Regulation of CBP and Tip60 coordinates histone acetylation at local and global level during Ras induced transformation

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ABSTRACT

Cell transformation is clearly linked to epigenetic changes. However, the role of the histone modifying enzymes in this process is still poorly understood. In this study we investigated the contribution of the Histone Acetyltransferase (HAT) enzymes to Ras mediated transformation. Our results demonstrated that lysine acetyltransferase 5 (KAT5), also known as Tip60, facilitates histone acetylation of bulk chromatin in Ras transformed cells. As a consequence, global H4 acetylation (H4K8ac and H4K12ac) increases in Ras transformed cells, rendering a more decompacted chromatin than in parental cells. Furthermore, low levels of CREB binding protein (CBP) lead to hypoacetylation of retinoblastoma 1 (*Rb1*) and cyclin-dependent kinase inhibitor 1B (*Cdkn1b or p27^Kip1*) tumour suppressor gene promoters to facilitate Ras mediated transformation. In agreement with these data, overexpression of *Cbp* counteracts Ras transforming capability in a HAT-dependent manner. Altogether our results indicate that CBP and Tip60 coordinate histone acetylation at both, local and global level to facilitate Ras induced transformation.

SUMMARY

The contribution of the histone modifying enzymes to Ras transformation is still poorly understood. This study demonstrates that two essential HAT enzymes, CBP and Tip60, coordinate histone acetylation at local and global level to facilitate Ras induced transformation.

INTRODUCTION

The transformation of a normal cell into a cancer cell is associated with a disruption of cellular controls that regulate cell division and/or cell death. At present, many of the genetic changes associated with this process are known. In addition to these genetic changes, increasing evidence suggests that epigenetic alterations are essential in establishing the transformed phenotype [1-3]. The initial indications of the epigenetic link to cancer came from studies of gene expression and DNA methylation (for review see [4]). For example, several tumour suppressors are silenced in human cancers by DNA methylation and aberrant changes in specific histone modifications have been described [2]. These modifications often target regulatory regions of proto oncogenes or tumour suppressor genes [2]. In addition to changes in the specific promoters, changes in global levels of individual histone modifications are also associated with cancer cells [5,6]. Specifically, alteration in H3K9me3, H4K16ac, H4K20me3, H3K56ac and H3K9ac levels are related to tumourigenesis [6-8]. In accordance with that, changes in the expression levels, mutations and translocations of histone acetyltransferases and methyltransferases have been linked to different types of cancer [1-3,9]. All these data remark the important contribution of epigenetic processes to cancer biology.

The *RAS* oncogene is mutated in an elevated proportion (30%) of human tumours [10]. In particular *RAS* mutations at codons 12, 13 or 61 significantly downgrade its GTPase ability, which are thus rendered constitutively active and able to transform mammalian cells. These mutations activate the Raf-MEK-ERK, Ral GDS-Ral, and PI3K-AKT pathways, which ultimately modulate different cellular functions [11,12]. *In vitro*, Ras transformed cells fail to activate many checkpoint controls: they lack contact inhibition and loss the requirement for mitogens and anchorage in order to proliferate [13]. These characteristics are similar to those

that contribute to the loss of controls that takes place in Ras induced tumours. Different epigenetic changes have been linked to Ras mediated transformation; in particular alteration of histone acetylation patterns have been reported [8,14]; moreover, activation of oncogenic Ras leads to increased DNA methyltransferase activity [15] and induces degradation of CBP in NIH3T3 cells [8,16]. Although changes on histone modifications have been correlated to Ras mediated oncogenic processes, the particular contribution of the histone modifying enzymes to cell transformation is still poorly understood. In this study we address this issue by studying the contribution of HAT enzymes to Ras mediated transformation. Our results indicate that Tip60 facilitates histone acetylation of bulk chromatin in Ras transformed cells rendering a more accessible chromatin than in parental cells. Meanwhile, low levels of CBP lead to hypoacetylation of *pRb* and *p27^{Kip1}* tumour suppressor gene promoter contributing to Ras mediated transformation. Altogether our results indicate that CBP and Tip60 coordinate histone acetylation at both local and global level to facilitate Ras induced transformation.

