Regulation of Krüppel-like Factor 6 Tumor Suppressor Activity by Acetylation

Dan Li, Steven Yea, Georgia Dolios, John A. Martignetti, Goutham Narla, Rong Wang, Martin J. Walsh, and Scott L. Friedman

Division of Liver Diseases, Department of Medicine; and Departments of Human Genetics and Pediatrics, Mount Sinai School of Medicine, New York, New York

Abstract

Kruppel-like factor 6 (KLF6) is a zinc finger transcription factor and tumor suppressor that is inactivated in a number of human cancers by mutation, allelic loss, and/or promoter methylation. A key mechanism of growth inhibition by wild-type KLF6 is through p53-independent up-regulation of p21WAF1/cip1 (CDKN1A), which is abrogated in several tumor-derived mutants. Here we show by chromatin immunoprecipitation that transactivation of p21WAF1/cip1 by KLF6 occurs through its direct recruitment to the p21WAF1/cip1 promoter and requires acetylation by histone acetyltransferase activity of either cyclic AMP-responsive element binding protein–binding protein or p300/CBP-associated factor. Direct lysine acetylation of KLF6 peptides can be shown by mass spectrometry. A single lysine-to-arginine point mutation (K209R) derived from prostate cancer reduces acetylation of KLF6 and abrogates its capacity to up-regulate endogenous p21WAF1/cip1 and reduce cell proliferation. These data indicate that acetylation may regulate KLF6 function, and its loss in some tumor-derived mutants could contribute to its failure to suppress growth in prostate cancer. (Cancer Res 2005; 65(20): 9216-25)

Introduction

Kruppel-like factor 6 (KLF6) is an ubiquitously expressed zinc finger protein belonging to the KLF family of transcription factors (1, 2). These proteins are DNA-binding transcriptional regulators that serve myriad roles in differentiation and development (3, 4). All KLF members possess a distinct NH2-terminus activation domain and a highly conserved COOH terminus zinc finger DNA-binding domain that interacts with “GC box” or “CACC” DNA motifs in responsive promoters (3, 4). In contrast to the conserved DNA-binding domain among KLFs, the divergent activation domain of each family member accounts for their diverse biological activities.

KLF6 has broad activity in regulating cell growth, tissue injury, and differentiation. Its transcriptional targets include a placental glycoprotein (5), HIV-1 (6), collagen α1(I) (2), transforming growth factor β1 (TGFβ1), types I and II TGFβ receptors (7), nitric oxide synthase (8), and urokinase type plasminogen activator (9). It is also an immediate early gene up-regulated in hepatic steatol cells during acute liver injury (1) and during the differentiation of preadipocytes into adipocytes (10), suggesting a generalized function of KLF6 in different biological contexts.

KLF6 has recently been identified as a tumor suppressor gene that is inactivated in primary prostate (1, 11), colon (12) nasopharyngeal (13), glial (14), and hepatocellular (15) cancers and is down-regulated in lung and prostate cancers (16, 17). Decreased KLF6 mRNA expression correlates with clinical outcome in prostate cancer (17). Somatic loss of heterozygosity (LOH) and DNA mutations result in functional deletion of the KLF6 gene in ~60% of prostate and 45% of colon tumors (1, 11, 12). KLF6 promoter silencing by methylation also has been uncovered in esophageal cancer cell lines (18).

One mechanism by which KLF6 reduces cell proliferation is through up-regulation of p21WAF1/cip1, a key cyclin-dependent kinase (cdk) inhibitor. This induction does not require p53, a well-established transactivator of p21WAF1/cip1, because p21WAF1/cip1 induction by KLF6 is preserved in p53-null cells (1). The molecular requirements for p21WAF1/cip1 up-regulation by KLF6 have not been defined, and information about transcriptional coactivators within KLF6 transcriptional complexes is limited. Heterologous interaction of KLF6 with p40 or Sp1 is required for keratin (19) or endoglin gene expression (20), respectively, but potential interacting proteins in that context have not been explored.

