The role of WDR5 in silencing human fetal globin gene expression

Zhen Xu,[1]* Yinghong He,[1]* Junyi Ju,[1] Gerhard Rank,[2] Loretta Cerruti,[2] Chi Ma,[1] Richard J. Simpson,[3] Robert L. Moritz,[3] Stephen M. Jane,[2,4] and Quan Zhao[1]

[1]Molecular Immunology and Cancer Research Center, The State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, China; [2]Department of Medicine, Monash University Central Clinical School, Prahran, Australia; [3]Ludwig Institute for Cancer Research, Parkville, Australia; [4]Alfred Hospital, Prahran, Australia

ABSTRACT

Background
Histone H3 lysine 4 (K4) methylation has been linked with transcriptional activity in mammalian cells. The WD40-repeat protein, WDR5, is an essential component of the MLL complex that induces histone H3 K4 methylation, but the role of WDR5 in human globin gene regulation has not yet been established.

Design and Methods
To study the role of WDR5 in human globin gene regulation, we performed knockdown experiments in both K562 cells and primary human bone marrow erythroid progenitor cells (BMC). The effects of WDR5 knockdown on γ-globin gene expression were determined. Biochemical approaches were also employed to investigate WDR5 interaction molecules. Chromosomal marks in the globin locus were analyzed by ChIP.

Results
We found that WDR5 interacted with protein arginine methyltransferase 5 (PRMT5), a known repressor of γ-globin gene expression, and was essential for generating tri-methylated H3K4 (H3K4me3) at the γ-globin promoter in K562 cells. Enforced expression of WDR5 in K562 cells reduced γ-globin gene expression, whereas knockdown of WDR5 increased γ-globin gene expression in both K562 cells and primary human bone marrow erythroid progenitor cells. Consistent with this, both histone H3 and H4 acetylation at the γ-globin promoter were increased, while histone H4R3 and H3K9 methylation were decreased, in WDR5 knockdown cells compared to controls. We found that WDR5 interacted with HDAC1 and a PHD domain-containing protein, ING2 (inhibitor of growth), an H3K4me3 mark reader, to enhance γ-globin gene transcriptional repression. In human BMC, levels of WDR5 were highly enriched on the γ-promoter relative to levels on other globin promoters and compared to the γ-promoter in cord blood erythroid progenitors, suggesting that WDR5 is important in the developmental globin gene expression program.

Conclusions
Our data are consistent with a model in which WDR5 binds the γ-globin promoter in a PRMT5-dependent manner; H3K4me3 induced at the γ-globin promoter by WDR5 may result in the recruitment of the ING2-associated HDAC1 component and consequent silencing of γ-globin gene expression.

Key words: WDR5, human globin gene regulation, silencing.

Citation: Xu Z, He Y, Ju J, Rank G, Cerruti L, Ma C, Simpson RJ, Moritz RL, Jane SM, and Zhao Q. The role of WDR5 in silencing human fetal globin gene expression. Haematologica 2012;97 (11):1632-1640. doi:10.3324/haematol.2012.061937

©2012 Ferrata Storti Foundation. This is an open-access paper.

*These authors contributed equally to this work.

Acknowledgments: we thank Robert Roeder and Yali Dou for the WDR5 wild-type and mutant cDNA plasmids, respectively. We are grateful to members of the Jane and Zhao laboratories for helpful discussions. This work was supported by National Natural Science Foundation of China NSFC#31071118, NSFC#31170716, NSFC#81121062, SBK201140017 (QZ), NSFC#81060248 (YH), the Fundamental Research Funds for the Central Universities, and the Doctoral Program of Higher Education of China RFDP#20090091110033, The Cooley's Anemia Foundation, and The National Health and Medical Research Council of Australia (SMJ).

Manuscript received on January 5, 2012. Revised version arrived on May 11, 2012. Manuscript accepted on May 28, 2012.

*Correspondence:
Quan Zhao, Molecular Immunology and Cancer Research Center, The State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, 210093, China.
Phone: international +86.25.83597251.
E-mail: qzhao@nju.edu.cn*

*Stephen M. Jane, Department of Medicine, Monash University Central Clinical School, Prahran, Vic, 3181, Australia.
Phone: international +61.3.99030640.
E-mail: stephen.jane@monash.edu*

The online version of this article has a Supplementary Appendix.

Introduction

The human β-globin gene cluster is located on chromosome 11 and encodes the five β-like globin genes: ε-, ^{G}γ-, ^{A}γ- δ-, and β-globin. These genes are expressed in a developmental stage- and tissue-specific manner, which is governed by a combination of transcriptional regulation and epigenetic tuning.[1,2] Increased fetal γ-globin (HbF) levels in adulthood are associated with symptomatic amelioration in sickle cell disease and β-thalassemia, and consequently, intense efforts have focused on the mechanisms regulating γ-globin gene expression to enable strategies for γ-globin reactivation in hemoglobinopathy patients.[3,4] HbF can be elevated in adults by chemicals such as hydroxyurea, azacytidines, and butyrates, although the exact molecular mechanisms mediating these responses remain unclear.[3,4] Nevertheless, epigenetic regulation is known to be involved. A large body of evidence has shown that epigenetic marks generated by modification of the amino-terminal tails of histones play important roles in altering chromatin structure and function, thereby controlling the transcription of genes, including globin. These marks often communicate with each other and can be read by histone modification binding effectors and their associated complexes, dictating both active and repressive histone codes.[5]

Histone lysines can be mono-, di-, or tri-methylated by the MLL or SET1 methyltransferase complex, and different modifications play diverse roles.[6] The WD40-repeat protein, WDR5, is a core subunit of the complex, and is required for complex assembly and methyltransferase activity.[6] Studies from yeast and mammals demonstrate that di- and tri-methylation of histone H3K4 (H3K4me2/3) are preferentially associated with transcribed regions of active genes, and are largely concurrent with the histone H3K36 methylation mark.[7] H3K4me1 is mainly enriched in more distal regions, such as enhancers and the 3'ends of genes.[8,9] H3K4 methylation can be regulated by H3R2 methylation in *cis* and by H2B monoubiquitination in *trans*.[10,11] However, the functions of histone H3K4 methylation still remain poorly understood.

Although H3K4 methylation is largely associated with transcription initiation and elongation, evidence is emerging that this mark could also be involved in gene repression. In yeast, H3K4me2/3 induced by Set1 directly contributes to repressive machinery on the PHO5, PHO84, and GAL10-GAL1 genes.[12,13] Recently, we have shown that the NuRD complex-associated protein PRMT5 can mediate the histone repressive mark, symmetric dimethyl H4R3 (H4R3me2s), which subsequently recruits DNMT3A, culminating in DNA methylation on the γ-globin promoter and gene silencing.[14,15] In this study, we demonstrate that WDR5 binds the γ-globin promoter in a PRMT5-dependent manner and plays a role in γ-globin gene silencing.

Design and Methods

Cell culture

K562 cells, 293T cells, human cord blood (CB) and bone marrow (BM) erythroid progenitors from healthy donors were cultured as described previously.[15] For BM culture conditions, isolated CD34^{+} cells were grown in StemSpan SFEM medium with 1X CC100 cytokine mix for six days, and then reseeded into the same medium supplemented with stem cell factor (SCF) (20 ng/mL), erythropoietin (EPO) (1 U/mL), IL-3 (5 ng/mL), dexamethasone (2 μM) and β-estradiol (1 μM) for two more weeks. Cell surface marker analyses with CD71 and glycophorin A indicated that cultured cells were greater than 90% erythroid lineage. Human BM and CB were collected under approval of the Melbourne Health Human Research Ethics Committees.

