Recruitment mechanism of Histone Deacetylase 3 to replication sites and role for histone deacetylases in DNA damage response

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Abstract

Histone Deacetylases (HDACs) are enzymes that modulate chromatin structure, mediate transcriptional repression and contribute to DNA damage repair. HDAC inhibitor compounds show promise as chemotherapeutic cancer treatments through disruption of chromatin structure and interference with cancer cell replication mechanisms.

HDACs are divided into four classes according to function, location and expression patterns. Class I HDACs include HDACs 1, 2 and 3, and are recruited to DNA replication sites through chromatin-associated proteins. HDACs 1 and 2 are recruited to DNA via the Chromatin Associated Factor 1 (CAF-1) complex, but the recruitment mechanism of HDAC3 is not well understood. Co-immunoprecipitation experiments found that unlike HDACs 1 and 2, HDAC3 is not associated with CAF-1. Instead, HDAC3 interacts with RbAp48 and msin3a, transcriptional corepressor proteins.

Previous research has confirmed the role of HDACs in DNA damage repair. Inhibition of HDAC function induces chromatin defects, indicating disrupted DNA repair pathways. Immunofluorescent analysis in HDAC-inhibited and control cells was performed to examine the role of HDACs in damage repair. Cells with inhibited HDAC activity displayed a significant increase in gamma-H2AX foci, an indicator of double-strand breaks. When DNA damage was induced, HDAC-inhibited cells also exhibited increased CAF-1 foci, indicators of the CAF-1 protein complex. A more thorough understanding of both HDAC recruitment mechanisms and role in DNA damage repair could lead to better, more targeted cancer chemotherapies.

Introduction

A. Histone function and chromatin structure

The primary function of chromatin, the molecular complex containing both genomic DNA and structural proteins, is to regulate the packaging and expression of genetic information in the eukaryotic cell. The basic structural unit of chromatin is the nucleosome, consisting of about 147 DNA base pairs wrapped as a left-handed superhelix around a histone octamer (Figure A). Two copies of the histone units H2A, H2B, H3 and H4 form the octamers, which provide the necessary electrostatic interactions to facilitate DNA coiling (Annunziato, 2008). The addition of the H1 histone unit allows multiple histones to form into a 30-nm diameter fiber capable of unwinding for transcriptional purposes. Further DNA packaging occurs via domains, loops, and protein scaffolds, possibly mediated by H1 phosphorylation; however, this higher-level structure is not well understood. Through this complex architecture, an estimated 2 m of linear DNA is folded into a 10-50 μM diameter cell (Annuziato, 2008).

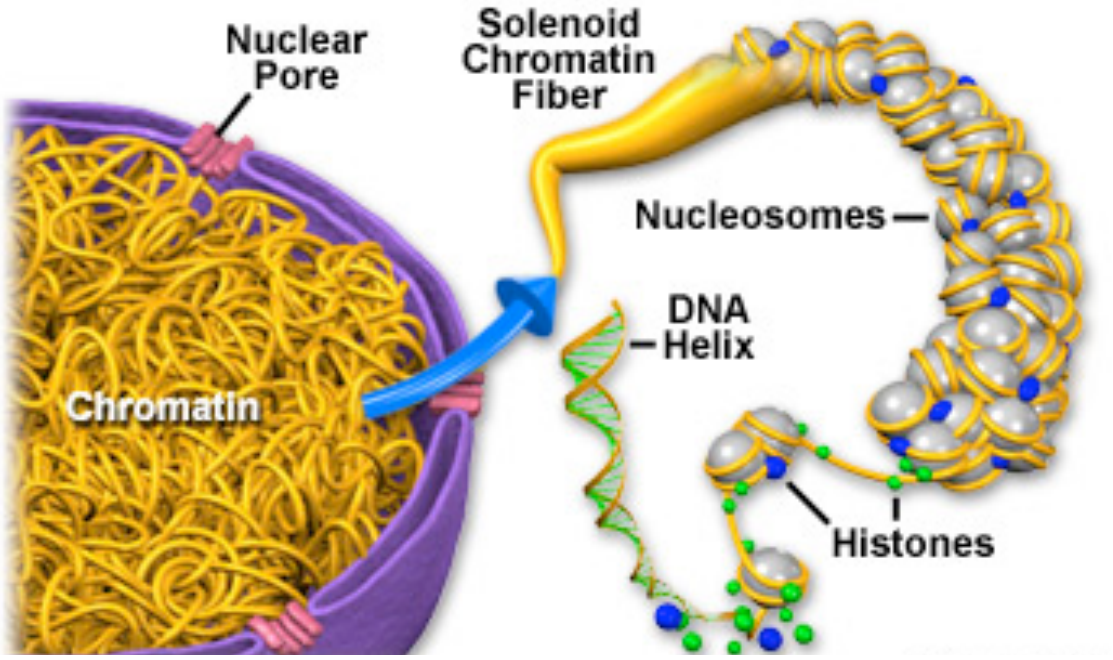

Figure A. Chromatin is the molecular complex that facilitates DNA packaging within the nucleus of a cell. Nucleosomes are the basic structures of chromatin, and are comprised of tightly wrapped histone protein octamers (Davidson, 2005).

