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Spatial Distribution of Histone Methylation During MHC Class II Expression

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Abstract

We have previously reported that MHC (Major Histocompatibility Complex) class II can be induced by histone deacetylase inhibitors (HDACi) in the absence of CIITA (class II transactivator) (Chou et al., 2005; Magner et al., 2000). Here we characterized the histone modifications associated with the CIITA-dependent (IFN- γ induced) and -independent (HDACi induced) MHC class II expression. We demonstrate that both IFN- γ and HDACi induced MHC class II expression exhibited enhanced histone H3, H4 acetylation and H3K4me3 at the MHC class II promoter while H3K9me3 was decreased. In contrast, high levels of H3K36me3 were detected at exons 3 and 5 but not at the promoter or the locus control region (LCR). Interestingly, high levels of H3K79me2 were only detected at the promoter and exon 3 of the B cell lines while the level remained low and unchanged despite active MHC class II expression induced by either IFN- γ or HDACi treatment. Constitutive expression of the CIITA protein by stable transfection of a CIITA deficient B cell line restored the H3K79me2 to a level comparable to its cell of origin. This data demonstrates that, although regulated by different pathways, both IFN- γ and HDACi treatments resulted in similar patterns of histone modifications and that HDACi induce both histone methylation and acetylation. In addition, the different spatial distribution of the lysine methylation markers along the gene suggests that these modifications play a distinctive role during different phases of the transcription process.

Keywords

CIITA; MHC class II; trichostatin A; histone acetylation; histone methylation

1. Introduction

Covalent modifications (acetylation, methylation, phosphorylation and ubiquitination) of various histone residues have been identified at both the histone tail and the core regions. Combinations of the different modifications may serve as a “histone code” and direct the recruitment of chromatin remodeling factors to promoters and determine the status of transcription (Strahl and Allis, 2000; Turner, 2000). For example, both H3K4me3 and H3K36me3 modifications are associated with genes that are actively expressed (Bannister et al., 2005; Hampsey and Reinberg, 2003; Santos-Rosa et al., 2002). High levels of H3K4me3

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are frequently detected at the promoter and 5' exons of transcribed genes while H3K36me3 is usually located at 3' exons (Bernstein et al., 2002; Morillon et al., 2005). Although histone lysine methylations were initially considered to be modifications associated with permanent silencing of genes, recent data indicated reversibility mediated by histone demethylation. Such observations coincide with findings suggesting that each methylation site is involved in distinctive phases of the transcription process (Hampsey and Reinberg, 2003; Morillon et al., 2005; Morris et al., 2005).

The enzymes involved in histone methylation in yeast are well established and H3K4 histone methyltransferase (HMT) SET1 and the H3K36 HMT SET2 are both recruited to the RNA pol II complex through an interaction with Paf1 (Krogan et al., 2003a; Krogan et al., 2003b). SET1 is associated specifically with the RNA pol II C-terminal domain (CTD) that is phosphorylated at serine 5, a hallmark of initiation (Hampsey and Reinberg, 2003; Ng et al., 2003), while SET2 is linked to the CTD heptapeptide phosphorylated at serine 2 in elongation (Hampsey and Reinberg, 2003; Li et al., 2003). H3K9 methylation, however, is found in locations where gene expression is suppressed and serves as a mark for the recruitment of heterochromatin protein 1 (HP1). Together, H3K9me3 and HP1 facilitate the repressive state of the chromatin which prohibits transcriptional initiation and gene expression (Peters et al., 2003). Unlike the other histone markers, H3K79 is located in the histone core (Luger et al., 1997). Methylation at K79 inhibits suppression mediated by the class III HDAC SIRT1 by limiting its recruitment to the K79 methylated region (Ng et al., 2002; van Leeuwen et al., 2002). Other reports also suggest the involvement of K79 methylation in epigenetic inheritance (Varga-Weisz and Dalgaard, 2002).

