Role of Chromatin Structure in Regulating Transcription

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Biology

To
The Honors College
Oakland University

In partial fulfillment of the
requirement to graduate from
The Honors College

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April 5, 2019

ABSTRACT

Histone chaperones are a class of proteins that assemble nucleosomes, a fundamental unit of chromatin, after DNA replication. Some of these chaperones play an important role in restoring chromatin structure, which is disrupted by DNA-dependent activities, including gene-transcription, in cells. Histone chaperones like Spt6 maintain the chromatin structure to keep genes and their functions stable by removing nucleosomal barriers and facilitating reassembly, to allow Polymerase II to read DNA. In this study, we observed the nucleosome changes upon the deletion of the Sh2 domain of Spt6 in *Saccharomyces cerevisiae*. In this study, we show that the Sh2 domain is important in regulating chromatin structure on a genome-wide scale. Without the Sh2 domain, higher occupancy of nucleosomes is observed even at highly transcribed genes. The Sh2 domain is also important in maintaining the targeting of Spt6 in the genome and without this domain present, we found there was an absence of Spt6 in highly transcribed genes. Loss of Sh2 domain function is adverse for nucleosome positioning, disrupting the original chromatin structure and shows that Spt6 is important in the organization of chromatin during transcription.

INTRODUCTION

Transcription is the process in which DNA is copied to make RNA, that is essential to making proteins in the human body (Clancy, 2008). In order to read the DNA, RNA polymerase II (RNAP II) moves down the DNA template to synthesize RNA, but it is halted by complexes called nucleosomes. Nucleosomes consist of an octamer composed of two copies of histones H3, H4, and a pair of the H2A-H2B dimer, which bind to and occlude DNA from DNA binding proteins (Pathak *et al*., 2018). Along the DNA strand there is a nucleosome free region (NFR) where no nucleosomes are present (Klein-Brill *et al*., 2019). At the NFR, proteins called transcription factors are able to bind to the DNA to assist in regulating transcription (Ozonov & Nimwegen, 2013). In most intragenic regions downstream of the NFR there is a nucleosome present, and thereafter are equally spaced nucleosomes (Klein-Brill *et al*., 2019). In order to expose the DNA wrapped around these nucleosomes, histone remodelers and chaperones can perform one of two different functions on nucleosomes. First, whole nucleosomes can be slid along the DNA and consequently removed from the DNA strand by the remodel structure of chromatin (RSC) complex (Hsu *et al*., 2019). Second, histones can be ejected to relax the DNA around the remaining histones so the DNA sequence can be read by polymerase II (Pol II) (Pathak *et al*., 2018). Some examples of histone remodelers are the RSC complex (remodeler of chromatin) and SWI/SNF and two examples of histone chaperones are the FACT (facilitates chromatin transcription) complex and Spt6 (Pathak *et al*., 2018; Herrera-Myano *et al*., 2014).

Spt6 is found across many domains of life, from yeast to human genes (Close *et al*., 2011). Its function is associated with the development of embryos in Zebrafish, *Drosophila*, and has been implicated in HIV progression (Close *et al*., 2011). In the cell, Spt6 works with the H3, H4, H2A-H2B histone dimers to act as the histone chaperone and can attach to histones and

