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The Role of Thd2 in Deposition-Related Deactylation and Chromatin Maturation

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The Role of Thd2 in Deposition-Related Deacetylation and Chromatin Maturation

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Abstract

During S phase of the cell cycle, newly synthesized histones are acetylated in the cytoplasm in patterns specific to DNA replication. Once incorporated into nucleosomes, these histones are rapidly deacetylated by enzymes known as histone deacetylases. Though common in all organisms, the significance of this molecular mechanism is not fully understood. Homologous to HDAC6 in humans and HDA1 in budding yeast, Tetrahymena histone deacetylase 2 (Thd2) has been identified as the only known histone deacetylase that performs this task in the unicellular eukaryote Tetrahymena thermophila. Localizing to the transcriptionally inactive germline nucleus, the micronucleus, Thd2 has been found to deacetylate histones H3 and H4 at K9 and/or K14. In order to gain further insight into the role of deposition-related deacetylation in chromatin maturation, the micronuclear morphology and modification status of H3K27, a known marker for heterochromatin in several eukaryotes, were examined in both vegetative and synchronized complete Δthd2 mutant cells. Immunofluorescence microscopy, DAPI staining and a western blot analysis revealed abnormal phenotypes and the conservation of H3K27 methylation in the absence of Thd2. These findings further indicate a role for Thd2 in the maintenance of chromatin structure and suggest the possibility of another mechanism required for deacetylation at H2K27. Essentially, this demonstrates the importance of deposition-related histone deacetylation in chromatin maturation after DNA replication and further maintenance of chromatin domains.

Introduction

Eukaryotic DNA packaging

In eukaryotic organisms, deoxyribonucleic acid (DNA) is the primary genetic material that serves as an instruction manual for all biological processes necessary to develop and sustain life (i.e. protein synthesis, cell differentiation and regeneration, gamete production, gene reparation etc.). The fundamental element of DNA, the nucleotide, is a molecule composed of a 5-carbon sugar, a nitrogenous base, and 1-3 phosphate groups.[5, 6] A single strand of DNA is composed of a sequence of four nucleotides (Adenine, Cytosine, Guanine and Thymine) connected by phosphodiester linkages and a complete DNA molecule consists of two anti-parallel polynucleotide strands bound together through hydrogen bonding to form a double helix.[5-7] Measuring between 1 and 2 meters in length, DNA molecules must be folded and compacted in order to fit inside a nucleus of only 10^{-5}m in diameter.[6]

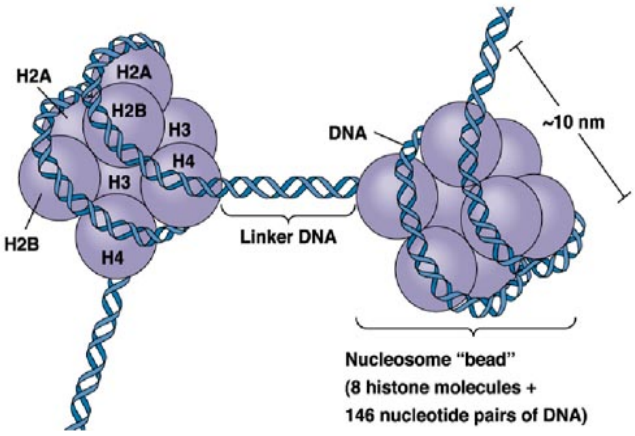

Figure 1. The nucleosome is the fundamental repeating unit of chromatin. DNA is wound around octamer of core histones forming the nucleosome. Linker DNA connects adjacent nucleosomes forming the appearance of "beads on a string".[1]

Within the nucleus, DNA is packaged into chromatin, the complex of a DNA molecule with both histone and non-histone proteins. Chromatin demonstrates a higher-order structure that is comprised of an intricate series of folding influenced by DNA-protein interactions. At the first level, a single molecule of double-stranded DNA is wrapped twice around an octamer of histone proteins, forming the fundamental repeating unit of chromatin, the nucleosome

(Figure 1). Each nucleosome contains 2 of each core histone (H2A, H2B, H3, and H4) and is surrounded by 146 to 147 bp of DNA. Furthermore, nucleosomes are connected together via linker DNA ranging from 20 to 100 bp in length. This level of DNA packaging decreases the size of a single DNA molecule by approximately 7 times the original length.[5, 6]

In addition to the core histones, in most eukaryotic cells, histone H1, also known as the linker histone, is incorporated into the chromatin structure through its binding to the linker DNA. Though the primary purpose of linker histones has yet to be proven, previous findings have suggested that the binding of H1 histones to the linker DNA, in conjunction with nucleosomal interactions, assists in the further compaction of nucleosomes into a 30 nm fiber (Figure 2).[5, 6, 8]

In regards to the exact structure of the 30 nm fiber, little is known due to the extremely compact nature of chromatin at this level and the difficulty of maintaining the integrity of DNA molecules during extraction. None the less, two models for the 30 nm fiber have been proposed: the solenoid model and the zig-zag model.[5, 8] In the solenoid model, it is suggested that connected nucleosomes are organized into a helical pattern creating a condensed and symmetrical structure (Figure 2).[5, 8] Alternatively, the zig-zag model proposes that linker DNA regions are twisted in irregular directions creating, as the name suggests, a zig-zag pattern between the nucleosomes.[5, 8] Together with the initial formation of nucleosomes, the 30 nm fiber further condenses the size of a DNA molecule by approximately 49 times the original length (Figure 2).