Mass spectrometry analysis and protein interaction studies

Proteins of interest were excised from SDS-PAGE and analyzed by mass spectrometry as described previously.[14] Immunoprecipitation, immunoblotting, and GST pulldown assays were performed as described previously.[16] Antibodies utilized in the immunoprecipitations were: FLAG (Sigma-Aldrich), HA (Roche), PRMT5, WDR5 and MLL2 (Abcam), HDAC1 and ING2 (Santa Cruz).

Size exclusion chromatography

Size exclusion chromatography was performed on a calibrated Superose 12 HR 10/30 gel filtration column, and 500 μL fractions were collected. Fractions were concentrated by TCA precipitation, electrophoresed on 12% SDS-PAGE, transferred to a PVDF membrane, and analyzed by Western blot with selected antibodies.

ChIP analysis

ChIP assays were performed as described previously.[14] Antibodies utilized were: H4R3me2s, H3K4me1, H3K4me2, H3K4me3, H3K9me3, H3ac, H4ac, PRMT5, WDR5 (Abcam), HDAC1, and ING2 (Santa Cruz). Each experiment was performed at least twice independently. The ChIP samples were analyzed by quantitative real-time polymerase chain reaction (PCR) using the FastStart Universal SYBR Green Master (Roche). A standard curve was prepared for each set of primers using serial titration of the input DNA. The percentage of ChIP DNA was calculated relative to the input DNA from primer-specific standard curves using the Rotor-Gene 6000 Series Software 1.7. Primers are listed in the *Online Supplementary Figure S1*.

RNA interference and viral infection

The siRNA target sequences for WDR5, RbBP5 and ING2 were inserted into the *Xho*I/*Hpa*I sites in the pLL3.7 lentiviral vector according to the manufacturer's recommendations (American Type Culture Collection, USA). The targeting oligonucleotides were:

WDR5-KD1 sequences: GTGGAAGAGTGACTGCTAA;
WDR5-KD2 sequences: GAATGAGAAATACTGCATA;
ING2-KD sequence: GCTTATGCAACCAAGTGTCTT.

Retrovirus or lentivirus production in 293T cells and infection of K562 cells or erythroid progenitors from BM were performed as described previously.[16] Erythroid progenitors from BM were infected with viral supernatants at Day 6 and transduced cells were selected for GFP expression by FACS at Day 12 followed by one more week of culture before collection. Transduced K562 cells were also selected for GFP expression by FACS.

RNA extraction and quantitative real-time PCR (Q-RT-PCR)

Total RNA was isolated from cells with Trizol reagent (Invitrogen). cDNA was generated using the reverse transcription system (Promega). The identities of the amplified bands were confirmed by sequencing. PCR conditions and primers have been described previously,[14] and all samples were run in triplicate. cDNA was analyzed by quantitative real-time PCR using the FastStart Universal SYBR Green Master (Roche). A standard curve

was prepared for each set of primers using serial titration of cDNA from K562 cells. The relative expression was calculated from primer-specific standard curves using the Rotor-Gene 6000 Series Software 1.7.

For BM samples, plasmid DNA encoding γ-globin or β-globin was used to generate the standard curve for determination of copy number. The number of molecules per nanogram total RNA from BM cells was calculated from standard curves using the Rotor-Gene 6000 Series Software 1.7. Q-RT-PCR primers for WDR5, ING2 were: WDR5 forward: TGATGGTCTCTGTCGCATCT;

WDR5 reverse: CTTCAGAGTGTTGTCCAGCG;

ING2 forward: ACGAGCCTCAGATAAAGCAAAG;

ING2 reverse: TGCCATGTGACATAAATCACGG.

Student's t-test was used to derive the significance of the differences between mean values.

Results

NF-E4 interacts with WDR5 on the human γ-globin promoter

In previous studies, we had shown that human erythroid transcription factor, NF-E4 could play a dual role, by either activating or repressing human γ-globin gene expression.[16,17] In a co-immunoprecipitation experiment followed by mass spectrometry in which PRMT5 was identified as an NF-E4 interacting protein,[14] another polypeptide, TLIDDDNPPVSFVK, corresponding to the human WDR5 protein was also isolated (Figure 1A). To confirm that NF-E4 interacted with WDR5, endogenous proteins from K562 cells or cell lysates from HA or Flag-tagged NF-E4 or WDR5 transfected 293T cells were analyzed by immunoprecipitation Western blot experiments. The two proteins were shown to interact in both of these settings (Figure 1B and C, and 2C). However, GST pull-down assays failed to demonstrate a direct interaction between NF-E4 and WDR5, indicating that the association of the two factors was likely to be mediated through an intermediary protein (Figure 1D). Despite this, WDR5 and NF-E4 were able to localize on the γ-globin promoter, but not the MyoD promoter in K562 cells as determined by ChIP analysis (Figure 1E).

WDR5 binding on γ-promoter is PRMT5-dependent

In view of our previous studies,[14] we postulated that PRMT5 might serve as the bridging protein between NF-E4 and WDR5. To evaluate this, K562 cell lysate was immunoprecipitated with PRMT5 antibody and blotted with WDR5 antibody. We showed that WDR5 could be immunoprecipitated by PRMT5 in these cells, but not by control IgG (Figure 2A). To determine whether this interaction was direct, we performed a GST pulldown assay. GST-PRMT5 was able to pull-down IVTT ^{35}S-labeled WDR5, whereas GST alone did not (Figure 2B). To examine whether the interaction of NF-E4 and WDR5 was PRMT5-dependent, we utilized K562 cells in which the expression of PRMT5 had been knocked-down (PRMT5-