Expression of MHC class II is tightly regulated at the transcriptional level. CIITA is particularly important in MHC class II expression since it interacts with and promotes the cooperative binding of multiple transcription factors, and serves as the scaffolding that stabilizes MHC class II enhanceosome formation (Harton and Ting, 2000). CIITA can, through its acidic domain, recruit cofactors (CBP/p300, PCAF and GCN5) with histone acetyltransferase (HAT) activity and is itself a HAT (Beresford and Boss, 2001; Harton and Ting, 2000; Raval et al., 2001; Wright and Ting, 2006). Histone acetylation resulting from the recruitment of HATs, leads to chromatin remodeling and enhanceosome formation. The MHC class II deficient RJ2.2.5 cell line (Rigaud et al., 1994), which has a functional defective CIITA, has reduced levels of histone acetylation at the MHC class II promoter when compared to its parental B cell line Raji (Beresford and Boss, 2001; Masternak et al., 2003).

Despite the generally known requirement for CIITA in MHC class II expression, previous reports have suggested a CIITA-independent MHC class II pathway (Chou et al., 2005; Collinge et al., 1998; Magner et al., 2000; Zhou et al., 1997). Several cell lines have been identified that are MHC class II negative but they can be induced by the HDACi trichostatin A (TSA) to express MHC class II in the apparent absence of CIITA (Chou et al., 2005; Magner et al., 2000). The Colon 26 cell line contains both a functional IFN- γ inducible and a CIITA-independent TSA activatable MHC class II pathway. Transfection of Colon 26 with a dominant negative CIITA expression plasmid substantially reduced the level of MHC class II induced by IFN- γ but not the TSA induced MHC class II expression. In addition, TSA treatment of primary kidney cultures generated from STAT-1 and IRF-1 knockout mice demonstrated robust MHC class II expression in the absence of CIITA (Chou et al., 2005; Magner et al., 2000).

In this report, we characterize the various histone modifications associated with active MHC class II expression. We find that both the CIITA-dependent (induced by IFN- γ) and – independent (induced by TSA) pathways lead to a similar pattern of histone modification at the MHC class II locus. Enhancement of acetylated histone H3, H4 and H3K4me3 were

detected at the promoter region while H3K36me3 marks were localized at exons 3 and 5 following activation of MHC class II. Reduced levels of H3K9me3 were demonstrated at the promoter following both IFN- γ and TSA treatment. However, H3K79me2 is detected at the promoter and exon 3 only in B cell lines constitutively expressing high levels of MHC class II. In the RJ2.2.5 B cell, H3K79 methylation was restored by the stable transfection of a functional CIITA. The data suggests that acetylation of histone H3 can initiate histone methylation at specific locations along the MHC class II gene which are required for gene expression.

2. Materials and Methods

2.1 Cell lines and culture conditions

Culture conditions for the mouse B cell line A20 and human epithelial cell line HeLa, B cell line Raji and Daudi were as described by the American Type Culture Collection. The human B cell line RJ2.2.5 was provided by Joseph. D. Fontes and culture conditions were as previously described (Magner et al., 2000). The mouse colon adenocarcinoma cell line Colon 26, provided by Elizabeth. A. Repasky, was cultured in DMEM (Invitrogen, Grand Island, NY, USA) with 10% FBS (Cellgro, Herndou, CA, USA). Each cell line was treated with the optimal concentration of IFN- γ (R&D systems, Minneapolis, MN, USA) or TSA (Wako, Osaka, Japan) and for durations at which maximal expression of MHC class II (IA α or DR α) was detected. Colon 26 cells were treated with either IFN- γ (100U/ml) or TSA (250nM) for 48 hr. HeLa cells were treated with 500U/ml of IFN- γ for 24 hr and RJ2.2.5 cells with 100U/ml IFN- γ or 100nM TSA for 24 hr. RNA was harvested and levels of mRNA measured as previously described (Magner et al., 2000).

