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Krüppel-like Factor 4 Is Acetylated by p300 and Regulates Gene Transcription via Modulation of Histone Acetylation*

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Colon cancer is the second leading cause of cancer death in the United States. Krüppel-like factor 4 (KLF4) is a transcription factor involved in both proliferation and differentiation in the colon. It is down-regulated in both mouse and human colonic adenomas and has been implicated as a tumor suppressor in the gut, whereas in breast cancer, KLF4 is an oncogene. KLF4 is also involved in reprogramming differentiated cells into pluripotent stem cells. KLF4 can act as a transcriptional activator or repressor, but the underlying mechanisms are poorly understood. We found that p300, a CREB-binding protein-related protein, interacts with KLF4 both *in vitro* and *in vivo* and activates transcription. We further made the novel observation that endogenous KLF4 is acetylated by p300/CBP *in vivo* and that mutations of the acetylated lysines resulted in a decreased ability of KLF4 to activate target genes, suggesting that acetylation is important for KLF4-mediated transactivation. Furthermore, we found that KLF4 differentially modulates histone H4 acetylation at the promoters of target genes. Co-transfection of KLF4 and HDAC3 resulted in a synergistic repression of a cyclin B_1 reporter construct. Our results suggest that KLF4 might function as an activator or repressor of transcription depending on whether it interacts with co-activators such as p300 and CREB-binding protein or co-repressors such as HDAC3.

Krüppel-like factor 4 (KLF4/GKLF/EZF) is a transcription factor involved in both the regulation of proliferation and differentiation in several tissues (1–4). As a negative regulator of the cell cycle, KLF4[3] activates the expression of $p21^{Cip1/WAF1}$, a cyclin-dependent kinase inhibitor (5), and represses the expression of cyclin B_1 (6). KLF4 is a key regulator of differentiation in the gut, as KLF4 knock-out mice are born with dramatically fewer goblet cells, and the remaining goblet cells are histologically and ultrastructurally abnormal (7). Consistent with the role of KLF4 in differentiation, there are several reports demonstrating that KLF4 activates the expression of intestinal alkaline phosphatase (IAP), an enterocyte-specific cell marker (8, 9). KLF4 appears to regulate differentiation in tissues other than gut epithelium as well. In the eye, conditional knock-out of KLF4 results in abnormal corneal epithelium, and the conjunctiva lack goblet cells (10). Moreover, mice lacking the *klf4* gene quickly die after birth due to malformations in the cornified envelope of the skin (11).

In gastrointestinal cancer, KLF4 appears to function as a tumor suppressor. In the APC^{min} mouse model of colorectal neoplasia, KLF4 is down-regulated in adenomas harvested from the colon (12). In both human colorectal tumors and cell lines, its expression is frequently down-regulated due to mutations, promoter methylation, and loss-of-heterozygosity (13). Recently, we have shown that expression of KLF4 inhibited the growth of xenograft tumors and induced the expression of mucin, a marker for goblet cell differentiation, further suggesting that KLF4 regulates normal intestinal homeostasis and suppresses tumor formation (14). In the gastric epithelium, KLF4 appears to play a similar role, as tissue-specific knock-out of KLF4 results in abnormal cellular proliferation and precancerous changes (15). However, in breast cancer KLF4 appears to function as an oncogene, as elevated expression of KLF4 could be detected in almost 70% of breast carcinomas (16), and increased nuclear staining for KLF4 appears to be associated with a more aggressive phenotype (17). Furthermore, expression of KLF4 by retroviral transduction in E1A-immortalized rat kidney cells resulted in transformation (18). As a possible explanation for this marked difference in the function of KLF4 between cancer types, it has been proposed that the role of KLF4 depends on the functional status of $p21^{Cip1/WAF1}$ (19). Finally, KLF4 appears to be important in the maintenance of stem cells, as the addition of only four factors, including KLF4, is sufficient to produce pluripotent stem cells from normal fibroblast cells (20).

It is not fully understood how KLF4 activates gene expression. However, some insight was gained by a study demonstrating a physical interaction between KLF4 and CREB-binding protein (CBP) (21). CBP and its close homologue, p300, are proteins that contain a catalytic histone acetyltransferase (HAT) domain (22) and act as transcriptional co-activators for many sequence-specific transcription factors (23). Acetylation of histones has long been associated with the activation of transcription (24). p300/CBP are recruited to specific regions of

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[3] The abbreviations used are: KLF4, Krüppel-like factor 4; IAP, intestinal alkaline phosphatase; HAT, histone acetyltransferase; HDAC, histone deacetylase; ChIP, chromatin immunoprecipitation; CBP, CREB-binding protein; RT, reverse transcription; GST, glutathione *S*-transferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, methyl thiazole tetrazolium; CREB, cAMP-response element-binding protein; IP, immunoprecipitation.

DNA by sequence-specific transcription factors, and this interaction serves to promote localized histone acetylation. For example, p53 interacts with p300 on both the GADD45 and *CDKN1A* (p21) promoter, and in both cases increases localized histone acetylation (25, 26). In a similar fashion, KLF6 interacts with p300 and promotes histone acetylation on the *CDKN1A* (p21) gene (27). Thus it is likely that p300/CBP is important for KLF4-mediated transactivation. p300 and CBP share 63% homology at the amino acid sequence level, and both are known to be able to acetylate histones. Given the strong similarity between these two HAT proteins, we postulated that KLF4 could directly interact with p300 as well.

Subsequent reports have shown that in addition to targeting histones for acetylation, p300/CBP can directly acetylate transcription factors as well (28). Acetylation can have diverse effects on transcriptional activity, such as increasing the DNA binding affinity of p53 (29), increasing binding between β-catenin and TCF4 (30), and delaying nuclear export of STAT3 (31). KLF1, another Krüppel-like factor family member, is acetylated by p300 (32), and acetylation of KLF1 increases its interaction with the SWI/SNF chromatin remodeling complex (33, 34).

Histone deacetylases (HDACs) appear to play an important role in the regulation of transcription, as treatment of cells with HDAC inhibitors such as trichostatin A or butyrate results in altered expression of a significant number of genes (35, 36). Butyrate is a physiologically relevant compound, as it is a normal by-product of metabolism of bacteria in the gut. In several colon cancer cell lines, butyrate treatment induces cell cycle arrest via induction of $p21^{Cip1/WAF1}$ expression (37, 38). Moreover, butyrate can induce differentiation (39, 40) and increase the expression of IAP (41), a marker of enterocyte differentiation. Since butyrate is a known inhibitor of HDACs, it seems likely that butyrate regulates histone acetylation and thus regulates the expression of multiple genes, including the KLF4 target genes $p21^{Cip1/WAF1}$ and IAP.