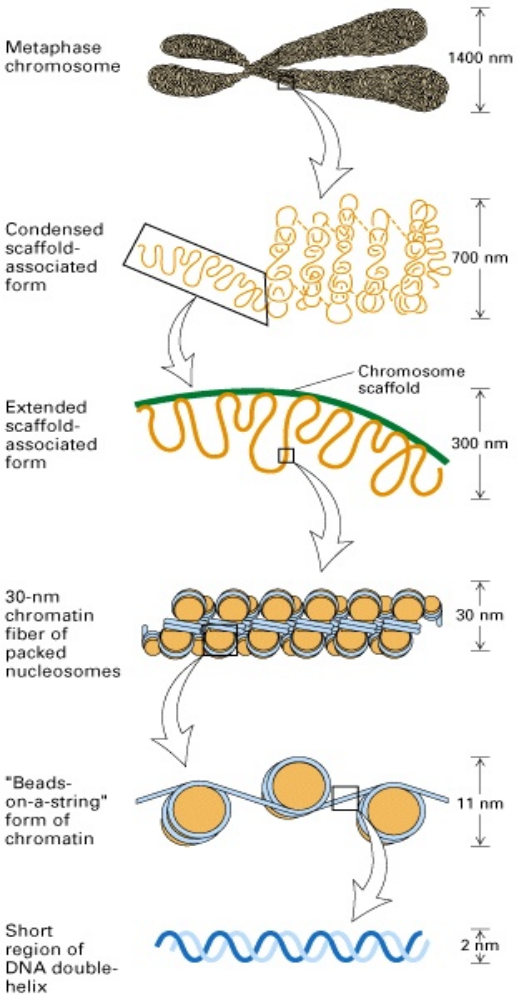

Figure 2. Higher order Structure of Chromatin. DNA double helix is complexed with histones forming nucleosomes. Nucleosomes are compacted into 30nm fiber (solenoid model depicted). Interactions with filamentous proteins in the nuclear matrix cause 30nm fiber to fold into a scaffold of radial loops (300nm) that further condense until develop into the chromosome.[2]

In the third level of DNA compaction, the nucleosomes of the 30nm fiber associate with a network of filamentous proteins within the nucleus called the nuclear matrix.[5] The nuclear matrix has two components: the nuclear lamina, a group of fibrous proteins lining the nuclear membrane, and the internal nuclear matrix, a group of filamentous proteins filling the interior cavity of the nucleus.[5, 6] The exact structure and nature of these nuclear proteins is not fully understood but, it has been suggested that the nuclear matrix may be composed of several different types of proteins that interact with the 30 nm fiber to form DNA into what is known as radial loop domains. During interphase, matrix-attachment regions (MARs), specific regions that are infused between genes, bind to specific nuclear matrix proteins forming radial loops ranging between 2.5×10^4 to 2.0×10^5 base pairs in length (Figure 2). The radial loop domains form a 300 nm zigzag scaffold that contributes to the further condensation and organization of DNA into the highest order of chromatin structure within the nucleus, the chromosome.[5, 6]

Chromatin may exist in two forms: heterochromatin and euchromatin. Heterochromatin is the highly condensed configuration of chromatin that is most often transcriptionally inactive whereas euchromatin is the less compact version that is usually active in transcription (Figure 3). The state of chromatin has been shown to have epigenetic significance that is often influenced by post-translational modifications that are mediated through the interactions of enzymatic proteins with the amino-terminal tails of histones.

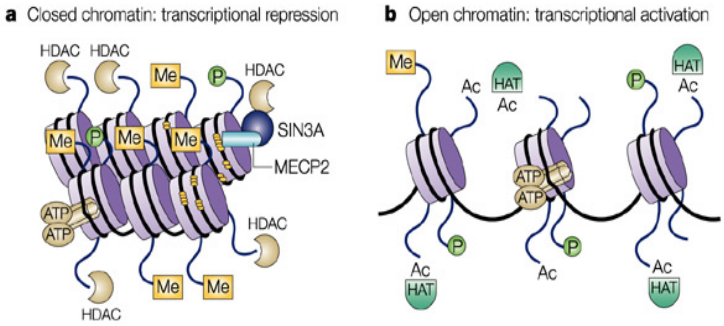

Figure 3. Chromatin domains are influenced by posttranslational modifications. (a)Heterochromatin is highly methylated by histone methyltransferases (HMTs), tightly condensed and inactive during transcription. (a)Euchromatin is highly acetylated by histone acetyltransferases (HATs), loosely packaged and usually active in transcription.[3]

Histones Tails

Ranging from yeast to humans, the amino acid sequences of the N-terminal tails of core histones are highly conserved evolutionarily indicating an essential role in cellular processes shared among eukaryotic species.[9, 10] It has been shown that the removal of the N-terminal tails of histones H3 and H4 does not affect the overall structure of the nucleosome demonstrating that the globular domain of core histones H2A and H2B alone is sufficient enough to maintain the structure of nucleosomes. These findings indicate that histone tails most likely serve a purpose separate from maintaining nucleosome structure. Furthermore, the highly basic nature of the amino acid tails of core histones suggests that they may be subjected to molecular mechanisms, such as posttranslational modifications,

that influence their interaction with the negative backbone of DNA that ultimately affect chromatin domains (Figure 3).[9-11]

Post-Translational Modifications

During DNA replication and transcription, specific lysine and arginine residues of the histone N-terminal tails are the targets of post-translational modifications, such as acetylation, phosphorylation, methylation, ubitquitination, and ADP riboslyation.[9] Placed in distinct patterns by enzymes, these modifications act as non-random epigenetic regulators for the metabolic processes of DNA (i.e. DNA repair, transcription, and the induction of apoptosis as well as chromosome condensation). [12-15] Acetylation and methylation are among the most well known histone modifications and have been shown to have significant correlations with the assembly of chromatin domains: euchromatin and heterochromatin.[10,16]

Histone acetylation is frequently associated with transcriptional activity because acetyl moieties are mostly found in higher concentrations on euchromatin (open chromatin) whereas heterochromatin (closed chromatin) tends to be hypoacetylated (Figure 3). Catalyzed by histone acetyltransferases (HAT), histone acetylation increases the alpha-helical content as well as decreases the basicity and affinity of histone tails for the negatively charged DNA molecule, allowing for a more relaxed chromatin structure. [11, 16] In contrast to acetylation, site-specific histone methylation is a process catalyzed by histone methylatransferases (HMT) and that likely increases the basicity and affinity of histone tails to the negatively charged backbone of the DNA molecule, thus promoting a more compact formation of chromatin (i.e. heterochromatin). [11]

DNA Replication and Histone Deacetylation

In eukaryotes, DNA replication occurs in interphase of the cell cycle. As the replication fork disassembles the parental strand, the daughter DNA strand is assembled by replication proteins. Parental histones are transferred to the daughter strand while new histones are synthesized in the cytoplasm. After transport to the nucleus, the new histones are incorporated onto both the daughter and parental DNA strands in a specific order: (1) $(H3/H4)_2$ tetramer, (2) two H2A/ H2B dimers, (3) then incorporation of linker histone, H1 with the assistance of histone chaperone, chromatin assembly factor 1, CAF-1.[10, 17, 18] Prior to assembly into nucleosomes, a significant amount of new histones are acetylated in the cytoplasm in replication specific patterns (i.e. K9 and/or K14). After transport to the nucleus and assembly onto the new DNA strand, these histone tails are rapidly deacetylated by histone deacetylase enzymes**.** Though occurring in all organisms, the significance of this process remains uncertain.