MATERIALS AND METHODS

Cell Culture

NIH3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% calf serum (CS, Invitrogen) or starved with 0.5% calf serum for 18h as indicated. Stable transfections using pCEFL-KZ-AU5, pCEFL-KZ-AU5-H-Ras (V12) and pCEFL-KZ-AU5-N-Ras (V12) [17] vectors were done using the calcium phosphate precipitation technique. Transient transfections were performed using Lipofectamin reagent (Invitrogen).

DNA constructs and antibodies

The plasmids pCEFL-KZ-AU5, pCEFL-KZ-AU5-H-Ras (V12), pCEFL-KZ-AU5-N-Ras (V12), pCEFL-KZ-AU5-H-Ras (V12S35), pCEFL-KZ-AU5-H-Ras (V12G37), pCEFL-

KZ-AU5-H-Ras (V12C40), pCEFL-KZ-AU5-N-Ras (V12S35), pCEFL-KZ-AU5-N-Ras (V12G37), pCEFL-KZ-AU5-N-Ras (V12C40) have been previously described [17,18]. pCDNA3-HA-CBP and pCDNA3-HA-CBPΔHAT have been described elsewhere [19]. pCDNA3-p27^{Kip1} was kindly provided by Dr. O. Bachs. pCDNA3-Tip60-HA was a gift of Dr. D. Trouche. Used antibodies are described in Supplementary Table 1.

Indirect immunofluorescence

Cells on cover slips were fixed in paraformaldehyde, permeabilized with methanol and incubated with antibodies as previously described [20,21].

RT Quantitative Real-Time PCR (qPCR)

Total cellular RNA was obtained with Ultraspec RNA Isolation System (Biotecx). cDNA was generated using Omniscript Reverse Transcription kit (Qiagen) and 2 μg of extracted RNA . Differences in the RNA content were determined by real time PCR using the ABI 7700 Sequence Detection System and SYBR Green Master Mix protocol (Applied Biosystems). PCR reactions were carried out triplicate in 20 μl with 2 μl from 1/5 reverse transcription dilution and 0,25 μM of specific primers at 95ºC for 10 min, followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC. The primer oligonucleotide sequences are described in Supplementary Table 2 and 3. All PCR products were approximately 50 bp to increase the efficiency of real-time reactions. Relative expression was calculated using the standard curve method. A standard curve was made for each gene of interest and the housekeeping by plotting in a base-10 semi-logarithmic graph the number of cycles at which the fluorescence crossed the threshold (Ct) against increasing amounts of DNA template. The relative quantification for the gene of interest is normalized to that of tubulin in the same sample and then the normalized numbers are compared between samples to get a fold change in expression.

FACS staining and analysis

FACS analysis was performed as described elsewhere [22]. Basically, cells transfected with the plasmid/s of interest and pCDNA3-GFP were harvested following trypsinization, washed twice in PBS and resuspended in 1 ml of DMEM. Non-permeabilized cells were stained during 30 min with 5 μg/ml Hoechst 33342 and DNA content of GFP population was analyzed by flow cytometry with a Becton Dickinson FACscan according to the manufactures' procedure.

Analysis of chromatin condensation with Micrococcal Nuclease (MNase)

The protocol is described in Supplementary Materials and Methods.

HAT/Histone deacetylase (HDAC) assays

In vitro acetylation/deacetylation assay was performed as described elsewhere [23,24].

Foci Formation Assay

Foci formation assays were performed as described elsewhere [18].

RNA interference by siRNA

Target sequences for small interfering RNAs are as follows. Tip60: 5´-ACGGAAGGUGGAGGUGGUU-dTdT-3´, control: 5´-CAUGUCAUGUGUCACAUCU-dTdT-3 (BioNova). The siRNAs were transfected using JetPei Kit (Polyplus).

Cell extract preparation. Immunoblotting

Total cell extracts were prepared in IPH buffer as previously described [25]. Histones were extracted by treatment of the whole cells with 0.25 M HCl at 4º C under rotation. After centrifugation at 12 000xg for 10 min at 4ºC, the histones were dialyzed against 0.1 M Acetic acid twice and against H_2O three times. The antibodies used are described in Supplementary Table 1. Immunoblotting was performed with standard procedures and visualized by means of an ECL kit (Amersham).

Chromatin immunoprecipitation (ChIP) analysis. Quantitative Real-Time PCR and ChIP quantification

ChIP assays were performed as described elsewhere [26]. A detailed protocol is described in Supplementary Materials and Methods.

