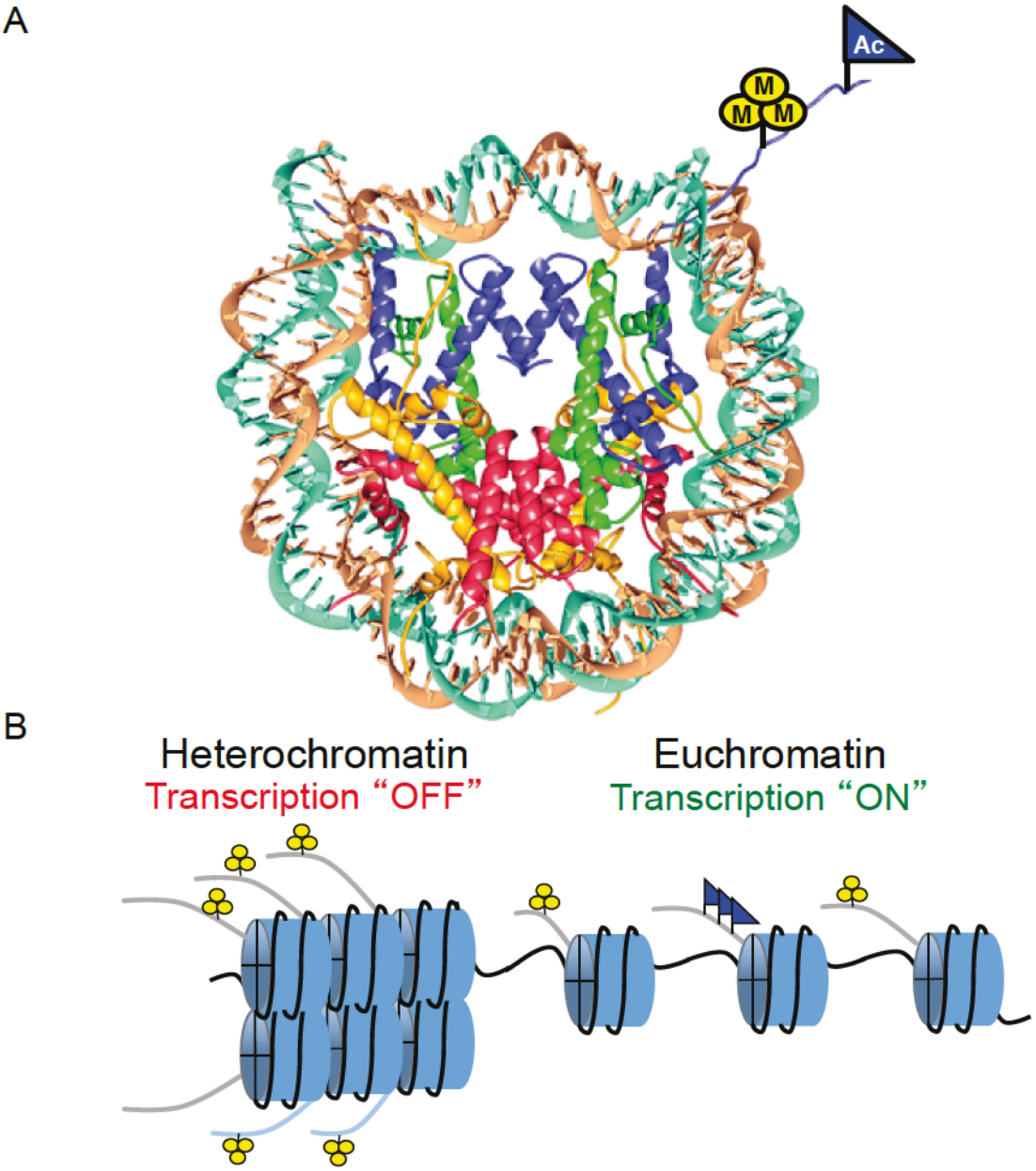


Figure 1 Nucleosome organization regulates transcription. A.) The basic unit of chromatin is the nucleosome, which is comprised of ~146bp of DNA coiled around an octamer of conserved core histone proteins H2A, H2B, H3, and H4 (PDB: 1AOI)[12]. Histone tails can be modified by a variety of chemical groups including acetylation and methylation[21,22]. B.) Nucleosomes are organized into compact heterochromatic regions, which are largely transcriptionally silent, and loose euchromatic regions, where the majority of transcription occurs[13,18]. Figure adapted from Dr. Romeo Papazyan.

mechanism of regulation for these processes[17]. For example, in euchromatic domains nucleosomes are loosely spaced and DNA is accessible to the cellular machinery, allowing for active gene transcription[13,18] **(Figure 1B)**. Conversely, in heterochromatic domains nucleosomes are tightly spaced and gene transcription is largely repressed[13,18] **(Figure 1B)**. Nucleosome organization is maintained by chromatin remodelers, histone chaperones, and other protein complexes that enzymatically modify histones[19,20].

1.2 Histone Post-Translational Modifications

Histones are basic globular proteins that contain flexible NH_2-terminal tails, which protrude away from the nucleosome core[12] **(Figure 1A)**. These tails are highly conserved across species and covalently modified by hundreds of diverse chemical groups including methylation, acetylation, phosphorylation, and ubiquitination[21,22] **(Figures 1A, 2A)**. Importantly, histone post-translational modifications (PTMs) exist in dynamic combinations that are discretely positioned in the genome, and thus histone PTMs provide a mechanism for epigenetic plasticity[21-23] **(Figures 2A, 2B)**. Furthermore, enzymatic complexes add ("write"), remove ("erase"), and bind ("read") histone PTMs in a context dependent manner, allowing their functional outputs to be tightly regulated[13,17,19,21] **(Figures 3A, 3B)**.

Histone PTMs function by directly altering chromatin structure and recruiting non-histone effector proteins[21]. For example, modifications that neutralize positive charge, such as acetylation, or add negative charge, such as phosphorylation, disrupt the electrostatic interactions between positively charged histones and

Figure 2

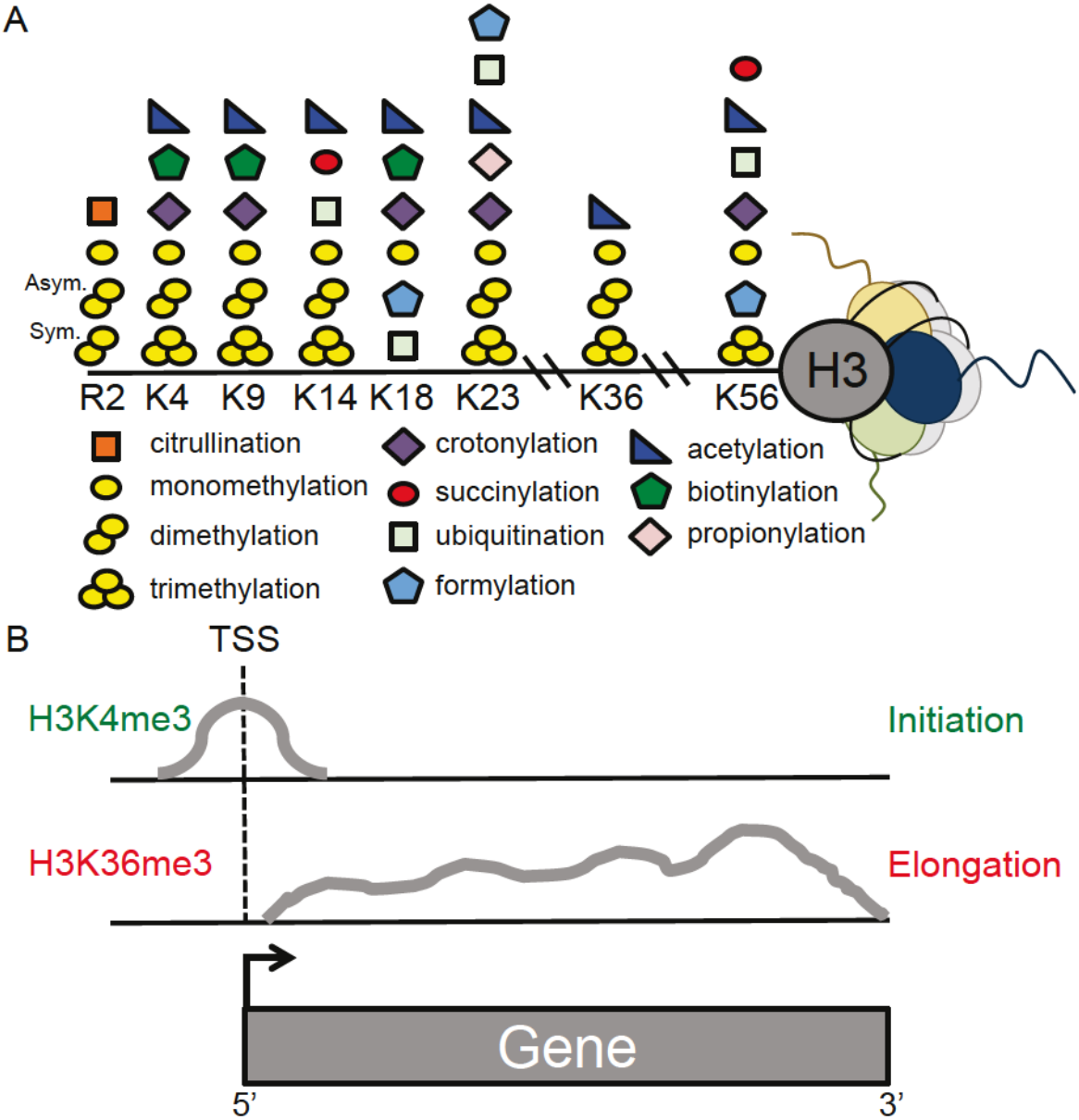


Figure 2 Histone PTMs exist in dynamic combinations that occupy discrete regions of the genome. A.) A subset of PTMs known to exist on histone H3[22]. The "histone code" hypothesis predicts that combinatorial histone PTMs act as binding sites for effector proteins that transduce downstream cellular activities[13,17,19]. Adapted from Xu, Du, and Lau[22]. B.) Histone PTMs have distinct localization patterns[31-33,52,53,268]. H3K4me3 is enriched at the 5'-ends of actively transcribed genes and is associated with transcription initiation[31-33,52,53,268]. H3K36me3 is enriched throughout the open reading frames of actively transcribed genes and is associated with transcription elongation[217,222,268]. Adapted from Kimura[268].

Figure 3

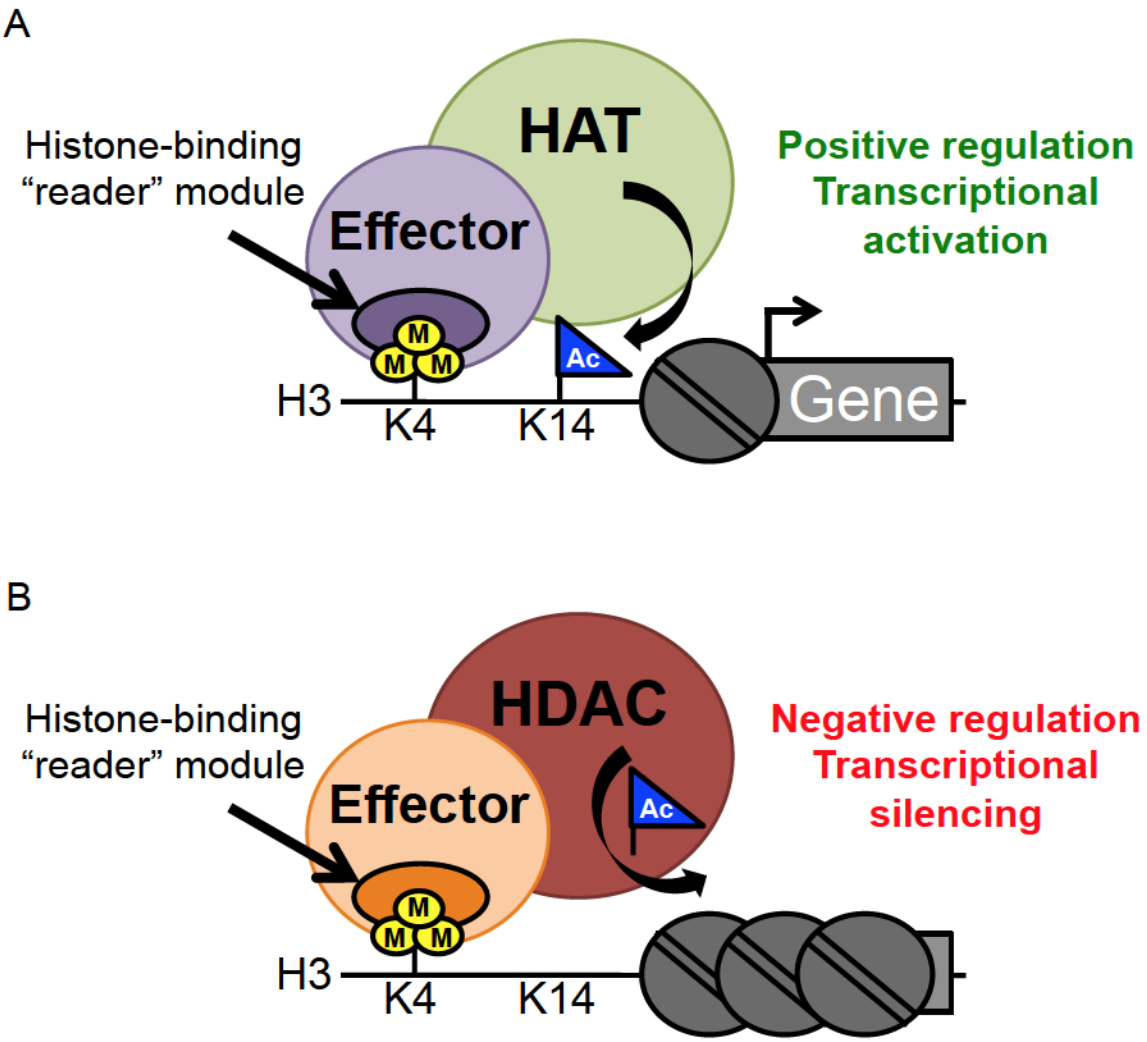


Figure 3 Enzymatic complexes bind, add, and remove histone PTMs in a context dependent manner to regulate downstream cellular functions. A/B.) Histone PTMs are recognized by specific histone-binding "reader" modules found within effector proteins[49]. Effector proteins recruit enzymatic proteins such as HATs and HDACs to discrete regions of the genome[5,17,19,21,49]. HATs acetylate chromatin to facilitate histone eviction and transcriptional activation[13,24,30,31,38-40]. HDACs deacetylate chromatin to facilitate nucleosome condensation and transcriptional silencing[37].

negatively charged DNA[24-28]. Additionally, the "histone code" hypothesis predicts that combinatorial histone PTMs act as binding, stabilization, and occlusion sites for effector proteins, which can transduce downstream activity through associated enzymatic complexes[13,17,19] **(Figures 3A, 3B)**. As site-specific acetylation and methylation are "written" and "read", respectively, by histone acetyltransferase (HAT) complexes to regulate gene expression, these modifications will be explained below in depth.

1.2A Lysine Acetylation

Lysine acetylation is associated with euchromatin and transcriptional activation[13,18,24,29,30]. It is globally enriched at the promoter regions and 5'-ends of open reading frames (ORFs)[31-33]. In this context, acetylation ablates electrostatic histone-DNA interactions and recruits effector proteins that contain acetyl-binding bromodomains. Through these mechanisms, acetylation opens chromatin so that genes remain accessible to the transcriptional machinery[25-27,30,34-36]. Acetylation is tightly regulated by the opposing enzymatic activities of HATs and histone deacetylases (HDACs)[37,38] **(Figures 3A, 3B)**.

Interestingly, the placement of acetylation at specific lysine residues can achieve diverse cellular functions. Type A nuclear HATs, such as Gcn5, acetylate nucleosomes at multiple positions, including lysine 14 of histone H3 (H3K14ac)[38-42]. Chromatin remodelers, such as RSC, bind H3K14ac and link acetylation to gene expression[43,44]. Type B cytoplasmic HATs, such as HAT1, acetylate H4K5 and H4K8[41,45,46]. The histone chaperone, CAF1, binds H4K5ac/H4K8ac and links acetylation to histone deposition[47,48]. Site-specific acetylation is further implicated in

multiple cellular processes including DNA repair, DNA replication, and chromosome condensation[21].

1.2B Lysine and Arginine Methylation

Unlike acetylation, methylation does not affect histone charge. Therefore, methylation functions primarily through the recruitment of effector proteins that contain methyl-binding "reader" domains[5,21,49]. Methylation is regulated by the opposing enzymatic activities of histone methyltransferases (HMTs) and histone demethylases (HDMs)[50,51]. Lysine and arginine methylation are associated with both gene activation and silencing, depending on the position and state (mono, di, tri) of methylation[5,21,52]. For example, the yeast Trithorax group HMT, Set1, trimethylates H3K4 at the 5'-ends of actively transcribed genes[31-33,52-54]. HATs, such as NuA3, and chromatin remodelers, such as NURF, bind H3K4me3 to promote transcription initiation[5,55,56]. Conversely, the mammalian Polycomb group HMT, EZH2, trimethylates H3K27 at the 5'-ends of transcriptionally silent genes[52,57]. The co-repressor complex, PRC1, binds H3K27me3 to repress transcription initiation[58]. Interestingly, H3K4me3 and H3K27me3 can be found together (on the same or nearby nucleosomes) in "bivalent domains" at the promoters and enhancers of developmentally regulated genes[59]. Here, these modifications may "poise" such genes for rapid activation or repression in response to developmental cues[59].

Similar to acetylation, the placement of methylation at specific lysine residues allows for diverse cellular functions. For example, the yeast HMT, Set2, methylates H3K36 throughout ORFs of actively transcribed genes[60-62]. Histone chaperones, such as Asf1, chromatin remodelers, such as Isw1b, and HATs, such as NuA3, bind

H3K36me3 to coordinate transcription elongation (as shown in **Chapters 2 and 3**)[63-66]. Interestingly, depending on which effector protein is bound, H3K36me3 is associated with both positive and negative regulation of the elongation pathway[63-66]. Lysine methylation is also implicated in other cellular processes such as DNA repair. For example, the yeast HMT, Set9, methylates H4K20 at DNA damage sites[67]. The cell-cycle checkpoint protein, Crb2, binds H4K20me to coordinate DNA repair[67-69]. Accordingly, a diverse family of methyl-binding domains have evolved to "read" histone methylation (see below)[49].

1.3 Histone Methyl-Binding "Reader" Domains

Histone PTMs are recognized by a variety of effector proteins that contain histone-binding "reader" domains[13,17,19,49,70,71]. Importantly, "reader" domains recognize both the chemical modification and surrounding histone sequence, allowing effector proteins to distinguish between closely related modifications[49,71]. Effector proteins often form chromatin-associated complexes, which propagate cellular functions through enzymatic or allosteric activities[71-73] **(Figures 3A, 3B)**. Of note, these complexes can be composed of multiple effector proteins, with multiple histone-binding domains[73,74]. Therefore, a combinatorial "histone code" may fine-tune complex localization and activity[13,17,19,73,74].

In general, methylation is bound by an aromatic amino acid "cage" that forms cation-π interactions between the methyl-ammonium group and aromatic residues[75,76]. To distinguish between methylated histone modifications, methyl-binding domains vary widely in both structure and recognition motif[49,70]. For

example, as methylation state increases (me1, me2, me3), hydrophobicity increases, and therefore each methyl state requires a "reader" with matched hydrophobicity[49]. Additionally, methyl-binding pockets have multiple sites of contact with the histone tail that determine specificity[49,71].

1.3A PHD Finger

The plant homeodomain (PHD) finger is defined structurally by a Cys_4-His-Cys_3 motif, three loops stabilized by two Zn^{2+} molecules, and a two-stranded antiparallel β-sheet[77]. PHD fingers are found in many chromatin-associated complexes, including HATs and HDACs, and therefore are associated with both gene activation and repression[55,56,78-83]. Examples of histone methylation marks engaged by PHD fingers include H3K4me2/3, H3K9me2/3, and unmodified histone H3, which bind with low micromolar affinities[55,56,74,80-83].

In addition to an aromatic "cage", PHD fingers achieve specificity through multiple contact points with the histone tail[49]. In general, the histone tail forms a third antiparallel β-sheet with the PHD finger and the spacing of histone residues is critical for recognition[49,55,56,80-83]. For example, the *S. cerevisiae* Yng1 PHD finger contains two binding pockets to target H3K4me3[55] **(Figure 4A)**. The first (Y157, S164, M168, and W180) acts as a "cage" to engage the trimethyl modification through cation-π interactions and the second (D172, E179, and W180) interacts with H3R2 through hydrogen bonding[55] **(Figure 4A)**. The molecular spacing R-X_1-K distinguishes H3K4me3 from H3K9me3[55,80,81]. Through this mechanism, Yng1 binds H3K4me3 with a K_d of 9.1μM[55].

Figure 4

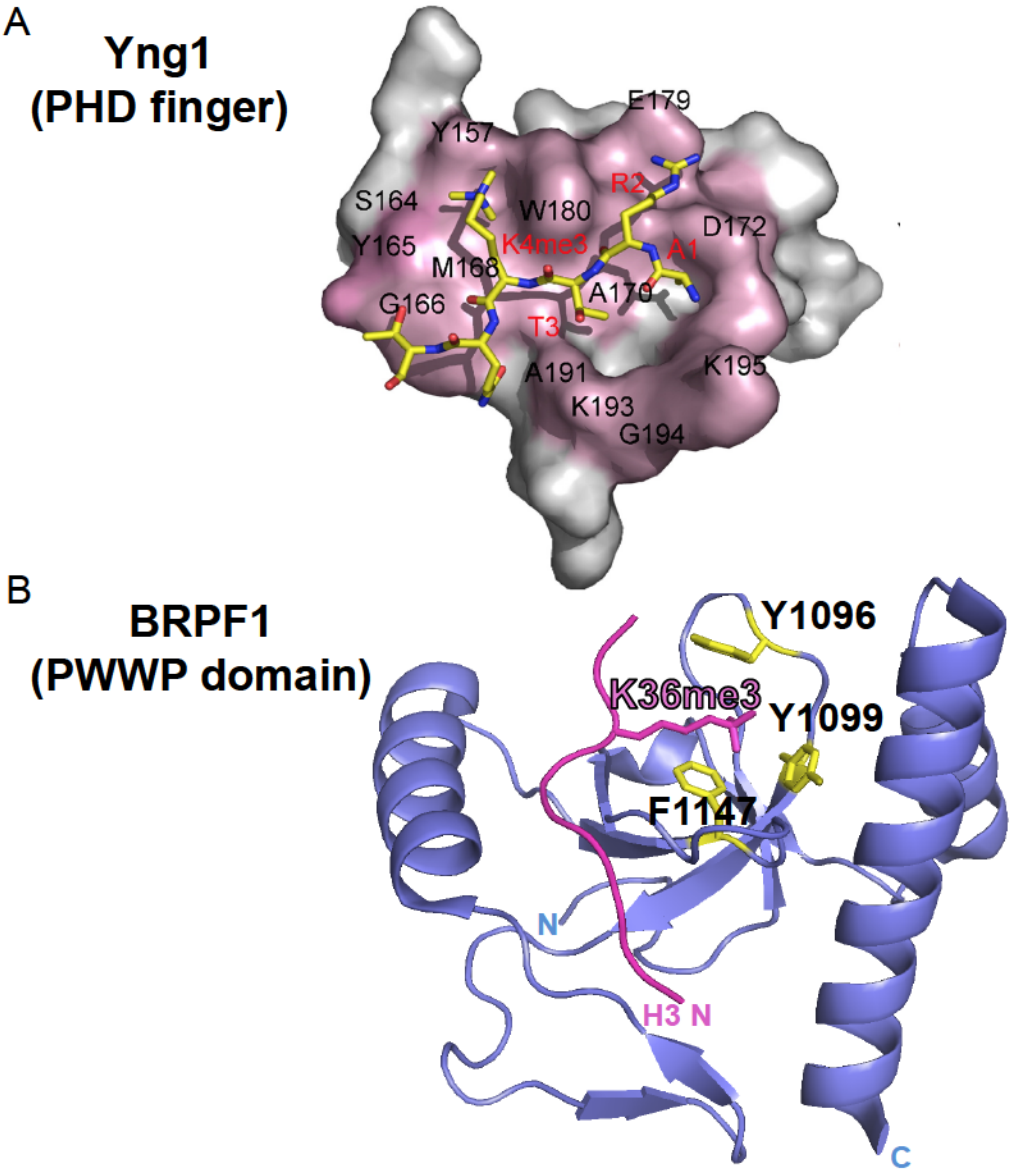


Figure 4 Histone methyl-binding domains interact with distinct histone tail residues to achieve specificity. A.) NMR structure of the *S. cerevisiae* Yng1 PHD finger bound to H3K4me3 peptide (PDB: 2JMI)[55]. An aromatic "cage" (Y157, S164, M168, and W180) engages the trimethyl residue through cation-π interactions[55]. A second groove (D172, E179, and W180) interacts with H3R2 through hydrogen bonding[55]. B.) Crystal structure of the *H. sapiens* BRPF1 PWWP domain bound to H3K36me3 peptide (PDB: 2X4W)[92]. The aromatic "cage" (Y1096, Y1099 and F1147) engages the trimethyl residue through cation-π interactions[92]. Hydrogen bonding and hydrophobic interactions with H3G33, H3G34, H3V35, and H3K36 further promote specificity[92].

1.3B PWWP Domain

The proline-tryptophan-tryptophan-proline (PWWP) domain belongs to the Tudor "royal" family[70,84-86]. PWWP domains are structurally defined by a five-stranded β-barrel core, an insertion motif between the second and third β-strands, and a variable C-terminal α-helix bundle[86,87]. Like PHD fingers, PWWP domains are found in many chromatin-associated complexes[73,74,86-89]. They are associated with diverse cellular functions including DNA methylation maintenance, DNA repair, transcriptional regulation, HIV infection, and alternative splicing[88,90-93]. To date, PWWP domains are known to bind H3K36me3, H3K79me3, and H4K20me1/3 with K_d's ranging from 56μM to low millimolar affinities[66,86,91-103]. In certain cases, affinities are greatly increased for nucleosomal substrates, suggesting PWWP domains synergistically bind methylated histones and DNA[87,100,102].

PWWP domains achieve histone-binding specificity in part from an aromatic "cage" characteristic of the Tudor "royal" family[70,86]. Furthermore, like PHD fingers, PWWP domains form multiple contact points with the histone tail[86,92]. For example, the *H. sapiens* BRPF1 PWWP domain contains three binding pockets to target H3K36me3[92] **(Figure 4B)**. The first (Y1096, Y1099 and F1147) acts as a "cage" to engage the trimethyl residue through cation-π interactions, the second (R1152 and W1154) interacts with H3G33, H3G34, and H3K36 through hydrogen bonding, and the third (V1127, L1130, and W1156) forms a hydrophobic groove surrounding H3V35[92] **(Figure 4B)**. Of note, the placement of G33 and G34 relative to K36 is unique to histone H3, and contributes greatly to BRPF1 targeting[92]. Through this mechanism, BRPF1 binds H3K36me3 with a relatively poor K_d of 2.7mM[92].

