RED CELLS, IRON, AND ERYTHROPOIESIS

Transcriptional and epigenetic basis for restoration of G6PD enzymatic activity in human G6PD-deficient cells

Kalliopi Makarona,[1] Valentina S. Caputo,[1] Joana R. Costa,[1] Binbin Liu,[2] David O'Connor,[1] Deena Iskander,[1] David Roper,[3] Lynn Robertson,[3] Neha Bhatnagar,[1] Evangelos Terpos,[4] Elisabeth Georgiou,[5] Maria Papaioannou,[6] D. Mark Layton,[1] Lucio Luzzatto,[7] Irene Roberts,[2] and Anastasios Karadimitris[1]

[1]Centre for Haematology, Department of Medicine, Hammersmith Hospital, Imperial College London, London, United Kingdom; [2]Department of Paediatrics, Children's Hospital and Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, Oxford University and John Radcliffe Hospital, Headington, Oxford, United Kingdom; [3]Blood Sciences Service, Hammersmith Hospital, Imperial College Healthcare National Health Service Trust, London, United Kingdom; [4]Department of Clinical Therapeutics, University of Athens School of Medicine, Athens, Greece; [5]Department of Biological Chemistry, School of Medicine, Aristoteleion University of Thessaloniki, Thessaloniki, Greece; [6]American Hellenic Educational Progressive Association Hospital, Aristoteleion University Medical School, Thessaloniki, Greece; and [7]Istituto Toscano Tumori, Firenze, Italy

Key Points

- Increase in HDAC binding is required for HDAC inhibitors to enhance gene transcription.
- G6PD deficiency in erythroid precursors can be restored by HDAC inhibitor-mediated increased transcription of the variant gene.

HDAC inhibitors (HDACi) increase transcription of some genes through histone hyperacetylation. To test the hypothesis that HDACi-mediated enhanced transcription might be of therapeutic value for inherited enzyme deficiency disorders, we focused on the glycolytic and pentose phosphate pathways (GPPPs). We show that among the 16 genes of the GPPPs, HDACi selectively enhance transcription of glucose 6-phosphate dehydrogenase (*G6PD*). This requires enhanced recruitment of the generic transcription factor Sp1, with commensurate recruitment of histone acetyltransferases and deacetylases, increased histone acetylation, and polymerase II recruitment to *G6PD*. These *G6PD*-selective transcriptional and epigenetic events result in increased *G6PD* transcription and ultimately restored enzymatic activity in B cells and erythroid precursor cells from patients with G6PD deficiency, a disorder associated with acute or chronic hemolytic anemia. Therefore, restoration of enzymatic activity in G6PD-deficient nucleated cells is feasible through modulation of *G6PD* transcription. Our findings also suggest that clinical consequences of pathogenic missense mutations in proteins with enzymatic function can be overcome in some cases by enhancement of the transcriptional output of the affected gene. (*Blood*. 2014;124(1):134-141)

Introduction

Histone acetylation mediated by histone acetyltransferases (HATs) is required for active gene transcription whereas deacetylation mediated by histone deacetylases (HDACs) is associated with transcriptional repression.[1,2] Histone deacetylase inhibitors (HDACi) are a diverse class of drugs currently in investigational and clinical use in the management of hemoglobinopathies and several types of malignancies.[3,4] The biochemical effects of HDAC inhibition include global increase in histone acetylation as well as acetylation of transcription factors (TFs) with consequent modulation of their function.[5,6] Despite their extensive clinical use, the exact mechanisms responsible for the effects of HDACi on transcription remain incompletely understood. Upregulation of gene expression through gene-specific histone hyperacetylation is probably important for the therapeutic effects of HDACi in hemoglobinopathies and cancer.[3] The same mechanism is responsible for the pronounced therapeutic effect of the HDACi sodium butyrate (NaBu) in inherited glycosylphosphatidylinositol deficiency.[7] In this autosomal recessive disorder, a mutation in the promoter of the housekeeping gene *PIGM* impairs promoter binding of the TF Sp1 leading to gene-specific histone hypoacetylation and polycomb-mediated transcriptional repression.[7,8]

Despite the global increase in histone acetylation in response to HDACi, changes in expression are seen in only a minority of genes, and nearly half of these genes are down- rather than upregulated.[9-11] In one of the few studies of the effect of HDACi on primary human cells, genome-wide epigenetic and gene expression profiling (GEP) analysis of $CD4^+$ T cells demonstrated high levels of HAT and HDAC co-occupancy in the most highly expressed genes.[12] Interestingly, only these genes, but not silent genes, underwent histone hyperacetylation in response to HDACi.[12] Taken together, these studies suggest that transcription of genes that are already active might be enhanced beyond basal levels by HDACi-mediated histone hyperacetylation at their promoters. This could have therapeutic potential, particularly in diseases resulting from enzyme defects or enzymopathies, in many of which there are no serious clinical manifestations as long as the level of enzyme activity is above a certain threshold.