Recent research has suggested a fractal model for DNA packaging. Fractal structures, characterized by the application of a power or scaling law, are self-similar and possess scaling independence. Moreno et al. proposed a multifractal map of the genome, classifying chromosomal fragments into low, medium and high multifractal units according to *Alu* (a Short Interspersed Element) content (Moreno et al., 2011). These units form an overall genomic structure far from equilibrium, and this non-linear classification holds promise for investigation of structural defects in the human genome.

During metaphase, chromatin forms into chromosomes, rigid higher-level coiled structures, to prevent DNA shearing and damage throughout replication. After division, chromosomes relax into less rigid chromatin fibers. Before

transcription, the coiled DNA must partially unwind to allow polymerases and transcription enzymes access to base pairs. This is accomplished through two pathways: modification of histones by the addition and removal of functional groups, and histone displacement by chromatin remodeling complexes (Annunziato, 2008).

B. Histone modification and histone deacetylases

Histone acetylation provides one pathway through which chromatin is modified for transcription. An addition of an acetyl group on the lysine residue at the N-terminus of a histone neutralizes the positive charge, therefore decreasing the histone's affinity for negatively charged DNA (Struhl, 1998). Conversely, the removal of this acetyl group increases binding affinity for DNA. This process plays a central role in gene regulation; acetylation allows for permissive chromatin and is conducive to transcription, whereas deacetylation favors repressive chromatin and denies polymerase access to genes. Acetylated lysine residues permit the binding of ATP-dependent chromatin remodeling complexes and the opening of promoters (Eberharter and Becker, 2002).

Histone deacetylase (HDAC) molecules catalyze the hydrolysis and removal of acetyl groups from lysine residues. Catalysis occurs by the polarization of substrate carbonyl groups by the Zn^{2+} ion and subsequent nucleophilic attack by a water molecule (Lombardi, 2011). Mammalian genomes contain 18 separate deacetylase molecules with highly conserved deacetylase domains. These are separated into Class I, IIa, IIb and IV according to function, localization, and expression patterns (Haberland et al., 2009). Research has shown that HDAC

function is crucial to the maintenance of genome stability and cell cycle progression. Significant loss of condensed nuclear heterochromatin is observed when HDAC function is inhibited, and cell cycle arrest results (Bhaskara et al., 2008).

C. Class I HDACs and HDAC 3 function

Class I HDACs, homologous to the yeast RPD3 histone deacetylase, include HDACs 1,2,3 and 8 which deacetylate residues both *in vitro* and *in vivo* (Bernstein et al., 2000). These enzymes are primarily localized to the nucleus and are expressed ubiquitously in cells. HDACs 1 and 2 are very similar and exist in transcriptional repression complexes, whereas HDAC3 exists in separate complexes. The protein complex of HDAC 8 is still unknown. However, deletion of any single Class I HDAC causes lethality in mice, suggesting that each performs a unique and crucial function in cells (Haberland et al., 2009).

Research suggests that HDAC3 plays roles in cell cycle progression, genome stability and DNA damage repair. When an inducible Cre-loxP system was used to delete HDAC3 function specifically in mouse embryonic fibroblast (MEF) cells, cell cycle progression was delayed and apoptosis was induced. DNA damage, correlative with inefficient DNA damage repair mechanisms, was observed prior to apoptosis (Bhaskara and Hiebert, 2011). Further experiments showed a decrease in the efficiency of spontaneous non-homologous end-joining (NHEJ) and double-strand break (DSB) repair with HDAC3 inhibition, suggesting HDAC3 involvement in both damage repair pathways. Additionally, metaphase spread analysis revealed increased chromosomal defects in dividing HDAC3-null cells following non-lethal IR

treatment (Bhaskara et al., 2010). A combination of conventional and immunoaffinity chromatography experiments have shown that HDAC3 forms a stable complex with the corepressors NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptors). Furthermore, co-immunoprecipitation using HDAC3-specific antibodies confirms association with both NCoR and SMRT (Li et al., 2001). These proteins provide a molecular base for various transcriptional repression complexes to target HDACs to gene promoters and enhancers.

D. HDAC inhibition and cancer

Recently, histone deacetylase inhibitors (HDACis) have emerged as a promising new chemotherapeutic option for cancer treatment. Cancer cells often upregulate the recruitment of HDACs to promoters of growth-regulating genes, preventing transcriptional complexes from accessing DNA and repressing transcription. Specific repression of growth-regulating genes allows the characteristic unchecked replication of cancer cells. For example, overexpression of the B-cell lymphoma 6 (BCL6) growth factor in lymphoma cells causes the recruitment of HDAC2 to repress CDKN1A, the gene encoding the cyclin-dependent kinase inhibitor p21 that mediates cell cycle arrest in healthy cells in response to stresses such as DNA damage (Pasqualucci et al., 2003).

Specifically, HDAC3 lies within a region of chromosome 5q31.3 that is often deleted in breast cancer (Johansdottir et al., 2006). Furthermore, the gene for NCoR, which associates with HDAC3, is located in a region of chromosome 17 that is

frequently deleted in hepatocellular carcinomas (Ebert, 2009). This correlates with the hypothesis that HDAC inhibition leads to increased histone acetylation, DNA damage and subsequent accumulation of mutations in cancer cells.

