# SIRT1 deacetylates SATB1 to facilitate MAR$^{HS2}$-MAR$^{\varepsilon}$ interaction and promote ε-globin expression

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## ABSTRACT

The higher order chromatin structure has recently been revealed as a critical new layer of gene transcriptional control. Changes in higher order chromatin structures were shown to correlate with the availability of transcriptional factors and/or MAR (matrix attachment region) binding proteins, which tether genomic DNA to the nuclear matrix. How posttranslational modification to these protein organizers may affect higher order chromatin structure still pending experimental investigation. The type III histone deacetylase silent mating type information regulator 2, S. cerevisiae, homolog 1 (SIRT1) participates in many physiological processes through targeting both histone and transcriptional factors. We show that MAR binding protein SATB1, which mediates chromatin looping in cytokine, MHC-I and β-globin gene loci, as a new type of SIRT1 substrate. SIRT1 expression increased accompanying erythroid differentiation and the strengthening of β-globin cluster higher order chromatin structure, while knockdown of SIRT1 in erythroid k562 cells weakened the long-range interaction between two SATB1 binding sites in the β-globin locus, MAR$^{HS2}$ and MAR$^{\varepsilon}$. We also show that SIRT1 activity significantly affects ε-globin gene expression in a SATB1-dependent manner and that knockdown of SIRT1 largely blocks ε-globin gene activation during erythroid differentiation. Our work proposes that SIRT1 orchestrates changes in higher order chromatin structure during erythropoiesis, and reveals the dynamic higher order chromatin structure regulation at posttranslational modification level.

## INTRODUCTION

Higher order chromatin structure plays an important role in eukaryotic gene expression regulation. This regulation permits selective cross talk between *cis*-regulatory elements that are spaced far apart in the DNA sequence, or even those present on separate chromosomes and therefore provides a transcription permissive or inhibitory environment for the RNA polymerase. Using Chromosome Conformation Capture (3C) (1) and related high-throughput technologies such as Hi-C (2), many functionally potent higher order chromatin structures have been identified, including α/β-globin active chromatin hub (3,4) and Igf-H19 imprinting structure (5,6). The nuclear matrix fibrous network within the cell nucleus acts as a scaffold where matrix attachment region (MAR) elements are anchored with the help of nuclear matrix binding proteins. The selective and dynamic association of different potential MARs to the nuclear matrix is closely related to the expression of associated genes and gene clusters (7), potentially through facilitated formation of the functional chromatin higher order architecture. SATB1 is a well-studied matrix binding protein that is actively involved in the formation of the higher order chromatin structure of cytokine gene cluster, MHC class I compounds (8–10) and acts as a platform to recruit chromatin remodeling complexes and histone modifiers (11). In embryonic fetal stage K562 cells, SATB1 binds to MARs located at hypersensitive site 2 (HS2) in the locus control region and at the 5′ regulatory region of the ε-globin gene. This binding activates ε-globin gene expression through the recruitment of the cAMP Response Element-Binding Protein (CREB) binding protein (CBP) (12). Our previous work further showed that SATB1 relocates the β-globin gene cluster to the nuclear matrix and mediates the formation of a long-range interaction among the MARs (inter-MAR association); both these

mechanisms contribute to transcriptional activation in the locus (13,14).

Posttranslational modifications of SATB1, including phosphorylation and acetylation, have been reported to modulate the *trans*-activity of SATB1 in T-cells (15,16). PCAF (p300/CBP-associated factor) acetylates SATB1 at lysine 136 and abolishes the ability of SATB1 to bind to DNA, and PKC-mediated SATB1 phosphorylation acts as a switch to determine the acetylation status and transcriptional activity of SATB1 (15). In addition to the above-mentioned SATB1 modulation at the posttranslational level in T-cells, our previous work showed that SATB1 acetylation decreased during hemin-induced erythroid differentiation of K562 cells, whereas no obvious protein abundance change was observed. Also, SATB1 deacetylation was accompanied by the strengthening of the 'inter-MAR association', which implies the involvement of deacetylase in the modulation of the higher order chromatin structure in the β-globin gene locus (14).

silent mating type information regulator 2, S. cerevisiae, homolog 1 (SIRT1) is a class III deacetylase that can reverse acetylated substrates in a $NAD^+$ dependent manner (17). As the closest homolog to the yeast Sir2 in mammals, SIRT1 plays important roles in many physiological processes, including aging (18), apoptosis (19), metabolism (20) and differentiation (17). SIRT1 fulfills these functions by deacetylating its substrates, which include FOXOs (18), p53 (19), PGC1-α (20) and Myo D (17). The present understanding of the broad physiological functions of SIRT1 indicates a potential role of the deacetylase on the regulation of higher order chromatin structure. In this study, we identify SATB1 as a substrate of SIRT1. We show that SIRT1 promotes ε-globin gene expression by binding to the MAR elements of the β-globin gene locus together with SATB1 and facilitating the inter-MAR association between $MAR^{HS2}$ and $MAR^\varepsilon$.

## MATERIALS AND METHODS

### Cell culture, retroviral infection and transfection

The 293A cells were maintained in α-MEM (GIBCO) medium containing 10% fetal bovine serum (FBS). K562 cells were maintained in RPMI 1640 (GIBCO) containing 10% FBS. Retrovirus packaging 293T cells were maintained in DMEM (GIBCO) containing 10% FBS. Umbilical cord blood (UCB) samples from normal full-term deliveries were obtained with the approval from the institutional and licensing committee and the informed consent from all subjects. Human primary hematopoietic cells were isolated using Anti-CD34-Microbeads (MACS, Miltenyi Biotec) following separation of mononuclear cells from UCB samples by density gradient centrifugation using a Percoll density gradient (Amersham Pharmacia Biotech). The purified human primary hematopoietic cells were cultured and induced for erythroid differentiation using Epo as described (21). 293T cells were transfected with recombinant retrovirus vectors using Lipofectamine 2000 reagent (Invitrogen). Media containing the retroviruses was

collected 48 h later, filtered and transferred onto target cells with polybrene (1 mg/ml) for 6 h. Stable polyclonal cell lines were selected by puromycin (1 μg/ml) for 48 h after infection.

### Coimmunoprecipitation, western blot analysis and antibodies

Cells were lysed at 4°C for 30 min in 1 ml of modified RIPA buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate, 1 mM PMSF and 1 μg/ml each of aprotinin, leupeptin and pepstatin. The lysates were centrifuged at 13 000 rpm for 15 min at 4°C to remove the cellular debris. The cell lysates were precleared by incubation at 4°C with control IgG and Protein A/G agarose beads (Santa Cruz). After being centrifuged at 4°C and 2500 rpm for 5 min, the supernatant was incubated with respective antibodies (anti-Myc antibody from Santa Cruz, anti-SATB1 antibody from Abcam and anti-SIRT1 antibody from Santa Cruz) and protein A/G agarose beads overnight at 4°C. The immunocomplex was washed with RIPA buffer five times, eluted by boiling in SDS–PAGE buffer for 5 min, and subjected to western blot with the appropriate antibodies [anti-SATB1 antibody from Abcam and BD, anti-SIRT1 antibody from Santa Cruz and Millipore, and anti-Acetylated Lysine antibody (anti-K-Ac) from Cell Signaling Technology].

### Recombinant protein production, purification and *in vitro* acetylation/deacetylation analysis

GST-SATB1-1-204 and GST-PCAF were produced and purified as described (22). Purified SIRT1 deacetylase, $NAD^+$ and Ac-CoA were purchased from Sigma. *In vitro* acetylation/deacetylation assays were performed as described (18) with minor modifications. Briefly, for the *in vitro* acetylation assay, 5 μg of GST-SATB1-1-204 was incubated with 1 μg GST-PCAF in the presence of acetyl-CoA (20 μM) at 30°C for 1 h. The reaction was performed in a buffer solution containing 50 mM Tris–HCl (pH 8.0), 0.1 mM EDTA, 1 mM Dithiothreitol (DTT) and 10% glycerol. The products of the reaction were resolved on a SDS–PAGE gel and analyzed by western blotting with an anti-K-Ac antibody (Cell Signaling Technology) or subjected to tandem mass spectrometry (MS/MS) analysis using a 4700 Proteomics Analyzer (Applied Biosystems). For the *in vitro* deacetylation assay, Ac-GST-SATB1-1-204 was incubated in deacetylation buffer containing 25 mM Tris–HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$ and purified recombinant human SIRT1 (Sigma, 3.5 U) in the presence or absence of NAD (Sigma, 60 μM) at 30°C for 1 h. The products of the reaction were resolved on a SDS–PAGE gel and analyzed by western blotting with an anti-K-Ac antibody (Cell Signaling Technology). The acetylated peptides were synthesized at the Beijing Genomics Institute and subjected to MS/MS after the *in vitro* deacetylation reaction using a 4700 Proteomics Analyzer (Applied Biosystems).