2.2 Plasmids and stable transfection

The CIITA expression plasmid pcDNA3.1-CIITA was generated by excising the full-length CIITA fragment from the pSVK-CIITA plasmid (Boss, JM) followed by insertion into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA), which contains a hygromycin resistant gene. Stable transfection of RJ2.2.5 was conducted by a standard electroporation procedure at 250mV and 960 μ F (Biorad, Hercules, CA, USA) with 20 μ g of the control vector pcDNA3.1 or pcDNA3.1-CIITA. The transfected RJ2.2.5 cells were then cultured in standard growth media and hygromycin B (350 μ g/ml) (Calbiochem, La Jolla, CA, USA) was added at 24 hr post-transfection. Seven days post-transfection, single RJ2.2.5 cells expressing CIITA were then selected by treatment with various concentrations of hygromycin (350, 450 and 550 μ g/ml) to generate clones with different levels of MHC class II expression. Flow cytometry was performed for the analysis of surface MHC class II expression and the levels of CIITA and MHC class II (DR α) mRNA expression were determined by RT-PCR as previously described (Chou et al., 2005; Magner et al., 2000).

2.3 Chromatin Immunoprecipitation (ChIP) assay

ChIP assays were performed following the standard protocol provided by Upstate Cell Signaling Solutions (Upstate Cell Signaling Solutions, Charlottesville, VA, USA) with some modification. For each experiment, 2×10^7 cells (or 4×10^7 cells for experiments examining multiple DR α regions) from each treatment were collected for formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) cross-linking. After cell lysis (10^7 cells/ml), supernatants were subjected to sonication to produce DNA fragments of 600-800bp. Cell lysates from each of the treatment samples were divided among five tubes with ChIP dilution buffer to a final volume of 1ml/tube. "Input DNA" samples (20 μ l/tube) were collected prior to immunoprecipitation and used as internal controls for starting DNA. Immunoprecipitations were carried out overnight at 4 $^{\circ}$ C with antibodies specific for the target proteins. Antibodies for acetylated histone H3 (06-599) and H4 (06-866), H3K9me3 (07-422) were obtained from Upstate Cell Signaling Solutions.

Antibodies for acetylated H3K9 (Ab4441), H3K4me3 (Ab8580), H3K36me3 (Ab9050) and H3K79me2 (Ab3594) were obtained from Abcam (Abcam, Cambridge, UK). Real-time quantitative PCR was performed to quantify the amount of DNA from both the ChIP and Input DNA fractions. DNA samples with no antibodies added were used as negative controls and samples from A20 or Raji cells were included as positive controls. Data presented are representative of multiple independent experiments.

2.4 Real-time Quantitative PCR

Analysis of chromatin immunoprecipitated products was performed using primers and probes specific for the DR α locus control region, promoter, exon 3 and exon 5 (sequences for the primers and probes will be given upon request). Serial dilutions of the untreated DNA were used to generate a standard curve for the determination of the amount of DNA in each sample. The amount of ChIP DNA (μ g) from each treatment was then divided by the amount of Input DNA (μ g) for normalization between the different treatments. The ChIP/Input ratios were compared between treatments to determine changes in the level of each histone modification. Triplicate wells of 1, 2 and 4 μ l samples were used to validate that the Ct values obtained from each sample were within the linear range of detection. Because input DNA samples represent only 2% of the total starting material, depending on the efficacy of the immunoprecipitation process, the ChIP/Input ratio value may range from 0-50.

2.5 Flow cytometry and cell sorting

The flow cytometric analysis was conducted by standard methods on a FACScan (Becton Dickinson, San Jose, CA, USA). R-Phycoerythrin-conjugated anti-HLA-DR monoclonal antibody (BD Pharmingen, San Diego, CA, USA) was used for the identification of pcDNA3.1-CIITA transfected RJ2.2.5 clones expressing surface MHC class II molecules. R-Phycoerythrin-conjugated mouse IgG2a antibody (Becton Dickinson) was used as an isotype control. Forward scatter versus side scatter gating was set to include all non-aggregated cells but to exclude apoptotic fragments.