RESULTS

Global HAT activity increases in Ras transformed cells

It has been previously described that chromatin from Ras transformed cell lines is more accessible than chromatin from normal cells [27]. This feature should correlate with epigenetic changes, in particular with new acetylation/deacetylation equilibrium. In order to gain further insight into this possibility we determined the global HAT and HDAC activities in control and Ras transformed NIH3T3 cells by *in vitro* assays. We used as a transformation model the well established NIH3T3 cell line stably transfected with *Hras* mutated at position 12 (RasV12), which makes Ras proteins constitutively active [16]. While originally NIH3T3 are immortal, they become transformed after constitutive activation of the *Hras* oncogene [[16] and Supplementary Figure 1]. Figure 1A shows that global HAT activity in Ras transformed cells was higher than in control cells while HDAC activity did not change. These results suggest that the equilibrium between HAT's and HDAC's is globally moved towards higher acetylation.

Global histone H4 acetylation (H4ac) increases in Ras transformed cells

Next, we analyzed whether the observed changes on HAT activity affect the acetylation status of bulk chromatin of Ras transformed cells. To this end, histones from NIH3T3 parental and Ras transformed cells were purified and H3 acetylation (H3ac) (K9 and K14 positions) and H4ac (K5, K8, K12 and K16 residues) were analyzed by immunoblot and immunostaining. Figure 1B and C shows that the level of H3ac remains unchanged during cell transformation, but H4ac increases (2-fold, P value = 0.04) in transformed cells. To gain further insight into this epigenetic change, we next tried to determine which positions on

histone H4 change their acetylation status. We used immunoblot analysis to determine the acetylation levels of K5, 8, 12 and 16 of histone H4 (Figure 1B and C). A significant increase in K8ac (2.4-fold, P value < 0.009) and in K12ac (1.8-fold, P value < 0.04) was detected in Ras transformed cells relative to the NIH3T3 parental cells. In agreement with previous maintenance of H3ac, also H3K9ac remains constant (Supplementary Figure 2A). The H3ac, H4ac, H4K12ac status was also analyzed by immunostaining and the results were found to be consistent with those observed above (Figure 1D). We then analyzed another active, H3K4me3, and repressive, H3K27me3, histone marks in control and Ras transformed cells. Supplementary Figure 2B and C shows that, in contrast to the global increase of H4ac, the levels of these methylation marks did not vary from control to Ras transformed cells.

Tip60 hyperacetylates histone H4 in Ras transformed cells

To try to identify the enzymes responsible for the observed changes on H4ac, we analyzed the levels of some HAT and HDAC enzymes in control and Ras transformed cells by immunoblot assays. While the level of analyzed HDACs remained constant (Figure 2A), in agreement with the results observed for global HDAC activity, some HAT enzymes underwent a strong regulation during the transformation process. We found clear differences for CBP, which was previously described to be degraded in Ras transformed cell lines [8,16], as well as for Tip60, whose levels increased (1,7 fold) upon *Ras* activation (Figure 2A). Then, we investigated whether these regulation contribute to Ras mediated transformation.

Given that global HAT activity is moved towards higher acetylation and we have observed increased protein levels for Tip60, we sought to determine whether Tip60 is responsible for the H4ac global increase observed in Ras transformed cells (Figure 1B and C). To do that, we analyzed the Tip60 HAT activity in control and in Ras transformed cells. In agreement with our previous observations, the HAT activity associated to Tip60 was higher in

Ras transformed cells than in parental cells (Figure 2B). In order to confirm the role of Tip60 in the observed H4ac increase, Tip60 was depleted using specific siRNA (Tip60 siRNA, Supplementary Figure 3) and the histone H4ac was analyzed in both cell lines by immunoblot. After Tip60 knockdown, H4ac levels were restored in Ras transformed cells (Figure 2C), pointing to an important role of this enzyme in the global acetylation increase observed upon Ras transformation.

Tip60 promotes global chromatin decompaction

Next, we aim to investigate the consequences of global acetylation increase mediated by Tip60 in Ras transformed cell biology. It has been demonstrated that nucleosomal organization of Ras transformed fibroblasts is more decondensed than that of normal parental fibroblasts and it is indispensable for cell transformation [27]. On the other hand, histone hyperacetylation correlates with gene activation and contributes to a more accessible chromatin state [28]. Thus, Tip60 could be responsible to render Ras transformed chromatin in a more decondensed state. In order to test this hypothesis, NIH3T3 cells were transfected with either the empty vector or Tip60-HA expressing plasmid and the chromatin condensation status was analyzed by Micrococcal Nuclease (MNase) enzyme accessibility. Results in Figure 2D showed that *Tip60* overexpression led to a more accessible chromatin, similar to the described for Ras transformed cells [29]. This data suggest that Tip60 mediated global acetylation could be responsible for an open chromatin state of transformed cells mediated by histone H4ac.

