Histone Dynamics and Transcription Elongation: Investigating the Co-Localization of Non-canonical Histone Variants H2A.Z and H3.3 in FACT mutations

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Introduction

Background:

The packaging of DNA within eukaryotic cells depends upon the association of genomic DNA with histone proteins, of which there are four basic varieties (H2A, H2B, H3 and H4) (1). These histone proteins assemble with DNA to form the nucleosome, which is a fundamental component of eukaryotic chromatin (1). Accompanying the four canonical histone subunits, are several types of histone variants, each of which play different roles in regulating various cellular activities (2). One particular histone variant is H2A.Z, a variant of H2A which has been shown to regulate transcriptional activity by maintaining the accessibility of DNA at promoter and enhancer regions in eukaryotic cells (3). One of the ways in which these regulatory regions are made accessible to DNA binding proteins is through the deposition of nucleosomes containing H2A.Z and H3.3; a non-canonical histone variant of H3 (3). Nucleosomes containing these two histone variants are significantly less stable than nucleosomes containing their canonical counterparts, H2A and H3 (4). The relative instability of H2A.Z/H3.3 containing nucleosomes results in the eviction of these nucleosomes, thus leading to so-called "naked" regions of DNA known as nucleosome free regions (NFRs) (4). The formation of NFRs greatly facilitates the binding of proteins that are associated with transcription, thus allowing transcription to occur more quickly (4). This evidence suggests that the localization of these particular histone variants is a fundamental part of what drives gene regulation in eukaryotic cells and as such a process must exist that regulates the genome wide expression of these histone variants to ensure that they do not become delocalized. Indeed studies have shown that the genome-wide expression of H2A.Z/H3.3 nucleosomes is generally restricted to expression within the promoters of transcriptionally active genes (4). However, the expression of H3.3 and H2A.Z, within separate nucleosomes is not quite so restricted. H3.3 expression can

be found extensively throughout eukaryotic chromatin, due largely to the fact that its deposition within nucleosomes occurs independently of DNA replication, unlike the canonical histone H3, which is only deposited into nucleosomes after DNA replication in S phase (5). Interestingly, H3.3 can even be deposited into active gene bodies in a manner that is transcription dependent (6). Similarly, a study by Hardy et al. first reported that H2A.Z was being randomly incorporated into nucleosomes in actively transcribed genes in both *S. Cerevisiae* and human cells. This study went on to further state that the amount of random H2A.Z incorporation in active genes is dependent on the level of transcription of the gene in question (7). This indicates that a transcription-based mechanism is responsible for the eviction of H2A.Z from intragenic nucleosomes.

Histone chaperones and chromatin remodelling complexes are protein complexes that function to assemble, disassemble and otherwise modify nucleosomes throughout eukaryotic chromatin (8). As these complexes modulate chromatin structure, they have been found to play an important role in dictating nucleosome structure and as such could potentially be responsible for the eviction of H2A.Z from actively transcribed regions of DNA that was described by Hardy et al. Indeed, a later paper by Jeronimo et al. discovered two separate strains of mutant yeast, one with a mutation in Spt16; a component of the histone chaperone FACT (Facilitates Chromatin Transcription) and another in Spt6; another histone chaperone. Mutations in these genes resulted in the deposition of H2A.Z into actively transcribed intragenic regions (9). These results, along with those of Hardy et al. suggest that these proteins play a transcription dependent role in mediating the removal of H2A.Z from the bodies of active genes (7), (9).

Experimental Question:

The mechanism for transcription mediated exclusion of H2A.Z as presented by Jeronimo et al. is likely to exist in mammalian systems given that all of the proteins examined in this study (Spt6, FACT and H2A.Z) have orthologs in higher eukaryotes (9). H3.3 is also deposited into gene bodies in a transcription dependent manner (6). As such co-localization of H2A.Z and H3.3 could potentially occur in nucleosomes within the active gene bodies of FACT or Spt6 knockout cells. This could result in the formation of H2A.Z/H3.3 nucleosomes within transcriptionally active genes. Given this information, I propose the following question:

"Do H2A.Z/H3.3 nucleosomes form in the active gene bodies of Spt6 or Spt16 (FACT) knock out cells?"

Relevance:

The formation of NFRs opens up genomic DNA to the potential for aberrant transcription known as cryptic transcription caused by binding of transcription factors and RNA polymerase to open sequences of DNA that would have normally been blocked by nucleosomes. (10). Cryptic transcription has been shown to be a driver of cancer metastasis (11) and as the formation of H2A.Z/H3.3 nucleosomes have the potential to produce NFRs within active gene bodies (4), this could potentially lead to cryptic transcription in mammalian cells. As such research in this direction could provide further insight into the role that nucleosome composition plays in the development of cancer. Interestingly, H2A.Z was found to be localized within gene bodies of highly expressed genes in mouse B-cell lymphoma (12). This suggests that changes in histone localization could contribute to the development of cancer. My study hopes to shed some kind of light on the possible contributions made by H2A.Z delocalization to cancer.

