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Oncogenic RAS alters the global and gene-specific histone modification pattern during epithelial–mesenchymal transition in colorectal carcinoma cells

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ABSTRACT

The presence of different forms of histone covalent modifications, such as phosphorylation, acetylation and methylation in localized promoter regions are markers for chromatin packing and transcription. Activation of RAS signalling pathways through oncogenic RAS mutations is a hallmark of colorectal cancer. Overexpression of Harvey-Ras oncogene induces epithelial–mesenchymal transition (EMT) in Caco-2 cells. We focused on the role of epigenetic modifications of histone H3 and its dependence on RAS signal transduction pathways and oncogenic transformation. Using cell lines stably overexpressing oncogenic Harvey-RAS with EMT phenotype, we studied the acquired changes in the H3 histone modification patterns. Two genes show inverse protein expression patterns after Ha-RAS overexpression: Cyclin D1, a cell cycle-related gene, and the EMT marker-gene E-cadherin. We report that these two genes demonstrate matching inverse histone repression patterns on their promoter, while histone markers associated with an active state of genes were affected by the RAS-activated signalling pathway MEK–ERK–MSK1. Furthermore, we show that though the level of methyltransferases enzymes was increased, the status of H3 three-methylation at lysine 27 (H3K27me^3), associated with gene repression on the promoter of Cyclin D1, was lower. Together, these results suggest that histone covalent modifications can be affected by oncogenic RAS pathways to regulate the expression of target genes like Cyclin D1 or E-cadherin and that the dynamic balance of opposing histone-modifying enzymes is critical for the regulation of cell proliferation.

1. Introduction

In colorectal cancer, alterations in the activity of tumor suppressor genes and oncogenes are accompanied by reversible epigenetic changes, which result in inheritable gene function modifications and lack of DNA sequence alterations. Different forms of epigenetic mechanisms have been shown to modify the expression of key genes during colorectal tumor progression. Global DNA hypomethylation (Feinberg and Vogelstein, 1983), as well as hypo- and hypermethylation of specific DNA regions in promoters of oncogenes (Nakamura and Takenaga, 1998), tumor suppressor (Di et al., 2002) and mismatch repair genes (Edelmann et al., 1997) contribute to tumor progression. Epigenetic changes have also been described to play a role in chromosomal instability (Suter et al., 2004) or in loss of gene imprinting, which occurs at the earliest stages of colorectal neoplasia causing abnormal gene expression (Sakatani et al., 2005) and is as well mediated by DNA methylation and covalent histone modifications. Histone covalent modifications such as acetylation, methylation and phosphorylation are distributed along promoters, on coding and intergenic regions modifying chromatin structure. Patterns of specific histone modifications constitute the "histone code" (Berger, 2002) that controls the recruitment of many factors in processes such as transcription, DNA repair or DNA replication. In general, acetylations of histone H4 and H3 on lysines 9 and 14 (H3K9/14ac), phosphorylation of histone H3 at serine 10 (H3S10p), and trimethylation of histone H3 at lysine 4 (H3K4me^3) are considered to be marks of gene expression activation. On the other hand, di- or trimethylations of histone H3 lysine 9 (H3K9me^2, H3K9me^3) and lysine 27 (H3K27me^3) have been linked to gene repression (Barski et al.,

Abbreviations: EMT, epithelial–mesenchymal transition; AP-1, activating protein 1; MAPK, mitogen-activated protein kinase; HAT, histone acetyltransferase; HDAC, histone deacetylase; ChIP, chromatin immunoprecipitation.

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2007). However, different modifications can have different effects depending on the chromatin context, while studies have presented a crosstalk among different histone modifications (Wang et al., 2008).

Epigenetic deregulation may affect different aspects of tumor progression and could be linked to patient prognosis. The global pattern of histone modifications has been reported as a risk-predictor for the tumor recurrence in prostate, gastric, breast and pancreas cancer patients (Seligson et al., 2005; Park et al., 2008a,b; Fraga et al., 2005). Moreover, changes in genes encoding proteins that participate in acetylation and methylation of H3 and H4 lysine residues have also been found in many types of tumors (Varambally et al., 2002; Kirmizis et al., 2003).

In the case of colorectal cancer, activation of signalling pathways induced by Ki-RAS or BRAF mutations regulates cell proliferation, apoptosis and several other cellular processes. Deregulation of these signalling pathways might result in epigenetic alterations, as well. In parallel, oncogenic Ha-RAS-transformed cells have been shown to acquire characteristics of cells that have undergone epithelial–mesenchymal transition (EMT) (Voulgari et al., 2008; Andreolas et al., 2008) and express stem cell markers (Morel et al., 2008). EMT is a developmental programme that has been related to cancer invasion and metastasis (Thiery and Sleeman, 2006). Constitutive activation of the RAS–MAPK pathway by oncoproteins leads to an increase in the H3S10p steady-state level through activation of ERK1/ERK2 or the p38 stress-signalling pathway (Dyson et al., 2005). Elevated level of H3S10p is associated with transcription and is observed in RAS-transformed cells (Chadee et al., 1999). Among several kinases, the stimuli-dependent MSK1/MSK2 (Drobic et al., 2004; Soloaga et al., 2003) and RSK2 (Trivier et al., 1996) have been described to be responsible for H3S10 phosphorylation. Moreover, several histone acetyltransferases (HATs), among which GCN5 or PCAF, have been shown to exhibit increased activity, both in vitro and in vivo, on H3 peptides which are phosphorylated on Ser-10 (Lo et al., 2000).

Cyclin D1 is overexpressed in adenocarcinomas (Sutter et al., 1997) and contributes to colonic tumor growth in colorectal cancer (Hulit et al., 2004). Various studies have characterized Cyclin D1 as a characteristic RAS target gene, while its promoter *cis*-elements have shown involvement in its RAS-dependent activation. The promoter region of the Cyclin D1 gene contains binding sites for AP-1 (Albanese et al., 1999), CREB, SP1 (Nagata et al., 2001; Hilton et al., 2005), ATF/CREB (Lee et al., 1999) and NFkB (Joyce et al., 1999), whose accessibility and occupancy by the cognate transcription factors depend on the chromatin structure and histone modifications. Another gene implicated in tumor growth is E-cadherin, whose loss of expression correlates with tumor progression and metastasis. Downregulation of the expression levels of E-cadherin has been associated with central cellular pathways such as TGFβ, β-catenin or the Wnt signalling, while hypermethylation of the E-cadherin promoter has been linked to promoter-silencing and therefore to carcinogenesis and EMT (Bolos et al., 2003; Comijn et al., 2001; Conacci-Sorrell et al., 2003).

