Cytometry Part A 66A:52–61 (2005)

# New Method to Detect Histone Acetylation Levels by Flow Cytometry

**Simona Ronzoni,[1] Mario Faretta,[1] Marco Ballarini,[1] PierGiuseppe Pelicci,[1] and Saverio Minucci[1,2]**
[1]European Institute of Oncology, Milan, Italy
[2]Department of Biomolecular Sciences and Biotechnologies, University of Milan, Milan, Italy

Received 25 May 2004; Revision Received 17 March 2005; Accepted 22 March 2005

**Background:** Reversible histone acetylation affects chromatin structural organization, thus regulating gene expression and other nuclear events. Levels of histone acetylation are tightly modulated in normal cells, and alterations of their regulating mechanisms have been shown to be involved in tumorigenesis.
**Methods:** We developed a new flow cytometric technique for detection of histone acetylation, based on a specific monoclonal antibody that recognizes acetylated histone tails. Bivariate analysis for histone acetylation levels and DNA were performed to study modulation of chromatin organization during the cell cycle and after induction of histone hyperacetylation by the histone deacetylase (HDAC) inhibitor trichostatin A (TSA). Histone acetylation and transcription levels were monitored during differentiation induced by retinoic acid alone or in combination with TSA. Blood samples from patients were analyzed with the described protocol to monitor the effects of HDAC inhibitors in vivo and validate the developed protocol for clinical usage.
**Results:** Flow cytometric detection of acetylation status can successfully detect modifications induced by HDAC inhibitor treatment in vivo as demonstrated by analysis of various blood samples from patients treated with valproic acid. Changes in acetylation levels during the cell cycle demonstrated a reproducible increase in histone acetylation during the replication phase that was subsequently decreased at the G2M entrance, thus paralleling the behavior of DNA replication and transcriptional activity.
**Conclusions:** Multiparameter analysis of histone acetylation and expression of molecular markers, DNA ploidy, and/or cell cycle kinetics can provide a quick and statistically reliable tool for the diagnosis and evaluation of treatment efficacy in clinical trials using HDAC inhibitors.


**Key terms:** flow cytometry; histone acetylation; leukemia; histone deacetylase inhibitor

In the eukaryotic cell nucleus DNA is wrapped around histones to constitute nucleosomes, the fundamental organizational element of chromatin. Modifications of chromatin structure by histone tail acetylation, phosphorylation, and methylation are involved in all processes regarding DNA such as gene expression, replication, and mitotic chromosome condensation (1). The pattern of histone posttranslational modifications is the result of an epigenetic code regulating gene transcription and silencing (2). In particular, histone acetylation can affect gene expression as evidenced by the observed positive correlation between histone 3 and 4 lysine acetylation and gene activation, while whereas hypo-acetylation is a mark of non-transcribed DNA regions (3).

The number of acetyl groups present on histones is regulated by the competing activities of different classes of enzymes. Histone acetyl transferases are part of coactivator complexes involved in chromatin remodeling to initiate transcription. Histone deacetylases (HDACs) regulate gene silencing by removing acetyl groups from histones and favoring chromatin condensation (4). Breakage of the equilibrium between the two activities can lead to dramatic effects on the regulation of transcriptional pathways, establishing the conditions for the development of cancer.

Recent discoveries have identified key molecular events in the pathogenesis of acute promyelocytic leukemia, caused by chromosomal rearrangements leading to the fusion of the retinoic acid receptor-α (RAR) gene with other cellular genes (5–7). The resulting protein chimera impairs retinoic acid (RA) response due to aberrant recruitment on RA-responsive promoters of transcriptional coregulators (such as SMRT/NCOR) in a HDAC associated complex (8). Maintenance of transcriptionally repressed chromatin state in the presence of physiologic

Contract grant sponsor: Gruppo Italiano Citometria.
*Correspondence to: PierGiuseppe Pelicci, European Institute of Oncology, Via Ripamonti 435, 20141, Milan, Italy.
E-mail: piergiuseppe.pelicci@ieo-research.it
Published online 24 May 2005 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cyto.a.20151

concentration of RA can be therefore proposed as a necessary event in the PML-RAR–mediated leukemogenesis (9). Further studies on other leukemogenic fusion proteins have shown that association and recruitment of HDAC complexes and consequent aberrant gene silencing can be a common theme in acute myelocytic leukemias (9,10). On the basis of the above-mentioned studies in the pathogenesis of leukemia, HDAC inhibitors (HDACi) have been used in combination with differentiation-inducing agents to relieve the block in hematopoietic differentiation. RA and HDACi combined treatment induced a clinical response in a patients with RA-resistant acute promyelocytic leukemia (11).

HDACi have also been shown to induce differentiation, growth arrest, and/or apoptosis in solid tumors, leading to their consideration as multipurpose antitumoral drugs. In fact, clinical trials have started to evaluate the efficacy of HDACi in nonleukemic patients (12).

Detection of histone acetylation levels in the cell nucleus can therefore provide an important tool in monitoring the cellular response to specific anticancer treatments and the production of monoclonal antibodies recognizing hyper-acetylated histones is consequently gaining enormous potential for research and diagnostic applications (13). In the present study, we developed a flow cytometric protocol for detection of hyper-acetylated histones and simultaneous cell cycle analysis based on a specific monoclonal antibody recognizing acetylated histone tails. After detection of their modulation in the cell cycle progression, histone acetylation and transcriptional levels were monitored during in vitro differentiation of leukemic cells induced by RA treatment alone or in combination with trichostatin A (TSA), showing that no global differences in transcriptional levels were caused by the presence of the HDACi.

To evaluate the effect for clinical investigations, the protocol was first tested on preclinical models of acute myeloid leukemia and then employed to analyze peripheral blood samples from patients who were affected by different types of cancer and enrolled in protocols making use of HDACi (valproic acid, VPA). We demonstrated that with the developed protocol histone hyper-acetylation can be successfully detected in patients, thus providing a potential marker of the activity of HDAC inhibitors in clinical trials.

## MATERIALS AND METHODS

### Cell Lines

Human U-937 and NB4 leukemic cell lines were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum.

