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Polycomb Recruitment at the Class II Transactivator

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ABSTRACT

The Class II Transactivator (CIITA), is the master regulator of Major Histocompatibility Class II (MHC II) genes. Transcription of CIITA is characterized by a decrease in specific trimethylation of histone H3 lysine 27 (H3K27me3), catalyzed by the histone methyltransferase Enhancer of Zeste Homolog 2 (EZH2). While EZH2 is the known catalytic subunit of the Polycomb Repressive Complex 2 (PRC2) and is present at the inactive CIITA promoter; the mechanisms of recruitment of PRC2 to mammalian promoters remains unknown. Here we identify two DNA-binding proteins, YY1 and Jarid2, which interact with and regulate PRC2 recruitment to CIITA promoters. Our study is the first to identify novel roles of YY1 and Jarid2 in the epigenetic regulation of a mammalian gene by the PRC2.

INDEX WORDS: PRC2, EZH2, Jarid2, YY1, H3K27me3, CIITA, Polycomb recruitment, Gene silencing
POLYCOMB RECRUITMENT AT THE CLASS II TRANSACTIVATOR GENE

by

NATHANIEL H. BOYD

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POLYCOMB RECRUITMENT AT THE CLASS II TRANSACTIVATOR

by

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LIST OF ABBREVIATIONS

PRC2 – Polycomb Repressive Complex 2

EZH2 – Enhancer of Zeste Homolog 2

Jarid2 – Jumonji, AT Rich Interactive Domain 2

YY1 – Yin-Yang 1

CIITA pIV – Class II Transactivator Promoter IV

MHC – Major Histocompatibility Complex

lncRNA – Long Non-Coding RNA

HOTAIR – HOX Antisense Intergenic RNA

IFN-γ - Interferon Gamma

HOX – Homeobox Gene

Pho - Pleiohomeotic

H3K27me3 – Histone H3 Lysine 27 Trimethylation

H3K4me3 – Histone H3 Lysine 4 Trimethylation

EED – Embryonic Ectoderm Development

SUZ12 – Suppressor of Zeste 12

RbAp46/48 – Retinoblastoma Associated Protein 46/48

E(z) – Enhancer of Zeste

PRE – Polycomb Response Element

HLA – Human Leukocyte Antigen

APC – Antigen Presenting Cell
1 INTRODUCTION

1.1 Epigenetics and Histone Modifications

A complete genome is an organismal blueprint which contains the necessary genes that encode proteins to produce a functioning organism. For appropriate gene expression to occur, chromatin must be regulated through reversible, dynamic changes [2, 3]. Epigenetics is a rapidly expanding field that studies heritable changes in gene expression that are controlled at the level of transcription by mechanisms other than DNA sequence alteration [4]. Three main epigenetic control mechanisms of gene expression include: DNA methylation, post-translational modifications (PTMs) of histones, and non-coding RNA based interactions [5]. The focal point of this study is on the regulation of a gene through histone modifying enzymes.

The eukaryotic cell contains large amounts of highly condensed nuclear DNA. Condensed DNA, or chromatin, is highly structured with multiple orders of condensation. Epigenetic studies currently focus on the “beads on a string” model of chromatin structure (Figure 1) which consist of DNA tightly wrapped one and a half times around nucleosomes [6]. Nucleosomes are multi-protein complexes composed of individual subunit proteins called histones which are highly conserved across eukaryotic species [7]. There are five types of histones designated as H1, H2A, H2B, H3 and H4 [8]. The nucleosome core forms an octamer containing 2 dimers each of H3/H4 [9, 10] and H2A/H2B [11]. H1 serves as a stabilizer of the linker DNA which is wrapped around the nucleosome core [12-14]. Each of these histone proteins has an N-terminal tail extending from the core nucleosome structure [15, 16] which interacts with DNA and other proteins. Interactions of histones and DNA are regulated through covalent PTMs to the histone tails [17, 18].

PTMs function to regulate chromatin dynamics by modifying histone-DNA interactions, altering higher order chromatin structure, and recruiting other chromatin factors [5]. There are multiple PTMs
Figure 1 Levels of Chromatin Compaction. The various levels of chromatin compaction: from the chromosome, the most compacted state of chromatin, to the individual DNA double helix. Most epigenetic studies are based on the “beads on a string” level of chromatin compaction as presented in Molecular Biology of the Cell, 4th ed. [1]
which regulate epigenetics, the most prevalent of which include acetylation, methylation, ubiquitination, sumoylation, phosphorylation, and ADP-ribosylation [19-24]. Histone methylation has been extensively studied, revealing that the location and degree of methylation on histone tail residues result in differential gene expression [25]. For example, the tri-methylation on lysine 27 of histone H3 (H3K27me3) is associated with repressed chromatin, while H3K4me3 is considered an activating mark [26, 27]. To explain this dynamic nature of histone methylation, a current model proposes “reader” molecules which recognize specific methylated histone marks and subsequently recruit chromatin remodeling enzymes and other non-histone proteins to further modify chromatin structure [18, 28].