Coactivators, specifically histone acetyltransferases (HAT), contribute to the transcriptional activity of other tumor suppressors (e.g., p53), where recruitment of HATs is vital to its function (21). HATs are chromatin-modifying proteins that directly regulate transcription through interaction with transcription factors (22), including p53 (23, 24), pRB (25), and BRCA1 (26), as well as the zinc finger transcription factors Sp1 (27) and EKLF (28, 29). Altered interactions between HATs and transcription factors may contribute to tumorigenesis. For example, disruption of the p300-p53 interaction may underlie the mechanism by which the viral oncoprotein E1A induces cell transformation (23, 24, 30).

In vivo, HATs function as part of large protein complexes that share a highly conserved acetyl-CoA binding motif but have different substrate specificities. Among the best studied HATs, cyclic AMP–responsive element binding protein–binding protein (CBP) and p300/CBP-associated factor (PCAF) mediate gene transcription through the acetylation of specific lysine residues on chromatin, and function as coactivators for a number of transcription factors regulating cell growth and development (31–34). In addition, acetylation of nonhistone proteins has emerged as a novel mechanism of posttranslational modification (35, 36).

Our identification of a KLF6 lysine-to-arginine mutation in primary prostate cancer raised the prospect that mutation of an acetylation substrate site might contribute to the loss of growth suppressive activity through the abrogation of p21WAF1/cip1 transactivation. In the present study, we have explored the role of CBP and PCAF in up-regulating p21WAF1/cip1 gene expression by KLF6. Loss of lysine through mutation impairs KLF6’s capacity to transactivate the p21WAF1/cip1 promoter or up-regulate endogenous...
p21^{WAF1/cip1}. These findings suggest that acetylation of KLF6 plays a critical role in its regulation of target gene expression. Given its role as a tumor suppressor, loss of KLF6 acetylation may be associated with tumorigenesis.

Materials and Methods

Expression plasmids. PCneo-KLF6 (human; previously known as "Z9") expression plasmid was constructed as previously described (1), HA-CBP was a gift from Dr. R.H. Goodman, Vumul University, Oregon Health Sciences University, Portland, OR (37). PCI-PCAF plasmid was a gift from Dr. Yoshioho Nakatani, Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA (38). p21^{WAF1/cip1} promoter-luciferase construct was a gift from Dr. Toru Ouchi, Department of Oncological Sciences, Mount Sinai School of Medicine, New York, NY (39). pRL-TK Vector used as a control for transfection efficiency was from Dual-luciferase Reporter Assay System (Promega, Madison, WI).

Site-directed mutagenesis. A lysine-to-arginine point mutant of KLF6 (pCIneoK209R) was constructed using Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA), as described below. pCIneo-KLF6 (human) was used as the template for the mutagenesis. The primers used for mutagenesis were K209R sense, 5'-CACCATTAAACGGCTGACGAGATTTACACCAAAAGC-3' and K209R antisense, 5'-GCTTTTGGTGTAAACTGCAGTGAG-3'. All mutated constructs were sequenced on both strands to verify these mutations and to confirm that no other alterations were introduced.

Cell culture. Prostate cancer 3 (PC3M) cells, 293 cells, and 293T cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in DMEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 units/ml penicillin and 100 units/ml streptomycin, and 2 mmol/L L-glutamine (Life Technologies, Gaithersburg, MD). Treatment with trichostatin A (Sigma-Aldrich, St. Louis, MO) was used at a final concentration of 0.5 

MD). Treatment with trichostatin A (Sigma-Aldrich, St. Louis, MO) with 50 

μg/mL of puromycin, and pooled clones of cells were used in subsequent experiments.

