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Lysine Acetylation Regulates Bruton's Tyrosine Kinase in B Cell Activation

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Bruton's tyrosine kinase (Btk) is essential for BCR signal transduction and has diverse functions in B cells. Although Btk has been extensively studied, the role of lysine acetylation in Btk regulation has not been reported. In this study, we show that BCR cross-linking induces histone lysine acetylation at the Btk promoter, correlating with marked recruitment of histone acetyltransferase E1A-associated 300-kDa protein (p300) to the locus. These effects enhance Btk promoter activity and increase the expression of Btk mRNA and protein. Consistent with these results, activated B cells display increased p300 expression and total histone acetyltransferase activity in vitro and in vivo, resulting in global histone acetylation. Interestingly, we found that BCR signaling induces Btk lysine acetylation mediated by p300. Moreover, lysine acetylation of Btk promotes its phosphorylation. Together, our results indicate a novel regulatory mechanism for Btk transcription and reveal a previously unrecognized posttranslational modification of the Btk protein and its association with phosphorylation in B cell activation. ***The Journal of Immunology*****, 2010, 184: 000–000.**

Bruton's tyrosine kinase (Btk) is a member of the nonreceptor tyrosine kinase Tec family and is expressed in many hematopoietic lineages, but not in T cells (1). It is well known that Btk is involved in multiple signaling pathways following the activation of diverse cell membrane receptors. The role of Btk in BCR activation, however, has been the most studied, and results indicate that Btk is critical for B cell survival, proliferation, and differentiation (2, 3). Btk was originally identified as a disease gene for human X-linked agammaglobulinemia (also referred to as Bruton's agammaglobulinemia) in which B cell development is blocked at the pre-B cell stage, resulting in a near total absence of peripheral B cells and a failure to mount Ab responses (4, 5). A spontaneous point mutation at arginine 28 to cysteine in the pleckstrin homology domain of murine Btk leads to the condition xid, which involves impaired B cell development and response and is similar to phenotypes observed in Btk knockout mice (6).

Upon BCR signaling, Btk is translocated to the plasma membrane by binding of its pleckstrin homology domain to PtdIns-3,4,5-P3. Btk is then phosphorylated by the Src kinase Lyn at tyrosine 551 (Y551) in its kinase domain, followed by autophosphorylation at Y223 in its SH3 domain. Phosphorylated Btk activates PLC-r2 by binding to BLNK and modulating Ca^{2+} influx, playing an important role in B cell function (1, 5, 7). In addition to phosphorylation, Btk expression is enhanced in response to BCR cross-linking. Several transcription factors, including Sp1, Sp3, Spi-B, PU.1, Bright, OBF-1, and NF-κB, have been identified as regulators of Btk transcription (8–12).

Lysine acetylation of histones in chromatin is regulated by histone deacetylases (HDACs) and histone acetyltransferases (HATs) and is essential for the activation of gene transcription (13–16). Mammalian HDACs are divided into four classes according to their homology with yeast counterparts. Among these, class I HDACs include HDAC-1, -2, -3, and -8, whereas class II HDACs include HDAC-4, -5, -6, -7, -9, and -10. The third class of HDACs is the sirtuin family with seven members (SIRT1-7). HDAC-11 is classified in the fourth class due to its similarity to both class I and II enzymes. HDACs decrease histone acetylation and generally mediate transcriptional repression. Many HATs have been identified and are divided into three major families: E1A-associated 300-kDa protein (p300)/CREB-binding protein, Gcn5-related *N*-acetyltransferases, and MYST proteins. These proteins acetylate histones and activate transcription. It has been reported that some HDACs and HATs modulate the lysine acetylation of nonhistone proteins such as p53, tubulin, GATA-1, STAT3, MyoD, and E2F1 (17–20). Therefore, the histone lysine-modified enzymes are also termed lysine deacetylases and lysine acetyltransferases, respectively (21, 22). Lysine acetylation has emerged as an important posttranslational modification for nonhistone proteins and could interact with other posttranslational modifications to constitute multisite modification programs and regulate cell function (17, 22).

In this study, we asked whether histone acetylation regulates Btk transcription, contributing to BCR-induced B cell activation. Our results demonstrate that the regulation of histone acetylation modulates Btk transcription and expression. In short, p300 promoted, whereas HDAC-1, -2, and -3 inhibited, the transcription and expression of Btk. Moreover, BCR signaling markedly recruited p300 to the Btk promoter and induced local histone acetylation. BCR-induced local acetylation, however, was not associated with reduced recruitment of HDAC-1 to the Btk locus. Consistent with these results, B cell activation in vitro with anti-μ or LPS and in vivo by OVA led to global histone acetylation, which was coordinated with an increase in p300 expression and total HAT activity, but was not a result of reduced HDAC-1 expression or reduced activity of total HDACs. These results indicate that histone acetylation regulates Btk transcription. Furthermore, we found that BCR signaling induces Btk lysine acetylation mediated by p300 through interaction

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Abbreviations used in this paper: Btk, Bruton's tyrosine kinase; ChIP, chromatin immunoprecipitation; HATs, histone acetyltransferases; HDACs, histone deacetylases; p300, E1A-associated 300-kDa protein; pol II, polymerase II; siRNA, small interfering RNA; TSA, trichostatin A.

with Btk and that Btk acetylation promotes its phosphorylation via Lyn signaling. These results identify a novel posttranslational modification of the Btk protein and an association between Btk acetylation and phosphorylation. Together, our study links lysine acetylation with Btk regulation and sheds new light on B cell activation.

Materials and Methods

Abs, chemicals, plasmids, primers, and small interfering RNAs

Abs against Btk, p300, and β-actin were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Abs against HDAC-1, -2, -3, AcH3 (K9/K14), AcH4 (K8), RNA polymerase II, and phosphotyrosine (4G10) were obtained from Upstate Biotechnology, Lake Placid, NY. The anti-Flag M2 Ab was from Sigma-Aldrich, St. Louis, MO. The anti-acetylated lysine Ab was from Cell Signaling Technology, Beverly, MA. Anti-GAPDH was from Kangchen Bio-tech (Shanghai, China). Anti–B220-APC was from BD Pharmingen, San Diego, CA. Anti-goat IgG-FITC, anti-rabbit IgG-Rhodamine, and F(ab′)2 anti-μ Abs were from Jackson ImmunoResearch Laboratories, West Grove, PA. Anti-pY551 was provided by Dr. Owen Witte (University of California, Los Angeles, Los Angeles, CA). LPS and IL-4 were from Sigma-Aldrich and PeproTech (Rocky Hill, NJ), respectively.

Trichostatin A (TSA), MS-275, actinomycin D, and curcumin were obtained from Sigma-Aldrich, dissolved in DMSO, and stored at −80°C until use.

A plasmid-expressing Btk was generated by subcloning murine Btk cDNA into the HindIII and XhoI sites of pcDNA3. Btk-600 luc and Btk-1351 luc were constructed by PCR amplification of −576/+24 and −1293/+58 of Btk promoter region, respectively, and ligated into the XhoI and HindIII sites of pGL3-basic. pBJ-HDAC-1-Flag and pBJ-ΔHDAC-1-Flag (H141A) were provided by Dr. Bradley Bernstein (Harvard Medical School, Boston, MA), and the HDAC-1-Flag and ΔHDAC-1-Flag sequences were subcloned into pcDNA3. pGEX-HDAC-1 plasmids (1-120, 120-242, and 332-482) were from Dr. Tony Kouzarides (University of Cambridge, Cambridge, U.K.). HDAC-2-Flag and HDAC-3-Flag in pcDNA3 were from Dr. Edward Seto (University of South Florida, Tampa, FL). pcDNA3-p300 and Δp300 were provided by Dr. Warner Greene (University of California, Los Angeles).

Primers for RT-PCR: Btk (5′-CTT GAA GGA GCT TGG GAC TG-3′; 5′-TCC CAC ATT AAA ACC CCA AA-3′); IBtk (5′-ACG GAA GCC AGA ATA GCA AA-3′; 5′-CAC CAT TCA GTC CCA GTG TG-3′); Lyn (5′-GTG ACA TTG TGG TGG CCT TA-3′; 5′-GCA TGC CTT CTC CAG TCT TC-3′); NF-κB (5′-GCT TTG CAA ACC TGG GAA TA-3′; 5′-AAC TCC GCC ATT TTC TTC CT-3′); RNA polymerase II (5′-TTT GCA TCT GCC ACA GTC TC-3′; 5′-GCA GAG GAG CCA GTT TTG TC-3′); HDAC-1 (5′-TGT TCC AGC CTA GTG CAG TG-3′; 5′-GGC AGC ATC CTC AAG TTC TC-3′); HDAC-2 (5′-CCG GTG TTT GAT GGA CTC TT-3′; 5′-GCG CTA GGC TGG TAC ATC TC-3′); HDAC-3 (5′-AAT GTG CCC TTA CGA GAT GG-3′; 5′-TGG CCT GCT GTA GTT CTC CT-3′); and β-actin (5′-TGT TAC CAA CTG GGA CGA CA-3′; 5′-TCT CAG CTG TGG TGG TGA AG-3′). Primers for chromatin immunoprecipitation (ChIP) assay: Btk promoter (−324/−44 bp) (5′-TTT GCC CCA GAG AGG GTA GT-3′; 5′-CAG CCA CTC AGT TCC CTT TT-3′); Btk promoter upstream (−2749/−2467 bp) (5′-GTT CCC TCT GTG TCG CTC TC-3′; 5′-TGC TGA GCT CCC TAC AGA CA-3′); and Btk exon 19 (5′-TGC TGA GCT CCC TAC AGA CA-3′; 5′-GTT CCC TCT GTG TCG CTC TC-3′).

HDAC-1 small interfering RNA (siRNA) was purchased from Dharmacon, Lafayette, CO. The following siRNAs were synthesized by Shanghai GenePharma Company, Shanghai, China: HDAC-2 (sense: 5′-GGC UAG GAU UGA AGA AGA CTT-3′; antisense: 5′-GUC UUC UUC AAU CCU AGC CTT-3′); HDAC-3 (sense: 5′-CCU CAU CGC CUG GCA UUG ATT-3′; antisense: 5′-UCA AUG CCA GGC GAU GAG GTT-3′); and p300 (sense: 5′-GAG GAU AUU UCA GAG UCU ATT-3′; antisense: 5′-UAG ACU CUG AAA UAU CCU CTT-3′).

