Final Project Proposal
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Characterizing the mechanism by which glutamine 5 serotonylation acts as a permissive histone post-translational modification

Background and Significance

Histone post-translational modifications (PTMs) are important regulators of gene expression involved in a variety of biological processes including development and cell fate determination.[1,2] These modifications occur by the covalent attachment of various biological molecules to histone residues, usually in the exposed N-terminal tails of the proteins.[3] Histone PTMs function both by mediating gene accessibility via regulation of chromatin structure and by recruiting effector proteins which can either directly or indirectly alter gene expression.[4,1] Upwards of 130 histone PTMs have been identified, including well-known modifications such as acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation, as well as more recently discovered modifications such as hydroxylation and crotonylation. Despite these advances in the identification of histone PTMs, there remains limited information characterizing most types of histone marks and their roles in regulating gene expression.

Serotonin (also known as 5-hydroxytryptamine (5-HT)) is best known for its canonical role as a neurotransmitter. However, serotonin has also been shown to participate in other forms of cell signaling and has most recently been implicated in gene expression regulation.[6] An extravesicular pool of serotonin exists in the nucleus of mammalian cells and a recent study has revealed that serotonin can be covalently linked to histone tails as a previously unidentified histone PTM.[6,7] Termed serotonylation, this histone PTM is found on glutamine 5 (Q5ser) in the H3 subunit of histones and is ubiquitously expressed across mammalian tissues with enrichment in those tissues which produce serotonin. Histones containing the Q5ser mark tend to be enriched in euchromatin and correlate with permissive gene expression. In addition, the Q5ser modification appears to play an important role in cell differentiation, with genes near serotonylated histones showing differential expression in cells before and after differentiation.[6]

Serotonylation occurs by a transamidation reaction catalyzed by tissue transglutaminase 2 (TGM2)[8] and *in vivo* is specifically targeted to histones marked by an H3 tri-methylated lysine 4 (H3K4me3) mark.[6] The H3K4me3 modification is a positive regulator of gene transcription which acts by recruiting the TAF3 subunit of the basal transcription factor TFIID.[9,10] It appears that the Q5ser modification works in conjunction with the H3K4me3 mark by potentiating the binding interaction of H3Kme3 with TAF3. However, questions still remain about the exact mode of action by which Q5ser marks increase TFIID binding to H3Kme3 and thus gene expression of target loci. In addition, it is unknown what mechanism regulates the targeting of the modification by TGM2 and whether there exist Q5ser-specific demonoaminylases that also play a role in controlling the prevalence and location of this histone PTM. Finally, it is an open question if there exist any Q5ser-specific “readers” which might play a role in modulating the activity of the modification either via chromatin remodeling or through interactions with H3Kme3 “readers” or even TFIID itself. Obtaining a better

understanding of the mechanism by which Q5ser promotes gene expression and uncovering the way in which its targeting in the genome is regulated will provide insight into various biological processes which may be transcriptionally controlled at least in part by this histone PTM. Specifically, cell differentiation is a likely candidate for transcriptional regulation by Q5ser and thus full characterization of this histone PTM will likely provide insight into how cell fate is determined and fulfilled.

Objectives

The goal of this proposal is to elucidate the mechanism by which the Q5ser histone PTM potentiates the binding of TFIID to H3K4me3 modifications and potentially contributes to permissive gene expression in other ways. As histone PTMs are known to fulfill their function through two types of mechanisms, I will take a two-pronged approach to try to elucidate which mode of action is at play. Specifically, I plan to probe how Q5ser modifications affect chromatin state and determine if such changes in chromatin state are a potential way in which Q5ser marks facilitate the TFIID-H3K4me3 binding interaction. In addition, I propose to identify interactors of Q5 serotonylation such as "readers," some of which may promote TFIID-H3K4me3 binding by directly interacting with TFIID or any of its binding partners or by indirectly altering the accessibility or properties of H3K4me3Q5ser-modified histones. These studies will help characterize the Q5ser histone PTM which is implicated as an important player in cell differentiation and thus is likely crucial to proper cell fate determination and development.

Specific Aims

Aim 1: Determine the effect of Q5ser histone PTM on chromatin compaction and the role this might play in potentiating TFIID binding

The degree of DNA compaction in different regions of the genome is known to play a crucial role in regulating gene expression. Genes in less compacted euchromatin regions tend to be more highly expressed than those in more compacted heterochromatin regions due to the greater accessibility of promoters and other positive regulatory elements, as well as the gene itself, to transcription machinery in euchromatin.[11] Histone PTMs are known to modulate chromosome compaction both by directly mediating DNA-histone binding affinity and nucleosome interactions and by recruiting PTM-specific "readers" which can remodel or otherwise affect chromatin compaction.[4] Therefore, I hypothesize that Q5ser modifications function at least in part by increasing the accessibility of the modified histone to TFIID, in this way promoting TFIID binding to the H34me3 mark leading to increased gene expression.