Western blotting. Cell extracts were harvested in radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, standard protocol). Protein samples (30 μg per sample) were separated on SDS-polyacrylamide gel (6% for CBP and 10% for KLF6 and PCAF) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were blocked in 5% dried milk in 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 0.1% Tween 20 (1× TBS-T) for 1 hour at room temperature. The membrane was incubated with primary antibody: anti-Z9/KLF6 antibody (R-173, Santa Cruz Biotechnology), or 4 μg of anti-HA antibody (Santa Cruz Biotechnology) for 1 hour at 4°C. The membrane was washed with 1× TBS for 20 minutes and incubated with the secondary antibody (horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG (Amersham Pharmacia, Piscataway, NJ) at 1:2,000 dilution followed by enhanced chemiluminescence protocol (Amersham Pharmacia). For Western blotting, cell extracts were harvested in radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, standard protocol). Protein samples (30 μg per sample) were separated on SDS-polyacrylamide gel (6% for CBP and 10% for KLF6 and PCAF) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were blocked in 5% dried milk in 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 0.1% Tween 20 (1× TBS-T) for 1 hour at room temperature. The membrane was incubated with primary antibody: anti-Z9/KLF6 antibody (R-173, Santa Cruz Biotechnology), or 4 μg of anti-HA antibody (Santa Cruz Biotechnology) for 1 hour at 4°C. The membrane was washed with 1× TBS for 20 minutes and incubated with the secondary antibody (horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG (Amersham Pharmacia, Piscataway, NJ) at 1:2,000 dilution followed by enhanced chemiluminescence protocol (Amersham Pharmacia). For Western blotting, cell extracts were harvested in radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, standard protocol). Protein samples (30 μg per sample) were separated on SDS-polyacrylamide gel (6% for CBP and 10% for KLF6 and PCAF) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were blocked in 5% dried milk in 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 0.1% Tween 20 (1× TBS-T) for 1 hour at room temperature. The membrane was incubated with primary antibody: anti-Z9/KLF6 antibody (R-173, Santa Cruz Biotechnology), or 4 μg of anti-HA antibody (Santa Cruz Biotechnology) for 1 hour at 4°C. The membrane was washed with 1× TBS for 20 minutes and incubated with the secondary antibody (horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG (Amersham Pharmacia, Piscataway, NJ) at 1:2,000 dilution followed by enhanced chemiluminescence protocol (Amersham Pharmacia).
Pharmacia) for 40 minutes, and exposed to X-ray film for autoradiography for ~10 minutes.

**Analysis of proliferation.** Proliferation was determined by estimating ³H-thymidine incorporation. BPH1 and PC3M cell lines stably expressing the appropriate expression vectors were plated at a density of 50,000 cells per well in 12-well dishes. Twenty-four hours after plating, 1 μCi/ml ³H-thymidine (Amersham Biosciences) was added. After 2 hours, cells were washed four times with ice-cold PBS and fixed in methanol for 30 minutes at 4°C. After methanol removal and cell drying, cells were solubilized in 0.25% sodium hydroxide/0.25% SDS. After neutralization with hydrochloric acid (1 N), disintegrations per minute were estimated by liquid scintillation counting. This process was repeated at 48 and 72 hours.

**Tumor samples.** The preparation of tumor samples was as previously described (1). Briefly, 5-μm sections stained with H&E were used as an accurate histologic reference for normal and tumor-derived tissue. Microdissection was done on sequential 20-μm sections and DNA subsequently extracted (Ambion paraffin block isolation kit, Austin, TX). DNA was isolated by proteinase K digestion overnight at 37°C incubator followed by heat inactivation at 95°C for 10 minutes. KLF6 sequence analysis of tumor samples was done as previously described (1).

**Peptide acetylation assay.** Four peptides were synthesized using commercially available resources (Invitrogen) covering the majority of lysine residues within the KLF6 molecule. The peptides’ sequences are as follows: (a) h68-87 ILAREKEESKISSSPPE, (b) h115-134 SSEELSPSTAKFSDPGEVL, (c) h204-223 FNGCBKVYTKSSHLKHQFR, and (d) h248-267 TRIFIHRKHTGPKFCSCHDR. For peptide acetylation assay, 5 μg of peptide were incubated at 30°C for 1.5 hours with 5 ng CBP or PCAF, 10 μL of 1 mmol/L acetyl CoA, 5 μL of 0.1 mol/L sodium butyrate, in the presence or absence of 10 μL ³H-acetyl CoA (0.5 μCi/μL, Amersham Pharmacia), in 1× HA assay buffer (Upstate Biotechnology). Following the reaction, the mixture was analyzed by scintillation counting to confirm ³H incorporation followed by mass spectrometry (MS) analysis. To assess ³H incorporation, 5 μL of reaction mixture were spotted onto a small square of filter paper followed by washing with 50 mmol/L Na2HPO4 (pH 9.0), then the filter paper was placed into scintillation fluid overnight for counting the next day.

