Transforming Growth Factor-β Stimulates p300-dependent RUNX3 Acetylation, Which Inhibits Ubiquitination-mediated Degradation*

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The Runt domain transcription factors (RUNXs) play essential roles in normal development and neoplasias. Genetic analyses of animals and humans have revealed the involvement of RUNX1 in hematopoiesis and leukemia, RUNX2 in osteogenesis and cleidocranial dysplasia, and RUNX3 in the development of T-cells and dorsal root ganglion neurons and in the genesis of gastric cancer. Here we report that RUNX3 is a target of the acetyltransferase activity of p300. The p300-dependent acetylation of three lysine residues protects RUNX3 from ubiquitin ligase Smurf-mediated degradation. The extent of the acetylation is up-regulated by the transforming growth factor-β signaling pathway and down-regulated by histone deacetylase activities. Our findings demonstrate that the level of RUNX3 protein is controlled by the competitive acetylation and deacetylation of the three lysine residues, revealing a new mechanism for the posttranslational regulation of RUNX3 expression.

The RUNX family of transcriptional regulators plays pivotal roles in various developmental differentiation processes ranging from sex determination, eye development, hematopoiesis, and segmentation in Drosophila to hematopoiesis, bone development, and neurogenesis in mammals. An important feature of these proteins is the Runt domain, a conserved 128-amino acid region that is necessary for binding specific DNA sequences (1). Three mammalian Runt domain transcription factors, RUNX1 (PEBP2α/B/CBFA2/AML1), RUNX2 (PEBP2αB/ CBFA1/AML3), and RUNX3 (PEBP2αC/CBFA3/AML2), have been identified (2). The RUNX1 gene regulates hematopoietic cell-specific genes and is required for definitive hematopoiesis (3). RUNX1 is the most frequent target for chromosomal translocation in leukemia and is responsible for about 30% of the cases of human acute leukemia (4). Haplo-insufficiency of RUNX1 causes an autosomal dominant congenital platelet disorder characterized by malformation of the clavicle and skull (9, 10). RUNX3 is required for the development of CD8-lineage T cells (11, 12) and TrkC-dependent dorsal root ganglion neurons (13, 14). RUNX3 also functions as a gastric tumor suppressor. About 60% of primary gastric cancer specimens express significantly lower levels of RUNX3 because of a combination of hemizygous deletion of the gene and hypermethylation of the RUNX3 promoter region (15).

Biochemical analysis has demonstrated that RUNX transcription factors physically interact with TGF-β-activated Smad proteins to mediate transforming growth factor-β (TGF-β) signaling (16). Bone morphogenetic protein (BMP), a member of the TGF-β superfamily, induces both the expression of RUNX2 and the interaction of RUNX2 with BMP-activated Smads, which facilitates the differentiation of mesenchymal cells into osteoblasts (17, 18). The tumor suppressor activity of RUNX3 also appears to be associated with TGF-β signaling. The gastric mucosa of RUNX3 knock-out mice develops hyperplasia because of the stimulation of proliferation and the suppression of apoptosis, processes that are accompanied by a decreased sensitivity to TGF-β (15).

Acetylation mediated by the transcriptional coactivator p300 is known to stimulate transcription either by modifying histones in chromatin to produce a transcriptionally active conformation or by directly targeting transcriptional activators such as p53, GATA-1, and E2F (19, 20, 21). It has been reported that p300 interacts with RUNX1 and stimulates RUNX1-dependent transcription (22). In contrast, histone deacetylase-6 (HDAC-6) interacts with RUNX2 and represses RUNX2-dependent transcription (23).

Despite these various observations, the underlying molecular mechanisms that are involved in the regulation of RUNX3 activity are poorly understood. Here we report that p300 histone acetyltransferase (HAT) activity can acetylate lysine residues in RUNX3. The TGF-β superfamily signaling pathway enhances the level of acetylation and the stability of RUNX3. Because the same RUNX3 lysine residues can be targeted by ubiquitin ligase Smurfs, TGF-β appears to suppress RUNX3 ubiquitination and degradation through competitive acetylation. HDACs deacetylate RUNX3 and allow the protein to be degraded through the ubiquitin-mediated pathway. Our results demonstrate previously unidentified links among p300, HDACs, and Smurfs in controlling RUNX3 stabilization and degradation.