HDACis enter all cells, but specifically induce cell differentiation and/or apoptosis in cancer cells (Bolden et al., 2006). As of 2012, 11 different HDACis were in clinical trials, but only two (Vorinostat and Robidepsin) were FDA-approved. Research has suggested that HDACis selectively induce apoptosis in tumor cells; one study observed a tenfold increase in sensitivity to HDACis in tumor cells relative to healthy cells (Dokmanovic and Marks, 2005). This selectivity may be due to the relative scarcity of HDAC-regulated genes, and the fact that most of them are involved in growth and cell cycle regulation (Wagner et al., 2010). For example, the retinoblastoma (Rb) repressor complex recruits HDACs to genes driving the progression of the cell cycle. In proliferating cells, such as dividing cancer cells, HDAC1 is stably bound to the Rb-E2F transcription factor throughout G1 and released at the G1-S phase transition (Ferreira et al., 2001). Disruption of similar processes with HDAC inhibition therapy could arrest the growth of cancer cells.

HDACis can function through the extrinsic and intrinsic apoptosis pathways. The extrinsic 'death-receptor' pathway recruits ligands to apoptosis-inducing receptors and activates caspases 8 and 10. The intrinsic mitochondrial pathway involves the release of cytochrome c to initiate the formation of apoptosomes and the activation of caspase 9 (Bolden et al., 2006).

Research suggests that HDACs are molecularly inhibited through the establishment of stable chelate rings with the HDAC Zn^{2+} ion (Lombardi et al., 2011).

These chelate ring structures imitate the transition state of HDAC enzymatic activity and therefore prevent the successful deacetylation of residues. Density functional theory calculations predict the formation of these hyperstable structures within the enzymes' active sites, but X-ray crystallography has yet to confirm this hypothesis (Botta et al., 2011).

More research into the mechanisms of HDAC structure will allow us to create more specialized and effective HDACis. Additionally, investigation of HDAC association and molecular complexes may provide further targets for cancer-fighting therapies. Because of their differences in function and molecular interactions, it is important to examine each HDAC individually to best tailor inhibitors for targeted cancer therapy.

E. Research questions

HDACs cannot directly bind to DNA and require interaction with protein partners such as transcriptional corepressors (Haberland et al., 2009). HDACs 1 and 2 associate with CAF-1 and are recruited to histones via interaction with the p48 and p150 subunits of this complex (Hoek et al., 2011). However, the recruitment mechanism of HDAC3 is still unknown.

I hypothesized that HDAC3 binds to chromatin through known HDAC-associated complexes such as CAF-1, NCoR, RbAp48, and sin3a proteins. To investigate the recruitment complex, I performed co-immunoprecipitation experiments followed by Western blot analysis. To explore the possibility of

transient interactions, I crosslinked proteins before immunoprecipitation and then compared the strength of the interactions with previous experiments.

HDACs 1 and 2 have been identified as key players in DNA damage repair pathways. Research suggests that HDACs are recruited to DSB sites to facilitate hypoacetylation of the H3K56 residue (Miller et al., 2010). Furthermore, much of HDACi function is dependent on the prevention of effective DNA damage repair in cancer cells. Normally, HDACs modulate signaling cascades involving ATM/ATR kinases and subsequent phosphorylation of gamma-H2AX, an indicator of DSBs. Inhibition of these deacetylases prevents proper restoration of chromatin structure after repair (Rajendran et al., 2011).

However, the specific mechanisms and protein interactions of this involvement, as well as the recruitment complexes at damage sites, are not well understood. To study this, I performed immunofluorescent staining and examined damage-associated proteins with and without HDAC inhibition. After linking HDAC inhibition with repair defects, I then aimed to investigate the proteins associated with this pathway.

Materials and Methods

A. Cell lines

Experiments were performed using the 293 human embryonic kidney (HEK) cell line and two versions of the 3T3 mouse embryonic fibroblast (MEF) cells. In one 3T3 line, the p150 subunit of the CAF-1 complex was tagged with the FLAG molecule to facilitate antibody interaction.

B. Co-immunoprecipitation protocol

Cells were removed from the incubator and washed three times with cold PBS. Cells were then centrifuged, resuspended and incubated for 8 min in 500 mL Buffer A solution with protease inhibitor and 20% Triton-X. After incubation, cells were resuspended in HERR buffer [KCl, 20 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 0.1% NP40, 10% glycerol] and sonicated for three one-second bursts. Depending on the desired stringency of protein interaction, either 50 mM KCl or 150 mM HERR buffer was used. Lysate was then centrifuged and supernatant was collected in a separate Eppendorf tube.

Protein A or Protein G agarose beads (for primary rabbit or mouse antibodies, respectively) were washed three times with HERR buffer. Beads and protease inhibitor were added to cell lysate and incubated on a rotator at 4°C for one hour to pre-clear solution.

After incubation, the sample was centrifuged and supernatant was collected into a new Eppendorf tube. 40 μL of sample was removed, mixed with sample buffer

and beta-mercaptoethanol, and stored at -20°C as input for gel electrophoresis. The remaining sample was divided into 2 tubes. Antibody was added to one sample and incubated on rotator for 3-6 h, depending on antibody. IgG was added to the second sample as a control. After incubation, sample buffer and beta-mercaptoethanol were added to the sample; tubes were stored at -20°C.