Previous studies have suggested that nucleosome deposition-related deacetylation may be necessary for further chromatin maturation after DNA replication. In the presence of sodium butyrate, a deacetylase inhibitor, newly synthesized chromatin acquired characteristics of immature chromatin. In addition to hyperacetylation of new histones relative to the control, there was an increase in chromatin sensitivity to DNaseI activity, an increase in magnesium solubility of newly synthesized core histones and a significant decrease in histone H1.[19, 20] Upon the complete removal of histone H1, DNaseI sensitivity and magnesium solubility of chromatin synthesized in sodium butyrate was eliminated. This data suggest that linker histone H1, may not be incorporated into chromatin until after deacetylation as a manner of regulating the deposition of linker histone H1[19].[19, 20]In another

related study, deacetylation of K91 was found to be necessary for stable binding of H3/H4 tetramer with H2a/H2B dimers during nucleosome formation after DNA replication. These findings indicate that deacetylation may be required for nucleosome formation and/or the facilitation of H1-mediated nucleosome condensation into higher order structure (ie.30 nm fiber).[21] The goal of this study is to further examine the role of deposition-related deacetylation in chromatin maturation after DNA replication.

Tetrahymena Thermophila

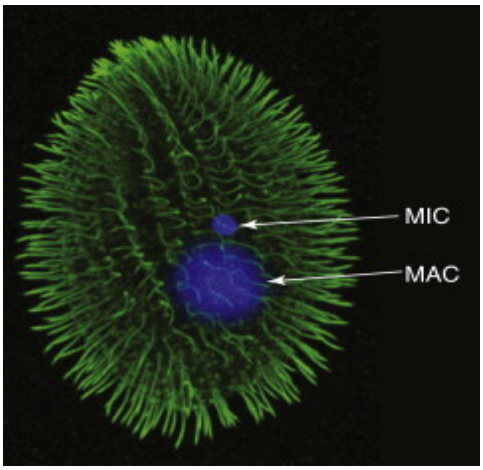

Figure 4. Nuclear Dimorphism of *Tetrahymena thermophila*. A ciliated eukaryote with two distint nuclei: the macronucleus and micronucleus.[4]

The use of *Tetrahymena thermophila* as a model system provides several advantages to this study. Most commonly found in lakes, streams and ponds, *Tetrahymena thermophila* is a unicellular organism that shares several molecular mechanisms with a large range of eukaryotes including budding yeast and human. [18, 22, 23] Additionally, *T. thermophila* is one of the fastest growing eurkaryotic species with a minimum doubling time of 2hours and cultures can be grown in several media types and temperatures (12^{o}C – 41^{o}C).[22] Most importantly, as a ciliated protozoan, *Tetrahymena thermophila* undergoes both asexual and sexual reproduction and possesses two distinct nuclei that differ in structure and function: the macronucleus and the micronucleus (Figure 4). [4, 18, 22, 24] The macronucleus (MAC) is the somatic nucleus that is mostly composed of hyperacetylated euchromatin (open conformation) and is actively transcribed and replicated during vegetative growth but, is not recycled during sexual conjugation.

Containing 45 copies of 200-300 transcriptionally active chromosomes derived from micronuclear DNA and 9000 mini chromosomes encoding ribosomal RNAs, the macronucleus is significantly larger than the micronucleus.[4, 22, 23] In contrast to the macronucleus, the micronucleus (MIC) is the germline nucleus that is composed of unacetylated heterochromatin (closed conformation) and contains 2 copies of 5 transcriptionally silent chromosomes. During vegetative cell division, the MIC is replicated by mitosis whereas in sexual conjugation, micronuclear DNA replicates by meiosis and forms zygotic nuclei that further differentiate into new macronuclei and micronuclei by a molecular mechanism that is not completely understood.[4, 23] In contrast to organisms with single nuclei, the dynamic nuclear dimorphism of *Tetrahymena thermophila* affords the opportunity to distinguish between transcription-related and nucleosome deposition-related deacetylation events. [4, 18, 24]

THD2 is a Histone Deacetylase

Tetrahymena histone deaetylase 2, Thd2, has been identified as a Class II histone deacetylase and is the only known HDAC to localize to the micronucleus in *Tetrahymena thermophila.*[18] Furthermore, Thd2 has two alternative splice variants, Thd2a and Thd2b, and is homologous to HDAC6 in humans and HDA1 in *S. ceravisae*.[18]

Though the micronuclei are completely unacetylated, temporary acetylation is detected during DNA replication. The transcriptional inactivity of the micronuclear chromatin throughout vegetative cell growth indicates that these temporary acetylation patterns and the localization of Thd2 to the micronucleus are correlated with nucleosome-deposition after DNA replication.[18]

Using complete mutant *Tetrahymena thermphila* cells, Smith et al. demonstrated that Thd2 was responsible for deacetylating micronuclear histone H3 after DNA replication. In the absence of Thd2, K9 and K14 of histone H3 retained acetylated and abnormal micronuclear phenotypes were observed. These findings implicate Thd2 in chromatin maintenance after DNA replication. In this study, I demonstrate that these abnormal phenotypes are passed through generations of cell divisions after original experiment further indicating Thd2 is involved in the maintenance of chromatin structure following DNA replication.

Materials and Methods

Cultures

All cultures were prepared using *Tetrahymena thermophila* wild type CU428 and mutant CU428 (Δ thd2) cells in a 2% super protease peptone yeast solution (PPYS: 0.01 g/mL proteose peptone, 0.002 g/mL glucose, 0.001 g/mL yeast extract, 0.02 g/mL $FeCl_3$) with 1X PSF antibiotic (Penicillin, Streptomycin, fungizone) and allowed to incubate in a C24 Incubator Shaker at room temperature (30^{o}C) until cells had reached mid-logarithmic growth phase, (approx. 3-7 x10^5 cells/mL).