Exponentially growing U-937 cells were treated for 4 h with 50 ng/ml of TSA (Sigma, St. Louis, MO, USA). Cells were then washed with cold phosphate buffer saline (PBS), counted, and fixed in 1% formaldehyde in PBS for 15 min. Fixed cells were then washed, resuspended in PBS, and stocked at 4°C.

Exponentially growing NB4 cells were treated for 2, 4, 24, and 48 h with 50 ng/ml of TSA and/or 1 μM RA to induce cell differentiation. To evaluate transcriptional activity, 5-bromouridine (BrU; Sigma) was added to cell culture medium to a final concentration of 4 mM during the last 30 min of treatment. Cells were subsequently collected and analyzed by flow cytometry.

### Blood Sample Preparation

Human peripheral blood (5 ml) was collected and spinned down at 1,500 rpm for 5 min. Cells were incubated in 3 ml of 0.1% NaCl on ice for 40 min. After addition of 3 ml of 1.6% NaCl and 10 ml of PBS, cells were spinned down at 13,000 rpm for 10 min.

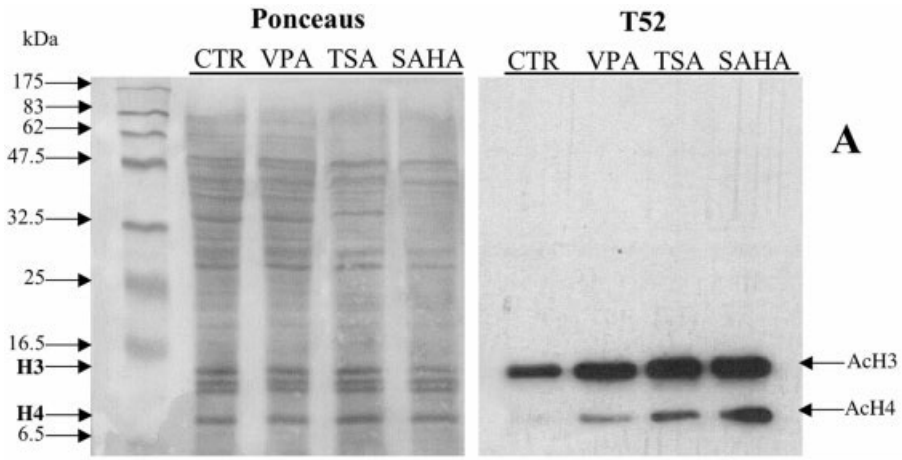
Cells were fixed in 1% formaldehyde in PBS for 15 min. Fixed cells were then washed and resuspended in PBS and stocked at 4°C to be processed for the histone acetylation levels detection according to the protocol below.

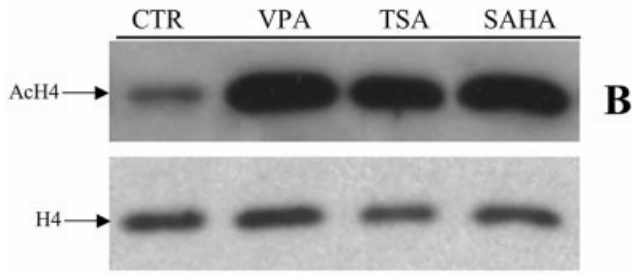
### Differentiation Analysis of Surface Markers

NB-4 cells, $5 \times 10^5$ per sample, were collected after 48 h of RA treatment (or left untreated as control), washed with PBS plus 1% bovine serum albumin (BSA), and incubated with the monoclonal mouse anti-human CD11c antibody (DakoCytomation, Glostrup, Denmark; 30 μg/ml) for 1 h at room temperature. Samples were washed and incubated with a fluorescein isothiocyanate (FITC)–conjugated affinity pure $F(ab')_2$ fragment of goat anti-mouse immunoglobulin (IgG; Sigma) for 1 h at room temperature in the dark. Cells were then resuspended in PBS for acquisition.

### Analysis of Histone Acetylation/DNA Content

Cells were fixed for 15 min in 1% formaldehyde in PBS on ice and subsequently refixed in 70% EtOH. Due to the large amount of HDAC proteins present in the cells, deacetylation activity is very high. Consequently, the prefixation steps became very important for maintaining histones in a hyper-acetylated state and avoiding artifacts. All washings were performed in a refrigerated centrifuge using cold PBS, and cells were finally fixed on ice. Removal of cell culture medium containing TSA and substitution with PBS can cause complete loss of the enhanced acetylation signal, even during the few minutes needed for the prefixation washings (data not shown). Ethanol fixation is not compatible with acetylated histone detection; consequently, a first incubation with 1% formaldehyde was needed before alcoholic treatment for optimal staining with propidium iodide (PI). Cells, $1 \times 10^6$ per sample, were washed with PBS + 1% BSA and permeabilized with 200 μl of 0.1% Triton X-100 in PBS for 10 min at room temperature. After washing with PBS + 1% BSA, the samples were incubated with 500 μl of 10% normal goat serum in PBS for 20 min. Histone acetylation levels were detected by incubation with the T52 mouse monoclonal antibody (5 μg/ml in PBS + 1% BSA), T25 (1 μg/ml), and anti-tetra-acetylated histone H4 (1:50 dilution; Upstate, Charlottesville, VA, USA). T52 and T25 were produced by




C

| Tested peptide | T52 reactivity | T25 reactivity |
|---|---|---|
| 5 monoAc-H4 | + | - |
| 8 monoAc-H4 | + | + |
| 12 monoAc-H4 | + | + |
| 16 monoAc-H4 | + | - |
| 5,12 diAc-H4 | ++ | + |
| 8, 16 diAc-H4 | ++ | + |
| 5, 12, 16 triAc-H4 | ++ | + |
| 5, 8, 12, 16 tetraAc-H4 | ++ | ++ |
| not acetylated H4 | - | - |
| tri-acetylated peptide | ++ | - |
| monoAc. non-H4 peptide 1 | + | - |
| monoAc. non-H4 peptide 2 | ++ | - |
| P53 non acetylated peptide | - | - |
| P53 acetylated peptide | + | - |