Interestingly, the human ZYMND11 PWWP domain requires histone variant H3.3 (yeast histone H3) to bind lysine 36 trimethylation[103]. Histone H3.3 contains the unique residue, S31, and therefore the placement of S31 relative to K36 also contributes to PWWP domain targeting[103]. Accordingly, BRPF1 may bind H3.3K36me3 with a lower dissociation constant.

1.4 NuA3 Histone Acetyltransferase Complex

The *S. cerevisiae* NuA3 complex was identified in 1998, as a 450kDa nucleosomal histone H3 acetyltransferase[104]. NuA3 is conserved in humans and "reads" and "writes" distinct histone PTMs to coordinate transcriptional activation[55,66,104-108]. Therefore, NuA3 represents a model to study the "histone code" hypothesis in the context of gene expression.

NuA3 stimulates transcription from nucleosome-assembled template *in vitro* and is required for the transcription of a subset of genes *in vivo*[55,109]. The catalytic subunit of NuA3 is the HAT, Sas3[105]. To date, five other complex members have been identified: Taf14, Yng1, Nto1, Eaf6, and Pdp3[55,66,105,106].

1.4A Sas3

Sas3 (ortholog of human MYST3) is essential for NuA3 complex integrity and specifically acetylates H3K14[55,105,110]. Sas3 is bound by the histone chaperone, FACT, and H3K14ac is bound by the chromatin-remodeler, RSC[43,44,105]. Together, these co-activators facilitate NuA3-directed transcription[44]. *SAS3* is synthetically lethal with the HAT, *GCN5*, suggesting NuA3 has overlapping functions with other yeast HAT complexes such as ADA and SAGA[110].

1.4B Yng1

Yng1 (ortholog of human ING5) is not essential for NuA3 complex integrity[106,111]. However, Yng1 and the H3K4me3 HMT, Set1, are required for NuA3 localization and HAT activity *in vivo*[106-108]. Specifically, the PHD finger of Yng1 binds H3K4me3 at the promoter regions and 5'ends of actively transcribed genes, allowing Sas3 to acetylate H3K14[55,107] **(Figure 5A)**. Mutation of the Yng1 PHD finger results in genome-wide mislocalization of NuA3 from promoter regions to ORFs, decreases H3K14ac at NuA3-target genes, and decreases the transcription of these genes[55]. Interestingly, the interaction between NuA3 and chromatin also depends on the N-terminus of Yng1 and the H3K36me3 HMT, Set2, indicating additional histone contacts contribute to NuA3 localization[66,108,112].

Yng1 expression decreases exogenous human p53-driven transcript levels in a yeast reporter assay[113]. Although in general NuA3 facilitates transcriptional activation, a subset of NuA3 proteins occupy heterochromatic barriers and contribute to barrier maintenance[114,115]. Therefore, NuA3 may have undefined gene-specific silencing functions[113-115].

1.4C Pdp3

A non-stable NuA3-associated protein, Pdp3 (Ylr455w), was previously detected using isotopic differentiation of interactions as random or targeted (I-DIRT) mass-spectrometry[55,116]. Pdp3 contains a PWWP domain homologous to BRPF1, a member of the conserved *H. sapiens* MOZ/MORF HAT complex[66,92,111] **(Figure 5B)**. Interestingly, in yeast, the functional domains of BRPF1 (PWWP domain, double

Figure 5

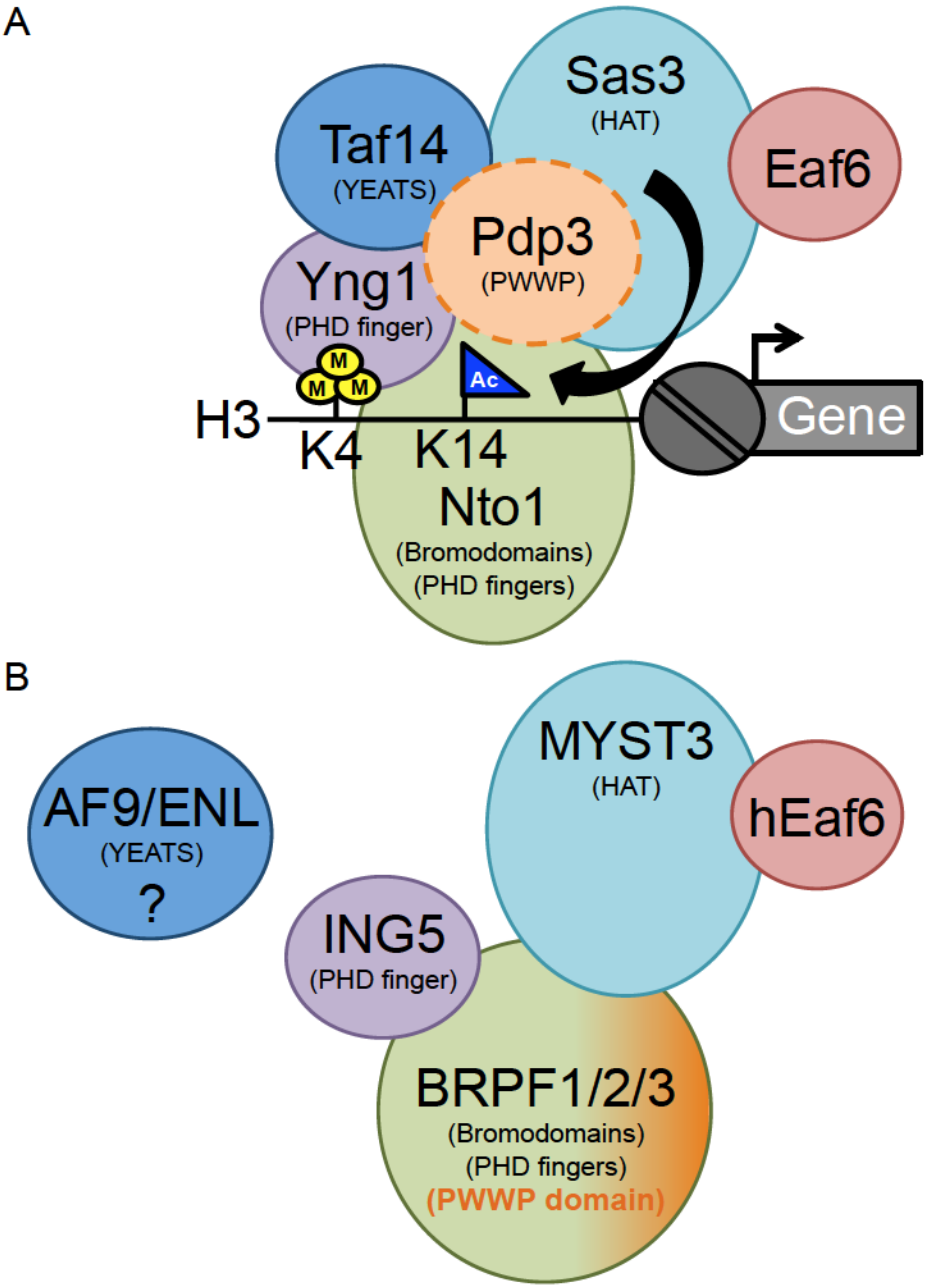


Figure 5 The NuA3 HAT complex initiates transcription and is conserved in humans. A.) Model of the *S. cerevisiae* NuA3 HAT complex[55,66]. The PHD finger of Yng1 binds H3K4me3[55,107,108]. The HAT domain of Sas3 then acetylates H3K14, leading to transcription initiation at a subset of genes[55,104-106]. B.) Model of the *H. sapiens* MOZ/MORF HAT complex[111]. BRPF1 contains an H3K36me3-binding PWWP domain that is absent in Nto1[111]. The BRPF1 PWWP domain is required for MOZ/MORF localization and HOX gene transcription[88,92].

PHD fingers, double bromodomains) are split between Pdp3 (PWWP domain) and Nto1 (double PHD fingers, double bromodomains)[111,117]. Additionally, Pdp3 co-localizes with Sas3 at heterochromatic barriers[115]. Together, these findings suggest that Pdp3 is a novel member of the NuA3 HAT complex. In **Chapters 2 and 3,** I detail our work characterizing the function of Pdp3 within NuA3.

1.4D Taf14

Taf14 (ortholog of human AF9 and ENL) is a YEATS (Yaf9, ENL, AF9, Taf14, Sas5) domain protein associated with many cellular processes including transcription initiation, transcription elongation, cell cycle progression, cytoskeletal-organization, post-replication repair, and chromosome stability[61,105,118-132]. Deletion of *TAF14* is not lethal, but results in sensitivity to UV light, γ-irradiation, and genotoxic agents. Deletion of *TAF14* also causes growth defects, cytoskeletal abnormalities, and decreased human p53 activation[120,128,133-136]. Although Taf14 has an important role in diverse cellular processes, its mechanism of action remains poorly understood[137,138]. Interestingly, AF9 binds H3K9ac through a conserved YEATS domain and recruits the H3K79me3 HMT, Dot1, to active chromatin[139]. Therefore, Taf14 may similarly bind acetylated histones and function as a scaffold for transcriptional regulators.

Taf14 is a member of multiple chromatin-associated complexes, including transcription factors TFIID and TFIIF, chromatin-remodelers Ino80, SWI/SNF, and RSC, and transcriptional co-activators NuA3 and Mediator[104,105,120,122,129,131,140-143]. In general, Taf14 interacts with the catalytic subunit of each complex through its C-terminus and assists in catalytic activity through undefined mechanisms[129,132]. The

C-terminus of Taf14 contains a conserved hydrophobic cluster known as an extra-terminal (ET) domain (also referred to as the Taf14 C-box)[128,144]. ET domains are predicted to generally function as protein-protein interaction modules[144]. In **Chapter 4,** I detail our work characterizing the ET domain of Taf14 within the NuA3 HAT complex and expand these findings to *H. sapiens* ET domain proteins[144].

1.5 NuA3 Human Homologs in Disease

The human homolog of NuA3 is the MOZ/MORF HAT complex[110,111]. MOZ/MORF is composed of the HAT MYST3 (ortholog of Sas3) and the scaffolding subunits BRPF1 (ortholog of Nto1 and Pdp3), ING5 (ortholog of Yng1), and hEAF6 (ortholog of Eaf6)[145,146] **(Figure 5B)**. MYST3, BRPF1, and ING5 contain conserved histone-binding "reader" domains that direct MOZ/MORF HAT activity[88,89,92,147,148]. For example, the PWWP domain of BRPF1 targets MOZ/MORF to H3K36me3-enriched HOX genes, which control hematopoietic stem cell development[92,149,150].

Chromosomal translocations and point mutations within MOZ/MORF result in epigenetic diseases[8,111,151-154]. For example, MOZ fuses to the HAT, p300/CBP, or the transcriptional co-activator, TIF-2, resulting in aberrant acetylation and overexpression of HOX genes[151,155-159]. Overexpression of HOX genes leads to hematopoietic stem cell transformation and leukemogenesis[8,160]. Importantly, MOZ-associated leukemia, such as acute myeloid leukemia (AML), has a very poor prognosis[161]. Over 50,000 people a year die from a lack of effective blood cancer treatments[8,161]. As MOZ fusions assemble into the MOZ HAT complex, inhibition of "reader" domains, such as the BRPF1 PWWP domain, may provide a treatment

mechanism for leukemia[8,88,92,158]. Additionally, null mutations of the tumor suppressor gene *ING5* are associated with squamous cell carcinoma[152-154]. Therefore, further mechanistic insight into MOZ/MORF localization and HAT activity may uncover novel drug targets.

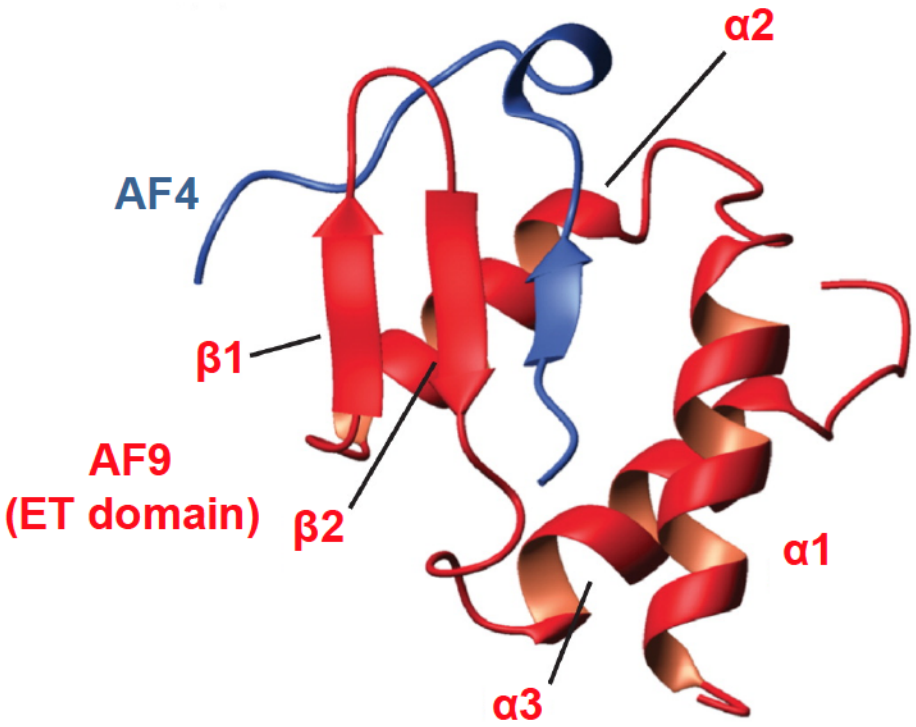
1.6 Taf14 Human Homologs in Disease

Human YEATS domain proteins AF9 and ENL (orthologs of Taf14) represent the two most common translocation partners of the *MLL* oncogene[162,163]. MLL-AF9 and MLL-ENL translocations aberrantly recruit elongation factors to HOX genes, resulting in HOX gene overexpression, hematopoietic stem cell transformation, and leukemogenesis[160,164-170]. Interestingly, MLL fusion products include only the C-terminus, and not the YEATS domain, of AF9 and ENL[138]. These C-termini each contain a conserved ET domain[144]. The ET domains of AF9 and ENL bind to transcriptional regulators including AF4, Dot1L, BCoR, hPC3, and Rnf2 to propagate pathogenesis[166,171-177] **(Figures 6A, 6B)**. As MLL translocations account for approximately 3% of AML and 10% of acute lymphoid leukemia (ALL), ET domains may represent a promising pharmacological target[178-180].

Similarly, the C-terminus of human BRD4 contains an ET domain that is conserved with Taf14[144]. The BRD4 ET domain binds transcriptional regulators including JMJD6, which activates the P-TEFb complex and releases paused RNAPII at coding regions via long-range enhancer interactions[181-184] **(Figure 6B)**. Aberrant RNAPII pause-release through BRD4 results in midline-carcinoma and hematological cancer[185-187]. Although BRD4 bromodomain inhibitors (JQ1, I-BET,

Figure 6

A



B

AF9 ET domain	BRD4 ET domain
AF4	ATAD5
BCoR	CHD4
Dot1L	GLTSCR1
hPC3	JMJD6
Rnf2	LANA-1
	MLV IN
	NSD3

Figure 6 Human ET domains mediate protein-protein interactions. A.) NMR structure of the *H. sapiens* AF9 ET domain bound to AF4 (PDB: 2LMO)[176]. B.) Regulatory proteins (blue) known to interact with the *H. sapiens* AF9 or BRD4 ET domains (red)[169,171-176,183,184]. ET domains are predicted to generally function as protein-protein interaction surfaces and therefore may represent a novel class of drug target[144].

etc.) have striking anti-cancer effects, the BRD4 ET domain remains pharmacologically uncharacterized[186-194].

1.7 Targeting Epigenetic Complexes for Therapeutic Development

Precise localization of chromatin-associated complexes is essential for transcriptional control and normal development[195]. The deregulation of chromatin-associated complexes results in many pathological conditions including cancer, heart disease, inflammation, and neurological disease[195-198]. Therefore, small molecule inhibitors of chromatin-modifying enzymes have therapeutic potential[195,199]. For example, the pan HDAC inhibitor, valproic acid, is used as a mood stabilizer and anticonvulsant, while the Class I/II HDAC inhibitor, Varinostat (SAHA), is used to treat advanced cutaneous T-cell lymphoma[200]. Valproic acid and Varinostat are currently in clinical trials for multiple cancer subtypes and neurological disorders[200]. Additionally, epigenetic imaging agents have diagnostic potential. For example, the Class I HDAC probe, Martinostat, is approved to quantitate HDAC complex distribution in humans[201].

Compounds targeting acetyl-lysine "reader" domains also have therapeutic potential[202]. For example, the bromodomain and extra-terminal (BET) family bromodomain inhibitors, JQ1 and I-BET, selectively target BRD4 with K_d's less than 100nM[188,189]. JQ1 treatment of midline carcinoma mouse xenograft models leads to cellular differentiation and apoptosis[188,191,202,203]. I-BET treatment of septic shock mouse models downregulates the expression of inflammatory genes[189,202]. JQ1 and

I-BET also show preclinical promise for treating multiple forms of leukemia, inflammation, and heart disease[190,191,195,204,205]. Currently, JQ1 and I-BET derivatives are in clinical trials for midline carcinoma, hematological cancer, and solid tumors[187].

In general, targeting “reader” domains of chromatin-associated complexes, as opposed to enzymatic subunits, may simultaneously increase the efficacy and decrease the toxicity of epigenetic drug treatments[202]. Therefore, understanding the basic molecular mechanisms of protein-protein interaction domains within chromatin-associated complexes is increasingly important from a therapeutic standpoint.

Chapter 2: Pdp3 is a Member of the NuA3 HAT Complex that Specifically Interacts with H3K36me3 through a Conserved PWWP Domain

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2.1 Introduction

Chromatin acts as a barrier to the transcriptional machinery, and precise coordination of chromatin structure is required for the passage of RNA polymerase II (RNAPII)[20,206,207]. Chromatin structure is regulated by chromatin remodelers, histone chaperones, and protein complexes that modulate histone post-translational modifications (PTMs)[19,20,49,208]. Together, these factors ensure that DNA remains accessible to the transcriptional machinery and facilitate gene expression[208]. These factors also restore chromatin structure following the passage of RNAPII to prevent aberrant transcription[20,209].

Histone PTMs occupy discrete genomic positions, such as promoter regions *versus* open reading frames (ORFs), and are associated with distinct functional

states, such as active *versus* silent transcription[17,19,31,33,53,210-212] **(Figure 2B)**. For example, histone H3 is often modified by trimethylation on lysine 4 (H3K4me3) and acetylation on lysine 14 (H3K14ac) at the 5'-ends of actively transcribed genes[23,31-33,52]. In *S. cerevisiae*, H3K4me3 is added by the histone methyltransferase (HMT), Set1[54]. NuA3, a conserved histone acetyltransferase (HAT) complex, specifically binds H3K4me3 through the plant homeodomain (PHD) finger in the Yng1 subunit[55,104-108,110,111] **(Figure 5A)**. NuA3 then acetylates H3K14 on the same histone tail through the HAT domain of Sas3[55,105,106] **(Figure 5A)**. Subsequently, regulatory complexes bind either NuA3 subunits directly or NuA3-catalyzed H3K14ac. For example, the histone chaperone, FACT, binds Sas3 through Spt16 and the chromatin-remodeler, RSC, binds H3K14ac through a tandem bromodomain in Rsc4[43,44,105,213-215]. RSC also interacts with NuA3 via Taf14[129,216]. Through such interactions, NuA3 promotes transcription initiation at a subset of genes[44,55].

Other histone PTMs are positioned within the ORFs of actively transcribed genes[20,209,211,217]. In *S. cerevisiae* H3K36 methylation is added by the HMT, Set2[60]. Set2 localizes to ORFs by physically binding the hyperphosphorylated C-terminal domain (CTD) of RNAPII and deposits H3K36me3 within gene bodies[33,60,61,218-221]. Accordingly, H3K36me3 is correlated specifically with the regulation of transcription elongation[61,62,218,219,222]. H3K36me3 facilitates transcriptional fidelity by recruiting complexes that collectively restore chromatin structure[20,209,217]. For example, the histone deacetylase (HDAC) complex, Rpd3S, engages H3K36me2/3 to generate a hypoacetylated environment behind elongating RNAPII[223-229]. Through this mechanism, Rpd3S compacts chromatin and represses intergenic transcription[223-

[229]. H3K36me3 also maintains chromatin structure by blocking *trans*-histone exchange[20,63,64,65]. For example, H3K36me3 precludes the histone chaperone, Asf1, from depositing newly synthesized histones and recruits the ATP-dependent remodeler, Isw1b, to preserve H3K36me3/hypoacetylated histones[63-65,230]. In addition to transcriptional functions, H3K36me3 is also associated with DNA methylation, dosage compensation, and alternative splicing[217].

It was previously determined that mutation of the Yng1 PHD finger results in the mislocalization of NuA3 from promoter regions to ORFs[55]. As H3K36me3 is enriched in ORFs, we speculated that NuA3 can engage this modification. H3K36me3 is recognized by proline-tryptophan-tryptophan-proline (PWWP) domain-containing proteins, such as the Ioc4 subunit of Isw1b[20,63,64]. As Pdp3 (Ylr455w), an uncharacterized PWWP domain-containing protein, co-purifies with NuA3 subunits, we hypothesized that Pdp3 is a member of NuA3 that interacts with H3K36me3[55,70,115,231].

Here, using mass spectrometric, biochemical, and biophysical analyses, we characterize Pdp3 as a novel subunit of the NuA3 HAT complex. Pdp3 specifically binds H3K36me3 *in vitro* and *in vivo* through a conserved PWWP domain. Collectively, our data suggest that while H3K36me3 generally acts as a repressive mark to protect chromatin integrity during transcription elongation, in yeast H3K36me3 may positively regulate transcription by recruiting HAT complexes into ORFs[20,209,217,232].

2.2 Methods

2.2A *S. cerevisiae* Strains

All strains used in **Chapter 2** are described in **Table 1**.

2.2B *E. coli* Plasmids

All constructs used in **Chapter 2** are described in **Table 2**.

2.2C Peptide Sequences

All peptides used in **Chapter 2** are listed in **Table 3**.

2.2D Mass Spectrometric Protein Identification for Cellular Pull Down Assays

Pdp3-TAP protein complex purification was performed with *S. cerevisiae* grown to log phase in YPD, as previously described to maintain complex integrity[115,116]. Proteins co-purifying with Pdp3 were subjected to tandem MS analysis of peptides with a Thermo LTQ-XL mass spectrometer coupled to an Eksigent nanoLC 2D system as previously described[44]. Tandem mass spectra were extracted by Thermo ExtractMSn version 1.0.0.8 and analyzed by Mascot (Matrix Science, London, UK; version 2.3.01). Scaffold (version Scaffold_4.0.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. All MS experiments and analyses were performed by the Alan J. Tackett laboratory at the University of Arkansas for Medical Sciences. I would like to acknowledge Drs. Alan J. Tackett and Stephanie D. Byrum for their many important contributions to this project.

Table 1
***S. cerevisiae* strains used in Chapter 2**

Strain	Genotype	Source
W303	*MATa; ura3-1; trp1Δ 2; leu2-3,112; his3-11,15; ade2-1; can1-100*	A. Tackett Lab
YNG1-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YNG1-TAP::HIS3*	Open Biosystems
PDP3-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PDP3-TAP::HIS3*	Open Biosystems
NTO1-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 NTO1-TAP::HIS3*	Open Biosystems
SAS3-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SAS3-TAP::HIS3*	Open Biosystems
TAF14-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TAF14-TAP::HIS3*	Open Biosystems
EAF6-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 EAF6-TAP::HIS3*	Open Biosystems
YNG1-TAP; *set2*Δ	*MATa leu2Δ0 met15Δ0 ura3Δ0 set2Δ::natMX YNG1-TAP::HIS3*	S. McDaniel, B. Strahl Lab
YNG1-TAP; *set1*Δ	*MATa leu2Δ0 met15Δ0 ura3Δ0 set1Δ::hph YNG1-TAP::HIS3*	S. McDaniel, B. Strahl Lab
YNG1-TAP; *pdp3*Δ	*MATa leu2Δ0 met15Δ0 ura3Δ0 pdp3Δ::hph YNG1-TAP::HIS3*	S. McDaniel, B. Strahl Lab
PDP3-TAP; *set2*Δ	*MATa leu2Δ0 met15Δ0 ura3Δ0 set2Δ::KanMX PDP3-TAP::HIS3*	S. McDaniel, B. Strahl Lab
PDP3-TAP; *set1*Δ	*MATa leu2Δ0 met15Δ0 ura3Δ0 set1Δ::Hygro PDP3-TAP::HIS3*	S. McDaniel, B. Strahl Lab
PDP3-TAP; *yng1*Δ	*MATa leu2Δ0 met15Δ0 ura3Δ0 yng1Δ::Hygro PDP3-TAP::HIS3*	S. McDaniel, B. Strahl Lab

Table 2
***E. coli* plasmids used in Chapter 2**
Source: T. Gilbert, S. Taverna Lab

Name	Description	Vector
PDP3 FL	Pdp3 aa 1-304	pET28 HisMBPFLAG
PWWP(1-74)	Pdp3 aa 1-74	pET28 HisMBPFLAG
PWWP(1-110)	Pdp3 aa 1-110	pET28 HisMBPFLAG
PWWP(2-150)	Pdp3 aa 2-150	pET28 HisMBPFLAG
PWWP(1-219)	Pdp3 aa 1-219	pET28 HisMBPFLAG
C-term(74-304)	Pdp3 aa 74-304	pET28 HisMBPFLAG
PDP3 F18A FL	Pdp3 aa 1-304	pET28 HisMBPFLAG
PDP3 W21A FL	Pdp3 aa 1-304	pET28 HisMBPFLAG
PDP3 F48A FL	Pdp3 aa 1-304	pET28 HisMBPFLAG
PDP3 FL	Pdp3 aa 1-304	pET32a
PDP3 F18A FL	Pdp3 aa 1-304	pET32a
PDP3 W21A FL	Pdp3 aa 1-304	pET32a

Table 3

Peptides used in Chapter 2

Sources: 5-FAM labeled peptides- Dr. B. Dancy, S. Taverna Lab; Biotin labeled peptides- C.D. Allis Lab and B. Strahl Lab

Tag	Name	Sequence
5-FAM	$H3_{(1-15)}$K4me3	H2N-ARTK(me3)QTARKSTGGKA(KFluorescein)-NH2
5-FAM	$H3_{(29-43)}$K36me0	H2N-APSTGGVKKPHRYKP(KFluorescein)-NH2
5-FAM	$H3_{(29-43)}$K36me1	H2N-APSTGGVK(me)KPHRYKP(KFluorescein)-NH2
5-FAM	$H3_{(29-43)}$K36me2	H2N-APSTGGVK(me2)KPHRYKP(KFluorescein)-NH2
5-FAM	$H3_{(29-43)}$K36me3	H2N-APSTGGVK(me3)KPHRYKP(KFluorescein)-NH2
5-FAM	$H3_{(70-88)}$K79me3	H2N-LVREIAQDFK(me3)TDLRFQSSA(KFluorescein)-NH2
5-FAM	$H4_{(11-29)}$K20me3	H2N-GKGGAKRHRK(me3)ILRDNIQGI(KFluorescein)-NH2
Biotin	$H3_{(1-20)}$Kme0	H2N-ARTKQTARKSTGGKAPRKQL(KBiot)-NH2
Biotin	$H3_{(1-20)}$K4me3	H2N-ARTK(me3)QTARKSTGGKAPRKQL(KBiot)-NH2
Biotin	$H3_{(1-20)}$K9me3	H2N-ARTKQTARK(me3)STGGKAPRKQL(KBiot)-NH2
Biotin	$H3_{(19-35)}$K27me3	H2N-QLATKAARK(me3)SAPATGGV(KBiot)-NH2
Biotin	$H3_{(15-43)}$Kme0	Ac-APRKQLATKAARKSAPSTGGVKKPHRYGG(KBiot)-NH2
Biotin	$H3_{(15-43)}$K36me3	Ac-APRKQLATKAARKSAPSTGGVK(me3)KPHRYGG(KBiot)-NH2
Biotin	$H4_{(11-27)}$Kme0	Ac-GKGGAKRHRKVLRDNIQ(Peg-Biot)
Biotin	$H4_{(12-27)}$K20me3	H2N-KGGAKRHR K(me3)VLRDNIQ(KBiot)-NH2

2.2E Protein Expression

PDP3 constructs were made with an N-terminal HIS6X-pfuMBP$_{(60-434)}$-FLAG tag (pET28a derivative vector obtained from the Greg Bowman Laboratory, Johns Hopkins University) and/or an N-terminal Thioredoxin-HIS6X-S•tag tag (pET32a vector, Millipore). Proteins were exogenously expressed in chemically competent BL21 *E. coli* (Invitrogen) after overnight induction with 1 mM IPTG at 18-20 ºC in LB medium. Point mutants were made with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and expressed as described.