### *In vivo* acetylation analysis

The 293A cells were cotransfected with Myc-SATB1 and pcDNA3.1 or pcDNA3.1-SIRT1. The cells were lysed and immunoprecipitated with an anti-Myc antibody. The immunocomplexes were resolved on SDS–PAGE and analyzed by western blotting with an anti-K-Ac antibody (Millipore). Acetylation of endogenous SATB1 in control and drug-challenged/genetic-modified K562 cells and in human HSC-derived primary erythroid cells were detected by immunoprecipitating the cell lysate with anti-SATB1 antibody (Abcam). The immunocomplexes were resolved on SDS–PAGE and analyzed by western blotting with an anti-K-Ac antibody (Cell Signaling Technology).

### RNA isolation and analysis

Total RNA was isolated using Trizol (Invitrogen). For real-time PCR analysis, cDNA was synthesized from total RNA by M-MuLV reverse transcriptase (New England Biolabs) with random primers (Takara). The resulting cDNA was subjected to PCR analysis with gene-specific primers using an IQ5 real-time PCR system (BIO-RED). The housekeeping gene GAPDH was used as the internal control. The PCR product was measured by SYBR Green.

The primers used for real-time PCR were as follows:

ε-globin forward primer: 5′-CTTTGGAAACCTGTCGT C-3′
ε-globin reverse primer: 5′-CTTGCCAAAGTGAGTAG C-3′
γ-globin forward primer: 5′-GGCAACCTGTCCTCTGC CTC-3′
γ-globin reverse primer: 5′-GAAATGGATTGCCAAAA CGG-3′
SATB1 forward primer: 5′-GATCTATGAATAAGCCT TTGGAG-3′
SATB1 reverse primer: 5′-TTTCGTCCTGGTATATTC GGT-3′
GAPDH forward primer: 5′-GGTCACCAGGGCTGCT TTTA-3′
GAPDH reverse primer: 5′-GAGGGATCTCGCTCCTG GA-3′

### Chromatin immunoprecipitation analysis

K562 cells were cross-linked in 1% formaldehyde for 15 min before being resuspended in lysis buffer [1% SDS, 10 mM EDTA and 50 mM Tris (pH 8.1)] containing protease inhibitors cocktail (1 mM PMSF and 1 μg/ml each of aprotinin, leupeptin and pepstatin). This solution was sonicated on ice until the cross-linked chromatin DNA was sheared to an average length of 500 bp. The sonicated cell supernatant was diluted 10-fold in ChIP dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl (pH 8.1) and 167 mM NaCl] containing protease inhibitors cocktail. After incubation with control IgG and protein A agarose, the precleared chromatin was immunoprecipitated with an anti-SIRT1 antibody (Santa Cruz), an anti-SATB1 antibody

(Abcam), or pre-immune goat serum as the control at 4°C overnight. The precipitated complexes were recovered by incubation with protein A-agarose at 4°C for 2 h, centrifuged at a low speed, and subjected to reverse cross-linking. DNA within the complexes was extracted with phenol–chloroform/isoamyl alcohol (25:24:1), precipitated with ethanol and used for real-time PCR analysis. For the quantification of ChIP signals, Cycle threshold ($C_t$) values were first obtained for input, IgG and ChIP samples from SYBR green-based real-time PCR. Fold occupancy of ChIP signal and IgG control with respect to input (1% of starting chromatin) was calculated respectively with the equation: $2^{CTinput-CTChIP}$ and $2^{CTinput-CTIgG}$.

The primers used were as follows:

MAR-HS2-forward primer: 5′-TGCCTCTACAAAAAG TACAAAAATTAGC-3′
MAR-HS2-reverse primer: 5′-CCCCACAAGAGTCCA AGTAAAAA-3′
MAR-ε-forward primer: 5′-CACTGTCACCACCTTTA AGGCA-3′
MAR-ε-reverse primer: 5′-GTTGGCTCCTCATCTATC TGCA-3′

### 3C analysis

The 3 C analysis was performed as previously described (14). Briefly, SIRT1 knockdown and control K562 cells were subjected to 1.5% formaldehyde cross-linking for 15 min. The cross-linked DNA was digested by a HindIII restriction enzyme, intramolecularly ligated and then reverse cross-linked. The ligation products were then analyzed by real-time PCR for the spatial proximity between the two fragments, each represented by one primer. The primers used were as follows:

Ercc3-forward primer: 5′-ACAGTAGTCAAGGCTGAG GAA-3′
Ercc3-reverse primer: 5′-ATGGTGCTGAGTGAAAGA AGT-3′
MAR-HS2 primer: 5′-TCTAATTTCTCGCATCCACAA TAT-3′
MAR-HS4 primer: 5′-CCTGGGAACAGTTAAACAGC AAC-3′
MAR-ε primer: 5′-CACTAAGGGTGAGGATGC TGAA-3′
MAR-γ primer: 5′-AGCCTACAGCATACAGGGTTCA T-3′

## RESULTS

### SIRT1 interacts with SATB1

As SIRT1 physically interacts with its substrates, we first tested for interaction between SATB1 and SIRT1. SATB1 and SIRT1 cDNAs were subcloned into vectors with or without a Myc tag. The Myc-tagged SATB1 and non-tagged SIRT1, or Myc-tagged SIRT1 and non-tagged SATB1, were then cotransfected into 293T cells and subjected to a coimmunoprecipitation (coIP) assay. As shown

in Figure 1A and B, the exogenously expressed SIRT1 and SATB1 coprecipitated reciprocally. A coIP assay of endogenous SATB1 and SIRT1 in K562 cells further confirmed an interaction between these two proteins (Figure 1C and D). Endogenous SIRT1 shows two bands in the coIP assay, one at ∼80 kDa and the other at ∼110 kDa. The SIRT1 protein immunoprecipitated by SATB1 is dominated by the 80 kDa moiety in the K562 cells (Figure 1D), although the 110 KD-SIRT1 moiety also interacts with SATB1 (Figure 1C). Confocal microscopy analysis of the 293T cells cotransfected with GFP-SATB1 and Cherry-SIRT1 revealed that SATB1 colocalized with SIRT1 (Figure 1E). These results demonstrate that SIRT1 interacts with SATB1. Expression of SIRT6 and SIRT7, the other two nuclear members of the sirtuin family, were also detected in K562 cells, but both of them show no interaction with SATB1 when exogenously expressed in 293T cells (Supplementary Figure S1).

