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Structure and biological importance of the Spn1-Spt6 interaction, and its regulatory role in nucleosome binding

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SUMMARY

Eukaryotic transcription and mRNA processing depend upon the coordinated interactions of many proteins, including Spn1 and Spt6, which are conserved across eukaryotes, are essential for viability, and associate with each other in some of their biologically important contexts. Here we report crystal structures of the Spn1 core alone and in complex with the binding determinant of Spt6. Mutating interface residues greatly diminishes binding *in vitro* and causes strong phenotypes *in vivo*, including a defect in maintaining repressive chromatin. Overexpression of Spn1 partially suppresses the defects caused by an *spt6* mutation affecting the Spn1 interface, indicating that the Spn1-Spt6 interaction is important for managing chromatin. Spt6 binds nucleosomes directly *in vitro*, and this interaction is blocked by Spn1, providing further mechanistic insight into the function of the interaction. These data thereby reveal the structural and biochemical bases of molecular interactions that function in the maintenance of chromatin structure.

INTRODUCTION

Spn1 and Spt6 are transcription factors that interact with one another and are each essential for viability in yeast (Clark-Adams and Winston, 1987; Fischbeck et al., 2002). *S. cerevisiae* Spn1 is a 410 residue, 46 kDa protein with a central core domain (residues 140–300) that is flanked on both sides by regions that are predicted to be disordered (Ward et al., 2004). Spt6 is a 1451 residue, 168 kDa protein whose core (residues 300–1250) likely resembles the structure of the bacterial Tex protein (Johnson et al., 2008) with an acidic N-terminal extension that is expected to be unstructured (Ward et al., 2004) and a C-terminal domain that adopts an SH2 fold (Dengl et al., 2009; MacLennan and Shaw, 1993). Spn1 and Spt6 interact stably with one another, and they and their interaction have been implicated in several aspects of gene expression (Krogan et al., 2002; Lindstrom et al., 2003; Yoh et al., 2007; Yoh et al., 2008).

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ACCESSION NUMBERS

Protein Data Bank: Coordinates and structure factor amplitudes have been deposited for the Spn1 (3o8z) and Spn1-Spt6 (3oak) crystal structures.

SUPPLEMENTAL INFORMATION

Supplemental information includes one table, four figures and supplemental experimental procedures.

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Spt6 was originally identified in a screen for factors that alter normal initiation of transcription (Clark-Adams and Winston, 1987; Denis, 1984; Neigeborn et al., 1987; Simchen et al., 1984). Subsequently, Spt6 was implicated in a variety of biological processes in organisms ranging from yeasts to humans, including embryogenesis in Zebrafish (Keegan et al., 2002; Kok et al., 2007), multiple stages of development in *Drosophila* (Ardehali et al., 2009), gut morphogenesis in *C. elegans* (Nishiwaki et al., 1993), signal transduction in mammals (Baniahmad et al., 1995; Shen et al., 2009), and HIV transcription regulation and mRNA processing in human cells (Vanti et al., 2009; Yoh et al., 2007). The role in transcription initiation has been ascribed to the ability of Spt6 to chaperone histones to promote reassembly of nucleosomes in the wake of RNA polymerase II (RNAPII), thereby reestablishing the default repressive chromatin state that prevents inappropriate initiation of transcription (Adkins and Tyler, 2006; Bortvin and Winston, 1996; Cheung et al., 2008; Kaplan et al., 2003). While of profound importance, maintaining repressive chromatin appears to be just one of Spt6's roles. For example, Spt6 also promotes elongation by RNAPII (Hartzog et al., 1998; Kaplan et al., 2005; Kaplan et al., 2000; Lindstrom et al., 2003) on nucleosome-free DNA templates *in vitro* (Endoh et al., 2004; Hartzog et al., 1998; Keegan et al., 2002; Yoh et al., 2007), as well as on chromatin templates *in vivo* (Ardehali et al., 2009). Together, these data indicate that Spt6 plays a number of mechanistically distinct roles during transcription.

The *SPN1* gene was originally identified as a key regulator of transcription from genes that are regulated post-recruitment of RNAPII (Fischbeck et al., 2002). Spn1 was also identified as a protein that interacts with Spt6 and has been reported to bind with Spt6 in some but not all of Spt6's functional states (Lindstrom et al., 2003; Yoh et al., 2007; Zhang et al., 2008). For example, Spt6 can be coimmunopurified with three distinct Spt4/5-RNAPII complexes, whereas Spn1 is found in only two of these complexes (Lindstrom et al., 2003). The *CYC1* gene of *S. cerevisiae* provides an example of how the Spn1-Spt6 interaction contributes to post-recruitment regulation (Zhang et al., 2008). RNAPII is constitutively bound to the *CYC1* promoter, but is kept from elongating because it interacts with Spn1, which in turn inhibits the Swi/Snf nucleosome remodeling complex from promoting transcription. During activation, Spt6 binds to Spn1, and repression of Swi/Snf recruitment is relieved.

Spn1 is also needed to achieve normal recruitment of the histone methyltransferase HYPB/Setd2 (Yoh et al., 2008) and the elongation factor TFIIS (Ling et al., 2006; Zhang et al., 2008) to RNAPII complexes traversing active genes. HYPB/Setd2 methylates histone H3K36, which in turn recruits Rpd3-type histone deacetylases to restore chromatin to the repressive hypoacetylated state and block inappropriate transcription (Yoh et al., 2008). In contrast to their antagonistic relationship in activating post-recruitment initiation, Spn1 and Spt6 each contribute towards restoration of repressive chromatin. Human Spn1/TWS1 also binds the protein arginine methyltransferase PRMT5, which methylates the elongation factor Spt5 and thereby regulates its interaction with RNAPII (Liu et al., 2007). Spn1 can additionally function through interactions with pathway-specific regulatory factors, such as the *Arabidopsis* steroid hormone responsive transcription factor BES1, which recruits Spn1 to the promoter and transcribed regions of activated genes (Li et al.). Spn1 therefore contributes in several ways to the appropriate functioning of RNAPII.

In addition to their roles in regulating transcription, Spt6 and Spn1 also collaborate to promote mRNA processing and export. Spt6 is required for proper 3' end formation by preventing premature 3' processing at upstream polyadenylation signals (Bucheli and Buratowski, 2005; Kaplan et al., 2005). Further, mammalian Spt6 can bind the Ser2-phosphorylated RNAPII C-terminal domain (CTD), enhancing recruitment of RNA processing/export factors (Yoh et al., 2007; Yoh et al., 2008), and *Drosophila* Spt6 copurifies with the RNA processing exosome complex (Andrulis et al., 2002). Both *SPN1*

and *SPT6* have also been implicated in mRNA splicing in *S. cerevisiae* (Burckin et al., 2005), and binding of human Spn1/IWS1 to the RNA export factor REF1/Aly is important for recruitment of REF1/Aly to the body of the *c-Myc* gene during transcription (Yoh et al., 2007).