2.2F Protein Purification

For pull down assays, BL21 cells (pET28a constructs) were resuspended in purification buffer (50 mM Tris pH 7.5, 500 mM NaCl, 40 mM imidazole, 10% glycerol, 2 mM β-ME, 1 mM PMSF, 2 mM benzamidine, pH 8.0) and lysed by sonication (Branson). Clarified lysate was nutated with Ni-NTA agarose resin (Invitrogen) for at least 1 hr at 4 °C. Resin was washed with purification buffer and protein was eluted with purification buffer containing 300 mM imidazole. Protein was frozen in liquid nitrogen and stored at −80 °C. For fluorescence polarization assays, BL21 cells (pET32a constructs) were resuspended in purification buffer (50 mM Tris pH 7.7, 500 mM NaCl, 10% glycerol, 5 mM DTT, 1 mM PMSF, 2 mM benzamidine) and lysed with a microfluidizer (Watts Fluidair). Clarified lysate was run through a BioScale Mini Profinity IMAC cartridge (BioRad) using an AKTA purifier system (GE Healthcare). The cartridge was washed with purification buffer containing 12 mM imidazole and protein was eluted with purification buffer containing 125 mM imidazole. Protein was exchanged into gel filtration buffer (50 mM Tris pH 7.5, 500

mM NaCl, 10% glycerol, 5 mM DTT) and separated by a Superdex 200 26/60 column (GE Healthcare) using an AKTA purifier system (GE Healthcare). Monomeric protein was frozen in liquid nitrogen and stored at −80 °C. I would like to acknowledge the laboratories of Drs. Herschel Wade and Daniel J. Leahy for their technical assistance and critical guidance.

2.2G Cellular Pull Down Assays

TAP-tagged *S. cerevisiae* strains were grown to mid-log phase in YPD, cryogenically lysed with a mixer mill (Retsch MM301), and stored at −80 °C. Cells (1 g per pull down condition) were homogenized (ProScientific) in 650 mM extraction buffer (650 mM NaCl, 20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM PMSF, 0.2% Triton X-100, 1% BSA, 40 mM imidazole) at a ratio of 1 mL buffer per 1 g yeast and nutated for 1 hr at 4 °C. Clarified extracts were diluted to 300 mM NaCl with 'no-salt' extraction buffer, mixed with 100 µL per sample of Ni-NTA agarose resin (Invitrogen) pre-coated with Pdp3 protein, and nutated for 30 min at 4 °C. Resin was washed 5 times with 300 mM wash buffer (300 mM KCl, 20 mM HEPES pH 7.9, 0.2% Triton X-100, 1% BSA, 40 mM imidazole) and 1 time with buffer containing 10 mM NaCl and 4 mM HEPES pH 7.9. Resin was incubated in 2X SDS-PAGE loading buffer containing 300 mM imidazole for 10 min to elute Pdp3-bound proteins. Samples were boiled for 5 min, resolved on 8% SDS-polyacrylamide gels, transferred to PVDF membrane, and probed with antibodies recognizing the PrA (DAKO P0450, 1/1500) and FLAG (SIGMA F3165, 1/1000) tags. Immunoblots were visualized using HRP-conjugated secondary antibody (NA931-1ML, 1/10,000)

and ECL solution (GE Healthcare). Inputs represent ~0.02-0.05% of total yeast lysate.

2.2H Peptides Used for Pull Down Assays

Peptides used for pull down assays were obtained from the C. D. Allis Laboratory (The Rockefeller University) and the University of North Carolina at Chapel Hill Peptide Synthesis and Arraying Core Facility[233].

2.2I Peptide Pull Down Assays

Steptavidin-coupled Dynabeads (Invitrogen) (25 µL per sample) were incubated with biotinylated histone peptides (1 µg per sample) in binding buffer (20 mM HEPES pH 7.9, 150 mM NaCl, 0.5 mM PMSF, 20% glycerol, 0.2% Triton X-100, 1% BSA) for 1 hr at room temperature. Unbound peptide was washed in binding buffer and beads were incubated with purified Pdp3 proteins (20 µg per sample) for 1 hr at room temperature. Beads were washed 3 times for 5 minutes each with binding buffer, and 1 time with buffer containing 4 mM HEPES pH 7.9, 10 mM NaCl, 0.5 mM PMSF, 20% glycerol, and 0.2% Triton X-100. Peptide-bound proteins were eluted in boiling 2X SDS-PAGE loading buffer. Samples were resolved on 15% SDS-polyacrylamide gels, transferred to PVDF membrane, and probed with antibodies recognizing the FLAG (SIGMA F3165, 1/1000) and streptavidin (Molecular Probes S-911, 1/10,000) tags. Immunoblots were visualized using HRP-conjugated secondary antibody (NA931-1ML, 1/10,000) and ECL solution (GE Healthcare). Inputs represent 0.5 µg of Pdp3 proteins.

2.2J Peptide Synthesis for Fluorescence Polarization Assays

Fluorescent peptides were synthesized using standard Fmoc-solid phase peptide chemistry on a Prelude Peptide Synthesizer (Protein Technologies). Peptides were made on a 0.05 mMol scale with 4 equivalents of amino acids using Rink Amide AM resin (Novabiochem) to generate peptide amides. 5-Carboxyfluorescein (5-FAM) (Chempep) was coupled to the peptides using Lys(ivDde) (Chempep). The ivDde protecting group was orthogonally removed using standard deprotection procedures. The resulting peptides were cleaved using TFA/thioanisole/water/triisopropylsilane/ phenol (87.5:2.5:2.5:2.5:5 v/v) and purified with a Varian Dynamax Microsorb C18 preparative column (Agilent). Purified peptide was lyophilized and its mass was confirmed with an Applied Biosystems Voyager DE-STR MALDI-TOF mass spectrometer (Life Technologies). Of note, to obtain the H3K79me3 5-FAM-linked peptide, it was necessary to install Fmoc-(FmocHmb)Phe-OH (Novabiochem) at F84. Peptides were synthesized and purified by Dr. Blair C.R. Dancy.

2.2K Fluorescence Polarization Assays

Binding assays were performed as previously described with the following modifications[234]. Full-length wild type Pdp3, F18A, and W21A proteins were exchanged into FP buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM DTT) and concentrated to ~260-430 µM using Amicon Centrifugal Filter Units MWCO 30,000 (Millipore). Binding assays were performed in a 60 µL volume with 96 well half area black flat bottom non-binding surface plates (Corning). Protein was serially diluted with FP Buffer in 2-fold increments and incubated with 120 nM of 5-FAM-labeled histone peptides. Following a 30 min equilibration period, fluorescence was detected

at room temperature with an Infinite M1000 plate reader (Tecan) using a 470 nm excitation filter and 527± 20 nm emission filter. Binding curves were analyzed by Prism 5.0 (GraphPad Inc.), using the total binding equation Y= B_{max}* X / (K_d + X) + NS * X + Background, where B_{max}= 1 and non-specific (NS) and background variables are constrained to be equal between peptides. Error bars represent the standard deviation of a representative experiment (n= 2) performed in triplicate. I would like to acknowledge Dr. Jungsan Sohn and Seamus Morrone for technical assistance with FP assays and data analysis.

2.2L Chromatin Association Assays

Strains were grown in YPD to an OD of ~0.8-1. 40-50 total ODs of cells were washed in SB buffer (1 M Sorbitol, 20 mM Tris.Cl pH 7.4), frozen in liquid nitrogen, and stored at −80 °C. Cells were resuspended in 1 mL of PSB buffer (20 mM Tris pH 7.4, 2 mM EDTA, 100 mM NaCl, 10 mM β-ME). 1 mL of SB buffer was then added and cells were spheroplasted with Zymolyase (Seikagaku Biobusiness) for 30 min at room temperature. Spheroplasts were pelleted at 2,000 x *g* and washed twice with LB buffer (0.4 M Sorbitol, 150 mM potassium acetate, 2 mM magnesium acetate, 20 mM PIPES pH 6.8). TritonX-100 was added to a final concentration of 1%. Cells were lysed for 15 min on ice. Chromatin was pelleted at 5,000 x *g* for 15 min. Supernatant was collected and saved as the “soluble” fraction. Chromatin was washed one time with LB buffer and resuspended in an equal volume to that of the “soluble” fraction. Volume equivalents were resolved on 15% SDS-polyacrylamide gels, transferred to PVDF, and probed with antibodies recognizing Protein A (Sigma Aldrich), G6PDH (Sigma Aldrich), and H4 (Abcam: ab10158). Immunoblots were

visualized using HRP-conjugated secondary antibodies and ECL Prime solution (GE Healthcare). Yeast strains were created by Stephen L. McDaniel in the laboratory of Dr. Brian D. Strahl at the University of North Carolina at Chapel Hill. Stephen L. McDaniel also performed all chromatin fractionation assays and the associated western blots. I would like to acknowledge Dr. Brian D. Strahl and Stephen L. McDaniel for their immeasurable contributions to this project.

2.3 Results

2.3A Pdp3 is a Member of the NuA3 HAT Complex

Eaf6, Nto1, Sas3, Taf14, and Yng1 were previously identified as stable members of the *S. cerevisiae* NuA3 HAT complex[55,104-106]. A non-stable NuA3-associated protein, Pdp3 (Ylr455w), was also detected using isotopic differentiation of interactions as random or targeted (I-DIRT) mass spectrometry[55,116]. Pdp3 contains a PWWP domain conserved with BRPF1, a member of the NuA3 *H. sapiens* homolog complex, MOZ/MORF[66,92,111]. Therefore, we reasoned that Pdp3 might function similarly to the PWWP domain of BRPF1 in the NuA3 complex. Endogenously TAP-tagged Pdp3 was isolated from *S. cerevisiae* using a method that preserves complex integrity[115,116]. Proteins co-purifying with Pdp3 were resolved by SDS-PAGE and subjected to tandem MS analysis. Pdp3 interacts with all stable members of NuA3 (Eaf6, Nto1, Sas3, Taf14, Yng1), core histones (H2A, H2B, H3, H4), and components of RNAPII (Rpb2, Rpb4)[55,104-106] **(Figure 7A)**. Although this assay cannot distinguish between direct and indirect interactions, these co-

Figure 7

A

Protein	Spectral Count	Complex
H2B2	231	Nucleosome
H4	215	Nucleosome
H2A2	89	Nucleosome
H3	42	Nucleosome
Pdp3	145	NuA3
Yng1	84	NuA3
Nto1	60	NuA3
Sas3	59	NuA3
Taf14	43	NuA3
Eaf6	18	NuA3
Rpb4	19	RNAPII
Rpb2	13	RNAPII

B

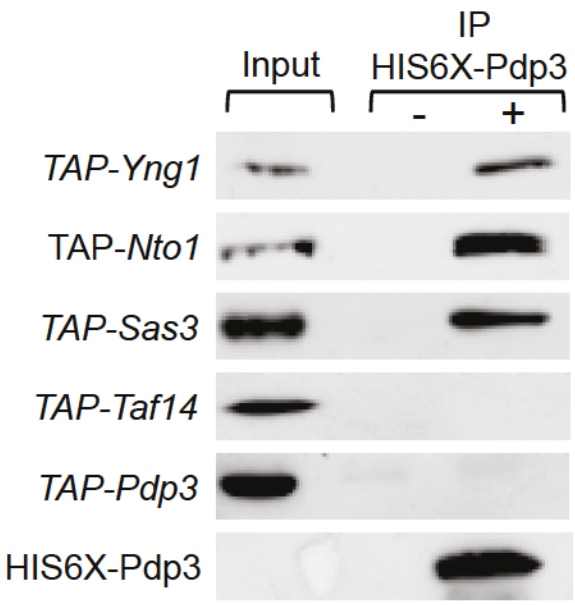


Figure 7 Pdp3 is associated with the NuA3 HAT complex, chromatin, and RNAPII. A.) Spectral counts are reported for endogenous *S. cerevisiae* proteins affinity purified by Pdp3-TAP, after searching with Mascot. This experiment was performed by Dr. Stephanie D. Byrum in the laboratory of Dr. Alan J. Tackett at the University of Arkansas for Medical Sciences. B.) Whole cell extracts (WCEs) from the indicated TAP-tagged yeast strains were immunoprecipitated with HIS6X-FLAG-Pdp3 treated (+) and untreated (-) Ni-NTA resin. WCEs (inputs) and immunoprecipitated samples (IP) were resolved by SDS-PAGE. The presence of NuA3 complex members was monitored by western blotting to TAP and FLAG.

purifications suggest that Pdp3 is a member of the NuA3 complex and that Pdp3 functions in the transcriptional regulation pathway.

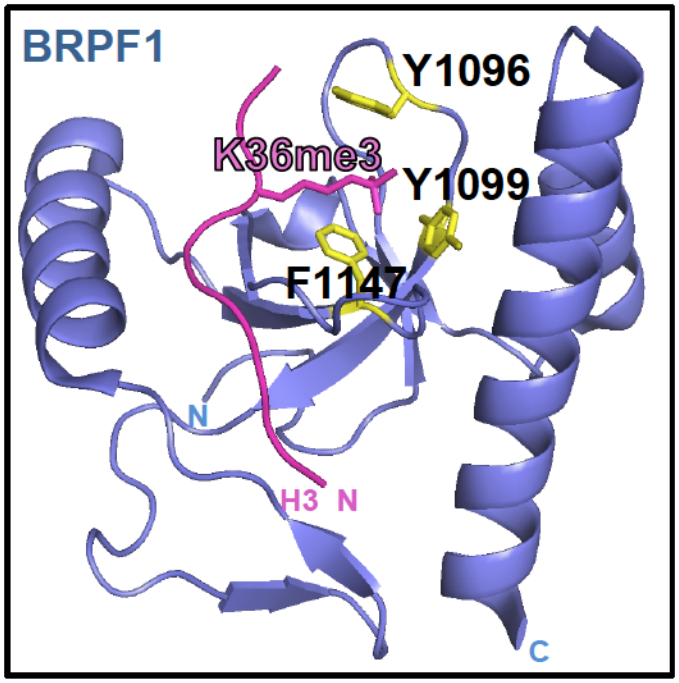
To confirm that Pdp3 is a member of NuA3, we tested the ability of full-length recombinant Pdp3 to pull down NuA3 proteins from cellular extract. Pdp3 was incubated with lysates from *S. cerevisiae* expressing endogenously TAP-tagged NuA3 subunits. Immunoprecipitated samples were resolved by SDS-PAGE and visualized by western blotting. Pdp3 interacts with Yng1, Nto1, and Sas3, supporting our MS results **(Figures 7A, 7B)**. Taf14 was not detected in Pdp3 pull downs, which may indicate that these proteins do not directly interact or that the TAP tag on Taf14 disrupts binding **(Figure 7B)**.

2.3B Pdp3 Specifically Engages H3K36me3

H3K36me3 localizes to the ORFs of actively transcribed genes and is associated with transcription elongation[32,33,61,62,218,219,222]. The PWWP domain of *H. sapiens* BRPF1 specifically engages H3K36me3[92,111] **(Figure 8A)**. Importantly, this PWWP domain is required for the recruitment of MOZ/MORF to H3K36me3-enriched HOX genes and for HOX gene transcription[88,92]. In *S. cerevisiae*, the histone-binding domains of BRPF1 (PWWP domain, double PHD fingers, double bromodomains) are split between Pdp3 (PWWP domain) and Nto1 (double PHD fingers, double bromodomains)[111,117]. Structural modeling predicts that the PWWP domain of Pdp3 contains conserved aromatic residues critical for methyl-lysine binding and conserved secondary structure with PWWP domains that bind methylated histones **(Figures 8B, 9A, 9B)**. Therefore, we reasoned that like BRPF1, Pdp3 might engage H3K36me3 and function as a transcriptional co-activator[88,92].

Figure 8

A



B

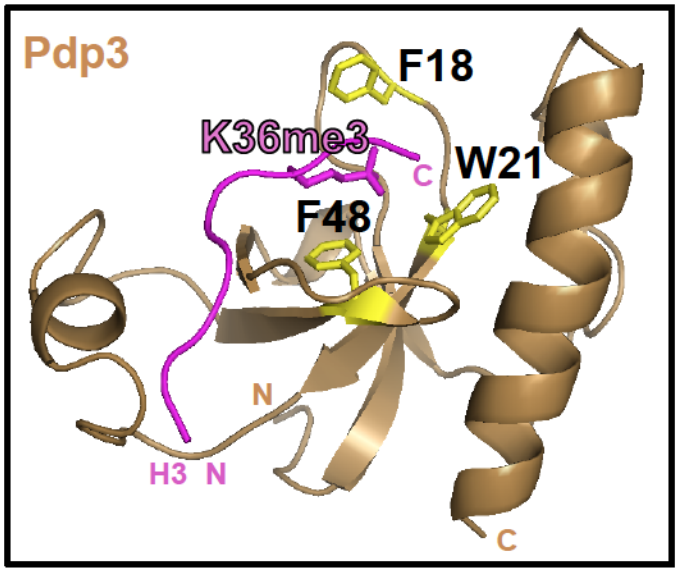


Figure 8 The Pdp3 PWWP domain is structurally conserved with human BRPF1. A.) Crystal structure of the *H. sapiens* BRPF1 PWWP domain (purple) bound to H3K36me3 peptide (pink) (PDB: 2X4W)[92]. B.) The predicted structure of the yeast Pdp3 PWWP domain (brown) bound to H3K36me3 peptide (pink). Note that Pdp3 is predicted to have conserved aromatic residues (yellow).The Pdp3 structure was modeled on 2X4W using Phyre2, minimized with MOE, and rendered with PyMOL in collaboration with Dr. Herschel Wade (JHMI).

Figure 9

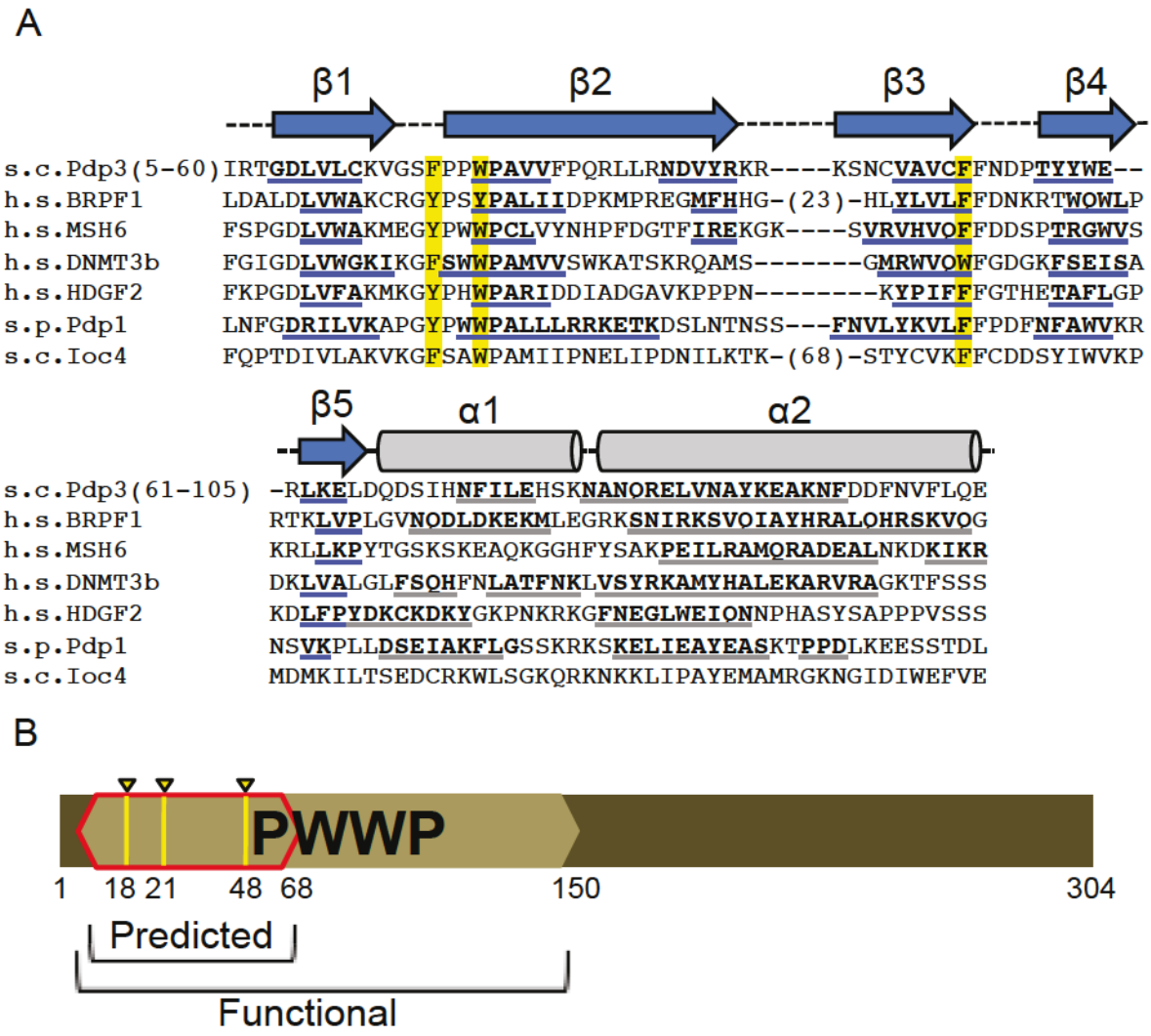


Figure 9 The Pdp3 PWWP domain shares conserved sequence and secondary structure with yeast and human PWWP domain proteins. A.) Clustal W alignment of *S. cerevisiae*, *S. pombe*, and *H. sapiens* PWWP domains. Beta sheets (blue arrows/underlined sequence) and alpha helices (grey cylinders/ underlined sequence) are annotated. Aromatic "cage" residues are highlighted in yellow. B.) Schematic representation of the Pdp3 protein. The predicted (red outline) and functional (tan hexagon) PWWP domains are annotated. Aromatic cage residues are highlighted in yellow.

To determine if Pdp3 engages H3K36me3, we tested the ability of histone peptides to pull down full-length Pdp3. Biotinylated peptides were immobilized on streptavidin resin and incubated with Pdp3. Immunoprecipitated samples were resolved by SDS-PAGE and visualized by western blotting. Pdp3 preferentially binds H3K36me3 peptide over all tested trimethylated and unmodified peptides **(Figure 10A)**.

2.3C Pdp3 Requires a Conserved Aromatic "Cage" for Binding H3K36me3

PWWP domains use an aromatic "cage" to interact with specific trimethylated lysine residues[49,70,71,92,98,235]. For example, BRPF1 requires aromatic residues Y1096, Y1099, and F1147 to coordinate the trimethylammonium group of H3K36me3 **(Figure 8A)**[92]. These aromatic residues are conserved in the PWWP domain of Pdp3 at positions F18, W21, and F48 **(Figures 8B, 9A, 9B)**. To determine whether Pdp3 uses an aromatic "cage" to bind H3K36me3, we tested the ability of H3K36me3 peptide to pull down full-length Pdp3 mutants F18A, W21A, or F48A. Each mutation independently blocks the interaction between Pdp3 and H3K36me3 peptide, suggesting Pdp3 requires a conserved aromatic "cage" to bind histones **(Figure 10B)**.

2.3D The PWWP Domain of Pdp3 is Necessary and Sufficient for Binding H3K36me3

We next wanted to determine whether the predicted PWWP domain of Pdp3 is sufficient to maintain an interaction with H3K36me3. Uniprot defined the Pdp3 PWWP domain as amino acid residues 7-68[236]**(Figure 9B)**. However, we found this

Figure 10

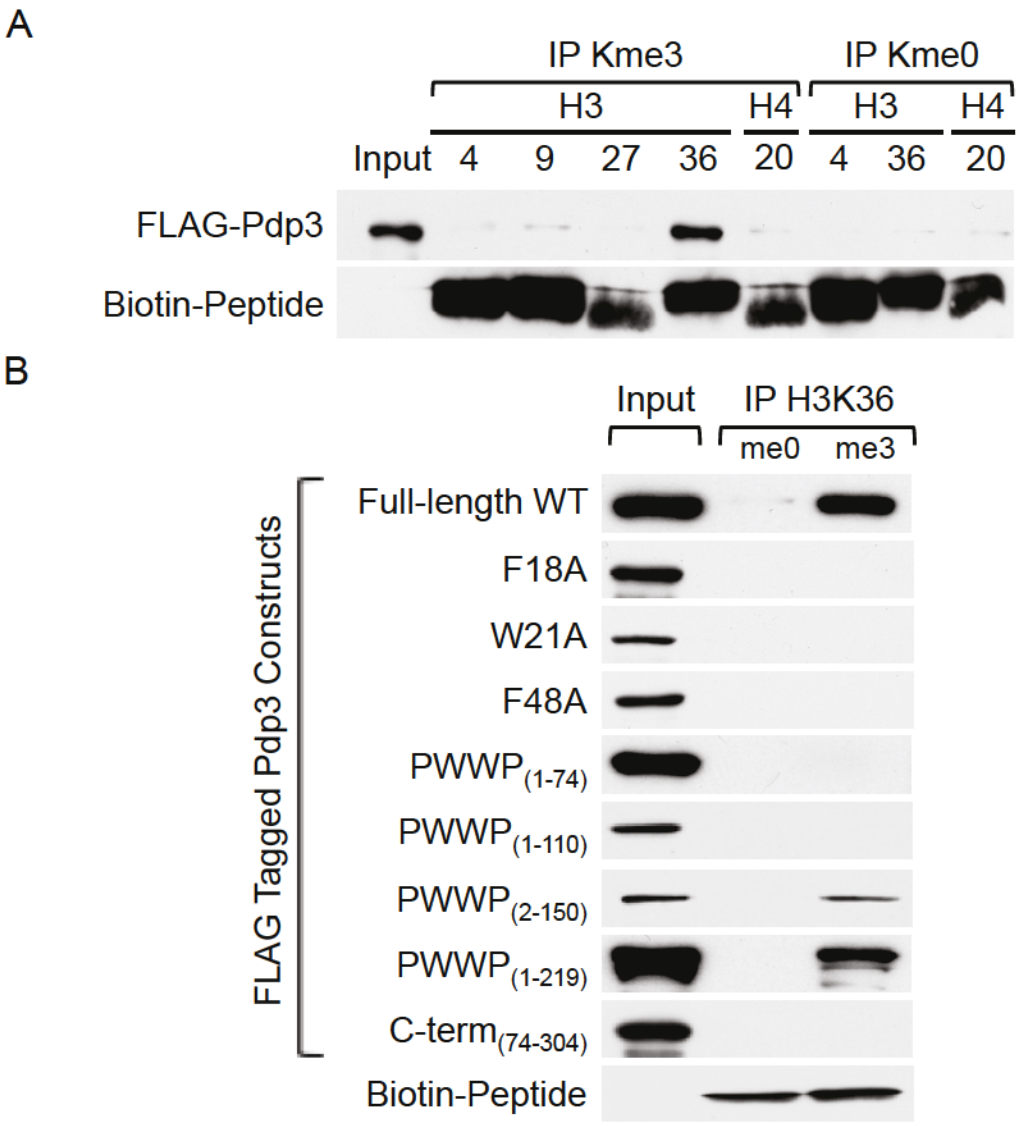


Figure 10 Pdp3 specifically interacts with H3K36me3 through a conserved PWWP domain. A.) Peptide pull down assays were performed with full-length FLAG-Pdp3 and biotinylated histone peptides. Purified protein (input) and immunoprecipitated samples (IP) were resolved by SDS-PAGE. Binding was monitored by western blotting to FLAG and streptavidin. B.) Peptide pull down assays were performed with FLAG-tagged full-length Pdp3, mutants: F18A, W21A, and F48A, truncations: $PWWP_{(1-74)}$, $PWWP_{(1-110)}$, $PWWP_{(2-150)}$, $PWWP_{(1-219)}$, and $C\text{-}term_{(74-304)}$, and biotinylated histone peptides. Purified proteins (inputs) and immunoprecipitated samples (me0 and me3) were resolved by SDS-PAGE. Binding was monitored by western blotting to FLAG and streptavidin.