In this study, we focused on understanding the role of global histone modifications in a model of colorectal cancer and how these modifications may be affected by pathways activated by oncogenes: Ha-Ras, Ki-Ras and BRAF. Transformed Caco-2 cells by these oncogenes showed aggressive phenotype with respect to Caco-2 parent cells. We found that two genes, Cyclin D1 and E-cadherin, showed inverse mRNA and protein expression levels after stable overexpression of their activated forms. We deliver evidence showing that the two genes Cyclin D1 and E-cadherin are reversely regulated by histone modifications in a RAS-dependent manner. Finally, we suggest that histone H3 K9/K14 acetylation levels on Cyclin D1 promoter are regulated by the MEK–ERK–MSK1 pathway.

2. Materials and methods

2.1. Cell lines

The Caco-2 cell line was supplied by the American Type Culture Collection (Manassas, VA, USA). Caco-2, an intermediate adenocarcinoma cell line, does not contain RAS mutations. Caco-2 stable-transfected cell lines with oncogenic Kirsten- (referred to as Caco-K6 and Caco-K15) Harvey-RAS (referred to as Caco-H1 and Caco-H2) (Roberts et al., 2006) BRAF genes (referred to as Caco-Br13 and Caco-Br23) and empty vector (referred to as Caco-neo) (Oikonomou et al., 2009) have been previously established in our lab. Cells were cultured in a humidified atmosphere at 37° C with 5% CO_2 and grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen LifeTechnologies, Carlsbad, CA) supplemented with 10% foetal bovine serum (FBS) (Invitrogen), 1% penicillin/streptomycin (Invitrogen) and non-essential amino acids (Invitrogen).

2.2. Protein kinase inhibitors

Caco-H2 cells were treated with MEK inhibitor UO126 (5–40 μM) (Alexis Biochemicals, CA) to block MEK–ERK pathway. Cells were incubated with inhibitor for 1–4 and 6 h. In parallel, Wortmanin (5 μM) (Alexis Biochemicals) was added to block AKT signalling pathway at the same time points. The cells were plated on 6-well plates in DMEM + 10% FBS and collected, and total lysates were loaded on a 10% PAGE gel. MSK1 kinase activity was blocked by adding H89 inhibitor (10 μM) (Alexis Biochemicals) and again total extracts were prepared as described in previous studies.

2.3. Cell extracts—Western blot

Total extracts were prepared from cells that were grown in 10-cm Petri dishes, washed 3 times with ice cold phosphate-buffered saline (PBS) and collected in ice cold lysis buffer (50 mM Tris–HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, 10 mM NaF, 1 mM EGTA, and 1% Triton X-100 plus protease inhibitors). Acid extractions of histone proteins were performed in resuspending cells, after a 3-time wash with PBS, in Triton extraction buffer (TEB; PBS containing 0.5% Triton X-100 (v/v), 2 mM PMSDF, 0.02% NaN_3) and kept on ice for 10 min. Nuclei were collected with a centrifugation at 6500 × *g* for 10 min at 4 °C and were resolved in TEB once more and after centrifugation they were resuspended in 0.2 N HCl. After an o/n incubation, the cells were centrifuged at 6500 × g for 10 min resulting in a supernatant containing the histone proteins. Extracts were resolved on SDS-PAGE (10 or 12% w/v acrylamide), transferred to nitrocellulose membranes (BioTrace NT; Pall Life Sciences, NY) and immunoblot with appropriate antibodies proceeded overnight. Standard chemiluminescence protocol was applied to visualize the proteins by using horseradish peroxidase conjugated anti-rabbit IgG or anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) and enhanced chemiluminescence detection system (ECL) (Amersham Biosciences, Buckinghamshire, UK).

2.4. Immunofluorescence

Cell cultures were fixed with 4% paraformaldehyde, washed, permeabilized with 0.3% Triton X-100 and blocked with 5% BSA prior to incubation with primary antibodies (H3K9–14ac, 1:500, Upstate; H3S10P, 1:200, Santa Cruz Biotechnology; H3K4me^3, 1:2000, Abcam; H3K9me^2, 1:500, Abcam; H4K12ac, 1:500 Abcam; H3K27me^3, 1:500, Abcam; p-MSK1, 1:500, Abcam; p-ERK, 1:500, Santa Cruz Biotechnology; Aurora A, 1:500, BD Biosciences; GCN5, 1:100, Santa Cruz Biotechnology; PCAF, 1:100, Santa Cruz Biotechnology, JMJD3, 1:200, Abgent; EZH2, 1:500, BD Biosciences). Cells

were then stained with Alexa488- or Alexa555-conjugated secondary antibodies (Molecular Probes). The nuclei counterstained with DAPI and the samples were mounted in Vectashield (Vector labs).

2.5. RNA extraction and real-time PCR

Total RNA extraction was isolated with TRIZOL reagent (Invitrogen) and reverse transcribed to cDNA with SuperscriptRT transcriptase II (Invitrogen). Real-time PCR was carried with specific primers and expression was normalized with GADPH. Primer pairs (5′–3′) were: PCAF: GCC CTA GCT GCT CAT GTT TC and GGT TTT TCA AAT GGG GGT TT; GCN5: CTA TGG GGC AAA CTC TCC AA and ATC CTC CAG GGT CAG GTT CT; EZH2: CAT AAT GTA TTC TTG GTC TCC C and AAA CCC ACA TTC TCT ATC CC; JMJD3: GAA ACC GAA GAT CAA CAC TG and TTG CTC TCC AGA TAG ATG CT; GADPH: GAA GGT GAA GGT CGG AGT and CAT GGG TGG AAT CAT ATT GGA A; HDAC9: GGC TCAG CTT CAG GAG CAT A and CTT CCT GTT CTT GCC TCT GC; primer used for HDAC4, HDAC5 and HDAC7 was in accordance with Ozdağ et al. (2006).

The reaction was carried in 96-well plates (BioRad, Hercules, CA, USA). PCR reactions were run in triplicate in the presence of SYBR-Green (BioRad). Melting curve analyses revealed a single product. Analysis was done using iCycler iQreal-time Detection System software (BioRad). The experiment was repeated twice for each gene and cell line. Standard deviation bars for each histogram were calculated automatically by the software of real-time machine based on the standard deviation of the triplicate both for GAPDH and for the target genes.