Submitted February 11, 2014; accepted April 14, 2014. Prepublished online as *Blood* First Edition paper, May 7, 2014; DOI 10.1182/blood-2014-02-553792.

The online version of this article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Most red-cell enzymopathies affect either glycolysis, a ubiquitous metabolic pathway capable of producing adenosine triphosphate under anaerobic conditions, or the pentose phosphate pathway, a shunt pathway that is closely linked to glycolyis and is indispensable for providing reductive potential.[13] Most inherited disorders of the glycolytic and pentose phosphate pathway (GPPP) are autosomal recessive, are caused by missense mutations that preserve some residual enzyme activity, and are characterized clinically by moderate to severe chronic hemolytic anemia.[13,14] Glucose-6-phosphate dehydrogenase (G6PD) deficiency, an X-linked disorder, is unique because of its very high frequency in areas where malaria is endemic. It is caused by polymorphic variants (eg, G6PDA− and G6PDMed) that entail a marked decrease in, but never complete lack of, enzyme activity.[15,16] G6PD deficiency is typically associated with episodic acute hemolytic anemia, triggered by infection, consumption of fava beans, or drugs, including the antimalarial agent primaquine (PQ).[17] In fact, G6PD deficiency is perhaps the single most important obstacle for PQ-based malaria elimination programs in endemic areas.[18,19] A rare, severe form of G6PD deficiency caused by sporadic mutations also exists, and like the rest of the GPPP disorders, it is associated with chronic hemolytic anemia.[14]

In this study, we tested the hypothesis that transcriptional upregulation of GPPP genes might be achieved by HDACi. For any of the GPPP genes for which this might be the case, these compounds could be of therapeutic value, even in the presence of disease-causing mutations.

Materials and methods

Normal and patient samples

Normal and patient peripheral blood (PB) samples and cord blood (CB) samples were collected under the auspices of a study approved by the National Research Ethics Service. All patients provided informed written consent. Patient characteristics, *G6PD* mutation status, and red-cell G6PD activity levels are described in supplemental Table 1, available on the *Blood* Web site. Epstein-Barr virus–transformed B-cell lines were generated by the European Collection of Cell Cultures (Salisbury, United Kingdom [UK]). Ethical approval was received by the National Research Ethics Service Committee London-Fulham. This study was conducted in accordance with the Declaration of Helsinki.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) combined with real-time quantitative polymerase chain reaction (ChIP-RQ-PCR) was performed as previously described.[8] Enrichment is expressed as percent of input. Typically, 1% of starting chromatin was used as input.

GEP and data analysis

Extraction of total RNA (from day 5, immunomagnetic bead–sorted $CD36^+$ erythroid cells, purity >90%; n = 3), complementary DNA synthesis, fragmentation, labeling, and hybridization on Affymetrix GeneChip Human Gene 1.0 ST Arrays (Affymetrix UK Ltd., High Wycombe, UK) were performed as previously described.[20] The GEP data are available online in ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) with accession number E-MTAB-2533.

GEP analysis was performed by using Affymetrix Expression Console Software (Affymetrix UK Ltd.) for robust multichip average normalization[21] and GenePattern Software (Broad Institute, Cambridge, MA) was used for differential gene expression analysis. For discriminative motif analysis, the MEME Suite (http://meme.nbcr.net/meme/)[22] was used. TOUCAN 2, release 5, sequence analysis software was used to retrieve promoters (800 bp and 600 bp upstream and 200 bp downstream of exon 1) of the 70 most upregulated genes, 70 most downregulated genes, and 70 genes whose expression was unchanged in response to NaBu treatment of erythroid cells. Differential expression of the $CD4^+$ T-cell data (GSE15735) was performed using Student *t* test after Robust Multichip Average normalization, with Bioconductor packages Affy, simpleaffy, and genefilter. All 3 samples at 12-hour and 0-hour time points were used for statistical testing. Multitesting was corrected using Benjamini-Hochberg method integrated into multtest. Genes with adjusted *P* values $< .05$ were considered significant.

Sequences of wild-type (WT) and mutagenized GC-rich boxes

The following is a list of motifs: Motif 1: 5′CCCCCGCCCCC3′ to 5′CCAACGATCCC3′; Motif 2: 5′GCCCCGCCCCG3′ to 5′GCTACGA CACGC3′; Motif 3: 5′GAGGGGTGGTG3′ to 5′GAGAAGTGGTG3′; Motif 4: 5′CGCCCCGCCCCG3′ to 5′CGCATCGATCCG3′; Motif 5: 5′GGGGCGGGGCC3′ to 5′GGTACTAGGCC3′; Motif 6: 5′GCAGGC GGGGA3′ to 5′GCAATCGAAGA3′; Motif 7: 5′GGCCCCGCCCAG3 to 5′GGCTATGCACAG3′.