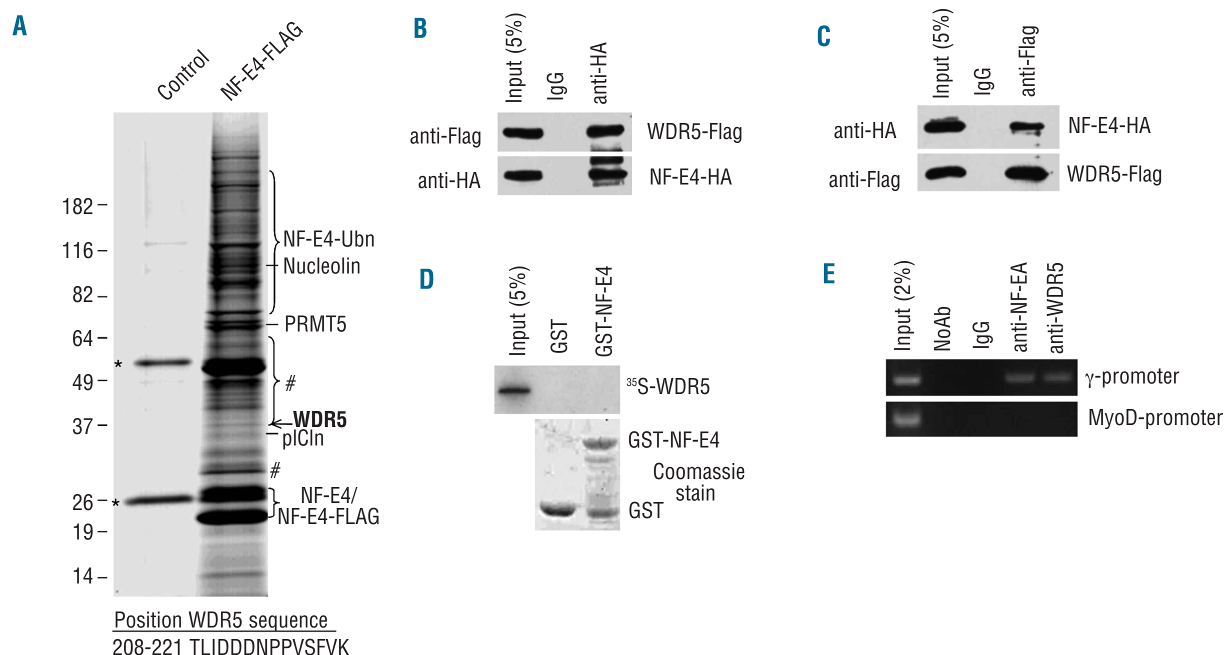

Figure 1. WDR5 associates with NF-E4 and binds the γ-globin promoter. (A) SimplyBlue Safestain of an SDS-PAGE gel of anti-Flag antibody immunoprecipitates from K562 cells transfected with NF-E4-Flag, or vector alone (control), before analysis by mass spectrometry. The bands corresponding to WDR5, PRMT5, NF-E4, polyubiquitinated NF-E4, and the known PRMT5 partner proteins, nucleolin and pICln, are shown. Hatch marks indicate bands that correspond to common background proteins including keratin, tubulins, and ribosomal proteins. Asterisks indicate immunoglobulin chains. The WDR5 peptide sequence identified is shown below. (B) HA antibody immunoprecipitation-Western blot analysis of WDR5 and NF-E4 from 293T cells transfected with both NF-E4-HA and WDR5-FLAG plasmids. Normal rabbit IgG served as the control. (C) Flag antibody immunoprecipitation-Western blot analysis of WDR5 and NF-E4 from 293T cells transfected with WDR5-FLAG plasmids. Normal rabbit IgG served as the control. (D) GST pull-down assay. Purified GST and GST-NF-E4 fusion proteins pre-adsorbed to glutathione-Sepharose beads were incubated with ^{35}S-labeled *in vitro* transcribed translated (IVTT) WDR5. Specifically bound protein was eluted from washed beads and visualized by autoradiography after SDS-PAGE. (E) Binding of the endogenous WDR5 and NF-E4 to the γ-promoter or the MyoD promoter analyzed by ChIP assays in K562 cells. No antibody or normal rabbit IgG served as the control.

KD) using a short hairpin (sh) RNA approach.[15] These cells demonstrated a reduction in PRMT5 levels to less than 20% of a scrambled (Scr) control shRNA transduced K562 cell line (Figure 2C). As expected, immunoprecipitation with anti-NF-E4 antisera resulted in 8-fold less PRMT5 in the precipitate from the PRMT5-kd cells compared with control (Figure 2C). Similarly, a 6-fold reduction in WDR5 was also evident in the immunoprecipitate from PRMT5-kd cells suggesting that PRMT5 was critical for the NF-E4/WDR5 interaction (Figure 2C). Consistent with this, ChIP experiments demonstrated that WDR5 binding to the γ-globin promoter was significantly decreased in PRMT5-kd cells compared to the scramble control, whereas NF-E4 binding remained similar in the two lines (Figure 2D). Taken together, these results indicated that the WDR5 interaction with NF-E4 was PRMT5-dependent.

WDR5 represses human γ-globin gene transcription

To determine the effect of WDR5 on γ-globin gene regulation, we established stable K562 cell lines transduced with either an empty vector, or a vector over-expressing WDR5 (WDR5-OE) (Figure 3A). Human γ-globin gene expression was quantified by Q-RT-PCR from total RNA in these cells, and demonstrated a 3-fold reduction compared with the control line (Figure 3B).

To examine the effects of reduced WDR5 expression, we generated two WDR5 knockdown (WDR5-KD) K562 cell lines using a specific shRNA, and a scrambled control construct. In the knockdown lines, WDR5 levels were reduced to approximately 30% of the Scr cells (Figure 3C), and Q-RT-PCR demonstrated a greater than 6-fold increase in γ-globin expression in these cells compared to control (Figure 3D). WDR5 has been shown to be a master regulator of HOX genes, such as HOXC8.[18] In agreement with a previous report,[18] enforced expression of WDR5 induced HOXC8 expression, whereas WDR5-KD cells showed reduced expression of HOXC8 (*Online Supplementary Figure S2A*).

WDR5 is a key component of the MLL complex, and has been shown to bind arginine 2 in histone H3 leading to methylation of lysine 4.[6] In K562 cells, co-immunoprecipitation experiments demonstrated that WDR5 could interact with the MLL2 methyltransferase (Figure 3E). To study the global effect of WDR5 on histones in K562 cells, Western blot analyses with specific antibodies were performed with WDR5-OE and WDR5-KD cells. As shown in *Online Supplementary Figure S3*, and consistent with previous reports,[18,19] histone H3K4 di- and tri-methylation were significantly increased in a cell line over-expressing WDR5 and were decreased in the knockdown line compared to the scrambled control, whereas a modest effect was observed with histone H3K4 mono-methylation. To test whether these global changes in histone marks induced by altered levels of WDR5 were evident on the γ-globin promoter, we performed ChIP experiments with the WDR5-OE and WDR5-KD cells. We observed that only H3K4 tri-methylation was substantially reduced in WDR5-KD cells, whereas mono- and di-methylation of H3K4 displayed little change (Figure 3F). To further confirm that methylation of H3K4 induced by WDR5 was directly linked with γ-globin gene expression, we established stable K562 cell lines expressing two WDR5 mutants (F133A or Y191F) (Figure 3G)[20] in addition to our WDR5-OE (WT) line. The F133A mutation disrupts MLL association with the complex, whereas the Y191F mutation maintains the complex, but significantly reduces histone methyltransferase activity on H3K4.[20] Consistent with previous studies, H3K4me3 was no longer increased at γ-globin promoter in F133A or Y191F cells compared to WDR5-OE (WT) cells (Figure 3H), and γ-globin expression