Cell culture and mice

The B cell line T347 (provided by Dr. Anne Davidson, Albert Einstein College of Medicine, Bronx, NY) and 293T cells were cultured in RPMI 1640 and DMEM medium, respectively, and supplemented with 10% FBS and antibiotics at 37°C and 5% CO_2. The chicken B cell line DT40, $Lyn^{-/-}$ DT40, and $Syk^{-/-}$ DT40 were obtained from RIKEN BioResource Center, Ibaraki, Japan, and cultured in RPMI 1640 medium containing 10% FBS and 1% chicken serum with 50 μM 2-mercaptoethanol at 39.5°C and 5% CO_2. C57BL/6 mice were purchased from the Shanghai Laboratory Animal Center of Chinese Academy of Sciences, Shanghai, China, and maintained under specific pathogen-free conditions. All animal studies were approved by the Institutional Animal Care and Use Committee. Splenic resting B cells (purity >95%) were isolated from C57BL/6 mice by negative selection using a B cell separation kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

Analysis of mRNA expression

Splenic B cells (2×10^6/ml) were treated with TSA, MS-275, or MC1568. In some experiments, B cells were activated by F(ab′)2 anti-μ Ab plus IL-4, or LPS alone, in the presence or absence of TSA. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA), and cDNA was prepared with the Promega cDNA Synthesis kit (Promega, Madison, WI). The mRNA levels were measured by RT-PCR using gene-specific primers.

Analysis of protein expression

293T or T347 cells (5×10^6/ml) were transfected using Lipofectamine 2000 (Invitrogen) with the following expression plasmids as indicated in each experiment: Btk, Flag-tagged HDAC-1, -2, and -3, ΔHDAC-1, HDAC1 fragments (1–120, 150–242, and 332–482), p300, and Δp300. DT40 or mutant DT40 cells were transfected by electroporation (550 V, 25 μF) with the Btk expression plasmid alone or together with either HDAC-1-Flag or p300 plasmids. An amount of empty vector was added that was equal to the amount of total DNA for each transfection. In some experiments, splenic B cells (2×10^6/ml) were activated in vitro and in vivo, as indicated in each experiment. Protein levels were determined by Western blot using the indicated Abs.

Gene silencing by siRNA

T347 cells (5×10^6/ml) were transfected with HDAC-1, -2, -3, or p300 siRNA (100 nM) using Lipofectamine 2000 for 48 h, according to the manufacturer's instructions. The mRNA and protein expression levels were analyzed by RT-PCR and Western blot, respectively.

Luciferase assay

T347 cells (5×10^6/ml) were transfected by electroporation (250 V, 960 μF) with Btk-1351 luc. At 16 h after transfection, TSA was added to the culture for 8 h. To study the effect of p300 or HDAC-1 on Btk promoter activity, T347 cells were electroporated with Btk-600 luc or Btk-1351 luc in combination with HDAC-1-Flag, ΔHDAC-1, p300, Δp300, or empty vector. Proteins were collected 24 h after transfection, and luciferase activity was measured using Promega luciferase assay reagents (Promega). Results were normalized to protein concentrations.

ChIP assay

Splenic B cells or T347 cells (1×10^7/ml) were treated with 1% formaldehyde and sonicated to break up the chromatin into 200- to 1000-bp fragments. Ten percent of the supernatant was kept for input, and the rest was precleared with salmon sperm DNA–protein A–agarose (Upstate Biotechnology), followed by the addition of 2 μg of the Abs or control mouse IgG. The DNA–protein–Ab complexes were precipitated using salmon sperm DNA–protein A–agarose. After washing, the complexes were eluted and reverse cross-linked. DNA was extracted with phenol–chloroform, and target DNA was amplified by PCR.

Immunofluorescence

Splenic B cells (2×10^6/ml) were labeled with B220-APC. After treatment with Cytofix/Cytoperm (BD Pharmingen), the cells were stained with goat anti-Btk Ab together with rabbit anti-p300, HDAC-1, AcH3 (K9/K14), or AcH4 (K8) Abs, followed by donkey anti-goat IgG-FITC and donkey anti-rabbit IgG-rhodamine. After washing, the cells were mounted with medium containing DAPI (Vector Laboratories, Burlingame, CA) and analyzed by confocal microscopy.

HDAC and HAT activity

Splenic B cells (2×10^6/ml) were treated with TSA or MS-275. In some cases, the B cells were activated in vitro or in vivo as indicated in each experiment. The cells were lysed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). A total of 50 μg of protein was analyzed for enzyme activity using a HAT or HDAC colorimetric assay kit (Biovision, Mountain View, CA).

Protein phosphorylation and acetylation

Cell lysates were immunoprecipitated with an anti-Btk Ab followed by the addition of Protein G PLUS-Agarose (Santa Cruz Biotechnology). Btk phosphorylation was measured with either anti-phosphotyrosine (4G10) or a pY551 Ab by Western blot. Btk acetylation was determined using an anti-acetylated lysine Ab. For analysis of Btk acetylation in vitro, Btk

protein (Abcam, Cambridge, MA) was incubated with either recombinant p300 HAT domain (Upstate Biotechnology) or PCAF (Biovision) in 50 μl of HAT buffer (250 mM Tris-HCl [pH 8.0], 0.5 mM EDTA, 5 mM DTT, and 50% glycerol) in the presence of 100 μM acetyl-CoA (Upstate Biotechnology). After 30 min at 30°C, the reaction products were separated by SDS-PAGE and analyzed for lysine acetylation with an anti-acetylated lysine Ab.

Btk kinase activity

Btk kinase activity was analyzed using an ELISA-based tyrosine kinase assay kit (Sigma-Aldrich). Btk was immunoprecipitated from cell lysates, which were prepared with lysis buffer containing an activated sodium vanadate solution, according to the kit instructions. Btk immunoprecipitates were determined to phosphorylate the exogenous substrate poly-Glu-Tyr. Phosphorylated polymer substrate was probed with an HRP-conjugated anti-phosphotyrosine Ab, followed by addition of the chromogenic substrate *o*-phenylenediamine. Color development was measured at OD492.

Statistical analysis

Statistical differences were determined with a Student two-tailed *t* test using GraphPad Prism software (GraphPad, San Diego, CA). A p value <0.05 was considered statistically significant.

Results

The HDAC inhibitor TSA causes Btk mRNA degradation independent of the inhibition of HDACs

To study the role of histone acetylation in Btk transcription, we first analyzed the effect of TSA on Btk mRNA expression. Instead of TSA-mediated upregulation of gene expression (23, 24), we found that TSA treatment dramatically reduced the level of Btk mRNA in splenic B cells (Fig. 1*A*, 1*B*). However, the mRNA levels of some Btk-related genes such as IBtk, Lyn, and NF-κB showed no significant decrease (Fig. 1*C*). A notable reduction in the level of Btk mRNA also was observed in B cells activated with either anti-μ plus IL-4 (Fig. 1*D*) or the polyclonal stimulator LPS (Fig. 1*E*). TSA-induced reduction of Btk mRNA expression led to an obvious decrease in the level of Btk protein (Fig. 1*F*). To confirm the effect of TSA on Btk mRNA, we injected C57BL/6 mice daily with TSA (5 mg/kg body weight i.p.) for 3 consecutive days. Compared with vehicle-treated control mice, the TSA-treated group exhibited a significantly reduced level of Btk mRNA (Fig. 1*G*). These results indicate that TSA markedly reduced the mRNA level of Btk in vitro and in vivo.

We next investigated whether TSA impaired Btk gene transcription resulting in the observed reduction in Btk mRNA levels. We performed reporter gene analysis and found that TSA did not reduce the activity of the Btk promoter in the T347 B cell line. In fact, promoter activity was increased by TSA treatment (Fig. 1*H*). We then analyzed acetylation of histone H3 (AcH3) on K9 and K14, which is associated with active transcription, at the Btk promoter using ChIP. Treatment with TSA promoted the level of AcH3 (K9/K14) of the Btk promoter (-324 to -44 bp) in splenic B cells (Fig. 1*I*). Furthermore, we examined the status of Btk gene transcription using RNA polymerase II (pol II)-ChIP (25). Consistent with the above results, TSA increased the recruitment of RNA pol II to the transcribed region of Btk in B cells (Fig. 1*J*, *left panel*). Moreover, the mRNA levels and protein expression of RNA pol II were enhanced in TSA-treated cells (Fig. 1*J*, *right panel*), suggesting an effect of TSA on global transcriptional activation.

The finding that Btk transcription is promoted in the presence of TSA implies that the TSA-induced Btk mRNA reduction could be caused by degradation. Therefore, we blocked cellular transcription with actinomycin D and determined the effect of TSA on the decay of Btk mRNA. As shown in Fig. 1*K*, TSA treatment resulted in increased Btk mRNA decay compared with vehicle-treated controls.

Although TSA may inhibit all HDACs, class I HDAC-8 and class III HDACs are only affected at high concentrations of TSA (26). To analyze which type of HDAC was involved in the TSA-induced Btk mRNA degradation, we treated splenic B cells with low concentrations of TSA as indicated in Fig. 2*A*. Application of 1 ng/ml (3.3 nM) TSA, which affects only class I HDAC-1 and -3 (26), reduced the level of Btk mRNA in 24-h cultures. To examine the effect of class II HDACs, we treated the B cells with the class II HDAC inhibitor MC1568 (also called 2f) (27, 28). However, the Btk mRNA level was not significantly decreased by MC1568 (Fig. 2*B*) as compared with TSA. We next determined the effect of the class I HDAC inhibitor MS-275 (26, 29). Unexpectedly, no reductions in Btk mRNA levels were observed in B cells treated with different concentrations of MS-275, as shown in Fig. 2*C*. In contrast, 10 or 50 ng/ml MS-275 increased Btk mRNA levels. These results suggest that the TSA-induced Btk mRNA reduction probably did not occur through the inhibition of HDAC activity. We, therefore, silenced class I HDAC-1, -2, and -3 with specific siRNAs in T347 cells expressing endogenous Btk and found that inhibition of HDAC-1, -2, or -3 actually increased the levels of Btk mRNA (Fig. 2*D*). Subsequently, we measured the effect of TSA and MS-275 on the activity of total HDACs and found that 1 or 2 ng/ml TSA did not significantly affect HDAC activity. The level of the HDAC activity, however, was obviously reduced in MS-275–treated cells, comparable to that of 100 ng/ml TSA-treated cells (Fig. 2*E*). Collectively, these results indicate that TSA-induced degradation of Btk mRNA is not associated with the inhibition of HDACs.

