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Heterochromatin Protein 1γ Is a Novel Epigenetic Repressor of Human Embryonic ϵ-Globin Gene Expression*[S]

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Production of hemoglobin during development is tightly regulated. For example, expression from the human β-globin gene locus, comprising β-, δ-, ϵ-, and γ-globin genes, switches from ϵ-globin to γ-globin during embryonic development and then from γ-globin to β-globin after birth. Expression of human ϵ-globin in mice has been shown to ameliorate anemia caused by β-globin mutations, including those causing β-thalassemia and sickle cell disease, raising the prospect that reactivation of ϵ-globin expression could be used in managing these conditions in humans. Although the human globin genes are known to be regulated by a variety of multiprotein complexes containing enzymes that catalyze epigenetic modifications, the exact mechanisms controlling ϵ-globin gene silencing remain elusive. Here we found that the heterochromatin protein HP1γ, a multifunctional chromatin- and DNA-binding protein with roles in transcriptional activation and elongation, represses ϵ-globin expression by interacting with a histone-modifying enzyme, lysine methyltransferase SUV4–20h2. Silencing of HP1γ expression markedly decreased repressive histone marks and the multimethylation of histone H3 lysine 9 and H4 lysine 20, leading to a significant decrease in DNA methylation at the proximal promoter of the ϵ-globin gene and greatly increased ϵ-globin expression. In addition, using chromatin immunoprecipitation, we showed that SUV4–20h2 facilitates the deposition of HP1γ on the ϵ-globin-proximal promoter. Thus, these data indicate that HP1γ is a novel epigenetic repressor of ϵ-globin gene expression and provide a potential strategy for targeted therapies for β-thalassemia and sickle cell disease.

The human β-globin gene cluster on chromosome 11 encodes five consecutive globins, 5′-ϵ-Gγ-Aγ-δ-β-3′, which are expressed in a development- and tissue-specific pattern (1–3). Increased fetal γ-globin levels in adults significantly ameliorate the severity of sickle cell disease and β-thalassemia (4–8). Thus, it is of great interest to improve our understanding of the basic mechanisms regulating γ-globin gene expression. Mouse models have helped provide an alternative strategy to reactivation of embryonic ϵ-globin gene expression, wherein reversing ϵ-globin gene silencing also ameliorates the symptoms of β-thalassemia (4, 9). Therefore, the precise molecular mechanisms controlling ϵ-globin gene silencing are wortho studying further.

Gene expression relies on the assembly of DNA into higher-order chromatin. In the context of heterochromatin, heterochromatin protein 1 (HP1)[2] proteins were originally identified (10–12). It is known that HP1 family proteins have additional functions, playing roles in transcriptional activation and elongation, cell cycle regulation, DNA repair, and RNA splicing (12–15). In contrast to HP1α and HP1β, HP1γ can also localize to euchromatin, thus functioning in transcriptional elongation and RNA processing (16). For example, HP1γ decreases the RNA polymerase II elongation rate, thus affect the alternative splicing of multiple genes (17). By searching the HP1γ ChIP sequencing data in the Gene Expression Omnibus (GEO, NCBI) datasets, we found that HP1γ is located on the β-globin gene locus in K562 cells. Consistently with this finding, our previous data have demonstrated that methylated histone H3K9, a marker specifically recognized by HP1 via the evolutionarily conserved chromodomain, is enriched at the γ-globin promoter (18). However, it is unclear whether HP1γ is involved in the regulation of the expression of the β-globin gene cluster and how this process works.

In this study, we reveal that HP1γ binds most strongly to the ϵ-globin promoter among those of the globin genes, thereby inducing coordinated repressive histone marks and DNA methylation. In K562 cells, HP1γ interacts with an H4K20me2/3 methyltransferase, SUV4–20h2, which promotes association of HP1γ with chromatin and results in silencing of ϵ-globin gene expression. These results demonstrate that HP1γ coordinates with SUV4–20h2 in repressing human embryonic globin expression.

Results

HP1γ Represses Human ϵ-Globin Gene Transcription in K562 Cells—Our previous studies have demonstrated that PRMT5 induces a range of coordinated repressive epigenetic

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[S] This article contains supplemental Tables 1–3.

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[2] The abbreviations used are: HP1, heterochromatin protein 1; KD, knockdown; Scr, scrambled; Q-RT-PCR, quantitative real-time PCR; DNMT, DNA methyltransferase; H4K20me2/3, dimethylation or trimethylation of histone H4 lysine 20; H3K9me3, trimethylation of histone H3 lysine 9; H3K9me2, dimethylation of histone H3 lysine 9.

