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# A Role for EZH2 in Silencing of IFN- $\gamma$ Inducible *MHC2TA* Transcription in Uveal Melanoma<sup>1</sup>

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We investigated the contribution of epigenetic mechanisms in *MHC2TA* transcriptional silencing in uveal melanoma. Although no correlation was observed between impaired CIITA transcript levels after IFN- $\gamma$  induction and DNA methylation of *MHC2TA* promoter IV (CIITA-PIV), an association was found with high levels of trimethylated histone H3-lysine 27 (3Me-K27-H3) in CIITA-PIV chromatin. The 3Me-K27-H3 modification correlated with a strong reduction in RNA polymerase II-recruitment to CIITA-PIV. Interestingly, we observed that none of these epigenetic modifications affected recruitment of activating transcription factors to this promoter. Subsequently, we demonstrated the presence of the histone methyltransferase EZH2 in CIITA-PIV chromatin, which is known to be a component of the Polycomb repressive complex 2 and able to triple methylate histone H3-lysine 27. RNA interference-mediated down-regulation of EZH2 expression resulted in an increase in CIITA transcript levels after IFN- $\gamma$  induction. Our data therefore reveal that EZH2 contributes to silencing of IFN- $\gamma$ -inducible transcription of *MHC2TA* in uveal melanoma cells. *The Journal of Immunology*, 2007, 179: 5317–5325.

Down-regulation of expression of MHC molecules is frequently noted on tumor cells. The low or lack of expression of both classes of MHC molecules impairs cellular immune recognition and frustrates an efficient T cell-mediated tumor eradication. The *MHC2TA* encoded CIITA, is essential for the transcriptional activation of *MHC-II* genes, whereas it plays an ancillary role in the regulation of *MHC-I* transcription (1, 2). Previous studies, including ours, have revealed that epigenetic modifications of chromatin play a critical role in the transcriptional silencing of *MHC2TA* and resulting *MHC-II* genes in cancer. Epigenetic modifications include methylation of DNA at CpG dinucleotides and posttranslational modifications of histone tails such as acetylation and methylation. It has been reported that the lack of IFN- $\gamma$ -induced *MHC2TA* transcription is associated with CpG dinucleotide methylation of CIITA-PIV and CIITA-PIII DNA in several cancer cell types (3–11). Besides CpG dinucleotide methylation, it has been suggested that the lack of IFN- $\gamma$ -induced transcription of *MHC2TA* in several cancer types is also associated with histone deacetylase activities (12–14).

Histone methylation plays an important role in chromatin dynamics and gene expression (15). The mechanisms that underlie gene repression by histone methylation are known to involve methylation of histone H3 at lysine 9 (K9-H3) and lysine 27 (K27-

H3), which is catalyzed by the conserved SET domain of various histone methyltransferases. The HMTase SUVAR39H1, principally catalyzes trimethylation of K9-H3 (3Me-K9-H3), whereas the HMTase G9a catalyzes monomethylation and dimethylation of K9-H3. The HMTase EZH2 catalyzes trimethylation of K27-H3 (3Me-K27-H3) (16–19). EZH2 is a component of the Polycomb repressive complex (PRC)<sup>2,3</sup>, which is involved in the initiation of gene silencing (20, 21). All these histone methylation modifications serve as recognition marks for recruitment of additional epigenetic modification enzymes to chromatin. The 3Me-K9-H3 mark, for instance, recruits DNA methyltransferase (Dnmt) 1 and 3a through HP1 interactions (22), and also EZH2 has been found to interact with Dnmts (23). In this way, the enzymatic methylation activities that modify histones and DNA are intimately linked (22, 23).

Uveal melanoma is the most common malignant tumor of the eye in adults (24). They frequently metastasize leading to a high incidence of tumor-related mortality early in disease (25). It has been documented that MHC-II molecule expression can be observed in uveal melanoma lesions, albeit at variable frequencies and levels of expression (26–29). However, in vitro growth of fresh tumor material after enucleation revealed that most of the established tumor cell lines did not express MHC-II molecules (28). Furthermore, ocular melanoma cell lines were found generally to lack IFN- $\gamma$ -mediated induction of *MHC-II* expression (30). This is in line with the notion that primary uveal melanocytes also lack IFN- $\gamma$ -mediated induction of *MHC-II* expression (30).

It is well established that transcriptional activation of *MHC2TA* by IFN- $\gamma$  requires the assembly of IRF-1, Stat-1, and USF-1 to their respective binding sites in CIITA-PIV (3, 31). On the one hand, the IFN- $\gamma$ -activated factor Stat-1 binds directly with the ubiquitously expressed factor USF-1, to the GAS/E box motif in

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<sup>3</sup> Abbreviations used in this paper: PRC, Polycomb repressive complex; Dnmt, DNA methyltransferase; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase.

CIITA-PIV. Indirectly, Stat-1 induces the transcription factor IRF-1, which subsequently participates in the activation of CIITA-PIV through binding to the ISRE in CIITA-PIV (3, 31). Recruitment of IRF-1, Stat-1, and USF-1 to CIITA-PIV following IFN- $\gamma$  exposure is severely impaired by CpG dinucleotide methylation as shown in trophoblast cell lines (3). Furthermore, the dynamics of CIITA-PIV chromatin acetylation modifications after IFN- $\gamma$  treatment are severely impaired, which correlates with the CpG dinucleotide methylation modification and lack of factor recruitment to CIITA-PIV (3).

In this study, we have evaluated the mechanisms that contribute to silencing of IFN- $\gamma$ -induced expression of MHC-II molecules in uveal melanoma. We show that in OMM1.3 cells the strongly reduced expression levels of CIITA after IFN- $\gamma$ -induction do not correlate with DNA methylation, but with histone H3-lysine 27 triple methylation and the presence of EZH2 in CIITA-PIV chromatin. Consistent with the transcriptionally silent state of *MHC2TA* was the lack of RNA polymerase II recruitment into CIITA-PIV chromatin in this cell line. RNA interference-mediated silencing of EZH2 expression resulted in an increment in CIITA expression levels after IFN- $\gamma$  induction. Together, these observations suggest that EZH2 is involved transcriptional down-regulation of IFN- $\gamma$ -induced expression of CIITA in uveal melanoma.

## Materials and Methods

### Cell culture

Mel285 and OMM1.3 cells were obtained from Dr. B. Ksander (Schepens Eye Research Institute, Harvard Medical School, Boston, MA), and OCM-1 and OCM-3 were provided by Dr. J. Kan-Mitchell (Wayne State University, Detroit, MI). All cell lines were derived from individual donors with different HLA-types. The cell lines Mel 285, OCM-1, and OCM-3 were established from the primary tumor, whereas OMM1.3 was established from a liver metastasis. All uveal melanoma cell lines used in this study expressed MHC-I molecules complexed with  $\beta_2m$  at the cell surface as detected by W6/32. The HeLa, Tera-2, JEG3, and JAR cell lines were purchased from the American Type Culture Collection. All cell lines were cultured in IMDM supplemented with 10% heat-inactivated FBS (Greiner), 100 U/ml streptomycin, and 100 U/ml penicillin.