**Mass spectrometry analysis.** KLF6 peptides and their acetylation products (1 pmol) were isolated and purified using Poros 20 R2 beads (Applied Biosystems, Foster City, CA) and ZipTipC₁₈ pipette tip (Millipore, Bedford, MA) following the manufacturer’s protocol. Molecular masses of the synthetic peptides before and after acetylation reaction were accurately measured by matrix-assisted laser desorption ionization-MS (MALDI-MS) using a QSTAR XL hybrid quadrapole time-of-flight mass spectrometer (Applied Biosystems), α-Cyano-4-hydroxy-cinnamic acid was used as matrix for sample preparation. To determine the acetylation site(s), fragment spectra of peaks corresponding to acetylated peptides were collected and analyzed by MALDI tandem MS (MS/MS) experiment using the same mass spectrometer.

**Results**

**KLF6 targets the p21/CIP1 (CDKN1A) locus and is associated with hyperacetylation of histone H3.** To determine if KLF6 interacts directly with the endogenous p21/CIP1 (CDKN1A) promoter, we used chromatin immunoprecipitation (Fig. 1), PCR reactions were carried out using primers encompassing −150 to −3 bp upstream of the start site of p21/WAF1/CIP1 transcription as shown (Fig. 1A), which contains multiple GC boxes predicted by sequence homology to interact with KLF6 and related family members. Chromatin immunoprecipitation analysis confirmed that KLF6 binds to this region of the endogenous p21/WAF1/CIP1 promoter, establishing p21/WAF1/CIP1 as a transcriptional target of KLF6 (Fig. 1B). Interestingly, we were unable to show the binding of Spl to the same region despite reports to the contrary based on electromobility shift assay (41). To confirm the ability of the Spl antibody to recognize Spl in the chromatin immunoprecipitation assay, a positive control was used to verify Spl on other loci (data not shown). Furthermore, ectopic expression of KLF6 showed increased recruitment of acetylated histone H3 encompassing the region between −150 to −3 of the KLF6 promoter when compared with the control expression vector (Fig. 1C).

**KLF6 interacts with CBP and PCAF in vivo.** Because CBP and PCAF interact with critical cellular proteins, including p53 (21) and p21/CIP1 (42) leading to altered function, we examined whether KLF6 interacted with endogenous KLF6 and CBP using a coimmunoprecipitation assay. As shown in Fig. 2A, CBP was associated with KLF6 when compared with the control expression vector (Fig. 1C). More specifically, ectopic expression of KLF6 showed increased recruitment of acetylated histone H3 encompassing the region between −150 to −3 of the KLF6 promoter when compared with the control expression vector (Fig. 1C).

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**Figure 1.** KLF6 is directly associated with the p21/WAF1/CIP1 (CDKN1A) locus and deposition of acetylated histone H3. A, schematic representation of p21/WAF1/CIP1 promoter. PCR for chromatin immunoprecipitation (ChIP) assay was done using primers (small arrows) positioned at −150 and −3 bp upstream of the transcriptional start site of p21/WAF1/CIP1 promoter. This GC-rich region contains five putative binding sites for Spl as well as for Spl, all of which are between −102 and −30 bp region, as shown in (A). B, chromatin immunoprecipitation assay at p21/WAF1/CIP1 promoter in 293 cells. Anti-KLF6 antibody (α-KLF6) was used for immunoprecipitation. Anti-histone H3 (α-H3) antibody was used as a positive control. The binding of Spl to p21/WAF1/CIP1 promoter is also examined using anti-Spl antibody (α-Spl). C, acetylation of the p21/WAF1/CIP1 (CDKN1A) locus within the gene promoter was monitored upon transfection with a KLF6 expression vector by chromatin immunoprecipitation analysis. Input levels of chromatin for immunoprecipitation reactions were monitored for total histone H3. HDAC inhibitors trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) at a final concentration of 0.5 μmol/L were used to compare acetylation of histone H3.
done from a lysate of $1 \times 10^6$ NIH 3T3 cells using a monoclonal anti-KLF6 antibody cross-linked to protein G agarose. Western blot using anti-CBP antibody revealed that CBP was coimmunoprecipitated with KLF6 (Fig. 2B). The interaction between KLF6 and PCAF was also detected using a similar approach (Fig. 2C).