## SIRT1 deacetylates SATB1

We then tested whether SATB1's acetylation status changes in response to an inhibitor or activator of SIRT1. 293 cells transfected with Myc-tagged SATB1 were treated with the class I and II histone deacetylase inhibitor TSA (1 μM), SIRT1 inhibitor Nicotinamide (NAM) (5 mM) or both for 10 h. The cell lysates were immunoprecipitated with an anti-Myc antibody, and western blotting was performed with an antibody against acetylated lysine. These analyses indicated that both TSA and NAM increased SATB1 acetylation, and the effect of NAM was greater than that of TSA (Figure 2A). To detect whether SATB1 was directly deacetylated by SIRT1, we performed an *in vitro* deacetylation assay. It has been reported that SATB1 is acetylated at lysine 136 within its PSD95/Dlg-A/ZO-1 (PDZ)-like domain by PCAF (15); therefore, we purified a truncated SATB1 protein containing amino acids 1–204 that covered the
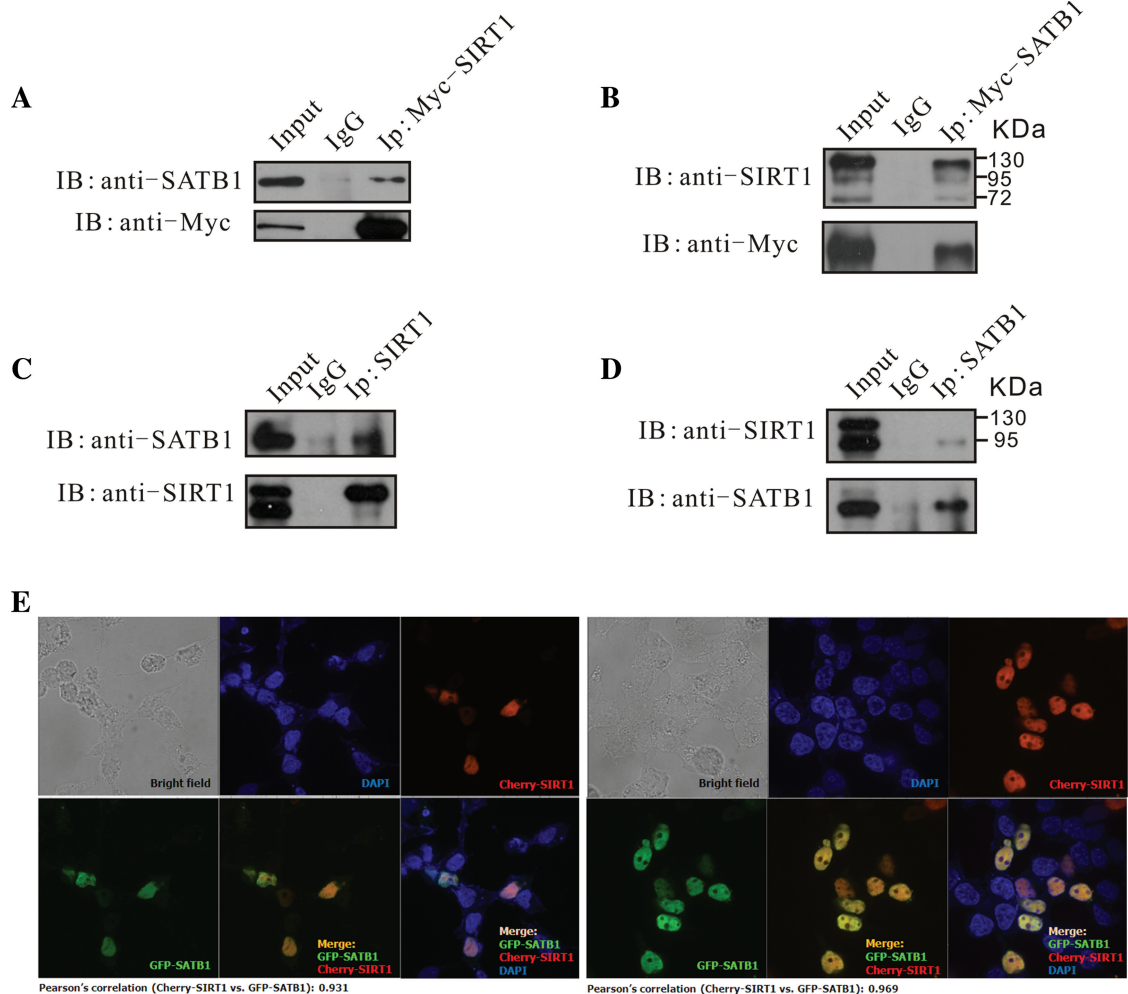


**Figure 1.** SIRT1 interacts with SATB1. (**A** and **B**) 293 cells were transfected with the indicated protein-expressing vectors, lysed and immunoprecipitated (IP) with an anti-Myc monoclonal antibody, and western blotting was performed with the indicated antibodies. (**C** and **D**) K562 cells were lysed, and endogenous proteins were IP with SATB1 or SIRT1 antibody before being probed with a SIRT1 or SATB1 antibody, respectively. (**E**) SIRT1 colocalizes with SATB1. 293T cells were cotransfected with GFP-SATB1 and Cherry-SIRT1. Cells were photographed under a confocal microscope. Two different microscopic fields were shown (Ultra*VIEW* VoX 3D LCI system, PerkinElmer Life Sciences Inc., USA). Pearson's correlation coefficients of GFP-SATB1 signal and Cherry-SIRT1 signal were shown beneath the panels.
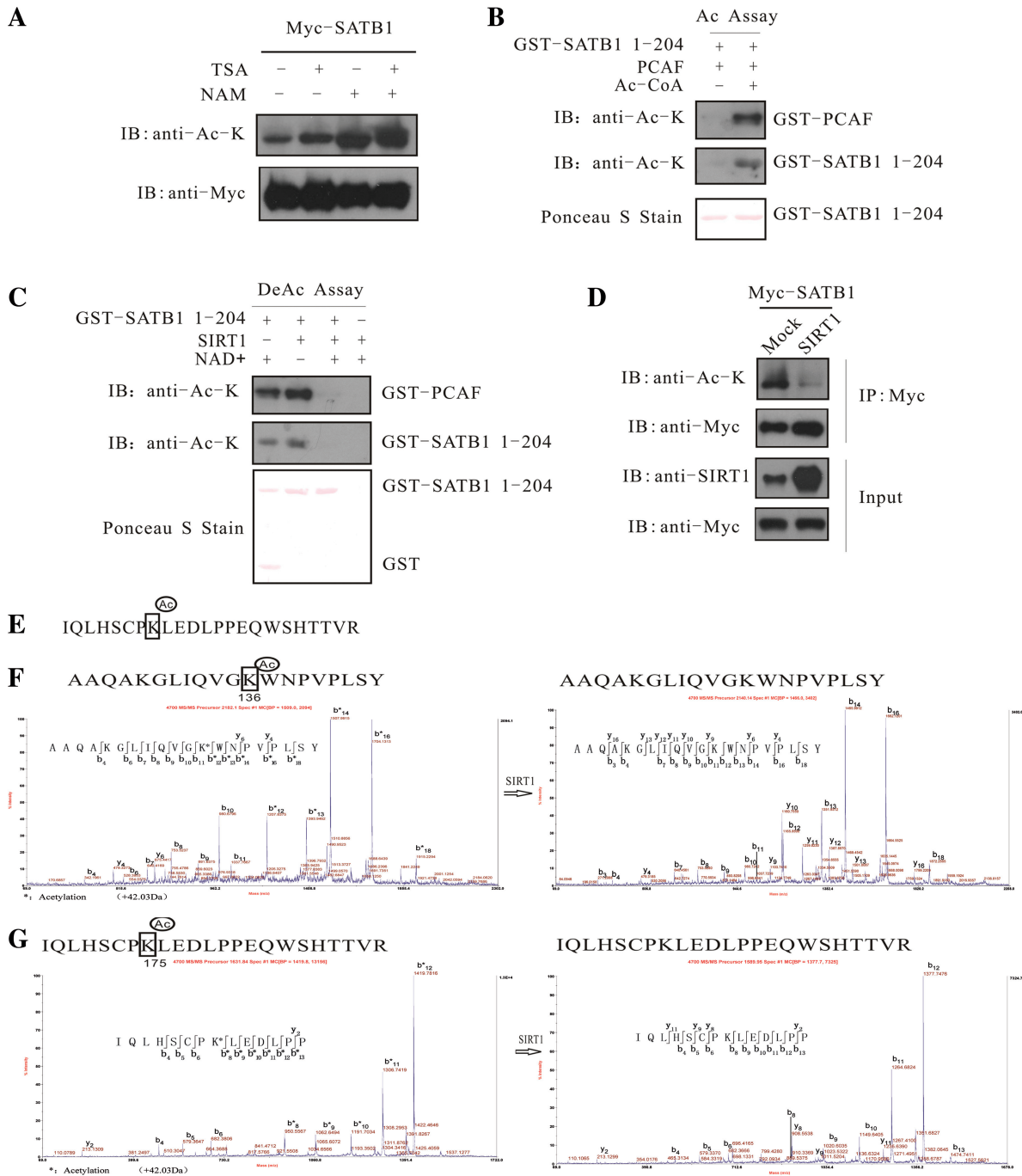
**Figure 2.** SIRT1 deacetylates SATB1. (**A**) The 293 cells were transfected with Myc-SATB1 and then treated with TSA and NAM as indicated. Immunoprecipitation was performed using a Myc antibody, and SATB1 acetylation levels were detected by western blotting with an acetylated-lysine antibody. (**B**) *In vitro* acetylation assay. Truncated GST-SATB1-1-204 was acetylated in the presence of PCAF and Ac-CoA. The self-acetylated PCAF was used as a positive control, and SATB1 acetylation was detected by western blotting with an acetylated-lysine antibody. The truncated GST-SATB1-1-204 protein was detected by Ponceau S staining. (**C**) *In vitro* deacetylation assay. *In vitro*-acetylated GST-SATB1 1-204 was deacetylated with SIRT1 and NAD$^+$. The self-acetylated PCAF was used as a positive control, and SATB1 acetylation was detected by western blotting with an acetylated-lysine antibody. The truncated GST-SATB1-1-204 protein was detected by Ponceau S staining. (**D**) The 293 cells were cotransfected with SIRT1 and Myc-SATB1-expressing vectors. Immunoprecipitation was then performed using a Myc antibody, and Myc-SATB1 acetylation levels were detected by western blotting with an acetylated-lysine antibody. The cell lysates were subjected to blotting with the indicated antibodies to show the expression of exogenous SIRT1 and Myc-SATB1. (**E**) Amino acid sequence of SATB1 containing the acetylated K175. (**F**) MS/MS spectrum analysis of the SATB1 peptide containing acetylated K136 before and after the *in vitro* deacetylation assay. (**G**) MS/MS spectrum analysis of the SATB1 peptide containing acetylated K175 before and after the *in vitro* deacetylation assay.