Histone acetylation at *pRb* and *p27^{Kip1}* tumour suppressor gene promoters

Our results show a Tip60 mediated global increase of H4ac upon Ras induced transformation. However, this result does not imply that H4ac increases at all particular gene

locations. Moreover, other and we have observed that CBP decreases after Ras transformation [[8,16] and Figure 2A], suggesting that CBP could contribute to Ras transformed cell biology in a different manner than Tip60. Many signals from the environment are thought to induce gene transcription by activating intracellular biochemical pathways that control the ability of transcriptions factors to recruit CBP to specific promoters. Thus, CBP could regulate histone acetylation at local level in response to Ras pathway activation; in particular, it could modulate acetylation of tumour suppressor and/or proto-oncogenes involved in Ras transformation.

It has been previously proposed that Ras activation induces downregulation of the $p27^{Kip}$ [30-32] and increases the cyclin dependent kinase inhibitor 1A (*Cdkn1a* or $p21^{Cip1}$) levels [30,33]. On the other hand, the principal function of Ras in G1–S-phase progression is to inactivate the tumour suppressor *pRb* and relieve cells from its growth inhibitory actions [30,34,35]. Then, we sought to test whether CBP could regulate histone acetylation at these tumour suppressor genes involved in Ras transformation. In order to do that, we first analyzed the expression of *pRb*, $p27^{Kip1}$ and $p21^{Cip1}$ upon Ras transformation. Given that we have described a decrease of CBP in Ras transformed cells, we also included this gene in our analysis together with the oncogene cell division cycle 6 (*Cdc6)* and interleukin2 (*Il2*), which are used as positive and negative controls respectively. As it has been previously shown [33], high Ras induction activation caused up regulation of $p21^{Cip1}$ (4-fold increase, P value= 0.0006) (Figure 3A). However, *pRb* and $p27^{Kip1}$ expression decreased (2.1-fold, P value= 0.0009, 2.0-fold, P value=0.0009) (Figure 3A), suggesting that they are Ras targets at the transcriptional level. In agreement with the mRNA levels, the pRb and $p27^{Kip1}$ protein levels also decreased in Ras transformed cells compared with the parental NIH3T3 cell line (Supplementary Figure 4). Next, we tested the potential contribution of histone acetylation to the expression of these tumour suppressor genes. To do that, we analyzed the H3ac and H4ac

status at their promoters in Ras transformed and parental cells by ChIP assays. We found that H3 and H4 acetylated signals decreased at $p27^{Kip1}$ and pRb promoters (Figure 3B and C). As expected, $p21^{Cip1}$ promoter acetylation increased, while Cbp and $Cdc6$ promoters did not show any significant change (Figure 3B and C). Given that transcriptional activity correlates with trimethylation of H3HK4 (H3K4me3) at promoters, we sought to analyze whether the histone acetylation decrease observed at pRb and $p27^{Kip1}$ promoters also correlated with a loss of H3K4me3. While a large increase of H3K4me3 was observed at $p21^{Cip1}$ promoter in Ras transformed cells, the opposite was found at pRb and $p27^{Kip1}$ promoters that displayed a clear decrease in H3K4me3 signals (Figure 3D).

CBP targets *pRb* and *p27^{Kip1}* tumour suppressor gene promoters

The observed decrease in histone acetylation at pRb and $p27^{Kip1}$ promoters might be linked to the low levels of CBP detected in Ras transformed cells. To test this hypothesis we analyzed whether CBP targets $p27^{Kip1}$ and pRb promoters in both Ras transformed and parental cells by ChIP assays. Results in Figure 3E showed that CBP directly targeted $p27^{Kip1}$ and pRb. On the other hand, CBP recruitment was partially lost at $p27^{Kip1}$ and pRb promoters in transformed cells correlating with low expression and histone hypoacetylation of these tumour suppressor gene promoters. Different results were observed at *Fos* (*c-fos*) promoter, whose CBP level remained constant, indicating that CBP redistribution is specific for some target promoters. Additionally the negative control *Neurogenin2* doesn't show any kind of enrichment for CBP.