Our flow cytometry analyses demonstrated that each 'positive' clone contained mixed populations of MHC class II expressing and non-expressing cells. To isolate the MHC class II expressing subgroup, each clone was subjected to cell sorting. 10^7 cells from each pcDNA3.1-CIITA clone were stained with R-Phycoerythrin-conjugated anti-HLA-DR monoclonal antibody as described but without the final fixation step. HLA-DR expressing cells were collected and cultured in RPMI growth media with 50% FBS for 24 hr before transferring to a standard growth media with hygromycin B.

3. Results

3.1 CIITA -dependent and -independent MHC class II induction pathways result in similar patterns of histone modification

We have previously identified Colon 26 as a cell line having both the IFN- γ induced CIITA-dependent and TSA induced CIITA-independent MHC Class II expression pathways (Chou et al., 2005; Magner et al., 2000). CIITA contains intrinsic HAT activity and the absence of CIITA has been shown to result in reduced histone acetylation at the MHC class I and II promoters (Beresford and Boss, 2001; Gialitakis et al., 2006; Raval et al., 2001). We therefore examined whether activation of MHC class II through the CIITA-independent pathway would enhance levels of histone acetylation and alter methylations along the MHC class II gene in a pattern similar to that shown by the CIITA-dependent pathway. Colon 26 cells were treated with either IFN- γ or TSA for 48 hr, a time point at which optimal levels of IA α expression were detected (Fig. 1A). The level of histone H3 and H4 acetylation was determined by a ChIP assay. The data demonstrated that both IFN- γ and TSA treated Colon 26 cells, as well as the

positive control B cell line A20, have enhanced levels of histone H3 and H4 acetylation at the IA α promoter region which is correlated with active IA α expression (Fig. 1B). However, the levels of histone acetylation in TSA treated Colon 26 were significantly greater than those treated with IFN- γ .

Previous studies have demonstrated the coordinated presence of histone methylation markers with lysine acetylation on nearby histone residues (Liang et al., 2004). We used ChIP assays to determine the changes in histone methylation following activation of IA α expression in Colon 26 by IFN- γ or TSA treatment. Enhancement of H3K4me3 was detected at the IA α promoter while the levels of H3K9me3 decreased after treatment with either reagent (Fig. 1C). Consistently, TSA treatment resulted in higher levels of modification than those induced with IFN- γ . These analyses demonstrated that the IFN- γ or TSA treatments similarly marked the IA α gene histones, and were associated with active transcription presumably by altering repressive chromatin. Our data also suggests that, although TSA is an HDAC inhibitor, it can also initiate changes in the status of histone methylations.

3.2 Differential spatial distributions of H3K4me3 and H3K36me3 at the MHC class II gene

Although changes in histone acetylation and methylation at the promoter play a major role during transcription initiation, histone marks located towards the 3' end of a gene can also affect gene expression. Previous work has demonstrated that the distribution of various histone modifications on genes may vary, depending on the markers examined. H3K4me3 is usually localized at the promoter and the 5' region of the actively transcribed genes while H3K36me3 tends to cluster towards the 3' end (Bannister et al., 2005; Santos-Rosa et al., 2002). We explored the level of various histone marks, as well as their spatial distribution following activation of MHC class II expression. We employed HeLa, a classical IFN- γ inducible cell and compared the results with those obtained from the human B cell line RJ2.2.5. RJ2.2.5 is a derivative of Raji having a defective CIITA gene and was shown to give a robust MHC class II response to TSA (Fig. 2A) (Chou et al., 2005). The MHC class II DR α gene consists of five exons in addition to the promoter and contains an upstream LCR. Real-time quantitative PCR primers and probes specific for the upstream LCR, promoter, exon 3 and exon 5 of the DR α gene were utilized in ChIP assays to determine the localization of histone modifications (Fig. 2B). As with the Colon 26 cells, DR α expression induced by IFN- γ in HeLa or TSA in RJ2.2.5 cells, was accompanied by enhanced histone H3K9 and H4 acetylation at the DR α promoter (Fig. 2A and Fig. 2C). The positive control B cell line Raji, which constitutively expresses DR α , also demonstrated high levels of histone H3K9 and H4 acetylation.