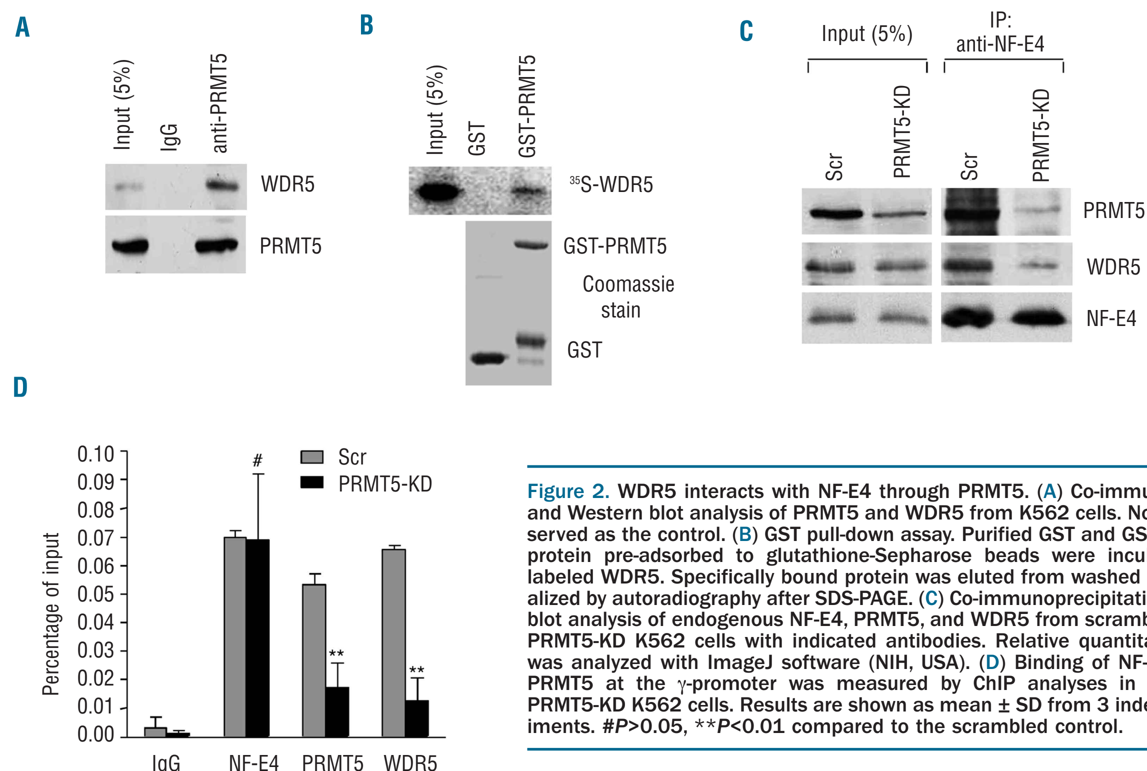

Figure 2. WDR5 interacts with NF-E4 through PRMT5. (A) Co-immunoprecipitation and Western blot analysis of PRMT5 and WDR5 from K562 cells. Normal rabbit IgG served as the control. (B) GST pull-down assay. Purified GST and GST-PRMT5 fusion protein pre-adsorbed to glutathione-Sepharose beads were incubated with ^{35}S-labeled WDR5. Specifically bound protein was eluted from washed beads and visualized by autoradiography after SDS-PAGE. (C) Co-immunoprecipitation and Western blot analysis of endogenous NF-E4, PRMT5, and WDR5 from scrambled control and PRMT5-KD K562 cells with indicated antibodies. Relative quantitation of protein was analyzed with ImageJ software (NIH, USA). (D) Binding of NF-E4, WDR5, and PRMT5 at the γ-promoter was measured by ChIP analyses in scrambled and PRMT5-KD K562 cells. Results are shown as mean ± SD from 3 independent experiments. #*P*>0.05, ***P*<0.01 compared to the scrambled control.

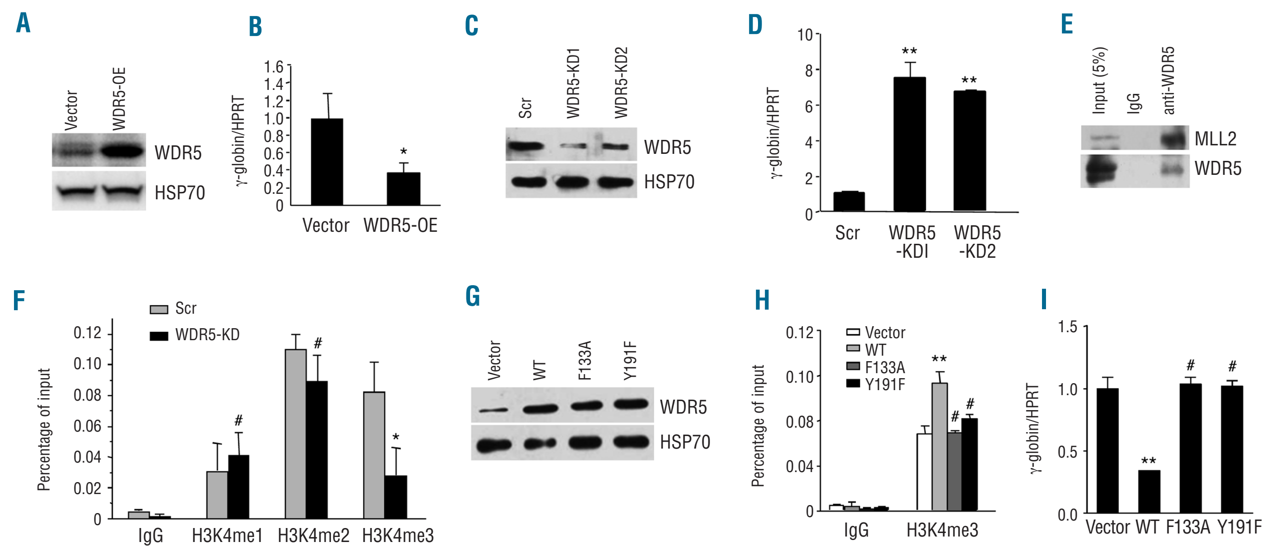

Figure 3. WDR5 represses γ-globin expression. (A) Western blot analyses of cellular extracts from WDR5-OE or vector only K562 cells with indicated antibodies. (B) γ-globin gene expression analysis by Q-RT-PCR of RNA from vector control or WDR5-OE K562 cells. Results are shown as mean±SD from 3 independent experiments. *P<0.05 compared to the vector control. (C) Western blot analyses of cellular extracts from WDR5-KD1 and WDR5-KD2 or scrambled control (Scr) K562 cells with indicated antibodies. (D) γ-globin gene expression analysis by Q-RT-PCR of RNA from WDR5-KD1, WDR5-KD2, and scrambled control (Scr) K562 cells. Results are shown as mean±SD from 3 independent experiments. **P<0.01 compared to the scrambled control. (E) Co-immunoprecipitation and Western blot analysis of WDR5 and MLL2 from K562 cells. Normal rabbit IgG served as the control. (F) Histone H3K4me1, H3K4me2, and H3K4me3 ChIP analyses at the γ-promoter were performed in scrambled control and WDR5-KD K562 cells. Results are shown as mean±SD from 3 independent experiments. #P>0.05, *P<0.05 compared to the scrambled control. (G) Western blot analyses of cellular extracts from vector control, WDR5 wild-type (WT), WDR5-F133A, and WDR5-Y191F K562 cells with indicated antibodies. (H) H3K4me3 ChIP analyses at the γ-promoter were performed in vector control, WDR5 wild-type (WT), WDR5-F133A, and WDR5-Y191F K562 cells. Results are shown as mean±SD from 3 independent experiments. #P>0.05, **P<0.01 compared to the vector control. (I) γ-globin gene expression analysis by Q-RT-PCR of RNA from vector control, WDR5 wild-type (WT), WDR5-F133A, and WDR5-Y191F K562 cells. Results are shown as mean±SD from 3 independent experiments. #P>0.05, **P<0.01 compared to the vector control.

was also maintained in the WDR5 mutant cells compared with the wild-type controls (Figure 3I). These results indicated that WDR5 mainly induced histone H3K4 tri-methylation on the human γ-globin promoter, which is important for modulating γ-globin expression.