PDZ-like domain and fusion expressed it with a GST tag. GST-SATB1-1-204 was acetylated *in vitro* by PCAF in the presence of acetyl-coenzyme A (acetyl-CoA). A western blot using an antibody against acetylated lysine confirmed the efficacy of the reaction (Figure 2B). Acetylated GST-SATB-1-204 was used as a substrate for SIRT1 in an *in vitro* deacetylation assay in the presence of the coenzyme $NAD^+$ (Figure 2C). The auto-acetylated PCAF, which is deacetylated by SIRT1 (23), served as a positive control. We showed that SIRT1 deacetylates GST-SATB1-1-204 *in vitro*. To determine whether SIRT1 deacetylates SATB1 *in vivo*, we cotransfected 293 cells with Myc-tagged SATB1, a SIRT1 overexpression construct or an empty vector and then immunoprecipitated the Myc-SATB1. By western blotting with an acetylated-lysine antibody, we found that SIRT1 overexpression decreased the amount of detected acetylated SATB1 (Figure 2D).

To further identify SIRT1 deacetylation sites on SATB1, we first detected acetylation sites on SATB1. PCAF acetylated GST-SATB1-1-204 was subjected to MS/MS. The analysis revealed that, besides the previously reported lysine 136, the lysine 175 residue of SATB1 was also acetylated by PCAF *in vitro* (Figure 2E). We then synthesized two peptides containing either acetylated lysine 136 or 175 as substrates and performed an *in vitro* deacetylation assay in the presence of SIRT1 and $NAD^+$. As shown in Figure 2F and G, MS/MS analysis demonstrated that both lysines 136 and 175 of SATB1 are deacetylated by SIRT1 *in vitro* (See also the enlarged MS/MS data in Supplementary Figure S2).

### SIRT1 promotes ε-globin gene expression

Given that SATB1 can activate the expression of the ε-globin gene in K562 cells (12–14), we hypothesized that SIRT1, which interacts with and deacetylases SATB1, could also influence ε-globin gene expression. K562 cells were treated with the SIRT1 activator resveratrol for 24 h or with the SIRT1 inhibitor NAM for 10 h. The expression of the ε-globin gene was monitored using real-time PCR. We found that resveratrol activated ε-globin gene expression in a concentration-dependent manner (Figure 3A), whereas NAM decreased the ε-globin gene expression (Figure 3B), accompanied with decreased SATB1 acetylation level upon resveratrol challenge and increased SATB1 acetylation level after NAM treatment (Figure 3A and B). We then determined whether overexpression or knockdown of SIRT1 could affect the mRNA abundance of the ε-globin gene. In agreement with the results obtained with resveratrol treatment, SIRT1 overexpression in K562 cells promoted ε-globin gene expression dramatically (Figure 3C). In contrast, the mRNA level of the ε-globin gene decreased significantly in K562 cells with stable knockdown of SIRT1 (Figure 3D), concomitant SATB1 acetylation level was shown decreased upon SIRT1 overexpression and increased in SIRT1-knockdown K562 cells (Figure 3C and D).

Subsequently, we tested whether SIRT1 could also promote the expression of the ε-globin gene in human primary erythroid cells. Human primary hematopoietic stem cells (HSCs) were isolated from cord blood and cultured in expansion medium for 1 week before being stimulated by erythropoietin (Epo) for another 3–9 days to induce erythroid differentiation. Similar to the K562 cells, treatment with resveratrol caused an upregulation of ε-globin gene expression, and treatment with NAM caused a downregulation of ε-globin gene expression in human primary erythroid cells (Figure 3E and F). In addition, human primary HSCs were infected with retrovirus-interfering SIRT1 or luciferase, subjected to selection with puromycin, and induced with Epo for 3 days. We found that the SIRT1 interference also impaired ε-globin gene expression in these primary erythroid cells, accompanied with increased SATB1 acetylation level (Figure 3G).

To determine whether SATB1 is required for the ε-globin expression induced by SIRT1, we employed a retrovirus-mediated gene transfer to establish stable strains of K562 cells carrying mock, wild-type SATB1, mutant SATB1 with a PDZ domain deletion (ΔPDZ SATB1), PDZ domain is the major partner-interacting domain of SATB1 (24), or SATB1 with point mutations at SIRT1 deacetylation sites (K136AK175A SATB1), the latter two SATB1 plasmids lost both SIRT1 deacetylation sites k136/k175, and are supposed to have impaired response to SIRT1 activity. The cells were then treated with a SIRT1 activator or inhibitor. As expected, the ΔPDZ-SATB1 exhibits very faint effect on the expression of ε-globin, consistent to a very weak activity of the construct for the loss of PDZ domain (25,26). Real-time PCR analysis further indicated that the expression of the ε-globin gene changed with the drug treatment in mock and wild-type SATB1 overexpressing cell strains, whereas the expression of the ε-globin gene in ΔPDZ SATB1 or K136AK175A SATB1 overexpressing K562 cells showed less or no response to resveratrol (Figure 4A and B) and NAM (Figure 4C and D) treatments, indicating that the acetylation sites in SATB1 provide a critical regulatory domain that is required for the regulation of ε-globin gene expression by SIRT1.