Spt6 and Spn1 and their interaction with one another therefore play pivotal roles in defining the composition of RNAPII elongation complexes, maintaining the structure of chromatin, and modulating the production of mature mRNA transcripts. To advance mechanistic understanding of their functions, we have determined the structural basis of the Spn1-Spt6 interaction. We also demonstrate the importance of this interface *in vitro* and *in vivo*, and show that Spn1 negatively regulates binding of Spt6 to nucleosomes.

RESULTS

Mapping of the Spn1-Spt6 Interface

Full length *S. cerevisiae* Spt6 and Spn1 proteins were poorly-behaved, but deletion of much of their presumably unstructured N-terminal regions (Ward et al., 2004) allowed us to observe co-elution of a complex of recombinant Spn1(120–410) and Spt6(206–1451) by size-exclusion chromatography in sodium chloride concentrations up to 300 mM (not shown). Spt6(239–1451) also bound Spn1 whereas Spt6(315–1451) did not. Further truncations revealed that Spt6(239–268) is sufficient for Spn1 binding (Figure 1A–B). This 30 residue segment of Spt6 is predicted to be unstructured, and comes from a region that is N-terminal to the region expected to resemble the structure of the bacterial protein Tex (Johnson et al., 2008). Spn1(148–293) includes most of the Spn1 residues that are predicted to be structured and retained the ability to bind Spt6. The slightly larger Spn1(141–305) fragment was previously shown to complement a deletion of *SPN1* (Fischbeck et al., 2002), indicating that this core domain provides the major function(s) of Spn1 *in vivo*. Isothermal titration calorimetry (ITC) was used to measure binding affinities of Spn1(148–293) for two different Spt6 constructs: Spt6(239–1117), the largest Spt6 construct that remained soluble at sufficient concentrations for these experiments, and Spt6(239–268), the smallest construct tried that retained full binding affinity. In both cases the binding displayed 1:1 stoichiometry and the mean binding constant (K_D) was 170 nM (Figure 1C–D and Table S1 available online). This indicates that the 30 residue segment of Spt6, Spt6(239–268), is sufficient to recapitulate the binding energy observed for larger Spt6 constructs.

Crystal structures of the Spn1 core

The crystal structure of Spn1(148–307) was determined by the single-wavelength anomalous diffraction method using data collected to 3.0 Å resolution from a selenomethionine-substituted crystal (Figure 2A, Table 1). This unrefined model was used in molecular replacement calculations with 2.15 Å data from a native crystal that belonged to a different space group, and the native structure was refined to R_{factor}/R_{free} values of 18.5/22.4%. Residues 148–295 were clearly observed in the electron density as were four non-native N-terminal residues that remained after TEV digestion. The twelve C-terminal residues were disordered and are not included in the final model.

Spn1 (148–307) forms a right-handed superhelical bundle of eight helices (named H1–8, Figure 2A). Surprisingly, this structure resembles the domains of the RNA processing factors Pcf11 (*S. cerevisiae*) (Meinhart and Cramer, 2004) and SCAF8 (human) (Becker et al., 2008) that bind the RNAPII CTD (Figure S1A). Spn1 is reported to associate with RNAPII (Zhang et al., 2008) and the structural similarity suggested that Spn1 might bind the RNAPII CTD. We have not, however, observed binding in a fluorescence polarization assay

between the Spn1 core and synthetic peptides, either phosphorylated or unphosphorylated, that span more than two heptad repeats of the RNAPII CTD (data not shown).

Crystal structure of an Spn1-Spt6 complex

The structure of an Spn1(148–293):Spt6(239–268) complex was determined by molecular replacement and refined to R_{factor}/R_{free} values of 18.6/24.4% against 2.15 Å resolution data (Figures 2B, S2, Table 1). There are two complexes in the asymmetric unit that superimpose closely with an RMSD of ~0.5 Å over all 170 native ordered C α atoms. Residues 148–292 of Spn1 are clearly visible in the electron density as are six non-native N-terminal residues that remain after TEV digestion. Residues 239–263 of Spt6 are also clearly defined in the electron density as well as four non-native N-terminal residues. There is no clear electron density for the five C-terminal residues of Spt6 and the one C-terminal residue of Spn1, and these are not included in the final model. Spn1 retains the same globular eight helix-bundle fold in the complex, showing no notable conformational changes upon binding Spt6 (RMSD ~0.5 Å on 135/145 pairs of C α atoms). Binding of Spt6 to Spn1 does not mimic the binding of RNAPII CTD peptides to Pcf11/SCAF8, as it involves a face of Spn1 that is structurally distinct from the binding surface of Pcf11/SCAF8 (Figure S1B).

The structure of the Spn1(148–293):Spt6(239–268) complex reveals an extensive interface in which the Spt6 residues drape across the structured Spn1 core domain as two helices, H1 (residues 239–249) and H2 (256–265) that are connected by a short extended segment. There are multiple contacts along the length of the Spt6 segment that bury a total of 1,790 Å² of accessible surface area upon complex formation. Spt6 H1 and the connecting segment, in particular, include a high fraction of conserved residues and contact a conserved patch on the Spn1 surface, thereby indicating that this interface is likely to be preserved across eukaryotes (Figures 2C–E, S1C–D)

Distinctive hydrophobic and polar interactions are made throughout the length of the Spt6 peptide (Figure 3). Starting at the Spt6 peptide N-terminus, the H1 residues M245, I248, and F249 contact a hydrophobic pocket formed by Spn1 residues L256, G262, I266, I286, and W289. The C-terminal carbonyl groups of Spt6 H1 form water-mediated hydrogen bonds with Spn1 R263 guanidinium, whose extensive network of contacts also includes direct hydrogen bonds with the carboxylate of D254 in the extended region of Spt6. The aromatic side chain of Spt6 Y255, from the extended central region, makes van der Waals contacts with Spn1 P227, V264, and F267, while its hydroxyl group forms a hydrogen bond with the carboxylate of Spn1 E226. The Spt6 H2 residues A258, L259, W257, and I261 contact two hydrophobic patches formed by Spn1 V264, F267, P227 and G231. Finally, the Spt6 E262 carboxylate hydrogen bonds with Spn1 S271 and R273, and K272 forms polar contacts with the amide of Spt6 N263. These extensive interactions are consistent with the strong specific binding observed between Spn1 and Spt6.