FIG. 1. **A:**. Evaluation of the acetylation levels of histones in U937 cells after 4 h of treatment with 1 mM VPA, 50 ng/ml TSA, and 4 μM SAHA by western blot analysis using T52 antibody against acetyl-lysine (right panel). Equal loading was verified by Ponceau staining of the membrane (left panel). **B:** Evaluation of the of histone H4 acetylation in U937 cells after 24 h of treatment with 1 mM VPA, 50 ng/ml TSA, and 4 μM SuberoylAnilide Hydroxamic Acid (SAHA) by western blot analysis using T25 antibody against acetyl-H4 (upper panel). Equal loading was verified using an antibody against histone H4 (Upstate; lower panel). **C:** Specificity of T52 clone for acetyl-lysine. Differentially acetylated H4/non H4 derived peptides, 18-aa long, were used in enzyme-linked immunosorbent assay to determine the specificity of the T52 antibody.

mouse immunization with a peptide derived from the tetra-acetylated histone H4 tail (Fig. 1). Detection was performed by incubation with a FITC-conjugated, affinity purified $F(ab')_2$ fragment of goat anti-mouse IgG (Sigma) for 1 h at room temperature in the dark. Cells were resuspended in 1 ml of PI solution containing 2.5 μg/ml of PI in PBS and 25 μl of RNAse (1 mg/ml in water) and stained overnight at 4°C.

### Analysis of Transcription/DNA Staining

For detection of transcription, cells were first fixed in 1% formaldehyde and subsequently in 70% EtOH. Cells, $1 \times 10^6$ per sample, were washed, permeabilized, and subsequently incubated with a solution of 5 μg/ml of mouse monoclonal anti-bromodeoxyuridine (anti-BrdUrd) antibody (Becton Dickinson Co., Franklin Lakes, NJ, USA) in PBS + 1% BSA for 1 h at room temperature in the dark. The anti-BrdUrd antibody recognizes 5′-BrU and 5′-BrdUrd. After subsequent incubation with FITC-conjugated affinity pure $F(ab')_2$ fragment of goat anti-mouse IgG, cells were finally resuspended in 1 ml of PI solution containing 2.5 μg/ml of PI in PBS and 25 μl of RNAase, 1 mg/ml in water, and stained overnight at 4°C in the dark.

### Flow Cytometric Analysis

Flow cytometric analysis was performed by a FACScan flow cytometer (Becton Dickinson), acquiring at least 15,000 events/sample. The instrument was equipped with a bandpass 530/30-nm optical filter for FITC fluorescence detection (FL1 channel) and a 650-nm longpass optical filter in front of the PI (FL3) channel detector. Cells doublets were discriminated by pulsed processor analysis, according to the comparison of peak and area electronic signals from the FL3. Cell cycle distribution analysis was performed with ModFit software (Verity Software House, Topsham, ME, USA).

To evaluate correlation between DNA content and transcription and histone acetylation levels, cell distribution was subdivided into different classes on the base of the

mean fluorescence of PI. G1 and G2M compartments were selected according to the peak positions and instrument linearity in the FL3 channel. Intermediate DNA content was further partitioned into 12 different areas spanning S phase. FL1 (transcription or acetylation) mean fluorescence was then calculated and plotted for each subclass. To better evaluate TSA effects, the ratio between acetylation-related mean fluorescence in treated and exponentially growing cells (fold increase) was calculated per DNA compartment to unmask dependence on cell cycle distribution.

### Western Blot Analysis of Histone Acetylation

U937 cells ($5 \times 10^6$) were lysed in 80 μl of lysis buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 4% β-mercaptoethanol, and 1% sodium dodecylsulfate [SDS]), sonicated, and centrifuged for 20 min at 13,000 rpm at 4°C. An equal volume of 2× SDS gel-loading buffer was added to 10 μl of supernatant, and the samples were boiled for 3 min. The proteins were separated by 15% SDS-polyacrylamide gel, transferred to nitrocellulose transfer membrane, and probed with the indicated antibodies.

## RESULTS

The monoclonal antibody T52 was produced by immunization of mice with a peptide corresponding to the histone H4 N-terminal tail carrying the four acetylated lysines (K5, K8, K12, and K16). Western blotting (Fig. 1A) and enzyme-linked immunosorbent assay (Fig. 1C) experiments showed that it recognizes acetyl-lysine with variable efficiency, with a preferential binding for histone H3 and H4 substrates, as opposed to a second monoclonal antibody (T25), which recognizes specifically histone H4 carrying K8/K12 acetylated (Fig. 1B and 1C).

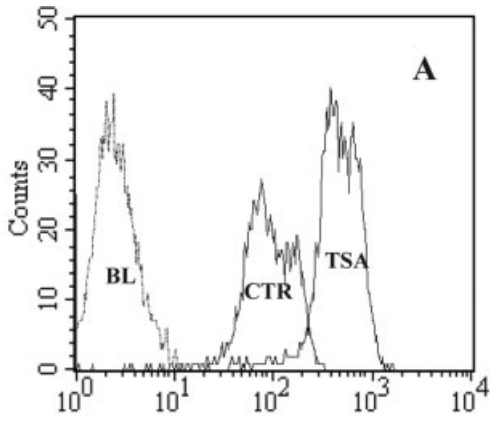
To test the possibility of performing histone acetylation level analysis by flow cytometry, exponentially growing U937 cells were subjected to treatment with TSA. The HDACi TSA binds to HDACs and inhibits their enzymatic activity (14). The block of acetyl-group removal from HDAC substrates leads to an overall increase in the number of acetylated proteins in the cell (of these proteins, the vast majority is composed of histones).