2.6. ChIP

Chromatin immunoprecipitation was performed essentially as described previously (Voulgari et al., 2008). Pre-cleared chromatin was incubated overnight by rotation with 2 μg of antibody (p-Jun, Santa Cruz Biotechnology; H3K9–14ac, Upstate; H3K27me^3, Upstate; H3K9me^2, Upstate; H3K9–14ac and H3K4me^3, Abcam; EzH2, BD Biosciences) or no antibody as a negative control. Immunoprecipitates were resuspended in 40 μl TE buffer whereas inputs were resuspended in 90 μl TE buffer. Inputs and immunoprecipitated DNA samples were quantified by real-time PCR. The reaction was carried in 96-well plates (BioRad) and PCR reactions were run in triplicate in the presence of SYBR-Green (BioRad). Cycling conditions include: denaturing step of 3 min at 95 °C followed by 40 cycles of 30 s at 95°; and annealing/elongation at 64 °C. Ct values of the immunoprecipitated samples were normalized with Ct input values. GADPH was used as an internal control. The experiment was repeated twice for each histone modification. Cyclin D1 promoter primers for real-time PCR were: 5′-ATG CCC AGG GCA AAT TCT AAA-3′ and 5′-ACA CAC CTC TGA ATG GAA AGC-3′; E-cadherin promoter primers for real-time PCR were: 5′-TCTGATCCCAGGTCTTAGTG-3′ and 5′-GGCTGGAGCGGGCTGGAG-3′

2.7. siRNA transfection assay

Plasmid DNA was transfected into cells by the calcium phosphate method that has been described previously (Chen and Okayama, 1987). For the transfection of siRNA, cells were treated with human siGenome ON-TARGET plus SMARTpool c-Jun (L-003268-00-0020) according to the manufacturer's protocol (Dharmacon, Chicago, IL). 48 h after transfection cells were harvested. Total protein extracts and RNA were extracted as described previously. To distinguish sequence-specific silencing from non-specific effects, negative controls were performed by using siCONTROL RISC-free siRNA (D-001210-03).

3. Results

3.1. Global levels of histone modifications in colon adenocarcinoma cell lines

To understand how patterns of global histone modifications are altered by activated oncogenes, we studied histone modifications in adenocarcinoma cell lines that have been stably transformed with the oncogenic form of Ha-RAS (Caco-H), Ki-RAS (Caco-K) and BRAF (Caco-BR). Immunostaining with antibodies specific for histone markers associated with active genes such as H3K9–14ac, H3S10P and H3K4me^3 indicated (Fig. 1A and D) a slight increase in the levels of H3S10P and H3K4me^3 levels in both Caco-H and Caco-K cells as compared to the non-transformed cell line Caco-2. On the other hand, immunostaining with antibodies specific for histone markers associated with repressed genes showed that the levels of H3K27me^3 were reduced in the Caco-H cell line as compared to the Caco-2 cells (Fig. 1B and D). Interestingly, at the same time, in the Caco-H cells, which are cells that have undergone an epithelial–mesenchymal transition (EMT), a unique distribution pattern of H3K27me^3 was observed, as the staining was lost in the perinuclear area and appeared as a granular pattern in the central nuclear area (Fig. 1C). To evaluate the differences on the global levels of histone modifications observed by immunostaining, we analyzed their levels by Western blot (Fig. 1D). Similarly, H3K27me^3 levels (associated with a repressed state of genes) were decreased in Caco-H cells while H3S10P and H3K4me^3 levels (associated with an active state of genes) were increased in the same cell line as compared to Caco-2 cells, indicating an inverse regulatory function of global histone modification levels in relation with the expression of oncogenic Ha-RAS and with EMT.

3.2. Levels of histone-modifying enzymes are affected by oncogenic Ha-RAS

In order to clarify the pathway followed for the changes in the global histone modifications in the cell lines studied, we determined the mRNA and protein levels of GCN5, PCAF, acetyltransferases, EzH2 histone methyltransferase and JMJD3 demethylase by Western blot and real-time PCR analysis (Fig. 2A and B). Notably, the H3K27me^3-specific methyltransferases EZH2 and JMJD3 were upregulated in the two cell lines overexpressing the oncogenic Ha-RAS protein (Caco-H1 and Caco-H2) both in protein and mRNA levels, indicating a possible involvement of these two histone methyltransferases in the regulation of the decreased global levels of H3K27me^3 in cells overexpressing the mutant Ha-RAS gene and thus undergoing EMT. GCN5, PCAF acetyltransferase enzymes also showed increased protein and mRNA levels overall in Caco-BR clones. In Fig. 2C we present a comparison between Caco-neo, empty vector containing cells, Caco-2 and Caco-H2 cells. In these cells we analyzed by immunofluorescence and confocal microscopy the protein levels of EzH2 and JmJD3 and the histone modification H3K27me^3. Caco-H2 cells showed variation, both for the proteins and the status of three-methylation of the lysine 27 of histone H3 as well as for EzH2 and JmJD3 and the histone markers H3K27me^3 and H3K9–14ac as compared to Caco-2 and Caco-neo cells.

3.3. MEK signalling regulates global H3K9/14ac levels in Ha-RAS transfected Caco-2 cells

In Caco-H cells the MEK/ERK and AKT pathways were shown to be highly activated (Fig. 3A) as compared to the Caco-2 cell line. In order to study the relation between the histone modification which is associated with increased gene expression (H3K9–14ac)

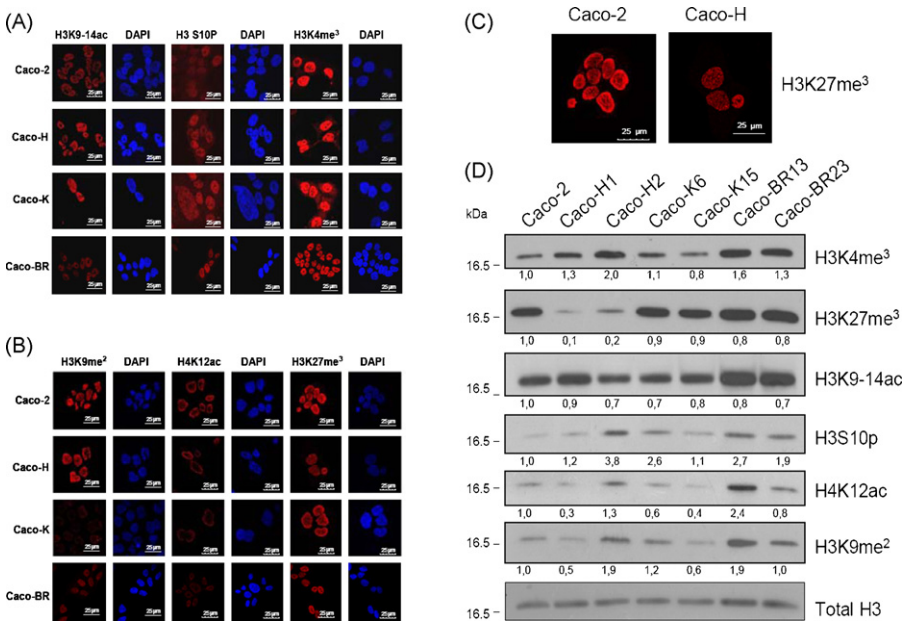

Fig. 1. Global histone modification levels in cell lines overexpressing the oncogenic Ha-RAS, Ki-RAS and BRAF protein. (A) Immunofluorescence confocal imaging of histone modification markers associated with activated genes in Caco-2, Caco-H, Caco-K and Caco-BR cells. (B) Immunofluorescence confocal imaging of histone modification markers associated with repressed genes in Caco-2, Caco-H, Caco-K and Caco-BR cells. Nuclei were stained with DAPI. Merged images are shown (magnification 63×). (C) Magnification of a Caco-2 versus Caco-H cell stained with the histone modification marker H3K27me^3 indicating the differential localization pattern. (D) Western blot analysis of the levels of histone markers in the above-mentioned cell lines. Numbers below the WB bands represent the value of quantification of the spots normalized to total histone H3.