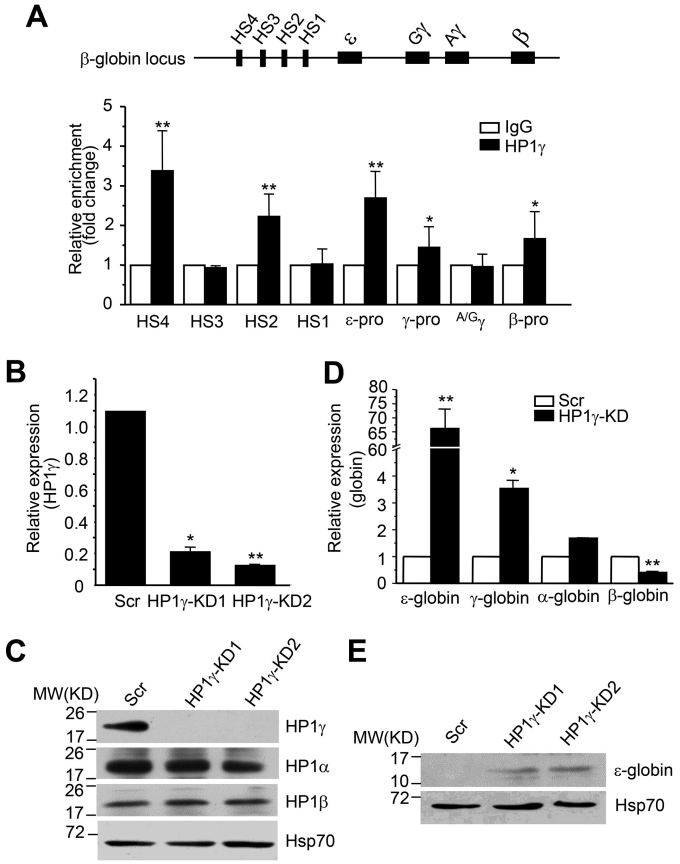

FIGURE 1. **HP1γ represses ε-globin gene expression in K562 cells.** *A*, *top panel*, schematic of the β-globin gene locus. *Bottom panel*, localization of HP1γ across the β-globin locus as measured by ChIP in chromatin fractions from K562 cells. The precipitated DNA was amplified with primers specific to the indicated regions of the β-globin locus. *HS*, hypersensitive site; *pro*, promoter; $^{G/A}\gamma$, intergenic regions between the Gγ- and Aγ-globin genes. The results are shown as the mean ± S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ compared with IgG control. *B*, HP1γ gene expression analysis by Q-RT-PCR of RNA extracted from HP1γ-KD1, HP1γ-KD2, and scrambled control K562 cells normalized to GAPDH mRNA. The results are shown as the mean ± S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ compared with the scrambled control. *C*, Western blotting analysis of cell lysates from HP1γ-KD1 and HP1γ-KD2 or Scr control K562 cells by using the indicated antibodies. *MW*, molecular weight. *D*, human ε-, γ-, β-, and α-globin gene expression analysis by Q-RT-PCR of RNA extracted from HP1γ-KD and scrambled control K562 cells normalized to GAPDH mRNA. The results are shown as the mean ± S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ compared with the Scr control. *E*, Western blotting analysis of ε-globin from cell lysates of HP1γ-KD and scrambled K562 cells using the indicated antibodies.

marks in erythroid cells, including H3K9me3, on the γ-promoter (18). Because the HP1 protein is an epigenetic reader of histone H3K9me3 via the conserved chromodomain, and HP1γ localizes to both the heterochromatic and euchromatic regions involved in gene expression regulation (12), we hypothesized that HP1γ might bind the globin genes participating in the modulation of globin gene expression. ChIP analysis across the β-locus by using HP1γ-specific antibodies demonstrated that HP1γ had a higher enrichment on the proximal promoter of ε-globin than that of the γ- or β-globins in K562 cells (Fig. 1*A*). We also found that HP1γ was enriched at the DNA hypersensitive sites HS4 and HS2 (Fig. 1*A*), two important enhancers of the β-globin gene (19).