Fixation

Wild type CU428 and Δ thd2 cells (10-15mL) were centrifuged in 15 ml conical tubes for 2 minutes at 2.5 setting on the Clay Adams Safeguard Centrifuge. The supernatants were immediately decanted and the cells were re-suspended in the remaining liquid. Cells were then washed with 5ml PHEM buffer (60 mM PIPES, 25mM HEPES, 10mM EGTA, 2 mM $MgCl_2$, pH = 6.9) and subsequently centrifuged 2 minutes at 2.5 setting. The supernatants were decanted and 1ml of paraformaldehyde fixative (3% paraformaldehyde, 0.2-0.5% Triton X-100 in PHEM buffer) was added to each cell type. Following an incubation period of 1 hour at room temperature, the cells were centrifuged at the same speed and the fixative was removed. Cells were then washed with 5ml of PHEM buffer and stored in the refrigerator.

Immunofluorescence and DAPI Staining

Fixed wild type and mutant Δthd2 mutant cells (100-400 μl) were centrifuged for 2 minutes at 2.2 rpm in the Eppendorf Centrifuge 5415 R and the PHEM buffer was decanted. After a 5 minute wash in 0.1% Bovine Serum Albumin (BSA) dissolved in 1X Phosphate

Buffered Saline (PBS: 1.9mM NaH_2PO_4, 8.1mM Na_2HPO_4, 154mM NaCl, pH= 7.2), the cells were centrifuged at the same speed and the supernatant was removed. Cells were then re-suspended in a 1% BSA-PBS solution with the primary antibody (Table 1). After an incubation period of 1 hour at room temperature, the cells were centrifuged at 2.5 setting for 2 minutes and the primary antibody solution was poured off. The cells were then washed twice with 400 μl of 0.1%BSA-PBS solution for 3 min per wash. Cells were then re-suspended in a 1% BSA-PBS solution with the secondary antibody (Table 1) and incubated at room temperature for 30-45 minutes. The cells were then centrifuged at the same speed and the secondary antibody solution was removed. Cells were washed twice with 400 μl of 0.1%BSA-PBS solution for 3 min per wash and then re-suspended in 200 μl of 1% BSA-PBS. After 1 μl of 1 μg/ml DAPI (4', 6-diamidino-2-phenylindole) was added to each micro-centrifuge tube, the cells were allowed to incubate for 10 minutes.

Table 1: Dilutions of anti-bodies with 1% BSA-PBS are listed in each specific experiment below.

Experiment	**1° Antibody**	**1° Dilution**	**2° Antibody**	**2° Dilution**
Chromatin Morphology	α-Micronuclear Linker Histone	1:500	Goat-α-rabbit	1:200
Methylation on H3K27	α-H3K27Me3	1:100	Goat-α-rabbit	1:200

Microscopy

Between 25-50 μl of each cell type were transferred onto square cover slips thinly layered with a poly-lysine solution using a pipette and allowed to sit for 20 minutes. Two 5 μl droplets of gel mount were added to a slide and allowed to dry for 2-3 minutes before the poly-lysine cover slips containing the cells were inverted onto it. The cells were viewed using fluorescence microscopy under the Canon ZoomBrowser EX microscope and images were taken using NIKON ACT-1 program.

Starvation

Wild type and mutant Δ thd2 cell cultures (200mL) were transferred into 50mL centrifuge tubes and centrifuged for 4 minutes at 2200 rcf using Beckman Coulter Allegra 25 R Centrifuge. The supernatant was decanted and the cells were washed in 10 mL of 10 mM Tris (pH = 7.5). The cells were centrifuged again at same speed, the supernatants were discarded. The cells were re-suspended in 200mL of 10 mM Tris, transferred to a 1000mL beaker and incubated at 28^{o}C for 12-24 hour period.

Nuclei Extraction

Starved wild type and mutant Δ thd2 cells were centrifuged at 2500 rcf for 5 minutes at room temperature and the supernatant was immediately poured off. In the cold room (4^{o}C), cells were suspended in Medium B (Medium A with 0.5% octanol at a 1:100 dilution with 100mM phenylmethanesunfonylfluoride, PMSF; Medium A (pH= 6.75): 3.4% sucrose, 2 mM $MgCl_2$, 4% gum arabic, 10 mM Tris (pH=7.5), 1mM iodoacetimide, 0.1 mM PMSF) to make a concentration of 1.5 x10^6 cells/mL. Cells were blended for 20-30 seconds in a blender and subsequently centrifuged in 50mL conical tubes at 3000 rcf for 5 minutes at 4^{o}C. Supernatant was poured back into blender and the remaining solution containing the isolated nuclei was re-suspended in 500 μl of a 1:100 dilution of I.S. Wash (0.25 M sucrose, 10mM Tris (pH=7.5), 3mM $CaCl_2$, 1mM $MgCl_2$, 10mM butyric acid, 1mM PMSF; pH = 7.5) with PMSF. Approximately 2-3 μl of the nucleic solution were placed onto a slide with 2-3 μl of methyl green and examined under Leica DM E microscope to ensure that the cells had fully lysed. The isolated nuclei were transferred to a conical tube on ice. Blending steps and the examination of nucleic solution were repeated2-3 additional times to ensure maximal macronuclear collection. Nuclei were washed with 5mL of the I.S. Wash- PMSF

solution, centrifuged at 3500 rcf at 4°C for 5 minutes and the supernatant was poured off. The nuclei were then re-suspended in 1mL of I.S. Wash-PMSF, transferred to a microcentrifuge tube and stored in -86°C freezer until further use.