### Fluorescent-activated cell sorter and Western blot analysis

Cells were harvested, washed with PBS containing 2% FBS, and stained for direct immune fluorescence detection of HLA-DR using PE conjugated mAb (BD Pharmingen). HLA-DQ was detected by indirect fluorescence with mAb SPVL3 and HLA-DP with the B7.21 mAb (32). The acquisition was performed on a FACScan, using the CellQuest program (BD Biosciences) for analysis.

For Western blot analysis, total protein samples were prepared by lysing  $1 \times 10^7$  cells/1 ml lysis buffer (250 mM Tris-HCl (pH 7.9), 150 mM NaCl, 5 mM EDTA, 0.5% NP40, and  $1 \times$  protease inhibitor mixture; Sigma-Aldrich) for 30 min on ice. After centrifugation the supernatant was used to determine protein concentrations using a bicinchoninic acid protein assay kit (Pierce). Equal amounts were used for SDS-PAGE analysis, and the gels were subsequently blotted and assessed for the presence of HLA-DR $\alpha$  and HLA-DR $\beta$  protein by Western blot as previously described (33).  $\beta$ -actin Ab (Ab-1; Oncogene Research Products) was used to ensure equal loading.

### RNA isolation and RT-PCR analysis

Total RNA was isolated from the melanoma cells using the RNeasy extraction method (Cinna Biotex Laboratories). RNA samples (2  $\mu$ g) were transcribed into cDNA using avian myeloblastosis virus reverse transcriptase (Promega). Quantification of panCIITA, HLA-DRA, 18S, and GAPDH transcripts was performed on an ICycler IQ system (Bio-Rad Laboratories) using the IQ SYBR Green Supermix and the following primers: panCIITA sense, 5'-CCGACACAGACACCATCAAC-3'; panCIITA antisense, 5'-CTTTTCTGCCCAACTTCTGC-3'; HLA-DRA sense, 5'-CAAAGAAGGAGACGGTCTGG-3'; HLA-DRA antisense, 5'-GGCTCTCTCAGTTCACAGG-3'; 18S sense, 5'-AACGGCTACCACATCCAA GG-3'; 18S antisense, 5'-ACCAGACTTGCCCTCCAATG-3'; GAPDH sense, 5'-GGTCGGAGTCAACGGATTTG-3'; GAPDH antisense, 5'-ATGAGCCCAGCCTTCTCCAT-3'; EZH2 sense, 5'-GCGCGGGACGAA

GAATAATCAT-3'; EZH2 antisense, 5'-TACACGCTCCGCCAACAA ACT-3';  $\beta_2m$  sense, 5'-TGCTGTCTCCATGTTTGATGTATCT-3'; and  $\beta_2m$  antisense, 5'-TCTCTGCTCCCCACCTCTAAGT-3'.

To compare the relative amount of target transcript in different samples, all values were normalized to the appropriately quantified 18S or GAPDH control.

Full-length cDNA synthesized from OMM1.3 CIITA isoform 4 mRNA was isolated by RT-PCR and cloned into pREP-4 as described previously (34) using the following primer pair: sense, 5'-ATGGCTAGCATGGAGT TGGGGCCCCTAGAAGGTGGCT-3'; antisense, 5'-ATGCTCGAGCAA GGTCAGCGTGGTTAGTGTCTCAG-3'.

### Promoter constructs and transient transfection

The pGL3-CIITA-PIV reporter constructs was previously described (5). Calcium phosphate transfections were performed with  $0.2 \times 10^6$  cells and 0.5  $\mu$ g of luciferase pGL3-reporter construct together with 0.1  $\mu$ g of  $\beta$ -actin-*Renilla*-reporter construct as an internal control. Twenty-four hours after transfection the cells were either treated with 500 U/ml IFN- $\gamma$  for 48 h or not treated. Functionality of OMM1.3 CIITA was evaluated by activation of the pGL3-DRA promoter-reporter in Tera-2 cells as previously described (34). Luciferase and *Renilla* luciferase activity were measured using the Dual Luciferase Assay kit according to the manufacturer's instructions (Promega) and promoter activity was normalized for transfection efficiency with the *Renilla* luciferase activity.

### Southern blot analysis

Cells were either untreated or treated with 500 U/ml IFN- $\gamma$  for 48 h before total genomic DNA isolation. DNA isolation, digestion, and Southern blot analysis were performed as described previously (8). The blots were hybridized with a [ $^{32}$ P]-labeled CIITA-PIV probe containing nucleotides -355 to +74 from the transcription start site.

### Bisulfite sequencing

Cells were either untreated or treated with 500 U/ml IFN- $\gamma$  for 48 h before total genomic DNA isolation. One microgram of genomic DNA was used to convert unmethylated CpGs using the EZ DNA Methylation kit from Zymo Research. CIITA-PIV promoter DNA was then amplified using BSP4.2 (forward primer, 5'-TGGGGATAAGTTTTTTGTAATTTA GGA-3') and BSP4.3 (reverse primer, 5'-CTACTAATAACCTCTCCC TCCCACCAA-3') spanning the regulatory region of CIITA-PIV. PCR conditions were: 3 mM MgCl $_2$ ,  $T_a$  of 55°C and 40 cycles. PCR products were purified using the Qiaquick kit (Qiagen), cloned into pGEMTeasy (Promega), and individual clones were sequenced at the Leiden Genome Technology Center.

### Chromatin immunoprecipitation (ChIP)

Melanoma cells were either untreated or treated with 500 U/ml IFN- $\gamma$  for 48 h before chromatin isolation. Firstly, crosslinking was performed by adding formaldehyde to a final concentration of 1% for 10 min at room temperature. The crosslinking was quenched by the addition of 0.125 M glycine for 5 min. After washing twice with PBS, the cells were harvested using 40% Trypsin/EDTA (Greiner) in PBS and scraping. The cells were then counted, washed once with PBS, and resuspended at a concentration of  $5 \times 10^6$  cells/ml in cell lysis buffer (5 mM PIPES (pH 8.0), 0.5% Nonidet P-40, and 85 mM KCl) containing protease inhibitors and kept on ice for 10 min. After centrifuging at 3000 rpm for 5 min at 4°C, the cell pellets were resuspended at a concentration of  $5 \times 10^6$  cells/ml in nuclear lysis buffer (50 mM Tris-HCl (pH 8.1), 10 mM EDTA, and 1% SDS) containing protease inhibitors and kept on ice for 10 min. Chromatin was then stored in 1 ml aliquots at -80°C until use.