As we observed an increase on $p21^{Cip1}$ promoter acetylation and on the levels of Tip60 enzyme in Ras transformed cells, we sought to investigate whether Tip60 contributes to $p21^{Cip1}$ regulation in these cells. In order to do that, we first depleted Tip60 on Ras transformed cells by using the Tip60 siRNA and the $p21^{Cip1}$ mRNA levels were analyzed by

qPCR 72h later. The results in Supplementary Figure 5A suggested that Tip60 was not involved on $p21^{Cip1}$ transcriptional control in response to Ras activation. Secondly, we overexpressed Tip60 on parental NIH3T3 cells and the activity of $p21^{Cip1}$ promoter was analyzed by qPCR. The results showed no changes on $p21^{Cip1}$ expression (Supplementary Figure 5B, upper panel). To discard the possibility that Ras signal could be involved in Tip60-mediated $p21^{Cip1}$ regulation, we repeated this experiment using HRas transformed cells instead of control NIH3T3 cells. The results indicated again that $p21^{Cip1}$ expression was not affected by Tip60 (Supplementary Figure 5B, lower panel). Finally, we tested whether Tip60 was recruited to $p21^{Cip1}$ promoter upon Ras transformation. ChIP results in Supplementary Figure 5C demonstrated that Tip60 was not targeted to this promoter during Ras transformation, in contrast with the recruitment observed at the *ncl* TSS, used as a positive control. Given that *cdc6* has been also described as a Tip60 target in other cellular contexts, we included this gene in the analysis. In this case a slight decrease of mRNA was observed after Tip60 depletion (Supplementary Figure 5A), however no changes upon Tip60 overexpression were observed in mRNA levels or Tip60 *cdc6* promoter recruitment (Supplementary Figure 5B and C), indicating possible indirect regulation upon the siTip60 interference. Altogether our results indicated that Tip60 was not involved in $p21^{Cip1}$ transcription regulation in NIH3T3 cells in response to Ras activation and strengthen the idea of a global Tip60 role on chromatin.

Both PI3K and Ral-GDS pathways contribute to CBP degradation

After Ras signalling pathway activation, there are three major routes that contribute to transmit Ras signal into the nucleus: Raf-MEK-ERK, Ral GDS-Ral and PI3K-AKT pathways. Specific point mutations at the effector domain of Ras can reduce the interaction with Raf-1 (E37G and Y40C mutations), with Ral-GDS (T35S and Y40C mutations), or with PI3K

(T35S and E37G mutations) [36] (Figure 4A). We sought to determine which of the three major pathways are required to Ras mediated CBP degradation. To this end, NIH3T3 cells were stably transfected with the hyperactive form, HRasV12, or their effector dominant mutants: HRasV12C40, HRasV12G37, HRasV12S35 that only activate PI3K, Ral-GDS and Raf-1, respectively [18,36] and CBP protein levels were analyzed by immunoblot. Overexpression of HRas V12S35 led to close normal CBP levels, however NIH3T3 clones of HRasV12G37 and HRas V12C40 showed lower CBP protein levels than the control cells (Figure 4B). These results suggest that both, Ral GDS-Ral A and PI3K-AKT pathways contribute to transmit the HRas signal into CBP coactivator. We have confirmed these results using the chemical PI3K inhibitor (Ly 294-002); after 20h of incubation with Ly 294-002, the levels of CBP were restored for HRasV12 cells and increased in control cells (Figure 4C). These results are in agreement and complementary to the observations from Liu and colleagues [8] where CBP is degraded after activation of PI3K pathway. Additionally, in the case of NRasV12 transformation we proposed Raf-MEK-ERK pathway as the major contribution to CBP degradation (Supplementary Figure 6A). These results were confirmed with the recovery of CBP protein levels after the incubation of cells with the Raf/ERK inhibitor PD98059 (Supplementary Figure 6B).

CBP overexpression counteracts Ras induced transformation of NIH3T3 fibroblasts

To understand how CBP loss of function could contribute to Ras induced cellular transformation, we tested the ability of CBP to affect Ras induced cell proliferation. To do that, we overexpressed CBP protein together with GFP into HRasV12 transformed NIH3T3 cells and analyzed the distribution of the cell cycle phases of green positive cells. We found that *Cbp* overexpression leads to a statistically significant delay of the G1-S transition of the cell cycle. The CBP induced G1 retention was 40-60% of the G1 delay due to *p27^{Kip1}*

overexpression. This effect was specific for CBP, as GFP did not induce such an arrest (Figure 5A and B). Similar results were obtained when we used cells transformed with oncogenic NRasV12 (Supplementary Figure 6C).