Statistical analysis

Descriptive statistics show mean ± standard error of the mean. Unpaired or paired Student *t* test or 1- or 2-way analysis of variance were used per requirements. Statistical analysis was performed, and graphs were created by using GraphPad Prism, v6.

Reagents, antibodies, flow cytometry, in vitro generation of erythroid precursors, quantitative reverse transcriptase PCR (qRT-PCR), western blotting, G6PD enzymatic activity assay, plasmids, mutagenesis, and reporter assays are described in supplemental Methods.

Results

Selective transcriptional upregulation of *G6PD* in response to HDACi

We first tested our hypothesis by measuring messenger RNA (mRNA) levels of all GPPP genes in non-G6PD–deficient Epstein-Barr virus–transformed B-cell lines after exposure to the HDACi NaBu. We found that among the 16 genes we tested, *G6PD* was the only one for which the mRNA level increased gradually with time in response to NaBu (Figure 1A). Similar results emerged from a GEP analysis of primary $CD4^+$ T cells treated with a combination of NaBu and trichostatin[12] (Figure 1B); results were also similar in cells of nonhematopoietic origin (supplemental Figure 1A). The effect of NaBu on *G6PD* transcription was direct, because it was observed as early as 4 hours after exposure, and it was not abrogated by the protein translation inhibitor cyclohexamide (supplemental Figure 1B).

Because the clinical phenotype of inherited GPPP defects manifests mainly in erythroid cells, we generated GEP of proerythroblasts (nucleated erythroid precursor cells) derived from CB $CD34^+$ cells in vitro (supplemental Figure 1C). We found that in these cells, 6 hours of treatment with NaBu causes a roughly similar proportion of genes to be down- or upregulated (2.7% and 3%, respectively; supplemental Table 2). Once again, as in B cells, among GPPP genes, only *G6PD* mRNA was significantly increased (Figure 1C), a finding validated by qRT-PCR in erythroid precursor cells treated with NaBu (Figure 1D). Thus, selective increase in *G6PD* expression in response to HDACi is a common feature of different cell types of both hematopoietic and nonhematopoietic origin.

Histone hyperacetylation and increased recruitment of HAT and HDAC underpin the selective *G6PD* transcriptional activation

To dissect the transcriptional and epigenetic basis of the selective responsiveness of *G6PD* to HDACi, we first assessed, at the promoter

