uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

Feras Al-Ghazawi

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Cellular and Molecular Medicine)

GRADE / DEGREE

Department of Cellular and Molecular Medicine

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Roles of Bromodomain in the Regulation of p300-Dependent Gene Expression

TITRE DE LA THÈSE / TITLE OF THESIS

Dr. Q. Li

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Dr. A. Blais

Dr. C. Kennedy

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Roles of Bromodomain in the Regulation of p300-Dependent Gene Expression

Feras Al-Ghazawi

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements for the M.Sc. program in

Cellular and Molecular Medicine
University of Ottawa

Your file Votre référence
ISBN: 978-0-494-51632-4
Our file Notre référence
ISBN: 978-0-494-51632-4

ABSTRACT

The transcriptional coactivator p300 displays an intrinsic histone acetyltransferase activity. It contains an evolutionarily conserved bromodomain serving as a specific acetyl-lysine binding module for histones or transcription factors to facilitate chromatin remodeling and transcriptional activation. The function of p300 is required by a diverse set of promoters. However, roles of bromodomain in the function of p300 are yet to be fully determined. In this study, we utilize cell lines expressing either wild-type or bromodomain truncated p300 to examine the expression of several p300-dependent genes that are involved in cell cycle regulation. The effects of histone acetylation on the expression of these genes were also examined by utilizing histone deacetylase inhibitors. Our results suggest that p300 regulates genes through different mechanisms and that bromodomain has roles in the recruitment of p300 to target promoters, providing an indication that the role of bromodomain in p300-dependent transcription is determined by individual promoter context.

Dedicated to my parents: Mustafa and Zainah

ACKNOWLEDGMENTS

I would like to acknowledge my advisor Dr. Qiao Li for her guidance, constant intellectual support and for providing excellent working environment for her students. I would also like to thank my laboratory colleagues for their continuous support and for the helpful discussions: Jihong Chen, Aliaa Higazi, Tanja Francetic, Blaire Langlais, Salim Fettaka, Mahmoud Abed and Jonathan St-Germain.

I would also like to acknowledge the Canadian Institutes of Health Research (CIHR) and the University of Ottawa for the financial support in the forms of CIHR Canada Graduate Scholarship and University of Ottawa admission and excellence scholarships.

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
ADP	Adenosine diphosphate
BWS	Beckwith-Wiedemann syndrome
CBP	Cyclic adenosine monophosphate response element-binding protein
CDK	Cyclin dependant kinase
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21)
CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57)
cDNA	Complementary DNA
CKI	Cyclin-dependent kinase inhibitor
ChIP	Chromatin immunoprecipitation
CT	Threshold cycle
CTD	Carboxy-terminal domain
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBSs	Egr1 binding sites
EDTA	Ethylene diaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EGR-1	Early growth response-1
GABA	Gamma-aminobutyric acid
GFP	Green fluorescent protein
GNAT	GCN5-related N-acetyltransferase
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitors
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
IP	Immunoprecipitation
MEF	Mouse embryonic fibroblast cells
MOZ	Monocytic leukemia zinc finger protein
mRNA	Messenger ribonucleic acid
NaB	Sodium butyrate
N-CoR	Nuclear receptor co-repressor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCAF	p300/CBP-associated factor
PCNA	proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PKB	Protein kinase B
PMSF	Phenylmethanesulphonylfluoride
PP2A	Protein phosphatase 2A
Rb	Retinoblastoma protein
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction

rRNA	Ribosomal ribonucleic acid
SAHA	Suberoylanilide hydroxamic acid
SCFA	Short chain fatty acids
SDS	Sodium dodecyl sulfate
SRC-1	Steroid receptor coactivator-1
TAF	TATA binding protein-associated factors
TGF-β	Transforming growth factor β
TSA	Trichostatin A
VPA	Valproic acid
WCE	Whole cell extract

TABLE OF CONTENTS

Page

Introduction

Gene expression regulation is a fundamental biological process which involves different genetic elements through which complex regulatory networks control multiple functions such as the synthesis of mRNA molecules and the production of proteins. The process of gene transcription is highly regulated at the level of transcription initiation and modulated by various cellular signals (reviewed in Sadhale *et al*, 2007). Transcription essentially begins with enhancing the accessibility of DNA and allowing the recruitment of a diverse set of transcriptional components to gene promoters (Luger *et al*, 1997). These components include chromatin remodeling complexes, transcription factors and coactivators such as p300 (reviewed in Narlikar, 2002; Orphanides and Reinberg, 2002).

Chromatin remodeling and transcription

Chromatin structure is dynamic and susceptible to changes, which affect the accessibility of DNA to transcription factors. Changes in chromatin dynamics do not randomly occur. In fact, they occur through highly regulated mechanisms (Eberharter *et al*, 2005). In principle, chromatin undergoes remodeling through two main mechanisms: ATP-dependent chromatin remodeling and post-translational modifications of the amino-terminal tails of histones (reviewed in Vignali *et al*, 2000).

Chromatin remodeling complexes utilize energy in the form of ATP to induce changes in the chromatin structure by destabilizing histone-DNA interactions and physically shifting nucleosomes into different positions in ATP-dependent manner (Pazin and Kadonaga, 1997). Thus, they expose regions of DNA that were initially associated

with nucleosomes and cover others. They have been classified into three main families based on the domain composition of their catalytic ATPase subunits: the SWI2/SNF2 family, SWI (ISWI) family and the Mi-2 family (Eisen *et al*, 1995; Pazin and Kadonaga, 1997).

The second mechanism which regulates chromatin remodeling, commonly known as the 'histone code', is a post-translational covalent modification of the amino-terminal tails of nucleosomal histones (reviewed in Strahl and Allis, 2000; Legube and Trouche, 2003). These modifications include phosphorylation, methylation, ubiquitination, ADP ribosylation, sumoylation, carbonylation, glycosylation and acetylation (reviewed in Kouzarides, 2007; Nightingale, 2006). These modifications are also thought to serve as epigenetic markers which regulate the expression of genes, either by changing the chromatin conformation or by acting as signals for the recruitment of coactivators or corepressors. Phosphorylation modifications of the histone tails add negative charges which would repel the negatively charged DNA and lead to chromatin decondensation (Roth and Allis, 1992). Acetylation of the positively charged lysine residues on histones associated with the negatively charged backbone of DNA neutralizes the positive charges and allows DNA to be more accessible for transcription. Histone acetylation plays an important role in the regulation of gene expression. In general, hyperacetylated chromatin regions are transcriptionally active, and hypoacetylated chromatin regions are repressed. Acetylation can also be incorporated as a signal for protein interactions, due to the fact that some proteins recognize acetylated histones through their bromodomains (Dhalluin *et al*, 1999). Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are the main regulators of the acetylation status of lysine residues on amino-terminal domains

of core histone proteins. They in fact regulate the process of selective acetylation or deacetylation, respectively (reviewed in Legube and Trouche, 2003). The balance of HAT/HDAC is critical in regulating histone acetylation status. It maintains a steady level of acetylation on core histones, and is controlled by regulating the two enzymes in terms of their quantity, activity, and availability (reviewed in Legube and Trouche, 2003).

Histone acetyltransferases catalyze acetylation of lysine residues on amino-termini of histone tails. They can be grouped into five families based on the composition of their catalytic domains (reviewed in Carrozza *et al* 2003; Lee and Workman, 2007). The GCN5-related N-acetyltransferases (GNATs) are enzymes that acetylate proteins on their primary amino functional group (reviewed in Dyda *et al*, 2000). GNATs transfer an acetyl group from acetyl coenzyme A to histones, aminoglycosides, and arylalkylamines. The GNAT superfamily includes the p300/CBP-associated factor (PCAF), which acetylates histones H3 and H4 in both free histones and mononucleosomes (Schiltz *et al*, 1999). The GNAT also includes GCN5 which acetylates free histones H3 and H4 but do not acetylate mononucleosomes (Yang *et al*, 1996). Members of the MYST histone acetyltransferases include MOZ, Ybf2/Sas3, Sas2, Tip60 HBO1 and MORF and are capable of acetylating free histones H4, H2A and H3 (Smith *et al*, 2005; Yamamoto and Horikoshi, 1997). They are also capable of acetylating nucleosomal histones H4 and H2A in their native complexes (Ikura *et al*, 2000). The coactivator p300 and closely related CREB binding protein (CBP) are versatile HATs which are capable of acetylating all four nucleosomal core histones. In addition to p300/CBP family, some members of the general transcription factors represent another family of HATs. They include TAF250 which is a component of the basic transcription complex TAFIID. TAF250 possesses a limited HAT

activity which assists their access and recruitment on transcriptionally repressed chromatin (Mizzen *et al*, 1996). The last family is the nuclear hormone-related HATs. Members of this family such as the steroid receptor coactivator 1 (SRC-1) and the activator of retinoid receptor ACTR (SRC3) possess a limited HAT activity (Lee and Workman, 2007).

Histone deacetylases (HDACs) are a diverse set of enzymes which function as a catalyst for the removal of acetyl groups from the amino-terminus of histone tails. The deacetylation enhances the association of histones to DNA and decreases the accessibility of DNA to transcription factors. HDACs are usually associated with several protein complexes such as corepressors as well as transcription factors such as Mad-1, BCL-6, and ETO at gene promoters and regulate their activity and accessibility for transcription (Wang *et al*, 1998). Studies up to date show that eighteen different HDACs were identified in human and classified into five different classes: I, IIa, IIb, III and IV (reviewed in Bolden 2006; De Ruijter *et al*, 2003). Members of HDAC class I are HDAC1, 2, 3 and 8. However, HDAC1 and 2 are functional only when they are associated with transcriptional repressor complexes, such as Sin3, NuRD and Co-REST (Zhang *et al*, 1999). The activity of HDAC1 and 2 is also regulated by phosphorylation, in which phosphorylation of specific sites remarkably increases their enzymatic activity (Galasinski *et al*, 2002). HDAC3 is associated with the silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) and the nuclear receptor co-repressor (N-CoR) to modulate its activity. On the other hand, HDAC8 has no known associations to date with co-repressor complexes. Next are the members of HDAC class IIa which consist of HDAC4, 5, 7 and 9. In fact, HDAC4, 5 and 7 are dependent on corepressors

such as SMRT/N-CoR for their activity and possess a special property that distinguishes them from other HDACs in their ability to shuttle from nucleus to cytoplasm during various stages of muscle cell differentiation, suggesting that they play roles in differentiation (Kao *et al*, 2001). HDAC9 has three isoforms: HDAC9a, HDAC9b and HDRP/HDAC9c and they are expressed at different ratios in different cell types. Members of class IIb are HDAC6 and 10. HDAC6 is expressed exclusively in the cytoplasm and acts on tubulin and thus has important roles in cell motility through microtubule formation control. HDAC6 is heavily expressed at the leading edge of the cell during the first steps of motility, and functions as a deacetylase in which deacetylation of microtubules decreases their stability and promote their depolymerization (Hubbert *et al*, 2002). HDAC10 has two isoforms and interacts with many HDACs and corepressor complexes such as HDACs 1-5, 7 and SMRT. Therefore, HDAC10 might have an additional function as a recruiter. Members of class III HDACs include $SIRT_{1-7}$ proteins, homologues of the yeast protein Sir2. SIRT deacetylases regulate gene expression in response to cellular redox status and dependent on NAD+ for their activity (Bolden *et al*, 2006). HDAC11 represents another class of HDACs, class IV. Although there is not much known about HDAC11, it had been shown that HDAC11 does not interact with other HDACs and corepressors such as Sin3, N-CoR and SMRT, which indicates possible unique functions of HDAC11 (Gao *et al*, 2002). Histones are not the only substrates to HDACs. Some proteins such as p53, E2F and MyoD are targets for deacetylation by HDACs, which suggests versatile roles of HDACs in many different functions in the cell (Juan *et al*, 2000 and reviewed in de Ruijter, 2003).

With the current knowledge that HATs and HDACs are involved in epigenetic regulation of a wide range of gene families, from tumor suppressors to cell cycle regulatory genes, new studies emerged to identify mechanisms of tumorigenesis that are related to abnormalities in HDAC and HAT expressions in cancer. A number of studies demonstrated an increased expression of several HDAC and HDAC-containing transcriptional repressor complexes in many cancers (Bolden *et al*, 2006). The basal expression of HDAC1 was shown to be increased significantly in many carcinomas such as prostate, gastric and breast carcinomas (Halkidou *et al* 2004; Choi *et al*, 2001 and Zhang, Z. *et al*. 2005 respectively). HDAC2 is overexpressed in cervical carcinomas (Huang *et al*, 2005); HDAC3 is overexpressed in colon carcinomas (Wilson *et al*, 2006) and HDAC6 is overexpressed in breast carcinomas (Zhang *et al*, 2004). Using the knowledge that HDACs are over expressed in many carcinomas, and their roles in epigenetic regulation, new avenues of research have been focused on elucidating the possibility of targeting HDACs as a new strategy to fight cancer.

Histone deacetylase inhibitors

HDAC inhibitors emerged in recent years as a new class of therapeutics against several cancers (reviewed in Bolden *et al*, 2006). HDAC inhibitors are promising agents as they selectively induce apoptosis in tumor cells, and pose minimal risks to normal cells. This was illustrated in preclinical animal studies and *in vitro* studies (reviewed in Drummond *et al*, 2005; Dokmanovic and Marks, 2005; Kelly and Marks, 2005). *In vitro* studies showed that transformed cells are more sensitive to these inhibitors which cause them to undergo one or more of growth arrest, differentiation, apoptosis, and reactive

oxygen species- associated and autophagocytic cell death. Studies on animal models show that HDAC inhibitors inhibit growth of solid tumors and hematological malignancies with very limited toxicity to normal cells and tissues. The applications of HDAC inhibitors as anticancer agents were assessed in several phase I and phase II clinical trials, with very promising preliminary results. In fact, some HDAC inhibitors have already been approved for cancer therapy such as suberoylanilide hydroxamic acid, which was approved by the U.S. Food and Drug Administration in 2006, and marketed under the name of vorinostat (Zolinza®; Merck & Co., Inc.) for the treatment of cutaneous T-cell lymphoma (Mann *et al*, 2007).

HDAC inhibitors affect molecular pathways which lead to the inhibition of cell growth and induce cycle arrest as well as apoptosis to transformed cells (Takai *et al*, 2004). HDAC inhibitors induce changes on the expression of a large number of genes by inhibiting the activity of HDACs on histone proteins which results in the accumulation of acetylated histones and enhanced accessibility of DNA template to transcription as a result of HDAC/HAT balance disruption. HDAC inhibitors can also affect global gene expression through regulating the acetylation of non-histone targets (reviewed in Johnstone and Licht, 2003). The activity and stability of several non-histone proteins are regulated by acetylation and association with HDAC complexes, which are affected by HDAC inhibitors. Some of these non-histone proteins directly and indirectly regulate transcription such as E2F, which is a transcription factor involved in cell cycle progression, and p53 which is a regulator of apoptosis (Martínez-Balbás *et al*, 2000; Somasundaram and El-Deiry, 1997). In a study by Glaser and colleagues (2003), global gene expression profiles of bladder and breast carcinomas treated with three different

HDAC inhibitors were examined. The study revealed that there is a common set of genes up- and down-regulated by the three different HDACs in both carcinomas. Interestingly, the genes that are upregulated are functionally related and involved in cell cycle arrest and apoptosis such as p16, p21, p27, Hep27 and TRPM-2. Whereas the downregulated genes such as thymidylate synthetase and CTP synthase, both of which are involved in DNA synthesis, cell division and survival. HDAC inhibitors do not only affect the expression of pro-apoptotic genes, they also target pro-survival genes. Results from our laboratory show that a key determinant for the susceptibility of cancer cells to histone deacetylase inhibitors is their ability to maintain cellular pro-survival or anti-apoptotic Akt/PKB (protein kinase B) activity (Chen, Ghazawi and Bakkar, 2006).

Although the potential use of HDAC inhibitors as therapeutic agents for cancer treatment is very promising, adverse side effects have been recorded in several clinical studies, which demonstrate the need for more careful studies on HDAC inhibitors to identify the administrative dosage limits and to provide a better understanding of their mechanisms of action (Bolden *et al*, 2006). There are many classes of natural and synthetic HDAC inhibitors. Short-chain fatty acid (SCFA) is a well studied class of HDAC inhibitors, (reviewed in Chen *et al*, 2003) which includes butyrate and valproic acid.

Butyrate is a SCFA which is naturally produced by anaerobic bacteria fermenting undigested dietary carbohydrates in colon. It was used in phase I clinical trials in patients with β-hemoglobinopathies and β-thalassemia syndromes as a method of gene therapy to reactivate the developmentally-silenced globin genes in these patients (Perrine *et al*, 1993). The first indication that butyrate has some anticancer activities appeared in a study

by Leder and colleagues (1975) in which it was revealed that the differentiation of immature red blood cells (erythroids) can be induced by butyrate treatment. Following this, a study by Riggs and colleagues (1977) has shown that butyrate induces a rapid and reversible increase in global histone acetylation levels, but butyrate targets were not identified. It is currently known that butyrate targets HDAC class I and IIa and inhibits their enzymatic activities (reviewed in Bolden *et al*, 2006). Several studies revealed that butyrate can induce cell cycle arrest and selective apoptosis of tumor cells. In fact, it was reported that butyrate is the most potent fatty acid in arresting the proliferation of colorectal carcinoma as shown in a study by Siavoshian and colleagues (1997). Thus, butyrate is a promising anti-cancer agent.

Valproic acid (VPA, 2-propylpetanoic acid) is another SCFA, which is commonly used in the treatment of epilepsy, bipolar disorders, migraine and neuropathic pain (reviewed in Johannessen and Johannessen, 2003). VPA is an effective drug against many seizures which acts by increasing levels and triggering the inhibitory effects of the main inhibitory neurotransmitter in the mammalian central nervous system, gamma-aminobutyric acid (GABA) (reviewed in Löscher, 1999). VPA inhibits HDAC class I and IIa (reviewed in Bolden et al, 2006). VPA has a significant anticancer activity, as it selectively causes transformed cells to undergo growth arrest and apoptosis (reviewed in Dokmanovic and Marks, 2005).

Hydroxamate compounds such as Suberoylanilide hydroxamic acid, Pyroxamide, Oxamflatin and Trichostatin A represent another important class of HDAC inhibitors. Trichostatin A (TSA) is an antifungal organic compound. A study by Yoshida and colleagues (1990) has shown that TSA is a potent inhibitor against histone deacetylases

in vivo as well as *in vitro*. TSA inhibits HDAC class I and IIa and possesses antiproliferative effects against tumor cells (reviewed in Bolden *et al*, 2006). Several recent studies illustrated that TSA can be used in the treatment of ovarian, prostate and pancreatic cancers (Zhou *et al*, 2006; Rokhlin *et al*, 2006 and Cecconi *et al*, 2007 respectively). TSA possesses selective anti-cancer activities towards tumors, however more research is needed to assess for any possible side effects associated with its therapeutic application.

Cyclic tetrapeptides represent another class of HDAC inhibitors. Apicidin is a potent HDAC inhibitor which was also associated with antiprotozoal activity (Darkin-Rattray *et al*, 1996). Apicidin acts as an antiproliferative agent by upregulating the expression of genes that are involved in cell cycle arrest such as p21 (Han *et al*, 2000). Depsipeptide is another cyclic tetrapeptide compound which acts as an inhibitor to class 1 HDACs (Furumaie al, 2002). Electrophilic ketones represent another class of HDAC inhibitors. Trifluoromethylketone compounds were shown to possess characteristics of HDAC inhibitors by inducing hyperacetylation of histone H4 and increase expression of p21 (Frey *et al*, 2002). Benzamides represent another class of HDAC inhibitors. Several benzamides possess HDAC inhibitor activity against class I HDACs. An example of these benzamides is MS-27-275 benzamide compound which specifically inhibits HDAC1 and 3 (Hu *et al*, 2003). Synthetic HDAC inhibitors are a new class of the inhibitors (reviewed in Riester*et al*, 2007). Using different model systems, screening of small synthetic molecules with inhibitory effects to HDAC and examining their anticancer effects is a novel approach for identifying new inhibitors as in the study by Wegener and colleagues (2008).

As discussed earlier, inhibiting HDACs disrupts the HAT/HDAC balance and results in the accumulation of histones in hyperacetylated state and modulate the acetylation status on non-histone proteins due to inhibiting the activity of HDACs which are responsible for deacetylation. In this study, three HDAC inhibitors namely, sodium butyrate, valproic acid and trichostatin A will be utilized and several aspects of their anticancer activities will be investigated.

Transcriptional coactivator p300

The adenovirus E1A-associated 300 kDa protein (p300), and closely related 265 kDa CREB binding protein (CBP) are structural and functional homologues and global transcriptional coactivators that are involved in the regulation of transcription of a wide array of genes and modulate the activity of a large number of transcription factors (reviewed in Vo and Goodman, 2001). The coactivator p300 was originally identified as an adenovirus E1A oncoprotein-associated factor by Eckner and colleagues (1994), in which it was shown that the 300 kDa protein is associated with E1A specifically through its carboxy-terminal region. Due to its large size, versatility of its domains and association with SV40 transcriptional enhancer, it was originally hypothesized that p300 has transactivation roles in transcription (Eckner *et al*, 1994). CBP was identified as a binding factor interacting with the activated kinase A-phosphorylated form of CREB (Chrivia *et al*, 1993). p300 and CBP share great similarity in DNA and amino acid sequences, and often have overlapping functions, and so they are commonly annotated as p300/CBP.

p300/CBP encompass a wide range of functions in the cell such as in differentiation, growth control, homeostasis, proliferation and apoptosis (reviewed in Goodman and Smolik, 2000). The major role of these coactivators is to serve as transcriptional integrators or adaptors to stabilize the transcriptional pre-initiation complex. They bind to a large number of regulatory proteins, nuclear hormone receptors, serum response factors, enhancer elements and general transcription factors (reviewed in Vo and Goodman, 2001). In addition to functioning as a physical bridge between various transcription factors and the basal transcriptional machinery, p300/CBP possess histone acetyltransferase activity, capable of adding an acetyl group to all four core histones *in vitro* (Ogryzko *et al*, 1996) and represent one of the five HAT families that were discussed earlier.

Accumulation of acetylated histones is a characteristic of transcriptionally active regions in the chromatin, and regions with an excess of hypoacetylated histones are generally silent (reviewed in Wolffe, 1994). However, histones are not the only targets of acetylation. p300/CBP acetylate components of the basal transcriptional machinery including general transcription factors (reviewed in Imhof *et al*, 1997). They also acetylate other chromatin bound non-histone proteins such as the architectural chromatin binding protein HMG I/Y (Munshi *et al*, 1998). Other targets of acetylation by p300/CBP are transcriptional activators such as GATA-1 and NF-Y (Boyes *et al*, 1998; Li *et al*, 1998 respectively). Recent evidence from several studies indicated that another target of acetylation by p300 is p300 itself, more specifically, on the HAT domain (Karanam *et al*, 2006). The significance of p300 autoacetylation was illustrated in a study by Thompson and colleagues (2004), in which the HAT enzymatic activities of hyperacetylated and

hypoacetylated forms of p300 were measured. The hyperacetylated forms of p300 were found to be much more active than hypoacetylated forms, by more than 10 fold. Thus, p300 contribute to the regulation of its own HAT activity. However, it is not clear yet whether p300 autoacetylation is intermolecular, through association with other HATs such as PCAF, or intramolecular, through a conformational change that allows the enzymatic reaction to occur (Karanam *et al*, 2006).

p300/CBP are involved in many developmental pathways in mammals (Reviewed in Goodman and Smolik, 2000). Evidences of p300/CBP roles in development came from gene knockout experiments in mice. In a study by Yao and colleagues (1998), mice lacking one or two alleles of the p300 gene were generated. Mice that were homozygous negative to p300 died between days 9 and 11.5 of gestation, displaying defects in heart development, neural tube closure, and cell proliferation. This indicates that p300 is exclusively required for functions that are not overlapped with CBP. Heterozygote mice to p300 also showed embryonic lethality. This highlights the importance of basal p300 levels for development. Remarkably, double heterozygotes for p300 and CBP also resulted in embryonic death, which suggests that minimal levels of both p300 and CBP are required for cell proliferation and development. In another study by Tanaka and colleagues (1997), heterozygous mice to CBP were generated and shown various skeletal abnormalities. The severity of the abnormality was correlated with the genetic background of CBP which further suggested the requirement of both CBP alleles in proper mammalian development.

Another significant property of p300/CBP is their roles as tumor suppressors. This was initially identified by Arany and colleagues (1995) when it was found that p300 and

CBP are both targets for viral oncoproteins, an indication of the central roles that both proteins play in the regulation of the cell cycle and tumorigenesis. In a study by Suganuma and colleagues (2002), several human carcinoma cell lines lacking normal p300 were transfected with either flag-tagged wild-type or mutant p300 to demonstrate the significance of p300 mutations in tumorigenesis. It was revealed that there is an apparent suppression of carcinoma cell growth by reintroduction of the normal functional form of p300. In another study by Gayther and colleagues (2000), mutations in the p300 gene in 193 epithelial cancer samples were examined. The study revealed that six common mutations lead to the expression of truncated, non-functional form of p300 in many primary tumors including colorectal and breast and pancreas. Additionally, incretion mutations were found in a primary breast cancer. Thus it is clear that p300/CBP play important roles in suppressing tumor growth, mainly by regulating the expression of many oncogenes (reviewed in Iyer *et al*, 2004).

p300/CBP regulate the activity of the apoptotic regulator and tumor suppressor p53 (reviewed in Grossman, 2001). p53 is a transcription factor, which upon activation during DNA damage, transactivates the transcription of a group of genes that are involved in DNA repair response. It also transactivates the transcription of apoptotic genes if the DNA damage proves to be irreparable (reviewed in Vousden, 2000). A number of studies illustrated that overexpression of p300/CBP enhances the transactivation activity of p53, which provided a clear link of roles of p300/CBP in p53 signaling (Avantaggiati *et al*, 1997; Gu *et al*, 1997; Lill *et al*, 1997 and Scolnick *et al*, 1997). In fact, in a study by Gu and Roeder (1997), it was revealed that p300/CBP acetylate p53 and stimulate its sequence-specific DNA-binding activity. Thus, the

modulation of p53 activity by p300/CBP is a one of the mechanisms by which these coactivators act as tumor suppressors.

Another tumor suppressor which is regulated by p300/CBP is the breast and ovarian cancer-specific tumor suppressor BRCA-1 (Pao *et al*, 2000). BRCA-1 was identified as a strong candidate for the breast and ovarian cancer susceptibility gene by Miki and colleagues (1994). Mutations in BRCA-1 are highly associated with increased risks of breast or ovarian cancer. p300/CBP are breast and ovarian tumor suppressors by regulating the activity of BRCA1. BRCA-1 interacts with the cAMP response element binding protein domain of p300/CBP through both its amino and carboxyl termini. BRCA-1 does not become acetylated by p300 (Hockings *et al*, 2008; Jeffy *et al*, 2005). However, BRCA-1 and p300 form a coactivator complex which associates on BRCA-1 regulated promoters and transactivate transcription. (Pao *et al*, 2000).

p300/CBP also function as tumor suppressors by inhibiting Wnt signalling pathway. Wnt signalling pathway involves a complex network of genes that regulate cell cycle and embryogenesis (reviewed in Polakis, 2000). Wnt signalling promotes tumor formation by affecting the expression of critical genes that regulate the cell cycle such as cyclin D1 (He *et al*, 1998). In a study by Li and colleagues (2007), it was shown that p300 inhibits Wnt signaling in a human colorectal cancer cell line. Thus p300/CBP also function as tumor suppressors by regulating signaling pathways and the cell cycle.