Overall our data suggest that low levels of CBP could contribute to Ras transformation modulating histone acetylation at local level, in particular at *p27*^{Kip1} and *pRb* tumour suppressor promoters. In that case, CBP should behave itself as a tumour suppressor gene. To test this hypothesis we analyzed the ability of *Cbp* overexpression to counteract Ras transforming foci formation in NIH3T3 fibroblasts. To this end we overexpressed *Cbp* together with *HRasV12* and the induced transforming foci were screened after cell staining (Figure 5C and D). We found that *Cbp* overexpression significantly protected the NIH3T3 fibroblasts from Ras transformation, giving a sharp reduction of the transforming activity [close to 30% versus the 100% of HRasV12 alone, see Figure 5D]. As we have previously observed for the cell cycle analysis (Supplementary Figure 6C), similar results were obtained when NRasV12 was used (Supplementary Figure 6D).

Our data supported the idea that CBP and Tip60 HATs enzymes coordinate their actions to facilitate Ras-transformation. Thus, we sought to test whether Tip60 also contributes to NIH3T3 cellular transformation as CBP does. To do that, we depleted Tip60 with Tip60 siRNA, 24h later we overexpressed *HRasV12* and analyzed the induced transforming foci. Unfortunately, although the interference of Tip60 allows experimental analysis of the cells after 72h, the depletion had strong effects on cellular proliferation on both NIH3T3 and HRasV12 transfected cells at longer incubation time, making impossible to quantify its effects on the Ras-induced foci. The results in Supplementary Figure 7 indicated that: (i) a decrease of Tip60 levels blocked proliferation of NIH3T3 cells; (ii) this growth arrest also took place upon transfection of *HRasV12* oncogene, and (iii) the combination of *Cbp* overexpression and Tip60 siRNA led to a complete blockade of cellular proliferation of

HRasV12 transfected cells. The fact that Tip60 is required for cell growth make difficult to conclude that Tip60 plays a direct role facilitating transformation however, it explains its contribution to Ras-mediated transformation.

Accurate CBP HAT activity is required to counteract Ras induced transformation

To test whether CBP HAT activity might be important to counteract Ras mediated transformation we repeated the focus formation assay with a mutant of CBP that lacks HAT activity (CBPΔHAT). The percentage of foci induced by H and NRasV12 was not significantly affected after *CbpΔHAT* mutant overexpression (Figure 5C and D and Supplementary Figure 6D). Similarly, *CbpΔHAT* mutants did not induce G1 arrest to the same degree than *Cbp* wild type (Figure 5A and B and Supplementary Figure 6C), suggesting that the antiproliferative effects induced by CBP were indeed mediated in part through its HAT activity. Accordingly with these results incubation of HRasV12 transformed NIH3T3 cells with Trichostatin A (TSA), a potent inhibitor of histone deacetylase enzymes, leads to a partial reversion of the Ras transformed morphology (Supplementary Figure 8). Although TSA inhibitors are not specific enough to justify the CBP effect on Ras induced transformation, this result indicates that the balance of HAT and HDAC is very important to protect the cell from cellular transformation. Taken together, all these results suggest a major role for CBP co activator in cellular protection from Ras oncogenic-induced transformation through its HAT function.

DISCUSSION

It is well established that epigenetic alterations are linked to cell transformation and to development of several types of human tumours [3]. However, the exact contribution of histone modifying enzymes to cell transformation is not completely understood. In the present

study, we demonstrated that the HAT enzymes Tip60 and CBP are regulated during Ras-mediated transformation contributing to transformation at different levels.

