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Elevated level of lysine 9-acetylated histone H3 at the MDR1 promoter in multidrug-resistant cells

Monika Toth,[1,4] Imre M. Boros[2] and Eva Balint[1,3,5]

[1]Institute for Plant Genomics, Human Biotechnology and Bioenergy (BAYGEN), Bay Zoltan Foundation for Applied Research, Szeged;
[2]Chromatin Research Group of University of Szeged–Hungarian Academy of Sciences, Department of Biochemistry and Molecular Biology, University of Szeged, Szeged, Hungary

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Failure of chemotherapy in breast cancer presents a major problem and is often due to elevated expression of ATP binding cassette (ABC)-type transporters, such as MDR1 protein. It has been shown that *MDR1/ABCB1* gene expression is regulated at the chromatin level by DNA methylation and histone acetylation. However, the modified histone residues have not been identified and the role of various histone acetyl transferases (HATs) is not fully understood. By studying a breast carcinoma model cell line and its *MDR1*-overexpressing derivative, we show that the histone 3 lysine 9 (H3K9) acetylation level is elevated 100-fold in the promoter and first exon of the *MDR1* gene in the drug-resistant cell line compared to the drug-sensitive cell line. The acetylation level of the other examined lysine residues (H3K4, H3K14, H4K8, and H4K12) is weakly or not at all elevated in the *MDR1* locus, although their acetylation is generally increased genome-wide in the drug-resistant cell. Downregulation of the expression of HATs PCAF and GCN5 by RNAi effectively reduces the expression of *MDR1*. Unexpectedly, treatment with a p300-selective inhibitor (HAT inhibitor II) further increases *MDR1* expression and drug efflux in the drug-resistant cells. Our data suggest that repeated exposure to chemotherapy may result in deregulated histone acetylation genome-wide and in the *MDR1* promoter. (*Cancer Sci* 2012; 103: 659–669)

The main reason for the failure of chemotherapy is the development of multidrug resistance in cancer cells, which is often caused by the elevated level of ATP binding cassette (ABC)-type transporter proteins that eliminate the chemotherapeutic drugs from the cell. The best characterized ABC transporter in cancer cells is P-glycoprotein (PgP), which is encoded by the *MDR1/ABCB1* gene. The two possible mechanisms that can contribute to the elevated expression of *MDR1* are: (i) genetic changes, meaning amplification or translocation of the *MDR1* gene;[1–3] or (ii) epigenetic changes, meaning increased expression of *MDR1* due to chromatin remodeling.[4,5] Epigenetic changes are preserved through cell division, thus the gene remains in active form after the cessation of chemotherapy.[6]

Breast cancer is usually initially responsive to chemotherapy, but acquisition of multidrug resistance is associated with worse prognosis. Numerous clinical studies detected PgP expression in a significant percentage of untreated breast cancers, showed that MDR1 mRNA expression increased after exposure to chemotherapy and correlated increased expression with a worse response to treatment.[7]

Expression of *MDR1* is induced rapidly by doxorubicin.[8] It can also be activated by drugs that are not PgP substrates, as well as by heat shock and genotoxic stress. The *MDR1* gene is transcribed from two promoters.[9] The majority of the transcripts originate from the downstream promoter (DSP),[10] whereas the upstream promoter (USP), located 112 kb upstream of the former, in the neighboring *RPIB9/RUNDC3B* gene, is active in some but not all multidrug-resistant cells.[11–13] The DSP is well characterized; several cis-regulatory elements and binding factors acting in positive or negative regulation of *MDR1* have been described.[14,15] An enhanceosome can form on an inverted CCAAT box binding NF-Y and recruiting p300/CREB binding protein (CBP)-associated factor (PCAF), a histone acetyltransferase (HAT).[16] The role of histone acetylation and deacetylation in the transcriptional regulation of *MDR1* has been established. Co-transfection of HATs p300, CBP, or PCAF co-activates the downstream *MDR1* promoter–luciferase construct.[16,17] Binding of these HATs to the *MDR1* promoter has been determined *in vivo*.[17–19]

Elevated levels of histone acetylation in the chromatin of the *MDR1* promoter in drug-resistant cells have been described before,[18,20,21] however, the acetylation of specific lysine residues has not been investigated. We examined the acetylation of various histone H3 and H4 residues in a breast carcinoma cell line and its *MDR1* overexpressing derivative. We found that H3K9 acetylation is elevated by two orders of magnitude in the major promoter and first exon of the *MDR1* gene in a drug-resistant cell line compared to a drug-sensitive cell line. Acetylation of the other examined lysine residues was weakly or not at all elevated in the *MDR1* locus, although their acetylation was globally increased in the drug-resistant cells. Simultaneous knockdown of PCAF and GCN5 decreased the expression of MDR1. Unexpectedly, treatment with HAT inhibitor II further increased *MDR1* expression and drug efflux in the drug-resistant cells. Our data suggest that repeated exposure to chemotherapy may result in altered regulation of histone acetylation, and that malignant cells accumulate multiple mechanisms to ensure elevated expression of *MDR1* and evasion of therapy.

Materials and Methods

Cell culture and drug treatment. Breast carcinoma cell line MCF7 was from ATCC (Manassas, VA, USA). The doxorubicin-resistant MCF7-KCR cell line had been developed from MCF-7 by stepwise selection, increasing the dose of doxorubicin from 10 nM to 1 μM.[22,23] Cells were maintained in RPMI medium supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 4 mM glutamine, and antibiotic–antimycotic solution (Gibco/Life Technologies, Carlsbad, CA, USA) at 37°C in a 5% CO_2 atmosphere. MCF7-KCR cells were maintained in cycles of 1 week in medium containing 1 μM doxorubicin followed by 1 week in drug-free medium. Before each experiment, cells were grown in drug-free medium for approximately

[3]To whom correspondence should be addressed.
E-mail: ebalint@bio.u-szeged.hu
[4]Present address: Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary.
[5]Present address: Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary.

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1 week. Doxorubicin (Sigma-Aldrich) was dissolved in PBS at 1.72 mM stock concentration (Fig. 1). For drug accumulation assay, cells were incubated with 25 μM doxorubicin for 4 h. After gentle trypsinization and washing, fluorescence of the cells was analysed by FACS on FL-2 channel using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA), 10 000 cells were counted and analyzed using CellQuestPro (Becton Dickinson). Trichostatin A (TSA) was dissolved in DMSO to 2 mg/mL; cells were treated for 24 h at the indicated concentrations (Figs 2,3).

RNA interference, analysis of mRNA expression, and quantitative PCR (qPCR). For RNA interference, siRNA targeting PCAF, GCN5, ADA2B, or non-targeting siRNA for control (Dharmacon/Thermo Fisher Scientific, Waltham, MA, USA, or Ambion/Life Technologies) was transfected into cells using DharmaFECT 1 (Dharmacon) transfection reagent following the manufacturer's protocol (Fig. 4). Expression of mRNAs and proteins was examined 48 and 72 h after transfection, respectively. Total RNA was purified from the cells using the RNeasy Plus kit (Qiagen, Hilden, Germany). Reverse transcription was carried out using TaqMan Reverse Transcription Reagents (ABI/Life Technologies) and random hexamers from 1 to 2 μg RNA. Real-time PCR was carried out with the SYBR-Green detection method on StepOne Plus Real-Time PCR System (ABI/Life Technologies). Primers used are summarized in Table S1. Thermal cycling was done for 10 min at 95°C followed by 50 cycles of 95°C for 15 s and 60°C for 45 s. Dissociation (melt) curves were analyzed after each run to confirm primer specificity. The 18S rRNA gene was used for normalization. HAT inhibitor II [2,6 bis-(3-bromo-4-hydroxybenzylidene), Calbiochem/Merck KGaA, Darmstadt, Germany] was dissolved in ethanol to 21.5 mM concentration; cells were treated for 24 h at the indicated concentrations (Figs 5,6).