### SIRT1 is involved in the hemin-induced activation of ε-globin gene expression in K562 cells

The molecular mechanisms by which hemin induces ε-globin gene activation in K562 cells remain largely unclear. We showed that SIRT1 deacetylates SATB1 and promotes ε-globin gene expression, and we previously observed that the acetylation of SATB1 decreases during hemin-induced K562 cell erythroid differentiation (14), suggesting that SIRT1 may participate in this process. We detected SIRT1 expression with hemin-induced K562 cell erythroid differentiation and found that SIRT1 protein abundance increases with the extension of hemin treatment (Figure 5A). We next determined whether SIRT1 was recruited to the MAR elements of the β-globin locus through its association with SATB1. We performed chromatin immunoprecipitation (ChIP) with a SIRT1 antibody in K562 cells before and after hemin treatment. We then performed real-time PCR by
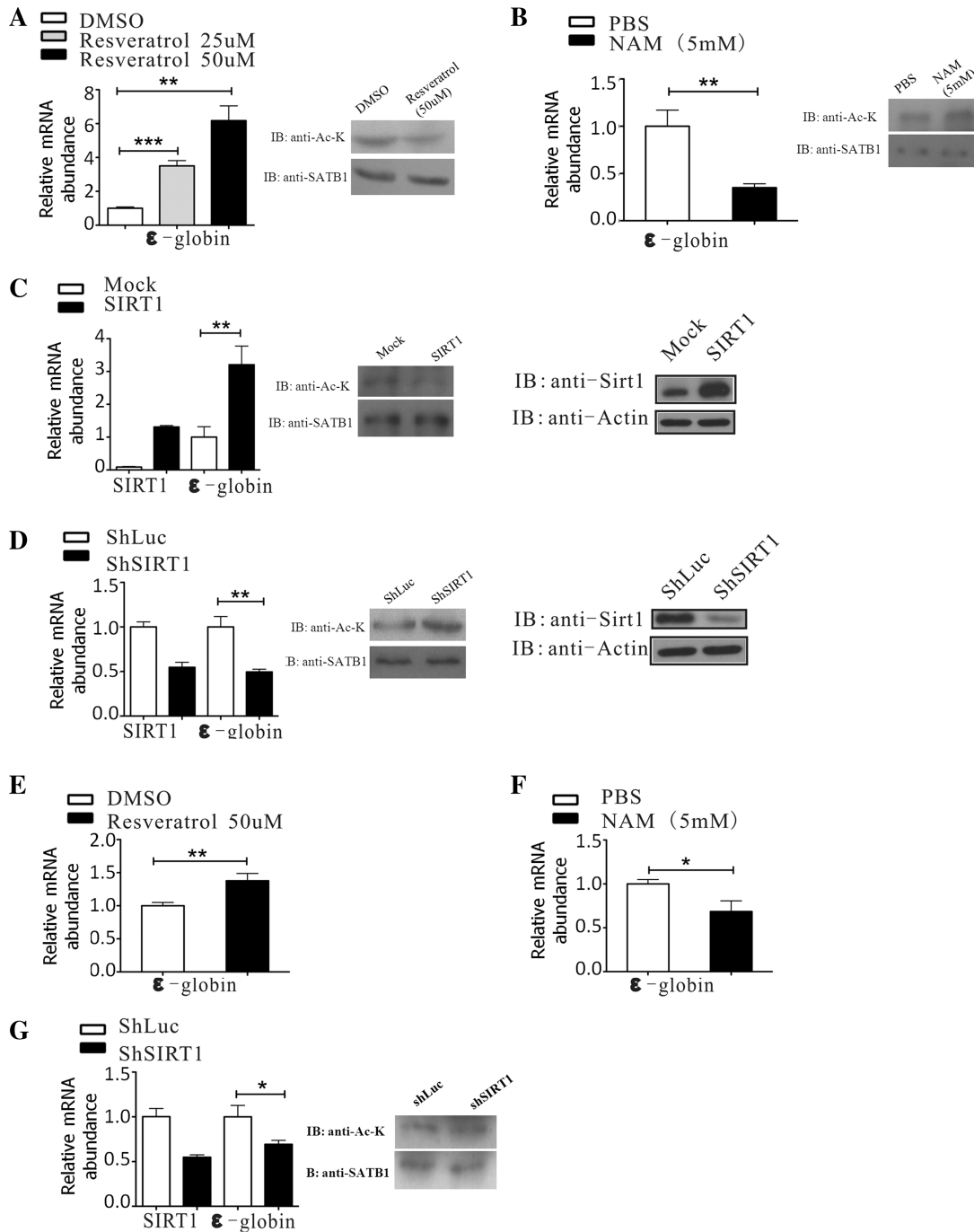
**Figure 3.** SIRT1 promotes ε-globin gene expression in K562 and primary erythroid cells. (**A** and **B**) K562 cells were treated with resveratrol or NAM, and ε-globin mRNA levels were detected by real-time PCR. The accompany changes of SATB1 acetylation level were detected by western blotting. Each error bar represents a standard deviation calculated from experiments performed in triplicate. Student's *t*-test was used for statistical analysis and *P*-values are indicated (\*\**P* < 0.01, \*\*\**P* < 0.001). (**C**) K562 cells were transfected with pcDNA3.1 or pcDNA3.1-SIRT1. ε-globin and SIRT1 mRNA levels were detected by real-time PCR. Each error bar represents a standard deviation calculated from experiments performed in triplicate. Student's *t*-test was used for statistical analysis and *P*-value is indicated (\*\**P* < 0.01). The SIRT1 protein levels and accompanying SATB1 acetylation levels were detected by western blot. (**D**) K562 cells were stably infected with a retrovirus carrying control shRNA or SIRT1 shRNA. ε-globin and SIRT1 mRNA levels in the stably infected cells were determined by real-time PCR. Each error bar represents a standard deviation calculated from experiments performed in triplicate. Student's *t*-test was used for statistical analysis and *P*-value is indicated (\*\**P* < 0.01). The SIRT1 protein levels and accompanying SATB1 acetylation levels were determined by western blotting. (**E** and **F**) HSCs were selected from cord blood, cultured in medium for 1 week, and treated with Epo for 3 days to induce erythroid differentiation. The primary erythroid cells were then treated with resveratrol or NAM, and ε-globin mRNA levels were determined by real-time PCR. Each error bar represents a standard deviation calculated from experiments performed in triplicate. Student's *t*-test was used for statistical analysis and *P*-value is indicated (\**P* < 0.05, \*\**P* < 0.01). (**G**) The selected HSCs were infected with a retrovirus carrying control shRNA or SIRT1 shRNA. Polyclones were selected by puromycin before being subjected to Epo induction for 3 days. ε-globin and SIRT1 mRNA levels were detected by real-time PCR. The accompany change of SATB1 acetylation level was detected by western blotting. Each error bar represents a standard deviation calculated from experiments performed in triplicate. Student's *t*-test was used for statistical analysis and *P*-value is indicated (\**P* < 0.05).
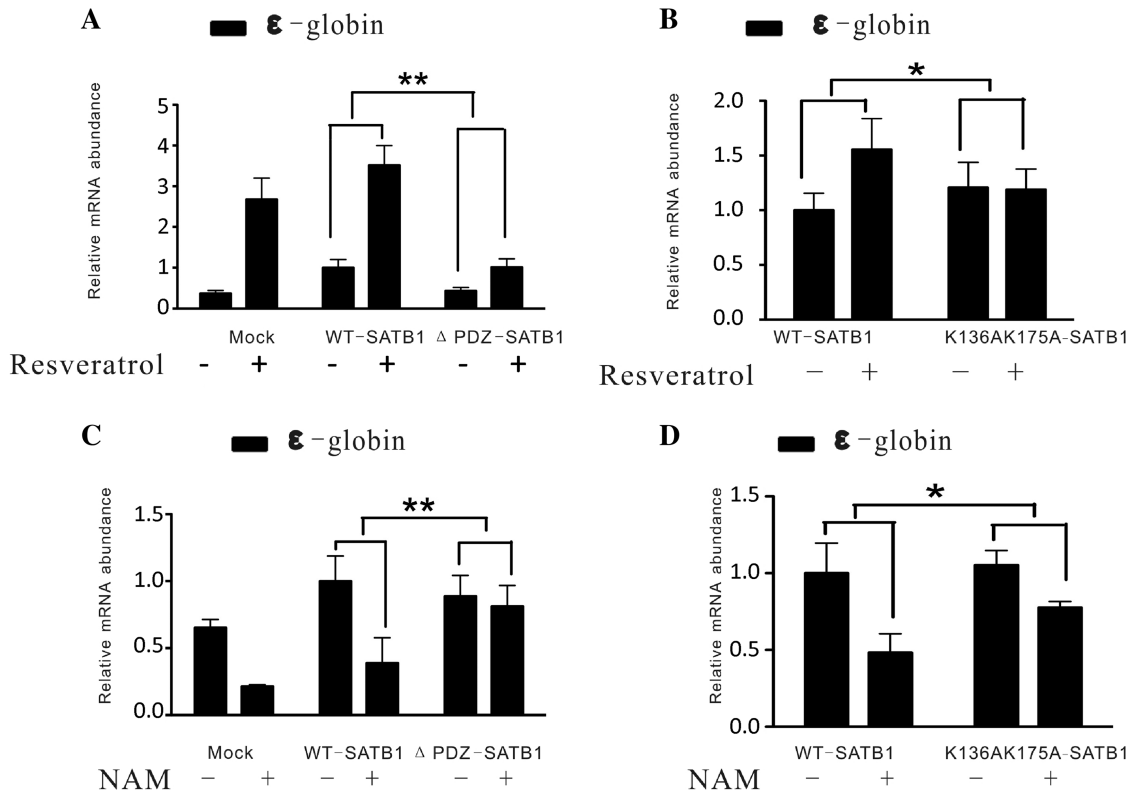
**Figure 4.** SATB1 is essential for the regulatory function of SIRT1 in ε-globin gene expression in K562 cells. (**A** and **B**) K562 cells were stably infected with a retrovirus carrying a control vector, wild-type SATB1, SATB1 with a PDZ-like domain deletion or SATB1 with point mutations at deacetylation sites (K136AK175A SATB1). Polyclones were selected by puromycin and then treated with resveratrol (50 μM if unspecified). ε-Globin mRNA levels were determined by real-time PCR. Each error bar represents a standard deviation calculated from experiments performed in triplicate. Student's *t*-test was used for statistical analysis and *P*-value is indicated (*$P < 0.05$, **$P < 0.01$). (**C** and **D**) K562 cells were stably infected with a retrovirus carrying a control vector, wild-type SATB1, SATB1 with a PDZ-like domain deletion or SATB1 with point mutations at deacetylation sites (K136AK175A SATB1). Polyclones were selected by puromycin and then treated with NAM (5 mM). ε-Globin mRNA levels were determined by real-time PCR. Each error bar represents a standard deviation calculated from experiments performed in triplicate. Student's *t*-test was used for statistical analysis and *P*-value is indicated (*$P < 0.05$, **$P < 0.01$).