nucleosomes and also functions in promoting modifications on histones (Pathak *et al.*, 2018; Lorch & Kornbert, 2017; Close *et al.*, 2011; Ivanovska *et al.*, 2010). Specifically, methylation of the lysine 36 of the histone H3 is promoted by Spt6 in order to reassemble the nucleosome after Pol II has passed (Duina, 2011). Spt6 can be divided into three parts: an N-terminal domain, a central portion, and a Sh2 located C-terminal domain (McCullough *et al*., 2015). The Sh2 domain contains an N-terminal and C-terminal that fold alongside one another and work together to allow Spt6 to bind to the linker part of Pol II (Close *et al*., 2011, Pathak *et al*., 2018). This is accomplished by the Sh2 domain recognizing the location on Pol II where it has been phosphorylated (Dengl *et al*., 2009; Close *et al*., 2011). Spt6 works with Pol II by replacing nucleosomes to their respective loci in the gene (Kato *et al*., 2013). The coordination of Spt6 and FACT is essential for optimal gene expression and in reassembling nucleosomes (Dronamarju *et al*., 2019). The FACT complex it is comprised of two subunits in yeast: Spt16 and Pob3 (Pathak *et al*., 2018). In the nucleosome, FACT works with H2A and H2B dimer pairs by removing one of the H2A- H2B dimers to allow transcription to proceed (Pathak *et al*., 2018). FACT also works to replace the removed dimer back into the nucleosome, reassembling the original nucleosome structure after Pol II has passed (Pathak *et al*., 2018; McCullough *et al*., 2015). Disrupting the original function of FACT results in shifted histone occupancies on the genome (Pathak *et al*., 2018). Along with histone chaperones, chromatin remodelers are also very important. RSC and SWI/SNF are histone remodelers in *S. cerevisiae* which can remove, move or change the conformation of nucleosomes (Kuryan *et al*., 2012; Klein-Brill *et al.*, 2019). The Rpb5 subunit of Pol II associates with RSC and it has been shown that without RSC present, the TATA-binding protein cannot bind to DNA, preventing transcription from occurring and cell death ensues (Kuryan *et al*., 2012; Klein-Brill *et al*., 2019).

In this study, we have localized Spt6, RSC and nucleosomes at a very high resolution genome-wide. In order to study these transcription factors, cells of *S. cerevisiae* are first cross-linked to create covalent bonds in the proteins associated with DNA to the DNA itself so the associated sequence can be isolated with Micrococcal nuclease (MNase) digestion and Chromatin Immunoprecipitation (ChIP) (ThermoFisher, n.d.b). Cells are then broken apart using a lysis buffer and are subjected to violent shaking with glass beads. This will break apart the cells without damaging the proteins and structures inside of the cell. From these lysed cells, we are able to extract DNA using MNase digestion (ThermoFisher, n.d.a). MNase is an endonuclease that cleaves/digests the DNA around the nucleosomes, releasing mononucleosome, dinucleosome or trinucleosome fragments (Chung *et al*., 2010). In the cell, a mononucleosome is a single nucleosome, in which a DNA fragment of about 147 base pair length is wrapped around this histone octamer (Pathak *et al*., 2018). In contrast, dinucleosomes and trinucleosomes consist of second and third nucleosomes where MNase failed to digest the DNA in between the nucleosomes. By purifying and sequencing the DNA fragments associated with nucleosomes, we are able to map the positions of nucleosomes by observing where the cuts were made along the DNA strand (Chung *et al*., 2010). ChIP uses antibodies to select for the protein associated DNA complexes of the chromatin chaperones that we want to study, in this case is Spt6 (Illumina, 2015). The cells are reverse cross linked to release the proteins from the DNA sequences we want to analyze on a genome wide scale (Illumina, 2015).

Two fundamental mysteries in the chromatin field of research are the mechanisms by which the chromatin modifying complexes recognize chromatin and how they subsequently modify histones. In this study, we transformed *S. cerevisiae* to generate strains in which the histone chaperone Spt6 was tagged and another which its Sh2 domain was deleted to determine

Spt6 localization and to study its role in regulating chromatin structure in transcription. We compared occupancy of Spt6 with those of the RSC complex in the WT (wild type) strains. This was subsequently compared to Rpb3, subunit of Pol II. Through this research we were able to find that the function of Spt6 is important in regulating nucleosome occupancy and positioning along the gene and that the Sh2 domain plays an important role in Spt6 function.

MATERIALS AND METHODS

Cell Cultivation and Cross Linking

S. cerevisiae cells were grown in yeast peptone dextrose media (YPD) to an optical density of 0.8-0.9. 90ml of cross linking solution (500mM EDTA, 5M NaCl, 1M Hepes-KOH, pH 7.5, H_2O) and 40ml of 37% formaldehyde was added to each sample and were allowed to cross link for 15 minutes. 150ml of 2.5M Glycine was used to quench the reaction. The cells were washed twice with chilled 1xTBS before being collected via centrifugation and stored at -80ºC until ready for experiments. Each vial of cells represented 100ml of culture.