To test if Q5 serotonylation regulates chromatin structure, I propose using a combination of primary-order chromatin structure techniques to probe the effect of Q5 serotonylation on accessibility. Specifically, I plan to use MNase-seq and ATAC (assay of transposase-accessible chromatin)-seq to indirectly and directly interrogate the primary-order chromatin structure of cells for which the Q5ser modification is either increased or decreased, relative to wild-type cells. For this analysis, undifferentiated cells, a rat-derived serotonergic cell line,[12] RN46A-B14, which was previously used in studies of the Q5ser histone PTM, will be used. To decrease the prevalence of the Q5ser mark, cells will be transduced with lentivirus expressing H3.3(Q5A) which cannot be serotonylated due to the mutation of Q5 to alanine.[6] To decrease Q5 serotonylation in another way, wild-type cells will be treated with the TGM2-specific inhibitor, LDN

27219, prior to chromatin structure analysis. Increasing the prevalence of the Q5ser histone PTM will be attempted by transducing cells with lentivirus expressing TGM2 under a strong promoter in the hopes that TGM2 overexpression will lead to increased serotonylation. MNase-seq and ATAC-seq will be performed as previously described[13,14] and analyzed using DANPOS and ZINBAS, respectively. Significance of changes in nucleosome accessibility between samples with different levels of Q5 serotonylation will be assessed at loci known to show Q5 serotonylation by Wilcoxon rank-sum test. Loci that were previously found to lack the Q5ser modification will be used as controls to set the p-value threshold. Because each chromatin structure technique utilizes a different methodology, each with its inherent biases, only those regions for which accessibility changes align and pass the significance threshold by both methods will be considered. The result of this analysis will be the identification of regions known to show the Q5ser modification for which changes in chromatin compaction are significant. Based on my hypothesis, the expectation is that there will be a significant decrease in accessibility (more hetorochromatic state) at Q5ser-modified loci in the case of abrogated serotonylation due to either the presence of H3.3(Q5A) or the inhibition of TGM2. Conversely, I expect to see a significant increase in accessibility (more euchromatic state) at these loci upon TGM2 overexpression, presumably stemming from an increase in Q5 serotonlyation. These results would indicate that Q5 serotonylation is indeed able to modify the chromatin state of nearby loci.

Following this analysis, I propose using ChIP (Chromatin immuno-precipitation)-PCR[15] in the same conditions as above to test if increased accessibility caused by Q5ser marks leads to increased TFIID binding. Target loci from the previous analysis will be selected such that a range of levels of change in accessibility are surveyed, and PCR primers will be designed for them. In addition, primers will be designed for regions not associated with Q5 serotonylation and for regions that are associated but do not show differences in chromatin accessibility to serve as negative controls. Using an antibody for TAF3, TFIID cross-linked to DNA will be pulled-down and the associated DNA will be analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Significance in differential binding will again be assessed by Wilcoxon rank-sum test. Correlation between differences in chromatin accessibility and differences in TFIID binding will be determined by linear regression.

Based on my hypothesis, I would expect to see a linear correlation between chromatin accessibility and TFIID binding, suggesting that a Q5 serotonylation-mediated increase in chromatin accessibility could be the mode of action by which TFIID binding to H3K4me3-modified histones is facilitated. To verify that increased TFIID binding at target loci is a function of increased TFIID binding to H3K4me3-modified histones, I would also try to do a two-step ChIP-PCR in the same conditions, first pulling down on TAF3 and then on histones with the combined H3K4me3Q5ser modification via antibodies that recognize each protein, respectively. Then qRT-PCR would be used to quantify the associated DNA fragments to determine differences in the amount of TFIID binding to H3K4me3Q5ser-modified histones under different conditions as before. Again, linear regression would be used to assess the level of correlation between TFIID binding to H3Kme3Q5ser-modified histones and the accessibility of the chromatin in that region due to Q5 serotonylation and if a linear relationship was observed as I expect, this would indicate that Q5 serotonylation indeed potentiates TFIID binding to

H3K4me3-modified histones by increasing the accessibility of the histone and surrounding DNA via changes in chromatin structure.

Unfortunately, some potential pitfalls that could prevent the acquisition of this data do exist. It is possible that significant differences in accessibility or TFIID binding are not observed between any of the conditions. In this case, the same experiment could be repeated using differentiated cells as large differences in Q5ser occupancy and related changes in gene expression have been observed between undifferentiated and differentiated cells. Another issue is that the overexpression of TGM2 may not necessarily lead to more Q5 serotonylation and could cause off-target effects since TGM2 catalyzes several reactions. However, even in the absence of this condition, conclusions could be drawn based on the experiments comparing reduced Q5 serotonylation conditions to wild-type. Finally, in general it is difficult to obtain definitive evidence that changes in chromatin state are directly caused by changes in Q5ser modification occupancy and directly lead to increased binding of TFIID. It is possible that each relationship will not show a linear correlation as expected and even if they do, this is still not proof of direct causation. Thus, this set of experiments could potentially be strengthened by adding a "rescue" condition in which chromatin accessibility is increased back to approximately normal levels by the mutation of silencing methylation sites in histones in a background of decreased Q5ser histone PTMs via either the H3.3(Q5A) mutant or the TGM2 inhibitor. If this was able to rescue TFIID binding to wild-type levels, this would provide strong evidence of Q5Aser modifications directly facilitating TFIID binding to H3K4me3-modified histones by increasing accessibility.