1.2 Polycomb Group and EZH2

A chromatin modifying complex currently under extensive study is the Polycomb Repressive Complex 2 (PRC2). PRC2 is a four-protein complex consisting of Embryonic Endoderm Development protein (EED), Suppressor of Zeste 12 (SUZ12), Enhancer of Zeste Homolog 2 (EZH2), and Retinoblastoma Associated Protein (RbAp46/48). EZH2 is the mammalian homolog of Drosophila Enhancer of Zeste [E(z)] [29, 30] and is responsible for catalyzing a tri-methyl group addition on lysine 27 of histone H3 (H3K27me3) in human cells [31-33]. SUZ12 binds to EZH2, stabilizes its methyltransferase activity, and is required for the PRC2 complex to function [34]. EED stabilizes the complex by binding to the product of PRC2 catalysis, H3K27me3, and creating a positive feedback loop by further stimulating the complex [35]. RbAp46/48 is a histone chaperone known to bind histones H3 and H4, which further stabilizes the complex [36].

PRC2 has been shown to be a critical player in the repression of homeobox (HOX) genes in insects and mammalian cells as well as in the regulation of differentiation in pluripotent cells [37]. The modulation of PRC2 expression and function has also been implicated in a variety of tumor types, including multiple myeloma, follicular lymphoma, and esophageal, renal, breast, prostate and ovarian cancers.
Polycomb Response Elements (PREs) in Drosophila DNA sequences first discovered in recruited proteins. An example of a polycomb recruitment protein in Drosophila is Pleiohomeotic (Pho), which serves as a recruiter of the Polycomb Repressive Complex 2 (PRC2) in Drosophila. Conserved PREs have yet to be defined in mammalian cells.
The discovery of the importance of PRC2 in many cellular processes has led to a growing effort to determine the mechanisms of PRC2 recruitment to target genes.

DNA binding domains among the polycomb complex proteins have yet to be identified in any species [35, 40]. In *Drosophila*, both PRC complexes are recruited to chromatin targets by DNA elements termed Polycomb Response Elements (PREs) (Figure 2), but these PREs are not commonly found in mammalian DNA, with only one human putative PRE described to date [41]. The PRE-binding protein in *Drosophila*, Pleiohomeotic (PHO), is related to the mammalian protein Yin-Yang 1 (YY1) [42-44], the only known mammalian homolog of the Drosophila PRE-binding proteins [41]. YY1 is a zinc-finger DNA (and RNA) binding protein which has been shown to both activate and repress gene transcription [45, 46]. In *Xenopus* embryos, YY1 has been found to associate with EED and is involved in neural differentiation [47]. Although these observations have led to speculation that YY1 has a similar function in mammalian cells as PHO in *Drosophila* [48], there is still little known about PRC1 and PRC2 tethering to target genes in mammalian cells [40]. Another protein with the potential to regulate EZH2 promoter binding is the Jumonji/ARID domain-containing protein 2 (JARID2), which has recently been shown to be a part of the PRC2 complex and to regulate PRC2 binding to target genes in human embryonic stem cells [49-53]. By studying the roles of YY1 and Jarid2 in PRC2 recruitment, we hope to elucidate novel mechanisms by which PRC2 is recruited to target promoters.

1.3 Model System

Major Histocompatibility Complex (MHC) or Human Leukocyte Antigen (HLA) molecules are essential for the function of the immune system as they serve as critical signaling molecules for immune surveillance. MHC molecules present processed antigen to T cells of the immune system and thus allow for the recognition and elimination of foreign antigen [54]. There are two classes of MHC, Class I and Class II, which are expressed from 11 loci on chromosome 6 [55]. The variability of each loci among humans ranges from low to very high, and the possible inherited combinations of each lead to vast antigen
recognition diversity. MHC Class I (MHC I) molecules are present on the surface of all nucleated cells and present peptides from intracellular origin [56]. MHC I molecules bind the T cell receptor on CD8 positive T cells, resulting in CD8 T cell activation and initiation of an immune response to an intracellularly derived antigen [57, 58]. MHC Class II (MHC II) molecules present peptides from extracellular origin, are constitutively expressed on antigen presenting cells (APCs) [56, 59] and are inducibly expressed on all nucleated cells in the presence of inflammatory cytokines, particularly interferon-\(\gamma\) (IFN-\(\gamma\)) [60-62]. MHC II molecules are important drivers of adaptive immune responses as they bind to CD4 positive T cells, and initiate inflammatory responses to extracellular antigens.

MHC II expression is tightly controlled at the level of transcription by the master regulator, Class II Transactivator, or CIITA [63]. CIITA binds to the MHC II enhanceosome complex assembled on active MHC II promoters (Figure 3) and activates transcription of MHC II genes by recruiting RNA polymerase II and driving elongation of MHC II genes [64-68]. In the absence of CIITA, MHC II is not transcribed resulting in a loss of expression of MHC II [69]. Dysregulated expression of MHC II is implicated in multiple diseases including the progression of cancer [70, 71] and initiation of autoimmune disease [72-74], thus it is imperative that we understand the mechanisms of CIITA expression. Because CIITA controls the expression of a universal immune molecule, it has four distinct promoters that are active in different cell types. Dendritic cells utilize promoter I for constitutive CIITA expression, B cells utilize promoter III for constitutive CIITA expression, and promoter IV is responsible for inducible CIITA expressed through IFN-\(\gamma\) in nucleated cells. Regulation of promoter II is less understood, and little is known about its function [75, 76].