MNase Digestion of *S. cerevisiae* cells

Cross linked cells were removed from -80ºC and resuspended in 400µl of FA lysis buffer with Protease Inhibitor Cocktail (PIC) (25µl Pepstatin, 25µl Leupeptin, 250µl PMSF, per 25 ml FA lysis buffer) along with ~800µl of glass beads. Cells were disrupted for 45 minutes at 4ºC. Whole cell extract was collected and washed once each with 1ml FA lysis buffer and PIC. Whole cell extracts were washed with 600µl NP-S buffer (36.3 µl Spermidine, 375 µl IGEPAL, 5ml 5M NaCl, 5ml 1M Tris-HCL pH 7.5, 5ml 0.5 M $MgCl_2$, 20ml DI H_2O) The cells were then thoroughly resuspended in 300µl of NPS + 0.007% BME. Before 12µl of MNase was added, samples were then split into three groups and were incubated at 32ºC at 1100rpms for 22 minutes, 26 minutes, 18 minutes, respectively. Samples were further resuspended while in the

thermomixer. 10µl of 0.5M EDTA was used to quench the reaction and cells were incubated on ice for 10 minutes. Samples were centrifuged for 10 minutes and the supernatant containing the chromatin was collected. The pellet was washed with 300µl NP-S + SDS 1% before being centrifuged. The supernatant from the wash was collected and pooled to the previous chromatin containing supernatant. Inputs to be sent for sequencing were created by adding: 100µl NP-S, 2µl EDTA, 4µl Tris pH 8.0, 10uL 10% SDS to 150µl chromatin. 5µl Proteinase K was added and samples were allowed to reverse cross link at 65ºC hot water bath overnight. The chromatin samples were stored in the -80ºC freezer for ChIP experiments.

Inputs were removed from the hot water bath and 50µl KoAc was added to each input. 271µl of chloroform was added to each tube and inputs were vortexed thoroughly. These samples were centrifuged for 8 minutes at 4ºC and max speed before the aqueous layer was extracted and transferred to new tubes. The original sample was back-extracted with 100µl of UP H_2O and centrifuged for 8 min at 4ºC at maximum speed. The aqueous layer was extracted and combined with the first extract. A second extraction was performed using 371µl chloroform. 1µl of glycogen and 1ml of 200 proof ethanol was added to each tube and DNA was precipitated over night at -80ºC.

Inputs were removed from -80ºC before being centrifuged. The pellet was washed once with 70% EtOH before being dried using the speed vac for 7 minutes. The pellets were resuspended in 75µl of 1xTE buffer and incubated at 37ºC for one hour to further resuspend. Inputs were then thoroughly resuspended by vortexing and wall washing before being quantified on NanoDrop 2000 spectrophotometer. Samples were then run on a 2% agarose gel at 120V to visualize the quality of MNase digested samples.

Sonication

Whole cell extract from cross linking was resuspended in 1ml FA lysis buffer and sonicated at 4°C for 30 seconds, allowed to rest for 30 seconds before the process was repeated for a total of 10 cycles. Chromatin is then collected by centrifugation and stored at -80ºC for ChIP.

Chromatin-Immunoprecipitation

After chromatin was produced, MNase and sonicated, the chromatin was subjected to ChIP. 60µl of magnetic mouse beads are washed with 1ml of PBS/BSA twice. The beads were resuspended in 100µl of PBS/BSA, 5µg of Anti-Myc antibody was added and beads were rotated for 3 hours at 4ºC. Beads were washed with 1 ml PBS/BSA twice before being resuspended in 60µl PBS/BSA and 40µl FA lysis/PIC. 50µl of chromatin prepared earlier and 100µl FA-PIC were added to samples, and samples were rotated again for 3 hours at 4ºC. Beads were then washed twice with 1ml FA-lysis buffer, twice with 1ml Wash Buffer II, twice with 1ml Washing Buffer III and once with 1ml TE before eluting DNA.

Elution of DNA

Beads were resuspended in 100µl of IP Elution Buffer and incubated at 65ºC for 15 minutes. Eluate was collected before beads were resuspended in 150µl Elution Wash Buffer and incubated at 65ºC for 10 minutes. Eluate was collected from the second wash and pooled with the first eluate. 5µl Proteinase K was added to eluted DNA and samples were allowed to reverse cross link at 65ºC before DNA was extracted and precipitates as described previously.

Data analysis

The samples were sent to the University of Michigan for sequencing with Illumina. The DNA sequences were aligned to the *S. cerevisiae* genome using Bowtie2 and normalized to compare each samples independent of the DNA sequencing reads. We used computational tool

BamR and R to plot heat maps shown in the figures. The sequences coming from each chromosome was set to 1, and this allows us to compare each sample without any issues of read counts.

