Activation of p21 by HDAC Inhibitors Requires Acetylation of H2A.Z

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Abstract

Differential positioning of the histone variant H2A.Z in a p53 dependent manner was shown to regulate p21 transcription. Whether H2A.Z is involved in p21 activity in the absence of p53 is not known. The p21 gene is repressed in estrogen receptor (ER) negative cell lines that are p53+/− and hormone independent for their growth. Here we demonstrate that class I and II pan Histone deacetylase inhibitors (HDACi) induce p21 transcription and reduce cell proliferation of MDA-MB231, an ERα-negative mammary tumor cell line, in a H2A.Z dependent manner. H2A.Z is associated with the transcription start site (TSS) of the repressed p21 gene. Depleting H2A.Z did not lead to transcription of p21 but annihilated the stimulating effect of HDACi on this gene. Acetylation of H2A.Z but not of H3K9 at the p21 promoter correlated with p21 transcription. We further show that HDACi treatment reduced the presence of the p400 chromatin remodeler at the p21 TSS. We propose a model in which association of p400 negatively affects transcription of p21 and p21 transcription by interfering with acetylation of H2A.Z.


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Introduction

Estrogen receptor negative breast cancer types are generally more aggressive and prone to metastasize. The absence of Estrogen receptor-alpha (ERα) correlates with hormone-independent growth of these mammary tumor cells and causes uncontrolled proliferation and insensitivity to anti-hormonal treatments. In ERα-negative cell lines, a subset of genes is epigenetically silenced [1,2], while the majority of genes involved in cell cycle control and proliferation are constitutively expressed [1,3,4]. Aberrant gene expression is frequently the result of chromatin modifications and composition, including histone post-translational modifications and/or incorporation of histone variants [5–9]. In particular, deregulation of enzyme complexes responsible for histone acetylation and deacetylation can be associated with breast cancer progression and an increase in tumor malignancy [5]. Thus, compounds that change chromatin modifications are a promising anti-cancer approach. Histone deacetylase (HDAC) inhibitors, such as Trichostatin A (TSA), Suberoylanilide hydroxamic acid (SAHA), Panobinostat (LBH598) and sodium butyrate (NaB) can inhibit cancer cell growth in vitro and in vivo [10–12] as a result of selective induction of endogenous genes that play significant roles in G1-S progression [5]. One of the major regulators of cell cycle progression is the cyclin-dependent kinase inhibitor p21 CIP1/WAF1, a gene of the CIP/KIP family, which inhibits CDK activity. p21 can be stimulated by p53 and its activity results in cell cycle arrest and/or apoptosis. Much of research on HDAC inhibitors has focused on the upregulation of p21. Activation of p21 involves acetylation of promoter chromatin, but the mechanism remains poorly understood [13,14].

The histone variant H2A.Z has been shown to bind to the promoter of p21 at the p53 binding sites in p53+/+ cells (U2OS) [15]. In response to stress, H2A.Z is evicted to allow p53 to bind which leads to p21 expression [15]. The p400 complex takes part in this pathway and was proposed to be responsible for H2A.Z deposition into the p21 promoter. Depleting p400 by siRNA increases p21 expression in a p53 dependent manner and induces premature senescence [16]. The mechanism of this activation is unclear.

In the ERα-negative breast cancer cell line MDA-MB231 p53 is mutated and non-functional. Here we show that activation of p21 in response to HDACi treatment of these ERα-negative cells requires H2A.Z acetylation and exchange at its transcription start site.