Given the clearly important role that histone acetylation and chromatin remodeling have in the regulation of transcription in general, we hypothesized that KLF4 might function as an activator or repressor of transcription via the differential regulation of histone acetylation mediated by its interaction with HAT proteins. In addition, the fact that several other transcription factors can be directly acetylated by p300/CBP led us to ask whether KLF4 was a target for acetylation as well.

EXPERIMENTAL PROCEDURES

Plasmid DNA Constructs—pCS2-KLF4 (N-terminal FLAG tag), FLAG-p300, and pRC-CMV-mCBP-HA have been described previously (14, 42–44). GST-p300 (CH3) was a gift from William Weis (45). HDAC4, -5, and -6 were kind gifts from Stuart L. Schreiber. Truncation mutants KLF4Δ1–154(ΔN), KLF4Δ155–399(ΔM), KLF4Δ402–483(ΔC), KLF4Δ367–483, KLF4Δ158–350, KLF4Δ393–483, KLF4Δ158–242, and KLF4Δ158–268 were generated by PCR and cloned into the pCS2 vector. Point mutations K225R/K229R, K225R, and K229R of KLF4 were introduced by site-directed mutagenesis using primers designed with the assistance of the software program SiteFind (46). Cyclin B_1-luciferase was produced by amplifying the proximal 2.4 kb of the cyclin B_1 gene from genomic DNA using PCR. The resultant 2.4-kb fragment was then inserted into the pGL3-basic (Promega) reporter. All constructs were verified by DNA sequencing. Primers for these constructs are available upon request.

Cell Culture and Transient Transfection—HEK293T, HCT116, and HT29 cells were grown in Dulbecco's modified Eagle's medium (Mediatech) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. LS174T cells were grown in RPMI medium (Mediatech) supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. Stable cell line LS174T-Tet-On-KLF4 has been described previously (14). HEK293T cells were transiently transfected using the calcium phosphate method as described previously (14).

Western Blot, Immunoprecipitation (IP), and GST Pulldown—Cells were lysed in the appropriate volume of lysis buffer (50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1% glycerol, 50 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, with protease inhibitors). For acetylation experiments described in Fig. 4, 5 mM sodium butyrate and 5 mM nicotinamide (Acros Organics) were added to cells 6 h prior to harvest and to lysis buffer to inhibit deacetylases. Western blot and immunoprecipitation were performed as described previously (14). GST and GST-p300 (CH3) were expressed in *Escherichia coli* and purified with GST beads (glutathione-Sepharose from Sigma). GST beads containing purified GST or GST-p300 (CH3 domain) proteins were then incubated with cell lysate containing FLAG-tagged KLF4 or KLF4 mutants at 4 °C overnight, and beads were washed three times with lysis buffer and boiled in 1× SDS sample buffer, followed by analysis via Western blot. For acetylation studies, KLF4 and its mutants were immunoprecipitated, eluted with 0.2 mg/ml FLAG peptide, and then analyzed by Western blot with an antibody that specifically recognizes acetylated lysine (Cell Signaling, catalog number 9441). For butyrate time course and ChIP assays, acetylated histone H4 antibody was used (Upstate, catalog number 06-598).

Time Course Assays—LS174T-Tet-On-KLF4 cells were plated at 1×10^5 cells/well in a 12-well plate. Starting the following day, doxycycline (1 μg/ml) was added to the appropriate wells such that all wells were harvested at the same time. Then Western blot was performed using a $p21^{Cip1/WAF1}$ (Cell Signaling, catalog number 4135), cyclin B_1 (Cell Signaling, catalog number 4135), β-actin (Sigma, catalog number A1978), or KLF4 antibody (14). For butyrate treatment, LS174T cells were plated on a 12-well plate, 1×10^5 cells/well. Starting the following day, cells were treated with 5 mM sodium butyrate for various time periods such that all cells were ready for harvest at the same time. Cells were then lysed, and Western blot was performed on the lysate as described above.

RT-PCR—LS174T-Tet-On-KLF4 cells were plated $\sim 1 \times 10^6$ cells per plate in a 10-cm dish. The following day, doxycycline (1 μg/ml) was added to the culture medium. After 48 h of incubation, RNA was isolated using the RNeasy kit (Qiagen). RT-PCR was performed as described previously (14). Densitometry was performed using the Quantity One software from Bio-Rad. The following primers were used: β-actin, 5′-CAACCGCGAGAAGATGAC-3′ and 5′-AGGAAGGCTGGAAGAGTG-3′; $p21^{Cip1/WAF1}$, 5′-CGACTGTGATGCGCTAATGG-3′ and 5′-AGAAGATCAGCCGGCGTTTG-3′; cyclin B_1, 5′-GCAGCACCTGGCTAAGAATG-3′

and 5′-GCCACAGCCTTGGCTAAATC-3′; IAP, 5′-CCATTGCCGTACAGGATGGAC-3′ and 5′-CGCGGCTTCTACCTCTTTGTG-3′; KLF4, 5′-CCGGTGACCGCATGTGCCCCAAGATTAAG-3′ and 5′-TTGAATTCTTAAGGTTTCTCGCCTGTGTGAGT-3′.

Luciferase and IAP Enzymatic Assays—IAP-luciferase reporter was used as described previously (41). HEK293T cells were transiently transfected in a 12-well plate with 0.2 μg of the appropriate reporter and 0.5 μg of empty vector (pCS2+) or the appropriate KLF4 construct with or without 0.5 μg of p300 plasmid DNA. Total DNA transfected in each well was 1.2 μg and was normalized using pCS2 DNA as needed. Two days later, cells were harvested, and luciferase activity was measured. All conditions were done in triplicate, and each experiment was carried out at least two times. The IAP enzymatic assay was performed as described previously (14). Briefly, HT29 cells were harvested after treatment with 5 mM sodium butyrate, and 40 μl of cell lysate was incubated with 200 μl of *p*-nitrophenyl phosphate (Sigma, catalog number A3469) in a 96-well plate for 1–3 h. The reaction was stopped by the addition of 50 μl of 3 M NaOH, and the yellow enzymatic product was quantitated by measuring absorbance in a 96-well plate reader (*V*max; Molecular Devices) at wavelength 405 nm.