The capacity to multi-function by p300/CBP allows them to be very efficient key regulators for RNA polymerase II-mediated transcription (Chen *et al*, 2005). p300/CBP function as coactivators of transcription through different modes of regulation. As discussed earlier, p300/CBP operate as scaffolding bridges to hold the transcriptional

apparatus. They can acetylate promoter-associated histone proteins to allow DNA to be more accessible for transcription, and they can acetylate transcription factors that bind to specific consensus nucleotide sequences on gene promoters (cis-elements) to enhance their activity, association or dissociation from transcriptional apparatus. p300 interacts and binds to many transcription factors such as ZBP89, ZF9, ZNF42, NRF2, CETS1p54, ETS1, ETS2, ELK1, GABP, SP1, BTEB3, CEBPB, CEBP, PAX-5, EGR1, EGR2, EGR3, NGFIC and WT1 (Smith *et al*, 2004). However, the specific mode of regulation by p300 and other coactivators depends on the presence and the arrangement of certain cis-elements, which define the mode of regulation by coactivators in gene transcription (Segal *et al*, 2003).

p300 interacts with regulatory proteins and transcription factors through several conserved functional domains (please refer to the diagram on the next page) that include three Cys/His-rich (C/H1, C/H2, and C/H3) domains, histone acetyltransferase (HAT) domain, CREB-binding domain (KIX), a Glutamine-rich (Q) domain, a bromodomain, and a recently discovered cell cycle regulated domain1 (CRD1), which partially-represses the HAT activity (Snowden *et al*, 2000 and reviewed in Vo and Goodman, 2001). Many studies identified a variety of DNA-binding transcriptional activator proteins and components of the basal transcriptional to almost each individual domain in p300 (Kraus *et al*, 1999). However, until a short time ago, the function of the bromodomain was largely unknown. Some early studies suggested that the bromodomain may be involved in protein-protein interactions and may play a role in assembly or activity of multi-component complexes involved in transcriptional activation (Tamkun *et al*, 1995).

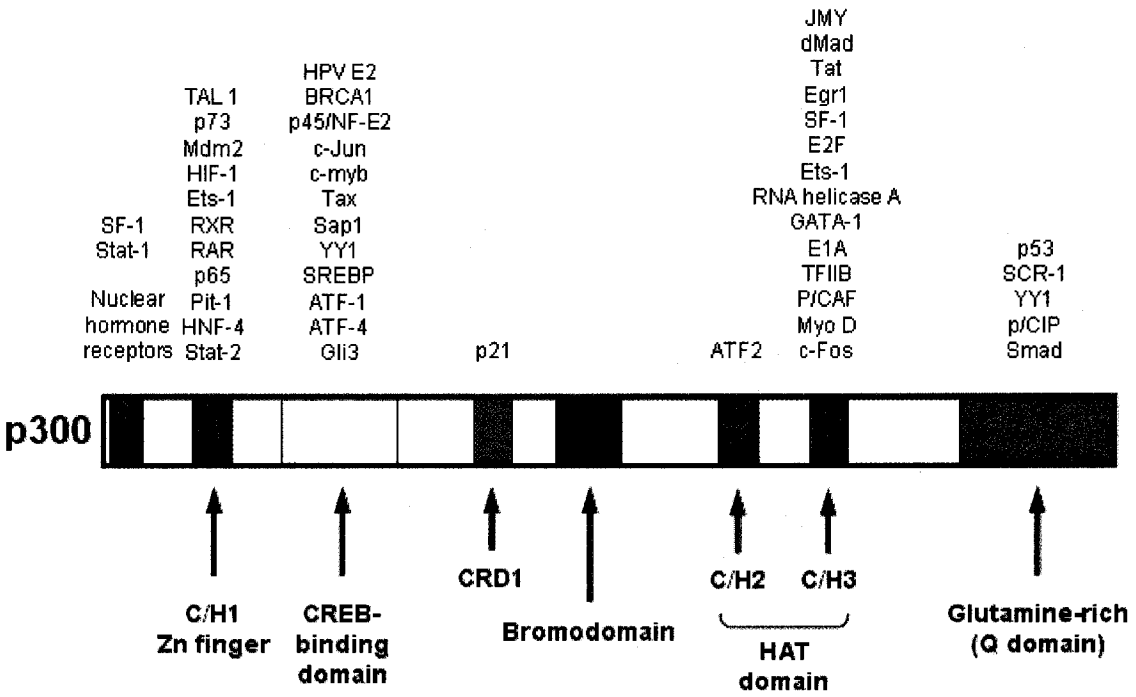


Diagram 1. The domain organization of p300. Many interacting proteins with p300 domains are shown at the top of the figure. Functional domains are depicted beneath. Adapted from (Vo and Goodman, 2001 Fig 1), (Goodman and Smolik, 2000 Fig 1), (Snowden et al, 2000 Fig 6).

The bromodomain was first reported in the Drosophila protein brahma (brm), hence the name (Tamkun *et al*, 1992). Bromodomains are found in a variety of mammalian, invertebrate and yeast DNA-binding proteins (Haynes *et al*, 1992). The 110 amino acid domain represents an extensive family of evolutionarily conserved protein module found in many chromatin-associated proteins, ATPase component of certain nucleosome remodeling complexes and in almost all known nuclear histone acetyltransferases (reviewed in Zeng and Zhou, 2002; Winston and Allis, 1999). The bromodomain has long been a domain in search of a meaningful *in vivo* function until a study by Dhalluin and colleagues (1999) that used a combination of structural and site-directed mutagenesis experimental approaches led to a novel finding that the

bromodomain of a human histone acetyltransferase, PCAF (p300/CBP associated factor), could interact specifically with acetylated lysine, making them the first known protein modules to do so. Several hypotheses were made based on this important finding, suggesting a functional relationship between bromodomain and the HAT activity of coactivators in the regulation of gene transcription. Nuclear magnetic resonance (NMR) analysis of a single bromodomain from the p300/CBP-associated factor P/CAF revealed a four-helix bundle with a left-handed twist (Dhalluin *et al*, 1999). The interaction between the P/CAF bromodomain and acetylated lysine residues were shown to occur on a hydrophobic pocket of bromodomain (Dhalluin *et al*, 1999). Interestingly, the nature of the interaction between bromodomain and acetylated lysines is similar to that between acetyl-CoA and the histone acetyltransferase, suggesting a convergent mechanism of acetyl recognition between bromodomains and histone acetyltransferases (Winston and Allis, 1999; Dutnall *et al*, 1998). A study by Jacobson and colleagues (2000) examined the double bromodomain module of TFIID, a multi-component transcription factor that recognizes and binds to TATA box on gene promoters by a TATA binding protein (TBP) subunit (reviewed in Goodrich and Tjian, 1994). The study showed a bromodomain-dependent increase in affinity for specific acetylated histones near the core promoter, which provided a link between histone acetylation and transcriptional activation by enhanced transcriptional preinitiation complex formation. In other studies, it was shown that deletion of the p300 bromodomain severely impairs p300 coactivator function for transcriptional enhancement by NF-kB p65 and Sp1 *in vitro* transcription assays (Kraus *et al*, 1999). Analysis of the HAT activity of a bromodomain mutant p300 protein revealed that it is impaired in its ability to acetylate nucleosomal histones in native

chromatin but not free histones (Kraus *et al*, 1999). A similar requirement for the bromodomain in the acetylation of nucleosomal histones was observed with yeast Gcn5p in the SAGA complex, an important protein for transcription *in vivo*, which possesses HAT activity (Sterner *et al*, 1999). Taken together, these studies suggest a possible requirement for the bromodomain for full HAT activity of p300 coactivator function. On the other hand, it is also proposed by many that the bromodomain may play a different role in anchoring HATs or other coactivators onto active chromatin by recognizing acetylated histones and/or other factors during chromatin remodeling (Manning *et al*, 2001). Thus, p300 bromodomain is an important domain for the function of p300. However, the precise function of bromodomain remains unknown. Additionally, it is not known whether the bromodomain is essential only to a limited set or to all the p300-regulated genes and the precise transcription factors to which bromodomains interact with remain largely unknown.

The above described facts and lack of solid understanding on bromodomain function prompted us to investigate and start the current study, in an attempt to elucidate possible roles of bromodomain in the function of p300. Based on the current knowledge and preliminary data from our laboratory, we hypothesize that *the bromodomain has important roles in regulating the recruitment of p300 to target promoters*. Given the importance of p300 in the transcriptional regulation of many gene families, and the evolutionary conserved nature bromodomain which is indicative of functional importance, further understanding of the roles of bromodomain in the function of p300 could yield valuable information which would ultimately enhance our understanding of the molecular mechanisms by which p300 functions.

As already discussed, p300 is a transcriptional regulator of a wide range of gene families and is involved in many cellular functions. However, roles of p300 in cell cycle control at the transcript level have not yet been fully elucidated. This stimulated our interest to examine the transcriptional regulation of several regulators of the cell cycle by the coactivator p300. In a study by Smith and colleagues (2004), the kinetics of many gene promoters was carefully examined and many genes under the specific control of p300 were identified, including a number of genes that are involved in cell cycle regulation. The expression of four p300-dependent genes that are involved in cell cycle control was examined in this study. These genes are the cyclin-dependent kinase inhibitor 1A (p21), early growth response 1 (Egr1), cyclin-dependent kinase inhibitor 1C (p57) and E2F transcription factor 1 (E2F1).

Roles of p300 in cell cycle regulation

The first study that identified the existence of different phases of the cell cycle was first conducted over half a century ago by Howard and Pelc (1951). In their study, the incorporation rate of P^{32} during DNA synthesis was monitored by autoradiography techniques. It was observed that P^{32} was incorporated only at a discrete period of interphase. Thus, a basis for a model of the cell cycle, which divides it into four different phases, was put forth. Following this model, considerable research has been focused on understanding the cell cycle and identifying main components that regulate it.

Eukaryotic cell cycle takes place in four phases. Progression through different phases of the eukaryotic cell cycle is mainly regulated by the expression of specific cyclin proteins, cyclin dependent kinase (cdk) complexes and other cdk regulators at

various stages of the cell cycle. The regulated expression of these proteins regulates the cellular decision to proliferate, differentiate, or arrest the cell cycle (reviewed in McInnes, 2008; Morgan, 1997; Snowden and Perkins, 1998; Nigg, 1995).

Cyclin dependent kinases (cdks) are serine/threonine protein kinases which phosphorylate components of the cell cycle in which different cdks phosphorylate and consequentially activate components of different phases of cell cycle cyclically. Cdks are dependent on functional partners Cyclin proteins (reviewed in Johnson and Walker, 1999). Cyclins were first discovered in marine invertebrates and were described as proteins that were expressed in cyclical fashion throughout the cell cycle, in which one is produced at each phase, while others are down-regulated and re-expressed during other phases of the cell cycle. Cyclin proteins bind to and activate cyclin dependent kinases through a cyclin box domain. There are four classes of cyclin proteins that are directly involved in cell cycle regulation: class A, B, D and E. Cyclin A regulates S phase entry and transition and activates cdk1 and 2. Levels of Cyclin B peak at M phase and activate cdk1. Cyclin D controls cell re-entry from G0 into cell cycle and activates cdk4 and cdk6. Cyclin E regulates G1-to-S–phase transition and activates cdk2 (Ohtsubo *et al*, 1995).

The activity of cdks is modulated by cyclin dependent kinase inhibitors (CKIs) and by phosphorylation. CKIs modulate cell cycle progression by inhibiting cdks in response to intracellular and extracellular signals (Harper & Elledge, 1996). There are two classes of CKIs: the Cip/Kip, which includes p21, p27 and p57; and Ink4, which includes p15, p16, p18, and p19. Phosphorylation of cdk is another mechanism by which their activity is up- or down-regulated, depending on the phosphorylation site (reviewed in Morgan, 1995).

Recent studies identified several direct and indirect roles of p300 in cell cycle regulation. One of the first observations that suggested the involvement of p300 in cell cycle was shown in a study by Yaciuk and colleagues (1991), which revealed that p300 is phosphorylated in a cell cycle-dependent manner. Following this observation, a number of studies emerged, which demonstrate roles of p300 in the expression and regulation of several components of the cell cycle. p300 is involved in cell cycle through activation or repression of several cell cycle regulatory genes through promoter interactions and thus regulates the transcription process, or through direct protein-protein interactions, to induce acetylation modifications to several components of the cell cycle.

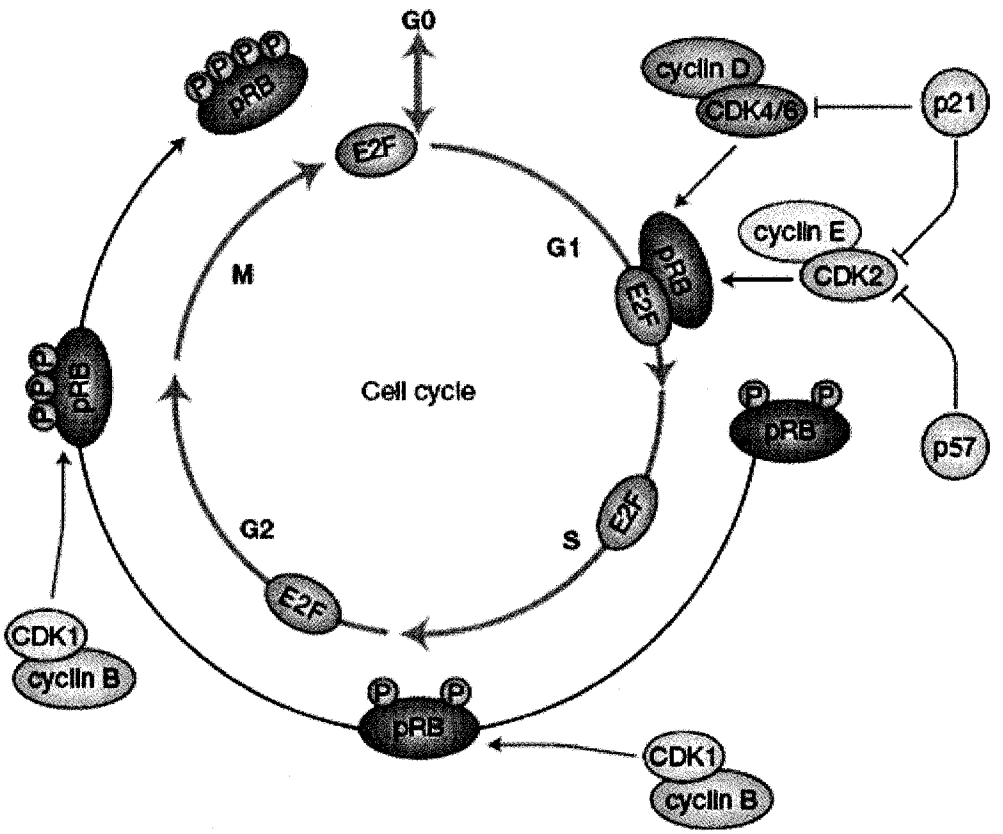


Diagram 2. General model of the cell cycle and its key regulators. Adapted from (Abukhdeir and Park, 2008 - Figure 1)

p300 and p21

The cyclin-dependent kinase inhibitor p21 (CDKN1A) is a 21 kDa protein which is composed of 164 amino acids in humans (Xiong *et al*, 1993). The main functions of p21 are to modulate cell cycle progression at G1 and initiate checkpoint arrest in response to apoptotic signals by inhibiting the activity of cyclin-cdk2 or -cdk4 complexes (reviewed in Abukhdeir and Park, 2008). Several studies illustrated that overexpression of p21 inhibits proliferation in mammalian cells (Kim *et al*, 2006; Lee *et* al, 1995; Xiong *et al*, 1993).

The transcriptional coactivator p300 is one of the main regulators of p21 gene expression (Xiao *et al*, 2000). In fact, first evidence of the importance of p300 in modulating p21 expression came from the observation that p300 was essential for p21 expression in keratinocyte terminal differentiation (Missero *et al*, 1995). The coactivator p300 mediates p21 transcription through collaboration with Sp1 and Sp3 transcription factors on the p21 promoter (Xiao *et al*, 2000). p300 also mediates transcription of p21 through a p53-mediated mechanism. Acetylation of the p53 protein by p300 enhances the transcriptional activity of p53 towards its targets, which include p21 (Somasundaram and El-Deiry, 1997).

The transcriptional activity of p300 is partially modulated by p21 (Snowden *et al*, 2000). p21 augments HAT activity of p300 through binding to the cell cycle regulated domain 1 (CRD1) domain of p300, which is a discreet domain in the amino-terminal domain that represses the activity of p300 at multiple promoters (Snowden *et al*, 2000). It is currently known that several anti-cancer drugs induce selective tumor death through p21-mediated cell cycle arrest (Lim *et al*, 2006; Ahmad *et al*, 2001). Thus, a better

understanding of the regulation of p21 expression in tumors would be valuable in anti-tumor drug design and therapy.

p300 and p57

The coactivator p300 regulates the transcription of other cyclin-dependent kinase inhibitors such as p27 and p57 (Snowden and Perkins, 1998). The cyclin-dependent kinase inhibitor p57 (CDKN1C) is a 57 kDa protein which is composed of 316 amino acids in humans, and is a distinct member of the p21 cdk inhibitor family (Matsuoka, *et al* 1995; Lee *et al*, 1995). p57 is mainly involved in modulating cell cycle progression at G1 and checkpoint arrest in response to apoptotic signals by inhibiting the activity of cyclin-cdk2 complex, in a similar manner as p21. Overexpression of p57 was shown to arrest cells in G1, thus it is a negative regulator of cell proliferation (Matsuoka, *et al* 1995).

Mutations or dysfunctions associated with p57 gene are linked to many cancers and syndromes. Mice lacking p57 gene show severe alteration in cell proliferation, apoptosis and differentiation profiles (Zhang *et al*, 1997). Genomic imprinting of p57 is directly associated with Beckwith-Wiedemann syndrome (BWS), which causes many growth defects such as macroglossia, visceromegaly and increased risks to a number of tumors, possibly due to deregulation of cell cycle events (Hatada *et al*, 1996).

p300 and E2F

Transcription factors of the E2F family play key roles in controlling cell cycle progression (reviewed in Sun *et al*, 2007; Attwooll *et al*, 2004). There are eight members

of the E2F gene family: E2F-1-8, in which E2F-1, -2, and -3 are the functional "activating" E2Fs in cell cycle regulation, while the remaining E2Fs act as suppressors. Activating E2F transcription factors bind target gene promoters on E2F binding sites, which consist of consensus DNA sequences, and transactivate transcription through a C-terminal transactivation domain (TAD) (Wells et al, 2002). E2F target genes encode proteins that drive S-phase entry, such as DNA polymerase, thymidine kinase (TK), dihydrofolate reductase (DHFR) and cdc6 which are involved in DNA replication (Marzio *et al*, 2000).

One of the mechanisms by which the activity of E2F is modulated is through interactions with negative regulators: members of the retinoblastoma tumor suppressor family (also known as pocket proteins), which consists of three genes: retinoblastoma protein (Rb), p107, and p130 (Dannenberg et al, 2004 and reviewed in Giordano et al, 2007). When the TADs of E2Fs are bound to pocket proteins, their transactivation activity is inhibited. Phosphorylation of pocket proteins by cdk complexes prevents their binding to E2F transcription factors, enabling them to activate the expression of genes that regulate entry into S phase. However, research in recent years has revealed that the regulation of E2F activity is not just simply through modulating the phosphorylation status of the pocket proteins but rather a complex one (reviewed in Blais and Dynlacht, 2007).

The coactivator p300 is an important regulator of E2F1 activity. In two separate studies by Marzio and colleagues (2000) and Martínez-Balbás and colleagues (2000), it was shown that p300/CBP and p300/CREB-binding protein (CBP)-associated factor (P/CAF) are coactivators of E2F-mediated transactivation. Tip60 was also identified as

another coactivator of E2F1 by Taubert and colleagues (2004). These coactivators regulate the activity of E2F1 by acetylation of three highly conserved lysine residues located at the N-terminus of E2F1 protein. The acetylation of E2F1 protein increases its stability, specific DNA-binding activity and transcriptional activity. E2F-mediated transactivation is also associated with histones H3 and H4 hyperacetylation at the E2F-target promoters, which could be linked to the HAT activity of the p300/CBP, P/CAF and Tip60 coactivators of E2F-mediated transactivation (Taubert *et al*, 2004).

p300 and Egr1

Immediate early growth response-1 gene (Egr1) encodes a transcription factor that is expressed in response to growth factors, cytokines, stress and irradiations (Sukhatme *et al*, 1988). Egr1 binds to promoters at two GC-rich Egr1 binding sites (EBSs), which are the consensus sequences 5'-GCGT/GGGGCG-3' and 5'-TCCT/ACCTCCTCC-3' to which Egr1 is recruited to regulate transcription (Wang and Deuel, 1992). Egr1 is involved in a wide range of cellular functions such as growth and cell cycle regulation as it regulates - or at least participates in the regulation of- the expression of a large number of genes (Ahmed, 2004). In an oligonucleotide-based microarray analysis by Fu and colleagues (2003), more than 300 genes were identified responsive to Egr1 overexpression in human endothelial cells, by 3 or more fold increase or decrease in expression. Among the responsive genes, a number of cell-cycle regulators were identified, such as cyclin D1 and the cyclin-dependent kinase inhibitor $p57^{kip2}$, which indicates that Egr1 has roles in modulating cell cycle in response to inducers to Egr1 such as growth factors and cytokines.

Egr1 also modulates irradiation-induced cell cycle arrest and apoptosis through regulating the expression of several genes such as p21, p53 and Rb. In a study by Das and colleagues (2000), various mouse embryonic fibroblast cells (MEF) that expressed wild type and Egr1 knock-outs showed that irradiation caused apoptosis to MEF cells that express Egr1 wild type, and are associated with increased levels of Egr1, p21 and p53 in response to the irradiation. However, Egr-$1^{-/-}$ cells were very resistant to apoptosis in response to irradiation, and that was accompanied by no changes in p21 and p53 expression levels. In another study by Day and colleagues (1993), it was shown that there are a number of EBSs on Rb gene promoter, which indicates another indirect mechanism of Egr1-dependent cell cycle arrest and apoptosis in response to irradiation through Rb, which is a regulator of p53 stability and apoptotic function (Hsieh *et al*, 1999).

Egr1 has important roles in the transactivation of p300/CBP genes, and is dependent on these coactivators for transcription and activity (reviewed in Adamson and Mustelin, 2005). In a study by Yu and colleagues (2004), it was found that there are 6 EBSs on p300 promoter, and 7 on CBP. Binding of Egr1 on EBSs on p300/CBP promoters was shown to induce the transcription of these coactivators. Endogenous levels of Egr1 are controlled by a negative feedback loop mechanism to prevent the presence of excessive levels of Egr1. Induced levels of Egr1 that transactivate the expression of p300/CBP are stabilized through acetylation by these coactivators. High levels of p300/CBP acetylate Egr1 and form a complex which downregulates the transcription of both Egr1 and p300/CBP.

As discussed earlier, HDAC inhibitors result in the accumulation of hyperacetylated histones, which is commonly correlated with transcriptional upregulation due to the enhancement of the accessibility of DNA template to transcription factors (reviewed in Narlikar, 2002; Orphanides and Reinberg, 2002). However, the hyperacetylation induced by HDAC inhibitors does not always result in transcriptional upregulation, which indicates that the mechanism of action by HDAC inhibitors is not a simple one. There is a growing interest to understand roles of HDAC inhibitors in regulating the acetylation status of non-histone proteins such as the acetyltransferase p300. However, the precise molecular mechanisms of their modes of action are still poorly understood.

The acetylation status of p300 modulates its transactivation activity (Stiehl *et al*, 2007). A number of studies illustrated that HDAC inhibitors enhance the acetylation and transactivation activity of p300 on p300-dependent promoters (Stiehl *et al*, 2007; Bouras *et al*, 2005; Simone *et al*, 2004; Mayo *et al*, 2003). One of the mechanisms by which HDAC inhibitors synergize the acetylation of p300 is by inhibiting the activity of HDACs which interact with and modulate the acetylation status of p300 (Stiehl *et al*, 2007). This allows an efficient p300 autoacetylation, which augments the activity of HAT up to 10 fold (Stiehl *et al*, 2007; Thompson *et al*, 2004). A number of studies identified several HDAC complexes that interact with and modulate the acetylation status of p300. HDAC1 was shown to interact at the C/H3 region of p300's HAT domain (Simone *et al*, 2004). SIRT1, a member of HDAC class III, was also shown to interact with and represses the transactivation activity of p300 (Bouras *et al*, 2005). Additionally, the inhibition of HDAC activity was associated with the recruitment of the p300 transcriptional co-activator to chromatin (Mayo *et al*, 2003).

Butyrate, valproic acid and trichostatin A HDAC inhibitors utilized in this study were shown to significantly increase the transactivation activity of p300 (Stiehl *et al*, 2007; Mayo *et al*, 2003). Thus, HDAC inhibitors can be utilized as an indirect approach to examine the regulation of p300-regulated genes by the coactivator p300. In a study by Bai and Merchant (2000), the upregulation of p21 expression by HDAC inhibitor was examined. The study revealed that butyrate inhibits HDAC1 activity at p21 promoter, which creates an environment of elevated histone acetylation by p300 and allows p300 to interact with the transcription factor ZBP89 to initiate transcription.

In this study, we will examine the transcriptional regulation of four p300-dependent genes in response to HDAC inhibitor treatments. The coactivator p300 was detected at all four gene loci, as shown by chromatin immunoprecipitation assays by Smith and colleagues (2004). A number of other studies further confirmed the requirement of p300 by p21, Egr1, p57 and E2F1 promoters as a coactivator for transcription (Xiao *et al*, 2000; Yu *et al*, 2004; Attia *et al*, 2007; Caretti *et al*, 2003, respectively). However, it is very important to keep in mind that the expression of these four genes is far from being simply regulated through p300 solely. Genes are often regulated by many proteins acting together and cooperate at multiple levels. This will be discussed in more depth in the discussion.

Advances in the analysis of DNA binding regulatory proteins and better understanding of the molecular mechanisms of HDAC inhibitors' mode of action will help in unmasking key elements of gene regulation, and hopefully would help in curing genetic diseases and cancer.

Materials and Methods

Cell culture and reagents

HeLa human adenocarcinoma cells and SiHa squamous carcinoma cells were obtained from American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO-Invitrogen) with 1% Pencillin/Streptomycin (GIBCO-Invitrogen) and 1% MEM Non-Essential amino acids (GIBCO-Invitrogen) and were supplemented with 10% Fetal Bovine Serum (Hyclone and WISENT). Cells were incubated at 37 °C in humidified air that contained 5 % CO_2.