Chromatin samples were sonicated into fragments with an average length of 0.5–3 kb using a Branson 250 sonifier and diluted 10 times using dilution buffer containing 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 7.9), 50 mM NaCl, and protease inhibitors. Sonicated chromatin was precleared using preblocked protein A agarose beads (Upstate Biotechnology). Five microgram of Ab (Table I), or no Ab as background control, was added to 2 ml precleared sonicated chromatin (equivalent to  $2 \times 10^6$  cells) and allowed to bind overnight at 4°C. Seventy microliters of 50% slurry of preblocked protein A agarose beads (Upstate Biotechnology) were added and allowed to bind for 1–4 h at 4°C. Beads were washed using low salt, high salt, LiCl, Tris-EDTA buffers, and chromatin complexes were released from the beads as described by the manufacturer. Immune-precipitated chromatin complexes were decrosslinked for 4 h at 65°C using 0.2M NaCl, and treated with 20  $\mu$ g/ml RNaseI for 15 min at 37°C. Proteins were removed by protein K digestion at 45°C for 1 h, followed by phenol/

Table I. Abs used in ChIP analysis

Name Ab	Supplier/Catalog no.
anti-acetyl-histone H3 recognizing acetylated Lys9 and 14	Upstate Biotechnology/06-599
anti-acetyl-histone H4 recognizing acetylated Lys5,8,12,16	Upstate Biotechnology/06-866
anti-trimethyl-histone H3 (Lys27)	Upstate Biotechnology/07-449 and T. Jenuwein Research Institute of Molecular Pathology (Vienna, Austria)
anti-trimethyl-histone H3 (Lys9)	Upstate Biotechnology/07-442
anti-IRF-1 (C-20)	Santa Cruz Biotechnology/sc497x
anti-STAT1 $\alpha$ p91 (C-24)	Santa Cruz Biotechnology/sc345x
anti-USF-1 (C-20)	Santa Cruz Biotechnology/sc229x
anti-Pol II (N-20)	Santa Cruz Biotechnology/sc899x
anti-EZH2	Upstate Biotechnology/07-689

chloroform extraction. Finally, after precipitation chromatin was resuspended in 30  $\mu$ l distilled H<sub>2</sub>O.

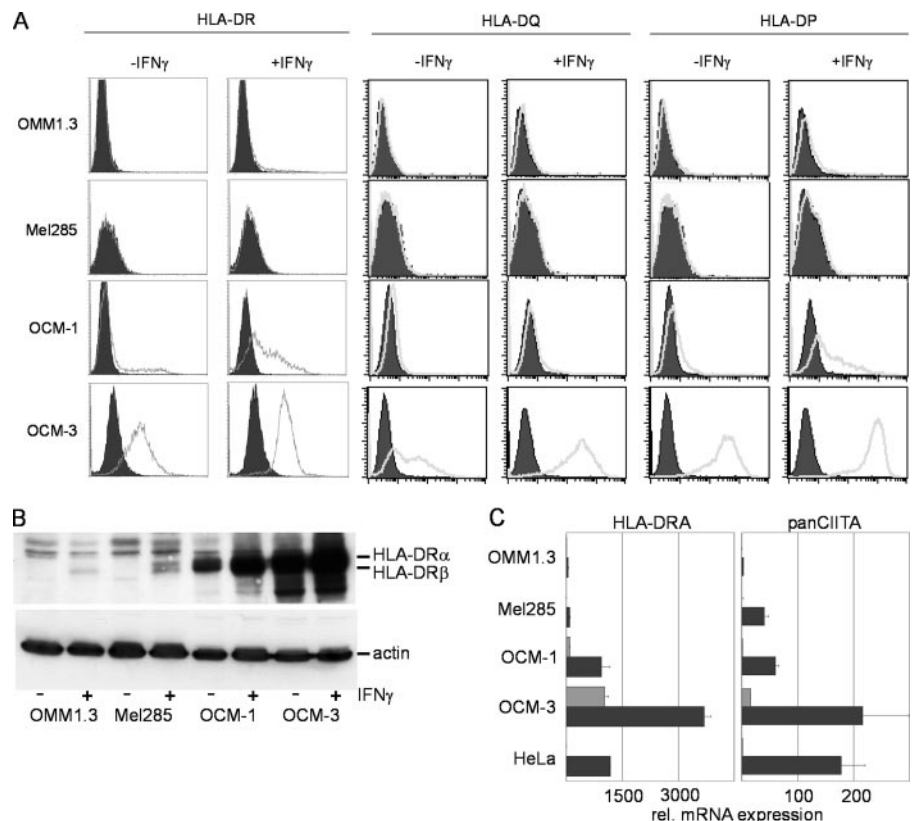
The ChIP for EZH2 was performed as described by Baguet et al. (35) with minor modifications. Cells were washed with PBS containing 2% FBS and cross-linked by addition of 1% formaldehyde, 9 mM NaCl, 91  $\mu$ M EDTA, 45  $\mu$ M EGTA, and 4.5 mM HEPES. Cross-linking was allowed to proceed at room temperature for 10 min and was terminated with 0.125 M glycine for 5 min. Cells were rinsed two times with ice-cold PBS containing 2% FBS and resuspended in buffer 1 (1% SDS, 5 mM EDTA, 50 mM Tris-HCl (pH 8) protease inhibitors, phosphatase inhibitors, and histone deacetylase (HDAC) inhibitors) at  $10 \times 10^6$  cells/ml. Cells were incubated on ice for 10 min and chromatin was sonicated using a Branson 250 sonifier. Chromatin was diluted 10-fold with 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8), protease inhibitors (Sigma-Aldrich), phosphatase inhibitors (Sigma-Aldrich), and HDAC inhibitors (Sigma-Aldrich), precleared with a 50% slurry of preblocked Fast Flow Protein A and G agarose beads (Upstate Biotechnology) for 30 min at 4°C, and rotated overnight at 4°C with 5  $\mu$ g anti-Ac-H3 or anti-EZH2 (see Table I). Chromatin complexes were extracted by incubation with Fast Flow Protein A and G agarose beads for 1–4 h at 4°C. Further washing and elution

was performed using the same buffers as described above containing protease inhibitors, phosphatase inhibitors, and HDAC inhibitors.

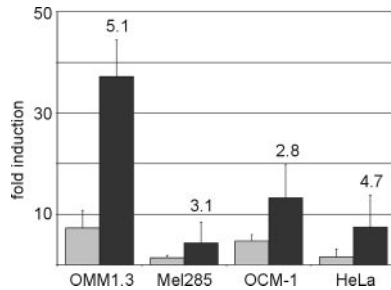
One-tenth of the immune precipitated chromatin was quantified by real-time PCR using an ICycler and SYBR Green Supermix from Bio-Rad, and the following primers: CIITA-PIV ChIP sense, 5'-TCCTGGC CCGGGGCTGG-3' (nt -246 till -229 from transcription start site); CIITA-PIV ChIP antisense, 5'-CTGTTCCCGGGCTCCCGC-3' (nt + 54 till + 72 from transcription start site);  $\beta_2$ m ChIP sense, 5'-CATGCCCT TCTTAAACATCACGAGAC-3' (nt -144 till -120 from transcription start site);  $\beta_2$ m ChIP antisense, 5'-CCCCAGCCAATCAGGACAA-3' (nt -10 till -27 from transcription start site); MYT ChIP sense, 5'-GCTGT GGGGAAAGGTAAGTC-3'; MYT ChIP antisense, 5'-ATGTCTCTCT GTCAGACGC-3' (primer set 9 from Kirmizis et al. (36)); GAPDH ChIP sense, 5'-TACTAGCGTTTTACGGGCG-3' (nt -145 till -126 from transcription start site); GAPDH ChIP antisense, 5'-TCGAACA GGAGGAGCAGAGAGCGA-3' (nt + 20 till -3 from transcription start site).

The data presented are derived from two independent ChIP analyses with real-time PCR performed in duplicate for each ChIP.