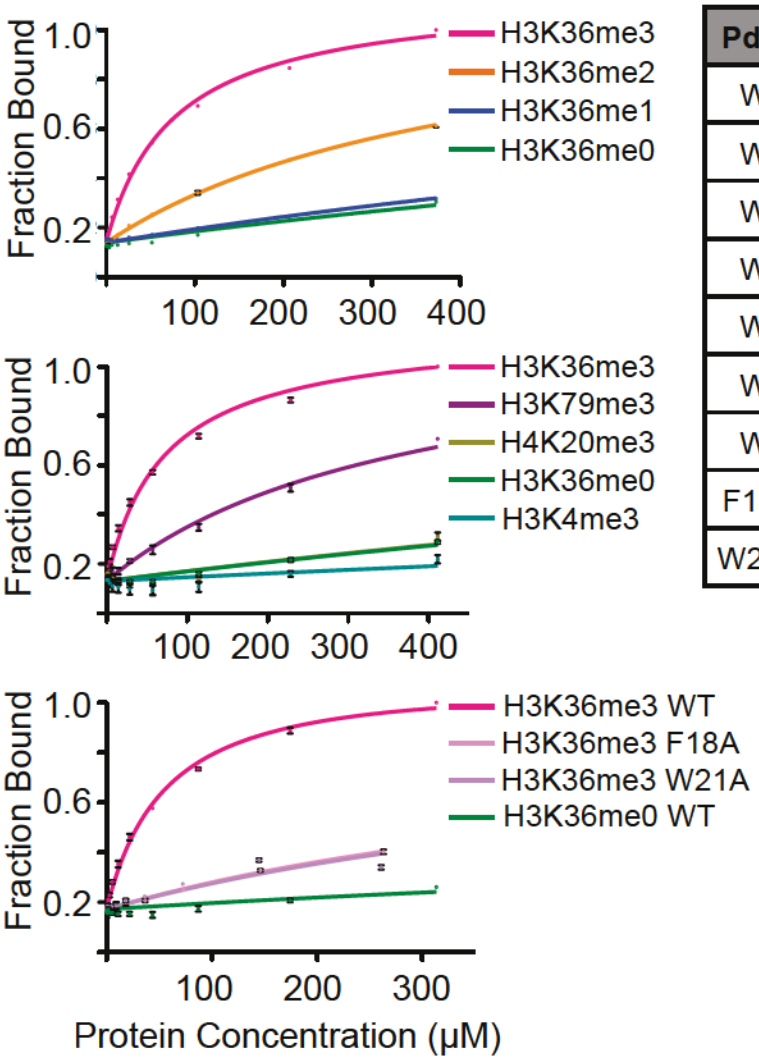
segment of Pdp3 ($PWWP_{(1-74)}$) is unable to bind H3K36me3 peptide **(Figure 10B)**. Our results suggest that residues beyond the predicted PWWP domain are necessary for Pdp3 function. Like BRPF1, our modeled Pdp3 structure contains two alpha helices proximal to the aromatic "cage" **(Figures 8A, 8B)**[92]. These alpha helices may stabilize the PWWP domain and hold the aromatic "cage" in a position that is favorable for histone binding. To test our hypothesis, we created a series of Pdp3 constructs that include increasing segments of the modeled alpha helices. We assayed the ability of H3K36me3 peptide to pull down $PWWP_{(1-110)}$, $PWWP_{(2-150)}$, and $PWWP_{(1-219)}$. $PWWP_{(2-150)}$ restores binding to H3K36me3 and thus represents the functional PWWP domain of Pdp3 **(Figures 9B, 10B)**. These data suggest Pdp3, and likely other PWWP domain proteins, require unannotated alpha helices for aromatic "cage" stability and function.

2.3E Biophysical Characterization of the Interaction between Pdp3 and H3K36me3

To biophysically quantitate the specificity of the interaction between Pdp3 and H3K36me3, we performed fluorescence polarization assays using full-length Thioredoxin-HIS6X-S•tag-Pdp3 and 5-FAM-labeled histone peptides. In agreement with our peptide pull down assays, Pdp3 favors binding to H3K36me3 over other known targets of PWWP domain proteins, such as H3K79me3 and H4K20me3 **(Figures 11A, 11B)**. Pdp3 engages H3K36me3 peptide with a K_d of 69.5 ± 3.7 μM **(Figure 11B)**[86,99]. Notably, this value is one of the lowest reported dissociation constants for PWWP domains and methylated histones[86,91,92,99,237]. As PWWP domains also bind DNA, the K_d of Pdp3 may be much lower in the context of the nucleosome[87,92,100,102].

Figure 11

A



B

Pdp3	Peptide	K_d(μM)
WT	H3K36me3	69.5 +/- 3.7
WT	H3K36me2	414 +/- 23
WT	H3K36me1	>>1000
WT	H3K36me0	>>1000
WT	H3K4me3	>>1000
WT	H3K79me3	434 +/-49
WT	H4K20me3	>>1000
F18A	H3K36me3	667 +/- 68
W21A	H3K36me3	703 +/- 73

Figure 11 Pdp3 specifically interacts with H3K36me3 peptide with a K_d of 69.5 μM. A.) Fluorescence polarization assays were used to measure binding affinities of full-length wild type S•tag-Pdp3 to the indicated 5-FAM-labeled histone peptides. Full-length F18A and W21A mutants were also tested. Error bars represent the standard deviation of a representative experiment (n= 2) performed in triplicate. B.) The total binding equation was used to calculate dissociation constants with Graphpad Prism software. K_d values were tabulated from two independent experiments performed in triplicate and averaged. Error represents the standard deviation of two independent experiments.

We then tested Pdp3 mutants, F18A and W21A, which are predicted to disrupt the aromatic "cage" required for H3K36me3 binding. F18A and W21A mutations reduce H3K36me3 peptide binding ~10 fold compared to wild type, further supporting our peptide pull down results **(Figures 10B, 11A, 11B)**. Pdp3 also weakly binds H3K36me2 peptide with a K_d of ~414 μM **(Figures 11A, 11B)**. The ~6 fold increase in specificity of Pdp3 for H3K36me3 (over H3K36me2) suggests that Pdp3 has a distinct function from complexes that preferentially bind H3K36me2, such as the transcriptionally repressive Rpd3S HDAC complex[223,226,229,238,239].

2.3F Pdp3 Requires H3K36me3 to Bind Chromatin *In Vivo*

To further understand the function of Pdp3, we determined whether Pdp3 binds H3K36me3 *in vivo* through a chromatin association assay that separates soluble and chromatin-bound proteins. The H3K36me3 HMT, *SET2,* was deleted in the *PDP3-TAP* background. The H3K4me3 HMT, *SET1*, and the NuA3 H3K4me3-binding protein, *YNG1,* were also deleted in the *PDP3-TAP* background. Strikingly, in the absence of H3K36me3, Pdp3 does not bind chromatin, indicating H3K36me3 is critical for Pdp3 localization *in vivo* **(Figure 12A)**. In contrast, Pdp3 binds chromatin in the absence of both H3K4me3 and Yng1, indicating Pdp3 directly targets NuA3 to ORFs via an interaction with H3K36me3[33,53,65] **(Figure 12A)**.

SET1, SET2, and *PDP3* were then deleted in the *YNG1-TAP* background. Yng1 remains bound to chromatin in the absence of H3K4me3, H3K36me3, and Pdp3 **(Figure 12B)**. This suggests that while Yng1 can engage chromatin through methyl-independent means (such as the Yng1 N-terminus or Pdp3), Pdp3 requires H3K36me3 for chromatin localization[107,108,112].

Figure 12

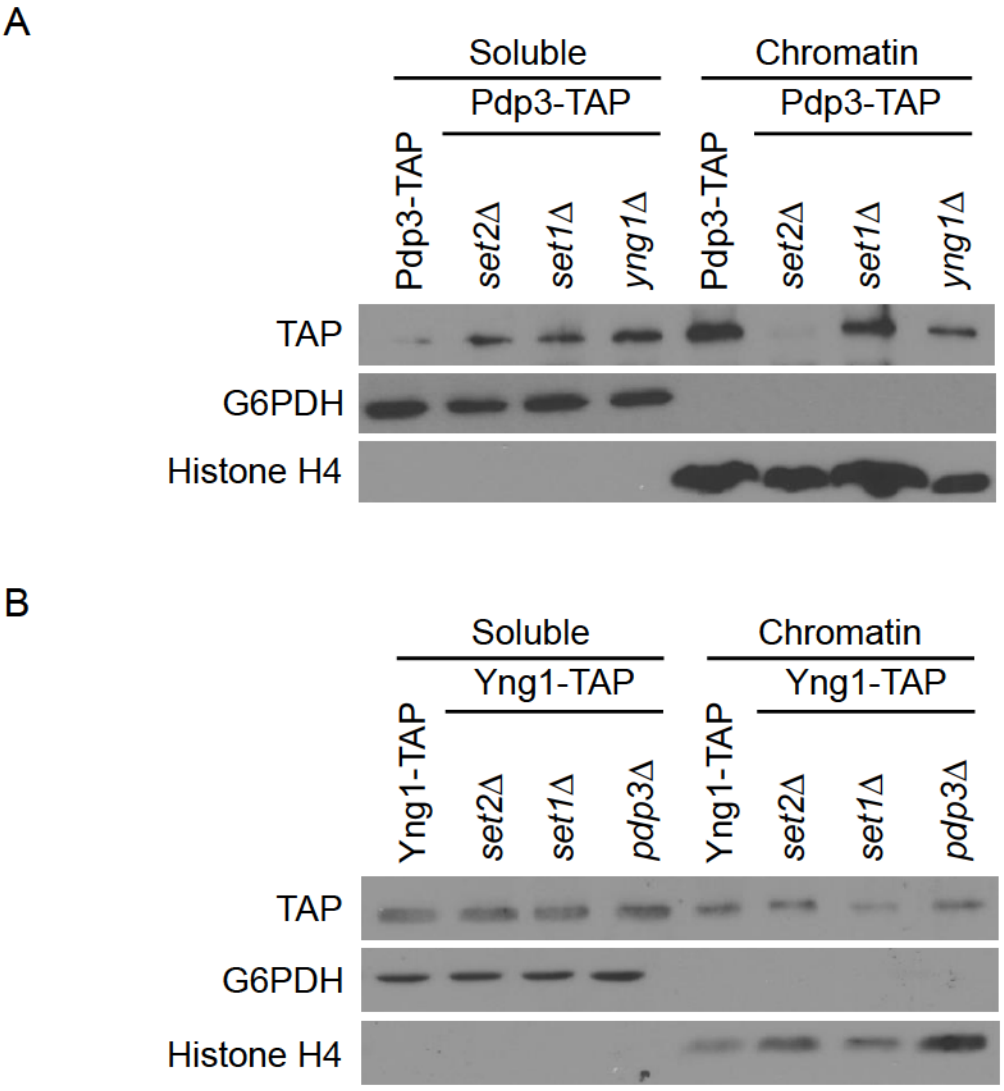


Figure 12 Pdp3, but not Yng1, requires H3K36me3 for chromatin association *in vivo.* A.) TAP-tagged *PDP3 S. cerevisiae* strains and B.) TAP-tagged *YNG1 S. cerevisiae* strains were biochemically fractionated into chromatin-associated proteins and soluble proteins. Fractions were probed for the presence of Pdp3 and Yng1 via the TAP tag. G6PDH (soluble) and histone H4 (chromatin-bound) serve as both loading and fractionation controls. These experiments were performed by Stephen L. McDaniel in the laboratory of Dr. Brian D. Strahl at the University of North Carolina at Chapel Hill.

2.4 Discussion

Here we define Pdp3 as a novel member of the NuA3 HAT complex. Ppd3 specifically interacts with H3K36me3 *in vitro* and *in vivo,* through a structurally conserved PWWP domain, and recruits NuA3 to H3K36me3-enriched chromatin. Therefore, we propose a new function for H3K36me3 in yeast- H3K36me3 may positively regulate transcription via HAT complex recruitment into ORFs. Until our study, only negative regulatory functions have been described for H3K36me3 in yeast, such as HDAC recruitment and blockage of *trans*-histone exchange[20,63-65,223,224]. In **Chapter 3** we continue to elucidate the positive role of Pdp3 in transcriptional regulation.

Mutations of the NuA3 human homolog, MOZ/MORF, are associated with multiple types of cancer[92,111,152-154,240]. For example, MOZ fuses to the HAT p300/CBP, resulting in aberrant acetylation and HOX gene overexpression[151,155-159]. HOX gene overexpression causes hematopoietic stem cell transformation and leukemogenesis[8,160]. Since MOZ fusions assemble into the MOZ HAT complex, inhibition of associated histone-binding "reader" domains may provide new treatments for leukemia[8,88,92,158]. Indeed, the PWWP domain of the BRPF1 subunit is required for MOZ/MORF localization and HOX gene transcription[88,92]. Therefore, small molecules targeting the aromatic "cage" within the BRPF1 PWWP domain may alleviate pathogenesis[92]. Additionally, our studies on the yeast homolog, Pdp3, reveal a novel characteristic of PWWP domains. We find that PWWP domains require unannotated alpha helices for aromatic "cage" stability and function. Therefore, it may be possible to inhibit PWWP domain binding by destabilizing these

alpha helices using constrained peptides and proteomimetics, which have shown recent promise in blocking helix-mediated interactions[241]. Future mechanistic studies into the PWWP domain of Pdp3 may further clarify how effector proteins contribute to human disease.

Chapter 3 The NuA3 HAT Complex Exists in Two Functionally Distinct Forms that Coordinate Transcription Initiation and Elongation Pathways

This research was originally published in Molecular and Cellular Proteomics. Tonya M. Gilbert, Stephen L. McDaniel, Stephanie D. Byrum, Jessica A. Cades, Blair C. R. Dancy, Herschel Wade, Alan J. Tackett, Brian D. Strahl, and Sean D. Taverna. A PWWP Domain-Containing Protein Targets the NuA3 Acetyltransferase Complex via Histone H3 Lysine 36 Trimethylation to Coordinate Transcriptional Elongation at Coding Regions. *Molecular and Cellular Proteomics*. 2014; 13: 2883-2895.

3.1 Introduction

The access of transcriptional machinery to DNA is dependent on chromatin structure[5,206]. Chromatin structure is regulated in part by chromatin-associated protein complexes that modulate histone post-translational modifications (PTMs)[13,17,19,20]. Histone PTMs are found throughout the genome in a context dependent manner[13,19,23,31-33,53,210] **(Figure 2B)**. For example, histone H3 is often both trimethylated on lysine 4 (H3K4me3) and acetylated on lysine 14 (H3K14ac) at the 5'-ends of transcriptionally active genes[23,31-33,53,54]. Alternatively, H3K36me3 is found throughout the open reading frames (ORFs) of active genes[32,33,53,209]. The conserved *S. cerevisiae* NuA3 histone acetyltransferase (HAT) complex can modulate its localization and function by engaging these differentially positioned

histone PTMs[55,104-108,110]. NuA3 binds H3K4me3 through the plant homeodomain (PHD) finger of the Yng1 subunit[55,106-108] **(Figure 5A)**. NuA3 then acetylates H3K14 and initiates transcription at a subset of genes through recruitment of the histone chaperone, FACT, and the chromatin-remodeler, RSC[43,44,55,105,106] **(Figure 5A)**. NuA3 also binds H3K36me3 through the proline-tryptophan-tryptophan-proline (PWWP) domain of Pdp3 (Ylr455w) **(See Chapter 2)**.

In yeast, H3K36me3 is correlated specifically with the regulation of transcription elongation[61,62,218,219,222]. H3K36me2/3 recruits the histone deacetylase (HDAC) complex, Rpd3S, which generates a hypoacetylated environment behind elongating RNAPII to represses intergenic transcription[223-229]. We find that H3K36me3 also recruits the NuA3 HAT complex into coding regions, for an undetermined function **(See Chapter 2)**. As other *S. cerevisiae* HAT complexes, such as NuA4 and SAGA, coordinate nucleosome eviction at ORFs through the acetylation of histones H3 and H4, we hypothesize that NuA3 may similarly facilitate transcription elongation and oppose Rpd3S HDAC activity[242-244].

Here, using biochemical and genetic analyses, we classify NuA3 into two functionally distinct forms: NuA3a and NuA3b. NuA3a uses the PHD finger of Yng1 to bind H3K4me3 at the 5'-ends of ORFs and initiates transcription through the acetylation of H3K14. NuA3b uses the PWWP domain of the unique member, Pdp3, to bind H3K36me3 throughout ORFs. We find that deletion of *PDP3* decreases NuA3-directed transcription and results in growth defects when combined with transcription elongation mutants, suggesting NuA3b positively regulates transcription elongation. We also determine that NuA3a, but not NuA3b, is synthetically lethal with

a deletion of the HAT, *GCN5*. This indicates that NuA3b has a role at ORFs that is independent of Gcn5 HAT activity, and therefore, NuA3b may acetylate a novel substrate(s).

3.2 Methods

3.2A Primer Sequences

Primers were designed by Primer3 to target the 5'-end of our genes of interest. All primer sequences used in **Chapter 3** are listed in **Table 4**.

3.2B *S. cerevisiae* Strains

All strains used in **Chapter 3** are described in **Table 5**.

3.2C *S. cerevisiae* Plasmids

All constructs used in **Chapter 3** are described in **Table 6**.

3.2D Relative Transcript Levels

Total RNA was prepared via Trizol (Life Technologies) from endogenously TAP-tagged *S. cerevisiae* strains: *YNG1*, *PDP3*, *PDP3;yng1*Δ, and *YNG1;pdp3*Δ. DNA was digested with Turbo DNase (Life Technologies). cDNA was synthesized with the Superscript III First Strand Synthesis System (Invitrogen) and diluted 1:20 for qPCR analysis. Samples were stored at −20 °C. Differences in transcript levels were measured by qPCR using Power SYBR Green PCR Master Mix (Life Technologies) and a Real Time PCR system (Applied Biosystems v2.1). Relative transcript levels of mutant strains as compared to wild type were calculated using the relative

Table 4
Primers used in Chapter 3
Source: T. Gilbert, S. Taverna Lab

Name	Sequence
YER124c F	ATTTTTCGTCAGCCTGCTATT
YER124c R	TCAACACCGTACAGTTTCCAA
YLL013c F	TCTTCAAGAAAACCGCCCTA
YLL013c R	AGTAAACGGGACCAGCAATG
YML062c F	ACCAAGCTGACAGGAAGCAT
YML062c R	TTGGCATGTTTCCCAATTTT
YGR157w F	ATTTCGCCAGTTTGTCGATT
YGR157w R	AATCTTTAACCACGCGATGC
Actin F	CTCCGTCTGGATTGGTGGTT
Actin R	TGGACCACTTTCGTCGTATTCTT
SSA1 F	AAGAACTTTACCCCAGAACAAATCT
SSA1 R	TCGTTAATAATACGCAAGACATTCA
SSB1 F	AATCTGTTCAAAAGGACATGAAGAC
SSB1 R	CGTTAAAGTAAGCTGGGACAGTAAT

Table 5

S. cerevisiae strains used in Chapter 3

Strain	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 [pRS316]*	S. McDaniel, B. Strahl Lab
set2Δ	*MATa leu2Δ0 met15Δ0 ura3Δ0 set2Δ::natMX [pRS316]*	S. McDaniel, B. Strahl Lab
eaf3Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 eaf3Δ::kanMX [pRS316]*	S. McDaniel, B. Strahl Lab
rco1Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rco1Δ::kanMX [pRS316]*	S. McDaniel, B. Strahl Lab
yng1Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 yng1Δ::kanMX [pRS316]*	S. McDaniel, B. Strahl Lab
pdp3Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pdp3::kanMX [pRS316]*	S. McDaniel, B. Strahl Lab
nto1Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 nto1Δ::kanMX [pRS316]*	S. McDaniel, B. Strahl Lab
sas3Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sas3Δ::kanMX [pRS316]*	S. McDaniel, B. Strahl Lab
Bur1 Shuffle	*MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200 bur1D::HIS3 lys2Δ202 [pRS316-Bur1]*	M. Keogh Lab
Bur1 Shuffle; *rco1Δ*	*MATa bur1Δ::HIS3 rco1Δ::KanMX ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202 [pRS316-Bur1]*	M. Keogh Lab
Bur1 Shuffle; *set2Δ*	*MATα, bur1Δ::HIS3, set2Δ::KanMX, ura3-52 or 3Δ0, leu2Δ1 or 2Δ0, trp1Δ63, his3Δ200 or 3Δ1, lys2Δ202 (pRS316-Bur1)*	M. Keogh Lab
Bur1 Shuffle; *yng1Δ*	*MATa bur1Δ::HIS3 ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202 yng1Δ::natMX [pRS316-Bur1]*	S. McDaniel, B. Strahl Lab
Bur1 Shuffle; *pdp3Δ*	*MATa bur1Δ::HIS3 ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202 pdp3Δ::kanMX [pRS316-Bur1]*	S. McDaniel, B. Strahl Lab
Bur1 Shuffle; *rco1Δ yng1Δ*	*MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202 bur1Δ::HIS3 rco1Δ::kanMX yng1Δ::natMX*	S. McDaniel, B. Strahl Lab
Bur1 Shuffle; *rco1Δ pdp3Δ*	*MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202 bur1Δ::HIS3 rco1Δ::kanMX pdp3Δ::natMX*	S. McDaniel, B. Strahl Lab
gcn5Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gcn5Δ::kanMX [pmk120]*	Open Biosystems
gcn5Δ pdp3Δ	*MATa leu2Δ0 met15Δ0 ura3Δ0 pdp3Δ::Nat gcn5Δ::KanMX [pMK144]*	S. McDaniel, B. Strahl Lab
gcn5Δ yng1Δ	*MATa leu2Δ0 met15Δ0 ura3Δ0 pdp3Δ::Nat gcn5Δ::KanMX [pMK144]*	S. McDaniel, B. Strahl Lab

Table 6
***S. cerevisiae* plasmids used in Chapter 3**
Source: M. Kuo Lab

Name	Description
pMK120	*CUP1-pr URA3*
pMK144	*CUP1-pr URA3 GCN5*
pMK144 E/H	*CUP1-pr URA3 GCN5 E175H*

standard curve method. Actin was used to normalize the amount of starting template between samples. Error bars represent the standard error of the mean of a representative experiment (n=4) performed in triplicate. Statistical significance was determined by an unpaired two-tailed t-test. I would like to acknowledge Dr. Karen L. Reddy, Teresa R. Luperchio, and Dr. Kimberly Stephens for their technical assistance with qPCR experiments and data analysis.

3.2E Yeast Strains and Cell Spotting Assays

S. cerevisiae strains were created using heterologous gene replacement[245]. *BUR1* delete shuffle strains were grown on synthetic complete (SC) -Ura plates to maintain the wild type *BUR1* plasmid prior to plating on 5-Fluoroorotic acid (5-FOA) treated medium[246]. For cell spotting assays, either 0.5 or 2 ODs of cells were 5-fold serially diluted, spotted onto the appropriate plates, and incubated at 30 °C for 2-3 days as indicated. Yeast strains/plasmids were created by Stephen L. McDaniel in the laboratory of Dr. Brian D. Strahl at the University of North Carolina at Chapel Hill. Cell spotting assays were also performed by Stephen L. McDaniel. I would like to acknowledge Dr. Brian D. Strahl and Stephen L. McDaniel for their immense contributions to this project.

3.2F *In Vitro* HAT Assays

HAT assays were performed as previously described with the following modifications[247]. Purifications of endogenously TAP-tagged *S. cerevisiae* NuA3a (*YNG1;pdp3*Δ) and NuA3b (*PDP3*) were performed to maintain complex integrity[115,116]. NuA3a and NuA3b were resuspended in HAT buffer (20 mM Tris pH 7.5, 50 mM NaCl, 5% glycerol) and mixed with 1 µg of recombinantly expressed *S.*

cerevisiae histone H3 and 30 μM acetyl CoA in a total volume of 60 μL. Acetyl CoA was omitted from the negative control reactions. All reactions were incubated for 30 min at 30 °C and frozen in liquid nitrogen. Samples were lyophilized and separated by acid urea gel electrophoresis as previously described[248]. Gels were stained with Simply Blue Safe Stain (Invitrogen). Recombinant histone H3 was obtained from Anne M. Cieniewicz. HAT reactions and acid urea gel electrophoresis were performed by Anne M. Cieniewicz.

3.3 Results

3.3A Pdp3 is Required for NuA3-Regulated Transcription

Mutation of the Yng1 PHD finger results in genome-wide mislocalization of NuA3 from promoter regions to ORFs, decreased H3K14ac, and decreased transcription of NuA3-regulated genes[55]. Since Pdp3 recruits NuA3 into ORFs via an association with H3K36me3, Pdp3 may also participate in transcriptional regulation. To further assess the function of Pdp3 *in vivo*, we performed RT-qPCR with wild type, *pdp3*Δ, and *yng1*Δ cells. We calculated the % transcription for each mutant relative to wild type. As expected, *yng1*Δ cells show a significant decrease in the % transcription of NuA3 target genes, confirming a positive role for Yng1 in NuA3 activity[55] **(Figure 13)**. Interestingly, *pdp3*Δ cells also show a significant decrease in the % transcription of NuA3 target genes **(Figure 13)**. These data indicate that NuA3 binding to both H3K4me3, via Yng1, and H3K36me3, via Pdp3, is required for NuA3-regulated transcription.