Western blot analysis and antibodies. Histone proteins were acid extracted according to the Abcam histone extraction protocol. Equal amounts of proteins were size-fractionated by 15% SDS-PAGE and transferred to nitrocellulose membrane. Antibodies were used at the following dilutions for immunoblotting: anti-H3 (Abcam, Cambridge, UK) 1:10 000; anti-H3K4acetyl (Millipore, Billerica, MA, USA) 1:5000; anti-H3K9acetyl (Millipore) 1:5000; anti-H3K14acetyl (Millipore) 1:5000; anti-H3K18acetyl (Abcam) 1:10 000; anti-H4K8acetyl (Abcam) 1:10 000; and anti-H4K12acetyl (Abcam) 1:500. Antibodies used for ChIP were the same, expect for the acetyl-H3K9-specific Ab (Diagenode, Liege, Belgium). Specific Abs for PCAF (#2760)[24] and GCN5 (2GC2c11)[25] have been described. The ADA2B Ab (#2459) was a generous gift from D. Devys (IGBMC, Illkirch, France).[26]

Chromatin immunoprecipitation. The ChIP analysis was done using the Acetyl-Histone H3 Immunoprecipitation Assay Kit (Millipore) according to the manufacturer's instructions. Lysis buffer was supplemented with protease inhibitor cocktail (Sigma-Aldrich) and 150 ng/mL TSA to preserve acetylation. One percent of each chromatin sample without immunoprecipitation provided the input DNA control. Immunoprecipitated DNA was analyzed by qPCR, percent of input was calculated using the ΔC_t method. Independent experiments were done between two and six times. Statistical analyses between groups was carried out by ANOVA and the Holm-Sidak *post hoc* test using SigmaStat (Systat Software Inc, Chicago, IL, USA) and Student's *t*-test using Excel (Microsoft, Redmond, WA, USA).

Results

Multiple mechanisms contribute to increased expression of MDR1 in the MCF7-KCR cell line. The MCF7 breast carcinoma cell line is sensitive to chemotherapeutic agents, but several drug-resistant sublines have been established from it.[27] We chose the MCF7-KCR cell line that had been established through stepwise treatment of MCF7 with doxorubicin.[22,23] Fluorescent assay showed lower intracellular doxorubicin accumulation in the MCF7-KCR cells than in the parental cells (Fig. 1a); the doxorubicin ID_{50} was 0.5 μM for MCF7 and 50 μM for MCF7-KCR cells (data not shown). To determine if drug resistance was caused by increased expression of drug transporters, we studied the mRNA expression of MDR1 and MRP1 by RT-qPCR. We found that the mRNA level of MDR1/ABCB1 was elevated 72 000-fold in the MCF7-KCR cell line compared to the parental cell line, whereas that of MRP1/ABCC1 was only elevated sevenfold (Fig. 1b). Expression of *RPIB9*, the gene partially overlapping *MDR1*, was also increased 10-fold, whereas the expression of control genes *GAPDH* and *c-FOS* was not significantly changed. The RT-PCR with primers specific to the −1 exon of *MDR1* showed that transcription was not initiated from the USP (data not shown). To determine if gene amplification was responsible for the enormous increase in the *MDR1* expression, we carried out qPCR on genomic DNA. The copy number of the *MDR1/ABCB1* locus in the MCF7-KCR cells was 40-fold that in the MCF7 cells (Fig. S1). These data suggest that, from each *MDR1* gene copy, approximately 2000-fold more mRNA is expressed in the drug-resistant cells than in the sensitive cells. Treatment of MCF7-KCR cells with MDR1-specific siRNA resulted in a strong reduction of MDR1 message and protein levels (data not shown). However, neither drug accumulation assay nor cell viability assay showed significant functional difference between non-targeting siRNA-treated control and MDR1 siRNA-treated cells, probably due to an effect of the transfection reagent on the cell membrane (data not shown). Thus, we conclude that in the MCF7-KCR cell line drug resistance is likely caused by a high level of expression of *MDR1*, and gene amplification is partially but not solely responsible for this. Other mechanisms, possibly epigenetic changes, might also contribute to the high MDR1 mRNA level in the resistant cells.

H3K9ac is highly enriched at downstream promoter and first exon of MDR1 in drug-resistant cell line. In order to study the epigenetic regulation of *MDR1* expression in the two cell lines, we carried out ChIP using Abs specific to acetylated histone lysine residues H3K4, H3K9, H3K14, H4K8, and H4K12. We analyzed the immunoprecipitated DNA by qPCR over the entire *MDR1* locus: using primer pairs specific to the USP, upstream first exon (minus exon); DSP, two regions of the downstream first exon (exon1-1 and 1-2); gene body (exon 7); and 3′-untranslated region (Fig. 1c). We also examined histone acetylation on the promoter of the *RPIB9/RUNDC3B* gene, which is transcribed from the other strand partially overlapping with *MDR1*. The *c-FOS* promoter and an intergenic region were used as controls for high and low levels of acetylated histones, respectively (see Table S1). In MCF7 cells, acetylation of H3K9 is very low in every studied region of the *MDR1* locus, 25-fold lower than that in the c-FOS promoter. Importantly, H3K9 acetylation is increased 60-fold in the DSP region and 110-fold in the downstream first exon in the drug-resistant cells compared to the level in the sensitive cells (Fig. 1e). The acetylation level of H3K4 over the *MDR1* gene locus in MCF7 cells is approximately sixfold lower than that in the c-FOS promoter (Fig. 1d). In MCF7-KCR cells, H3K4 acetylation is slightly increased in the *MDR1* locus, in particular at the DSP, first exon and gene body region. Similarly, acetylation values of H4K8 in MCF7 cells are approximately threefold lower at the studied regions of *MDR1* than at the c-FOS promoter; in MCF7-KCR cells, in the *MDR1* DSP and along the gene body, they reach the level of that at the c-FOS promoter (Fig. 1g). Furthermore, acetylation values of H4K12