Sodium butyrate, Valproic acid and Trichostatin A were obtained from Sigma-Aldrich (please refer to supplementary table #1 for reagent details). The following antibodies were obtained from Santa Cruz biotechnology: anti-p300 (N-15) rabbit polyclonal, anti-p21(F-2) mouse monoclonal, anti-p57(C-20) rabbit polyclonal, anti-Egr1 (C-19) rabbit polyclonal, anti-E2f1(KH95) mouse monoclonal, anti-Ac-Histone H3 (Lys 9/14) rabbit polyclonal. Anti-β-Actin (AC-15) mouse monoclonal was obtained from Sigma. The following secondary antibodies for Western were obtained from GE Healthcare: anti-mouse IgG horseradish peroxidase conjugate and anti-rabbit IgG horseradish peroxidase conjugate (please refer to supplementary table #2 for antibodies details).

Cell synchronization and Flow cytometry assays

HeLa cells were grown in 10 cm tissue culture plates (Falcon). For late G1-phase block, double thymidine block was performed in the following procedure: at 30%

confluency cells were treated with 2 mM thymidine (Sigma-Aldrich) for 18 h. Cells were then washed twice with PBS and released in thymidine-free DMEM medium for 9 hours. After releasing, cells were blocked with 2 mM thymidine for 17 h. At that point the major population of cells was synchronized at late G1-phase and then cells were released back to the cell cycle by washing them twice with PBS and grown in DMEM media.

For mitotic block, thymidine-nocodazole block was performed in the following procedure: at 40% confluency, cells were treated with 2 mM thymidine for 24 hours, then washed with PBS and released in DMEM media for 3 h. Following the release, cells were treated with nocodazole (Sigma-Aldrich) for 16 h. At that point the major population of cells was synchronized at M-phase and then cells were released back to the cell cycle by washing them twice with PBS and grown in DMEM media.

Cells were washed and harvested in PBS. In order to fix the cells in ethanol, cells were resuspended in 300 μL PBS and 700 μL of 95% ethanol was added drop by drop, the suspension was then incubated in -20°C overnight. Fixed cells were then extracted by simple centrifugation and then incubated with PBS containing 50 μg/ml of RNase A and 50 μg/ml of propidium iodide for 30 minutes at 37°C. DNA contents of the cells were then profiled by fluorescence activated cell analyzer (Beckman Coulter) to determine the distribution of cells in different phases of the cell cycle.

Western blot analysis

Cells were grown in 10 cm tissue culture plates and treated accordingly. Cells were washed once and harvested in PBS. Whole cell protein extraction was done by adding whole cell extraction buffer (10 % glycerol, 50 mM Tris-HCl pH 7.6, 400 mM

NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 % NP-40) for 30 minutes at 4°C. Protein concentration was quantified using Bradford protein assay by Ultrospec3000 spectrophotometer (Pharmacia Biotech). The optical density (OD) of samples which were diluted in Bio-Rad reagent (Bio-Rad) along with a blank which consisted of 1 μL of whole cell extraction buffer added to 1 mL of Bradford reagent, were measured at 595 nm wavelength. Equal amounts of protein (30-100μg) were added to 2x Laemmli reducing buffer (25 % glycerol, 125 mM Tris-HCl pH 6.8, 4 % SDS, 10% β-mercaptoethanol, 0.01% bromophenol blue), boiled for 5 minutes and loaded onto SDS-polyacrylamide gel (375 mM Tris-HCl pH 8.8, 0.1 % SDS, 0.05 % APS, 0.05 % TEMED) which contained 4, 6, 8 or 12% acrylamide depending on the protein of interest. Proteins were transferred overnight from gel to an Immun-Blot PVDF membrane (Bio-Rad), blocked with 5% nonfat milk in PBST for 1 h and then incubated overnight with diluted antibody in 1% of non-fat milk with agitation for overnight at 4°C. The dilutions used were 1:500 for anti-p300, 1:200 for anti-p21, 1:200 for anti-p57, 1:200 for anti-E2F1, 1:500 for anti-β actin, and 1:500 for anti-Egr1. The membrane was then washed 3 times in PBST for 10 minutes and then incubated in proper secondary antibody diluted in PBST with 1 % milk at room temperature, then washed again for three times. For protein signal detection, Western Lightning™ Chemiluminescence reagents kit (Perkin Elmer) was used. For reprobing (as to probe for loading control), membranes were stripped 30 minutes at 50 °C in stripping buffer (2 % SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM β-mercaptoethanol) blocked with 5% nonfat milk in PBST for 1 h and then incubated with antibody of interest. Quantification of Western blot results was performed using Scion Image computer software (Scion Corporation).

Immunoprecipitation

Cells were grown in 10 cm tissue culture plates. Cells were washed, harvested in PBS and whole cell protein extraction was performed as already described. 750 μg of whole cell protein were diluted to adjust NaCl salt concentration to 150 mM by adding a dilution buffer (10% glycerol, 20 mM Tris-HCl pH 8.0, 0.5 EDTA pH 8.0, 1 mM DTT, 1 mM PMSF, and 1mg/ml of BSA). IP samples were agitated for 2 hours with 30 μL of protein agarose A beads, 10 μL of anti-p300 (N-15) rabbit polyclonal primary antibody. Agarose A beads were washed three times with 1 mL of washing buffer (10 % glycerol, 100 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.5 mM EDTA pH 8.0, 1 mM DTT, 1 mM PMSF, 0.1 % NP-40). 20 μL of 2x of Laemmli reducing buffer were added to the beads then boiled for 5 minutes, vortexed gently for 30 seconds, boiled again for 5 minutes and then samples were loaded on 6% SDS-PAGE gel, and proceeded on with same procedure described above for Western.

Reverse Transcription and Real-Time Polymerase Chain Reaction

Cells were grown in 6 well tissue culture plate (Falcon) and treated accordingly. Total RNA was isolated using RNeasy Mini kit (Qiagen) and manufacturer's protocol was followed. Multiskan Spectrum spectrophotometer (Thermo) was used to quantify total RNA concentrations and their quality. Equal amounts of RNA were used in reverse transcription in which High Capacity cDNA Archive Kit - Multiscribe Reverse Transcriptase (Applied Biosystems) was utilized to reverse transcript RNA to cDNA according to the manufacturer's protocol.

The quantitative PCR amplification was performed using the Applied Biosystems 7500 Fast Real-Time PCR System. Gene transcript levels were quantified using the

TaqMan® reporter assay. Primers for four genes used for amplification were pre-designed (TaqMan® primers by Applied Biosystems). Using appropriate cDNA dilution for consistent loading, the Real-Time PCR protocol was followed to perform 40 cycles of amplification. 18S rRNA primers were used to calibrate for any inconsistencies in cDNA input, as a reference control outgroup. The amount of target, normalized to the 18S endogenous reference and relative to a calibrator (control) is calculated using the arithmetic formula: $2^{-\Delta\Delta CT}$ in which the threshold cycle (CT) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. ΔCT represents the difference between target's threshold cycle number and control's and ΔΔCT represents the difference between ΔCT and threshold cycle number of the18S endogenous reference.

Chromatin Immunoprecipitation

Cells were grown in 20 cm plates (Falcon) or in two 10 cm plates per sample, treated accordingly and then crosslinked by adding formaldehyde 37% (BDH) dropwise to a concentration of 1% at room temperature for 20 minutes and the reaction was stopped by 200 mM glycine. Cells were then washed twice and collected in ice-cold PBS. Cells were washed once with 1 mL buffer A (100 Mm Tris-HCl pH 9.4, 10 Mm DTT), once with 1 mL buffer B (10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES PH 6.5, 0.25% Triton X-100), once with 1 mL buffer C (10 Mm EDTA, 0.5 mM EGTA, 10 mM HEPES PH 6.5, 200 mM NaCl) and lysed in 300 μL buffer D (10 mM EDTA, 50 mM Tris-HCl pH 8, 1% SDS) supplemented with protease inhibitor cocktail (Calbiochem). Lysate was then sonicated using Bioruptor system (Diagenode) for 10 minutes at 30 seconds on/off intervals and 'High' setting to achieve shearing chromatin to fragments in

the range of 300-1000 bp in length, and then centrifuged at 13,000 rpm for 10 min at 4°C. Supernatants were diluted 7 folds in ChIP dilution buffer (20 mM Tris-HCl pH 8.0, 1% Triron X-100, 2 mM EDTA, 150 mM NaCl) and supplemented with protease inhibitors, PMSF and DTT, and incubated overnight at 4°C with 1 μg of primary antibodies (anti-p300 N-15 or anti-Ac K9/14 Histone 3). 30 μL of Salmon Sperm DNA/Protein A agarose (Upstate-Milipore) were then added for 2 h at 4°C, washed for 10 minutes in low salt wash buffer (20 mM Tris-HCl pH 8.0, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), washed once for 10 minutes in high salt wash buffer (20 mM Tris-HCl pH 8.0, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl), washed for 10 minutes in LiCl wash buffer (20 mM Tris-HCl pH 8.0, 0.25 M LiCl, 1% NP-40, 1% dioxycholate, 1 mM EDTA) and washed twice for 10 minutes in TE buffer (10 mM Tris-HCL pH 8.0, 1 mM EDTA). Immunocomplexes were eluted off the beads in 300 μL of elution buffer (1% SDS, 0.1 M $NaHCO_3$) and crosslinks were reversed by incubating eluates with 5 μL of proteinase K (Rouche) in 65°C overnight. Eluates were then purified using QIAquick PCR Purification Kit (QIAGEN) and manufacturer's protocol was followed. Polymerase chain reaction was then performed to amplify promoter sequences with pre-designed primers (please refer to supplementary table #3 for primer sequences) spanning regions of p21, E2F1, Egr1 and p57 promoters to which p300 binds to (Smith *et al*, 2004). ChIP on HeLa and SiHa were performed independently for three times each using anti-Ac H3, independently for two times each using anti-p300, and together side by side once using anti-p300. However, all PCR reactions of HeLa and SiHa were performed together in the same reaction. Equal volumes of purified DNA were used for PCR reaction performed with GoTaq® flexi DNA polymerase PCR Kit (Promega), with

the following PCR reaction components (1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM forward and reverse primers, 10 μL of purified DNA and 1 unit of Taq DNA polymerase per reaction in a total volume of 25 μL) for 30 cycles. PCR products were visualized by electrophoresis in 2% agarose gel (Rouche) in TAE stained with ethidium bromide (0.001%) and scanned on AlphaImager™ (Alpha Innotech).

Statistical analysis

Results are presented as means ± standard deviation. Statistical comparisons between two groups were performed using the unequal variance (two tailed) Student's *t*-test. *P* values less than 0.05 or 0.001 were considered to be statistically significant.

Results

Effects of HDACi on the expression of p300-regulated genes in HeLa.

HDAC inhibitors affect the expression of many genes which are involved in cell cycle regulation and ultimately induce growth arrest to cancer cells (Takai *et al*, 2004). However, the mechanisms of their action in the context of the p300 functions are not yet fully understood. To understand roles of HDAC inhibitors in p300-mediated cell cycle regulation we employed HeLa cells, originated from cervical carcinoma (Masters, 2002), which express wild type p300 (Ohshima *et al*, 2001). HeLa had been utilized for numerous studies on cell cycle regulation (Stauffer *et al*, 2007; Wang *et al*, 2008). It had also been utilized for a number of important studies on p300, such as in studying roles of p300 in chromatin assembly and as a tumor suppressor (Asahara *et al*, 2002; Suganuma *et al*, 2002 respectively). In fact, the first study which elucidated that p300 possesses histone acetyltransferase activity, which is arguably the most crucial function of p300, was discovered using an E1A pull-down assay from HeLa nuclear extract (Ogryzko *et al*, 1996). Thus, it is a very useful cell line in p300 and cell cycle regulation studies.

In order to elucidate effects of HDAC inhibitors on the expression of genes that are involved in cell cycle regulation and transcriptionally regulated by p300, we examined the expression of several genes, namely, cyclin-dependent kinase inhibitor 1A (p21), early growth response 1 (Egr1), cyclin-dependent kinase inhibitor 1C (p57), E2F transcription factor 1 (E2F1). To assess the effects of HDAC inhibitors on the expression of those p300-regulated genes and to learn some indications on whether the magnitude of the effects brought by HDAC inhibitors are dose-dependent, two different concentrations

of sodium butyrate and valproic acid (2 and 5 mM) treatments were applied on asynchronized HeLa to measure their effects on the protein expression levels (Figure 1A and 1B) as well as on the mRNA expression levels (Figure 1C). The results suggest that the magnitude of the effects brought by HDAC inhibitors are dose-independent, as the differences between the effects seen between 2 and 5 mM are statistically insignificant, except for Egr1 in response to VPA treatment. However, including more time points and the use of an array of increasing concentrations of the HDAC inhibitors are necessary to further validate the dose-independent effect and further pharmacokinetic analysis is necessary. Next, to assess the effects of HDAC inhibitors on the protein expression of those p300-regulated genes, we treated asynchronized HeLa with 5 mM of NaB, 5 mM of VPA and 200 nM of TSA for 4, 8 and 16 hours. This was done to assess the short and long-term effects of HDAC inhibitors. Through Western blot analysis (Figure 2A and 2B), it was observed that the three HDAC inhibitors upregulated the protein expression of p21 in time-dependent manner. As shown by the Western blot analysis, NaB treatment produced the most prominent effect. The three HDAC inhibitors upregulated the protein expression of Egr1 in time-dependent manner. However, VPA treatment produced the most prominent effect. Even though NaB treatment upregulated the protein expression of p57, VPA and TSA did not have any significant effects. This suggests that the treatment effects on gene expression may be different among the various HDAC inhibitors, perhaps due to different specificities between the different inhibitors. Last of all, the three HDAC inhibitors had no significant effects on the protein expression of E2F1.

Next, to determine whether the changes on protein expression levels by HDAC inhibitors come from protein modifications that either stabilize or degrade the proteins or

from changes brought by HDAC inhibitors at transcriptional level which translate into changes at the protein level, we looked at the effects of HDAC inhibitors at mRNA transcript levels. Quantitative Real Time PCR was performed on asynchronized HeLa cells, in which they were treated with sodium butyrate (5 mM), valproic acid (5 mM for all but 2 mM for E2F1) and trichostatin A (200 nM) HDAC inhibitors for 4 and 16 hours to measure short and long-term effect of the inhibitors on transcript levels of the p300-regulated genes. Total mRNA was isolated, reverse transcribed, and cDNA was generated. Equal amounts of cDNA in addition to specific Real-Time PCR primers were used to amplify and quantify the relative expression levels of the four genes in the study. As shown (Figure 3A, 3B, 3C and 3D), changes in mRNA levels show resemblance and similar patterns as of the protein levels (Figure 2A and 2B). The HDAC inhibitors up-regulated p21 (Figure 3A) and Egr1 (Figure 3B). NaB and TSA upregulated mRNA expression of p57 (Figure 3C) with VPA showing no effect, consistent with Western blot data (Figure 2A and 2B), and did not have significant effects on E2F1 mRNA expression (Figure 3D).

Together, these results indicate that HDAC inhibitors upregulate the expression of the cyclin dependent kinase inhibitors p21 and p57, which are correlated to cell cycle arrest and are known to initiate a number of sequential events leading to cell cycle arrest. They upregulated the expression of Egr1, which transactivates the histone acetyltransferase activity of p300 in response to the disruption of HDAC/HAT balance by the HDAC inhibitors. They also have no significant effect on E2F1, which regulates cell cycle progression from G1 to S, and is unaffected by HDAC inhibitors, at least at the gene expression level. Additionally, HDAC inhibitors affect gene expression of p300-

regaulated genes by affecting mRNA transcription, in which they translate into changes at the protein level.

Figure 1. Effects of two different concentrations of HDACi on both protein and mRNA expression of p300-regulated genes in HeLa.

(A) Protein expression of p300-regulated genes as measured by Western blot analysis in asynchronized HeLa in control (lane 1) and treated with sodium butyrate (2 and 5 mM) and valproic acid (2 and 5 mM) harvested at 4, 8 and 16 hours after treatment. Western blot analysis was performed with a series of antibodies against cyclin-dependent kinase inhibitor 1A (p21), early growth response 1 (Egr1), cyclin-dependent kinase inhibitor 1C (p57) and E2F transcription factor 1 (E2F1). β-actin was used as an internal loading control **(B)** Quantification of Western blot results. Values represent fold difference in reference to control (untreated cells). The amount of protein loaded on SDS-PAGE gel was normalized by comparison with levels of β-actin. Quantification was performed using Scion Image computer software and represent mean ± standard deviation (error bar) of three or more independent experiments. **(C)** mRNA expression of p21, Egr1 and p57 as measured by quantitative Real-Time PCR in HeLa following 4 and 16 hours treatments with valproic acid (2 and 5 mM). Statistical significance of differences as compared with the lower concentration of the treatments (2 mM) were denoted by * or ** to indicate $p < 0.05$ or $p < 0.001$ respectively.

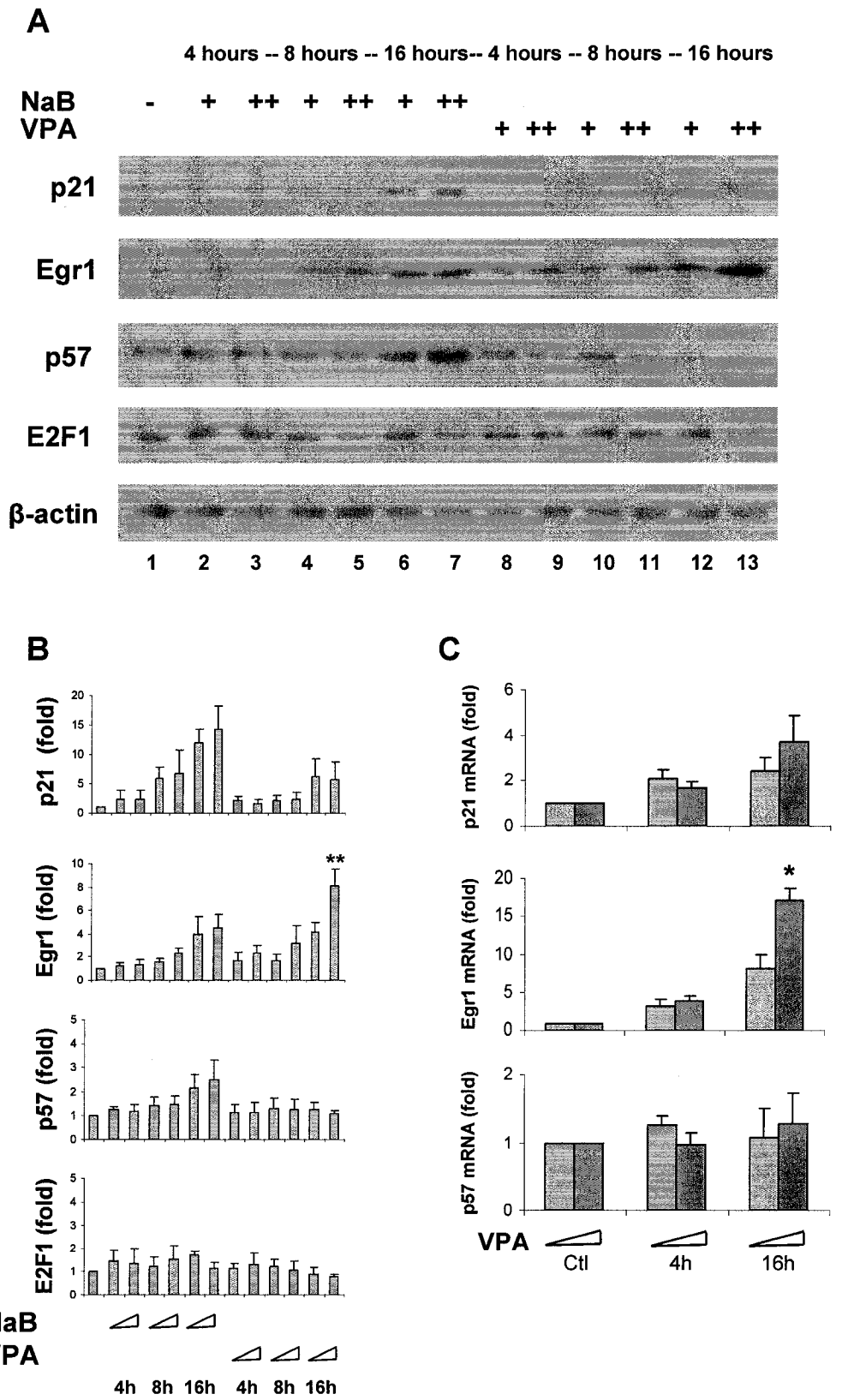

A
4 hours -- 8 hours -- 16 hours-- 4 hours -- 8 hours -- 16 hours
NaB
VPA
p21
Egr1
p57
E2F1
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13
B
p21 (fold)
Egr1 (fold)
p57 (fold)
E2F1 (fold)
**
NaB
VPA
4h 8h 16h 4h 8h 16h
C
p21 mRNA (fold)
Egr1 mRNA (fold)
p57 mRNA (fold)
*
VPA
Ctl
4h
16h

Figure 1.

Figure 2. Effects of HDACi on the protein expression of p300-regulated genes in HeLa.

(A) Protein expression of p300-regulated genes as measured by Western blot analysis in asynchronized HeLa in control (lane 1) and treated with sodium butyrate (5 mM), valproic acid (5 mM) and trichostatin A (200 nM) harvested at 4, 8 and 16 hours after treatment. Western blot analysis was performed with a series of antibodies against cyclin-dependent kinase inhibitor 1A (p21), early growth response 1 (Egr1), cyclin-dependent kinase inhibitor 1C (p57) and E2F transcription factor 1 (E2F1). β-actin was used as an internal loading control **(B)** Quantification of Western blot results. Values represent fold difference in reference to control (untreated cells). The amount of protein loaded on SDS-PAGE gel was normalized by comparison with levels of β-actin. Quantifications were performed using Scion Image computer software and represent mean ± standard deviation (error bar) of three or more independent experiments. Statistical significance of differences as compared with untreated controls were denoted by * or ** to indicate $p < 0.05$ or $p < 0.001$ respectively.

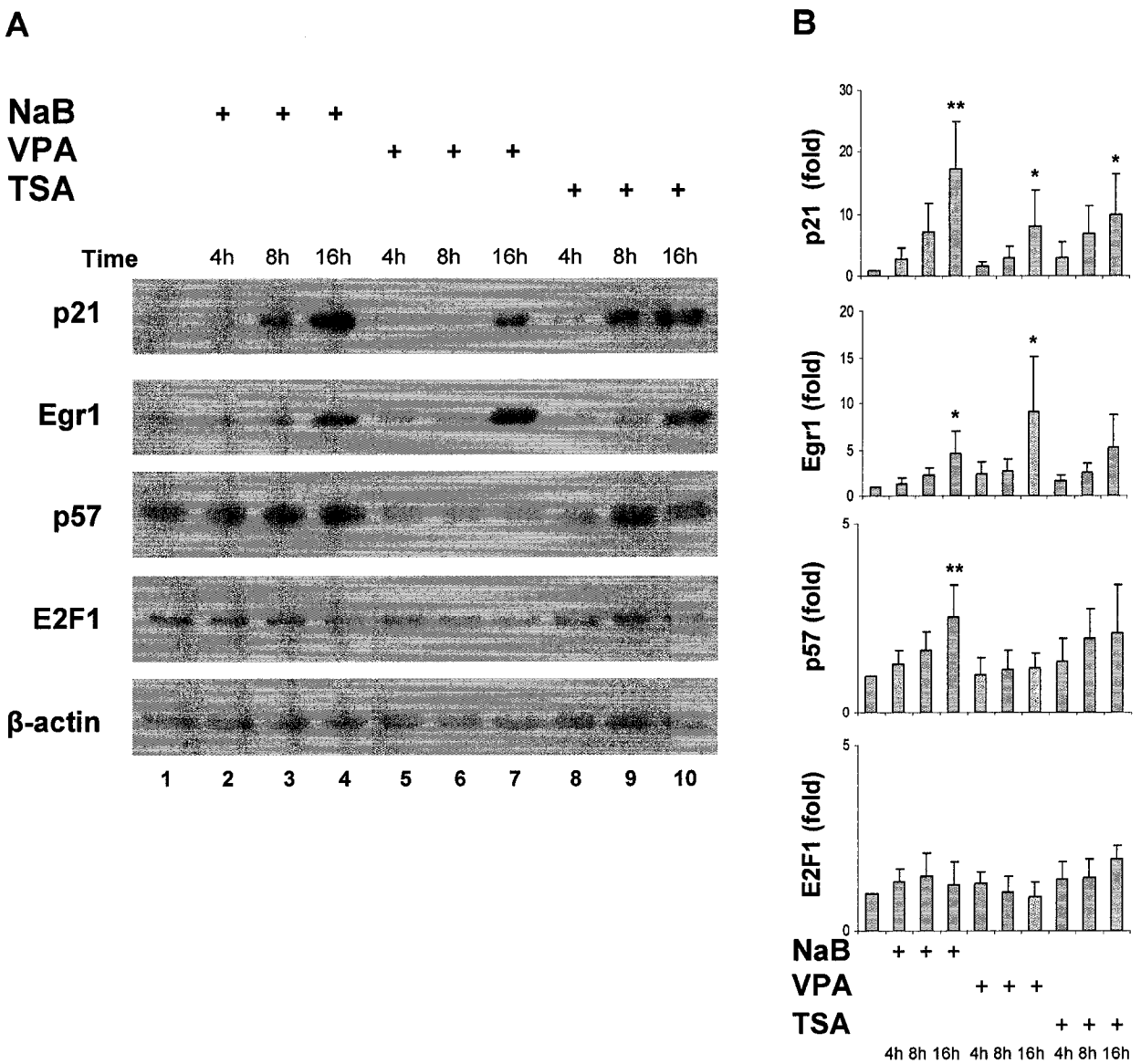

A
NaB + + +
VPA + + +
TSA + + +
Time 4h 8h 16h 4h 8h 16h 4h 8h 16h
p21
Egr1
p57
E2F1
β-actin
1 2 3 4 5 6 7 8 9 10
B
p21 (fold)
Egr1 (fold)
p57 (fold)
E2F1 (fold)
NaB + + +
VPA + + +
TSA + + +
4h 8h 16h 4h 8h 16h 4h 8h 16h

Figure 2.

Figure 3. Effects of HDACi on the mRNA transcription of p300-regulated genes in HeLa.

Total RNA was extracted from HeLa following 4 and 16 hours treatments with butyrate (5 mM), valproic acid (5 mM for all but 2mM for E2F1) and TSA (200 nM), and reverse transcribed. Relative mRNA levels of **(A)** p21, **(B)** Egr1, **(C)** p57 and **(D)** E2F1 were measured by quantitative Real-Time RT-PCR on the reverse transcribed cDNA using Taqman® primers and probes specific to each gene. 18S rRNA was used as an internal control, to which amounts of mRNA in all samples were normalized. Values represent fold difference in reference to control (untreated cells). Each experiment was repeated three times in duplicates. Error bars represent the standard deviation of at least three independent experiments. Statistical significance of differences as compared with untreated controls were denoted by * or ** to indicate $p < 0.05$ or $p < 0.001$ respectively.