and Ha-RAS overexpression, we followed with the analysis of the involvement of the MEK/ERK pathway in this specific phenomenon. Treatment of Caco-H cells with the MEK inhibitor UO126 (40 μM) in a time-course of 6 h resulted in a decrease in p-ERK protein levels after 1 h of treatment (Fig. 3C, E and F). To demonstrate that off-target effects due to high concentration of UO126 do not affect histone modifications, we treated Caco-H cells with different concentration of UO126 (5, 10, 20 and 40 μM) for 1 and 2 h. For each point we performed a Western blot analysis and the expression levels of p-ERK, p-MEK, Cyclin D1 and H3K9–14ac are presented in Fig. 3B. The inhibition of p-ERK begins also with concentration as low as 5 μM, which however is not sufficient to block p-ERK for a long time in Caco-H cells. Indeed, after 1 h, and perhaps also earlier, the status of p-ERK becomes normal. No changes in p-MEK, Cyclin D1 and H3K9–14ac appeared within 2 h of treatment with any concentration of UO126. In fact, the levels of H3K9/14ac decreased after 3 h of UO126 treatment at 40 μM, following the downregulation of p-ERK (Fig. 3C). At the same time, to investigate the effect of MEK–ERK–MSK1 pathway on H3K9–14ac, we inhibited the kinase activity of MSK1 by treating the cells by specific MSK1 inhibitor H89 (10 μM). In this case, global levels of H3K9/14ac decreased after 6 h of MSK1 inactivation (Fig. 3D). Under the same conditions, PI3 kinase had no effects on the global levels of H3K9–14ac (data not shown) indicating that the specific participation of the MEK/ERK pathway in the regulation of the active global histone modification levels.

3.4. Two Ha-RAS target genes show inverse histone repression patterns on their promoter

Cyclin D1 and E-cadherin are two genes that are inversely regulated between Caco-2 and Caco-H cell lines as observed both at protein level by Western blot analysis and at mRNA level through real-time PCR analysis (Fig. 4A, left and right). Therefore, they were selected as 'marker-genes' in order to analyze in detail the specific histone modification alterations on gene promoters during cancer progression by ChIP analysis. Interestingly, the analysis of the histone markers that are associated with the repressed state of genes (H3K27me^3 and H3K9me^2), when comparing the intermediate adenoma cell line Caco-2 and its EMT-like derivative Caco-H, showed an inverse pattern on the two promoters (Cyclin D1 and E-cadherin) (Fig. 4B, left). On the Cyclin D1 promoter, whose expression is increased in Caco-H cells, the levels of H3K27me^3 and H3K9me^2 were significantly lower on its promoter, as compared to Caco-2 cells. We performed further ChIP analysis for the histone methylase association EzH2 on the Cyclin D1 promoter. The results obtained confirm and help to demonstrate that although in Caco-H cells there is an increase of EzH2 protein expression, the three-methylation level in K27 of histone H3 in the promoter region of Cyclin D1 is lower with respect to Caco-2 cells. In fact as shown in Fig. 4B right, there is an increase of EZH2 on the promoter region of Cyclin D1 in Caco-2 cells with respect to Caco-H. On the other hand, on the promoter of E-cadherin, the expression of which is almost abolished in Caco-H cells, the levels of the histone marks associated with repressed genes were significantly higher in Caco-H cells as compared to Caco-2 cells. At the same time, the analysis of the histone marks associated with the active state of genes (H3K4me^3 and H3K9/14ac) was indeed, as expected, downregulated on the promoter of E-cadherin in Caco-H cells as compared to Caco-2 cells (Fig. 4B, left). Surprisingly in the case of the Cyclin D1 promoter the levels of the active histone markers were slightly downregulated, indicating a more complicated regulation pattern of the active/repressed state of this particular gene in Caco-H cells. To clarify the reason for downregulation of active histone markers on Cyclin D1 promoter we analyzed the expression of 4 class II histone deacetylase (HDAC) by real-time PCR. In Fig. 4C are shown the results of expression analysis for HDAC4, HDAC5, HDAC7 and HDAC9. In Caco-H cells all these 4 histone deacetylase enzymes are upregulated. In particular, HDAC7 and HDAC9 are strongly induced in Caco-H cells, where the value in the case of HDAC9 in Caco-H1 cells reaches 80-fold as compared with Caco-2 cells.

