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HDAC-mediated deacetylation of KLF5 associates with its proteasomal degradation

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Abstract

Krüppel-like factor 5 (KLF5) is a basic transcription factor that regulates diverse cellular processes during tumor development. Acetylation of KLF5 at lysine 369 (K369) reverses its function from promoting to suppressing cell proliferation and tumor growth. In this study, we examined the regulation of KLF5 by histone deacetylases in the prostate cancer cell line DU 145. While confirming the functions of HDAC1/2 in KLF5 deacetylation and the promotion of cell proliferation, we found that the knockdown of HDAC1/2 upregulated KLF5 protein but not *KLF5* mRNA, and the increase in KLF5 protein level by silencing HDAC1/2 was at least in part due to decreased proteasomal degradation. Deacetylase activity was required for HDAC1/2-mediated KLF5 degradation, and mutation of KLF5 to an acetylation-mimicking form prevented its degradation, even though the mutation did not affect the binding of KLF5 with HDAC1/2. Mutation of K369 to arginine, which prevents acetylation, did not affect the binding of KLF5 to HDAC1 or the response of KLF5 to HDAC1/2-promoted degradation. These findings provide a novel mechanistic association between the acetylation status of KLF5 and its protein stability. They also suggest that maintaining KLF5 in a deacetylated form may be an important mechanism by which KLF5 and HDACs promote cell proliferation and tumor growth.

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Author Contributions

R.T. designed and performed experiments, and analyzed the data; B.Z. designed and performed some experiments, and wrote the manuscript. Y.L. performed some analyses. J.L.K., R.T., S.X. and C.S. designed some experiments, provided comments, and edited the manuscript; and J.T.D. conceived the idea, designed some of the experiments, provided overall guidance, and wrote and finalized the manuscript.

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Keywords

KLF5; acetylation; histone deacetylases (HDACs); protein stability

INTRODUCTION

The transcription factor Krüppel-like factor 5 (KLF5) regulates a variety of biological processes including cell proliferation, apoptosis, angiogenesis, stemness and the epithelial-mesenchymal transition (EMT) in cancer cells [1–6]. In regulating cell proliferation and tumor growth, KLF5 has been shown to play opposing roles depending on TGF- β and likely other signaling molecules [7]. In prostate cancer cells, KLF5 suppresses tumor growth in the presence of TGF- β , which induces the acetylation of KLF5 at lysine 369 (K369) [8–10], but promotes tumor growth when KLF5^{K369} acetylation is interrupted [7]. Mechanistically, KLF5^{K369} acetylation is essential for the transcriptional regulation of KLF5 downstream target genes. For example, acetylation of KLF5 is critical for KLF5 to bind and activate the p15 promoter [10]. Moreover, acetylation of KLF5 is also required to transcriptionally activate PDGF-A [11]. Acetylation of KLF5 is also necessary for KLF5 to bind to the promoter of p21 and form a repressive complex [12].

The coactivator/acetylase p300 interacts with the DNA-binding domain of KLF5 to acetylate KLF5 at K369 [11]. On the other hand, HDAC1 [13] and HDAC2 [12] have been shown to remove the acetyl group from KLF5. In addition, the oncogenic regulator SET, which masks histone lysines from being acetylated, also masks KLF5 from being acetylated [11]. In our previous study, we found that TGF- β treatment resulted in the recruitment of p300 to acetylate KLF5 [8]; and our unpublished data suggests that oncogenic signaling from RAS or the inactivation of PTEN also affects the acetylation of KLF5 at K369. These findings indicate that both acetylase and deacetylase regulate the acetylation of KLF5 to alter its transcriptional activities. Our previous studies have shown that KLF5 is an unstable protein with a short half-life [14] and is degraded at least through the ubiquitination-proteasome pathway [14]. WWP1, FBW7, SMURF2 and EFP have been identified as E3 ligases that target KLF5 for ubiquitination and subsequent degradation [15–18]. However, whether the acetylation of KLF5 affects its protein stability is unknown at present.

In this study, we investigated whether and how the deacetylases HDAC1 and HDAC2, which regulate KLF5 acetylation, regulate KLF5 protein stability. We found that acetylated KLF5 is more stable than deacetylated KLF5, and that HDACs not only control the acetylation of KLF5 but also affect KLF5 protein stability.