CBP and PCAF synergize with KLF6 in transactivating p21$^{WAF1/cip1}$. To establish the functional significance of the interaction between KLF6 and CBP and/or PCAF, we explored whether CBP and PCAF potentiated the transactivation of p21$^{WAF1/cip1}$ by KLF6 (Fig. 3). As shown in Fig. 3A, KLF6 transactivated the p21$^{WAF1/cip1}$ (CDKN1A) promoter 3-fold, which was further increased to 12-fold in the presence of CBP. When a HDAC inhibitor, trichostatin A was added, the transactivation of p21$^{WAF1/cip1}$ by KLF6 was further increased, with the maximal transactivation >40-fold achieved in the presence of CBP plus trichostatin A.

In addition, we also examined the synergistic effects of KLF6 and PCAF in driving p21$^{WAF1/cip1}$ transactivation (Fig. 3B). In the presence of PCAF, the transactivation of p21$^{WAF1/cip1}$ by KLF6 was enhanced 14-fold. Again, trichostatin A maximally increased the synergistic effects of KLF6 and PCAF. These results suggest the functional involvement of CBP and PCAF as acetyltransferases, these results suggested that enhanced acetylation of KLF6 by trichostatin A promoted the transactivation of p21$^{WAF1/cip1}$ promoter. Previous studies showed that trichostatin A itself enhances transactivation of the p21$^{WAF1/cip1}$ promoter (41). This is consistent with the results shown in Fig. 3, in which the luciferase activity in empty vector control was also increased in the presence of trichostatin A. It is likely that this effect is due to the enhanced acetylation by trichostatin A on endogenous transcription factors that transactivate the exogenous p21$^{WAF1/cip1}$ promoter construct.

KLF6 is acetylated by CBP and PCAF, in vitro and in vivo. The data above suggested that KLF6 and either CBP or PCAF functionally interact on the p21$^{WAF1/cip1}$ promoter and raised the possibility that acetylation of KLF6 by either these two HATs promoted p21$^{WAF1/cip1}$ transactivation. To test this hypothesis, we did an in vitro acetylation assay. As shown in Fig. 4A, KLF6 was acetylated in vitro by both CBP and PCAF but not by a CBP mutant lacking the HAT domain. To verify that KLF6 was also acetylated in vivo, we immunoprecipitated KLF6 from 293T cells and blotted with an anti-acetylated lysine antibody (Fig. 4B).
and a b-type fragment in the spectra. As shown in the fragment mass spectrum of 2,361.3 (nonacetylated form; Fig. 5E, top), a series of peaks were observed that corresponded to y-ion (y4-y11). Acetylation of any of the three lysine residues was easily identified. In the fragment mass spectrum of 2,403.3 (singly acetylated form; Fig. 5E, middle), we observed two peaks corresponding to y11 ions. The major one (labeled with y11*) indicated the acetylation of Lys213, and the minor one indicated the acetylation of 209, respectively. In the fragment mass spectrum of 2,445.3 (doubly acetylated form; Fig. 5E, bottom), we observed similar y-series peaks as in the singly acetylated peptide 3, which indicated the acetylation of Lys209 and Lys213. Two additional y-ion peaks were also detected (labeled y8* and y9*) in the same spectrum. Because no mass shift was observed for peaks of y6 and y7, we interpreted this observation to have resulted from acetylation of His216, together with the acetylation of Lys213 or Lys209 (most likely 213). Based on the mass spectrometric analysis, the data indicate that the acetylation accessibility of the three lysine residues on KLF6 peptide 3 is in the order of 213, 209, and 218. The histidine residue can also be acetylated by CBP.

