DISSERTATION

SPN1, A MULTIFUNCTIONAL PLAYER IN THE CHROMATIN CONTEXT

Submitted by

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ABSTRACT

SPN1, A MULTIFUNCTIONAL PLAYER IN THE CHROMATIN CONTEXT

Spn1 was initially identified as a transcription factor that copurified with Spt6. Spn1 functions in transcription initiation and elongation, mRNA processing and export, histone modification, as well as in heterochromatic silencing. Our recent study demonstrated that Spn1 could bind histones and assemble nucleosomes *in vitro*. Therefore, Spn1 is a new member of the histone chaperone family. Here we found that Spt6 regulates Spn1-nucleosome interaction and conversely, Spn1 regulates Spt6-H2A-H2B interaction. Co-regulation between Spn1 and Spt6 enables them to be independent histone chaperones in nucleosome assembly. In addition, abrogation of Spn1-Spt6 interaction does not generate cryptic transcripts at certain genes. Furthermore, we identified a new interaction between Spn1 and the histone chaperone Nap1. Spn1, Nap1 and histones can form a large complex. We also found Spt6 could compete Nap1 for Spn1 binding, therefore disrupting Spn1-Nap1 interaction and releasing Nap1. In sum, Spn1 plays a multifunctional role in the chromatin context via dynamic interactions with its binding partners.

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CHAPTER 1

REVIEW OF LITERATURE

1.1 RNA POLYMERASE II OVERCOMES THE NUCLEOSOME BARRIER DURING TRANSCRIPTION ELONGATION

Eukaryotic organisms efficiently package genetic information into a nucleoprotein complex called chromatin. The basic unit of chromatin is the nucleosome, which is composed of 146 bp of DNA bound around histone octamer proteins, consisting of two H2A-H2B dimers and one H3-H4 tetramer (Luger et al., 1997).

Despite the important requirement for packing DNA into the nucleus, the nucleosome presents a natural barrier for any cellular process that requires DNA access, such as gene expression. RNA polymerase II (Pol II) proceeds through genes that could contain several to thousands of nucleosomes. Therefore the nucleosomes must first be disassembled (either partially or entirely) to allow transcription machineries to access DNA. Following disassembly, nucleosomes are reassembled in the wake of Pol II. Disregulation of nucleosome dynamics leads to aberrant transcription (Kaplan et al., 2003). Notably, the average elongation rate at Pol II-transcribed genes is ~3.8 kb/min *in vivo*, which is comparable to the elongation rate on naked DNA *in vitro* (Singh and Padgett, 2009). Therefore, studies on discovering protein factors that regulate nucleosome dynamics and facilitate transcription have risen. Histone chaperones, chromatin remodelers, histone modifiers and histone variants are such proteins that regulate nucleosome dynamics during transcription (Venkatesh and Workman, 2015). Here I will focus on histone chaperones.

1.2 HISTONE CHAPERONES

The term “histone chaperone” came out to reveal the function of nucleoplasmin that could prevent histone-DNA aggregation during nucleosome assembly (Laskey et al., 1978). Histone chaperones, are nowadays defined as a group of proteins that bind histones and shield nonspecific interactions between the positively charged histones and the negatively charged DNA, therefore allow the correct nucleosome formation in an ATP-independent manner (Burgess and Zhang, 2013; Ransom et al., 2010).

Nucleosome assembly and disassembly follows a stepwise fashion (Figure 1.1) (Das et al., 2010). Two H3-H4 dimers could be sequentially deposited onto DNA and then assembled into H3-H4 tetramer. Alternatively, H3-H4 tetramer is assembled and formed before being deposited onto DNA. As a result, an intermediate structure called a tetrasome (H3-H4 tetramer on the DNA) is observed. Then the first H2A-H2B dimer is incorporated into tetrasome, forming another intermediate structure called a hexasome. Finally, addition of a second H2A-H2B dimer into hexasome makes up nucleosome. In each step of the assembly pathway, histone chaperones are required and they bind histones to prevent non-specific interactions with DNA. The stepwise assembly process is reversed to allow the disassembly of nucleosomes.

Prior to the assembly and disassembly process in the nucleus, some histone chaperones associate with histones upon their synthesis in cytoplasm and transport them into nucleus. Two such histone chaperones are Nap1 and Asf1, which bind karyopherins and transport H2A-H2B and H3-H4, respectively (Campos et al., 2010; Mosammaparast et al., 2002). Additionally, histone chaperones directly or indirectly modulate histone

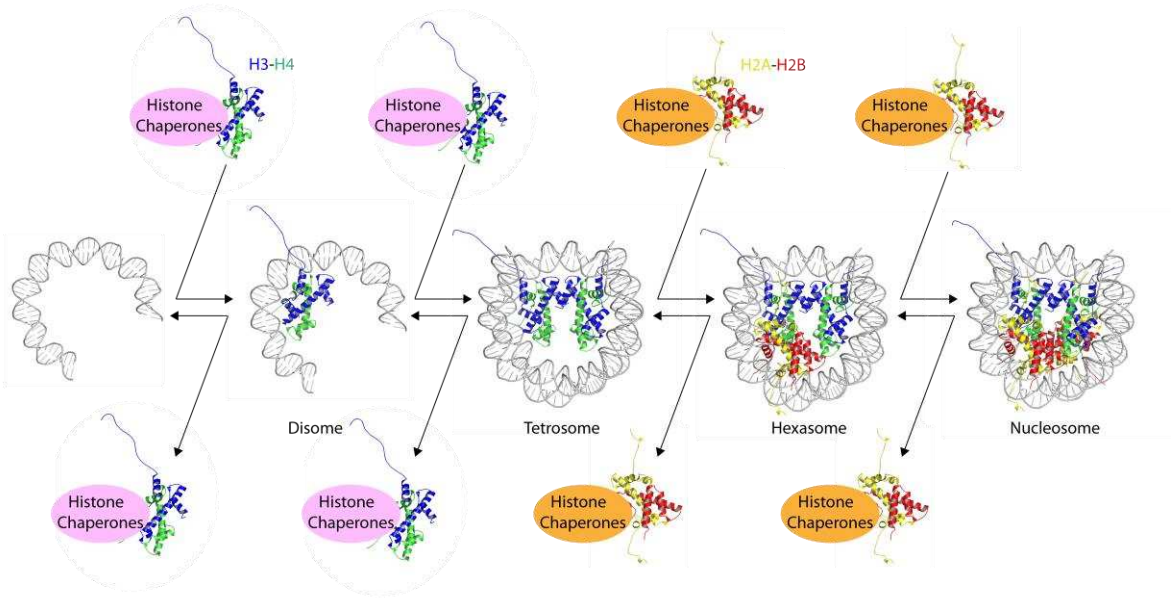


Figure 1.1 Nucleosome (PDB ID: 1AOI) assembly and disassembly are assisted by histone chaperones (adapted from Das. *et a*l, 2010). Yellow indicates H2A, red indicates H2B, blue indicates H3 and green indicates H4.

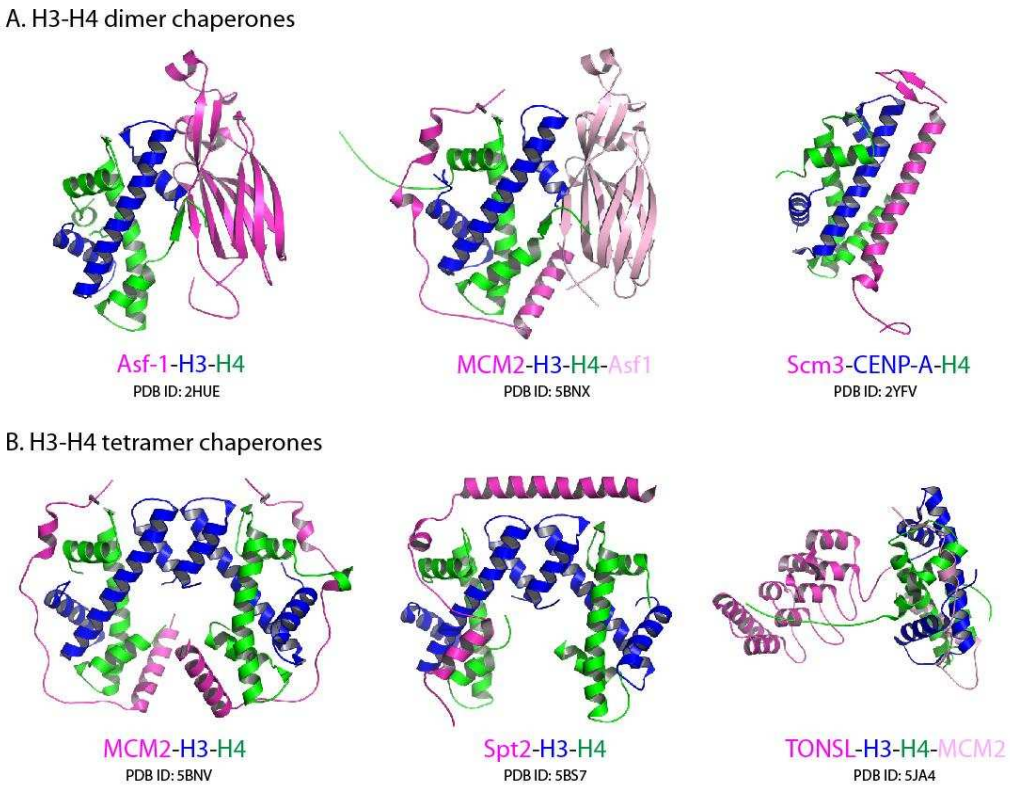


Figure 1.2 Histone H3-H4 chaperones. (A) H3-H4 dimer chaperones. (B) H3-H4 tetramer chaperones. Blue indicates H3, green indicates H4 and magenta indicates histone chaperones.

posttranslational modifications (Avvakumov et al., 2011). For example, mutation in histone chaperone Spt6 causes a loss of histone H3 trimethylation at Lys-36 (H3K36me3) (Youdell et al., 2008).

1.3 HISTONE H3-H4 CHAPERONES

Given that histone chaperones have preferential binding to histone H2A-H2B or H3-H4, histone chaperons are generally classified into two groups: H2A-H2B chaperones and H3-H4 chaperones. However, the criterion is not stringent, as some histone chaperones bind both H3-H4 and H2A-H2B, such as Nap1, Spt6, and FACT (Andrews et al., 2008; McCullough et al., 2015; Tsunaka et al., 2016). In addition to canonical histones, histone variants can also be recognized by unique histone chaperones, such as ANP32E and HJURP (Scm3 in yeast) (Cho and Harrison, 2011; Hu et al., 2011; Obri et al., 2014). Histone variants have one or a few amino acids differences compared with their conventional counterparts and confer nucleosome with novel structure and function (Boulard et al., 2007).

The positioning of histone H3-H4 into the nucleosome is important, because H3-H4 must be deposited on the DNA prior to H2A-H2B and removed from DNA after H2A-H2B. Therefore here I will focus on H3-H4 chaperones. Histone H3-H4 exists as dimer or tetramer. Therefore, despite the fact that all H3-H4 chaperones bind H3-H4, they do so in versatile ways. I classify them into two groups: (I) H3-H4 dimer chaperones and (II) H3-H4 tetramer chaperones (Figure 1.2, Table 1.1). MCM2 belongs to group (I) in that it can bind H3-H4 dimer in the presence of Asf1, as well as group (II) in that two MCM2 molecules bind an H3-H4 tetramer. Notably, the newly discovered TONSL was the first

chaperone reported to solely bind histone H4 tail rather than globular domain. In addition to its histone chaperone activity, TONSL is also a histone reader recognizing H4K20me0 (Figure 1.2B) (Saredi et al., 2016). Further comparing chaperones within the subgroup, they bind to different sites on H3-H4. For example, in group (II), a pair of MCM2 molecules wrap around the lateral surface of an H3-H4 tetramer, while Spt2 binds to the top surface (H3-H3' four-helix bundle interface) of an H3-H4 tetramer (Figure 1.2B).

1.3 HISTONE CHAPERONES IN TRANSCRIPTION REGULATION

Here I focus on three chaperones Nap1, Spt6 and Spn1 (identified in the thesis) in *Saccharomyces cerevisiae* that have been implicated in transcription regulation (Table 1.2).

Nap1

Nap1 (nucleosome assembly protein 1) is conserved among all eukaryotes from yeast to humans. Nap1 is a histone chaperone and assists in nucleosome assembly, therefore widely being used as a standard reagent for *in vitro* chromatin assembly assays. Yeast Nap1 has a molecular weight of 48 kD and binds both histone H2A-H2B and H3-H4 *in vitro*. However, Nap1 preferentially binds H2A-H2B *in vivo* and regulates H2A-H2B dynamics (Andrews et al., 2010; Chen et al., 2016; Miyaji-Yamaguchi et al., 2003). In addition to canonical histones, Nap1 also exchanges H2A with its variant H2A.Z (Mizuguchi et al., 2004).

Nap1 is composed of a structured central domain and disordered N- and C-terminal regions. The central domain (74-365) of Nap1 is highly conserved and it forms a dome-

Table 1.1 H3-H4 chaperones whose structures with H3-H4 have been solved. See text for references.

H3-H4 cargo	Chaperone	Complex PDB ID	Ratio H3:H4: chaperone	Functions	Note
H3-H4 dimer	Asf1 (*S. cerevisiae*)	2HUE	1:1:1	Histone donor for CAF-1, HIRA and MCM2	H3 (*X. laevis*)
	DAXX (*H. sapiens*)	4HGA	1:1:1	Deposition factor independent of DNA synthesis: telomere maintenance, ribosomal DNA, pericentric heterochromatin	H3.3
	HJURP (*H. sapiens*)	3R45	1:1:1	Deposition factor, centromere maintenance	CENP-A
	Scm3 (*S. cerevisiae*)	2YFV	1:1:1		
	MCM2 (*H. sapiens*)	5BNX	1:1:1	DNA replication	H3.3; cocrystallization with Asf1
		5BO0	1:1:1	DNA replication	H3.2; cocrystallization with Asf1
	TONSL (*H. sapiens*)	-	1:1:1	DNA replication; H4K20me0 reader	Compatible in a co-chaperone complex with MCM2 and Asf1; solely binds H4 tail
	UBN1 (HIRA) (*H. sapiens*)	4ZBJ	1:1:1	Deposition factor independent of DNA synthesis	H3.3; cocrystallization with Asf1
H3-H4 tetramer	FACT (*H. sapiens*)	4Z2M	2:2:1	Transcription	H3.1
	MCM2 (*H. sapiens*)	5BNV	2:2:2	DNA replication	H3.3
	Rtt106 (*S. cerevisiae*)	-	2:2:2	Heterochromatic silencing; DNA damage response	Rtt106 dimerizes; experimental and model-derived complex structure
	Spt2 (*H. sapiens*)	5BS7	2:2:1	Transcription	H3 (*X. laevis*)
	TONSL (*H. sapiens*)	5JA4	2:2:2	DNA replication; H4K20me0 reader	H3.3; cocrystallization with MCM2; solely binds H4 tail

Table 1.2 Summary of histone chaperones studied in the thesis. See text for references.

Histone chaperone	Year (activity)	Histone cargo	Associated transcription factors	Functions
Nap1	1983	H2A-H2B (preferred) H3-H4 H2A.Z-H2B	FACT, Chd1, Elongator complex, PAF complex, TFIIS, Ctk1, Spn1	Histone shuttling Transcription H2A.Z exchange
Spt6	1996	H3-H4 H2A-2B	Pol II, FACT, Spt5, PAF complex, Spt2, Spn1	Transcription mRNA processing and exportation Histone modification H2A.Z distribution
Spn1	2016	H3-H4	Pol II, Spt6, TBP, Spt 4/5, TFIIS, PAF complex, Nap1	Transcription mRNA processing and exportation Histone modification

shaped architecture via a long dimerization helix (Park and Luger, 2006a) (Figure 1.3 A and B). The central domain is not only important for Nap1 dimerization, but is also necessary and sufficient for histone binding and nucleosome assembly (Figure 1.3C) (Aguilar-Gurrieri et al., 2016; D'Arcy et al., 2013; Fujii-Nakata et al., 1992; Park et al., 2005). The C-terminal region is required for nucleosome sliding (Park et al., 2005). SEC-MALs (size exclusion chromatography with inline multi-angle light scattering) analysis revealed that Nap1 exists a dominant tetrameric formation (via β-hairpin extension) in equilibrium with a small portion of dimer under physiological condition (Bowman et al., 2014). Monomer is only obtained in the presence of high concentration of denature reagent guanidine hydrochloride and the secondary structure is also lost (McBryant and Peersen, 2004).

Nap1 is also involved in histone shuttling between the cytoplasm and nucleus. Nap1 also changes its localization during the cell cycle: in S phase Nap1 is found in nucleus and G2 phase in the cytoplasm. Nap1 interacts with Kap114p, a nuclear import receptor that imports H2A-H2B (Mosammaparast et al., 2002; Mosammaparast et al., 2001). The import relies on the Nap1 NLS (nuclear localization sequence, 290-295aa), which protrudes from the main structure; the export relies on NES (nuclear export sequence, 88-102aa) which is located at the loop connecting α1 and α2 (Park and Luger, 2006a). The NES is partially masked by adjacent accessory domain (141-180aa) and therefore masking and unmasking may regulate exportation (Figure 1.3 B).

Nap1 also plays roles in transcription. Nap1 physically interacts with histone chaperone FACT and chromatin remodeler Chd1; genetically interacts with several transcription

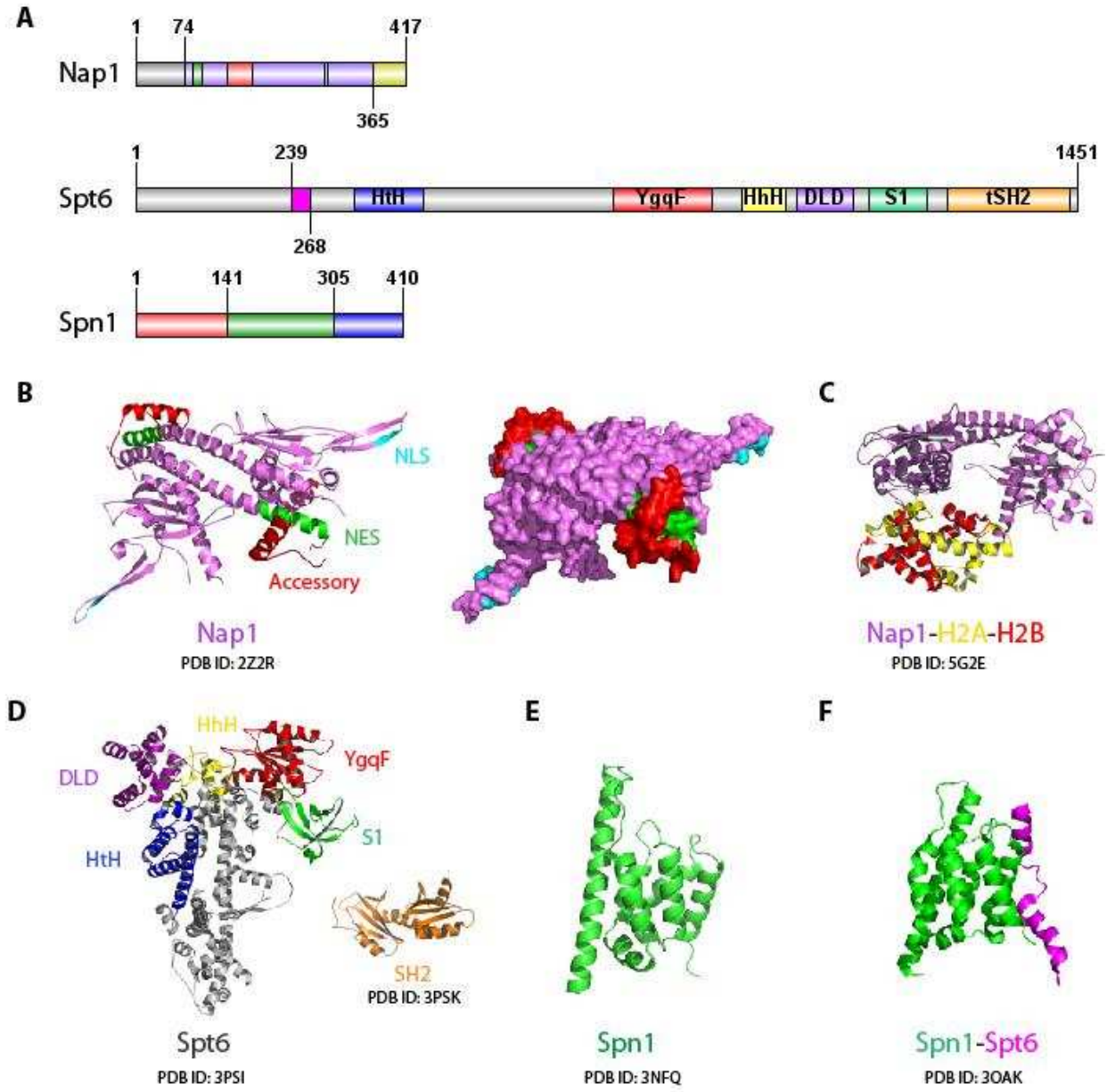


Figure 1.3 Histone chaperones studied in this thesis. (A) Schematic of protein domains. (B) Nap1 structure. (C) Nap1 with H2A-H2B complex structure. (D) Spt6 structure. (E). Spn1 structure. (F) Spn1-Spt6 complex structure.

elongation factors, such as Elongator complex, TFIIS and PAF complex (Krogan et al., 2006; Walfridsson et al., 2007; Xue et al., 2013). Nap1 is recruited to promoters and open reading frames (ORFs), and the recruitment is upregulated during transcription (Del Rosario and Pemberton, 2008). Loss of *NAP1* suppressed cryptic transcription within the ORFs in a strain with a *CTK1* (Pol II kinase) deletion (Xue et al., 2013).

Spt6

Spt6 was originally identified in a genetic screen looking for factors that alter normal initiation of transcription in *Saccharomyces cerevisiae* (Clark-Adams and Winston, 1987). Spt6 is conserved and essential from yeast to humans. Spt6 has also been identified as a histone chaperone and could assemble nucleosome *in vitro* (Bortvin and Winston, 1996). In the past two decades, Spt6 is classified as an H3-H4 chaperone (Bortvin and Winston, 1996). However, recent study shows that Spt6 binds both H3-H4 and H2A-H2B with similar affinity (McCullough et al., 2015).