RESULTS

In order to test and compare polymerase occupancy, heat maps were generated comparing Spt6, nucleosomes and Pol II occupancies in WT and Spt6myc tagged cells in which over 6000 genes have been aligned by their transcription start sites. This includes ChIP data for Rpb3 occupancy which is a core subunit of Pol II. Figure 1A shows that most of DNA sequence read lengths are around 150 base pairs, the relative length of a DNA fragment wrapped around a nucleosome. We can see a minimal peak at ~300 base pairs, showing that the MNase occasionally missed a cut between two nucleosomes to produce a dinucleosome length fragment (Figure 1A). The genes of all heat maps were sorted according to the level of Pol II occupancy in

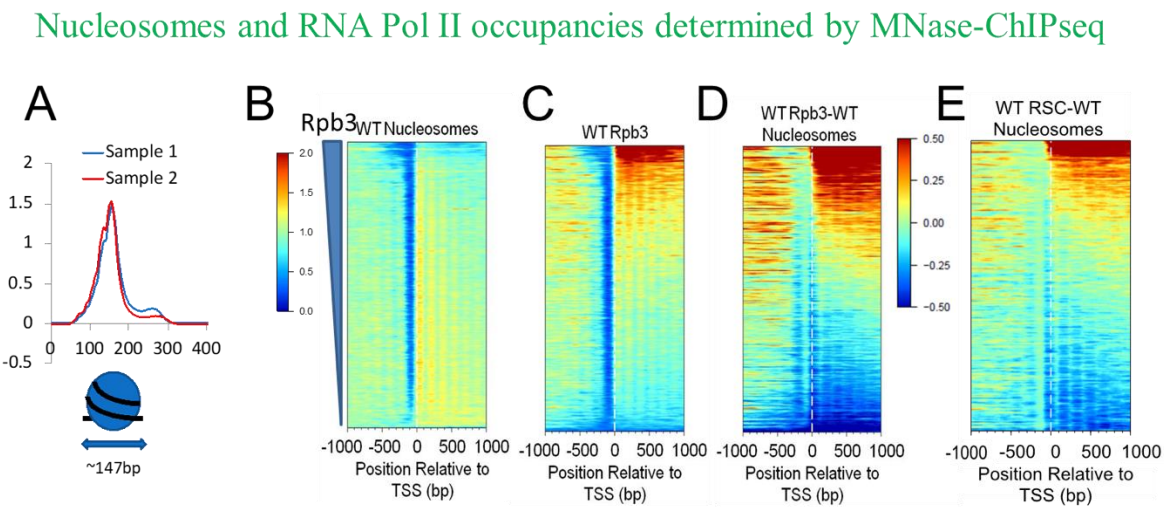

Figure 1. Fragment length analysis of Mnase- digested samples where most fragments are close to the 147 bp length of DNA (A). Heat maps show coverage for 6000 genes, in descending order of Rpb3 occupancy B-D), WT nucleosome occupancy (B), RpB3 occupancy (C), Rpb3 normalized to nucleosomes density (D) and nucleosome-normalized RSC (E) are shown.

the coding regions, from highest to lowest. All genes show nucleosome depleted regions upstream of their transcription start sites (TSS) where transcription factors are implicated in binding to DNA to aid in transcription initiation. Also present in the NDR is the RNAP II TATA box, where TATA binding protein (TBP) binds (Addgene, n.d.). We observe that there are more nucleosomes in lowly transcribed genes than in highly transcribed genes in our WT cells (Figure 1B). Genes which are actively transcribing are associated with lower nucleosome occupancies since the nucleosomes are disassembled to allow for Pol II to traverse the sequence (Pathak *et al.*, 2018). We can also observe that Rpb3 (Pol II) is present in highly transcribed genes (Figure 1C), presumably due to its function during transcription in reading the DNA sequence. When we perform a meta-gene analysis on both the Rpb3 and nucleosomes, we find definitively that Rpb3 is present in highly transcribed genes (Figure 1D), as there are more Rpb3 per dyad locus at the same locations where less occluding nucleosomes are present. When analyzing RSC, a