MATERIAL AND METHODS

Cell lines and reagents

The DU 145 prostate cancer cell line and the 293T cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and propagated as previously described [7]. Trichostatin A (TSA), z-Leu-Leu-Leu-al (MG132) and cycloheximide (CHX) were purchased from Sigma (St. Louis, MO).

Establishment of DU 145 cells expressing KLF5, KLF5^{K369R} and KLF5^{K369Q}

The CRISPR-cas9 system was used to eliminate KLF5 protein according to the protocol from the Zhang laboratory [19]. Single clones without KLF5 expression were identified by Western blotting, and truncation of both *KLF5* genes was confirmed by sequencing. Retroviruses expressing wild-type *KLF5*, the acetylation-deficient mutant KLF5^{K369R} and the acetylation-mimicking mutant KLF5^{K369Q} were packaged and applied to infect a KLF5-null clone of DU 145 cells according to the protocol described in our previous study [7].

shRNAs and lentiviruses

PLKO.1 lentiviral vectors expressing shRNAs targeting HDAC1 (TRCN0000004814 and TRCN0000004818) and HDAC2 (TRCN0000004819 and TRCN0000004821) mRNA were purchased from Sigma, and were used following the lentiviral protocols described on the Addgene website (<http://www.addgene.org/lentiviral/protocols-resources/>). PLKO.1 empty vector (Sigma, SHC001) was used as the control.

Immunoprecipitation

Plasmids of Flag-HDAC1 or Flag-KLF5 were co-transfected into 293T cells using the JetPrime polyplus transfection reagent (Radnor, PA) according to the manufacturer's protocol. Empty vector pcDNA3.1 was used as a negative control. Immunoprecipitation was performed following a standard protocol using the KLF5 antibody from R&D Systems with protein G sepharose (Sigma).

Statistical analysis

Results from all experiments were expressed as means $\pm$ standard errors. The statistical significance of differences between two groups was determined by unpaired Student *t* test. Two-way ANOVA tests were used for analyses of protein degradation curves.

RESULTS

Silencing HDAC expression enhances KLF5 acetylation

Previously we found that acetylation of KLF5 determines its functions in cell proliferation and transcriptional regulation in normal epithelial cells. TGF- β treatment induces KLF5 acetylation at K369, which reverses the role of KLF5 in cell proliferation from promotion to suppression [8–10]. To investigate whether TGF- β also regulates the acetylation of KLF5 in cancer cells, we treated DU 145 prostate cancer cells with TGF- β for 24 hours, and found that TGF- β treatment clearly induced the acetylation of KLF5 (Fig. 1A, 1B). Acetylation of KLF5 is also regulated by HDACs [11, 12], so we further tested whether HDACs also regulate KLF5 acetylation in DU 145 cells. As expected, knockdown of HDAC1 or HDAC2 by RNA interference (RNAi) increased KLF5 acetylation; and the knockdown further enhanced the acetylation of KLF5 induced by TGF- β treatment (Fig. 1A, 1B). Interestingly, silencing HDAC1 or HDAC2 also increased the protein level of total KLF5 without TGF- β treatment (Fig. 1A, 1C), suggesting that acetylation could enhance the stability of KLF5 protein in prostate cancer cells. However, when TGF- β treatment was applied, the increase in KLF5 protein caused by silencing HDAC1 or HDAC2 was ablated (Fig. 1A, 1C), which

could further suggest an association of KLF5 stability with its acetylation, since TGF- β induced acetylation of KLF5 could have been sufficient to stabilize KLF5 before HDAC1 or HDAC2 knockdown.

Silencing HDACs increases the protein but not mRNA level of KLF5 and suppresses cell growth

To test whether the increase in KLF5 protein level that results from silencing HDACs involves an increased *KLF5* mRNA level, we knocked down HDAC1 or HDAC2 in DU 145 cells and measured both protein and RNA levels of KLF5. While the knockdown of HDAC1 or HDAC2 upregulated the protein level of KLF5 (Fig. 2A, 2B), it did not increase the mRNA level of *KLF5* (Fig. 2C, 2D). In fact, knockdown of HDAC1 decreased the *KLF5* mRNA level (Fig. 2C), suggesting that upregulation of KLF5 protein is attributed to its protein synthesis or stability. We also overexpressed HDAC1 and HDAC2 in 293T cells, which express a higher level of KLF5, and found that ectopic expression of HDAC1 or HDAC2 downregulated the protein level of KLF5 (Fig. 2E, 2F), consistent with the upregulation of KLF5 by silencing HDACs.