Spt6 has a molecular weight of 168 kD and it harbors several characterized domains (Figure 1.3A and D) (Close et al., 2011). N-terminal region (~first 300aa) of Spt6 is acidic and disordered, however a small peptide (239–268) forms two helices in the presence of Spn1(148–293) (Figure 1.3, F) (McDonald et al., 2010). Notably, this small peptide also binds nucleosomes in the presence of Nhp6 (HMGB family member), and therefore there is a competition between Spn1 and nucleosomes (McDonald et al., 2010). HtH domain (336-442aa) resembles a DNA-binding motif, possibly serves as protein-protein interaction motif as well (Close et al., 2011). YqgF domain (735-887aa) is less well described and it is predicted to have ribonucleases or resolvases activity (Close et al., 2011). HhH (helix-hairpin-helix) domain (933-1002aa) is known for double-strand DNA

binding (Close et al., 2011). DLD (dealth-like domain) (1019-1104aa) mediates intermolecular interaction (Close et al., 2011). S1 domain (1129-1219aa) is possibly used for interactions that do not contain nucleic acids (Close et al., 2011). tSH2 domain (1250-1440aa) specifically interacts with phosphorylated CTD (C-terminal domain) of Pol II (Close et al., 2011).

In addition, Spt6 also physically interacts with histone chaperones FACT and Spt2, genetically interacts with the histone modifier PAF complex and chromatin remodelers Swi/Snf and RSC (Chen et al., 2015; Du et al., 1998; Kaplan et al., 2005; Lindstrom et al., 2003b; Neigeborn et al., 1986; Nourani et al., 2006). As a result, Spt6 plays roles in each stage of transcription, including initiation, elongation and termination (Ardehali et al., 2009; Endoh et al., 2004; Hartzog et al., 1998; Kaplan et al., 2005; Zhang et al., 2008). Mutation in Spt6 results in aberrant transcripts due to a failure to maintain repressive chromatin; mutation also causes 6-azauracil-sensitive (6-AU) phenotype indicative of transcription elongation defects (Hartzog et al., 1998; Ivanovska et al., 2011; Kaplan et al., 2003).

Spt6 also couples transcription with mRNA processing and export, as well as post-translational modification of histones (Ardehali et al., 2009; Yoh et al., 2007; Yoh et al., 2008). In this process, Spt6 does not work alone, instead it recruits Spn1 and forms a megacomplex with Pol II, mRNA export adaptor and histone methyltransferase.

Spn1

S. cerevisiae Spn1 was originally identified in a genetic screen for transcription factors that were capable of suppressing a defective TBP allele and in copurification of Spt6 (Fischbeck et al., 2002). Spn1 is an essential and highly conserved factor involved in

transcription (Fischbeck et al., 2002; Krogan et al., 2002). Spn1 physically interacts with Pol II and co-localizes with it along ORFs (Krogan et al., 2006; Mayer et al., 2010; Zhang et al., 2008). Moreover, Spn1 genetically interacts with transcription factors TBP, Spt4/5 and TFIIS, and histone modifier PAF complex and chromatin remodeling Swi/Snf complex (Fischbeck et al., 2002; Lindstrom et al., 2003b; Zhang et al., 2008).

During transcription initiation, Spn1 recruits Spt6, followed by recruitment of Swi/Snf complex upon activation at the *CYC1* gene (Zhang et al., 2008). During transcription elongation, Spt6 recruits Spn1, followed by mRNA export factor and histone modifier and accomplished mRNA processing at *c-Myc*, *HIV-1* and *PABPC1* genes (Yoh et al., 2008). Therefore, Spn1 and Spt6 do not always stay in the complex and their recruitment to promoters or ORFs are in a sequential fashion. In addition, Spn1 and Spt6 are not always codependent. For example, mutations in Spn1 do not affect Spt6-mediated chromatin assembly at *PHO5* gene (Adkins and Tyler, 2006).

The *S. cerevisiae* Spn1 (containing 410 residues) has an unstructured N-terminal region, structured central domain and unstructured C-terminal region (Figure 1.3, A and D). The N-terminal region (first 140 residues, 1-140aa) is acidic, while the C-terminal region (last 105 residues, 306-410aa) is basic. Interestingly, our analytical ultracentrifugation (AUC) and circular dichroism (CD) data suggested that oppositely charged N- and C-terminal regions do not interact and they spread out in solution (Adam Almeida, unpublished). The central domain (middle 165 residues, 141-305aa) is basic and highly conserved from yeast to humans. The central domain covers the essential functions of wild type Spn1 under optimal growth condition, but showing growth defects when combined with other

chromatin factors, indicating the importance of N- and C-terminal regions (Catherine Radebaugh, unpublished). The central domain also binds Spt6, and mutations in the Spn1-Spt6 interface cause a defect in maintaining repressive chromatin (McDonald et al., 2010).

Given that Spn1 associates with numerous chromatin factors during transcription, we set out to discover direct interaction between Spn1 and nucleosomes, the basic units of chromatin. We found that Spn1 preferentially binds histone H3-H4 rather than H2A-H2B and directly interacts with nucleosomes and DNA. Therefore, we proposed that Spn1 is a new member of histone chaperone family. We will focus on histone chaperone activity of Spn1 through chapters 2-4.

CHAPTER 2 [1]

SPN1 IS A CHROMATIN BINDING PROTEIN

2.1 INTRODUCTION

Eukaryotic organisms efficiently package the genetic information by compacting DNA into chromatin. The basic unit of chromatin is the nucleosome, which is composed of 146 bp of DNA bound around histone octamer proteins, consisting of two H2A-H2B dimers and one H3-H4 tetramer (Luger et al., 1997). The disassembly of nucleosomes permits RNA polymerase II to access the DNA, whereas assembly of nucleosomes prevents aberrant transcription. Histone chaperones are a group of proteins that assist in nucleosome disassembly and/or assembly. Histone chaperones mediate histone eviction and deposition during transcription (Petesch and Lis, 2012).

Spt6 is known to associate with RNA polymerase II and facilitate transcription elongation (Ardehali et al., 2009; Endoh et al., 2004; Hartzog et al., 1998). Tagging and purification of yeast Spt6 resulted in co-purification of a previously uncharacterized protein Spn1 (also called Iws1) (Krogan et al., 2002). Spn1 was also identified in a genetic screen for transcription factors that were capable to suppress a post-recruitment defective TBP allele (Fischbeck et al., 2002). The Spn1 gene is essential and highly conserved from yeast to humans (Fischbeck et al., 2002; Krogan et al., 2002; Lindstrom et al., 2003a; Yoh et al., 2007). Spn1 functions in transcription initiation and elongation, mRNA processing and export, histone modification, as well as heterochromatic silencing

[1] I would like to thank Uma M. Muthurajan for initiating characterization of Spn1-nucleosomes and Spn1-DNA interactions using EMSA.

(Kiely et al., 2011; Mayer et al., 2010; Yoh et al., 2007; Yoh et al., 2008; Zhang et al., 2008). Moreover, Spn1 genetically interacts with histone modifier PAF1 complex and chromatin remodeling complexes Ino80 and Swi/Snf (Chen et al., 2010; Collins et al., 2007; Zhang et al., 2008). Furthermore, Spn1 collaborates with its partner Spt6 in several processes and mutations on the Spn1-Spt6 interface caused a defect in maintaining repressive chromatin (Kiely et al., 2011; McCullough et al., 2015; McDonald et al., 2010; Yoh et al., 2007; Yoh et al., 2008; Zhang et al., 2008). These studies suggest that Spn1 plays a role in regulating chromatin structure.

In this study, we investigated the histone binding activity of Spn1 and found that Spn1 specifically binds histone H3-H4. In addition, we also demonstrated that Spn1 binds nucleosomes and DNA. Considering these results, we concluded that Spn1 is a histone chaperone that regulates chromatin through directly interacting with nucleosomal components.

2.2 RESULTS

Spn1 preferentially binds histone H3-H4

To investigate histone-binding activity of Spn1, we used size-exclusion chromatography. We incubated recombinant *S.cerevisiae* Spn1 with either recombinant *X. laevis* H3-H4 or H2A-H2B under physiological conditions (150 mM NaCl), and obtained the elution profiles. H3-H4 with Spn1 mixture resulted in a significant change in the elution profile compared with Spn1 alone. In contrast, Spn1 failed to interact with H2A-H2B (Figure 2.1A and B). When fractions were evaluated by SDS-PAGE, preferential binding of H3-H4 to Spn1 was confirmed.

The central domain of Spn1, designated Spn1(141-305), is highly conserved and complemented a deletion of *SPN1* under optimized conditions (YPD medium at 30℃) (Fischbeck et al., 2002). The central domain crystal structure has been determined at 1.85 Å resolution (McDonald et al., 2010; Pujari et al., 2010). Therefore, we next analyzed its histone H3-H4 binding activity. Interestingly, central domain did not bind H3-H4 (Figure 2.1C). This suggests that histone H3-H4 binding requires N-terminal and/or C-terminal regions of Spn1.

Spn1 binds nucleosomes and DNA

We next tested the nucleosome-binding activity of Spn1 using electrophoretic mobility shift assays (EMSA). Nucleosomes were formed with 147 bp Widom 601 fragment and *X.laevis* octamer. We analyzed three different Spn1 constructs: full length Spn1, Spn1(141-305) and Spn1-K192N (Figure 2.2A). Full length Spn1 could bind nucleosomes (Figure 2.2A, lanes 3-4). Central domain Spn1(141-305) could not bind nucleosomes, indicating N-terminal and/or C-terminal regions are required (Figure 2.2A, lanes 5-6). Spn1-K192N (lysine residue at position 192 was substituted with asparagine) could also bind nucleosomes. Since Spn1-K192N fails to interact with Spt6, this suggests that the Spn1-nucleosome interaction is independent of Spt6 (Zhang et al., 2008). In addition, we also tested DNA binding using the 147 bp nucleosomal DNA (Figure 2.2B). Likewise, we observed only the central domain was defective for binding.

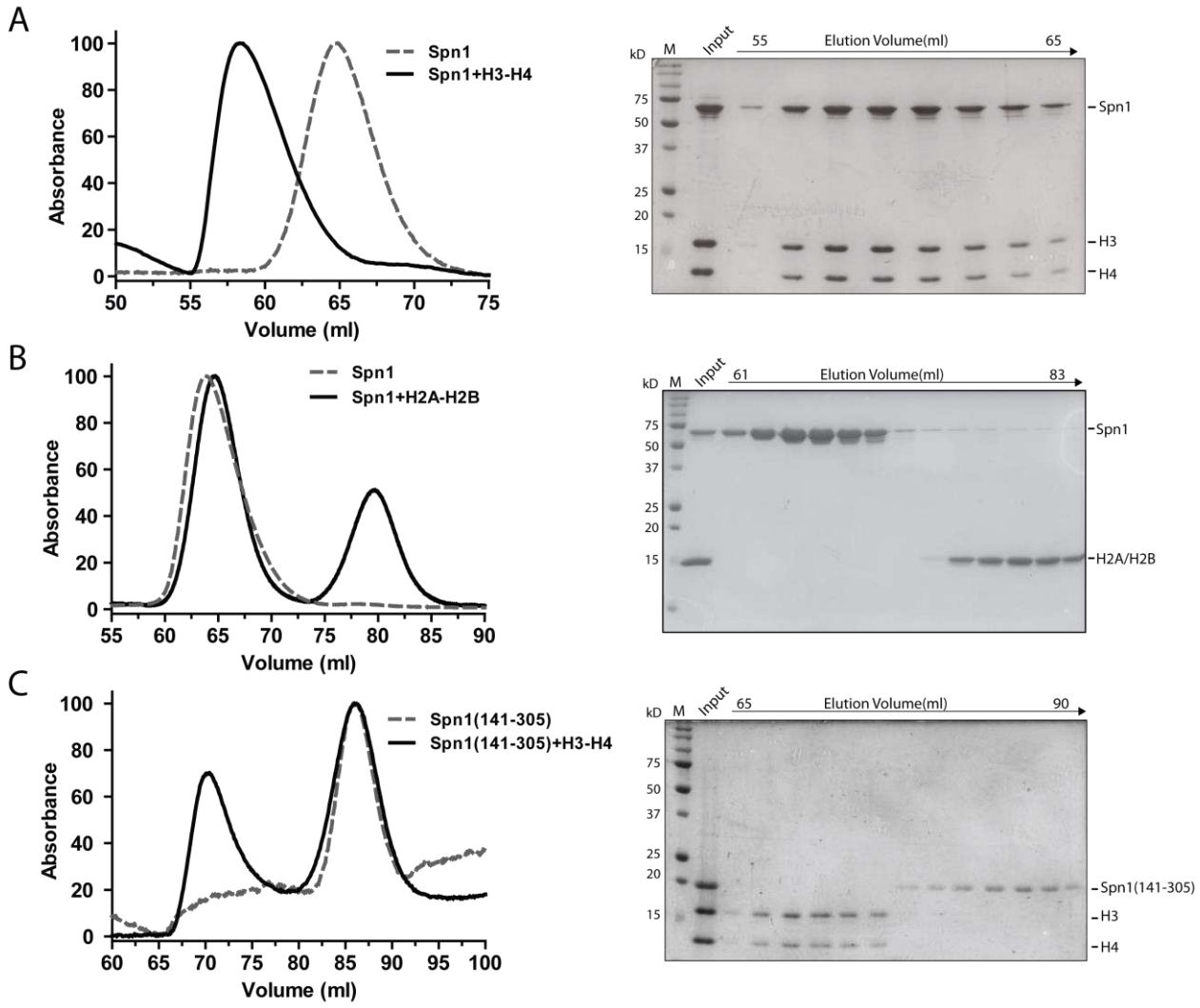


Figure 2.1 Overlays of size-exclusion chromatograms with Spn1 alone (grey, dashed lines) and Spn1-histone binding experiments (black, solid lines). The x-axis shows elution volume (the void volume is 40 ml). The y-axis shows normalized absorbance at 280nm. Fractions collected from each binding experiment were analyzed on 15% SDS-PAGE (Spn1 alone fractions were not shown). (A) Spn1 and histone H3-H4 co-eluted. (B) Spn1 and H2A-H2B could not interact and elute separately. Spn1 has a 2-fold higher molar extinction coefficient than H2A-H2B, therefore Spn1 peak appears approximately 2 times higher than the H2A-H2B peak at equimolar concentration. (C) Spn1(141-305) and H3-H4 could not interact and elute separately.

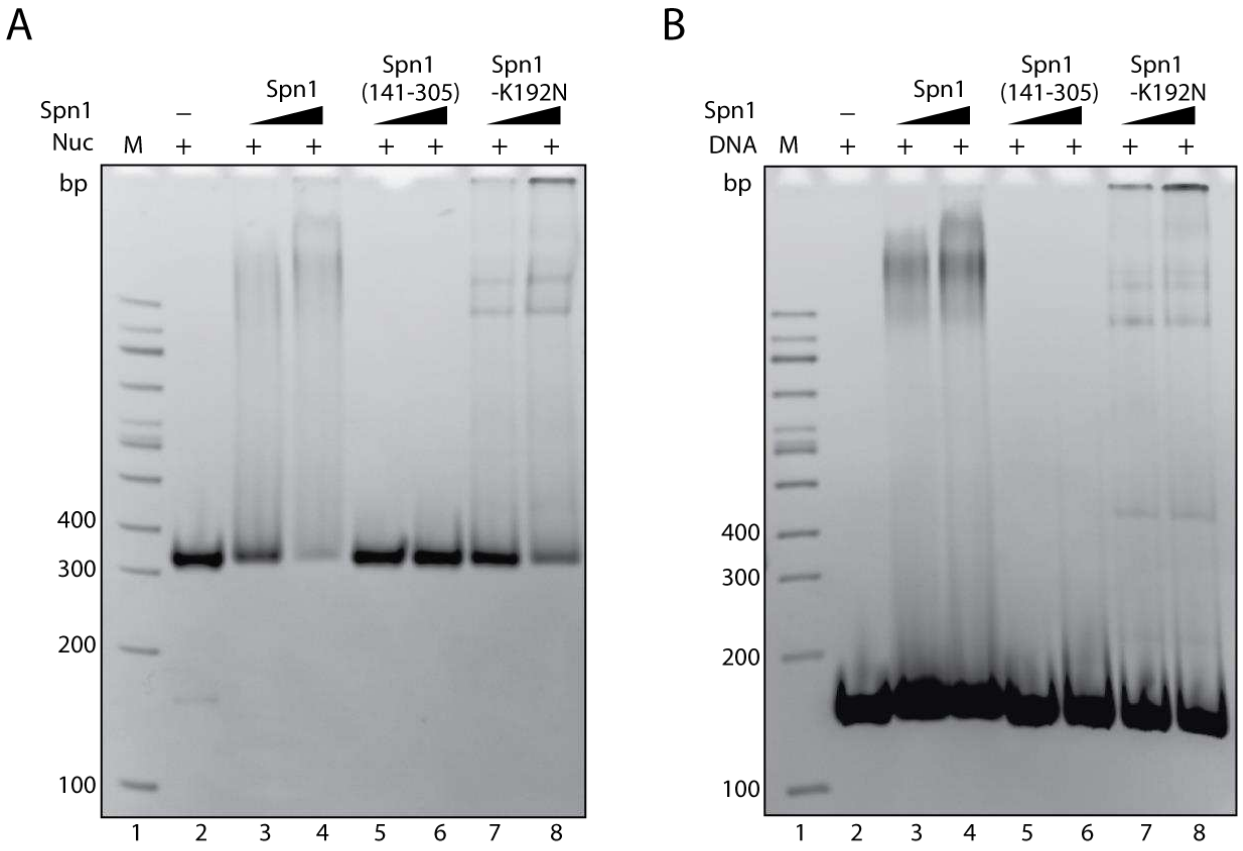


Figure 2.2 Spn1 and Spn1-K192N can bind nucleosomes and DNA, whereas central domain Spn1(141-305) cannot. 5 μM of nucleosomes or DNA were incubated with a 2 or 4 fold molar excess of Spn1 constructs as indicated for 30 minutes at room temperature. Complexes were analyzed by electrophoresis through a 5% native PAGE. The gels were stained with ethidium bromide.

2.3 DISCUSSION

In eukaryotes, different chromatin factors perform various functions to access the genomic DNA for transcription, replication, repair and recombination. Histone chaperones have been identified to relieve the nucleosome barrier and increase DNA accessibility. Histone chaperones bind histones to assemble and/or disassemble nucleosomes.

We found Spn1 binds histone and promotes nucleosome assembly *in vitro* (Ling Zhang, unpublished). Unlike the known histone chaperones such as Nap1 and Spt6 that bind both H3-H4 and H2A-H2B, Spn1 preferentially binds H3-H4 (Andrews et al., 2008; McCullough et al., 2015). In addition, the central domain (141-305) of Spn1 could not bind histone H3-H4, suggesting N-terminal and/or C-terminal regions would be required for histone binding. Spn1 N-terminal region (1-140aa) is acidic and C-terminal region (306-410aa) is basic, however, these two disordered regions extend away from central domain and don't appear to interact (Adam Almeida, unpublished). Therefore, it is likely that the N-terminal region binds histones in that many histone chaperones contain acidic patches that help stabilize interactions with basic histones.

We also found that Spn1 binds nucleosomes and DNA. It is not necessary for histone chaperones to bind nucleosomes or DNA, for example, Nap1 does not bind either. Deletion of both N- and C-terminal regions of Spn1 resulted in loss of binding, suggesting the N-and/or C-terminal regions play an essential role in these activities. It is likely that C-terminal region is responsible for nucleosome and/or DNA binding since it has a basic overall charge (consistent with other DNA binding proteins) and it is separated from histone binding. Moreover, Spn1-K192N, which cannot interact with Spt6 still binds

nucleosomes and DNA. These results make Spn1 unique among histone chaperones, because others, like Nap1, lack nucleosome- and DNA-binding activities. Chaperone Spt6 could bind nucleosomes only in the presence of the small HMGB family member Nhp6 (McDonald et al., 2010). Binding between Spt6 and DNA is much weaker and the Spt6-DNA complex is barely detectable on EMSA (Close et al., 2011).

The features of Spn1 presented here differ from those of other known histone chaperones, because it binds H3-H4 and directly binds to nucleosomes and DNA. Notably, we have shown Spn1 is a multifunctional chaperone capable of binding each component of the nucleosomes as well as the final assembled product.

2.4 METHODS

Protein purification

S. cerevisiae Spn1 and Spn1(141-305) were purified as described (Pujari et al., 2010). *X. laevis* histones H2A, H2B, H3 and H4 were purified and refolded into H2A-H2B dimer or H3-H4 tetramer, or octamer that assembled into nucleosomes as described (Dyer et al., 2004).

Size-exclusion chromatography binding assay

Purified recombinant proteins were mixed at equimolar concentrations (20μM, H3-H4 was calculated as tetramer) and incubated for 15min at 4℃. The protein mixture was chromatographed on a 120 ml Superdex 200 16/60 column (GE Healthcare) in 20 mM Tris pH7.5, 150 mM NaCl, 10% glycerol and 5 mM 2-mercaptoethanol. Fractions were collected and heated at 95℃ for 5min prior to 15% SDS-PAGE.

EMSA of nucleosome and DNA binding

601-147 bp DNA was purified as described (Lowary and Widom, 1998). 5 μM of nucleosome or DNA were incubated with a 2 or 4-fold molar excess of Spn1 constructs for 30 minutes at room temperature. The binding buffer was 50 mM Tris pH7.5, 50 mM NaCl, 2 mM Arginine. The binding reactions were analyzed on 5% native PAGE in 0.2X TBE at 300V and 4℃ for 3h. The gels were stained with ethidium bromide.

CHAPTER 3 [2]

CO-REGULATION BETWEEN SPN1 AND SPT6 IS IMPORTANT FOR THEIR INDEPENDENT HISTONE CHAPERONE ACTIVITY

3.1 INTRODUCTION

Spn1 is an essential and highly conserved factor involved in transcription initiation and elongation, mRNA export, histone modification and the formation of repressive chromatin (Fischbeck et al., 2002; Hainer et al., 2011; Mayer et al., 2010; Yoh et al., 2007; Yoh et al., 2008; Zhang et al., 2008). Our recent study has showed that Spn1 is also a histone H3-H4 chaperone that could assemble nucleosome *in vitro*. We also found Spn1 directly binds nucleosomes and DNA (Chapter 2).

The *Saccharomyces cerevisiae* Spn1 (containing 410 residues) has unstructured N-terminal region, structured central domain and unstructured C-terminal region. The N-terminal region (first 140 residues, 1-140aa) is acidic, while the C-terminal region (last 105 residues, 306-410aa) is basic; these charge features are conserved from yeast to humans. The central domain (middle 165 residues, 141-305aa) is basic and the charge as well as the primary amino acids sequence are highly conserved from yeast to humans (Fischbeck et al., 2002). The central domain covers the essential functions of wild type Spn1 under optimal growth condition, but strains harboring this allele exhibit growth defects when combining with other chromatin factors, indicating the importance of N- and C-terminal regions (Fischbeck et al., 2002). Moreover, binding to histone

[2] Liangqun (Lillian) Huang performed northern blot shown in Figure 3.4, Garrett Edwards performed AUC shown in Figure 3.5D and I performed the rest of the experiments. I would like to thank Yajie Gu and Pamela Dyer for providing labeled histone proteins.

methyltransferase HYPB/Setd2 has been mapped to a region within N-terminal and mRNA export adaptor REF1/Aly has been mapped to a region within C-terminal of human Spn1 (Yoh et al., 2008).