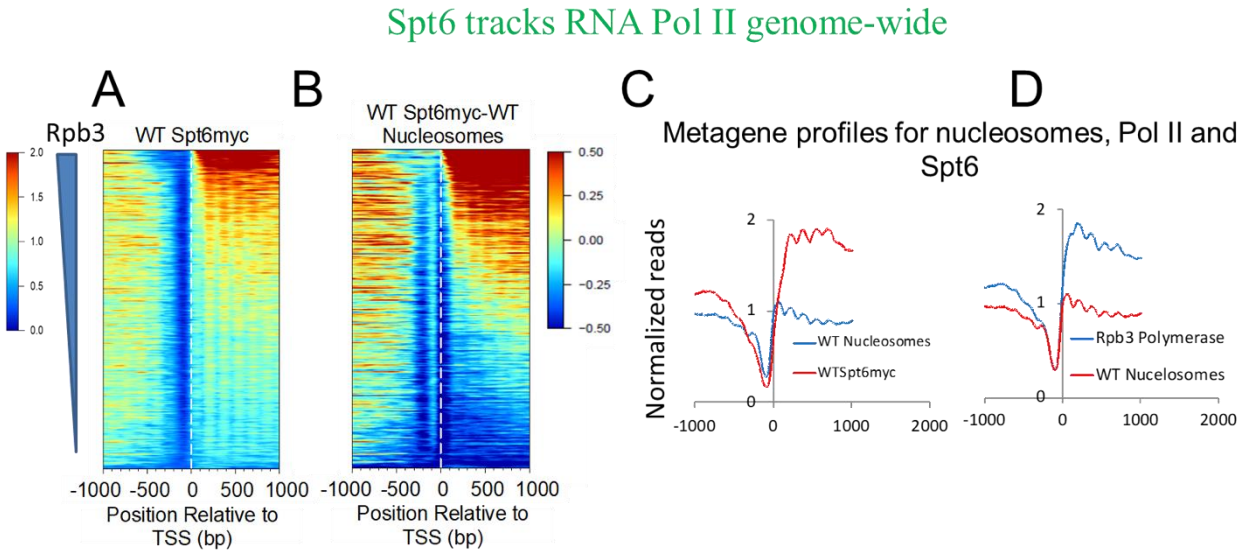

Figure 2. Heat map showing Spt6 (A) and nucleosomes-normalized Spt6 occupancies (B) over 6000 genes sorted from high to low Rpb3 occupancy. C-D) Metagene plots showing the occupancies of Spt6 (C) and Pol II (D) compared to the nucleosomes in top 25% transcribed genes. Data from Figure 1A used to generate normalized counts in Figure B.

chromatin remodeler, we can see that RSC is also present at highly transcribed genes (Figure 1E).

Similar to Pol II, when we analyzed Spt6 in the WT cells, we observed more Spt6 present in highly transcribed genes than in lower transcribed genes, presumably due to its association with Pol II and the transcription process (Figure 2 A-B). As observed in Figure 2C, there is a higher Spt6 occupancy in the top 25% of transcribed genes than that observed for nucleosomes in the same genes. A similar trend was observed for Pol II occupancies. Our findings are supported by Burugula *et. al*. (2014), who found that Spt6 occupancy is associated with Pol II occupancy in highly transcribed genes due to the tandem Src homology 2 domain (tSH2) working with the Pol II C-terminal domain (CTD). It appears that Spt6 does not associate with +1 nucleosomes in the WT cell, instead associating with nucleosomes further downstream of the TSS. This is a novel and unpublished finding.