The abbreviations used are: TGF-β, transforming growth factor-β; HDAC, histone deacetylase; HAT, histone acetyltransferase; CBFβ, core binding factor β; TSA, trichostatin A; HA, hemagglutinin; a.a., amino acid(s); E3, ubiquitin-protein isopeptide ligase.

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REGULATION OF RUNX3 BY ACETYLATION AND UBIQUITINATION

EXPERIMENTAL PROCEDURES

Cell Culture—All tissue culture media and antibiotics were from Invitrogen. 293 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics and antimycotics at 37 °C under a 5% CO2 atmosphere.

Plasmids and Antibodies—Myc-, HA-, and green fluorescent protein (GFP)-tagged RUNX1, RUNX2, RUNX3 full-length and deletion mutants were constructed in a CMV promoter-derived mammalian expression vector (pCS4-3Myc, -3HA, -GFP). Mutations affecting the RUNX3 acetylation site were introduced by PCR, and mutant cDNAs were cloned into the pCS4-3Myc vector. The pCMV β-galactosidase plasmid was included as an internal control for transfection efficiency.

Immunoprecipitation and Immunoblot Analysis—After transfection, 293 cells were lysed in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% Na deoxycholate, 1% glycerol, 25 mM NaF, 1 mM EDTA, 1 mM Na3VO4, 250 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and cleared by centrifugation. The supernatants were immunoprecipitated with appropriate antibodies, washed, and then incubated with horseradish peroxidase-coupled secondary antibodies. After washing, the blots were developed with Western blotting chemiluminescence luminol reagent (Amersham Biosciences). For trichostatin A (TSA) (Sigma) treatment, 293 cells were cultured in fresh medium for 1 day after transfection and then treated with the indicated concentrations of TSA for 16 h.

Immunofluorescence Studies—Cells grown on 22-mm coverslips (Fisher) were transfected as indicated, washed with phosphate-buffered saline, fixed with 4% formaldehyde for 15 min at room temperature, and then blocked with 10% fetal bovine serum, phosphate-buffered saline, 0.1% Triton X-100 for 45 min. To detect Myc-HDAC5, cells were stained with 1:200-diluted 9E10, an anti-Myc monoclonal antibody. Immunostained cells were then incubated with appropriate antibodies, washed, and stained with 1:200-diluted 9E10, an anti-Myc monoclonal antibody.
Regulation of RUNX3 by Acetylation and Ubiquitination

Acetylation of RUNX3 mediated by p300 and mapping of the acetylation sites. A, acetylation of RUNX3 by p300. 293 cells were transfected with plasmids expressing p300 and Myc-tagged full-length and truncated RUNX cDNAs. The cell lysates were analyzed by immunoprecipitation using an antibody against acetylated lysine followed by Western blotting using an anti-Myc antibody. Arrowheads indicate bands representing acetylated RUNX proteins. B, mapping of the lysine residues in RUNX3 that are targeted for p300-dependent acetylation. 293 cells were transfected with plasmids expressing p300 and Myc-tagged RUNX3 lysine mutants. Cell lysates were analyzed by immunoprecipitation using an antibody against acetylated lysine followed by Western blotting using an anti-Myc antibody (upper panel). To examine the level of expression of the RUNX mutants, the same lysates were analyzed by Western blotting using the anti-Myc antibody (lower panel). C, schematic diagram of RUNX3 and lysine residues. All the residues are conserved among the three RUNX proteins. Circles indicate experimentally determined acetylation sites. D, sequence-specific DNA binding by RUNX3-K148R,K186R,K192R mutants. Electrophoretic mobility shift assay was performed with the TGF-β-responsive element probe, and nuclear lysates were prepared from 293 cells transfected with Myc-tagged RUNX3 or the RUNX3-K148R,K186R,K192R mutant. Double stranded oligonucleotides, TGF-β-responsive element, and M1 were used as specific competitors and M2 as a nonspecific competitor. The arrows indicate the specific TGF-β-responsive element binding complex. The arrowhead indicates a nonspecific band. E, physical interaction of RUNX3-KR mutants with PEBP2α/CBFα, Smad3, and p300. Myc-tagged RUNX3 and RUNX3-KR mutants were cotransfected with HA-tagged PEBP2α/CBFα, Smad3, or p300 into 293 cells. Each cell lysate was analyzed by immunoprecipitation followed by Western blotting using the indicated antibody.