Spt6 is also an essential and highly conserved factor participating in transcription initiation and elongation, as well as mRNA exportation and histone modification(Mayer et al., 2010; Yoh et al., 2007; Yoh et al., 2008). Spt6 is also a histone chaperone that can promote nucleosome assembly and disassembly *in vitro* (Bortvin and Winston, 1996; Kuryan et al., 2012). Spt6 is a multifunctional factor capable of binding RNA polymerase II and each component of the nucleosome (Bortvin and Winston, 1996; Krogan et al., 2002; McDonald et al., 2010; Yoh et al., 2007). The *Saccharomyces cerevisiae* Spt6 (containing 1451 residues) has multiple well-characterized domains (Close et al., 2011). Spt6 N-terminal region is acidic (first ~300 residues) and unstructured. Despite its inherent disorder, Spt6(239-268) forms two helices upon binding with Spn1 (McDonald et al., 2010). Spt6(239-268) is also the region that is required for nucleosome binding, thus Spn1 competes with nucleosomes for Spt6 binding (McDonald et al., 2010). It has been shown that Spn1 and Spt6 interaction is important *in vivo* and mutations within the Spn1-Spt6 interface causes growth defects (McCullough et al., 2015; McDonald et al., 2010).

Despite the fact that Spn1 and Spt6 collaborate in several cellular processes, it is not clear how they each contribute to as chaperones. It is possible that Spn1 and Spt6 would form a co-chaperone complex and enhance chromatin interactions. On the other hand, it is also possible that each chaperone works independently. Here we have dissected Spn1 domains and mapped the nucleosome and histone binding regions, which are distinct

from Spt6 binding region. We also found Spt6 could regulate Spn1-nucleosome interaction and conversely, Spn1 could regulate Spt6-H2A-H2B interaction. In addition, abrogation of Spn1-Spt6 interaction did not produce cryptic transcripts, suggesting that Spn1 and Spt6 work independently during nucleosome assembly. Therefore, dynamic association and disassociation between Spn1 and Spt6 confer them redundant and independent functions.

3.2 RESULTS

Spt6 and nucleosomes could not simultaneously bind to Spn1

Deletion of both N- and C-terminal regions of Spn1 resulted in the loss of nucleosome binding, indicating N- and/or C-terminal regions are required (Chapter 2). The nucleosome-binding region is distinct from Spt6 binding, as the crystal structure of Spn1(148-293)-Spt6(239-268) has been determined indicating that the central domain of Spn1 is required for Spt6 binding. Therefore, we asked whether Spn1 could simultaneously bind to both nucleosomes and Spt6. Before answering this question, we first determined which terminal region was required for nucleosome binding. We generated a series of truncation mutants and performed electrophoretic mobility gel shift assay (EMSA) using nucleosomes that were assembled with 147bp Windom 601 dsDNA (Figure 3.1A and B). We found that the C-terminal region (306-410) is sufficient for nucleosome binding (Figure 3.1B, lanes 13-14) and N-terminal region does not bind nucleosomes (Figure 3.1B, lanes 11-12). Next we set to examine the potential complex that could be formed with Spn1, Spt6 and nucleosomes, using an EMSA capable of resolving these distinct complexes. We allowed fluorescent-labeled Spn1, fluorescent-

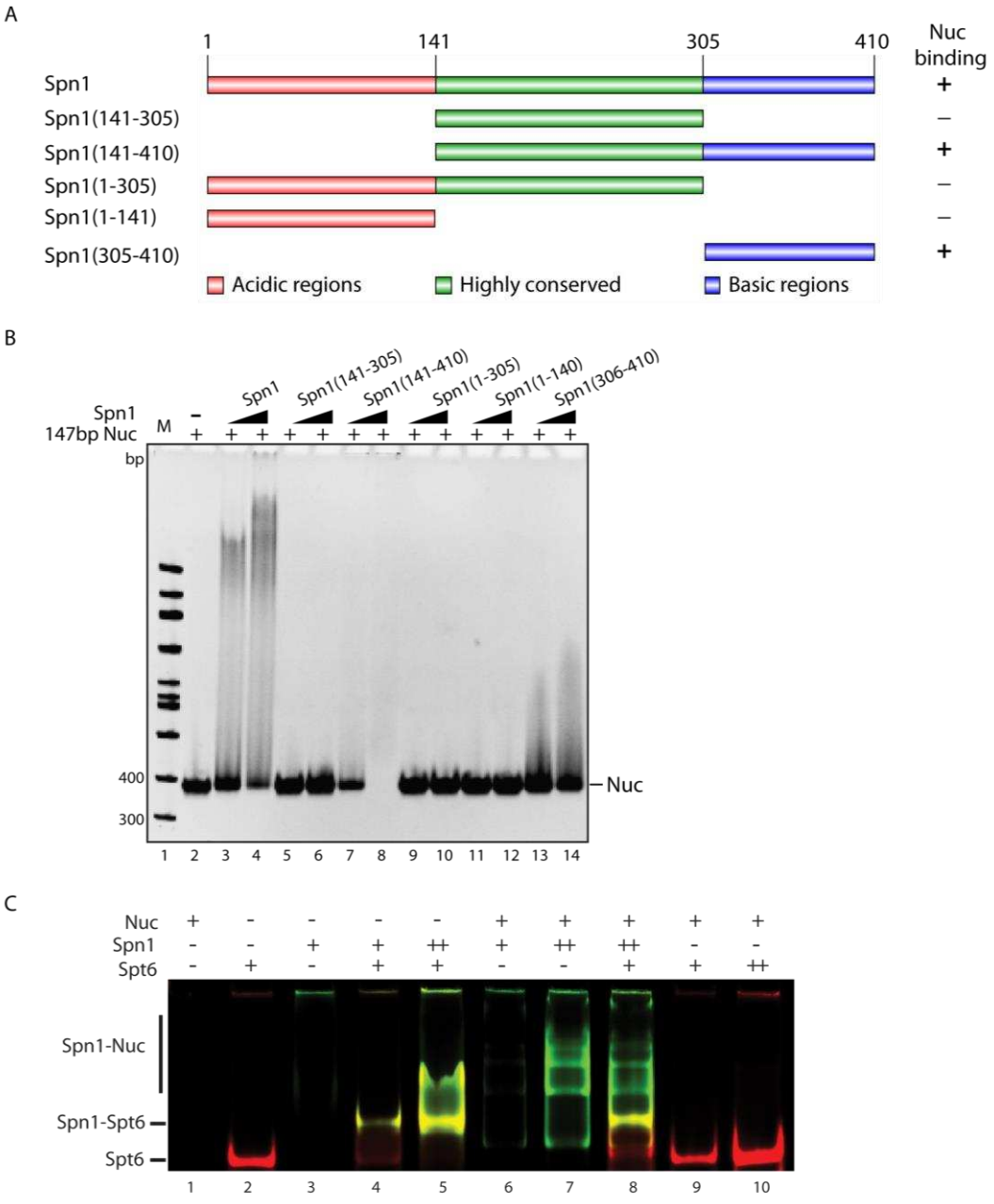


Figure 3.1 Spn1 doesn't bind Spt6 and nucleosomes at the same time. (A) Schematic of Spn1 constructs. The nucleosome binding results from (B) was summarized on the right. (B) EMSA of Spn1 and nucleosomes binding. (C) Spn1 (Atto 532 labeled), Spt6 (Alexa-488 labeled) and nucleosomes (unlabeled) were incubated with each other at room temperature for 15mins prior on native PAGE. The gel was scanned by fluorescence. "+" indicates 0.8 μM and "++" indicates 1.6 μM.

labeled Spt6 and unlabeled nucleosomes to interact with each other, and then separated the complexes by native PAGE. We observed Spn1-Spt6 complex and Spn1-nucleosome complex (Figure 3.1C, lanes 4-5 and 6-7, respectively). Notably, Spn1 per se could not enter the native PAGE, unless in association with Spt6 or nucleosomes. Unlike Spn1, Spt6 per se does not bind nucleosomes unless adding the small HMGB family member Nhp6 (Figure 3.1C, lanes 9-10) (McDonald et al., 2010). Unexpectedly, we did not observe a larger complex with all three components. Instead, we only observed Spn1 in association with one at a time, either Spn1-Spt6 or Spn1-nucleosome (Figure 3.1C, lane 8). This indicated competitive interactions between Spt6 and nucleosome for Spn1. We speculate that the competition is due to steric occlusion. Since Spt6 (theoretical MW 168.2 kD) and nucleosome (~206 kD) are big molecules and their binding regions on Spn1 are adjacent, there is possibly not enough physical room on Spn1 to simultaneously position both Spt6 and nucleosome.

Spt6(239-268) binds H2A-H2B as well as Spn1

A previous study found that Spn1 could block the Spt6-nucleosome interaction in that Spn1 competes with nucleosomes for the same binding region (239-268aa) on Spt6, suggesting that Spn1 could play a regulatory role for Spt6-nucleosome interaction (Figure 3.2A) (McDonald et al., 2010). Our study complements this study and we conclude Spt6 could also play a role in regulating Spn1-nucleosome interaction. Unlike Spt6, however, Spn1 has distinct regions for binding partners (Figure 3.2A). The small region spanning amino acids 239-268 on Spt6 is sufficient to recapitulate the binding of full length Spt6 to Spn1 (Figure 3.2B) (McDonald et al., 2010). We used synthesized Spt6(239-268) peptide (unlabeled) to compete full length Spt6(unlabeled) for Spn1(fluorescent-labeled) binding

using EMSA. As expected, the detectable fluorescent band indicating Spn1-Spt6 complex diminished upon Spt6 peptide titration (Figure 3.2C, compare lanes 1-7 to 8). Since this peptide is located within the acidic N-terminal region of Spt6, we next asked whether it also binds histones.

Histone H2A-H2B (fluorescently labeled) could not enter native PAGE either (Figure 3.2D, lane1), but addition of Spt6 forms a complex that was detectable (Figure 3.2D, lane 4). Dramatically decreased amount of Spt6-H2A-H2B complex was observed upon Spt6(239-268) titration, suggesting that Spt6-H2A-H2B interaction was disrupted by Spt6(239-268)-H2A-H2B interaction (Figure 3.2D, lanes 5-6). Likewise, we also observed decreased amount of Nap1-H2A-H2B complex (Figure 3.2D, compare lanes 8-9 to 7). Since Spt6 could not interact with Nap1 (Chapter 4), therefore Spt6(239-268)-H2A-H2B interaction also competed Nap1-H2A-H2B interaction. Thus we conclude that the small Spt6 peptide also binds histone H2A-H2B. So it is unlikely to obtain a ternary complex with Spn1, Spt6 and H2A-H2B in that Spn1 and H2A-H2B bind to the same regions on Spt6.

A small conserved region within the N-terminal region of Spn1 is important for H3-H4 binding

We next mapped the H3-H4 binding region on Spn1. The known histone chaperones use their acidic domains for histones binding, thus we speculated that the acidic N-terminal region of Spn1 binds H3-H4. We generated a series of deletion mutations and used size-exclusion chromatography to evaluate their binding abilities (Figure 3.3A and B). As expected, deletion of the N-terminal region disrupts H3-H4 binding, as

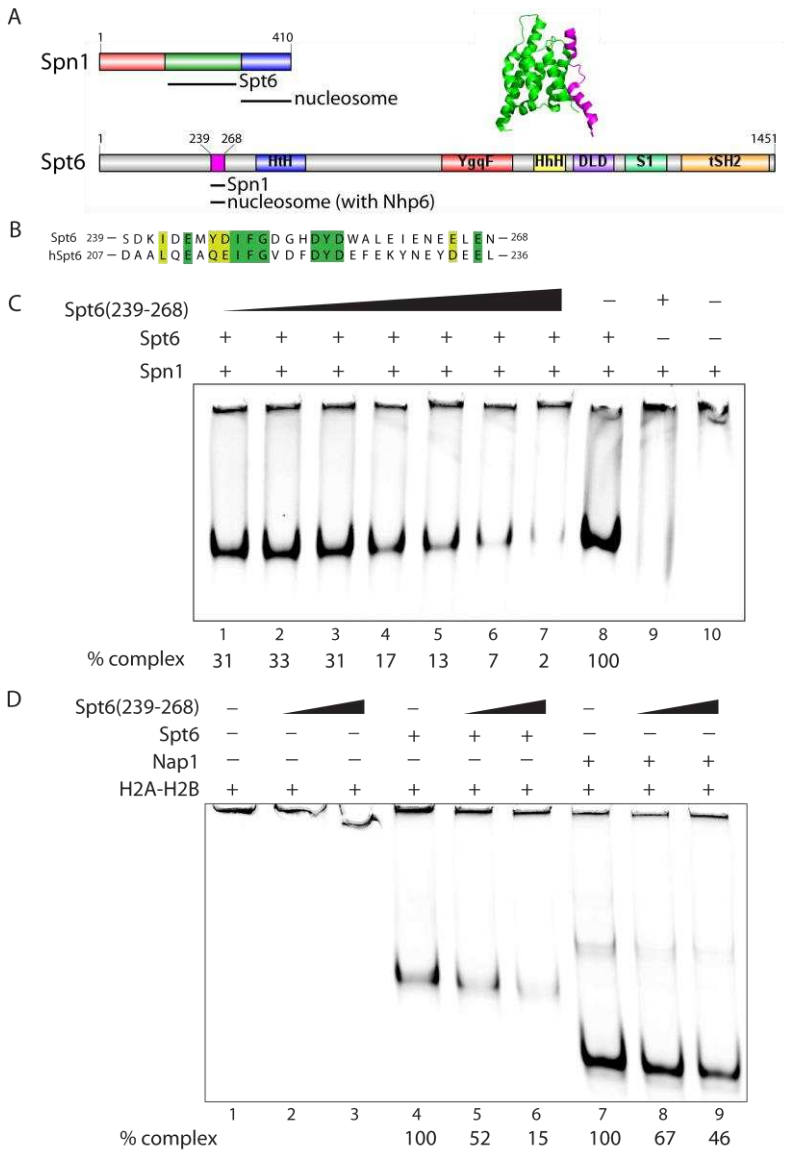


Figure 3.2 H2A-H2B and Spn1 binding to Spt6 are mutually exclusive. (A) Schematic of Spn1 and Spt6 domains. The binding partners interaction regions are mapped (results were from Figure 3.1). The crystal structure is Spn1-Spt6 complex (PDB ID 3OAK). Spn1 in green contains amino acids 148-293. Spt6 in magenta contains amino acids 239-268. (B) Spt6 peptide sequence. It is aligned with human Spt6. Green indicates conserved and yellow indicated semi-conserved. (C) Spt6 peptide could compete full length Spt6 for Spn1 binding. "+" indicates 0.8 μM. Spt6 peptide was titrated (lanes 1-7: 16,32,49,65,81,97,114 μM, lane 8:32 μM). (D) H2A-H2B also binds Spt6 peptide. Both Spt6-H2A-H2B and Nap1-H2A-H2B complex were diminished upon Spt6 peptide titration. H2A-H2B remained at 1 μM. Spt6 remained at 2 μM. Nap1(calculated as monomer) remained at 1.25 μM. Spt6 peptide has two concentrations: 16 and 49 μM.

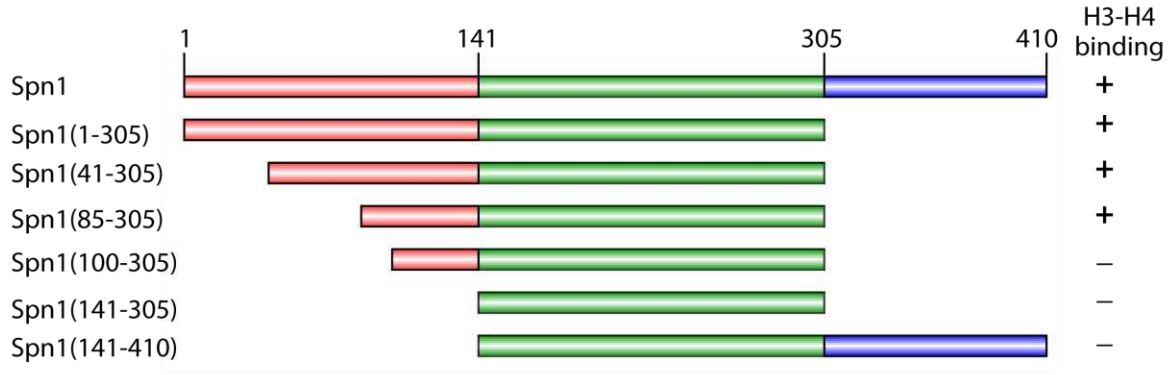
Spn1(141-410) does not co-elute with H3-H4 (Figure 3.3B). In addition, the first 84 amino acids within N-terminal region are not required, but a further deletion of the first 99 amino acids results in loss of binding (Figure 3.3B). This indicated that a small region spanning 85-99aa is important for binding. We aligned the sequences between yeast and humans, and found that this region is highly conserved (Figure 3.3C).

The majority of histone chaperones bind to the globular domain of histones, but some histone chaperones such as Nap1 and FACT bind to both globular domain and histone tails (McBryant et al., 2003; Tsunaka et al., 2016). Interestingly, a newly discovered histone chaperone TONSL only binds the H4 tail (Saredi et al., 2016). Therefore, we asked where Spn1 binds on H3-H4. We made two versions of H3-H4 constructs for binding experiments in size-exclusion chromatography: one contains full length H3 and tailless H4 (H3-H4TL, deletion of the first 20aa of H4), the other contains tailless H3 and full length H4 (H3TL-H4, deletion of the first 27aa of H3). We found Spn1(85-305) could coelute with H3-H4TL, but not with H3TL-H4 (Figure 3.3D). This indicated that histone H3 tail is required for Spn1 binding.

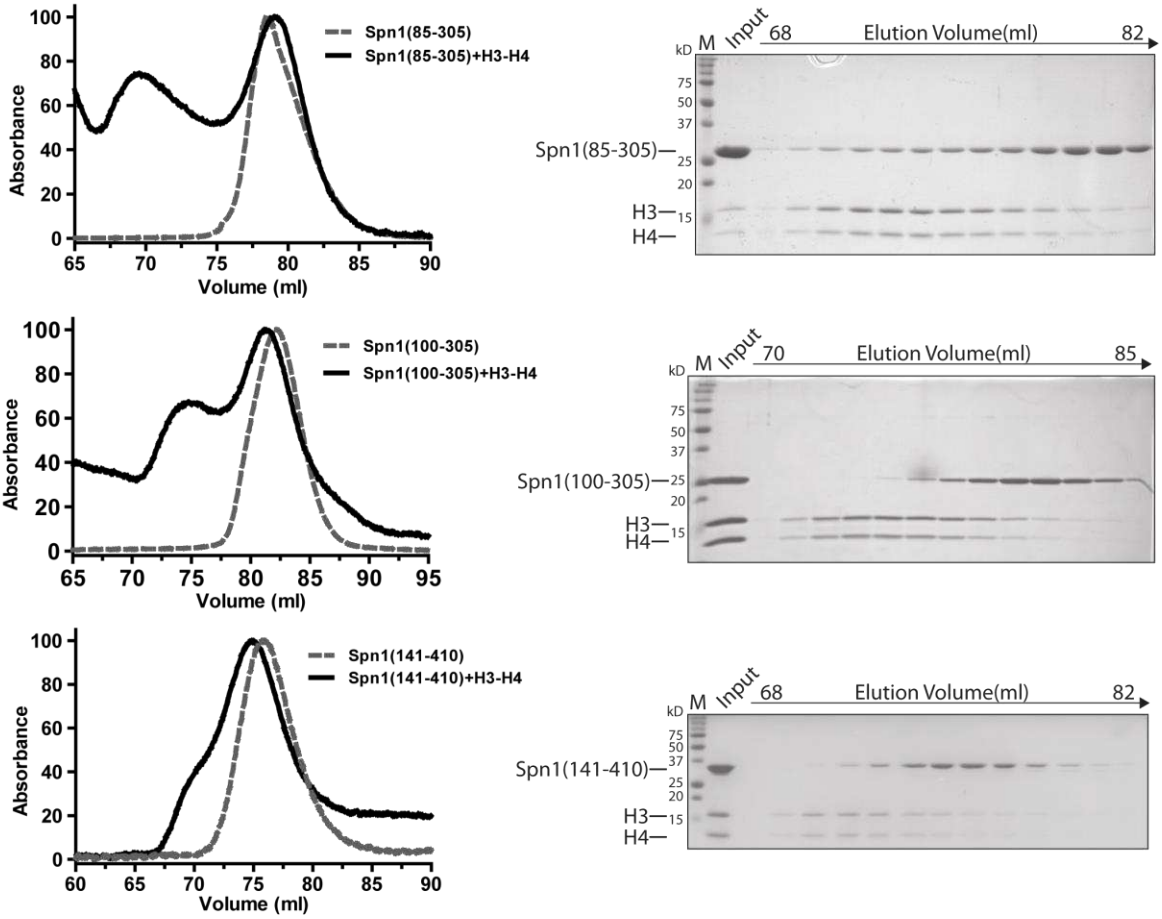
Spn1-Spt6 interaction is not required for nucleosome reassembly *in vivo*

Next we performed northern blotting for known cryptic genes, *STE11*, *SPB4* and *FLO8* in *SPN1* mutants (Cheung et al., 2008). The failure to reassemble nucleosomes in the wake of elongating Pol II allowed transcription initiation factors to bind to and activate cryptic promoters. In contrast to the deletion of *RCO1* and *EAF3* (two components of Rpd3S histone deacetylase complex)（Figure 3.4, lanes 5-6), three different *SPN1* mutants

A


1
141
305
410
H3-H4 binding
Spn1
Spn1(1-305)
Spn1(41-305)
Spn1(85-305)
Spn1(100-305)
Spn1(141-305)
Spn1(141-410)
+
+
+
+
–
–
–

B


Spn1(85-305)
Spn1(85-305)+H3-H4
Absorbance
Volume (ml)
Input
Elution Volume(ml)
kD
M
Spn1(85-305)
H3
H4
Spn1(100-305)
Spn1(100-305)+H3-H4
Spn1(141-410)
Spn1(141-410)+H3-H4

C


Spn1 85— S T D F S D D D L E K E E H N —99
hSpn1 488— F T G F N Q E D L E E E K G E —502

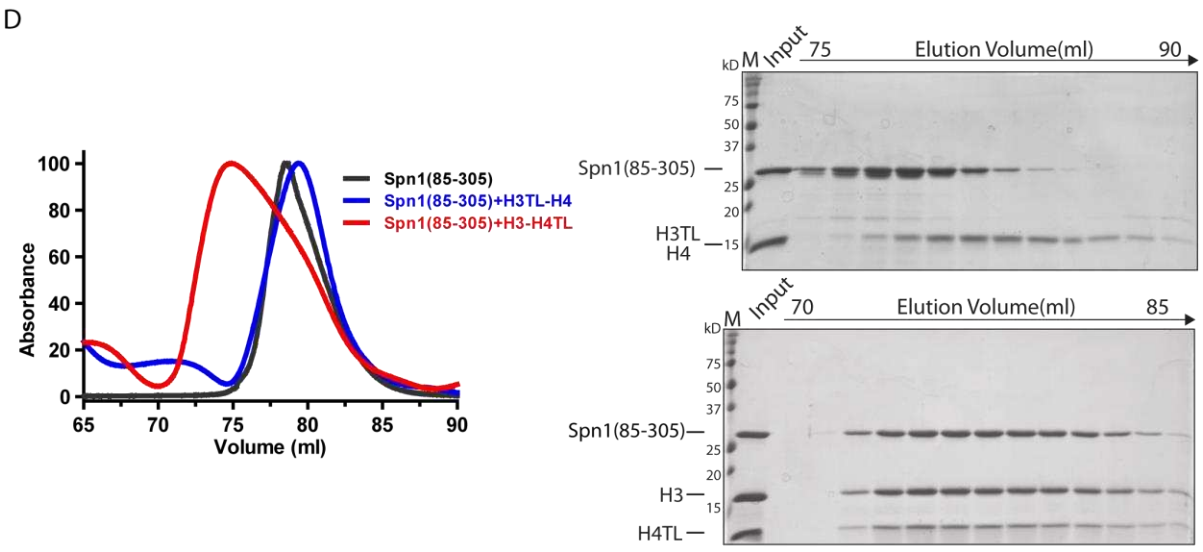


Figure 3.3 Histone H3-H4 binding region is within the N-terminal of Spn1. (A) Schematic of the constructs tested for H3-H4 binding by size-exclusion chromatography. (B) Representative size-exclusion chromatograms and SDS-PAGEs of the complex fractions. (C) Alignment of yeast Spn1 region spanning 85-99 amino acids with human. The green indicates conserved amino acids and yellow indicates semi-conserved amino acids. (D) Spn1(85-305) requires H3 tail for binding. Spn1(85-305) could coelute with H3-H4TL, but not with H3TL-H4. H3-TL means deletion of 1-27aa and H4-TL means deletion of 1-20aa.