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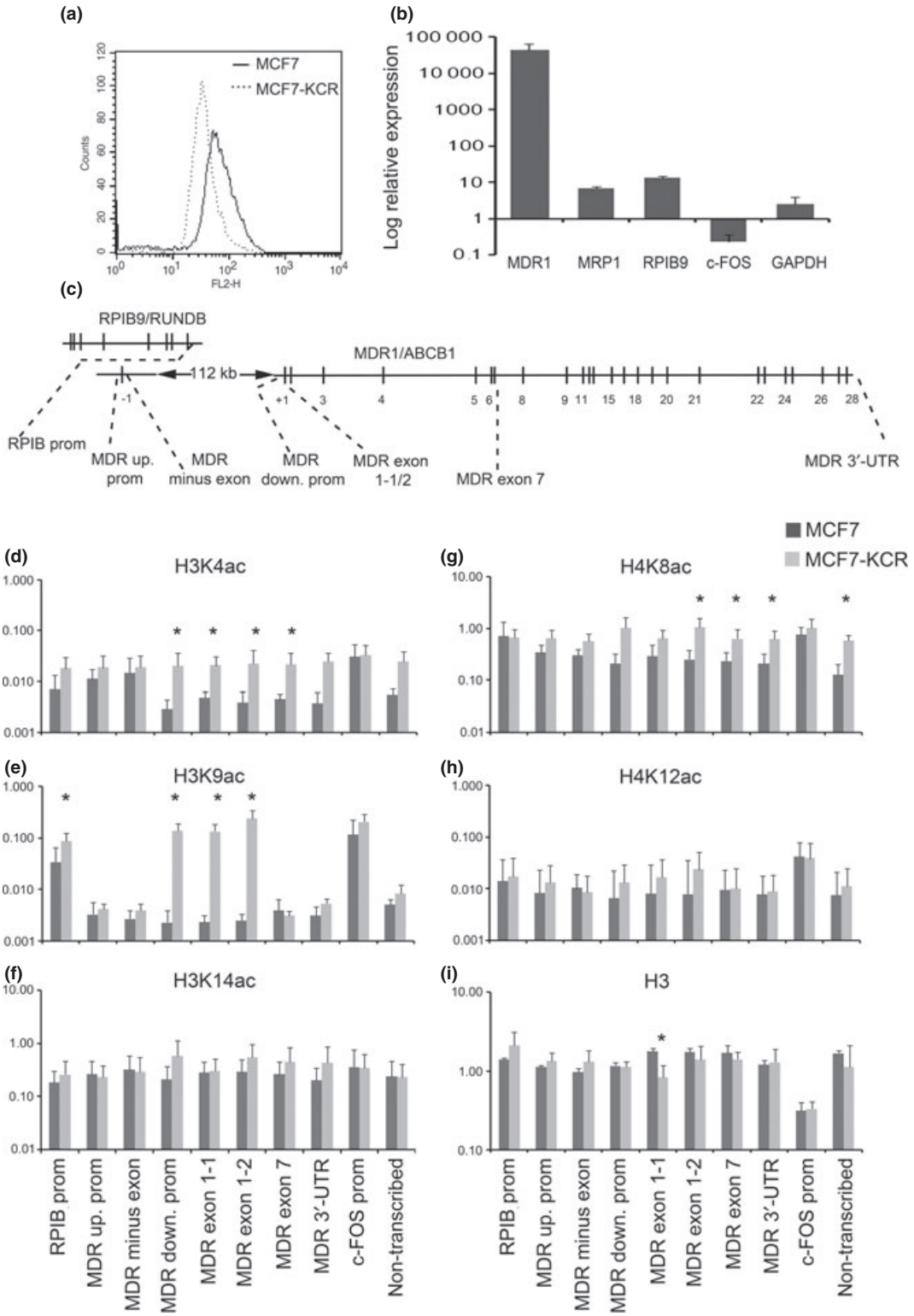

Fig. 1. Comparison of MCF7 and MCF7-KCR breast cancer cell lines for drug accumulation, drug transporter gene expression, and histone acetylation at the *MDR1*/*ABCB1* locus. (a) Cells were treated with 25 μM doxorubicin for 4 h and fluorescence was determined by FACS. MCF7-KCR cells accumulated less drug, indicating highly elevated ATP binding cassette (ABC) transporter activity. (b) The mRNA levels of MDR1, MRP1, RBIP9, c-FOS, GAPDH, and 18S for internal control, were quantified by quantitative PCR (qPCR) and are shown in the MCF7-KCR cell line relative to that in MCF7 cells. (c) Physical map of the *MDR1* locus. Exons are indicated as crossing lines, some exons are numbered. Upper line indicates the *RPIB9*/*RUNDC3B* gene transcribed in the opposite direction. The positions of qPCR primers used in ChIP experiments are indicated. c-FOS-specific primers on chromosome 14 and primers for a non-transcribed region on chromosome 6 are not shown. The ChIP experiments were carried out using anti-H3K4ac (d), anti-H3K9ac (e), anti-H3K14ac (f), anti-H4K8ac (g), anti-H4K12ac (h), and anti-H3 pan (i) Abs. The quantities of precipitated DNA corresponding to the indicated regions were determined by qPCR, and are expressed as the percent of input. The scales are logarithmic and different in different experiments. Values represent the means of 2–5 experiments. Error bars indicate SD. Down., downstream; prom, promoter; up., upstream; UTR, untranslated region. *$P < 0.05$, significantly different in MCF7-KCR versus MCF7 cells.

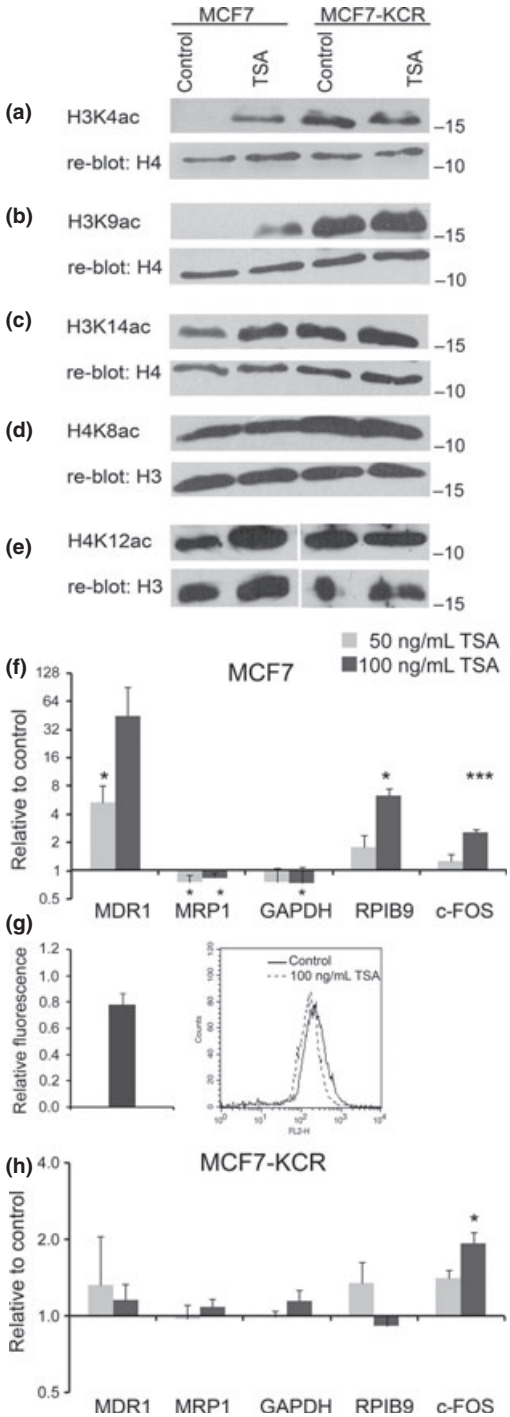

Fig. 2. Analysis of changes in genome-wide histone acetylation, mRNA expression level, and drug accumulation after treatment with trichostatin A (TSA). (a–e) Analysis of genome-wide acetylation levels of specific histone lysine residues in drug-sensitive and drug-resistant cells. Histones were extracted from mock-treated cells (control) or cells treated with 50 ng/mL TSA for 24 h. Western blot analysis was carried out with the indicated acetyl-lysine-specific Abs, and each membrane was reblotted with a control Ab. (f,h) MCF7 and MCF7-KCR cells were mock-treated or treated with 50 ng/mL (gray bars) or 100 ng/mL (black bars) TSA for 24 h. The level of the indicated transcripts and 18S RNA (as internal control) was quantified by RT-quantitative PCR. Expression of each gene is shown relative to that in the mock-treated control cells. $*P < 0.05$; $***P < 0.005$. (g) MCF7 cell were treated with 100 ng/mL TSA and drug accumulation was determined by FACS. Bar graph shows average and SD of three independent experiments, curves are from one representative experiment.