RESULTS

p300 Physically Interacts with RUNX3—To detect interactions between p300 and the RUNX proteins, RUNX1, RUNX2, and RUNX3 were transfected into 293 cells and then analyzed by immunoprecipitation and Western blotting. In this transient analysis, the endogenous p300 protein was detected in RUNX immunoprecipitates (Fig. 1C, left panel), whereas RUNX3 protein was detected in p300 immunoprecipitates (Fig. 1C, right panel). These results indicate that p300 interacts with RUNX2 and RUNX3 as well as RUNX1.

To identify the regions in p300 and RUNX3 that are involved in the interaction, RUNX3 and p300 (full-length or deletion derivatives) were co-expressed in 293 cells and analyzed by immunoprecipitation and Western blotting. A schematic representation of the RUNX3 and p300 proteins and their deletion derivatives is shown in Fig. 1, A and B. The binding data show that p300 interacts with the Runt domain and the C-terminal region of RUNX3 (Fig. 1D). It was previously reported that the C-terminal region of RUNX1 is responsible for the induction of cell differentiation and for interaction with the N-terminal region of p300 (13). Additionally, we found that RUNX3, as well as RUNX1, interacts with p300. The regions responsible for the interaction of p300 and RUNX3 were mapped to the N terminus (p300–1: a.a. 1–565) and C terminus (p300–4: a.a. 963–2369) of p300 and to the Runt domain and C terminus of RUNX3 (Fig. 1E).

p300 Acetylates RUNX Proteins—Based on our observation that the C-terminal region of p300, which contains the HAT domain, interacts with RUNX proteins, we asked whether p300 could also acetylate RUNX3. Lysates of cells expressing p300...
and Myc-tagged RUNX3 were immunoprecipitated with anti-acetyl-lysine antibodies, and the immunoprecipitates were Western blotted using an anti-Myc antibody. This experiment revealed that p300 acetylates RUNX3 (Fig. 2A). RUNX3 contains eight highly conserved lysine residues that are potential acetylation sites: one in the N-terminal region, six in the Runt domain, and one in the C-terminal region (Fig. 2C). To identify which lysine residue(s) is/are acetylated in a p300-dependent manner, a series of RUNX3 deletion mutants was co-expressed with p300 in 293 cells and monitored by immunoprecipitation assay using an anti-acetyl-lysine antibody. Acetylation was detected within the Runt domain and the C-terminal region of RUNX3 (Fig. 2A). To identify the precise targets of acetylation, RUNX3 mutants with lysine to arginine (KR) substitutions were tested for p300-dependent acetylation in 293 cells. Immunoprecipitation and Western blot analysis showed that the doubly mutated RUNX3-K148R,K192R and RUNX3-K186R,K192R proteins exhibited a marked decrease in acetylation (Fig. 2B). This result suggests that the Lys-148, -186, and -192 residues of RUNX3 are the major targets of the acetyltransferase activity of p300. In support of this result, mutation of these three lysine residues (RUNX3-K148R, K186R, and K192R) abrogated the acetylation signal (Fig. 2B). In Fig. 2C, the circles represent the RUNX3 acetylation sites. These mutations apparently do not affect the ability of RUNX3 to interact with DNA (Fig. 2D) or with other proteins, including PEBP2β/CBFβ, Smad3, and p300 (Fig. 2E).

RUNX3 Acetylation Is Controlled by the HDAC-dependent Pathway—Our finding that RUNX3 is acetylated by p300 raised the possibility that there is a deacetylation mechanism. Therefore, we examined whether any of the HDACs (HDAC1, -2, -4, and -5) could remove the acetyl moiety from acetylated RUNX3. Immunoprecipitation and Western blotting analysis revealed that RUNX3 interacts with HDAC1, -2, and -5 and to a lesser degree with HDAC4 (Fig. 3A). The effect of these HDACs on RUNX3 acetylation was then analyzed by immunoprecipitation and Western blotting. The level of RUNX3 expression was measured by Western blotting.