**FIGURE 1.** Protein and RNA expression of MHC class II and its transactivator in uveal melanoma cell lines. *A*, Uveal melanoma cell lines were cultured without IFN- $\gamma$  (-IFN- $\gamma$ ) or treated with IFN- $\gamma$  (+IFN- $\gamma$ ) for 48 h and cell surface expression of HLA-DR, -DQ, and -DP was determined by FACS analysis. The filled curves represent the isotype controls and the open curves represent MHC-II staining. *B*, Western blot analysis of the levels of total HLA-DR $\alpha$  and HLA-DR $\beta$  proteins in uveal melanoma without (-) or with (+) IFN- $\gamma$  treatment for 48 h. *C*, Quantitative PCR analysis of HLA-DRA and CIITA transcript levels in the uveal melanoma cell lines. RNA was isolated from cells without (light bars) or with (dark bars) 48 h treatment with IFN- $\gamma$ . Values were normalized to 18S RNA and the amount of HLA-DRA and panCIITA transcripts in HeLa without IFN- $\gamma$  treatment were set at 1.







**FIGURE 2.** Transcriptional activity of exogenous CIITA-Promoter-IV in uveal melanoma. Uveal melanoma and HeLa cells were transiently transfected with a CIITA-PIV promoter reporter construct without (light bars) or with (dark bars) IFN- $\gamma$  treatment for 48 h. Numbers above the dark bars represent the fold induction in CIITA-PIV activity after IFN- $\gamma$  treatment. pGL3-basic, which lacks a promoter upstream of the luciferase gene, was used as background control. Promoter activity is indicated as fold induction relative to the control. Error bars, SD of three independently performed assays.

### RNA interference

Twenty-four hours before transfection cells were plated at  $15 \times 10^3$  cells/cm<sup>2</sup>. Dharmacon siRNAs were introduced according to the manufacturer with a final concentration siRNA of 100 nM in DharmaFECT 3. The transfection was repeated after 24 h to enhance EZH2 silencing. Twenty-four hours after the second transfection cells were either incubated or not with 500 U/ml IFN- $\gamma$  and 48 h after the second transfection cells were harvested for RNA and protein analysis. Used siRNAs were: EZH2 Duplex (Dharmacon catalog no. P-002079-01-05) and siGLO Lamin A/C (Dharmacon catalog no. D-001620-01-05). The level of EZH2 mRNA and protein knockdown was assessed respectively by real-time PCR analysis and Western blot analysis using anti-EZH2 (BD Biosciences catalog no. 612666) and anti- $\beta$ -actin (Ab-1, Oncogene) as described above. IFN- $\gamma$ -mediated induction of CIITA and  $\beta_2m$  mRNA following EZH2 knock-down was quantified by real-time PCR analysis as described above.

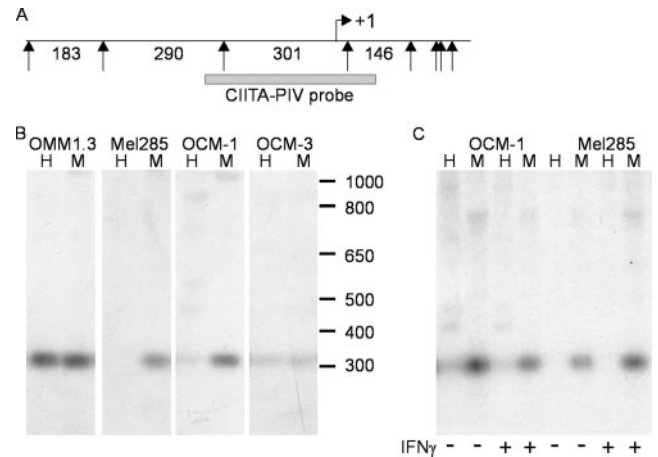
## Results

### Uveal melanoma cell lines display varying MHC-II molecule expression characteristics

A panel of established uveal melanoma cell lines was selected on the basis of their MHC-II expression as determined by FACS analysis. Fig. 1A shows that OMM1.3 and Mel285 cells lack detectable levels of HLA-DR expression at the cell surface following exposure to IFN- $\gamma$ . OCM-3 cells abundantly express HLA-DR in a constitutive fashion, which contrasts with the weak constitutive expression of HLA-DR in OCM-1 cells. Both of these cells manifested an increase in IFN- $\gamma$ -induced cell surface expression of HLA-DR (Fig. 1A). In all cell lines, constitutive and IFN- $\gamma$ -induced HLA-DQ and HLA-DP surface expression correlates with HLA-DR surface expression (Fig. 1A).

To rule out the possibility that the lack of HLA-DR surface expression in Mel285 and OMM1.3 cells is due to impaired protein transport to the cell surface, a Western blot analysis on whole cell lysates was performed (Fig. 1B). This analysis revealed that OMM1.3 and Mel285 cells do not produce the HLA-DR protein in the absence of IFN- $\gamma$ . OMM1.3 cells expressed minute amounts of HLA-DR after IFN- $\gamma$  induction, while this expression was more abundant in Mel285 cells. Furthermore, both OCM-1 and OCM-3 cells expressed the HLA-DR protein in the absence of IFN- $\gamma$  induction, albeit to varying levels, which could be up-regulated by IFN- $\gamma$  congruent with the results obtained by FACS analysis.

Subsequently, we investigated the mRNA expression levels of CIITA and HLA-DRA in these uveal melanoma cell lines by real-time RT-PCR analysis. In contrast to OCM-3 cells, CIITA



**FIGURE 3.** Methylation pattern of the genomic CIITA-PIV region in uveal melanoma. *A*, Schematic overview of the restriction sites (indicated as vertical arrows) of the methylation-insensitive *MspI* (M) enzyme and the methylation sensitive *HpaII* (H) enzyme at the CIITA-PIV promoter region. Possible generated DNA fragment sizes are indicated as well as the location of the probe used. The transcriptional start site is also depicted. *B*, CIITA-PIV methylation pattern as determined by Southern blot analysis on *HindIII/HpaII* (H), or *HindIII/MspI* (M) digested genomic DNA of the uveal melanoma cell lines OMM1.3, Mel285, OCM-1, and OCM-3. *C*, Mel285 and OCM-1 cells were treated without (-) or with IFN- $\gamma$  (+) for 48 h before genomic DNA was isolated. CIITA-PIV methylation pattern was as determined as described under *B*.