Materials and Methods

Cell Lines, Transfection and Western Blotting

MDA-MB231, Hs-578T and HeLa cells were purchased from ATCC (used up to 15 passages). All cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with Glutamax containing 50 mg/ml gentamicin, 1 mM sodium pyruvate and 10% heat-inactivated fetal calf serum (FCS) (Invitrogen). MDA-MB231 cells were treated with 50 or 100 ng/ml TSA (Sigma-Aldrich) and with LBH589 5 × 10−8 or 5 × 10−9 M for the indicated times. 4 × 106 MDA-MB231 cells where mock-trans-
fected (pcDNA3.1) or transfected with 2 μg of pcDNA3.1/Tip60 (gifts from Dr. Didier Trouche) with Amaxa Cell Line Nucleofactor Kit V program X-013 according to the manufacturer’s protocol. MDA-MB231 cells were mock-transfected or transfected with H2A.Z siRNA ON-TARGET plus SMARTpool or scrambled (sc) siRNA (Dharmacon Thermo Scientific) with Interferine (Ozyme) according to the manufacturer’s protocol. Tip60 siRNA [17] was purchased from Eurogentec, and transfected with Interferine (Ozyme) according to the manufacturer’s protocol. For western blotting total cell extracts were isolated and proteins levels of p21 and GAPDH was analyzed by immunoblotting on gel SDS-page 15% with anti-p21 (SantaCruz Biotechnology, sc-397), anti-Tubulin (SantaCruz Biotechnology, sc-806), and anti-GAPDH (Millipore, ab379).

RNA Analysis
Total RNA was extracted using RNeasy mini-kit (Qiagen) and eluted with 35 μl of RNAase-free water. First strand cDNA was generated using 1 or 2 μg of total RNA in a reaction containing random oligonucleotides as primers with the ThermoScript RT-PCR system (Invitrogen). Real-time PCR was performed on a Mastercycler® ep realplex (Eppendorf) using the platinum SYBR Green q-PCR SuperMix (Invitrogen) according to the manufacturer’s instruction. Amplification conditions: 1 min at 95°C, 3 min at 95°C followed by 40 cycles (20 s at 95°C, 20 s at 60°C, 20 s at 72°C); q-PCR for RPLP0 mRNA was used as an internal control. The primers used in q-PCR: CDKN1A (p21, Cip1): 5'-GGAGACATTGTGGACAGCTGT-3' and 5'-GGATTAGGGGTCTCCTGTTG-3', H2AFZ: 5'-CCCTTTCTCTGCCTGCTTG-3' and 5'-CGGTGAGG-TACTCAGGAGT-3', RPLP0: 5'-TGCGAGCATCTACAACCTGAA-3' and 5'-CAGGGACAGCTGATGGGAATAC-3', H2AFZ: 5'-AGAGAGGCATCCTCCAGACA-3’, and 5'-AGCTGAGCCTGGCCGAGT-3’. q-PCR for RPLP0, RPLP0 and GAPDH was performed using random primers with the Thermocycler, 1 min at 95°C, 3 min at 95°C followed by 40 cycles (20 s at 95°C, 20 s at 60°C, 20 s at 72°C). Primer sequences for q-PCR to amplify fragment (Eppendorf): 5'-AGGATCCAGGATCTCTCCAGCAAACCTGAA-3' and 5'-CAGGGACAGCTGATGGGAATAC-3', 5'-CGGTGAGG-TACTCAGGAGT-3', RPLP0: 5'-TGCGAGCATCTACAACCTGAA-3' and 5'-CAGGGACAGCTGATGGGAATAC-3', 5'-AGAGAGGCATCCTCCAGACA-3’, and 5'-AGCTGAGCCTGGCCGAGT-3’. Primers designed to amplify the p21 promoter region in p53 positive cells, such as ERα-positive MCF-7 (data not shown) or U2OS cells [15]. The ERα- MDA-MB231 cell line bears a mutated, non-functional p53 gene. We thus asked whether H2A.Z was also associated with the p21 promoter in these cells as was shown to be the case in the p53- osteosarcoma, SaOS cell line [15]. Using chromatin immunoprecipitation (ChIP), we determined that H2A.Z was present at the transcription start site (TSS) of the p21 gene. The amplified fragment (#4, Fig. 2) is adjacent to a set of six putative Sp1 binding sites that were shown to mediate p21 transcription in a reporter assay [13]. The amount of H2A.Z detected at upstream sequencings, including the p53 recognition elements (fragment #2), was significantly less abundant than at the TSS (Fig 2b). According to the hypothesis that H2A.Z containing nucleosomes direct p53 binding [15], it was not surprising that H2A.Z was absent from these sites. As previously described in yeast [24], [25], decondensed chromatin is found at promoters of inactive but inducible genes. Based on this observation, we reduced the cellular pool of available H2A.Z by small interfering RNAs directed against H2A.Z and assessed p21 mRNA expression (Fig. 2c). p21 transcription was insignificantly reduced (Fig. 2c). ChIP experiments further confirmed that despite a reduction in H2A.Z association with the TSS, polymeerase II (pol II) was not recruited to this inactive gene (Fig 2d). Thus, p21 transcriptional regulation does not only depend on the amount of H2A.Z associated with its TSS. Interestingly, treatments with 50 ng/ml TSA reduced H2A.Z binding to the activated p21 (Fig 2e, 1b). Release of H2A.Z was accompanied by recruitment of pol II and, strikingly, by an increase in the presence of...
acetylated H2A.Z at the p21 TSS [Fig. 2f]. This increase in acetylation was not seen for histone H3K9 (Fig. 2g). Moreover, the amount of acetylated H2A.Z and acetylated H3K9 present at the TSS did not vary in siH2A.Z transfected cells in which p21 remained repressed (Fig. 2h–2i). We propose that acetylation of H2A.Z rather than its presence correlates with p21 transcription activation in ERα-breast cancers. We postulated that the anti-proliferative effect of HDACi via p21 expression depends on acetylation of H2A.Z. We thus asked whether this effect would be abolished in siH2A.Z treated cells.