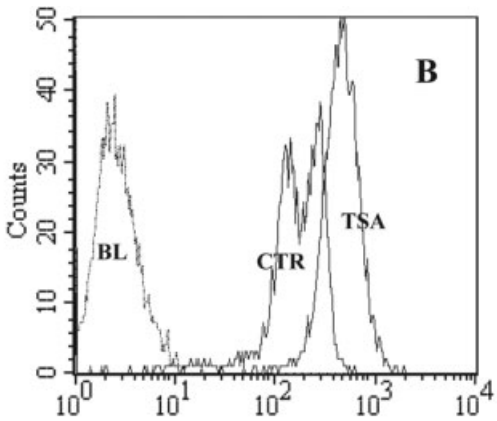
T52, T25, and a commercial anti tetra-acetylated histone H4 polyclonal antibody (Upstate) were employed, and after optimization of the working dilutions, the obtained results were compared (Fig. 2). All tested antibodies showed a fourfold increase in the mean fluorescence of TSA-treated cells in comparison with control, untreated, exponentially growing cells. Basal levels of acetylation were measurable (compare curves BL and CTR in Fig. 2) in these cells, as expected from the presence of acetylated histones in cells under growing conditions and confirmed by western blotting experiments carried out in parallel (data not shown). TSA dosage was chosen to maximize acetylation effects at early time points but minimize cell toxicity because increased concentration showed relevant cell death starting from 24 h of incubation (data not shown). Due to the comparable performances of the tested reagents, all studies in the present work were performed using the T52 antibody. Even if the antibody can react with all acetylated histone isoforms and -acetylated lysine residues, in whole cell assays, the amounts of acetylated, non-histone proteins is negligible, and for practical purposes the antibody can be considered as an anti-acetylated histone antibody as demonstrated by the obtained results.

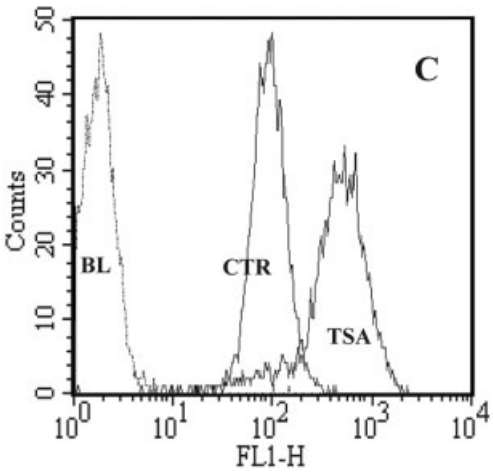
Biparametric T52/DNA analysis was then performed to correlate histone acetylation levels with cell cycle (Fig. 3). For parallel analysis of transcription, U937 cells were exposed to the halogenated riboside BrU, which permeates the cells and is assembled into mRNA, providing a measure of the global transcriptional activity (15–18). Commercial antibodies available for detection of BrdU exhibits cross-reactivity toward BrU and they can efficiently recognize BrU-containing RNA. Denaturation of nucleic acids (usually performed for detection of replicated DNA) was omitted because the compound is part of single-stranded RNA. Transcription levels showed a striking correlation with cell cycle progression (Fig. 3A and 3D). Transcriptional activity increased from G1 to S phase, and during replication transcriptional levels paralleled the increase in DNA content as demonstrated by the high correlation coefficient of the linear fit between DNA and RNA mean fluorescence. As expected, G2/M cells showed diminished BrU incorporation levels due to the reduced transcription rate during the last phases of the cell cycle (Fig. 3D).

Histone acetylation levels presented similar distributions during the cell cycle, demonstrating the strict correlation between chromatin modifications and RNA transcription/DNA replication (Fig. 3B and 3E). Fluorescence from T52 staining increased during S phase in proportion to the PI signal (Fig. 3E), thus paralleling the growing amount of histones during cell cycle progression. Similarly to the observed transcriptional behavior, when cells proceeded through late S and G2M phases, the signal intensity showed a decrement, indicating the enhanced activity of HDACs in these phases of the cell cycle.

Figures 3C and 3F show how TSA treatment caused a complete redistribution of acetylation signal throughout the cell cycle. After 4 h of treatment of asynchronous cells, when changes in cell cycle progression were not evident (data not shown), cells presented an evident increase in the mean acetylation level (Fig. 3E–G). Moreover, acetylation level modifications showed a striking cell cycle dependence with enhanced effects in G1 and G2M: TSA fold increase (TSA treated vs. untreated cell mean fluorescence) was almost constant through the S phase as expected for a signal dependent mainly on the total amount of histone present in the cells. The marked increase in G1 and in G2M (even if less pronounced) cells unmasked the already hypothesized enhanced HDAC activity in these phases. As a further remark, histone acetylation fluorescence from TSA-treated cells showed a high linear correlation with all the DNA fluorescence classes as a result of a now complete dependence from the total amount of histone (assumed to be proportional to the DNA content) including the G1 and G2M sub-phases,




| D | BL | CTR | TSA |
|---|---|---|---|
| Panel **A** T52 (5μg/ml) | 3 | 90 | 470 |
| Panel **B** T25 (1μg/ml) | 3 | 185 | 472 |
| Panel **C** Anti-Acetyl-H4 (dilution 1:50) | 3 | 98 | 509 |

FIG. 2. Monoparametric analysis of histone acetylation levels. **A:** Stained with mouse T52 antibody (5 μg/ml). **B:** Stained with mouse T25 antibody (1 μg/ml). **C:** Stained with rabbit anti–acetyl-histone H4 (dilution 1:50l Upstate). **D:** Table of mean fluorescence values (arbitrary units). BL, blank sample of U937 cells stained only with FITC-conjugated secondary antibody; CTR, U937 exponentially growing cells (control); TSA, U937 cells treated with 50 ng/ml of TSA for 4 h.

which were displaced in their acetylation level intensity with respect to the linear fit in an exponentially growing population.

The NB4 acute promyelocytic leukemia cell line can differentiate to granulocytes after RA treatment, due in part to RA-induced release of HDAC from the fusion protein PML-RAR (19). NB4 cells were treated with RA, TSA, and RA + TSA, and the effects on differentiation, histone acetylation, transcription, and cell cycle were analyzed by biparametric flow cytometry.

Surface differentiation-marker analysis showed that RA alone or in combination with TSA induced marked differentiation after 48 h, whereas TSA administration showed no effect (data not shown). Cell cycle progression was transiently altered by treatment with TSA alone or in combination with RA: cells showed a slight accumulation in the G1 phase of the cell cycle at 24 h (Fig. 4B), which disappeared at late time points. Transcription levels were not dramatically affected during treatment with TSA, RA, or RA + TSA at any examined time points as shown in Figure 4A (rows 2–4), with mean BrU fluorescence level essentially unchanged throughout the duration of drug treatments (Fig. 4C).