WDR5 interacts with ING2-HDAC1 enhancing their binding at the γ-promoter

Although histone H3K4 tri-methylation is regarded as an active marker in most contexts, it can also be recognized by the ING2 protein, which associates with the repressive Sin3A-HDAC1 complex.[20] To assess the effect of WDR5 on other histone modifications, we performed ChIP assays with antibodies against acetylated H3 and acetylated H4, and the H4R3me2s and H3K9me3 marks. We showed that both H3 acetylation and H4 acetylation were significantly increased in the WDR5-KD line compared to the scrambled control (Figure 4A), consistent with the repressive role of WDR5 on γ-gene expression. The histone mark H4R3me2s mediated by PRMT5 was also reduced. We also observed a significant reduction in H3K9me3 in the WDR5-KD line compared to control, commensurate with the repressive effect of WDR5 (Figure 4A). Intriguingly, PRMT5 binding to the γ-globin promoter was not changed in WDR5-kd cells compared to the scramble control (Figure 4B), suggesting that the PRMT5 methyltransferase activity or complex might be perturbed under conditions of reduced levels of WDR5. Histone acetylation has been shown to play a critical role in globin gene regulation.[2] To test whether WDR5 can form a complex with HDAC1 and ING2, we performed immunoprecipitation studies using WDR5 or ING2 antibodies on K562 cell lysates. We found that WDR5 could interact with both ING2 and HDAC1 (Figure 4C and D). This is consistent with our previous observation that NF-E4 can also interact with HDAC1.[17] To confirm that WDR5 and ING2 formed a multi-protein complex with PRMT5 *in vivo*, we performed gel filtration chromatography with cellular extracts from K562 cells expressing Flag-tagged PRMT5, and analyzed fractions by Western blot (Figure 4E). Both WDR5 and ING2 could be co-eluted with PRMT5, although the peak of the elution profiles did not exactly overlap, suggesting that PRMT5 also participates in another complex in these cells.[15] To determine whether the interaction of ING2 and HDAC1 with WDR5 occurred on the γ-promoter, ChIP experiments with specific antibodies were performed in WDR5-OE K562 cells. We found that ING2 and HDAC1 binding to the γ-promoter were markedly increased in the overexpression line compared to the vector control cells (Figure 4F). Binding of HDAC1 on the HOXC8 promoter showed no change in the WDR5-OE line compared to the control cells, indicating specificity (*Online Supplementary Figure S4*). To examine ING2 function in γ-globin gene regulation, we generated an ING2 knockdown K562 cell line using a specific shRNA (ING2-KD). The expression of ING2 in these cells was reduced to 25% of the control (Figure 4G). The expression of the γ-gene assessed by Q-RT-PCR was increased 5-fold in this line compared to the scrambled control (Figure 4H). In contrast, ING2 did not affect

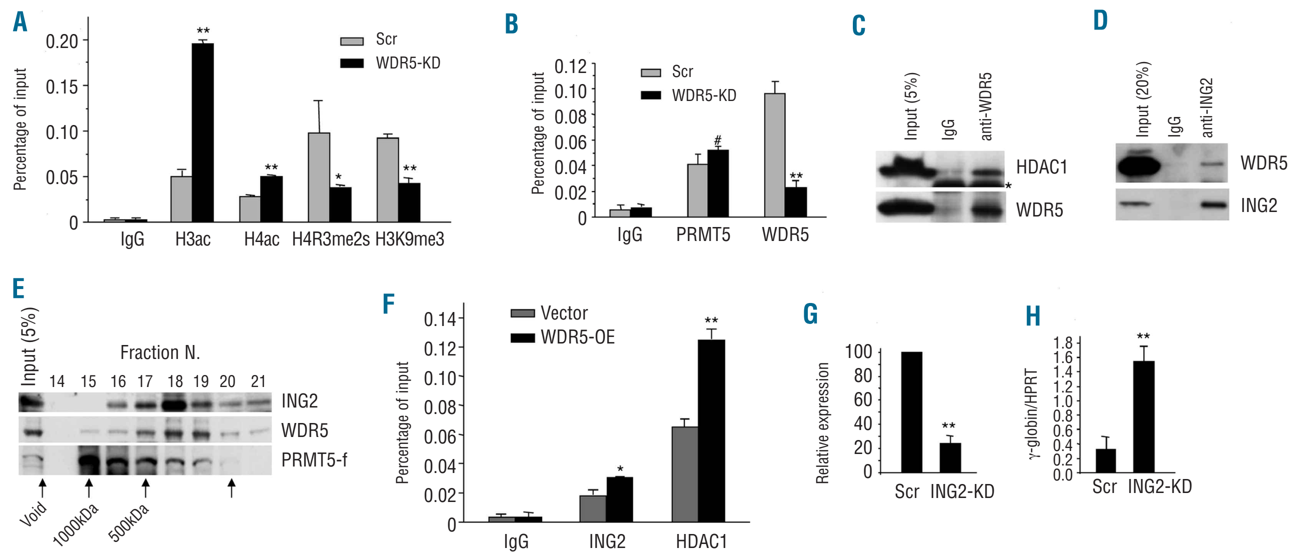

Figure 4. WDR5 associates with the ING2-HDAC1 complex at the γ-promoter. (A) Histone H3ac, H4ac, H4R3me2s, and H3K9me3 ChIP analyses at the γ-promoter were performed in K562 and WDR5-KD cells. Results are shown as mean ± SD from 3 independent experiments. **P<0.01, *P<0.05 compared to the scrambled control. (B) Binding of WDR5, and PRMT5 at the γ-promoter was measured by ChIP analyses in scrambled and WDR5-KD K562 cells. Results are shown as mean ± SD from 3 independent experiments. #P>0.05, **P<0.01 compared to the scrambled control. (C) WDR5 antibody co-immunoprecipitation and Western blot analysis of endogenous WDR5 and HDAC1 from K562 cells with indicated antibodies. The asterisk indicates IgG heavy chains. Normal rabbit IgG served as the control. (D) ING2 antibody co-immunoprecipitation and Western blot analysis of endogenous WDR5 and ING2 from K562 cells. Normal rabbit IgG served as the control. (E) Western blot analysis of extract from K562 cells expressing Flag-tagged PRMT5 (PRMT5-f) fractionated by Superose 12 gel filtration. Column fractions were concentrated and analyzed using the antibodies indicated. (F) ING2 and HDAC1 ChIP analyses at the γ-promoter from WDR5 over-expressing or vector control K562 cells. Results are shown as mean ± SD from 3 independent experiments. **P<0.01, *P<0.05 compared to the vector control. (G) ING2 gene expression analysis by Q-RT-PCR of RNA from ING2 knockdown (ING2-KD) and scrambled control (Scr) K562 cells. Results are shown as mean ± SD from 3 independent experiments. **P<0.01 compared to the scrambled control. (H) γ-globin gene expression analysis by Q-RT-PCR of RNA from ING2 knockdown (ING2-KD) and Scr K562 cells. Results are shown as mean ± SD from 3 independent experiments. **P<0.01 compared to the scrambled control.

HOXC8 expression at this reduced level (*Online Supplementary Figure S2B*). Hence, we hypothesized that the HDAC1-containing ING2 protein complex might recognize the histone H3K4me3 mark induced by WDR5 in this context, acting to repress γ-globin gene expression.

Knockdown of WDR5 reactivates γ-globin gene expression in human adult erythroid cells

Human γ-globin gene expression gradually declines after birth, and is almost absent in adult erythroid progenitors from bone marrow. To ascertain whether WDR5 played a role in the developmental globin gene expression program, we compared WDR5 binding to the γ-promoter in human erythroid progenitor cells from cord blood (CB, γ-globin 'on' state) and adult bone marrow (BM, γ-globin 'off' state)[14] utilizing a WDR5 antibody for ChIP analysis. WDR5 levels on the γ-promoter in BM erythroid progenitors were more than 7-fold higher than in CB (Figure 5A), which is consistent with the repressive role of WDR5 on γ-globin genes. In keeping with this, NF-E4 occupancy on the γ-promoter in BM erythroid progenitors was significantly higher than in CB (*Online Supplementary Figure S5*). We then examined the distribution of WDR5 across the β-globin locus in BM erythroid progenitors using primer pairs spanning key regions of the locus. We assessed the hypersensitive sites (HS1-4) of the locus control region (LCR), the ε-, γ-, and β-globin promoter, and the intergenic region between the ^{G}γ- and ^{A}γ-genes. Increased WDR5 binding was observed on the ε-, γ-, and β-globin promoters, and on the intergenic region between the ^{G}γ- and ^{A}γ-genes compared to the HSs or the adjacent olfactory receptor 51B4 (OR51B4) promoter (Figure 5B). We also observed a similar binding pattern of WDR5 across the β-globin locus in CB (*Online Supplementary Figure S6*), suggesting that other key factors such as BCL11A, SOX6, or EKLF, may contribute to developmental switching.[21] However, a significant differential between the γ-promoter and the other enriched globin sequences was also evident (Figure 5B).