Class I HDACs downregulate, whereas p300 upregulates, Btk protein expression

Because silencing of HDAC-1, -2, or -3 led to increased levels of Btk mRNA, we analyzed the expression of the Btk protein to further verify the inhibitory effect of the HDACs on Btk expression. Consistent with the mRNA results, Btk protein expression in T347 cells was enhanced when HDAC-1, -2, or -3 was silenced by its corresponding siRNA (Fig. 3*A*). In contrast, overexpression of HDAC-1, -2, or -3 reduced Btk protein levels in T347 cells (Fig. 3*B*). Accordingly, the protein expression of exogenous Btk in 293T cells was inhibited by forced expression of HDAC-1, -2, or -3, as shown in Fig. 3*C*. HDAC-1 with a catalytic domain deletion, however, failed to reduce Btk protein levels in 293T cells (Fig. 3*D*). Consistent with this finding, Btk expression was not significantly affected by HDAC-1 fragments, as indicated in Fig. 3*E*. These results indicate that the intact catalytic domain of HDAC-1 is required for inhibition of Btk protein expression and that the LXCXE domain-mediated function is not associated with this effect of HDAC-1.

We next analyzed the role of histone acetyltransferase p300 in Btk protein expression. p300 inhibition using a specific siRNA markedly reduced Btk protein levels in T347 cells (Fig. 3*F*), suggesting p300-mediated upregulation of Btk expression. This concept was supported by the results that p300 overexpression increased the protein levels of endogenous and exogenous Btk in T347 (Fig. 3*G*) and 293T cells (Fig. 3*H*), respectively. Similar to findings obtained for HDAC-1, a mutation in the catalytic domain of p300 abrogated its effect (Fig. 3*I*). Overall, these results define a regulatory role for lysine acetylation-modifying enzymes in Btk protein expression.

BCR signaling induces histone acetylation at the Btk promoter by recruiting p300, leading to Btk transcriptional activation

We investigated the mechanism underlying the effects of these enzymes on Btk expression by analyzing the effect of histone acetylation on Btk transcription in the setting of BCR-induced B cell activation. We first employed a ChIP assay to determine whether HDAC-1 and p300 were recruited to the Btk promoter in

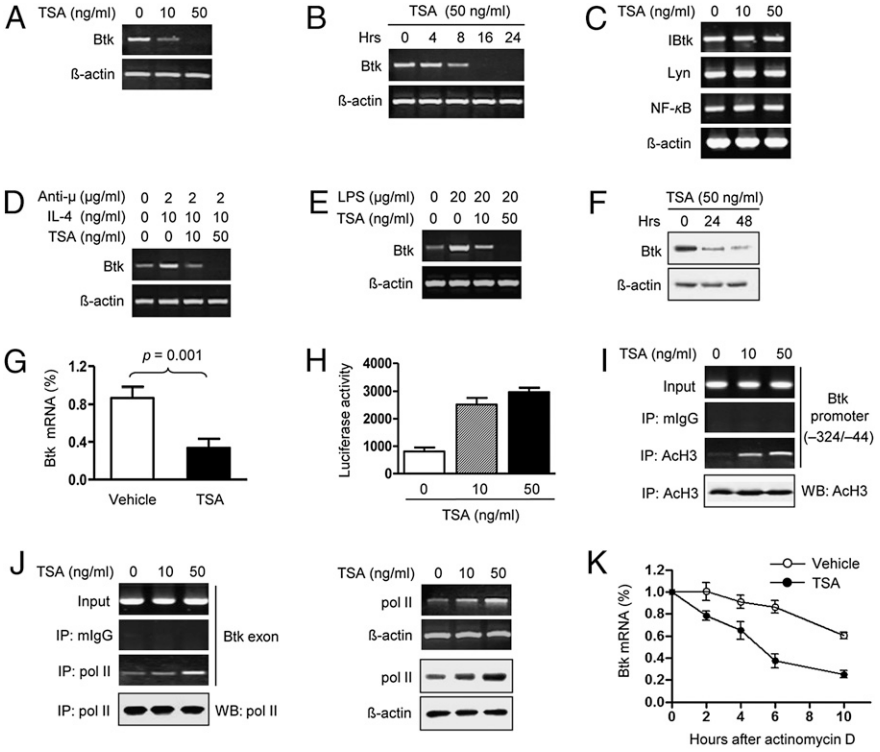

FIGURE 1. TSA reduces the level of Btk mRNA by degradation. *A–F*, TSA greatly reduced the level of Btk mRNA in vitro. Splenic B cells were treated either with different doses of TSA for 24 h (*A*) or with 50 ng/ml TSA for different time periods (*B*). Btk mRNA levels were measured by RT-PCR. The mRNA expression of the indicated genes in B cells treated as in *A* was determined (*C*). Splenic B cells were stimulated for 24 h with anti-μ plus IL-4 (*D*) or LPS (*E*) in the absence or presence of TSA, and Btk mRNA levels are shown. Btk protein expression in B cells treated with TSA as indicated was analyzed by Western blot (*F*). β-actin was used as a control. *G*, TSA significantly decreased the level of Btk mRNA in vivo. C57BL/6 mice were injected i.p. with TSA (5 mg/kg, $n = 4$) or vehicle (DMSO, $n = 3$) once a day for 3 consecutive days. The RNA samples were collected from splenic cells 24 h after the last injection. Data are expressed as the mRNA ratio of Btk to β-actin. Bars indicate the mean ± SD of each group. The p value represents the difference between the two groups. *H*, TSA promoted the activity of the Btk promoter. T347 B cells were electroporated with 20 μg Btk-1351 luc and cultured for 24 h with or without the addition of TSA for the last 8 h. Luciferase activity of each group from three experiments is shown. *I*, TSA increased the level of AcH3 at the Btk promoter. Splenic B cells were treated with or without TSA for 24 h. The AcH3 level of the Btk promoter (−324 to −44 bp) was analyzed by ChIP. An anti-AcH3 (K9/K14) Ab was used to immunoprecipitate AcH3, and mouse IgG served as the negative control. The amount of DNA containing the Btk promoter in the input and precipitates was measured by PCR. Immunoblotting of the precipitated AcH3 (K9/K14) was used as a control for the IP. *J*, TSA enhanced the recruitment of RNA pol II to a Btk exon. Splenic B cells treated as indicated in *I* were analyzed by RNA Pol II-ChIP (*left panel*). DNA immunoprecipitated by an anti-RNA pol II Ab was amplified by PCR with primers located in exon 19 of Btk. Mouse IgG served as the negative control. The levels of the precipitated RNA pol II were determined by Western blot for the IP control. The mRNA (*upper right panel*) and protein (*lower right panel*) levels of RNA pol II in B cells treated with or without TSA were analyzed by RT-PCR and Western blot, respectively. *K*, TSA treatment resulted in increased Btk mRNA decay. Cellular transcription was blocked by 5 μg/ml actinomycin D, followed by the addition of TSA (50 ng/ml) or vehicle after 4 h. The levels of Btk mRNA at the indicated times were measured by RT-PCR. The mRNA ratios of Btk to β-actin at each time are shown. Data represent the mean ± SD of three independent experiments.

vivo. As shown in Fig. 4*A* (*left panel*), HDAC-1 was recruited to the Btk locus (−324 to −44 bp) in naive splenic B cells. The amount of HDAC-1 bound to the locus, however, was not significantly changed in response to BCR cross-linking with anti-μ. In contrast, the level of p300 recruited to the Btk locus was very low in unactivated B cells and was dramatically increased by BCR signaling. This recruitment of p300 was also observed in T347 cells (Supplemental Fig. 1). The difference in p300 recruitment between the naive and activated B cells was not due to a bias in the p300 immunoprecipitation assay (Fig. 4*A*, *left panel*). We did not detect the segment upstream of the Btk promoter (−2749 to −2467 bp) in the p300 or HDAC-1 immunoprecipitates (Fig. 4*A*, *right panel*), indicating the specificity of the observed recruitment of both p300 and HDAC-1.

We next analyzed whether BCR cross-linking induced histone acetylation at the Btk promoter. As shown in Fig. 4*B*, BCR ligation by anti-μ markedly increased the amount of AcH3 (K9/K14) at the Btk locus (−324 to −44 bp). This result was correlated with dramatic recruitment of p300 in the activated B cells, suggesting that p300 mediates BCR-induced local histone acetylation. Indeed, inhibition of p300 with curcumin (30, 31), a p300/CREB-binding protein inhibitor, significantly reduced the histone acetylation at the Btk locus induced by BCR engagement (Fig. 4*B*). Moreover, overexpression of p300 caused an increase in the AcH3 level at the locus (Fig. 4*C*). Consistent with the finding that inhibition of HDACs by TSA promoted local histone acetylation (Fig. 1*I*), overexpression of HDAC-1 decreased the AcH3 level at the locus (Fig. 4*D*). These results indicate that HDAC-1 can downregulate local acetylation. However, HDAC-1 appeared to not be correlated with BCR-induced local acetylation because we did not observe any reduction in the level of HDAC-1 recruitment at the locus on BCR signaling as compared with naive B cells in several independent experiments.