To determine whether HP1γ regulates ε-globin gene expression, we generated two HP1γ stable knockdown K562 cell lines by using lentiviral vectors containing specific shRNAs. In the knockdown lines (HP1γ-KD), HP1γ mRNA levels were decreased to ~20% that of scramble (Scr) K562 cells (Fig. 1*B*). Reduced protein levels were confirmed by Western blotting analysis using an anti-HP1γ-specific antibody without changes of HP1α and HP1β (Fig. 1*C*). Interestingly, Q-RT-PCR showed a more than 65-fold increase in ε-globin expression, whereas HP1γ knockdown resulted in only a 3.5-fold increase in γ-globin expression and a reduction in β-globin expression (Fig. 1*D*). No significant induction of α-globin gene expression was found in response to knocking down HP1γ. Western blotting analysis confirmed that ε-globin protein levels were markedly increased in the HP1γ-KD cells compared with Scr cells (Fig. 1*E*). These results indicate that HP1γ represses human ε-globin gene transcription in K562 cells.

HP1γ Coordinates with SUV4–20h2 and Functions at the ε-Proximal Promoter—The HP1 protein is an epigenetic reader of histone H3K9me3 that relies on a conserved chromodomain (12). Interestingly, we found that, when HP1γ was knocked down, the H3K9me2/3 histone marks in the region of the proximal promoter of the ε-globin gene were significantly decreased in a feedback mechanism (Fig. 2*A*). Additionally, we found that the H4K20me2/3 histone marks in the same region were also significantly reduced in HP1γ knockdown cells compared with Scr cells (Fig. 2*B*). These results are consistent with the scenario occurring on the γ-promoter, in which histone marks such as H3K9 methylation and H4K20 methylation are coordinately regulated (18). The results also strongly suggest that HP1γ might closely associate with a methyltransferase that, in turn, deposits a histone mark, *e.g.* methylated H4K20. At the heterochromatin level, HP1γ has been shown to interact with SUV4–20h2 and, consequently, recruit SUV4–20h2 to heterochromatin (20). Indeed, our co-immunoprecipitation experiments confirmed that HP1γ interacted with SUV4–20h2 in erythroid K562 cells (Fig. 2*C*). Because antibodies suitable for ChIP analysis of human SUV4–20h2 are currently not available, we were not able to perform ChIP experiments to determine the enrichment of endogenous SUV4–20h2 at the ε-promoter. Hence, we used a K562 cell line stably overexpressing HA-tagged SUV4–20h2 and performed ChIP analysis using an anti-HA antibody. SUV4–20h2 was enriched in the region of the proximal promoter of the ε-globin gene (Fig. 2, *D* and *E*), which is the same region in which HP1γ also bound the ε-globin gene (Fig. 2, *D* and *F*). These results indicate that HP1γ coordinates with SUV4–20h2, thus potentially resulting in modulation of histone methylation on the ε-globin gene.

SUV4–20h2 Represses Human ε-Globin Gene Expression—To determine the potential role of SUV4–20h2 in ε-globin gene regulation, we generated two stable SUV4–20h2 knockdown K562 cell lines by using lentiviral vectors containing specific shRNAs. SUV4–20h2 mRNA levels and protein levels were examined by Q-RT-PCR and Western blotting analysis, respectively. The SUV4–20h2 expression levels were reduced in these two cell lines (SUV4–20h2-KD) to ~40% that in cells transduced with scrambled control RNA (Fig. 3, *A* and *B*). ε-Globin gene expression was quantified by Q-RT-PCR, and the ε-globin mRNA level was 60-fold higher in SUV4–20h2-KD cells