at the *MDR1* locus are approximately eightfold lower than at the c-FOS promoter in both cells (Fig. 1h). In contrast, acetylation of H3K14 is uniform in both cells (Fig. 1f). The striking differences between the levels of particular modified histone forms in drug-resistant and drug-sensitive cells are unlikely to have resulted from differences in nucleosome densities in the *MDR1* locus, because immunoprecipitation carried out with the H3 pan Ab resulted in similar values in the two cell lines (Fig. 1i). These data reveal that highly elevated levels of acetylated H3K9 at the DSP and first exon of the *MDR1* gene correlate best with increased *MDR1* expression in the drug-resistant MCF7-KCR line.

Global histone acetylation levels higher in drug-resistant than in drug-sensitive cell lines. The observed alteration in the histone acetylation levels at the *MDR1* locus in the MCF7-KCR cells prompted us to study the global acetylation of specific histone lysine residues in the two cell lines. Western blot analysis showed that, although to different extent, each examined acetylated forms of histone H3 and H4 were present in higher levels in the drug-resistant cell line than in the drug-sensitive counterpart (Fig. 2a–e). Most strikingly, although K4 and K9 acetylated forms of H3 were hardly detectable in the parental cells, they were present at highly elevated levels in the drug-resistant cells (Fig. 2a,b, tracks 1,3). A less dramatic, but still noticeable, increase in the levels of H3K14, H4K8, and H4K12 was detectable in the drug-resistant cells compared to the drug-sensitive line (Fig. 2c–e, tracks 1,3). In order to further characterize the regulation of histone acetylation, we treated the two cell lines with TSA, a histone deacetylase (HDAC) inhibitor, for 24 h. The TSA treatment induced a large increase of H3K4 and H3K9 acetylation and a moderate increase of H3K14, H4K8, and H4K12 acetylation in MCF7 cells (Fig. 2a–e, track 2), but the acetylation level of the studied residues did not increase any further in MCF7-KCR cells (Fig. 2a–e, track 4). These data show that global increases in the levels of the studied acetylated forms of H3 and H4 are characteristic features of the drug-resistant cell line, and HDAC inhibition causes no further increase.

Inhibition of HDACs upregulates MDR1 expression and increases H3K9 acetylation at the first exon of MDR1 in drug-sensitive but not drug-resistant cells. In order to obtain further proof of the role of histone acetylation in *MDR1* expression, we treated the two studied cell lines with TSA and examined changes in mRNA expression. *MDR1* expression increased fivefold or 45-fold after treatment with 50 or 100 ng/mL TSA, respectively, in the drug-sensitive line (Fig. 2f), however, it did not change in the drug-resistant line compared to untreated cells (Fig. 2h). Fluorescent assay showed decreased drug accumulation in MCF7 cells (Fig. 2g) but not in MCF7-KCR cells (data not shown), indicating that increased MDR1 expression results in drug efflux. Expression of RPIB9 increased sixfold in MCF7 cells, but did not change in MCF7-KCR cells. However, the expression of MRP1 and GAPDH did not change in either cell line, supporting the model of Wang *et al.*[28] that HDAC inhibitor treatment induces expression of only certain "primed" genes. Therefore, we were interested in whether TSA had any local effect on the chromatin structure of the *MDR1* gene, and carried out ChIP analysis. In the MCF7 cells we detected large increases in the level of H3K9 acetylation: 3–10-fold at the RPIB9 promoter, USP, exon −1, DSP and exon 1 of *MDR1* (Fig. 3b). Furthermore, acetylation of H3K4 increased threefold at the gene body (Fig. 3a), and that of H4K12 increased twofold at the USP and exon 1 (Fig. 3e). However, acetylation levels of H3K14 and H4K8 did not significantly change in the studied regions (Fig. 3c,d). After treatment of the drug-resistant cells with TSA, neither acetylation of H3K9, nor that of H3K4, H3K14, or H4K8 changed at any of the studied regions regardless of whether they were low or

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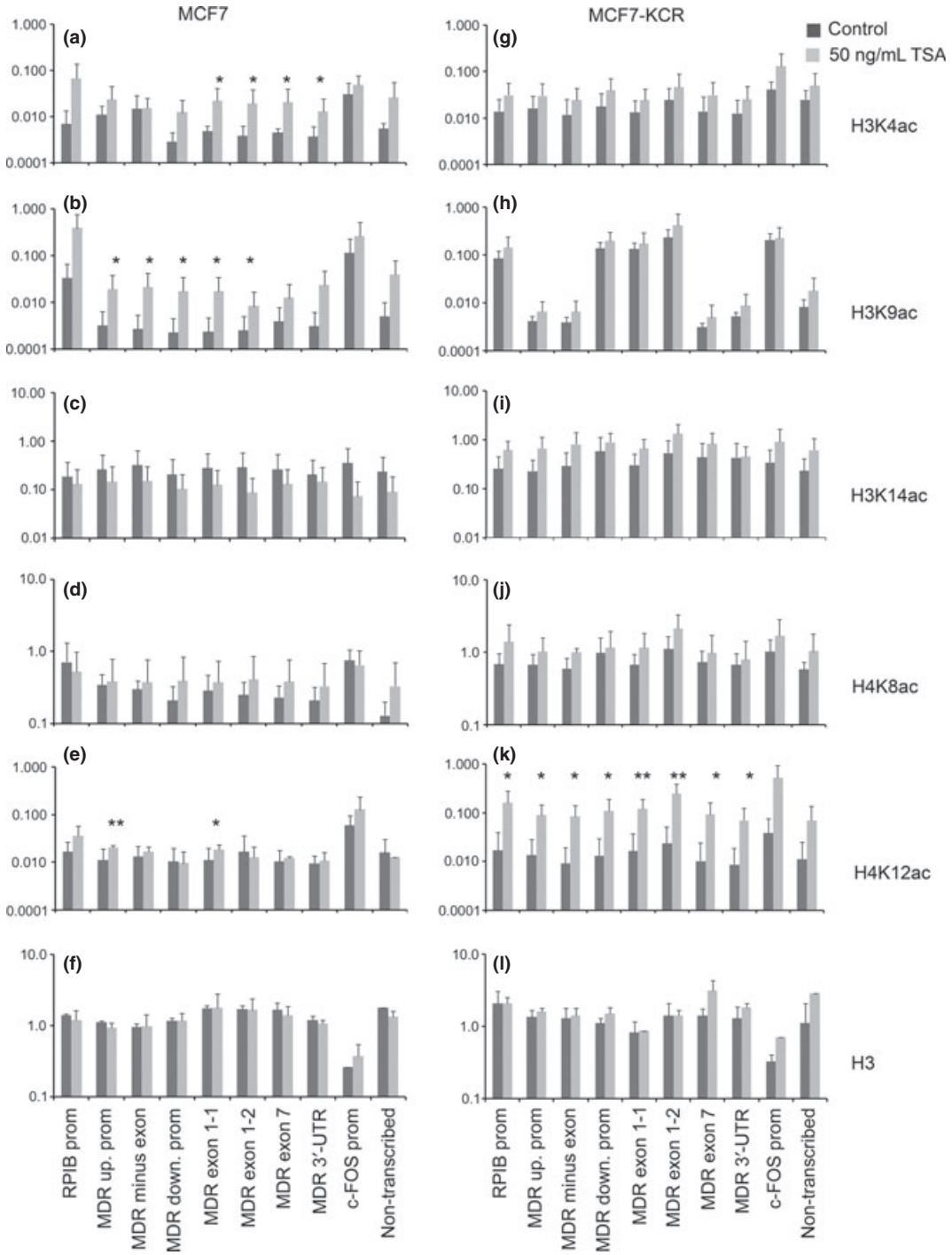