Fig. 3. The deacetylation of RUNX3 is mediated by HDAC5. A, physical interaction between RUNX3 and HDACs. HA-RUNX3 was co-expressed with Myc-HDACs in 293 cells as indicated. The interaction between RUNX3 and HDAC was analyzed by co-immunoprecipitation using an anti-HA antibody and Western blotting using an anti-Myc antibody (left panel). The level of expression of each HDAC was analyzed by Western blotting using the anti-Myc antibody (right panel). B, deacetylation of RUNX3 by HDACs. HA-RUNX3 was co-expressed with HDACs and p300 in 293 cells as indicated. The effect of HDACs on RUNX3 acetylation was analyzed by co-immunoprecipitation using an anti-acetylated lysine antibody and Western blotting using the anti-HA antibody. C, co-localization of RUNX3 and HDAC5. Green fluorescent protein-RUNX3 was co-expressed with HDAC5 in 293 cells. Exogenous HDAC5 was visualized by immunostaining with the anti-Myc (9E10) monoclonal antibody. The nucleus was detected by 4,6-diamidino-2-phenylindole (DAPI) staining.

Fig. 4. The p300-dependent acetylation of RUNX3 is stimulated by TGF-β signaling. Acetylation of RUNX3 by TGF-β stimulation or TSA treatment of 293 cells. Myc-tagged RUNX3 was expressed in 293 cells with or without p300 and HDAC5 and treated with TGF-β (0.5 ng/ml, 1 h) and/or TSA (0.5 μM, 16 h). Protein extracts were analyzed by immunoprecipitation using an anti-acetylated lysine antibody and Western blotting using an anti-Myc antibody. The level of RUNX3 expression was measured by Western blotting.

Regulation of RUNX3 by Acetylation and Ubiquitination
decreased by the HDACs (Fig. 3B). RUNX3 was more effectively deacetylated by Class II HDACs (HDAC4, -5) than by class I HDACs (HDAC1, -2). Interestingly, for reasons that remain unclear, HDAC4 showed only very weak affinity to RUNX3 yet quite high RUNX3 deacetylation activity comparable with HDAC5 (Fig. 3A and B). Further analysis of the precise binding domains between HDACs and RUNX3 may provide a clue to resolve this discrepancy. We examined whether RUNX3 colocalizes with HDAC5 by an immunohistochemical study. HDAC5 was detected in both the cytoplasm and nucleus, whereas RUNX3 was found exclusively in the nuclei of 293 cells. When HDAC5 and RUNX3 were cotransfected, nuclear HDAC5 was found to colocalize with RUNX3 (Fig. 3C). Interestingly, in some cells that strongly expressed RUNX3, HDAC5 was detected only in the nucleus. These results suggest that HDAC5 and RUNX3 physically interact and colocalize in the nucleus in vivo.

The TGF-β Signaling Pathway Stimulates the p300-mediated Acetylation of RUNX3—Runt domain transcription factors are important targets for TGF-β superfamily signaling. To determine whether the TGF-β signaling pathway influences the acetylation and/or deacetylation of RUNX3, 293 cells were transfected with RUNX3 in the presence or absence of p300 and treated with TGF-β or the HDAC inhibitor TSA. Notably, the level of RUNX3 acetylation was enhanced not only by p300 coexpression but also by TGF-β and TSA treatment (Fig. 4A). Combining these effectors further increased the level of RUNX3 acetylation. RUNX3 acetylation by TGF-β was effectively reversed by HDAC5 (Fig. 4B). On the other hand, the RUNX3 K148R,K186R,K192R mutants showed a significantly reduced level of acetylation by p300 and/or TGF-β (Fig. 4B). This result suggests that p300 and the HDACs are the physiological effectors that control RUNX3 acetylation and that the TGF-β superfamily signaling pathway favors acetylation over deacetylation.