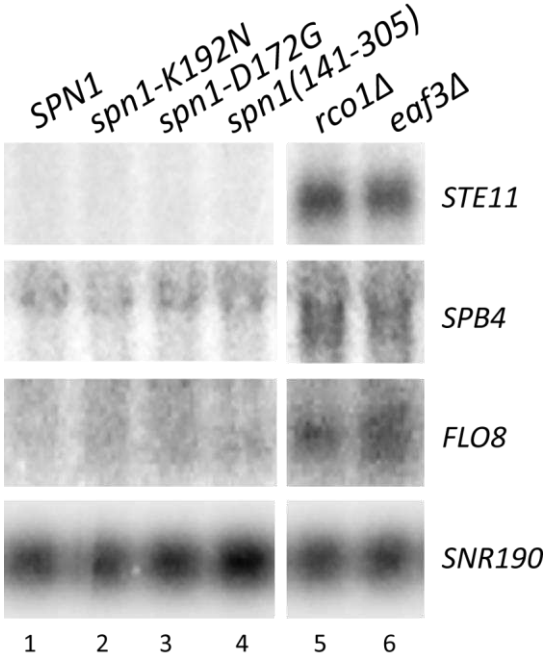


Figure 3.4 Abrogation of Spn1 and Spt6 interaction does not generate cryptic transcripts. Total RNA from the indicated strains were subjected to northern blotting, probing the *STE11, SPB4* and *FLO8* genes (*SNR190* was used as a loading control).

did not generate cryptic transcripts (Figure 3.4, lanes 1-4). *spn1-K192N* and *spn1-D172G* were defective for Spt6 binding, but not for histone binding; *spn1(141-305)* was defective for histone binding, but not for Spt6 binding (Chapter 2) (Pujari et al., 2010). Thus, the Spn1-Spt6 interaction is not required for nucleosome reassembly *in vivo* at certain gens.

Spn1 binds H3-H4 tetramer with a 2:1 stoichiometry in solution

Histone H3-H4 exists within the nucleosome as a tetramer through the H3-H3' four-helix bundle interaction. There are two groups of histone H3-H4 chaperones that bind either H3-H4 dimer or H3-H4 tetramer (Burgess and Zhang, 2013). One group of histone chaperones, such as Asf1 binds to H3 interface that is involved in tetramer formation; another group of histone chaperones, such as MCM2 and Spt2 bind to the H3-H4 tetramer (Chen et al., 2015; English et al., 2006; Huang et al., 2015). Despite both MCM2 and Spt2 binding to the H3-H4 tetramer, their binding sites are quiet different; a pair of MCM2s wrap around the lateral surface, while Spt2 binds to the top surface which forms helix bundle. Therefore, we decided to determine how Spn1 binds H3-H4. We included two H3-H4 constructs in addition to wild type in FRET assays: one is $H3^{C110A,\ L126R,\ I130R}$-H4 mutant that disrupts the H3-H3' four-helix bundle and precludes H3-H4 tetramer formation, the other is cross-linked (XL) H3-H4 that keeps H3-H4 as tetramer (Winkler et al., 2012b).

Spn1 doesn't have any endogenous cysteine residues and therefore we expressed and purified $Spn1^{T185C}$ (threonine at position 185 is mutated to cysteine) for fluorescence labeling purpose (see Appendix VII for phenotypic analysis). Full length Spn1 was labeled with Atto-647N (acceptor) and titrated into a constant amount of Alexa-488 (donor)

labeled histones. Our results showed that Spn1 binds wild type H3-H4 with lower nanomolar affinity (Kd^{app} ~10nM), which is similar to other histone chaperones with different histone complexes under similar conditions (Andrews et al., 2008; Dechassa et al., 2014). We also found Spn1 could interact with both $H3^{C110A,\ L126R,\ I130R}$-H4 dimer ($Kd^{app}$~40 nM) and XL-H3-H4 ($Kd^{app}$~5 nM), and the interactions were quite tight compared to H2A-H2B binding (Kd^{app}~500 nM) (Figure 3.5 A, B and C).

We next performed analytical ultracentrifugation (AUC) experiment to determine the stoichiometry of Spn1 and H3-H4. Instead of using smallest H3-H4 binding construct Spn1(85-305) (theoretical MW 27.37 kD) whose molecular weight is closer to H3-H4 dimer (theoretical MW 26.51 kD), or full length Spn1(theoretical MW 48.25 kD) whose molecular weight is closer to H3-H4 tetramer (theoretical MW 53.02kD), we chose Spn1(1-305) (theoretical MW 36.63kD) for an easy separation. The sedimentation coefficient ($S_{(20,W)}$) of Spn1(1-305) alone is 2.23. Addition of H3-H4 produced two complexes which increased the $S_{(20,W)}$ of Spn1(1-305) to 3.78 and 4.55, respectively. One complex has MW 53.89 kD (closer to one Spn1 for one H3-H4 dimer, MW 63.14kD) and the other has MW 121.03 kD (closer to two Spn1 with one H3-H4 tetramer, MW 126.28kD). As H3-H4 dimer exists in equilibrium with H3-H4 tetramer under physiological conditions, therefore, one Spn1 could bind H3-H4 dimer (1:1 ratio) or H3-H4 tetramer (2:1 ratio) (Figure 3.5F).

3.3 DISCUSSION

A previous study found that Spn1 and nucleosome compete for Spt6 binding in that Spn1 and nucleosome binding regions on Spt6 overlap, indicating that Spn1 regulates the Spt6-

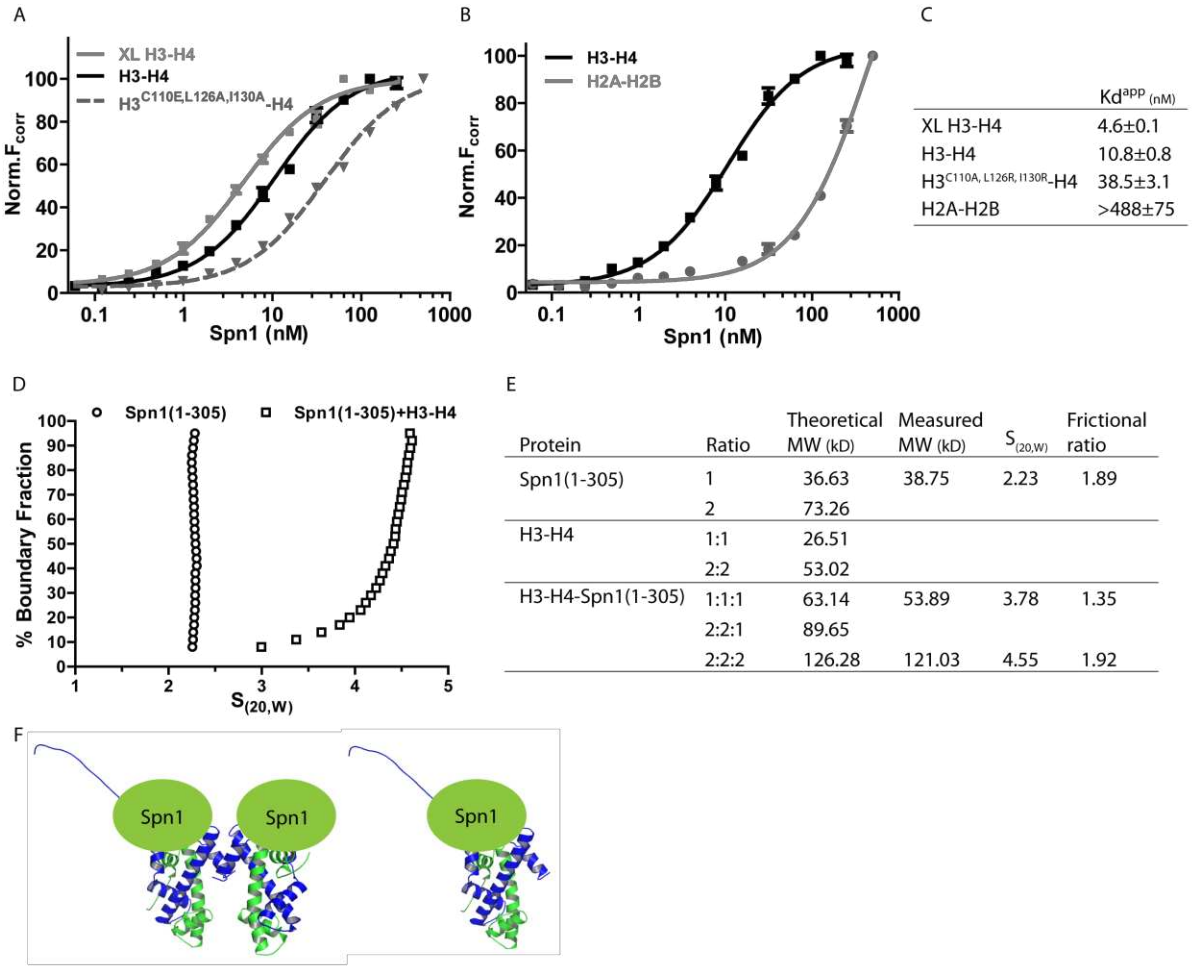


Figure 3.5 Histone-binding activity of Spn1. (A) Spn1 binds both H3-H4 tetramer and H3-H4 dimer with high affinity. The corrected fluorescent change (y axis) was plotted against increasing Spn1 concentration (log[Spn1]) (x axis) to produce a binding curve where the Spn1 concentration at 50% fluorescent change equals the apparent dissociation constant (Kd^{app}) for the Spn1-histone complex. (B) Spn1 prefers binding to histone H3-H4. (C) The data points in (A) and (B) were fit with a non-linear regression curve to establish the apparent dissociation constant. Hill coefficient was set to 1. Each data set has R^2 >0.98. (D) Sedimentation velocity analytical ultracentrifugation (SV-AUC) analysis of Spn1(1-305) alone and Spn1(1-305)-H3-H4 complex under 150mM NaCl. (E) Molecular weight, Sedimentation coefficient and frictional ratio were obtained from (D). (F) Model of Spn1-H3-H4 complex. Left: two Spn1 bind one H3-H4 tetramer; right: one Spn1 binds one H3-H4 dimer.

nucleosome interaction (McDonald et al., 2010). Our study also observed the phenomenon that Spn1, Spt6 and nucleosomes could not form a ternary complex, although Spt6 and nucleosomes bind to distinct regions on Spn1. Spt6 binds to the central domain of Spn1, while nucleosomes bind to the C-terminal region of Spn1. Since the central domain and C-terminal region of Spn1 are adjacent, we speculated that the competition between Spt6 and nucleosomes for Spn1 binding was caused by steric occlusion. In sum, our study, in which we identified distinct Spt6 and nucleosome binding sites on Spn1 and a previous study (where overlapping Spn1 and nucleosome binding sites on Spt6 were found) complement each other. It appears that mutual regulation between Spn1 and Spt6 is important for their independent interactions with nucleosomes. One explanation is that Spt6 releases Spn1 from nucleosomes to initiate multiple rounds of nucleosome assembly (based on our study), and on the other hand, Spn1 releases Spt6 from nucleosomes to allow nucleosome assembly (based on the previous study).

In addition, Spn1 also regulates Spt6-H2A-H2B interaction in that Spn1 and H2A-H2B bind to the same region on Spt6. It is interesting that the small 30aa peptide of Spt6 is sufficient for Spn1, nucleosomes as well as H2A-H2B binding. During nucleosome assembly, association with Spn1 would allow Spt6 to release H2A-H2B for deposition. In addition, we found abrogation of Spn1-Spt6 interaction doesn't produce cryptic transcripts, suggesting that the Spn1-Spt6 interaction was not required for nucleosome assembly *in vivo*. In contrast, Spt6 mutant *spt6-1004* (deletion of 931-994aa, within the helix-hairpin-helix domain) has been shown to produce cryptic transcripts. This deletion is not contained within the Spn1-Spt6 interface (Kaplan et al., 2003).

We also mapped the H3-H4 binding region on Spn1 and found a small conserved region spanning amino acids 85-99 is important for binding. This small region is within the acidic N-terminal of Spn1 and does not overlap with Spt6 binding site. Thus we cannot exclude the possibility of a ternary complex with Spn1, Spt6 and H3-H4. We also found that the histone H3 tail is required for Spn1 binding. Another study using a peptide pull-down found that human Spn1 preferentially binds to H3K4me2 (Chan et al., 2009). H3K4me2 can be recognized by the ATP-dependent chromatin remodeler Chd1 and regulated by PAF1 complex; association of Chd1 with PAF1 complex and H3K4 methyltransferase core complex serves to facilitate pre-mRNA splicing (Flanagan et al., 2005; Sims et al., 2007). In addition, Spn1 genetically interacts with both Chd1 and the PAF1 complex (Collins et al., 2007).

We also determined that Spn1 could bind H3-H4 dimer with a 1:1 ratio, or H3-H4 tetramer with a 2:1 ratio, since H3-H4 dimer and H3-H4 tetramer coexist in equilibrium under physiological condition. Spn1 does not dimerize *in vivo*, thus it appears one Spn1 binding to one H3-H4 dimer is independent of a second Spn1 binding to the second H3-H4 dimer (Pujari et al., 2010). Connecting with the known histone chaperones, it is possible that Spn1 could adapt an earmuff-binding mode similar to MCM2 on H3-H4 (Huang et al., 2015).

In sum, Spn1-Spt6 is an inactive co-chaperone complex for nucleosome assembly and the two proteins must be separated for their independent activities. Once the nucleosome is formed, Spn1 and Spt6 could associate and colocalize with Pol II for the next assembly/disassembly cycle. Studies with human Spn1 and Spt6 suggested that Spn1-

Spt6-CTD of Pol II form a megacomplex which is required for mRNA processing and exportation (Yoh et al., 2008). Therefore, dynamic association and dissociation between Spn1 and Spt6 are important for their distinct as well as redundant functions.

3.4 METHODS

Protein purification and fluorescent labeling

Spn1(141-305) was purified as described (Pujari et al., 2010). All the other Spn1 constructs followed similar procedure and the histidine tag within each construct was not removed. Spt6 was purified as described (Close et al., 2011). Spt6 was fluorescently labeled at its native surface-exposed cysteine residues. Spt6(239-268) (crude grade) was purchased from NeoScientific and resuspended in buffer containing 20 mM Tris pH7.5, 150 mM NaCl. The concentration of the peptide was determined by its extinction coefficient and absorbance at 280 nm obtained from NanoDrop. Histones were purified as described and H2A-H2B^{T118C} and H3-H4^{T71C} were used for labeling purpose (Dyer et al., 2004). All the labeled proteins were purified followed the protocol as described (Winkler et al., 2012a).

Sedimentation velocity analytical ultracentrifugation

Spn1(1-305)-H3-H4 was purified from 120 ml Superdex 200 16/60 column (GE Healthcare) in 20 mM Tris pH7.5, 150 mM NaCl, and 0.2 mM TCEP. The fractions were collected and concentrated to 400ul, A280~0.4. Sedimentation velocity analytical ultracentrifugation experiments were performed using a Beckman Coulter Optima XL-A analytical ultracentrifuge. The reactions (Spn1(1-305) alone and complex) and were spun in an AN-60 Ti rotor at 50,000 rpm at 20℃. Partial specific volumes of sam ples were determined using UltraScan 3 version 2.0. Time invariant and radial invariant noise were

subtracted from the sedimentation velocity data by 2-dimensional Spectrum Analysis (2DSA) followed by genetic algorithm refinement and Monte Carlo analysis. Sedimentation coefficient distribution G(s) were obtained with enhanced van Holde-Weischet analysis. Calculations were performed on the UltraScan LIMS cluster at the Bioinformatics Core Facility at University of Texas Health Science Center at San Antonio and Lonestar cluster at Texas Advanced Computing Center. The data was plotted by Graphpad Prism.

FRET

We followed the procedure as described (Winkler et al., 2012a). Each assay was done using a reaction buffer of 20mM Tris-HCl at pH 7.5, 150mM NaCl, 1mM EDTA, 5% Glycerol, 1mM DTT, 0.01% NP40, and 0.01% CHAPS. Reactions were incubated for 30mins at room temperature. The plates were scanned on a Typhoon Trio variable mode imager. The data points were normalized and plotted in GraphPad Prism.

Size-exclusion chromatography binding assay

Purified recombinant proteins were mixed and incubated for 15min at 4℃. The protein mixture was chromatographed on a 120 ml Superdex 200 16/60 column (GE Healthcare) in 20 mM Tris pH7.5, 150 mM NaCl, 10% glycerol and 5 mM 2-mercaptoethanol. Fractions were collected and heated at 95℃ for 5 min prior to 15% SDS-PAGE.

EMSA

The binding buffer was 20 mM Tris pH7.5, 150 mM NaCl, 2 mM Arginine, 1mM DTT, 0.5 mM EDTA. The binding reactions were analyzed on 5% native PAGE in 0.2X TBE at 150V and 4℃ for 1h.

CHAPTER 4 [3]

THE HISTONE CHAPERONES SPN1 AND NAP1 INTERACT *IN VITRO* AND *IN VIVO*

4.1 INTRODUCTION

Several RNA polymerase II elongation factors of *Saccharomyces cerevisiae* were identified through tandem affinity purification (TAP). Tagging and purification of Spt6 resulted in copurification of a previously uncharacterized protein Spn1 (also named Iws1, interacts with Spt6) (Krogan et al., 2002) . Since then, a number of studies have revealed that Spn1 coordinates with Spt6 in transcription initiation and elongation, mRNA processing and export, histone modification, as well as heterochromatic silencing (Kiely et al., 2011; Mayer et al., 2010; Yoh et al., 2007; Yoh et al., 2008; Zhang et al., 2008). Subsequently, structures of Spn1 and Spn1-Spt6 complex have been determined and mutations on the interface result in growth defects (McCullough et al., 2015; McDonald et al., 2010; Pujari et al., 2010). Our recent study demonstrated that Spn1 is a new member of the histone H3-H4 chaperone family that could assemble nucleosomes *in vitro*; Spn1 is also multifunctional in that it directly binds nucleosomes and DNA. In order to discover other Spn1 functions, we set out to characterize the physical interaction web of Spn1 with other histone chaperones. We followed a TAP-tagged Spn1 purification procedure and identified Nap1 by immunoblotting.

[3] This chapter is the result of collaboration. All authors contributed extensively to the work, including Liangqun(Lillian) Huang (Figures 4.1A, 4.2B, 4.3A and D, and 4.4B), Kenneth Lyon (Figures 4.2G, 4.3B and E), Catherine Radebaugh (Figure 4.1B) and myself (Figures 4.2C-F and 4.4A and C). I would like to thank Daniel Krzizike and Hataichanok(Mam) Scherman for providing Nap1 proteins, and Yajie Gu and Pamela Dyer for providing labeled histone proteins. I would also like to thank Ling Zhang for initiating the characterization of Spn1-Nap1 interaction.

The nucleosome assembly protein Nap1 is a conserved histone chaperone. In yeast, the *NAP1* gene is not essential, but its deletion in *Drosophila* and mouse leads to embryonic lethality (Lankenau et al., 2003; Ohkuni et al., 2003; Rogner et al., 2000). Nap1 interacts with different histones *in vitro*, but it primarily associated with H2A-H2B *in vivo* and therefore is an H2A-H2B histone chaperone (Andrews et al., 2010; Andrews et al., 2008; Chen et al., 2016). Nap1 plays roles in histone trafficking, nucleosome assembly and disassembly (Andrews et al., 2010; Ishimi and Kikuchi, 1991; Kuryan et al., 2012; Park and Luger, 2006b; Zlatanova et al., 2007). Nap1 is also involved in transcription, as its recruitment to both promoters and open reading frames is upregulated during transcription (Del Rosario and Pemberton, 2008).

However, it is not clear how Nap1 is recruited to chromatin and how it functions during transcription. A previous study has found that Nap1 could be recruited by Yra1 (a conserved essential component of the TREX mRNA transcription and export complex), but there exists other factors that would contribute (Del Rosario and Pemberton, 2008). Since multiple lines of evidence have shown that Spn1 binds to RNA polymerase II and travels along during transcription, we speculate that Nap1 could also be recruited by Spn1 (Fischbeck et al., 2002; Mayer et al., 2010; Yoh et al., 2008). Here we found that Spn1, Nap1 and histones could form a complex, suggesting that Spn1 could maintain Nap1-histones in the nucleus during nucleosome assembly. In addition, Nap1 and Spt6 compete for Spn1 binding due to steric occlusion, therefore disruption of Spn1-Spt6 interaction no longer allows Spt6 to compete Nap1 from Spn1 binding.