Hypothesis:

Based on the evidence presented above, I hypothesize that de-localization of H2A.Z in mammalian cells caused by knockdown of FACT should result in the formation of H2A.Z/H3.3 nucleosomes within active gene bodies. If this is the case, then we should see an increase in the number of H2A.Z/H3.3 nucleosomes in actively transcribed genes in FACT depleted cells.

Prediction:

If H2A.Z/H3.3 nucleosomes are immunoprecipitated and the associated DNA is sequenced, then we should see H2A.Z/H3.3 enrichment in the active gene bodies of Spt16 or Spt6 depleted cells as compared to untreated cells.

Experimental Design

RNAi treatment and validating the results of Jeronimo et al. in a mammalian system:

Prior to performing these experiments, the results seen in the Jeronimo et al. study will need to be validated in mammalian cells. To do this an RNA knockdown of Spt16 will be performed on mouse embryonic fibroblasts (MEFs). For the purposes of simplifying these experiments, Spt16 knockdown will be the focus of this study as it is highly expressed in embryonic cells (13). Additionally, a study by Garcia et al. used RNA knockdown on Spt16 in MEFs, as such Spt16 knockdown should work in this study as well (13). Furthermore, RNAi appears to be the best method for facilitating the knockdown of FACT in MEFs. RNAi induces the transient knockdown of Spt16, this would avoid potential complications that may be associated with generating stable Spt16 knockout lines in MEFs. FACT is highly expressed in pluripotent cells such as MEFs (13) and as such it may be important to MEF function. This could potentially make

it difficult to maintain a stable FACT knockout line. Additionally, for the purposes of this experiment, FACT knockdown should only need to be analyzed for 72 hours at most. As such, a transiently transfected system should be suitable for this purpose. To generate siRNA capable of inhibiting Spt16, DNA plasmids that encode for shRNA specific to Spt16 will be ordered from Sigma-Aldrich (14). A negative control plasmid containing the anti-sense strand of Spt16-shRNA will also be purchased to control for any effect the size of the transcript may have on the results. To provide information regarding the transfection efficiency and onset of siRNA-induced inhibition of the gene, a GFP containing reporter plasmid with the siRNA recognition sequence for Spt16-siRNA will be purchased as well (14). The advantages provided by this GFP-construct is two-fold, first this will prove that transfection has occurred and secondly, this will provide information regarding the speed at which inhibition occurs, thus providing information that can be used to optimize the experimental procedure later. Chip-seq will then be used to immunoprecipitate H2A.Z in both Spt16 knockdown cells and wild-type cells transfected with a plasmid containing the anti-sense Spt16 shRNA as a negative control. This will be analyzed to determine if a similar de-localization of H2A.Z is seen in these cells as was seen in yeast (9).

The Model System: Transgenic Mouse Embryonic Fibroblasts

To control the expression of H3.3 in mammalian cells so that H2A.Z/H3.3 nucleosomes may be more easily isolated, a cell line developed by Kraushaar et al. will be used. This is a MEF cell line that has been stably transfected with an inducible transgene that encodes a Hemagglutinin-FLAG-tagged H3.3 histone (HA-H3.3) that can be induced by treating the cells with doxycycline (15). The added HA-tag will simplify the isolation of H3.3 by chIP. Furthermore, to minimize the potential for S-phase induced histone disassembly, cells will be treated with DNA polymerase inhibitor aphidicolin for 18 hours prior to induction of HA-H3.3 (15). This will result in the

significant upregulation of H3.3 into active gene bodies over time and thus increase the likelihood of H2A.Z/H3.3 formation upon treatment with Spt16-shRNA.

To verify that aphidicolin treated cells do not enter S-phase, bromodeoxyuridine (BrdU) staining will be used. This stain incorporates itself into replicating DNA and can then be identified using immunocytochemistry (15). The stained cells will be fixed with formaldehyde and prepared for microscopy (16). If aphidicolin treated cells show no sign of BrdU incorporation when compared to untreated cells, then entry into S-phase will have been inhibited.

After treatment with aphidicolin, HA-H3.3 expression in cells will be activated by treatment with doxycycline and then transfected with Spt16-shRNA using lipofectamine to facilitate transfection (14). As mentioned in the previous section anti-sense Spt16-shRNA will be used as a negative control. It would be ideal for doxycycline treatment and shRNA activation to occur around the same time so that the incubation time could be identical for both treatments, to maximize the potential for production of H2A.Z/H3.3 nucleosomes. Cells will then be incubated for 12, 24, 48 and 72 hrs. At each of these time points data will be collected for the double-chIP-seq experiment. Harvesting cells over several time points will provide more information on the formation of H2A.Z/H3.3 nucleosomes.