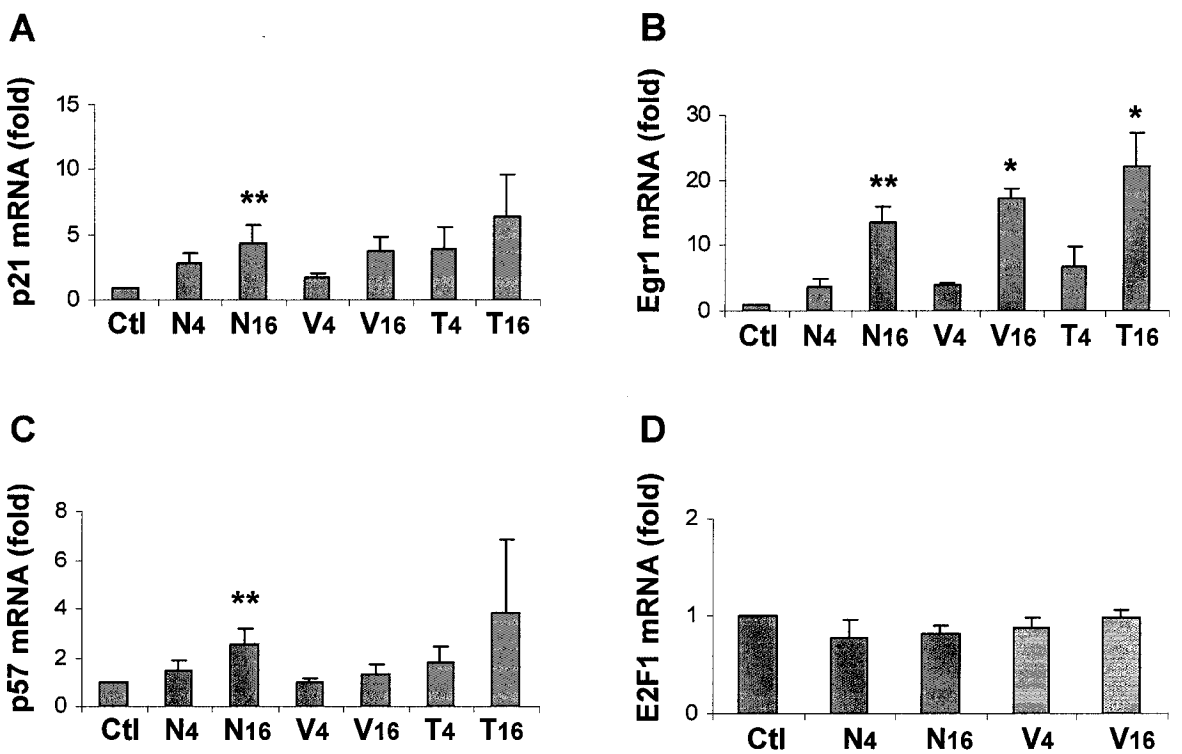

A
p21 mRNA (fold)
15
10
5
0
**
Ctl
N4
N16
V4
V16
T4
T16
B
Egr1 mRNA (fold)
30
20
10
0
**
*
*
Ctl
N4
N16
V4
V16
T4
T16
C
p57 mRNA (fold)
8
6
4
2
0
**
Ctl
N4
N16
V4
V16
T4
T16
D
E2F1 mRNA (fold)
2
1
0
Ctl
N4
N16
V4
V16

Figure 3.

Protein profiles of p300-regulated genes in synchronized HeLa.

There has been controversy over which phase of the cell cycle is affected by HDAC inhibitors (Noh and Lee, 2003). Additionally, it is not clear whether the differences between the effects brought by the three distinct HDAC inhibitors utilized in this study come from different specificities between the three inhibitors towards different phases. These facts prompted us to investigate the protein expression profiles of p300-regulated genes during different phases of the cell cycle in control and in response to HDAC inhibitors. After carefully studying the effects of HDAC inhibitors in asynchronized HeLa cells, we examined the protein expression profile of p21, Egr1, p57 and E2F1 in synchronized HeLa cells in control and in response to HDAC inhibitors. Cells were either synchronized by a double thymidine block in order to enrich for cells in late G1, just prior to S phase entry (Fig 4), or blocked with thymidine for 24h then nocodazole for 16h in order to enrich for cells in M phase (Fig 5). Treating cells with excessive amounts of thymidine blocks DNA replication and results in reversible cell cycle synchronization in late G1, just prior to S phase entry, which could be reversed by releasing the cells in untreated DMEM medium (Studzinski and Lambert, 1969). Treating cells with nocodazole, a microtubule depolymerising agent, would arrest them in M phase at the metaphase stage, as the spindle apparatus fail to assemble as the main microtubule components fail to polymerize (de Brabander *et al*, 1976). Synchronized HeLa cells were treated with HDAC inhibitors and released back into the cell cycle (Fig 4A, 5A). The typical timeline scheme of cell cycle progression into different phases in HeLa is well established (Whitfield *et al*, 2002), and the time frame examined at each synchronization protocol used in this study were represented in (Fig 4C, 5C). The M and late G1 time

points were chosen in this study as they represent two different important time points to which we can correlate the effects of the HDAC inhibitors and different phases of cell cycle. The time line used in this study allows for examining cell cycle progression through different phases of the cell cycle (as indicated in Fig 4C, 5C). Additionally, synchronization protocols at these time-points in HeLa are feasible and well established. Flow cytometry analysis of cells stained with propidium iodide was performed to assess the synchronization protocols' efficiency (Fig 4D, 5D).

Effects of HDAC inhibitors on expression of p21 and p57, regulators of cell cycle progression at G1/S were seen at 4 and 8 hours as cells enter into S phase and progress back into G1 (Fig 4A). However, they were not seen in M phase and early G1, which is consistent with the knowledge that p21 and p57 regulates cell cycle at middle and late G1 phase. Egr1 is up-regulated by HDAC inhibitor treatments up to 4 hours of release in S phase, and up-regulated as released in M phase. It also appears to possess a modification at M phase, marked by the appearance of another band with slower migration on the SDS PAGE gel (Fig 5A). Levels of E2F1 protein in control and in HDAC treated cells slightly (but not statistically significant) decrease at 8 hours after release into S phase (Fig 4), which is further shown as cells are released from M phase (Fig 5A). It is interesting to note that the effects of HDAC inhibitors on asynchronized cells are more prominent than synchronized ones, most likely due to the limited toxic side effects or drug-specific cell stress brought by the synchronization agents (reviewed in Davis *et al*, 2001). This is evident by comparing the sub-G1 cell numbers, an indicative of apoptosis, between asynchronized and synchronized cells, in which there is always a larger number of the sub-G1 in the synchronized cells.

Together, there seems to be no major differences in the specificities between the three inhibitors used in this study towards different phases of cell cycle. However, the magnitude of effects between the three inhibitors on the expression of several genes at distinct phases of cell cycle vary in quantitative terms, perhaps due to different specificities between the three inhibitors towards various HDACs as different genes are regulated by distinct HDAC complexes. A more detailed discussion on the different specificities of the distinct HDAC inhibitors was provided in the discussion.

Figure 4. Effects of HDACi on the protein expression of p300-regulated genes in synchronized HeLa in late G1 phase.

(A) HeLa cells were arrested in **late G1 phase** by double thymidine block and protein expression was examined in asynchronized control (lane 1), synchronized control (lane 2) and released cells. Cells were released and harvested at 4 and 8 hours after the release. Extracts prepared from control and treated asynchronized cells (lanes 1, 11-14) were also examined. **(B)** Quantification of Western blot results. The amount of protein was normalized by comparison with levels of β-actin, and represent fold difference in reference to control (untreated cells). The quantification was performed using Scion Image computer software and represent means ± standard deviations (error bars) of three independent experiments. **(C)** Standard timeline scheme of cell cycle phase transitions in asynchronized HeLa. The arrow represents the timeline (0 - 8h) as cells progress from late G1 to G1-re-entry timeframe examined in this experiment. **(D)** Examining the efficiency of synchronization protocol by flow cytometry. All experiments were performed three times with consistency.

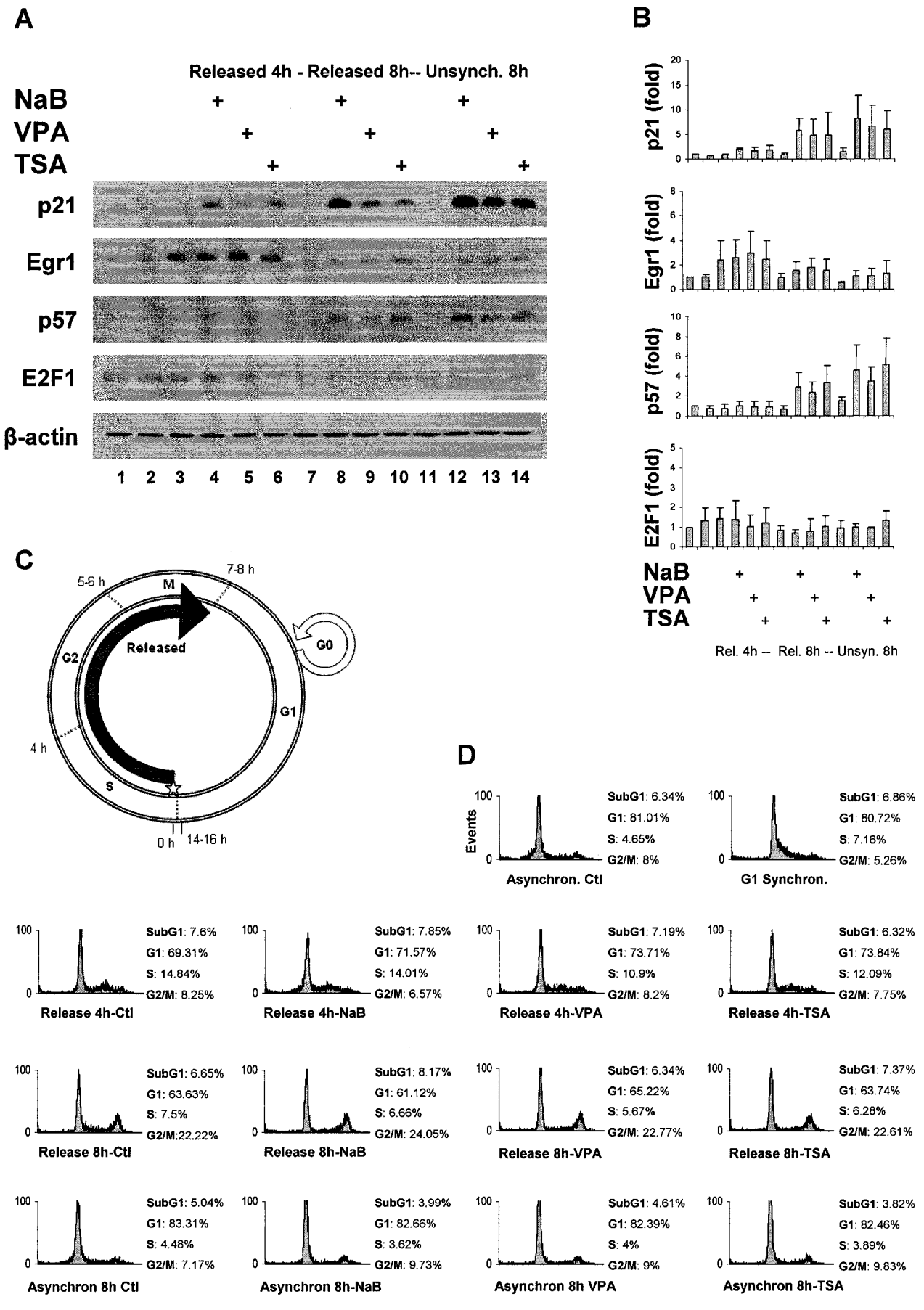

A
Released 4h - Released 8h-- Unsynch. 8h
NaB
VPA
TSA
p21
Egr1
p57
E2F1
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
p21 (fold)
Egr1 (fold)
p57 (fold)
E2F1 (fold)
NaB
VPA
TSA
Rel. 4h -- Rel. 8h -- Unsyn. 8h
C
5-6 h
7-8 h
M
Released
G2
G0
G1
4 h
S
0 h
14-16 h
D
Events
Asynchron. Ctl
SubG1: 6.34%
G1: 81.01%
S: 4.65%
G2/M: 8%
G1 Synchron.
SubG1: 6.86%
G1: 80.72%
S: 7.16%
G2/M: 5.26%
Release 4h-Ctl
SubG1: 7.6%
G1: 69.31%
S: 14.84%
G2/M: 8.25%
Release 4h-NaB
SubG1: 7.85%
G1: 71.57%
S: 14.01%
G2/M: 6.57%
Release 4h-VPA
SubG1: 7.19%
G1: 73.71%
S: 10.9%
G2/M: 8.2%
Release 4h-TSA
SubG1: 6.32%
G1: 73.84%
S: 12.09%
G2/M: 7.75%
Release 8h-Ctl
SubG1: 6.65%
G1: 63.63%
S: 7.5%
G2/M:22.22%
Release 8h-NaB
SubG1: 8.17%
G1: 61.12%
S: 6.66%
G2/M: 24.05%
Release 8h-VPA
SubG1: 6.34%
G1: 65.22%
S: 5.67%
G2/M: 22.77%
Release 8h-TSA
SubG1: 7.37%
G1: 63.74%
S: 6.28%
G2/M: 22.61%
Asynchron 8h Ctl
SubG1: 5.04%
G1: 83.31%
S: 4.48%
G2/M: 7.17%
Asynchron 8h-NaB
SubG1: 3.99%
G1: 82.66%
S: 3.62%
G2/M: 9.73%
Asynchron 8h VPA
SubG1: 4.61%
G1: 82.39%
S: 4%
G2/M: 9%
Asynchron 8h-TSA
SubG1: 3.82%
G1: 82.46%
S: 3.89%
G2/M: 9.83%

Figure 4.

Figure 5. Effects of HDACi on the protein expression of p300-regulated genes in synchronized HeLa in M phase.

(A) HeLa cells were arrested in **M phase** by thymidine-nocodazole block and protein expression was examined in asynchronized control (lane 1), synchronized control (lane 2) and released cells. Cells were released and harvested at 3 and 4 hours after the release. Extracts prepared from control and treated asynchronized cells (lanes 1, 11-14) were also examined **(B)** Quantification of Western blot results. The amount of protein was normalized by comparison with levels of β-actin, and represent fold difference in reference to control (untreated cells). The quantification was performed using Scion Image computer software and represent means ± standard deviations (error bars) of three independent experiments. Statistical significance of differences as compared with asynchronized controls is denoted by * to indicate $p< 0.05$. **(C)** Standard timeline scheme of cell cycle phase transitions in asynchronized HeLa. The arrow represents the timeline (0 - 4h) as cells progress from M to G1 timeframe examined in this experiment. **(D)** Examining the efficiency of synchronization protocol by flow cytometry. All experiments were performed three times with consistency.

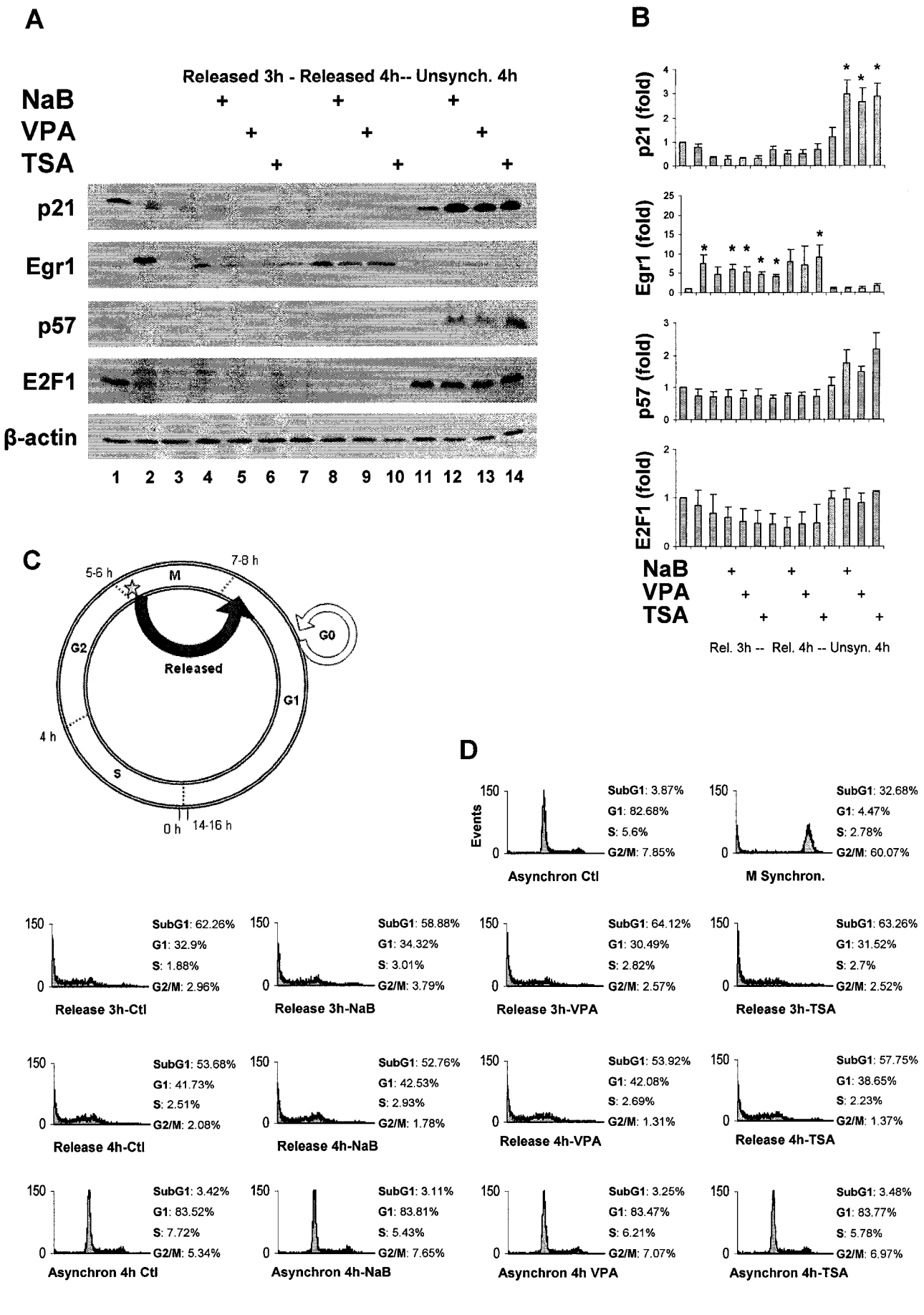

A
Released 3h - Released 4h-- Unsynch. 4h
NaB
VPA
TSA
p21
Egr1
p57
E2F1
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
p21 (fold)
Egr1 (fold)
p57 (fold)
E2F1 (fold)
NaB
VPA
TSA
Rel. 3h -- Rel. 4h -- Unsyn. 4h
C
5-6 h
7-8 h
M
G2
Released
G0
G1
4 h
S
0 h
14-16 h
D
Events
Asynchron Ctl
SubG1: 3.87%
G1: 82.68%
S: 5.6%
G2/M: 7.85%
M Synchron.
SubG1: 32.68%
G1: 4.47%
S: 2.78%
G2/M: 60.07%
Release 3h-Ctl
SubG1: 62.26%
G1: 32.9%
S: 1.88%
G2/M: 2.96%
Release 3h-NaB
SubG1: 58.88%
G1: 34.32%
S: 3.01%
G2/M: 3.79%
Release 3h-VPA
SubG1: 64.12%
G1: 30.49%
S: 2.82%
G2/M: 2.57%
Release 3h-TSA
SubG1: 63.26%
G1: 31.52%
S: 2.7%
G2/M: 2.52%
Release 4h-Ctl
SubG1: 53.68%
G1: 41.73%
S: 2.51%
G2/M: 2.08%
Release 4h-NaB
SubG1: 52.76%
G1: 42.53%
S: 2.93%
G2/M: 1.78%
Release 4h-VPA
SubG1: 53.92%
G1: 42.08%
S: 2.69%
G2/M: 1.31%
Release 4h-TSA
SubG1: 57.75%
G1: 38.65%
S: 2.23%
G2/M: 1.37%
Asynchron 4h Ctl
SubG1: 3.42%
G1: 83.52%
S: 7.72%
G2/M: 5.34%
Asynchron 4h-NaB
SubG1: 3.11%
G1: 83.81%
S: 5.43%
G2/M: 7.65%
Asynchron 4h VPA
SubG1: 3.25%
G1: 83.47%
S: 6.21%
G2/M: 7.07%
Asynchron 4h-TSA
SubG1: 3.48%
G1: 83.77%
S: 5.78%
G2/M: 6.97%

Figure 5.

Basal levels of p300-regulated transcripts are higher in SiHa.

The expression of four p300-regulated genes in response to HDAC inhibitors was examined in HeLa, which expresses wild type p300. Next, we thought it would be very interesting to investigate the expression of the same four genes in another cell line, which expresses a mutant form of p300, in an attempt to understand some of the effects of p300.deregulation. A number of cell lines which express mutant forms of p300 were readily available and economical for us, however one cell line (SiHa) was of a particular interest to be employed in this study. SiHa was established from a surgically removed carcinoma of the uterine cervix. A homozygous p300 deletion of exons 15–18, which results in an in-frame deletion that causes specific loss of the bromodomain was identified in the SiHa cervical carcinoma cell line by Ohshima and colleagues (2001). In fact, a number of studies utilized SiHa to serve as indicators for the effect of bromodomain-deficiency in the function of p300 (Suganuma *et al*, 2002; Ohshima *et al*, 2001). We utilized this cell line to learn more on how the four p300-dependent genes respond to HDAC inhibitors and to gain insights into possible roles of p300 bromodomain and how the cell compensates for the loss of p300 bromodomain in regulating the expression of p300-dependent genes. However, we recognize that a different and more reliable system should be utilized when studying the precise roles of p300 bromodomain, which would ideally consist of $p300^{-/-}$ cell lines transfected with either wild-type or bromodomain truncated p300 expression constructs (Iyer *et al*, 2007).

To examine the basal gene expression profiles of the p300-regulated genes in HeLa and SiHa cell lines, Real-Time PCR as well as Western blot analysis was performed. For Real-Time PCR analysis, mRNA was isolated, reverse transcribed, and

cDNA was generated. Equal amounts of cDNA in addition to specific Real-Time PCR primers were used to amplify and quantify the relative expression levels of the genes in the study between HeLa and SiHa (Figure 6A). These results indicate that basal levels of p21, Egr1 and p57 are significantly higher in SiHa. For Western analysis, same amounts of whole cell lysates from HeLa and SiHa were migrated on SDS-PAGE, and probed with primary antibodies corresponding to our genes of interest. Western blots (Figure 6B and 6C) show that only basal levels of p21 are significantly higher in SiHa than in HeLa. Thus, the basal levels of some p300-regulated gene expression appear to be higher in SiHa than HeLa at the mRNA transcriptional level.

Figure 6. Relative levels of gene expression between HeLa and SiHa.

(A) Quantitative Real-Time PCR analysis of endogenous p300, p21, Egr1, p57 and E2F1 mRNA in HeLa and SiHa. 18S rRNA was used as an internal control, to which amounts of mRNA in all samples were normalized. Values represent fold difference in reference to HeLa **(B)** Western blot analysis of the five genes in HeLa and SiHa. Same amounts of protein from control (untreated) HeLa and SiHa whole cell extract were loaded on SDS-PAGE and probed with proper antibodies accordingly. Anti-β-actin was used for loading control **(C)** Quantification of Western blot analysis of HeLa and SiHa proteins using Scion Image computer software. The amount of protein was normalized by comparison with levels of β-Actin, and represents fold difference in reference to HeLa. Error bars represent the standard deviation of three or more independent experiments. Statistical significance of differences as compared with HeLa is denoted by * to indicate $p < 0.05$.

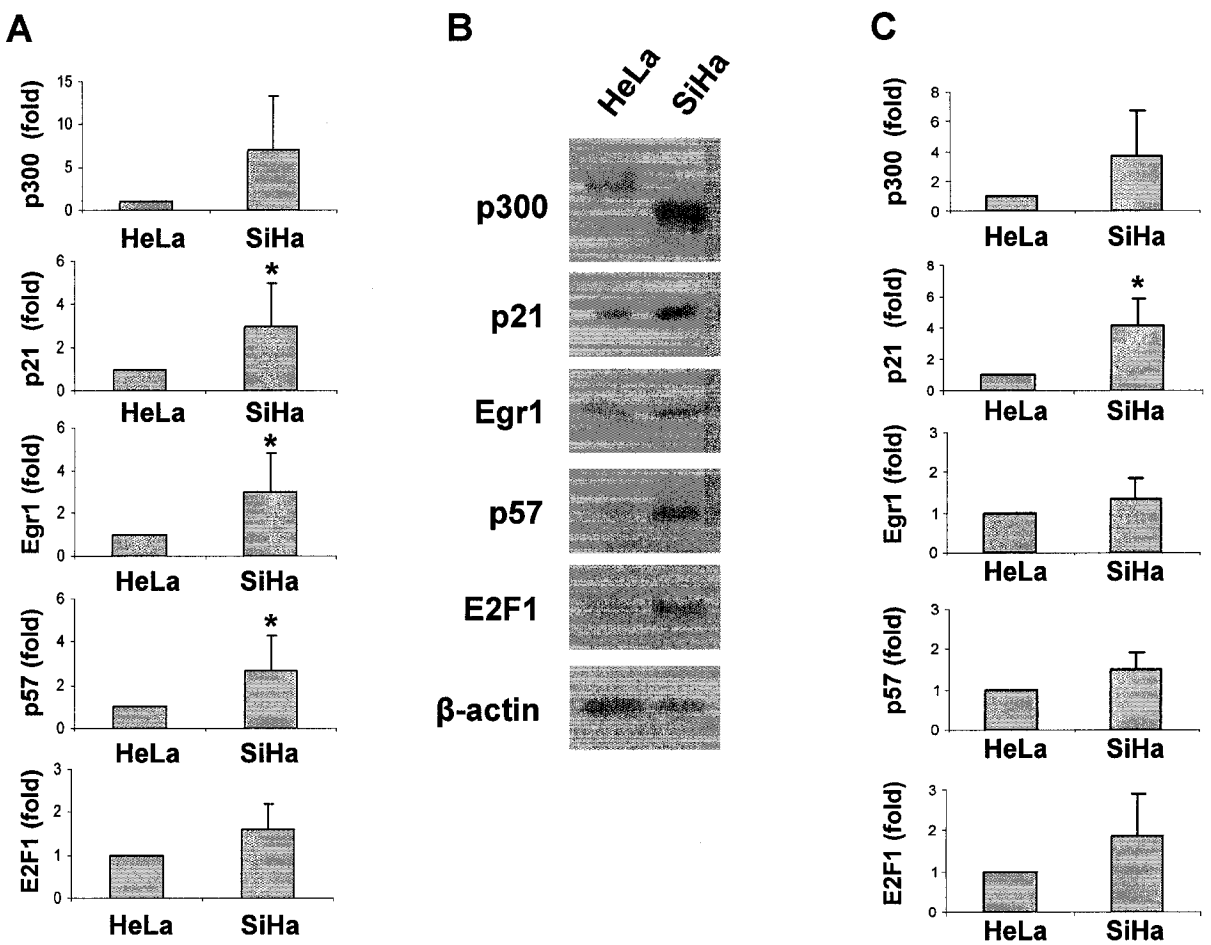

A
p300 (fold)
HeLa
SiHa
p21 (fold)
Egr1 (fold)
p57 (fold)
E2F1 (fold)
B
HeLa
SiHa
p300
p21
Egr1
p57
E2F1
β-actin
C
p300 (fold)
p21 (fold)
Egr1 (fold)
p57 (fold)
E2F1 (fold)

Figure 6.