Fig. 3. Changes in histone acetylation pattern in the *MDR1/ABCB1* locus after treatment with trichostatin A (TSA) were analyzed by ChIP. MCF7 and MCF7-KCR breast cancer cells were untreated (black) or treated with 50 ng/mL TSA (gray) for 24 h. The ChIP assays were carried out with anti-H3K4ac (a,g), anti-H3K9ac (b,h), anti-H3K14ac (c,i), anti-H4K8ac (d,j), anti-H4K12ac (e,k), and anti-H3 pan Ab for control (f,l). Quantities determined by qPCR are expressed as percent of input. Scales are logarithmic and values represent means of 2–5 experiments. Error bars are SD. $*P < 0.05$ and $**P < 0.01$ are significantly different in treated versus untreated cells. Down., downstream; prom, promoter; up., upstream; UTR, untranslated region.

high in the untreated cells (Fig. 3a–j). In contrast, acetylation of H4K12 (Fig. 3k) increased 8–16-fold in MCF7-KCR cells. The amount of H3 histone did not change significantly in the studied regions in either cell line (Fig. 3f,l). These data suggest that elevated H3K9 acetylation at both promoters and first exons of the *MDR1* gene correlates with its increased expression in MCF7 after treatment with TSA.

Simultaneous downregulation of PCAF and GCN5 reduces the level of MDR1 mRNA. The highly increased acetylation level of H3K9 observed around the transcription start site (TSS) of *MDR1* and its elevated expression in MCF7-KCR cells prompted us to investigate which HAT(s) might contribute to them. It has been shown previously that PCAF participates in the regulation of *MDR1* expression.[16] Initial RT-qPCR and

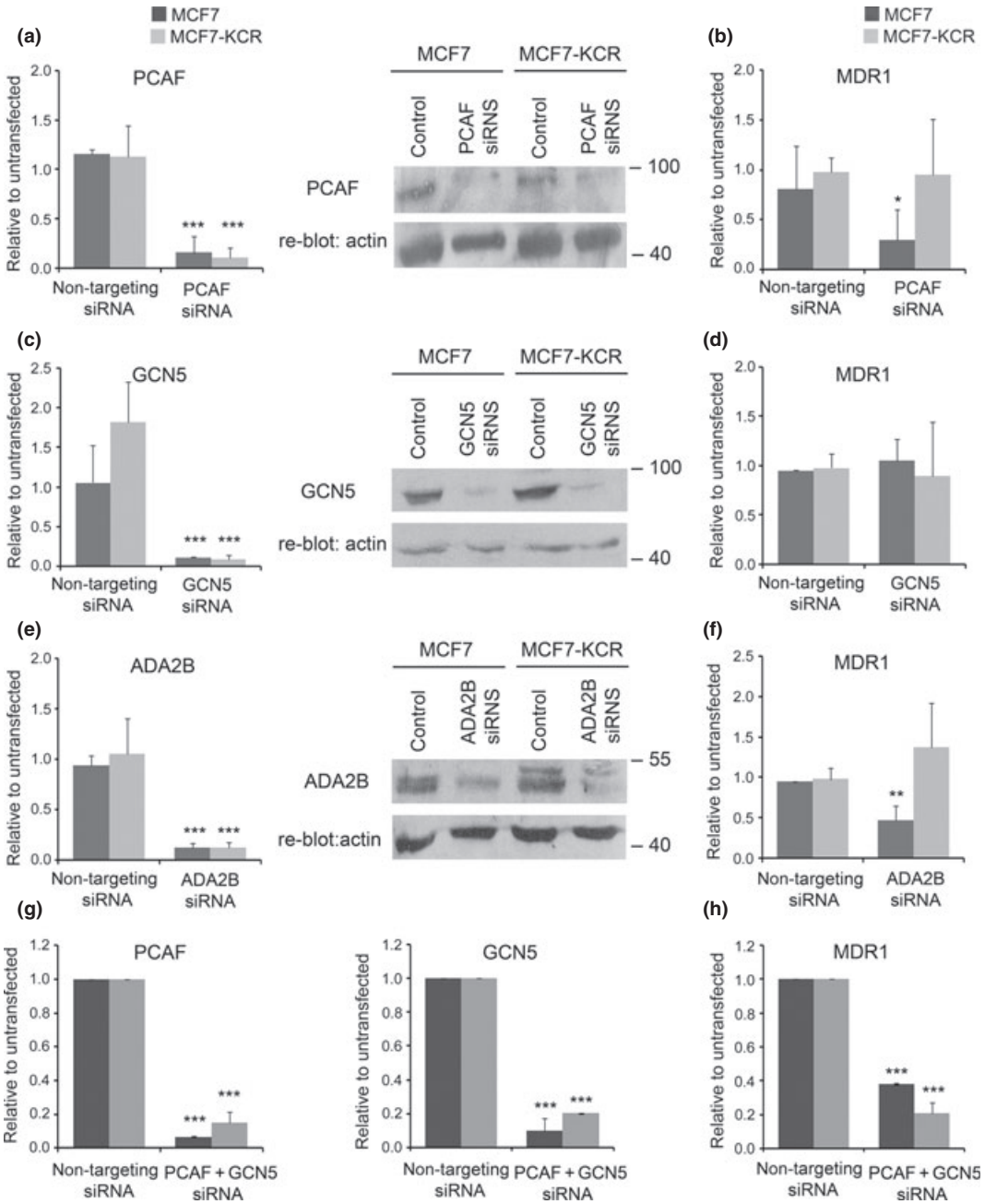

Fig. 4. Effect of downregulation of individual subunits of SAGA-type HAT complexes on the expression level of *MDR1*. MCF7 (black) and MCF7-KCR (gray) breast cancer cells were untransfected, or transfected with non-targeting siRNA for control, or siRNA for the indicated gene. The expression levels of PCAF, GCN5, or ADA2B (left panels of a,c,e, respectively, and g) were determined by RT-quantitative PCR. Levels of PCAF, GCN5, or ADA2B proteins (right panels of a,c,e, respectively) were determined by Western blotting. Each blot was re-probed for β-actin for internal control. The level of MDR1 mRNA was determined by RT-quantitative PCR (b,d,f,h). Expression of each gene is shown normalized to that in the untransfected control cells. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