Acetylation of RUNX3 Inhibits Its Degradation—Interestingly, we observed that the level of RUNX3 expression increased in proportion to the level of acetylation (Fig. 4A). To test whether p300-dependent acetylation affects the stability of RUNX3, RUNX3 was expressed in 293 cells in the presence or absence of p300; the level of protein, as measured by Western blotting, was found to be much higher in the presence of p300 (Fig. 5A, left panel). To confirm these observations, a fixed amount of RUNX3 was expressed alone or with increasing amounts of p300. In this transient assay, the level of RUNX3 dramatically increased in proportion to the amount of input p300 (Fig. 5A, right panel). To elucidate the mechanism underlying this phenomenon, the stabilities of wild-type RUNX3 and RUNX3-K148R,K186R,K192R were examined. Wild-type RUNX3 was quickly degraded, whereas p300 protected RUNX3 from degradation (Fig. 5B, left panel). In contrast, the RUNX3 KR protein was much more stable than wild-type RUNX3 and co-expression of p300 did not further increase its stability (Fig. 5B, right panel). These results suggest that these lysine residues are responsible for RUNX3 degradation and that acetylation by p300 protects the protein by masking these residues from ubiquitination. This interpretation led us to examine whether the deacetylation of RUNX3 by HDACs would decrease its stability. In support of our hypothesis, co-expression of HDAC5 destabilized RUNX3 but co-expression of p300 counteracted this effect and increased the stability of RUNX3 (Fig. 5C).

Acetylation Protects RUNX3 from Smurf-mediated Degradation—C-terminal truncation of RUNX3 was also observed to stabilize the protein. A RUNX3 derivative that terminated at amino acid 374 (RUNX3Δ374) was expressed at a level similar to that of wild-type RUNX3, and its expression was significantly increased in the presence of p300 (Fig. 6A). However, a derivative lacking residues beyond amino acid 187 (RUNX3Δ187) exhibited significantly enhanced stability, and the presence of p300 did not further increase the level of this mutant protein (Fig. 6A). This result indicates that the region from amino acids 187 to 374 is required for degradation of

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**Fig. 5.** The p300-mediated acetylation of lysine residues stabilizes RUNX3. A, left panel, Myc-tagged RUNX3 was expressed in 293 cells in the absence or presence of p300, and the levels of expression were measured by Western blotting using the anti-Myc antibody. An anti-β-actin antibody was used as a loading control (lower panel). Right panel, 293 cells were cotransfected with a fixed amount of Myc-tagged RUNX3 (0.1 μg) and increasing amounts of HA-tagged p300 (0–1 μg) as indicated, and the levels of RUNX3 expression were measured with the anti-Myc antibody. B, 293 cells transfected with Myc-tagged RUNX3 and RUNX3-K148R,K186R,K192R were treated with cycloheximide (40 μg/ml) and cultured for the indicated periods. Cell lysates were prepared, and the levels of expression of RUNX3 and the KR mutants were measured by Western blotting using the anti-Myc antibody. The experiment was performed in the absence (left panel) and presence (right panel) of p300. C, the effect of HDAC5 and p300 on the stability of RUNX3. Myc–RUNX3 was coexpressed with HDAC5 and p300 in 293 cells as indicated and cultured for the indicated periods in the presence of cycloheximide (40 μg/ml). Protein extracts were resolved by SDS-PAGE, and RUNX3 expression was analyzed by Western blotting using an anti-Myc antibody.
RUNX3. This region contains a conserved PPXY motif (P-box; a.a. 309–312) that is recognized by the Smurf WW domain (24). Based on this observation, we asked whether Smurf ubiquitin ligases are involved in the RUNX3 degradation pathway and whether p300 interferes with this pathway. Co-immunoprecipitation and ubiquitination assays revealed that Smurf1 and Smurf2 interact with and ubiquitinate RUNX3 (Fig. 6, B and C). Myc-tagged RUNX3 was used as substrate in in vivo ubiquitination reactions in the presence or absence of Smurf and HA-ubiquitin. RUNX ubiquitination was determined by co-immunoprecipitation using the anti-Myc antibody and Western blotting using the anti-HA antibody. D, Myc-tagged RUNX3 was cotransfected with increasing amounts of Smurf1 (0–2 μg) in the absence or presence of p300 (0.5 μg) as indicated. The levels of expression were measured by Western blotting using the anti-Myc antibody. E, Myc-tagged RUNX3, RUNX3-P309R, RUNX3-del-309–312, and RUNX3-K148R,K186R,K192R were cotransfected with increasing amounts of Smurf1 or Smurf2 (0–2 μg) as indicated. Their levels of expression were measured by Western blotting using the anti-Myc antibody.