Effects of HDACi on the expression of p300-regulated genes in SiHa.

Next, to examine roles of p300 bromodomain in the function of p300, we assessed the effects of histone hyperacetylation induced by HDAC inhibitors on the protein expression of the p300-regulated genes in asynchronized SiHa. Cells were treated with 5 mM of NaB, 5 mM of VPA and 200 nM of TSA for 4, 8 and 16 hours, to assess for the short and long-term effects of HDAC inhibitors. Through Western blot analysis (Figure 7A and 7B), it was observed that butyrate upregulated the protein expression of p21. However, the fold induction of ~4 fold was much less than ~15 folds in HeLa (Figure 2A and 2B). Similarly, HDAC inhibitors upregulated the protein expression of Egr1 less than ~2 fold induction, as compared to ~10 folds seen in HeLa. An interesting observation was that HDAC inhibitors were associated with a downregulation of p57 protein expression in SiHa, in contrast to the upregulation observed in HeLa. In fact, NaB had no significant effect on p57 in SiHa, in contrast to upregulation in HeLa. VPA and TSA had downregulated expression of p57 as opposed to no changes seen in HeLa. Lastly, HDAC inhibitors had no significant effects on E2F1 in SiHa, which was also observed in HeLa.

To measure the effects of HDAC inhibitors on mRNA transcript levels, Quantitative RT-PCR was performed on asynchronized SiHa cells. They were in fact treated with sodium butyrate (5 mM), valproic acid (5 mM for all but 2 mM for E2F1) and trichostatin A (200 nM) for 4 and 16 hours to measure short and long-term effects of the treatments on transcript levels of the p300-regulated genes. It came to no surprise that changes in mRNA levels show resemblance and similar patterns as of the protein levels which indicates that changes on protein levels originate from changes that occurred at transcript levels. The HDAC inhibitors slightly up-regulated p21 (Figure 8A) as well as

Egr1 (Figure 8B), downregulated mRNA expression of p57 (Figure 8C) and did not have a significant effect on E2F1 mRNA expression (Figure 8D). To conclude, these results suggest that HDAC inhibitors fail to significantly upregulate the expression of cyclin dependent kinase inhibitor p21. They also downregulate the expression of cyclin dependent kinase inhibitor p57 and fail to significantly upregulate the expression of Egr1. Additionally, HDAC inhibitors affect gene expression of p300-regulated genes by affecting mRNA transcription, in which they translate into changes at the protein level.

Figure 7. HDACi have less prominent effects on the protein expression of p300-regulated genes in SiHa.

(A) Protein expression of p300-regulated genes as measured by Western blot analysis in asynchronized SiHa in control (lane 1) and treated with sodium butyrate (5 mM), valproic acid (5 mM), and trichostatin A (200 nM) harvested at 4, 8 and 16 hours after treatment. Western blot analysis was performed with a series of antibodies against p21, Egr1, p57 and E2F1. β-actin was used as an internal loading control **(B)** Quantification of Western blot results. Values represent fold difference in reference to control (untreated cells). The amount of protein loaded on SDS-PAGE gel was normalized by comparison with levels of β-actin. Quantification was performed using Scion Image computer software and represent means ± standard deviations (error bars) of three or more independent experiments. Statistical significance of differences as compared with untreated controls is denoted by * or ** to indicate $p < 0.05$ or $p < 0.001$ respectively.

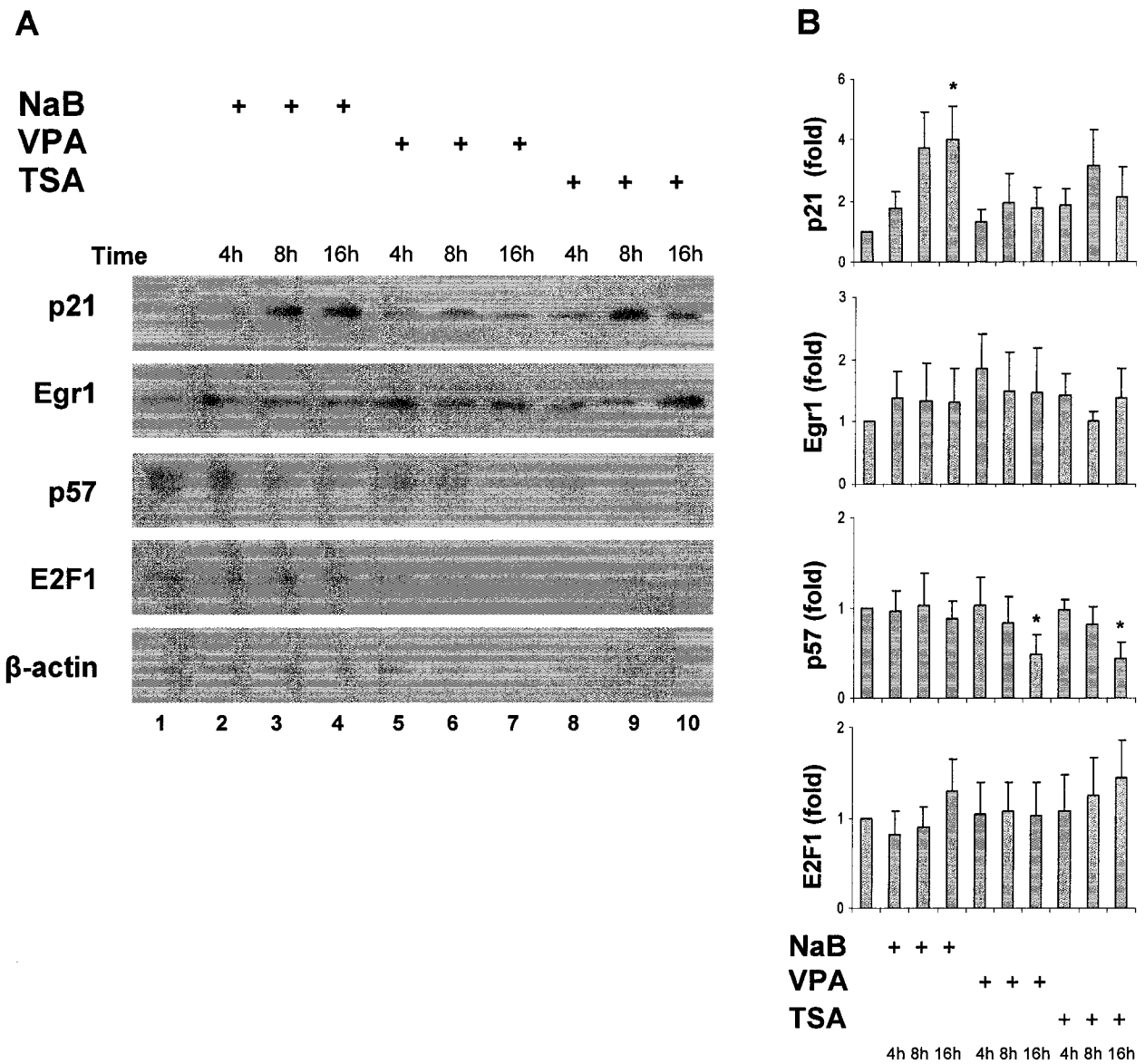

A
NaB + + +
VPA + + +
TSA + + +
Time 4h 8h 16h 4h 8h 16h 4h 8h 16h
p21
Egr1
p57
E2F1
β-actin
1 2 3 4 5 6 7 8 9 10
B
p21 (fold)
Egr1 (fold)
p57 (fold)
E2F1 (fold)
NaB + + +
VPA + + +
TSA + + +
4h 8h 16h 4h 8h 16h 4h 8h 16h

Figure 7.

Figure 8. Effects of HDACi on the mRNA transcription of p300-regulated genes in SiHa.

Total RNA was extracted from SiHa following 4 and 16 hours treatments with butyrate (5 mM), valproic acid (5 mM for all but 2mM for E2F1) and TSA (200 nM), and reverse transcribed. Relative mRNA levels of **(A)** p21, **(B)** Egr1, **(C)** p57 and **(D)** E2F1 were measured by quantitative Real-Time RT-PCR on the reverse transcribed cDNA using Taqman® primers and probes specific to each gene. 18S rRNA was used as an internal control, to which amounts of mRNA in all samples were normalized. Values represent fold difference in reference to control (untreated cells). Each experiment was repeated three times in duplicates. Error bars represent the standard deviation of three or more independent experiments. Statistical significance of differences as compared with untreated controls is denoted by * or ** to indicate $p < 0.05$ or $p < 0.001$ respectively.

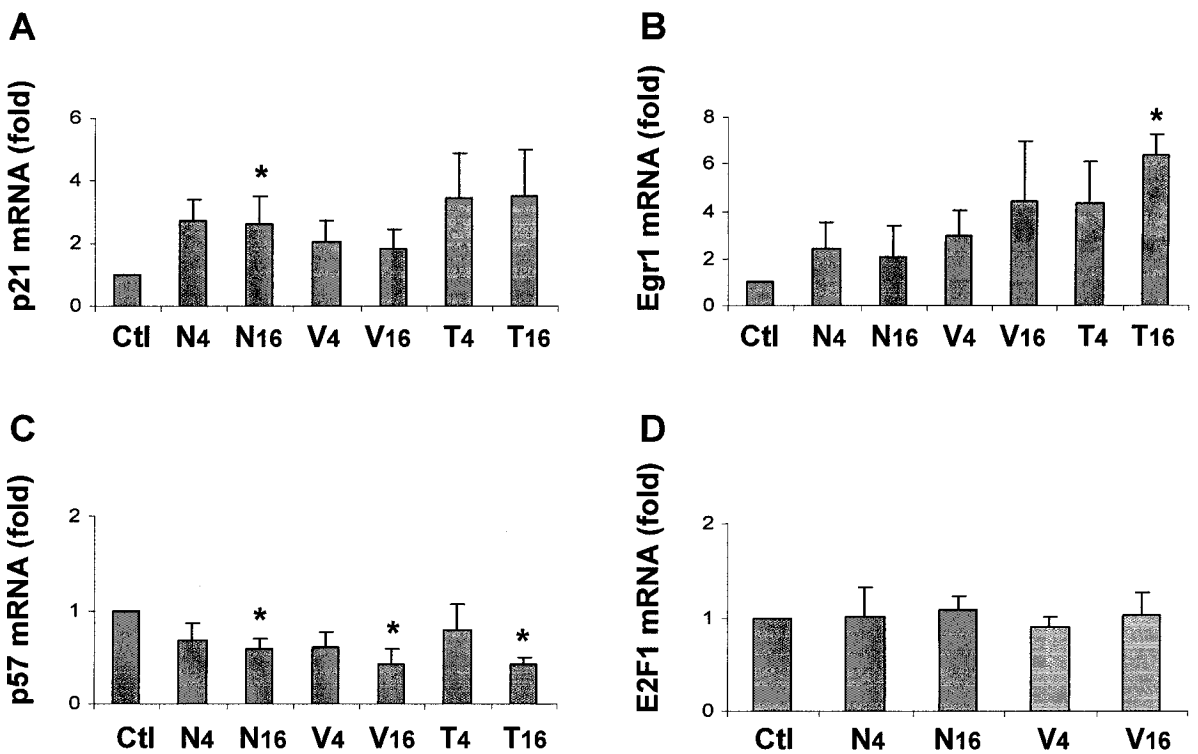

A
p21 mRNA (fold)
0
2
4
6
*
Ctl
N4
N16
V4
V16
T4
T16
B
Egr1 mRNA (fold)
0
2
4
6
8
*
Ctl
N4
N16
V4
V16
T4
T16
C
p57 mRNA (fold)
0
1
2
*
*
*
Ctl
N4
N16
V4
V16
T4
T16
D
E2F1 mRNA (fold)
0
1
2
Ctl
N4
N16
V4
V16

Figure 8.

HDACi induce the acetylation of histones H3 (Lys 9/14).

After measuring the effects of NaB, VPA and TSA on the expression of the four genes in SiHa, we found that the effects of HDAC inhibitors are less prominent. We next compared the acetylation levels of histone 3 of all four gene promoters in response to the HDAC inhibitors to the basal acetylation levels of histone 3 in control, in an attempt to investigate for the possibility that the difference in responses seen in SiHa is due to less acetylation, which would leave promoters with less accessibility for transcription. First, to investigate the global acetylation levels between the two cell lines, we treated asynchronized HeLa and SiHa with 5 mM of NaB, 5 mM of VPA and 200 nM of TSA for 4 hours. Through Western blot analysis using antibody against acetylated lysines 9 and 14 on histone 3 and comparing H3Ac between control (basal H3Ac) and in response to treatments (induced H3Ac), it is apparent that HDAC inhibitors increase global acetylation levels of histone 3, in both HeLa and SiHa (Figure 9A and 9C) to ≈2 fold in HeLa and ≈2.5 fold in SiHa. It was also apparent that the basal levels of H3Ac are comparable between HeLa and SiHa (Figure 9E).

Next, we examined the histone hyperacetylation levels on the promoters of p21, Egr1, p57 and E2F1. Following 4h of treatment with 5 mM of NaB, 5 mM of VPA and 200 nM of TSA, Chromatin immunoprecipitation (ChIP) assay was performed. It was performed using the same antibody against acetylated lysines 9 and 14 on histone 3 which was used in Western, to pull down acetylated promoters. Promoter regions to which p300 binds to, and which the PCR primers covered were pre-designed by Smith and colleagues (2004). As shown (Figure 10), there is a remarkable increase in H3 acetylation in the promoters of the four genes, in both cell lines. However, in contrast to

HeLa in which the basal H3 acetylation level is almost undetectable, there is a high basal acetylation level SiHa. HDAC inhibitors also induced the acetylation levels of H3 histones which are associated with p300-regulated promoters in SiHa by comparable levels to HeLa. The results obtained were preliminary as more repeats and additional experiments are required before any conclusions can be made. However, our results suggest that histone H3 becomes hyperacetylated in response to HDAC inhibitors. Our results also indicate that basal acetylation levels of histone 3 are higher in SiHa than HeLa. This suggests higher transcription levels in SiHa since H3Ac is a marker of transcriptional upregulation. This could explain the lower fold induction by HDAC inhibitors since the basal transcription level for the inducible p300-regulated genes is higher in SiHa (also indicated in Figure 6A). A plausible explanation for the acetylation observed in SiHa in spite of the bromodomain-deficiency of p300 is through functional redundancy with other HATs, which will be discussed in more depth in the discussion section.

Figure 9. Global histone H3 (Lys 9/14) acetylation in HeLa and SiHa in control and in response to HDACi by Western blotting.

(A) The global acetylation levels of histone 3 in HeLa in control and in response to HDAC inhibitors are assessed through Western blot analysis. Following 4 hours treatments with butyrate (5 mM), valproic acid (5 mM) and TSA (200 nM), Western blot analysis was performed using antibody against acetylated lysine 9/14 resides of histone 3. Bars represent fold induction as compared to basal levels of acetylated lysine 9/14 on H3 (lane 1). β-actin was used as an internal loading control. **(B)** Quantification of Western blot results. The amount of protein loaded on 12% SDS-PAGE gel was normalized by comparison with levels of β-actin. Quantification was performed using Scion Image computer software. Values represent fold difference in reference to control (untreated cells) and represent means ± standard deviations (error bars) of three independent experiments. **(C-D)** Experimental setup as in panels (A-B) however performed on SiHa cell line. **(E)** Comparison of basal endogenous acetylation levels of histone 3 proteins between HeLa and SiHa. Statistical significance of differences as compared with untreated controls in panels B, D and E are denoted by * or ** to indicate $p < 0.05$ or $p < 0.001$ respectively.

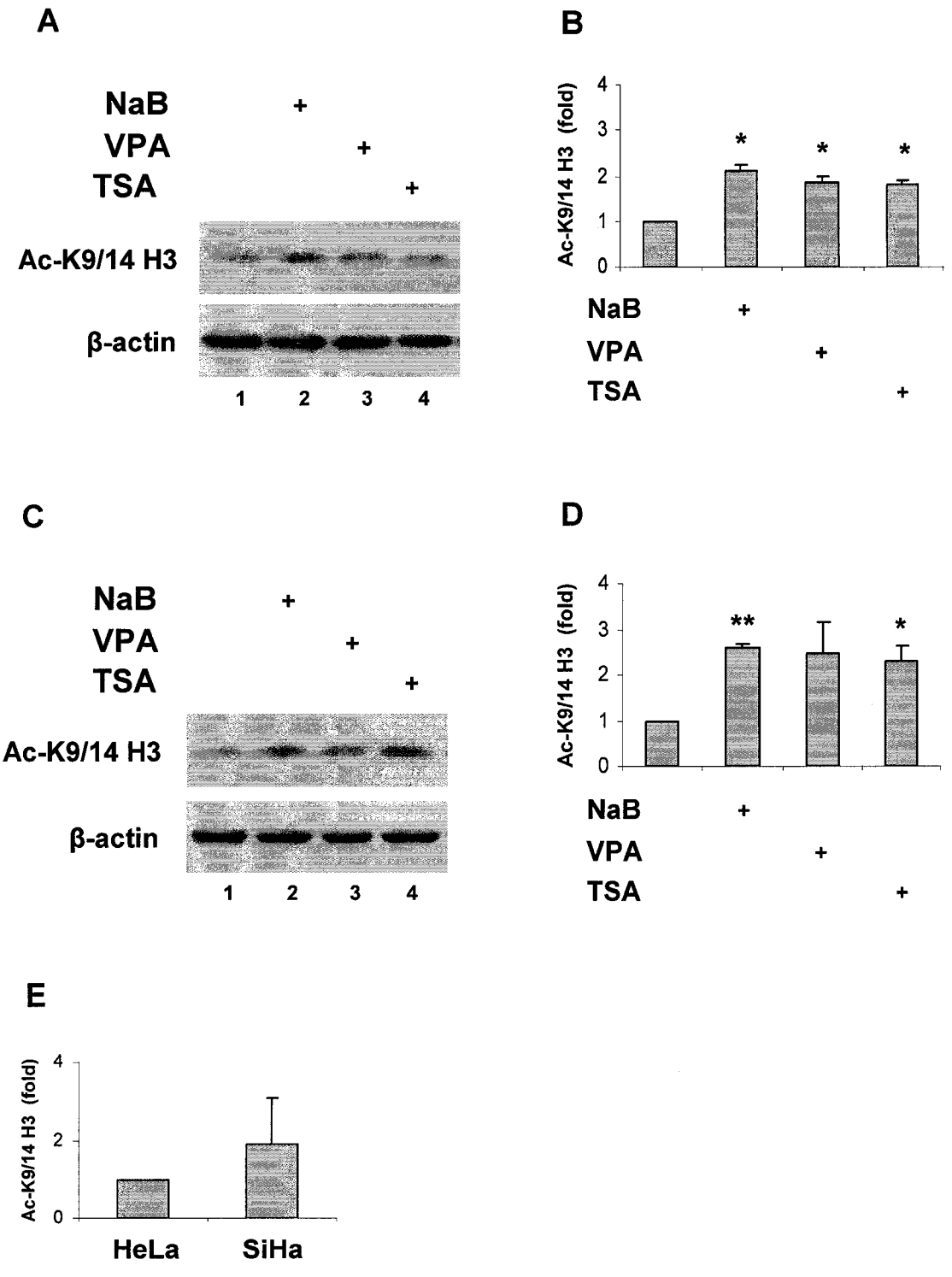

A
NaB
VPA
TSA
Ac-K9/14 H3
β-actin
1 2 3 4
B
Ac-K9/14 H3 (fold)
0 1 2 3 4
* * *
NaB
VPA
TSA
C
NaB
VPA
TSA
Ac-K9/14 H3
β-actin
1 2 3 4
D
Ac-K9/14 H3 (fold)
0 1 2 3 4
** *
NaB
VPA
TSA
E
Ac-K9/14 H3 (fold)
0 2 4
HeLa
SiHa

Figure 9.

Figure 10. HDACi induce acetylation of histones H3 (Lys 9/14) which are associated with promoters of p300-regulated genes in both HeLa and SiHa.

The acetylation statues of histone 3 associated with promoters of the p300-dependent genes: p21, Egr1, p57 and E2F1 in asynchronized HeLa and SiHa were assessed by chromatin immunoprecipitation (ChIP). Following treatment for 4 hours with 5 mM butyrate, 5 mM valproic acid, or 200 nM trichostatin A HDAC inhibitors, ChIP was performed in which equal amounts of DNA from both cell lines were immunoprecipitated. Primers mapped the following regions on the gene promoters: p21 (from -308 to -205), Egr1 (from -483 to -345), p57 (from +49 to +196) and E2F1 (from -580 to -448) in reference to transcription start site. Antibody used in this assay was anti-Ac-Histone 3 which recognizes acetylated lysine 9 and 14 on histone 3.

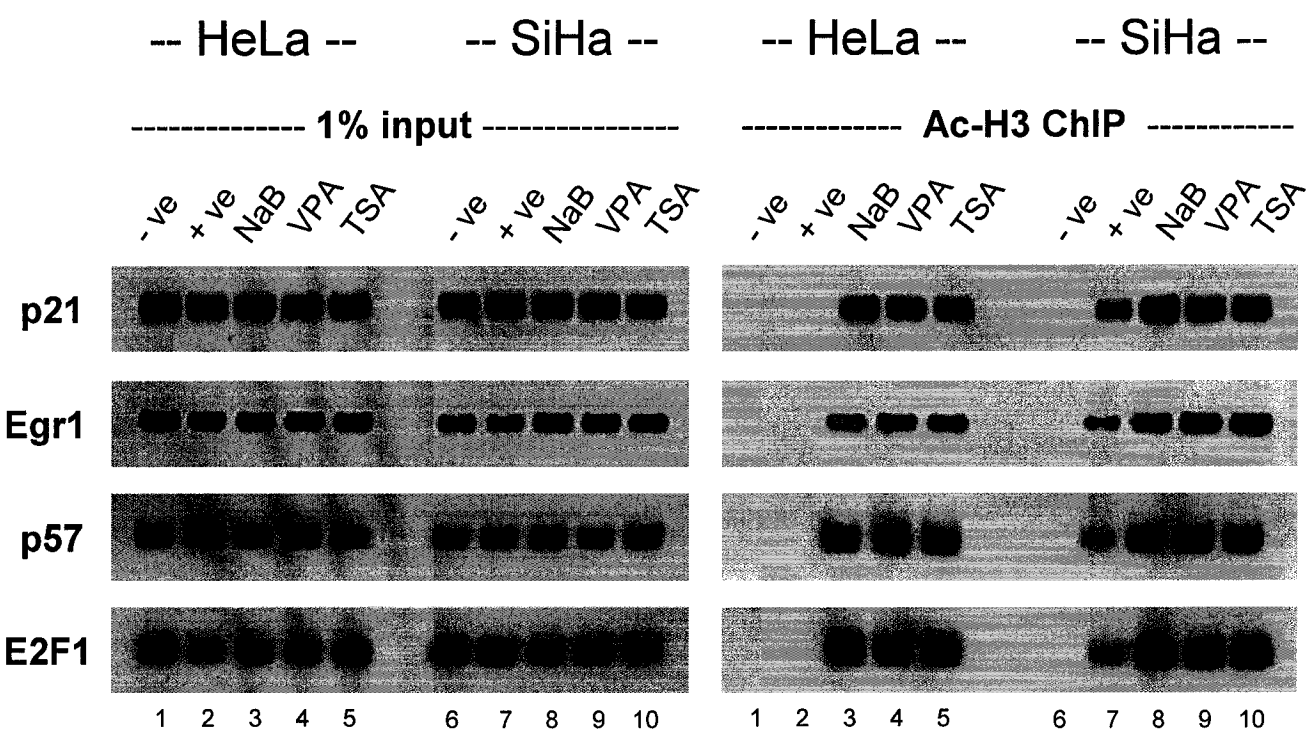

-- HeLa --
-- SiHa --
-- HeLa --
-- SiHa --
1% input
Ac-H3 ChIP
- ve
+ ve
NaB
VPA
TSA
p21
Egr1
p57
E2F1
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10

Figure 10.

Recruitment profiles of p300 in HeLa and SiHa.