Western blot analysis showed that PCAF expression is not elevated, but in fact slightly decreased in the MCF7-KCR cells compared to the drug-sensitive cells (data not shown; Fig. 4a, right). We knocked down the expression of PCAF; as well as GCN5, a HAT closely related to PCAF; and ADA2B, an essential subunit of the SAGA-type HAT complexes;[24] in order to test their role in the regulation of *MDR1* expression. Treatment of the cells with PCAF-specific siRNA resulted in a strong reduction of PCAF message and protein levels in both cell lines (Fig. 4a), and a concomitant 70% reduction of MDR1 mRNA level in MCF7 cells, but no change in MCF7-KCR cells (Fig. 4b). GCN5-specific siRNA treatment resulted in a 90% reduction of GCN5 mRNA and a similarly strong reduction of GCN5 protein level (Fig. 4c), but the MDR1 mRNA level did not significantly change in either cell line (Fig. 4d). Treatment of the cells with ADA2B-specific siRNA resulted in an 88% reduction of ADA2B message level in both cell lines (Fig. 4e), and a concomitant 50% reduction of MDR1 mRNA level in MCF7 cells, but no change in MCF7-KCR cells (Fig. 4f). However, simultaneous knockdown of the expression of PCAF and GCN5 (Fig. 4g) resulted in a strong reduction of MDR1 mRNA levels in both cell lines (Fig. 4h).

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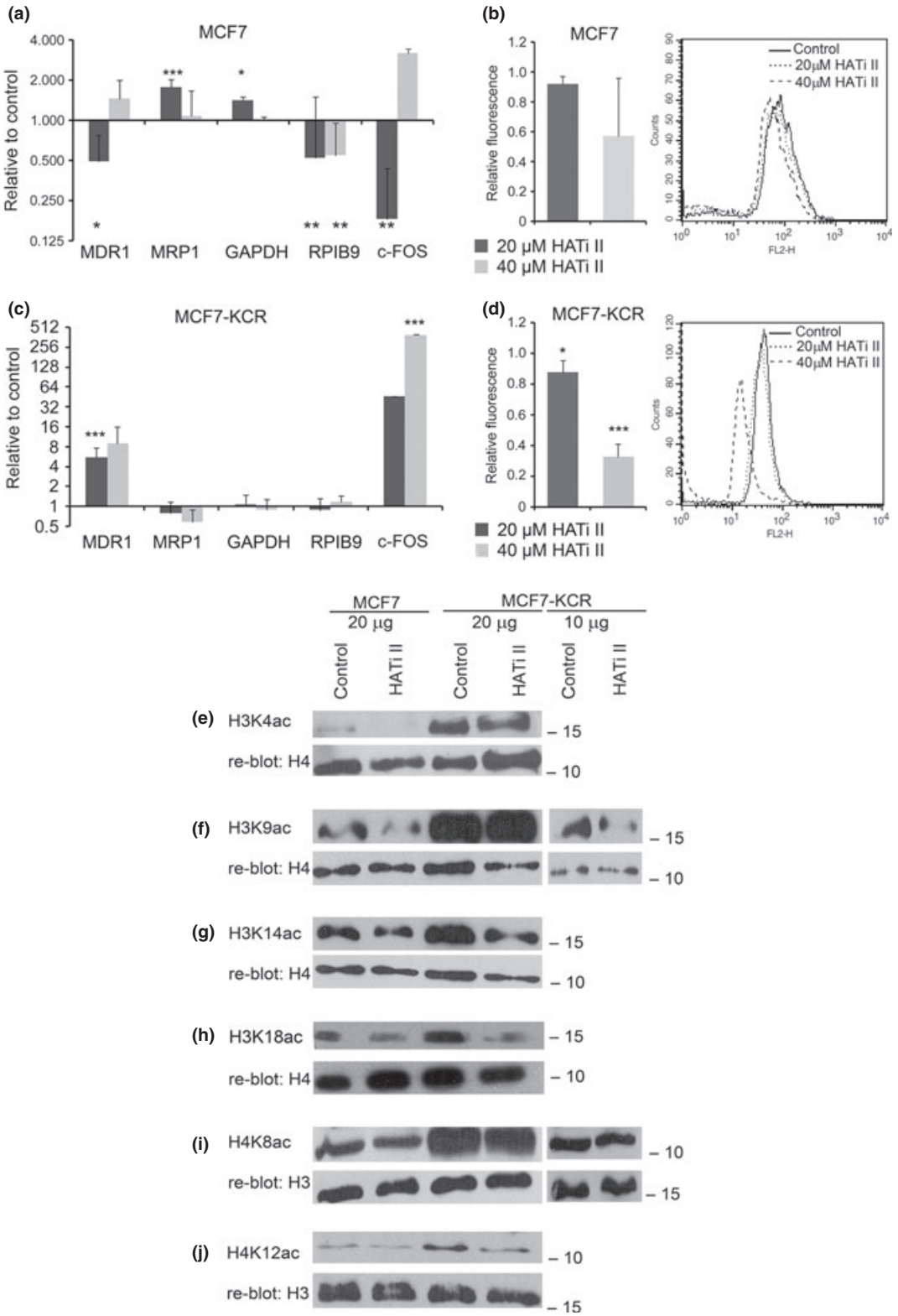

Fig. 5. Analysis of the changes in mRNA expression, drug accumulation and genome-wide histone acetylation levels in MCF7 and MCF7-KCR breast cancer cells after treatment with histone acetyl transferase (HAT) inhibitor II (HATi II). MCF7 (a,b) and MCF7-KCR (c,d) cells were treated with 20 or 40 μM HATi II for 24 h. (a,c) The level of the indicated transcripts and 18S RNA (as internal control) was quantified by RT-quantitative PCR. Expression of each gene is shown relative to that in the mock-treated control cells. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$. (b,d) Drug accumulation was determined by FACS. Bar graphs show the average and SD of three independent experiments; curves are from one representative experiment. (e–j) Analysis of the acetylation level of specific histone lysine residues. MCF7 and MCF7-KCR cells were mock-treated (control) or treated with 20 μM HATi II for 24 h. Histones were extracted, 20 μg (tracks 1–4) was analyzed by Western blotting with the indicated acetyl-lysine-specific Abs, and each membrane was re-blotted with a control Ab. In order to see the change and not to overexpose the film we also analyzed 10 μg histone extract from MCF7-KCR cells (f,i, tracks 5,6).