Mutations that disrupt Spn1-Spt6 complex formation *in vitro*

To validate the relevance of the interface seen in the crystal structure, Spn1(148–293) and Spt6(239–268) variants were expressed, purified, and binding affinities were measured by ITC (Figures 4, S3, Table S1). The Spt6-F249K protein bound Spn1 with a mean K_D of 11 μM, a reduction in affinity compared to the WT interaction of about 60-fold. The affinity of the Spn1-R263D protein for Spt6 (mean K_D of 30 μM) was reduced about 170-fold compared to WT. No binding was detected for the Spn1-F267E protein at concentrations expected to give a reliable estimate of affinity around 40 μM. These observations validate the crystallographic interface and demonstrate that single point mutations are sufficient to significantly reduce binding affinity *in vitro*.

Mutating the Spn1-Spt6 interface causes profound effects *in vivo*

To determine the physiological importance of the Spn1-Spt6 interaction, we introduced *spt6-F249K*, *spn1-R263D*, and *spn1-F267E* mutations into the genomes of yeast cells such that each mutant protein was expressed from its native promoter as the sole source of the affected protein. *spt6-F249K* caused a moderate growth defect at low temperatures, and this was strongly enhanced at elevated temperatures (Figure 5A). Strains with the *spn1-R263D* mutation grew normally at all temperatures, while those with the *spn1-F267E* mutation were normal at low temperatures but failed to grow at high temperatures. Each of the three mutations caused a defect in transcription initiation site selection due to defective chromatin repression, as indicated by the Spt⁻ phenotype (growth of a strain with the *lys2-128Δ* allele on medium lacking lysine (Simchen et al., 1984), Figure 5A–C). The strength of the defect was different for each mutation, being weakest for *spt6-F249K*, stronger for *spn1-R263D*, and strongest for *spn1-F267E*. This order correlates precisely with the level of perturbation of binding observed with these mutant proteins *in vitro* (Figure 4, Table S1), strongly supporting the importance of the Spn1-Spt6 binding interface detected in our crystal structure in maintaining a repressive chromatin state *in vivo*.

Each mutation in the Spn1-Spt6 binding interface disturbed the interaction to a different extent *in vitro* (Figure 4, Table S1), but each individual mutation was tolerated *in vivo* (Figure 5). If the phenotypes caused by individual mutations result from partial disruption of binding, then combining the mutations should lead to enhanced defects. Consistent with this prediction, cells with both *spt6-F249K* and *spn1-R263D* mutations were viable but severely impaired for growth (Figure 5A), and cells with both *spt6-F249K* and *spn1-F267E* were inviable (Figure S4D). The severity of the defect caused by combining mutations therefore correlates with the level of disruption of binding by the individual mutations, suggesting that the Spn1-Spt6 interaction is essential for viability. We were unable to detect an interaction between Spt6 and Spn1-F267E *in vitro* (Figure 4), but the viability of the *spn1-F267E* strain suggests that this mutant retains some binding. Combining *spt6-F249K* with *spn1-K192N* was also lethal (Figure S4D); this mutation does not directly affect the Spn1-Spt6 interface (Figure 2B–C) but has been shown to decrease the interaction between Spn1 and RNAPII (Zhang et al., 2008), and our results with the recombinantly expressed protein suggest that Spn1-K192N protein is unstable (not shown). Synthetic growth defects therefore support the importance of the Spn1-Spt6 interaction, but defects outside this interface can also cause additive growth defects.

Another strategy for determining the importance of the Spn1-Spt6 interface is to test the effect of overexpressing one partner. If decreased affinity of Spt6-F249K protein for Spn1 is responsible for growth defects *in vivo*, these defects might be suppressed by increasing the level of Spn1 protein. We tested this by transforming an *spt6-F249K* strain with high copy plasmids containing variants of *SPN1*. As shown in Figure 5C, increasing the level of normal *SPN1* suppressed the temperature sensitivity and partially corrected the Spt⁻ phenotype caused by *spt6-F249K*. Consistent with retention of partial binding, overexpression of *spn1-R263D* also had an effect but suppressed less efficiently, rescuing growth at 36° but not at 38° and having no effect on the Spt⁻ phenotype. Spn1-K192N is not active at elevated temperatures ((Zhang et al., 2008) and Figure 5A) and overexpression of this allele also did not rescue the phenotypes caused by *spt6-F249K*. Elevated *SPN1* copy number did not suppress the temperature sensitivity or Spt⁻ phenotypes caused by the *spt6-1004* allele (data not shown), which is a deletion of the helix-hairpin-helix domain within the Tex-like core of Spt6 (Kaplan et al., 2005). The suppression of *spt6* defects by increased Spn1 is therefore at least partly specific for a mutation that alters the Spn1-Spt6 interface, and supports the importance of this interaction in an essential function *in vivo*.

Spt6 binds nucleosomes directly and is inhibited by Spn1

Spt6 has been shown to bind both (H3–H4)₂ tetramers and H2A–H2B dimers (Bortvin and Winston, 1996), leading to models in which Spt6 acts as a histone chaperone during nucleosome eviction and redeposition. Following our earlier prediction (Johnson et al., 2008) that the N-terminal region of Spt6 binds nucleosomes, we examined purified Spt6 constructs for nucleosome-binding activity in an electrophoretic mobility shift assay (EMSA). Interestingly, we found that intact Spt6 does bind nucleosomes in this assay (Figure 6), but does so only in the presence of the small HMGB family member Nhp6 (Stillman, 2010). This is similar to the requirement for addition of Nhp6 to observe complexes between nucleosomes and a different histone chaperone, FACT (Formosa et al., 2001). As with FACT, this suggests that Spt6 may form stable complexes with nucleosomes only after the nucleosome has been partially destabilized by Nhp6. This requirement appears to be physiologically relevant, because loss of Nhp6 *in vivo* exacerbated the growth defects caused by any of several mutations in Spt6 (Figures 5B, S4A). Histone chaperones do not necessarily bind intact nucleosomes, in fact the Asf1-(H3–H4) interaction is incompatible with histone contacts within the nucleosome (Antczak et al., 2006; English et al., 2006; Natsume et al., 2007). Binding to both free histones and to nucleosomes therefore might indicate that Spt6 makes multiple distinct contacts with nucleosomes and their components during different steps in chromatin maintenance. Spt6(239–1451) bound nucleosomes while Spt6(315–1451) did not (Figure 6, compare lanes 2 and 3). Thus, the region of Spt6 required for nucleosome binding (residues 239–314) contains the region required for Spn1 binding (239–268), suggesting that these two interactions may be mutually exclusive.