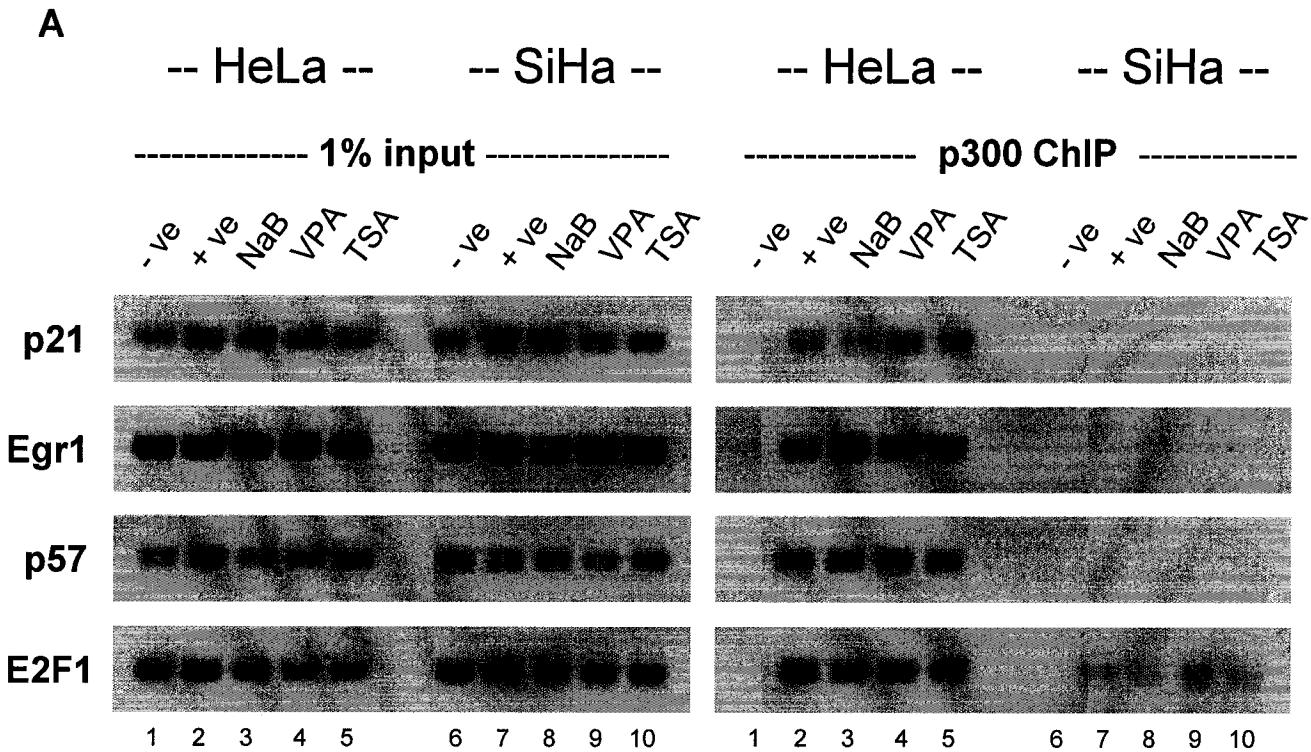
Following the examination of the acetylation levels of histone 3 in HeLa and SiHa in control and in response to HDAC inhibitors at both protein and promoter levels. We next compared the recruitment profiles of p300 to the promoters of p21, Egr1, p57 and E2F1. In fact, p300 is recruited at p300-dependent promoters and functions as a transcriptional coactivator. However, it is not clear whether bromodomain-deficient p300 in SiHa is recruited in the same manner as the wild type p300 in HeLa. Additionally it is not fully clear if HDAC inhibitors affect the recruitment of p300 by enhancing or repressing the association with proximal promoters and thus affect gene transcription. Following 4h of treatment with 5 mM of NaB, 5 mM of VPA and 200 nM of TSA, ChIP assay was performed with anti-p300 (N-15) used to pull-down p300-associated promoters from two cell lines that were immunoprecipitated. Our results indicate that wild type p300 in HeLa is recruited to the four gene promoters (Figure 11A). This is in agreement with another study which showed that p300 is recruited to the four gene promoters in Jurkat T cells which also express wild type p300 (Smith *et al*, 2004). Our results also suggest that recruitment of wild type p300 in HeLa is significantly more than in SiHa (Figure 11A). To rule out the possibility that the decrease of p300-promoter association seen in SiHa is not as a result of a natural decrease of the affinity of the protein to the antibody used in the ChIP assay against the bromodomain-deficient p300, we performed an immunoprecipitation experiment in which equal amounts of whole cell protein lysates of control (untreated) HeLa and SiHa cells were immunoprecipitated using the same antibody used in ChIP, anti-p300 (N-15). The results (Figure 11B) show that the antibody binds to p300 in HeLa and to bromodomain-deficient p300 in SiHa with comparable

affinities. This is evident by comparing the input and immunoprecipitated proteins, in which larger amounts of p300 were immunoprecipitated from SiHa. This is consistent with the input, which shows that p300 is more abundant in SiHa than HeLa (also shown in Figure 6B). The results also show that the recruitment profiles of p300 to the proximal promoters of p21, Egr1, p57 and E2F1 are similar between untreated and treated cells, with no significant differences in p300 association in response to HDAC inhibitors. HDAC inhibitors do not appear to affect the recruitment of either wild type or bromodomain-deficient p300. Thus, HDAC inhibitors may perhaps impose their effects through regulating other factors such as HDAC complex formation, and deregulation of HAT activity of coactivators such as p300. Collectively, our data suggest that bromodomain-deficiency may affect the recruitment of p300 to p300-regulated promoters. This suggests possible important roles of bromodomain in p300 recruitment. However, the results are preliminary and more repeats as well as additional experiments are required to determine the precise role of bromodomain in the function of the coactivator p300.

Figure 11. Recruitment profiles of p300 to the promoters of the four genes in control and in response to HDACi in HeLa and SiHa.

(A) The recruitment profiles of p300 protein to p21, Egr1, p57 and E2F1 promoters in asynchronized HeLa and SiHa were assessed by chromatin immunoprecipitation (ChIP). Following treatment for 4 hours with 5 mM butyrate, 5 mM valproic acid, or 200 nM trichostatin A HDAC inhibitors, ChIP was performed in which equal amounts of DNA from both cell lines were immunoprecipitated. Primers mapped the following regions on the gene promoters: p21 (from -308 to -205), Egr1 (from -483 to -345), p57 (from +49 to +196) and E2F1 (from -580 to -448) in reference to transcription start site. Antibody used in this assay was anti-p300 (N-15), which recognizes the N-terminus of p300.

(B) Immunoprecipitation of p300 using anti-p300 (N-15) of equal amount (750 μg) of whole cell protein from untreated HeLa and SiHa, compared side by side to assess the binding efficiency of the antibody in the two cell lines.


A
-- HeLa --
-- SiHa --
-- HeLa --
-- SiHa --
---------- 1% input ----------
---------- p300 ChIP ----------
- ve
+ ve
NaB
VPA
TSA
p21
Egr1
p57
E2F1
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10

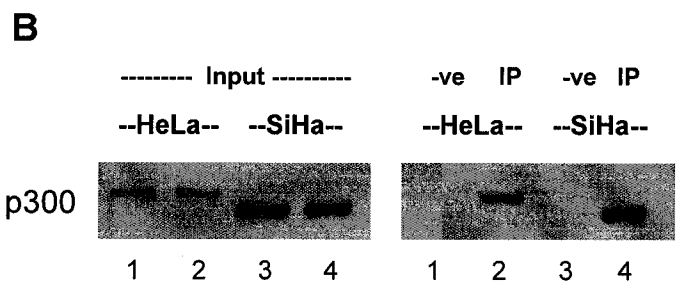

B
--------- Input ---------
-ve IP
-ve IP
--HeLa-- --SiHa--
--HeLa-- --SiHa--
p300
1 2 3 4
1 2 3 4

Figure 11.

Figure 12. General model of p300, p21, p57, Egr1 and E2F1 interactions.

Histone deacetylase inhibitors inhibit the activity of deacetylases that restrict p300 autoacetylation. p300 regulates transcription of Egr1, which is a positive regulator of the p300 gene. Excess levels of Egr1 are stabilized through acetylation by p300. High levels of p300 acetylate Egr1 and form a complex which downregulates the transcription of both Egr1 and p300. The coactivator p300 transactivates E2F1, p21 and p57 gene expression. p21 and p57 proteins inhibit the activity of cyclin dependent kinases and thus prevent phosphorylation of protein retinoblastoma (pRb) which is associated with E2F1. Failure to phosphorylate the pRb-E2F1 complex prevents the release of E2F1 which is necessary for cell cycle progression.

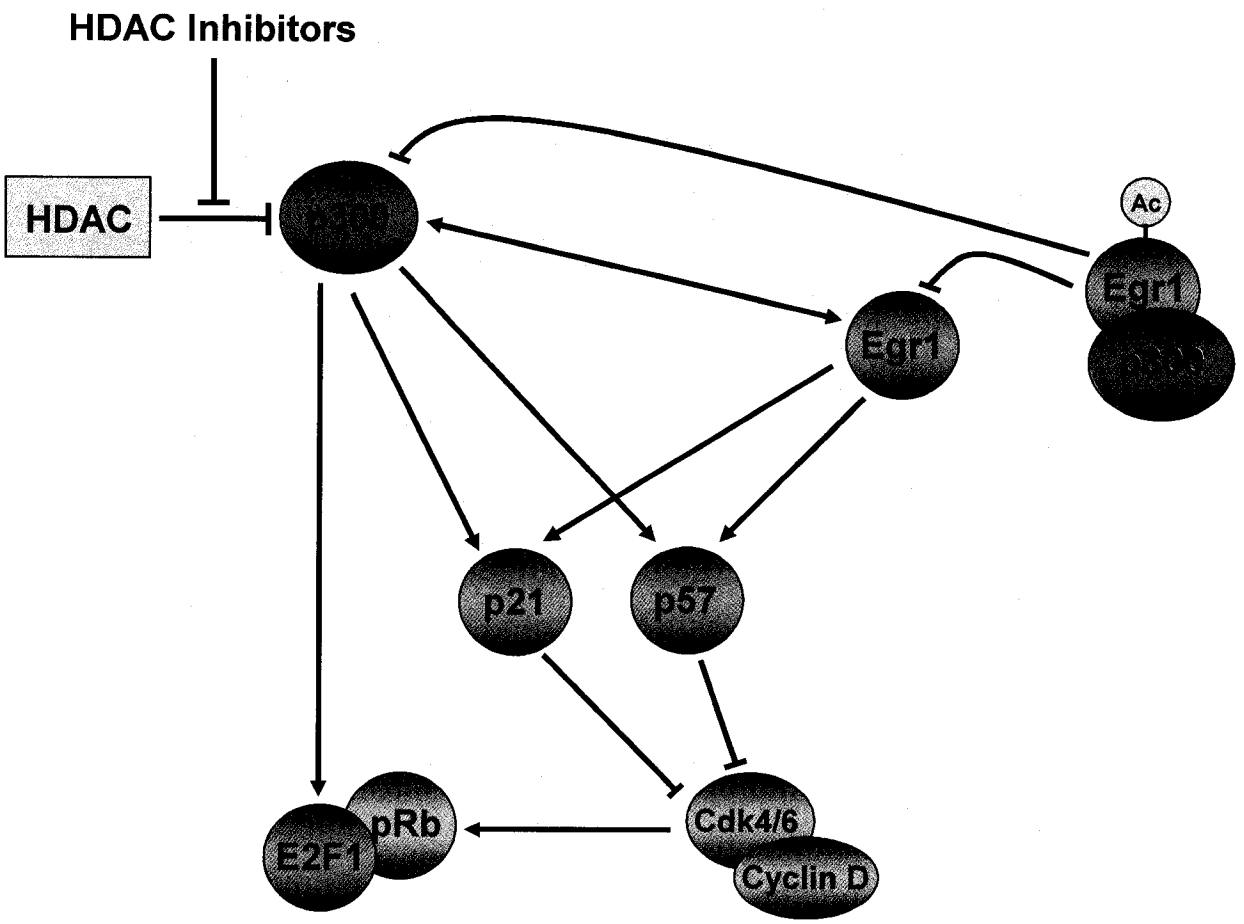

HDAC Inhibitors
HDAC
Ac
Egr1
Egr1
p21
p57
pRb
E2F1
Cdk4/6
Cyclin D

Figure 12.

Discussion

In this study, we examined the effects of HDAC inhibitors on the expression of several p300-dependent genes which are involved in cell cycle regulation. We also examined the effects of HDAC inhibitors on a cell line which expresses a mutant form of p300 with bromodomain truncation. Our results demonstrated that HDAC inhibitors upregulate the expression of a number of genes. However, they did not result in transcriptional upregulation to all genes. Moreover, through chromatin immunoprecipitation assays, we observed that the recruitment of p300 to target promoters was affected in the bromodomain-deficient cell line, which suggests role of bromodomain in the recruitment of p300 to target promoters.

Acetylation and gene expression

Acetylation of histone and non-histone proteins by histone acetyltransferases such as p300 plays significant role in regulating gene expression. In general, accumulation of acetylated histones is a characteristic of transcriptionally active regions in the chromatin. Similarly, regions with excess hypoacetylated histones are generally transcriptionally silent (reviewed in Wolffe, 1994). Acetylation of histone proteins which are associated with gene promoters induce conformational changes in chromatin and become more accessible for transcription factors. Although acetylation can be generally linked to transcriptional activation, it is not sufficient to induce transcription in absence of other factors. As indicated by our results, histone 3 associated with the gene promoter of E2F1 was extensively acetylated by HDAC inhibitors, however, there was no significant

increase in transcription in response to HDAC inhibitors. Therefore acetylation alone is not sufficient to induce transcription.

Histone acetylation serves another function which is providing signals for coactivators such as p300 to recruit and transactivate gene transcription. The 'histone code' hypothesis postulates that acetylation of specific lysine residues on histone tails play key role in epigenetic regulation by serving as a flag mark to initiate transcription. A number of studies suggested an obscure link between acetylation of histone tails at specific lysine residues and transcriptional activation. This was the case until a study by Dhalluin and colleagues (1999) revealed that bromodomain recognizes and binds specifically to certain acetylated lysine residues on histone tails. Acetylated lysines are recognized by the bromodomain of many chromatin-remodeling complexes and histone acetyltransferases such as p300. Consequently, they initiate the assembly of transcription factors and RNA polymerase II to initiate transcription (Chen and St-Germain, 2005).

Acetylation of non-histone proteins by p300 and other HATs is another important mechanism by which transcription is directly and indirectly regulated (reviewed in Glozak *et al*, 2005; Sterner and Berger, 2000). In fact, p300 acetylates various non-histone protein targets which results in either an increase in their transcriptional activity, or enhances their binding affinity to chromatin or other components that are related to transcription. On the other hand, p300 acetylates and activates general transcription factors and transcriptional activators such as GATA-1, which has crucial role in the expression of genes involved in the differentiation of blood cells (Boyes *et al*, 1998). Additionally, p300 acetylates NF-Y, which has important role in transactivation of genes involved in cell cycle progression (Li *et al*, 1998). Also, p300 acetylates the architectural

chromatin binding protein HMG I/Y and increases its binding affinity to chromatin (Munshi *et al*, 1998). Also, p300 regulates the activity of p53 through acetylation (Somasundaram and El-Deiry, 1997). Moreover, p300 regulates E2F1 activity by acetylation, which increases protein stability as well as specific DNA-binding and transcriptional activity (Marzio *et al*, 2000; Martínez-Balbás *et al*, 2000). In fact, one of the main targets of acetylation by p300 is p300 itself. p300 undergoes autoacetylation to enhance its own HAT activity by up to 10 fold (Thompson *et al*, 2004). Other targets of p300 acetylation include, but not limited to, nuclear receptor coactivators such as ACTR, TIF2 and SRC-1, transcriptional activators such as c-Myb, EKLF, MyoD, dTCF and non-histone chromatin proteins such as Sin1 (reviewed in Sterner and Berger, 2000). Thus, acetylation is an important mechanism which is extensively involved in transcriptional regulation at many different levels.

Effects of HDACi on cell cycle

Inhibition of HDAC enzymatic activities disrupts HAT/HDAC balance. The result of inhibiting histone deacetylase enzyme is the accumulation of hyperacetylated histones. Although histone hyperacetylation is usually linked to transcriptional upregulation, recent evidences emerged in recent years suggest that this is not always the case. Results that came from our Western and Real-Time PCR analyses show that HDAC inhibitors induce the expression of cyclin kinase inhibitors p21 and p57 in HeLa, in time-dependent manners. Induction of cyclin kinase inhibitors transcript levels results in cell cycle arrest in G1, which ultimately lead to growth arrest (reviewed in Johnson and Walker, 1999). In fact, p21 and p57 block cell cycle through two ways. Firstly, by inhibiting the association

of several cyclin dependent kinases with cyclins such as cdk2/cyclin E and cdk4/cyclin D. They are required for Rb activation through phosphorylation to release E2F and transactivate transcription of genes that are required for G1-S progression. Secondly, p21 prevents S phase entry by binding to the proliferating cell nuclear antigen (PCNA) which inhibits DNA synthesis (reviewed in Johnson and Walker, 1999). Our results are in agreement with many studies which indicated the upregulation in the cyclin kinase inhibitors expression in response to HDAC inhibitors in many carcinomas (Glaser *et al*, 2003; Frey *et al*, 2002 and Takai *et al*, 2004). Additionally, our results from synchronization experiments show that p21 responds to HDAC inhibitors as cells enter G1 and G2 while p57 respond to HDAC inhibitors as cells enter G1, which is consistent with what is known about the expression of p21 and p57 (reviewed in Morgan, 1997). Our results indicate that the basal expression levels of p21 and p57 in HeLa and SiHa cervical carcinomas is very low, which is consistent with what is known about the basal expression of these genes in other cancer cell lines such as breast, colon, liver and epithelial ovarian cancers (Jiang *et al*, 1997; Liu *et al*, 2005; Ito *et* al, 2001; Anttila *et al*, 1999 respectively). HDAC inhibitors significantly upregulate the expression of p21 and p57 in cancer cells and this could account for some of the anticancer effects of these HDAC inhibitors (Gui *et* al, 2003; Cucciolla *et* al, 2008 respectively).

Our results also show that HDAC inhibitors induce the expression of Egr1 in a time-dependent manner. It is reported that Egr1 is a transactivator of p300 (reviewed in Adamson and Mustelin, 2005). The levels of Egr1 rapidly increase in response to HDAC inhibitors as shown by our Western and Real-Time PCR results. They increased in response to HDAC inhibitors perhaps to induce transcription of p300. In addition, cell

synchronization results revealed that Egr1 levels are induced at G1 and G2. They are also modified at M phase, which is apparent by the appearance of another band with slower migration on the SDS-PAGE, possibly due to acetylation. Results also showed no significant expression of Egr1 at M phase. In fact, high levels of p300/CBP acetylate Egr1 and form a complex which downregulates the transcription of both Egr1 and p300/CBP (Yu *et al*, 2004).

Our results also show that E2F1 levels do not change significantly in response to HDAC inhibitors. p300 regulates E2F1 protein activity through acetylation (Marzio *et al*, 2000; Martínez-Balbás *et al*, 2000). However, the exact mechanisms by which p300 regulate E2F1 transcription at the promoter level are not fully understood yet. Synchronization results show that E2F1 levels are slightly upregulated (although not statistically significant) during S-phase, consistent with its role of promoting S-phase entry. Results also show that E2F1 appears to be modified at M phase, which is apparent by the appearance of another band with slower migration on the SDS-PAGE, possibly due to phosphorylation. It has been reported that E2F1 is phosphorylated by Cyclin A/Cdk2, which inhibits its DNA-binding activity at late G2 just in prior to M entry (Xu *et al*, 1994). Phosphorylated and unphosphorylated E2F1 levels decrease as cells progress from M to G1. Western and Real-Time PCR results show trivial changes in E2F1 expression in response to all three HDAC inhibitors, suggesting that p300 regulates E2F1 transactivation on the promoter possibly through an indirect mechanism or different mode of regulation. In fact, indications for the indirect role of p300 on E2F1 promoter come from chromatin immunoprecipitation assay in the p300 bromodomain-deficient cell line, SiHa. The assay showed that among the four genes under the study, E2F1 had the

smallest difference in p300 promoter occupancy between HeLa and SiHa. To conclude, HDAC inhibitors target genes that modulate cell cycle perhaps through different modes of regulation and they do not always result in gene expression upregulation.

Requirement of the bromodomain by p300-dependent genes

Our work on SiHa cell line which expresses mutant form of p300 which lacks bromodomain has revealed several indications on some of the roles of bromodomain in the function of p300. Western blot results in SiHa show that the gene expression of p21 is induced by less fold difference (≈2-4 fold) in comparison to (≈5-16 fold) in HeLa. HDAC inhibitors also did not induce p21 expression in SiHa to levels seen in HeLa, possibly due to p300 bromodomain-deficiency. Evidence for the significance of bromodomain for transactivation of p21 was illustrated in a study by Ohshima and colleagues (2001). In their study, a transient reporter assay was performed in which SiHa cells were transfected with a luciferase reporter construct mediated by the p21 promoter. The promoter activity was in fact measured in control and in response to cotransfection with a plasmid encoding a constitutively active form of transforming growth factor β (TGF-β) type I receptor, which is a known inducer of p21. The study revealed that TGF-β failed to activate the promoter p21 in the luciferase construct in SiHa, and upon reintroduction of p300 wild type, TGF-β was able to induce the expression of p21 in the construct in a p300-dependent manner. Thus bromodomain has important roles in the coactivator function of p300 at the p21 promoter. On the other hand, p57 is downregulated in SiHa, where as its upregulated in HeLa. This is also possibly due to

p300 bromodomain-deficiency, which suggests that bromodomain may be important for p300 coactivation function on p57 promoter.

Our results also show that HDAC inhibitors induce the expression of Egr1 in less fold difference (≈1-2 fold) as compared to (≈4-8 fold) observed in HeLa. This implies that p300 might not fully induced the transcription of Egr1 in SiHa. It is known that high levels of both Egr1 and p300 would lead to Egr1 acetylation by p300 and p300-complex formation, which functions in a negative loop mechanism to attenuate the expression of both proteins (Yu *et al*, 2004). Endogenous levels of p300 protein in SiHa are abundantly more than 3 fold times as compared to HeLa (Figure 6B). On the other hand, it has been reported that p300 bromodomain maintains the HAT function, at least on non-nucleosomal histone protein targets (Manning *et al*, 2001). Thus, more abundant p300 in SiHa could rapidly acetylate Egr1 and consequently attenuate the expression of Egr1 in negative loop mechanism, and as a result, it is not surprising that Egr1 levels are induced in a limited manner.

Our findings within the study also show that E2F1 levels in SiHa do not change significantly in response to HDAC inhibitors. This is in consistence with HeLa, which further indicate that E2F1 is regulated by p300 in an indirect manner and may not be dependent on p300 bromodomain for transcription. Given all the observations on the effects of bromodomain-deficiency on the gene expression of the four genes, it is likely that bromodomain requirements vary between different p300-regulated genes.

Roles of bromodomain in p300 recruitment

Bromodomains are found in many HAT proteins such as GCN5, ring3, Br140, $TAF_{II}250$, PCAF and p300 (reviewed in Tamkun, 1995). Our results suggest that bromodomain has roles in the recruitment of p300 to p300-regulated gene promoters. As shown by chromatin immunoprecipitation results, there is a remarkable decrease in p300 recruitment to p300 regulated promoters in SiHa (Figure 11).

Based on what is currently known in literature, there seem at least three possible roles of bromodomain in the recruitment of p300 to target promoters. First of all, it is an acetyl-binding domain, which recognizes acetylated lysine residues on histone proteins that are associated with gene promoters. Loss of bromodomain, which guides p300, would result in less recruitment of p300 as it fails to recognize acetyl signals. To rule out the possibility that there is a defect in global acetylation as well as in the acetylation of lysines to which bromodomains bind, we assessed the global acetylation levels and found that it is comparable between the two cell lines as shown by Western blot results. To further confirm that acetylation occurs at lysine residue to which bromodomains bind we performed ChIP using antibody that binds to acetylated lysine 9/14 on histone 3 to which bromodomains specifically bind to. According to a study by Agalioti and colleagues (2002), bromodomains recognize and bind to acetylated lysine 8 on histone H4 and acetylated lysine 9 and acetylated lysine 14 on histone H3. Our results indicate that acetylation of lysine 9 and 14 on histone 3 occur in SiHa in response to HDAC inhibitors and yet p300 fails to efficiently bind on gene promoters as shown by ChIP results (Figure 11). This indicates that bromodomain may have roles in the recruitment of p300 perhaps by guiding it to its targets. The second possible role of bromodomain is in p300

anchoring on chromatin. In a study by Manning and colleagues (2001), p300-chromatin interaction assay was developed in which the affinities of wild type and bromodomain-truncated p300 mutant to chromatin were assessed. The main conclusion of the study by Manning and colleagues (2001) was that deletion of the bromodomain significantly reduced the ability of p300 to bind on chromatin. Additionally, it was found that isolated p300 bromodomain can bind to free histones but not to nucleosomal histones (Kraus et al, 1999). Thus, bromodomain is likely required for the binding of p300 to chromatin, more specifically, to nucleosomal histones where promoters reside. This is in agreement to our chromatin immunoprecipitation results which indicated that bromodomain-deficient p300 binds at much lower degree as compared to normal p300 to p300-regulated gene promoters. Thus it is possible that bromodomain may act as an anchoring domain which tethers p300 to the chromatin template (reviewed in Zeng and Zhou, 2002). The third possible role of bromodomain in the function of p300 is that it may contribute to full p300 HAT activity, which will be described in further details.

Significance of bromodomain in p300 HAT function

There is a growing evidence that bromodomain contributes either directly or indirectly in the histone acetyltransferase or acetyltransferase activity of p300. A number of studies suggested a contribution of bromodomain to histone acetylation by tethering transcriptional HATs to specific sites at the chromatin and stabilizing HAT/chromatin binding (reviewed in Brownell and Allis, 1996). In a study by Kraus and colleagues (1999), the transcriptional activities of various segments of p300 were examined in the context of transcription activation process. The study reveals that bromodomain deletion

impairs p300's ability to acetylate nucleosomal histones in native chromatin. These results suggest that the bromodomain contributes to the ability of HATs to acetylate nucleosomal histones. Therefore, bromodomain contributes to p300 transcriptional activity, perhaps in part by supporting p300 HAT activity. Furthermore, the bromodomain is important for the stable interaction of p300 with chromatin, thus contributing to its ability to form a template-committed complex with chromatin. Overall, this could contribute to p300's ability to associate with chromatin prior to activator-mediated targeting and to enhance transcription initiation by stable association with the promoter through multiple rounds of transcription. However the exact mechanism by which bromodomain contributes to the HAT function of p300 has not been elucidated yet.

Our results suggest that in the p300 bromodomain-deficient SiHa, the HDAC inhibitors can still induce transcription of p21 and Egr1 in SiHa, although in much less fold difference as compared to HeLa. Also, there is a comparable basal level of acetylated histone 3 between HeLa and SiHa at protein and promoter levels, however it was observed that there is a high basal level of H3Ac associated with p300-dependent promoters in SiHa, compared to the almost non-detectable basal acetylation on H3 in HeLa (Figure 10). A plausible explanation for such observations could be based on functional redundancies and compensating mechanisms by other histone acetyltransferase family members (Arany *et al*, 1995). Numerous studies illustrated that functional redundancies exist between histone acetyltransferase proteins such as p300, the functional homologue of p300 CBP, PCAF, GCN5 and Tip60 which ultimately regulate the fine-tuned acetylation balance *in vivo* (Wittschieben *et al*, 2000; Yamauchi *et al*,

2000; Arany *et al*, 1995). Thus, it is possible that in SiHa, other HATs could compensate for the affected recruitment of p300 or to the possible inefficiency of p300 HAT activity due to bromodomain deficiency. However, the exact molecular details of such compensatory mechanisms in the context of the regulation of the four genes under this study are yet to be determined and in need of further experimental evidences.

Short chain fatty acids versus hydroxamate HDACi

Various HDAC inhibitors differ from each other in many aspects. Since every inhibitor inhibits a limited number of HDACs, the regulatory pathways affected by these inhibitors can be different (Noh and Lee, 2003). Butyrate, valproic acid and trichostatin A inhibit HDAC class I and IIa. However, they differ slightly in terms of inhibitory activities and target specificities (reviewed in Bolden et al, 2006). This is also supported by our Western and Real-Time PCR results which demonstrate different degrees of induction or suppression among the three HDAC inhibitors used in this study. Furthermore, we observed that in HeLa, butyrate and trichostatin A upregulated the protein expression of p57, while valproic acid did not have any significant effects (Figure 2). This provides an example where closely related HDAC inhibitors can have different effects on the expression of some genes.