Chromatin Immunoprecipitation (ChIP)—ChIP assays were performed according to the protocol developed by Nowak *et al.* (47) with some modifications. 1×10^6 LS174T-Tet-On-KLF4 cells were plated on 10-cm dishes. The following day doxycycline was added to a final concentration of 1 μg/ml, and cells were grown for an additional 36 h. The cells were cross-linked with disuccimidyl glutarate (Pierce, catalog number 20593) and formaldehyde at room temperature. Cells were pelleted at 3000 rpm for 1 min and resuspended in 900 μl of L1 buffer (50 mM Tris, 2 mM EDTA, 0.1% IGEPAL, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture, pH 8.0) and allowed to sit on ice for 15 min. After centrifugation at 4000 rpm for 5 min, supernatant was removed, and the nuclear pellet was resuspended in 500 μl of ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.0, protease inhibitor mixture). Cell lysate was sonicated four times for 10 s on ice at setting 5 on a Branson Sonifier 150, with a 30-s break between sonications. After centrifugation at 13,200 rpm for 10 min at 4 °C, supernatant was transferred to a fresh tube. Absorbance at 260 nm (A_{260}) of a 1:50 dilution of each sample was measured using a spectrophotometer to estimate DNA content. For each assay, 100 μl of the most dilute sample was used, and the more concentrated samples were diluted in lysis buffer so that each condition received the same amount of total DNA. Lysate was diluted and incubated with 4 μg of the appropriate antibody overnight, followed by incubation with 100 μl of protein A-agarose/salmon sperm DNA 50% slurry (Upstate, catalog number 16-157) for 3 h at 4 °C. Beads were then washed with a series of washes, and bound DNA-protein complexes were eluted and decross-linked. DNA was then purified by phenol/chloroform extraction and ethanol precipitation. Pelleted DNA was resuspended in 20 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and 1 μl was used for PCR. Optimal cycling parameters to ensure operation in the linear range were primer-specific, but typically 32–40 cycles were done. The following primers were used: cyclin B_1, 5′-TCTTGCCCGGCTAACCTTTCCAGG-3′ and 5′-TTCCGCCGCAGCACGCCGAGAAGA-3′; IAP, 5′-CCACAAGACACTGTGAGCCACACC-3′ and 5′-AAGTGGGGACACCAGGAACCGGCT-3′; GAPDH, 5′-ATGCCAGGAGCCAGGAGATG-3′ and 5′-TGAGAGGCGGGAAAGTTGGG-3′. For ChIP assays, antibodies used include p300 (Santa Cruz Biotechnology, catalog number sc-584), HDAC3 (BD Biosciences, catalog number 611124), and KLF4 (14).

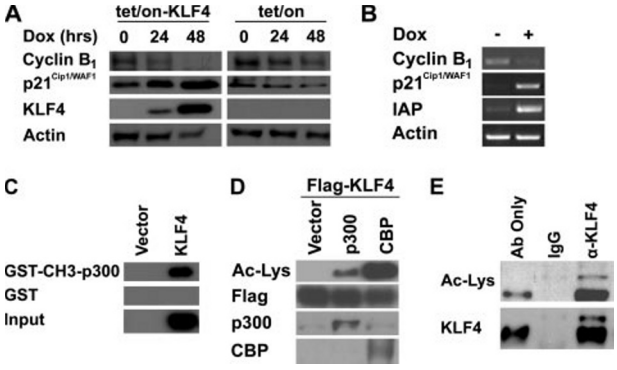

FIGURE 1. **KLF4 interacts with and is acetylated by p300/CBP.** *A*, KLF4 represses cyclin B_1 and activates p21 expression in an LS174T doxycycline-inducible cell line. LS174T-Tet-On-KLF4 cells were treated with 1 μg/ml doxycycline (*Dox*) for various time periods prior to harvest. Western blot was then performed on the cell lysate. *B*, RT-PCR for several KLF4 target genes in LS174T-Tet-On-KLF4 cells after treatment with doxycycline for 48 h. *C*, KLF4 interacts with the CH3 domain of p300. GST-p300 (CH3) protein was incubated with FLAG-tagged KLF4 overnight. After pulldown with GST beads, KLF4 was detected via Western blot with FLAG antibody. *D*, KLF4 is acetylated by p300/CBP. KLF4 and full-length p300 or CBP were overexpressed in 293T cells. After IP with FLAG antibody, a 55-kDa protein was detected via Western blot using an acetyl-lysine-specific (*Ac-Lys*) antibody. Western blots confirmed similar levels of expression of KLF4 in each condition and the expression of p300 and CBP where indicated. *E*, endogenous KLF4 is acetylated. Endogenous KLF4 was immunoprecipitated from HCT116 cells using KLF4 antibody and probed via Western blot with anti-KLF4 and anti-acetylated lysine antibody. Upper band is KLF4. Anti-KLF4 antibody alone (without cell lysate) and mouse IgG (with cell lysate) were included as negative controls.

RESULTS

KLF4 Interacts with and Is Acetylated by p300/CBP—Previous reports implicated KLF4 as a negative regulator of the cell cycle. To test this function in colon cancer cells, we established a doxycycline-inducible KLF4 stable LS174T cell line and monitored the expression of $p21^{Cip1/WAF1}$ and cyclin B_1 after treatment with doxycycline over a time course. As shown in Fig. 1*A*, expression of KLF4 increased after doxycycline treatment. Expression of $p21^{Cip1/WAF1}$ increased in a similar manner, whereas expression of cyclin B_1 was strongly down-regulated at 48 h, suggesting that KLF4 represses the cell cycle by differentially regulating expression of these genes. To demonstrate treatment with doxycycline does not have a direct effect on the expression of these proteins, we performed the same experiment using the parental cell line, LS174T-Tet-On, and found no effect on either $p21^{Cip1/WAF1}$ or cyclin B_1 expression. We also examined the effect of KLF4 overexpression on the level of mRNA for both target genes using semi-quantitative RT-PCR (Fig. 1*B*). After 48 h of treatment with doxycycline, $p21^{Cip1/WAF1}$ mRNA was significantly increased, whereas the level of cyclin B_1 mRNA decreased, suggesting that KLF4 regulates the expression of these genes by directly modulating their tran-