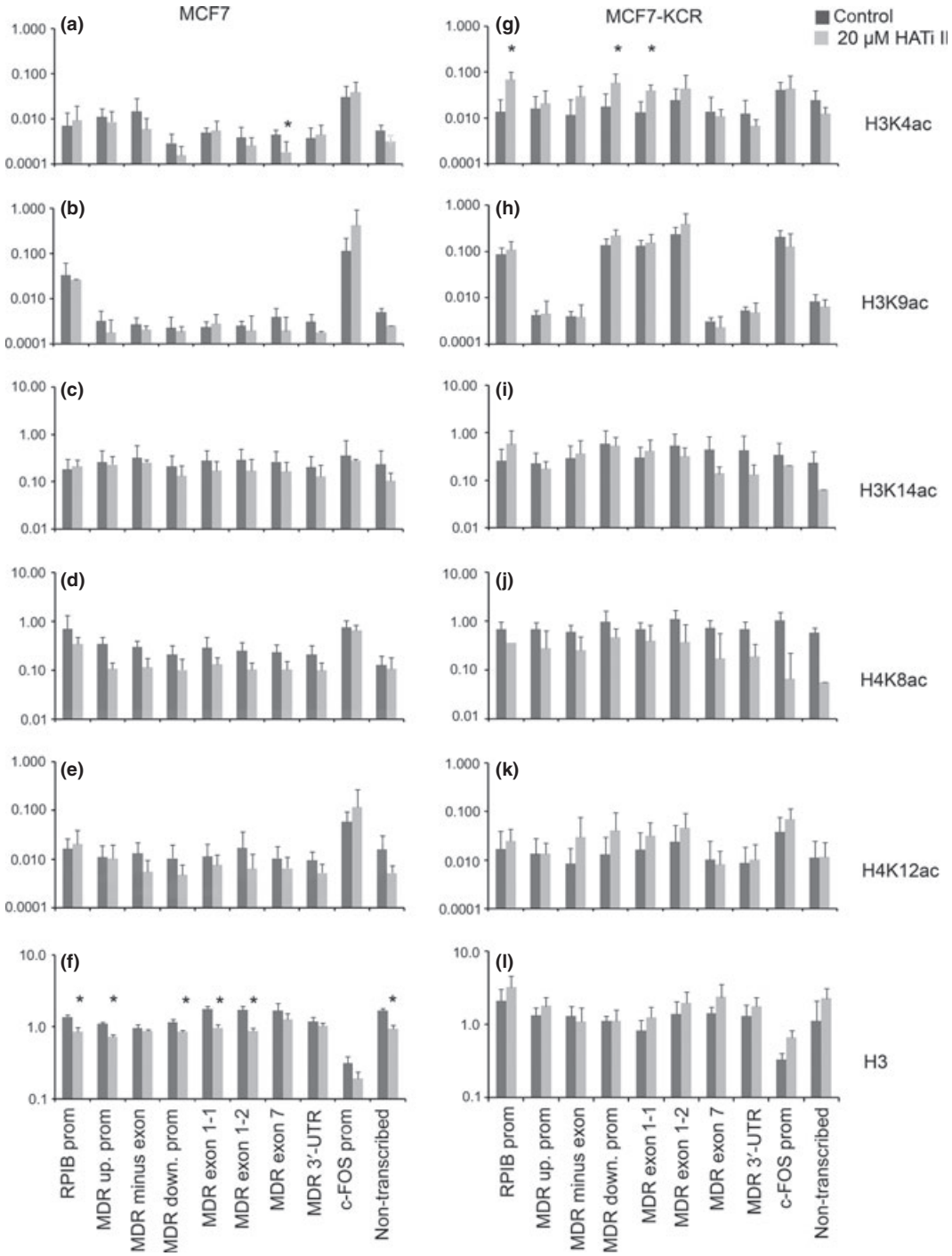

Fig. 6. Changes in histone acetylation pattern in the *MDR1/ABCB1* locus after treatment with histone acetyl transferase (HAT) inhibitor II (HATi II), analyzed by ChIP assay. MCF7 (left) and MCF7-KCR breast cancer cells (right) were untreated or treated with 20 μM HATi II for 24 h. Quantities determined by qPCR are expressed as percent of input. Scales are logarithmic and values represent means of 2–5 experiments. Error bars are SD. $*P < 0.05$ is significantly different in treated versus untreated cells. The ChIP assay was carried out using anti-H3K4ac (a,g), anti-H3K9ac (b,h), anti-H3K14ac (c,i), anti-H4K8ac (d,j), anti-H4K12ac (e,k), and anti-H3 pan Abs for control (f,l). Down., downstream; prom, promoter; up., upstream; UTR, untranslated region.

These data suggest that a complex containing PCAF and ADA2B plays a role in the regulation of the basal expression of *MDR1* in the drug-sensitive cell line. The finding that downregulation of PCAF or GCN5 individually is not able to downregulate MDR1 expression in the drug-resistant cells, but their simultaneous downregulation is, suggests that these HATs substitute each other in maintaining the elevated expression of MDR1.

Inhibition of p300 HAT activity increases MDR1 expression in drug-resistant but not drug-sensitive cells. P300 was also shown to participate in the activation of *MDR1* expression.[17] Therefore, we treated the cells with a novel p300 selective inhibitor,

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HATi II [2,6-bis-(3-bromo-4-hydroxybenzylidene)],[29] and examined changes in gene expression. After treatment of MCF7 cells for 24 h with 20 μM HATi II, *MDR1* expression slightly decreased, whereas that of MRP1 increased twofold (Fig. 5a). Fluorescent assay showed no significant change in doxorubicin accumulation in these cells (Fig. 5b). Unexpectedly, treatment of the MCF7-KCR cells with HATi II resulted in a sevenfold increase in the expression of *MDR1* (Fig. 5c). Furthermore, fluorescent drug accumulation assay also showed that the intracellular doxorubicin level in the HATi II-treated cells was lower than in the untreated MCF7-KCR cells, suggesting that the MDR1 protein produced is functional (Fig. 5d). Interestingly, although expression of the other genes did not change, that of *c-FOS* increased 300-fold. This suggests that the increased expression after treatment with HATi II is not unique to *MDR1* in the drug-resistant cells, supporting the hypothesis that the functioning of HATs is altered there. Thus, like PCAF inhibition, inhibition of p300 does not downregulate expression of *MDR1* in drug-resistant cells; on the contrary, it results in an increase in *MDR1* expression and drug efflux.

Global acetylation in drug-resistant cells effectively decreased by treatment with HATi II. The surprising finding of increased MDR1 and c-FOS mRNA levels after HAT inhibitor treatment made us wonder how HATi II affected acetylation of specific lysine residues genome-wide and at specific regions in the *MDR1* locus. Although global acetylation of H3K4 and H3K9 were almost undetectably low in MCF7 cells (Fig. 2a,b), we could still see a reduction in their levels after HATi II treatment (Fig. 5e,f, track 2). A less dramatic reduction in the amount of H3K14, H4K8, and H4K12 acetylated histones was clearly detectable in the drug-sensitive cells (Fig. 5g,j, track 2). A recent study showed that p300 specifically acetylates H3K18.[30] Therefore, we examined the acetylation level of that residue and found it also decreased (Fig. 5h). Similarly, acetylation of all six examined histone lysine residues was reduced to various extents in the drug-resistant cell line (Fig. 5e–j, tracks 3–6). These data show that HATi II treatment was effective in reducing genome-wide acetylation in both cell lines, and failure to reduce the expression of *MDR1* in MCF7-KCR cells was not due to inactivation of the drug. Next, we examined the specific changes in histone acetylation in the *MDR1* locus. Interestingly, the level of H3K4 acetylation increased fivefold at the RPIB9 promoter, DSP, and exon 1 of *MDR1* in the drug-resistant but not drug-sensitive cells (Fig. 6a,g). There was no statistically significant change in the acetylation levels of H3K9, H3K14, H4K8, or H4K12 in either cell line (Fig. 6b–e,h–k). There was some decrease in histone occupancy around the TSS in MCF7 cells (Fig. 6f,l). These data show that although HATi II effectively reduces histone acetylation genome-wide, it does not affect or even increase the acetylation level of certain histone residues at the *MDR1* locus. Specifically, the increase in H3K4 acetylation coincides with the increase of *MDR1* expression in the drug-resistant cell line after treatment with HATi II.