TSA treatment led to HDAC inhibition/increased acetylation levels with a very fast kinetics. Histone acetylation increased in all cell cycle phases after short incubation with TSA (2 h; Fig. 5A, column 2), reaching a maximal effect after 4 h of treatment (Fig. 5A, column 3). No effects on global acetylation were observed at the same time point or later in RA-treated NB4 cells (Fig. 5A). Simultaneous administration of RA and TSA marginally increased histone acetylation levels if compared with TSA treatment. Although TSA was maintained in the medium, acetylation levels returned to basal conditions in 24 h, indicating that the perturbation of the cell cycle and the acetylation modifications (Fig. 4B) were temporally uncoupled. Drug effects on acetylation levels completely disappeared after 48 h of treatment (Fig. 5, column 4).

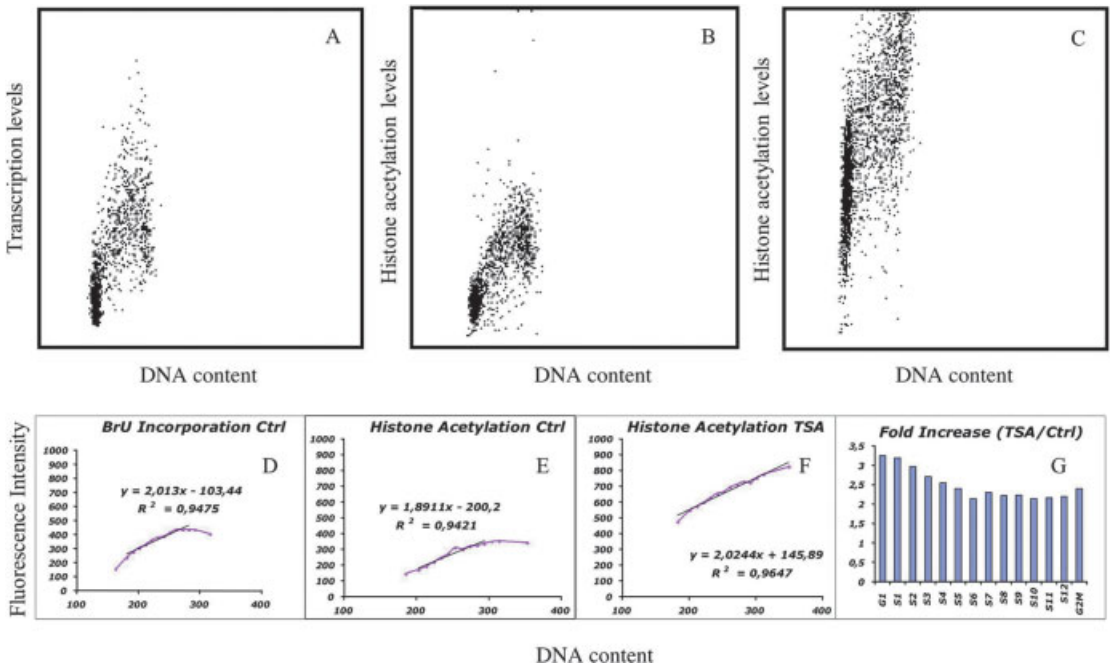



FIG. 3. Analysis of transcription and acetylation levels in correlation to cell cycle. **A:** Detection of transcription levels and cell cycle in exponentially growing U937 cells. Cells were incubated with 4 mM (−)-5-BrU for 30 min before fixation. Biparametric analysis of histone acetylation levels and cell cycle in (**B**) U937 exponentially growing cells and (**C**) U937 cells treated with 50 ng/ml of TSA for 4 h. **D–F:** Correlation between transcription (anti-BrU; D), histone acetylation (T52; E, F) mean fluorescence and DNA content in exponentially growing and TSA-treated cells. Values were calculated according to the procedure explained in Materials and Methods. **G:** Ratios between histone acetylation levels in TSA-treated versus untreated cells were then calculated for each DNA compartment showing an increased action of the drug in the G1–S early and late S–G2M phases.

Detailed analysis of the correlation between histone acetylation and cell cycle progression revealed the same pattern previously observed in U937 cells: TSA-treated cells loosed the typical downregulation of the acetylation signal in the G1–early S and late S–G2M cell cycle phases. Cells treated with TSA and with TSA + RA showed an almost perfect linear correlation with DNA (histone) content. The maximal increase in histone acetylation fluorescence is reached after 4 h of treatment in G1–S early compartment. Simultaneous administration of RA and TSA produced a slightly increased de-acetylation in the first hours of treatment (Fig. 5C, TSA/CTRL and TSA + RA/CTRL curves). Ratio between histone acetylation mean fluorescence in treated and control cells confirmed the transient effect of TSA and its disappearance at late time points.

HDACi currently represent one of the most promising new treatments for anticancer therapy. Consequently, new assays to test their activity in vivo are strongly desired. After a first preclinical validation phase conducted on leukemic mice treated with HDACi (data not shown), we decided to test the developed protocol for clinical use. We analyzed blood samples from patients affected by different tumors (solid tumors and leukemias) and treated with VPA, a drug used for years in the cure of epilepsy whose HDAC inhibition activity has been recently demonstrated (20). In the case of leukemias, analysis of blast cells from peripheral blood or bone marrow ago-aspirates will provide direct indication of the degree of HDAC inhibition induced by the HDACi. Although the analysis of normal cells from peripheral blood will not necessarily correlate with the acetylation status of cancer cells in the case of solid tumors, it may complement pharmacokinetic data by providing functional clues on the action of the drug and therefore acting as a possible surrogate marker. Figure 6 shows representative samples from patients treated with a standard dosage of VPA (30 mg/kg/day) and affected by breast cancer (Fig. 6A and 6B), colon cancer (Fig. 6C and 6D), melanomas (Fig. 6E and 6F), and leukemia (myeloproliferative disease in Fig. 6G and acute myeloid leukemia in Fig. 6H). Histone acetylation levels can be successfully measured with variable responses to VPA observed from case to case.

## DISCUSSION

In this study, we report for the first time the development of a flow cytometric protocol for detection of histone acetylation levels employing a mouse monoclonal antibody against acetylated lysine (T52). Selective signal increase after inhibition of HDACs demonstrated how the antibodies can detect variations in the mean number of acetyl groups (on all acetylated proteins for T52 and exclusively on histone H4 for T25, not shown) in a cell population.