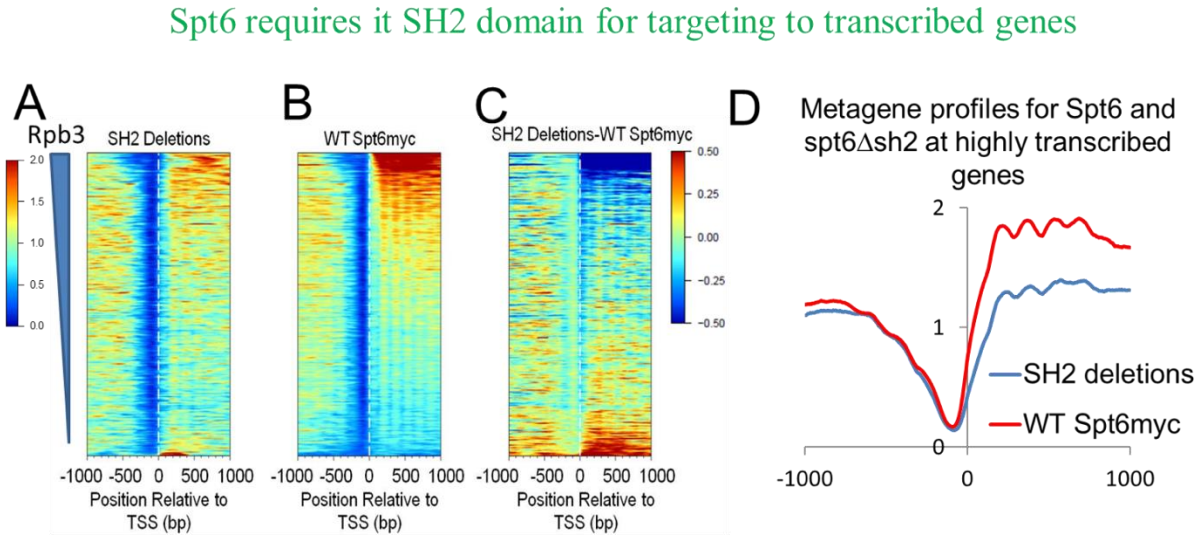

Figure 3. Heat map showing Spt6 occupancy of spt6Δsh2 mutants (A), wild type cells (B) and mutant Spt6 occupancy normalized to WT occupancy (C). Metagene plot showing occupancies of Spt6 at top 25% transcribed genes (D).

According to Sun *et. al*. (2010), Spt6 is used to make sure that nucleosomes are put in the correct regions of transcribed genes by interacting with the H3 histone. When we deleted the Sh2 domain from Spt6, Spt6 is positioned in a random manner on the genes (Figure 3A, 3C), with a depletion of Spt6 in the top transcribed genes of our Spt6 mutants. This leads to support the claim from Sun *et al.*, (2010), that the Sh2 domain is important for correct targeting of Spt6 genome-wide.

Functional inhibition of Spt6 can lead to a decrease in nucleosomes in transcribed genes (McCullough *et al*., 2015). To observe this, we deleted the 202nd amino acid in Sh2 to render the subunit nonfunctional. When we observed nucleosome occupancy in our nonfunctional Sh2 mutants, the nucleosomes were dispersed in a random pattern at a genome-wide scale (Figure 4A). Further normalization using our WT cells (Figure 4C) supported this observation, in