4.2 RESULTS

Nap1 associates with Spn1 *in vivo*

S. cerevisiae Spn1 was originally identified in copurification with Spt6 and in a genetic screen for transcription factors that were capable to suppress a defective TBP allele (Fischbeck et al., 2002; Krogan et al., 2002). In order to identify other known histone chaperones that interact with Spn1, we used the same strategy and set out to purify TAP-tagged Spn1. As a result, we detected the presence of Nap1 by immunoblotting. Nap1 specifically copurified with tagged Spn1, compared to untagged strain (Figure 4.1A). We further tested whether Nap1 genetically interacts with Spn1. *SPN1* is essential, and a strain with allele *spn1(141-305)* combined with deletion of *NAP1* was generated. The region within Spn1(141-305) is highly conserved and fails to interact with histone H3-H4, nucleosomes or DNA (Chapter 2). The *spn1(141-305)* strain exhibits an equally healthy growth compared to the wild type *SPN1* strain on YPD plate containing 10mM caffeine (Figure 4.1B, left image). However, when *spn1(141-305)* allele is combined with *nap1Δ*, growth defects were observed (Figure 4.1B, right image). This suggests that Nap1 has genetic interaction with Spn1 *in vivo*.

The C-terminal region of Spn1 binds to the central domain of Nap1

To investigate whether Spn1 and Nap1 interact directly, we performed a series of EMSAs using Spn1 and Nap1 constructs (Figure 4.2A). In each reaction, we incubated constant amount of Nap1 construct with 1-2 fold molar excess of Spn1 construct and visualized the results on native PAGE. Here we have one representative gel to reveal that C-terminal region (306-410) of Spn1 sufficiently binds to full length Nap1(Figure 4.2B, lanes 19-20).

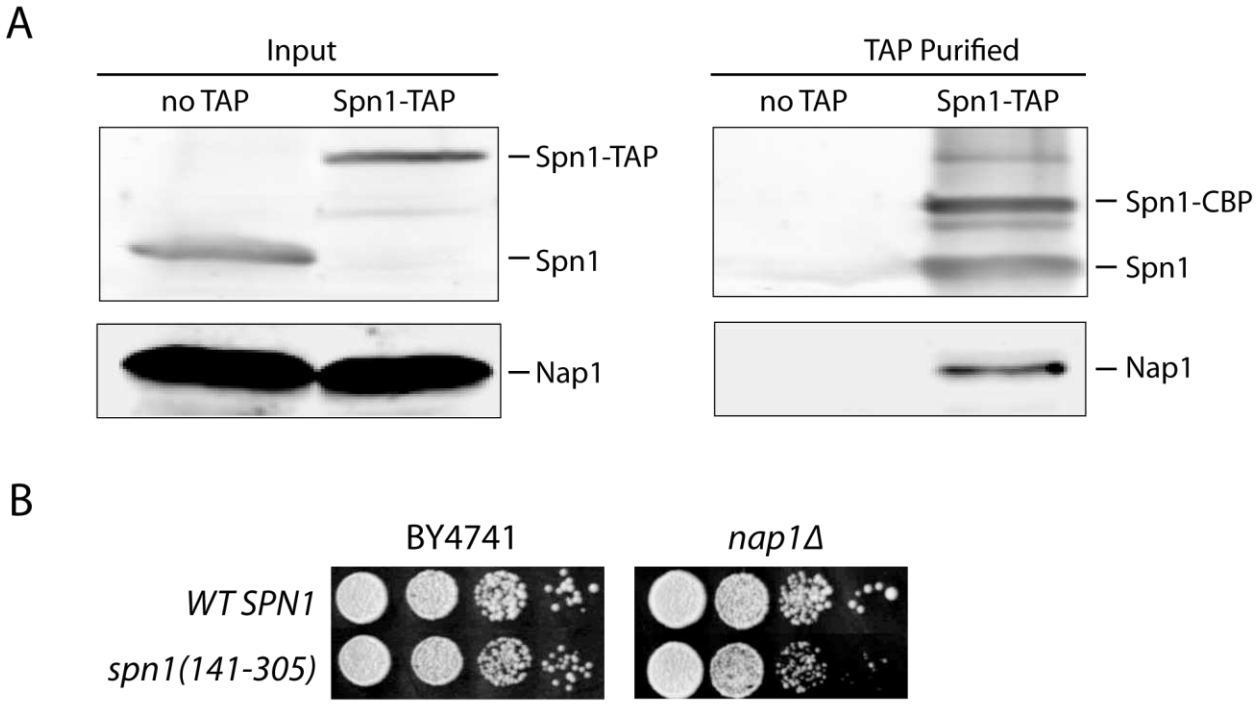
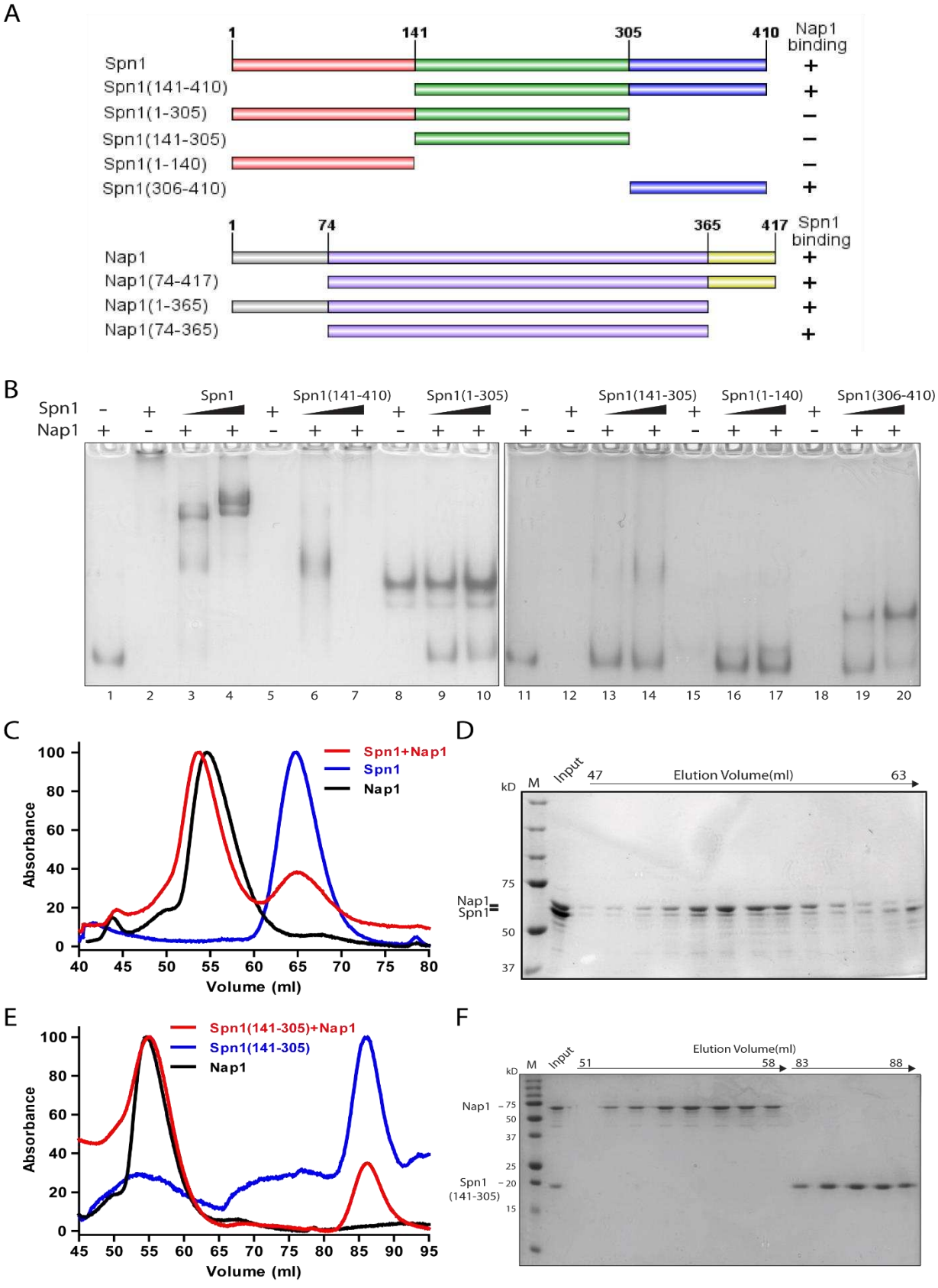
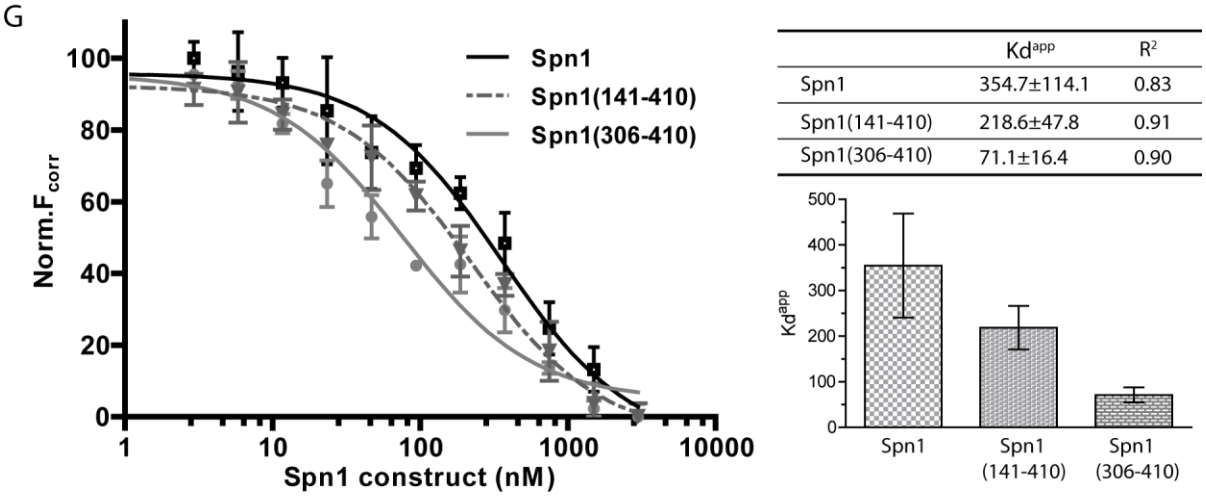


Figure 4.1 Nap1 associates with Spn1 *in vivo*. (A) Whole-cell lysates from a Spn1-TAP and an untagged strain were incubated with IgG Sepharose (input, left panel) and then the IgG-bound protein complex was released by using TEV protease (purified, right panel). Spn1 and Nap1 were detected by immunoblotting with the indicated antibodies. (B) Phenotypic analysis of strain with wild type *SPN1* (or allele *spn1(141-305)*) combined with deletion of *nap1*. The cells were spotted at 10-fold serial dilutions and grown on YPD plates containing 10mM caffeine.

Additional constructs were tested to determine the binding regions for Spn1-Nap1 interaction in a similar way; Nap1 constructs all bind Spn1 and the smallest one is Nap1(74-365) which lacks the disordered N-and C-terminal regions. To further confirm these interactions, we used size-exclusion chromatography and tested one “positive” interaction of Spn1-Nap1 (Figure 4.2B, lanes 3-4) and one “negative” interaction of Spn1(141-305) and Nap1(Figure 4.2B, lanes 13-14). Since Nap1 self-associates and forms a tetramer (theoretical tetramer MW 191.5 kDa) at 150mM NaCl condition, we only observed a modest shift in Nap with Spn1 (theoretical MW 46.08kDa) elution profile compared with Nap1 alone (Figure 4.2C); meanwhile, a significant change was observed compared with Spn1 alone (Bowman et al., 2014). We also found that central domain (141-305) of Spn1 couldn’t interact with Nap1 (Figure 4.2E). When fractions were evaluated by SDS-PAGE, requirement of Spn1 terminal regions was again observed (Figure 4.2D and F).

Next we used a fluorescence quenching assay to measure the binding affinity of Spn1 construct and Nap1. Nap1 was fluorescently labeled and kept constant, while each Spn1 construct was titrated. The measured fluorescence changed upon increasing Spn1 construct, indicating a direct interaction between Spn1 and Nap1. We found that Spn1 C-terminal region (306-410) binds Nap1 with ~5-fold higher affinity than full-length Spn1 (Kd ~70nM and ~350nM, respectively), which was consistent with our native PAGE observation above that C-terminal region is sufficient for Nap1 binding. Deletion of acidic N-terminal of Spn1 slightly enhanced the binding affinity (Kd ~220nM). Moreover, neither N-terminal region nor central domain of Spn1 binds Nap1, suggesting that the N-terminal


A
1
141
305
410
Nap1 binding
Spn1
Spn1(141-410)
Spn1(1-305)
Spn1(141-305)
Spn1(1-140)
Spn1(306-410)
1
74
365
417
Spn1 binding
Nap1
Nap1(74-417)
Nap1(1-365)
Nap1(74-365)
B
Spn1
Nap1
Spn1
Spn1(141-410)
Spn1(1-305)
Spn1(141-305)
Spn1(1-140)
Spn1(306-410)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
C
Spn1+Nap1
Spn1
Nap1
Absorbance
Volume (ml)
D
kD
M
Input
47
Elution Volume(ml)
63
75
Nap1
Spn1
50
37
E
Spn1(141-305)+Nap1
Spn1(141-305)
Nap1
Absorbance
Volume (ml)
F
Elution Volume(ml)
kD
M
Input
51
58
83
88
Nap1
75
50
37
25
Spn1
(141-305)
20
15



	Kd^{app}	R^2
Spn1	354.7±114.1	0.83
Spn1(141-410)	218.6±47.8	0.91
Spn1(306-410)	71.1±16.4	0.90

Figure 4.2 Spn1 and Nap1 physically interact. (A) Schematic illustration of Spn1 and Nap1 truncation constructs. The EMSAs results were summarized on the right indicating interaction (+) or no interaction (-). (B) Representative native PAGE showed Nap1 interacted with various Spn1 constructs. A constant amount of Nap1 (5 μM, calculated as monomer) was incubated with increasing amount of Spn1 construct (5 and 10 μM, respectively). Protein-protein complexes were visualized by Coomassie staining. (C) and (E) Overlays of size-exclusion chromatograms with Spn1(or Spn1(141-305))alone (blue), Nap1 alone (black), and Spn1(or Spn1(141-305))/Nap1 mixture (red). The mixture contained 20 μM Spn1(or Spn1(141-305) and 20 μM Nap1 (calculated as monomer). The x-axis shows elution volume (the void volume is 40 ml). The y-axis shows normalized absorbance at 280nm. (D) and (F) SDS-PAGE analysis of the Spn1/Nap1 mixture or Spn1(141-305)/Nap1 mixture chromatographic fractions. (G) C-terminal region of Spn1 binds Nap1 with ~5-fold higher affinity than full length Spn1. The normalized fluorescence changed upon titration of Spn1 construct (0-3000 nM) into fluorescently labeled Nap1 (10 nM in reactions with Spn1 or Spn1(306-410), 5 nM in reaction with Spn1(141-410)). Each curve is one experiment, and each data point is the mean of quartic measurements within a replicate. The Hill coefficient was set to 1. The Kd^{app}s and R^2s were listed in the right and the Kd^{app}s were demonstrated in bar graph below.

and central domain may hinder Nap1 binding. There is a possible intramolecular interaction between N-terminal (and/or central domain) and C-terminal regions.

Spn1, Nap1 and histones form a complex

Unlike Nap1, which binds both H2A-H2B and H3-H4 with similarly high affinity (Kd ~1nM), Spn1 prefers binding to H3-H4 (Kd ~10nM) (Andrews et al., 2008; D'Arcy et al., 2013) (Chapter 3). H3-H4 binds to the N-terminal region of Spn1, while Nap1 binds to the C-terminal region of Spn1. Therefore we asked whether Spn1, Nap1 and H3-H4 could form a complex. We used EMSAs to visualize the complex directly. H3-H4 is positively charged and could not enter the gel (Figure 4.3A, lane 7), whereas it forms distinct bands in the presence of Nap1 (Figure 4.3A, lane 5). We did not observe any complex when Spn1 was added to Nap1-H3-H4, nor did we observed free Nap1, thus the addition of Spn1 might change solubility state or electric charge of the Nap1-H3-H4 complex (Figure 4.3A, lane 4). To test this, we used C-terminal (306-410) of Spn1 and indeed slower migrating bands were observed (Figure 4.3A, lane 11). The bands represented higher ordered complexes of Spn1, Nap1 and H3-H4. Next, we applied the HI-FI competition assay to examine whether Spn1 would affect interaction between Nap1 and H3-H4 (Hieb et al., 2012; Winkler et al., 2012a)..

$H3\text{-}H4_{Donor}$ was combined with $Nap1_{Acceptor}$, and increasing amounts of unlabeled Nap1 (or Spn1 construct) was added to compete with the $H3\text{-}H4_{Donor}$-$Nap1_{Acceptor}$ complex (Figure 4.3G). $H3\text{-}H4_{Donor}$ (10 nM) concentration was 5-fold below $Nap1_{Acceptor}$ (50 nM) concentration in order to eliminate the possibility of free $H3\text{-}H4_{Donor}$ in the reaction. The

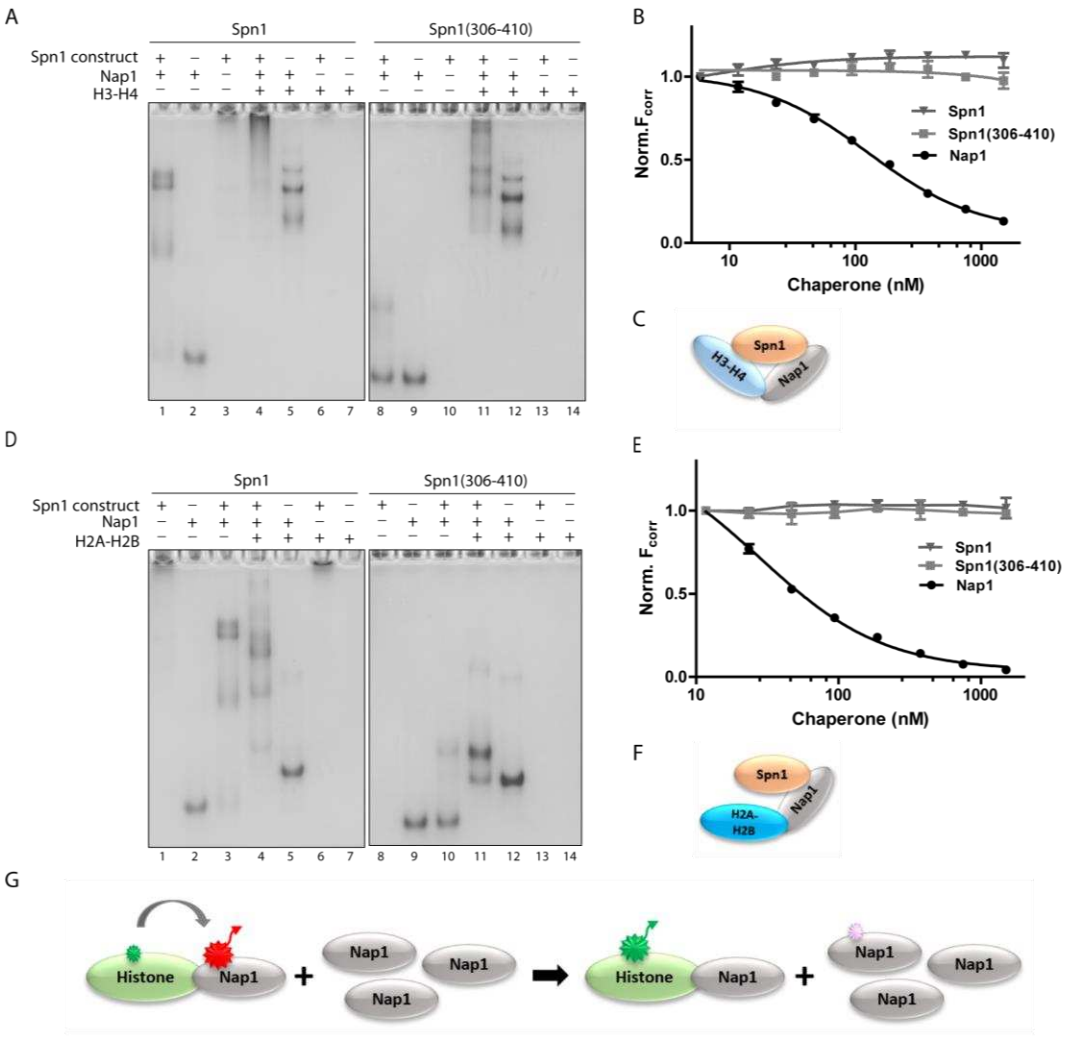


Figure 4.3 Spn1, Nap1 and histone H3-H4 or H2A-H2B could form a ternary complex. (A) EMSA was shown Spn1(and Spn1(306-410)), Nap1 and histone H3-H4 complex. Protein-protein complexes were visualized by Coomassie staining. "+" indicates 5 μM for both (A) and (C). (B) FRET competition of unlabeled Nap1, Spn1, Spn1(306-410) to the H3-H4$_{Donor}$-Nap1$_{Acceptor}$ Complex. H3-H4$_{Donor}$ and Nap1$_{Acceptor}$ were kept constant at 10 nM and 50 nM, respectively, while the unlabeled proteins were titrated(6-1500 nM). Points and error bars represent the average of one experiment with two replicates. (C) EMSA was shown Spn1(and Spn1(306-410)), Nap1 and histone H2A-H2B complex. (D) FRET competition of unlabeled Nap1, Spn1, Spn1(306-410) to the H2A-H2B$_{Donor}$(10 nM)-Nap1$_{Acceptor}$ (50 nM) Complex. The unlabeled proteins were titrated (12-1500 nM).

competition was monitored by a loss of FRET between H3-H4$_{Donor}$ and Nap1$_{Acceptor}$. As expected, Nap1$_{Acceptor}$ was competed with unlabeled Nap1 (Figure 4.3B). In contrast, neither Spn1 or Spn1(306-410) could compete, suggesting that Spn1 doesn't interfere H3-H4$_{Donor}$-Nap1$_{Acceptor}$ interaction. Since Spn1 and Nap1 binding affinities to H3-H4 are comparable (Kd ~10nM vs ~1nM), so if they bind the same surface of histone, Spn1 would hijack the limited amount of H3-H4$_{Donor}$ from H3-H4$_{Donor}$-Nap1$_{Acceptor}$ complex and cause FRET loss. Therefore Spn1 binds to a surface on Nap1 or H3-H4 that is not the surface of Nap1-H3-H4 interaction (Figure 4.3C). Despite Nap1 indiscriminately binds both H3-H4 and H2A-H2B, the H3-H4 interaction is less studied and more studies suggested that Nap1 has more biological functions with H2A-H2B (Andrews et al., 2010; Chen et al., 2016). Therefore, we followed same protocol and determined that Spn1, Nap1 and H2A-H2B could form a ternary complex (Figure 4.3D, lanes 4 and 11). Since Spn1 could not interact with H2A-H2B, Nap1 bridges Spn1 and H2A-H2B together and accommodates them at different sites (Figure 4.3D and 4.3E).