Consistent with this possibility, adding Spn1(120–410) to the nucleosome-binding assay inhibited the formation of Spt6-nucleosome complexes (Figure 6, compare lanes 2 and 5). Spn1 did not form stable complexes with nucleosomes itself, so the inhibition is unlikely to be caused by competition between Spn1 and Spt6 for a common binding site on nucleosomes. Spn1 did not interact with Nhp6 genetically (Figure S4B–C), and inhibition of Spt6-nucleosome complex formation by Spn1 could not be overcome by increasing the concentration of Nhp6 (not shown), making it unlikely that the inhibition is caused by sequestering of Nhp6 by Spn1. Instead, Spn1 appears to prevent Spt6 from binding to nucleosomes directly by blocking the Spt6 binding domain. Supporting this interpretation, the Spn1-R263D variant with reduced affinity for Spt6 did not block formation of Spt6-nucleosome complexes efficiently (Figure 6, lane 7). Further, the Spt6-F249K mutation affects a region important for both nucleosome binding and Spn1 interaction (Figure 4 and Figure 6, compare lanes 2 and 8). Spt6-F249K protein was impaired for nucleosome binding but the residual binding was partially resistant to the addition of Spn1 (Figure 6, lanes 8 and 9). These results show that Spt6 residue F249 contributes to both nucleosome binding and to Spn1 binding, and that Spn1 binding can block an interaction between Spt6 and nucleosomes. The Spn1-Spt6 interaction can therefore provide a switch that controls the interaction of Spt6 with nucleosomes, and the proper functioning of this switch is important for maintaining normal chromatin structure.

DISCUSSION

We have determined a crystal structure of the ordered central domain of Spn1 and shown that it binds a 30-residue segment of Spt6. We have also determined a crystal structure of an Spn1-Spt6 complex that reveals that Spt6 binds in an extended/helical conformation that drapes the Spt6 residues along one face of Spn1. We further used site-directed mutations to disrupt this interaction and demonstrated that the interaction observed in solution depends on residues located at the interface seen in the crystal structure. Binding is not accompanied by conformational changes in Spn1. In contrast, Spt6(239–268) is almost certainly unstructured in isolation and becomes ordered upon binding Spn1. Indeed, the first 300

residues of Spt6 are likely to be unstructured in isolation (Ward et al., 2004) whereas much of the remainder of the protein appears to comprise multiple recognizable structural domains that likely fold against each other to form an elongated structure (Johnson et al., 2008). The extended and inherently flexible nature of the Spn1 binding sequence of Spt6 presumably explains why dramatic mutations in the interface substantially weaken but do not completely eliminate this interaction, as localized perturbations can be accommodated by conformational changes that do not propagate across the entire interface. Moreover, the ~35 residues separating the Spn1-binding residues from the ordered region of Spt6 likely provide a tether whose flexibility may be required to allow the Spn1-Spt6 complex to form in multiple functional contexts.

Spt6 and Spn1 were found to co-purify in high-throughput screens (Gavin et al., 2002; Krogan et al., 2002), and further studies suggested that their interaction promotes normal activation of genes regulated after recruitment of RNAPII (Fischbeck et al., 2002; Yoh et al., 2007; Zhang et al., 2008). However, Spt6 and Spn1 have also been implicated in several other distinct and overlapping roles, and each has been implicated in interactions with multiple factors. We have verified a functional interplay between Spt6 and Spn1 proteins in *S. cerevisiae* by demonstrating that cells cannot tolerate certain partial loss-of-function alleles of both genes simultaneously, and by showing that the defects caused by the *spt6-F249K* allele, which impairs binding with Spn1, can be suppressed by overexpressing *SPN1*. Our results therefore show that a short region of Spt6 is important for interacting with Spn1 during at least one of the essential processes mediated by these proteins.

One of the primary functions ascribed to Spt6 is as a histone chaperone that promotes the reassembly of nucleosomes following passage of RNAPII (Adkins and Tyler, 2006; Bortvin and Winston, 1996; Cheung et al., 2008; Kaplan et al., 2003). Spt6 was previously shown to bind histones and to promote nucleosome deposition *in vitro* (Bortvin and Winston, 1996), and we now show that it can also bind to intact nucleosomes. The dependence of this interaction upon the presence of Nhp6 is consistent with our genetic studies and with an equivalent dependence of the unrelated FACT histone chaperone for its interaction with intact nucleosomes (Formosa et al., 2001). We found that nucleosome binding requires a section of Spt6 that overlaps with the Spn1 binding site, that Spn1 antagonizes Spt6-nucleosome binding *in vitro*, that either *SPT6* or *SPN1* mutations that affect the Spn1-Spt6 interaction cause the Spt⁻ phenotype, and that the effects of the *spt6-F249K* allele can be suppressed by overexpressing Spn1. These results show that the Spn1-Spt6 interaction disrupts Spt6-nucleosome binding and that this disruption has a positive role in maintaining normal chromatin. An attractive explanation is that Spn1 is actively engaged in the nucleosome reassembly process, possibly by disengaging Spt6 from nucleosomes to allow multiple rounds of reassembly (see the graphical abstract available online). Another possibility is that Spn1 binding influences the balance between Spt6's functional roles in nucleosome reassembly and mRNA processing. Regardless of the precise mechanistic details, Spn1 appears to be important for Spt6-mediated nucleosome reassembly *in vivo*.

Nucleosome reassembly is just one of several processes in which Spt6 and Spn1 have been implicated. For example, Spn1, in complex with Spt6, has been reported to interact with a variety of other factors that function in mRNA processing, nucleosome modification, or transcription, including REF1/Aly, Setd2, and RNAPII (Yoh et al., 2007; Yoh et al., 2008; Zhang et al., 2008). Our structural data provide insight into how Spn1 might accommodate simultaneous interactions. Spt6 binds against a region of the Spn1 surface that is rich in conserved residues, but other regions of Spn1 display a similar level of conservation and are therefore good candidates for binding surfaces for other proteins (Figures 2C, S1C). This includes residues that are immediately adjacent to the Spt6 binding surface but extend beyond contacts with Spt6, which may indicate that other factors can bind Spn1

cooperatively or competitively with Spt6. Finally, K192, whose mutation to asparagine impairs Spn1 function (Zhang et al., 2008), and appears to function in interactions with RNAPII, is located in a conserved pocket that is formed at the ends of H1, H2, and H4 (Figure 2B–C) suggesting another potential binding surface.