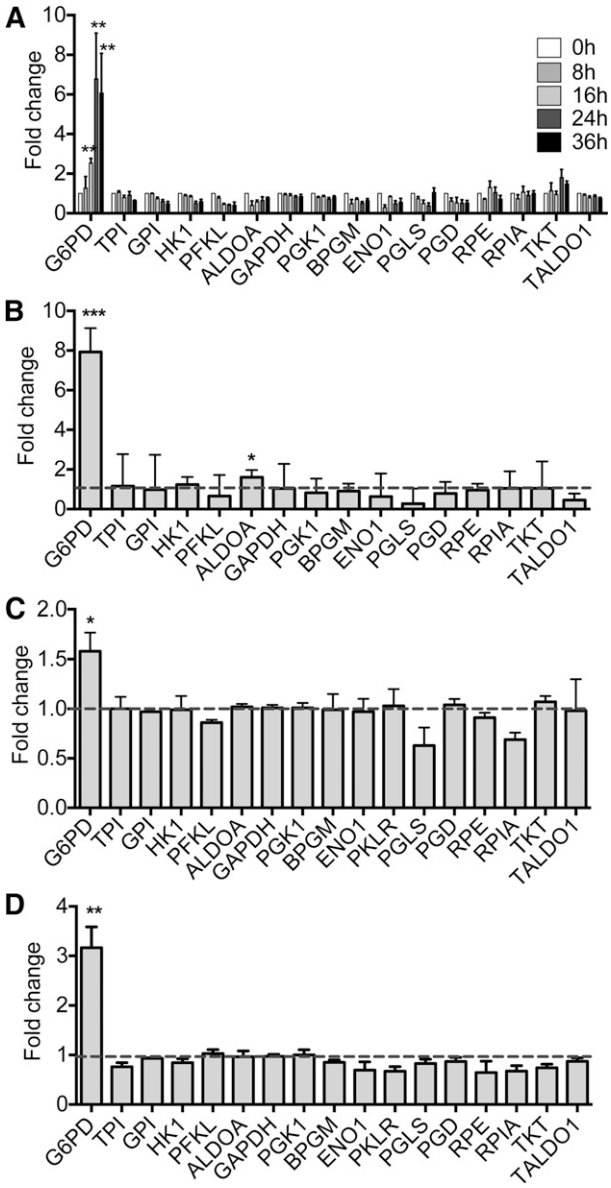

Figure 1. mRNA expression of GPPP genes in response to HDACi. (A) GPPP mRNA levels in response to NaBu as assessed by qRT-PCR. (A) WT (ie, non-G6PD–deficient) B-cell line was treated with 3 mM NaBu. Data are normalized to t = 0 levels (n = 3). *HK1*, *GPI*, *PFKL*, *ALDOA*, *TPI*, *GAPDH*, *PGK1*, *PGAM*, *ENO1*, *PK*, and *BPGM* are part of the glycolytic pathway, and *G6PD*, *PGLS*, *PGD*, *RPE*, *TKT*, and *TALDO1* are part of the pentose phosphate pathway. (B) GPPP mRNA expression extracted from the GSE15735 data set. $CD4^+$ T cells were treated with 100 ng/mL trichostatin (TSA) and 2 mM NaBu for 12 hours (n = 3). (C) mRNA levels of GPPP genes as assessed by GEP analysis of day 5 CB $CD34^+$-derived proerythroblasts after 6-hour treatment with 1 mM NaBu (n = 3). (D) mRNA levels of GPPP genes in CB $CD34^+$-generated erythroblasts after NaBu treatment for 48 hours. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$.

regions of GPPP genes, the extent of histone acetylation, a modification that is associated with transcriptional activation.[1,2] We found increased baseline levels of H3 and H4 acetylation in the core promoters of all GPPP genes tested, consistent with their status as housekeeping genes. However, an increase in histone acetylation in response to NaBu was observed only in *G6PD* and in none of the other GPPP genes (Figure 2A).

Levels of histone acetylation affecting transcription depend upon dynamic recruitment and antagonistic activity of a multitude of HATs and HDACs.[12] By using ChIP-RQ-PCR, we found that baseline binding of HDAC1, -3, -4, -5, -7, and -6 and of HAT-p300, -CBP, and -GCN5[23] was similar at the *G6PD* promoter and the promoters of the rest of the GPPP genes; however, in response to HDACi, occupancy of HDAC and HAT increased only at the promoter of *G6PD* (Figure 2B-C). Specifically, significant increases were observed in the binding levels of all 3 HATs tested and, among the HDACs, of HDAC1 and HDAC6.

The suggestion that these epigenetic changes in response to HDACi mediate increased transcription is supported by our further finding, that Polymerase II (Pol II) binding to the core promoter and gene body of *G6PD*, but not of the other genes, is significantly increased (Figure 2D). Thus, in GPPP genes, increased transcription in response to HDACi is underpinned by histone hyperacetylation and concomitant increase in the recruitment of HATs and HDACs, and it is highly selective for *G6PD*.

HDACi preferentially modulate transcription of active genes

Counter to the previous understanding of HDACs as transcriptional repressors,[1] it was reported that HDAC binding is highest in the most active genes and that in response to HDACi concomitant with enhanced histone acetylation, HDAC binding increases further in these genes.[12] However, the implication of these observations, namely that active rather than silent genes would be more likely to respond to HDACi by modulating their transcription, was not investigated.

To address this, we compared basal mRNA levels of genes in erythroid precursor cells that responded to HDACi (by being either up- or downregulated; fold change >1.5; $P < .05$) and in those that were unaffected (Figure 3A). We found a highly significant difference ($P < .0001$), in that basal mRNA levels were higher overall for genes showing change than for genes showing no change. Furthermore, it was of interest that within the former group, downregulated genes had higher basal mRNA levels than upregulated genes (Figure 3A). The same pattern of gene response to HDACi was found in the $CD4^+$ T cells treated with NaBu and trichostatin (Figure 3B). These data suggest that it is the genes that are already active, rather than inactive genes, that are more likely to enhance their expression beyond basal levels in response to HDACi.

To search for *cis* DNA elements that determine increased transcription in response to HDACi, we used an unbiased discriminative motif analysis, whereby we searched for motifs that appear in the promoters of genes that are upregulated but not in the promoters of genes that show no change or that are downregulated in response to NaBu in erythroid cells. In both searches, we identified an identical GC-rich motif (termed Motif A) as being highly enriched in the promoters of the upregulated genes but not in those of the other two groups of genes (Figure 3C).

Sp1-dependent increase in *G6PD* transcription in response to HDACi

Downstream motif identification analysis showed that Motif A corresponded to an Sp1 binding motif (supplemental Figure 2A) and was very similar to several GC-rich boxes (also potential Sp1 binding sites) upstream of the transcription start sites in the promoter of *G6PD* (Figure 4A and "Materials and methods"). Of these, Motifs 3 and 4 had been shown previously to be part of the essential core promoter[24] and had been validated as bona fide Sp1 binding sites in vitro.[25]

To dissect the role of *cis* DNA elements in *G6PD* transcription, we first carried out a promoter deletion analysis. In both the absence and the presence of NaBu, *G6PD* promoter activity depended on a proximal 562-bp promoter fragment containing 7 GC-rich motifs,