H2A.Z Controls HDACi Induced Growth Arrest via p21 Expression

MDA-MB231 cells were grown in standard medium, treated or not with siH2A.Z for 24 hours before adding TSA or LBH. Surviving cells (MTT test, Fig. 3a) and dead cells (trypan blue, Fig. 3b) were counted before treatment as well as 24 h and 48 h following HDACi addition. Reduced cell growth in TSA treated cells was partly rescued in cells previously transfected with siH2A.Z (Fig. 3a). In particular, significant counts of dead cells were determined as soon as 24 h post treatment (Fig. 3b). While siH2A.Z transfected cells also showed a 2-fold increase in cell death compared to untreated cells, these cells were much less sensitive to TSA (Fig. 3b). Cell death at 48 h was almost similar in untreated cells compared to siH2A.Z transfected and TSA exposed cultures. Furthermore, p21 mRNA levels did not vary in siH2A.Z transfected cells upon HDACi treatment compared to control cells treated only with TSA or LBH (Fig. 3c, S1c). H2A.Z is also required for p21 activation upon TSA treatment in ERα-negative, p53−/− Hs-598T cells (Fig. S2c). In contrast, knock-down of H2A.Z had no effect on p21 activation in HeLa cells (Fig. S2a). Thus, H2A.Z specifically regulates p21 in ERα-negative breast cancers following HDAC inhibitor treatment. We further found that acetylation of H2A.Z bound to the p21 TSS was greatly reduced in siH2A.Z treated cells exposed to TSA (Fig. 3d). Pol II recruitment and elongation was abolished in TSA treated MDA-MB231 cells, from which H2A.Z was depleted (Fig. 3e). Thus, H2A.Z appears essential to mediate the anti-proliferative effect of HDACi by regulating p21 expression. Notably, acetylation of H2A.Z was necessary for this regulation. We next wanted to gain insight into the mechanisms of H2A.Z acetylation at the p21 promoter in ERα-cells.