Spn1, Spt6 and Nap1 could not form a ternary complex

The structure of Spn1(148-293)-Spt6(239-268) complex has been determined and the 30 amino acids peptide (239-268) of Spt6 is sufficient to bind central domain of Spn1 (McDonald et al., 2010). Spt6 binding site is not overlapped with Nap1 binding site, which is within the C-terminal region (306-410) of Spn1. Therefore, it seems likely that Spn1, Spt6 and Nap1 could form a ternary complex. We labeled each protein with a distinct fluorescent dye and analyzed the protein-protein complexes using EMSAs. We pre-incubated Spn1 and Nap1 together and they formed Spn1-Nap1 complex (Figure 4.4A, lane 6), then titrated in Spt6 (Figure 4.4A, lanes 7-10). Unexpectedly, we didn't detect a

slower mobility band that would suggest a larger complex. Instead we observed that Spn1-Nap1 complex was diminished and the Spn1-Spt6 complex appeared (Figure 4.4A, lanes 4 and 7-10). Meanwhile, released free Nap1 was visible on the gel (smear band below Spt6 band, Figure 4.4A, lanes 8-10). Additionally, we also found Spt6 and Nap1 could not interact (Figure 4.4A, lane 5).

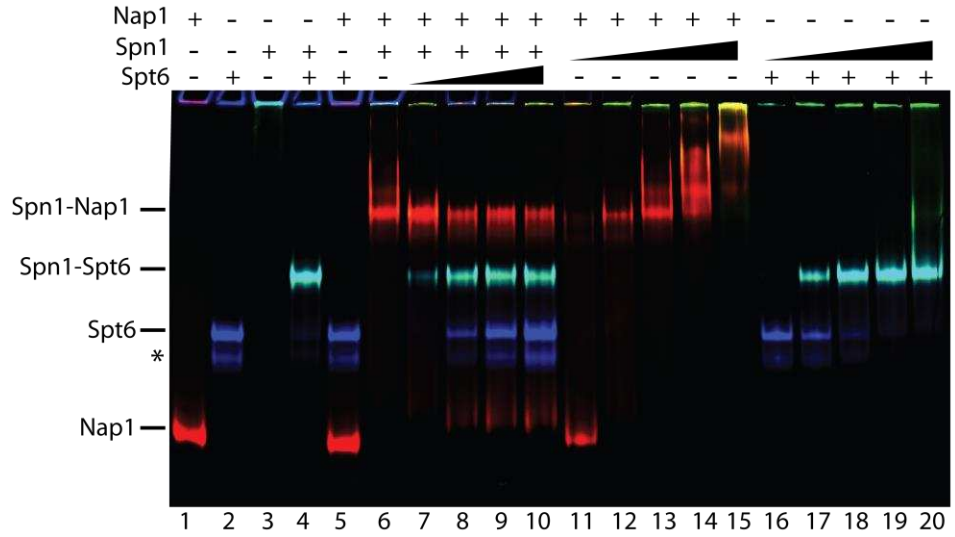
We next purified TAP-tagged Spn1-K192N (lysine residue at position 192 was substituted with asparagine) (Figure 4.4B). Spn1-K192N does not bind Spt6, therefore we speculate Spt6 no longer competes Nap1 for Spn1 binding (Zhang et al., 2008). As expected, but also surprising, we got ~30 fold amount of Nap1 co-purified with Spn1 compared with wild type Spn1 purification. It appears that Spn1 “trapped” Nap1. It suggests that Spn1 functions in two independent complexes: Spn1-Nap1 or Spn1-Spt6.

We further used the Spt6 peptide spanning amino acids 239-268 which is sufficient to recapitulate the binding of Spt6 to Spn1 and repeated the competition assay (Figure 4.4C). Unlike full length Spt6 (Figure 4.4C, lanes 6-8), Spt6(239-268) barely competed Nap1 for Spn1 binding (Figure 4.4C, lanes 9-11). We did not observe released free Nap1 and Spn1-Nap1 complex was unchanged (Figure 4.4C, lanes 5 and 9-11).

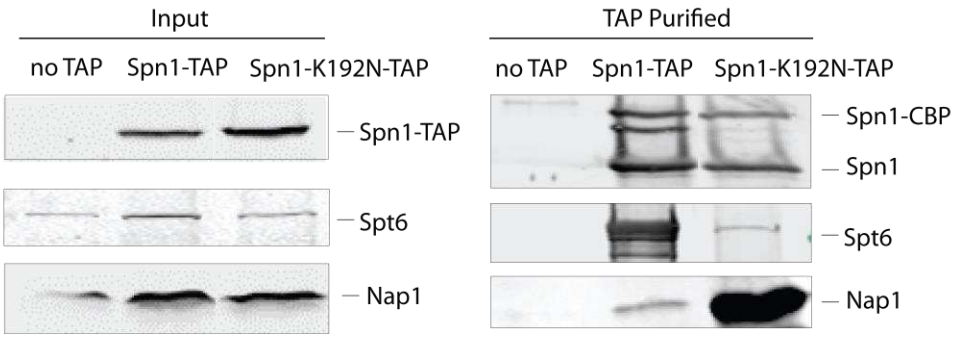
4.3 DISCUSSION

In the Spn1-TAP purification, we found that histone chaperone Nap1 co-purified with Spn1. We also observed that *spn1(141-305) nap1*Δ strain exhibited poor growth, suggesting Spn1 and Nap1 have genetic interaction within the cell. In addition to our study, previous studies indicated that Spn1 and Nap1 could interact. For example, Spn1

A


Nap1
Spn1
Spt6
Spn1-Nap1
Spn1-Spt6
Spt6
*
Nap1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

B


Input
TAP Purified
no TAP
Spn1-TAP
Spn1-K192N-TAP
Spn1-TAP
Spt6
Nap1
Spn1-CBP
Spn1
Spt6
Nap1

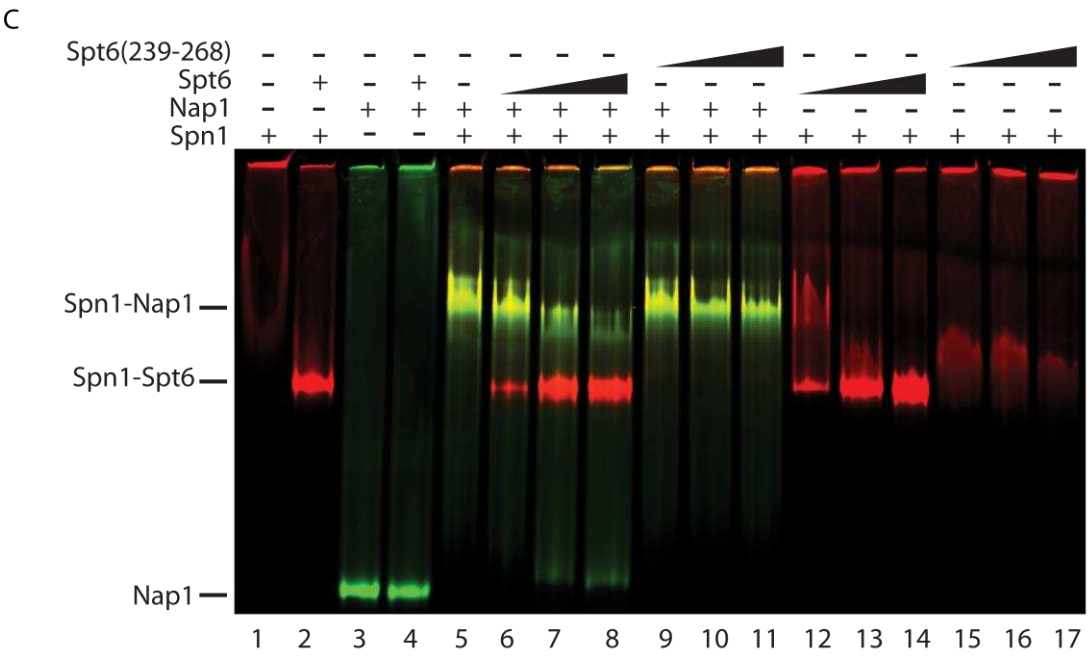


Figure 4.4 Spt6 could compete Nap1 for Spn1 interaction, whereas a small segment (239-268) of Spt6 could not. (A) Spn1, Nap1 and Spt6 could not form a ternary complex and Spt6 competed Nap1 for Spn1 binding. Spn1(Atto 532), Spt6(Alexa 488) and Nap1(Atto 647N) were labeled with distinct fluorescent dyes. Nap1 was kept at 1 μM, Spn1 was at 1.5 μM (0.5, 1, 1.5, 2, 3 μM for lanes 11-14 and 16-20) and Spt6 was at 1 μM (0.5, 1, 1.5, 2 μM for lanes 7-10). *indicates Spt6 degradation product. (B) Whole-cell lysates from Spn1-TAP, Spn1-K192N-TAP and an untagged strain were incubated with IgG Sepharose (input, left panel) and then the IgG-bound protein complex was released by using TEV protease (purified, right panel). Spn1 and Nap1 were detected by immunoblotting with the indicated antibodies. (C) Spt6(239-268) could not compete Nap1 for Spn1 binding. Only Spn1(Atto 532) and Nap1(Atto 647N) were labeled, therefore Spt6 or Spt6(239-268) alone was invisible on the gel. Nap1 was kept at 0.8 μM, Spn1 was at 1.6 μM, Spt6 was at 1 μM (0.5, 1, 2 μM for lanes 6-8 and 12-14) and Spt6(239-268) was titrated (13.6, 25.6 and 51.1 μM for lanes 9-11 and 15-17). Protein colors were determined by Typhoon settings and could not be changed.

and Nap1 genetically interact with Mft1, one subunit of THO complex which is involved in transcription elongation and mRNA export from the nucleus (Collins et al., 2007; Del Rosario and Pemberton, 2008); both Spn1 and Nap1 physically interact with Nab2, which is nuclear polyadenylated RNA-binding protein and required for nuclear mRNA export and poly(A) tail length control (Batisse et al., 2009). Therefore, Spn1 and Nap1 may coordinate in transcription elongation, chromatin assembly and mRNA exportation.

We further mapped Spn1 and Nap1 binding surface and we found that Spn1(306-410) interacts with Nap1(74-365). We also demonstrated that Spn1, Nap1 and histone H3-H4 or H2A-H2B could form a ternary complex. Since Spn1 is a nuclear protein and Nap1 is a nucleocytoplasmic shuttling protein, it is possible that Spn1 is responsible for navigating the ternary complex and maintains the complex in the nucleus, followed by proper positioning histones and assemble nucleosomes in the wake of RNA polymerase II (Mosammaparast et al., 2002).

The export of Nap1 from nucleus to cytoplasm relies on NES (nuclear export sequence, 88-102aa) which is located at the loop connecting α1 and α2 (Park and Luger, 2006a). It has been demonstrated that NESs are recognized by the export karyopherin Crm1 and Nap1 is mediated export via Crm *in* vivo (Mosammaparast et al., 2002). However, The NES is partially masked by adjacent accessory domain (141-180aa). We have shown that Spn1 binds to a different surface on Nap1 rather than Nap1-histone interface; therefore, it is possible that Spn1 binds to NES or its neighboring regions. As a result, NES is completely masked and Nap1 is located in nucleus.

Despite Nap1 and Spt6 binding to different regions on Spn1, we could not obtain a ternary complex. Given that both Nap1 (tetramer formation, theoretical MW 191.5kDa) and Spt6 (theoretical MW 168.2kDa) are big molecules, it is likely due to steric occlusion that there is not enough physical room on Spn1 to simultaneously position both of them. We also speculate that there is a possible intramolecular interaction between the N-terminal region and the C-terminal region of Spn1. Thus it is also likely that Nap1 could disrupt the intramolecular interaction and the weakened intramolecular interaction is disfavored for Spt6 interaction. Although it is not clear what prevents the formation of a ternary complex, it is clear that Spn1-Spt6 complex and Spn1-Nap1 complex function independently. Although Nap1 and Spt6 do not physically interact, the "trapped" Nap1 in nucleus would be rescued by Spt6. Spt6 would compete Nap1 for Spn1 binding, thus releasing Nap1 from Spn1. Interestingly, previous studies suggest that Nap1 and Spt6 have opposite roles. For example, Nap1 could incorporate Htz1 within canonical nucleosomes and Spt6 could not; loss of Nap1 suppressed cryptic transcription and mutation of Spt6 caused cryptic transcription (Cheung et al., 2008; Jeronimo et al., 2015; Park et al., 2005; Xue et al., 2013). Therefore, Nap1 and Spt6 likely cooperate in a certain way.

4.4 METHODS

TAP purification

2 liters of cells were cultured in rich medium and harvested at mid-log phase. Cells were flash frozen, and then broken by grinding in liquid nitrogen using mortar and pestle. The broken cells were resuspend in 40 mL of Hepes lysis buffer (40mM Hepes-KOH, pH7.5, 10% Glycerol, 150 mM NaCl, 0.1% Tween 20, 1 mM EDTA, 1 mM DTT) plus protease inhibitor cocktails. The suspension was sonicated for 7 cycles with 1 minute on and 2

minutes off on ice. The cell lysates were clarified by centrifuging at 4000 rpm for 20 min. The supernatants were first pre-absorbed with 400 µL of sepharose CL-4B (50% slurry) at 4℃ for 1 hour, and then 400 µL of IgG sepharose 6 fast flow (50% slurry, (GE 17-0969-01)) was added to the pre-absorbed supernatant and incubated overnight at 4℃ by rotating. IgG sepharose beads were pelleted down and washed with Hepes lysis buffer 5 times and then TEV cleavage buffer (10 mM Tris-HCl pH 7.5, 10% Glycerol, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT, and 1 mM PMSF) twice. Beads bound TAP proteins were released by TEV protease and precipitated with TCA. The precipitated proteins were subjected to SDS-PAGE and western blotting. Antibodies used include anti-Nap1 (Santa Cruz, sc-7165), anti-Spn1, anti-TAP (Thermo Fisher Scientific, CAB1001) and anti-CBP.

Protein purification and fluorescent labeling

Spn1(141-305) was purified as described (Pujari et al., 2010). All the other Spn1 constructs followed similar procedure and the histidine tag within each construct was not removed. Spn1 doesn't contain cysteine residue and we made Spn1^{T185C} for labeling purpose. Nap1 and its constructs were purified and Nap1^{D201C} was used for labeling purpose (Andrews et al., 2008). Spt6 was purified and labeled (uniformly labeled using its native cysteine residues) as described (Close et al., 2011). Spt6(239-268) (crude grade) was purchased from NeoScientific and resuspended in buffer containing 20 mM Tris pH7.5, 150 mM NaCl. The concentration of the peptide was determined by its extinction coefficient and absorbance at 280 nm obtained from NanoDrop. Histones were purified as described and H2A-H2B^{T118C} and H3-H4^{T71C} were used for labeling purpose

(Dyer et al., 2004). All the labeled proteins were purified as described ((Winkler et al., 2012a).

EMSAs

EMSAs were done in native conditions using a reaction buffer consisting of 20 mM (or 50 mM) Tris-HCl at pH 7.5, 150mM NaCl for protein-protein interactions and 50mM NaCl for protein-nucleosome(or DNA) interactions, 0.5mM EDTA, 1mM DTT, and 10% Glycerol. Reactions were incubated for 15-30mins at room temperature and then ran on 5% native PAGE in 0.2X TBE. Two sizes of gels were used, one size was 20x20 cm (Figure 4.3) and the other was 10x8 cm (the rest of the figures). The big sized gels were ran at 300V and 4℃ for 3 h, while the small sized gels were ran at 150V and 4° C for 1 h. The fluorescent gels were scanned on a Typhoon FLA 9500 (GE Healthcare) and the coomassie stained gels were scanned on ImageQuant LAS 500 (GE Healthcare).

High-throughput interactions by fluorescence intensity assays (HI-FI)

Each assay was done using a reaction buffer of 20mM Tris-HCl at pH 7.5, 150mM NaCl, 1mM EDTA, 5% Glycerol, 1mM DTT, 0.01% NP40, and 0.01% CHAPS. Reactions were incubated for 30mins at room temperature. The plates were scanned on a Typhoon Trio variable mode imager. The data points were normalized and plotted in GraphPad Prism.

CHAPTER 5

SUMMARY AND PERSPECTIVE

SUMMARY

My dissertation focuses on characterization of Spn1, a new member of the histone chaperone family, and its interactions with two other chaperones Spt6 and Nap1. Spn1 was initially identified as a transcription factor that copurified with Spt6 (Fischbeck et al., 2002; Krogan et al., 2002). Spn1 is involved in transcription initiation and elongation, mRNA processing and export, histone modification, as well as heterochromatic silencing. During these processes, Spn1 extensively interacts with chromatin factors, including transcription factors, histone modifiers, chromatin remodelers and mRNA export factors (Kiely et al., 2011; Mayer et al., 2010; Yoh et al., 2007; Yoh et al., 2008; Zhang et al., 2008). Therefore, we set out to examine whether Spn1 directly interacts with nucleosome and its components (histones and DNA), and the short answer is yes. Spn1 recognizes nucleosomes through nucleosomal DNA, possibly as well as through histone tails. We also found that Spn1 is an H3-H4 specific chaperone and a small region (spanning amino acids 85-99) within the acidic N-terminal region of Spn1 is required for the interaction. In addition, the histone H3 tail, but not the H4 tail is required for Spn1 binding.

Previous research found that Spn1 disrupts Spt6-nucleosome binding owing to Spn1 and nucleosome bind to the same region of Spt6, suggesting Spn1 serves as a switch for Spt6-nucleosome interaction (McDonald et al., 2010). Given that both Spn1 and Spt6 could bind nucleosomes, histones and DNA, we extended the characterization. Unlike Spt6, Spn1 separates the regions for Spt6 and nucleosome binding. However, we did not

observed a ternary complex either possibly due to steric occlusion. Instead we found coexisted Spn1-nucleosome and Spn1-Spt6 complex, suggesting that Spt6 regulates Spn1-nucleosome interaction. Therefore, we conclude that regulation between Spn1 and Spt6 are mutual and bidirectional. Unexpectedly, we also found that Spt6 peptide (239-268aa) could compete Spt6 for H2A-H2B binding, suggesting that Spt6 peptide could bind H2A-H2B as well. This raises an interesting question that Spn1 and H2A-H2B could compete each other for Spt6 binding. It is beneficial for Spt6 being able to release H2A-H2B for deposition upon interaction with Spn1. We are not clear whether Spn1, Spt6 and H3-H4 could form a ternary complex or not. If they could not form a ternary complex, one possibility is that H3-H4 also binds to Spt6 peptide which making the interactions mutually exclusive; another possibility comes from steric occlusion that there is not enough room on Spn1 to simultaneously position two partners. However, we also know that Spn1-Spt6 interaction is not required for nucleosome assembly, since we did not observe cryptic transcripts in either Spn1-Spt6 interaction intact or abrogated strains. Therefore, we prefer a speculation that Spn1, Spt6 and H3-H4 could not form a ternary complex.

Based on our current data, we propose a model at a gene whose transcription is regulated by both Spn1 and Spt6 (Figure 5.1). Spn1 and Spt6 associate and travel with Pol II. Once Spn1-Spt6 complex lands on the nucleosome ahead, they would dissociate and act as independent histone chaperones. Spn1 could bind H3-H4 and Spt6 could bind H2A-H2B (as well as H3-H4). In the wake of Pol II, Spn1 and Spt6 reassociate to unload their histone cargos. Once the nucleosome is formed, the Spn1-Spt6 complex again travels with Pol II and get ready for the next nucleosome assembly/disassembly cycle. It has

been demonstrated that Spn1-Spt6-Pol II complex regulates mRNA processing and histone modification (Yoh et al., 2008).

Moreover, we found a new interaction between Spn1 and the histone chaperone Nap1. Spn1 and Nap1 physically interact, as well as share functional similarity *in vivo*. We speculate that Nap1 is possibly recruited by Spn1 to the gene body. We also found that Spn1, Nap1 and histones form a complex, and that Spn1 binds to a distinct surface on Nap1 rather than Nap1-H2A-H2B (or H3-H4) interaction surface. Since Nap1 is a shuttling factor, it is possible that Spn1 guides the ternary complex and maintains it in the nucleus by masking the Nap1 NES (nuclear export sequence). In the nucleus, Nap1 deposits H2A-H2B onto the hexasome. Upon interaction with Spt6, Spn1 dissociates from Nap1 and no longer masks NES. NES becomes accessible to karyopherin Crm1 which would aid Nap1 to return to cytoplasm (Figure 5.2).

PERSPECTIVE

There are still some follow-up questions that need to be answered.

1. We proposed that Spn1 and Spt6 dissociate for their independent chaperone activities, therefore Spn1 and Spt6 together would not enhance the nucleosome assembly. To examine this, a supercoiling assay could be used. Furthermore, we are also interested to exam the disassembly activity of Spn1 and Spt6 using a transcription based assay (Appendix III).

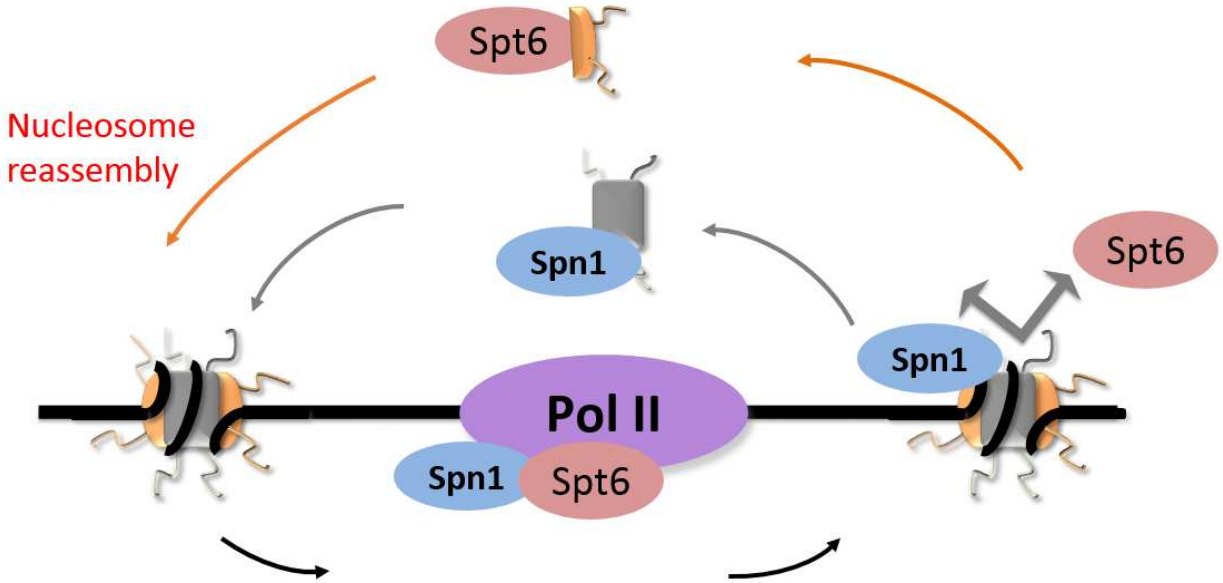


Figure 5.1 Proposed model for association and disassociation of Spn1 and Spt6 during transcription elongation. Above the DNA template shows nucleosome reassembly/disassembly process. Spn1 and Spt6 disassociate (Spn1, Spt6 and nucleosomes could not form a ternary complex) and function independently. Spn1 binds H3-H4 and Spt6 binds H2A-H2B. The Spn1-Spt6 complex is not required for nucleosome assembly process. Below the DNA template shows the nucleosome is formed and Spn1 and Spt6 reassociate. The association of Spn1 and Spt6 allow each other to unload their histone cargos. Spn1-Spt6 complex is required for mRNA processing. As Pol II proceeds and encounters the next nucleosome barrier, Spn1 and Spt6 dissociate and get ready for a new round of nucleosome assembly/disassembly.