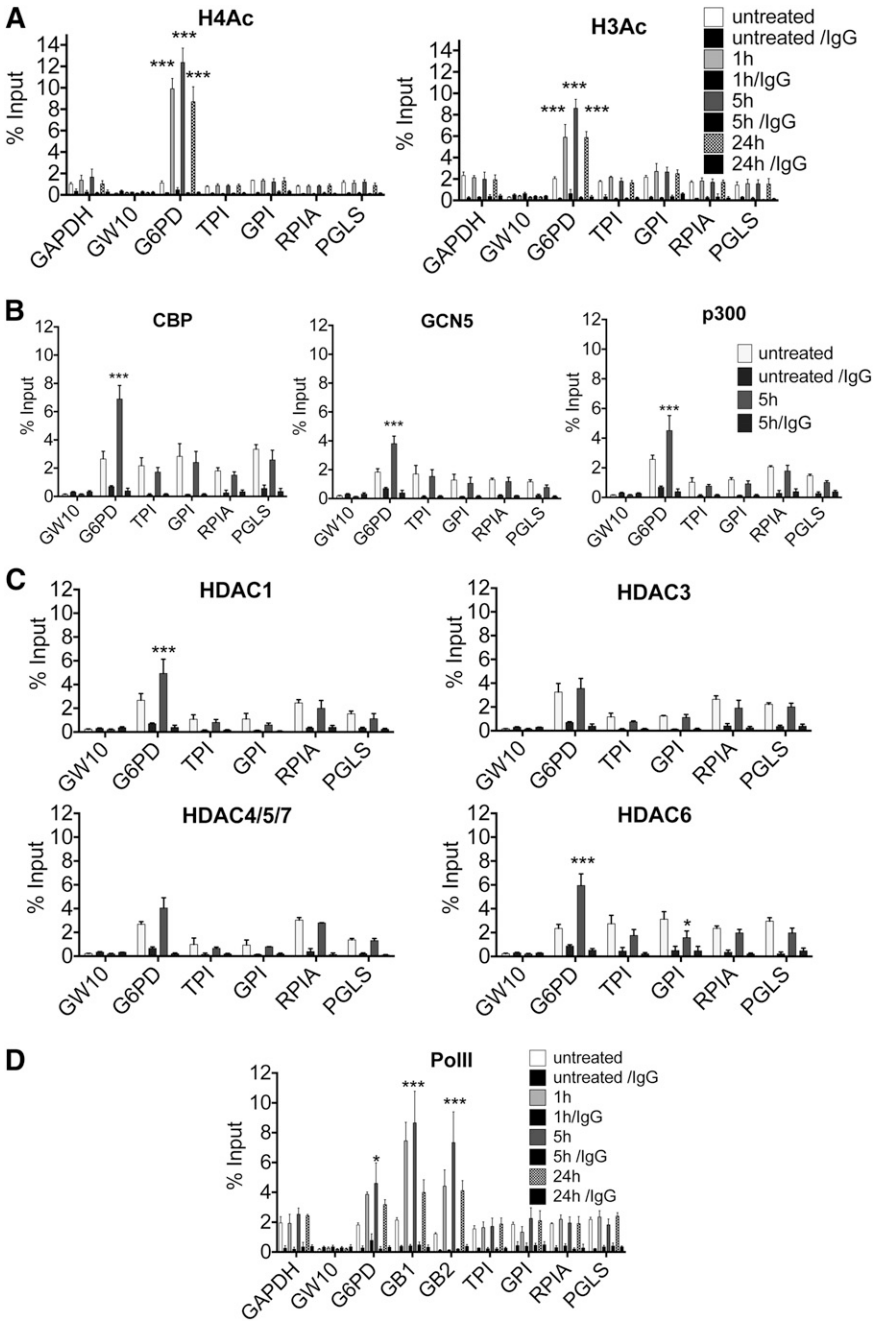

Figure 2. Epigenetic changes in GPPP gene promoters in response to NaBu treatment. (A) Histone 3 and histone 4 acetylation (H3Ac; H4Ac) levels assessed by ChIP-RQ-PCR in WT B cells at baseline and after NaBu treatment. A *GAPDH* promoter amplicon is used as a positive control. GW10, an amplicon in a region devoid of genes on chromosome 10 is shown as a negative control (n = 3). (B-C) HAT and HDAC binding, respectively, at the GPPP gene promoters in WT B cells in the presence of NaBu. CBP, GCN5, and p300 binding assessed by ChIP-RQ-PCR after 5-hour treatment with NaBu (n = 3). (D) Pol II binding on the GPPP gene promoters and 2 gene body areas of *G6PD* (GB1 and GB2) in WT B cells after 0 to 24 hours of treatment with NaBu (n = 3). Enrichment of binding in the target areas using specific antibody and immunoglobulin G (IgG) controls is calculated as percentage of input throughout (n = 3). $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$