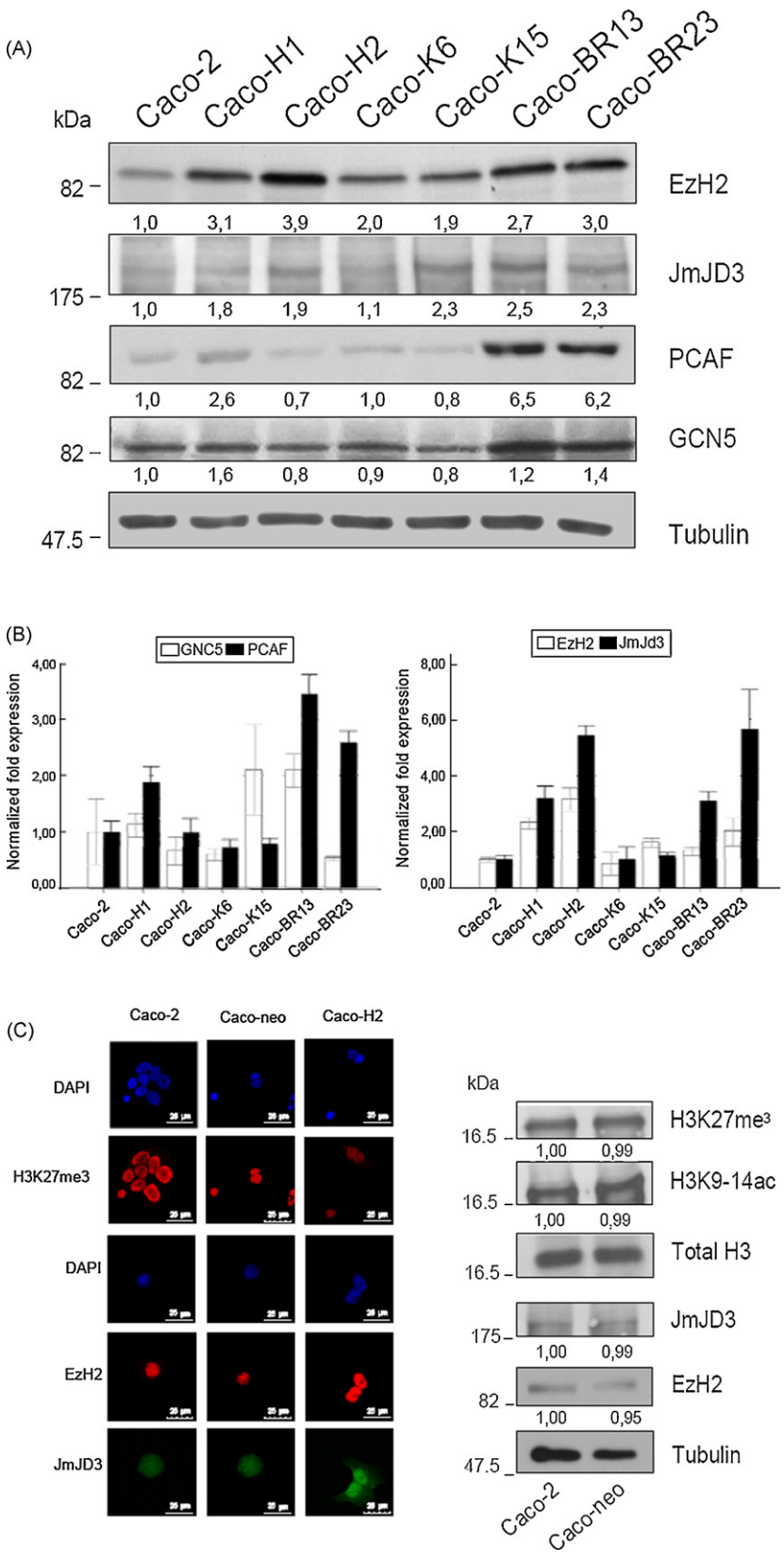

Fig. 2. The levels of certain histone-modifying enzymes change upon oncogenic Ha-RAS overexpression. Total protein (A and B) mRNA levels of GCN5, PCAF, acetyltransferases and EzH2 and JMJD3 histone methyltransferases by Western blot and real-time PCR analysis, respectively. Values were normalized to GAPDH. (C) Immunofluorescence confocal imaging and Western blot analysis comparison between Caco-2 parent cell lines and Caco-neo cells. Numbers below the WB bands represent the value of quantification of the spots normalized to tubulin and total histone H3.

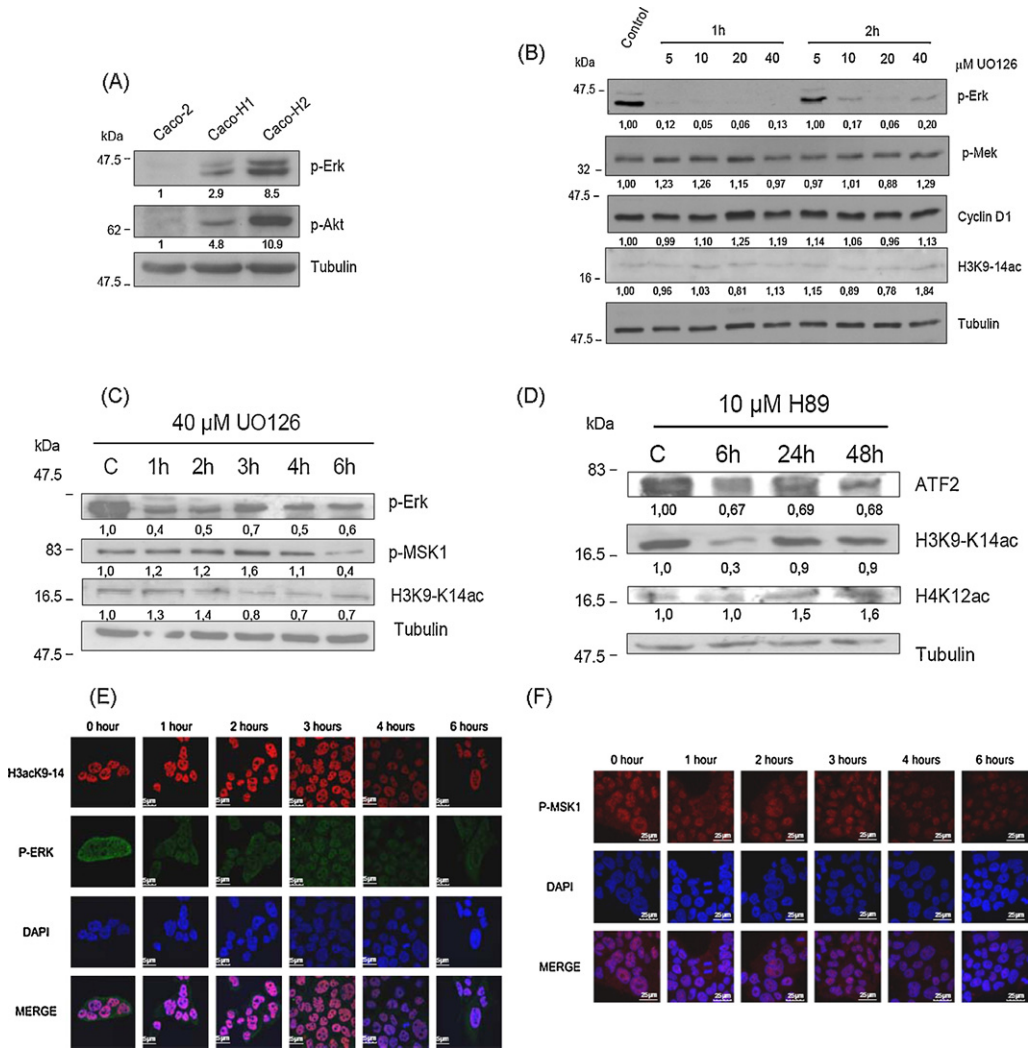

Fig. 3. H3K9–14ac global levels are altered by RAS-activated MEK–ERK signalling pathway. (A) Transfected Caco-2 cell line with oncogenic Ha-RAS (Caco-H) showed increased levels of p-AKT and p-ERK in total protein extracts. (B) Dose–response curve of ERK1/2 inhibitor UO126. (C) UO126 (40 μM) was added to the cells and extracts were prepared after 1–4 and 6 h of incubation. Reduction of H3K9–14ac in the Caco-2 and Caco-H cell line was observed after 3-h incubation. (D) Western blot indicates alteration of global H3K9–14ac acetylation after 6 h of inhibition with H89 (10 μM) of MSK1 kinase activity. ATF2, a MSK1 target, has been used as control. (E and F) Reduction of H3K9–14ac, p-ERK and p-MSK1 is shown, followed by immunocytochemistry and confocal microscopy observation. Numbers below the WB bands represent the value of quantification of the spots normalized to tubulin.