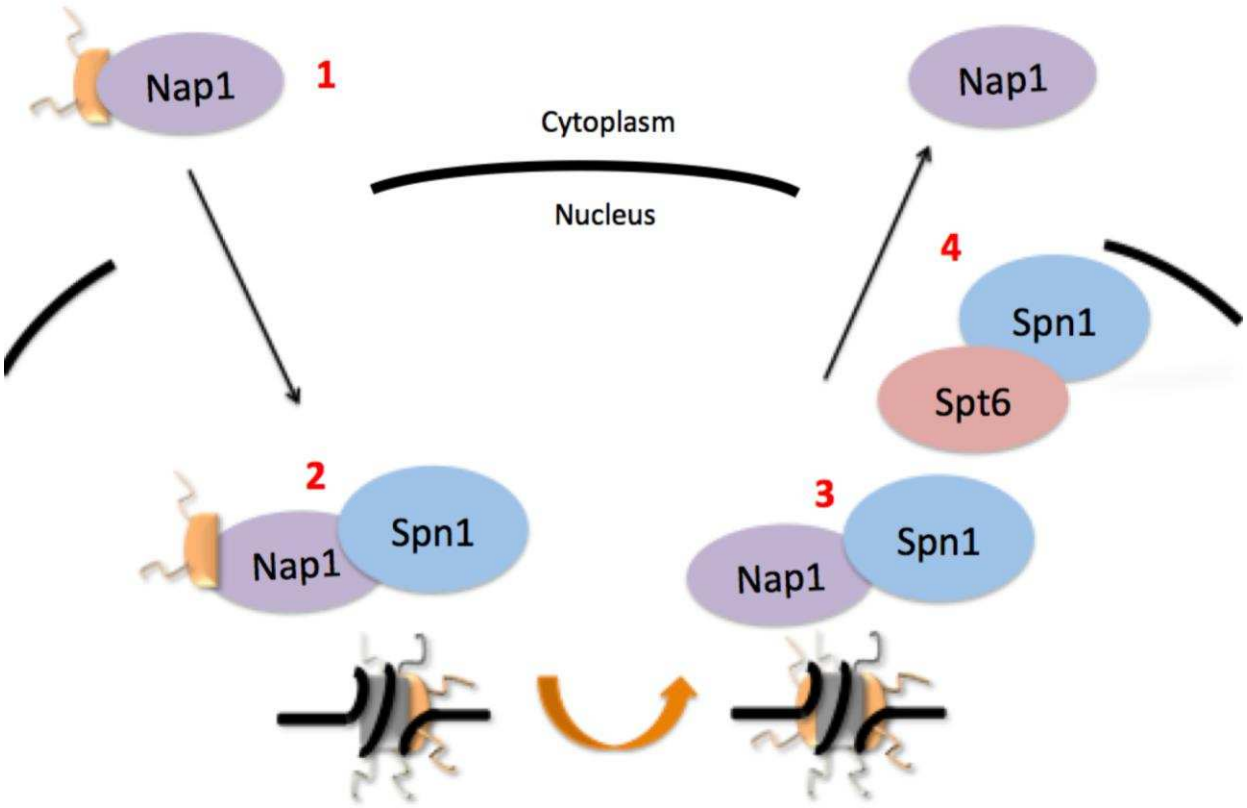


Figure 5.2 Proposed model for Nap1 nuclear exportation that is regulated by both Spn1 and Spt6. Step 1: H2A-H2B synthesis in the cytoplasm and binds to Nap1 to prevent non-specific interactions. Step 2: Nuclear localization sequence (NLS) of Nap1 is accessible to the Kap114p protein, resulting in nuclear transport. Spn1 would bind the Nap1-H2A-H2B complex and navigates the complex to the hexasome for assembly. Spn1 would mask the nuclear exportation sequence (NES) of Nap1 and maintain Nap1-H2A-H2B complex in the nucleus. Step 3: Nap1 deposits H2A-H2B onto hexasome and gets ready to export for next cycle. However, burial of NES by Spn1 would not allow the exportation. Step 4: Spt6 competes Nap1 for Spn1 binding, therefore releasing Nap1. Spn1 and Spt6 remains in nucleus.

2. We found that histone H3 tail is required for Spn1 binding, however, we do not know whether the tail is sufficient. So we will compare the binding affinities between full length H3-H4 and tailless H3-H4. In addition, we will also examine whether Spn1 could bind tailless nucleosome and understand how Spn1 recognizes nucleosomes.
3. We are not clear where H3-H4 binds on Spt6. It is likely the same peptide. If it were, then we would not obtain a ternary complex. However, we cannot exclude another region within the acidic N-terminal region of Spt6. Histone chaperone FACT has separated regions for H2A-H2B and H3-H4: C-terminal region of Spt16 binds H2A-H2B, while Mid (middle domain)-AID domain of Spt16 binds H3-H4 (Kemble et al., 2015; Tsunaka et al., 2016). We could not either exclude the possibility of forming a ternary complex based on the Spn1 domain dissection experiments.
4. We propose that Spn1 might recruit Nap1 and we can test this on ChIP assay. We also propose that Spn1 might keep Nap1 in nucleus during nucleosome assembly/disassembly, therefore we plan to isolate nuclei for further examination by western blot.

Furthermore, we also need to elucidate these questions in general. Histone chaperones tightly interact with histones. How do histones dissociate from histone chaperones? We found that through an interaction with another protein and therefore compete off histones based on the Spn1-Spt6 studies. Is this a general mechanism? The details on molecular level are still not clear.

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APPENDIX I [4]

SPN1 AND H3-H4 CRYSTALLIZATION

We began crystallization trials with full-length Spn1 and full-length H3-H4. Spn1 and H3-H4 were individually purified, and then mixed prior to size-exclusion chromatography for obtaining complex. Preliminary trials had yielded nothing. Next we tried Spn1(1-305) construct and yield needle-like crystals (Table S1 and Figure S1). We optimized the condition and made more than 100 solutions for high-quality crystals. These optimizations yielded larger and 3-dimensional crystals (Figure S1). To exclude the possibility of salt crystals, we did pre-diffraction on X-ray generator in CSU. The X-ray pattern suggested the protein nature of the crystals, however these crystals diffracted poorly. Further optimization included additive screen and seeding. Recently, we also set up two sets of trials, one was Spn1(85-305) with H3-H4TL(in which H4 is tailless) and the other was Spn1(1-305) with H3-H4TL. We have collected data from Synchrotron sources and efforts to analyze the data are ongoing.

[4] This project was conducted under the supervision of Francesca Mattiroli. The work shown here was a result of collaboration. I completed protein preparation and purification and Francesca completed crystal harvesting and data collection.

Table S1. Spn1(1-305) and H3-H4 crystallization trials.

List	Screen kit	Complex concentration (mg/ml)	protein: crystallization solution ratio	Initial hit
1	MIDAS	10	2:1	No
2		10	3:1	No
3	Morpheus	10	1:1	No
4		10	2:1	No
5		10	3:1	No
6	PGA	10	2:1	Yes
7	Classics Suite	10	2:1	No
8		10	3:1	No
9	Cryos Suite	11	2:1	No
10	JCSG+ Suite	10	1:1	No
11	JCSG Core Suite I	10	2:1	No
12		16	2:1	No
13	JCSG Core Suite II	10	2:1	No
14		16	2:1	No
15	JCSG Core Suite III	10	2:1	No
16		16	2:1	No
17	JCSG Core Suite IV	10	2:1	No
18		16	2:1	No
19	MbClass II Suite	11	2:1	No
20	MPD Suite	11	2:1	No
21	PACT Suite	10	1:1	No
22	PEGs Suite	10	2:1	No
23		10	3:1	No
24	Protein Complex Suite	10	1:1	No

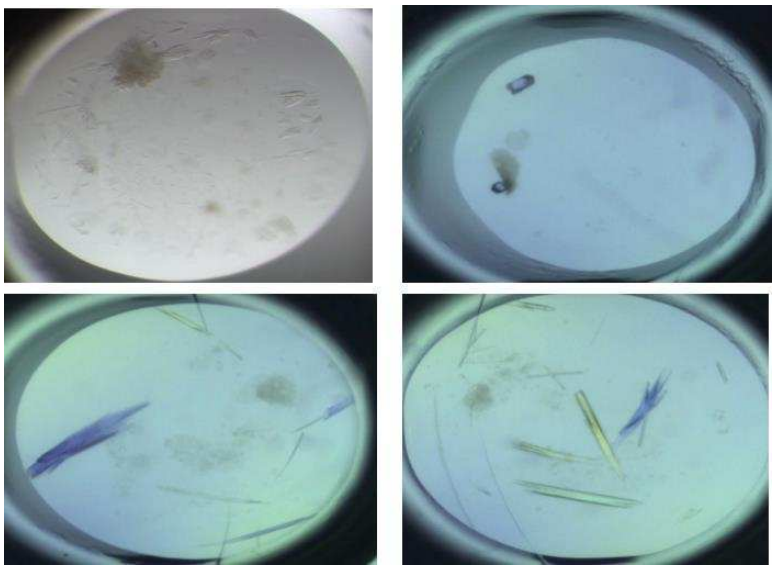

Figure S1. Crystal images of Spn1(1-305)-H3-H4 complex. Top left is the initial hit from PGA screen. The rest three are from optimization screens.

APPENDIX II [5]

NUCLEOSOME ASSEMBLY ASSAY

We used a newly developed nucleosome assembly assay to evaluate Spn1's activity. Unlike widely used supercoiling assay, this new assay saves manpower and time. We included Nap1 as a positive control and we obtained increased amount of newly formed nucleosomes or tetrasomes upon Nap1 titration (Figure S2). Unfortunately, we are not able to evaluate Spn1's activity, since Spn1 binds nucleosome and DNA. Even if nucleosome is efficiently assembled by Spn1, Spn1 would immediately bind the product and form Spn1-nucleosome complex.

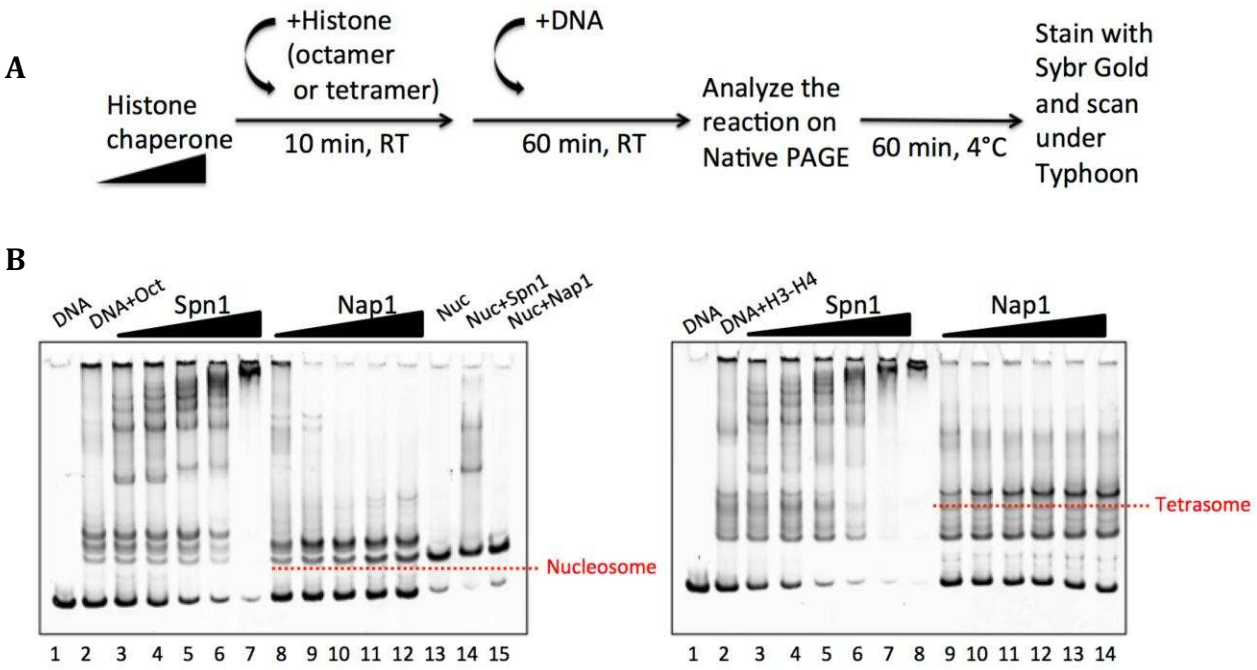

Figure S2. Nucleosome assembly assay. (A) Schematic of the assay. (B) Left: nucleosome assembly assay. Right: tetrasome assembly assay. The red lines underlined the band indicate newly formed nucleosome or tetrasome. Upon titration of Nap1, an increased amount of nucleosome or tetrasome was observed. In contrast, Spn1 forms complex with nucleosome or tetrasome.

[5] This assay was developed by Serge Bergeron.

APPENDIX III [6]

IN VITRO TRANSCRIPTION ASSAY

We used a newly developed *in vitro* transcription system to access the efficiency of histone chaperones and chromatin remodeler RSC on promoting passage of Pol II through a nucleosome (Kuryan et al., 2012). In the system, the DNA template is composed of a single-stranded C tail attached to a 601 positioning sequence. The C tail serves as a binding site for Pol II. Upon addition of nucleoside triphosphates (NTP), Pol II transcribes into the double-stranded 601 DNA containing the nucleosome assembled from recombinant *X. laevis* octamers. By quantifying the amount of transcripts produced, we are able to compare the histone chaperones of interest in the disassembly process (Figure S3). We included Nap1 as a positive control, and examined Spn1 with RSC and Spn1 with Nap1. Consistent with literature, Nap1 and RSC dramatically disassemble the nucleosome and produced lots of transcripts. In contrast, Spn1 and RSC (compare to RSC alone) did not generate significant increased amount of transcripts, suggesting that Spn1 and RSC do not coordinate during nucleosome disassembly process. Since we identified Spn1-Nap1 complex (chapter 4), we also asked whether Spn1 and Nap1 together would enhance (or decrease) the disassembly. As a result, the amount of transcripts was almost unchanged compared with Spn1 alone or Nap1 alone, suggesting Spn1 and Nap1 don't cooperate. Next we will test Spn1 and Nap1 together with RSC to further confirm their independence (or collaboration) in the process.

[6] Hataichanok(Mam) Scherman performed this assay.

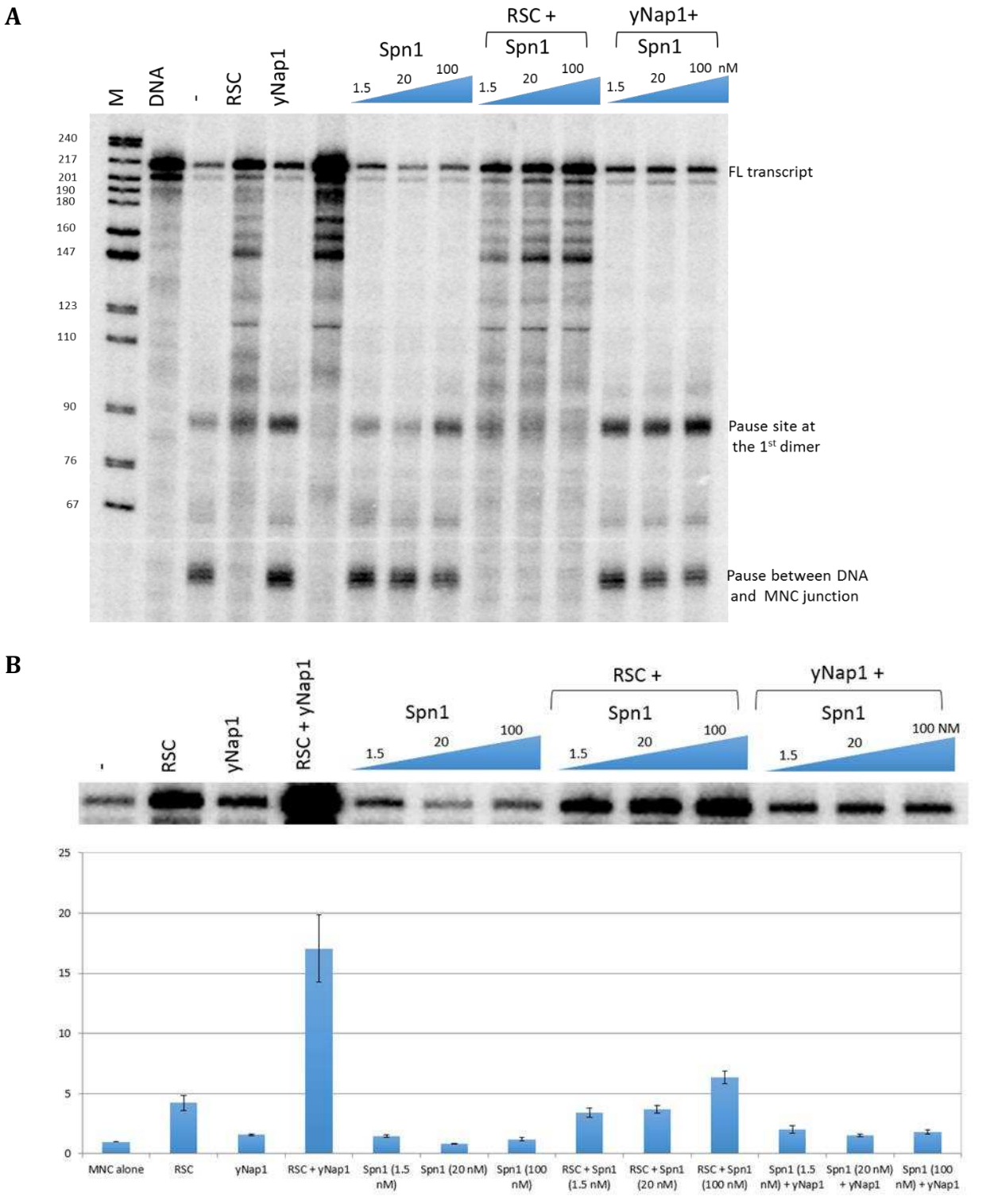

Figure S3. *In vitro* transcription assay to evaluate disassembly efficiency of Spn1 and Nap1 in the presence of RSC. (B) Quantification of full-length transcripts from (A).

Appendix IV

ANCHORING AWAY SPN1 CAUSES GROWTH DEFECT

SPN1 is an essential gene for yeast viability. Therefore we applied anchor-away technique for a depletion of Spn1. In the anchor-away system, the nuclear protein will be translocated to cytoplasm in the presence of rapamycin (Figure S4) (Haruki et al., 2008). We used the anchor-away parental strain that carries an FKBP12 tag to the ribosomal protein RPL13A. The RPL13A protein anchors C-terminally FRB-tagged Spn1 to the cytoplasm upon rapamycin treatment. The parental strain also contains a mutation in *TOR1* and a deletion of *FPR1*, making it rapamycin-insensitive and FKBP12-sensitive (Table S2). Next we compared the growth of parental strain (*WT*) and Spn1 anchor away strain (*SPN1-FRB*) on YPD medium with or without rapamycin. Rapamycin treatment of *SPN1-FRB* barely grew (Figure S5). To verify the observed growth defect was directly caused by anchoring away Spn1, we complemented *SPN1* plasmid into *SPN1-FRB* and repeated the spot assay. As a result, co-expression of Spn1 completely rescued the growth defect.

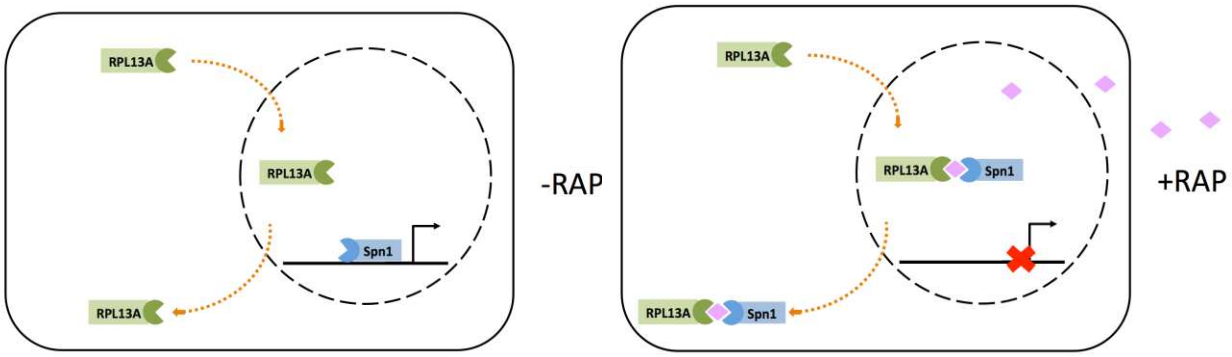

Figure S4. Schematic of anchor away technique.

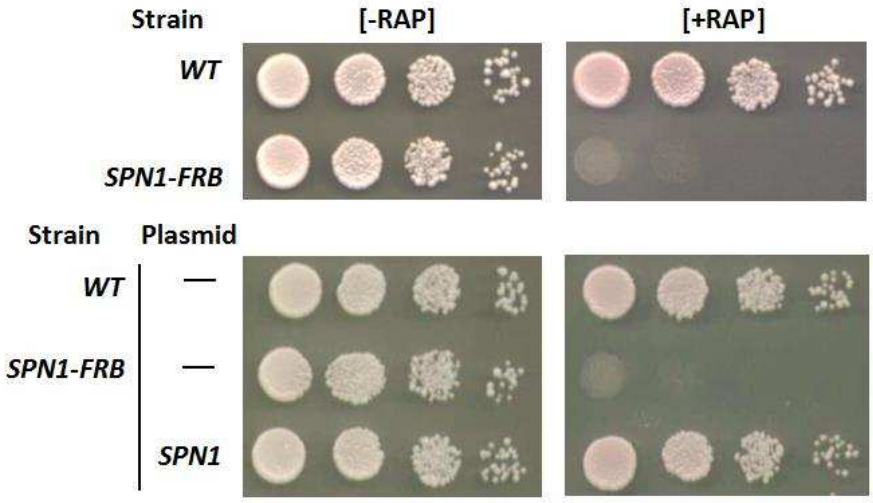

Figure S5. Anchoring away Spn1 makes cells barely grew. If complementing *SPN1-FRB* strain with untagged *SPN1* plasmid, the growth defect was completely rescued. Spot assay on YPAD (with extra adenine in YPD) plates in the absence or presence of 1μg/ml rapamycin (RAP).