Understanding the details of PRC2 and EZH2 involvement in transcriptional control of CIITA promoter IV (CIITA pIV) is of great significance to human health. EZH2 is implicated in the proliferation of many types of tumors and recent findings further demonstrate metastatic ability decreases in pancreatic cancer when EZH2 siRNA is used to suppress EZH2 expression [77]. MHC Class II expression has also been shown to be decreased in breast cancer, B-cell lymphoma, and Hodgkin lymphoma, which may
The MHC II promoter consists of three DNA elements known as the X1, X2, and Y boxes which bind the components of the MHC II enhanceosome. Regulatory Factor X, Cyclic AMP Response Element Binding Protein, and Nuclear Factor Y form the enhanceosome complex and bind to the X1, X2, and Y boxes respectively. The Class II Transactivator (CIITA) binds to the MHC II enhanceosome complex and recruits RNA Polymerase II as well as Cyclin Dependent Kinases 7 and 9 (CDK7/9) to phosphorylate RNA Polymerase II and initiate transcription of MHC II.

**Figure 3 The MHC II Promoter.** The MHC II promoter consists of three DNA elements known as the X1, X2, and Y boxes which bind the components of the MHC II enhanceosome. Regulatory Factor X, Cyclic AMP Response Element Binding Protein, and Nuclear Factor Y form the enhanceosome complex and bind to the X1, X2, and Y boxes respectively. The Class II Transactivator (CIITA) binds to the MHC II enhanceosome complex and recruits RNA Polymerase II as well as Cyclin Dependent Kinases 7 and 9 (CDK7/9) to phosphorylate RNA Polymerase II and initiate transcription of MHC II.
correlate with increased EZH2 binding [71, 78-80]. Once the mechanism of PRC2 recruitment is understood in greater detail, modulation of MHC II gene expression may be achieved by blocking the specific recruitment of EZH2 to CIITA promoters using small molecule inhibitors. Broad applications include treatment of diseases involving dysregulated expression of MHC II. Here we detail the binding patterns of two putative PRC2 recruitment proteins, YY1 and Jarid2, and their effects on the expression of the inducible gene CIITA pIV.

2 MATERIALS AND METHODS

2.1 Cells

HeLa (human epithelial) cells from ATCC (Manassas, VA) were cultured and passaged in Dulbecco Modified Eagle Medium (DMEM) (Mediatech, Inc., Herndon, VA) containing 10% Fetal Bovine Serum (FBS), 5 mM L-glutamine, and 5 mM penicillin-streptomycin at 37°C and 5% carbon dioxide.

2.2 Antibodies

Antibodies recognizing MYC-tag, FLAG-tag, and JARID2 were purchased from Abcam (Cambridge, MA). Antibodies recognizing YY1, HA-tag, and HRP conjugated rabbit antibody were purchased from Santa Cruz (Santa Cruz, CA). Antibody recognizing H3K27me3 was purchased from Active Motif (Carlsbad, CA). Antibody recognizing EZH2 was purchased from Cell Signaling Technology (Danvers, MA) and HRP conjugated mouse antibody was purchased from Promega (Madison, WI).

2.3 Plasmids

The Myc-EZH2-pCDNA3 plasmid construct was described previously and was kindly provided by Jer-Tsong Hsieh [81]. The pCMV-HA-JARID2 construct was described previously [52]. Flag-YY1 was described previously and was kindly provided by Myra Hurt [82]. The CIITA pIV luciferase construct was
described previously and was kindly provided by Bernard Mach [83]. The pcDNA control plasmid was described previously [84].

2.4 Primers

Primers and probes for CIITA pIV and for the GAPDH promoter used in ChIP assays are as previously described [85-87] as well as primers and probes for CIITA pIV RNA, HLA-DRA RNA and GAPDH mRNA [86, 87]. Primers and the probe for Jarid2 mRNA expression are as follows: Jarid2 sense CACCGTCCTCCCCAATAACA, Jarid2 antisense CTCGAGACCAGCATGAGGTAGA, and Jarid2 probe 6 FAM-AGGGTCCATCCTGCACCTCG-TAMRA.

2.5 siRNA Constructs and Transient Transfections

Short interfering RNA duplexes (siRNA) pre-designed for YY1, JARID2 or scrambled All Star Control (Qiagen) were used to knock down expression of YY1 or JARID2. HeLa cells were transfected with 10 nM of YY1 or JARID2 specific siRNA or All Star scrambled control sequence according to the HiPerfect Transfection Reagent protocol (Qiagen). Cells were lysed in NP40 cell lysis buffer with EDTA free complete protease inhibitor (Roche) and were analyzed by western blot or RNA expression levels for efficiency and specificity of knock down.