separately targeting MAR$^{HS2}$ and MAR$^{ε}$, where SATB1 binding has been previously identified (14). Our results confirmed the binding of SIRT1 to these two MARs in uninduced K562 cells, and the binding increased after the hemin induction of the K562 cells (Figure 5B and C). Very weak re-ChIP signals of SATB1 and SIRT1 at both MARs were detected in hemin-induced K562 cells, implying that SIRT1 can bind to the MARs in complex with SATB1 (Supplementary Figure S5). These observations suggest that SIRT1 may participate in the hemin-induced activation of ε-globin gene expression in K562 cells. To further test this hypothesis, we treated SIRT1 downregulated K562 cells and control cells with increased amounts of hemin for 2 days. Real-time PCR showed that ε-globin gene expression increased with increasing amounts of hemin in control K562 cells, whereas the mRNA level of the ε-globin gene was much lower in the SIRT1 down-regulated K562 cells than in control cells, and the induction of ε-globin in response to hemin was significantly impaired (Figure 5D). These results altogether indicate that SIRT1 plays an important role in the hemin-induced activation of ε-globin gene expression in K562 cells. Furthermore, we detected
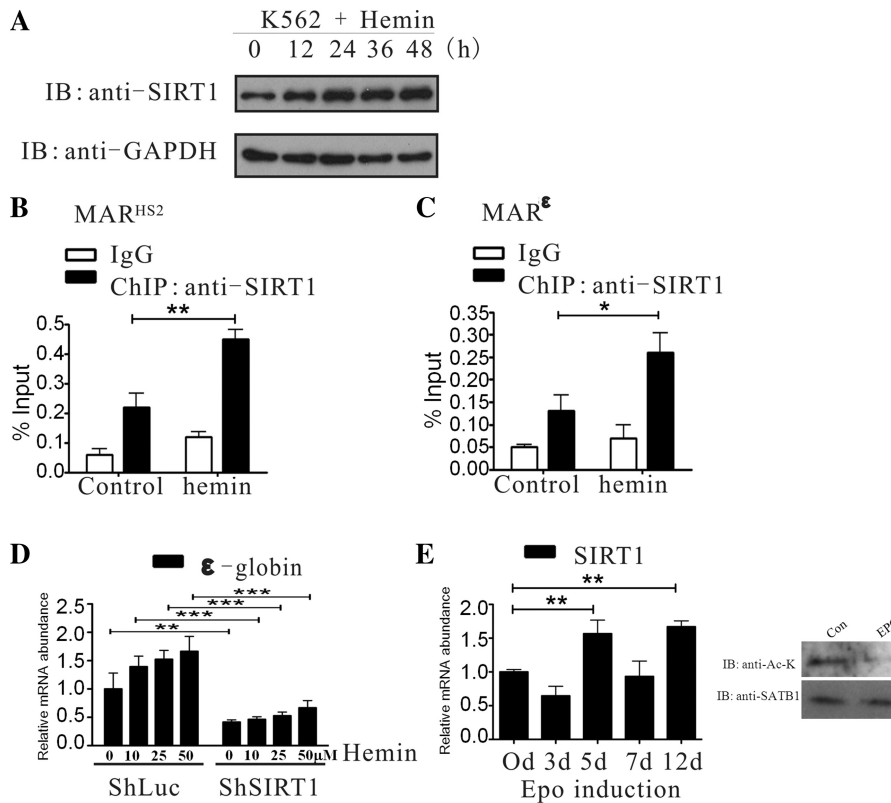
SIRT1 expression during Epo-induced erythroid differentiation of umbilical HSCs. SIRT1 mRNA abundance fluctuated but increased overall during erythroid differentiation, concomitant decrease in SATB1 acetylation level was also detected (Figure 5E).

### SIRT1 increases the binding of SATB1 to the MARs and facilitates the formation of the inter-MAR association in the β-globin gene locus

To further investigate the molecular mechanisms of SIRT1's regulatory function on ε-globin gene expression through SATB1, the ChIP assay was applied to detect SATB1's binding to MAR$^{HS2}$ and MAR$^{ε}$ in control and SIRT1-interfered K562 cells. Both semi-quantitative and real-time PCR showed that SATB1's binding to MAR$^{HS2}$ and MAR$^{ε}$ decreased in SIRT1-interfered K562 cells (Figure 6A and B), the observation agrees with a previous report that acetylated-SATB1 binds DNA less efficiently (15).

Our previous work demonstrated that SATB1 mediates the long-distance interaction between MAR$^{HS2}$ and MAR$^{ε}$ and that this higher order chromosome organization contributes to the transcriptional activity of the

**Figure 5.** SIRT1 promotes ε-globin gene expression during hemin-induced K562 cell erythroid differentiation. (**A**) Hemin induction of K562 cells increases SIRT1 protein expression. K562 cells were treated with hemin. The cells were harvested at different time periods and then subjected to western blotting using a SIRT1 antibody. (**B** and **C**) Hemin increases SIRT1 binding to the MARs of the β-globin gene cluster. K562 cells were treated with hemin for 24 h. The ChIP assay was performed with an anti-SIRT1 antibody to detect the binding of SIRT1 to MAR$^{HS2}$ and MAR$^ε$ before and after hemin induction. Each error bar represents a standard deviation calculated from experiments performed in triplicate. Student's *t*-test was used for statistical analysis and *P*-value is indicated (*$P < 0.05$, **$P < 0.01$). (**D**) SIRT1 interference impaired the hemin-induced activation of the ε-globin gene in K562 cells. Stable control shRNA and SIRT1 shRNA K562 cells were treated with increased concentration of hemin (0, 10, 25 and 50 μM) for 24 h, and ε-globin mRNA levels were detected by real-time PCR. Each error bar represents a standard deviation calculated from experiments performed in triplicate. Student's *t*-test was used for statistical analysis and *P*-value is indicated (**$P < 0.01$, ***$P < 0.001$). (**E**) HSCs were selected from cord blood, and treated with Epo to induce erythroid differentiation. The cells were harvested at different time periods and SIRT1 mRNA levels were determined by real-time PCR. SATB1 acetylation levels before and after 6d Epo induction were detected by western blotting. Each error bar represents a standard deviation calculated from experiments performed in triplicate. Student's *t*-test was used for statistical analysis and *P*-value is indicated (**$P < 0.01$).

ε-globin gene (14). Thus, we examined the association between MAR$^{HS2}$ and MAR$^ε$ in control and SIRT1 knockdown K562 cells. A 3C assay with HindIII fragments of the β-globin gene cluster indicated that the association between MAR$^{HS2}$ and MAR$^ε$ decreased upon SIRT1 knockdown (Figure 6C).

## DISCUSSION

This study establishes the MAR binding protein SATB1 as a new substrate of the class III deacetylase SIRT1 and supports that SIRT1 participates in ε-globin gene activation during erythroid differentiation. Moreover, we provide evidence that SIRT1 exerts its regulatory effect on ε-globin gene expression by enhancing SATB1's binding to MAR$^{HS2}$ and MAR$^ε$ and strengthening the association between the two MARs. These results suggest that SIRT1 is a regulator of the higher order chromatin structure.