Before gel electrophoresis, samples were thawed at room temperature and heated at 95°C for 5 min, then centrifuged at 3000 rpm at room temperature. 6%, 10% or 13% polyacrylamide gels were prepared, according to size of protein studied. Protein ladder, IgG and antibody input, and IgG and antibody sample were loaded into the wells. Gels were run for 2 h at 100 V.

A Western blot was performed to transfer proteins to PVDF paper. Transfer was run at 300 mA for 2.5 h. After transfer, membranes were blocked in 5% milk-PBS solution for 1 h. Primary antibody (see dilutions below) was added to 1% milk in PBS, applied to membrane and incubated for 3 h. Membranes were then washed in 1X PBS-Tween-20 solution 3-10 times, depending on primary antibody. After washes, secondary antibody was added to 2% milk in PBS, applied to membrane and incubated for 1 h. Membranes were then washed 3-10 times in 1X PBS-Tween-20.

Pierce ECL Western blotting substrate was then applied to membranes and incubated for 5 min. Membranes were then exposed to X-ray film for 10 sec – 10 min, depending on strength of signal, and then developed.

Antibody			
αFLAG	**Mouse**	**ab49763**	**1:500**
αmsin3a	**Rabbit**	**Don Ayer Lab**	**1:1000**
αNCOR	**Rabbit**	**ab58396**	**1:500**
αRbAp48	**Rabbit**	**ab1765**	**1:500**
αHdac3	**Rabbit**	**ab7030**	**1:500**
αp150/CAF-1	**Mouse**	**ab7655**	**1:500**

Table 1. Antibodies used in co-immunoprecipitation protocol.

C. Immunofluorescence protocol

Cells were removed from incubator and washed in cold PBS three times, then resuspended in 500 µL PBS. Cells were placed onto slides using a cytospin machine for 5 min. Slides were placed in a 1:1 methanol-acetone solution at 4°C for 20 min to fix and permeablize cells. A blocking solution of 10% normal goat serum (NGS) in PBS was added to slides and incubated for 30 min. Primary antibody was added to 10% NGS solution and incubated for 1 h at room temperature. Antibody was then aspirated and slides were washed three times with PBS. A 1:600 dilution of secondary fluorescent antibody in 10% NGS was added to slides and incubated, covered with lightproof foil, for 45 min at room temperature. Slides were then washed three times with PBS. A 1:1000 dilution of Hoescht stain in PBS was added to slides and incubated, covered, for 10 min at room temperature. Slides were then washed three times with PBS and cover slips were mounted using VectaShield.

Primary antibody			
αFLAG	**Mouse**	**ab49763**	**1:2000**
αH2AX	**Mouse**	**ab11174**	**1:5000**
Secondary antibody			
Alexa 488	**Mouse**		**1:600**
Alexa 546	**Rabbit**		**1:600**

Table 2. Antibodies used in immunofluorescence protocol.

Results

To examine the hypothesized protein interaction of HDAC3 with CAF-1, we first performed co-immunoprecipitation (IP) pulldowns using either anti-HDAC3 or anti-FLAG antibodies to immunoprecipitate the p150:FLAG subunit of the CAF-1 complex. For the FLAG-tagged p150 pulldown, Western blot membranes were probed with RbAp48, HDAC3, and FLAG antibodies.

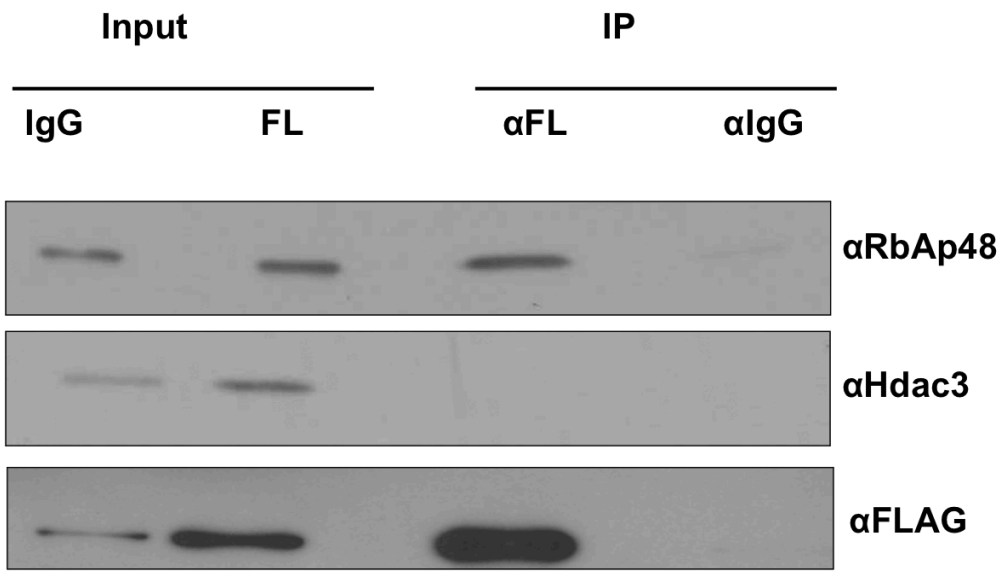

Figure 1. Western blot of co-immunoprecipitation with FLAG in FLAG-tagged p150 3T3 cells. Pre-IP input samples were collected before addition of IgG or FLAG antibodies. Rabbit IgG antibody was added to one IP sample as a control. The blot was probed with anti-RbAp48, anti-HDAC3 and anti-FLAG antibodies.