Correlative analysis of DNA–histone acetylation levels allowed for characterization of the acetylation process during cell cycle. The entry in the replication phase was associated with an increasing mean value in the anti-acetylated histone signal from T52 monoclonal antibody, paralleling the growth in histone content associated with DNA duplication.

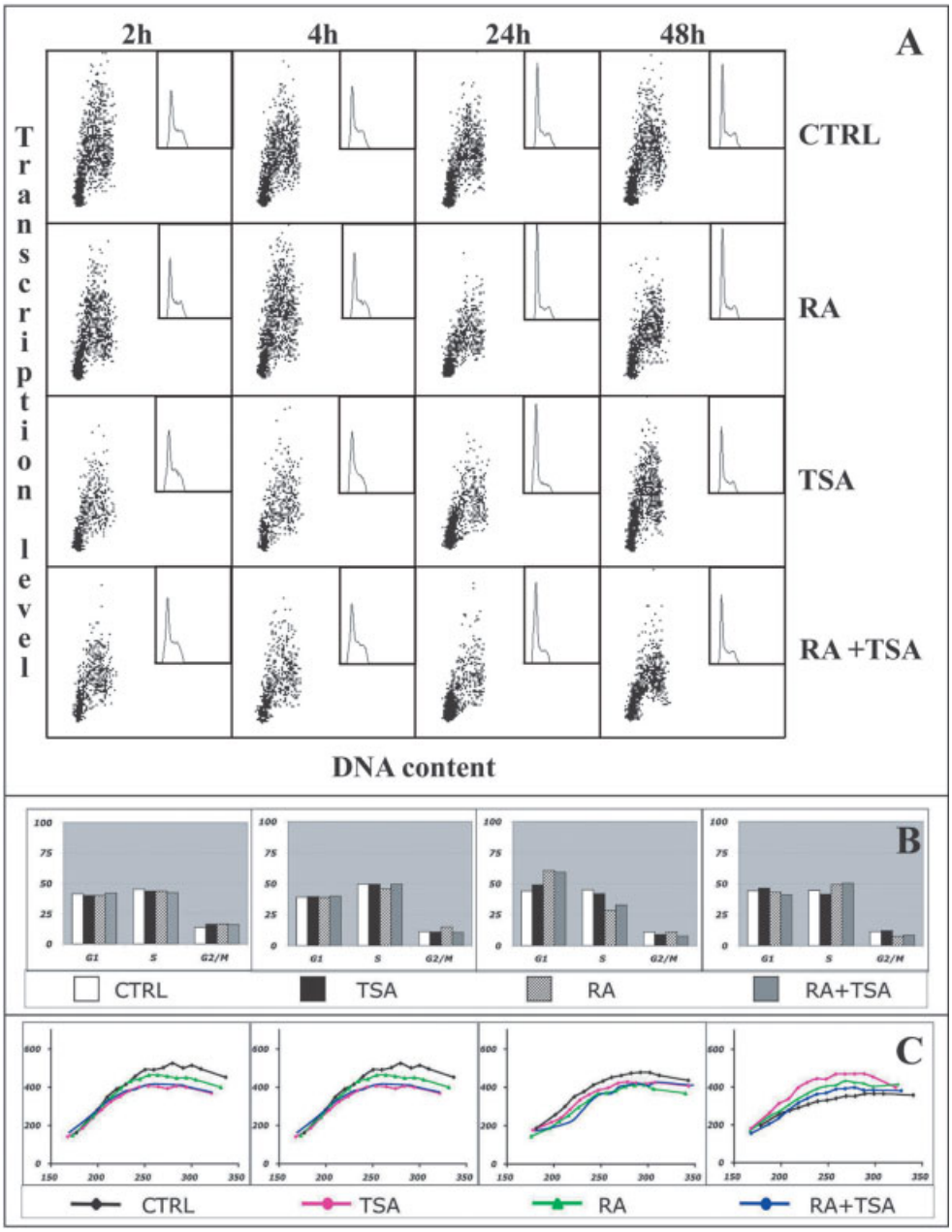



FIG. 4. Biparametric analysis of transcription levels and cell cycle. During the last 30 min of treatment, cells were incubated with 4 mM (−)-5-BrU. **A:** Exponentially growing NB4 cells (row 1), NB4 cells treated with 1 μM RA (row 2), NB4 cells treated with 50 ng/ml TSA (row 3), and NB4 cells treated with 50 ng/ml TSA and 1 μM RA (row 4) for 2, 4, 24, and 48 h. **B:** Cell cycle phase distributions. **C:** Correlation between transcription (anti-BrU) mean fluorescence and DNA content in exponentially growing and treated cells. Values were calculated according to the procedure explained in Materials and Methods.

Previously published fluorescence microscopy studies showed a specific regulation of acetylation signals during DNA replication. Maintenance of a heterochromatic state requires recruitment of acetyltransferase CAF-1 on replication foci, causing specific H4 acetylation on lysines 5 and 12 (21). At the end of S phase, an increased HDAC activity was instead observed in concomitance of recruitment of HDAC2 on newly synthesized DNA (22). A precise timing in the synthesis of expressed and silenced genes has been consequently demonstrated, with the first and middle S phases devoted to replication of actively transcribed, hyper-acetylated chromatin (euchromatin), whereas DNA synthesis ends with silenced and hypo-acetylated chromatin (heterochromatin) reconstitution (23). Our flow cytometric data well fit into this scheme, as demonstrated by a first increase in histone acetylation signal detected in the G1 to S phase transition and by the appearance of a subpopulation characterized by lower acetylation signal close to the tetraploid DNA content (late S to G2M cell cycle phase) which can not simply related to the variation of the total amount of histone present in the cells. Strikingly a perfect correlation between acetylation-related fluorescence and DNA content (considered as an indirect measure of histone content) is achieved after inhibition of HDAC activity, demonstrating that only in this case the T52 signal could reflect the total amount histone protein