Histone Isolation

Wild type and Δ thd2 nuclei were thawed on ice and counted using a hemocytometer. Approximately 1 x 10^7 nuclei per cell type were centrifuged in Beckman Coulter Allegra 25 R Centrifuge at 7000 rcf in 4°C for 10 minutes and the supernatant was discarded. The pellets were re-suspended in 300 μl of 0.4 N H_2SO_4 and incubated on shaker in 4°C room for 2 hours. After another centrifugation at 7000rcf in 4°C for 10 minutes, the acid-soluble supernatants containing histones were res-suspended in 100% Trichloroacetic Acid (TCA) and stored in a -86° freezer while the pellet was then discarded. Immediately preceding gel electrophoresis, the histones were thawed on ice, centrifuged at 13,000 rcf in 4°C for 10 minutes and the supernatant was removed using a pipette. Following the addition of 500 μl of 0.1% HCl in Acetone, the histones were vortexed using Scientfic Industries Vortex Genie2 on setting 9 for 3-5 seconds and centrifuged in the same conditions. The 0.1% HCl in acetone solution was discarded and the pellet was re-suspended in 500 μl of pure acetone (100%) using a vortex. After the acetone was removed, the tubes containing the pellets were left open to allow the histone extracts to desiccate for 2-minutes.

SDS-Polyacrylimide Gel Electrophoresis

Laemmli Sample buffer (80 μl) was added to each tube of histone extract and small holes were made in the caps of the tubes using a PrecisionGlide Needle (18G1 ½) The tubes

were allowed to boil with the caps up for approximately 5 minutes to allow the histones to dissolve into sample buffer.

A pre-made sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) BIO-RAD Mini PROTEAN TGX Precast Gel kit was assembled and the mini Protean Tetra Cell was filled with SDS-PAGE buffer. Equal amounts of protein extracts (15-20 μl) were loaded into each lane to ensure that the histones were equally loaded in each lane. SDS-PAGE ladder was loaded into the first lane and Laemmlie Sample buffer was loaded into the last lane as the control. The gel was electrophoresed at 150V for 45minute to 1 hour.

The SDS-polyacrylimide gel was removed from the cassette and placed into Transfer Buffer (Tris-Glycine Electrophoresis Buffer: 25 mM Tris, 250 mM glycine (electrophoresis grade pH 8.3), 0.1% (w/v) SDS) for 3-5 minutes. Using the Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell, histones were transferred to a NitroBind Pure Nitrocellulose 0.45 micron membrane.

A 0.5% Ponceau S stain was used to quantify the amount of total protein loaded. Between 2-4 washes with de-ionized water were performed to remove the excess Ponceau S stain. The membrane was then suspended in Tris-Buffered Saline (TBS: 0.9% NaCl in 100mM Tris, pH=7.5), covered with plastic wrap and stored in the refrigerator.

Histone Immunoblot

Membranes containing histones were incubated in a 5% milk powder in TBS solution on a shaker for 30 minutes. The milk powder-TBS solution was poured off and the membrane was washed twice with TBS for 5 minutes on the shaker. The membrane was then re-suspended in a 1% BSA dissolve in TBS solution with the primary antibody (Table 2) and incubated in the cold room (4^{o}), on the shaker for 12-14 hours. The primary

antibody solution was removed and the membrane was washed twice with TBS for 5 minutes on the shaker. The membrane was then incubated in a 1%BSA-TBS solution with the secondary antibody (Table 2) for 1 hour and washed 3 times with TBS for 5 minutes on a shaker. A SuperSignal West Dura Trial Kit and autoradiography film were used to detect and develop images of the secondary antibodies. Length of exposure ranged between 1 and 120 seconds.

Table 2: Dilutions of anti-bodies with 1% BSA-TBS are listed in each specific experiment below.

Experiment	**1° Antibody**	**1° Dilution**	**2° Antibody**	**2° Dilution**
Acetylation on H3K27	α-H3K27Ac	1:2000	Goat-α-rabbit	1:3000
Methylation on H3K27	α-H3K27Me3	1:2000	Goat-α-rabbit	1:3000
Histone H3	α-H3	1:2000	Goat-α-rabbit	1:3000

Results

Chromatin deformations in absence of Thd2

An effective method for examining the role of Thd2 in chromatin formation and maturation is by observing the micronuclear morphology in complete vegetatively grown Δthd2 mutants. In a previous experiment by Smith et al., complete Δthd2 mutant cells of *Tetrahymena thermophila* were engineered through the transformation of a genetically modified plasmid replacing Thd2 with a gene coding for paromomycin resistance into wild type cells.[18] Subsequent immunofluorescence procedures using an antibody for micronuclear linker histone-1 (Mlh1), a protein only found to localize in the micronucleus of *T. thermophila*, revealed that the micronuclear chromatin of mutant cells displayed elongated phenotypes. These results were indicative of a role for Thd2 in the maintenance of chromatin structure. Though elongated micronuclei are typical in *T. thermophila* during anaphase of cell division, Δthd2 mutant micronuclei displayed approximately 10 times as many elongated phenotypes as in wild type cells and several micronuclei were located in close proximity of the macronucleus, an atypical characteristic of anaphase micronuclei.[18]

To test whether Δthd2 mutant cells would display a greater number of elongated phenotypes than wild type cells following ~200 cell divisions after the initial published experiments, DAPI staining and immunofluorescence with antibody for micronuclear linker histone-1 was performed on both normal vegetative and starved cells. An antibody for micronuclear linker histone-1 binds specifically to the linker DNA between nucleosomes in the micronucleus allowing illumination of the outline of the micronuclear DNA (Figure 6). Micronuclei were examined by fluorescence microscopy and the observed phenotypes were recorded. Observed micronuclei were recorded from cells within randomly selected

fields as to provide unbiased collection of phenotypes. An unpaired t-test was performed to determine significance. The level of significance was measured at $p<0.05$.

Several micronuclear phenotypes were observed during this experiment. Both wild type and Δthd2 mutant cells displayed normal, elongated and enlarged phenotypes (Figure 6). Furthermore, several Δthd2 mutant micronuclei displayed variable sizes in comparison to the wild type cells. Overall, there was a significantly greater percentage of abnormal micronuclei in Δthd2 mutant cells in comparison to wild type cells ($p = 0.0229515$; Figure 7). More specifically, Δthd2 mutant cells demonstrated a significantly greater percentage of elongated phenotypes in comparison to wild type cells ($p = 0.039364$; Figure 7). However, there was not a significant difference in the number of enlarged phenotypes between Δthd2 mutant and wild type cells ($p = 0.079966$; Figure 7).