First, we performed a pulldown of FLAG-tagged p150 protein to examine its interactions. Although HDAC1 and HDAC2 have been shown to associate with the p150 subunit of the CAF-1 complex, we did not find an association between p150 and HDAC3. We confirmed previous research associating RbAp48 and p150 (Ahmad et al. 1999), an important positive control for successful pulldown of p150.

Next, we performed a pulldown of HDAC3 protein to examine its interactions and confirm the previous conclusions. For the HDAC3 pulldown, Western blot membranes were probed with HDAC3, RbAp48, NCoR, and p-150 antibodies.

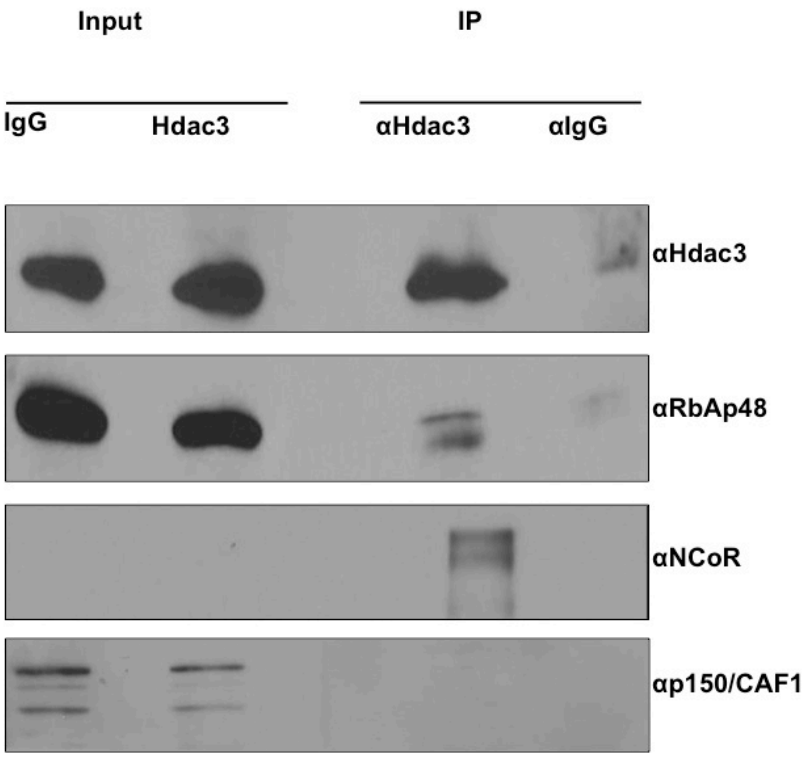

Figure 2. Western blot of co-immunoprecipitation with rabbit HDAC3 and rabbit IgG antibody in 3T3 cells. Pre-IP input samples were collected before addition of HDAC3 or IgG antibodies. Rabbit IgG antibody was added to one IP sample as a control. Blots were probed with anti-HDAC3, anti-RbAp48, anti-NCoR and anti-p150 antibodies.

As in FLAG-tagged p150 3T3 cells, we did not find an association between p150 (CAF-1) and HDAC3, suggesting a different recruitment complex than HDAC1 and HDAC2. We also confirmed previous research associating HDAC3 with NCoR (Codina et al., 2005), a positive control for successful pulldown of HDAC3. Additionally, we found an association of RbAp48 and HDAC3, suggesting an RbAp48-containing complex is involved with recruitment of HDAC3 to replication sites. This led to later experiments to investigate proteins known to interact with RbAp48 (see below).

To further characterize HDAC3 protein interactions, we crosslinked 3T3 cells with formaldehyde for 10 min to stabilize weak or transient interactions and then performed immunoprecipitation with HDAC3 antibody.