3.5. Histone H3 K9/K14 acetylation levels on the Cyclin D1 promoter are regulated by MEK–ERK–MSK1 pathway

Further on, we wanted to understand in more detail the interrelation between the Ha-RAS-activated pathways and the presence of the active mark H3K9/14ac on the promoter of Cyclin D1. With these means, we analyzed the dependence of H3K9/14ac presence on the promoter of Cyclin D1, on a pathway highly activated in Ha-Ras overexpressing cells (Caco-H), namely the MEK–ERK–MSK pathway. Thus, we blocked the MEK–ERK pathway in Caco-H cells using the UO126 inhibitor at a final concentration of 40 μM. To follow Cyclin D1 expression we used Western blots (Fig. 5A, right), while the H3K9/K14ac levels on the promoter region were determined by ChIP (Fig. 5A, left and central). The results obtained indicate that blocking the MEK–ERK pathway reduced both the acetylated H3K9/14ac levels on the Cyclin D1 promoter as well as the Cyclin D1 expression levels. In order to obtain further confirmation for the role of MEK–ERK–MSK1 pathway, we used the H89 inhibitor (10 μM) to inhibit MSK1 activity, which resulted similarly in decreased levels of H3K9/14ac on the Cyclin D1 promoter (Fig. 5B, right) verifying the hypothesis of a MEK–ERK–MSK1 pathway related regulation of the H3K9–14ac levels on the promoter of Cyclin D1 (Fig. 5B).

3.6. H3K9–14ac binding on Cyclin D1 promoter partially depends on c-Jun

Cyclin D1 is a gene of which promoter activity has been shown to be closely related to its potential consensus AP-1 binding sites (Herber et al., 1994; Shen et al., 2008). Particularly c-Jun, the predominant AP-1 protein and the major dimerizing partner has been shown, in previous studies, to bind on the AP-1 sites of Cyclin D1 suggesting a regulatory function on its transcription (Bakiri et al., 2000; Shaulian and Karin, 2001).

Interested in identifying a possible interrelation between c-Jun levels and histone modifications on the Cyclin D1 promoter in our EMT model, where both c-Jun and Cyclin D1 were shown to be upregulated, we followed ChIP analysis in cells with reduced expression levels of c-Jun. In this means, after reducing c-Jun expression via c-Jun-specific siRNA, we followed ChIP analysis on the Cyclin D1 promoter comparing H3K9/14ac levels under the conditions of normal c-Jun expression (siControl) and reduced c-Jun

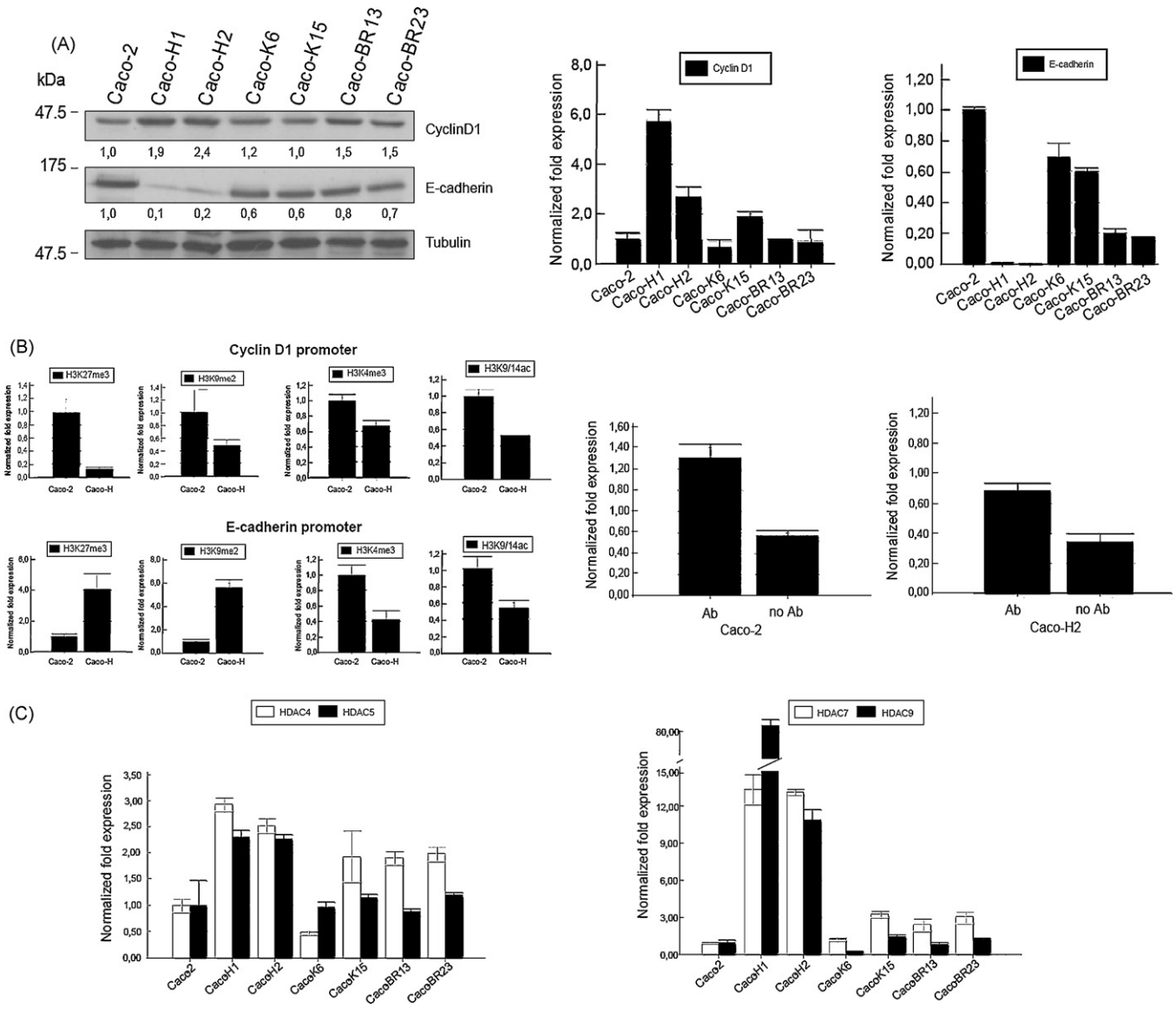

Fig. 4. Repressive and active histone modifications on the Cyclin D1 and E-cadherin promoter in the Caco-H cell line. (A) Western blot (left) and total RNA levels (right) of Cyclin D1 and E-cadherin in cell lines overexpressing oncogenic RAS. Reverse pattern of expression between Cyclin D1 and E-cadherin in Caco-H cells as compared to Caco-2 was shown in both, mRNA and protein levels. (B) (left panel) Real-time PCR analysis of ChIP experiments on the promoter of Cyclin D1 and E-cadherin with chromatin extracted from Caco-2 and Caco-H cells. Repressive histone modification patterns of H3K27me^3 and H3K9me^2 and active histone modification patterns of H3K4me^3 and H3K9/14ac were analyzed. Real-time PCR analysis of ChIP experiments on the promoter of Cyclin D1 and E-cadherin with chromatin extracted from Caco-2 and Caco-H cells. All ChIPs were performed with indicated antibodies or, as a negative control, in the absence of any antibody. Results are normalized to Inputs. (B) (right panel) Real-time PCR analysis of ChIP experiments on the promoter of Cyclin D1 to verify the occupancy of the promoter by methyltransferase enzymes EzH2. IP was performed using EzH2 antibody. (C) Expression analysis of class II HDACs. In Caco-H cells all HDACs analyzed displayed a strong upregulation whereas in Caco-K and Caco-BR clones the induction was less. Numbers below the WB bands represent the value of quantification of the spots normalized to tubulin.

expression levels (c-Jun siRNA). Upon c-Jun downregulation (Fig. 6, left and central), the levels of H3K9/14ac on the Cyclin D1 promoter region decreased (Fig. 6, right) indicating a c-Jun contribution and involvement in the recruitment or stabilization of acetyltranferase complexes on the Cyclin D1 promoter, thus regulating Cyclin D1 expression upon RAS activation.