Short chain fatty acid (SCFA) HDAC inhibitors are weaker inhibitors and their biological effects are detected through their use at a milli-molar scale. In contrast, TSA is effective at a nano-molar scale and appear to be more specific than SCFA inhibitors, which appear to have a ubiquitous range of HDAC inhibitory activities. TSA is also a potent inhibitor, whereas butyrate and valproic acid are weaker and appear to be less

specific in terms of their substrate binding. In two studies by Guardiola and colleagues (2002) and Gurvich and colleagues (2004) it was shown that valproic acid and butyrate do no inhibit HDAC6 and HDAC10 in contrast to TSA. The differences in HDAC specificities could explain differences in induction or suppressor by the use of these different inhibitors on the gene expression of the four genes under this study. In fact, in another study by Siavoshian and colleagues (2000), the effects of butyrate and TSA on proliferation and differentiation of human intestinal epithelial cells were compared. Butyrate was found to block cell cycle at the G1 phase whereas TSA blocks cell cycle at G1 and G2. Also, it was found that after six hour of treatment with either inhibitor, histone H4 hyperacetylation was observed. However, the hyperacetylation was maintained even after 24 hours following butyrate treatment, whereas acetylation levels induced by TSA dropped to basal levels after 24 hours. Thus, it is apparent that various HDAC inhibitors have distinct roles. This is either possibly due to difference in their affinities, or perhaps due to differences to accessibilities to their targets because of their chemical composition. Understanding different effects and specificities of various HDAC inhibitor compounds is necessary for their potential use as therapeutic agents against cancer.

Therapeutic significance of HDACi anticancer activities

Histone deacetylase inhibitors induce apoptosis and differentiation to transformed cells of a wide range of cell types including cervix, bladder, skin, ovaries, colon, breast and leukemia, with minimum toxic side effects to normal cells (reviewed in Bolden *et al*, 2002; Dokmanovic and Marks, 2005). HDAC inhibitors possess a wide range of

anticancer activities. They induce cell cycle arrest to cancer cells through induction of cyclin dependent kinase inhibitors such as p21 and p57. In fact, it is known that the upregulation of p21 and p57 induces cell cycle arrest at G1/S and prevents progression and cell division (reviewed in Johnson and Walker, 1999). Thus, HDAC inhibitor-mediated cell cycle arrest is a crucial mechanism by which these compounds target tumor cells.

HDAC inhibitors inhibit histone deacetylases and disrupt the HAT/HDAC balance. They also augment the HAT activity of p300, which is a tumor suppressor protein (Arany *et al*, 1995). p300 suppresses the growth of several carcinoma cell lines including pancreatic, breast and colon (Gayther et al, 2000). On the other hand it also controls the activity of the apoptotic regulator and tumor suppressor p53 (reviewed in Grossman, 2001). It also regulates the activity of other tumor suppressors such as breast and ovarian cancer-specific tumor suppressor BRCA-1, and inhibits signalling pathways that induce cancer such as Wnt signalling pathway. Thus, an additional mechanism by which HDAC inhibitors target cancer cells is mediated through p300, which possesses a range of anticancer activities.

HDAC inhibitors initiate apoptosis to many cancer cells through direct and indirect mechanisms. HDAC inhibitors modulate the activity of the proapoptotic factor p53 indirectly by enhancing its activity by acetylation. HDAC inhibitors initiate apoptosis through direct mechanisms too. Previous results from our laboratory demonstrated that butyrate and valproic acid induce apoptosis through activation of caspase-dependent pathway by inducing the caspase-8 and -9 transcript levels (Chen, Ghazawi and Bakkar, 2006). HDAC inhibitors have many more anticancer activities by inducing extrinsic

death-receptor pathway, intrinsic mitochondrial death pathway, regulate reactive oxygen species activity and affect the activity of diverse kinase-mediated signal transduction pathways (reviewed in Bolden *et al*, 2002). Therefore, understanding the exact molecular mechanisms by which different HDAC inhibitors exert their effects in the context of anticancer activities is crucial to design effective therapeutics against many cancers.

Roles of HDACi in p300-mediated cell cycle regulation

HDAC inhibitors induce histone hyperacetylation not only in cancer but in normal cells too (Zhao *et al*, 2005). This further confirms that histone acetylation and changes in chromatin structure are not the only mechanism by which HDAC inhibitors regulate gene expression. One plausible hypothesis for a possible mechanism by which HDAC inhibitors lead to the transactivation of several genes is that they may allow HAT proteins such as p300 to increase their association with promoters. However, as shown by our chromatin immunoprecipitation results, there were no significant changes in the recruitment profile of p300 in response to HDAC inhibitors. This was also illustrated in a recent study by Balakrishnan and Milavetz (2008), in which the p300 recruitment profile on SV40 in control and in response to treatment with butyrate and trichostatin A were assessed. No changes on p300 association on SV40 minichromosomes were observed after the treatments. In fact this was also illustrated in another study by Gui and colleagues (2004). In agreement with our results, chromatin immunoprecipitation assays by Gui and colleagues revealed that the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) does not cause any significant differences in the recruitment profile of p300 to p21 promoter. However, it was also shown that SAHA causes

dissociation of HDAC1 protein from p21 control after 3 hours of treatment. In another study by Kobayashi and colleagues (2004), it was shown that the transcription factors ZBP-89 and Sp1 are recruited to p21 promoter following butyrate treatment. In another study by Xiao and colleagues (2000) it was shown that p300 is indispensable to p21 promoter activation in response to TSA treatment. Collectively, p300 may act as a coactivator at some promoters whenever repressor complexes such as HDACs are dissociated by various cellular signals or mechanisms or through exogenous HDAC inhibitors. This allows promoter kinetics to change in favor of p300 coactivator function in terms of acetylating transcription factors or as an anchoring protein which acts as an adaptor for the transcriptional pre-initiation complex. Moreover, it is currently known that HDAC inhibitors activate p300 by modulating the acetylation status of p300 itself (Stiehl *et al*, 2007). Inhibiting the activity of several HDACs allows an efficient p300 autoacetylation, which augments the p300 HAT activity significantly (Stiehl *et al*, 2007; Bouras *et al*, 2005; Simone *et al*, 2004; Mayo *et al*, 2003; Thompson *et al*, 2004).

In summary, HDAC inhibitors may cooperate with p300 in regulating the cell cycle by a) stimulating p300 HAT activity, through inhibition of HDAC counterpart b) affecting the promoter kinetics of several p300 regulated genes, as in dissociating HDACs from repressor complexes in favor of the coactivator function of p300 and c) regulating the activity of non-histone proteins such as transcription factors that are also involved in p300-mediated cell cycle regulation.

Conclusions

In this study we examined the effects of HDAC inhibitors on the expression of several p300-dependent genes which have several roles in cell cycle, in an attempt to understand some of their anticancer effects and understand several aspects of the p300-dependent gene regulation. We found that HDAC inhibitors do not affect the expression of all p300-dependent genes. Our data on p300-dependent expression in a cell line which expresses bromodomain-deficient p300 suggest that in contrast to what we and others observed in cell lines which express wild type p300, the recruitment of bromodomain-deficient p300 to target promoters was reduced. Thus, we conclude that p300 bromodomain may have a role in the recruitment of the coactivator to target genes. More research is necessary to characterize precise roles of HDAC inhibitors and bromodomain in modulating global gene expression to fully understand the molecular pathways by which these inhibitors selectively induce cell cycle arrest to cancer cells, and by which bromodomain precisely contributes to the function of p300.

References

Abukhdeir AM, Park BH. P21 and p27: roles in carcinogenesis and drug resistance. Expert Rev Mol Med. 2008 Jul 1;10:e19. Review.

Adamson ED, Yu J, Mustelin T. Co-factors p300 and CBP catch Egr1 in their network. Prostate. 2005 Jun 1;63(4):407-10.

Agalioti T, Chen G, Thanos D. Deciphering the transcriptional histone acetylation code for a human gene. Cell. 2002 Nov 1;111(3):381-92.

Ahmad N, Adhami VM, Afaq F, Feyes DK, Mukhtar H. Resveratrol causes WAF-1/p21-mediated G(1)-phase arrest of cell cycle and induction of apoptosis in human epidermoid carcinoma A431 cells. Clin Cancer Res. 2001 May;7(5):1466-73.

Ahmed MM. Regulation of radiation-induced apoptosis by early growth response-1 gene in solid tumors. Curr Cancer Drug Targets. 2004 Feb;4(1):43-52.

Anttila MA, Kosma VM, Hongxiu J, Puolakka J, Juhola M, Saarikoski S, Syrjänen K. p21/WAF1 expression as related to p53, cell proliferation and prognosis in epithelial ovarian cancer. Br J Cancer. 1999 Apr;79(11-12):1870-8.

Arany Z, Newsome D, Oldread E, Livingston DM, Eckner R. A family of transcriptional adaptor proteins targeted by the E1A oncoprotein. Nature. 1995 Mar 2;374(6517):81-4.

Asahara H, Tartare-Deckert S, Nakagawa T, Ikehara T, Hirose F, Hunter T, Ito T, Montminy M. Dual roles of p300 in chromatin assembly and transcriptional activation in cooperation with nucleosome assembly protein 1 in vitro. Mol Cell Biol. 2002 May;22(9):2974-83.

Attia M, Rachez C, De Pauw A, Avner P, Rogner UC. Nap1l2 promotes histone acetylation activity during neuronal differentiation. Mol Cell Biol. 2007 Sep;27(17):6093-102.

Attwooll C, Lazzerini Denchi E, Helin K. The E2F family: specific functions and overlapping interests. EMBO J. 2004 Dec 8;23(24):4709-16.

Avantaggiati ML, Ogryzko V, Gardner K, Giordano A, Levine AS, Kelly K. Recruitment of p300/CBP in p53-dependent signal pathways. Cell. 1997 Jun 27;89(7):1175-84.

Bai L, Merchant JL. Transcription factor ZBP-89 cooperates with histone acetyltransferase p300 during butyrate activation of p21waf1 transcription in human cells. J Biol Chem. 2000 Sep 29;275(39):30725-33.

Balakrishnan L, Milavetz B. HDAC inhibitors stimulate viral transcription by multiple mechanisms. Virol J. 2008 Mar 19;5:43.

Baluchamy S, Rajabi HN, Thimmapaya R, Navaraj A, Thimmapaya B. Repression of c-Myc and inhibition of G1 exit in cells conditionally overexpressing p300 that is not dependent on its histone acetyltransferase activity. Proc Natl Acad Sci U S A. 2003 Aug 5;100(16):9524-9.

Blais A, Dynlacht BD. E2F-associated chromatin modifiers and cell cycle control. Curr Opin Cell Biol. 2007 Dec;19(6):658-62. Review.

Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov. 2006 Sep;5(9):769-84. Review.

Bouras T, Fu M, Sauve AA, Wang F, Quong AA, Perkins ND, Hay RT, Gu W, Pestell RG. SIRT1 deacetylation and repression of p300 involves lysine residues 1020/1024 within the cell cycle regulatory domain 1. J Biol Chem. 2005 Mar 18;280(11):10264-76.

Boyes J, Byfield P, Nakatani Y, Ogryzko V. Regulation of activity of the transcription factor GATA-1 by acetylation. Nature. 1998 Dec 10;396(6711):594-8.

Brownell JE, Allis CD. Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation. Curr Opin Genet Dev. 1996 Apr;6(2):176-84. Review.

Caretti G, Salsi V, Vecchi C, Imbriano C, Mantovani R. Dynamic recruitment of NF-Y and histone acetyltransferases on cell-cycle promoters. J Biol Chem. 2003 Aug 15;278(33):30435-40.

Carrozza MJ, Utley RT, Workman JL, Côté J. The diverse functions of histone acetyltransferase complexes.Trends Genet. 2003 Jun;19(6):321-9. Review.

Cecconi D, Donadelli M, Rinalducci S, Zolla L, Scupoli MT, Scarpa A, Palmieri M, Righetti PG. Proteomic analysis of pancreatic endocrine tumor cell lines treated with the histone deacetylase inhibitor trichostatin A. Proteomics. 2007 May;7(10):1644-53.

Chang BD, Watanabe K, Broude EV, Fang J, Poole JC, Kalinichenko TV, Roninson IB.Effects of p21Waf1/Cip1/Sdi1 on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases. Proc Natl Acad Sci U S A. 2000 Apr 11;97(8):4291-6.

Chen J, Ghazawi FM, Bakkar W, Li Q. Valproic acid and butyrate induce apoptosis in human cancer cells through inhibition of gene expression of Akt/protein kinase B. Mol Cancer. 2006 Dec 11;5:71.

Chen J, St-Germain JR, Li Q. B56 regulatory subunit of protein phosphatase 2A mediates valproic acid-induced p300 degradation. Mol Cell Biol. 2005 Jan;25(2):525-32.

Chen JS, Faller DV, Spanjaard RA. Short-chain fatty acid inhibitors of histone deacetylases: promising anticancer therapeutics? Curr Cancer Drug Targets. 2003 Jun;3(3):219-36. Review.

Chen PL, Scully P, Shew JY, Wang JY, Lee WH. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell. 1989 Sep 22;58(6):1193-8.

Choi JH, Kwon HJ, Yoon BI, Kim JH, Han SU, Joo HJ, Kim DY. Expression profile of histone deacetylase 1 in gastric cancer tissues. Jpn J Cancer Res. 2001 Dec;92(12):1300-4.

Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH. Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature. 1993 Oct 28;365(6449):855-9.

Cucciolla V, Borriello A, Criscuolo M, Sinisi AA, Bencivenga D, Tramontano A, Scudieri AC, Oliva A, Zappia V, Della Ragione F. Histone deacetylase inhibitors upregulate p57Kip2 level by enhancing its expression through Sp1 transcription factor. Carcinogenesis. 2008 Mar;29(3):560-7.

Dannenberg JH, Schuijff L, Dekker M, van der Valk M, te Riele H. Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. Genes Dev. 2004 Dec 1;18(23):2952-62.

Darkin-Rattray SJ, Gurnett AM, Myers RW, Dulski PM, Crumley TM, Allocco JJ, Cannova C, Meinke PT, Colletti SL, Bednarek MA, Singh SB, Goetz MA, Dombrowski AW, Polishook JD, Schmatz DM. Apicidin: a novel antiprotozoal agent that inhibits parasite histone deacetylase. Proc Natl Acad Sci U S A. 1996 Nov 12;93(23):13143-7.

Das A, Chendil D, Dey S, Mohiuddin M, Mohiuddin M, Milbrandt J, Rangnekar VM, Ahmed MM. Ionizing radiation down-regulates p53 protein in primary Egr-1-/- mouse embryonic fibroblast cells causing enhanced resistance to apoptosis.
J Biol Chem. 2001 Feb 2;276(5):3279-86.

Davis PK, Ho A, Dowdy SF. Biological methods for cell-cycle synchronization of mammalian cells. Biotechniques. 2001 Jun;30(6):1322-6, 1328, 1330-1. Review.

Day ML, Wu S, Basler JW. Prostatic nerve growth factor inducible A gene binds a novel element in the retinoblastoma gene promoter. Cancer Res. 1993 Dec 1;53(23):5597-9.

De Brabander MJ, Van de Veire RM, Aerts FE, Borgers M, Janssen PA. The effects of methyl (5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl) carbamate, (R 17934; NSC 238159), a new synthetic antitumoral drug interfering with microtubules, on mammalian cells cultured in vitro. Cancer Res. 1976 Mar;36(3):905-16.

de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB. Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J. 2003 Mar 15;370(Pt 3):737-49. Review.

Dhalluin C, Carlson JE, Zeng L, He C, Aggarwal AK, Zhou MM. Structure and ligand of a histone acetyltransferase bromodomain. Nature. 1999 Jun 3;399(6735):491-6.

Dokmanovic M, Marks PA. Prospects: histone deacetylase inhibitors. J Cell Biochem. 2005 Oct 1;96(2):293-304. Review.

Drummond DC, Noble CO, Kirpotin DB, Guo Z, Scott GK, Benz CC. Clinical development of histone deacetylase inhibitors as anticancer agents. Annu Rev Pharmacol Toxicol. 2005;45:495-528. Review.

Dutnall RN, Tafrov ST, Sternglanz R, Ramakrishnan V. Structure of the histone acetyltransferase Hat1: a paradigm for the GCN5-related N-acetyltransferase superfamily. Cell. 1998 Aug 21;94(4):

Dyda F, Klein DC, Hickman AB. GCN5-related N-acetyltransferases: a structural overview. Annu Rev Biophys Biomol Struct. 2000;29:81-103. Review.

Eberharter A, Ferreira R, Becker P. Dynamic chromatin: concerted nucleosome remodelling and acetylation. Biol Chem. 2005 Aug;386(8):745-51. Review.

Eckner R, Ewen ME, Newsome D, Gerdes M, DeCaprio JA, Lawrence JB, Livingston DM. Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. Genes Dev. 1994 Apr 15;8(8):869-84.

Edwards MC, Wong C, Elledge SJ. Human cyclin K, a novel RNA polymerase II-associated cyclin possessing both carboxy-terminal domain kinase and Cdk-activating kinase activity. Mol Cell Biol. 1998 Jul;18(7):4291-300.

Eisen JA, Sweder KS, Hanawalt PC. Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions. Nucleic Acids Res. 1995 Jul 25;23(14):2715-23. Review.

Elledge SJ. Cell cycle checkpoints: preventing an identity crisis. Science. 1996 Dec 6;274(5293):1664-72. Review.

Frey RR, Wada CK, Garland RB, Curtin ML, Michaelides MR, Li J, Pease LJ, Glaser KB, Marcotte PA, Bouska JJ, Murphy SS, Davidsen SK. Trifluoromethyl ketones as inhibitors of histone deacetylase. Bioorg Med Chem Lett. 2002 Dec 2;12(23):3443-7.

Furumai R, Matsuyama A, Kobashi N, Lee KH, Nishiyama M, Nakajima H, Tanaka A, Komatsu Y, Nishino N, Yoshida M, Horinouchi S. FK228 (depsipeptide) as a natural prodrug that inhibits class I histone deacetylases. Cancer Res. 2002 Sep 1;62(17):4916-21.

Fu M, Zhu X, Zhang J, Liang J, Lin Y, Zhao L, Ehrengruber MU, Chen YE. Egr-1 target genes in human endothelial cells identified by microarray analysis. *Gene.* 2003; 315: 33–41.

Galasinski SC, Resing KA, Goodrich JA, Ahn NG. Phosphatase inhibition leads to histone deacetylases 1 and 2 phosphorylation and disruption of corepressor interactions. J Biol Chem. 2002 May 31;277(22):19618-26.

Galbiati L, Mendoza-Maldonado R, Gutierrez MI, Giacca M. Regulation of E2F-1 after DNA damage by p300-mediated acetylation and ubiquitination. Cell Cycle. 2005 Jul;4(7):930-9

Gao L, Cueto MA, Asselbergs F, Atadja P. Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. J Biol Chem. 2002 Jul 12;277(28):25748-55.

Gayther SA, Batley SJ, Linger L, Bannister A, Thorpe K, Chin SF, Daigo Y, Russell P, Wilson A, Sowter HM, Delhanty JD, Ponder BA, Kouzarides T, Caldas C. Mutations truncating the EP300 acetylase in human cancers. Nat Genet. 2000 Mar;24(3):300-3.

Gediya LK, Khandelwal A, Patel J, Belosay A, Sabnis G, Mehta J, Purushottamachar P, Njar VC. Design, synthesis, and evaluation of novel mutual prodrugs (hybrid drugs) of all-trans-retinoic acid and histone deacetylase inhibitors with enhanced anticancer activities in breast and prostate cancer cells in vitro. J Med Chem. 2008 Jul 10;51(13):3895-904.

Giordano A, Rossi A, Romano G, Bagella L. Tumor suppressor pRb2/p130 gene and its derived product Spa310 spacer domain as perspective candidates for cancer therapy. J Cell Physiol. 2007 Nov;213(2):403-6. Review.

Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG, Davidsen SK. Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther. 2003 Feb;2(2):151-63.

Glozak MA, Sengupta N, Zhang X, Seto E. Acetylation and deacetylation of non-histone proteins. Gene. 2005 Dec 19;363:15-23. Review.

Goodman RH, Smolik S. CBP/p300 in cell growth, transformation, and development. Genes Dev. 2000 Jul 1;14(13):1553-77. Review.

Goodrich JA, Tjian R. TBP-TAF complexes: selectivity factors for eukaryotic transcription. Curr Opin Cell Biol. 1994 Jun;6(3):403-9. Review.

Gregory DJ, Garcia-Wilson E, Poole JC, Snowden AW, Roninson IB, Perkins ND. Induction of transcription through the p300 CRD1 motif by p21WAF1/CIP1 is core promoter specific and cyclin dependent kinase independent. Cell Cycle. 2002 Sep-Oct;1(5):343-50.

Grossman SR. p300/CBP/p53 interaction and regulation of the p53 response. Eur J Biochem. 2001 May;268(10):2773-8. Review.

Gu W, Roeder RG. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell. 1997 Aug 22;90(4):595-606.

Gu W, Shi XL, Roeder RG. Synergistic activation of transcription by CBP and p53. Nature. 1997 Jun 19;387(6635):819-23.

Gui CY, Ngo L, Xu WS, Richon VM, Marks PA. Histone deacetylase (HDAC) inhibitor activation of p21WAF1 involves changes in promoter-associated proteins, including HDAC1. Proc Natl Acad Sci U S A. 2004 Feb 3;101(5):1241-6.

Gurvich N, Tsygankova OM, Meinkoth JL, Klein PS. Histone deacetylase is a target of valproic acid-mediated cellular differentiation. Cancer Res. 2004 Feb 1;64(3):1079-86.

Halkidou K, Gaughan L, Cook S, Leung HY, Neal DE, Robson CN. Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. Prostate. 2004 May 1;59(2):177-89.

Han JW, Ahn SH, Park SH, Wang SY, Bae GU, Seo DW, Kwon HK, Hong S, Lee HY, Lee YW, Lee HW. Apicidin, a histone deacetylase inhibitor, inhibits proliferation of tumor cells via induction of p21WAF1/Cip1 and gelsolin. Cancer Res. 2000 Nov 1;60(21):6068-74.

Harper JW, Elledge SJ. Cdk inhibitors in development and cancer. Curr Opin Genet Dev. 1996 Feb;6(1):56-64. Review.

Hatada I, Inazawa J, Abe T, Nakayama M, Kaneko Y, Jinno Y, Niikawa N, Ohashi H, Fukushima Y, Iida K, Yutani C, Takahashi S, Chiba Y, Ohishi S, Mukai T. Genomic imprinting of human p57KIP2 and its reduced expression in Wilms' tumors. Hum Mol Genet. 1996 Jun;5(6):783-8.

Haynes SR, Dollard C, Winston F, Beck S, Trowsdale J, Dawid IB. The bromodomain: a conserved sequence found in human, Drosophila and yeast proteins. Nucleic Acids Res. 1992 May 25;20(10):2603.

He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. Identification of c-MYC as a target of the APC pathway. Science. 1998 Sep 4;281(5382):1509-12.

Hockings JK, Degner SC, Morgan SS, Kemp MQ, Romagnolo DF. Involvement of a specificity proteins-binding element in regulation of basal and estrogen-induced transcription activity of the BRCA1 gene. Breast Cancer Res. 2008;10(2):R29

Howard A, Pelc SR. Nuclear incorporation of P32 as demonstrated by autoradiographs. Exp. Cell Res. 1951. 2:178–187

Hsieh JK, Chan FS, O'Connor DJ, Mittnacht S, Zhong S, Lu X. RB regulates the stability and the apoptotic function of p53 via MDM2. Mol Cell. 1999 Feb;3(2):181-93.

Hu E, Dul E, Sung CM, Chen Z, Kirkpatrick R, Zhang GF, Johanson K, Liu R, Lago A, Hofmann G, Macarron R, de los Frailes M, Perez P, Krawiec J, Winkler J, Jaye M. Identification of novel isoform-selective inhibitors within class I histone deacetylases. J Pharmacol Exp Ther. 2003 Nov;307(2):720-8.

Huang BH, Laban M, Leung CH, Lee L, Lee CK, Salto-Tellez M, Raju GC, Hooi SC. Inhibition of histone deacetylase 2 increases apoptosis and p21Cip1/WAF1 expression, independent of histone deacetylase 1. Cell Death Differ. 2005 Apr;12(4):395-404.

Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A, Yoshida M, Wang XF, Yao TP. HDAC6 is a microtubule-associated deacetylase. Nature. 2002 May 23;417(6887):455-8.

Ianari A, Gallo R, Palma M, Alesse E, Gulino A. Specific role for p300/CREB-binding protein-associated factor activity in E2F1 stabilization in response to DNA damage. J Biol Chem. 2004 Jul 16;279(29):30830-5.

Ikura T, Ogryzko VV, Grigoriev M, Groisman R, Wang J, Horikoshi M, Scully R, Qin J, Nakatani Y. Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. Cell. 2000 Aug 18;102(4):463-73.

Imhof A, Yang XJ, Ogryzko VV, Nakatani Y, Wolffe AP, Ge H. Acetylation of general transcription factors by histone acetyltransferases. Curr Biol. 1997 Sep 1;7(9):689-92. Review.

Ito Y, Takeda T, Sakon M, Tsujimoto M, Monden M, Matsuura N. Expression of p57/Kip2 protein in hepatocellular carcinoma. Oncology. 2001;61(3):221-5

Iyer NG, Ozdag H, Caldas C. p300/CBP and cancer. Oncogene. 2004 May 24;23(24):4225-31. Review.

Iyer NG, Xian J, Chin SF, Bannister AJ, Daigo Y, Aparicio S, Kouzarides T, Caldas C. p300 is required for orderly G1/S transition in human cancer cells. Oncogene. 2007 Jan 4;26(1):21-9.

Jacobson RH, Ladurner AG, King DS, Tjian R. Structure and function of a human TAFII250 double bromodomain module. Science. 2000 May 26;288(5470):1422-5.

Jeffy BD, Hockings JK, Kemp MQ, Morgan SS, Hager JA, Beliakoff J, Whitesell LJ, Bowden GT, Romagnolo DF. An estrogen receptor-alpha/p300 complex activates the BRCA-1 promoter at an AP-1 site that binds Jun/Fos transcription factors: repressive effects of p53 on BRCA-1 transcription. Neoplasia. 2005 Sep;7(9):873-82.

Jiang M, Shao ZM, Wu J, Lu JS, Yu LM, Yuan JD, Han QX, Shen ZZ, Fontana JA. p21/waf1/cip1 and mdm-2 expression in breast carcinoma patients as related to prognosis. Int J Cancer. 1997 Oct 21;74(5):529-34.

Johannessen CU, Johannessen SI. Valproate: past, present, and future. CNS Drug Rev. 2003 Summer;9(2):199-216. Review.

Johnson DG, Walker CL. Cyclins and cell cycle checkpoints. Annu Rev Pharmacol Toxicol. 1999;39:295-312. Review.

