Exploring the Role of Histone Deacetylase 3 in DNA Methylation in *Neurospora crassa*

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Introduction

DNA methylation is thought to have important implications in developmental, cell, and cancer biology. It is hypothesized that DNA methylation is one modification involved in tumor formation. Aside from its deleterious effects, DNA methylation plays a normal role in murine X-chromosome inactivation, gene silencing, silencing of transposons and retrotransposons, and possibly formation of centromere and telomere heterochromatic structure. In *Neurospora crassa*, DNA methylation may form in response to deacetylation and methylation of various histone tail residues. The most important modified residue in *N. crassa* is thought to be lysine 9 of histone 3 since modification of this residue alone has genome-wide implications for DNA methylation in *Neurospora*. Tri-methylation of Lysine 9 of H3 by histone methyltransferase DIM5 is essential for genome-wide DNA methylation by DNA methyltransferase DIM2 (Tamaru et. al, 2003). Histone modifications thought to promote DNA methylation, such as histone hypoacetylation and methylation, have been linked to heterochromatin formation and gene silencing in a variety of organisms, including *Neurospora*. *N. crassa* is an

ideal organism in which to study DNA methylation because it is of haploid constitution, is easy to culture, and its DNA methylation is dispensable, unlike in mammals (Selker et. al., 2003).

This research focuses on the role of Histone Deacetylase 3 in DNA methylation *N. crassa*. HDA3 mutants are particularly difficult to study as the gene *hda*-3 is thought to be essential because its yeast homolog *clr6* is essential in that organism. Previous research in the Selker lab also points towards this conclusion, with *hda*-3 mutants producing 20% inviable white ascospores. These mutants have difficulty forming vegetative macroconidia under regular conditions (37 °C). These growth defects lend more evidence to the theory that *hda*-3 is essential, implicating HDA3 as an important protein in *N. crassa*. *Neurospora's* closest homolog to the *Sacchromyces cerevisae* protein RPD3, HDA3, may have many characteristics of other RPD3 homologs, including complexing with DNA methyl-binding proteins (Nan et. al., 1998) and DNA methyltransferases (Dobosy and Selker, 2001). This would directly implicate HDA3 as a regulator of DNA methylation.

To study these issues, I will employ a null *hda-3*^{RIP} mutant. To form this mutant, Dr. Joe Dobosy transformed *N. crassa* his-3 strain 1673 with an ectopic copy of the *hda-3* allele targeted to the his-3 region. Correct transformation and recombination should yield a functional his-3, which can be used to select for the transformed strains. The presence of the ectopic *hda*-3 in the *N. crassa* genome will pre-meiotically induce Repeat Induced Point Mutation (RIP), a mechanism in *N. crassa* that deaminates and methylates DNA to create C to T transition mutations in both the duplication and the original copy of repetitive genes. These point mutations yield a wide range of mutants, from leaky to lethal (Tamaru & Selker,

2003).

DNA methylation status was examined using isochisomers DpnII and Sau3AI. DpnII is not sensitive to methylated cytosine nucleotides, where as Sau3AI is. Testing various regions methylated during RIP for DNA methylation is important, not only because these are the majority of methylated regions in *N.* crassa, but also because it can yield information about regions influenced either directly or indirectly by HDA3 specifically as opposed to other histone deacetylases. Although determination of changes in DNA methylation of particular regions is not enough to elucidate the mechanism by which HDA3 acts, DNA methylation loss in a *hda-3*^{RIP} null mutant is enough to determine some regions in which HDA3 may act. With these experiments, I hope to further elucidate the role of HDA3 in DNA methylation.