with 50% of transcriptional activity depending upon a fragment containing the GC-rich Motifs 1 through 4 (Figure 4B). Next, we proceeded to confirm the relative functional importance of Motifs 1 through 7 in the regulation of baseline *G6PD* transcriptional activity and its enhancement following NaBu treatment by using *G6PD* promoter constructs with individually mutated motifs (Figure 4C). We found that inactivation of Motifs 6 and 7 had little or no effect on the transcriptional activity of the corresponding promoter constructs. By contrast, mutagenesis of each of Motifs 1 through 5 resulted in significant reduction of transcriptional activity, with the most pronounced reduction (80%) observed with Motif 1 mutant (Mut1; Figure 4C), consonant with previous data.[24] Transcriptional response to NaBu was not completely abrogated by any of the 7 mutants but was blunted down to 35% to 40% of maximum (ie, of that achieved by the promoter fragment F5) by each of Mut1 through Mut5 (Figure 4C), highlighting the importance of the corresponding core promoter motifs in transcriptional regulation of *G6PD* and its responsiveness to HDACi.

Finally, we confirmed binding of Sp1 at the promoter of *G6PD* and of 4 other GPPP genes in vivo at equivalent levels (Figure 4D). Interestingly, upon NaBu treatment and similar to histone acetylation and HAT and/or HDAC and Pol II binding, Sp1 binding also increased selectively at the promoter of *G6PD* (Figure 4D). Further supporting the importance of Sp1 in the transcriptional regulation of *G6PD*, mithramycin A, an agent that prevents TF binding to GC-rich boxes[26] nearly completely abrogated transcription of *G6PD* (supplemental Figure 2B), as did a dominant-negative form of Sp1 (Figure 4E) under basal conditions and following NaBu treatment.

Taken together, these findings identify the GC-rich Motifs 1 through 5 in the *G6PD* core promoter, the binding to them of Sp1,

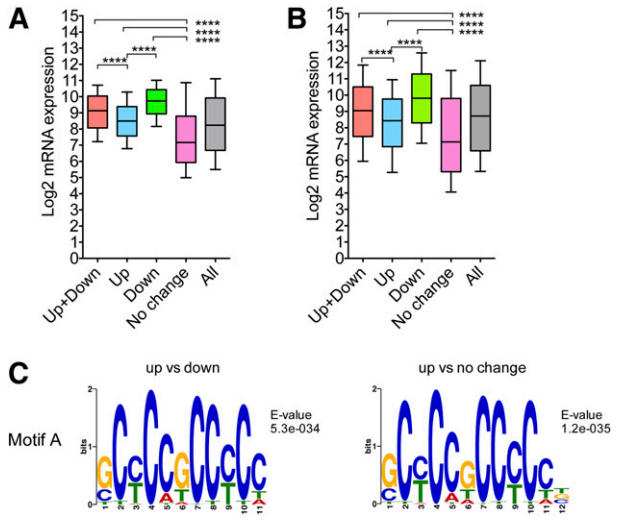

Figure 3. Modulation of transcription of active vs inactive genes by HDACi. (A) Comparison in erythroid cells of basal mRNA expression levels of groups of genes modulated (up- and downregulated, all genes together or as individual groups) with genes that show no change in response to HDACi. The basal expression level of all genes (All) is also shown. Genes were ranked according to a cutoff of 1.5-fold change and $P < .05$. (B) The same analysis as in (A) of the GSE15735 data set. $CD4^+$ T cells were treated with 100 ng/mL TSA and 2 mM NaBu for 12 hours. Box blots show 25% to 75% quartiles; horizontal lines show the mean, and whiskers show the 10th to 90th percentiles. One-way analysis of variance (ANOVA) corrected for multiple comparisons; ****$P < .0001$. (C) De novo motif identification in the promoters of the 70 most up- vs downregulated and most upregulated vs no change genes after NaBu treatment. A GC-rich motif (Motif A) was the most highly enriched in the promoters of the upregulated genes in both group comparisons. In downstream analysis, Motif A was identified as an Sp1 binding site (supplemental Figure 2A).

dynamic recruitment of HAT and HDAC, promoter hyperacetylation, and enhanced Pol II binding as central to enhanced *G6PD* transcription in response to HDACi.

HDACi increase *G6PD* expression and G6PD enzyme activity in cells from patients with G6PD deficiency

Next, we tested whether HDACi-mediated enhanced *G6PD* transcription would result in increased protein levels and ultimately in increased enzymatic activity, even in the presence of a pathogenic mutation in *G6PD*.