4. Discussion

Epigenetic changes, among them, histone modification are considered to be markers for a variety of specific cellular processes such as transcription activation and DNA repair, or more global functions like DNA replication and chromosome condensation. During tumor progression, epigenetic events co-exist with genetic alterations. Consequently, a significant interest in the search and identification of new markers of histone modifications that are altered during tumorigenesis has developed over the last few years. Different parameters, as tumor progression stage, grade of tumor differentiation, or gene mutations could associate with changes in the global patterns of histone modifications in tumor cells. Here we demonstrate, using cancer cell lines that some histone markers of euchromatin i.e. H3K27me^3 and H3S10p are altered at global level as the tumor progresses. In the present study we used a model of transformed colon cell lines that have undergone epithelial–mesenchymal transition (EMT) in the case of Caco-H or present alteration in the cell proliferation control in the case of Caco-K and Caco-BR (Oikonomou et al., 2009). We decided to focus on the EMT process because it represents an advanced process of cancer cells associated with the metastasis and has recently been reported to gain stem cell properties. Activating signalling

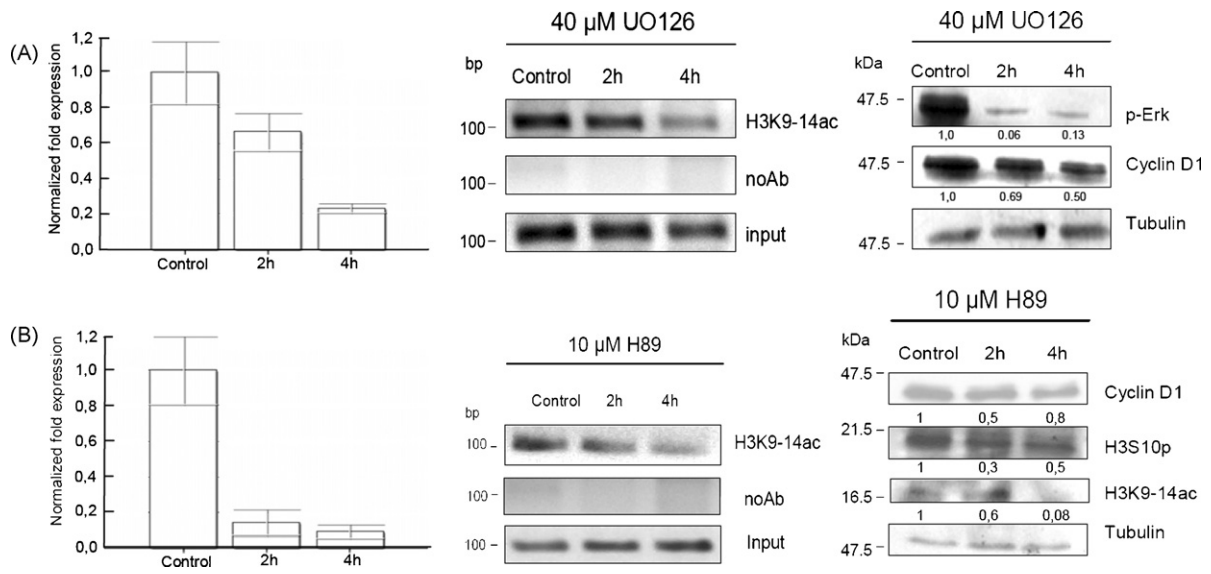

Fig. 5. MEK–ERK RAS-activated signalling pathway upregulates Cyclin D1 expression and alters histone modification patterns on its promoter region. (A) Real-time PCR analysis (left panel) and end-point PCR analysis (central panel) of ChIP experiment analysing the H3K9–14ac modification on the promoter of Cyclin D1 after inhibition of the MEK–ERK pathway. Western blot after inhibition of MEK–ERK pathway with U0126 at a final concentration of 40 μM (right panel). (B) Real-time PCR analysis (left panel) and end-point PCR analysis (central panel) of ChIP experiment analysing the H3K9–14ac modification on the promoter of Cyclin D1 after inhibition of the MEK–ERK–MSK1 pathway. Western blot analysis after inhibition of the MSK1 kinase activity with H89 at a final concentration of 10 μM (right panel). All ChIP results are normalized to Inputs. Numbers below the WB bands represent the value of quantification of the spots normalized to tubulin.

pathways through oncogenic Ha-RAS transformation leads to cells presenting EMT phenotype. In stem cells the chromatin is globally de-condensed and is enriched in acetylated histones, while repressive heterochromatic marks like H3K9me^3, are reduced (Meshorer and Misteli, 2006; Kimura et al., 2004). The open and dynamic status of chromatin is necessary for stem cell differentiation. Hence, H3K9/14ac could be a signature for both stem cell differentiation and tumor progression.

Changes in the chromatin appearance have been reported during oncogene transformation (Chadee et al., 1999) and associated with the metastatic potential of tumor cells. Histone acetylation as one of the modifications related to the opened or closed state of chromatin.

Our results indicate that at global level the H3K9/14ac is slightly lower between Caco-2 intermediate adenoma cell line and Caco-H-transformed cells. We show a downregulation of this histone marker when we analyzed at local level on the promoters of Cyclin D1 and E-cadherin, two genes involved in tumor progression. Furthermore, we revealed that a possible explanation for the reduction of acetylation in Caco-H cells was the strong induction of HDACs enzymes. According to our results in a recent paper, using human breast epithelial cell lines (MCF10A) transformed with Ha-RAS it has been demonstrated (Park et al., 2008a,b) that the inhibition of histone deacetylase enzymes with the specific inhibitor apicidin results in an increase of global acetylation of histone H3 and H4 and a decrease of Cyclin D1. Here we have also shown that the MAPK–ERK–MSK1 signalling pathway is linked to the global levels of H3K9/14ac as after blocking the Ha-RAS-activated MEK and MSK1 kinases the acetylation levels on the Cyclin D1 promoter were reduced.