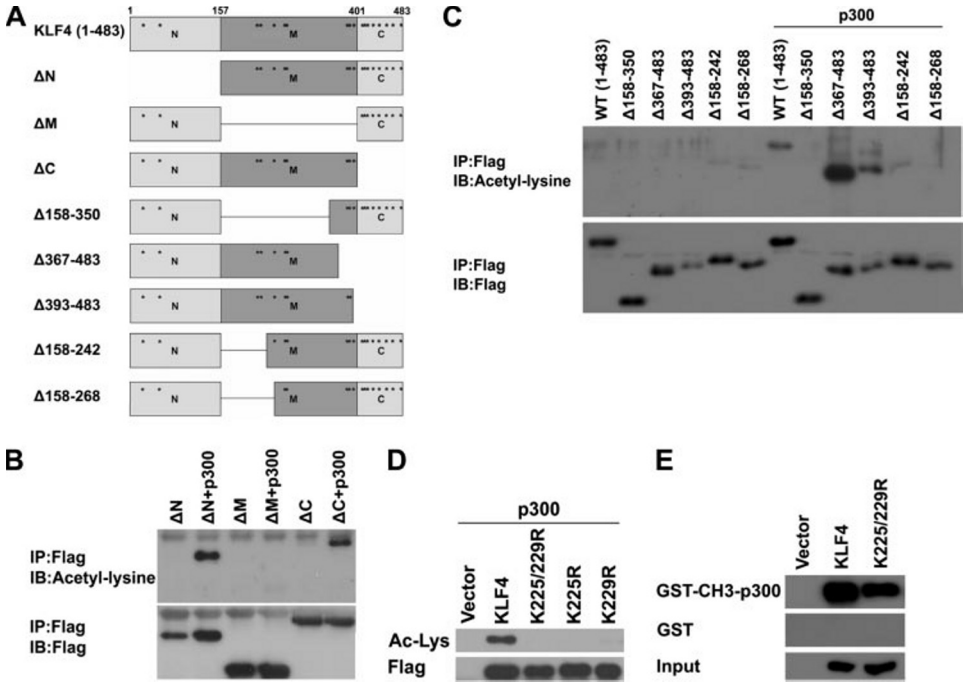

FIGURE 2. **KLF4 is acetylated by p300/CBP at Lys-225/229.** *A*, diagram of constructs used for mapping acetylated lysine residues in KLF4. All lysines found within the wild-type sequence are marked with an *asterisk*. *B*, KLF4 is acetylated within in the middle region (residues 155–399). KLF4 deletion mutants ΔN, ΔM, and ΔC were overexpressed with full-length p300 in 293T cells. After IP with FLAG antibody, a Western blot with an acetyl-lysine-specific antibody was performed. *C*, KLF4 is acetylated within the proximal 193 amino acids of the middle region. The same experiment was performed as in *B*, this time using constructs Δ158–350, Δ367–483, Δ393–483, Δ158–242, and Δ158–268. *D*, KLF4 is acetylated at Lys-225/229. Mutation of either lysine 225 or 229 abolished recognition of KLF4 with an acetyl-lysine-specific antibody. *E*, point mutation does not affect interaction with p300. GST-p300 (CH3) was incubated with FLAG-tagged KLF4-K225R/K229R. After pulldown with GST beads, KLF4 was detected via Western blot using a FLAG antibody. *IB*, immunoblot.

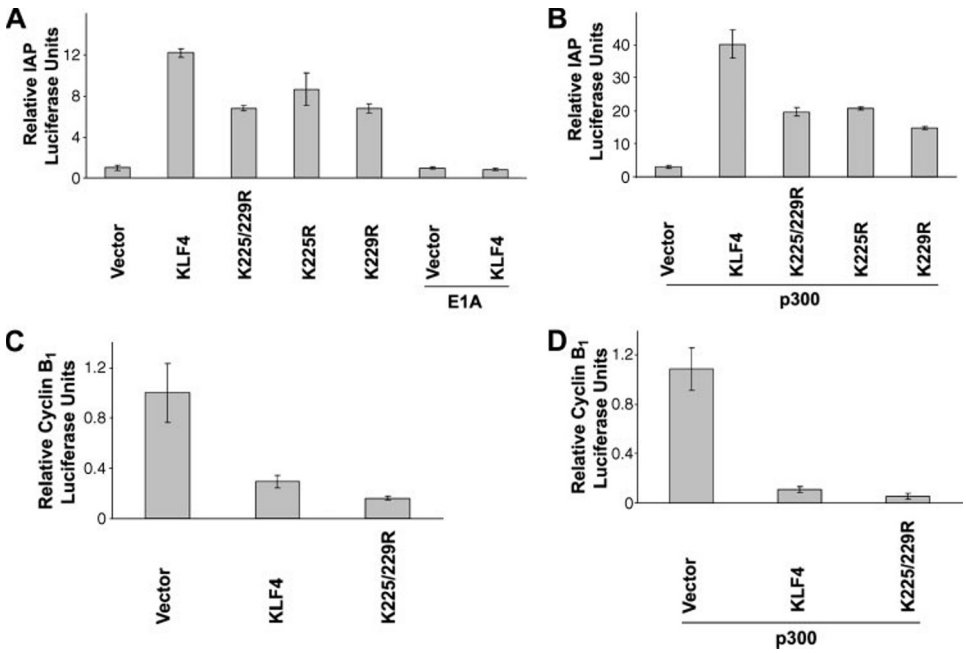

FIGURE 3. **Acetylation by p300 regulates KLF4-mediated transactivation.** *A*, lysine mutant KLF4 is less effective at activating the IAP-luciferase reporter. Empty vector, wild-type, or lysine mutant KLF4 was co-transfected into 293T cells with IAP luciferase plasmid. After 48 h, luciferase activity was measured. Co-transfection of KLF4 with E1A, which also binds the CH3 domain of p300, sequesters p300, abolishes KLF4-mediated activation of the reporter. *B*, addition of p300 results in super-activation of the IAP reporter. Full-length p300 plasmid was co-transected into 293T cells, and luciferase activity measured as in *A*. *C*, lysine mutant KLF4 is still a strong repressor of the cyclin B_1 luciferase reporter. Wild-type or lysine mutant KLF4 was co-transfected into 293T cells. After 48 h, luciferase activity was measured. *D*, addition of p300 had no effect on KLF4-mediated repression of the cyclin B_1 gene. Same experiment as in *C*, but with the addition of full-length p300.

scription. Furthermore, expression of KLF4 also resulted in a strong induction of IAP, a known target of KLF4 and a marker of enterocyte differentiation (41).

An earlier report demonstrated a direct interaction between KLF4 and the histone acetyltransferase CBP via a GST pulldown assay with *in vitro* radiolabeled KLF4 and GST-tagged CBP (21). We hypothesized that p300, a protein highly similar to CBP, might interact with KLF4 as well. To test this, we incubated purified GST-p300 protein (CH3 domain) with lysate from HEK293T cells overexpressing FLAG-tagged KLF4. After GST-CH3-p300 protein was pulled down with GST beads, we were able to detect KLF4 in the elution fraction via Western blot (Fig. 1*C*), confirming that KLF4 and the CH3 domain of p300 interact *in vitro*. As a control, we performed the same experiment with GST protein alone, which did not interact with KLF4.