To examine the effects of altered expression of WDR5 and ING2 in BM erythroid progenitors, we utilized specific shRNAs in lentiviral vectors to knock down the levels of the individual proteins. WDR5 expression was reduced to 40% of a scrambled control (Figure 5C), and ING2 was reduced to 10% of control (Figure 5D). The expression of γ-genes was increased 2.5-fold in the WDR5-KD cells and almost 4-fold in the ING2-KD cells, reflecting the degree of knockdown achieved with the respective shRNAs. Expression of the β-globin genes in these cells was not altered (Figure 5E). These results suggest that WDR5 may play an important role in the human globin gene expression program.

Discussion

Histone methylation marks have been correlated with both gene activation and silencing, and are dependent on context.[5] WDR5 is a key component of the MLL complex, which methylates histone H3K4. In the current study, we

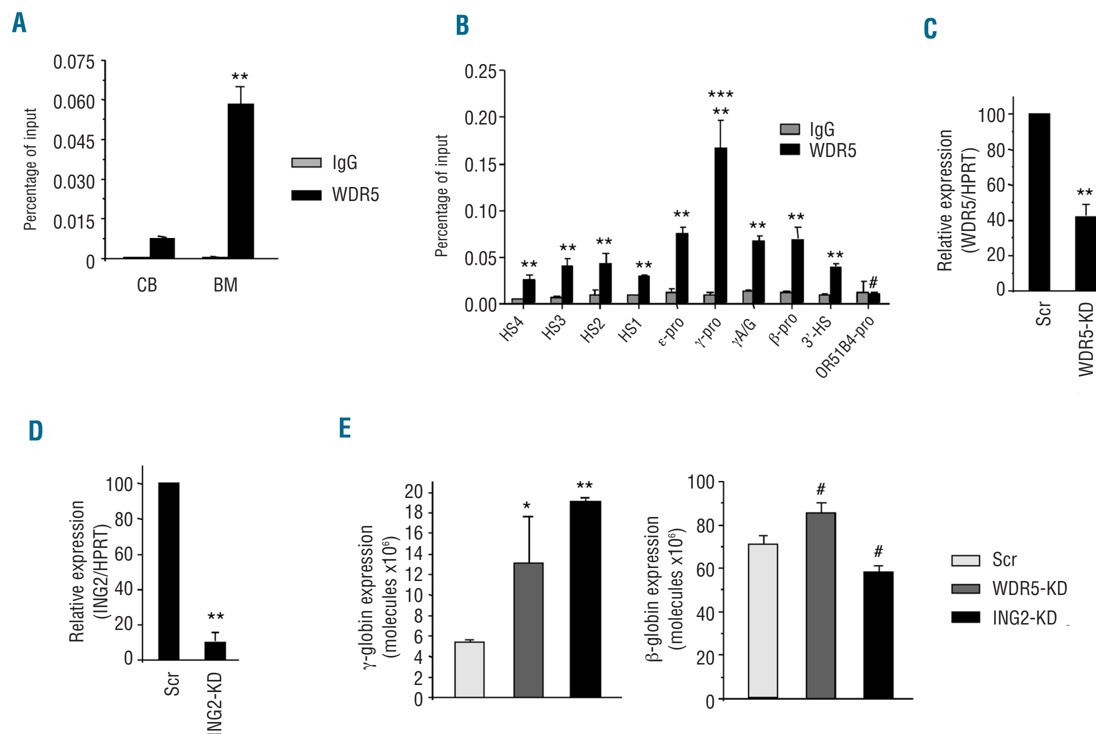

Figure 5. Role of WDR5 in developmental globin gene silencing. (A) WDR5 ChIP analysis at the γ-promoter in erythroid progenitor cells from cord blood (CB) and adult bone marrow (BM). Results are shown as mean±SD from 3 independent experiments. ***P*<0.01 compared to CB. (B) Localization of WDR5 across the β-globin locus measured by ChIP in chromatin fractions from erythroid progenitors from adult bone marrow. The precipitated DNA was amplified with primers specific for the indicated regions of the β-globin locus. HS: hypersensitive site; pro: promoter; $^{G/A}\gamma$: intergenic region between $^{G}\gamma$- and $^{A}\gamma$-globin genes; OR51B4: Olfactory receptor 51B4 gene. Results are shown as mean±SD from 3 independent experiments. #*P*>0.05 compared to the IgG control, ***P*<0.01 compared to OR51B4-pro, and ****P*<0.05 compared to all other loci. (C) Expression of WDR5 and (D) ING2 was analyzed in erythroid progenitors from BM expressing specific shRNAs or scrambled control (Scr). Results are shown as mean±SD from 3 independent experiments. ***P*<0.01 compared to the scrambled control. (E) Q-RT-PCR analysis of γ-globin and β-globin gene expression in WDR5-KD, ING2-KD, and Scr BM cells. Results are shown as mean±SD from 3 independent experiments. #*P*>0.05, **P*<0.05, and ***P*<0.01 compared to the scrambled control.

showed that WDR5 could induce γ-globin gene transcriptional repression through tri-methylation of histone H3K4. The H3K4me3 mark can be recognized by the PHD finger protein ING2, which co-ordinates with HDAC1 to deacetylate histones, thereby condensing the local chromatin. Although histone H3K4 methylation is preferentially distributed at the 5' end of the coding regions of many genes, and generally correlates with transcriptional activation, numerous silenced genes have been shown to display this mark. In genome-wide studies from human T cells, H3K4me3 islands were detected in approximately 60% of silent promoters.[9] H3K4me3 is enriched around transcription start sites of both active and repressed genes in mouse embryonic stem (ES) cells,[22] and in differentiated mouse erythroleukemia (MEL) cells, H3K4me3 is decreased at the β^{maj}-globin promoter[23] affirming that H3K4me is not always linked to gene activation.

The bivalent modification proposed during differentiation in ES cells may partially explain this. In mammalian ES cells, concurrent H3K4me3 and H3K27me3 (polycomb-related histone mark) on the same nucleosomal DNA maintains the gene in a state poised for activation even if it is silenced temporarily.[24] This bivalency is prevalent in circumstances in which differentiation may be inhibited, but a quick response to reprogramming may be needed.[25,26] However, these two marks appear to be separated and show spatial and temporal aspects in Xenopus embryos, demonstrating a hierarchy in epigenetic and transcriptional regulation.[27] In the current study, we did not find co-occurrence of H3K4me3 and H3K27me3 on the γ-promoter (*data not shown*). This is consistent with the fact that HP1 protein was not found in the PRMT5 complex in K562 cells.[15] However, we observed co-occurrence of H3K4me3 and H3K9me3. Although H3K4 methylation and H3K9 methylation often display reciprocal states,[28] it has been reported that H3K4me3 and H3K9me3 can colocalize in the globin coding region.[29,30] At the clock protein Rev-erbα promoter, knockdown of WDR5 also resulted in reduction of both histone H3K4 and H3K9 methylation.[31] Indeed, peptide binding and pulldown assays have shown that methylated H3K9 peptide has the potential to bind WDR5.[18,32] Intriguingly, LSD1, an H3K4 methyl eraser,[33] is capable of demethylating H3K9 in an androgen receptor-dependent manner.[34,35] It would be interesting to determine which H3K9 methyltransferase or demethylase is functional for the mark in the globin context. The methylation of histone H3K9 and H3K4 seem to function distinctly in a setting where they co-occur on the promoter. In fact, the levels of both H3K4me3 and K3K9me3 at the γ-promoter are low when γ-globin is actively transcribed.[30]

The physiological relevance of this kind of bivalent modification awaits further investigation.