A tumor-derived K-to-R mutant has impaired ability to up-regulate p21WAF1/cip1 and suppress growth. We have shown a high frequency of LOH and point mutations of KLF6 in human prostate cancer (1). Importantly, in addition to the point mutants of KLF6 originally identified from prostate cancer (1), several additional lysine mutations were identified in prostate, colon, and hepatocellular cancers (11, 12, 15). Of these, we focused on a single new mutation not previously reported, K209R (Fig. 6A), to explore the potential effect of loss of an acetylation site on KLF6 function. Given that K209 can be acetylated by CBP as confirmed by MS (Fig. 5), a K209R mutation would be predicted to abrogate acetylation at this site. To assess the ability of K209R to transactivate the p21WAF1/cip1 promoter, we cotransfected the mutant with a p21WAF1/cip1 promoter reporter construct into PC3M cells. As shown in Fig. 6B, compared with wild-type KLF6, K209R completely lost the ability to transactivate the p21WAF1/cip1 promoter. To correlate this change with p21WAF1/cip1 expression in vivo, K209R was introduced into PC3 cells and the endogenous p21WAF1/cip1 level was assessed by Western blot. As shown in Fig. 6C, K209R lost the ability to up-regulate endogenous p21WAF1/cip1.

Loss of growth suppression by the K209R mutant was also shown in stably transfected prostate cancer cell lines. K209R was stably expressed in both PC3M cells (a metastatic prostate cancer line) and BPH1 cells (a line derived from benign prostate). In contrast to cells expressing wild-type KLF6, the K209R mutant was unable to decrease cell proliferation in either BPH1 or PC3M cell lines (Fig. 6D). Moreover, in PC3M cells but not BPH cells, the K209R mutant actually increased proliferation when compared with cells stably expressing the pCIneo empty vector. These results were validated in two independent sets of stable cell lines.

Figure 3. CBP and PCAF potentiate p21WAF1/cip1 up-regulation by KLF6, which is further enhanced by trichostatin A (TSA). A, CBP potentiates KLF6 in transactivating the p21WAF1/cip1 promoter. Left columns, PC3 cells were cotransfected with KLF6 and CBP constructs, along with a p21WAF1/cip1 promoter reporter. Right columns, the same cotransfection were done in the presence of an HDAC inhibitor, trichostatin A. B, PCAF potentiates KLF6 in transactivating p21WAF1/cip1 promoter. Left columns, PC3 cells were cotransfected with KLF6 and PCAF constructs, along with a p21WAF1/cip1 promoter reporter. Right columns, the same cotransfection were done in the presence of the HDAC inhibitor, trichostatin A. Transfection efficiencies were normalized using Renilla luciferase assay measured in the same lysate at the same time.
Acetylation Regulates KLF6 Tumor Suppressor

Discussion

KLF6 has been established as a tumor suppressor gene involved in key intracellular pathways in a number of human cancers (1, 12, 14, 15, 43, 44). Among several target genes transcriptionally regulated by KLF6, p21WAF1/cip1 has been particularly relevant to prostate cancer given p21 WAF1/cip1's central role in growth regulation, especially as a mediator of p53-stimulated cell cycle arrest. The acetylation status of the human p21WAF1/cip1 (CDKN1A) locus has been extensively characterized in cellular contexts that are tied to cancer development (45). These activities require specific GC-rich elements (46) in the p21WAF1/cip1 promoter, which are tied to cancer development (45). These activities require specific GC-rich elements (46) in the p21WAF1/cip1 promoter, which are consensus target sequences for KLF6 binding (1). However, little is known about either the biochemical requirements for transcriptional activity of KLF6, or the complexes through which KLF6 regulates target gene activity.

An important goal in studying KLF6 has been to understand the physiologic significance of protein complexes that modify its function in regulating the activity of its target genes. Recently, we showed that KLF6 sequesters cyclin D1 to reduce cyclin D/cdk4 interactions and enhance the phosphorylation of pRb, thereby promoting G1 cell cycle arrest (47). This activity alters the equilibrium of p21WAF1/cip1 levels, with induction and titration onto cdk2 complexes and further growth suppression (47). These findings suggest that covalent modifications may differentially regulate KLF6 activity.