Coordinated enhancement of both H3K4me3 and H3K36me3 was detected following activation of DR α by both IFN- γ and TSA (Fig. 3A and Fig 3B). The level of H3K4me3 peaked at the DR α promoter region in both the CIITA-dependent and -independent activation pathways, but also appeared in the LCR of TSA treated RJ2.2.5 (Fig. 3A). In contrast, high levels of H3K36me3 were detected at exons 3 and 5 of the DR α gene after transcription activation by both agents, with the peak situated at exon 3 (Fig. 3B). In all experiments, the defective CIITA expression in the RJ2.2.5 cell line prevented IFN- γ induction of DR α expression and the associated changes in histone modifications. However, its DR α expressing cell of origin, Raji, demonstrated high levels of H3K4me3 at the promoter and H3K36me3 at both exons 3 and 5. The HMTs responsible for the methylation of a specific lysine residue have been shown to associate with particular stages of the transcription process (Hampsey and Reinberg, 2003). Thus, the different spatial distribution of H3K4me3 and H3K36me3 suggests that histone modifications are differentially involved in the various stages of transcription and elongation.

3.3 Association of H3K79me2 with constitutive, but not IFN- γ or TSA induced MHC class II expression

As mentioned above, unlike H3K4 and H3K36, H3K79 is a residue of the histone H3 core region positioned at the surface of the histone structure which does not interact with the DNA backbone (Bannister et al., 2005; Luger et al., 1997). These characteristics differentiate H3K79 methylation from the other histone methylation marks that modulate gene expression by altering their interactions with DNA. We therefore examined the levels of H3K79me2 and its distribution in comparison with the other modifications discussed above. ChIP assays were performed in HeLa, Raji and RJ2.2.5 cells. Interestingly, the levels of H3K79me2 remained low and unchanged at all regions tested, despite robust DR α induction by IFN- γ in HeLa and TSA in RJ2.2.5 respectively (Fig. 4A). However, the Raji B cell line exhibited high levels of H3K79me2 at both the DR α promoter and exon 3 with low levels at exon 5 and the LCR (Fig. 4A). The high levels of H3K79me2 at the promoter and exon 3 were not unique to the Raji cells as Daudi, another MHC class II expressing B cell line, demonstrated a similar pattern (Fig. 4B). Overall, our data points to differences in the regulation of H3K79 methylation between the constitutive and induced MHC class II expression pathways.

The high levels of H3K79me2 detected in the B cell lines Raji and Daudi suggest that the constitutive expression of CIITA may be required for the recruitment and/or activity of the enzyme DOT1L (the human homolog of DOT1) which methylates H3 on lysine 79. To test this hypothesis, RJ2.2.5 cells were transfected with a CIITA expression plasmid pcDNA3.1-CIITA and clones were selected with hygromycin B. Each selected clone (3D and 4B) constitutively expressed high levels of CIITA and DR α mRNA (Fig. 5A) as well as surface MHC class II molecules (Fig. 5B). ChIP assays were performed with two independent MHC class II expressing clones (pcDNA3.1-CIITA 3D and 4B) to detect the level of H3K79me2 at multiple regions of the DR α gene. Three RJ2.2.5 clones transfected with the empty vector pcDNA3.1 (pcDNA3.1 5B, 5C and 5D) were also included as negative controls. The constitutive expression of CIITA by stable transfection restored the level of H3K79me2 in RJ2.2.5 to that comparable to the positive control Raji (Fig. 5C). High levels of H3K79me2 were localized at both the DR α promoter and exon 3, but remained low in the RJ2.2.5 clones transfected with the empty vector (Fig. 5C). Constitutive expression of CIITA in RJ2.2.5 also led to high levels of H3K36me3 at the DR α gene and this histone modification remained restricted to the 3' region of DR α (Fig. 5C). Together, the data suggest that the continuous presence of high levels of CIITA may be crucial for the recruitment of enzymes with histone methylating activity to the DR α gene following IFN- γ activation. However, CIITA is dispensable for H3K4 and H3K36 methylation which are induced by TSA treatment in its absence.