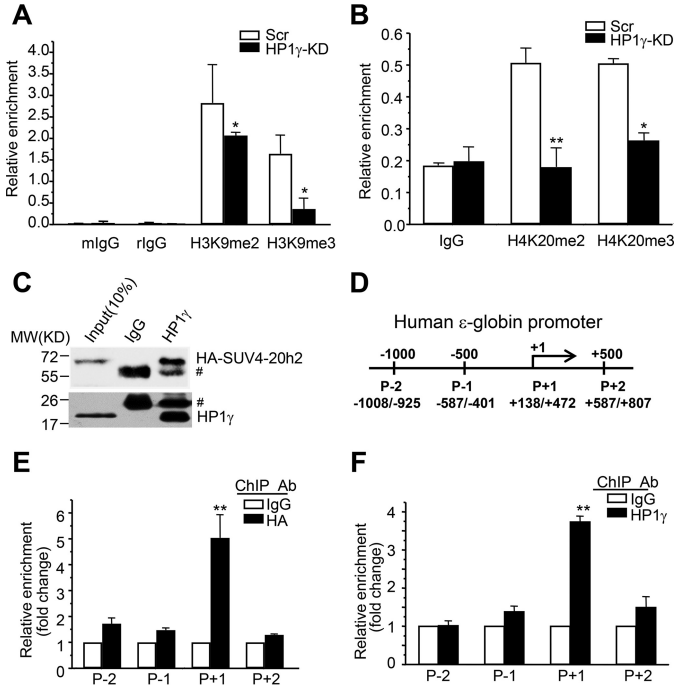

FIGURE 2. **HP1γ coordinates with SUV4–20h2 and acts at the ε-proximal promoter.** *A*, histone H3K9me2 and H3K9me3 ChIP analyses at the ε-proximal promoter were performed in Scr and HP1γ-KD K562 cells. The results are shown as the mean ± S.D. of three independent experiments. *, $p < 0.05$ compared with the Scr control. *rIgG*, rabbit IgG; *mIgG*, mouse IgG. *B*, histone H4K20me2 and H4K20me3 ChIP analyses at the ε-proximal promoter from HP1γ-KD or Scr K562 cells. The results are shown as the mean ± S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ compared with the Scr control. *C*, co-immunoprecipitation of endogenous HP1γ and HA-SUV4–20h2 from SUV4–20h2-overexpressing K562 cells. The heavy and light chains of IgG are indicated by the *pound symbol* (#). *MW*, molecular weight. *D*, Schematic of the four primers designed for ChIP spanning the ε-promoter. *E*, SUV4–20h2 binds to the ε-globin proximal promoter, as demonstrated by ChIP analysis. IgG from rabbit served as a control. The results are shown as the mean ± S.D. of three independent experiments. **, $p < 0.01$ compared with IgG control. *Ab*, antibody. *F*, HP1γ binds to the ε-globin proximal promoter, as demonstrated by ChIP analysis. Mouse IgG served as a control. The results are the mean ± S.D. of three independent experiments. **, $p < 0.001$ compared with the IgG control.

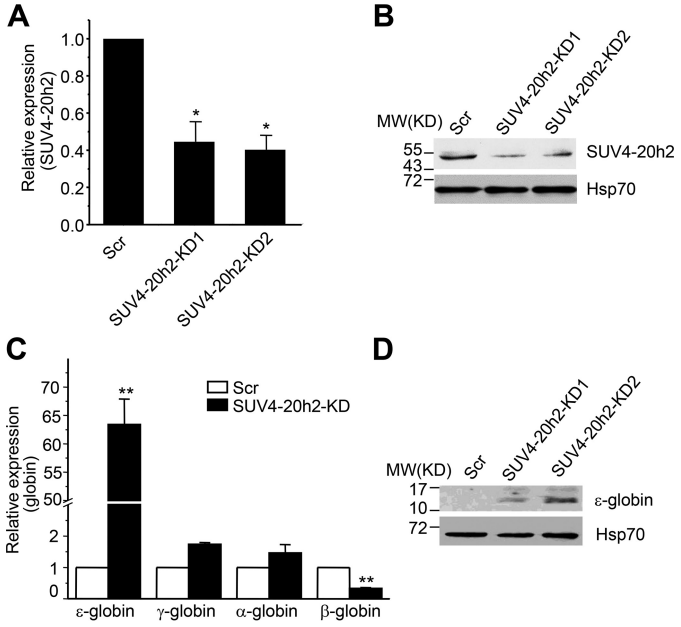

FIGURE 3. **SUV4–20h2 represses human ε-globin gene expression.** *A*, SUV4–20h2 gene expression analysis by Q-RT-PCR of RNA from SUV4–20h2-KD1, Suv4–20h1-KD2, and scrambled control K562 cells. The results are shown as the mean ± S.D. of three independent experiments. *, $p < 0.05$ compared with the scrambled control. *B*, Western blotting analyses of cellular extracts from SUV4–20h2-KD1 and SUV4–20h2-KD2 or scrambled control K562 cells using the indicated antibodies. *MW*, molecular weight. *C*, ε-, γ-, β-, and α-globin gene expression analysis by Q-RT-PCR of RNA from SUV4–20h2-KD or scrambled control K562 cells. The results are shown as the mean ± S.D. of three independent experiments. **, $p < 0.01$ compared with the Scr control. *D*, Western blotting analysis of ε-globin and GAPDH from cell lysates of SUV4–20h2-KD1, SUV4–20h2-KD2, and scrambled control K562 cells using the indicated antibodies.

than in Scr control cells (Fig. 3*C*). In contrast, no significant induction of either γ-globin or the α-globin was observed (Fig. 3*C*). This was accompanied by a reduction of β-globin expression (Fig. 3*C*). The protein levels of ε-globin were confirmed to be markedly induced in SUV4–20h2 knockdown cells compared with Scr control cells (Fig. 3*D*). These results indicate that SUV4–20h2 represses ε-globin gene transcription in K562 cells.