Figure1: *N.crassa* HDA3 Homologs

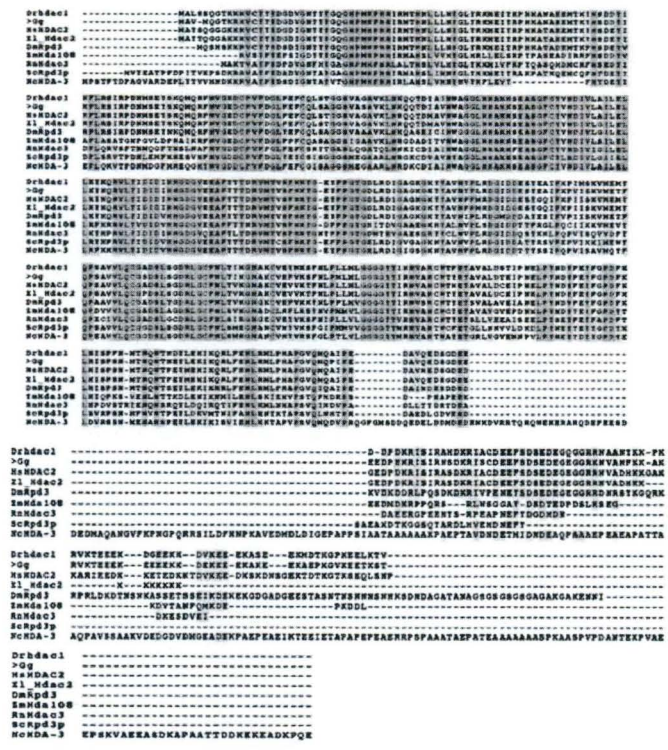
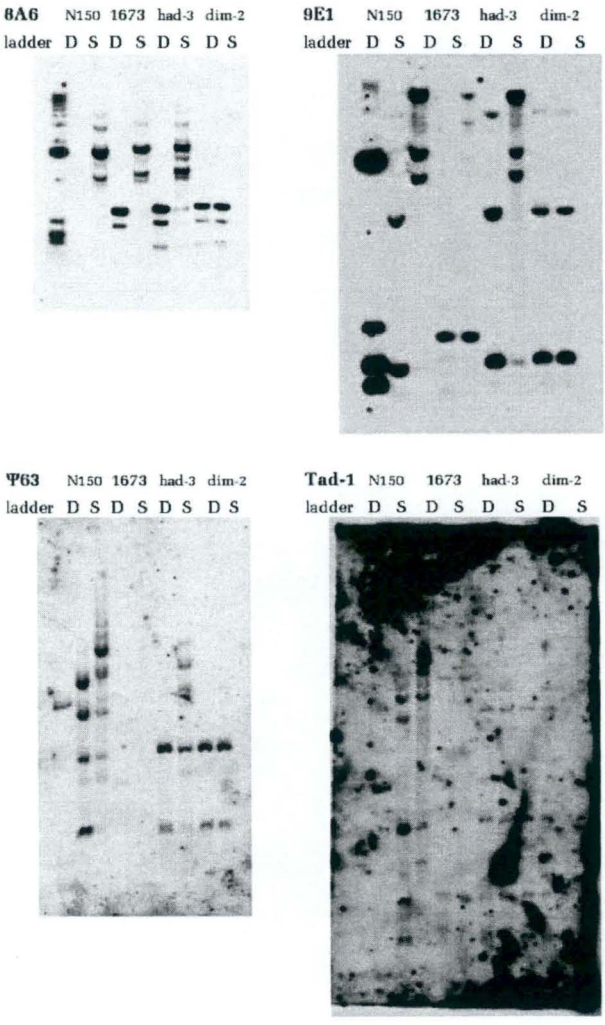


Figure 2: Regions that Lost Methylation



Materials and Methods

DNA methylation analysis was performed on *hda-3* Selker mutant strain 2666 using isochisomers and Southern blot probing for regions methylated during RIP. These regions include 8A6, ψ-63, 11E5, Tad-1, 8G3, 8F8, 8F10, 5B8, and 9E1 (Table 1). 2666 is an *hda-3* null RIP mutant created by Dr. Joe Dobosy. It was grown in Vogel's minimal liquid media without shaking at 37°C for seven days. Even under these conditions, growth was poor. Conidiation did not occur, and mycelia were brown and gray instead of the wild type bright orange.