## SIRT1 interacts with and deacetylates SATB1

SATB1 is a well-defined chromatin organizer that is involved in the expression regulation of multiple gene clusters (27). SATB1 recruits subunits of the nucleosome-mobilizing complexes CHRAC and ACF and the NURD chromatin remodeling complex, including histone deacetylases HDAC1 and HDAC2 (11). Using exogenous and endogenous co-IP assays and fluorescent cellular colocalization analysis, we found that the class III histone deacetylase SIRT1 also binds to SATB1 in both the 293 and the erythroid K562 cell lines and deacetylates SATB1.

The PDZ-like domain is adjacent to the N-terminal end of SATB1 and is mainly responsible for the homodimerization and protein partner association of SATB1 (25). In addition, PDZ-like domain mediated dimerization is required for the high-affinity DNA binding of SATB1 (26). This domain also includes the previously reported PCAF acetylation site lysine 136 and PKC

**A** ChIP: anti-SATB1



**B** ChIP: anti-SATB1



**C**
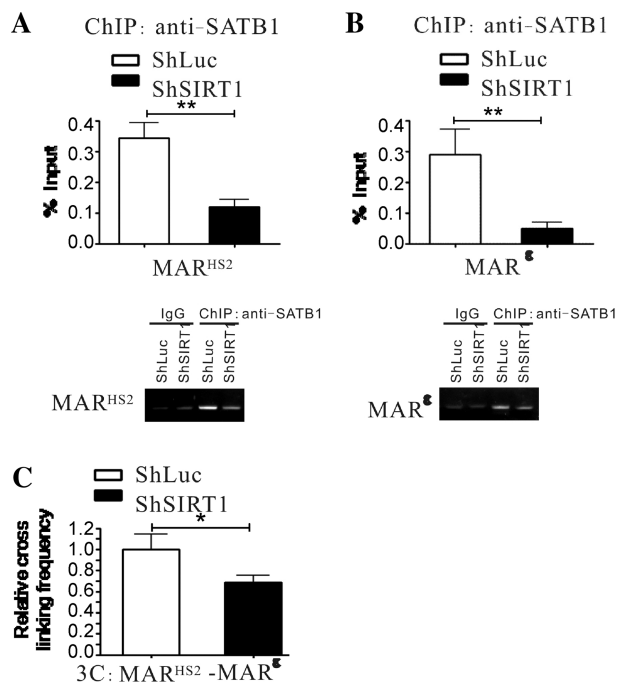


**Figure 6.** SIRT1 facilitates the formation of the inter-MAR association. (**A** and **B**) Knockdown of SIRT1 by RNA interference decreases SATB1 binding to the MARs of the β-globin gene cluster. Stable control shRNA and SIRT1 shRNA K562 cells were subjected to ChIP assay with an anti-SATB1 antibody. The coprecipitated chromatin DNA was analyzed by semi-quantitative PCR and real-time PCR using primers that amplify $MAR^{HS2}$ and $MAR^{\epsilon}$. Each error bar represents a standard deviation calculated from experiments performed in triplicate. Student's *t*-test was used for statistical analysis and *P*-values are indicated (**$P < 0.01$). (**C**) Knockdown of SIRT1 by RNA interference decreases the inter-MAR association in the β-globin gene cluster. Stable control shRNA and SIRT1 shRNA K562 cells were subjected to the 3C assay with HindIII digestion. The spatial proximity between $MAR^{HS2}$ and $MAR^{\epsilon}$ was analyzed using real-time PCR. Each error bar represents a standard deviation calculated from experiments performed in triplicate. Student's *t*-test was used for statistical analysis and *P*-value is indicated (*$P < 0.05$).

phosphorylation site serine 185 (15). Therefore, we focused on this PDZ domain in our further screening of the SIRT1 deacetylation sites on SATB1. In addition to lysine 136, we found lysine 175 as a new PCAF acetylation site on SATB1; we also found that SIRT1 could deacetylate SATB1 at both sites *in vitro*.

### SIRT1 is a new regulator of ε-globin gene expression

SATB1 is a known activator of the ε-globin gene (12); therefore, we detected the effect of SIRT1 on ε-globin gene expression. The drug challenges and genetic modifications of SIRT1 expression in the erythroid K562 cell line and human primary erythroid cells confirmed the positive linkage between SIRT1 and ε-globin gene expression. The activity of SIRT1 also extends to other globin genes, as we found that SIRT1 also increased the expression of the γ-globin gene (Supplementary Figure S3). Although no SIRT1 substrate, except SATB1, is known to participate in globin gene regulation, we cross-checked whether the regulatory effect of SIRT1 on ε-globin gene expression

depended on SATB1 acetylation. K562 cells that expressed mutant SATB1 proteins that lack the SIRT1 targeting sites (K136 and K175) became insensitive to treatment with a SIRT1 activator (resveratrol) and an inhibitor (NAM), which have a strong impact on ε-globin expression in normal K562 cells. A mild positive effect of SIRT1 on SATB1 expression was also observed (Supplementary Figure S4), which could synergize with the SATB1 deacetylation effect of SIRT1 in regulating ε-globin gene expression.

Our observation that both protein abundance of SIRT1 and its recruitment to the SATB1 specific $MAR^{HS2}$ and $MAR^{\epsilon}$ increased during hemin induction of K562 cells suggests the involvement of SIRT1 in K562 erythroid differentiation. This is further supported by the analysis showing that SIRT1 interference greatly impaired hemin-induced ε-globin gene activation in K562 cells. Moreover, our observation of primary erythroid cells revealed a fluctuating increase of SIRT1 and concomitant decrease in SATB1 acetylation during erythroid differentiation in umbilical HSCs. We also found that resveratrol, the activator of SIRT1, stimulates the ε-globin gene expression in the primary erythroid cells, whereas the SIRT1 inhibitor, NAM, and direct SIRT1 knockdown in these primary cells inhibited the ε-globin gene expression. Collectively, these results suggest an active role of SIRT1 in the physiological process of ε-globin gene activation during erythropoiesis.

### SIRT1 regulates the inter-MAR association of the β-globin gene cluster via posttranslational modification of SATB1

Recent studies have identified multiple SIRT1 substrates with various physiological roles, including P53 (19), FOXO (18), PGC1-α (20) and HIF-2α (28). SIRT1 functions by deacetylating these factors and regulating their transcriptional activities, protein stability or cellular localization. In addition to acting on transcriptional factors, SIRT1 also participates in heterochromatin organization by interacting with DNA methyltransferase 3b (29) or by deacetylating histone H1 (30) and SUV39H1 (31,32), a histone methyl transferase. However, there are no reports concerning the involvement of SIRT1 in the regulation of long-range higher order chromatin structure.

Our previous work identified an association between $MAR^{HS2}$, $MAR^{HS4}$, $MAR^{\epsilon}$ and $MAR^{\gamma}$ in the β-globin gene cluster, and we showed that SATB1 mediates this inter-MAR association (14). SATB1, presumably as part of a macro–protein complex, coordinately mediates the formation of the inter-MAR association in the β-globin gene cluster, and the polymerization characteristic of the SATB1 protein makes it the potential core element in organizing the inter-MAR association. Modifications to the components of the protein complex may accordingly affect the formation of the inter-MAR association and the globin gene expression.

In this study, we found that SIRT1 deacetylates SATB1, facilitates the inter-MAR association between $MAR^{HS2}$ and $MAR^{\epsilon}$ and promotes ε-globin gene expression. This work establishes the linkage between SIRT1 and higher

order chromatin structure regulation. It also provides an important example of how posttranslational modifications on protein components affect the organization of long-range higher order chromatin structure and fine regulation of targeted gene expression. Other posttranslational modifications of SATB1, including phosphorylation (15) and sumoylation (33), may also participate in the regulation of the inter-MAR association. Further investigations on the posttranslational modifications to these higher order chromatin structure organizers are still needed to comprehensively understand the dynamic transcriptional regulation at the 3D chromatin organization level.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Materials and Methods and Supplementary Figures 1–5.