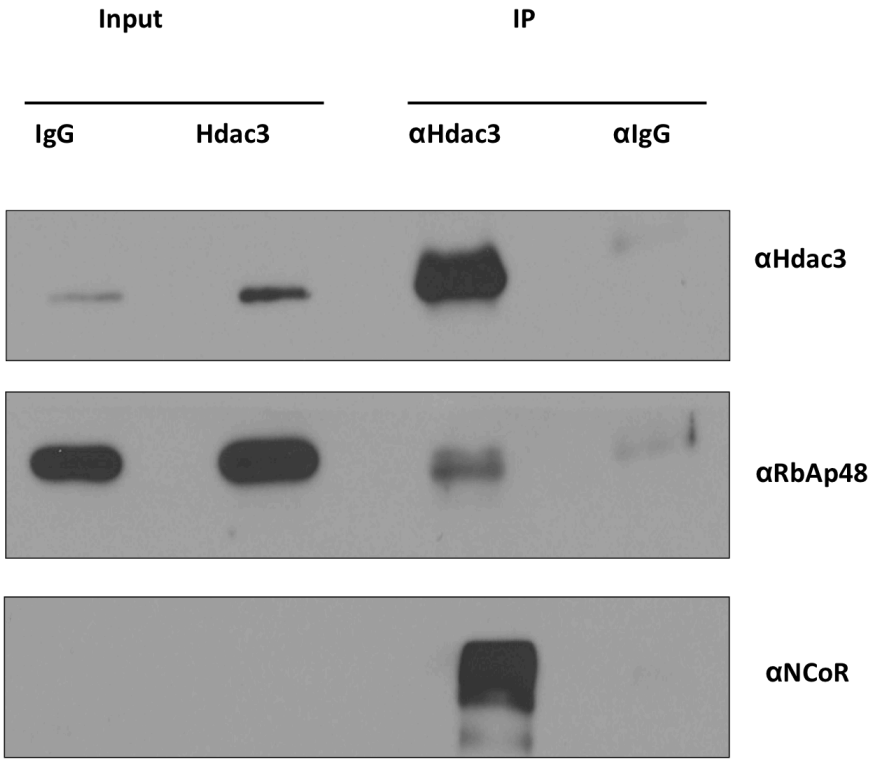

Figure 3. Western blot of co-immunoprecipitation with HDAC3 antibody and IgG antibody in crosslinked 3T3 cells. Crosslinking treatment with formaldehyde stabilizes protein association via primary amino group interactions. Rabbit IgG antibody was added to one IP sample as a control. Blots were probed with anti-HDAC3, anti-RbAp48 and anti-NCoR antibodies.

Like in non-crosslinked cells (Figure 2), we found an association of RbAp48 and HDAC3, as well as NCoR and Hdac3. Darker bands in crosslinked cells, compared to non-crosslinked cells, suggest the interaction between HDAC3 and RbAp48 is transient and can be stabilized by crosslinking with formaldehyde.

RbAp48 is a component of the CAF-1 complex, but can also associate with other proteins. Because we ruled out CAF-1 interaction, we examined the alternative RbAp48-containing complexes. The sin3 family of corepressor proteins, known to interact with HDACs 1 and 2, presented a likely option for association with HDAC3.

Consequently, we investigated possible HDAC3 interactions with both RbAp48 and sin3 proteins. A Western blot of an HDAC3 pulldown was probed with various proteins in the sin3 family and an interaction with msin3a was found.

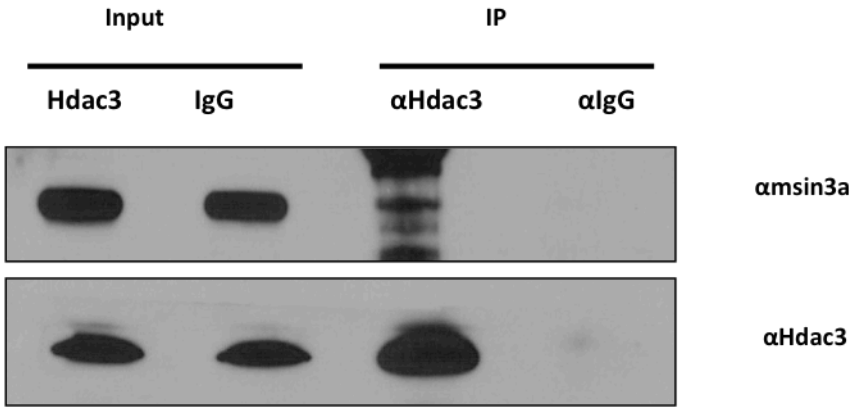

Figure 4. Western blot of co-immunoprecipitation with HDAC3 antibody and IgG antibody in HeLa cells. Rabbit IgG antibody was added to one sample as a control. Blots were probed with anti-msin3a and anti-HDAC3 antibodies.

When a pulldown of HDAC3 was performed, an interaction with the msin3a protein was found. Msin3a, an essential corepressor protein involved in embryonic development, cell cycle regulation and DNA damage repair, has been known to interact with RbAp48 (Ridgway and Almouzni 2000). This provides a plausible mechanism for the association of HDAC3 with RbAp48.

To confirm this association, I then performed a reciprocal immunoprecipitation experiment with msin3a antibody and probed the blot with HDAC3 and msin3a antibodies.

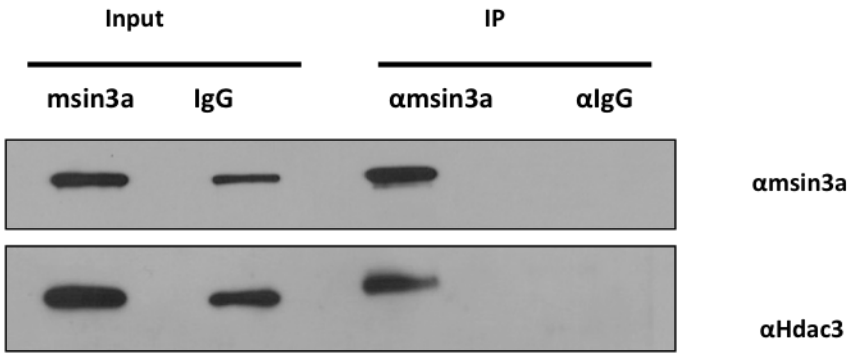

Figure 5. Western blot of co-immunoprecipitation with msin3a antibody and IgG antibody in HeLa cells. Rabbit IgG was added to one IP sample as a control. Msin3a antibody was developed by the Don Ayer Lab at the Huntsman Cancer Institute. Blots were probed with anti-msin3a and anti-HDAC3 antibody.

The interaction between HDAC3 and msin3a was confirmed when we pulled down msin3a from HeLa cell lysate. Probing the blot with msin3a antibody also served as a positive control of successful pulldown of the protein itself.