We further determined the effect of p300 on the activity of the Btk promoter. Fig. 4*E* shows that forced expression of p300 in T347 cells induced a 10.8- or 9.8-fold increase in the activity of the Btk promoter using 600- and 1351-bp detected fragments, respectively. Mutant p300, however, failed to enhance this activity. In line with these data, overexpression of intact, but not mutant, p300 increased

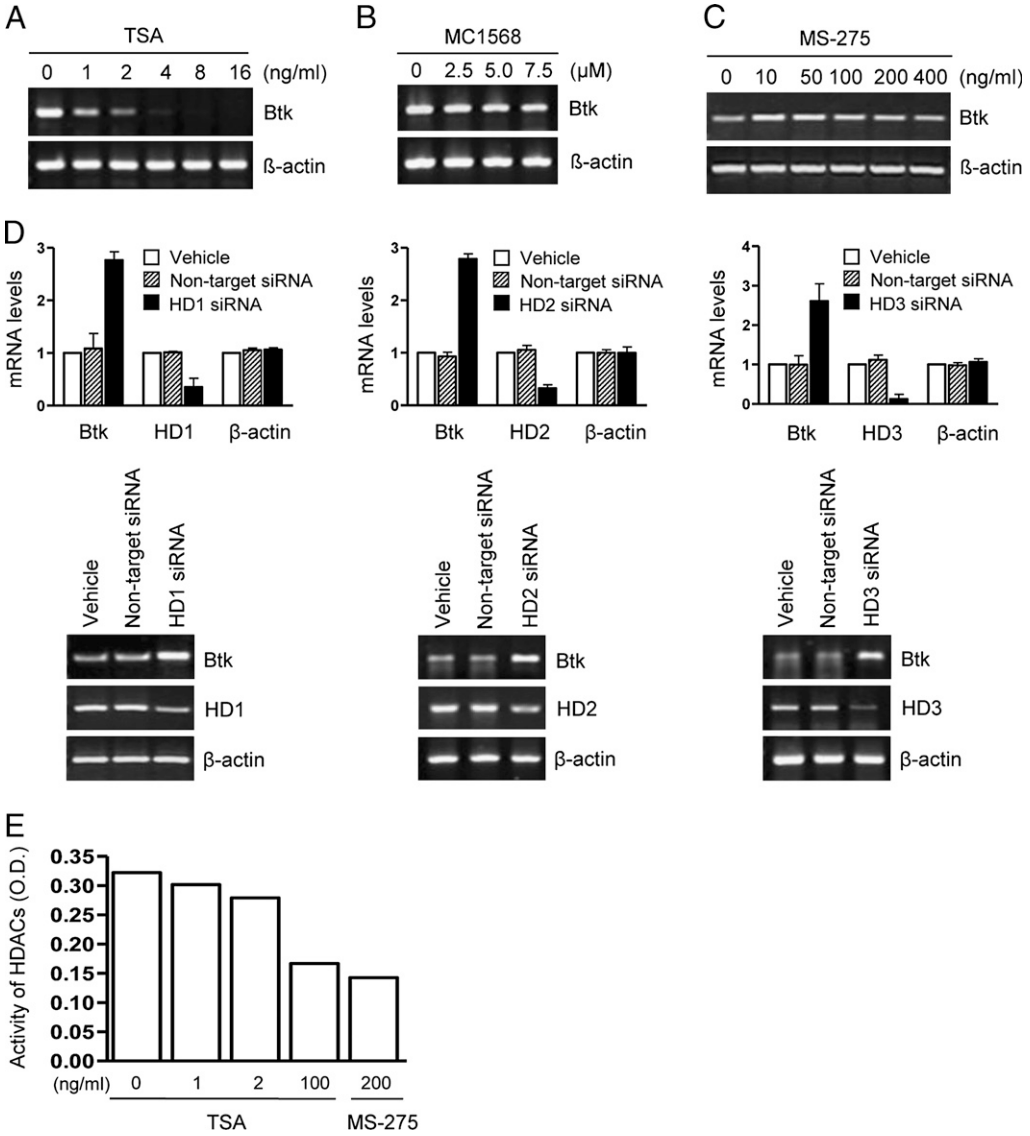

FIGURE 2. TSA-induced Btk mRNA degradation is independent of histone deacetylase inhibition. *A–C*, TSA, but not the other HDAC inhibitors tested, caused a reduction in the level of Btk mRNA. Splenic B cells were treated with TSA (*A*), MC1568 (*B*), or MS-275 (*C*) as indicated. The mRNA levels were determined by RT-PCR 24 h after treatment. *D*, Silencing of HDAC-1, -2 or -3 increased Btk mRNA expression. To silence HDAC-1, -2, or -3, 100 nM specific siRNA was transfected into T347 cells. Vehicle and nontarget siRNAs were used as negative controls. The mRNA levels were measured 48 h after transfection. The mean mRNA level from each vehicle group was set at one. Data represent the mean ± SD of three independent experiments (*upper panels*). Representative gel images are shown (*lower panels*). *E*, HDAC activity. Splenic B cells were treated with or without the inhibitors as indicated. The HDAC activity in total cellular extracts was measured 24 h after treatment, as described in the *Materials and Methods.*

the level of Btk mRNA (Fig. 4*F*). We also analyzed the role of HDAC-1 in Btk promoter activity. In contrast to the effect of p300, overexpression of HDAC-1 resulted in a 2.9- or 2.6-fold reduction in activity, as indicated in Fig. 4*G*. This effect led to reduced Btk mRNA expression (Fig. 4*H*). No significant change in activity or mRNA levels was observed in the group expressing the mutant HDAC-1 as compared with the controls (Fig. 4*G*, 4*H*).

Taken together, these results show that p300 promotes, and HDAC-1 inhibits, Btk transcription via recruitment to the promoter and regulation of local histone acetylation.

B cell activation in vitro and in vivo results in global histone acetylation, which correlates with HAT activity

The finding that p300 and HDAC-1 are recruited to the Btk promoter where they regulate the local histone acetylation promoted us to investigate the expression of p300 and HDAC-1 and their role in global histone acetylation. We cultured splenic B cells with or without anti-μ and stained them with the indicated Abs (Fig. 5*A*). Immunofluorescence analysis showed that HDAC-1 was expressed in unstimulated splenic B cells, but the expression level was not reduced by BCR signaling. Interestingly, p300 expression was low in the naive B cells and markedly increased by BCR cross-linking (Fig. 5*A*). These results were confirmed by immunoblotting experiments (Fig. 5*B*). Moreover, the increase in p300 expression was associated with increased levels of AcH3, AcH4, and Btk in the activated B cells (Fig. 5*A*, 5*B*). When we treated the activated B cells with curcumin, it inhibited p300 expression and reduced the levels of AcH3 and AcH4; moreover, Btk expression was attenuated (Fig. 5*B*).

We then determined whether B cell activation by other pathways would lead to a similar protein expression profile. We activated splenic B cells with LPS. As shown in Fig. 5*C*, the expression of HDAC-1 was not significantly affected in LPS-activated B cells compared with naive B cells; however, p300 expression was markedly induced by LPS. Consistent with these results, expression levels of the acetylated

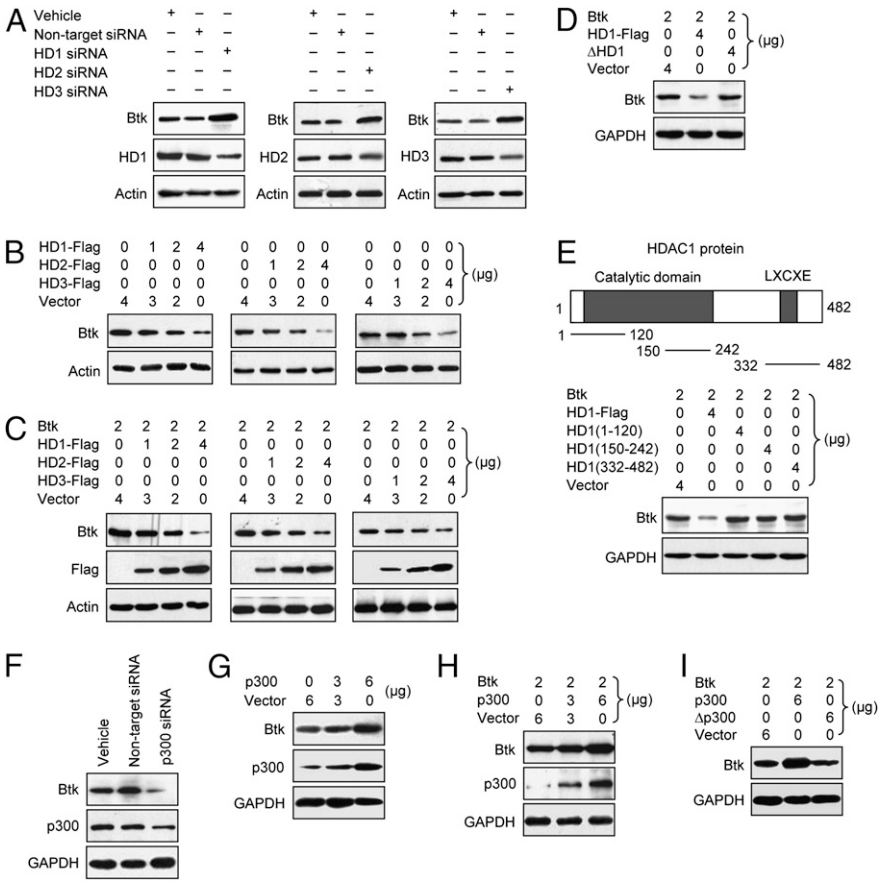

FIGURE 3. HDACs and p300 negatively and positively regulate the expression of the Btk protein, respectively. *A–C*, HDAC-1, -2, and -3 inhibited the protein expression of endogenous and exogenous Btk. T347 cells were transfected with 100 nM siRNAs for HDAC-1, -2, or -3. Vehicle or nontarget siRNAs were used as negative controls (*A*). T347 cells were transfected with Flag-tagged HDAC-1, -2, or -3 expression plasmids (*B*). 293T cells were transfected with a plasmid encoding Btk alone or in combination with plasmids expressing Flag-tagged HDAC-1, -2, or -3 (*C*). *D* and *E*, The effect of HDAC-1 on Btk protein expression requires an intact catalytic domain. 293T cells were transfected with a Btk expression plasmid alone or together with plasmids encoding HDAC-1-Flag or Δ HDAC-1 (*D*). 293T cells were transfected with a Btk expression plasmid alone or together with plasmids encoding HDAC-1-Flag or the HDAC-1 fragments as indicated (*E*). *F–H*, p300 increased the protein expression of endogenous and exogenous Btk. T347 cells were transfected with 100 nM p300 siRNA. Vehicle or nontarget siRNAs were used as negative controls (*F*). T347 cells were transfected with a p300 expression plasmid (*G*). 293T cells were transfected with a Btk expression plasmid alone or with the p300 expression plasmid (*H*). *I*, The effect of p300 on Btk protein expression requires its catalytic domain. 293T cells were transfected with a Btk expression plasmid alone or in combination with plasmids expressing either p300 or Δp300. Protein samples were collected 48 h after transfection, and the protein levels were measured by Western blot using the corresponding Abs. Either β-actin or GAPDH was used as a control.

histones and Btk were increased in the activated B cells. Subsequently, we determined protein expression in splenic B cells from C57BL/6 mice immunized with or without OVA. The expression pattern was similar to that observed in B cells activated in vitro by anti-μ or LPS (Fig. 5*D*). Thus, these findings show that global histone acetylation in B cells activated in vitro and in vivo is related to p300, but not HDAC-1, supporting the findings that p300 upregulates Btk expression.