Histone acetyltransferase proteins can acetylate transcription factors directly (28, 30). Indeed, STAT3 (31, 48), p53 (29), and even another Krüppel-like factor family member, KLF1 (32), have been shown to be acetylated by histone acetyltransferases. Given our result that KLF4 can interact with p300, we postulated that KLF4 could be directly acetylated by p300/CBP as well. To test this, we overexpressed FLAG-tagged KLF4 and p300 or CBP in HEK293T cells and immunoprecipitated KLF4 using a FLAG-specific antibody conjugated to agarose beads, followed by Western blot with an acetyl-lysine-specific antibody. We found that KLF4 was indeed acetylated by both p300 and CBP (Fig. 1*D*). Western blots confirmed the expression of p300 and CBP when co-transfected with KLF4.

To assess the physiologic relevance of our results, we immunoprecipitated endogenous KLF4 from HCT116 cells (Fig. 1*E*). After IP with an anti-KLF4 antibody, KLF4 was detected as a band slightly heavier than the IgG band also pres-

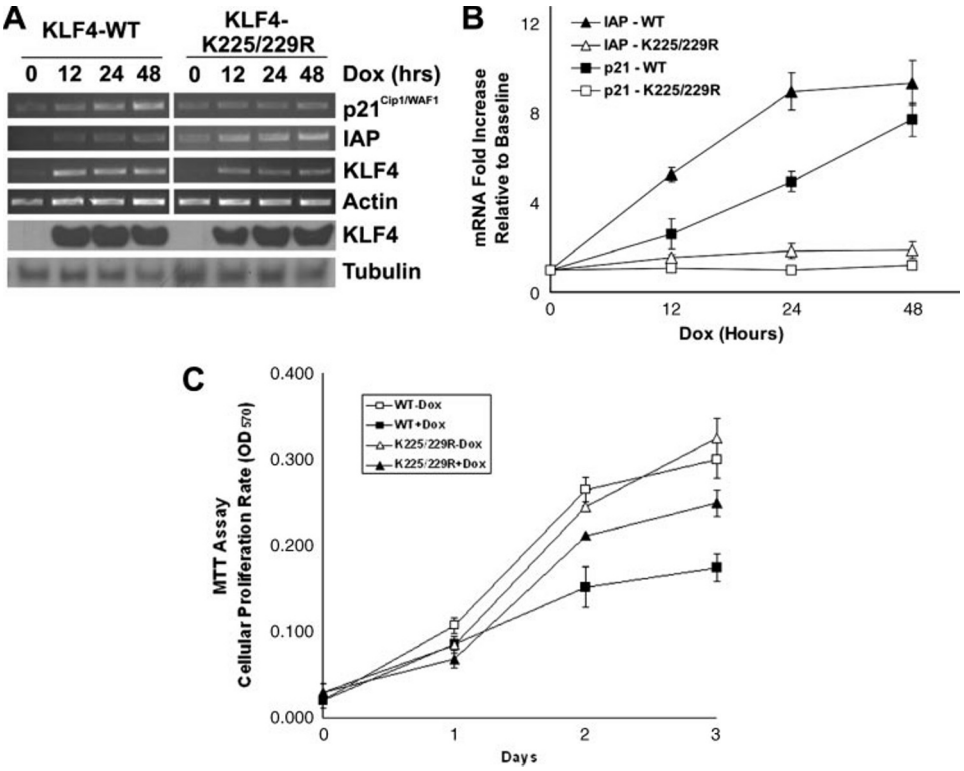

FIGURE 4. **Lysine mutant KLF4 is defective at activation of endogenous gene expression and repression of proliferation.** *A*, RT-PCR for $p21^{Cip1/WAF1}$, IAP, KLF4, and actin in wild-type KLF4 and lysine mutant KLF4 stable cell lines after treatment with doxycycline (*Dox*) for the specified time periods. Overexpression of wild-type KLF4 strongly increased the endogenous levels of $p21^{Cip1/WAF1}$ and IAP mRNA, whereas lysine mutant KLF4 only weakly activated these genes. Western blots were performed using anti-FLAG antibody to confirm similar levels of expression of wild-type and mutant KLF4, anti-tubulin was used as a loading control. *B*, graphical representation of data from *A*, using densitometric analysis of images taken of ethidium bromide-stained agarose gels from at least three separate PCR runs. *C*, MTT assay. Wild-type KLF4 and lysine mutant KLF4 stable cell lines were plated in a 96-well plate and treated with doxycycline for the specified time periods. At each time point, cells were incubated with MTT reagent, and resultant blue product was measured using an absorbance plate reader at A_{570}. Wild-type KLF4 effectively repressed cellular proliferation after treatment for 3 days, whereas lysine mutant KLF4 was significantly less effective at repressing cellular proliferation.

ent due to the immunoprecipitation step, whereas when we used mouse IgG or anti-KLF4 (rabbit) without lysate for the IP, we were unable to detect KLF4. Via Western blot with an acetylated lysine-specific antibody, we were able to detect KLF4 acetylation, confirming that KLF4 is indeed acetylated *in vivo*.

Our next step was to identify the lysines acetylated by p300/CBP. As the KLF4 amino acid sequence contains 18 lysines, our strategy was to design three deletion mutants, lacking either the N-terminal transactivation domain, middle region, or the C-terminal DNA binding domain (see Fig. 2*A*). We then performed the same FLAG-IP/acetyl-lysine Western blot with each KLF4 mutant as was done with wild-type KLF4 as shown in Fig. 1*D*. Acetylation was still detectable in the ΔN and ΔC mutants, whereas acetylation in the ΔM mutant was absent, suggesting that the middle region contains the acetylation target (Fig. 2*B*). KLF4ΔM lacks a total of 8 lysines, so we produced several more deletion mutants encompassing this domain. Acetylation was not detected in the KLF4Δ158–350 construct, which lacks five lysines within in the middle region, whereas mutants KLF4Δ367–483 and KLF4Δ393–483 could still be acetylated (Fig. 2*C*). KLF4Δ158–242 and KLF4Δ158–268, lacking the first two and three lysines of the M region, respectively, were also unable to be acetylated. Thus, we conclude that the acetylation target is Lys-225 and/or Lys-229, as these are the only two lysines absent in KLF4Δ158–242. Using full-length KLF4, we mutated each lysine (Lys-225 and Lys-) to arginine, individually as well as simultaneously, and we found that all three point mutants could not be acetylated by p300 (Fig. 2*D*), suggesting that the presence of both lysine residues is required for acetylation by p300. Similar results were found with CBP (data not shown). To exclude the possibility that these point mutations did not affect the ability of KLF4 to interact with p300, we incubated GST-CH3-p300 protein with cell lysate containing the KLF4 double point mutant and found that KLF4-K225R/K229R could still interact with the CH3 domain of p300 *in vitro* (Fig. 2*E*).