Figure 13

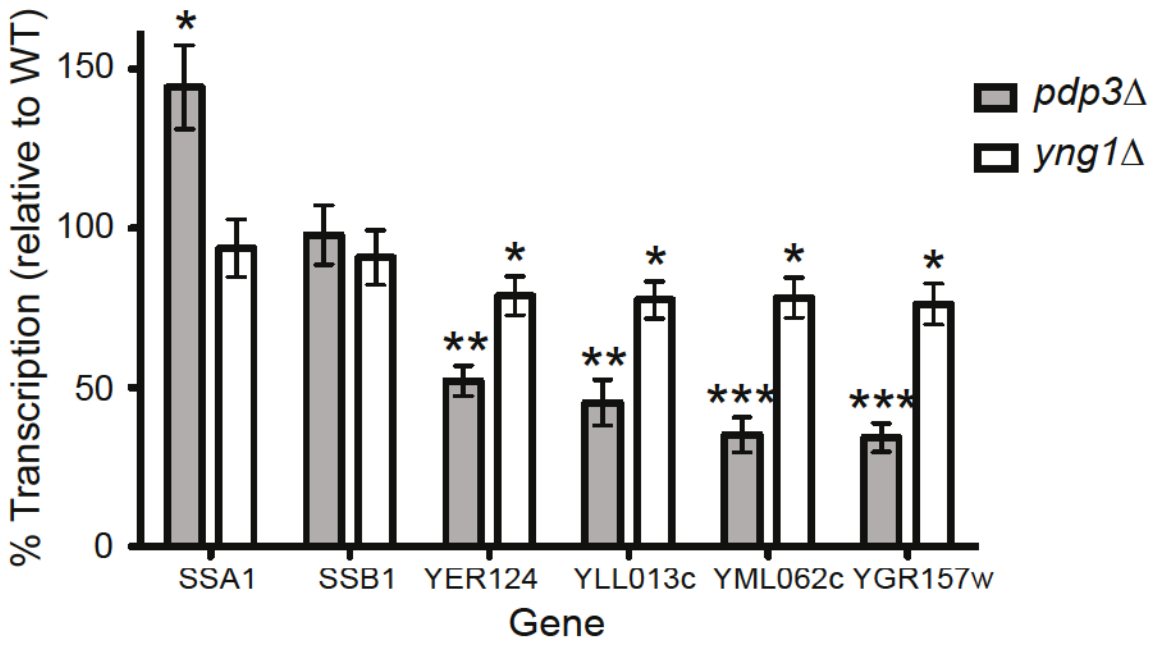


Figure 13 NuA3-regulated transcription requires both Yng1 and Pdp3. RT-qPCR analysis of WT, *yng1Δ,* and *pdp3Δ* cells to determine the relative transcript levels of NuA3-target genes and non-target genes (SSA1 and SSB1). Transcript levels were normalized to Actin expression. Error bars represent the standard error of the mean of a representative experiment (n=4) performed in triplicate. Asterisks indicate statistical significance as determined by an unpaired two-tailed t-test.
* = $p < .05$, ** = $p < .01$, *** = $p < .001$

3.3B *PDP3* has Genetic Links to *SET2* and Promotes Transcription Elongation

Since Pdp3 binds H3K36me3 and is required for proper NuA3-regulated transcription, Pdp3 may facilitate transcription elongation. The *BUR1* bypass assay is a genetic tool that measures the influence of H3K36 methylation-dependent interactions on transcription elongation. Bur1, a cyclin-dependent kinase, acts as an essential positive regulator by phosphorylating several components of elongating RNAPII[249-251]. Although deletion of *BUR1* is lethal, deletion of *BUR1* combined with deletion of *SET2* or *RCO1* (a unique member of the Rpd3S HDAC complex) bypasses lethality[218,224]. To explore whether Pdp3 or Yng1 contribute to transcription elongation, we evaluated deletions of *PDP3* and *YNG1* in the *bur1*Δ background. Neither *bur1*Δ*pdp3*Δ nor *bur1*Δ*yng1*Δ cells are viable **(Figure 14A)**. These results suggest either *PDP3* and *YNG1* do not function in the elongation pathway, or Pdp3 and Yng1 act as positive elongation factors and thus do not display a bypass phenotype. To distinguish between these possibilities, we created strains with *PDP3* or *YNG1* deleted in the *bur1*Δ*rco1*Δ background. Since this background shows a bypass phenotype, we could now observe how deletion of *PDP3* or *YNG1* affects transcription elongation. Significantly, both *bur1*Δ*rco1*Δ*pdp3*Δ and *bur1*Δ*rco1*Δ*yng1*Δ cells show a decrease in growth as compared to the *bur1*Δ*rco1*Δ background **(Figure 14A)**. Our results suggest that Pdp3 and Yng1 positively regulate transcription elongation.

To further connect NuA3 to the transcription elongation pathway, we performed a 6-azauracil (6-AU) growth assay. 6-AU is a transcription elongation

Figure 14

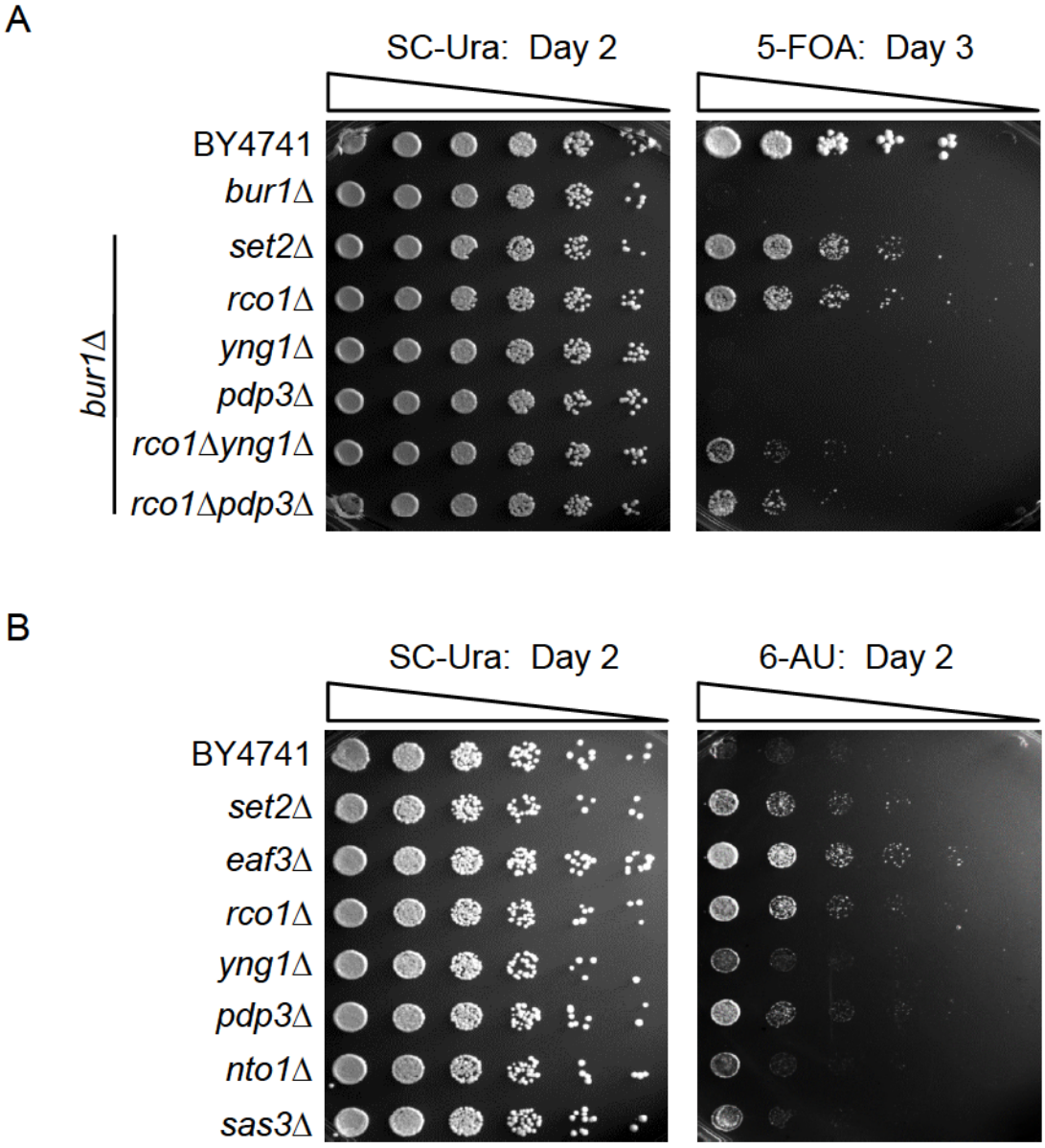


Figure 14 *PDP3* functions in the *SET2* genetic pathway to facilitate transcription elongation. A.) 2 ODs of the indicated *S. cerevisiae* strains were 5-fold serially diluted on SC-Ura (left) or SC-Ura + 5-FOA (right) plates and grown at 30 °C for two or three days, respectively. B.) 0.5 ODs of the indicated *S. cerevisiae* strains were 5-fold serially diluted onto SC-Ura (left) or SC-Ura + 150 µg/mL 6-AU (right) plates and grown at 30 °C for two days. These experiments were performed by Stephen L. McDaniel in the laboratory of Dr. Brian D. Strahl at the University of North Carolina at Chapel Hill.

inhibitor. Strains containing a deletion of *SET2,* or deletions of downstream H3K36me3 effectors such as *RCO1* and *EAF3*, show a resistance phenotype[218,224]. Of all the tested NuA3 deletions, only *pdp3*Δ cells display 6-AU resistance **(Figure 14B)**. This suggests that Pdp3 may directly regulate transcription elongation, while Yng1 may indirectly facilitate transcription elongation by initiating transcription.

3.3C *PDP3* is Not Synthetically Lethal with *GCN5*

It has previously been determined that NuA3 subunits, *SAS3* and *YNG1*, display synthetic lethality with the HAT, *GCN5*[110]. NuA3 and Gcn5-associated complexes (such as SAGA) acetylate H3K14 and localize to both promoter regions and ORFs[242,244,252]. Therefore, Sas3 and Gcn5 may collaborate to promote transcription initiation and elongation. Given the positive role of Pdp3 in the transcription elongation pathway, we wanted to determine if *PDP3* is synthetically lethal with *GCN5*. We performed a *GCN5* plasmid shuffle assay in *gcn5*Δ*yng1*Δ and *gcn5*Δ*pdp3*Δ backgrounds. Surprisingly, *gcn5*Δ*pdp3*Δ cells show no deleterious phenotype, indicating *PDP3* does not genetically interact with *GCN5* **(Figure 15)**. These results suggest that Pdp3-containing NuA3 either does not acetylate H3K14 or that Pdp3-directed H3K14ac has a distinct function from the role of Gcn5 at ORFs. We performed an *in vitro* HAT assay with endogenous NuA3 complexes (*YNG1*-TAP;*pdp3*Δ and *PDP3*-TAP) and recombinant *S. cerevisiae* histone H3. We observe multiple acetylation states from each complex, suggesting Pdp3-containing NuA3 may target novel sites **(Figure 16)**. Further MS analysis is required to determine the specificity of Pdp3-containing NuA3.

Figure 15

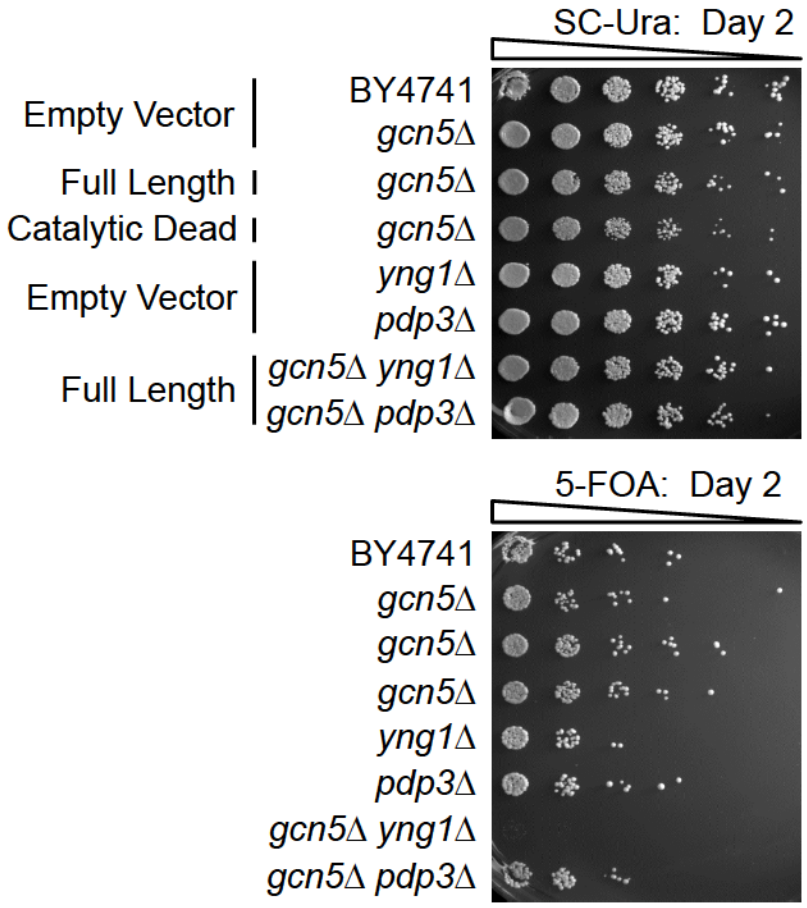


Figure 15 *YNG1*, but not *PPD3*, is synthetically lethal with *GCN5*. 0.5 ODs of the indicated *S. cerevisiae* strains were 5-fold serially diluted onto SC-Ura (top) or SC-Ura + 5-FOA (bottom) plates and grown at 30 °C for two days. This experiment was performed by Stephen L. McDaniel in the laboratory of Dr. Brian D. Strahl at the University of North Carolina at Chapel Hill.

Figure 16

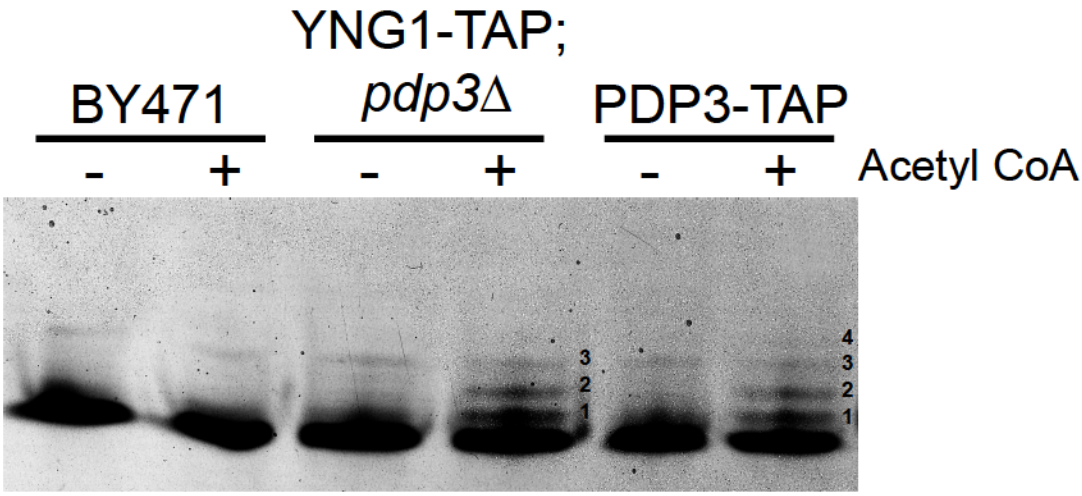


Figure 16 Pdp3-containing NuA3 may acetylate novel targets. *In vitro* HAT assays were performed with endogenous NuA3 complexes (*YNG1*-TAP;*pdp3*Δ and *PDP3*-TAP), recombinant *S. cerevisiae* histone H3, and acetyl CoA. Reactions were separated by acid urea gel electrophoresis and stained with Simply Blue Safe Stain. The *YNG1*-TAP;*pdp3*Δ complex acetylated three distinct states and the *PDP3*-TAP complex acetylated four distinct states. MS analysis will determine which histone residues each complex acetylated. HAT reactions and acid urea gel electrophoresis were performed by Anne M. Cieniewicz.

3.4 Discussion

Using biochemical and genetic approaches, we find that the *S. cerevisiae* NuA3 HAT complex exists in two functionally distinct forms: NuA3a and NuA3b. This nomenclature has previously been used to distinguish subtypes of related complexes, such as the Isw1a and Isw1b ATP-dependent chromatin remodelers. While these complexes contain the same catalytic subunit, Isw1, Isw1b uniquely contains the PWWP domain protein, Ioc4, which targets remodeling activity to H3K36me3-enriched nucleosomes[63,64]. Similarly, we hypothesize that while NuA3a and NuA3b contain the same catalytic subunit, Sas3, NuA3b uniquely contains the PWWP domain protein, Pdp3. We predict that NuA3a specifically associates with H3K4me3 through the PHD finger of Yng1 to acetylate H3K14 and facilitate transcription initiation[55,105-108] **(Figure 17A)**. Conversely, NuA3b specifically associates with H3K36me3 through the PWWP domain of Pdp3 to facilitate transcription elongation via an undetermined mechanism **(Figure 17B)**.

Altogether our data support the model that NuA3b is compositionally distinct from NuA3a[55]. However, we cannot exclude the alternative possibility that Pdp3 is a member of both NuA3a and NuA3b. Pdp3 activity may be allosterically modulated, allowing NuA3b to bind H3K36me3 in a context dependent manner. Notably, the conserved human BRPF1 protein resembles a fusion of yeast Nto1 and Pdp3[146]. Therefore, in order for yeast NuA3a/b and human MOZ/MORF HAT complexes to display similar genomic localization patterns, the BRPF1 PWWP domain may be regulated by unknown protein-protein interactions.

Figure 17

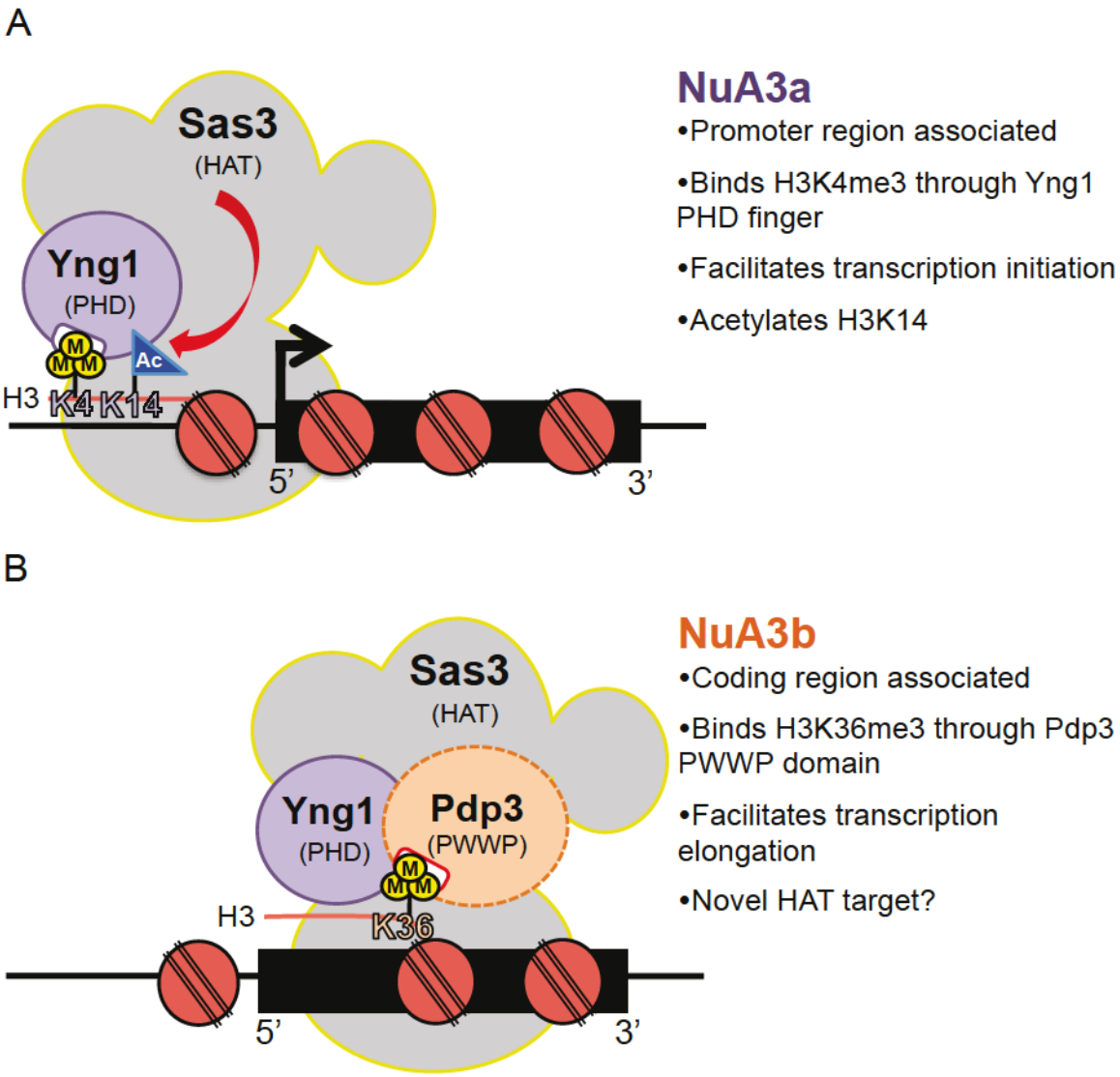


Figure 17 The NuA3 HAT complex exits in two functionally distinct forms that facilitate transcription. A.) Model of the NuA3a HAT complex[66]. NuA3a binds H3K4me3 at promoter regions through the PHD finger of Yng1[55,107,108]. Sas3 then acetylates H3K14, leading to transcription initiation of a subset of genes[55,104-106]. B.) Model of the NuA3b HAT complex[66]. NuA3b contains a unique member, Pdp3. NuA3b binds H3K36me3 at ORFs through the PWWP domain of Pdp3 to positively regulate transcription elongation. Although the function of NuA3b in the elongation pathway is not fully defined, we speculate that Sas3 may acetylate histones or non-histone proteins to evict nucleosomes.

We find that Pdp3, and thereby NuA3b, positively regulate transcription elongation, yet the mechanism of NuA3b activity remains unclear. Since NuA3-directed transcription is decreased in *pdp3Δ* cells, NuA3b may participate in acetylation-dependent nucleosome eviction, similar to the proposed function of Gcn5 at ORFs[242-244,253]. However, *PDP3* is not synthetically lethal with *GCN5*, suggesting NuA3b may acetylate a different histone residue or a non-histone protein to signal for transcription elongation **(Figure 17B)**. Interestingly, a related human HAT complex, HBO1, can switch between histone H4 and H3 acetylation depending on the association of JADE *versus* BRPF subunits[254]. Although speculative, NuA3b may similarly change HAT specificity with the addition of Pdp3. Future work is needed to determine the mechanism of NuA3b activity in the transcription elongation pathway.

Finally, how specific histone PTMs regulate transcription remains an important question in chromatin biology. For example, although H3K36me2 and H3K36me3 are both linked to transcription elongation, these marks are associated with different functions[20,209,217]. In yeast H3K36me2 suppresses intergenic transcription via HDAC complex recruitment, while H3K36me3 facilitates transcription elongation via HAT complex recruitment[223,224,228,232]. We propose that differential methyl states of H3K36 may act as a "switch" to regulate the progression of RNAPII. For example, on lowly transcribed genes, the H3K36me2 state may predominate and recruit Rpd3S to maintain a repressive chromatin environment. Conversely, on highly transcribed genes, the H3K36me3 state may predominate and recruit NuA3b (and other HATs such as NuA4) to facilitate nucleosome disruption in front of elongating RNAPII[232]. Alternatively, H3K36me2 and H3K36me3 may

coordinate HDAC and HAT activities within the same ORF to modulate transcript levels. Future studies are required to test these models.

Chapter 4: The Conserved ET Domain of Taf14 Mediates an Interaction with Yng1 and is Important for NuA3-Directed HAT Activity

4.1 Introduction

Chromatin acts as a barrier to multiple cellular processes, including transcription[16]. Therefore, nucleosome organization provides a mechanism for the regulation of gene expression[17]. Nucleosome organization is coordinated in part by chromatin-associated protein complexes that modulate histone post-translational modifications (PTMs)[19,20]. These complexes often contain both an enzymatic subunit(s) and an effector protein(s) that binds specific histone PTMs, allowing for the precise targeting of chromatin-templated activity[13,17,19,21,49,73,98].

Histone PTMs are deposited throughout the genome in a context dependent manner[13,19,23,33,53,210] **(Figure 2B)**. For example, histone H3 is often both trimethylated on lysine 4 (H3K4me3) and acetylated on lysine 14 (H3K14ac) at the 5'-ends of transcriptionally active genes[23,31-33,52,53,133]. Alternatively, H3K36me3 is found throughout the open reading frames (ORFs) of expressed genes[32,33,53,209]. The conserved *S. cerevisiae* NuA3 histone acetyltransferase (HAT) complex can modulate its localization and function by engaging ("reading") and adding ("writing") these differentially positioned histone PTMs[55,66,104-108,110]. NuA3a specifically binds H3K4me3 through the plant homeodomain (PHD) finger of the Yng1 subunit[55,107] **(Figure 17A)**. NuA3a then acetylates H3K14, leading to transcription initiation at a

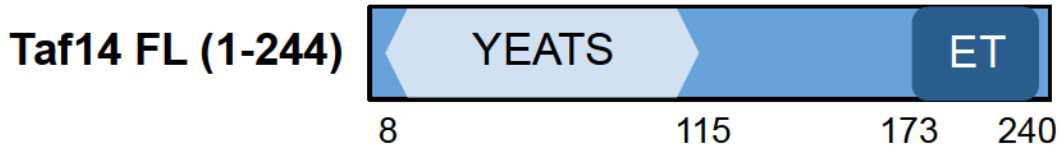
subset of genes[55,105,106] **(Figure 17A)**. NuA3b contains the unique member, Pdp3, a proline-tryptophan-tryptophan-proline (PWWP) domain protein[66] **(Figure 17B)**. NuA3b binds H3K36me3 through the PWWP domain of Pdp3 to facilitate transcription elongation[66] **(Figure 17B)**.

In addition to Yng1 and Pdp3, NuA3 contains other effector proteins, including the YEATS (named for Yaf9, ENL, AF9, Taf14, and Sas5) domain protein, Taf14[105]. Taf14 is a subunit of multiple chromatin-associated complexes (TFIID, TFIIF, Ino80, SWI/SNF, RSC, Mediator, and NuA3) and has links to transcription initiation, transcription elongation, cell cycle progression, cytoskeletal-organization, post-replication repair, and chromosome stability[104,105,118-132,140-143,255]. While it is known that C-terminal residues 124-244 are required for Taf14's engagement with chromatin-associated complexes *in vivo*, the mechanism of Taf14-directed interactions remains poorly defined[129,132,216].