2.6 Dual Luciferase Assay and Transient Transfection

HeLa cells were plated at 1X10^5 cells/well in 6-well tissue culture plates. Eight hours after plating, cells were transfected with 5µg of plasmid (CIITA-Luc, Flag-YY1, HA-Jarid2, Myc-EZH2, or pcDNA control) using Genejuice transfection regent (Novagen) according to manufacturer’s protocol. Each sample was also transfected with renilla luciferase as a control for protein levels. 18 hours after transfection, cells were treated with 500 U/mL IFN-γ and harvested 6 hours after stimulation as indicated. Cells were lysed using 1x lysis reagent (Promega), scraped from plates, and a dual luciferase was run per manufacturer’s protocol.
2.7 Co-Immunoprecipitation Assay and Transient Transfections

HeLa cells were plated at a cell density of $8 \times 10^5$ in 10cm$^2$ tissue culture plates. Cells were allowed to adhere to plate overnight. Cells were then transfected with 5µg of plasmid (Flag-YY1, HA-Jarid2, Myc-EZH2, or pcDNA control) using GeneJuice (Novagen) per manufacturer’s protocol. Cells were harvested 18 hours later in NP40 lysis buffer with EDTA free complete protease inhibitor (Roche). Lysates were centrifuged, normalized for protein concentration, and precleared with protein G beads (Pierce). After preclear, lysates were immunoprecipitated overnight with anti-Myc, anti-HA, or anti-Flag agarose beads (Sigma) or protein G mock bead control. For endogenous Co-IP, lysates were incubated with anti-Jarid2 antibody (Abcam) overnight, and the following morning incubated with Protein G beads for 1 hr. Bead-immune complexes were washed with dilute NP40 to remove background and subsequently eluted from beads and denatured by boiling for 5 minutes in Laemmli gel loading buffer with beta-mercaptoethanol (Bio-Rad). The collected supernatant was analyzed by western blot.

2.8 Chromatin Immunoprecipitation (ChIP)

HeLa cells were plated at a density of $2 \times 10^6$ in 10cm$^2$ tissue culture plates. Cells were treated with IFN-$
\gamma$ (500 U/mL) as indicated. After IFN-$\gamma$ stimulation, cells were cross-linked with 37% formaldehyde. Crosslinking was stopped with 0.125M glycine and cells were harvested and lysed in cell lysis buffer (5 mM PIPES, 85 mM KCl) with IGEPAL (sigma) and e-complete protease inhibitors (Roche) for 15 minutes on ice. Lysates were spun to isolate nuclei, and re-suspended in SDS lysis buffer with protease inhibitors and lysed on ice for 30 minutes. Lysates were then flash frozen twice in liquid N$_2$ and subsequently sonicated in a water bath sonicator (3x 10 min cycles, 30 sec on/30 sec off) (Bioruptor, Diagenode). Lysates were pre-cleared for 1 hour at 4°C with salmon sperm DNA/protein A agarose beads (Millipore). After pre-clear, antibodies for indicated protein (or mock control) were added to each sample and incubated overnight with rotation at 4°C. Following overnight incubation, immunoprecipitation was performed with salmon sperm DNA/protein A which were added and incubat-
ed for 1 hour with rotation at 4°C. Following immunoprecipitation, samples were washed for 3 minutes at 4°C with each of the following buffers as described previously: low salt buffer, high salt buffer, LiCl buffer, and 1X TE. Samples were then eluted with SDS elution buffer and crosslinks were reversed overnight with 5M NaCl at 65°C; proteins were degraded using proteinase K (Invitrogen). Immunoprecipitated DNA was isolated using phenol:chloroform:isoamyl alcohol (Invitrogen) as per manufacturer’s protocol. Isolated DNA was analyzed by qPCR using primers and probes for indicated gene of interest.

2.9 RNA Expression and Transient Transfections

HeLa cells were plated at a density of 8X10^5 cells per 10 cm^2 tissue culture plate, and allowed to adhere to plate overnight. Cells were transfected with 5µg Myc-EZH2, Flag-YY1, HA-Jarid2, or pcDNA as indicated. 18 hours after transfection, cells were stimulated with IFN-γ as indicated and harvested with Qiazol RNA extraction reagent (Qiagen) per manufacturer’s protocol. An Omniscript Reverse Transcription Kit (Qiagen) was used to generate cDNA from the extracted RNA. DNA generated was quantified by qPCR using primers and probes for CIITA pIV and GAPDH mRNA.

2.10 RNA Expression in siRNA Treated Cells

Cells were plated at a density of 8X10^5 cells per 10 cm^2 tissue culture plate. Cells were transfected with indicated siRNA construct as described above and incubated for 72 hours. Subsequently, an RNA extraction was performed as described above.

3 RESULTS

3.1 Ectopic Expression of EZH2, YY1 or Jarid2 Suppress CIITA-mediated MHC II mRNA Transcription

Previous studies in our lab and others have demonstrated that the HLA-DRA and CIITA pIV genes are epigenetically regulated [89-91]. In particular, CIITA pIV is regulated by bivalent epigenetic marks
and its activation is characterized by a rapid loss of the suppressive modification H3K27me3 [89, 92]. The H3K27me3 histone mark is conferred by the histone methyltransferase EZH2, which is part of the larger Polycomb Repressive Complex 2 (PRC2) [31, 93, 94]. While PRC2 is known to be involved in gene silencing [31, 94], little is known of the mechanism by which PRC2 is recruited to target genes. Two possible recruitment proteins have been chosen as candidates for recruitment of this complex, YY1 and Jarid2. YY1 is the human homolog of a PRC2 recruitment protein discovered in *Drosophila* and Jarid2 has recently been implicated as a regulator of the PRC2 complex in human stem cells. To explore the effects of YY1 and Jarid2 on CIITA pIV, transient transfection was used to observe the effects of elevated expression of EZH2, YY1 and Jarid2 on the activity of the human CIITA promoter IV associated with a luciferase reporter gene. HeLa cells were co-transfected with a CIITA pIV-luciferase reporter plasmid and either Myc-EZH2, Flag-YY1, or HA-Jarid2 plasmids. Cells were stimulated with the cytokine IFN-γ and incubated for 6 hours to activate the CIITA pIV. Data is presented as fold increase in CIITA pIV luciferase activity, with increases measured relative to unstimulated sample.