To obtain a global view of the regulation of histone acetylation, we measured the activity of total HATs and HDACs. Consistent with the above results, levels of HAT activity were significantly increased in B cells activated either in vitro or in vivo, whereas HDAC activity levels were comparable between the activated and control B cells (Fig. 5*E*). Therefore, we concluded that B cell activation-induced global histone acetylation is related to increased HAT activity but not reduced HDAC activity.

Lysine acetylation-modifying enzymes modulate Btk phosphorylation through Lyn and Syk

It has been proposed that lysine acetylation-modifying enzymes could modulate phosphorylation of nonhistone proteins as well as histones (22). Our results indicate that BCR cross-linking increases the expression of both p300 and Btk and that p300 promotes Btk expression. We were interested in determining whether p300 could affect Btk phosphorylation; thus, we cotransfected p300 and Btk in 293T cells and immunoprecipitated Btk. Forced expression of p300 increased the level of pan-tyrosine phosphorylation of Btk as measured by the 4G10 Ab (Fig. 6*A*). Moreover, phosphorylation at the Y551 site detected with a specific Ab was also increased (Fig. 6*A*). In line with these results, silencing of p300 with a specific siRNA reduced the level of Btk phosphorylation in T347 cells (Fig. 6*B*). We further analyzed the role of HDACs in Btk phosphorylation. Overexpression of HDAC-1, -2, or -3 caused reduced pan-tyrosine phosphorylation and Y551 phosphorylation in immunoprecipitated Btk (Fig. 6*C*), whereas inhibition of HDAC-1, -2, or -3 with siRNA led to increased phosphorylation (Fig. 6*D*). These results indicate that p300 promotes Btk phosphorylation, whereas HDACs repress Btk phosphorylation.

Because Lyn plays an essential role in Btk phosphorylation, we determined whether Lyn signaling was involved in the modulation of Btk phosphorylation by these enzymes. We transfected wild-type or Lyn-deficient chicken DT40 B cells with Btk and p300 or HDAC-1. Consistent with the results obtained with 293T cells,

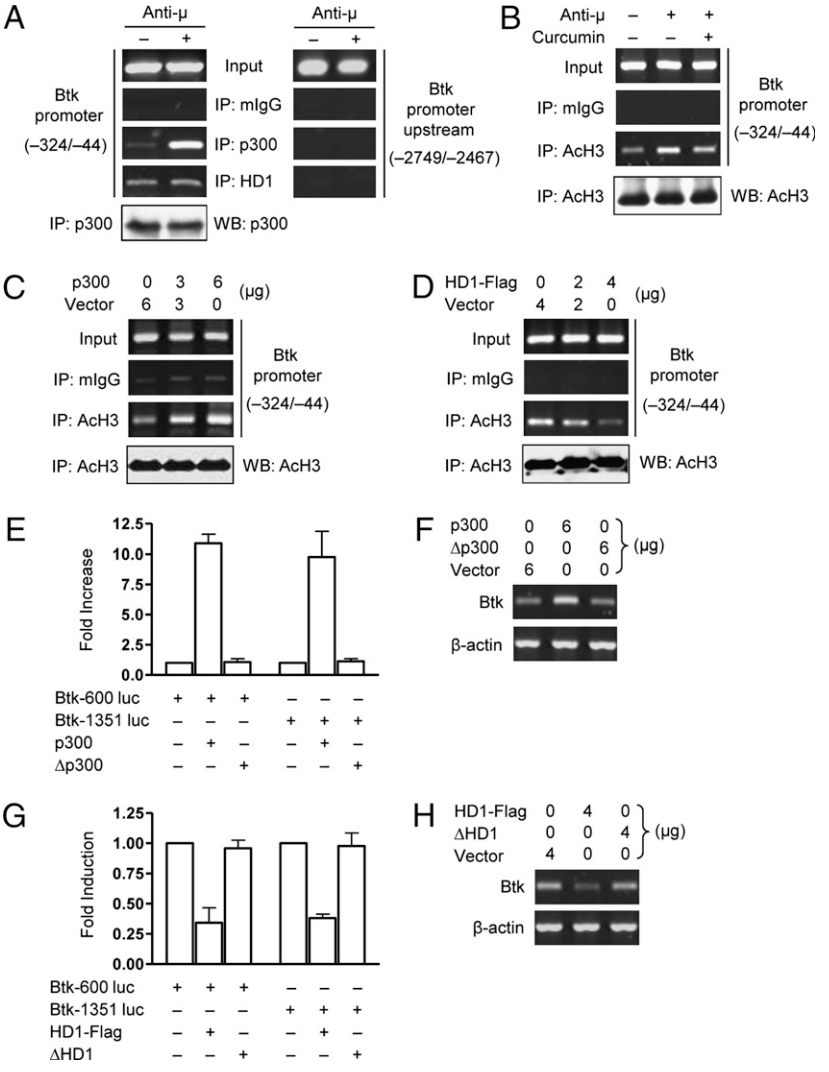

FIGURE 4. BCR signaling recruits p300 to the Btk promoter, leading to histone acetylation and transcriptional activation. *A*, BCR cross-linking recruited p300 to the Btk promoter. Splenic B cells were activated with or without 10 μg/ml anti-μ for 24 h. The recruitment of p300 or HDAC-1 to the Btk locus (−324 to −44 bp) was analyzed by ChIP using specific Abs. Mouse IgG served as a negative control. The precipitated p300 was determined by immunoblotting for an IP control. PCR measurement of the region upstream of the Btk promoter (−2749 to −2467 bp) was used as a control for the specificity of protein recruitment. *B*, BCR cross-linking promoted histone acetylation at the Btk promoter, which was inhibited by curcumin. Splenic B cells were treated as indicated in *A*, in the absence or presence of 100 nM curcumin. The levels of AcH3 (K9/K14) at the Btk locus (−324 to −44 bp) were analyzed by ChIP. *C* and *D*, p300 increased, but HDAC-1 decreased, the AcH3 level of the Btk promoter. T347 cells were transfected with plasmids expressing p300 (*C*) or HDAC-1-Flag (*D*) as indicated for 48 h. The levels of AcH3 (K9/K14) at the promoter (−324 to −44 bp) were analyzed by ChIP. *E* and *G*, p300 promoted, but HDAC-1 inhibited, the activity of the Btk promoter. T347 cells were transfected for 24 h with Btk-600 luc or Btk-1351 luc alone or in combination with p300, Δp300 (*E*), HDAC-1-Flag, or ΔHD1 (*G*) as indicated. Luciferase activity is shown as fold increase or reduction compared with the activity of the group transfected with Btk-600 luc or Btk-1351 luc (set at a value of one). Data represent the mean ± SD of three independent experiments. *F* and *H*, p300 enhanced, but HDAC-1 reduced, Btk mRNA expression. T347 cells were transfected with or without p300, Δp300 (*F*), HDAC-1-Flag, or ΔHDAC-1 (*H*) as indicated. The mRNA levels were measured 48 h after transfection by RT-PCR.

p300 and HDAC-1 increased and decreased, respectively, the level of Btk phosphorylation in wild-type DT40 cells (Fig. 6*E*). Remarkably, Lyn deficiency prevented these effects (Fig. 6*E*). The regulation of Btk protein expression by p300 and HDAC-1 was not affected in Lyn-deficient DT40 cells (Supplemental Fig. 2). It has been reported that Syk also contributes to Btk phosphorylation (32). We examined the effect of Syk on Btk phosphorylation using Syk-deficient DT40 cells. Syk deficiency partially blocked the up- and downregulation of Btk phosphorylation by p300 and HDAC-1, respectively (Fig. 6*E*). Taken together, these results indicate that Syk and, in particular, Lyn are required for the regulatory effect of p300 and HDAC-1 on Btk phosphorylation. We further investigated the role of p300 and HDAC-1 in the expression of Lyn and Syk proteins. No significant change was observed in the levels of Lyn or Syk upon over-expression of p300 or HDAC-1 (Supplemental Fig. 3). These results suggest that the regulatory effects of p300 and HDAC-1 on Btk phosphorylation are not associated with the expression of Lyn and Syk.

BCR signaling induces Btk lysine acetylation mediated by p300, which is correlated with Btk phosphorylation

The above results suggest that Btk could be lysine-acetylated. We first analyzed whether BCR cross-linking induces Btk lysine acetylation. BCR ligation by anti-μ significantly induced Btk acetylation, as measured by an anti-acetylated lysine Ab in precipitated Btk (Fig. 7*A*). To confirm this result, we examined Btk lysine acetylation in vitro. As shown in Fig. 7*B*, Btk was acetylated by p300 in a concentration-dependent manner; however, Btk could not be acetylated by PCAF. We then overexpressed p300 and Btk in 293T cells to determine the effect of p300 on Btk lysine acetylation in vivo. p300 promoted Btk acetylation, whereas mutant p300 failed to acetylate Btk (Fig. 7*C*). Further, we analyzed the role of p300 in BCR signaling-induced Btk acetylation. Fig. 7*D* shows that inhibition of p300 by curcumin strongly decreased the level of Btk acetylation in B cells activated by anti-μ. Thus, these results indicate that Btk is modified posttranslationally by lysine acetylation and that p300 plays an important role in BCR signaling-induced Btk modification.

We have shown that lysine acetylation-modifying enzymes modulate Btk phosphorylation. Therefore, we examined the association between Btk acetylation and phosphorylation in BCR-induced B cell activation. As shown in Fig. 7*D*, BCR cross-linking by anti-μ induced both Btk phosphorylation and acetylation. Moreover, curcumin inhibited Btk phosphorylation and acetylation. Consistent with these results, overexpression of intact, but not mutant, p300 increased the levels of both Btk acetylation and phosphorylation in 293T cells (Fig. 7*C*). These results indicate that Btk acetylation is correlated with Btk phosphorylation.