A previous study showed that acetylated KLF5 suppresses while deacetylated KLF5 promotes cell proliferation [7], so we tested whether HDACs silencing suppresses cell growth by enriching acetylated KLF5. Silencing HDAC1 or HDAC2 indeed suppressed the growth of DU 145 cells (Fig. 2G, 2H).

Increase in KLF5 protein by silencing HDACs involves reduced proteasomal degradation

The increase in KLF5 protein expression rather than mRNA expression resulting from the silencing of HDACs suggests that knockdown of HDACs affects KLF5 protein stability. To test this possibility, we treated DU 145 cells with cycloheximide (CHX) for different times to block the synthesis of KLF5 protein and measured the KLF5 protein level. Silencing either HDAC1 or HDAC2 delayed the protein degradation of KLF5 (Fig. 3A, 3B).

It has been shown that the protein stability of KLF5 is regulated by the ubiquitin proteasome pathway (UPP) [17, 18]. We thus treated DU 145 cells with MG132, a proteasome inhibitor, and tested whether HDACs regulate KLF5 expression through proteasome-dependent protein degradation. Consistent with our original reports, as expected, MG132 treatment increased the protein level of KLF5 in the shRNA control group (Fig. 3C). Interestingly, knockdown of either HDAC1 or HDAC2 significantly enhanced the upregulation of KLF5 caused by MG132 treatment (Fig. 3C, 3D), indicating that silencing HDACs increased the KLF5 protein level by reducing proteasome-dependent protein degradation.

Acetylation at K369 stabilizes KLF5 protein but does not affect its interaction with HDACs

We asked whether the deacetylase activity of HDACs is required for their regulation of KLF5 protein stability. To address this question, we treated DU 145 cells with trichostatin A (TSA), a specific HDAC inhibitor, while silencing HDAC1 or HDAC2. We found that with TSA treatment, knockdown of HDAC1 or HDAC2 failed to increase KLF5 protein level (Fig. 4A), suggesting that the regulation of KLF5 protein level by HDACs depends on their deacetylase activities.

To test whether HDAC-induced KLF5 degradation depends on the deacetylation of KLF5 at K369, we prepared DU 145 cells in which the endogenous *KLF5* was deleted, and the acetylation-preventing KLF5^{K369R} (KR) or acetylation-mimicking KLF5^{K369Q} (KQ) mutant was stably expressed. Interestingly, knockdown of HDAC1 or HDAC2 still increased KLF5 protein levels in KR cells but had no such effect on KQ cells (Fig. 4B, 4C). These results suggest that it is the deacetylated KLF5 that is sensitive to proteasomal degradation, and that the regulation of KLF5 protein stability by HDAC1/2 depends on the deacetylation of KLF5 at K369. The failure of HDAC silencing to increase KLF5 protein levels in KQ cells (Fig. 4B, 4C) further suggests that acetylation of KLF5 at K369 prevents proteasome-mediated KLF5 degradation.

We also treated KLF5^{K369R} and KLF5^{K369Q} cells with CHX for different times, and then tested the effect of HDAC silencing on KLF5 protein level. Consistently, while knockdown of HDAC1 or HDAC2 delayed the protein degradation of KLF5^{K369R}, the knockdown did not affect KLF5 protein levels in KQ cells (Fig. 4D, 4E). In addition, KLF5^{K369Q} was significantly more stable than KLF5 and KLF5^{K369R} (Fig. 4F), further indicating that acetylation of KLF5 at K369 is necessary for its stability. Consistently, TSA treatment led to more increase in the expression of wildtype KLF5 and KLF5^{K369R} than KLF5^{K369Q} (Fig. 4G). In addition, TSA treatment reduced the extent of degradation of wildtype KLF5 and KLF5^{K369R} but not that of KLF5^{K369Q}, as indicated by the fold changes of KLF5 protein levels after CHX treatment (Fig. 4G). These findings further indicate the role of KLF5 acetylation at K369 in maintaining KLF5 protein stability.

KLF5 interacts with HDACs, as demonstrated in previous reports [12, 13]. To test whether the acetylation of KLF5 affects its interactions with HDACs, we pulled down protein complexes of KLF5, KLF5^{K369R} (KR) and KLF5^{K369Q} (KQ) from 293T cells expressing different forms of KLF5 using immunoprecipitation and detected the presence of HDAC1 in these complexes. HDAC1 was detected in each of the KLF5 complexes regardless of KLF5 acetylation status (Fig. 4H), suggesting that the binding between KLF5 and HDAC1 is independent of KLF5 acetylation status.