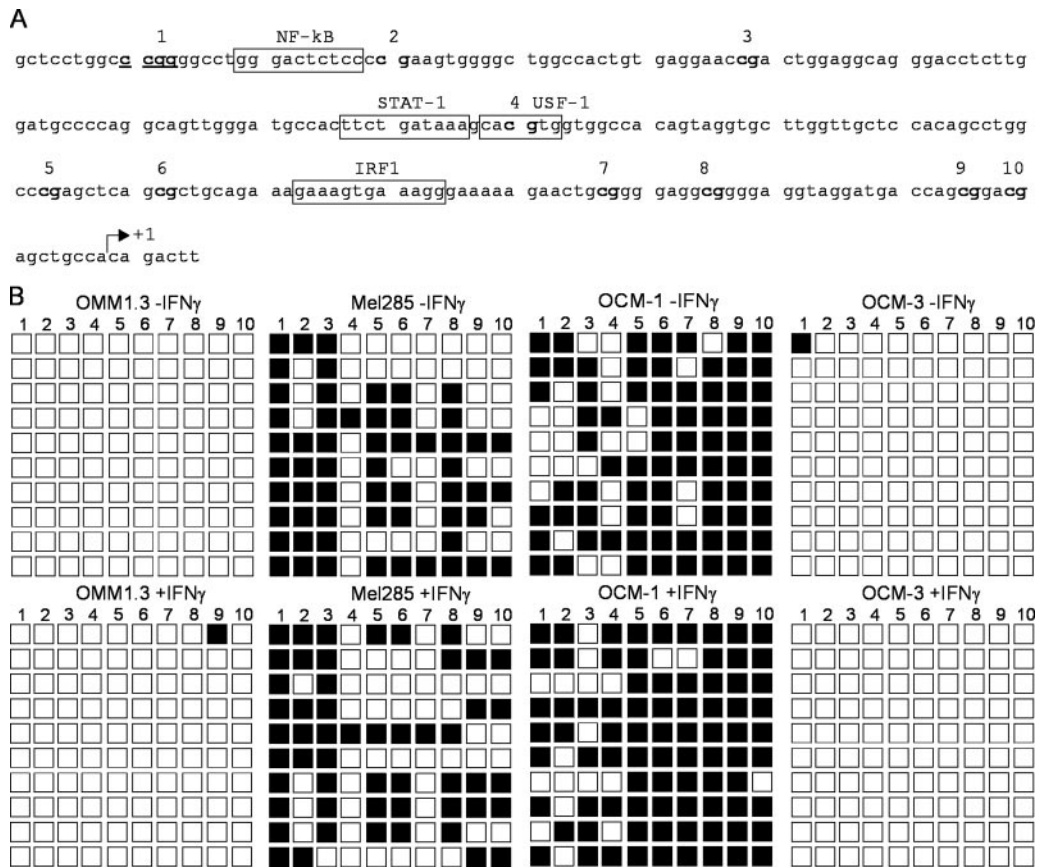
transcripts in the absence of IFN- $\gamma$  were hardly detectable in OMM1.3, Mel285, and OCM-1 cells. However, when compared with Mel285, OCM-1, and OCM-3 cells, which showed a clear increase in IFN- $\gamma$ -induced CIITA expression, CIITA transcript levels in OMM1.3 cells remained strongly reduced (Fig. 1C). CIITA transcript levels correlated with the expression of HLA-DRA mRNA, with the exception of Mel285 cells, which hardly displayed any induced HLA-DRA transcription (Fig. 1C). The finding that Mel285 cells lack expression of HLA-DRA mRNA and protein expression is in line with the observation that the CIITA transcripts expressed in Mel285 cells predominantly encode for nonfunctional CIITA protein (T. M. Holling and P. J. Van den Elsen, unpublished observations). Moreover, OMM1.3 cells were found to express functional CIITA transcripts as determined by cloning of full length OMM1.3-CIITA in the eukaryotic expression vector pREP-4, which was capable to activate a DRA promoter-reporter (data not shown).

### Transient promoter-luciferase reporter analyses

To examine whether the severely reduced CIITA transcript levels in OMM1.3 cells, after IFN- $\gamma$  exposure, are due to the lack of transcription factors involved in the activation of the principal IFN- $\gamma$ -responsive *MHC2TA* promoter, CIITA-PIV, we performed a transient transfection assay with a CIITA-PIV promoter-luciferase construct. The results of these assays revealed that OMM1.3 cells display IFN- $\gamma$ -responsiveness of the CIITA-PIV promoter to levels similar to those observed in Mel285 and OCM-1 cells (Fig. 2). These experiments show that the uveal melanoma cell lines tested contain all the necessary transcription factors and signaling pathways to activate CIITA-PIV.

### Southern blot and bisulfite sequence analysis

The inability of IFN- $\gamma$  to induce CIITA expression in OMM1.3 cells could result from CpG methylation in promoter DNA as had previously been found for other tumor cell types. To examine this,



**FIGURE 4.** CpG dinucleotide methylation pattern in Mel285 and OMM1.3 cells. **A**, Schematic overview of the CIITA-PIV region. CpG nucleotides are indicated in bold and numbered. Boxes, Known transcription factor binding sites. **B**, Bisulfite sequencing of the template strand of genomic DNA from OMM1.3, Mel285, OCM-1, or OMM1.3 cells without IFN- $\gamma$  treatment (upper row) or with 48 h IFN- $\gamma$  treatment (lower row). Numbers refer to the CpG nucleotide numbers in **A**. At least 10 bisulfite treated CIITA-PIV amplicons were examined in each case. Each sequenced amplicon is represented by a horizontal row of boxes. Methylated CpGs are indicated by black boxes, unmethylated CpGs are indicated by white boxes.

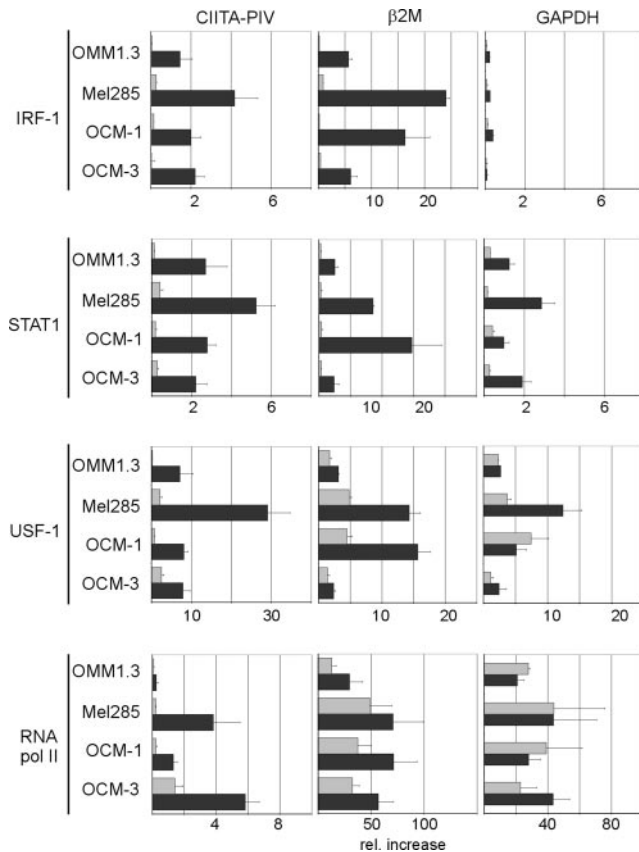
we first digested genomic DNA from OMM1.3, Mel285, OCM-1, and OCM-3 uveal melanoma cells with the restriction enzymes *HpaII* (methylation sensitive) and *MspI* (methylation insensitive), which cleave CIITA-PIV promoter DNA at the same restriction sites located outside the transcription factor binding sites for IFN- $\gamma$ -induced CIITA-PIV activation (Figs. 3A and 4A). The Southern blot analysis with the CIITA-PIV probe shows that in OMM1.3 cells these sites are unmethylated, which is similar to that in OCM-3 cells (Fig. 3B). Surprisingly, these sites in Mel285 and OCM-1 cells, which do express CIITA mRNA after IFN- $\gamma$  induction, were found to be methylated (Fig. 3B). We then investigated the possibility that exposure of Mel285 and OCM-1 cells to IFN- $\gamma$  would influence the methylation status of the promoter IV region of *MHC2TA*. However, IFN- $\gamma$  exposure to these uveal melanoma cells did not significantly influence the methylation status of the promoter IV region of *MHC2TA* (Fig. 3C).