SUV4–20h2 Contributes to the Deposition of HP1γ on the ε-Globin Gene—SUV4–20h2 is a lysine methyltransferase that has been linked to gene repression through the establishment of H4K20me2/3 histone marks (20). To examine whether SUV4–20h2 functions on histones associated with the ε-globin gene, we performed ChIP analyses using specific antibodies against polymethylated histone H4K20 (H4K20me2/3). As expected, the enrichment of H4K20 methylation, particularly H4K20me3, was significantly reduced at the ε-globin promoter in SUV4–20h2-KD cells compared with Scr cells (Fig. 4*A*). At constitutive heterochromatin, after stable binding of the HP1 molecule to trimethylated histone H3K9 nucleosomes, HP1 recruits the SUV4–20h enzyme, which methylates H4K20 (20). We then tested whether SUV4–20h2 also affects HP1γ binding to chromatin. We lysed Scr and SUV4–20h2-KD cells with NETN300 solution (0.5% Nonidet P-40, 2 mM EDTA, 10 mM Tris-HCl (pH 8.0), and 300 mM NaCl), from which loosely chromatin-bound proteins were eluted from genomic DNA through 300 mM NaCl treatment (S1 fraction in Fig. 4*B*). The chromatin proteins tightly associated with genomic DNA were thus released into the soluble fraction after the genomic DNA was digested by benzonase (C1 fraction in Fig. 4*B*). We found that significantly less HP1γ was released into the soluble fraction in SUV4–20h2 knockdown cells than in Scr cells after the genomic DNA was digested with benzonase (21, 22) (Fig. 4*B*). These results suggest that depletion of SUV4–20h2 resulted in less HP1γ deposition on chromatin. In agreement with this finding, the enrichment of HP1γ on the ε-promoter was significantly reduced after SUV4–20h2 was knocked down in K562 cells (Fig. 4*C*). Interestingly, we observed that the enrichment of H3K9me2 on the ε-promoter was also significantly reduced in SUV4–20h2 knockdown cells compared with Scr cells. The enrichment of H3K9me3 was not changed in this context (Fig. 4*D*). Together, these results suggest that SUV4–20h2 facilitates the localization of HP1γ on the ε-globin gene.

The Loss of HP1γ Decreases DNA Methylation at the ε-Globin Gene—In addition to histone modification, DNA methylation has also been shown to be important in modulating globin gene expression (23–25). We have demonstrated previously that the levels of DNA methylation of three CpG dinucleotides in the

