Assessing Acetylation Outcomes of Putative Proteins in *Cryptococcus Neoformans*

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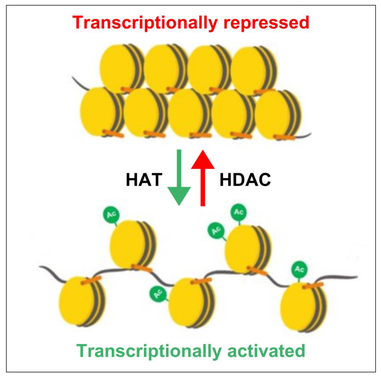
Introduction / Background

Histone acetyltransferases (HATs) are enzymes associated with the transcriptional activation of genes. p300, a known HAT enzyme in humans, acetylates histone tails at lysine and arginine residues, making DNA more prone to transcriptional activation. Therefore, identifying different HATs can aid in the understanding of gene regulation. A previous study by the Price lab demonstrated high expression levels of SAS3 in CSF-grown *C. neoformans*, suggesting a potential role in human pathogenesis. Based on protein sequence data, SAS3 has a putative HAT domain, although no definitive proof exists concerning its enzymatic activity in *C. neoformans*.

Similarly, four other genes in *C. neoformans* (ESA1, CNAG_06451, CNAG_05429, and CNAG_03583) have homology to enzymes with histone acetylation functions in other organisms, but little is known about the function of these genes in *C. neoform*ans. In addition to these five putative HAT genes, one putative HDAC gene (CNAG_04786) is being analyzed for potential histone deacetylation activity.

Therefore, our goal is to determine if SAS3, CNAG_03583, CNAG_05429, and CNAG_04786 are involved in histone acetylation.

Mechanism of Histone Acetylation



Methods/Aims

Our plan is to assess the various proteins possessing putative histone acetylation activity via the following aims:

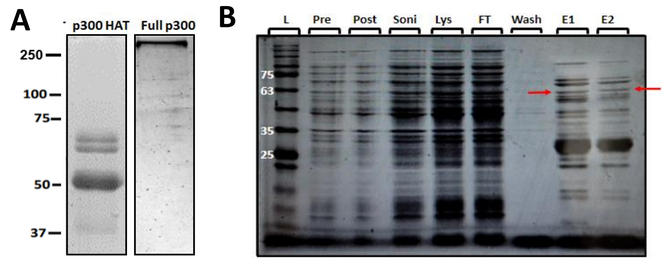
Protein purifications with transformed genes of interest → Isolation of an appropriate HAT/HDAC substrate → HAT and HDAC assays to assess for specific functions of the isolated proteins

The selected genes of interest will first be cloned into GC5 and BL21 E.coli cells. Once transformed, maxi preps of the DNA plasmid were performed in order to generate high protein yields. These proteins were then isolated via His-tag protein purification via Ni-NTA resin. Prior to using these proteins in downstream assays, dialysis was performed to remove any remaining imidazole that could interfere with the following HAT and HDAC assays. To further analyze our proteins of interest, SDS-PAGE gels were performed and stained with Coomassie to further quantify the proteins prior to incorporating them into the working HAT assay protocol.

The current HAT assay protocol tests histone acetylation levels via two antibodies: one polyclonal antibody detecting all acetylated lysines and a monoclonal antibody that only detects acetylated histone lysine-9 and lysine-14. The fluorescent secondary antibody used to generate the resulting western blot signals was a fluorescent-dye-conjugated anti-rabbit antibody. Although past HAT assays have been performed using human core histones (isolated from HeLa nuclear cell extract via AKTA-go FPLC), future HAT assays will be performed using purified recombinant *C. neoformans* histones as the substrate.

The final aim of performing an HDAC assay will be accomplished by modifying the parameters of the current HAT assay protocol in order to analyze the reverse reaction.

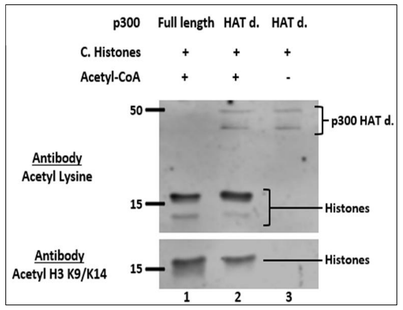
Proof of Principle: Protein Purification



(A) This is an image of the purified p300 HAT domain construct, shown to be 50 kDa in size. **(B)** Proteins found in BL21 cells transformed with ESA1 transfected into pet45b(+) were detected in various stages of protein production and isolation. The BL21 cells were induced to produce ESA1 with IPTG. Ni-NTA resin was then used to isolate the His-tagged protein. The expected band from the predicted weight for ESA1 was obtained at 63 kDa, with bands larger than 63 kDa being non-specific contamination and bands less than 63 kDa being possible ESA1 fragments.