These results suggest that p300-mediated acetylation inhibits RUNX3 ubiquitination by Smurf proteins. Interestingly, deletion of the P-box (RUNX3-del-309–312) or substitution of one of the proline residues with arginine (RUNX3-P309R) confers resistance to Smurf-mediated degradation. Similarly, the RUNX3-K148R,K186R,K192R mutant is also resistant to Smurf-mediated degradation (Fig. 6E). Taken together, our results suggest that the acetylation of lysine residues by p300 protects RUNX3 from Smurf-mediated degradation.

Acetylation Increases RUNX3 Transactivation Activity—To further investigate the functional consequences of RUNX3 acetylation and deacetylation, we analyzed the effect of p300 and HDAC5 on RUNX3-mediated transcriptional activation and found that inhibition of HDAC by TSA increased the transactivation activity of RUNX3 (Fig. 7A). In the absence of RUNX3, TSA did not affect reporter activity (data not shown). p300 also increased RUNX3-mediated transactivation activity. The p300-dependent increase in RUNX3 activity is suppressed by the co-expression of HDAC5, and this suppression in turn is reversed by TSA treatment (Fig. 7A). Semiquantitative reverse transcriptase-PCR experiments revealed that the mRNA levels of endogenous and exogenous RUNX3 were not affected by expression of HDAC5 and p300 and treatment of TSA in 293 cells (Fig. 7B). These findings suggest that acetylation is responsible for the increase in RUNX3-dependent transactivation activity. Notably, although mutation of the lysine residues targeted by p300 stabilizes the mutant proteins, these alterations significantly decrease RUNX3 transactivation activity (Fig. 7C). Coexpression of p300 does not increase the activity of the mutant RUNX3 proteins relative to the effect of p300 alone on pGL3–12-RXE-luc reporter plasmid.

In agreement with the increase in RUNX3 acetylation medi-
ated by TGF-β, the transactivation activity of RUNX3 was up-regulated by TGF-β treatment (Fig. 7D). The effect of p300 on RUNX3 transactivation activity was also further up-regulated by TGF-β. Cotransfection with HDAC5 significantly reduced the effect of TGF-β. On the other hand, the RUNX3-KR mutant showed only a minimal response to TGF-β.

**DISCUSSION**

The RUNX family of transcriptional regulators controls lineage-specific gene expression by interacting with transcription factors such as PEBP2β/CBFβ, Smad, C/EBP, ETS, ALY, TLE, HDAC, and p300 (16, 22, 25–30). Depending on the specific promoter context, RUNX proteins can function as transcriptional activators or suppressors. Although p300 and HDAC6 have been implicated in regulating this RUNX activity (22, 23), the molecular mechanisms by which they do so are poorly understood.

The multifaceted transcriptional co-activator p300 plays a central role in coordinating and integrating multiple signaling events. Previously, it was thought to act mainly as a scaffold that connects different transcription factors or transcription machines, but it was recently discovered to have additional properties, such as HAT and E3 ligase activities (20, 31). The HAT activity of p300 allows it to influence chromatin conformation and transcriptional activity by acetylating histones or transcription factors such as GATA-1, MyoD, E2F-1, Smad7, and p53 (21, 32). The functional consequences of acetylation are diverse and include the enhancement of protein stability and, thus, transcriptional activity. For example, p53 is specifically acetylated at multiple lysine residues by p300/cAMP-response element-binding protein-binding protein, which inhibits its ubiquitination-mediated degradation by Mdm2 (33). HDACs are often associated with co-repressor complexes and...
can exert their repressive effects on both histone and non-histone proteins by removing acetyl groups (34). Deacetylation by HDACs also serves as an important step in the Mdm2-mediated degradation of p33 (34).