4. Discussion

Acetylated and methylated lysines can sometimes act in opposite directions in the control of gene expression (Gialitakis et al., 2006; Liang et al., 2004; Peters et al., 2003). Previous studies have shown the coordinated change in H3 lysine 4 methylation with demethylation of H3 lysine 9 accompanied by enhanced acetylation of histone H3 (Gialitakis et al., 2006). Our work confirms and extends these studies by demonstrating the location of methylation at the lysine residues (H3K36 and H3K79) and the differential spatial distribution of these modifications along the MHC class II gene. The potential competition between acetylation and methylation is also reflected in our analyses showing the coordinated enhancement of H3K9 acetylation with the decrease of H3K9me3 following induction of MHC class II expression. The constitutive level of H3K9me3 found at the IA α promoter was somewhat surprising since this is a heterochromatic mark. However, focal areas of H3K9 methylation have been identified in euchromatin (Schultz et al., 2002) and these sites may represent a potentially repressed state

which allows more rapid gene induction upon activation, similar to those suggested for memory marks (Bernstein et al., 2006). The data presented here depict the histone modifications associated with MHC class II during 'steady state' expression, and further study of the kinetics of the various histone modifications may allow a better understanding of their role during MHC class II transcription.

The distinct spatial distribution of different histone modifications, shown here and observed by others (Krogan et al., 2003a; Krogan et al., 2003b; Morillon et al., 2005), indicate that each mark may have unique consequences during the various phases of transcription. For example, transcription initiation by the methylated H3K4 has been shown to mediate recruitment of ISWI, a chromatin remodeling ATPase in *S. cerevisiae*, to the 5' end of the gene (Morillon et al., 2005; Santos-Rosa et al., 2003). Recognition of H3K4me3 by the ISWI containing NURF complex is mediated by a homeodomain (PHD) finger (Wysocka et al., 2006). Trimethylation of H3K4 is important for the proper loading of the RNA pol II complex at the 5' promoter region as suggested by the observation that mutation of SET1 leads to aberrant recruitment and altered distribution of RNA pol II at the promoter (Morillon et al., 2005; Santos-Rosa et al., 2003). The PHD domain of human BPTF (bromodomain and PHD domain transcription factor) and the bromodomain binding acetylated lysine residues have been demonstrated to stabilize the NURF complex on chromatin resulting in nucleosome sliding and alteration of chromatin structure as transcription proceeds (Morillon et al., 2005; Santos-Rosa et al., 2003).

Trimethylation by SET2 at H3K36 is required for the proper processing of RNA transcripts, and mutation or deficiencies of SET2 have been shown to result in the accumulation of RNA pol II at the 3' end of the gene and stalling of termination (Kizer et al., 2005). The SET2 enzyme directs the restoration of repressive chromatin after RNA pol II passage during elongation by recruitment of the HDAC Rpd3 complex to gene coding regions (Keogh et al., 2005). Rpd3, by removing histone acetylation, provides the repression state required to prevent spurious transcription initiation from intragenic sites (Carrozza et al., 2005). Deletion of SET2 also leads to a reduction of H4K8-Ac at the promoter due to a defect in Nu4A recruitment (Morillon et al., 2005). H3K36 methylation may therefore serve as a mark for 'trans-tail' signaling between histones H3 and H4. Thus, methylation appears to modulate both the acetylation and deacetylation required for continuous transcriptional activation.