Recently, residues 173-240 of the Taf14 C-terminus have been classified as an extra-terminal (ET) domain[144] **(Figure 18A)**. Of note, this domain has previously been annotated as the Taf14 C-box[128]. ET domains are structurally defined by hydrophobic clusters containing 3 α-helices, a characteristic α_2/α_3 loop, and acidic patches that are predicted to act as protein-protein interaction surfaces[144,176,256] **(Figure 6A)**. ET domains bridge the previously unrelated YEATS family and bromodomain and extra-terminal (BET) family proteins[144]. For example, the Taf14 ET domain shows hydrophobic cluster conservation with YEATS proteins Sas5 (*S. cerevisiae*), AF9 (*H. sapiens*), and ENL (*H. sapiens*), as well as BET proteins Bdf1 (*S. cerevisiae*), Bdf2 (*S. cerevisiae*), BRD2 (*H. sapiens*), BRD3 (*H. sapiens*), and

Figure 18

A



B

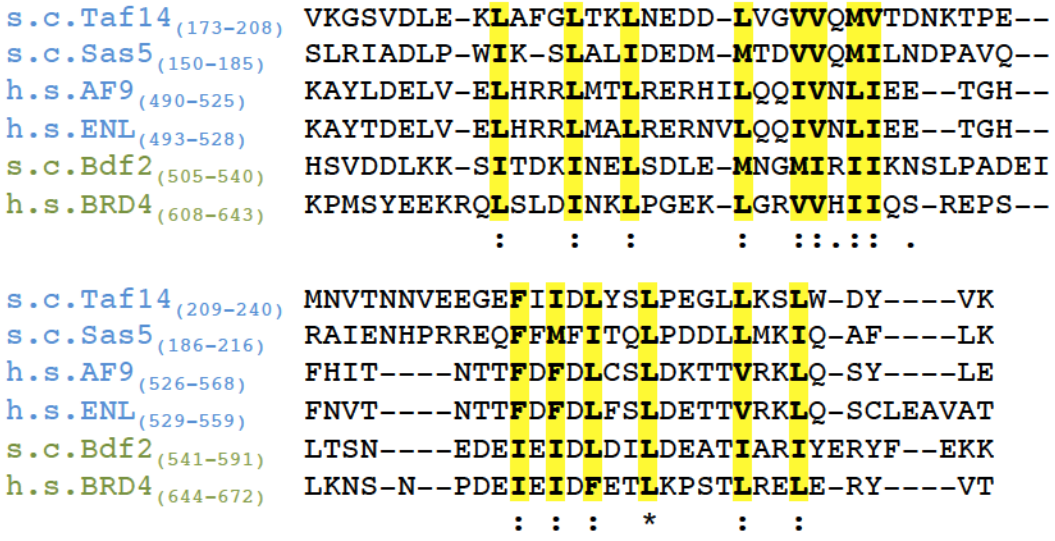


Figure 18 Taf14 contains an ET domain that is conserved in YEATS and BET family proteins. A.) Schematic representation of the *S. cerevisiae* Taf14 protein. The YEATS domain (light blue hexagon) spans residues 8-115. The ET domain (dark blue rectangle) spans residues 173-240[144]. B.) Clustal W alignment of conserved ET domains from YEATS (blue) and BET (green) family proteins[144]. Hydrophobic clusters are highlighted in yellow. Adapted from Faure and Callebaut[144].

BRD4 (*H. sapiens*)[144] **(Figure 18B)**. Interestingly, the ET domain of mammalian AF9 directly binds specific regulators of transcription, including AF4, Dot1L, BCoR, hPC3, and Rnf2[166,171-177] **(Figures 6A, 6B)**. Likewise, the ET domain of mammalian BRD4 directly binds a distinct set of regulators, including GLTSCR1, ATAD5, NSD3, JMJD6, CHD4, LANA-1 and MLV-IN[183,257-259] **(Figure 6B)**. Therefore, ET domains may generally function to modulate protein-protein interactions[144].

Importantly, the ET domains of AF9, ENL, and BRD4 are found in multiple cancer-associated chromosomal translocations[162,163,185,260-262]. MLL-AF9 and MLL-ENL fusions aberrantly recruit elongation factors to HOX genes, resulting in the transformation of hematopoietic stem cells and acute leukemia[75,160,164,166-170,263]. For example, AF9 and ENL ET domains bind to AF4, which activates the P-TEFb complex and releases paused RNA polymerase II (RNAPII) at coding regions, allowing transcription elongation to proceed[164,166,176]. Inappropriate release of paused RNAPII propagates downstream pathogenesis via the overexpression of HOX genes and subsequent hematopoietic stem cell renewal[160,169]. Disruption of the AF9-AF4 interaction promotes cell death in MLL cancer cell lines, suggesting ET domains represent promising pharmacological targets[178,180].

Similarly, the ET domain of BRD4 interacts directly with JMJD6, which activates the P-TEFb complex and releases paused RNAPII at coding regions via long-range enhancer interactions[181-184]. Aberrant enhancer-driven P-TEFb activation by BRD4-NUT or other BRD4 pathologies is implicated in many diseases including midline-carcinoma, acute myeloid leukemia (AML), multiple myeloma, lymphoma, sepsis, and heart disease[185-187,190,191,204]. BRD4 BET bromodomain inhibitors, such

as JQ1 and I-BET, have striking anti-cancer effects via the downregulation of oncogenes including *MYC*, *BCL2*, and *CDK6*[186-194]. However, toxicity and drug resistance remain a persistent problem for developing bromodomain-targeted therapies[264]. To date, the ET domain of BRD4 remains pharmacologically uncharacterized and may provide a novel target for BRD4 pathologies.

Here, using mass spectrometric, biochemical, and genetic analyses we further classify ET domains as protein-protein interaction modules. In the *S. cerevisiae* NuA3 HAT complex, the ET domain of Taf14 directly engages residues 105-125 of Yng1. Deletion of the Taf14 ET domain is synthetically lethal with a deletion of the HAT, *GCN5*, suggesting the ET domain is required for NuA3 HAT activity. Interestingly, the *H. sapiens* ET domain proteins AF9 and BRD4 also engage residues 105-125 of Yng1. Therefore, this *S. cerevisiae* Yng1 sequence may contain a conserved ET domain recognition motif and provide a mechanism to inhibit pathological ET domain interactions.

4.2 Methods

4.2A *S. cerevisiae* Strains

All strains used in **Chapter 4** are described in **Table 7**.

4.2B *S. cerevisiae* Plasmids

All constructs used in **Chapter 4** are described in **Table 8**.

4.2C *E. coli* Plasmids

All constructs used in **Chapter 4** are described in **Table 9**.

Table 7
S. cerevisiae strains used in Chapter 4

Strain	Genotype	Source
BY471	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
TAF14-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TAF14-TAP::HIS3*	Open Biosystems
taf14ΔET-TAP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 TAF14Δ180-244-TAP::HIS3*	A. Raman, S. Taverna Lab
gcn5Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gcn5Δ::kanMX [pmk120]*	Open Biosystems
gcn5Δ; taf14Δ	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 taf14Δ::hph gcn5Δ::kanMX [pmk120]*	S. McDaniel, B. Strahl Lab

Table 8
***S. cerevisiae* plasmids used in Chapter 4**

Name	Description	Source
pMK120	*CUP1-pr URA3*	M. Kuo Lab
Vector	pRS313-3HA	B. Strahl Lab
pTaf14	pRS313-Taf14-3HA	S. McDaniel, B. Strahl Lab
pTaf14ΔET	pRS313-Taf14ΔET-3HA	S. McDaniel, B. Strahl Lab

Table 9
***E. coli* plasmids used in Chapter 4**
Source: T. Gilbert, S. Taverna Lab

Name	Description	Vector
MBP Alone	HIS6X-pfuMBP(aa 60-434)-FLAG	pET28 HisMBPFLAG
Eaf6 FL	Eaf6 aa 1-113	pET28 HisMBPFLAG
Pdp3 FL	Pdp3 aa 1-304	pET28 HisMBPFLAG
Yng1 FL	Yng1 aa 1-219	pET28 HisMBPFLAG
Taf14 ET	Taf14 aa 173-240	pET28 HisMBPFLAG
Sas5 ET	Sas5 aa 150-216	pET28 HisMBPFLAG
Bdf2 ET	Bdf2 aa 505-591	pET28 HisMBPFLAG
AF9 ET	AF9 aa 490-568	pET28 HisMBPFLAG
ENL ET	ENL aa 493-559	pET28 HisMBPFLAG
BRD4 ET	BRD4 aa 608-672	pET28 HisMBPFLAG
N-term(1-140)	Yng1 aa 1-140	pET28 HisMBPFLAG
PHD finger(141-219)	Yng1 aa 141-219	pET28 HisMBPFLAG
Hinge(105-155)	Yng1 aa 105-155	pET28 HisMBPFLAG
Hinge(105-140)	Yng1 aa 105-140	pET28 HisMBPFLAG
Hinge(105-130)	Yng1 aa 105-130	pET28 HisMBPFLAG
Hinge(105-125)	Yng1 aa 105-125	pET28 HisMBPFLAG
Hinge(120-140)	Yng1 aa 120-140	pET28 HisMBPFLAG
Yng1 W180E FL	Yng1 aa 1-219	pET28 HisMBPFLAG
Yng1 P108A FL	Yng1 aa 1-219	pET28 HisMBPFLAG
Yng1 L116W FL	Yng1 aa 1-219	pET28 HisMBPFLAG
Yng1 L117S FL	Yng1 aa 1-219	pET28 HisMBPFLAG
Yng1 K119A FL	Yng1 aa 1-219	pET28 HisMBPFLAG
Yng1 L122W FL	Yng1 aa 1-219	pET28 HisMBPFLAG
Pho23 FL	Pho23 aa 1-330	pET28 HisMBPFLAG
S•tag Alone	Thioredoxin-HIS6X-S•tag	pET32a
Taf14 FL	Taf14 aa 2-244	pET32a
Taf14 ΔET	Taf14 aa 2-179	pET32a
Yng1 FL	Yng1 aa 2-219	pET32a
Hinge(105-130)	Yng1 aa 105-130	pET32a

4.2D Mass Spectrometric Protein Identification for Cellular Pull Down Assays

BY471 *S. cerevisiae* were grown to mid-log phase in YPD, cryogenically lysed with a mixer mill (Retsch MM301), and stored at −80 °C. Cells (2 g per pull down condition) were resuspended in extraction buffer (20 mM HEPES pH 7.9, .1% Tween-20, 2 mM $MgCl_2$, 300 mM NaCl, 1 mM PMSF, 2 mM Benzamidine) at a ratio of 10 mL buffer per 2 g yeast. Cells were treated with ~.002% DNaseI for 10 min at room temperature. Cells were homogenized (ProScientific) and nutated for 1 hr at 4 °C. Extracts were sonicated (Branson) on the lowest setting for 12 rounds of 30 sec on/off in 20 mL aliquots. Clarified extracts were mixed with 125 µL per sample of Protein G Dynabeads (Life Technologies) that were pre-coated with either the appropriate ET domain (Taf14 ET, Sas5 ET, or Bdf2 ET) or the HIS6X-pfuMBP$_{(60-434)}$-FLAG tag alone control. Extracts were nutated for 4 hr at 4 °C. Resin was washed 5 times with extraction buffer and 1 time with buffer containing 20 mM HEPES pH 7.9, .1% Tween-20, 2 mM $MgCl_2$, and 10 mM NaCl. Resin was resuspended in 125 µL per sample of FLAG peptide (100 µg/mL in TBS) (F3290, Sigma Aldrich) and incubated for 10 min at room temperature with gentle agitation to elute ET domain/tag-bound proteins. Supernatant was collected and the FLAG peptide elution was repeated 4 times. Eluates were lyophilized, resuspended in 2X NuPAGE LDS sample buffer (Life Technologies), boiled for 5 min, and resolved on 12% NuPAGE Novex Bis-Tris gels (Life Technologies). Gels were stained with Simply Blue Safe Stain (Invitrogen) and lanes were excised for tandem MS analysis of peptides with a Thermo LTQ-XL mass spectrometer coupled to an Eksigent nanoLC 2D system as previously described[44]. Tandem mass spectra were extracted

by Thermo ExtractMSn version 1.0.0.8 and analyzed by Mascot (Matrix Science, London, UK; version 2.3.01). Scaffold (version Scaffold_4.0.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. BCMB rotation student Raiha Tahir assisted in yeast preparation and Anne M. Cieniewicz excised bands for MS analysis. All MS experiments and analyses were performed by the laboratory of Dr. Alan J. Tackett at the University of Arkansas for Medical Sciences. I would like to acknowledge Dr. Alan J. Tackett, Dr. Stephanie D. Byrum, and Lisa M. Orr for their many important contributions to this project.

4.2E Protein Expression

Constructs were made with an N-terminal HIS6X-pfuMBP$_{(60\text{-}434)}$-FLAG tag (pET28a derivative vector obtained from the Greg Bowman Laboratory, Johns Hopkins University) and/or an N-terminal Thioredoxin-HIS6X-S•tag tag (pET32a vector, Millipore). Proteins were exogenously expressed in chemically competent BL21 (Invitrogen) or Rosetta2(DE3) (EMD Millipore) *E. coli* after overnight induction with 1 mM IPTG at 18-20 ºC in LB medium. Point mutants were made with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and expressed as described.

4.2F Protein Purification

E. coli cells were resuspended in purification buffer (50 mM Tris pH 7.5, 500 mM NaCl, 40 mM imidazole, 10% glycerol, 5 mM DTT, 1 mM PMSF, 2 mM benzamidine, pH 8.0- pET28a constructs) or (50 mM Tris pH 7.5, 500 mM NaCl, 10 mM imidazole, 10% glycerol, 5 mM DTT, 1 mM PMSF, 2 mM benzamidine, pH 8.0- pET32a constructs) and lysed by sonication (Branson). Clarified lysate was nutated with Ni-

NTA agarose resin (Invitrogen) for at least 1 hr at 4 °C. Resin was washed with purification buffer and protein was eluted with purification buffer containing 300 mM imidazole. Protein was frozen in liquid nitrogen and stored at −80 °C. BCMB rotation student Raiha Tahir assisted with protein purification.

4.2G *In Vitro* Pull Down Assays

Protein G Dynabeads (Life Technologies) (50 µL per sample) were incubated with FLAG (SIGMA F3165) or S•tag (Abcam 18588) antibody (2 µg per sample) in PBS for 1 hr at room temperature. Beads were washed with PBS and incubated with "bait" proteins (20 µg per sample) in binding buffer (20 mM HEPES pH 7.9, 150 mM NaCl, 1 mM PMSF, 2mM Benzamidine, .05% Tween-20, 10% glycerol, .25% BSA) for 1 hr at room temperature. Unbound "bait" was washed in binding buffer and beads were incubated with "prey" proteins (20 µg per sample) for 1 hr at room temperature. Beads were washed 3 times with binding buffer and 1 time with buffer containing 4 mM HEPES pH 7.9, 10 mM NaCl, .05% Tween-20, and 5% glycerol. Proteins were eluted in boiling 2X SDS-PAGE loading buffer. Samples were resolved on 15% or 12% SDS-polyacrylamide gels, transferred to PVDF membrane, and probed with antibodies recognizing the FLAG (SIGMA F3165, 1/1000) and S•tag (Abcam 18588, 1/1000) tags. Immunoblots were visualized using HRP-conjugated secondary antibodies (NA931-1ML, 1/10,000 and NA934-1ML, 1/7,500) and ECL solution (GE Healthcare). Inputs represent 0.25-0.5 µg of protein. BCMB rotation student Raiha Tahir performed a second biological replicate of pull down experiments. I would like to recognize Raiha Tahir for her hard work and dedication to this project.

4.2H Yeast Strains and Cell Spotting Assays

S. cerevisiae strains were created using heterologous gene replacement[245]. For cell spotting assays, 0.5 ODs of cells were 5-fold serially diluted, spotted onto the appropriate plates, and incubated at 37 °C or 30 °C as indicated for 2 days. Yeast strains/plasmids were created by Stephen L. McDaniel in the laboratory of Dr. Brian D. Strahl at the University of North Carolina at Chapel Hill and Ana Raman. Cell spotting assays were performed by Stephen L. McDaniel. I would like to acknowledge Dr. Brian D. Strahl and Stephen L. McDaniel for their thoughtful contributions to this project.

4.3 Results

4.3A The ET Domain of Taf14 Directly Engages Yng1 in the NuA3 HAT Complex

Taf14 is a *S. cerevisiae* YEATS domain protein found in the NuA3 HAT complex and other chromatin-associated complexes[105,129]. The C-terminus of Taf14 is required for its interaction with these complexes *in vivo*[129,132,216]. Recently, C-terminal residues 173-240 of Taf14 have been classified as an ET domain, which is distinguished by conserved hyrdrophobic clusters that bridge the previously unrelated YEATS and BET family proteins[144] **(Figures 18A, 18B)**. ET domains are predicted to act as protein-protein interaction surfaces and may function as molecular scaffolds[144,176,256]. As the NuA3 effector proteins Yng1 and Pdp3 modulate transcription initiation and elongation pathways, respectively, we wanted to probe the biological role of the Taf14 ET domain in NuA3-directed transcription.

To determine if the ET domain of Taf14 mediates its interaction with NuA3, we first tested the ability of full-length Taf14 to pull down full-length NuA3 subunits Pdp3, Eaf6, and Yng1. Immunoprecipitated samples were resolved by SDS-PAGE and visualized by western blotting. Taf14 directly binds Yng1, but not Pdp3 or Eaf6 **(Figure 19)**. We were unable to recombinantly express full-length Nto1 and Sas3, and therefore we could not assess direct interactions between Taf14 and these subunits. However, previous studies suggest that Taf14 binds Sas3[129].

To establish that the ET domain is required for the interaction between Taf14 and Yng1, we created a $Taf14_{(2\text{-}173)}$ (Δ ET) construct. Taf14 ΔET does not pull down Yng1, indicating the ET domain is necessary for binding **(Figure 20A)**. To verify that the ET domain is sufficient for the interaction between Taf14 and Yng1, we created a MBP-HIS6X-FLAG-tagged $Taf14_{(173\text{-}240)}$ (ET) construct. Taf14 ET pulls down Yng1, indicating the ET domain is sufficient for binding **(Figure 20B)**. Of note, only MBP-containing Taf14 ET constructs are stable, suggesting yeast ET domains may be intrinsically disordered like the human AF9 ET domain[176].

4.3B A Conserved Motif within Yng1 Binds Yeast and Human ET Domains

To further confirm that the ET domain of Taf14 engages Yng1, we tested the ability of full-length Yng1 to pull down Taf14 ET. Yng1 directly binds Taf14 ET, supporting our previous findings **(Figures 20B, 21)**. To investigate the potential for a conserved binding mechanism, we created MBP-HIS6X-FLAG-tagged *S. cerevisiae* ET domain constructs: $Sas5_{(150\text{-}216)}$ and $Bdf2_{(505\text{-}591)}$, as well as *H. sapiens* ET domain constructs: $AF9_{(490\text{-}568)}$, $ENL_{(493\text{-}559)}$, and $BRD4_{(608\text{-}672)}$. Again, the MBP tag was required for protein stability. To our surprise, in addition to Taf14, Yng1 directly binds

Figure 19

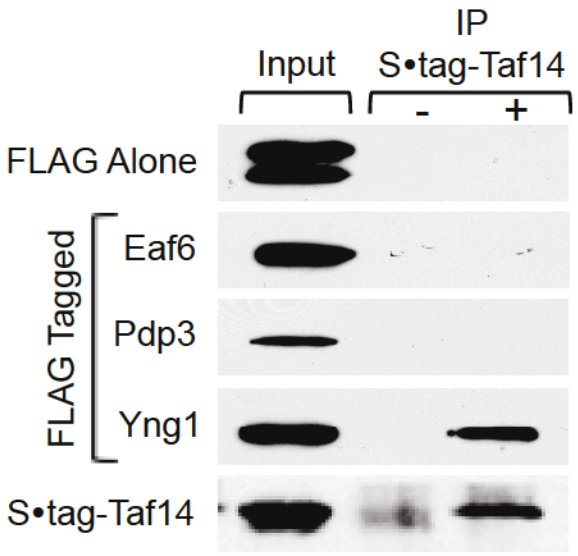


Figure 19 Taf14 directly interacts with the Yng1 subunit of NuA3. Pull down assays were performed with purified S•tag alone (-) or full-length S•tag-Taf14 (+) coated resin and purified full-length FLAG-tagged NuA3 subunits: Eaf6, Pdp3, and Yng1. Purified proteins (input) and immunoprecipitated samples (IP) were resolved by SDS-PAGE. Binding was monitored by western blotting to FLAG and S•tag.

Figure 20

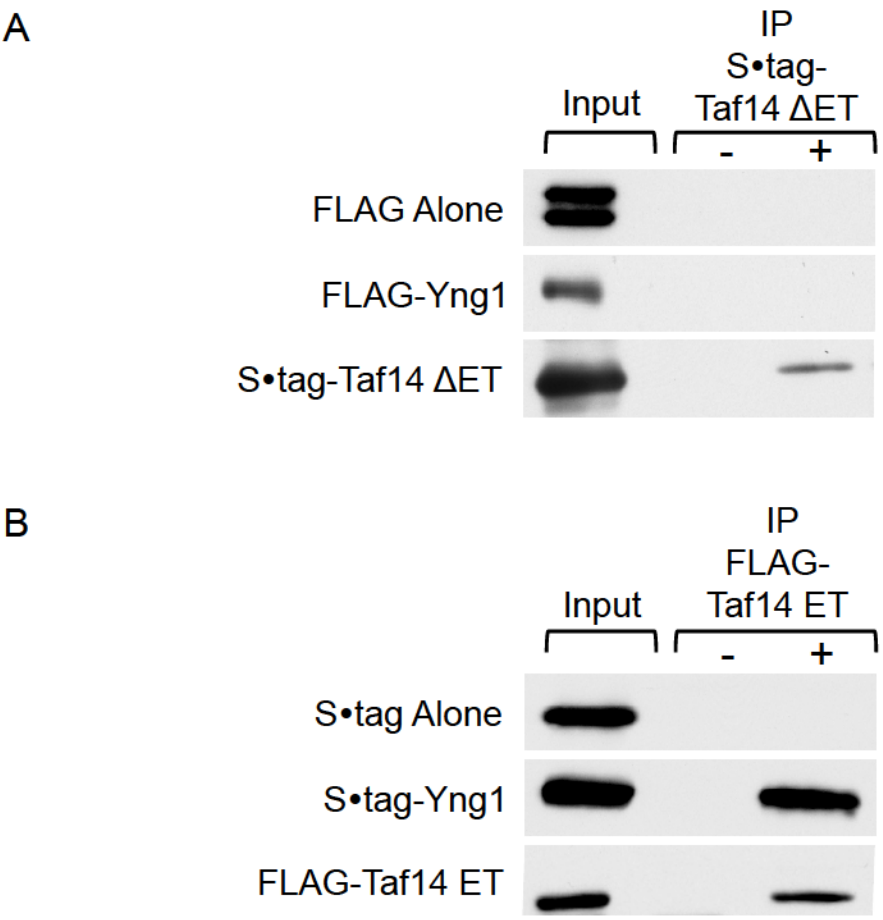


Figure 20 The ET domain of Taf14 is necessary and sufficient to interact with Yng1. A.) Pull down assays were performed with purified S•tag alone (-) or S•tag-Taf14 ΔET (+) coated resin and purified full-length FLAG-Yng1. Purified proteins (input) and immunoprecipitated samples (IP) were resolved by SDS-PAGE. Binding was monitored by western blotting to FLAG and S•tag. B.) Pull down assays were performed with purified FLAG alone (-) or FLAG-Taf14 ET (+) coated resin and purified full-length S•tag-Yng1. Purified proteins (input) and immunoprecipitated samples (IP) were resolved by SDS-PAGE. Binding was monitored by western blotting to S•tag and FLAG.

Figure 21

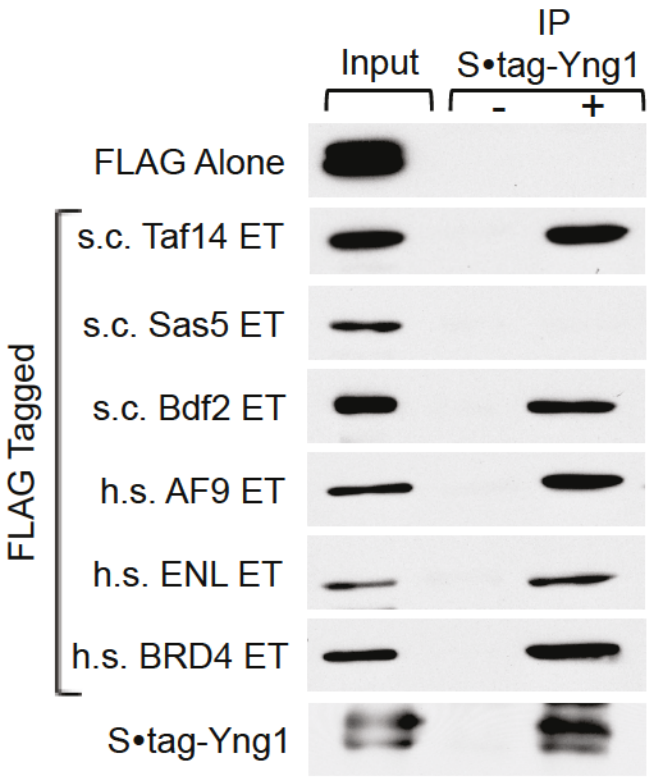


Figure 21 Yeast and human ET domains interact with Yng1. Pull down assays were performed with purified S•tag alone (-) or full-length S•tag-Yng1 (+) coated resin and purified FLAG-tagged ET domains: Taf14, Sas5, Bdf2, AF9, ENL, and BRD4. Purified proteins (input) and immunoprecipitated samples (IP) were resolved by SDS-PAGE. Binding was monitored by western blotting to FLAG and S•tag.

the ET domains of yeast Bdf2 and human AF9, ENL, and BRD4 **(Figure 21)**. This suggests that a subset of ET domains recognize a conserved signature contained within the Yng1 protein.

We next wanted to define a recognition motif within Yng1. We tested the ability of full-length Taf14 to pull down a series of Yng1 truncation constructs: N-term$_{(1\text{-}140)}$, PHD finger$_{(141\text{-}219)}$, Hinge$_{(105\text{-}155)}$, Hinge$_{(105\text{-}140)}$, Hinge$_{(105\text{-}130)}$, Hinge$_{(105\text{-}125)}$, and Hinge$_{(120\text{-}140)}$. Hinge$_{(105\text{-}125)}$ is sufficient to bind Taf14, suggesting a critical recognition motif exists between residues 105-125 of Yng1 **(Figure 22A)**. Importantly, Hinge$_{(105\text{-}130)}$ retains an interaction with Taf14 ET, further supporting this idea **(Figure 22B)**.