In summary, we have determined the structural basis for the interaction between Spn1 and Spt6, and shown that this interaction is important *in vivo* and that it regulates Spt6-nucleosome binding *in vitro*. Overall, our data indicate that Spn1 is important for Spt6-mediated nucleosome reassembly, perhaps by regulating the process or by providing a switch that drives disengagement. This does not, however, seem to encompass all of the functional roles in which these two proteins and their interaction with each other participate (Lindstrom et al., 2003; Yoh et al., 2007; Yoh et al., 2008; Zhang et al., 2008). Sequence conservation indicates that other surfaces on the Spn1 core domain are good candidates for mediating functionally important interactions. Moreover, interactions with Setd2 and REF1/Aly have been mapped to the N- and C-terminal regions, respectively, which extend beyond the Spn1 core domain and are predicted to be unstructured (Ward et al., 2004; Yoh et al., 2007; Yoh et al., 2008). This use of inherently flexible segments, including the N-terminal region of Spt6 that binds the Spn1 core, may provide a mechanism that allows flexibility in a crowded transcriptional environment.

EXPERIMENTAL PROCEDURES

See the Supplemental Information for protein expression and purification, strains used, strain construction, plasmid construction, and tests of the effects of combining *spn1* and *spt6* mutations.

Crystallization and Structure Determination

All crystals were grown at 20°C by sitting drop vapor diffusion. Se-Spn1(148–307) drops comprised 2 μL of 8 $\text{mg}\cdot\text{mL}^{-1}$ protein with 2 μL of well solution (0.1 M Bis-Tris Propane pH 7.0, 1.4 M Li_2SO_4). Native Spn1(148–307) drops comprised two parts 7.5 $\text{mg}\cdot\text{mL}^{-1}$ protein and one part well solution (0.01 M MgCl_2 , 0.05 M HEPES pH 7.0, 1.6 M $(\text{NH}_4)_2\text{SO}_4$). Spn1(148–293)-Spt6(239–268) drops comprised two parts 13 $\text{mg}\cdot\text{mL}^{-1}$ protein and one part well solution (0.2 M $\text{Mg}(\text{CH}_3\text{CO}_2)_2$, 0.1 M MES pH 6.5, 20% PEG 8000). Crystals were cryoprotected in a solution of the reservoir made up with 30% glycerol, and cooled by plunging into liquid nitrogen.

All data were processed using HKL2000 (Otwinowski and Minor, 1997). Phases were determined for Se-Spn1(148–307) by single-wavelength anomalous diffraction. Phenix (phenix. autosol) (Adams et al., 2010) located 4 out of 6 possible selenium positions and computed a map into which a model was built. This unrefined model was used in molecular replacement using PHASER (McCoy et al., 2005) to determine the structure of native Spn1(148–307) at 2.15 Å resolution. This subsequently refined model was used to determine the Spn1(148–293):Spt6(239–268) structure by molecular replacement. In all cases, model-building, refinement and validation were performed using Coot (Emsley et al., 2010), Phenix (Adams et al., 2010), and MolProbity (Chen et al., 2010), respectively. Refinement of the Spn1(148–293)-Spt6(239–268) complex included TLS restraints (Painter and Merritt, 2006).

Electrostatic potential surfaces were calculated using APBS (Baker et al., 2001). Figures of molecular structures were generated using PyMol (DeLano, 2002).

Size-Exclusion Chromatography Binding Assay

Purified recombinant proteins were mixed at equimolar concentrations (2 μM) and incubated for 2 hours at 4°C. The protein mixture was concentrated to 15 μM and chromatographed on a 120 mL superdex 200 16/60 column (GE Healthcare) in 15 mM Tris pH 7.5, 200 mM NaCl, 5% glycerol, 0.5 mM EDTA, and 2 mM 2-mercaptoethanol.

Isothermal Titration Calorimetry

Amino-acid substitutions were made by site-directed mutagenesis and verified by DNA sequencing. Purified recombinant proteins were dialyzed overnight at 4°C against 2 L of degassed ITC buffer (20 mM Tris pH 7.5, 150 mM NaCl, 5% glycerol, 2 mM 2-mercaptoethanol, 0.5 mM EDTA). Titrations for all reactions were done at 25°C on an iTC200 (Microcal), including an initial injection of 0.4 μL (which was omitted from data analysis), and all injections were spaced 180 s apart. For Spt6(239–1117) reactions, the titrations were carried out with 18 injections of 1.8 μL 76 μM Spn1(148–293) into 8.2 μM Spt6(239–1117). For Spt6(239–268) reactions, the titrations were with 18 injections of 2 μL 74 μM Spt6(239–268) into 8 μM Spn1(148–293). For Spt6-F249K reactions, the titrations were with 18 injections of 1.8 μL 1.52 mM Spt6(239–268)-F249K into 168 μM Spn1(148–293). For Spn1-R263D reactions, the titrations were with 18 injections of 1.8 μL 3.5 mM Spt6(239–268) into 389 μM Spn1(148–293)-R263D. For Spn1-F267E reactions, the titrations were with 18 injections of 2.0 μL 5.2 mM Spt6(239–268) into 578 μM Spn1(148–293)-F267E). In all cases, three independent experiments were performed. Data were analyzed using Origin software (Microcal), and the stoichiometry (N), association constant (K_A), and change in enthalpy (ΔH) were obtained by fitting the isotherm to the one-site binding model. Other thermodynamic parameters were calculated using the following relationships:

$$K_A^{-1} = K_D \text{ and } -RT \ln K_A = \Delta H - T\Delta S$$

Nucleosome Preparation and Gel Mobility Shift Binding Assay

A 146 bp sea urchin 5S rDNA fragment labeled with Cy5 was generated by PCR and gel purified. *Xenopus laevis* histone H2A-S113C was labeled with Oregon Green488-maleimide and then assembled into nucleosome core particles as described (Xin et al., 2009). 150 fmoles of nucleosome was mixed with 2 μM Spt6 or Spn1 protein in 10 μL reactions containing 100 mM NaCl, 0.8 mg/ml HSA, 9.7% glycerol, and 3 μM *S. cerevisiae* Nhp6a. Following incubation at 30° for 15 min, samples were subjected to electrophoresis on native polyacrylamide gels (4.5% acrylamide (acr:bis, 37.5:1), 0.5 \times TBE, 5% glycerol, 2 mM MgCl_2 at 160 V for 6 hrs at 4°C). The gels were scanned using a Typhoon imager at 670 BP30/Red(633 nm) for Cy5-DNA and 520 BP40/Blue(488 nm) for Oregon Green488-H2A, and the amount of signal in the bound form quantified with ImageQuant Software (GE Health Sciences).