Acetylation by p300 Regulates KLF4-mediated Transactivation—Given that acetylation of transcription factors often has an effect on its transcriptional activity (28), we decided to investigate the ability of lysine mutant KLF4 to activate one of its known target genes, IAP. Co-transfection of an IAP-luciferase construct with wild-type KLF4 in 293T cells resulted in strong (~12-fold) activation of the reporter (Fig. 3*A*). The addition of p300 resulted in a synergistic activation of the IAP-luciferase construct (Fig. 3*B*), with an ~40-fold increase of luciferase activity relative to base line. However, acetylation-deficient mutant KLF4-K225R/K229R was only able to activate the reporter ~50% of the wild-type KLF4, a result maintained after co-transfection with p300. A similar decrease in activation was found for both single point mutants as well. Co-transfection of wild-type KLF4 with E1A, a protein known to sequester p300 by binding to the CH3 domain (49), completely abolished KLF4-mediated activation of the reporter, suggesting that p300 is required for KLF4-mediated transcription of the IAP gene.

These results suggest that acetylation of KLF4 by p300 is important for transactivation; however, it is still unclear whether acetylation is important for KLF4-mediated repression. Since cyclin B_1 is negatively regulated by KLF4, we decided to use a cyclin B_1-luciferase construct to study whether KLF4-K225R/K229R could still repress transcription of this gene as effectively as wild-type. As seen in Fig. 3*C*, wild-type KLF4 repressed the cyclin B_1-luciferase reporter. KLF4-K225R/K229R was similarly effective at repressing

this reporter. Furthermore, the addition of p300 appeared to have minimal effect on the ability of wild-type or lysine mutant KLF4 to repress the cyclin B_1 reporter, suggesting that p300 is important for KLF4-mediated activation but not for repression.

Lysine Residues 225 and 229 of KLF4 Are Important for Induction of Endogenous p21$^{Cip1/WAF1}$ and IAP—To further test the functional significance of these lysine residues in transcriptional activation by KLF4, we established a doxycycline-inducible stable cell line from LS174T-Tet-On cells, expressing KLF4-K225R/K229R. After treating these cells with doxycycline for various amounts of time, we harvested the RNA and performed RT-PCR. We found that overexpression of wild-type KLF4 resulted in an ~10-fold induction of IAP mRNA at 48 h post-treatment, whereas overexpression of KLF4-K225R/K229R resulted in only an ~2-fold induction of IAP (Fig. 4, *A* and *B*). Similarly, wild-type KLF4 resulted in an ~8-fold induction of p21$^{Cip1/WAF1}$, whereas KLF4-K225R/K229R produced almost no change. We performed RT-PCR for KLF4 mRNA as well. KLF4 mRNA was almost undetectable prior to treatment, but quickly increased at 12 h post-treatment. β-Actin was used as a loading control. Western blotting for wild-type and mutant KLF4 gave results similar to our RT-PCR data, confirming similar levels of expression of wild-type and mutant KLF4 in our two cell lines.

Lysine Residues 225 and 229 of KLF4 Are Important for KLF4-mediated Inhibition of Cellular Proliferation—We previously demonstrated that overexpression of KLF4 repressed cellular proliferation using an MTT assay (14). Thus, we decided to test whether double lysine mutant KLF4 could repress cellular proliferation as well. Without treatment with doxycycline, our lysine mutant stable cell line proliferated at a rate similar to our wild-type KLF4 stable cell line (Fig. 4*C*). Induction of wild-type KLF4 with doxycycline treatment for 3 days approximately halved the rate of proliferation, as was seen previously. However, double lysine mutant KLF4 was significantly less effective at repressing cellular proliferation, suggesting these residues are important for KLF4-mediated inhibition of proliferation.

KLF4 Differentially Modulates Histone Acetylation at the Promoters of Its Target Genes—Inhibition of p300 by E1A completely blocked KLF4-mediated transcriptional activation (see Fig. 3*A*). However, mutation of Lys-225/229 to arginine only partially inhibited this activity, suggesting that p300 interacts with KLF4 and activates KLF4 target genes by more than one mechanism. Since a major function of p300 is to acetylate histones (22) and histone acetylation can have a potent effect on gene expression (24), we hypothesized that KLF4 might activate gene expression via recruitment of p300 to the target promoter. To test this, we performed ChIP using both a KLF4-specific antibody and an acetylated histone H4-specific antibody in our KLF4-inducible stable cell line. As shown in Fig. 5*A*, the induction of KLF4 expression via doxycycline treatment resulted in KLF4 binding to the proximal promoter of the IAP gene. Furthermore, we found an increased level of acetylated H4 on this same promoter, suggesting that KLF4 increases histone acetylation at the IAP promoter. After induction of KLF4 expression via doxycycline treatment, KLF4 binds the cyclin B_1 gene as well, but intriguingly, this resulted in an overall decrease of histone H4 acetylation at its promoter. As a control, we also performed PCR after ChIP for the housekeeping gene *GAPDH*; KLF4 binding could not be detected, although we were able to detect this gene in the input fraction. Furthermore, ChIP assays using a p300 antibody demonstrated that overexpression of KLF4 resulted in increased occupancy of p300 at the IAP promoter, whereas occupancy of p300 did not change at the cyclin B_1 promoter (Fig. 5*B*). These results further suggest that KLF4 differentially regulates histone acetylation at the promoter of its target genes.