Another histone modification, H3K27me^3 has been found to be downregulated in some types of cancer, like ovarian, breast and pancreatic cancers (Wei et al., 2008). In this study, H3K27me^3 antibody was found to stain the periphery of the interphase nuclei in different cell lines in agreement with the role of H3K27me^3 as a marker for facultative heterochromatin. Interestingly, the distribution pattern of H3K27me^3 in the nucleus of Caco-H cells was modified, as its expression was lost from the peripheral nuclei area while a granular pattern of expression was observed in the nucleus. We suggest that this could be related either to the degree of differentiation or to the EMT phenotype induced by Ha-RAS. EzH2 and JMJD3 are two methyl-protein-modifying enzymes that have been described as H3K27me^3 specific. EzH2 is a histone H3K27 methylase, which is highly expressed in metastatic prostate cancers, lymphomas and breast cancer and has been shown to provide a proliferative advantage in primary cells (Kleer et al., 2003). Our

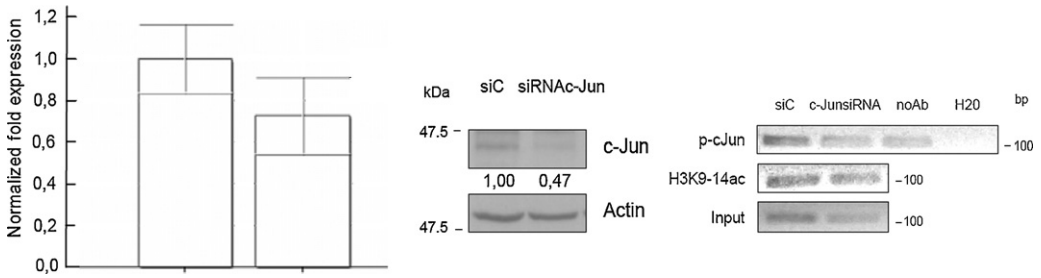

Fig. 6. c-Jun contributes to stabilization/recruitment of acetyltransferases in CycD1 promoter. Real-time PCR analysis (left panel) and end-point PCR analysis (central panel) of ChIP experiment analysing the levels of H3K9/14ac modification on the promoter of Cyclin D1 after reduction of the c-Jun levels (siRNAc-Jun) in Caco-H cells as compared to cells treated with siRNA control (siC). Results are normalized to inputs. (right panel) Western blot analysis of the reduction levels of c-Jun in cells treated with c-Jun-specific siRNA. Numbers below the WB bands represent the value of quantification of the spots normalized to actin.

experiments show that proliferative cells as Caco-H express high RNA and protein levels of EzH2 and this does not correlate with lower global levels of H3K27me^3. Interestingly, we followed by ChIP the presence of EzH2 on the promoter of Cyclin D1 and show that although EzH2 protein is overexpressed, the presence of EzH2 on the promoter of Cyclin D1 is reduced in Caco-H cells. Recently, negative regulation of EzH2 function by AKT-mediated phosphorylation of a highly conserved serine residue, S21 of EzH2 has been reported (Cha et al., 2005). This mechanism facilitates the reduction of H3K27 trimethylation by decreasing the affinity of phosphorylated EzH2 to chromatin but without affecting the composition of the polycomb–repressor complex. In fact, the AKT pathway was observed in a more active state in Caco-H as compared to Caco-2 cells. Furthermore, additional independent functions have as well been suggested for EzH2 that do not involve H3K27 methylase (Martin et al., 2006). The presence of H3K27me^3 on DNA contributes to chromatin compaction (Margueron et al., 2008) and protects the chromatin from demethylases like the H3K27me^3-specific demethylase JMJD3 (Hong et al., 2007).

The dynamic balance of opposing histone-modifying enzymes is critical for the regulation of cell proliferation. It has been shown previously that HDAC1, HDAC2 and HDAC3 are highly expressed in a subset of colorectal carcinomas and associated with proliferative properties of tumors and poor survival. Preferential deacetylation of H3K9 has been reported for HDAC1 and HDAC3 in knock-down experiments (Zhang et al., 2004) while growth reduction was observed after knock-down of these enzymes in several colon cancer cell lines (Wilson et al., 2006; Zhu et al., 2004; Spurling et al., 2008). However, the expression analysis of different HATs and the class I HDACs (data not shown) in different cell lines does not provide a clear explanation for the increased H3K9/14ac levels in cell lines that express oncogenic RAS. Here, we report that the RAS effect on H3-acetylation is possibly due to the action of human class II HDAC enzymes. Increased expression levels of HDAC 4,5,7,9 in Caco-H cells could be important for the decrease in H3-acetylation observed in Caco-H cells, as reported by Ozdağ et al. (2006).

It is well known that Ras proteins have essential roles in controlling the activity of multiple downstream effector-pathways that regulate normal cellular proliferation. Moreover, Ras activation and more specifically Ha-RAS activation, and herewith cancer progression and EMT, results in increased levels of Cyclin D1 and at the same time in decreased levels of E-cadherin. Importantly, regulation of the Cyclin D1 expression by different transcription factors is well reported but the interplay between transcription factors and histone modifications is not yet well understood. Similarly, the expression of E-cadherin and therefore its promoter activity is regulated by interplay between CpG island methylation and histone modifications (Dumont et al., 2008; Lombaerts et al., 2006).

Here, we present evidence supporting the idea that oncogenic Ha-RAS affects cell proliferation and cancer progression through alteration of histone modification patterns on gene promoters involved in these processes. Thus, the epigenetic modification marking repressed gene promoters H3K27me^3 was found decreased on the promoter of Cyclin D1 in Caco-H cells as compared to Caco-2 cells though EzH2 was upregulated, while the same histone modification was found increased on the promoter of E-cadherin, matching their inverse expression patterns in these cells. Similarly, the other mark for repressed gene promoters, H3K9me^2, showed similar results indicating that indeed the presence of these repressive marks can be associated with the expression levels of certain genes.

The presence of histone modifications for active gene promoters did not absolutely correlate with the inverse expression profile of the two genes E-cadherin and Cyclin D1. Nevertheless, the decreased levels of marks of active gene promoters, H3K9me^2 and H3K9–14ac, matched the low expression of E-cadherin in Caco-H cells as compared to Caco-2 cells. On the other hand, Cyclin D1 promoter occupancy by the same active marks did not correlate, as both were slightly decreased in Caco-H cells as compared to Caco-2. Interestingly, it has been reported that the presence of active marks may serve as a way to inhibit transcription silencing of a gene. Importantly, the protein levels of Cyclin D1 vary during cell cycle progression, composing the further study of the histone modifications on the promoter of Cyclin D1 on specific time points during the G1- to S-phase transition of the cell cycle, of great importance.

Further on, deciphering the regulation of Cyclin D1 on the promoter level, deregulation of Cyclin D1 expression through MEK–ERK pathway was shown to be mediated by changes in the levels of active histone mark H3K9/14ac. This might present a supplementary mechanism for the regulation of the expression of Cyclin D1 in this particular EMT model.

All this together highlights the complexity of gene regulation and demonstrates how effectors of EMT, such as Ha-RAS, might regulate specific gene expression by epigenetic rearrangement.

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