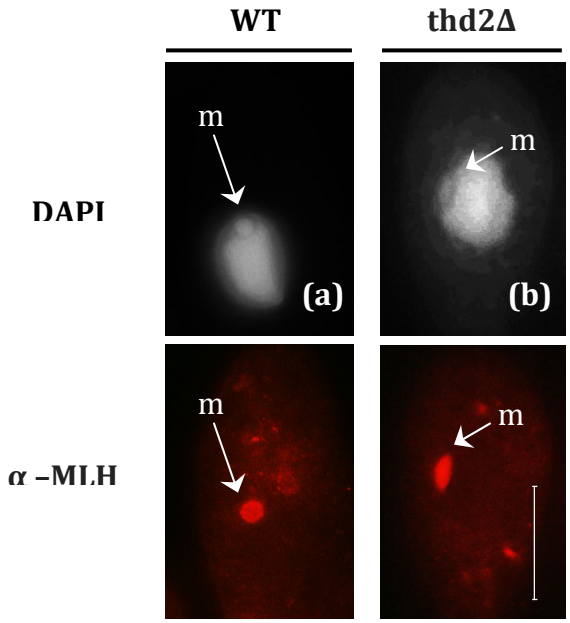

Figure 6. **Analysis of micronuclear chromatin phenotypes in the absence of Thd2.** Immunofluorescence and DAPI staining were performed on vegetative wild type (n = 195) and Δthd2 mutant (n = 175) cells using α-MLH antiserum to observe morphology of micronuclear chromatin in absence of Thd2. Images a & b display the normal (WT) and elongated phenotypes respectively. Refer to Figure 9 for image of enlarged micronuclei. Scale bar represents 10μm.

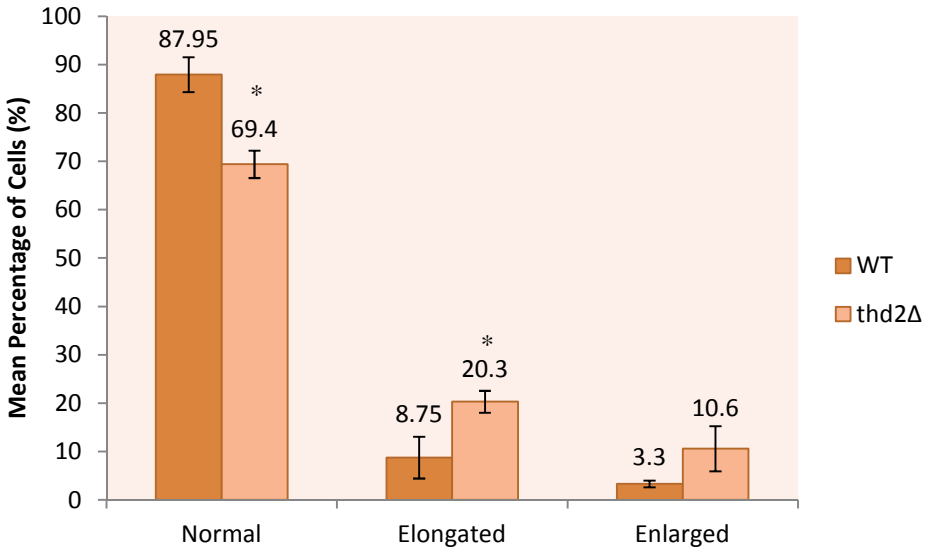

Figure 7. Observed micronuclear chromatin phenotypes in vegetative wild type (n = 195) and Δthd2 mutant (n = 175) cells.

Cell synchronization is beneficial to examining whether abnormal phenotypes appear at certain stages within the cell cycle. [25] As previously mentioned, elongated micronuclei are typical during anaphase of the cell cycle but, the close proximity of micronuclei to macronuclei is atypical at this stage. In order to test whether observed elongated phenotypes were the result of cell cycle arrest in anaphase or due to a decrease in chromatin maintenance by Thd2, wild type and Δthd2 mutant cells were synchronized using starvation methods. Immunofluorescence and DAPI staining using an antibody for micronuclear linker histone-1 were performed and cells were viewed by fluorescent microscopy. Phenotypes of observed micronuclei were recorded from cells within randomly selected fields as to provide unbiased collection of phenotypes. If Δthd2 mutant cells were halting in anaphase, we would expect to see elongated micronuclei located farther from macronucleus.

The results showed that starved Δthd2 mutant cells displayed a greater percentage of total abnormal micronuclei than in starved wild type cells (Figure 8 & 9). More specifically, there were 2 times as many enlarged micronuclei and approximately 5 times as many elongated micronuclei in Δthd2 mutant cells compared to wild type cells (Figure 8 & 9). The majority of elongated micronuclei were again located closely to the macronuclei indicating that they are not halting in anaphase. Furthermore, starved Δthd2 mutant cells displayed about 2 times as many elongated micronuclei compared to vegetative mutant cells (Figure 7 & 9). This experiment was only performed once and therefore must be repeated in order to validate these findings.