DISCUSSION

In this study, we examined the regulation of KLF5 protein by HDACs. While confirming that KLF5 acetylation can be interrupted by the HDAC1 and HDAC2 deacetylases but induced by TGF- β treatment in the prostate cancer cell line DU 145 (Fig. 1), we found that knockdown of HDAC1 and HDAC2 increased the protein level of KLF5, and this increase involved the attenuation of proteasome-dependent protein degradation of KLF5 (Fig. 2, 3). In addition, TSA treatment also increased KLF5 protein level even after CHX treatment (Fig. 4G). These findings suggest that deacetylation leads to the susceptibility of KLF5 to proteasome-mediated degradation. Further supporting this conclusion, we found that while deacetylation of KLF5 by the K369R mutation did not slow down KLF5 degradation, the K369Q mutant that mimics acetylated KLF5 became more stable and thus irresponsive to HDAC silencing-enhanced KLF5 stability (Fig. 4B–F). Consistently, TSA also prevented wildtype KLF5 and K369R mutant from degradation, while the resistance of K369Q to protein degradation was not affected by TSA treatment (Fig. 4G).

The inability of the K369R deacetylation mutant to increase KLF5 stability further indicates that lysine 369 or its ubiquitination is not directly responsible for the degradation of KLF5 mediated by UPP, because the K369R mutant cannot be ubiquitinated. It is unknown how acetylation at K369 interferes with the ubiquitination of KLF5 and its proteasomal degradation. We noticed that the acetylation status of KLF5 at K369 did not affect its binding to HDAC1 (Fig. 4H), Therefore one possibility is that acetylation of KLF5, while still allowing binding to HDACs (Fig. 4), prevents the binding of the involved E3 ubiquitin ligase to KLF5 to prevent the degradation of KLF5 by the E3 ligase.

KLF5 has been identified as an unstable protein that is ubiquitinated by WWP1 [17], FBW7 [18], SMURF2 [15] and EFP [16]. KLF5^{K369Q} could be detached from the proposed E3 ligase constitutively (Fig. 4I right), leading to its resistance to HDAC-mediated degradation (Fig. 4E). It is possible that the recruitment of p300 to KLF5, which has been demonstrated previously [8, 10], blocks the interaction between a ubiquitin E3 ligase and KLF5, which could be responsible for the observed lack of effect of silencing HDAC1 or HDAC2 on KLF5 protein expression in the presence of TGF- β treatment (Fig. 1A).

However, the acetylation deficient mutant KLF5^{K369R} was still stabilized after the knockdown of HDAC1/2 or TSA treatment and the mutation did not affect its interaction with HDAC1 (Fig. 4), which was similar to that of wildtype KLF5. It is thus possible that the binding of HDACs to KLF5 is a required event for an E3 ligase to mediate KLF5's degradation (Fig. 4I). Another possibility is that the involved E3 ubiquitin ligase has an acetyl group regulated by HDACs, and the acetylation of this E3 ligase leads to its dissociation from KLF5 and releases KLF5 from being degraded. Interestingly, EFP has some potential acetylation sites at 273, 320, 402 and 567 according to the protein modification summary from Phosphosite (<https://www.phosphosite.org/>), supporting the idea that the activity of the E3 ligase could be regulated by HDACs.

Our previous study has demonstrated that TGF- β -activated SMADs enter the nucleus to recruit the acetylase p300 to KLF5 via protein interactions, leading to the acetylation of KLF5 at K369 and the reassembly of the KLF5 transcriptional complex on the promoter of p15 [10]. As a result, acetylation at K369 reverses the function of KLF5 in gene regulation, cell proliferation, and tumor growth, with deacetylated KLF5 promoting but acetylated KLF5 slowing cell proliferation and tumor growth [7, 8, 10]. The KLF5 protein level is controlled by the UPP in both normal and cancer cells, and UPP-mediated KLF5 degradation occurs in cells that express a high level of KLF5, are actively proliferating, and are not treated with TGF- β [14, 20]. HDACs in general promote cell proliferation to play an oncogenic role in prostate cancer [21], and silencing HDAC1 or HDAC2 indeed slowed cell proliferation in DU 145 cells (Fig. 2). Taken together, it seems that HDACs interact with KLF5 to prevent KLF5 from being acetylated, which contributes to the promotion of cell proliferation and tumor growth while keeping the KLF5 protein level fine-tuned by the UPP system.