In order to determine if decreased CIITA promoter IV activity results in decreased CIITA mRNA levels, mRNA expression was measured in IFN-γ stimulated HeLa cells transiently transfected with Myc-EZH2, Flag-YY1, or HA-Jarid2. RNA was extracted following transfection and 18 hours of IFN-γ stimulation, and cDNA was generated from extracted RNA using a reverse primer specific for CIITA pIV mRNA in reverse transcription. Quantification of this cDNA was achieved by qPCR using primers and probes specific for CIITA pIV mRNA. Data is presented as relative CIITA mRNA molecules quantified using a standard curve in qPCR. Figures 4A and B indicate that when YY1 and Jarid2 proteins are expressed at levels higher than physiological norms, CIITA pIV activity is suppressed and results in a suppression of CIITA pIV mRNA expression.
To determine if YY1 and Jarid2 suppression of CIITA promoter activity and CIITA mRNA transcription results in a decrease in MHC II mRNA, HeLa cells were transfected as described above and RNA was quantified using qPCR with primers and probe specific for MHC II mRNA. Significant decreases in expression of EZH2, YY1, and Jarid2 suppress CIITA and MHC transcription.

**Figure 4** Ectopic expression of EZH2, YY1, and Jarid2 suppress CIITA and MHC transcription. (A) EZH2, YY1, and Jarid2 suppress CIITA pIV luciferase activity. pcDNA, Myc tagged EZH2, Flag tagged YY1, and HA tagged Jarid2, and a CIITA pIV-Luc reporter construct were co-transfected into HeLa cells. Cells were harvested following 6 hours of IFN-γ stimulation and a luciferase assay was performed in triplicate with data being presented as fold increase in luciferase activity. (B) EZH2, YY1, and Jarid2 suppress CIITA mRNA transcription. Myc-EZH2, Flag-YY1, and HA-Jarid2 were transfected into HeLa cells. Cells were harvested following 18 hours of IFN-γ treatment and a Trizol extraction of mRNA was performed. cDNA was generated using a reverse primer specific for CIITA pIV and was quantified using qPCR with primers and probes specific for CIITA pIV. A western blot showing transfection efficiency is shown. (C) EZH2, YY1, and Jarid2 suppress MHC II mRNA transcription. Transfections, IFN-γ treatments, Trizol extraction, and qPCR were performed as above with the exception that primers and probes specific to MHC II promoter were used. A western blot showing transfection efficiency is shown. Error bars indicate SEM of 3 (A, B) and 4 (C) independent experiments with p values shown as a result of students T Test. *p≤0.05 **p≤0.01 ***p≤0.001 ****p≤0.0001.
MHC II mRNA transcription were observed in cells overexpressing EZH2, YY1, or Jarid2 following 18 hours of IFN-γ stimulation (Figure 4C).

3.2 Binding Dynamics of EZH2, YY1 and Jarid2 at CIITA pIV

We have previously observed that the suppressive histone modification H3K27me3 rapidly decreases at CIITA pIV following IFN-γ activation (Figure 5A) [91]. HeLa cells were subjected to a chromatin immunoprecipitation (ChIP) after 1 hour, 18 hours, or no treatment of IFN-γ to stimulate CIITA pIV activity. Antibody for H3K27me3 was used to immunoprecipitate the H3K27me3 modification. Data is presented as percent input of total precipitated DNA resulting from quantification using qPCR and primers and probe for CIITA pIV. Levels of H3K27me3 were also quantified at the GAPDH promoter to determine specificity (Figure 5B).

If YY1 and Jarid2 are involved in recruitment of the PRC2 to CIITA pIV, it would be reasonable to assert that YY1 and Jarid2 binding at CIITA pIV likely follow the same trend as the H3K27me3 suppressive modification. To evaluate the binding profile of YY1 and Jarid2 at CIITA pIV, HeLa cells were subjected to a ChIP assay using antibodies for either YY1 (Figure 5C), Jarid2 (Figure 5D), or EZH2 (Figures 5C and 5D). Data is presented as percent input of total DNA.

3.3 EZH2, YY1 and Jarid2 are Binding Partners

If YY1 and Jarid2 are involved in recruitment of PRC2, it is likely these proteins interact with EZH2 (the active enzyme in the complex). To address this question, we examined the interactions using via co-immunoprecipitation. HeLa cells were transfected with either pcDNA control, Myc-EZH2, Flag-YY1, or HA-Jarid2. Cells were lysed, incubated with antibodies, and the immunoprecipitated lysate was transferred and probed with antibody for potential binding partners. In Figure 6A, YY1 immunoprecipitated with EZH2 in cells overexpressing tagged versions of these proteins. Figure 6B shows EZH2
immunoprecipitated with endogenous Jarid2 in cells overexpressing tagged EZH2. Finally, in Figure 6C, YY1