To examine the effects of HDAC inhibition on DNA damage, we treated 293 HEK cells for 24 h with either the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) or DMSO as a control. Cells were then treated with 5 μg/mL bleocin, an intercalating agent, for 24 h to induce DNA damage. We stained cells with Hoescht stain to identify nuclei and gamma-H2AX primary antibody to identify and quantify DSB sites within cells.

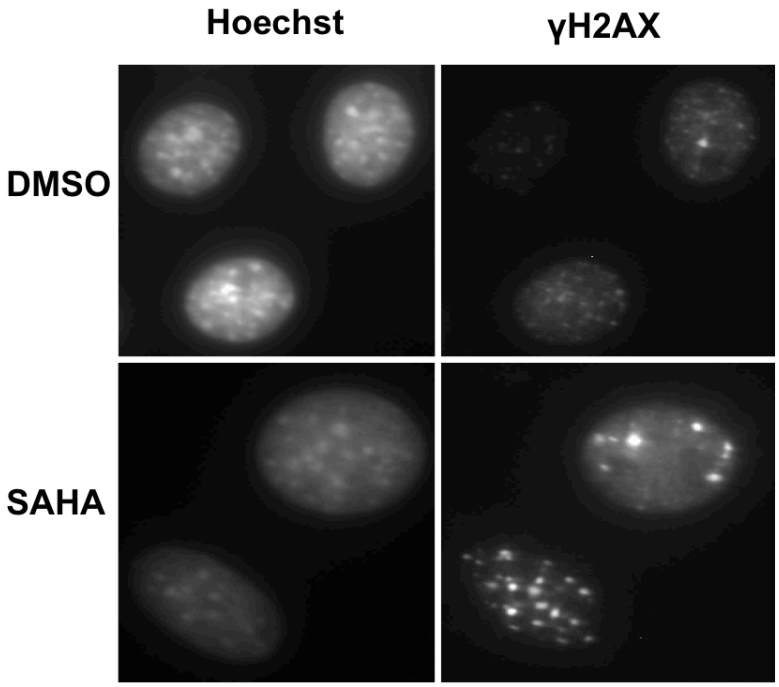

Figure 6. Immunofluorescent analysis of 293 HEK cells stained with Hoescht and gamma-H2AX antibodies after treatment with bleocin and either DMSO or SAHA. DMSO was used as a control. Fluorescent foci were quantified in 20 cells for each treatment.

SAHA-treated cells presented significantly more gamma-H2AX foci than DMSO-treated cells after bleocin-induced DNA damage. This supports the hypothesis that HDACs are involved in DNA damage repair and contribute to repair defects when inhibited.

I then aimed to examine the mechanism through which HDACs are recruited to DNA damage sites. Because CAF-1 has been associated with HDAC recruitment to nascent chromatin sites, I hypothesized that CAF-1 was involved in recruitment to DSB sites. I treated 3T3 cells with bleocin to induce DNA damage, then stained SAHA-treated or DMSO-treated FLAG-tagged 3T3 cells with FLAG antibody and Hoescht stain.

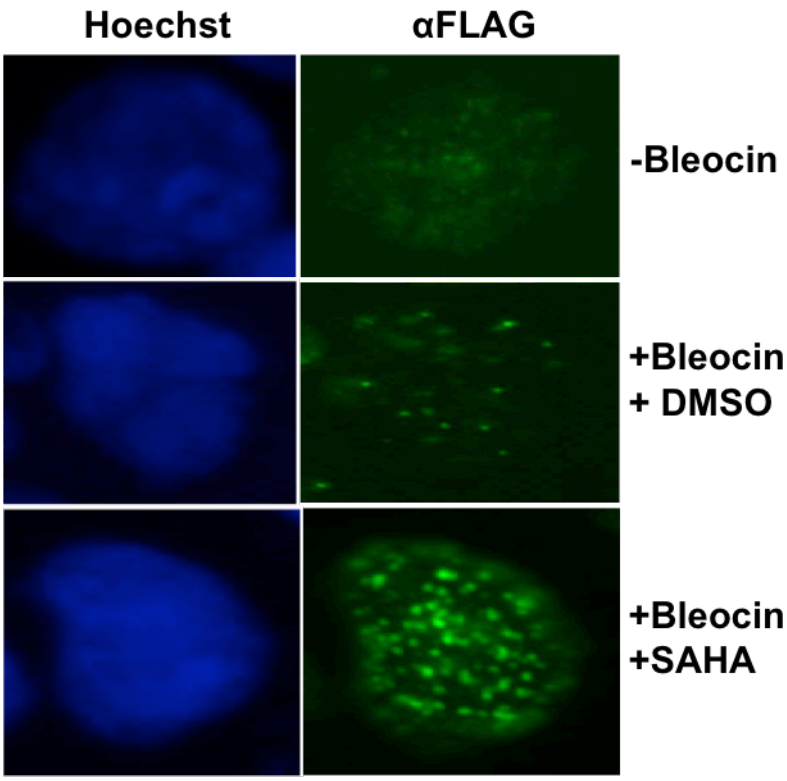

Figure 7. Immunofluorescent analysis of FLAG-tagged p150 3T3 cells treated with and without bleocin; bleocin-treated cells were treated with DMSO or SAHA. Fluorescent foci were quantified in 20 cells for each treatment. The blue Hoechst stain indicates one cell's nucleus, and the corresponding green foci indicate FLAG-tagged p150 foci within that nucleus.