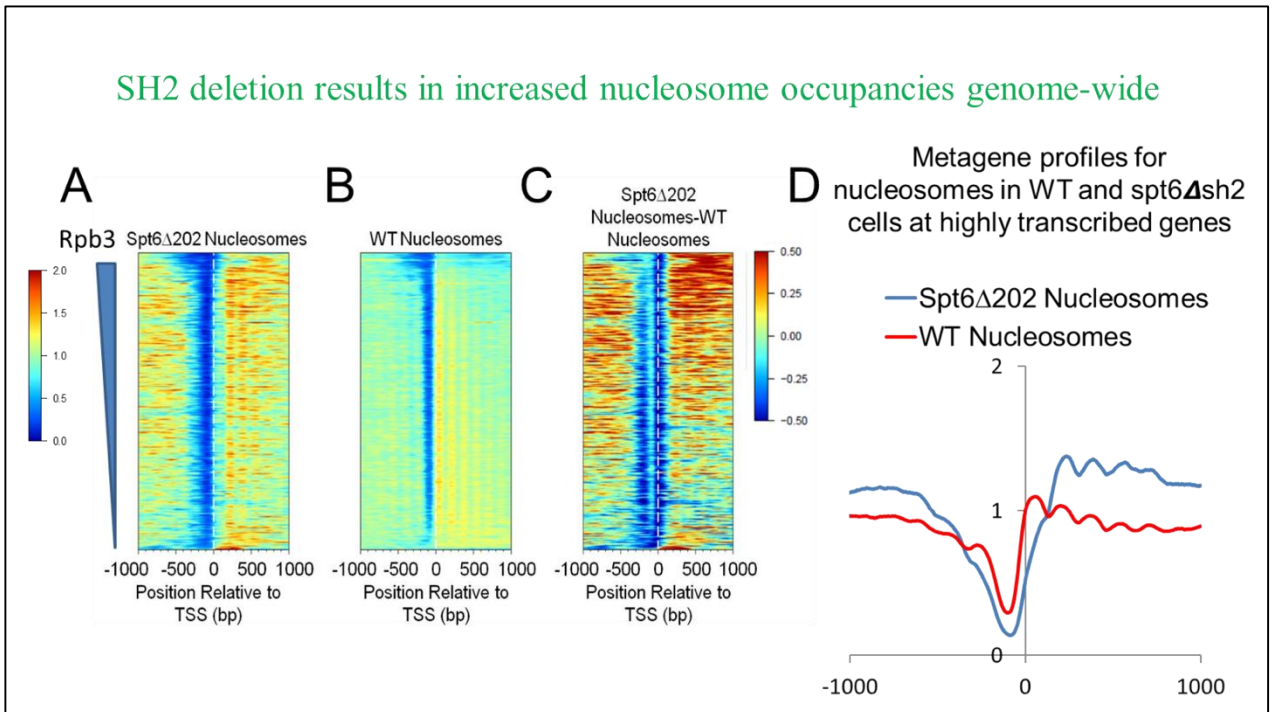

Figure 4. Heat maps showing nucleosome occupancy in non-functional Sh2 mutants (A), in WT cells (B), and WT-nucleosome normalized occupancies in the mutant (C). Metagene plot showing nucleosome occupancies in WT and spt6 mutant cells at top 25% transcribed genes (D).

addition to elucidating a depletion in the +1 nucleosome (Figure 4D). In the research conducted by Ivanovka *et al*. (2010), they found that Spt6 was responsible for the position of the +1 nucleosome in support of our observations. Without Spt6 proper function, there is a depletion of nucleosomes at the +1 position. We can also conclude that the nucleosomal positioning was not as precise in the Spt6 mutants due to the fuzzy peaks observed when compared to the WT nucleosomes (Figure 4D).

DISCUSSION

We show that Spt6 is a very important histone chaperone that facilitates upkeep of the chromatin structure. Kato *et al.* (2013), showed that with decreased amounts of Spt6, there is a decrease in the H3 histone which is associated with the repression of transcription. In our data, we found that without Spt6, the nucleosomes are not positioned correctly and in a random pattern, in support of the findings by Kato *et al.* (2013). Without correct nucleosomal positioning, transcription could result in a loss of information about gene expression from the DNA strand and could cause the chromatin structure to lose its stability (Kato *et al*., 2013). The exact mechanisms that cause Spt6 to have less nucleosomal occupancy in highly transcribed genes when it is mutated is unknown, calling more attention to this area of research. It is also found that if the Helix-turn-helix motif is removed in Spt6 there will be problems with the reassembly of nucleosomes, transcription and lower levels of Spt6 present in a gene (Dronamarju *et al*., 2018). Not only will nucleosomes be in their incorrect positions, but cryptic transcription will occur in which the intergenic regions are transcribed, which are not normally transcribed (Dronamarju *et al*., 2019; Lefers & Holmgrem, 2004; Arigo *et al*., 2006). Since these transcripts are unstable and are often destroyed, the significance of them are unknown (Arigo *et al*., 2006).

Spt6 can be found in mammalian cells, specifically in *homo sapiens* cells, allowing research that is conducted on *S. cerevisiae* to be applied to that of human cell functioning (Uniprot, 2019). Human cytomegalovirus (HCMV) is a herpes virus, that generally produces no symptoms in healthy infected individuals (Cygnar *et al.,* 2012). However, this virus can cause adverse effects in infants that include jaundice and seizures, as well as cause a decrease in vision and pneumonia in individuals who have a weakened immune systems or just had an organ transplant (Mayo Clinic, 2019; Cygnar *et al.,* 2012). Viral protein UL69 has been shown to be essential for the replication of this virus, gene expression, translation and communication with RNA (Cygnar *et al.,* 2012). UL69 interacts with Spt6 and disruption of this interaction causes adverse effects for the virus such as, loss of growth of the virus and dysfunction of moving the virus to the cytoplasm from the nucleus (Cygnar *et al.,* 2012). Taking out the Spt6 protein completely prevents the wild type HCMV from replicating and prevents UL69 from performing its proper function (Cygnar *et al.,* 2012). Research on this front can develop new treatments for those with HCMV and the breakdown of its function in the human cells.