To further evaluate the methylation pattern of CIITA-PIV in more detail, a bisulfite sequence analysis was performed with genomic DNA of all cell lines. The CIITA-PIV region contains several CpG dinucleotides of which one (CpG no.4) is located in the E-box, which is a USF-1 binding site. The binding sites for Stat-1 (GAS-box) and IRF-1 (ISRE) lack CpG dinucleotides and their binding is unlikely to be affected by methylation (Fig. 4A). In line with the Southern blot analysis, Mel285 and OCM-1 cells displayed an extended CpG dinucleotide methylation pattern, which was not significantly influenced by IFN- $\gamma$  exposure (Fig. 4B). Also in line with the Southern blot analysis, the promoter

region in OMM1.3 and OCM-3 cells was found unmethylated and also not affected by IFN- $\gamma$ . Furthermore, no nucleotide abnormalities were observed in the analyzed promoter DNA sequence of all the cell lines investigated.

*Impaired IFN- $\gamma$ -induced MHC2TA transcriptional activation in OMM1.3 cells does not correlate with IFN- $\gamma$ -induced IRF-1 and Stat-1 binding, but is associated with strongly reduced RNA polymerase II binding to CIITA-PIV chromatin*

To examine whether factors involved in the IFN- $\gamma$ -signaling pathway and mediating CIITA-PIV activation are recruited to CIITA-PIV, we investigated the presence of IRF-1, Stat-1, and USF-1 at CIITA-PIV in the uveal melanoma cell lines OMM1.3, Mel285, OCM-1, and OCM-3 by ChIP assays and real-time PCR analyses. ChIP assays with the promoter of the constitutively expressed and IFN- $\gamma$ -responsive  $\beta_2m$  gene and of *GAPDH* in these cell lines were used as control (37). Fig. 5 shows that, in IFN- $\gamma$ -uninduced cells, these factors were almost absent in CIITA-PIV chromatin. IFN- $\gamma$  stimulation resulted in recruitment of IRF-1, Stat-1, and USF-1 to CIITA-PIV chromatin in all uveal melanoma cell lines investigated. Because the CpG site in the E-box is mostly unmethylated or partially methylated (as shown for Mel285 and OCM-1 cells), it seems that these factors were recruited to CIITA-PIV, independent of the overall methylation status of CIITA-PIV in the uveal melanoma cell lines investigated.

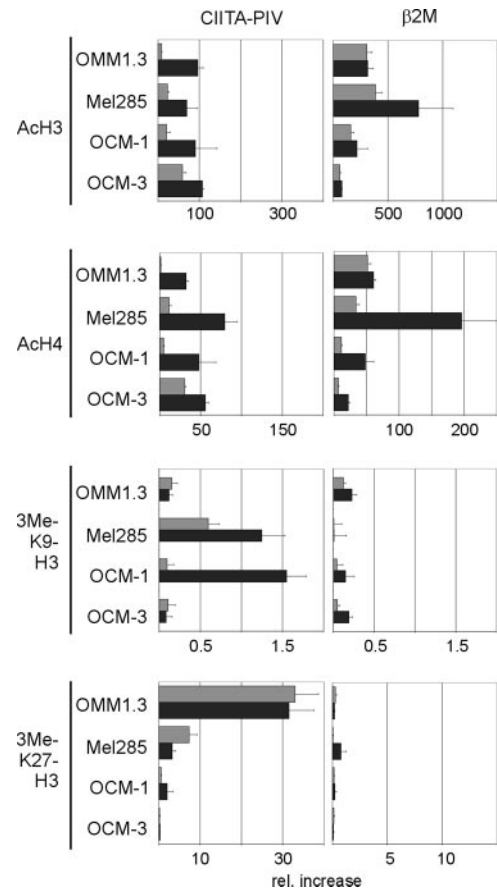


**FIGURE 5.** In vivo transcription factor and RNA polymerase II binding at the CIITA-PIV region in uveal melanoma. ChIP analysis was performed on genomic DNA of the four uveal melanoma cell lines, without (light bars) or with 48 h IFN- $\gamma$  treatment (dark bars). IRF-1, STAT1, USF-1, or RNA polymerase II (RNA polII) binding was determined on the CIITA-PIV region (*left column*), the  $\beta$ -2-microglobulin ( $\beta$ <sub>2</sub>M) promoter region (*middle column*), or the GAPDH promoter region (*right column*). Protein binding is indicated as relative increase. Error bars, SEM.

Next, we investigated the recruitment of RNA polymerase II to CIITA-PIV chromatin as a measure for assembly of the basal transcription initiation complex. Although IFN- $\gamma$  treatment induces the recruitment of the essential transcription factors involved in CIITA-PIV activation in OMM1.3 cells, RNA polymerase II could barely be detected (Fig. 5). In contrast, in Mel285 and OCM-1 cells, the presence of RNA polymerase II could readily be detected after IFN- $\gamma$  activation. In OCM-3 cells, RNA polymerase II is already constitutively present in CIITA-PIV chromatin and is increased upon IFN- $\gamma$  activation. These findings are in agreement with the real-time PCR analyses of CIITA transcripts in these cell lines (Fig. 1C). We conclude from these ChIP analyses that the strongly reduced CIITA expression levels after IFN- $\gamma$ -induction in OMM1.3 cells correlate with impaired RNA polymerase II assembly at CIITA-PIV in vivo.

*Lack of RNA polymerase II binding is associated with histone H3-lysine 27 trimethylation and recruitment of EZHZ to CIITA-PIV chromatin*

De-acetylation of histones at the promoter region has been demonstrated in tumor cells that show reduced CIITA expression (9, 12–14). In uninduced uveal melanoma cells, we could detect by ChIP varying levels of acetylated histone H3-lysines 9 and 14 and acetylated histone H4-lysines 5, 8, 12, and 16 in CIITA-PIV chromatin, which corresponded with transcript levels of CIITA in IFN-



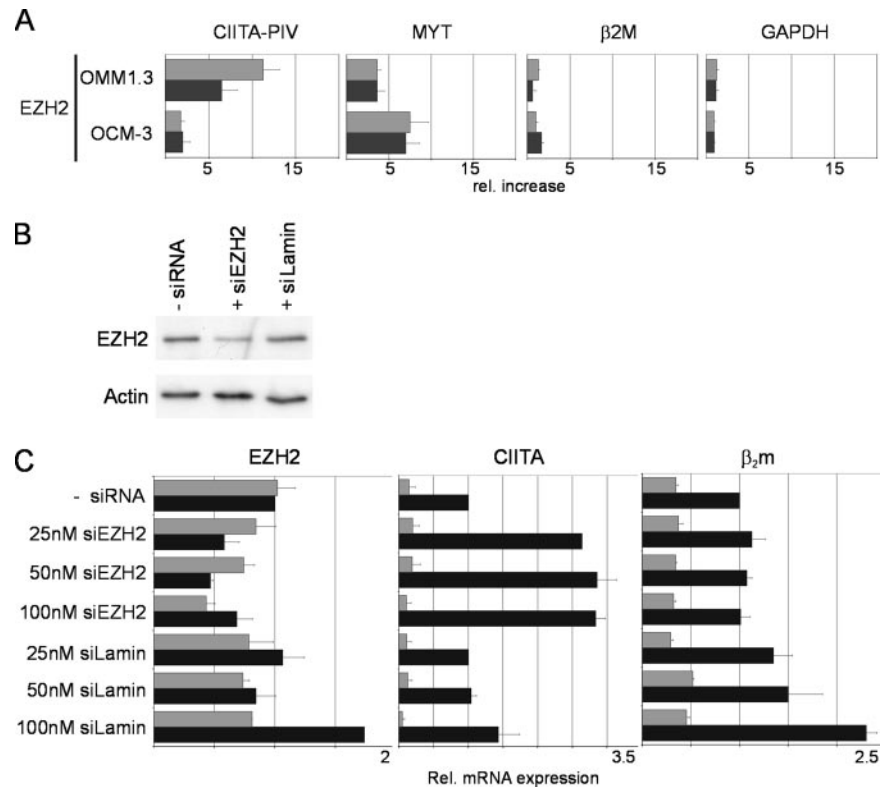
**FIGURE 6.** In vivo modifications of histone H3 and H4 at the CIITA-PIV region in uveal melanoma. ChIP analysis was performed on genomic DNA of the uveal melanoma cell lines indicated, without (light bars) or with 48 h IFN- $\gamma$  treatment (dark bars). Modifications of histones analyzed were: acetylated histone H3 (AcH3), acetylated histone H4 (AcH4), trimethylation of lysine 9 of histone H3 (3Me-K9-H3), and trimethylation of lysine 27 of histone H3 (3Me-K27-H3). ChIP and RT-PCR analysis were performed as described for Fig. 5.