We observed a general increase of cellular HAT activity in Ras transformed cells compared with parental cells (Figure 1A). In particular, we showed increased expression and HAT activity of Tip60 that is responsible for the observed global H4 hyperacetylation (Figure 2B). Interestingly, we did not detect any contribution of Tip60 facilitating $p21^{Cip1}$ activation during transformation, different than it has been showed in other cellular contexts, particularly those where DNA damage response has been activated [37-43]. Nevertheless, other examples in the literature are in agreement with our results showing that other enzymes different that Tip60 must be responsible for $p21^{Cip1}$ promoter acetylation [44,45]. How Tip60-mediated global H4 hyperacetylation could affect cell transformation biology? Our results indicate that Tip60 promotes chromatin decompaction and accessibility to MNase enzyme (Figure 2D). These data are in concordance with previous observations showing that nucleosomal organization of c-Ha-Ras oncogene transformed fibroblasts is more decondensed than that of normal parental fibroblasts [27]. Several groups have also reported a global increase in phosphorylation of histone H3 at serine 10 in transformed cells [29,46,47] that may also be related to a more open chromatin conformation [46] and it is indispensable for cell transformation [29]; more recently Liu and colleagues have shown that Ras-PI3K signalling down regulates H3K56ac, which is associated to alteration on proliferation and cell migration of tumour cells [8]; interestingly they showed that Ras-PI3K-AKT pathway specifically targets H3K56ac via CBP/p300 degradation mediated by MDM2 ubiquitin ligase [8]. Our results are in good agreement with these findings and suggest an important contribution of global state of chromatin during transformation. It establishes a link between Ras signalling pathway and the epigenetic programme. Specifically, we found that oncogenic HRas reduces CBP levels throughout Ral GDS-Ral A and PI3K-AKT pathways; however, the NRasV12

elicited CBP degradation is absolutely depending of the Raf-MEK-ERK pathway. These differences between both isoforms of Ras proteins could be due to the specific subcellular localization of the proteins, that determines the effectors that could be recruited, and their different molecular and biological outputs [48].

In addition to the Tip60 increase, other and we have reported a decrease in CBP HAT enzyme levels in Ras transformed cells ([8,16] and Figure 2A). In the present work we addressed the contribution of CBP degradation during cell transformation. We demonstrated that CBP directly targets and acetylates $p27^{Kip1}$ and pRb tumours suppressor promoters, keeping them in an active state in the control cells (Figure 3E). Upon Ras transformation these genes are dramatically repressed and they become hypoacetylated, suggesting that CBP is required to activate these tumour suppressor promoters. To confirm the importance of CBP counteracting Ras transformation we have demonstrated that overexpression of Cbp, but not a mutant lacking HAT activity, leads to partially block of cell cycle progression and Ras mediated colony formation (Figure 5B and D)

Interestingly, we have not observed global changes on acetylation of lysine residues, which are modified by CBP in another cellular context, including H3K9, H3K14, H4K16 and H4K5 (data not shown). A possible explanation of this result is that CBP HAT activity is regulated by different factors [49,50], such as post translational modifications [51,52], viral oncoproteins [49,53], specific transcription factors recruitment [54-57], or cell type specific factors [50]. All these factors might explain why low CBP levels affect only some specific promoters in Ras transformed NIH3T3 cells. Another plausible explanation is that other histone acetyltransferases could compensate the observed reduction of CBP enzyme. In any case, these results are in agreement to previous studies showing that depletion of CBP only affects some histone residues at specific promoters [58].

Taken together, our results demonstrate that Tip60 and CBP are targeted by the oncogenic Ras pathway. Both enzymes contribute to the oncogenic activity at different and complementary levels: (i) modulation of the chromatin accessibility at global level and (ii) regulation of transcription of tumour suppressors. Understanding the mechanisms underlying Ras-mediated transformation may serve as tumour development prevention.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Global HAT activity and H4ac increase in Ras transformed cells

(A) Whole cell extracts were prepared from NIH3T3 control (Ctrl) and H RasV12 transformed (HRasV12) cell lines. Increased amounts of total cell extracts were used for *in vitro* HAT and HDAC assay. The graphics show the average and standard deviations of three independent experiments.

(B) Histones were purified from Ctrl and HRasV12 transformed cell lines and the acetylation levels of histone H3, H4 and acetylation at positions K5, K8, K12 and K16 in H4 were analyzed by immunoblot.

(C) Quantification of acetylation levels in samples from (B). The histogram shows normalized levels relative to H3 obtained from three independent immunoblotting experiments.

(D) Histones from Ctrl and HRasV12 transformed cell lines were immunostained with the indicated antibodies (in red). Cells were co-stained with DAPI to reveal the DNA (blue). The images were taken under the same setting conditions in a conventional fluorescent microscope. Scale bar: 30 μm.