**Figure 5 Binding Dynamics at CIITA pIV.** (A) H3K27me3 levels rapidly decrease upon IFN-γ stimulation. HeLa cells were stimulated with IFN-γ and were cross-linked, lysed, and sonicated. A chromatin immunoprecipitation (ChIP) was performed using antibodies specific for H3K27me3. Associated DNA was analyzed by qPCR with primers and probes specific for CIITA pIV. (B) H3K27me3 levels at GAPDH are not affected by IFN-γ stimulation. To determine if IFN-γ stimulation affects global H3K27me3 levels, a ChIP assay was performed as previously described. Associated DNA was analyzed by qPCR using primers and probes for GAPDH. (C) EZH2 and YY1 dissociate from the CIITA pIV upon IFN-γ stimulation. A ChIP assay was performed as previously described using antibodies specific for YY1 and EZH2. (D) Jarid2 association with CIITA pIV increases upon IFN-γ stimulation. A ChIP assay was performed as previously described with antibodies specific for Jarid2 and EZH2. Error bars in (B), (C), and (D) represent SEM of three independent experiments with p values shown as a result of students T Test. *p≤0.05 **≤0.01 ***p≤0.001 ****p≤0.0001. Error bars in (A) are SEM of experimental replicates and are representative data of all experiments performed. qPCR values were normalized to the total amount of DNA in the reaction (Input). The control IgG values were 0.005 ± 0.001 and were subtracted from experimental values.
EZH2, YY1, and Jarid2 are binding partners. (A) EZH2 and YY1 associate in HeLa cells. HeLa cells were transfected with Myc-EZH2 and Flag YY1. Cells were harvested and lysates were used in a co-immunoprecipitation assay. Antibody-conjugated agarose beads (IP) for Flag, Myc, and a mock bead control were incubated with rotation overnight. Beads were washed, eluted, and subjected to western blot with respective antibodies (IB). Lane 1 contains the positive control, Lane 2 the negative control, and Lane 3 is the experimental sample. (B) HeLa cells were transfected with Myc-EZH2. A co-immunoprecipitation assay was performed as previously described with the exception that antibodies for Myc, Jarid2, and a mock bead control were used. (C) HeLa cells were transfected with Flag YY1 and HA-Jarid2. A co-immunoprecipitation was performed as previously described with the exception that antibodies for Flag, HA, and a mock bead control were used. Data shown is representative of three independent experiments.
immunoprecipitated with Jarid2 in cells overexpressing tagged YY1 and Jarid2. Blots showing equal transfection efficiency are shown.

3.4 Knocking Down YY1 or Jarid2 Significantly Decreases H3K27me3 and EZH2 Binding at CIITA pIV

If YY1 and Jarid2 recruit PRC2, knocking down YY1 or Jarid2 may disrupt binding of PRC2 to the CIITA pIV and reduce H3K27me3 silencing. To explore the effects of knocking down YY1 and Jarid2 on polycomb recruitment, ChIP assays were performed to determine levels of EZH2 and H3K27me3 levels at CIITA pIV in cells treated with siRNA duplexes specific for YY1 or Jarid2. Cells were left untreated in order for the promoter to remain in an inactive state, with maximum PRC2 occupancy. In Figure 7A, we observed a significant decrease in EZH2 binding at the CIITA pIV in the presence of YY1 specific siRNA, as compared to a scrambled control siRNA. Mirroring this observation, there was a significant decrease of the suppressive histone modification, H3K27me3. Figure 7B is a western blot showing efficient and specific knockdown of YY1. Treating cells with siRNA specific to Jarid2 resulted in effects on EZH2 and H3K27me3 binding, with a significant decrease of EZH2 and H3K27me3 in the presence of Jarid2 siRNA (Figure 7C). mRNA expression assays were used to determine an efficient and specific knockdown of Jarid2. In Figure 7D, expression of Jarid2 mRNA significantly decreased with treatment of Jarid2 specific siRNA but did not affect GAPDH mRNA expression.