Utilizing the same methods of protein purification illustrated above, the remaining proteins of interest will also be purified for use in future downstream assays.

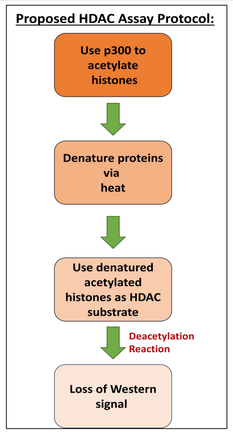
Proof of Principle: HAT Assay



Once purified, the p300 HAT domain was incorporated into a HAT assay as a positive control. The absence of acetyl-CoA, the acetyl group source for the p300 enzyme to catalyze the acetylation reaction, served as the negative control. Core histones purified from HeLa nuclei served as the substrate. The p300 HAT domain catalyzed acetylation (lane 2) but failed to do so in the absence of acetyl-CoA (lane 3), demonstrating successful histone acetylation function.

The current HAT assay protocol will be modified to incorporate *C. neoformans* histones as the substrate and will be applied to the remaining putative HATs to determine their potential HAT activity.

Development of HDAC Assay



From the current HAT assay protocol, an HDAC assay can be developed by modifying the utilized substrates and controls.

1. *Core histones will first be acetylated to create the HDAC substrate.*
2. *The proteins will then be denatured to destroy p300 activity.*
3. *To confirm that the denatured proteins can still be epigenetically modified, a negative control of p300 and denatured core histones will be run to show baseline acetylation. A positive control of denatured p300 and unacetylated histones was also used to show non-acetylated histones due to p300 denaturing.*

If the putative HDAC does possess HDAC activity, the histones will be deacetylated and no longer bind to the fluorescent antibodies, resulting in a loss of signal.

Isolation of Native *C. neoformans* Histones

One of the issues our lab has had to troubleshoot is translating eukaryotic proteins in bacteria, given that their codon frequencies are different. However, performing codon optimization allows for isolating higher protein yields by maximizing *E.coli*'s translation machinery functions.

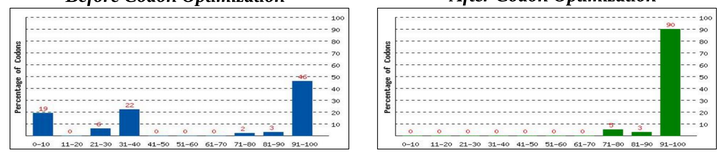


Figure 1. Bar graphs indicating success of performed codon optimization. The x-axis represents the codon usage frequency of any given codon in native *E. coli* genes, and the y-axis represents the percentage of codons in histone H2A that have that usage frequency. After performing codon optimization, the frequency of uncommon codons present in the histone gene was reduced to zero.

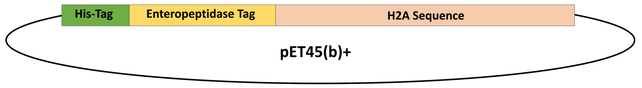


Figure 2. pet45b(+) vector with His-tag and enteropeptidase region illustrated. The histones will be induced and isolated using the same protein purification methods described for ESA1, and the histone tag will be cleaved via enteropeptidase, which targets its specific cleavage site attached between the his-tag and the gene sequence.

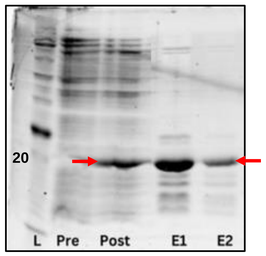


Figure 3. SDS-PAGE of purified H2A *C. neoformans* histones. Samples were taken before and after induction of transformed BL21s. The last two lanes show the first two elutions. The histone bands are marked by red arrows across induction and elution bands. Unlike native *C. neoformans* H2A histones, these histones run at a higher molecular weight due to their His-tag and their enteropeptidase tag. Similar procedures were followed for the other three nucleosome subunits (H2B, H3, and H4).

Future Goals/Acknowledgements

Future Goals:

1. Perform co-immunoprecipitation experiments to detect proteins complexed with confirmed HAT enzymes.
2. Send *C. neoformans* histones and confirmed HAT enzymes to Pocono Rabbit Farm & Laboratory for novel antibody production.
3. Perform chromatin immunoprecipitation (ChIP) assays to determine where confirmed HAT enzymes bind to the genome.

Acknowledgements:

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