Significantly more p150 foci were observed in bleocin and SAHA-treated cells compared to bleocin and DMSO-treated cells. This suggests that when HDACs are inhibited, more p150 is recruited to DNA damage sites and repair mechanisms are defective. The association between HDAC inhibition and p150 recruitment provides a possible pathway for HDAC involvement in damage repair. However, more experiments are needed to confirm the hypothesized correlation of increased p150 recruitment with defective repair mechanisms.

Discussion

A. HDAC3 does not associate with CAF-1 as a recruitment factor

Since previous research suggests that HDAC1 and HDAC2 are recruited to chromatin via association with CAF-1, I hypothesized that HDAC3 might be recruited by CAF-1 as well. However, my results contradicted that hypothesis. Immunoprecipitation with FLAG in FLAG-tagged p150 cells did not show association with HDAC3 (Figure 1). Conversely, immunoprecipitation with HDAC3 did not show association with p150, even when crosslinked with formaldehyde to detect a weak interaction. This is an interesting result because it contradicts previous assumptions that HDAC3 follows the same recruitment pathway as other Class I HDACs.

B. HDAC3 associates with RbAp48 and msin3a

In my exploration of alternate pathways of HDAC3 recruitment, I found an association with RbAp48 and HDAC3 (Figure 2). Although RbAp48 is a component of the CAF-1 complex and shows interaction with FLAG-tagged p150 (Figure 1), the lack of HDAC3 association with the p150 subunit of CAF-1 led us to conclude that HDAC3 interacts with other RbAp48-containing complexes. Our crosslinking treatment would have shown an interaction between HDAC3 and CAF-1 if HDAC3 was indeed interacting with RbAp48 as part of the CAF-1 complex, but the lack of co-immunoprecipitation allows us to conclude RbAp48 is acting with an alternative complex.

Further immunoprecipitation experiments with HDAC3 showed an association with msin3a (Figure 4), a protein known to interact with RbAp48 (Ridgway and Almouzni, 2000). We confirmed this interaction with immunoprecipitation of msin3a, which showed interaction with HDAC3 (Figure 5). Msin3a is a component of the sin3a family of corepressor proteins, known to interact with other Class I HDACs in transcriptional repression pathways. This interaction suggests a plausible complex for the recruitment of HDAC3 to chromatin. Further experiments are needed to fully identify other possible components of this recruitment complex.

C. HDACs play a role in DNA damage repair

When we compared HDAC-inhibited cells to normal cells via immunofluorescent analysis, a significant increase in the DSB marker gamma-H2AX was observed in HDAC-inhibited cells (Figure 6). This suggests that HDACs play a role in DNA damage pathways and cells are unable to effectively repair chromatin when HDACs are inhibited. The involvement of HDACs in DNA damage repair holds great promise for cancer therapies; if inhibition of HDACs prevents DNA damage repair, this could be harnessed for chemotherapeutic purposes, especially in cancer cells that upregulate their DNA damage response and resist other cancer treatments.

D. p150 recruitment to DSB sites is increased with HDAC inhibition

In an effort to examine the pathways through which HDACs are recruited to DNA damage sites, we first induced DNA damage and then fluorescently labeled the p150 subunit of CAF-1 in HDAC-inhibited and control cells. Significantly more p150 foci were observed in HDAC-inhibited cells (Figure 7). We can infer that less foci means less DNA damage, and the non-SAHA treated cells are more effectively repairing DNA damage. The SAHA-treated cells present more p150 foci, suggesting that the role of HDACs in damage repair is dependent upon p150/CAF-1 interaction.

E. Further directions

More research is needed to effectively identify the recruitment complex of HDAC3. To examine the interaction with msin3a, we could perform starvation-induced cell cycle synchronization. Then, we could co-immunoprecipitate HDAC3 and msin3a at different points in the cell cycle and probe Western blots for interaction of these proteins. Furthermore, we could perform chromatin fractionation to isolate molecules that are bound to chromatin at any point in the cell cycle.

Another approach to identify all co-precipitated proteins is to apply a silver stain to the gel. Each stained band corresponds with a protein, and could be cut out from the gel and identified via MALDI-TOF analysis. Depending on the quality of protein band separation, we could potentially identify every interacting protein through this technique.

Furthermore, there is a possibility that proteins other than msin3a and RbAp48 comprise part of the HDAC3 recruitment complex. To investigate this, further immunoprecipitation analysis could be performed. By pulling down HDAC3, RbAp48, and msin3a, and probing the subsequent Western blots with possible associated proteins, we can form a more complete model of the HDAC3 recruitment complex.

Although we confirmed that HDAC inhibition hampers the DNA damage repair pathway, the role of HDACs is not well understood. Further immunofluorescent staining could be conducted to examine this pathway. To confirm the role of p150/CAF-1 in DSB repair, cells could be stained with both anti-p150 and anti-gamma-H2AX antibodies. If these two molecules showed significant colocalization, this would support our hypothesis that CAF-1 is recruited to DNA damage sites. Furthermore, colocalization experiments could be performed with individual HDAC antibodies and anti-gamma-H2AX antibody. This would identify the specific HDACs that are recruited to damage sites.

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