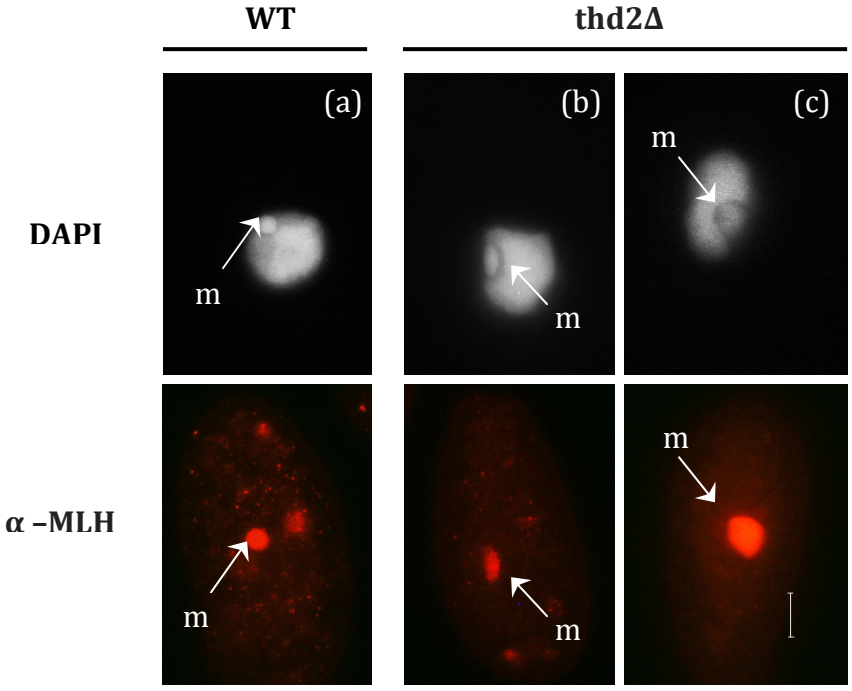

Figure 8. Analysis of micronuclear chromatin phenotypes of synchronized cells in the absence of Thd2. Immunofluorescence and DAPI staining were performed on starved wild type (n = 101) and Δthd2 mutant (n = 105) cells using α-MLH antiserum to observe morphology of micronuclear chromatin in absence of Thd2. Scale bar represents 10μm.

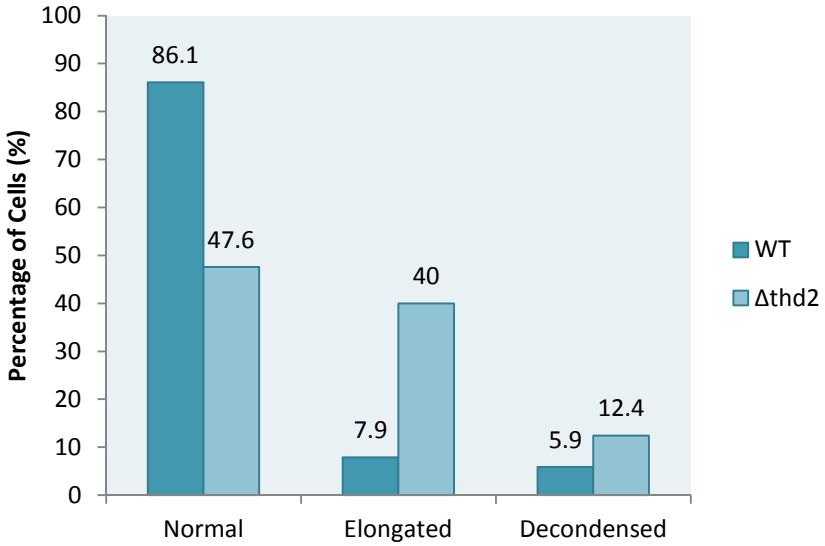

Figure 9. Distribution of observed micronuclear chromatin phenotypes in starved wild type (n = 101) and Δthd2 mutant (n = 105) cells.

THD2 is not responsible for deacetylation at H3K27

Tri-Methylation on histone H3 at lysine residue 27 (H3K27) is known to be a marker for heterochromatin in several eukaryotes including *Tetrahymena thermophila*.[26, 27] Previous studies have demonstrated that Thd2 is responsible for deacetylating newly synthesized histone H3 at K9 and/or K14 but, it is uncertain whether this enzyme plays a role in the deacetylation of H3K27.[18] As the only known histone deacetylase to localize to the normally hypoacetylated micronucleus, I sought to test whether Thd2 is responsible for deacetylation specifically at H3K27 prior to tri-methylation by histone methyltransferases (HMT).

DAPI staining and immunofluorescence using an antibody for tri-methylation at H3K27 was performed on both wild type and Δthd2 mutant cells to test whether deacetylation by Thd2 at H3K27 is required for further tri-methylation at this residue. If deacetylation by Thd2 is an essential mechanism that facilitates the tri-methylation of

H3K27, we would expect not to see no signal for H3K27 tri-methylation in Δthd2 mutant cells.

The results were not supportive of the hypothesis and showed that despite the absence of Thd2, tri-methylation was detected at H3K27 in Δthd2 mutant cells (Figure 10).

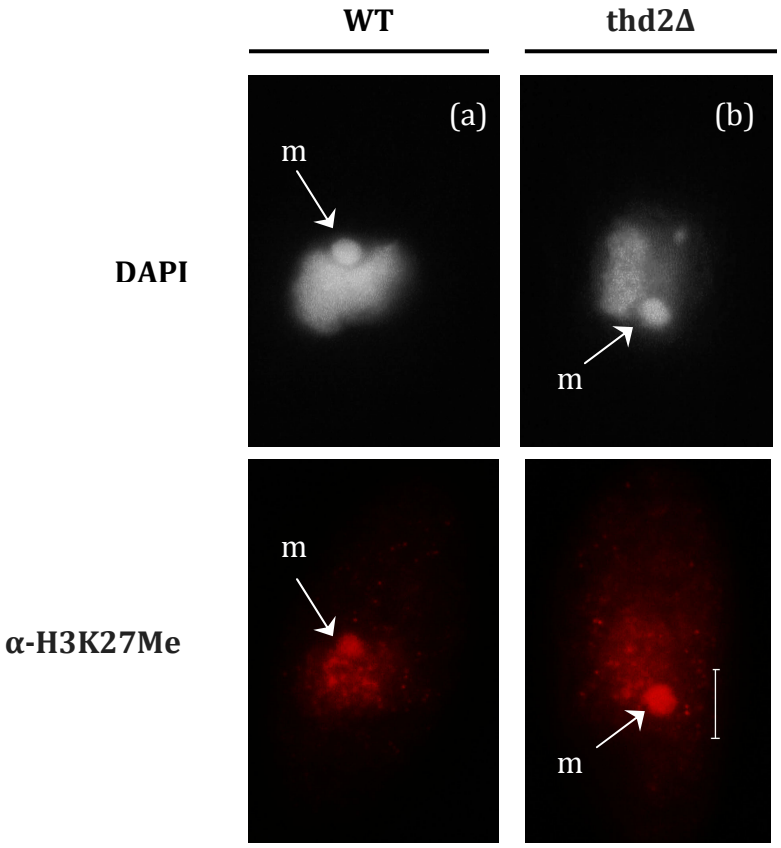

Figure 10. Analysis of methylation markers at H3K27 in Δthd2 mutants.
Immunofluorescence and DAPI staining were performed on vegetative wild type and Δthd2 mutant cells using α-H3K27Me antiserum to observe relative amount of methylation on histone H3 lysine residue in absence of Thd2. Scale bar represents 10μm.