We further analyzed the effect of p300-mediated Btk acetylation on the tyrosine kinase activity of Btk. Overexpression of p300 enhanced the level of Btk kinase activity. Mutant p300, however, did not significantly affect this activity. Moreover, BCR signaling-

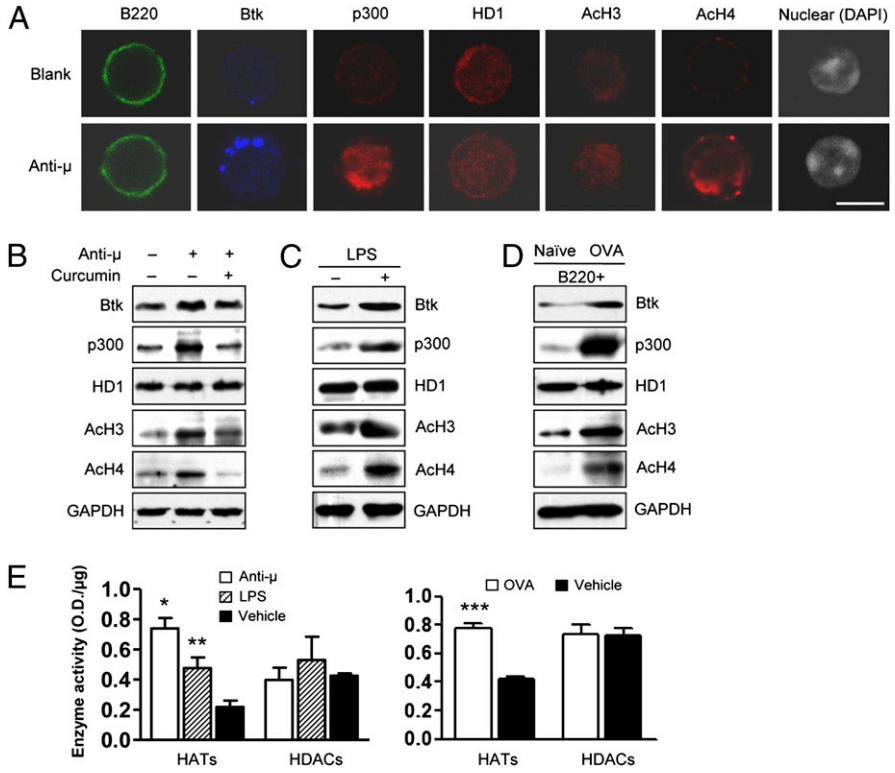

FIGURE 5. B cell activation in vitro and in vivo leads to global histone acetylation, coordinated with increased p300 expression and HAT activity. *A*, BCR cross-linking increased the levels of AcH3, AcH4, and Btk, which were associated with marked expression of p300. Splenic B cells were cultured with or without 10 μg/ml anti-μ for 2 h. The expression of the indicated proteins was analyzed by confocal microscopy with Abs against B220, Btk, p300, HDAC-1, AcH3 (K9/K14), or AcH4 (K8), as described in the *Materials and Methods*. Scale bar represents 5 μM. *B*, Curcumin suppressed the induction of AcH3, AcH4, and Btk by BCR signaling. Splenic B cells were activated with 10 μg/ml anti-μ for 24 h, in the absence or presence of 100 nM curcumin. Protein levels were measured by Western blot with the corresponding Abs, as indicated in *A*. *C*, LPS induced the expression of AcH3, AcH4, Btk, and p300. Splenic B cells were activated with 20 μg/ml LPS for 24 h. Protein expression levels were analyzed by Western blot. *D*, OVA immunization resulted in increased expression of AcH3, AcH4, Btk, and p300. C57BL/6 mice were i.p. injected with OVA (100 μg) or vehicle (DMSO) for 10 d. Splenic $B220^+$ cells were sorted by flow cytometry, and protein expression levels were analyzed by Western blot. *E*, B cells activated in vitro and in vivo showed increased HAT activity. Splenic B cells activated as indicated in *B*, *C*, or *D* were analyzed for HAT or HDAC activity as described in the *Materials and Methods*. Data represent the mean ± SD of three independent experiments for each group. Statistically significant differences compared with the vehicle group are shown as p values. $*p = 0.0142$; $**p = 0.0271$; $***p = 0.0022$.

induced Btk activity was suppressed by curcumin (Fig. 7*E*). These results suggest that Btk acetylation regulated by p300 promotes Btk kinase activity.

To better understand the effect of p300 on Btk acetylation, we studied whether p300 interacts with Btk in splenic B cells using coimmunoprecipitation. p300 was detected in Btk immunoprecipitates derived from naive B cells; however, the level of coprecipitated p300 was markedly increased in B cells activated by anti-μ (Fig. 7*F*, *left panel*). In line with this result, Btk was observed in p300 immunoprecipitates from naive B cells, and B cell activation promoted the association of Btk with p300 (Fig. 7*F*, *right panel*).

Discussion

In this study, we demonstrate that the regulation of histone acetylation modulates Btk transcription and expression. Our results show that p300 promotes histone acetylation at the Btk locus, leading to Btk transcriptional activation. Consequently, the expression of Btk mRNA and protein is enhanced. In contrast, HDAC-1 impairs local histone acetylation and decreases Btk transcription and expression. Consistent with these results, inhibition of HDACs by TSA increases local histone acetylation and activates Btk transcription. Remarkably, we found that BCR cross-linking recruits p300 to the Btk locus and induces local histone acetylation. BCR signaling-induced local acetylation, however, does not seem to be related to HDAC-1 because the recruitment of HDAC-1 to the Btk locus was not reduced in activated B cells. Instead, HDAC-1 was consistently recruited to the locus, and its levels were comparable to those observed in naive B cells. In contrast, the level of p300 bound to the locus was very low in unactivated B cells and dramatically increased in response to BCR signaling. Therefore, we propose that HDAC-1 anchored at the promoter serves as a corepressor for Btk transcription in naive B cells. After B cell activation, p300 was markedly recruited to the locus and functioned as a Btk transcription coactivator by promoting local histone acetylation and remodeling the chromatin structure (Fig. 7*G*). These findings reveal a novel mechanism underlying the regulation of Btk transcription and indicate an important role of histone acetylation in BCR-induced Btk expression.

It has been reported that histone acetylation is associated with several critical events in B cells such as V(D)J arrangement, class switching recombination, and somatic mutation (33–36). However, global histone acetylation, especially its regulation in B cells, remains largely a mystery. Our results indicate that B cell activation in vitro with anti-μ or LPS and in vivo by the T cell dependent Ag OVA results in increased levels of acetylated histone H3 and H4. This global histone acetylation in B cells activated via different pathways is not associated with a reduction in the level of HDAC-1, but is instead correlated with an increase in p300 expression. Moreover, inhibition of p300 by curcumin reduces the increased levels of acetylated histones and Btk induced by BCR

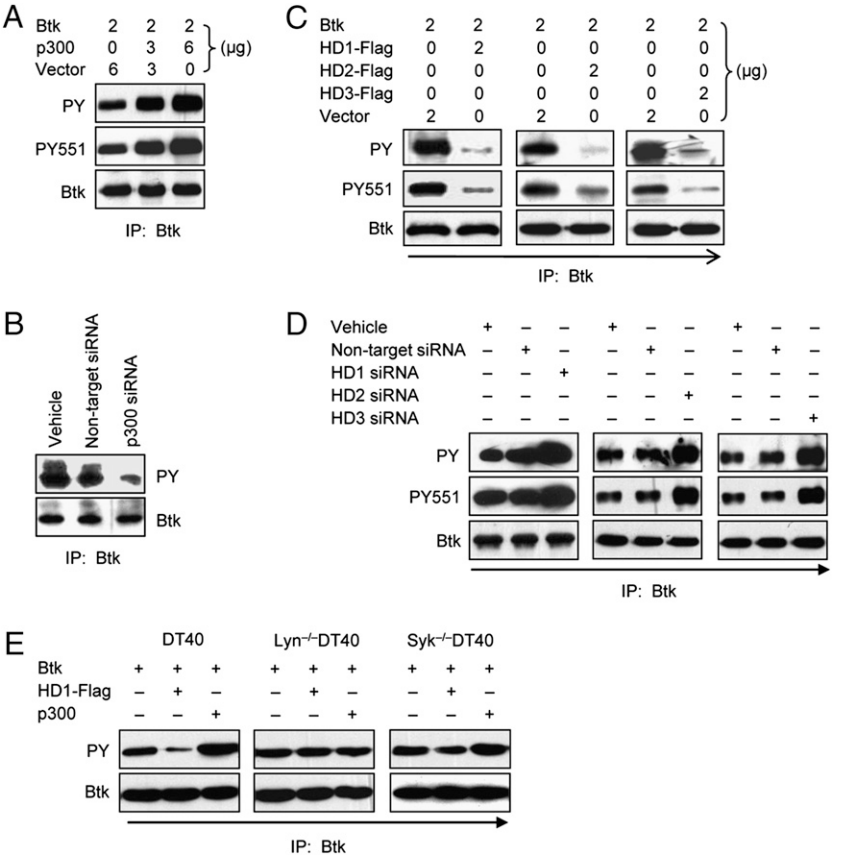

FIGURE 6. p300 and HDACs modulate Btk phosphorylation via Lyn and Syk. *A* and *B*, p300 increased the level of Btk phosphorylation. 293T cells were transfected with the Btk expression plasmid alone or together with the p300 plasmid (*A*). T347 cells were cultured with vehicle or transfected with 100 nM of either nontarget siRNA or p300 siRNA (*B*). Btk was immunoprecipitated 48 h after transfection and analyzed for the phosphorylation level of tyrosine residue(s) by immunoblotting using a 4G10 anti-phosphotyrosine (PY) or an anti-pY551 Ab. *C* and *D*, HDAC-1, -2, and -3 decreased the level of Btk phosphorylation. 293T cells were transfected with the Btk expression plasmid alone or with Flag-tagged HDAC-1, -2, or -3 (*C*). T347 cells were transfected with 100 nM siRNAs for HDAC-1, -2, or -3, respectively. Vehicle and nontarget siRNA were used as controls (*D*). Btk was immunoprecipitated 48 h after transfection and blotted with either a 4G10 anti-phosphotyrosine or an anti-pY551 Ab. *E*, Lyn and Syk were required for the effect of HDAC1 or p300 on Btk phosphorylation. DT40, $Lyn^{-/-}$ DT40, or $Syk^{-/-}$ DT40 cells were electroporated with 6 μg Btk expression plasmid alone or in combination with 18 μg HDAC-1-Flag or p300 plasmid. The phosphorylation level of precipitated Btk was measured 48 h after transfection by immunoblotting using a 4G10 anti-phosphotyrosine Ab.

cross-linking, indicating a significant role for p300 in global histone acetylation and Btk expression. Consistent with our results, the loss of p300 has been shown to attenuate BCR-induced gene expression (37). Notably, the patterns of p300 and HDAC-1 expression resemble those observed in the ChIP assay of protein recruitment, suggesting that similar regulation of local histone acetylation could be adopted for other genes in B cell activation. Our further studies on the regulation of global histone acetylation show that B cell activation-induced histone acetylation is coordinated with HATs, whereas HDACs can reduce histone acetylation in naive B cells. These results indicate a difference in the regulation of histone acetylation before and after B cell activation and significantly advance the understanding of B cell activation events (Fig. 7*G*). These findings could also be implicated in other systems such as T cell activation.