H3K79 is a residue located in the histone core which unlike the other residues does not directly contact the DNA phosphate backbone (Luger et al., 1997). Thus, the mechanisms of H3K79 mediated regulation are likely different from those directed by the histone modifications discussed above. The H3K79 HMT, DOT1, is unique in that it lacks the classical SET domain found in other HMTs (Feng et al., 2002; Lacoste et al., 2002). It has been suggested that methylation by DOT1 may serve as a physical barrier to prevent H3K79 occupancy by SIRT1, a NAD⁺ dependent class III HDAC, thereby foiling repression by SIRT and allowing activation of gene expression (Hampsey and Reinberg, 2003; van Leeuwen et al., 2002). Unexpectedly, our data demonstrated that treatment with both IFN- γ and TSA induced significant levels of MHC class II expression in the absence of H3K79me2, suggesting a requirement for SIRT in the IFN- γ and TSA induced DR α expression unlikely. In addition, treatment of both HeLa and RJ2.2.5 cells with the SIRT1 inhibitor Sirtinol failed to activate DR α expression (data not shown). The observation in this study that H3K79me2 was restored by transfection of CIITA in the CIITA deficient B cell RJ2.2.5 suggests that CIITA may be crucial for the recruitment and/or functional activity of DOT1L. Given that the CIITA constitutively expressed in the B cell lines and that used for transfection in RJ2.2.5 in these studies are B cell specific (type III CIITA) the question arises whether other types of CIITA induce the functional activity of DOT1L. However, since the robust transcription of MHC class II in HeLa following treatment by IFN- γ is mainly via type IV CIITA, and occurs in the absence of H3K79me2, suggests that the induction of MHC class II via type III and IV CIITA does not require H3K79me2. It is

unclear therefore what the function of lysine 79 methylation might be in the constitutive expression of MHC class II by B cells.

One possible explanation for the observed distribution of H3K79 methylation may result from the exchange of H3 variants (Ahmad and Henikoff, 2002) as outlined in the H3 barcode hypothesis (Hake and Allis, 2006). During gene transcription, the chaperone protein HIRA is thought to mediate H3.3 variant associated nucleosome assembly and replace H3.1. Since methylation of H3K79 appears to target histones that are in the context of assembled nucleosomes (Lacoste et al., 2002), the newly deposited H3.3 may be deficient in K79 methylation during the time course of our experiments. In the Raji and Daudi B cells, the expression of DR α is in a constitutive steady state, which may allow the accumulation of H3K79 methylation. The detection of H3K79me₂ at the DR α promoter and exon 3 of the stable pcDNA3.1-CIITA transfected RJ2.2.5 further supports the notion that long term, constitutive expression of CIITA may be required for the accumulation of H3K79 methylation.

Experiments previously conducted in our lab demonstrated that tumor cells treated with HDACi upregulate MHC class II and CD40 and can be used as an effective anti-tumor vaccine (Khan et al., 2004; Tomasi et al., 2006). The finding that HDACi can alter repressed immune genes in tumor cells is currently being tested in animal models and analyzed for their potential in clinical trials. Inhibitors including siRNA directed at the specific enzymes involved in chromatin alteration as described here could potentially be tailored for the restoration of genes silenced during tumor immune escape.

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Abbreviations

CIITA	class II transactivator
MHC	major histocompatibility complex
HDAC	histone deacetylase
LCR	locus control region
HMT	

	histone methyltransferase
CTD	C-terminal domain
HP1	heterchromatin protein 1
HAT	histone acetyltransferase
TSA	trichostatin A
ChIP	chromatin immunoprecipitation

Figure 1. CIITA-dependent and -independent MHC class II expression pathways are associated with similar patterns of histone modifications

A. CIITA-dependent and -independent IA α expression in the Colon 26 cell line. Colon 26 cells were treated with either IFN- γ (100U/ml) or TSA (250nM) for 48 hr and RNA was recovered for analysis of IA α and CIITA expression by real-time quantitative RT-PCR. Expression was represented as level of induction relative to the untreated Colon 26. **B and C.** Chromatin Immunoprecipitation assays were performed to determine the level of histone modifications associated with IA α expression. Antibodies specific to the acetylated histone H3 and H4 (B), H3K4me3 and H3K9me3 (C) were used to immunoprecipitate crosslinked chromatin fragments. The immunoprecipitates were then analyzed for the abundance of IA α promoter DNA by real-time quantitative PCR. ChIP DNA samples from the B cell line A20 were included as positive controls and samples with no antibodies added were included as negative controls.