Interestingly, Hinge$_{(105\text{-}130)}$ also interacts with AF9 ET and BRD4 ET **(Figure 23A)**. We aligned residues 105-125 of Yng1 with known AF9 and BRD4 binding partners[176,183]. Yng1 contains a motif previously annotated in AF9 interactors, L-X-L-X-I-X-L, as well as an upstream L-X-X-P motif[176] **(Figures 23B, 23C)**. To determine the importance of these motifs in ET domain recognition, we tested the ability of Taf14 to pull down full-length Yng1 mutated at residues P108, L116, L117, K119, L122, and W180. W180E served as a negative control, as this residue is only critical for the interaction between the Yng1 PHD finger and H3K4me3. Full-length Pho23 was tested as an additional negative control, as Pho23 contains a PHD finger that binds H3K4me3, but does not contain either the L-X-L-X-I-X-L or L-X-X-P motifs. L116W, L117S, K119A, and L122W mutations independently abolish or reduce the interaction between Yng1 and Taf14 **(Figure 24)**. These results suggest that the

Figure 22

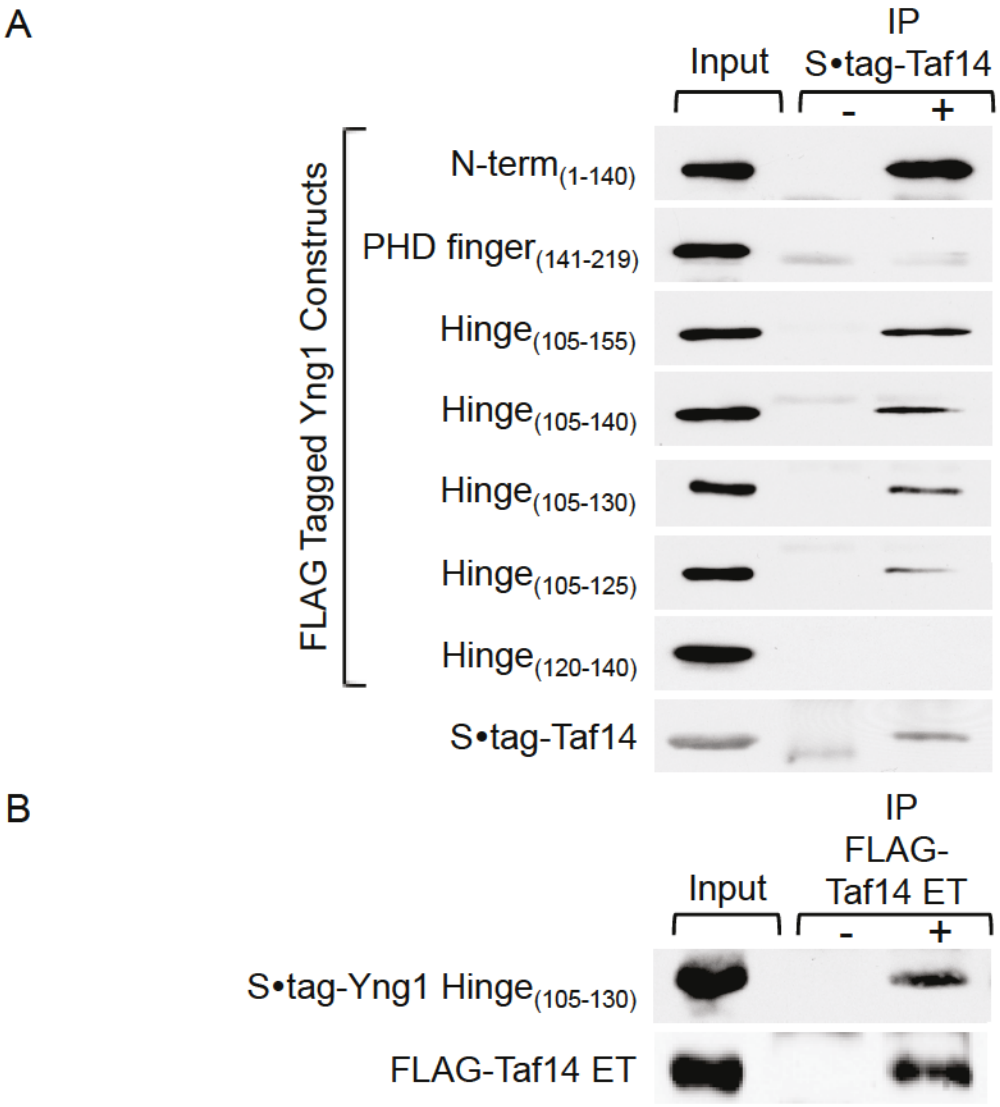
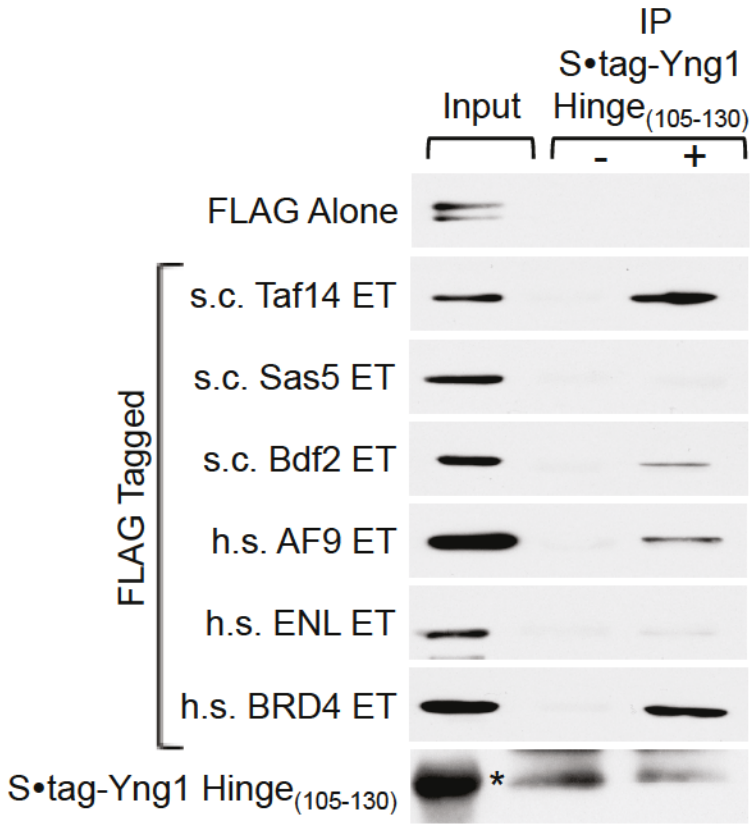


Figure 22 A small region of Yng1 is sufficient to interact with the Taf14 ET domain. A.) Pull down assays were performed with purified S•tag alone (-) or full-length S•tag-Taf14 (+) coated resin and purified FLAG-tagged Yng1 truncations: N-term$_{(1-140)}$, PHD finger$_{(141-219)}$, Hinge$_{(105-155)}$, Hinge$_{(105-140)}$, Hinge$_{(105-130)}$, Hinge$_{(105-125)}$, and Hinge$_{(120-140)}$. Purified proteins (input) and immunoprecipitated samples (IP) were resolved by SDS-PAGE. Binding was monitored by western blotting to FLAG and S•tag. B.) Pull down assays were performed with purified FLAG alone (-) or FLAG-Taf14 ET (+) coated resin and purified S•tag-Hinge$_{(105-130)}$. Purified proteins (input) and immunoprecipitated samples (IP) were resolved by SDS-PAGE. Binding was monitored by western blotting to S•tag and FLAG.

Figure 23

A



B

```
s.c.Yng1(105-125)    --LEEPGAY--KEPKLLLKINLKKAK---
h.s.BCoR(1181-1207) NHLEDPHYS--ELTNLKVCIELTGLHPKK
h.s.AF4(746-771)     TKLLSPLRDTPPPQSLMVKITLDLLS---
h.s.Dot1L(866-891)   -PISIPLSTV-QPNKLPVSIPLASVVLP-
                       :  *        .* : * *
```

C

```
s.c.Yng1(105-125)   LEEPGA----------------YKEPKLLLKINLKKA
h.s.JMJD6(188-225) IDPLGTSAWNALVQGHKRWCLFPTSTPRELIKVTRDEG
                   ::  *:                  . *: *:*:. .:.
```

Figure 23 A small conserved region of Yng1 is sufficient to interact with yeast and human ET domains. A.) Pull down assays were performed with purified S•tag alone (-) or S•tag-Hinge$_{(105-130)}$ (+) coated resin and purified FLAG-tagged ET domains: Taf14, Sas5, Bdf2, AF9, ENL, and BRD4. Purified proteins (input) and immunoprecipitated samples (IP) were resolved by SDS-PAGE. Binding was monitored by western blotting to FLAG and S•tag. * The S•tag alone and S•tag-Hinge$_{(105-130)}$ proteins are very close in MW, and therefore these bands appear side by side. B/C.) Clustal W alignments of Yng1 with binding partners of AF9 and BRD4, respectively[169,171-176,183,184]. A potential ET domain recognition motif is highlighted in red[176].

Figure 24

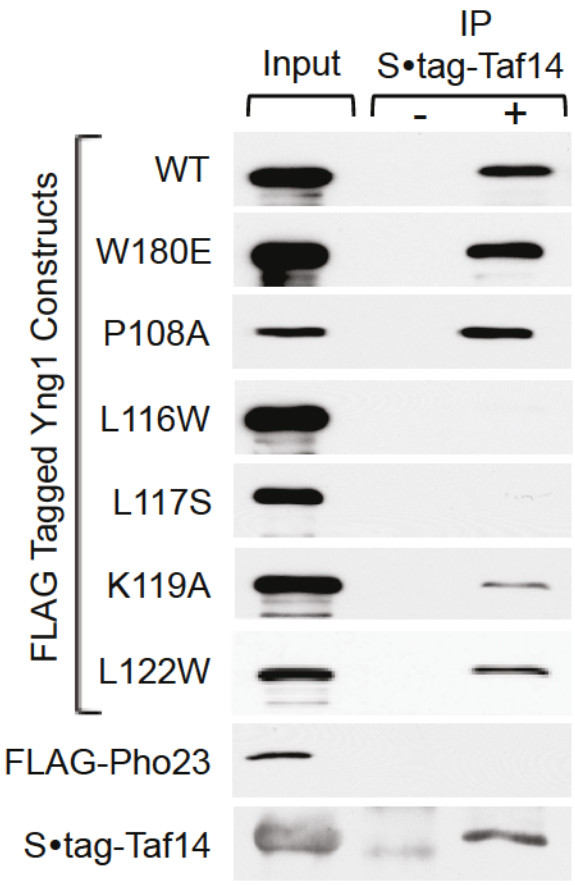


Figure 24 Mutation of a conserved motif within Yng1 disrupts Taf14 binding. Pull down assays were performed with purified S•tag alone (-) or full-length S•tag-Taf14 (+) coated resin and purified full-length FLAG-tagged Yng1 constructs: WT, W180E, P108A, L116W, L117S, K119A, and L122W. Full-length FLAG-Pho23 was also tested. Purified proteins (input) and immunoprecipitated samples (IP) were resolved by SDS-PAGE. Binding was monitored by western blotting to FLAG and S•tag.

region of Yng1 surrounding and including the L-X-L-X-I-X-L motif is critical for binding Taf14. Conversely, as P108A has no affect on Taf14 binding, the L-X-X-P motif may generally be dispensable for ET domain interactions **(Figure 24)**.

Since the ET domains of AF9 and BRD4 propagate aberrant protein-protein interactions in many diseases including midline-carcinoma, leukemia, sepsis, and heart disease, a shared recognition signature (such as L-X-L-X-I-X-L) may provide a novel mechanism to block downstream pathogenesis[164,185,204]. We hypothesize that with optimization a biologic could be constructed to specifically and potently block ET domain interactions.

4.3C NuA3 Requires the ET domain of Taf14 for Functional Activity

To further understand the biological role of ET domains, we investigated the importance of the Taf14 ET domain *in vivo*. We find that *taf14ΔET* has growth defects that phenocopy *taf14Δ*, indicating the ET domain is critical for Taf14 function **(Figure 25A)**. It has previously been determined that *SAS3* and *YNG1* display synthetic lethality with the HAT, *GCN5,* as NuA3 and Gcn5-associated complexes both acetylate H3K14 to promote transcription[110,242,244,252]. To determine if Taf14 regulates NuA3 HAT activity, we performed a *GCN5* plasmid shuffle assay in the *gcn5Δtaf14Δ* background. Interestingly, both *gcn5Δtaf14Δ* and *gcn5Δtaf14Δ* + pTaf14ΔET cells show synthetic lethality, indicating *TAF14* genetically interacts with *GCN5* **(Figure 25B)**. These data suggest that the ET domain of Taf14 is required for NuA3-directed H3K14ac HAT activity. We speculate that through an interaction with Yng1, Taf14 links NuA3 to other chromatin-associated complexes, which collectively facilitate transcription.

Figure 25

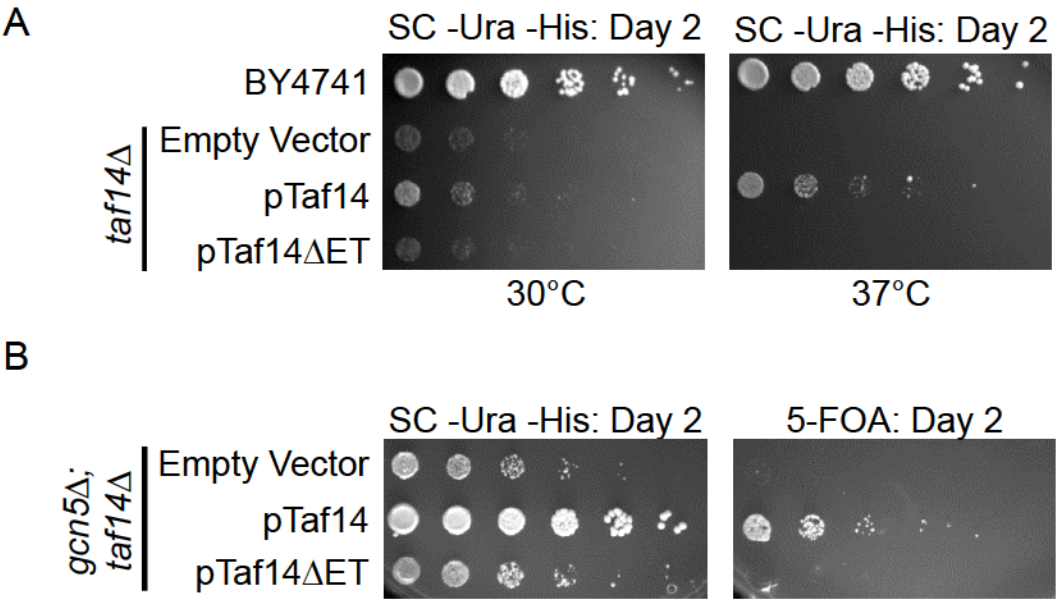


Figure 25 The ET domain of Taf14 is required for NuA3 H3K14ac HAT activity. A.) 0.5 ODs of the indicated *S. cerevisiae* strains were 5-fold serially diluted onto SC -Ura -His plates and grown at 30 °C (left) or 37 °C (right) for two days. B.) 0.5 ODs of the indicated *S. cerevisiae* strains were 5-fold serially diluted onto SC -Ura -His (left) or SC -Ura -His + 5-FOA (right) plates and grown at 30 °C for two days. These experiments were performed by Stephen L. McDaniel in the laboratory of Dr. Brian D. Strahl at the University of North Carolina at Chapel Hill.

4.3D Yeast ET Domains Facilitate Protein-Protein Interactions

As ET domains are predicted to act as protein-protein interaction modules and the Taf14 ET domain is required for NuA3 HAT activity, we performed a screen to search for novel yeast ET domain interactors[144]. Purified Taf14 ET, Sas5 ET, and Bdf2 ET were used as "baits" and incubated with wild type *S. cerevisiae* lysate. The MBP-HIS6X-FLAG tag alone was used as a control for non-specific binding. Immunoprecipitated samples were resolved by SDS-PAGE and subjected to tandem MS analysis. Relative spectral counts were calculated for each ET domain and compared to the tag alone control. Enriched ET domain binding partners were categorized by gene ontology (GO) analysis. Recombinant ET domains from Taf14, Sas5, and Bdf2 interact with many diverse protein families, including members of biosynthetic, metabolic, translation, complex subunit organization, transcription initiation, and gene expression pathways **(Figures 26A, 26B, 26C)**.

As NuA3 regulates transcriptional activation, we focused on interactions relating to transcription initiation and gene expression pathways[55,66]. Taf14 ET interacts with known Taf14-associated complexes: TFIID, TFIIF, Ino80, and SWI/SNF **(Figure 27)**. Taf14 ET also interacts with many transcriptional activators including RNAPII/III, CDK, the pre-initiation complex, elongation factors, and ATP-dependent RNA helicases **(Figure 27)**. Sas5 ET interacts with the transcriptional activators Ino80 and RNAPI/II/III, as well as the transcriptional repressors CCR-NOT and Iswi1 **(Figure 28)**. Bdf2 ET interacts with Ino80, RNAPI/II/III, Swr1, SAGA/SILK, elongation factors, ATP-dependent RNA helicases, and linker histone H1 **(Figure 29)**. Although further validation is needed, our results suggest that ET domains may

Figure 26

A

Taf14 ET-Associated Biological Processes	Number of Hits	P-Value
Metabolic process	181	1.974e-21
Biosynthetic process	121	3.845e-20
Translation	37	3.728e-08
Protein folding	18	2.599e-06
DNA-templated transcriptional preinitiation complex assembly	11	5.667e-06
Macromolecular complex assembly	36	2.612e-04
Gene expression	72	8.765e-04
Macromolecular complex subunit organization	46	3.135e-03

B

Sas5 ET-Associated Biological Processes	Number of Hits	P-Value
Biosynthetic process	66	9.293e-09
Metabolic process	101	2.210e-08
Translation	22	8.243e-05
Regulation of DNA-templated transcription initiation	6	1.749e-04
Proteasome assembly	6	2.762e-03
Macromolecular complex assembly	23	3.150e-03
Ubiquitin-dependent protein catabolic process	13	5.871e-03
Protein complex subunit organization	20	7.071e-03

C

Bdf2 ET-Associated Biological Processes	Number of Hits	P-Value
Metabolic process	239	4.740e-19
Biosynthetic process	140	7.166e-13
Translation	49	7.250e-10
Protein folding	24	4.634e-08
Posttranscriptional regulation of gene expression	20	2.306e-03
Ribonucleoprotein complex subunit organization	20	4.453e-03

Figure 26 Gene Ontology analysis of proteins co-purifying with yeast ET domains. A/B/C.) Endogenous proteins co-purifying with recombinant *S. cerevisiae* Taf14 ET, Sas5 ET, and Bdf2 ET "baits" were identified by tandem MS analysis. Spectral count enrichment was calculated relative to the tag alone control. Enriched proteins (2-fold or greater) were categorized by gene ontology analysis. Biological processes with $P < .01$ are listed.

Figure 27

Name	Spectral Count Enrichment	Taf14 ET-Associated Complex
Taf2	32.00	TFIID
Taf8	12.46	TFIID
Taf4	8.73	TFIID
Taf12	7.87	TFIID
Taf5	7.67	TFIID
Taf6	7.52	TFIID
Ino80	6.34	Ino80
T2fb	6.16	TFIIF
Taf10	5.31	TFIID
Rpb11	4.36	RNAPII
Rpc1	4.00	RNAPIII
Lsm3	3.88	Lsm8p
Taf9	3.78	TFIID
Sub2	3.70	TREX
Rvb1	3.61	Ino80/Swr1/R2TP
Cdk1	3.55	CDK
Snf2	3.20	SWI/SNF
Snf5	2.87	SWI/SNF
Sas5	2.67	Sas2/4/5
Pab1	2.65	PABP
Utp10	2.63	SSU processome
Swi3	2.54	SWI/SNF
Smd2	2.54	snRNP
Tbp	2.38	Preinitiation complex
Ies4	2.25	Ino80
Dbp2	2.18	ATP-dependent RNA helicase
Spt6	2.17	Elongation factor
Ded1	2.16	ATP-dependent RNA helicase
Rpb1	2.14	RNAPII
Rpac2	2.04	RNAPI/III

Figure 27 The ET domain of Taf14 mediates protein-protein interactions. Endogenous proteins co-purifying with recombinant *S. cerevisiae* Taf14 ET "bait" were identified by tandem MS analysis. Spectral count enrichment was calculated relative to the tag alone control. Enriched proteins (2-fold or greater) involved in DNA replication, transcription, and RNA processing are listed.

Figure 28

Name	Spectral Count Enrichment	Sas5 ET-Associated Complex
Not3	6.71	CCR4-NOT
Rpc2	5.85	RNAPIII
Rvb1	5.28	Ino80/Swr1/R2TP
Iswi1	4.97	Iswi1
Rpa34	4.92	RNAPI
Rpc1	4.04	RNAPIII
Utp10	3.33	SSU processome
Rpb11	3.32	RNAPII
Rpa12	3.02	RNAPI
Rfc2	2.88	Replication Factor C
Pds5	2.52	Cohesion Maintenance Factor
Rpb1	2.31	RNAPII
Rpa2	2.15	RNAPI
Ino80	2.09	Ino80

Figure 28 The ET domain of Sas5 mediates protein-protein interactions. Endogenous proteins co-purifying with recombinant *S. cerevisiae* Sas5 ET "bait" were identified by tandem MS analysis. Spectral count enrichment was calculated relative to the tag alone control. Enriched proteins (2-fold or greater) involved in DNA replication, transcription, and RNA processing are listed.

Figure 29

Name	Spectral Count Enrichment	Bdf2 ET-Associated Complex
Mbf1	8.29	Multiprotein Bridging Factor
Pab1	5.05	PABP
Smt3	4.92	SUMO family
Nhp6a	4.87	HMG protein family
Spt6	3.92	Elongation factor
Rvb1	3.81	Ino80/Swr1/R2TP
Rad23	3.37	NEF2
Sub2	3.25	ATP-dependent RNA helicase
Rpc1	3.07	RNAPIII
Swr1	3.01	Swr1
Sas5	3.00	Sas2/4/5
Dbp2	2.79	ATP-dependent RNA helicase
Rpac2	2.70	RNAPI/III
PCNA	2.69	PCNA
Dbp5	4.46	ATP-dependent RNA helicase
Spt5	2.41	DSIF
Utp10	2.39	SSU processome
Rpb11	2.36	RNAPII
Rvb2	2.36	Ino80/Swr1/R2TP
Top2	2.21	Topoisomerase II
Prp43	2.18	RNA helicase
Def1	2.18	RNAPII degradation factor
H1	2.16	Linker histone
Ded1	2.11	ATP-dependent RNA helicase
Chd1	2.06	SAGA/SILK
Rpa12	2.05	RNAPI

Figure 29 The ET domain of Bdf2 mediates protein-protein interactions. Endogenous proteins co-purifying with recombinant *S. cerevisiae* Bdf2 ET "bait" were identified by tandem MS analysis. Spectral count enrichment was calculated relative to the tag alone control. Enriched proteins (2-fold or greater) involved in DNA replication, transcription, and RNA processing are listed.

generally serve as scaffolds that facilitate interactions between chromatin-associated complexes.

4.4 Discussion

Here we further characterize ET domains as protein-protein interaction modules. We determine that the ET domain of *S. cerevisiae* Taf14 engages multiple chromatin-associated complexes including TFIID, TFIIF, Ino80, SWI/SNF, and NuA3. Within the NuA3 complex, Taf14 ET binds residues 105-125 of Yng1. Disruption of this interaction results in growth defects and synthetic lethality with the HAT, *GCN5,* suggesting the ET domain of Taf14 facilitates NuA3 H3K14ac HAT activity. We also find that the ET domains of *S. cerevisiae* Sas5 and Bdf2 interact with multiple transcriptional activators. Therefore, ET domains may generally contribute to transcriptional regulation by bridging chromatin-associated complexes.

Interestingly, the ET domains of *H. sapiens* AF9 and BRD4 also engage residues 105-125 of Yng1, which contain a conserved recognition motif: L-X-L-X-I-X-L[176]. We hypothesize that with optimization, a biologic could be constructed to specifically and potently inhibit ET domain interactions. For example, disruption of the AF9-AF4 interaction with a peptide mimetic promotes cell death in MLL cancer cell lines[178,180]. Such inhibitors may provide a novel treatment mechanism to block pathogenesis in ET-domain associated diseases[164,178,180,185,204].

Recently, “reader” domain targeted therapies have demonstrated clinical benefits. For example, the BET bromodomain inhibitors JQ1 and I-BET selectively target BRD4 and show promise for treating multiple forms of leukemia, inflammation,

and heart disease in mouse models[190,191,195,204,205]. JQ1 and I-BET derivatives are currently in phase I clinical trials for midline carcinoma, hematological cancer, and solid tumors[187,265]. However, such “reader” targeted therapies have serious toxicities. Recent studies from Genentech and Astra Zeneca determined that JQ1 treatment results in significant weight loss, immunosuppression, and gastrointestinal atrophy, which makes JQ1 treatment intolerable for long-time use[264]. Therefore, alternatively targeting the BRD4 ET domain may improve tolerance by specifically blocking aberrant protein-protein interactions.

Many questions remain about the function of ET domains. For example, do ET domains act solely as scaffolding modules or do they allosterically regulate the activity of their associated protein? Within NuA3, Taf14 ET does not bind the Yng1 PHD finger and conversely, mutation of the Yng1 PHD finger does not disrupt Taf14 binding. Therefore, the ET domain of Taf14 likely does not allosterically regulate the Yng1-H3K4me3 interaction. However, we determined that the ET domain of Taf14 is necessary for NuA3-directed HAT activity. Based on our MS data, we speculate that the ET domain of Taf14 functions as a molecular scaffold, which bridges NuA3 to TFIID, TFIIF, Ino80, SWI/SNF, and other chromatin-associated complexes[129]. Interestingly, Yng1 shares conserved hydrophobic residues with multiple subunits of Taf14-containing complexes including RSC30 of RSC, Snf5 and Snf11 of SWI/SNF, and Tfg2 of TFIIF. Taf14-containing complexes may physically interact by Taf14 dimerization or by exchanging conserved subunits, to facilitate transcriptional activation. Although intriguing, these models require future testing.

Finally, a human homolog of Taf14, AF9, binds H3K9ac through a conserved YEATS domain[139,265]. Therefore, Taf14 may also bind acetylated histones. We speculate that via its direct interaction with H3K4me3-binding Yng1, Taf14 may contribute to a combinatorial histone "code" to target NuA3-directed H3K14 acetylation.

Chapter 5: General Discussion

Although each cell in the human body contains the same DNA, different cell types express unique gene signatures that can be inherited through epigenetic mechanisms[1]. Epigenetic mechanisms are propagated in part through post-translational modifications (PTMs) of histone proteins[2,3,5,6]. As the disruption of epigenetic mechanisms leads to aberrant gene expression and epigenetic diseases including cancer, heart disease, inflammation, and neurological disease, understanding the function of histone PTMs at a molecular level is critical for therapeutic development[7,8,195-198].

Histone PTMs occupy discrete genomic positions, such as promoter regions *versus* open reading frames (ORFs), and are associated with distinct cellular processes, such as gene expression *versus* gene silencing[17,19,31,33,53,210-212]. For example, histone H3 is often both trimethylated on lysine 4 (H3K4me3) and acetylated on lysine 14 (H3K14ac) at the 5'-ends of transcriptionally active genes[23,31-33,52,53,133]. Alternatively, H3K36me3 is found throughout the ORFs of active genes[32,33,53,209]. The "histone code" hypothesis predicts that combinatorial histone PTMs act as binding sites for effector proteins, which can transduce downstream activity through chromatin-associated complexes[13,17,19]. As the conserved *S. cerevisiae* NuA3 histone acetyltransferase (HAT) complex modulates its localization and activity by engaging ("reading") and adding ("writing") these differentially

positioned histone PTMs, we use NuA3 as a model to study the "histone code" in the context of transcriptional activation[55,66,104-108,110].

Here we classify NuA3 into two functionally distinct forms: NuA3a and NuA3b[66]. NuA3a specifically binds H3K4me3 through the plant homeodomain (PHD) finger of the Yng1 subunit[55,107]. NuA3a subsequently acetylates H3K14, leading to transcription initiation at a subset of genes[55,105,106]. NuA3b contains the unique member, Pdp3, a proline-tryptophan-tryptophan-proline (PWWP) domain protein[66]. NuA3b binds H3K36me3 through the PWWP domain of Pdp3 to facilitate transcription elongation[66]. The target of NuA3b HAT activity is not yet known, but is likely independent of H3K14[66].