Although TSA treatment promotes Btk transcription, it also results in a severe reduction in Btk mRNA levels via degradation. Unexpectedly, we found that the effect of TSA on Btk mRNA was not in accordance with that of the HDACs. First, a low concentration of TSA (1 ng/ml), reported to affect only HDAC-1 and -3, showed minor effects on HDAC activity, but reduced the level of Btk mRNA. Second, other HDAC inhibitors (the class I HDAC inhibitor MS-275 and the class II HDAC inhibitor MC1568) did not decrease Btk mRNA levels, although HDAC activity was markedly suppressed by MS-275. Importantly, silencing of HDAC-1, -2, or -3 by specific siRNAs did not reduce, but rather increased, the levels of Btk mRNA and protein. These results indicate a contrast in the effects of TSA and the HDACs on the expression of Btk mRNA. It is known that TSA reduces the mRNA levels of many genes (38–40); however, the effect of TSA is thought to act through HDACs. This speculation is contradictory to the fact that TSA inhibits HDACs and promotes histone acetylation. Based on the current knowledge of inhibitors, our results show a nonspecific effect of TSA and suggest potential targets of TSA in addition to the HDACs. These results may provide insight into TSA-mediated mRNA reduction.

Lysine acetylation has emerged as an important posttranslational modification for histones and nonhistone proteins (17, 22, 41, 42). We have demonstrated that Btk can be modified by lysine acetylation. Our results indicate that p300 acetylates Btk in vitro and in vivo. Moreover, BCR cross-linking induces Btk lysine acetylation mediated by p300 through interaction with Btk. These results identify p300 as a modulator of Btk acetylation, which is correlated with p300-mediated Btk transcription. It was recently reported that IL-2 inducible T cell kinase, another Tec family member, is acetylated by HDAC inhibitors in a human acute myeloid leukemia cell line (43). These results, together, indicate a novel posttranslational modification in the Tec kinase family. It has been shown that several signaling pathways regulate protein acetylation. For instance, type I IFN induces lysine acetylation of multiple components (44). To our knowledge, our results show for the first time that BCR signaling induces lysine acetylation of nonhistone proteins. It may represent a novel model of lysine acetylation in signal transduction (Fig. 7*G*).

Btk lysine acetylation could play diverse physiological roles. We found that lysine acetylation-modifying enzymes modulate Btk tyrosine phosphorylation, which is promoted by p300, but inhibited by HDAC-1, -2, and -3. Moreover, BCR-induced B cell activation induces both Btk acetylation and phosphorylation, and curcumin suppresses the acetylation and phosphorylation by BCR cross-linking. Furthermore, these results are supported by the finding that overexpression of p300 enhances both Btk acetylation and phosphorylation. Thus, Btk acetylation is correlated with its phosphorylation, further regulating Btk kinase activity (Fig. 7*G*). Although it is well documented that lysine acetylation of histones cross-regulates histone phosphorylation, much less is known about this interplay in nonhistone proteins (22). Moreover, the mechanism underlying such cross-talk remains to be clarified. Our results

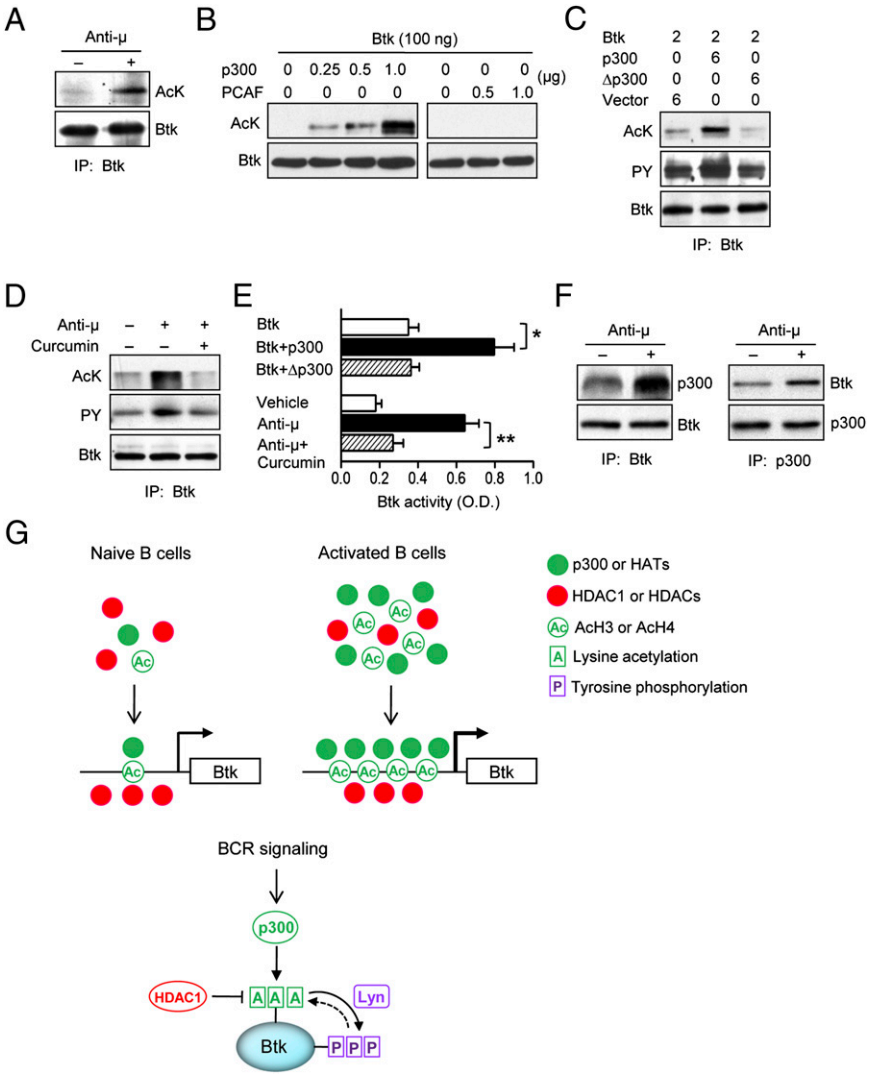

FIGURE 7. BCR cross-linking induces Btk lysine acetylation by p300 via interaction with Btk, which is associated with Btk phosphorylation. *A*, BCR signaling induced Btk lysine acetylation. Splenic B cells were activated with 10 μg/ml anti-μ for 24 h, and the immunoprecipitated Btk was analyzed for lysine acetylation by Western blotting with an anti-acetylated lysine Ab. *B*, Btk was lysine-acetylated by p300, but not by PCAF, in vitro. Btk protein was incubated with or without either p300 HAT or PCAF protein in HAT buffer containing Acetyl-CoA for 30 min at 30°C as indicated. The reaction products were subjected to SDS-PAGE and analyzed for lysine acetylation using the anti-acetylated lysine Ab. Btk served as a loading control. *C*, Overexpression of p300 promoted Btk acetylation and phosphorylation. 293T cells were transfected with a Btk expression plasmid alone or in combination with either p300 or Δp300. Btk was immunoprecipitated 24 h after transfection and analyzed for acetylation or phosphorylation using an anti-acetylated lysine or a 4G10 anti-phosphotyrosine Ab, respectively. *D*, Curcumin inhibited BCR signaling-induced Btk acetylation and phosphorylation. Splenic B cells activated as indicated in *A* were treated with or without 100 nM curcumin. The level of acetylation or phosphorylation in immunoprecipitated Btk was determined using the corresponding Abs. *E*, p300 increased Btk kinase activity. Samples prepared as indicated in *C* or *D* were analyzed for tyrosine kinase activity, as described in the *Materials and Methods*. Data represent the mean ± SD of three independent experiments for each group. Statistically significant differences between the groups are shown as *p* values. *F*, Association of p300 with Btk. Splenic B cells were activated as indicated in *A*. Btk was immunoprecipitated and coprecipitated p300 was measured with an anti-p300 Ab (*left panel*). Conversely, p300 was immunoprecipitated, and Btk in the immunoprecipitate was determined by Western blot (*right panel*). *G*, Working models for the role of lysine acetylation in Btk regulation. In naive B cells, a balance between HDAC and HAT activities maintains a low level of global histone acetylation, contributing to the quiescent status of the cells. Similarly, the dominant recruitment of HDAC-1 versus p300 to the Btk promoter limits local histone acetylation, sustaining basal Btk transcription. On BCR signaling, the expression and activity of HATs are markedly induced, leading to increased global histone acetylation and regulating B cell activation. Meanwhile at this stage, p300 is recruited to the Btk locus and promotes local histone acetylation. These events result in local chromatin remodeling and recruit other molecules such as transcription factors, finally resulting in an increase in Btk transcription (*upper panel*). BCR cross-linking induces the expression of both p300 and Btk and enhances the interaction of p300 with Btk. These effects lead to Btk lysine acetylation by p300, which further promotes Lyn-mediated Btk tyrosine phosphorylation and Btk kinase activity. Conversely, HDAC-1 represses Btk lysine acetylation by interacting with Btk (Supplemental Fig. 4), downregulating Btk phosphorylation and its function. In another way, Btk phosphorylation may modulate its acetylation (*lower panel*). $*p = 0.0137$; $**p = 0.0359$.