3.5 Loss of Jarid2 Increases CIITA mRNA Expression

As a loss of YY1 or Jarid2 showed decreased PRC2 recruitment to CIITA pIV, we next investigated the effect of YY1 and Jarid2 knockdown on CIITA mRNA expression. If YY1 or Jarid2 recruits PRC2, a loss of either protein would decrease the amount of PRC2 present at the CIITA pIV promoter, resulting in less epigenetic suppression and elevated levels of CIITA mRNA expression. To explore this possibility, cells were transfected using siRNA specific for YY1, Jarid2, or scrambled control siRNA and CIITA pIV mRNA expression was measured following the addition IFN-γ for 1, 2, 3, or 4 hours. As shown in figure 8A,
Knockdown of YY1 or Jarid2 results in a significant loss of EZH2 binding and H3K27me3 at CIITA pIV. (A) and (C) knockdown of YY1 or Jarid2 decreases binding of EZH2 and H3K27me3 levels at CIITA pIV. HeLa cells were transfected with YY1 (A) or Jarid2 (C) siRNA and were incubated for 72 hours. Cells were harvested and a ChIP assay was performed as previously described using antibodies for EZH2 and H3K27me3. (B) YY1 siRNA is efficient and specific. A western blot demonstrates efficiency and specificity of the YY1 siRNA construct. (D) Jarid2 siRNA is efficient and specific. RNA was extracted and quantified using qPCR to determine the efficiency and specificity of the knockdown. Primers and probes specific to either Jarid2 or GAPDH were used in qPCR. Error bars in (A) and (C) represent SEM of four independent experiments with p values shown as a result of students T Test. *p≤0.05 **p≤0.01 ***p≤0.001 ****p≤0.0001. Error bars in (D) are SEM of experimental replicates and are representative data of all experiments performed. qPCR values were normalized to the total amount of DNA in the reaction (Input). The control IgG values were 0.009 ± 0.002 and were subtracted from experimental values.
Figure 8 Knockdown of Jarid2 significantly increases CIITA mRNA in the presence of IFN-γ stimulation. (A) Knockdown of Jarid2 increases CIITA mRNA transcription. HeLa cells were transfected with Jarid2 siRNA and were incubated for 72 hours. RNA was extracted and was quantified using qPCR as previously described using primers and probe specific for CIITA RNA. (B) Jarid2 siRNA is efficient. Primers and probe specific for Jarid2 RNA were used in qPCR to determine that at each time point, a significant decrease in Jarid2 mRNA was observed. (C) Jarid2 siRNA is specific. Primers and probe specific for GAPDH RNA were used to determine that, at each time point, there was no significant change in GAPDH mRNA levels. Error bars in (A) represent SEM of three independent experiments with p values shown as a result of students T Test. *p≤0.05 **≤0.01 ***p≤0.001 ****p≤0.0001. Error bars in (B) and (C) are SEM of experimental replicates and are representative data of all experiments performed.
there was a significant increase in CIITA mRNA transcription at each timepoint of IFN-γ stimulation in the presence of Jarid2 siRNA as compared to scrambled control siRNA. In contrast, knockdown of YY1 failed to show a significant change in CIITA mRNA expression levels (Data not shown). Figure 8B shows the results of Jarid2 mRNA quantification to determine the efficiency of the Jarid2 siRNA. GAPDH mRNA expression was also measured to determine the specificity of Jarid2 siRNA duplexes in Figure 8C.

4 DISCUSSION

CIITA is the master regulator of MHC-II gene expression, and as such it modulates the levels of MHC-II in the cell [63]. CIITA is a tightly controlled gene that, when dysregulated, is capable of causing devastating immune related problems. Therefore, it is imperative that we understand the epigenetic mechanisms by which the CIITA gene is modulated. Indeed, as MHC II expression is controlled by CIITA [63], dysregulation of CIITA is correlated with a host of complications, from autoimmune diseases caused by an overexpression of MHC II [72-74] to tumor development implicated from decreased MHC II expression [70, 71].

Epigenetic regulation plays an important role in CIITA pIV activation and has been shown to be effectively silenced by EZH2, the active member of the PRC2, and the silencing H3K27me3 modification it confers. Previous work from our lab demonstrated that knocking down EZH2 not only increases CIITA pIV mRNA transcription during IFN-γ stimulation, but also results in aberrant expression of silent CIITA pIV in the absence of cytokine stimulation. Because CIITA pIV is no longer silenced with knockdown of EZH2, it is likely that EZH2 and the PRC2 acts alone in the suppression of CIITA pIV, similar to its role in controlling gene expression during stem cell differentiation [95]. Results from these previous studies describe EZH2 as a crucial player in the silencing of CIITA pIV [90, 91].

To date, there is much speculation regarding the specific mechanism by which PRC2 is tethered to target chromatin. Many studies on PRC2 have focused on developmental genes and others that re-
quire permanent or near-permanent silencing by PRC2 [50-53, 96-101]. Alternatively, our study aims to elucidate PRC2 mechanisms in a fluctuating system that requires rapid activation from a silent state and equally rapid deactivation in the absence of IFN-γ. Through information from current literature, two proteins were chosen as candidates for involvement in the recruitment of PRC2 to CIITA plIV, YY1 and Jarid2. YY1 is the mammalian homolog of Pleiohomeotic (Pho), which binds to conserved DNA elements in *Drosophila* DNA and is responsible for tethering the *Drosophila* equivalent of the PRC2 to these elements [42, 44]. Because of this homology, it is possible that YY1 has retained its PRC2 recruiting ability from *Drosophila* to mammalian cells. Similarly, Jarid2 has recently been found to associate with the PRC2 in *Drosophila* [102]. Likely in keeping with a semi-conserved function through evolution, Jarid2 co-occupies chromatin sites with PRC2 and has been implicated as a major player in recruitment of PRC2 in mammalian cells [50-53, 99, 100]. We sought to determine if CIITA plIV was a novel co-occupation site for Jarid2 and PRC2, and if Jarid2 was responsible for regulating the binding of PRC2 to CIITA plIV.