Results

The amino acid sequence of the *N. crassa* HDA3 protein proves to be highly homologous to many eukaryotic strains (Figure 1), indicating that it is a very old strain. Former research has found homologs in the prokaryotes and archaea as well, indicating that ancestral organisms found the molecule useful even before the divergence of the kingdoms. Joe's *hda-3*^{RIP} strain 2666 was used to determine DNA methylation changes in various regions of the *N. crassa* genome. These digests were compared to those of

Figure 3: Regions with No Change in DNA Methylation

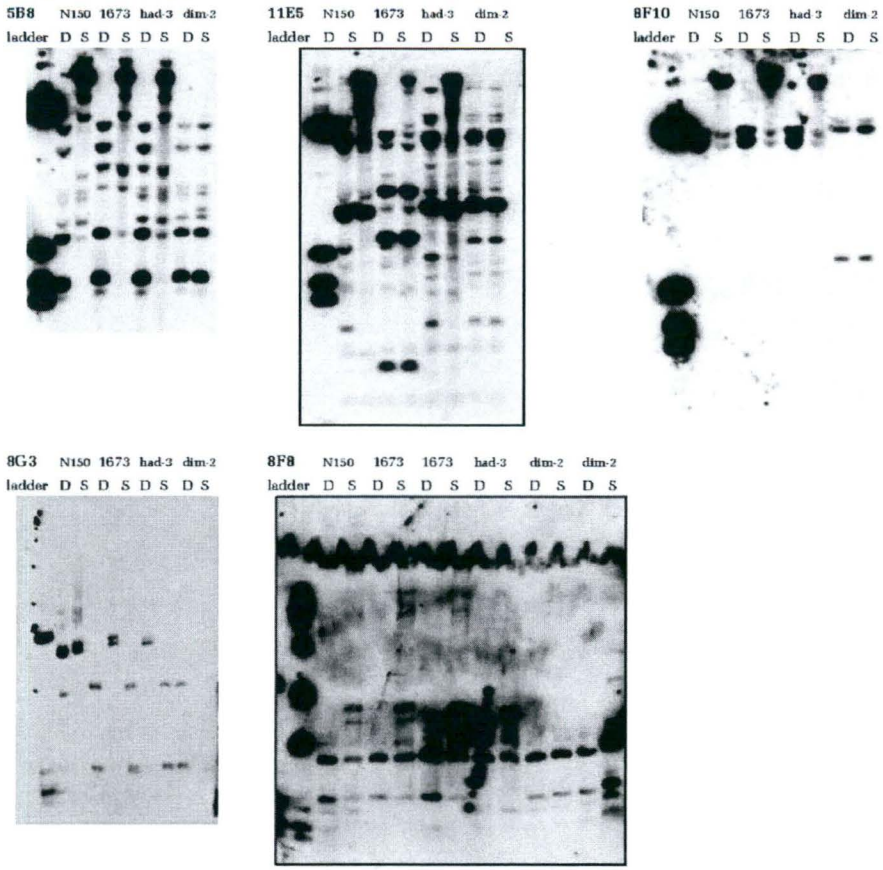


Table 1

hda-3/RIP DNA Methylation Summary

Region of Interest	Loss of DNA Methylation	Comments	# Repeats in Genome	Normal % Methylation	Linkage Group
8A6	Partial	Transposon (Dh Minos)	12	~90	V
9E1	Partial	RIP-mutated repeat	4	~90	--------
63	Partial	5s rDNA and RIPed Transposon	---------	---------	---------
Tad1	Full	Retrotransposon/telomere	---------	---------	---------
8F5	No loss	Helicase homolog/ centromeres	~30	~50	---------
8F10	No loss	---------	1	~50	III
5B8	No loss	Hs Tigger	~5	>90	II
11E5	No loss	RIP-mutated repeat	~8	~60	IV
8G3	No loss	NH-like trasposase	~9	>90	II

strain 1877, a *dim-2* null allele, and our control for complete demethylation, strain 1673, the parental wild type of *hda-3* mutant strain 2666, and N150, another nonparental wild type strain.