indicate that p300 and the HDACs fail to modulate Btk tyrosine phosphorylation when Lyn is deficient, showing a critical role for Lyn in the acetylation-phosphorylation cassette and identifying a mediator of the interplay between lysine acetylation and tyrosine phosphorylation (Fig. 7*G*). Collectively, our findings have identified a previously unrecognized lysine acetylation modification of Btk and indicate that this acetylation promotes Btk phosphorylation. These results delineate a novel aspect of Btk regulation and introduce a new way to investigate Btk function in physiology and pathology.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Khan, W. N. 2001. Regulation of B lymphocyte development and activation by Bruton's tyrosine kinase. *Immunol. Res.* 23: 147–156.
2. Satterthwaite, A. B., and O. N. Witte. 2000. The role of Bruton's tyrosine kinase in B-cell development and function: a genetic perspective. *Immunol. Rev.* 175: 120–127.
3. Rawlings, D. J., and O. N. Witte. 1994. Bruton's tyrosine kinase is a key regulator in B-cell development. *Immunol. Rev.* 138: 105–119.
4. Tsukada, S., D. J. Rawlings, and O. N. Witte. 1994. Role of Bruton's tyrosine kinase in immunodeficiency. *Curr. Opin. Immunol.* 6: 623–630.
5. Lindvall, J. M., K. E. Blomberg, J. Väliaho, L. Vargas, J. E. Heinonen, A. Berglöf, A. J. Mohamed, B. F. Nore, M. Vihinen, and C. I. Smith. 2005. Bruton's tyrosine kinase: cell biology, sequence conservation, mutation spectrum, siRNA modifications, and expression profiling. *Immunol. Rev.* 203: 200–215.
6. Khan, W. N., F. W. Alt, R. M. Gerstein, B. A. Malynn, I. Larsson, G. Rathbun, L. Davidson, S. Müller, A. B. Kantor, L. A. Herzenberg, et al. 1995. Defective B cell development and function in Btk-deficient mice. *Immunity* 3: 283–299.
7. Rawlings, D. J., A. M. Scharenberg, H. Park, M. I. Wahl, S. Lin, R. M. Kato, A. C. Fluckiger, O. N. Witte, and J. P. Kinet. 1996. Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. *Science* 271: 822–825.
8. Himmelmann, A., C. Thevenin, K. Harrison, and J. H. Kehrl. 1996. Analysis of the Bruton's tyrosine kinase gene promoter reveals critical PU.1 and SP1 sites. *Blood* 87: 1036–1044.
9. Webb, C. F., Y. Yamashita, N. Ayers, S. Evetts, Y. Paulin, M. E. Conley, and E. A. Smith. 2000. The transcription factor Bright associates with Bruton's tyrosine kinase, the defective protein in immunodeficiency disease. *J. Immunol.* 165: 6956–6965.
10. Brunner, C., and T. Wirth. 2006. Btk expression is controlled by Oct and BOB.1/OBF.1. *Nucleic Acids Res.* 34: 1807–1815.
11. Yu, L., A. J. Mohamed, O. E. Simonson, L. Vargas, K. E. Blomberg, B. Björkstrand, H. J. Arteaga, B. F. Nore, and C. I. Smith. 2008. Proteasome-dependent autoregulation of Bruton tyrosine kinase (Btk) promoter via NF-kappaB. *Blood* 111: 4617–4626.
12. Müller, S., A. Maas, T. C. Islam, P. Sideras, G. Suske, S. Philipsen, K. G. Xanthopoulos, R. W. Hendriks, and C. I. Smith. 1999. Synergistic activation of the human Btk promoter by transcription factors Sp1/3 and PU.1. *Biochem. Biophys. Res. Commun.* 259: 364–369.
13. Jenuwein, T., and C. D. Allis. 2001. Translating the histone code. *Science* 293: 1074–1080.
14. Sterner, D. E., and S. L. Berger. 2000. Acetylation of histones and transcription-related factors. *Microbiol. Mol. Biol. Rev.* 64: 435–459.
15. Hassig, C. A., and S. L. Schreiber. 1997. Nuclear histone acetylases and deacetylases and transcriptional regulation: HATs off to HDACs. *Curr. Opin. Chem. Biol.* 1: 300–308.
16. Kouzarides, T. 2007. Chromatin modifications and their function. *Cell* 128: 693–705.
17. Glozak, M. A., N. Sengupta, X. Zhang, and E. Seto. 2005. Acetylation and deacetylation of non-histone proteins. *Gene* 363: 15–23.
18. Cheung, W. L., S. D. Briggs, and C. D. Allis. 2000. Acetylation and chromosomal functions. *Curr. Opin. Cell Biol.* 12: 326–333.
19. Gu, W., and R. G. Roeder. 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* 90: 595–606.
20. Hubbert, C., A. Guardiola, R. Shao, Y. Kawaguchi, A. Ito, A. Nixon, M. Yoshida, X. F. Wang, and T. P. Yao. 2002. HDAC6 is a microtubule-associated deacetylase. *Nature* 417: 455–458.
21. Allis, C. D., S. L. Berger, J. Cote, S. Dent, T. Jenuwien, T. Kouzarides, L. Pillus, D. Reinberg, Y. Shi, R. Shiekhattar, et al. 2007. New nomenclature for chromatin-modifying enzymes. *Cell* 131: 633–636.
22. Yang, X. J., and E. Seto. 2008. Lysine acetylation: codified crosstalk with other posttranslational modifications. *Mol. Cell* 31: 449–461.
23. Avila, A. M., B. G. Burnett, A. A. Taye, F. Gabanella, M. A. Knight, P. Hartenstein, Z. Cizman, N. A. Di Prospero, L. Pellizzoni, K. H. Fischbeck, and C. J. Sumner. 2007. Trichostatin A increases SMN expression and survival in a mouse model of spinal muscular atrophy. *J. Clin. Invest.* 117: 659–671.
24. Gray, S. G., T. Yakovleva, W. Hartmann, M. Tally, G. Bakalkin, and T. J. Ekström. 1999. IGF-II enhances trichostatin A-induced TGFbeta1 and p21 (Waf1,Cip1, sdi1) expression in Hep3B cells. *Exp. Cell Res.* 253: 618–628.
25. Sandoval, J., J. L. Rodríguez, G. Tur, G. Serviddio, J. Pereda, A. Boukaba, J. Sastre, L. Torres, L. Franco, and G. López-Rodas. 2004. RNAPol-ChIP: a novel application of chromatin immunoprecipitation to the analysis of real-time gene transcription. *Nucleic Acids Res.* 32: e88.
26. Hess-Stumpp, H., T. U. Bracker, D. Henderson, and O. Politz. 2007. MS-275, a potent orally available inhibitor of histone deacetylases—the development of an anticancer agent. *Int. J. Biochem. Cell Biol.* 39: 1388–1405.
27. Mai, A., S. Massa, R. Pezzi, S. Simeoni, D. Rotili, A. Nebbioso, A. Scognamiglio, L. Altucci, P. Loidl, and G. Brosch. 2005. Class II (IIa)-selective histone deacetylase inhibitors. 1. Synthesis and biological evaluation of novel (aryloxopropenyl) pyrrolyl hydroxyamides. *J. Med. Chem.* 48: 3344–3353.
28. Inoue, S., A. Mai, M. J. Dyer, and G. M. Cohen. 2006. Inhibition of histone deacetylase class I but not class II is critical for the sensitization of leukemic cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. *Cancer Res.* 66: 6785–6792.
29. Hu, E., E. Dul, C. M. Sung, Z. Chen, R. Kirkpatrick, G. F. Zhang, K. Johanson, R. Liu, A. Lago, G. Hofmann, et al. 2003. Identification of novel isoform-selective inhibitors within class I histone deacetylases. *J. Pharmacol. Exp. Ther.* 307: 720–728.
30. Marcu, M. G., Y. J. Jung, S. Lee, E. J. Chung, M. J. Lee, J. Trepel, and L. Neckers. 2006. Curcumin is an inhibitor of p300 histone acetylatransferase. *Med. Chem.* 2: 169–174.
31. Morimoto, T., Y. Sunagawa, T. Kawamura, T. Takaya, H. Wada, A. Nagasawa, M. Komeda, M. Fujita, A. Shimatsu, T. Kita, and K. Hasegawa. 2008. The dietary compound curcumin inhibits p300 histone acetyltransferase activity and prevents heart failure in rats. *J. Clin. Invest.* 118: 868–878.
32. Baba, Y., S. Hashimoto, M. Matsushita, D. Watanabe, T. Kishimoto, T. Kurosaki, and S. Tsukada. 2001. BLNK mediates Syk-dependent Btk activation. *Proc. Natl. Acad. Sci. USA* 98: 2582–2586.
33. McMurry, M. T., and M. S. Krangel. 2000. A role for histone acetylation in the developmental regulation of VDJ recombination. *Science* 287: 495–498.
34. Nambu, Y., M. Sugai, H. Gonda, C. G. Lee, T. Katakai, Y. Agata, Y. Yokota, and A. Shimizu. 2003. Transcription-coupled events associating with immunoglobulin switch region chromatin. *Science* 302: 2137–2140.
35. Wang, L., N. Whang, R. Wuerffel, and A. L. Kenter. 2006. AID-dependent histone acetylation is detected in immunoglobulin S regions. *J. Exp. Med.* 203: 215–226.
36. Woo, C. J., A. Martin, and M. D. Scharff. 2003. Induction of somatic hypermutation is associated with modifications in immunoglobulin variable region chromatin. *Immunity* 19: 479–489.
37. Xu, W., T. Fukuyama, P. A. Ney, D. Wang, J. Rehg, K. Boyd, J. M. van Deursen, and P. K. Brindle. 2006. Global transcriptional coactivators CREB-binding protein and p300 are highly essential collectively but not individually in peripheral B cells. *Blood* 107: 4407–4416.
38. Lin, H. Y., C. S. Chen, S. P. Lin, J. R. Weng, and C. S. Chen. 2006. Targeting histone deacetylase in cancer therapy. *Med. Res. Rev.* 26: 397–413.
39. Chittur, S. V., N. Sangster-Guity, and P. J. McCormick. 2008. Histone deacetylase inhibitors: a new mode for inhibition of cholesterol metabolism. *BMC Genomics* 9: 507.
40. Drummond, D. C., C. O. Noble, D. B. Kirpotin, Z. Guo, G. K. Scott, and C. C. Benz. 2005. Clinical development of histone deacetylase inhibitors as anticancer agents. *Annu. Rev. Pharmacol. Toxicol.* 45: 495–528.
41. Motta, M. C., N. Divecha, M. Lemieux, C. Kamel, D. Chen, W. Gu, Y. Bultsma, M. McBurney, and L. Guarente. 2004. Mammalian SIRT1 represses forkhead transcription factors. *Cell* 116: 551–563.
42. Tang, Y., J. Luo, W. Zhang, and W. Gu. 2006. Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. *Mol. Cell* 24: 827–839.
43. Choudhary, C., C. Kumar, F. Gnad, M. L. Nielsen, M. Rehman, T. C. Walther, J. V. Olsen, and M. Mann. 2009. Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* 325: 834–840.
44. Tang, X., J. S. Gao, Y. J. Guan, K. E. McLane, Z. L. Yuan, B. Ramratnam, and Y. E. Chin. 2007. Acetylation-dependent signal transduction for type I interferon receptor. *Cell* 131: 93–105.