## FUNDING

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## REFERENCES

1. Dekker,J., Rippe,K., Dekker,M. and Kleckner,N. (2002) Capturing chromosome conformation. *Science*, **295**, 1306–1311.
2. Lieberman-Aiden,E., van Berkum,N.L., Williams,L., Imakaev,M., Ragoczy,T., Telling,A., Amit,I., Lajoie,B.R., Sabo,P.J., Dorschner,M.O. *et al.* (2009) Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science*, **326**, 289–293.
3. Tolhuis,B., Palstra,R.J., Splinter,E., Grosveld,F. and de Laat,W. (2002) Looping and interaction between hypersensitive sites in the active beta-globin locus. *Mol. Cell*, **10**, 1453–1465.
4. Zhou,G.L., Xin,L., Song,W., Di,L.J., Liu,G., Wu,X.S., Liu,D.P. and Liang,C.C. (2006) Active chromatin hub of the mouse alpha-globin locus forms in a transcription factory of clustered housekeeping genes. *Mol. Cell Biol.*, **26**, 5096–5105.
5. Murrell,A., Heeson,S. and Reik,W. (2004) Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops. *Nat. Genet.*, **36**, 889–893.
6. Zhao,Z., Tavoosidana,G., Sjölinder,M., Göndör,A., Mariano,P., Wang,S., Kanduri,C., Lezcano,M., Sandhu,K.S., Singh,U. *et al.* (2006) Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. *Nat. Genet.*, **38**, 1341–1347.
7. Labrador,M. and Corces,V.G. (2002) Setting the boundaries of chromatin domains and nuclear organization. *Cell*, **111**, 151–154.
8. Cai,S., Han,H.J. and Kohwi-Shigematsu,T. (2003) Tissue-specific nuclear architecture and gene expression regulated by SATB1. *Nat. Genet.*, **34**, 42–51.
9. Cai,S., Lee,C.C. and Kohwi-Shigematsu,T. (2006) SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. *Nat. Genet.*, **38**, 1278–1288.
10. Kumar,P.P., Bischof,O., Purbey,P.K., Notani,D., Urlaub,H., Dejean,A. and Galande,S. (2007) Functional interaction between PML and SATB1 regulates chromatin-loop architecture and transcription of the MHC class I locus. *Nat. Cell Biol.*, **9**, 45–56.
11. Yasui,D., Miyano,M., Cai,S., Varga-Weisz,P. and Kohwi-Shigematsu,T. (2002) SATB1 targets chromatin remodelling to regulate genes over long distances. *Nature*, **419**, 641–645.
12. Wen,J., Huang,S., Rogers,H., Dickinson,L.A., Kohwi-Shigematsu,T. and Noguchi,C.T. (2005) SATB1 family protein expressed during early erythroid differentiation modifies globin gene expression. *Blood*, **105**, 3330–3339.
13. Gong,H., Wang,Z., Zhao,G.W., Lv,X., Wei,G.H., Wang,L., Liu,D.P. and Liang,C.C. (2009) SATB1 regulates beta-like globin genes through matrix related nuclear relocation of the cluster. *Biochem. Biophys. Res. Commun.*, **383**, 11–15.
14. Wang,L., Di,L.J., Lv,X., Zheng,W., Xue,Z., Guo,Z.C., Liu,D.P. and Liang,C.C. (2009) Inter-MAR association contributes to transcriptionally active looping events in human beta-globin gene cluster. *PLoS One*, **4**, e4629.
15. Pavan Kumar,P., Purbey,P.K., Sinha,C.K., Notani,D., Limaye,A., Jayani,R.S. and Galande,S. (2006) Phosphorylation of SATB1, a global gene regulator, acts as a molecular switch regulating its transcriptional activity in vivo. *Mol. Cell*, **22**, 231–243.
16. Purbey,P.K., Singh,S., Notani,D., Kumar,P.P., Limaye,A.S. and Galande,S. (2009) Acetylation-dependent interaction of SATB1 and CtBP1 mediates transcriptional repression by SATB1. *Mol. Cell Biol.*, **29**, 1321–1337.
17. Fulco,M., Schiltz,R.L., Iezzi,S., King,M.T., Zhao,P., Kashiwaya,Y., Hoffman,E., Veech,R.L. and Sartorelli,V. (2003) Sir2 regulates skeletal muscle differentiation as a potential sensor of the redox state. *Mol. Cell*, **12**, 51–62.
18. Brunet,A., Sweeney,L.B., Sturgill,J.F., Chua,K.F., Greer,P.L., Lin,Y., Tran,H., Ross,S.E., Mostoslavsky,R., Cohen,H.Y. *et al.* (2004) Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science*, **303**, 2011–2015.
19. Vaziri,H., Dessain,S.K., Ng Eaton,E., Imai,S.I., Frye,R.A., Pandita,T.K., Guarente,L. and Weinberg,R.A. (2001) hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell*, **107**, 149–159.
20. Rodgers,J.T., Lerin,C., Haas,W., Gygi,S.P., Spiegelman,B.M. and Puigserver,P. (2005) Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature*, **434**, 113–118.
21. Sankaran,V.G., Menne,T.F., Xu,J., Akie,T.E., Lettre,G., Van Handel,B., Mikkola,H.K., Hirschhorn,J.N., Cantor,A.B. and Orkin,S.H. (2008) Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor BCL11A. *Science*, **322**, 1839–1842.
22. Golemis,E. (2002) *Protein-Protein Interactions: a Molecular Cloning Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 44–47.
23. Pediconi,N., Guerrieri,F., Vossio,S., Bruno,T., Belloni,L., Schinzari,V., Scisciani,C., Fanciulli,M. and Levrero,M. (2009) hSirT1-dependent regulation of the PCAF-E2F1-p73 apoptotic pathway in response to DNA damage. *Mol. Cell Biol.*, **29**, 1989–1998.
24. Notani,D., Ramanujam,P.L., Kumar,P.P., Gottimukkala,K.P., Kumar-Sinha,C. and Galande,S. (2011) N-terminal PDZ-like domain of chromatin organizer SATB1 contributes towards its function as transcription regulator. *J. Biosci.*, **36**, 461–469.
25. Galande,S., Dickinson,L.A., Mian,I.S., Sikorska,M. and Kohwi-Shigematsu,T. (2001) SATB1 cleavage by caspase 6 disrupts PDZ domain-mediated dimerization, causing detachment from chromatin early in T-cell apoptosis. *Mol. Cell Biol.*, **21**, 5591–5604.
26. Purbey,P.K., Singh,S., Kumar,P.P., Mehta,S., Ganesh,K.N., Mitra,D. and Galande,S. (2008) PDZ domain-mediated dimerization and homeodomain-directed specificity are required for high-affinity DNA binding by SATB1. *Nucleic Acids Res.*, **36**, 2107–2122.
27. Galande,S., Purbey,P.K., Notani,D. and Kumar,P.P. (2007) The third dimension of gene regulation: Organization of dynamic chromatin loopscape by SATB1. *Curr. Opin. Genet. Dev.*, **17**, 408–414.

28. Dioum,E.M., Chen,R., Alexander,M.S., Zhang,Q., Hogg,R.T., Gerard,R.D. and Garcia,J.A. (2009) Regulation of hypoxia-inducible factor 2alpha signaling by the stress-responsive deacetylase sirtuin 1. *Science*, **324**, 1289–1293.
29. Kashiwagi,K., Nimura,K., Ura,K. and Kaneda,Y. (2011) DNA methyltransferase 3b preferentially associates with condensed chromatin. *Nucleic Acids Res.*, **39**, 874–888.
30. Vaquero,A., Scher,M., Lee,D., Erdjument-Bromage,H., Tempst,P. and Reinberg,D. (2004) Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin. *Mol. Cell*, **16**, 93–105.
31. Vaquero,A., Scher,M., Erdjument-Bromage,H., Tempst,P., Serrano,L. and Reinberg,D. (2007) SIRT1 regulates the histone methyl-transferase SUV39H1 during heterochromatin formation. *Nature*, **450**, 440–444.
32. Bosch-Presegué,L., Raurell-Vila,H., Marazuela-Duque,A., Kane-Goldsmith,N., Valle,A., Oliver,J., Serrano,L. and Vaquero,A. (2011) Stabilization of Suv39H1 by SirT1 is part of oxidative stress response and ensures genome protection. *Mol. Cell*, **42**, 210–223.
33. Tan,J.A., Sun,Y., Song,J., Chen,Y., Krontiris,T.G. and Durrin,L.K. (2008) SUMO conjugation to the matrix attachment region-binding protein, special AT-rich sequence-binding protein-1 (SATB1), targets SATB1 to promyelocytic nuclear bodies where it undergoes caspase cleavage. *J. Biol. Chem.*, **283**, 18124–18134.