Despite clear evidence that KLF6 transcriptionally activates p21WAF1/cip1 (1, 12), the biochemical mechanisms underlying this observation have not been clarified. Here, we establish that KLF6 activity is associated with increased complexing of acetylated histones within the p21WAF1/cip1 (CDKN1A) locus. Furthermore, we show that KLF6 interacts with the CBP/p300 complex and its acetylation by CBP contributes to normal KLF6 activity. Moreover, MS shows acetylation of a specific lysine residue of KLF6 that is mutated in a primary prostate tumor. Collectively, the findings provide evidence that acetylation of KLF6 is biologically significant, and its dysregulation in human cancer may contribute to loss of KLF6's growth suppressive activity.

Our findings also provide an important functional link between protein acetyltransferases and KLF6 by showing that KLF6 recruits CBP and PCAF to the p21WAF1/cip1 locus. In contrast to the evidence that KLF6 occupies the p21WAF1/cip1 promoter using chromosomal immunoprecipitation, the lack of promoter occupation by Sp1 was unexpected. One potential explanation may be the weak affinity of different antisera used against Sp1 from cross-linked material to detect the protein in these experiments. Alternatively, Sp1 may represent a low-abundance protein involved primarily in stimulated rather than basal expression of p21WAF1/cip1. This possibility is supported by studies showing that p21WAF1/cip1 transactivation by Sp1 is greatly enhanced by TGFB signaling, for example (48). Although our study does not exclude the possible participation of Sp1-like factors in regulating the expression of p21WAF1/cip1 (49), it may indicate a greater role for KLF6 than Sp1 in regulating p21WAF1/cip1 within this specific cellular context. Additionally, Sp1 may use different GC boxes in the p21WAF1/cip1 promoter from those used by KLF6 under our experimental conditions. In other contexts, there may be cooperation between KLF6 and Sp1, as suggested by their transcriptional synergy in regulating the expression of TGFB and of endoglin, a TGFB-binding protein (20). Thus, there may be a precise stoichiometry between KLF6 and Sp1 that is promoter and context specific in regulating their target genes.

The identification of several point mutations of KLF6 in human cancer affecting lysine residues led us to explore the potential role of lysine modification in regulating KLF6 activity. These have included mutations in prostate (K186R; ref. 1), colon (K74R; ref. 12), and hepatocellular carcinoma (K182R; ref. 15). Here we tested the functional activity of an additional prostate cancer–derived mutant of KLF6, K209R, for its capacity to both transactivate the p21waf1/cip1 promoter, and to function as a substrate for protein acetyltransferase activity by CBP. Our findings indicate that acetylation of a specific Lys209 is necessary for KLF6 to up-regulate its activity. Despite clear evidence that KLF6 transcriptionally activates p21WAF1/cip1 (1, 12), the biochemical mechanisms underlying this observation have not been clarified. Here, we establish that KLF6 activity is associated with increased complexing of acetylated histones within the p21WAF1/cip1 (CDKN1A) locus. Furthermore, we show that KLF6 interacts with the CBP/p300 complex and its acetylation by CBP contributes to normal KLF6 activity. Moreover, MS shows acetylation of a specific lysine residue of KLF6 that is mutated in a primary prostate tumor. Collectively, the findings provide evidence that acetylation of KLF6 is biologically significant, and its dysregulation in human cancer may contribute to loss of KLF6's growth suppressive activity.