A Role for p400 but not Tip60 in p21 Transcription Regulation

We first tested the impact of depleting or overexpressing Tip60, a histone acetyltransferase frequently found in complex with p400 and known to participate in H2A.Z-mediated transcription regulation [15,26]. Modulation of Tip60 mRNA levels did not alter p21 expression levels, which remained almost undetectable (Fig. 4a). Accordingly, association of H2A.Z, acetylated H2A.Z and pol II did not vary at the p21 TSS in Tip60 depleted cells (Fig. 4b). We next investigated which cofactor could be responsible for TSA induced activation of p21. Tip60 did not seem to be associated with the p21 TSS in MDA-MB231 cells treated or not with TSA (Fig. 4c). In contrast, we detected significant amounts of the p400 remodeler at the p21 TSS. Association of p400 decreased in TSA treated cells in which p21 was activated (Fig. 4c). Reducing the available pool of p400 alone was able to activate p21 expression (Fig. 4d) suggesting that the presence of p400 at the p21 TSS represses this gene. Reduction of p400 allowed recruitment of the p300 acetyltransferase (Fig. 4e). Concomitantly, acetylation levels of H2A.Z markedly increased, due to eviction of a fraction of H2A.Z and an increase in acetylated H2A.Z at the p21 TSS (Fig. 4e). We propose that TSA controls cell growth by modulating p21 expression, p21 activation requires release of p400 and H2A.Z, and an increase in acetylation of H2A.Z.

Discussion

Lack of regulation of the p21 gene whose expression is needed for cells to respond to insults by arresting proliferation is frequently observed in cancer. This loss is exacerbated by the absence of the functional tumor suppressor p53 protein in more aggressive tumor types. Histone acetyltransferase inhibitors have been shown to activate p21 independently of p53. TSA (500 ng/ml) reduced growth of MG63 osteosarcoma, p53−/− cells and activated various p21 promoter constructs driving a luciferase reporter gene [27]. We demonstrate that the endogenous p21 gene is also activated by TSA or LBH589 in ERα- mammary tumor cells whose growth rate is insensitive to hormones and antihormones. In this study we provide a comprehensive analysis of the activation of the p21 gene in ERα- MDA-MB231 cells which provides a mechanistic link between histone acetylation, H2A.Z variant incorporation and p21 mediated growth arrest.

Unlike in p53 positive cells, U2OS osteosarcoma or MCF-7 cells, where the histone variant H2A.Z is associated with the p53 binding sites of the inactive p21 gene, H2A.Z was present at the...
TSS in ERα cells. H2A.Z binding to the TSS was frequently been observed in yeast [25] and human cells [28] where is thought to create a chromatin structure that is responsive to stimuli and cofactor binding. Its presence in promoter chromatin was also shown

**Figure 2. Acetylation of H2A.Z at the p21 promoter is necessary for its transcription.**

a) Schematic representation of the p21 promoter region showing PCR amplified fragments (1–4). b) Binding of H2A.Z to the p21 promoter in MDA-MB231 cells. c) mRNA expression of H2AFZ (left) and p21 (right) in MDA-MB231 cells transfected with a smartpool siH2A.Z for 72h. d) H2A.Z and polymerase II (pol II) binding to the p21 TSS (fragment #4) in cells transfected with siH2A.Z or scramble siRNAs. e) H2A.Z binding to the p21 TSS (fragment #4) in cells treated with TSA (50 ng/ml) for 48 h. f–g) acetylated H2A.Z, polymerase II (pol II) (f) and acetylated H3K9 (g) binding to the p21 TSS (fragment #4) in cells treated with TSA. h–i) acetylated H2A.Z (h) and H3K9 (i) amount at p21 TSS in cells transfected with siH2A.Z or scramble siRNA.

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to be important for activation of ERα target genes such as TFF1, PGR or CCND1 [29], [30]. Association of H2A.Z with promoter sequences could thus be related to an alternative pathway of gene activation in the absence of cognate transcription factors, including p53 or ERα. However, loss of H2A.Z binding was not sufficient to stimulate p21 expression in MDA-MB231 cells. This observation correlates with previous studies showing that a 50% reduction H2A.Z association was not sufficient to induce changes in gene regulation [31].