HIGHLIGHTS

- Spn1 core region binds directly to a segment of Spt6
- Crystal structures and biochemical analysis reveal the basis for this interaction
- Spn1-Spt6 binding is important *in vivo* for maintaining repressive chromatin
- Spt6 binds nucleosomes directly and this interaction is inhibited by Spn1

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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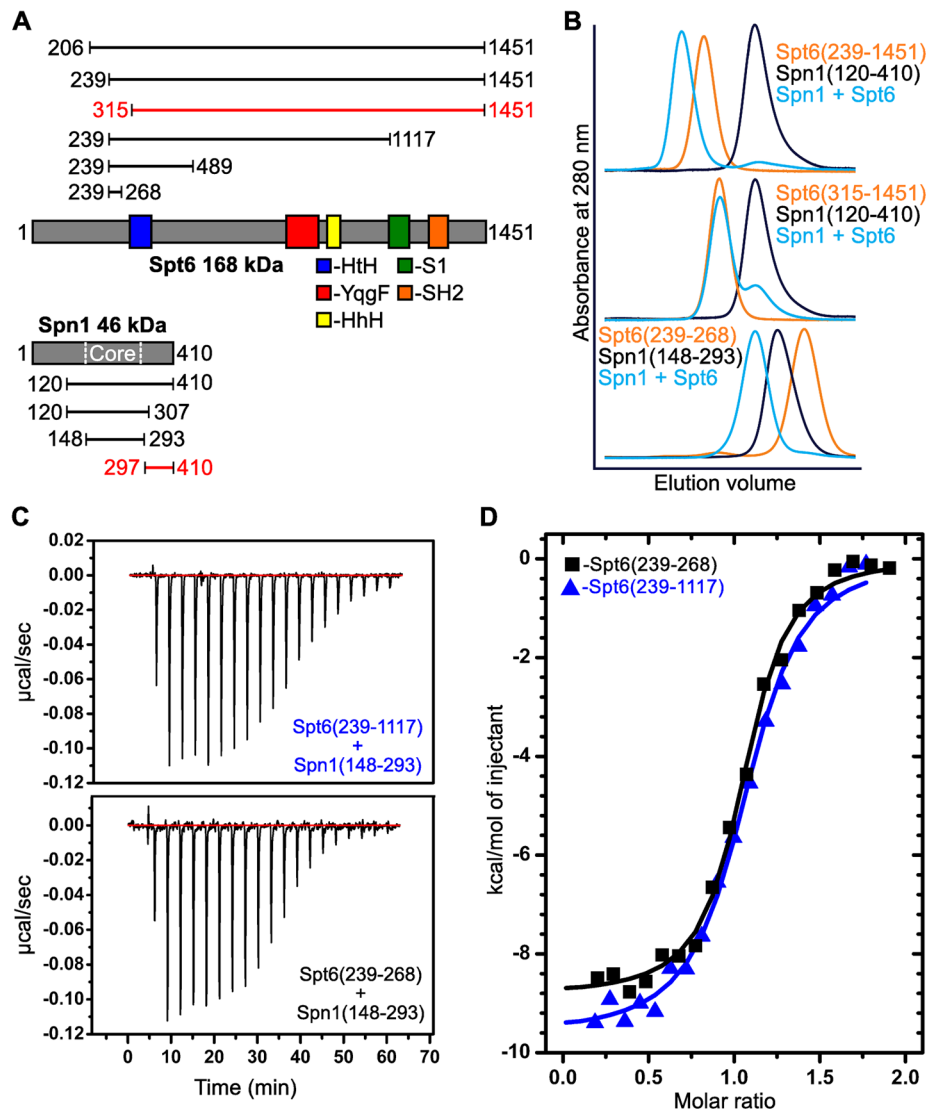


Figure 1. The Spn1 core binds 30 residues in the Spt6 N-terminal region

(A) Spt6 and Spn1 domain organization and their interacting regions. Constructs assayed for binding are indicated above (Spt6) or below (Spn1) with their N- and C-terminal residues given. Constructs that showed binding are colored black while those that did not show binding are colored red.

(B) Overlays of size-exclusion chromatograms with Spn1 black, Spt6 orange, and binding experiments blue. Chromatograms are scaled on the y-axis to allow comparison of the elution volume profiles of each protein or binding experiment. Spn1 has a 6-fold lower molar extinction coefficient, and so at equimolar concentrations, the Spn1 peak appears approximately 6 times smaller than the Spt6 peak (blue, middle panel). The x-axis shows the 50–100 mL elution volume.

(C) Raw ITC data of the titration of Spn1(148–293) into Spt6(239–1117) (top) and the titration of Spt6(239–268) into Spn1(148–293) (bottom).

(D) Representative binding isotherms of the titration experiments from (C). See text and Table S1 for thermodynamic parameters.

(E) Same as panel D but for Spt6. High conservation (red), medium conservation (yellow).

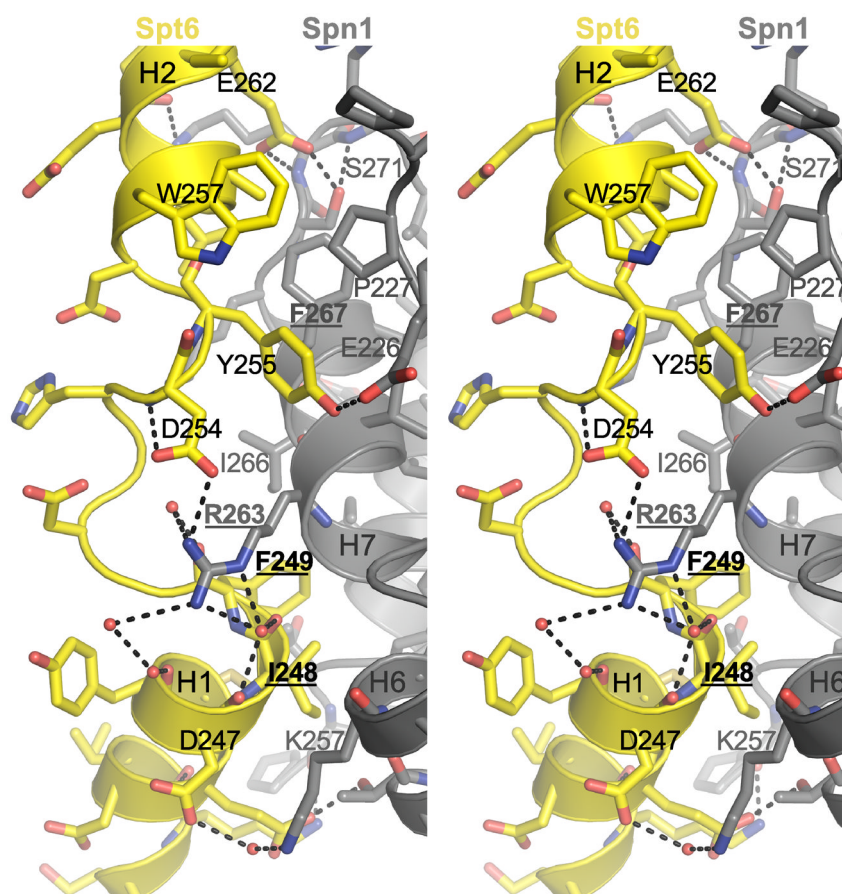


Figure 3. Details of the Spn1-Spt6 interface

Stereoview of the Spn1-Spt6 interface. Residues mutated in this study are indicated in bold font and underlined. Water molecules are shown as red spheres and polar interactions are indicated by black dashes.