Both YY1 and Jarid2 have been implicated to be involved in PRC2 recruitment at some genes, but details remain obscure. To determine novel roles for these proteins at an inducible promoter, CIITA plIV, cells were transfected to ectopically express EZH2, YY1, or Jarid2. In cells that overexpress YY1 or Jarid2, there is an approximately 6 and 4 fold decrease in CIITA plIV luciferase activity, respectively, as compared to activity in cells transfected with the CIITA plIV-Luc construct only. Results from these reporter assays suggest that overexpression of EZH2 alone was insufficient to suppress CIITA plIV activity. However, when cells expressed higher levels of either YY1 or Jarid2, there was a significant decrease in the activity of the promoter (Figure 4A), suggesting CIITA plIV suppression. The same trend was observed when we measured levels of CIITA plIV mRNA and MHC II mRNA (Figure 4B and C). In contrast, the overexpression of EZH2 had more of a negative effect on CIITA plIV and MHC II mRNA expression than on CIITA plIV luciferase activity, suggesting that EZH2 and the PRC2 require elements additional to the promoter of CIITA plIV genes in order to bind and silence.
We have previously reported that following IFN-γ stimulation, EZH2 and H3K27me3 levels rapidly decrease at CIITA pIV [91]. ChIP experiments indicate YY1 follows a similar trend of association with CIITA pIV (Figure 5C), albeit at lower levels. This observation supports the hypothesis of YY1 tethering PRC2 to CIITA pIV when silenced, and dissociating from the promoter once activated by IFN-γ. Interestingly, Jarid2 significantly increases its presence at the CIITA pIV after IFN-γ stimulation (Figure 5D). This could be due to Jarid2 playing a role in the rapid activation of the promoter by recruiting other enzymes to regulate PRC2 removal. A recent study indicates that overexpression of Jarid2 in Drosophila results in a reduction of H3K27me3 [102]; it is possible Jarid2 acquired dual roles for PRC2 recruitment and removal in mammalian cells. Further study is needed to elucidate mechanisms responsible for the interesting binding pattern of Jarid2 at the CIITA pIV.

We sought to determine whether YY1 and Jarid2 interact with the PRC2 in differentiated cells. Both YY1 and Jarid2 were found to associate with the PRC2 complex through co-immunoprecipitation. This study is the first to our knowledge to show YY1 exhibiting a physical association with EZH2 in human cells (Figure 6A). Additionally, YY1 is a physical binding partner of Jarid2 (Figure 6C), suggesting a novel YY1-Jarid2-PRC2 regulatory complex. Existence of this complex in differentiated cells may explain why the ablation of YY1 expression has limited effects on an inducible promoter but affects gene expression in undifferentiated myoblasts [48]. This complex is supported by previous studies linking YY1 to the PRC2 complex through the association of YY1 and EED, another member of the PRC2 complex. [41, 48, 103, 104].

Knocking down YY1 or Jarid2 results in decreased levels of EZH2 and PRC2 at CIITA pIV (Figure 7A and C), suggesting decreased polycomb recruitment when these proteins are expressed at sub-physiological amounts. To corroborate this observation, CIITA mRNA levels increase when Jarid2 is knocked down (Figure 8A), which indicate Jarid2 as important in CIITA pIV suppression. In comparison, knockdown of YY1 failed to consistently increase CIITA pIV mRNA expression. It also possible that Jarid2
Figure 9 Model: YY1 and Jarid2 action on Polycomb Repressive Complex 2 at CIITA pIV. CIITA pIV is silenced through recruitment of PRC2 by the concurrent binding of YY1 and Jarid2 with EZH2 at the promoter. Upon activation, Jarid2 binding increases at CIITA pIV and possibly facilitates removal of PRC2 through interactions with YY1 or other unknown mechanisms.
is more important in the regulation of this promoter, with YY1 having a more peripheral role in regulating biphasic gene expression. YY1 is strongly implicated in PRC2 recruitment to HOX genes and the inactive X chromosome, two permanently silenced genes likely regulated in a different manner than the inducible CIITA gene. This is supported by observations that YY1 has binding sites in a proposed human PRE among the HOX gene cluster [41], as well as associating with Xist, a long non-coding RNA (lncRNA) responsible for silencing the inactive X chromosome [105].

At this time, it is currently thought that PRC2 recruitment to target genes is controlled by a combination of recruitment proteins YY1, Jarid2, AEBP2, and adding further complexity, cell-type specific long non-coding RNA (lncRNA) which act as scaffolding for these recruitment proteins [50-53, 99, 100, 103, 104, 106-108]. At this point, PRC2 recruitment to mammalian genes is poorly understood, and observations from the current study add to the complexity. Though knockdown of YY1 did not have a conclusive effect on CIITA pIV mRNA expression, YY1 remains a strong recruitment candidate in current literature because of its association with EED [103] and Xist [105]. Another lncRNA, HOTAIR, recruits PRC2 to HOX genes to silence in a similar H3K27me3-mediated manner. LncRNA was initially investigated in the current study, however knockdown of HOTAIR proved to have no effect on the expression of CIITA pIV mRNA (Data not shown).

It is likely that PRC2 recruitment is gene specific, and that CIITA pIV requires different machinery to regulate its rapid activation than genes such as HOX or the X chromosome that are permanently silenced during development. Our study is the first to link YY1 and Jarid2 with epigenetic regulation of a dynamic promoter that requires tight control of rapid activation and silencing. Figure 9 depicts a model summarizing the interactions of the PRC2 with YY1 and Jarid2 at CIITA pIV. CIITA pIV in the silenced state shows interaction between YY1, Jarid2, and EZH2 and with the activation of CIITA pIV, increased
recruitment of Jarid2 may aid in the removal of the PRC2 through interaction of YY1 or through other unknown mechanisms. Further study is needed to define the fine-tuned mechanisms of each of these proteins’ involvement in the precise manipulation of CIITA pIV by PRC2.
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