Double chIP-Seq and RNA-Seq:

To specifically analyze chIP-seq data from nucleosomes that inhabit regions over active genes, RNA-seq will be performed. First mRNA will be extracted from MEFs. The cells used here will be MEFs that are not expressing the transgene. The role of these cells is to provide information on which genes are being actively transcribed in MEFs so that I can align this data with the reads I obtain from chIP-seq to determine when nucleosomes were found on transcriptionally

active genes. For this reason, the cells do not need to be treated experimentally; however, they do need to be from the same cell line to rule out any mutations that may result in differential gene expression in other types of MEFs. The extracted mRNA will be converted into cDNA by using a reverse transcriptase reaction and a primer that will recognize the polyA tail of mRNA, this will allow for the conversion all mRNA isolated into cDNA. This cDNA will be sequenced and the sequences will be compared to a reference genome obtained from the UCSC genome database (17). This will allow me to align the isolated mRNA with the mouse genome so that it may act as a reference for which genes are being expressed. To specifically extract H2A.Z/H3.3 nucleosomes, a double-chIP procedure will be performed on cells from each time point specified earlier (4). For this procedure, a crude subcellular fractionation will be used to specifically isolate nuclei from cells (18). This method will crudely purify cellular extracts though a series of lysis steps designed to lyse cellular and organellar membranes, while leaving the nucleus intact, thus making the final purification of nucleosomes by chIP easier. Nuclear extracts will be treated with Micrococcal Nuclease to digest only inter-nucleosomal DNA, whereas DNA that is bound tightly to the nucleosomes will be protected from this treatment. Then, nucleosomes shall be crosslinked with formaldehyde to ensure that H2A.Z/H3.3 nucleosomes remain together for the duration of the chIP procedure. Immunoprecipitation will be performed against HA-H3.3 using an anti-hemagglutinin antibody. As a negative control, anti-IgG antibody will added to some of the nuclear extract to control for antibody addition. The nucleosomes isolated from this reaction will be purified by affinity chromatography (4). The next step will use an anti-H2A.Z antibody to select specifically for H2A.Z/H3.3 nucleosomes. Anti-IgG will be used once more as a negative control. DNA will then be extracted from these nucleosomes and sequenced. This sequences provided here will be aligned to the RNA-seq data to indicate if H2A.Z/H3.3 nucleosomes are significantly associated with active genes. The chIP-seq data obtained for each active gene can then be

formatted into an average gene plot. The average gene plot contains reads from all genes complied via chIP-seq. This will allow for the analysis of an overall trend across all transcriptionally active genes. This should allow me to compare the number of H2A.Z/H3.3 reads that occurred over active gene bodies between Spt16 depleted cells and control cells.

Discussion:

If the hypothesis is proven correct then the gene plot derived from the active genes of Spt16 knockdown cells should show a significant increase in the number of H2A.Z/H3.3 nucleosomes when compared to the control. If the hypothesis is proven to be incorrect, however, then the gene plot derived from the active genes of Spt16 knockdown cells should resemble that of the control plot. A positive result would indicate that NFRs may be forming in active gene regions, thus potentially implicating a role for FACT in cryptic transcription. To elucidate this further, additional experiments could potentially probe for the presence of cryptic transcription at active gene sites in Spt16 knockdown cells. Similarly, the study conducted by Jeronimo et. al. found evidence of H2A.Z induced cryptic transcription within the gene bodies of yeast with mutations in Spt16 (9). Additionally, and quite intriguingly, H2A.Z was found to relocate substantially within the active gene bodies of B-cell Lymphoma mice (12). Considering the evidence above, positive results in this experiment serve to implicate a novel role for the FACT complex in driving cancer development.

A negative result here may indicate that H2A.Z/H3.3 nucleosome formation is being prevented by some other mechanism. One might be tempted to implicate Spt6 in this role as I did not test for it in my experiments, however in the paper by Jeronimo et al. they used separate FACT and Spt6 mutants, both of which resulted in similar phenotypes (9). So if the mechanism

carries over into mammalian cells it seems unlikely that Spt6 would play a role in rescuing the Spt16 knockdown phenotype.

Due to the artificially induced expression of HA-H3.3 in experimental cells, the biological relevance of this experiment is questionable. However, the benefits of the HA-H3.3 system are greater than the potential for misleading results as it allows for H3.3 to be isolated far more effectively thus making the extraction of H3.3 far more sensitive than it would be otherwise. Later experiments could potentially attempt to analyze H3.3/H2A.Z content using only aphidicolin to clarify the influence that ectopic expression of H3.3 may have had on the results.

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