$\gamma$ -uninduced cells (Fig. 6). The levels of these acetylated histone H3 and histone H4 lysine residues in CIITA-PIV chromatin was strongly enhanced after IFN- $\gamma$  treatment both in uveal melanoma cells with constitutive and IFN- $\gamma$ -induced expression of CIITA and in OMM1.3 cells with severely impaired CIITA expression levels (Fig. 6). Our data therefore indicate that, in the OMM1.3 cell line, histone hypoacetylation of CIITA-PIV chromatin does not correlate with reduced CIITA transcript levels after IFN- $\gamma$ -induction.

Next, we investigated histone modifications of CIITA-PIV chromatin that are associated with silent chromatin and transcriptionally inactive genes, with an emphasis on the trimethylation of histone H3-lysine 9 and of histone H3-lysine 27 (3Me-K9-H3 and 3Me-K27-H3, respectively). The 3Me-K9-H3 modification was negligible in CIITA-PIV chromatin of uninduced and IFN- $\gamma$ -induced OMM1.3 and OCM-3 cells (Fig. 6). In uninduced OCM-1 cells, this modification was also negligible in CIITA-PIV chromatin whereas in uninduced Mel285 cells, low levels of this modification could be detected. In both OCM-1 and Mel285 cells, the levels of 3Me-K9-H3 in CIITA-PIV chromatin were enhanced after IFN- $\gamma$ -induction, but overall the levels of this histone methylation modification remained relative low (Fig. 6). In contrast, the 3Me-K27-H3 modification was abundantly present in CIITA-PIV chromatin in OMM1.3 cells when compared with Mel285,



**FIGURE 7.** EZH2 binding and involvement in expression of CIITA RNA. **A**, In vivo binding of EZH2 at the CIITA-PIV region in uveal melanoma. ChIP analysis was performed on genomic DNA of the uveal melanoma cell lines indicated, without (light bars) or with 48 h IFN- $\gamma$  treatment (dark bars). Analyzed was the presence of EZH2 at the CIITA-PIV, MYT (control for EZH2 ChIP),  $\beta_2m$ , and GAPDH promoter region. ChIP and RT-PCR analysis were performed as described for Fig. 5. **B**, Western blot analysis of the level of total EZH2 after transfection of siRNAs in OMM1.3 cells. Forty-eight hours after the second transfection cells were harvested for protein analysis. -siRNA, cells transfected without siRNA; +siEZH2, cells transfected with 100 nM siEZH2 duplex; +siLamin, cells transfected with 100 nM siGLO Lamin A/C. **C**, Quantitative PCR analysis of CIITA transcript levels in OMM1.3 cells transfected with or without siRNAs. Total RNA was isolated from cells without (gray bars) or with (black bars) 24 h treatment with IFN- $\gamma$ . Values of EZH2, panCIITA, and  $\beta_2m$  were normalized to GAPDH. The amount of transcripts in OMM1.3 cells transfected in the absence of siRNA were set at 1. Error bars, SEM.



OCM-1, and OCM-3 cells (Fig. 6). The 3Me-K9-H3 and 3Me-K27-H3 modifications were hardly detectable in  $\beta_2m$  promoter chromatin in any of the uveal melanoma cell lines investigated (Fig. 6).

Next, we investigated by ChIP whether the high levels of 3Me-K27-H3 correlated with enhanced levels of EZH2 in CIITA-PIV chromatin. As shown in Fig. 7A, OMM1.3 cells displayed a significant increase in EZH2 levels in CIITA-PIV chromatin when compared with OCM-3 cells. In both cell lines, EZH2 is recruited to the MYT promoter, whereas hardly any association of EZH2 is found with the  $\beta_2m$  and GAPDH promoters. Together, in line with the observed 3Me-K27-H3 modification, EZH2 was found to be associated with CIITA-PIV chromatin in OMM1.3 cells.

*RNA interference mediated the down-regulation of EZH2 results in enhanced CIITA expression levels after IFN- $\gamma$  induction in OMM1.3 cells*

Next, we determined whether EZH2 is involved in the expression of CIITA after IFN- $\gamma$  induction in OMM1.3 cells. For those purposes, OMM1.3 cells were transfected with EZH2 siRNA duplex and down-regulation of EZH2 was evaluated by Western blot and real-time PCR analysis. The optimized transfection procedure resulted in a reduction in EZH2 protein expression of 56% by the introduced EZH2 siRNA duplex (Fig. 7B, lane +siEZH2), while transfection medium alone (-siRNA) or an irrelevant siRNA smart-pool targeting Lamin A and C (+siLamin) did not affect the EZH2 protein expression levels. Next, we determined the amount of EZH2, CIITA, and  $\beta_2m$  mRNA transcripts in siRNA transfected OMM1.3 cells. As shown in Fig. 7C, we observed a dose-dependent down-regulation of constitutive EZH2 expression in OMM1.3 cells, which was influenced by IFN- $\gamma$ . OMM1.3 cells with down-regulated EZH2 mRNA levels displayed an increase in the levels of CIITA transcripts after IFN- $\gamma$  treatment, while no increase in CIITA mRNA levels was observed in cells transfected

with the irrelevant siLamin. Moreover, in the EZH2 knock-down samples, the transcript levels of  $\beta_2m$  were not affected, which is in line with the ChIP analysis where we did not observe any EZH2 or the 3Me-K27-H3 modification in the  $\beta_2m$  promoter region.

Together, these observations suggest a role for EZH2 in the silencing of CIITA expression in IFN- $\gamma$ -treated OMM1.3 uveal melanoma cells.