Figure 2. Enhanced histone acetylation at the DRα promoter is associated with active DRα expression

HeLa cells were treated with 500U/ml IFN-γ for 24 hr and RJ2.2.5 cells were treated with 100U/ml IFN-γ or 100nM TSA for 24 hr. **A.** Levels of CIITA and DRα mRNA expressed in RJ2.2.5 and HeLa cells were measured by real-time quantitative RT-PCR. **B.** A schematic of the location of the various DRα regions examined is shown. Positions of the primer and probe sets used for ChIP assay analysis are labeled as grey lines. **C.** ChIP assays were performed with antibodies specific for the acetylated H3K9 and histone H4. The immunoprecipitates were then analyzed, with primers and probes specific for the DRα promoter region, and the abundance of DRα promoter fragment quantified by real-time quantitative PCR. The DRα

expressing Raji B cell line was included as positive controls and samples with no antibodies added were included as negative controls.

Figure 3. Localization of H3K4me3 at the DR α promoter and H3K36me3 at the 3' exons following activation of DR α expression

HeLa cells were treated with 500U/ml IFN- γ for 24 hr and the RJ2.2.5 cells were treated with 100U/ml IFN- γ or 100nM TSA for 24 hr. ChIP assays were performed with antibodies specific for the H3K4me3 (A) and H3K36me3 (B). The immunoprecipitates were then analyzed for the abundance of the DR α upstream LCR, promoter, exons 3 and 5 DNA by real-time quantitative PCR. DR α -expressing Raji B cell line was included as positive controls and samples with no antibodies added were included as negative controls.

Figure 4. The absence of H3K79me2 at the DR α locus of induced cells and its association with the DR α promoter and exon 3 during constitutive expression

A. Spatial distribution of H3K79me2 following activation of DR α expression. HeLa cells and the RJ2.2.5 cells were treated as in Fig. 2. ChIP assays were performed with antibodies specific for the H3K79me2. The immunoprecipitates were then analyzed for the abundance of the DR α s in Fig. 3. **B.** Presence of H3K79me2 at the DR α promoter and exon 3 of the MHC class II expressing B cell lines. ChIP assays were performed with the DR α expressing B cell lines Raji and Daudi and untreated, DR α negative, RJ2.2.5 as above.

Figure 5. Constitutive expression of CIITA restored the levels of H3K79me2 and H3K36me3 in RJ2.2.5

A. CIITA and DRα mRNA expression in the selected RJ2.2.5 clones. RNA was harvested from RJ2.2.5 cells stably transfected with empty plasmid pcDNA 3.1 (5B, 5C and 5D) or pcDNA3.1-CIITA (3D and 4B) for analysis of DRα and CIITA mRNA expression by RT-PCR. CIITA expressing B cell line Raji was included as positive control. **B.** Analysis of surface HLA-DR expression of the selected RJ2.2.5 clones. RJ2.2.5 clones described above were stained with antibodies specific for surface HLA-DR molecules (black line). Staining with the mouse IgG2a antibodies were included as isotype controls (grey line). DRα expressing Raji B cell line was included as positive controls. **C.** ChIP assays were performed with the selected RJ2.2.5 clones to determine the level of H3K79me2 and H3K36me3 at the various regions of the DRα gene. The DRα expressing B cell line Raji was included as positive controls and samples with no antibody were included as negative controls.