Therefore, we studied the impact of NaBu on *G6PD* mRNA and protein levels in B cells from a patient with chronic hemolytic anemia that was due to a rare *G6PD* mutation (an in-frame single codon deletion in exon 13, G6PD Brighton).[27] We found that in both normal and G6PD Brighton B cells, protein levels also increased (Figure 5A-B) commensurate with the time-dependent increase in mRNA levels (Figure 5A). Most importantly, G6PD enzymatic activity in normal and in G6PD Brighton cells increased similarly upon NaBu addition until at 36 hours, it reached a plateau at about 2.5-fold to threefold over baseline (Figure 5C). These findings show that, at least in this case, the mutation-related protein change does not prevent the increased level of protein produced from providing a substantial net increase in enzyme activity. Similar results were obtained by using suberoylanilide hydroxamic acid, a different class of HDACi (supplemental Figure 3), suggesting that enhancement in *G6PD* expression is not limited to a single class of HDACi.

There is no a priori reason to expect that a coding mutation such as that of G6PD Brighton should alter the epigenetic regulation of *G6PD*. We confirmed this by reproducing all the experiments performed in normal cells in G6PD Brighton B cells, demonstrating that NaBu induced increased transcription, histone hyperacetylation (supplemental Figure 4A), increased recruitment of HAT (supplemental Figure 4B) and HDAC (supplemental Figure 4C), and increased Sp1 and Pol II binding (supplemental Figure 4D-E).

We next tested the response of *G6PD* expression and activity in primary erythroid precursor cells generated from 3 patients, each one with a different type of severe G6PD deficiency that caused chronic nonspherocytic hemolytic anemia (CNSHA): G6PD Brighton, G6PD Harilaou,[28] and G6PD Serres[29] (see Table 1 and supplemental Table 1). In erythroid precursor cells from normal patients, *G6PD* mRNA protein and enzymatic activity, whether obtained from PB or from CB $CD34^+$ cells, increased markedly in response to NaBu treatment (Table 1). In erythroid precursor cells from the 3 severely deficient patients with CNSHA, the results were similar: most importantly, the G6PD enzyme activity was restored to levels in the same range as that seen in erythroid precursor cells from normal patients (Table 1 and supplemental Figure 5).

G6PDA− and G6PDMed are two of the most common polymorphic G6PD variants, with highest prevalence in areas where malaria is endemic.[19] We therefore tested the impact of HDACi on expression and enzymatic activity in erythroid precursor cells from hemizygous individuals with G6PDMed and G6PDA− (Table 1 and supplemental Table 1). Similar to the CNSHA-associated variants, we found that in G6PDA− and G6PDMed erythroid cells generated from PB, treatment with NaBu resulted in increased mRNA and protein levels (supplemental Figure 5) and restoration of G6PD enzymatic activity to the same levels as in normal controls (Table 1).

Potential impact of HDACi on other inherited disorders

Among genes that were upregulated (fold change >1.5; $P < .05$) in erythroid cells in response to NaBu, we identified 65 that are implicated in Mendelian inherited disorders (supplemental Table 3). Twenty of these genes encode proteins with enzymatic function, implying that if the pathogenic mutation is associated with residual enzyme activity, the activity might be significantly increased by the increased mRNA and protein level achieved in response to HDACi.

Discussion

Focusing on GPPP, we provide proof-of-principle for our previous hypothesis[30] that pathogenic missense mutations in proteins with enzymatic function might be overcome by simply increasing the transcriptional output of the affected gene, especially when the mutation does not entirely abrogate enzymatic activity. For at least one of the genes in the GPPP, G6PD, HDACi produce a marked increase in transcriptional output, such that enzyme activity is restored in G6PD-deficient primary erythroid precursor cells.

We found that the basal state epigenetic profile (high level of promoter histone acetylation, and HAT and Pol II binding) is similar in the 16 GPPP genes analyzed and consistent with their housekeeping function. What was not expected was the high level of HDAC binding. In this respect we note that, although HDACs are traditionally thought to act as transcriptional repressors (reviewed in Shahbazian and Grunstein[1] and Perissi et al[31]), recent work has challenged this view. In primary human T cells, the highest levels of HDAC binding are found on active rather than on silent genes.[12] We have now validated, for the first time, the functional importance of this finding at the level of an individual gene and provided support for the notion that a dynamic HAT-HDAC balance is required for