Aim 2: Identify "readers" specific to the Q5ser histone PTM

Most characterized histone PTMs have been shown to have a set of specific "readers" which are recruited to the sites of these histone marks. These "readers" are able to promote changes in chromatin compaction and interact with other types of gene expression regulators, providing an indirect mechanism by which histone PTMs can control gene expression.[3] As of yet, no Q5ser-specific histone PTM "readers" have been identified, but it is likely that at least some exist and could be important in Q5 serotonylation's role as a permissive histone mark. Therefore, I propose using immunoprecipitation experiments followed by mass spectrometry analysis (IP-MS) to identify putative "readers" of the Q5ser histone PTM.

IP-MS analysis will be done as described in Farelly et al. with a few modifications. Briefly, histones from undifferentiated RN46A-B14 cells will be immunoprecipitated using antibodies which recognize the following specific histone PTMs: H3K4me3, H3K4me3Q5ser, and Q5ser. The IPs will be analyzed by nano-LC–MS/MS using a Fusion Lumos and the resulting data will be searched again Uniprot's human database with peptide matches being filtered by a Percolator. Samples will be compared using a t-test. The first IP will serve as the negative control to identify which observed interactors are specific to H3K4me3 rather than Q5ser or generally nonspecific and thus should be discarded. The comparison of the other two IPs will help deconvolute which interactors can be recruited by Q5ser alone and which require Q5ser in combination with H3Kme3. Interacting proteins significantly enriched in both the Q5ser and H3Kme3Q5ser IPs relative to the negative control will be prioritized based on level of significance and likelihood of being a "reader" based on BLAST analysis.

Following this analysis, I plan to characterize top hits by looking at their effect on chromatin compaction, TFIID binding, and gene expression at target loci. This will be done by generating overexpression and knockout RN46A-B14 cell lines using lentivirus and CRISPR/Cas9, respectively, and comparing them to wild-type cells. Chromatin compaction and TFIID binding will be quantified in these lines as described in the experiments in Aim 1. In addition, gene expression will be measured using RNA-seq[6] and analyzed for differential expression using the DESeq2 package, with significance being determined via Fischer's exact tests. For those interactors which are indeed "readers," I expect to see differences between the overexpression, knockout, and wild-type lines in gene expression at least, and likely correlated changes in chromatin state and TFIID binding. On top of providing the validation of certain hits as "readers," these experiments will provide insight into the mechanism by which validated "readers" function, whether through chromatin remodeling or interactions with transcription machinery.

However, there are certain problems that may disrupt the progress of these proposed experiments. Many interactions are transient and could be lost in the wash steps of the IP. In the case that very few or no interactors unique to Q5ser are identified, strategies can be utilized to try to stabilize natural interactions such as crosslinking of cells prior to IP. In addition, antibodies can sometimes be nonspecific or be ineffective in IP due to low affinity binding. This is unlikely to be an issue as the described antibodies have previously been used to IP histones, however an alternative is to stably express (using lentivirus) epitope-tagged histones in cells such that you can pull down on this epitope which often is more specific and has higher binding affinity. Validation and characterization of "readers" is also likely to be more complicated than described due to challenges in generating stable overexpression and knockout lines. To overcome such barriers, transient overexpression and knockdown via siRNA, respectively, could be utilized. Finally, it will be difficult to definitively characterize all of the identified hits from this analysis. Even those hits validated as "readers" will require further analysis using both *in vitro* activity assays and data describing function *in vivo.* In addition, this study will likely identify interactors of Q5ser which are not "readers" such as regulators which remove or add marks based on different conditions to promote various cellular processes via changes in gene expression. Though outside the scope of this proposal, these hits will be important to follow up on in the future in order to elucidate the regulation of Q5 serotonylation and its effects on gene expression.

Conclusion

In summary, this proposal aims to make progress in the understanding of Q5 serotonylation of histones and its role in regulating gene expression. The focus of this study is on the role this histone PTM may play in chromosome compaction and its recruitment of "readers," with the goal of uncovering information about the mechanism by which Q5 serotonylation acts as a permissive histone mark and potentiates TFIID binding. These data will prove valuable in uncovering how gene expression is modulated in biological processes regulated by Q5ser modifications such as cell differentiation and potentially other pathways that are not yet known to be controlled by this newly identified histone PTM.

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