Molecular effectors that read the epigenetic H3K4me3 mark are emerging. In the setting of transcriptional activation, PHD finger proteins, BPTF, RAG2, and Yng1 can read H3K4me3 specifically, and positively regulate their target genes.[5,36-38] Recently, the TAF3 subunit of TFIID complex has been shown to bind H3K4me3, thereby directly connecting the H3K4me3 mark with transcription.[39] Conversely, H3K4me3 can also associate with gene repression. A genetic screen in *Caenorhabditis elegans* found that Set1 can be involved in gene silencing by an ncRNA-mediated mechanism.[40] In addition, it has been shown that H3K4me2/3 induced by Set1 in yeast can recruit RPD3S, inhibiting pre-initiation complex formation and attenuating GAL1 induction.[13] We present data here indicating that at the γ-promoter H3K4me3 induced by WDR5 can be recognized by ING2 and can be coupled with deacetylation of both histone H3 and H4. This resembles cyclin D, which is silenced by the Sin3A-HDAC1 complex containing protein ING2 coupled with H3K4me3 binding.[20,41] Thus, H3K4 methylation can either up-regulate or down-regulate transcription, providing a two-way regulatory mechanism where this single histone mark can be translated with opposing activities. This phenomenon may be a common feature for the cell to rapidly adapt to environmental or physiological changes such as developmental or stress signals.[5]

The SET1/MLL complex is responsible for histone H3K4 methylation in mammals, and the plasticity of the WDR5 molecule plays an important role in the scaffolding for the SET1 complexes in regulating various developmental programs.[42] In addition to WDR5, several other components such as RbBP5 and Ash2 are also important for SET1/MLL methyltransferase activity.[6] Interestingly, recent studies indicated that WDR5-RbBP5-Ash2 could act separately from SET1/MLL methyltransferase as seen in the regulation of the nuclear receptor co-regulator-interacting factor 1 (NIF-1).[43] In addition, WDR5 can function as a subunit of other non-methyltransferase-related nuclear complexes, such as CHD-containing chromatin remodeling complex or ATAC and MOF histone acetyltransferase complexes.[44-46] Our study demonstrates that WDR5 can directly bind to PRMT5 and associate with the HDAC1-ING2 components at the γ-promoter. We showed that WDR5 binding on the γ-promoter is PRMT5-dependent. At the γ-promoter, WDR5 mediates histone H3K4me3, potentially through methyltransferase MLL2. The H3K4me3 mark, together with WDR5, couples the recruitment of ING2-HDAC1 to the promoter and coordinates subsequent events such as histone deacetylation at the promoter. This is consistent with the fact that inactive genes primed by MLL-mediated methylation of H3K4 undergo a dynamic shift of acetylation and deacetylation, which determines Pol II recruitment on or off the promoter.[47] This notion is further supported by the recent finding of a peptidyl prolyl isomerase, cyclophilin CyP33, which can regulate MLL1 function through HDAC recruitment, switching the overall functional balance of MLL1 toward gene repression.[48] However, we cannot exclude the possibility that WDR5 plays an active role in β-globin gene expression.

The binding pattern of WDR5 at the β-globin locus in human bone marrow cells displays a scenario reminiscent of the behavior of MLL2 in mouse MEL cells,[23] suggesting a model of 'spreading or tracking' between the LCR and the β-globin genes. The higher enrichment of WDR5 at the γ-promoter compared to its enrichment at the β-promoter may provide localized chromatin conformational changes due to histone modifications leading to gene silencing. The significance of WDR5 binding on other regions of the locus is unclear. Perhaps the relatively low levels of WDR5 binding may contribute to different methylation states of histone H3K4 and may function to maintain the locus in a transcriptionally potent configuration.[49] Further studies with co-ordinated epigenetic complex regulators and histone marks should provide additional clues to β-globin gene regulation during development.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Ginder GD, Gnanapragasam MN, Mian OY. The role of the epigenetic signal, DNA methylation, in gene regulation during erythroid development. Curr Top Dev Biol. 2008;82:85-116.
2. Kiefer CM, Hou C, Little JA, Dean A. Epigenetics of beta-globin gene regulation. Mutat Res. 2008;647(1-2):68-76.
3. Atweh GF, DeSimone J, Saunthararajah Y, Fathallah H, Weinberg RS, Nagel RL, et al. Hemoglobinopathies. Hematology Am Soc Hematol Educ Program. 2003:14-39.
4. Perrine SP. Fetal globin stimulant therapies in the beta-hemoglobinopathies: principles and current potential. Pediatr Ann. 2008; 37(5):339-46.
5. Ruthenburg AJ, Li H, Patel DJ, Allis CD. Multivalent engagement of chromatin modifications by linked binding modules. Nat Rev Mol Cell Biol. 2007;8(12):983-94.
6. Trievel RC, Shilatifard A. WDR5, a complexed protein. Nat Struct Mol Biol. 2009;16(7):678-80.
7. Shilatifard A. Molecular implementation and physiological roles for histone H3 lysine 4 (H3K4) methylation. Curr Opin Cell Biol. 2008;20(3):341-8.
8. Robertson AG, Bilenky M, Tam A, Zhao Y, Zeng T, Thiessen N, et al. Genome-wide relationship between histone H3 lysine 4 mono- and tri-methylation and transcription factor binding. Genome Res. 2008;18(12):1906-17.
9. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, et al. High-resolution profiling of histone methylations in the human genome. Cell. 2007;129(4):823-37.
10. Vitaliano-Prunier A, Menant A, Hobeika M, Geli V, Gwizdek C, Dargemont C. Ubiquitylation of the COMPASS component Swd2 links H2B ubiquitylation to H3K4 trimethylation. Nat Cell Biol. 2008;10(11):1365-71.
11. Guccione E, Bassi C, Casadio F, Martinato F, Cesaroni M, Schuchlautz H, et al. Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive. Nature. 2007;449(7164):933-7.
12. Carvin CD, Kladde MP. Effectors of lysine 4 methylation of histone H3 in Saccharomyces cerevisiae are negative regulators of PHO5 and GAL1-10. J Biol Chem. 2004;279(32):33057-62.
13. Pinskaya M, Gourvennec S, Morillon A. H3 lysine 4 di- and tri-methylation deposited by cryptic transcription attenuates promoter activation. EMBO J. 2009;28(12):1697-707.
14. Zhao Q, Rank G, Tan YT, Li H, Moritz RL, Simpson RJ, et al. PRMT5-mediated methylation of histone H4R3 recruits DNMT3A, coupling histone and DNA methylation in gene silencing. Nat Struct