Table S2. Strains, plasmids and Primers used in the study

Strains		
Name	Relevant genotype	Reference
W303-1B	*MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	(Haruki et al., 2008)
HHY168	*Isogenic to W303-1B except tor11 fpr1::NAT rpl13A-2×FKBP12::TRP1*	(Haruki et al., 2008)
Plasmids		
Name	Description	Reference
pFA6a-FRB-His3MX6	FRB tagging	(Haruki et al., 2008)
pRS316	empty Vector/Complementation Assay	this study
pCR611	*SPN1* vetor/Complementation Assay	this study
Primers		
Name	Sequence	Description
SPN1-FW	GTACAAGAGGTTGACTTCAAGATTAAACAAGAACASTAAACGGATCCCCGGGTTAATTAA	*FRB* tagging
SPN1-RV	ATACATATCTCAAAGCATTACGGAATTACCTGTTTTGTTAGAATTCGAGCTCGTTTAAAC	*FRB* tagging

Appendix V [7]

HUMAN SPN1 CHARACTERIZATION

To investigate whether histone chaperone activity of Spn1 is conserved in human, we successfully expressed and purified human Spn1 (hSpn1) (Figure S6 A). cDNA of hSpn1 was purchased from Harvard PlasmID Database. It was cloned into MultiBac vector pACEBac1 for expression in insect cells (sf 21). The his-tag was inserted at the C-terminus for purification purpose. We did a couple of tests listed below. Unfortunately, hSpn1 seems nonfunctional (possibly the protein is not correctly folded) and we have not observed the interactions between hSpn1 and objects. Therefore, we paused any other experiments on full-length hSpn1 (1-819aa). We have made a smaller construct (523-819aa), which could be expressed in *E.coli* and the solubility is about 40-50%. We will start to characterize the small construct in the future.

1. Does hSpn1 bind DNA and nucleosome? No (Figure S6 C and D).

   We also found that the purified hSpn1 is phosphorylated (Figure S6 B). It is possible that dephosphorylated hSpn1 could bind DNA.
2. Does hSpn1 bind hFACT and hNap1? No.
3. Does hSpn1 get acetylated by P300? No.

[7] I would like to thank Yajie Gu and Whitney Luebben (Baldwin) for assistance on building the expression system and performing acetylation assay, respectively. I would also like to thank Hataichanok(Mam) Scherman, Tao Wang and Keda Zhou for providing hNap1, hFACT and human nucleosome, respectively.

Appendix VI [8]

SPN1 AND GCN5 DON'T FORM A STABLE COMPLEX

Gcn5 is one of the well-characterized histone acetyltransferases (HATs). Gcn5 acetylates histones, as well as non-histone proteins such as transcription factors (Orphanides et al., 1998). Previous research has suggested that Gcn5 plays a role in transcription elongation (Govind et al., 2007). To determine whether the acetyltransferase activity of Gcn5 has impact on Spn1, we generated strains lacking Gcn5 and expressing Spn1 mutants (*spn1-K192N* or *spn1(141-305)*). We found both stains showed severe growth defects under several stressed conditions (Catherine Radebaugh, unpublished). This suggests that Spn1 and Gcn5 have genetic interaction. Further, we performed a standard in vitro HAT assay to test whether Gcn5 could acetylate Spn1. The following Mass Spectrometry (MS) results showed that 5 lysine residues within Spn1 were acetylated (Hataichanok (Mam) Scherman, unpublished). Lastly, we examined whether Spn1 and Gcn5 have physical interaction using size-exclusion chromatography (Figure S7). We observed the proteins mixture eluted as a broad peak that was located between Spn1 alone and Gcn5 alone. Elution fractions were collected and resolved on SDS-PAGE. As shown on gel, Spn1 eluted earlier and Gcn5 eluted later. We concluded that Spn1 and Gcn5 don't have stable interaction. The HAT activity would be accomplished via transient interaction.

[8] I would like to thank Hataichanok(Mam) Scherman for providing Nap1 and Gcn5 proteins.

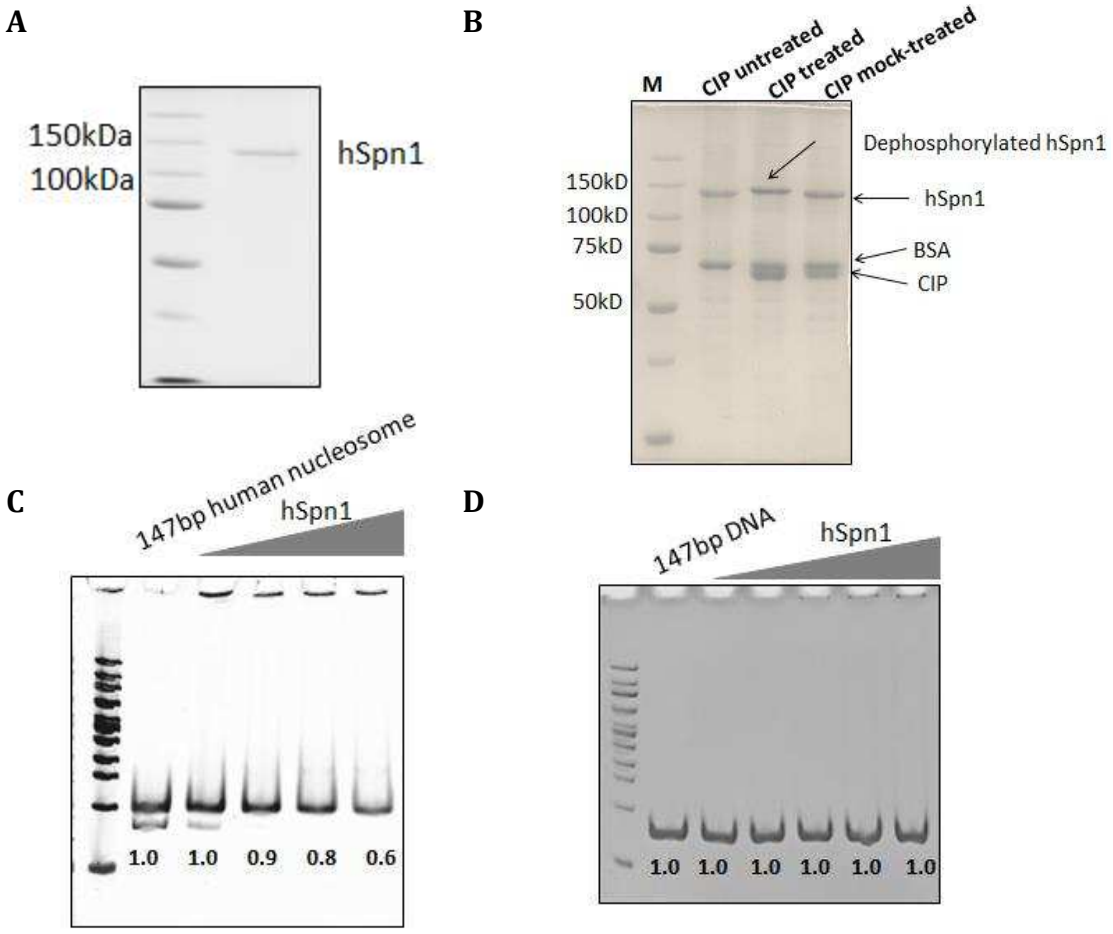

Figure S6. (A) Purified hSpn1. (B) Dephosphorylation assay using CIP (alkaline phosphatase, calf intestinal) was performed following manufacturer's (NEB) protocol. (C) hSpn1 does not bind nucleosome. (D) hSpn1 does not bind DNA. The numbers below the bands indicate the intensity. The nucleosome(or DNA) alone band is normalized to 1.

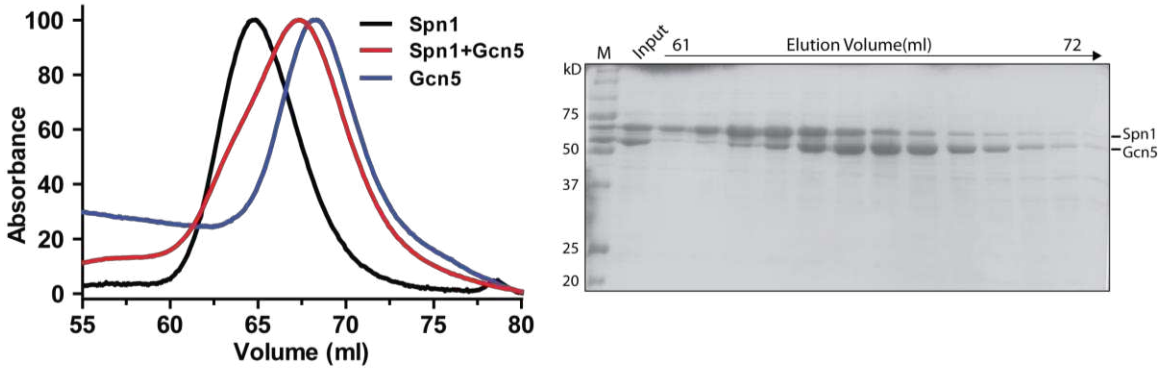

Figure S7. Overlays of size-exclusion chromatograms with Spn1 alone (black line), Gcn5 alone (blue line), and Spn1/Gcn5 binding experiment (red line). The binding reaction contained equimolar Spn1 and Gcn5 (15 μM) in 20 mM Tris pH7.5, 150 mM NaCl and 5mM BME. The fractions evaluated on SDS-PAGE were collected from Spn1/Gcn5 binding experiment.

Appendix VII [9]

SPN1-T185C PHENOTYPIC ANALYSIS

We made Spn1-T185C for labeling purpose. Here we did a phenotypic analysis and we found this substitution exhibited normal growth.

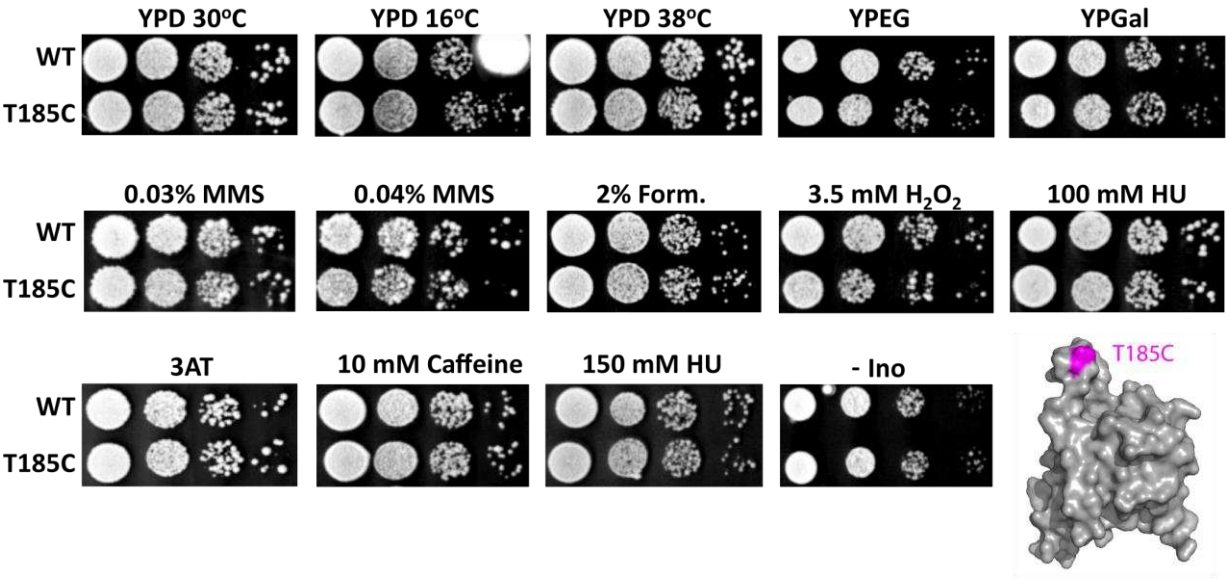

Figure S8. Phenotypic analysis of wild type *SPN1* and *SPN1-T185C*. The location of T185 was colored in magenta in the crystal structure (PDB ID: 3NFQ).

[9] Catherine Radebaugh performed this phenotypic analysis.

Appendix VIII

SPN1-SPT6 COMPLEX DISSOCIATES IN THE PRESENCE OF DNA

We examined how Spn1-Spt6 complex functions in the presence of DNA. We incubated Spn1 and Spt6 together and they formed a distinct complex on the gel (Figure S9, lane 3), then we titrated in DNA (Figure S9, lanes 4-10). Spn1-Spt6 complex dissociated and Spn1 binds DNA (Figure S9, lanes 4-10). The Spn1-DNA complex formed in the presence of Spt6 is not different from Spn1-DNA complex formed in the absence of Spt6 (Figure S9, lanes 11-17). In contrast, Spt6 failed to associate with DNA owing to its low binding affinity (Kd~100 μM) (Close et al., 2011).

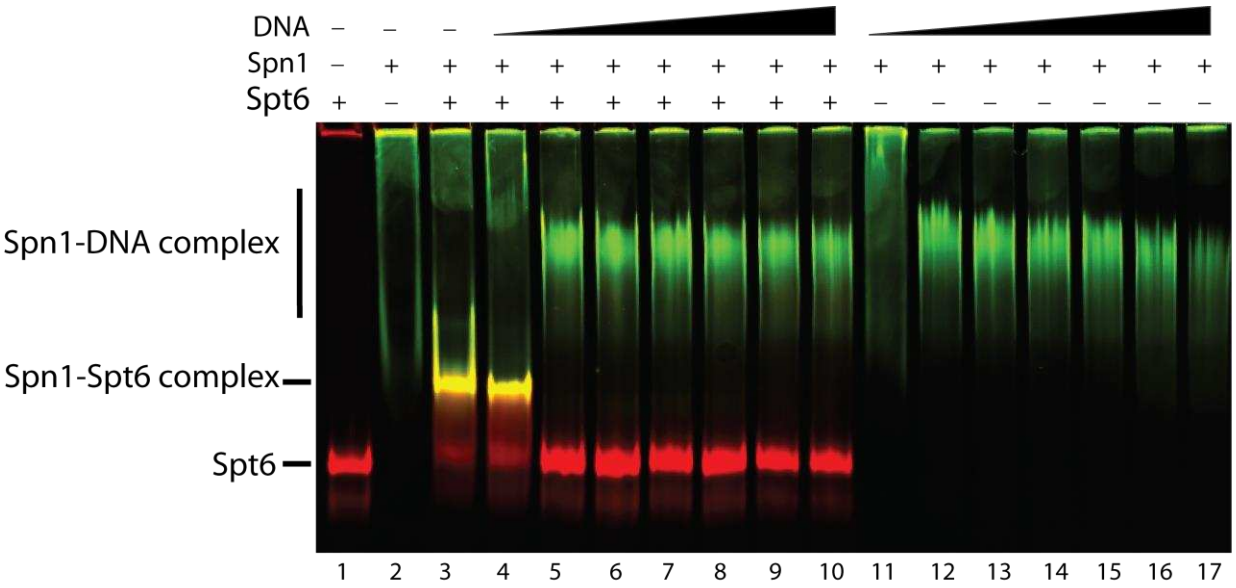

Figure S9. Spn1-Spt6 complex dissociates in the presence of DNA. 1uM Atto 532 labeled Spn1 (green shown in lane 2, Spn1 does not enter native PAGE under the normal electrophoresis conditions) and 1uM Alexa-488 labeled Spt6 (red shown in lane 1) were incubated at room temperature for 15mins (lanes 3-10) and they form a distinct Spn1-Spt6 complex (yellow shown in lane 3). Then an increased amount of DNA were added (lanes 4-10) for another 15mins, resulting the disappearance of Spn1-Spt6 complex. Free Spn1 binds DNA and formed Spn1-DNA complex (lanes 4-10), which was identical compared with Spn1 and DNA alone reactions (lanes 11-17). The samples were analyzed by 5% native PAGE and visualized by fluorescence.

Appendix IX [10]

NAP1 AND NUCLEOSOME (OR DNA) BINDING TO SPN1 ARE MUTUALLY EXCLUSIVE

Since both Nap1 and nucleosome (or DNA) bind to C-terminus (306-410) of Spn1, we further explore whether the binding is mutually exclusive. We used EMSAs to detect the complexes on native PAGE. We fluorescent-labeled Nap1 and Spn1 and stained the gel with SYBR Gold prior to Typhoon scan. SYBR Gold is fluorescent stain for detection of nucleosomal DNA or free DNA and therefore we do not need to label them. We found Spn1-nucleosome complex partially dissociated in the presence of Nap1 (Figure 4.4A, lanes 6 and 10), and released free nucleosome (Figure S10, lane 10). Spn1 associated with Nap1 (Figure S10, lanes 7 and 9). Notably, Nap1 and nucleosome migrated at the same position on gel (Figure S10, lanes 2-4), thus Spn1-Nap1 complex and Spn1-nucleosome complex co-migrate on the gel (Figure S10, lanes 9-10). Thus we conclude that binding to Nap1 and nucleosome is mutually exclusive for Spn1. Next we tested DNA that also binds to C-terminus of Spn1. We found Spn1-Nap1 interaction was disrupted in the presence of DNA, and Spn1 associated with DNA (Figure S10, lanes 5-6 and 9-10). We again do not observe a ternary complex.

[10] Kenneth Lyon performed this experiment.

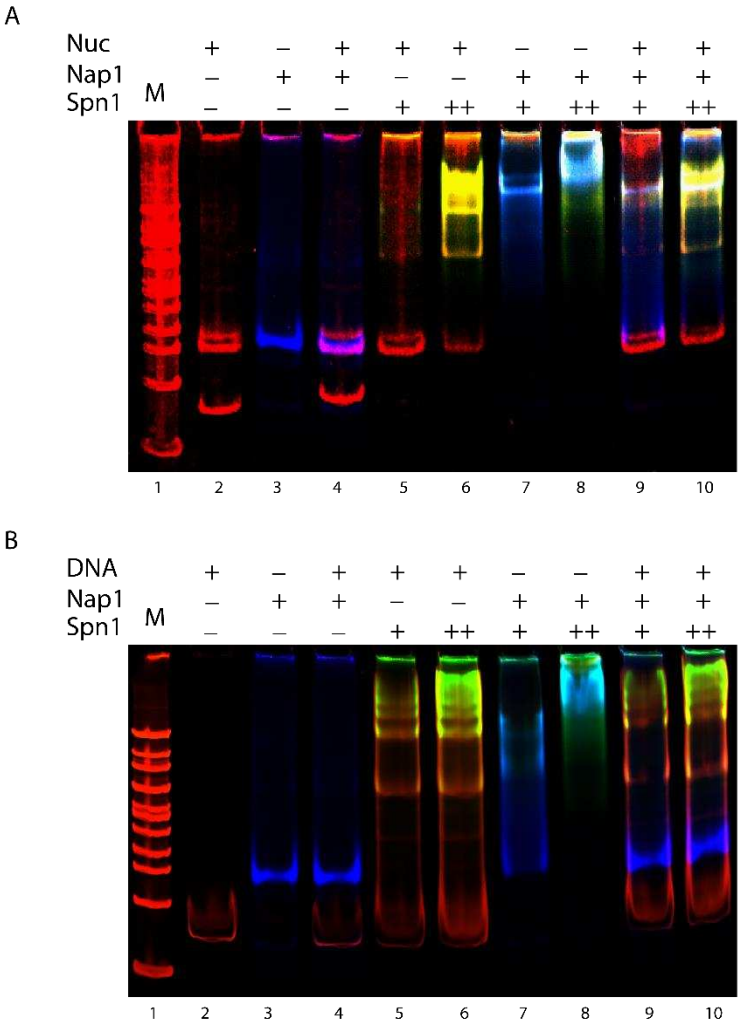

Figure S10. Nap1 and nucleosome (or DNA) binding to Spn1 are mutually exclusive. (A) Nap1 and nucleosome binding to Spn1 are mutually exclusive, and Spn1-nucleosome complex coexists with Spn1-Nap1 complex. (B) DNA disrupts Spn1-Nap1 interaction. "+" indicates 1 μM and "++" indicates 2 μM.

Appendix X

SPN1 DNA-BINDING REGION MAPPING

We tested the constructs below and tried to map the DNA binding regions on Spn1. We used EMSA and found constructs that could bind nucleosome also bind DNA (Figure S11). One interesting phenomenon is that N-terminal region Spn1(1-140) and central domain(141-305) neither bind DNA; however, Spn1(1-305) could bind, suggesting disruption of the boundary region between N-terminal and central domain is detrimental.

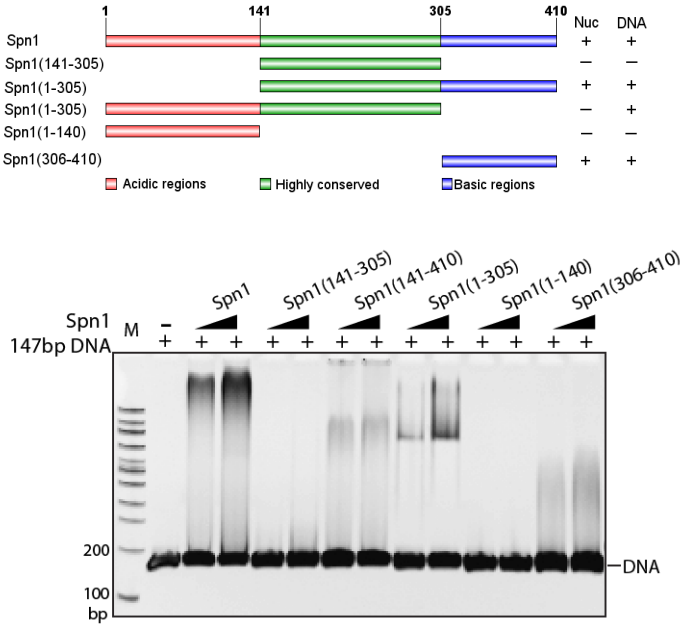

Figure S11. Identification of DNA binding regions on Spn1. DNA (5 uM) was mixed with different titrated Spn1 constructs(5 and 10 uM). The samples were incubated for 30min at room temperature and then analyzed by 5% native PAGE in 0.2XTBE buffer. The gel was visualized by ethidium bromide staining.