Johnstone RW, Licht JD. Histone deacetylase inhibitors in cancer therapy: is transcription the primary target? Cancer Cell. 2003 Jul;4(1):13-8. Review.

Juan LJ, Shia WJ, Chen MH, Yang WM, Seto E, Lin YS, Wu CW. Histone deacetylases specifically down-regulate p53-dependent gene activation. J Biol Chem. 2000 Jul 7;275(27):20436-43.

Kao HY, Verdel A, Tsai CC, Simon C, Juguilon H, Khochbin S. Mechanism for nucleocytoplasmic shuttling of histone deacetylase 7. J Biol Chem. 2001 Dec 14;276(50):47496-507.

Karanam B, Jiang L, Wang L, Kelleher NL, Cole PA. Kinetic and mass spectrometric analysis of p300 histone acetyltransferase domain autoacetylation. J Biol Chem. 2006 Dec 29;281(52):40292-301.

Kobayashi H, Tan EM, Fleming SE. Acetylation of histones associated with the p21WAF1/CIP1 gene by butyrate is not sufficient for p21WAF1/CIP1 gene transcription in human colorectal adenocarcinoma cells. Int J Cancer. 2004 Mar 20;109(2):207-13.

Kraus WL, Manning ET, Kadonaga JT. Biochemical analysis of distinct activation functions in p300 that enhance transcription initiation with chromatin templates. Mol Cell Biol. 1999 Dec;19(12):8123-35.

Kelly WK, Marks PA. Drug insight: Histone deacetylase inhibitors--development of the new targeted anticancer agent suberoylanilide hydroxamic acid. Nat Clin Pract Oncol. 2005 Mar;2(3):150-7. Review.

Kim EJ, Shin HK, Cho JS, Lee SK, Won MH, Kim JW, Park JH. trans-10,cis-12 conjugated linoleic acid inhibits the G1-S cell cycle progression in DU145 human prostate carcinoma cells. J Med Food. 2006;9(3):293-9.

Kouzarides T. Chromatin modifications and their function. Cell. 2007 Feb 23;128(4):693-705. Review.

Leder A, Orkin S, Leder P. Differentiation of erythroleukemic cells in the presence of inhibitors of DNA synthesis. Science. 1975 Nov 28;190(4217):893-4.

Lee KK, Workman JL. Histone acetyltransferase complexes: one size doesn't fit all. Nat Rev Mol Cell Biol. 2007 Apr;8(4):284-95. Review.

Lee MH, Reynisdóttir I, Massagué J. Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes Dev. 1995 Mar 15;9(6):639-49.

Legube G, Trouche D. Regulating histone acetyltransferases and deacetylases. EMBO Rep. 2003 Oct;4(10):944-7. Review.

Li J, Sutter C, Parker DS, Blauwkamp T, Fang M, Cadigan KM. CBP/p300 are bimodal regulators of Wnt signaling. EMBO J. 2007 May 2;26(9):2284-94

Li Q, Herrler M, Landsberger N, Kaludov N, Ogryzko VV, Nakatani Y, Wolffe AP. Xenopus NF-Y pre-sets chromatin to potentiate p300 and acetylation-responsive transcription from the Xenopus hsp70 promoter in vivo. EMBO J. 1998 Nov 2;17(21):6300-15.

Li Q, Su A, Chen J, Lefebvre YA, Haché RJ. Attenuation of glucocorticoid signaling through targeted degradation of p300 via the 26S proteasome pathway. Mol Endocrinol. 2002 Dec;16(12):2819-27.

Lill NL, Grossman SR, Ginsberg D, DeCaprio J, Livingston DM. Binding and modulation of p53 by p300/CBP coactivators. Nature. 1997 Jun 19;387(6635):823-7.

Lim JH, Park JW, Kim MS, Park SK, Johnson RS, Chun YS. Bafilomycin induces the p21-mediated growth inhibition of cancer cells under hypoxic conditions by expressing hypoxia-inducible factor-1alpha. Mol Pharmacol. 2006 Dec;70(6):1856-65.

Liu BP, Chong EY, Cheung FW, Duan JA, Che CT, Liu WK. Tangutorine induces p21 expression and abnormal mitosis in human colon cancer HT-29 cells. Biochem Pharmacol. 2005 Jul 15;70(2):287-99.

Löscher W. Valproate: a reappraisal of its pharmacodynamic properties and mechanisms of action. Prog Neurobiol. 1999 May;58(1):31-59. Review.

Luger K, Mäder AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature. 1997 Sep 18;389(6648):251-60.

Mann BS, Johnson JR, Cohen MH, Justice R, Pazdur R. FDA approval summary: vorinostat for treatment of advanced primary cutaneous T-cell lymphoma. Oncologist. 2007 Oct;12(10):1247-52.

Manning ET, Ikehara T, Ito T, Kadonaga JT, Kraus WL. p300 forms a stable, template-committed complex with chromatin: role for the bromodomain. Mol Cell Biol. 2001 Jun;21(12):3876-87.

Martínez-Balbás MA, Bauer UM, Nielsen SJ, Brehm A, Kouzarides T. Regulation of E2F1 activity by acetylation. EMBO J. 2000 Feb 15;19(4):662-71.

Marzio G, Wagener C, Gutierrez MI, Cartwright P, Helin K, Giacca M. E2F family members are differentially regulated by reversible acetylation. J Biol Chem. 2000 Apr 14;275(15):10887-92

Masters JR. HeLa cells 50 years on: the good, the bad and the ugly. Nat Rev Cancer. 2002 Apr;2(4):315-9.

Matsuoka S, Edwards MC, Bai C, Parker S, Zhang P, Baldini A, Harper JW, Elledge SJ. p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene.Genes Dev. 1995 Mar 15;9(6):650-62.

Mayo MW, Denlinger CE, Broad RM, Yeung F, Reilly ET, Shi Y, Jones DR. Ineffectiveness of histone deacetylase inhibitors to induce apoptosis involves the transcriptional activation of NF-kappa B through the Akt pathway. J Biol Chem. 2003 May 23;278(21):18980-9.

McInnes C. Progress in the evaluation of CDK inhibitors as anti-tumor agents. Drug Discov Today. 2008 Oct;13(19-20):875-81

Michelson AM. Deciphering genetic regulatory codes: a challenge for functional genomics. Proc Natl Acad Sci U S A. 2002 Jan 22;99(2):546-8.

Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science. 1994 Oct 7;266(5182):66-71.

Missero C, Calautti E, Eckner R, Chin J, Tsai LH, Livingston DM, Dotto GP. Involvement of the cell-cycle inhibitor Cip1/WAF1 and the E1A-associated p300 protein in terminal differentiation. Proc Natl Acad Sci U S A. 1995 Jun 6;92(12):5451-5.

Mizzen CA, Yang XJ, Kokubo T, Brownell JE, Bannister AJ, Owen-Hughes T, Workman J, Wang L, Berger SL, Kouzarides T, Nakatani Y, Allis CD.The TAF(II)250 subunit of TFIID has histone acetyltransferase activity. Cell. 1996 Dec 27;87(7):1261-70.

Morgan DO. Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol. 1997;13:261-91. Review.

Morgan DO. Principles of CDK regulation. Nature. 1995 Mar 9;374(6518):131-4. Review.

Munshi N, Merika M, Yie J, Senger K, Chen G, Thanos D. Acetylation of HMG I(Y) by CBP turns off IFN beta expression by disrupting the enhanceosome. Mol Cell. 1998 Oct;2(4):457-67.

Narlikar GJ, Fan HY, Kingston RE. Cooperation between complexes that regulate chromatin structure and transcription. Cell. 2002 Feb 22;108(4):475-87. Review.

Nigg EA. Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. Bioessays. 1995 Jun;17(6):471-80. Review.

Nightingale KP, O'Neill LP, Turner BM. Histone modifications: signalling receptors and potential elements of a heritable epigenetic code. Curr Opin Genet Dev. 2006 Apr;16(2):125-36.

Noh EJ, Lee JS. Functional interplay between modulation of histone deacetylase activity and its regulatory role in G2-M transition. Biochem Biophys Res Commun. 2003 Oct 17;310(2):267-73.

Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell. 1996 Nov 29;87(5):953-9.

Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM, Pagano M. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol Cell Biol. 1995 May;15(5):2612-24.

Ohshima T, Suganuma T, Ikeda M. A novel mutation lacking the bromodomain of the transcriptional coactivator p300 in the SiHa cervical carcinoma cell line. Biochem Biophys Res Commun. 2001 Feb 23;281(2):569-75.

Orphanides G, Reinberg D. A unified theory of gene expression. Cell. 2002 Feb 22;108(4):439-51. Review.

Pao GM, Janknecht R, Ruffner H, Hunter T, Verma IM. CBP/p300 interact with and function as transcriptional coactivators of BRCA1. Proc Natl Acad Sci U S A. 2000 Feb 1;97(3):1020-5.

Pazin MJ, Kadonaga JT. SWI2/SNF2 and related proteins: ATP-driven motors that disrupt protein-DNA interactions? Cell. 1997 Mar 21;88(6):737-40. Review.

Peng J, Zhu Y, Milton JT, Price DH. Identification of multiple cyclin subunits of human P-TEFb. Genes Dev. 1998 Mar 1;12(5):755-62.

Perrine SP, Ginder GD, Faller DV, Dover GH, Ikuta T, Witkowska HE, Cai SP, Vichinsky EP, Olivieri NF. A short-term trial of butyrate to stimulate fetal-globin-gene expression in the beta-globin disorders. N Engl J Med. 1993 Jan 14;328(2):81-6.
Polakis P. Wnt signaling and cancer. Genes Dev. 2000 Aug 1;14(15):1837-51. Review.

Ramji DP, Foka P. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J. 2002 Aug 1;365(Pt 3):561-75. Review.

Riester D, Hildmann C, Schwienhorst A. Histone deacetylase inhibitors: turning epigenic mechanisms of gene regulation into tools of therapeutic intervention in malignant and other diseases. Appl Microbiol Biotechnol. 2007 Jun;75(3):499-514.

Riggs MG, Whittaker RG, Neumann JR, Ingram VM. n-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. Nature. 1977 Aug 4;268(5619):462-4.

Rokhlin OW, Glover RB, Guseva NV, Taghiyev AF, Kohlgraf KG, Cohen MB. Mechanisms of cell death induced by histone deacetylase inhibitors in androgen receptor-positive prostate cancer cells. Mol Cancer Res. 2006 Feb;4(2):113-23.

Roth SY, Allis CD. Chromatin condensation: does histone H1 dephosphorylation play a role? Trends Biochem Sci. 1992 Mar;17(3):93-8. Review.

Sadhale P, Verma J, Naorem A. Basal transcription machinery: role in regulation of stress response in eukaryotes. J Biosci. 2007 Apr;32(3):569-78. Review.

Santoso B, Kadonaga JT. Reconstitution of chromatin transcription with purified components reveals a chromatin-specific repressive activity of p300. Nat Struct Mol Biol. 2006 Feb;13(2):131-9.

Schiltz RL, Mizzen CA, Vassilev A, Cook RG, Allis CD, Nakatani Y. Overlapping but distinct patterns of histone acetylation by the human coactivators p300 and PCAF within nucleosomal substrates. J Biol Chem. 1999 Jan 15;274(3):1189-92.

Scolnick DM, Chehab NH, Stavridi ES, Lien MC, Caruso L, Moran E, Berger SL, Halazonetis TD. CREB-binding protein and p300/CBP-associated factor are transcriptional coactivators of the p53 tumor suppressor protein. Cancer Res. 1997 Sep 1;57(17):3693-6.

Segal E, Shapira M, Regev A, Pe'er D, Botstein D, Koller D, Friedman N. Module networks: identifying regulatory modules and their condition-specific regulators from gene expression data. Nat Genet. 2003 Jun;34(2):166-76.

Sherr CJ. Cancer cell cycles. Science. 1996 Dec 6;274(5293):1672-7. Review.

Siavoshian S, Blottière HM, Le Foll E, Kaeffer B, Cherbut C, Galmiche JP. Comparison of the effect of different short chain fatty acids on the growth and differentiation of human colonic carcinoma cell lines in vitro. Cell Biol Int. 1997 May;21(5):281-7.

Siavoshian S, Segain JP, Kornprobst M, Bonnet C, Cherbut C, Galmiche JP, Blottière HM. Butyrate and trichostatin A effects on the proliferation/differentiation of human intestinal epithelial cells: induction of cyclin D3 and p21 expression. Gut. 2000 Apr;46(4):507-14.

Simone C, Stiegler P, Forcales SV, Bagella L, De Luca A, Sartorelli V, Giordano A, Puri PL. Deacetylase recruitment by the C/H3 domain of the acetyltransferase p300. Oncogene. 2004 Mar 18;23(12):2177-87.

Smith AT, Tucker-Samaras SD, Fairlamb AH, Sullivan WJ Jr. MYST family histone acetyltransferases in the protozoan parasite Toxoplasma gondii. Eukaryot Cell. 2005 Dec;4(12):2057-65.

Smith JL, Freebern WJ, Collins I, De Siervi A, Montano I, Haggerty CM, McNutt MC, Butscher WG, Dzekunova I, Petersen DW, Kawasaki E, Merchant JL, Gardner K. Kinetic profiles of p300 occupancy in vivo predict common features of promoter structure and coactivator recruitment. Proc Natl Acad Sci U S A. 2004 Aug 10;101(32):11554-9.

Snowden AW, Anderson LA, Webster GA, Perkins ND. A novel transcriptional repression domain mediates p21(WAF1/CIP1) induction of p300 transactivation. Mol Cell Biol. 2000 Apr;20(8):2676-86. Mol Cell Biol 2000 Jul;20(14):5360.

Snowden AW, Perkins ND. Cell cycle regulation of the transcriptional coactivators p300 and CREB binding protein. Biochem Pharmacol. 1998 Jun 15;55(12):1947-54.

Somasundaram K, El-Deiry WS. Inhibition of p53-mediated transactivation and cell cycle arrest by E1A through its p300/CBP-interacting region.Oncogene. 1997 Mar 6;14(9):1047-57.

Stauffer D, Chang B, Huang J, Dunn A, Thayer M. p300/CREB-binding protein interacts with ATR and is required for the DNA replication checkpoint. J Biol Chem. 2007 Mar 30;282(13):9678-87.

Sterner DE, Berger SL. Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev. 2000 Jun;64(2):435-59. Review.

Sterner DE, Grant PA, Roberts SM, Duggan LJ, Belotserkovskaya R, Pacella LA, Winston F, Workman JL, Berger SL. Functional organization of the yeast SAGA complex: distinct components involved in structural integrity, nucleosome acetylation, and TATA-binding protein interaction. Mol Cell Biol. 1999 Jan;19(1):86-98.

Stiehl DP, Fath DM, Liang D, Jiang Y, Sang N. Histone deacetylase inhibitors synergize p300 autoacetylation that regulates its transactivation activity and complex formation. Cancer Res. 2007 Mar 1;67(5):2256-64.

Strahl BD, Allis CD. The language of covalent histone modifications. Nature. 2000 Jan 6;403(6765):41-5.

Studzinski GP, Lambert WC. Thymidine as a synchronizing agent. I. Nucleic acid and protein formation in synchronous HeLa cultures treated with excess thymidine. J Cell Physiol. 1969 Apr;73(2):109-17.

Suganuma T, Kawabata M, Ohshima T, Ikeda MA. Growth suppression of human carcinoma cells by reintroduction of the p300 coactivator. Proc Natl Acad Sci U S A. 2002 Oct 1;99(20):13073-8.

Sukhatme VP, Cao X, Chang LL, Tsai-Morris C-H, Stamenkovich D, Ferreira PCP, Cohen DR, Edwards SA, Shows TB, Curran T, Le Beau MM, Adamson ED. A zinc-finger encoding gene corregulated with c-Fos during growth and differentiation and after depolarization. *Cell.* 1988; 53: 37–43

Sun A, Bagella L, Tutton S, Romano G, Giordano A. From G0 to S phase: a view of the roles played by the retinoblastoma (Rb) family members in the Rb-E2F pathway. J Cell Biochem. 2007 Dec 15;102(6):1400-4. Review.

Takai N, Desmond JC, Kumagai T, Gui D, Said JW, Whittaker S, Miyakawa I, Koeffler HP. Histone deacetylase inhibitors have a profound antigrowth activity in endometrial cancer cells. Clin Cancer Res. 2004 Feb 1;10(3):1141-9.

Tamkun JW. The role of brahma and related proteins in transcription and development. Curr Opin Genet Dev. 1995 Aug;5(4):473-7. Review.

Tamkun JW, Deuring R, Scott MP, Kissinger M, Pattatucci AM, Kaufman TC, Kennison JA. brahma: a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. Cell. 1992 Feb 7;68(3):561-72.

Tanaka Y, Naruse I, Maekawa T, Masuya H, Shiroishi T, Ishii S. Abnormal skeletal patterning in embryos lacking a single Cbp allele: a partial similarity with Rubinstein-Taybi syndrome. Proc Natl Acad Sci U S A. 1997 Sep 16;94(19):10215-20.

Taubert S, Gorrini C, Frank SR, Parisi T, Fuchs M, Chan HM, Livingston DM, Amati B. E2F-dependent histone acetylation and recruitment of the Tip60 acetyltransferase complex to chromatin in late G1. Mol Cell Biol. 2004 May;24(10):4546-56.

Thompson PR, Wang D, Wang L, Fulco M, Pediconi N, Zhang D, An W, Ge Q, Roeder RG, Wong J, Levrero M, Sartorelli V, Cotter RJ, Cole PA. Regulation of the p300 HAT domain via a novel activation loop. Nat Struct Mol Biol. 2004 Apr;11(4):308-15.

Vignali M, Hassan AH, Neely KE, Workman JL. ATP-dependent chromatin-remodeling complexes. Mol Cell Biol. 2000 Mar;20(6):1899-910. Review.

Vo N, Goodman RH. CREB-binding protein and p300 in transcriptional regulation. J Biol Chem. 2001 Apr 27;276(17):13505-8.

Vousden KH. p53: death star. Cell. 2000 Nov 22;103(5):691-4. Review.

Wang H, Larris B, Peiris TH, Zhang L, Le Lay J, Gao Y, Greenbaum LE. C/EBPbeta activates E2F-regulated genes in vivo via recruitment of the coactivator CREB-binding protein/P300. J Biol Chem. 2007 Aug 24;282(34):24679-88.

Wang J, Hoshino T, Redner RL, Kajigaya S, Liu JM. ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. Proc Natl Acad Sci U S A. 1998 Sep 1;95(18):10860-5.

Wang X, Pan L, Feng Y, Wang Y, Han Q, Han L, Han S, Guo J, Huang B, Lu J. P300 plays a role in p16(INK4a) expression and cell cycle arrest. Oncogene. 2008 Mar 20;27(13):1894-904.

Wang ZY, Deuel TF. An S1 nuclease-sensitive homopurine/homopyrimidine domain in the PDGF A-chain promoter contains a novel binding site for the growth factor-inducible protein EGR-1. Biochem Biophys Res Commun. 1992 Oct 15;188(1):433-9.

Wegener D, Hildmann C, Riester D, Schober A, Meyer-Almes FJ, Deubzer HE, Oehme I, Witt O, Lang S, Jaensch M, Makarov V, Lange C, Busse B, Schwienhorst A. Identification of novel small-molecule histone deacetylase inhibitors by medium-throughput screening using a fluorigenic assay. Biochem J. 2008 Jul 1;413(1):143-50.

Wells J, Graveel CR, Bartley SM, Madore SJ, Farnham PJ. The identification of E2F1-specific target genes. Proc Natl Acad Sci U S A. 2002 Mar 19;99(6):3890-5.

Wittschieben BO, Fellows J, Du W, Stillman DJ, Svejstrup JQ. Overlapping roles for the histone acetyltransferase activities of SAGA and elongator in vivo. EMBO J. 2000 Jun 15;19(12):3060-8.

Whitfield ML, Sherlock G, Saldanha AJ, Murray JI, Ball CA, Alexander KE, Matese JC, Perou CM, Hurt MM, Brown PO, Botstein D. Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol Biol Cell. 2002 Jun;13(6):1977-2000.

Wilson AJ, Byun DS, Popova N, Murray LB, L'Italien K, Sowa Y, Arango D, Velcich A, Augenlicht LH, Mariadason JM. Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. J Biol Chem. 2006 May 12;281(19):13548-58.

Winston F, Allis CD. The bromodomain: a chromatin-targeting module? Nat Struct Biol. 1999 Jul;6(7):601-4.

Wolffe AP. Nucleosome positioning and modification: chromatin structures that potentiate transcription. Trends Biochem Sci. 1994 Jun;19(6):240-4. Review.

Xiao H, Hasegawa T, Isobe K.p300 collaborates with Sp1 and Sp3 in p21(waf1/cip1) promoter activation induced by histone deacetylase inhibitor. J Biol Chem. 2000 Jan 14;275(2):1371-6.

Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. Nature. 1993 Dec 16;366(6456):701-4.

Xu M, Sheppard KA, Peng CY, Yee AS, Piwnica-Worms H. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol Cell Biol. 1994 Dec;14(12):8420-31.

Yaciuk P, Carter MC, Pipas JM, Moran E. Simian virus 40 large-T antigen expresses a biological activity complementary to the p300-associated transforming function of the adenovirus E1A gene products. Mol Cell Biol. 1991 Apr;11(4):2116-24.

Yamamoto T, Horikoshi M. Novel substrate specificity of the histone acetyltransferase activity of HIV-1-Tat interactive protein Tip60. J Biol Chem. 1997 Dec 5;272(49):30595-8.

Yamauchi T, Yamauchi J, Kuwata T, Tamura T, Yamashita T, Bae N, Westphal H, Ozato K, Nakatani Y. Distinct but overlapping roles of histone acetylase PCAF and of the closely related PCAF-B/GCN5 in mouse embryogenesis. Proc Natl Acad Sci U S A. 2000 Oct 10;97(21):11303-6.

Yang XJ, Ogryzko VV, Nishikawa J, Howard BH, Nakatani Y. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature. 1996 Jul 25;382(6589):319-24.

Yao TP, Oh SP, Fuchs M, Zhou ND, Ch'ng LE, Newsome D, Bronson RT, Li E, Livingston DM, Eckner R. Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. Cell. 1998 May 1;93(3):361-72.

Yoshida M, Kijima M, Akita M, Beppu T. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem. 1990 Oct 5;265(28):17174-9.

Yu J, de Belle I, Liang H, Adamson ED. Coactivating factors p300 and CBP are transcriptionally crossregulated by Egr1 in prostate cells, leading to divergent responses. Mol Cell. 2004 Jul 2;15(1):83-94.

Zeng L, Zhou MM. Bromodomain: an acetyl-lysine binding domain. FEBS Lett. 2002 Feb 20;513(1):124-8. Review.

Zhang Y, Ng HH, Erdjument-Bromage H, Tempst P, Bird A, Reinberg D. Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev. 1999 Aug 1;13(15):1924-35.

Zhang P, Liégeois NJ, Wong C, Finegold M, Hou H, Thompson JC, Silverman A, Harper JW, DePinho RA, Elledge SJ. Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. Nature. 1997 May 8;387(6629):151-8.

Zhang Z, Yamashita H, Toyama T, Sugiura H, Omoto Y, Ando Y, Mita K, Hamaguchi M, Hayashi S, Iwase H. HDAC6 expression is correlated with better survival in breast cancer. Clin Cancer Res. 2004 Oct 15;10(20):6962-8.

Zhang Z, Yamashita H, Toyama T, Sugiura H, Ando Y, Mita K, Hamaguchi M, Hara Y, Kobayashi S, Iwase H. Quantitation of HDAC1 mRNA expression in invasive carcinoma of the breast. Breast Cancer Res Treat. 2005 Nov;94(1):11-6.

Zhao Y, Tan J, Zhuang L, Jiang X, Liu ET, Yu Q. Inhibitors of histone deacetylases target the Rb-E2F1 pathway for apoptosis induction through activation of proapoptotic protein Bim. Proc Natl Acad Sci U S A. 2005 Nov 1;102(44):16090-5.

Zhou C, Qiu L, Sun Y, Healey S, Wanebo H, Kouttab N, Di W, Yan B, Wan Y. Inhibition of EGFR/PI3K/AKT cell survival pathway promotes TSA's effect on cell death and migration in human ovarian cancer cells. Int J Oncol. 2006 Jul;29(1):269-78.

Supplementary Table 1. Reagent details.

Reagent	**Source**	**Catalog Number**
Dulbecco's Modified Eagle Medium	GIBCO-Invitrogen	12800-017
Pencillin/Streptomycin	GIBCO – Invitrogen	15140
MEM Non-Essential amino acids	GIBCO – Invitrogen	11140
Fetal Bovine Serum	Hyclone	SH30396.03
Fetal Bovine Serum	WISENT	080450
Sodium butyrate	Sigma-Aldrich	B5887
Valproic acid	Sigma-Aldrich	P4543
Trichostatin A	Sigma-Aldrich	T8552
Thymidine	Sigma-Aldrich	T9250
Nocodazole	Sigma-Aldrich	M1404
Bradford reagent	Bio-Rad	500-0006
PVDF membrane	Bio-Rad	162-0177
Western Lightning Chemiluminescence	Perkin Elmer	NEL105
RNeasy Mini kit	Qiagen	74104
High Capacity cDNA Archive Kit	Applied Biosystems	4322171
Formaldehyde	BDH	BDH0500
Protease inhibitor cocktail	Calbiochem	539134
Salmon Sperm DNA/ Protein A agarose	Upstate-Milipore	16-157
Proteinase K	Rouche	03115879001
QIAquick PCR Purification Kit	QIAGEN	28106
GoTaq® flexi DNA polymerase PCR Kit	Promega	PRM8295
Agarose	Rouche	1685 678

Supplementary Table 2. Antibodies details.

Antibody	Source	Catalog Number
Anti-mouse IgG horseradish peroxidase conjugate 2^{ry} Ab	GE Healthcare	NA931
Anti-rabbit IgG horseradish peroxidase conjugate 2^{ry} Ab	GE Healthcare	NA9340
Anti-p300 (N-15)	Santa cruz	sc-584
Anti-p21(F-2)	Santa cruz	sc-6246
Anti-p57(C-20)	Santa cruz	sc-1040
Anti-Egr1 (C-19)	Santa cruz	sc-189
Anti-E2f1(KH95)	Santa cruz	sc-251
Anti-Ac-Histone H3 (Lys 9/14)	Santa cruz	sc-8655
Anti-β-Actin	Sigma-Aldrich	A5441

Supplementary Table 3. Oligonucleotide sequences (from 5' to 3') of the primers used for PCR analysis in ChIP assays

Gene	Forward Primer	Reverse Primer
p21	AGTGCCAACTCATTCTCCAAGTAA AAA AAGCC	TCCCTCCTCCCCCAGTCCCTC
Egr1	GGAACCA GGGAGGAGGGAGGGAG	GCCGGGCCATATCGGGCCAC
p57	GGACGAGACAGGCGAACCCGAC	GAGAAGAAGGGGAAAGGAGAGG AGGAGAG
E2F1	CCCCTCGCCATTT CCAGGCAC	CACAGCCCAGCCATCAGCCAC