Figure 2. Tip60 hyperacetylates histone H4 in HRas transformed cells

(A) Whole cell extracts were prepared from parental (Ctrl) and HRasV12 cell lines. Total protein amount was adjusted and the CBP, Tip60, HDAC1 and HDAC 2 protein levels were analyzed by immunoblot using specific antibodies. The tubulin levels were used as a loading control. The figure shown is representative of at least three independent experiments.

(B) Whole cell extracts were prepared from Ctrl and HRasV12 cell lines. Total protein amount was adjusted and Tip60 was immunoprecipitated. The HAT activity associated to the immunopellet was determined by *in vitro* HAT assay.

(C) Ctrl and HRasV12 cell lines were transfected with 200 pmol Tip60 siRNA or control siRNA (C siRNA). After 4 days histones were purified and the H4ac levels were analyzed by immunoblot. The histogram shows normalized H4ac levels relative to H3 levels obtained from three independent immunoblot experiments.

(D) NIH3T3 cells overexpressing Tip60-HA or empty vector were incubated with Micrococcal Nuclease enzyme (MNase), DNA was extracted and analyzed in agarose gels.

Figure 3. Histone acetylation at promoters of Ras-target genes

(A) Total cellular RNA from Ctrl and HRasV12 cell lines was obtained and passed to cDNA. Differences in the content of RNA from the two cell lines were determined by qPCR. *p21^{Cip1}*, *pRb*, *p27^{Kip1}*, *Cbp*, *Cdc6* and *Interleukin 2* (*Il2*) RNA values were normalized to those obtained for tubulin RNA.

(B-D) Comparative ChIP-analysis was performed in parallel with the same number of Ctrl and HRasV12 cells using the indicated antibodies (described in Supplementary Table 1). The immunoprecipitates were analyzed by qPCR using specific primers for *p21^{Cip1}*, *pRb*, *p27^{Kip1}*, *Cbp*, *Cdc6* and *Il2* promoters. ChIP enrichments are presented as percentage (%) of

bound/input signal. ChIP with antibody against HA tag served as negative control. The graphics show the average and standard deviations of three independent experiments.

(E) The same as in (D), but using specific antibody against CBP and specific primers for *pRb*, *p27^{Kip1}*, *c-fos* and *Ngn2* promoters.

Figure 4. Ras pathways that contribute to CBP degradation

(A) Schematic representation of Ras mutations that regulate effector interactions.

(B) Total cell extracts from either Ctrl or NIH3T3 transfectants clones overexpressing HRasV12, V12C40, V12G37 or V12S35 mutants were prepared. Total protein amount was adjusted and the CBP levels were determined by immunoblot using anti CBP antibody. The same blot was probed with anti tubulin antibody as a loading control.

(C) Ctrl and HRasV12 cell lines were treated with Ly294 for 20h. Total protein extracts were prepared and the CBP levels were determined by immunoblot using anti CBP antibody. The results showed correspond to a representative experiment; similar results were obtained in four biological independent experiments.

Figure 5. *Cbp* overexpression inhibits Ras induced transformation

(A) HRasV12 cells were transfected with pCDNA3-GFP and pCDNA3-HA-CBP, pCDNA3-HA-CBPΔHAT or pCDNA3-p27^{Kip1} (1 μg of each plasmid). 36 h after transfection, cells were harvested and stained with Hoechst. The DNA content of the GFP population was analyzed by flow cytometry.

(B) Quantification of G1 cells from data in (A). The histogram shows % cells in G1 relative to the G1 retention induced by p27^{Kip1}. The data correspond to the average and standard deviation of the results from four biological independent experiments.

 (C) NIH3T3 fibroblasts were cotransfected with 25 ng of pCEFL-KZ-AU5-HRasV12 and with 4μg of the corresponding pCDNA3, pCDNA3-HA-CBPΔHAT or pCDNA3-HA-CBP. As negative control were cotransfected both empty vectors (pCEFL-KZ-AU5 and pCDNA3). After 14 days the dishes were stained with Giemsa to score the transformed foci. All plasmids DNAs produced similar numbers of marker selectable colonies.

(D) Focus formation was then quantified and expressed as a percentage of each HRasV12-induced focus formation. Histograms correspond to the average and standard deviation of three separate assays performed in triplicate.

FIGURE 1

A

B

C

D

FIGURE 2

FIGURE 3

FIGURE 4

A

B

C

FIGURE 5