Mol Biol. 2009;16(3):304-11.
15. Rank G, Cerruti L, Simpson RJ, Moritz RL, Jane SM, Zhao Q. Identification of a PRMT5-dependent repressor complex linked to silencing of human fetal globin gene expression. Blood. 2010;116(9):1585-92.
16. Zhao Q, Zhou W, Rank G, Sutton R, Wang X, Cumming H, et al. Repression of human gamma-globin gene expression by a short isoform of the NF-E4 protein is associated with loss of NF-E2 and RNA polymerase II recruitment to the promoter. Blood. 2006;107(5):2138-45.
17. Zhao Q, Cumming H, Cerruti L, Cunningham JM, Jane SM. Site-specific acetylation of the fetal globin activator NF-E4 prevents its ubiquitination and regulates its interaction with the histone deacetylase, HDAC1. J Biol Chem. 2004;279(40):41477-86.
18. Wysocka J, Swigut T, Milne TA, Dou Y, Zhang X, Burlingame AL, et al. WDR5 associates with histone H3 methylated at K4 and is essential for H3 K4 methylation and vertebrate development. Cell. 2005;121(6):859-72.
19. Dou Y, Milne TA, Ruthenburg AJ, Lee S, Lee JW, Verdine GL, et al. Regulation of MLL1 H3K4 methyltransferase activity by its core components. Nat Struct Mol Biol. 2006;13(8):713-9.
20. Shi X, Hong T, Walter KL, Ewalt M, Michishita E, Hung T, et al. ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. Nature. 2006;442(7098):96-9.
21. Sankaran VG, Xu J, Orkin SH. Advances in the understanding of haemoglobin switching. Br J Haematol. 2010;149(2):181-94.
22. Goldberg AD, Banaszynski LA, Noh KM, Lewis PW, Elsaesser SJ, Stadler S, et al. Distinct factors control histone variant H3.3 localization at specific genomic regions. Cell. 2010;140(5):678-91.
23. Demers C, Chaturvedi CP, Ranish JA, Juban G, Lai P, Morle F, et al. Activator-mediated recruitment of the MLL2 methyltransferase complex to the beta-globin locus. Mol Cell. 2007;27(4):573-84.
24. Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, et al. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell. 2006;125(2):315-26.
25. Pan G, Tian S, Nie J, Yang C, Ruotti V, Wei H, et al. Whole-genome analysis of histone H3 lysine 4 and lysine 27 methylation in human embryonic stem cells. Cell Stem Cell. 2007;1(3):299-312.
26. Zhao XD, Han X, Chew JL, Liu J, Chiu KP, Choo A, et al. Whole-genome mapping of histone H3 Lys4 and 27 trimethylations reveals distinct genomic compartments in human embryonic stem cells. Cell Stem Cell. 2007;1(3):286-98.
27. Akkers RC, van Heeringen SJ, Jacobi UG, Janssen-Megens EM, Francoijs KJ, Stunnenberg HG, et al. A hierarchy of H3K4me3 and H3K27me3 acquisition in spatial gene regulation in Xenopus embryos. Dev Cell. 2009;17(3):425-34.
28. Cheng X, Blumenthal RM. Coordinated chromatin control: structural and functional linkage of DNA and histone methylation. Biochemistry. 2010;49(14):2999-3008.
29. Vakoc CR, Mandat SA, Olenchock BA, Blobel GA. Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. Mol Cell. 2005;19(3):381-91.
30. Kim A, Kiefer CM, Dean A. Distinctive signatures of histone methylation in transcribed coding and noncoding human beta-globin sequences. Mol Cell Biol. 2007;27(4):1271-9.
31. Brown SA, Ripperger J, Kadener S, Fleury-Olela F, Vilbois F, Rosbash M, et al. PERIOD1-associated proteins modulate the negative limb of the mammalian circadian oscillator. Science. 2005;308(5722):693-6.
32. Schuetz A, Allali-Hassani A, Martin F, Loppnau P, Vedadi M, Bochkarev A, et al. Structural basis for molecular recognition and presentation of histone H3 by WDR5. EMBO J. 2006;25(18):4245-52.
33. Forneris F, Battaglioli E, Mattevi A, Binda C. New roles of flavoproteins in molecular cell biology: histone demethylase LSD1 and chromatin. FEBS J. 2009;276(16):4304-12.
34. Metzger E, Wissmann M, Yin N, Muller JM, Schneider R, Peters AH, et al. LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature. 2005;437(7057):436-9.
35. Wissmann M, Yin N, Muller JM, Greschik H, Fodor BD, Jenuwein T, et al. Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression. Nat Cell Biol. 2007;9(3):347-53.
36. Wysocka J, Swigut T, Xiao H, Milne TA, Kwon SY, Landry J, et al. A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. Nature. 2006;442(7098):86-90.
37. Ramon-Maiques S, Kuo AJ, Carney D, Matthews AG, Oettinger MA, Gozani O, et al. The plant homeodomain finger of RAG2 recognizes histone H3 methylated at both lysine-4 and arginine-2. Proc Natl Acad Sci USA. 2007;104(48):18993-8.
38. Martin DG, Baetz K, Shi X, Walter KL, MacDonald VE, Wlodarski MJ, et al. The Yng1p plant homeodomain finger is a methyl-histone binding module that recognizes lysine 4-methylated histone H3. Mol Cell Biol. 2006;26(21):7871-9.
39. Vermeulen M, Mulder KW, Denissov S, Pijnappel WW, van Schaik FM, Varier RA, et al. Selective anchoring of TFIID to nucleosomes by trimethylation of histone H3 lysine 4. Cell. 2007;131(1):58-69.
40. Ketting RF, Plasterk RH. A genetic link between co-suppression and RNA interference in C. elegans. Nature. 2000;404(6775):296-8.
41. Pena PV, Davrazou F, Shi X, Walter KL, Verkhusha VV, Gozani O, et al. Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. Nature. 2006;442(7098):100-3.
42. Zhang P, Lee H, Brunzelle JS, Couture JF. The plasticity of WDR5 peptide-binding cleft enables the binding of the SET1 family of histone methyltransferases. Nucleic Acids Res. 2012;40(9):4237-46.
43. Garapaty S, Xu CF, Trojer P, Mahajan MA, Neubert TA, Samuels HH. Identification and characterization of a novel nuclear protein complex involved in nuclear hormone receptor-mediated gene regulation. J Biol Chem. 2009;284(12):7542-52.
44. Mendjan S, Taipale M, Kind J, Holz H, Gebhardt P, Schelder M, et al. Nuclear pore components are involved in the transcriptional regulation of dosage compensation in Drosophila. Mol Cell. 2006;21(6):811-23.
45. Suganuma T, Gutierrez JL, Li B, Florens L, Swanson SK, Washburn MP, et al. ATAC is a double histone acetyltransferase complex that stimulates nucleosome sliding. Nat Struct Mol Biol. 2008;15(4):364-72.
46. Wang YL, Faiola F, Xu M, Pan S, Martinez E. Human ATAC Is a GCN5/PCAF-containing acetylase complex with a novel NC2-like histone fold module that interacts with the TATA-binding protein. J Biol Chem. 2008;283(49):33808-15.
47. Wang Z, Zang C, Cui K, Schones DE, Barski A, Peng W, et al. Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. Cell. 2009;138(5):1019-31.
48. Wang Z, Song J, Milne TA, Wang GG, Li H, Allis CD, et al. Pro isomerization in MLL1 PHD3-bromo cassette connects H3K4me readout to CyP33 and HDAC-mediated repression. Cell. 2010;141(7):1183-94.
49. Schneider R, Bannister AJ, Myers FA, Thorne AW, Crane-Robinson C, Kouzarides T. Histone H3 lysine 4 methylation patterns in higher eukaryotic genes. Nat Cell Biol. 2004;6(1):73-7.