These findings have established a novel association between the deacetylation of KLF5 and its proteasomal degradation. Deacetylases HDAC1 and HDAC2 interact with KLF5, and the interaction not only prevents KLF5 acetylation but also leads to its proteasomal degradation.

Loss of HDACs results in increased levels of acetylated KLF5, which become insensitive to proteasomal degradation and have different activities in gene transcription [10, 11] and tumor growth in prostate cancer cells [7], supporting the biological effects of HDACs knockdown (Fig. 2G and 2H). Our study provides a molecular mechanistic explanation for how KLF5 acetylation affects its protein stability. KLF5, as a basic transcription factor, plays diverse roles in tumor development. Acetylation of KLF5 at K369 is essential to determine its regulation of target genes, tumor growth, and other steps during tumor development. Therefore, our findings could impact future studies targeting KLF5's function for therapeutic development.

In this study, we found that silencing HDAC1 or HDAC2 increased the protein levels of both total KLF5 and acetylated KLF5 but not the mRNA level of *KLF5*, while overexpression of HDAC1 or HDAC2 decreased KLF5 protein level. The increase in KLF5 protein level by silencing HDACs was at least in part due to reduced protein degradation in a proteasome dependent manner. In addition, deacetylase activity was required for HDAC-mediated KLF5 degradation, and degradation occurred only of deacetylated KLF5 but not acetylated KLF5, even though the acetylation status of KLF5 did not affect its binding to HDAC1. Collectively, these data indicate that HDACs maintain KLF5 in the deacetylated form, which is susceptible to proteasomal degradation while more active in the promotion of cell proliferation and tumor growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Silencing HDAC1/2 increased KLF5 protein by preventing proteasomal degradation.
- Deacetylase activity was required for HDAC1/2-mediated KLF5 degradation.
- The acetylation-mimicking form of KLF5 was prevented from degradation.
- The binding of KLF5 with HDAC1/2 was not affected by its acetylation status.

Figure 1. Silencing HDAC1 and HDAC2 enhances TGF-β-promoted KLF5 acetylation

(A) Detection of indicated proteins by Western blotting in whole cell lysates (WCL) and endogenous KLF5 proteins immunoprecipitated (IP) from DU 145 prostate cancer cells with or without TGF-β treatment. shCon, control shRNA; shHDAC1, shRNA mixture for HDAC1; shHDAC2, shRNA mixture for HDAC2. Numbers below HDAC panels indicate ratios of HDAC protein levels to β-actin. (B) Relative protein level of acetylated KLF5 (Ac-KLF5) in reference to total input KLF5 level, as calculated based on band intensities from panel A. Ac-KLF5 was detected by acetylated lysine antibody after IP with KLF5 antibody. (C) Relative protein level of KLF5 in reference to β-actin level for each sample, as calculated according to band intensities from panel A.

Figure 2. Silencing HDAC1 and HDAC2 increases the protein level but not the mRNA level of KLF5 while attenuating cell proliferation

(A–B) Detection of KLF5, HDAC1 and HDAC2 by Western blotting after HDAC1 and HDAC2 were knocked down by a mixture of shRNAs in DU 145 cells. (C, D) mRNA level of *KLF5* in DU 145 cells after knockdown of *HDAC1* (C) and *HDAC2* (D), as detected by real-time qPCR. (E, F) Detection of KLF5, HDAC1 and HDAC2 by Western blotting after HDAC1 and HDAC2 were overexpressed in 293T cells. β-actin is the internal control. Numbers below lanes indicate KLF5 protein levels relative to β-actin level. (G, H) Knockdown of *HDAC1* (G) or *HDAC2* (H) decreased cell proliferation in DU 145 cells, as detected by SRB assay. ***, $P < 0.001$.

Figure 3. HDAC1- and HDAC2-mediated downregulation of KLF5 protein involves the proteasome-dependent protein degradation

(A, B) Knockdown of *HDAC1* and *HDAC2* delayed protein degradation, as determined by the cycloheximide (CHX) assay. (C, D) The proteasome inhibitor MG132 increased KLF5 protein level after the knockdown of *HDAC1* and *HDAC2*. Panels B and D show relative protein levels of KLF5 calculated from duplicated experiments for panels A and C, respectively. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.