Decrease in H3K27 acetylation in the absence of Thd2

In order to obtain a more quantitative analysis of the amount of methylation and acetylation at H3K27 in complete Δthd2 mutant cells, western immunoblot analyses were performed using antiserums for H3K27 acetylation and methylation. Similar to the

previous hypothesis, if Thd2 is responsible for deacetylating at H3K27 and this process is required for further tri-methylation, we would expect to see a signal for acetylation but, no signal for tri-methylation at H3K27.

The results demonstrated that the level of tri-methylation was conserved between wild type and Δthd2 mutant cells (Figure 12). In contrast, there was a decrease in the level of acetylation in Δthd2 mutant cells compared to wild type cells (Figure 12). It also must be noted that there was a greater amount of H3 protein loaded for mutant cells compared to wild type cells (Figure 11 & 12).

In support of previous findings, antibodies for H3K27 acetylation and histone H3 revealed bands representing $H3_f$, the proteolytically cleaved and faster migrating form of histone H3. [18] These bands were found just below histone H3, also known as $H3_s$, but, were not found in Δthd2 mutant cells or when probed with an antibody for H3K27 methylation (Figure 12).

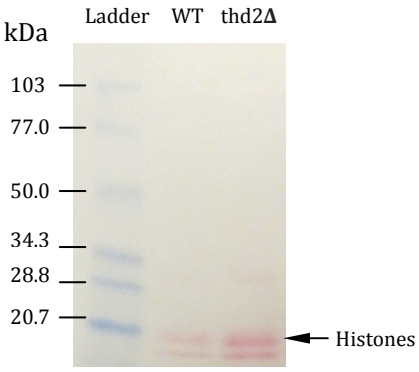

Figure 11. Ponceau S staining of relative amount of protein loaded. Slightly more mutant (thd2Δ) protein loaded than wild type (WT).

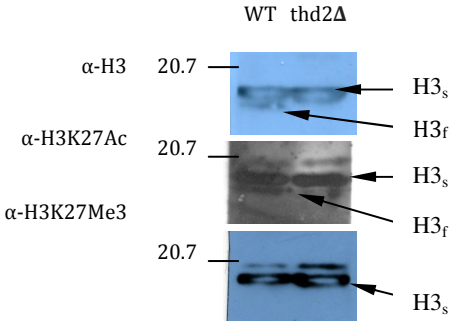

Figure 12. Western blot analysis of H3K27 modifications in thd2Δ cells. Antiserum α-H3 was used to observe and quantify the relative amount of histone H3 (top) loaded for both wild type and mutant cells (thd2Δ). Antiserums α-H3K27Ac and α-H3K27Me3 respectively, were used to quantify the relative amounts of acetylation (middle) and tri-methylation (bottom) at histone H3 lysine 27 in the absence of Thd2.

Discussion

The acetylation of newly synthesized core histones and their subsequent deacetylation after nucleosome deposition is a process that is conserved among all organisms. However, the purpose behind this dynamic process and its relation to further chromatin maturation after DNA replication is not completely understood. In this study, wild type and Δthd2 mutant cells from *Tetrahymena thermophila* were utilized in immunofluorescence and immunoblot experiments to examine the possible involvement of Thd2 deacetylation activity in micronuclear chromatin structure as well as to test for the possible antagonistic roles of acetylation and methylation in chromatin maturation and/or maintenance after DNA replication.

Consistent with previous studies by Smith et al., vegetative Δthd2 mutant cells displayed defective micronuclear phenotypes. The presence of elongated and enlarged micronuclei in Δthd2 mutant cells demonstrates that these phenotypes have persisted through generations of cell divisions following the original experiment.[18] Additionally, these phenotypes suggest a role for Thd2 in the maintenance of chromatin structure after DNA replication. Given the data from previous experiments, perhaps these results are an indication that Thd2 deacetylation activity is a mechanism that influences chromatin condensation by regulating the incorporation of histone H1 into newly synthesized chromatin (i.e. H1-mediated nucleosome condensation into 30 nm fiber).[19,20] Future projects should include investigation into the relationship between histone deacetylation and H1–linker DNA interactions.

Elongation of micronuclei is a typical characteristic in anaphase of the cell cycle but, the close proximity of elongated micronuclei to the macronucleus is atypical. Starved

mutant cells also displayed elongated and enlarged micronuclei and the vast majority of these abnormal micronuclei were located in close proximity to the macronucleus. Previous research has indicated that the doubling time of Δthd2 mutant cells is approximately 3 times as long as the doubling time of wild type cells. This could indicate that Δthd2 mutant cells take longer to perform cytokinesis and thus remain in anaphase (elongated micronuclei) for an extended period of time but, given the fact that the cell cycle was not closely monitored in this experiment, it is difficult to determine whether elongated nuclei were the result of cell cycle arrest in anaphase. This experiment was only performed once and therefore, should be repeated several more times using synchronized cells in order to validate the these findings revealed in this study.

Immunofluorescence and immunoblot experiments revealed that in the absence of Thd2, Δthd2 mutant cells retained methylation at H3K27. These findings demonstrate that Thd2 is most likely not involved in the specific deacetylation of H3K27 and that this proposed mechanism is not required for further tri-methylation at this site. Interestingly, immunoblot analyses demonstrated that there was a decrease in H3K27 acetylation in Δthd2 mutant cells. Future experiments should include a co-immunoprecipitation in order to investigate whether Thd2 co-localizes to the micronucleus in a complex of proteins. This may reveal whether another enzyme is responsible for deacetylation of H3K27 during DNA replication in the absence of Thd2.

Due to the difficulty of separating macro- and micronuclei, both nuclei types were loaded together in the western blot. This limits the experiment in that we are unable to distinguish H3K27Me signal in micronuclei from the signal originating from the macronuclei. Future experiments should include complete isolation of micronuclei from

macro nuclei prior to Western blot experiments to allow for a more accurate quantification of relative H3K27 methylation and acetylation in the micronuclei of Δthd2 mutant cells.

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