## Discussion

In the current study, we show that silencing of IFN- $\gamma$ -inducible MHC-II molecule expression in malignant uveal melanoma cells was associated with distinct epigenetic modifications involving chromatin of the principal IFN- $\gamma$ -responsive *MHC2TA*-promoter IV (CIITA-PIV). This is illustrated by the observation that CIITA transcript levels after IFN- $\gamma$  exposure in the uveal melanoma cell line OMM1.3 are strongly reduced when compared with the other cell lines investigated. The impairment in CIITA transcript levels is associated with relatively high levels of tri-methylated histone H3-lysine 27 (3Me-K27-H3) and EZH2 in CIITA-PIV chromatin (see Table II), whereas the 3Me-K9-H3 and CpG dinucleotide methylation modifications were absent. Notably, OMM1.3 cells did not display hypoacetylation at CIITA-PIV after IFN- $\gamma$ -induction, but manifested an increase in histone acetylation similar to the other cell lines investigated. It suggests dominance of the 3Me-K27-H3 modification in the IFN- $\gamma$ -induced dynamic chromatin environment. Moreover, regardless of the presence of methylated CpG dinucleotides and low levels of the 3Me-K9-H3 modification in CIITA-PIV chromatin in Mel285 and OCM-1 cells, transcription of the PIV-isoform of CIITA was still found after IFN- $\gamma$  induction. However, these cell lines did not manifest the 3Me-K27-H3 modification as observed in OMM1.3 CIITA-PIV chromatin. OCM-3 cells, which display constitutive and IFN- $\gamma$ -induced transcription of *MHC2TA*, lacked the 3Me-K27-H3 histone modification, in addition to lack of CpG dinucleotide methylation in CIITA-PIV chromatin (Table II). We also observed that



TABLE II. Overview of constitutive and IFN $\gamma$ -induced *MHC-II* expression and epigenetic modifications at the *CIITA-PIV* region in uveal melanoma cell lines<sup>a</sup>

Cell line	OMM1.3		Mel285		OCM-1		OCM-3	
IFN $\gamma$	-	+	-	+	-	+	-	+
HLA-DR protein expression	-	-	-	-/+	-	+	+	++
DNA methylation	-	-	+	+	+	+	-	-
Transcription factor binding	-	+	-	+	-	+	-	+
RNA polymerase II binding	-	-	-	+	-	+	+	++
Acetylated histone H3/H4	-	++	-/+	++	-/+	++	+	++
3Me-K9-H3 modification	-	-	+	++	-	++	-	-
3Me-K27-H3 modification	++	++	-/+	-	-	-	-	-
EZH2 binding	++	+	ND	ND	ND	ND	-/+	-/+

<sup>a</sup> -, absent; +, detectable; ++, strongly enhanced; -/+, barely detectable.

none of the above-mentioned epigenetic modifications interfered with assembly at *CIITA-PIV* of transcription factors essential for IFN- $\gamma$ -induced transcription of *MHC2TA* (Table II). The 3Me-K27-H3 modification correlated with recruitment of EZH2 to *CIITA-PIV* chromatin in OMM1.3 cells. The observed increase in *CIITA* transcript levels after IFN- $\gamma$  treatment of OMM1.3 cells with siRNA-down-regulated EZH2 expression suggests that this histone methyl transferase plays a role in *MHC2TA* transcriptional silencing in OMM1.3 cells.

On the basis of the results obtained with OMM1.3 cells, our observations suggest a correlation between the 3Me-K27-H3 modification and impaired RNA polymerase II recruitment to *CIITA-PIV*. In contrast, the CpG dinucleotide methylation modification in conjunction with low levels of the histone H3-lysine 9 modification in *CIITA-PIV* DNA as seen in Mel285 and OCM-1 cells do not greatly interfere with RNA polymerase II recruitment into *CIITA-PIV* chromatin. The tri-methylated histone H3-lysine 27 modification therefore seems to be the most important epigenetic regulator, which affects *CIITA* transcription levels after IFN- $\gamma$ -induction, in uveal melanoma cells.

In all cell lines investigated, recruitment after IFN- $\gamma$  induction of regulatory factors to *CIITA-PIV* (i.e., IRF-1, Stat-1, and USF-1) is not impaired by the 3Me-K27-H3 modification or by CpG dinucleotide methylation. It should be noted that of the critical regulatory elements in *CIITA-PIV* (i.e., ISRE, GAS-box and E-box), only the E-box contains a CpG dinucleotide. In vitro methylation of this E-box CpG affects binding of USF-1 as has been demonstrated in EMSA (3). Therefore, it is of interest to note that of the various CpG dinucleotides in *CIITA-PIV*, the E-box CpG apparently is not or partially methylated in vivo (Fig. 4).

Promoter DNA methylation is regarded as a major epigenetic mechanism for silencing gene expression (38). There are two general mechanisms by which methylation-modified DNA inhibits gene expression. As mentioned above, methylation-modified CpG dinucleotides in cognate binding sites of specific DNA binding factors could interfere in promoter assembly of these factors. In contrast, the methylation modified CpG dinucleotides in DNA form binding sites of methyl-DNA binding proteins (e.g., MeCP2), which act as transcriptional repressors through their interaction with histone deacetylases. However, recently it has been documented that gene transcription in some cases occurs despite fully methylated promoter DNA (39, 40). Furthermore, similar observations have been made with respect to histone deacetylases, which may act for some genes as transcriptional activators rather than repressors (41). As mentioned above, we do not observe interference in the recruitment of activating transcription factors critical for IFN- $\gamma$ -induced transcriptional activation of *CIITA-PIV* in uveal melanoma cells in the presence of the observed epigenetic modifications. This is seemingly in contrast to the observation

made by Morris et al. (3), who showed impaired factor recruitment to *CIITA-PIV* after IFN- $\gamma$  exposure in the choriocarcinoma cell lines JEG3 and JAR. However, it should be noted that the uveal melanoma cell panel studied did not contain a cell line displaying both triple methylated lysine 27 in histone H3 in the presence of CpG dinucleotide methylation. Therefore, we cannot exclude the possibility that a combination of the above-mentioned epigenetic modifications not only affects RNA polymerase II recruitment, but also recruitment of IFN- $\gamma$ -induced activation factors to *CIITA-PIV*. This notion is underscored by our recent observation that besides dense CpG dinucleotide methylation, *CIITA-PIV* chromatin in JEG3 and JAR cells also contains high levels of the 3Me-K27-H3 modification (T. M. Holling and P. J. Van den Elsen, unpublished observations).

As mentioned before, the enzymatic activities that modify DNA and histones by methylation are intimately linked (22, 23). The 3Me-K9-H3 mark recruits Dnmt 1 and 3a through HP1 interactions (22), and also EZH2 has been found to interact with Dnmts (23). Therefore, the lack of CpG dinucleotide methylation and absence of the 3Me-K9-H3 mark at *CIITA-PIV* chromatin in OMM1.3 cells would also infer absence of EZH2-associated Dnmts in OMM1.3 cells. This notion is in line with the recent observation that the 3Me-K27-H3 modification premarks genes for de novo methylation in cancer (42). It could therefore be argued that the epigenetic make-up of the *CIITA-PIV* region in OMM1.3 cells indeed reflects premarking for de novo methylation of DNA, and that this is an intermediate epigenetic state in the complete shut down of Ag presentation functions.

Together, the results from our studies with uveal melanoma cell lines suggest that the trimethylation of histone H3-lysine 27 is an important epigenetic modification, which contributes to strongly reduced *CIITA* expression levels after IFN- $\gamma$  induction and which would explain the lack of MHC-II molecule expression at the cell surface.

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## Disclosures

The authors have no financial conflict of interest.

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