These findings contribute to the "histone code" hypothesis in two ways. First, we ascribe a new function to H3K36me3 in yeast. In addition to repressing intergenic transcription through the recruitment of histone deacetylase (HDAC) complexes, H3K36me3 also positively regulates transcription elongation through the recruitment of HAT complexes[63-66,223,224,228]. The ability of a single modification to promote opposing cellular processes, suggests that additional binding mechanisms are required to properly target these complexes. Accordingly, NuA3a/b contains multiple effector proteins, including Yng1 and Taf14. Yng1 and Taf14 may bind H3K4me2/3 and histone acetylation, respectively, to fine tune NuA3b localization at specific ORFs[55,107,139]. Such combinatorial patterns could direct differential HAT and HDAC recruitment at H3K36me3-enriched chromatin. Second, we demonstrate that NuA3a/b changes HAT specificity based on subunit composition. This phenomenon is conserved in humans, as the related HBO1 HAT complex can switch between

histone H4 and H3 acetylation depending on the association of JADE *versus* BRPF subunits[254]. Therefore, subunit exchange may generally increase the plasticity of chromatin-associated complexes.

Finally, we characterize extra-terminal (ET) domains as mediators of protein-protein interactions. Specifically, the ET domain of Taf14 engages residues 105-125 of Yng1 and this interaction is critical for NuA3-directed H3K14ac HAT activity. We speculate that the Taf14 ET domain functions as a molecular scaffold, which bridges NuA3 to TFIID, TFIIF, Ino80, SWI/SNF, and other chromatin-associated complexes[129]. Interestingly, the *H. sapiens* ET domain proteins AF9 and BRD4 also engage residues 105-125 of Yng1. Therefore, this Yng1 sequence may contain a conserved ET domain recognition motif and provide a novel mechanism to block pathogenesis in ET-domain related diseases[164,178,180,185,204]. We hypothesize that targeting the "reader" domains and protein-protein interaction surfaces of chromatin-associated complexes, as opposed to the enzymatic subunits, may increase the overall efficacy of epigenetic drug treatments[202].

Appendix

A.1 The YEATS Domain of Taf14 Binds Histones *In Vitro*

TAF14 is not an essential gene, but deletion of all three *S. cerevisiae* YEATS domain-containing genes (*TAF14*, *SAS5*, *YAF9*) is lethal[128]. Therefore, YEATS domains serve a required (yet undetermined) function in yeast. Since YEATS domains can bind histones *in vitro*, we tested the ability of full-length Taf14 and the Taf14 YEATS domain to pull down endogenous total core histones from *T. thermophila*[138,175]. Immunoprecipitated samples were resolved by SDS-PAGE and visualized by western blotting to histone H3. The YEATS domain of Taf14 is sufficient to bind histones *in vitro* **(Figure 30A)**. In this manner, Taf14 may contribute to a combinatorial histone "code" to target NuA3-directed H3K14 acetylation **(Figure 30B)**.

A.2 Disruption of the Spt16-Sas3 Interaction Enhances 6-Azauracil Sensitivity Under Stress Conditions

The histone chaperone, FACT, facilitates both transcription initiation and elongation by exchanging histone H2A/H2B dimers[266]. Sas3 is known to directly interact with FACT through Spt16[44,105]. To connect Sas3 to the transcription elongation pathway, we performed a 6-azauracil (6-AU) growth assay. 6-AU is a

Figure 30

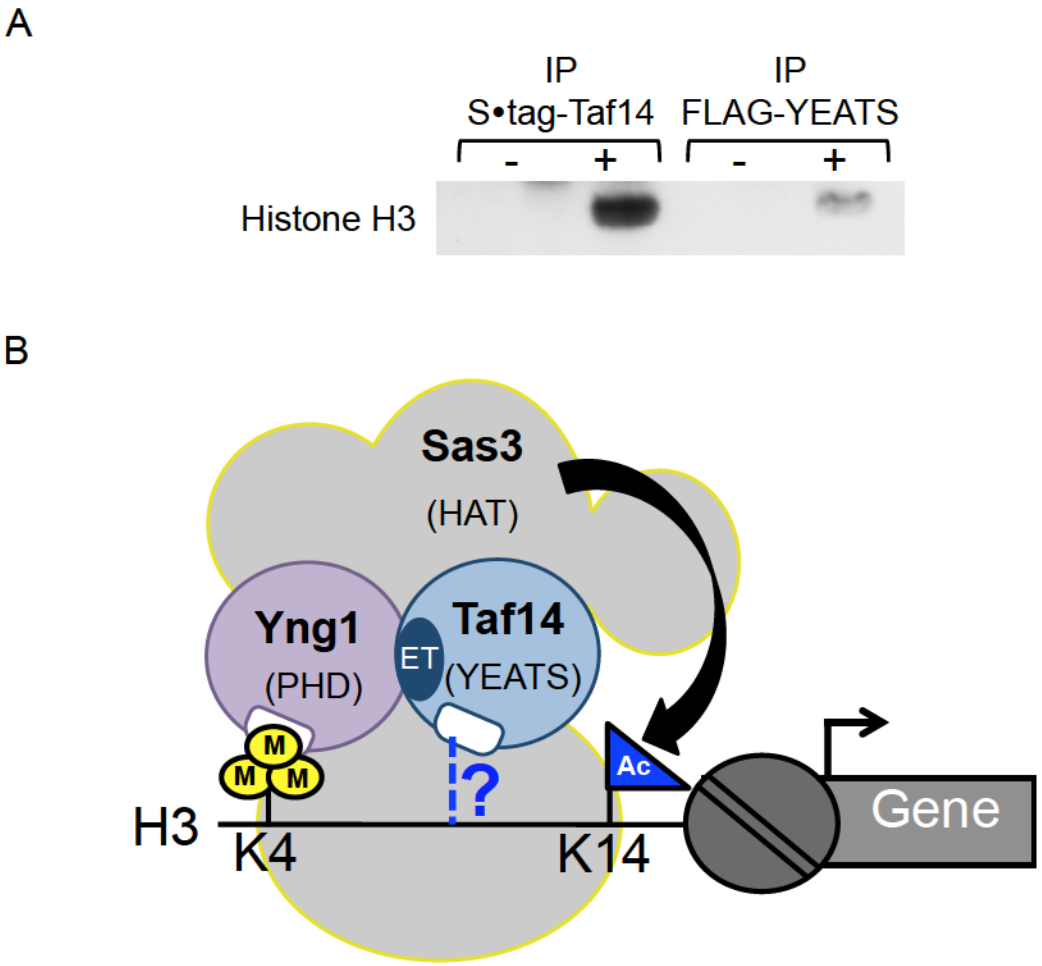


Figure 30 The YEATS domain of Taf14 is sufficient to interact with histones *in vitro* and may contribute to NuA3 targeting. A.) Pull down assays were performed with purified full-length S•tag-Taf14 (+) coated resin or FLAG-YEATS (+) coated resin and endogenous total core histones from *T. thermophila*. Immunoprecipitated samples (IP) were resolved by SDS-PAGE. Binding was monitored by western blotting to histone H3. Histones were obtained from Dr. Romeo Papazyan. B.) Hypothetical model of the NuA3 HAT complex. The YEATS domain of Taf14 may engage histones[139]. In this manner, Yng1 and Taf14 may "read" a combinatorial "histone code" to target NuA3-directed H3K14ac at specific regions of the genome.

transcription elongation inhibitor that shows distinct growth phenotypes with mutants of elongation machinery. We find that a mutant of Spt16 (*spt16-11*), which blocks the Spt16-Sas3 interaction, displays 6-AU sensitivity under stress conditions **(Figure 31)**. Similarly, deletion of the Sas3 C-terminus (*sas3Δ657*), which also blocks the Spt16-Sas3 interaction, displays 6-AU sensitivity **(Figure 31)**. These results suggest that under stress conditions, FACT recruitment via Sas3 facilitates transcription elongation. Yeast strains were designed and created by the Alan J. Tackett laboratory at the University of Arkansas for Medical Sciences.

A.3 Disruption of the Yng1 PHD Finger and the Pdp3 PWWP Domain Synergistically Enhances 6-Azauracil Resistance Under Stress Conditions

The PHD finger of Yng1 and the PWWP domain of Pdp3 are known to facilitate transcription initiation and elongation, respectively, by recruiting the NuA3 HAT complex to chromatin[55,66,106]. *S. cerevisiae* strains containing a deletion of *SET2,* or downstream H3K36me3 effectors, show a 6-AU resistance phenotype[218,224]. Using genetic spotting assays, we find that combined mutation of the Yng1 PHD finger (*yng1ΔPHD)* and the Pdp3 PWWP domain (*pdp3W21A*) synergistically enhances 6-AU resistance under stress conditions **(Figure 32)**. Therefore, the Yng1 PHD finger may contribute to NuA3 targeting at ORFs during stress response. Yeast strains were created by Ana Raman.

Figure 31

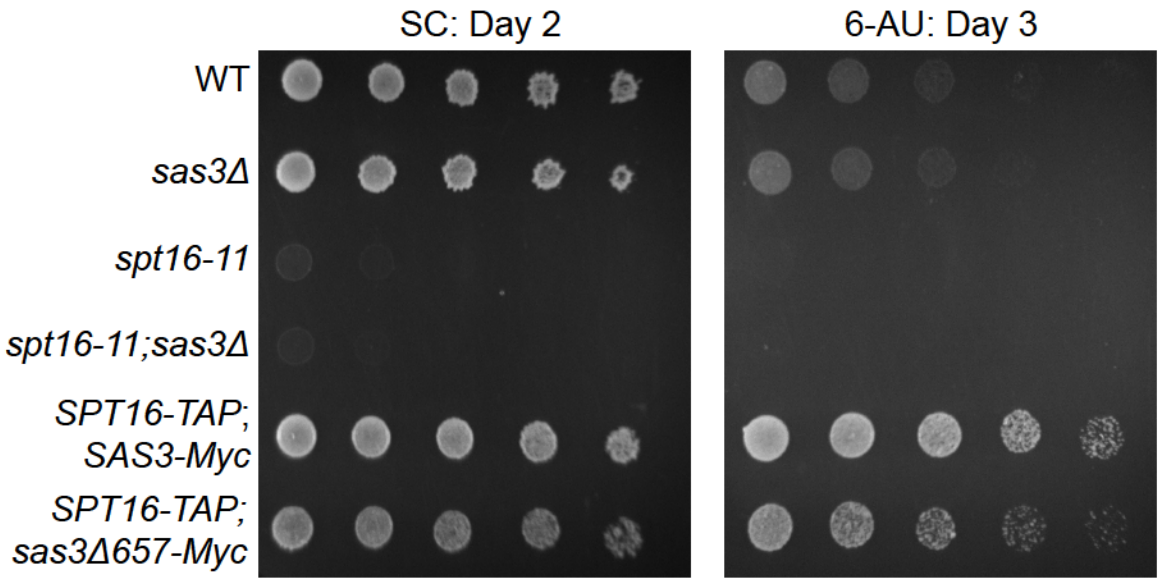


Figure 31 Disruption of the Spt16-Sas3 interaction enhances 6-azauracil sensitivity under stress conditions. Three-fold serial dilutions of the indicated *S. cerevisiae* strains were spotted onto SC media in the absence (left) or presence (right) of 100 ug/mL 6-azauracil (6-AU). The plates were incubated at 37 °C for two or three days as indicated.

Figure 32

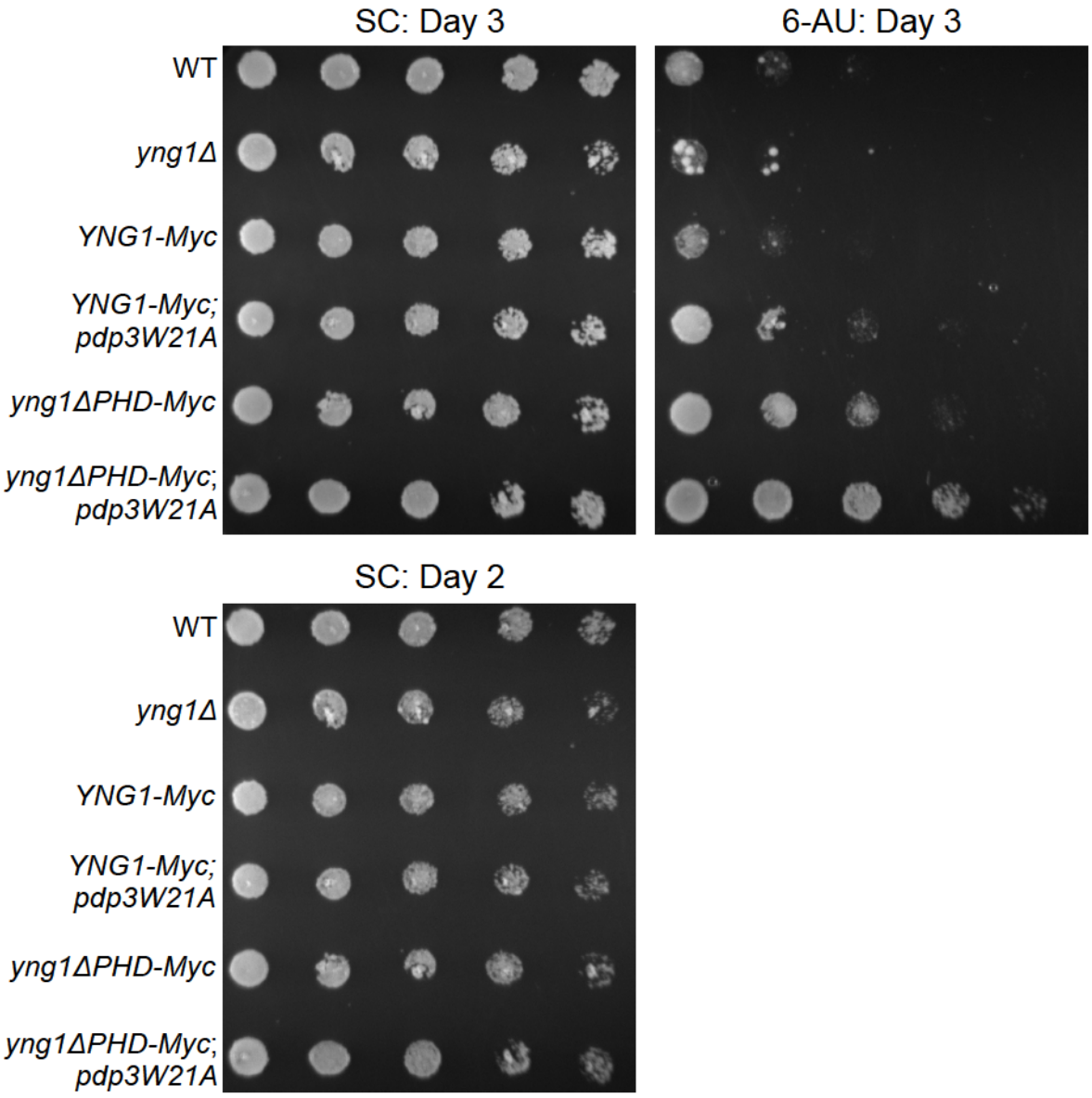


Figure 32 Disruption of the Yng1 PHD finger and the Pdp3 PWWP domain synergistically enhances 6-azauracil resistance under stress conditions. Three-fold serial dilutions of the indicated *S. cerevisiae* strains were spotted onto SC media in the absence (left) or presence (right) of 150 ug/mL 6-azauracil (6-AU). The plates were incubated at 37 °C for three days.

A.4 Yng1 Dimerizes *In Vitro*

A human homolog of Yng1, ING4, contains a dimerization domain[267]. We therefore tested the ability of full-length Yng1 to dimerize *in vitro.* Immunoprecipitated samples were resolved by SDS-PAGE and visualized by western blotting. Yng1 dimerizes with itself, but not with the related PHD finger protein Pho23 **(Figure 33)**. This suggests that like the NURF chromatin-remodeling complex, NuA3 may bridge two histones or two nucleosomes[74,268].

Figure 33

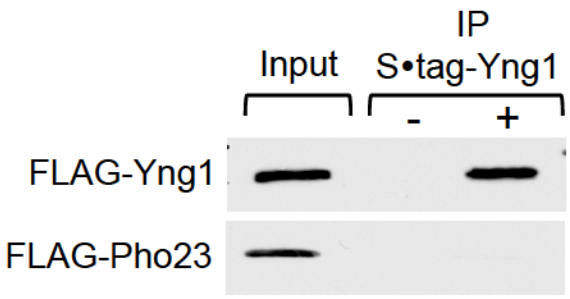


Figure 33 Yng1 dimerizes *in vitro*. Pull down assays were performed with purified S•tag alone (-) or full-length S•tag-Yng1 (+) coated resin and purified full-length FLAG-tagged Yng1 and Pho23. Purified proteins (input) and immunoprecipitated samples (IP) were resolved by SDS-PAGE. Binding was monitored by western blotting to FLAG.

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Curriculum Vitae

Tonya M. Gilbert, Ph.D. Candidate

Educational History:

Ph.D. Expected	2014	Program in Biochemistry, Cellular, and Molecular Biology (BCMB) Dept. of Pharmacology and Molecular Sciences Johns Hopkins University School of Medicine (JHUSOM) Mentor: Sean D. Taverna, Ph.D.
B.S.	2008	Biochemistry (*summa cum laude*) State University of New York (SUNY) at Geneseo Mentor: Kazushige Yokoyama, Ph.D.

Research Experience:

Doctoral Student	08/09-12/14	Laboratory of Sean D. Taverna, Ph.D. JHUSOM Dept. of Pharmacology and Molecular Sciences Center for Epigenetics
NSF Fellow	07/11-07/14	Graduate Research Fellowship

- Determined the mechanisms for chromatin localization and activity of a histone acetyltransferase complex that differentially regulates transcription initiation and elongation pathways by targeting distinct histone post-translational modifications
    - Designed and developed experimental procedures in a yeast model using biochemical, biophysical, and genetic techniques including: FPLC protein purification, peptide synthesis, western blotting, immunoprecipitation assays, enzymatic activity assays, fluorescence polarization assays, molecular cloning, RT-qPCR, and cell survival assays
    - Translated findings into a human model to identify novel targets for inhibition of AF9 and BRD4 pathologies

Research Technician	01/09-06/09	Laboratory of Michael D. Bulger, Ph.D. University of Rochester Dept. of Biochemistry and Biophysics

- Investigated the role of histone hyperacetylated domains in mouse erythroid cell development
    - Described novel hypersensitive sites using hypersensitive site mapping, ChIP, and RT-qPCR

Summer Scholar	05/08-08/08	Graduate Education in the Biological Sciences Laboratory of Michael D. Bulger, Ph.D. University of Rochester Dept. of Biochemistry and Biophysics

Research Student	08/06-12/08	Laboratory of Kazushige Yokoyama, Ph.D. SUNY at Geneseo Dept. of Chemistry

- Applied silica sol-gel technology to regulate the rate of drug diffusion
    - Determined the effect of particle size on host-guest chemistry using UV-Vis spectroscopy
- Conducted preliminary studies to characterize the oligomerization of Amyloid-β protein
    - Measured thermodynamic properties using isothermal titration calorimetry

Other Professional Experience:

Preparing Future Faculty Teaching Academy	09/13-12/14	JHUSOM
Hopkins Biotech Network	12/13-12/14	JHUSOM
Institute for Excellence in Education Summer Teaching Camp	08/13	JHUSOM

Scholarships, Fellowships, and Other External Funding:

National Science Foundation Graduate Research Fellowship, 07/11-07/14, $126,000.

The Center for Innovation in Graduate Biomedical Education Biomedical Careers Initiative Travel Award, 08/14, $500.

JHUSOM Department of Pharmacology and Molecular Sciences Scheinberg Travel Award, 05/13, $500.

SUNY at Geneseo Foundation Undergraduate Research Grant, 10/08, $600.

SUNY at Geneseo Foundation Scholarship, 12/06-12/08, $4,000.

Rotary Club Scholarship, 08/05-08/08, $4000.

Academic Honors:

05/14	Oral Presentation Award	JHUSOM BCMB Colloquium
02/14	Poster Presentation Award	JHUSOM BCMB Poster Symposium
10/12	Oral Presentation Award	JHUSOM BCMB Retreat
05/12	Oral Presentation Award	JHUSOM BCMB Colloquium
12/08	Award for Excellence in Biochemistry	SUNY at Geneseo
08/08	Presidential Scholar Award	SUNY at Geneseo

Publications, Peer-reviewed:

Gilbert, T.M.*, McDaniel, S.L.*, Byrum, S.D., Cades, J.A., Dancy, B.C., Wade, H., Tackett, A.J., Strahl, B.D. and Taverna, S.D. (2014) A PWWP Domain-Containing Protein Targets the NuA3 Acetyltransferase Complex via Histone H3 Lysine 36 trimethylation to Coordinate Transcriptional Elongation at Coding Regions. *Molecular & Cellular Proteomics* 11:2883-2895. PMCID: PMC2881130

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Cieniewicz A.M., Moreland L., Ringel A.E., Mackintosh S.G., Raman A., **Gilbert T.M.**, Wolberger C., Tackett A.J. and Taverna S.D. (2014) The bromodomain of Gcn5 regulates site-specificity of lysine acetylation on histone H3. *Molecular & Cellular Proteomics* 11: 2896-2910. PMCID: PMC2666593

Burdine, M.S., Smart, S.K., Larson, S., Byrum, S.D., **Gilbert, T.M.**, Raman, A., Tackett, A.J. and Taverna, S.D. Transient interactions between the Sas3 histone acetyltransferase and yFACT are required for proper transcriptional activity at NuA3 targeted genes. *Submitted.*

Yokoyama, K., Swana, J.R., **Gilbert, T.M.,** Chen, D.D., Chen, L. and Kogan, P. (2009) The nanoscale description of acid penetration to the gold colloids encapsulated in silica sol-gel matrix. *Journal of Sol-Gel Science and Technology* 50: 48-57.

Abstracts and Presentations:

Gilbert, T.M., McDaniel, S.L., Byrum, S.D., Cades, J.A., Dancy, B.C., Wade, H., Tackett, A.J., Strahl, B.D. and Taverna, S.D. (2014) An H3K36me3 binding PWWP protein targets the NuA3 acetyltransferase complex to coordinate transcriptional elongation at coding regions. Poster Presentation. BCMB Retreat, Harbourtowne, St. Michaels, MD, October 12-13, 2014.

Gilbert, T.M., McDaniel, S.L., Byrum, S.D., Cades, J.A., Dancy, B.C., Wade, H., Tackett, A.J., Strahl, B.D. and Taverna, S.D. (2014) An H3K36me3 binding PWWP protein targets the NuA3 acetyltransferase complex to coordinate transcriptional elongation at coding regions. Poster Presentation. Discovery on Target 2014, Westin Boston Waterfront, Boston, MA, October 8-10, 2014.

Gilbert, T.M. (2014) Histone methylation and transcriptional elongation: Hang on to your HAT complex! Oral Presentation. TCNP Investigators 2014 Meeting, National Institutes of Health, Bethesda, MD, March 26-27, 2014.

Gilbert, T.M., McDaniel, S.L., Byrum, S.D., Cades, J.A., Dent, S.Y.R., Wade, H., Tackett, A.J., Strahl, B.D. and Taverna, S.D. (2013) Mechanisms contributing to chromatin localization of the HAT complex NuA3. Poster Presentation. Chromatin: Structure and Function, Westin Grand Cayman Seven Mile Beach Resort and Spa, Grand Cayman Island, November 11-14, 2013.

Gilbert, T.M. (2013) Mechanisms contributing to chromatin localization and function of the HAT complex NuA3. Oral Presentation. BCMB Retreat, Harbourtowne, St. Michaels, MD, October 6-7, 2013.

Gilbert, T.M. (2012) The mechanism of NuA3 chromatin localization and its role in gene transcription. Oral Presentation. BCMB Retreat, Sheppard Pratt Conference Center, Baltimore, MD, October 12, 2012.

Gilbert, T.M., Papazyan, R., Wade, H. and Taverna, S.D. (2011) The mechanism of NuA3 chromatin localization and its role in transcriptional activation. Poster Presentation. BCMB Retreat, Sheppard Pratt Conference Center, Baltimore, MD, October 7, 2011.

Gilbert, T.M. (2008) Nanoscale description of acid penetration to the gold colloids encapsulated in silica sol-gel matrix. Poster Presentation. 236th ACS National Meeting & Exposition, Philadelphia, PA, August 17-21, 2008.

Gilbert, T.M. and Yokoyama, K. (2008) Study of gold colloidal nanoparticles embedded in a silica based sol-gel. Oral Presentation. 53rd Annual Undergraduate Research Symposium of the Rochester Section of the American Chemical Society, SUNY Geneseo, Geneseo, NY, April 26, 2008.

Gilbert, T.M. and Yokoyama, K. (2007) Investigation of gold colloid nanoparticles embedded in a silica based sol-gel. Poster Presentation. 21st National Conference on Undergraduate Research, Dominican University of California, San Francisco, CA, April 12-14, 2007.

Service and Leadership:

12/13-12/14 **Advisory Committee Member, Biomedical Careers Initiative**
Created and organized professional development events for JHUSOM graduate students.

10/13-11/13 **Course Director, Effective Science Communication (Spring)**
Designed syllabus, scheduled guest-lectures, developed/delivered lectures, created/graded assignments, and maintained website for a JHUSOM graduate course surveying topics in science communication. Materials included grammar and writing style, writing research manuscripts and grants, designing scientific figures, oral and poster presentations, communicating science to the lay public, and communicating science through social media.

06/12-08/13 **Mentor, The Leadership Alliance Summer Research Early Identification Program**
Instructed under-represented undergraduate students in molecular biology techniques and development of independent research projects. Also focused on improving critical thinking, organization, professional development, public speaking, and poster presentation.

05/13-06/13 **Teaching Assistant, Effective Science Communication (Fall)**
Assisted in organization, development of select lectures, and assignment grading for a JHUSOM graduate course.

12/11-02/13 **Graduate Student Recruitment Volunteer Manager, BCMB Program**
Organized recruit transportation, meals, interviews, and social activities (~70 people), managed volunteer duties (~ 60 people), and monitored budget provided by the BCMB graduate program.

12/10-01/13 **Lead Volunteer, Incentive Mentoring Program**

Mentored a female student at risk for dropping out of Paul Laurence Dunbar High School. Led a team of mentors to improve academic achievement, professional development, and self-motivation through tutoring and social activities. Coordinated a summer internship at the Johns Hopkins University School of Nursing.

11/12-12/12 **Tutorial Leader, Genetics**
Led group discussions, created review material, and assisted in exam preparation for JHUSOM graduate course.

06/11-08/12 **Mentor, Boys Hope Girls Hope of Baltimore**
Taught basic laboratory skills to high school student facing psychosocial challenges. Interviewed applicants for summer research program.

08/07-12/08 **Volunteer, FACE AIDS SUNY at Geneseo Chapter**
Organized fundraising activities to provide healthcare, shelter, and education for those living with HIV and AIDS in Africa. Coordinated and hosted an external lecture on HIV vaccine development.

08/06-08/07 **Executive Board Member, Biology Club**
Organized academic, social, and fundraising activities for SUNY at Geneseo students.

08/06-12/06 **Teaching Assistant, General Chemistry**
Led review sessions and graded exams for SUNY at Geneseo undergraduate course.