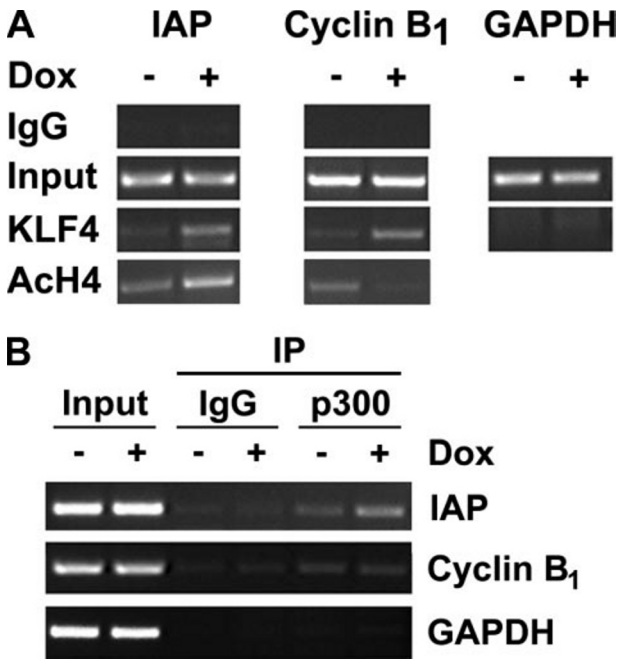

FIGURE 5. **KLF4 differentially modulates histone acetylation at the promoters of its target genes.** *A*, KLF4 physically binds the proximal promoter of IAP and cyclin B_1 genes and modulates local histone H4 acetylation. LS174T-Tet-On-KLF4 cells were treated with doxycycline for 48 h, DNA was cross-linked and sonicated, and IP was performed using the labeled antibodies. Eluted DNA-protein complexes were decross-linked, and PCR was performed using primers targeting the proximal promoter of the labeled genes. KLF4 did not bind an unrelated gene, *GAPDH*. *B*, KLF4 recruits p300 to the IAP promoter but not the cyclin B_1 promoter. The same experiment as in *A* was performed, but a p300-specific antibody was used instead. *Dox*, doxycycline.

Our finding that overexpression of KLF4 differentially modulates histone acetylation was compelling, so we decided to investigate the role of HDACs using the HDAC inhibitor sodium butyrate. Previous reports have shown that butyrate can inhibit proliferation and induce differentiation, and thus it seemed logical that inhibition of deacetylation should have a similar effect as KLF4 on the expression of IAP, cyclin B_1, and p21$^{Cip1/WAF1}$. We treated HT29 cells with butyrate and measured the enzymatic activity of IAP. Treatment with butyrate for 48 h resulted in a dramatic increase in IAP enzymatic activity (Fig. 6*A*). Furthermore, we treated LS174T cells with butyrate for various times and measured the changes in p21$^{Cip1/WAF1}$ and cyclin B_1 expression via Western blot. Butyrate treatment resulted in a gradual increase in p21$^{Cip1/WAF1}$ expression, peaking at 24 h (Fig. 6*B*). Conversely, butyrate had a minimal effect on cyclin B_1 expression for the first 12 h; however, after 24 h

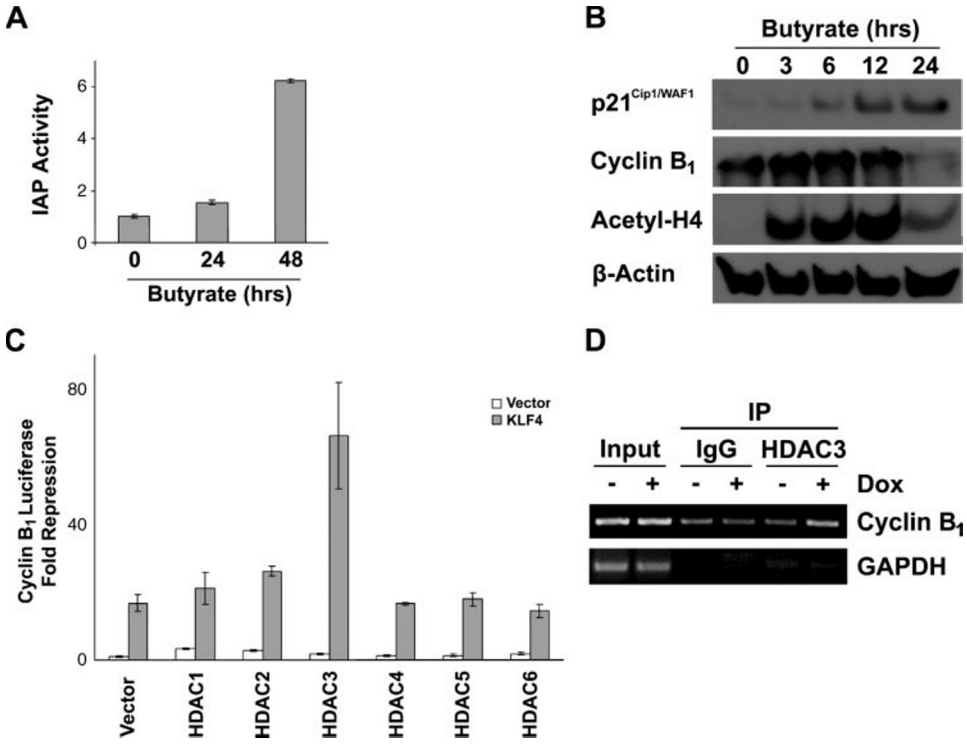

FIGURE 6. **KLF4 recruits HDAC3 to repress cyclin B_1 expression.** *A*, treatment with butyrate, a histone deacetylase inhibitor, results in increased alkaline phosphatase enzymatic activity. HT29 cells were treated with sodium butyrate for various time periods, and then cell lysate was incubated with *p*-nitrophenyl phosphate, an artificial substrate of IAP that produces a yellow color when cleaved. Absorbance was then measured at 405 nm. *B*, inhibition of deacetylation produces a similar pattern of expression as KLF4. LS174T cells were treated with butyrate for various time periods, and Western blot was performed using labeled antibodies. Actin was included as a loading control. *C*, KLF4 synergizes with HDAC3 in repressing the cyclin B_1 reporter. Empty vector (*clear bars*) or KLF4 (*shaded bars*) was co-transfected with the cyclin B_1-luciferase reporter, along with various HDAC plasmids. Co-transfection of HDAC3 with KLF4 in synergistic repression of the reporter. No synergy was seen with other HDACs. *D*, KLF4 recruits HDAC3 to the cyclin B_1 promoter. ChIP was performed as in Fig. 5, but using an HDAC3-specific antibody. Overexpression in KLF4 in LS174T cells resulted increased occupancy of HDAC3 on the cyclin B_1 promoter. GAPDH was included as a negative control.