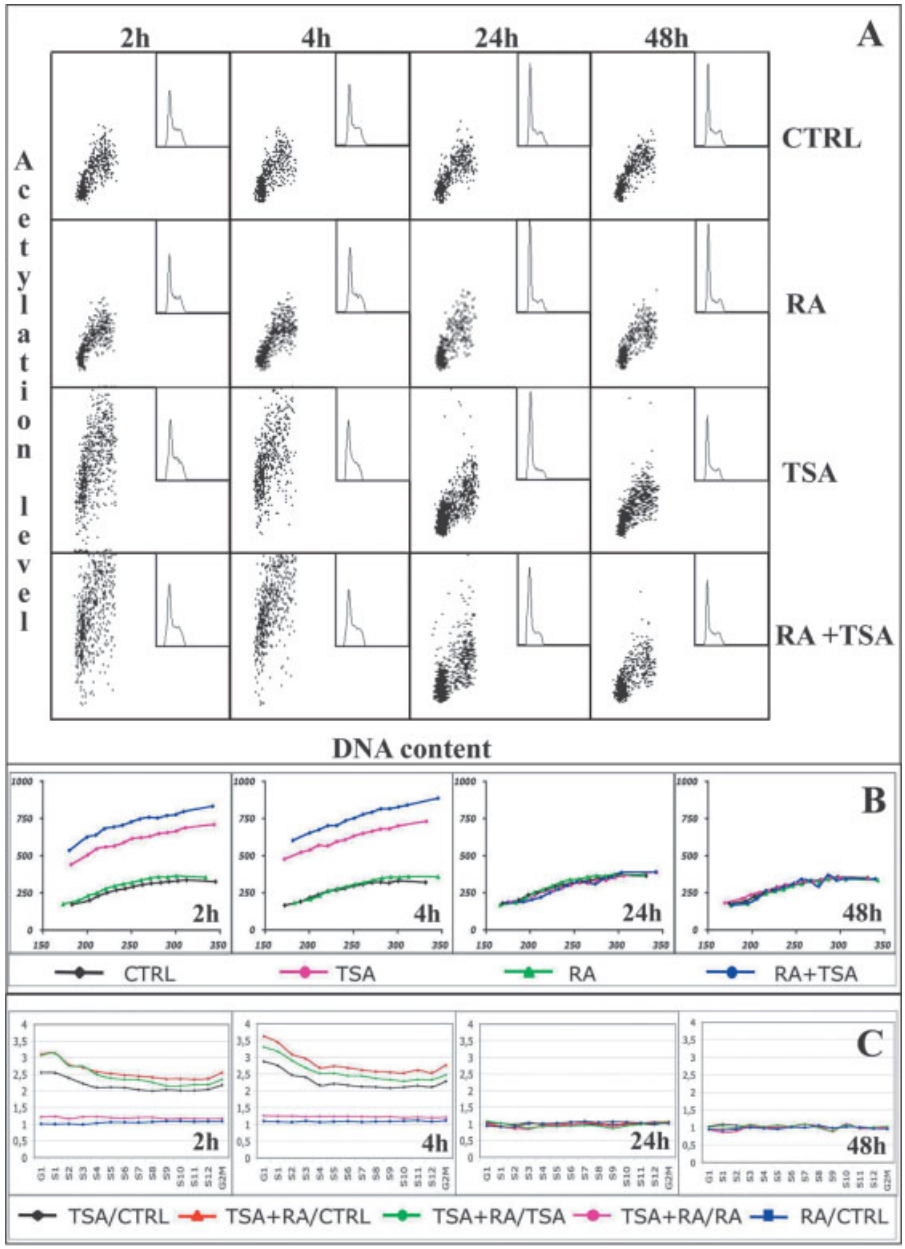



FIG. 5. Biparametric analysis of histone acetylation levels and cell cycle. **A:** Exponentially growing NB4 cells (row 1), NB4 cells treated with 1 μM RA (row 2), NB4 cells treated with 50 ng/ml TSA (row 3), and NB4 cells treated with 50 ng/ml TSA and 1 μM RA (row 4) for 2, 4, 24, and 48 h. **B:** Correlation between histone acetylation (T52) mean fluorescence and DNA content in exponentially growing and treated cells at selected time points during treatment. Values were calculated according to the procedure explained in Materials and Methods. **C:** Measurement of acetylation variation in response to TSA, RA, and TSA + RA treatments calculated as the ratio between histone acetylation mean fluorescence in treated and untreated cells in function of the mean DNA content. Each panel reports the measurement at the indicated time point.

present in the cells throughout the entire cell cycle. A parallel analysis on transcriptional activity measured by BrdUrd incorporation levels provided a further correlation of histone acetylation levels with transcription and replication, as demonstrated by the large amount of newly transcribed RNA during DNA synthesis.

The developed flow cytometric protocol allowed for characterization of global transcription and chromatin organization during hematopoietic differentiation. Analysis of data obtained from the NB4 in vitro model showed how creation of a chromatin environment favorable to transcription by histone hyper-acetylation is not paralleled by an increase in the effective transcription rate. HDACi treatment does not consequently imply general unspecific gene activation. Hyper-acetylation effects are located in a very narrow temporal window in the first hours of treatment; repeated treatments could be consequently requested to maintain HDAC inhibition over time.

The possibility to directly measure the activity of HDAC inhibitors in vivo through analysis of peripheral blood cell acetylation levels can provide a new parameter to complement classic analysis of drug treatment such as measurement of compound levels in serum. Direct comparison of side effects, drug concentration, and indirect drug activity based on acetylation level detection in blood cells could provide a more complete picture to be used in the design of treatment plans and to frequently monitor compound activity during clinical trials. Studies are in progress to test