In 2007 Gevry et al. showed that p400 and H2A.Z associate with the repressed p21 gene in U2OS cells [15]. In response to DNA damage, p400 and H2A.Z were evicted to allow TIP60 recruitment and subsequent p21 activation [15]. More recently Park et al., however, demonstrated that p400 inhibits TIP60 activity by direct binding to TIP60 via its SANT domain [26].

In ER-negative, p53 mutant breast cancer cell lines, TSA treatment activates p21. Under these conditions, binding of p400 and H2A.Z to the p21 promoter was reduced, but the concomitant increase in H2A.Z acetylation was independent of TIP60.
Furthermore, depleting p400 also stimulated p21 expression independently of TIP60 suggesting that p400 exerts a repressive effect. In contrast, depleting H2A.Z was not sufficient to mediate p21 repression under normal conditions but it played a central role in p21 activation upon TSA treatment. We propose that acetylation of H2A.Z rather than H2A.Z per se is important to drive proper p21 gene expression. A ratio in favor of acetylated H2A.Z was associated with p21 activation upon TSA treatment. This observation corroborates findings by Valdes-Mora and colleagues who recently correlated aberrant gene expression in prostate cancer cells with H2A.Z acetylation at specific promoters [32]. It is tempting to speculate that p400 favors p21 activation by catalysing H2A.Z eviction and allowing the recruitment of HATs such as p300.

The histone-acetyltransferase CBP/p300 has been shown to act on the p21 promoter at several Sp1 sites and independently of p53 as part of a multiprotein complex which also contains PR and Sp1 [33] in T47 mammary tumor cells. Here, in triple negative MDA-MB231 cells (ER-, PR-, HER-), p300 was also present at the p21 promoter at levels proportional to transcriptional activity. Hence, the cofactors required for p21 activation are distinct in p53 negative compared to positive cells [34]. These alternative pathways in cells in which regulation of p21 does not obey to the classical pathways, open new avenues for growth control therapies.

Hua et al. [35] identified the histone variant H2A.Z as a potential epigenetic marker since its hormone-dependent expression correlates with increased probability of metastasis and decreased patient survival in a large scale study. For it to serve as a prognostic factor in breast cancer, the mechanisms unraveled by our study are relevant in ER- p53−/− cells that are otherwise difficult to act upon.

Supporting Information

Figure S1 p21 is activated in response to pan HDAC class I and II inhibitors. a) MTT assay to quantify proliferation rates in the presence of LBH589 of MDA-MB231 cells cultured in rich medium. Two different concentrations (5*10−9 M and 5*10−8 M) were used. b) q-PCR analysis of p21 mRNA expression levels. c) p21 mRNA expression level in MDA-MB231 treated or not with LBH589 (5*10−9 M) for 48 h and/or transfected with a smartpool siH2A.Z (72 h) as indicated. d) Western blot analysis of p21 protein levels after 24 h of TSA treatment at the indicated doses.

Figure S2 H2A.Z specifically regulates p21 in ER-negative breast cancers following HDAC inhibitor treatment. a, b, c, d) q-PCR analysis of p21 and H2A.Z mRNA expression in Hela (a, b) and in Hs-578T (c, d) cells. Cells were treated with siH2A.Z or scramble siRNA and treated for 24 h with two different concentrations of TSA.

Figure 4. p400 but not Tip60 functions in p21 expression in the absence of p53. a) Tip60 and p21 mRNA expression. b) H2A.Z, Acetyl-H2A.Z and RNA pol II binding to the p21 TSS in cell transfected with siTip60 or scramble siRNA. c) ChIP analysis of Tip60 and p400 recruitment to the p21 TSS (fragment #4) in cells treated with TSA. d) p21 mRNA expression in MDA-MB231 transfected with siRNA against p400 and treated or not with TSA. e) H2A.Z, Acetyl-H2A.Z and p300 enrichment at p21 TSS (fragment #4) in MDA-MB231 transfected with si p400.

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