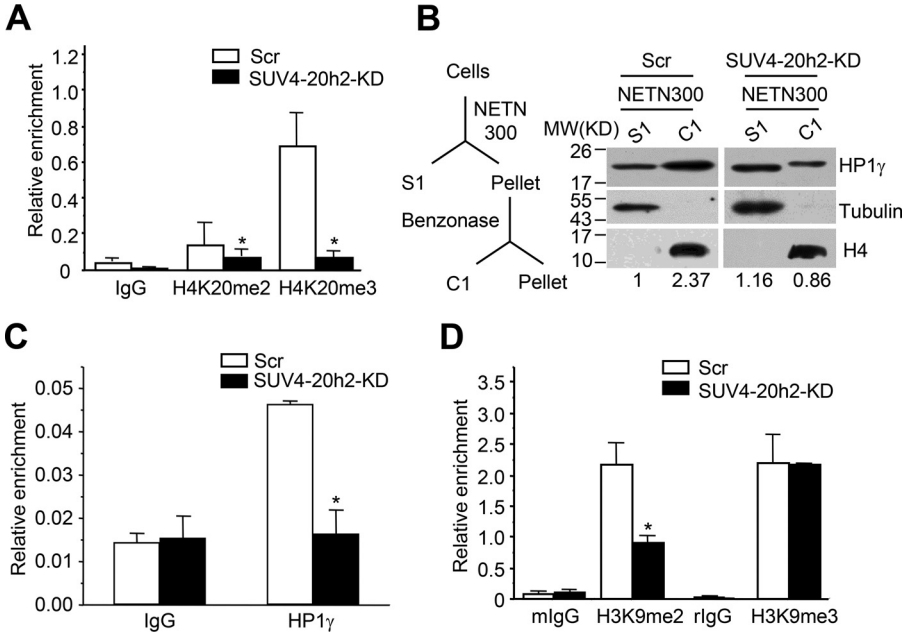

FIGURE 4. **SUV4–20h2 facilitates the deposition of HP1γ at the ϵ-proximal promoter.** *A*, ChIP analysis of the histone mark H4K20me2/3 on the ϵ-globin proximal promoter from SUV4–20h2 knockdown cells or scrambled control cells. The results are shown as the mean ± S.D. of three independent experiments. *, $p < 0.05$ compared with the IgG control. *B*, SUV4–20h2-KD or Scr K562 cells were lysed by NETN300 (lysis buffer with 300 mM NaCl). After harvesting the soluble fractions, the pellets were digested with benzonase to extract the chromatin fraction. Each fraction was examined by Western blotting with the indicated antibodies. Tubulin and histone H4 were used as loading controls for the soluble fraction and chromatin fraction, respectively. Relative quantification of HP1γ protein with ImageJ software (National Institutes of Health) is shown at the bottom. *C*, ChIP analysis of the HP1γ on the ϵ-globin proximal promoter of SUV4–20h2-KD cells or scrambled control cells. The results are shown as the mean ± S.D. of three independent experiments. *, $p < 0.05$ compared with the indicated control. *D*, ChIP analysis of H3K9me2/3 on the ϵ-globin promoter from the SUV4–20h2-KD cells or scrambled control cells. The results are shown as the mean ± S.D. of three independents experiments. *, $p < 0.05$ compared with the indicated control.

proximal promoter of the ϵ-globin gene were greatly reduced in SUV4–20h1 knockdown cells compared with scrambled control cells (26). In view of this, we set out to examine whether DNA methylation of the ϵ-globin gene was affected by HP1γ by using bisulfite DNA sequencing. After HP1γ was knocked down, the methylation of CpG dinucleotides in the proximal promoter of the ϵ-globin gene in the cells was also significantly reduced compared with that in Scr cells (Fig. 5*A*). Interestingly, these methylation sites were largely coincident with the HP1γ binding site on the ϵ-globin gene (Figs. 2*F* and 5*A*). Consistent with these results, the enrichment of DNMT3A on the related gene region was also significantly decreased in HP1γ-KD cells (Fig. 5*B*). These data are consistent with the induction of ϵ-globin gene expression in HP1γ knockdown cells, thus suggesting that HP1γ, SUV4–20h2, and DNMT3A exert coordinated functions in ϵ-gene silencing.

Discussion

Among HP1 family members, HP1γ is unique in that it is located at both euchromatin and heterochromatin (12, 17). In this study, we found that HP1γ coordinates with SUV4–20h2 and, consequently, induces deposition of repressive histone marks and DNA methylation, thereby silencing ϵ-globin gene expression.

Constitutive heterochromatin is characterized by the presence of nucleosomes rich in H3K9me3 and H4K20me3. The current model suggests that the H3K9me3 mark triggered by the H3K9 methyltransferase SUV39h stabilizes HP1 binding at heterochromatin. HP1 then recruits SUV4–20h, which generates the histone mark H4K20me3 (20, 21). Our results show