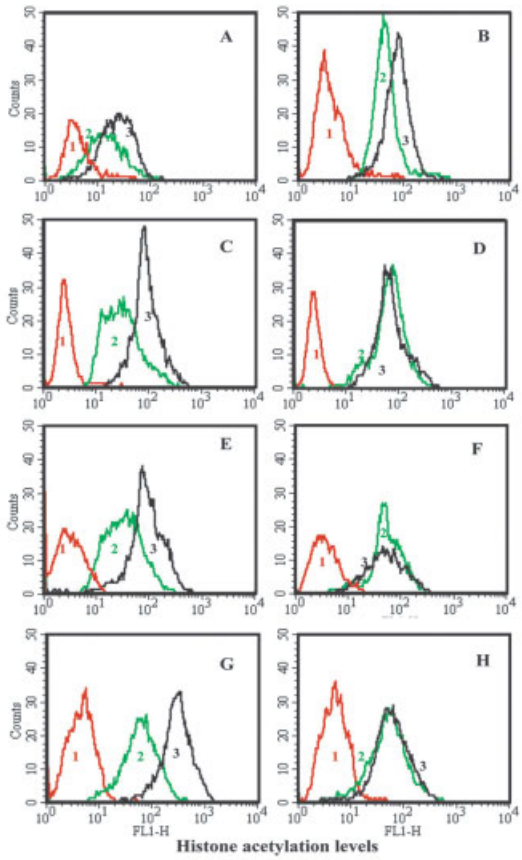



FIG. 6. Histone acetylation levels in peripheral blood cells from patients affected by breast cancer (**A, B**), colon cancer (**C, D**), melanomas (**E, F**), and leukemia (**G:** myeloproliferative disease, **H:** acute myeloid leukemia) treated with the HDACi VPA. Blood samples were taken before beginning therapy (curve 2) or after 2 weeks of treatment (curve 3; 30 mg/kg/day administered orally). **I:** Table of mean fluorescence values (arbitrary units).

**Histone acetylation levels**

| I | Blank sample stained only with FITC-conjugated antibody **Curve 1** mean value (A.U.) | Blood samples were taken before beginning of the therapy **Curve 2** mean value (A.U.) | Blood samples were taken after2 weeks of treatment **Curve 3** mean value (A.U.) |
|---|---|---|---|
| Breast cancer **Panel A** | 4 | 23 | 34 |
| Breast cancer **Panel B** | 7 | 60 | 77.3 |
| Colon cancer **Panel C** | 4 | 40 | 96 |
| Colon cancer **Panel D** | 3 | 73 | 78 |
| Melanoma **Panel E** | 10 | 41 | 109 |
| Melanoma **Panel F** | 13 | 64 | 65 |
| Leukaemia**Panel G** | 5 | 69 | 318 |
| Leukaemia**Panel H** | 7.7 | 70 | 80 |

the ability to directly measure HDAC inhibition in tumor samples. Moreover, flow cytometric analysis of histone acetylation levels can open new promising perspectives in the field of tumor characterization and prognostic marker identification. The protocol we developed can be efficiently employed for multivariate analysis to correlate histone acetylation and DNA ploidy and/or expression of known oncogenic factors.

## ACKNOWLEDGMENTS

We thank Daniele Piccini, Marco Maccarana, and Liliana Areces for the development of T52 mouse monoclonal antibody. Simona Ronzoni received a prize from the Gruppo Italiano Citometria for protocol development.

## LITERATURE CITED

1. Berger SL. Histone modifications in transcriptional regulation. Curr Opin Genet Dev 2002;12:142–148.
2. Jenuwein T, Allis CD. Translating the histone code. Science 2001;293: 1074–1080.
3. Grunstein M. Histone acetylation in chromatin structure and transcription. Nature 1997;389:349–352.
4. Kornberg RD, Lorch Y. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. Cell 1999;98:285–294.
5. Minucci S, Pelicci PG. Retinoid receptors in health and disease: coregulators and the chromatin connection. Semin Cell Dev Biol 1999; 10:215–225.
6. Gelmetti V, Zhang J, Fanelli M, Minucci S, Pelicci PG, Lazar MA. Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. Mol Cell Biol 1998;18:7185–7191.
7. Faretta M, Di Croce L, Pelicci PG. Effects of the acute myeloid leukemia–associated fusion proteins on nuclear architecture. Semin Hematol 2001;38:42–53.
8. Minucci S, Maccarana M, Cioce M, De Luca P, Gelmetti V, Segalla S, Di Croce L, Giavara S, Matteucci C, Gobbi A, et al. Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation. Mol Cell 2000;5:811–820.

9. Minucci S, Nervi C, Lo Coco F, Pelicci PG. Histone deacetylases: a common molecular target for differentiation treatment of acute myeloid leukemias? Oncogene 2001;20:3110–3115.
10. Alcalay M, Orleth A, Sebastiani C, Meani N, Chiaradonna F, Casciari C, Sciurpi MT, Gelmetti V, Riganelli D, Minucci S, et al. Common themes in the pathogenesis of acute myeloid leukemia. Oncogene 2001; 20:5680–5694.
11. Warrell RP Jr, He LZ, Richon V, Calleja E, Pandolfi PP. Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. J Natl Cancer Inst 1998;90:1621–1625.
12. Johnstone RW. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat Rev Drug Discov 2002;1:287–299.
13. Turner BM. Cellular memory and the histone code. Cell 2002;111: 285–291.
14. Finnin MS, Donigian JR, Cohen A, Richon VM, Rifkind RA, Marks PA, Breslow R, Pavletich NP. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature 1999;401:188–193.
15. Halicka HD, Bedner E, Darzynkiewicz Z. Segregation of RNA and separate packaging of DNA and RNA in apoptotic bodies during apoptosis. Exp Cell Res 2000;260:248–256.
16. Jensen PO, Larsen J, Larsen JK. Flow cytometric measurement of RNA synthesis based on bromouridine labelling and combined with measurement of DNA content or cell surface antigen. Acta Oncol 1993;32:521–524.
17. Jensen PO, Larsen J, Christiansen J, Larsen JK. Flow cytometric measurement of RNA synthesis using bromouridine labelling and bromodeoxyuridine antibodies. Cytometry 1993;14:455–458.
18. Jensen PO, Larsen J, Larsen JK. Analysis of RNA synthesis by cytometry. Methods Cell Biol 2001;64:129–138.
19. Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F, Berger R. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood 1991;77: 1080–1086.
20. Gottlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG, et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 2001;20:6969–6978.
21. Taddei A, Roche D, Sibarita JB, Turner BM, Almouzni G. Duplication and maintenance of heterochromatin domains. J Cell Biol 1999;147: 1153–1166.
22. Rountree MR, Bachman KE, Baylin SB. DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. Nat Genet 2000;25:269–277.
23. Jasencakova Z, Meister A, Walter J, Turner BM, Schubert I. Histone H4 acetylation of euchromatin and heterochromatin is cell cycle dependent and correlated with replication rather than with transcription. Plant Cell 2000;12:2087–2100.