Figure 4. KLF6 is acetylated in vitro and in vivo. A, KLF6 is acetylated in vitro by CBP and PCAF. Equivalent molar amount of CBP (wild type), CBP (HAT–), and PCAF (wild type) were immunoprecipitated from cells expressing HA-tagged CBP, HA-tagged PCAF (HAT–), and FLAG-tagged PCAF (wild type). In vitro translated KLF6 was incubated with immunoprecipitation-purified CBP (wild type), CBP (HAT–), and PCAF (wild type), together with [3H]-acetyl CoA. KLF6 was synthesized with [3H]-leucine in vitro and immunoprecipitated as a control. Immunoprecipitated products were resolved on SDS/PAGE followed by autoradiography. B, KLF6 is acetylated in vivo. 293T cells expressing FLAG-tagged KLF6 were immunoprecipitated with anti-FLAG agarose, followed by SDS-PAGE and Western blot analysis. Immunoprecipitated KLF6 was detected by anti-acetylated lysine antibody. Moreover, using lysates from the same cell line, FLAG-tagged KLF6 was immunoprecipitated by anti-acetylated lysine antibody and detected by anti-FLAG antibody using Western blot.
Figure 5. Determination of key acetylation sites on KLF6. A, the location of four synthetic peptides covering the majority of lysine residues on KLF6 molecule (*K73, this residue is only present in the normal human but not murine KLF6 sequence). B and C, peptide acetylation assays by CBP and PCAF. CBP acetylates peptides 3 and 4 encompassing most lysine residues on the DNA-binding domain. PCAF acetylates none of the lysine residues on the four synthetic peptides. Histone H4 peptide fragment was used as a positive control. D, MALDI-MS spectra of peptide 3 before (top) and after CBP acetylation (bottom). Peaks corresponding to protonated molecular ions were labeled with measured monoisotopic protonated molecular masses and denoted with M+H*, M-Ac+H*, M-2Ac+H*, and M-3Ac+H* for nonacetylated, single, double, and triple acetylations, respectively. E, fragment ion spectra resulted from MS/MS analysis of peptide ions 2361.3, 2403.3, and 2445.3 (top, middle, and bottom). Peaks corresponding to y-series of fragment ions were labeled and aligned with reversed sequence of peptide 3 (NH2-terminal is indicated by -NH2).
p53’s interaction with other cellular and viral factors, thereby
altering protein p53 levels (52–55).

Acetylation of specific lysine residues by different acetyltrans-
ferases may lead to divergent functional outcomes. For example,
acetylation of pRb at specific lysine residues by p300 has been
linked to inhibition of cell cycle progression (25), whereas acety-
lation of pRb by PCAF of key Lys873/874 near the COOH terminus
correlates with cell differentiation (56). These divergent outcomes
may also reflect differences between p300 and PCAF in promoting
cell differentiation in vivo (57). It seems possible that similar, subtle

Figure 6. A lysine (K) to arginine (R) mutant (K209R) identified from prostate cancer has impaired ability to up-regulate p21WAF1/cip1 expression. A, a KLF6 mutation (K209R) affecting an acetylation site in primary prostate cancer. Sequencing chromatogram of DNA derived from microdissected primary prostate cancer is shown, along
with normal sequence from the surrounding unaffected sequence. B, K209 tumor mutant has reduced ability to transactivate p21WAF1/cip1 promoter. Wild-type KLF6
and K209R were cotransfected with a p21WAF1/cip1 promoter reporter, respectively. Empty expression vector was transfected as a control for baseline luciferase activity. Transfection efficiencies were normalized using Renilla luciferase assay measured in the same lysate at the same time. C, K209R tumor mutant has reduced ability to
up-regulate endogenous p21WAF1/cip1. Wild-type KLF6 and K209R were transfected into PC3 cells, respectively. Thirty-six hours after transfection, cells were lysed and
loaded onto SDS-PAGE followed by Western blot analysis. Anti-p21WAF1/cip1 antibody was used to detect the expression of endogenous p21WAF1/cip1 protein. The protein
expression of transfected KLF6 and mutants constructed was detected by anti-KLF6 antibody. Tubulin was blotted as a control for protein loading. D, impaired growth
suppression by K209R mutant in stable prostate cell lines. Both PC3M and BPH cell lines were stably transfected with either pCI-neo empty vector, KLF6 wild type,
or K209R mutant as described in Materials and Methods, and cell proliferation was assessed by estimating 3H-thymidine incorporation at 24, 48, and 72 hours after
replating equal numbers of cells. The differences were statistically significant, as indicated by Ps. *, P<0.05; **, P<0.01.
that KLF6 serves as a substrate for CBP HAT activity. Combined
with the loss of growth suppressive activity of a tumor-derived
lysine mutant of KLF6, these findings point to acetylation as a key
modification in regulating its growth- and tumor-suppressive
activities.

Acknowledgments

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Dan Li, Steven Yea, Georgia Dolios, et al.


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