Discussion

Histones are subject to various post-translational modifications that can affect transcription. Histone lysine acetylation was proposed to create a more amenable chromatin structure for transcription by neutralizing the positive charge of histones. Moreover, occurring on various residues, acetylation also represents "letters" of the histone code that are interpreted by the transcriptional machinery. Altered genome-wide acetylation levels of H3K9, H3K18, H4K12, and H4K16 have been found in prostate, lung, kidney, breast, pancreatic, and gastric cancers.[31–34] In particular, H3K18 acetylation was shown to be a prognostic factor in several studies, with high histone acetylation correlating with better prognosis.[34] In contrast, a study involving non-small-cell lung cancer patients found that higher levels of H3K9 acetylation correlated with shorter survival.[35] These contrasting findings suggest that acetylation on various lysine residues may have different functions in different types of cancer. We investigated whether altered genome-wide histone acetylation might accompany drug resistance. We found that global acetylation levels of H3K4, H3K9, H3K14, H4K8, and H4K12 are higher in drug-resistant MCF7-KCR cells than in drug-sensitive MCF7 cells. Similar to our findings, increased global acetylation has been reported for the OV1/VCR multidrug-resistant ovarian carcinoma cell line.[36]

Increased histone H3 and H4 acetylation in the chromatin of the *MDR1* promoter, has been found in drug-resistant cell lines expressing elevated levels of MDR1.[18] When compared to a T-cell leukemia line, two cell lines expressing increased levels of MDR1 mRNA showed 3–30-fold levels of acetylated H3 in the *MDR1* promoter, first exon and transcribed region.[18,20,21] Interestingly, of doxorubicin-resistant sarcoma cell lines generated from the same parental cell, some showed elevated H3 acetylation at the DSP, but others at the USP only.[11] The individual acetyl-lysine-specific Abs were not available at the time of those studies, so the exact residues acetylated have not been identified. We revealed that acetylation of H3 lysine 9 is elevated by two orders of magnitude in the promoter and first exon of the *MDR1* gene in a drug-resistant cell line, whereas H3K4, H3K14, H4K8, and H4K12 acetylation increased only mildly or not at all, compared to the drug-sensitive parental cell line.

Although most studies correlate H3K4 methylation with transcriptional activity of a given gene, Wang *et al.*[37] reported enrichment of H3K4 acetylation around the TSS of genes with an intermediate level of expression. We found elevated genome-wide acetylation of H3K4 and a slight increase of H3K4ac in the first exon of *MDR1* in MCF7-KCR cells. Furthermore, Wang *et al.* characterized active genes with a combinatorial pattern of modifications, including H3K4ac, H3K9ac, H4K8ac, and, for genes with the highest expression, H4K12ac along the gene body. Interestingly, we found H3K9ac alone extremely highly elevated around the TSS, and H4K12ac unchanged along the gene body of *MDR1* in the drug-resistant cells.

Trichostatin A is a potent inhibitor of HDACs and it induces the expression of several genes.[28] It is a promising therapeutic candidate against cancer because it induces differentiation, cell cycle arrest, and apoptosis.[38,39] However, several studies have shown that TSA may induce drug resistance, limiting its therapeutic potential.[16,40] In acute myeloid leukemia cells, TSA treatment increased the expression not only of *MDR1* but also of genes encoding other drug transporters, *BCRP/ABCG2* and *MRP8/ABCC11*, thereby inducing a very broad drug resistance phenotype.[41] In contrast, other reports showed that TSA did not induce *MDR1* transcription, although it resulted in a significant increase in the level of acetylated histones at the *MDR1* promoter.[18,42] Demethylation of promoter DNA was required for TSA-inducibility of *MDR1* expression in these cells.[42,43] Here, we report that TSA induces *MDR1* expression in drug-sensitive MCF7 cells, but not in drug-resistant MCF7-KCR cells. Three studies reported findings similar to ours.[21,44] In a drug-sensitive small-cell lung carcinoma line, TSA induced *MDR1* expression, but decreased it in the drug-resistant derivative line. The extent and temporal kinetics of H4 and H3 acetylation at the *MDR1* promoter was different in the two cell lines.[18] These data suggest that TSA can be a therapeutic candidate for multidrug-resistant cancers or those with altered histone acetylation.

The finding that H3K9 acetylation around the TSS of *MDR1* is very high in our drug-resistant cell line prompted us to investigate which HAT might be responsible for generating this epigenetic mark. The HATs that may participate in the

regulation of *MDR1* expression have not been extensively studied. One candidate, PCAF, has been shown to co-activate an *MDR1* promoter–luciferase reporter construct,[16] and to bind to the endogenous promoter.[18] Our RNA knockdown studies suggest that a SAGA-type complex involving PCAF and ADA2B,[24] but not GCN5, participates in the basal expression of *MDR1* in MCF7 cells. Individual downregulation of PCAF, GCN5, or ADA2B did not reduce the very high expression of *MDR1* in MCF7-KCR cells, however, simultaneous downregulation successfully reduced it. This suggests that PCAF and GCN5 substitute each other in maintaining the elevated expression of *MDR1* and they may be responsible for generating the elevated H3K9 acetylation pattern at the *MDR1* TSS. Both PCAF and p300 can acetylate H3K9 *in vitro*, although this is not their primary substrate.[45,46] Inhibition of p300 by a specific inhibitor, HATi II,[29] resulted in genome-wide reduction of acetylation on all six studied histone lysine residues in both cell lines. Nevertheless, expression of *MDR1* further increased in MCF7-KCR but not MCF7 cells. The acetylation level of H3K4 increased fivefold at the RPIB9 promoter and around the TSS of *MDR1* in the drug-resistant but not drug-sensitive cells, whereas acetylation of the other examined residues remained relatively unchanged. There could be several possible explanations for the unexpected induction of *MDR1* expression in the drug-resistant cells. One possibility is that HATi II is sensed by the cell as a xenobiotic, and HATi II activates the pathway that normally induces *MDR1* expression.[47] However, the fact that HATi II does not induce *MDR1* expression in MCF7 argues against this hypothesis. It is more likely that the various HATs and HDACs co-regulate each other and that these interactions are deregulated in MCF7-KCR. Inhibition of p300 may result in activation of (an) unidentified HAT(s) and these in turn acetylate H3K4 and further induce *MDR1* expression. However, it is questionable whether increased expression of *MDR1* is really due to increased acetylation of H3K4. As p300 acetylates transcription factors besides histones, the possibility exists that inhibition of p300 results in a reduced level of acetylation of another factor that regulates *MDR1* expression in the drug-resistant cell line. The role of HDACs in the altered histone acetylation in MCF7-KCR remains to be explored.

In summary, the drug-resistant MCF7-KCR cell line, developed by treatment of MCF7 cells with increasing concentrations of doxorubicin, seems to use multiple mechanisms that ensure elevated expression of *MDR1*. These mechanisms include gene amplification and epigenetic alterations, including highly elevated H3K9 acetylation around the TSS of *MDR1*. Both PCAF and GCN5 seem to be responsible for the increased H3K9 acetylation and elevated expression of MDR1. Unexpectedly, inhibition of p300 activity results in a further increase of MDR1 mRNA levels in the drug-resistant cells, suggesting that there might be additional mechanisms deregulated. Our findings are valuable for the understanding of the role and deregulation of histone modifications in cancer.

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Disclosure Statement

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. *MDR1* gene is amplified over 40-fold in drug-resistant cells.

Table S1. Primers used for quantitative PCR.

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