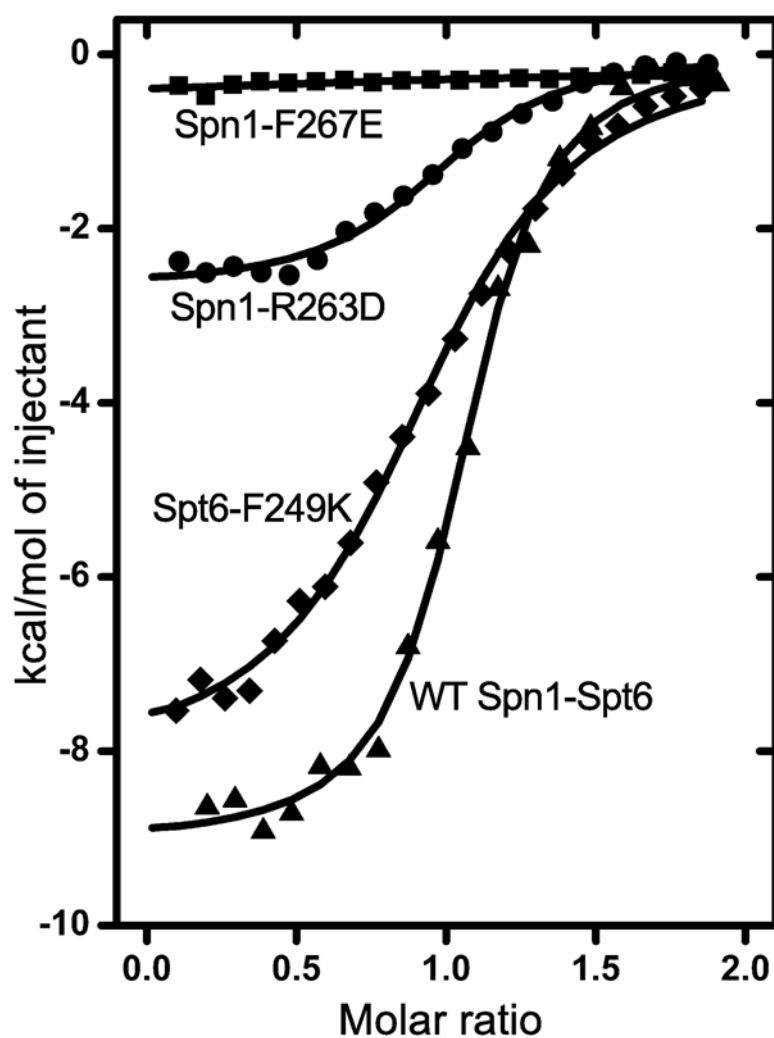


Figure 4. Mutations at the Spn1-Spt6 interface disrupt binding *in vitro*
Representative ITC binding isotherms for the indicated mutant Spt6(239–268) and Spn1(148–293) proteins, Spt6-F249K (diamonds), Spn1-R263D (circles), and Spn1-F267E (squares). A WT isotherm is shown for reference (triangles). See text and Table S1 for thermodynamic parameters.