Along with HCMV, Spt6 function is important in the expression of genes. Upon infection, HIV can integrate itself into the host genome (Nakamura *et al.*, 2012). At the HIV-1 promoter, Spt6, FACT and Chd1 have been shown to work with this site controlling transcription, as well as delaying HIV-1 replication (Nakamura *et al.,* 2012; Vanti *et al*., 2009). PAAF1 is a factor associated with ATPase and protects the Spt6 protein from wearing down (Nakamura *et al.,* 2012). Degradation of PAAF1 or Spt6 caused a decreased number of histones on the HIV-1 chromatin, creating transcripts that were unable to produce usable proteins from this chromatin (Nakamura *et al.,* 2012). In addition, knockdown of FACT, Spt6 and Chd1 caused more expression of the HIV promoter (Vanti *et al.,* 2009). It was also found that Spt6 and Chd1 are

important to control whether HIV-1 is consequently expressed or not at all (Vanti *et al*., 2009). Knockdown of Spt6 or PAAF1 can cause cancer genes like MET and NOV to be more highly expressed (Nakamura *et al.*, 2012). In addition, if Spt6 is knocked down alone, the regulation of tumor suppressor genes like BRCA1 became inhibited showing that Spt6 is important in gene regulation of expression for those genes that may cause cancer (Nakamura *et al.*, 2012).

Histone chaperones are essential in making more copies of the DNA and the expression of genes not only in *S. cerevisiae* but also in mammalian cells (Gurard-Levin *et al*., 2014). If there are mutations in histone chaperones, cancer can be the end result. In the research conducted by Gurard-Levin *et al*., (2014), mutations on the human histone chaperones DAXX and ATRX that are associated with the H3 and H4 octamers, can be found in 44% of nervous system tumors due to a misplacement of histones. Along with nervous system tumors, DAXX or ATRX mutations were found in almost one half of tumors found in the pancreas caused by neuroendocrine tumors (Rafiei & Bremner, 2014). Dysfunction of DAXX and ATRX cause irregular telomere structures, where mutations in ATRX are associated with tumors present in the brain (Rafiei & Bremner, 2014). ASF1b, a histone chaperone in human cells that helps to maintain chromosome integrity, has been associated with breast cancer (Gurard-Levin et al., 2014; Rafiei & Bremner, 2014).

Dysfunction in histone chaperones not only cause some forms of cancer but they may also cause developmental disorders. HIRA is a human histone chaperone that is homologous to Hir1p and Hir2p in *S. cerevisiae,* and it is found that without this histone chaperone, developing embryos perish (Li & Jiao, 2017). HIRA is found to be involved with DiGeorge Syndrome, in which it can cause difficulties in learning, comprehension and can cause a higher chance of individuals with DGS in developing Schizophrenia (Li & Jiao, 2017). One of the functions of

HIRA is to replace histones to the DNA, and this is found to also affect the flexibility of neurons and effect the act of thinking and understanding in both adult and child brains (Li & Jiao, 2017). Not only are histone chaperones important but chromatin remodelers are, as well. In neurons, the BRG1 Associated Factor complex (nBAF), is seen to have an influence throughout an individual's life on gene expression (López & Wood, 2015). ARID and SMARC are genes that provide proteins for the nBAF complex (López & Wood, 2015). If there is a dysfunction in SMARC, Coffin- Siris Syndrome can occur where affected individuals develop unusual joints and intellectual disabilities (López & Wood, 2015). Part of ARID, ARIDb mutations can cause autism, difficulties with speech and intellectual disabilities (López & Wood, 2015). Not only are the histone chaperones and remodelers important, but the associated factors to these complexes also play an important role in the function of the human body.

Overall the role and function of Spt6 is shown to affect nucleosomal positioning and help upkeep chromosomal integrity. Research into this histone chaperone and other chaperones is necessary, due to the implications it can create in cancer research and disease treatments. What other diseases could be caused from the dysfunction of Spt6, and could targeting this protein be a viable treatment for HIV or HCMV? Research in the field of epigenetics is necessary in discoveries to help prolong the human life.

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