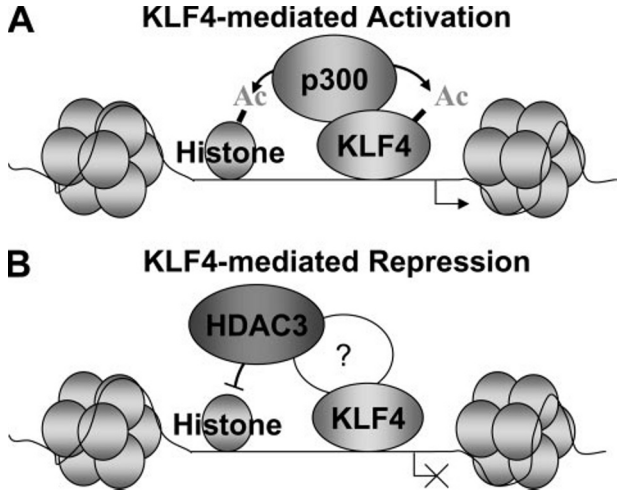

FIGURE 7. **Model of KLF4-mediated transcription.** *A*, model of KLF4-mediated activation. KLF4 binds its target sequence and then recruits the co-activator p300. p300 acetylates KLF4 in addition to acetylating neighboring histones such has histone H4. Histone acetylation promotes localized unwinding of DNA and allows other transcription factors and the basal transcriptional machinery to bind. *B*, model of KLF4-mediated repression. KLF4 binds its target sequence and then recruits co-repressor proteins, including HDAC3, to the promoter, resulting in an overall decrease in transcription of the target gene.

of treatment, the expression of cyclin B_1 was dramatically reduced, suggesting that butyrate had induced cell cycle arrest. Furthermore, inhibition of histone acetylation by butyrate resulted in a rapid increase in histone H4 acetylation, peaking at 6–12 h. To probe the role of histone deacetylases more specifically, we investigated the ability of various HDACs to synergize with KLF4 on the repression of our cyclin B_1-luciferase reporter (Fig. 6*C*). Co-transfection of the reporter and KLF4 alone resulted in ~17-fold repression of the reporter. Co-transfection of the reporter and HDACs 1–6 individually resulted in modest repression (1.3–3.3-fold repression), and co-transfection of KLF4 and most HDAC plasmids resulted in no further increase in repression. However, co-transfection of HDAC3 and KLF4 resulted in 66-fold repression of the reporter, suggesting that KLF4 synergizes with HDAC3 in repressing the cyclin B_1 reporter. To confirm this interaction on native chromatin, we performed a ChIP using an HDAC3-specific antibody. As seen in Fig. 6*D*, overexpression of KLF4 in our doxycycline-inducible cell line resulted in increased occupancy of HDAC3 on the cyclin B_1 promoter, suggesting that KLF4 can recruit HDAC3 to native chromatin.

DISCUSSION

In this study, we made the novel observation KLF4 interacts with, and is acetylated by, the HAT proteins p300 and CBP. This observation is physiologically relevant, as we found that KLF4 is acetylated *in vivo* as well. Furthermore, we found that KLF4 physically binds the IAP promoter, recruits p300, and increases localized histone H4 acetylation. Acetylation of KLF4 appears to be important for full transactivation of the IAP gene, whereas it is dispensable for repression of the cyclin B_1 gene. However, KLF4 may repress cyclin B_1, in part, by promoting localized deacetylation of histone H4. The precise mechanism for the latter remains to be elucidated. However, our reporter studies (Fig. 6*C*) and ChIP data (Fig. 6*D*) suggest that HDAC3 may play a role. A recent report that found a direct interaction between KLF4 and MUC1 on the p53 promoter and that MUC1-C can recruit HDAC3 to the same promoter, resulting in localized histone deacetylation, appears to confirm this possibility (50). We anticipate that our results are part of a general mechanism of KLF4-mediated activation and repression; however, more work needs to be done to fully understand this.

Given the recent finding that KLF4 can repress the Wnt pathway (14), a key pathway in the early stages in colon cancer development (51), clearly a greater understanding of the molecular mechanisms of the tumor suppressor protein KLF4 will give further insight into its role in colon carcinogenesis.

Although several other intensely studied transcription factors have been shown to be acetylated by HAT proteins, the acetylation of KLF4 has not been reported previously. Moreover, we found that KLF4 is acetylated *in vivo*, as after immunoprecipitation of endogenous KLF4 from HCT116 colon cancer cells, we could detect KLF4 with an acetyl-lysine-specific antibody. Through deletion mutation and site-directed mutagenesis of full-length KLF4, we have identified the acetylated lysines as Lys-225 and Lys-229. However, both single point mutants, K225R and K229R, were unable to be acetylated by p300. This was not due to the mutation preventing interaction between KLF4 and p300, as a double lysine mutant (K225R/K229R) could still interact with GST-tagged p300. We speculate that both lysines constitute a recognition sequence for p300 and that mutation of either residue abolishes this recognition. This seems plausible, given that in p300-targeted acetylation of Tat, mutation of specific residues surrounding the lysine targeted for acetylation prevented acetylation (52). Moreover, p300 acetylates only certain lysine residues within the C termini of the histone H2A, H2B, H3, and H4 proteins, despite the presence of entire clusters of lysines, implying a high degree of substrate specificity (53).

Acetylation of KLF4 appears to be important in KLF4-mediated transactivation as mutation of either Lys-225 or Lys-229 within the KLF4 amino acid sequence resulted in decreased activation of an IAP reporter construct and significantly blocked KLF4-mediated induction of IAP and $p21^{Cip1/WAF1}$ mRNA, as measured by RT-PCR. However, acetylation does not seem to be important for KLF4-mediated repression as double lysine mutant KLF4 is equally effective at repressing a cyclin B_1 reporter construct. It is unclear specifically how acetylation of KLF4 results in increased transactivation. One possibility is that KLF4 mediates protein-protein interactions with other transcriptional co-activators and that the acetyl group on these key lysines is required for such interactions.

Our ChIP experiments are in agreement with previous reports that KLF4 physically binds the proximal promoters of both IAP and cyclin B_1. In addition, we report the novel finding that binding of KLF4 to these gene targets has a differential effect on localized histone H4 acetylation. KLF4 recruits p300 to the IAP promoter and results in an increase in histone H4 acetylation. Conversely, overexpression of KLF4 results in a decrease of histone H4 acetylation and no change in p300 occupancy at the cyclin B_1 gene promoter. Given that histone acetylation is typically associated with gene activation and deacetylation with repression, this presents a possible mechanism for how KLF4 can function as both an activator and repressor of gene transcription. We propose a model (Fig. 7*A*) where, in the context of gene activation, KLF4 recruits p300 to the promoter, is directly acetylated by p300, and results in p300-mediated histone acetylation. In the context of gene repression (Fig. 7*B*), KLF4 recruits co-repressor proteins to the promoter, including HDAC3. Future experiments will test this model and attempt to identify whether KLF4 interacts with HDAC3 directly or possibly through other co-repressors. The molecular mechanisms of KLF4-mediated gene expression will have important implications within several fields, including gastrointestinal biology, cancer biology, and stem cell biology.

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