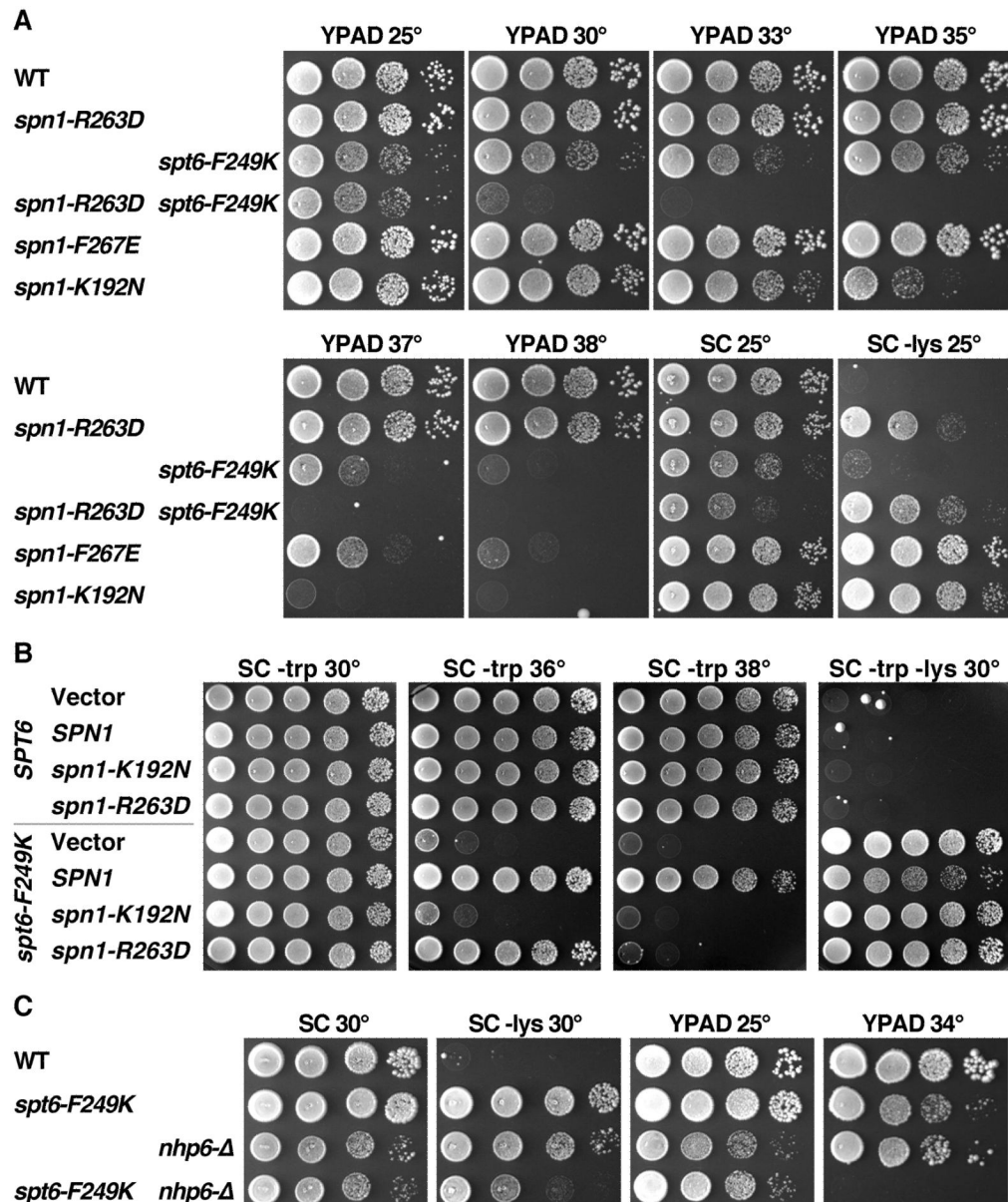


Figure 5. The Spn1-Spt6 interaction is significant for the essential activities of each protein *in vivo*

(A) Isogenic strains from the A364a genetic background and with the relevant genotypes indicated (Supplemental Information) were grown to saturation in rich medium, then aliquots of 10-fold serial dilutions were placed on solid medium and incubated as labeled. SC is synthetic medium (complete or lacking tryptophan or lysine as noted) and YPAD is rich medium. Growth on medium lacking lysine reveals the Spt⁻ phenotype, reporting here on aberrant transcription initiation from the *lys2-128Δ* allele. The strain with the *spt6-F249K* allele grows slowly on SC – lys at 25°, but the Spt⁻ phenotype is more robust at 30° (panel B).

(B) As in panel A. *nhp6-Δ* indicates deletion of both *NHP6A* and *NHP6B*.

(C) WT and *spt6-F249K* strains were transformed with a high copy number vector, or the same vector carrying the version of *SPN1* noted (Supplemental Information). Multiple transformants were tested to insure that the phenotypes detected are typical, then one clone

of each was grown to saturation in synthetic medium lacking tryptophan to select for retention of the plasmids. Aliquots of 10-fold serial dilutions were placed on solid synthetic medium as in panel A and incubated as indicated.

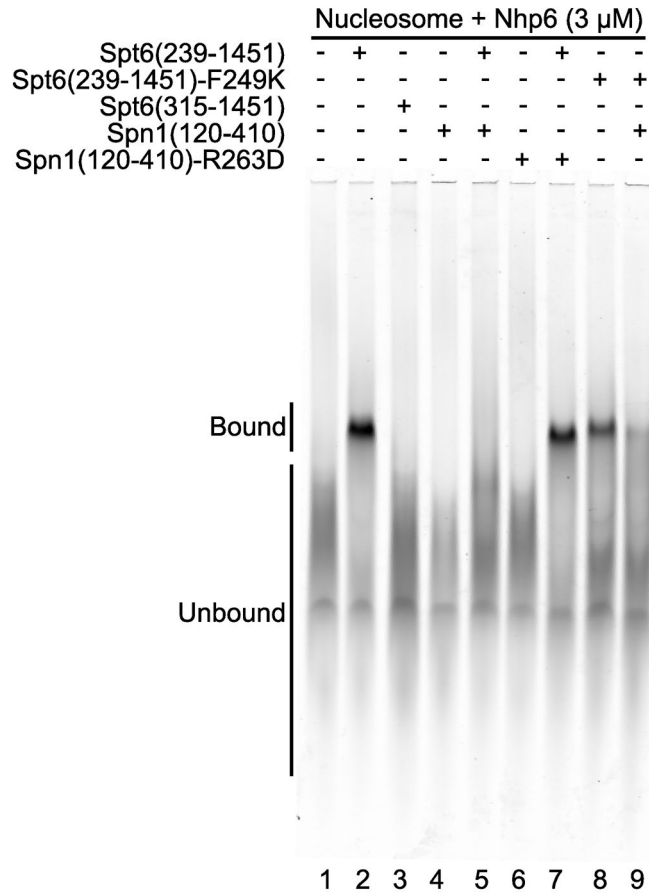


Figure 6. Spt6 binds nucleosomes and is competed by Spn1

Electrophoretic mobility shift assay visualizing the signal from the labeled DNA incorporated into the nucleosomes (Cy5, 633 nm). All reactions included 150 fmoles of nucleosomes, 3 μ M Nhp6, and 2 μ M of the indicated Spt6 or Spn1 proteins. Migration positions of bound and unbound nucleosomes are indicated. Addition of Spn1 reduced the amount of Spt6-nucleosome complex from 37% of the total signal in lane 2 to 3.2% in lane 5 (9% remaining), but Spn1-R263D only reduced it to 28% (76% remaining). Spt6-F249K formed a lower amount of stable complex (18%) but Spn1 was less effective at inhibiting this (6% bound, or 36% of the original signal remaining comparing lanes 8 and 9) than it was with WT Spt6 (9% of the original signal remaining).

Table 1

Data Collection and Refinement Statistics

	Spn1 Core SeMet	Spn1 Core Native	Spn1-Spt6 Complex
Data Collection			
Beamline	SSRL 9-2	Home source	Home source
Space group	P3 ₁ 12	P3 ₂ 12	P2 ₁ 2 ₁ 2
Unit cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	61.9, 61.9, 240.4	61.3, 61.3, 116.05	105.9, 68.7, 73.9
α , β , γ (°)	90, 90, 120	90, 90, 120	90, 90, 90
Resolution (Å)	35-3.0	30-2.15	30-2.15
Wavelength (Å)	0.97923 (Peak)	1.54178	1.54178
<i>I</i> / σ ₁	23.2 (2.8)	22 (4.1)	19.7 (3.1)
Completeness (%)	90.8 (92.3)	99.8 (98.4)	100.0 (100.0)
<i>R</i> _{sym} (%)	5.2 (47.6)	6.1 (31.9)	6.4 (54.4)
Redundancy	4.2 (4.1)	5.8 (3.8)	5.3 (5.0)
Refinement			
Resolution (Å)		29.6-2.15	27.7-2.15
No. of reflections		13,847	30,096
<i>R</i> _{work} / <i>R</i> _{free} (%)		18.5/22.4	18.6/24.4
No. protein atoms		1,216	2,903
No. solvent atoms		144	268
RMSD Bond lengths (Å)/angles (°)		0.007/1.001	0.012/1.279
ϕ / ψ most favored/allowed (%)		99.3/100.0	99.2/100.0

Values in parentheses are for the highest-resolution shell