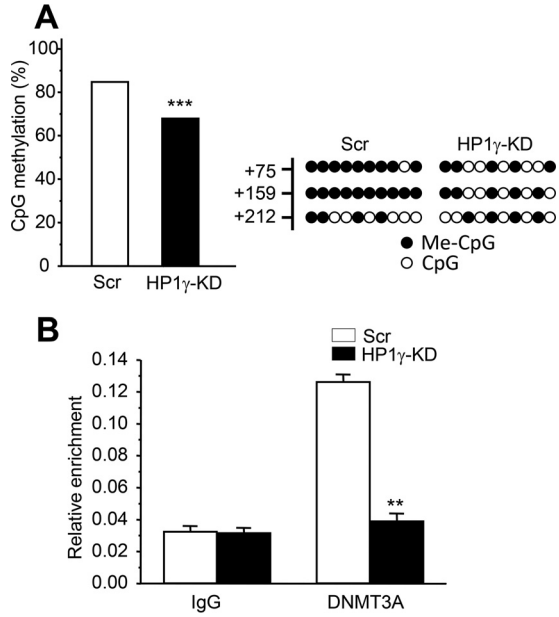

FIGURE 5. **Loss of HP1γ decreases DNA methylation at the ϵ-globin gene.** *A*, DNA methylation at the human ϵ-gene in HP1γ-KD and Scr control cells (*left panel*). Each row shows the methylation status of individual CpG dinucleotides, as derived from sequence analysis of 40 individual cloned PCR products of the ϵ-gene after bisulfite modification from knockdown or Scr control K562 cells (*right panel*). The differences between the knockdown line and the scrambled control were highly significant (Fisher's exact test; ***, $p < 0.0001$). The numbers on the *right* represent the positions of the CpG dinucleotides relative to the transcriptional start site of the ϵ-gene. *B*, ChIP analysis of DNMT3A on the ϵ-globin proximal promoter from HP1γ-KD cells or scrambled control cells. Normal mouse IgG served as the control. The results are shown as the mean ± S.D. of three independent experiments. **, $p < 0.01$ compared with the indicated control.

that HP1γ interacts with SUV4–20h2 and that its loss leads to a decrease in the histone marks H3K9me3 and H4K20me2/3 at the ε-promoter. These observations support this model and represent a novel feedback mechanism that occurs beyond heterochromatin modification. Furthermore, we show that SUV4–20h2 can also help affect the deposition of HP1γ on the chromatin and at the ε-promoter, thus providing an important complement for the model. These results indicate that the methylations of H3K9 and H4K20 are functionally linked. Further systematic studies are needed to address whether the H3K9 methyltransferase SUV39h or G9a and two other HP1 family members, HP1α or HP1β, affect human globin expression. We also showed that HP1γ represses γ-globin gene expression to a lesser extent. It would be interesting to determine how HP1γ regulates γ-globin gene expression.

In our previous studies, we have shown that SUV4–20h1 plays a role in repressing ε-globin and γ-globin gene expression (18, 26), similar to SUV4–20h2 in this study. However, we found that SUV4–20h1 and SUV4–20h2 behave differently in globin gene regulation. First, we observed that the extent of repression of ε-globin and γ-globin expression by the two proteins was different. In SUV4–20h1 knockdown K562 cells, the ε-globin and γ-globin expression levels were induced by over 400-fold and 10-fold, respectively (18, 26), whereas, in SUV4–20h2 knockdown K562 cells, the ε-globin expression levels were increased by 60-fold, and no induction of γ-globin expression was observed, although the knockdown efficiencies of the two were not the same. In addition, the ε-globin gene appeared to be more sensitive to the SUV4–20h proteins than the γ-globin gene (18, 26). These results suggest that SUV4–20h1 and SUV4–20h2 have distinct roles in gene regulation. In fact, genetic studies have demonstrated that SUV4–20h1 and SUV4–20h2 are not redundant: mice deficient in SUV4–20h2 are viable, whereas SUV4–20h1 knockout mice die perinatally and show overall growth retardation (27). Second, SUV4–20h1 may recruit different multiprotein repressors from SUV4–20h2. In previous studies, we found that SUV4–20h1 interacts with the PRMT5 complex, including DNMT3A, thus resulting in deposition of the H4K20me3 mark at the ε-promoter (18, 26, 28). Intriguingly, we did not find the HP1 protein in association with the PRMT5 multiprotein repressor complex (18). In this study, we identified an interaction between SUV4–20h2 and HP1γ, thus suggesting that there may be at least two independent complexes located at the ε-promoter and that the recruitment of SUV4–20h2 in this setting may occur through a similar mechanism as that observed at pericentric heterochromatin.

DNA methylation and histone methylation play essential and coordinated roles in globin gene silencing (23–25). Direct links between these processes are emerging, including interactions observed among the DNA methyltransferase, arginine methyltransferase, and methyl-CpG binding proteins (3, 28, 29). In this study, we show that histone lysine methylation and DNA methylation are directly linked in the context of human ε-globin gene silencing. The histone lysine methyltransferase SUV4–20h2 deposits the repressive histone H4K20me2/3 mark, which may act as a necessary trigger for subsequent H3K9 methylation. Methylated H3K9 is a template for the direct binding of HP1γ and the recruitment of DNA methyltransferase (DNMT) to these sites, thus enhancing cytosine methylation.

In summary, our study reveals a novel role of HP1γ in the regulation of the ε-globin gene. The identification of both HP1γ and SUV4–20h as key regulators of ε-globin and γ-globin silencing (18, 26) provides alternative potential therapeutic targets for the treatment of β-thalassemia and sickle cell disease.