Here we provide evidence that the Runt domain transcription factor RUNX3 is acetylated by p300 in response to TGF-β superfamily signaling and is deacetylated by HDACs. Acetylation protects RUNX3 from Smurfl-dependent degradation, which indicates that competition between acetylation and deacetylation may control the ubiquitination-dependent degradation of RUNX3. We have mapped the RUNX3 acetylation sites to three conserved lysine residues (Fig. 2C).

Both acetylation by p300 and substitution of these critical lysine residues with arginine markedly increase the expression of RUNX3 (Fig. 5). However, in the absence of acetylation, these RUNX3 residues confer instability because they are targeted by ubiquitination. Evidence supporting a link between the acetylation and the ubiquitination of RUNX3 comes from previous observations that RUNX1 is degraded by the ubiquitin-proteasome pathway (35) and that RUNX2 is degraded by the ubiquitin ligase Smurfl (36), together with our observation that Smurfl and Smurfl2 interact with and ubiquitize RUNX3 (Fig. 6, B and C). We also found that the PXXY motif is critical for Smurfl-mediated RUNX3 degradation. Even a single amino acid substitution (proline to arginine) in the motif confers strong resistance to Smurfl-mediated degradation of RUNX3 (Fig. 6E).

The Smurfl-mediated decrease in the level of RUNX3 protein was reversed by co-expression of p300, suggesting that acetylation by p300 stabilizes RUNX3, presumably by protecting lysine residues from ubiquitination by Smurf proteins or other E3 ligases (Fig. 6D). Furthermore, lysine to arginine substitutions significantly stabilize RUNX3, probably by blocking ubiquitination (Fig. 6E). These results strongly suggest that the competition between ubiquitination and acetylation of the same lysine residues regulates the stability of RUNX3. Our findings that HDACs deacetylate (Fig. 2) and therefore destabilize RUNX3 (Fig. 5) further support the proposed link between acetylation/deacetylation and ubiquitination. Moreover, it has been demonstrated that several members of the HDAC6 superfamily complex are involved in the ubiquitination signaling pathway (37).

Under normal cell growth conditions, the acetylation of RUNX3 protein is almost undetectable. However, acetylation becomes more evident after treatment with the HDAC inhibitor TSA (Fig. 4A). Furthermore, treatment with TSA results in RUNX3-dependent transcription (Fig. 7A). These results indicate that RUNX3 acetylation is a dynamic process regulated by acetylation and deacetylation and that HDACs are involved in this process. More importantly, the acetylation of RUNX3 is significantly increased by treatment with TGF-β and further augmented by treatment with TSA or by the transient expression of p300. An increase in the extent of RUNX3 acetylation by TGF-β leads to a increase in RUNX3 transcription activity. On the other hand, HDAC5 decreases TGF-β-dependent RUNX3 acetylation and reverses the effect of TGF-β. These results indicate that the TGF-β signaling pathway positively regulates p300-dependent RUNX3 acetylation and RUNX3 activity. It is not yet clear whether the increase in transcription activity is independent of the increased stability or depends on the interaction of RUNX with other nuclear transcriptional modulators, although there is no obvious difference between wild-type RUNX and the KR mutant proteins with respect to their physical interactions with PEBP2α/CBFβ, Smad3, and p300 (Fig. 2E).

The molecular mechanism by which TGF-β superfamily sig-

naling promotes p300-dependent RUNX3 acetylation remains to be determined. One possibility is that Smad proteins mediate RUNX acetylation in response to TGF-β stimulation. However, it is worth noting that TGF-β mediates the phosphorylation of Smad3 and potentiates the association between Smad proteins and p300 (38). Because all RUNX proteins physically interact with TGF-β and bone morphogenetic protein-activated Smad proteins, it would be interesting to examine whether receptor-activated Smad proteins facilitate RUNX acetylation by p300 while suppressing the E3 ligase activity of p300. Collectively, our results reveal a previously unknown mechanism of RUNX stability, namely, acetylation and the deacetylation mediated by p300 and HDACs, respectively, that controls the ubiquitin-mediated degradation of RUNX3.

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