Probing for 8A6 revealed a slight loss of DNA methylation in the *hda-3* mutant seen by the presence of the 600 bp fragment in the Sau3AI digest (Fig.2). The DpnII lane of N150 is not present due to a leak in that lane during gel electrophoresis. 9E1 also showed slight loss of methylation as indicated by the light band around 400 bp. This band is not present in either of the wild type strains but is strong in the DpnII digest as well as in both lanes of the *dim-2* mutant. ψ-63 had slight loss of methylation, as indicated by the smallest band in the Sau3AI lane being darker than in wild type. This darkening is indicative of DNA methylation loss, as it is present and strong in the *dim-2* digests. The lighter large bands in the ψ-63 Sau lane are a good indicator that our darker small band is not darker simply due to stronger probing. Wild type 1673 is not present in this gel because it lacks the ψ-63 region. Although the Tad-1 gel contains a lot of

background, the strength and size of the visible larger fragments indicate *hda-3*^{RIP} has had complete loss of methylation in Tad-1. 5B8, 11E5, 8F10, 8F8, and 8G3 did not lose DNA methylation in the *hda-3* mutant (Figure 3). Table 1 summarizes the pattern of DNA methylation in the 2666 hda3RIP mutant.

Discussion

Although there is no correlation between where these regions are in the genome, what they are, and whether or not their DNA methylation is regulated by HDA3, one could be found if more regions were analyzed. Since HDA3 seems to be a global deacetylase, it probably acts on a wide range of regions. It is interesting to note that two transposons (8A6 and ψ63) partially lost DNA methylation when *hda-3* was knocked out (Figure 2). Tad-1, a retrotransposon, had a complete loss of DNA methylation. Transposons and retrotransposons are viral relics possibly silenced by DNA methylation to prevent their continued proliferation from the host genome. The RIPed mutated repeat 9E1 also had partial loss of methylation. This indicates HDA3 may be necessary for maintaining DNA methylation and silencing of the RIP mutated fragments. Loss of methylation in RIPed regions could lead to aberrant expression of this mutated region, producing dysfunctional and harmful RNA or protein byproducts.

Regions where methylation is not affected include a transposase (8G3), an enzyme that aids transposons in cutting themselves out of the genome and reinserting elsewhere in the genome (Figure 3). We could theorize that this region may be highly regulated by a number of histone deacetylases because its silencing is crucial for the viability of the organism.

Dr. Dobosy thought 5E6 region might have a loss of methylation, although he notes the stains on the gel indicate this pattern may be attributed to restriction fragment length polymorphisms rather than changes in DNA methylation. Dobosy's find of partial loss of methylation in am^{RIP8} is useful in that TSA is found to have the same effect on this region. Since TSA is a direct inhibitor of HDA activity, we conclude that HDAs, including HDA3, do act in this region, and their effect on DNA methylation is directly correlated with their ability to deacetylate neighboring histone lysine residues.

To elucidate a mechanism for HDA regulation of DNA methylation, it is important to determine which HDAs affect different lysine residues on various histones; how they modify them; what regions of DNA interact with these modified residues; and finally, what changes in methylation occur in *hda* mutants in these regions. Regions of DNA without changes in DNA methylation in *hda*-3^{RIP} strains may be regulated by HDA3, but DNA methylation loss is prevented by histone acetyltransferase (HAT) reacetylation. Without analyzing histone modifications, it is difficult to determine all of the regions a HDA may be regulating. To determine the regions HAD may be regulating, ChIP should be conducted to determine what regions of the chromosome HDA3 targets. ChIP should be followed by a microarray analysis of what regions of DNA the HAD3 bound histones bind to.

Finally, the mechanism of HDAC3 protein should be elucidated by determining with what proteins it complexes. Specifically, we would like to know if HDAC3 protein binds DIM-5 protein, thus directly linking histone deacetylation with histone methylation. This could be found with affinity purification of HDAC3 and a Western blot. Using an antibody specific to DIM-5, as well as the

antibody for the Histidine 6 or FLAG tagged HDA3 protein, it can be determined whether or not DIM-5 is in complex with HDA3 by "pulling down" each protein and whether or not the other co-precipitates.

Epigenetic DNA methylation studies have wide reaching applications in understanding development, from gene imprinting and dosage compensation to tumor formation and carcinogenesis. This study will provide new data regarding links between histone modification and formation of silent heterochromatin. It should help fill in the most critical gaps in current understanding of the epigenetic regulation of gene transcription, specifically, the role of histone deacetylases in this regulation.

Sources

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Tamaru et. al, (2003). "Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in *Neurospora crassa*;" *Nature Genetics*, 34, 75-79.