Experimental Procedures

Antibodies and Reagents—The primary antibodies used in this study included mouse anti-HA monoclonal antibody (12CA5, Roche), rabbit anti-Hsp70 antibody (A01236, GenScript, Piscataway, NJ), mouse anti-GAPDH monoclonal antibody (sc-47724, Santa Cruz Biotechnology), rabbit anti-ε-globin antibody (AP19854c, Abgent, San Diego, CA), mouse anti-HP1γ antibody (05-690, Millipore, Billerica, MA), rabbit anti-HP1α antibody (sc-28735, Santa Cruz Biotechnology), mouse anti-HP1β antibody (MAB3448, Millipore), rabbit anti-H4K20me2 antibody (07-367, Millipore), mouse anti-H3K9me2 antibody (ab1220, Abcam, Cambridge, MA), mouse anti-H4K20me3 antibody (ab9053, Abcam), rabbit anti-H3K9me3 antibody (ab8898, Abcam), and mouse anti-DNMT3A antibody (ab13888, Abcam). DMEM, RPMI 1640 medium, and fetal bovine serum were purchased from Thermo Fisher (Waltham, MA). Decitabine was purchased from Sigma.

Cell Culture—K562 cells were grown as described previously (18, 30). Human K562 cells were maintained in RPMI 1640 medium supplemented with 10% FBS (Thermo Fisher).

Cell Lysis, Immunoprecipitation, and Western Blotting—For the immunoprecipitation assays, K562 cells were lysed with ice-cold NETN300 buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 300 mM NaCl). The soluble fractions were collected and then directly subjected to electrophoresis or immunoprecipitation with the indicated antibodies, which was followed by Western blotting analysis with the indicated antibodies.

ChIP Analysis—ChIP assays were performed as described previously (18, 30). The genomic DNA isolated from the K562 cells was sonicated to an average size of 200–600 bp. The solubilized chromatin was immunoprecipitated with antibodies against HA, HP1γ, H3K9me2, H3K9me3, H4K20me2, H4K20me3, or DNMT3A. Antibody-chromatin complexes were pulled down using protein A/G-Sepharose, washed, and then eluted. After cross-link reversal and proteinase K treatment, the immunoprecipitated DNA was extracted with phenol/chloroform, ethanol-precipitated, and dissolved in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer. Each experiment was performed at least twice independently. The ChIP DNA concentrations were qualified by quantitative real-time PCR using the FastStart Universal SYBR Green Master (Roche). A standard curve was prepared for each set of primers on the basis of serial titration of the input DNA. The percentage of ChIP DNA was calculated relative to the input DNA from the primer-specific standard curves by using Rotor-Gene 6000 series software. The primers are listed in supplemental Table 1.

RNA Interference—The shRNA target sequences for SUV4–20h2 and HP1γ were inserted into the XhoI/HpaI sites in the

pL.L3.7 lentiviral vector according to the instructions of the provider (American Type Culture Collection). The targeting oligonucleotides were as follows: SUV4–20h2-KD1 sequence, GCGTGAAGGTGCTCCGGGA; SUV4–20h2-KD2 sequence, GGCGCTATGGGCTGCCTTA; HP1γ-KD1 sequence, GAAGTGTCCTCAAATTGTA; and HP1γ-KD2 sequence, GAGGCAGAGCCTGAAGAAT.

Isolation of Genomic DNA and Bisulfite Sequencing Analysis—Genomic DNA isolation and bisulfite treatment were performed as described previously (18). The primers used to amplify the bisulfite-treated ε-promoter are provided in supplemental Table 2.

Q-RT-PCR—RNA was isolated from cells by using TRIzol reagent (Life Technologies) according to the protocol of the manufacturer. cDNA was synthesized with the SuperScript first-strand synthesis system (Thermo Fisher) and analyzed by quantitative real-time PCR using FastStart Universal SYBR Green Master Mix in a Rotor-Gene 6000 (Corbett Research). Cycling conditions were 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The primers used are listed in supplemental Table 3.

Statistical Analysis—All experiments were performed in triplicate and were repeated at least three times to verify the reproducibility of the experimental findings. The data were analyzed using GraphPad Prism 5.0 software (San Diego, CA). A two-way comparison was performed using Student's *t* test, and a *p* value of less than 0.05 was considered to be significant. Fisher's exact test was used in Fig. 5*A*. The results are given as the means ± S.D.

Author Contributions—Yadong Wang, Ying Wang, L. M., M. N., J. J., M. L., Y. D., B. Y., T. G., X. L., C. G., and C. M. performed the experiments and analyzed the results. R. T. provided ideas, reagents, and discussions. Q. Z. designed the project and wrote the manuscript with Yadong Wang.

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