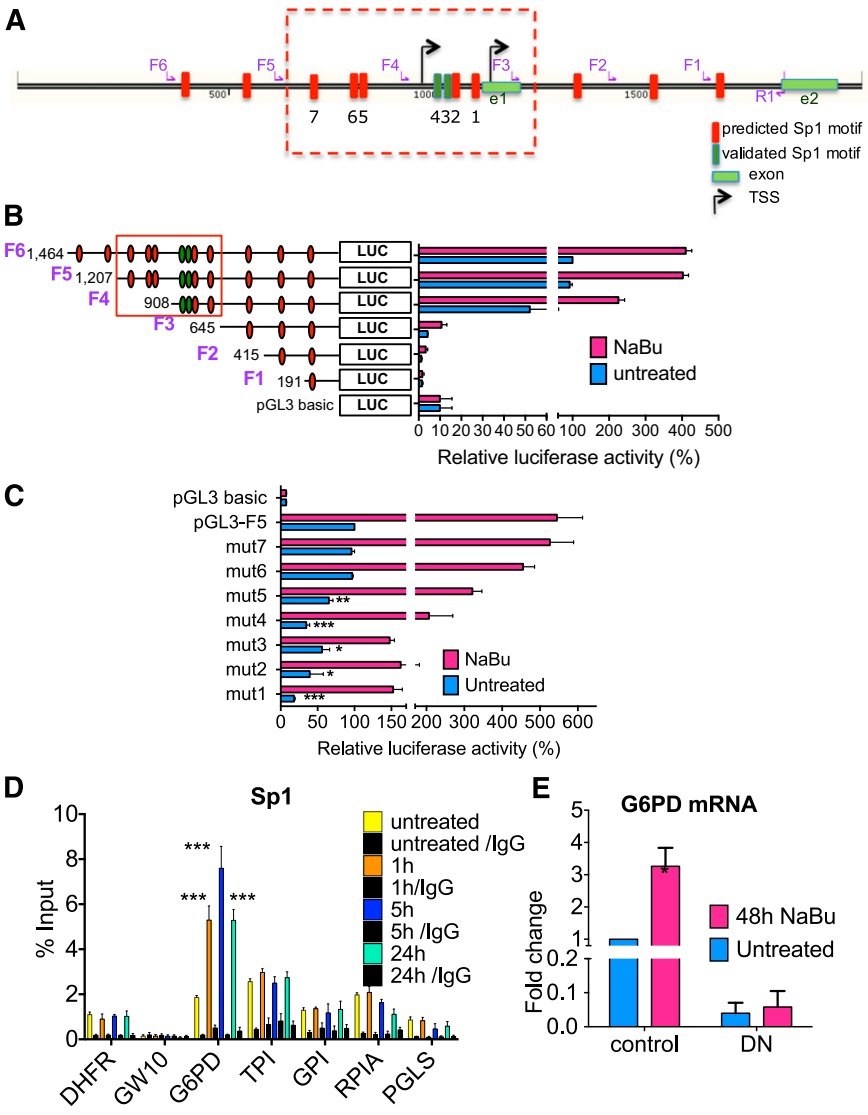

Figure 4. Sp1-dependent increase in *G6PD* transcription in response to HDACi. (A) Schematic of the *G6PD* promoter drawn to scale. Vertical boxes represent Sp1 motifs; predicted motifs are shown in red; two previously validated Sp1 binding sites (Motifs 3 and 4) are shown in dark green. The Sp1 motifs that are included in the 562-bp promoter region, also boxed in Figure 4B, are numbered 1 through 7. R1 is the reverse primer and F1 through F6 are the forward primers used to generate promoter fragments F1 through F6 shown in (B). There are 2 transcription start sites (TSS). *NEMO*, a gene whose promoter overlaps with that of *G6PD* is not shown. (B) Transcriptional activity of *G6PD* promoter assessed by luciferase (Luc) reporter assays. Luciferase activity was measured at baseline and upon 3 mM NaBu treatment of 24 hours. Activity is normalized against promoterless pGL3 basic and is shown relative to the baseline levels of the longest promoter fragment, F6 (n = 3). (C) Luciferase reporter assays conducted as in (B), using *G6PD* promoter constructs containing mutated putative Sp1 binding sites as shown, at baseline and upon 3 mM NaBu treatment for 24 hours. Mean ± standard error of the mean (SEM); n = 3. One-way ANOVA was used to compare pGL3-F5 to the mutated construct activities. (D) Sp1 binding in the GPPP gene promoters assessed by ChIP-RQ-PCR in a WT B-cell line. *DHFR* was used as a positive control for Sp1 binding. Data are shown as mean ± SEM for 3 independent experiments. Paired Student *t* test was performed to compare untreated vs 5-hour and 24-hour treatment with NaBu (n = 3). (E) The effect of a dominant negative (DN) form of Sp1 on *G6PD* transcription. In all, 293 T cells were transfected either with a plasmid containing a complementary DNA (cDNA) encoding a DN form of Sp1 or with a no-insert control plasmid. *G6PD* mRNA levels were assessed 48 hours later by qRT-PCR (n = 3).

transcription of active genes through modulation of histone acetylation levels.

It was quite remarkable that NaBu would enhance transcription of only *G6PD* among the 16 genes we tested. Whereas the basal epigenetic landscape was similar for all GPPP genes, NaBu produced a sharp difference. Histone acetylation was increased only in *G6PD*, a direct effect of HDAC inhibition followed by enhanced binding of Pol II and increased transcription rate. This highly selective response of *G6PD* to HDACi was marked by increased binding of both HAT and HDAC above basal levels. This novel observation suggests that HAT and HDAC, once recruited to *G6PD* (but not to other GPPP genes), set up a fine dynamic balance that sustains enhanced histone acetylation levels and brings about the NaBu-induced increase in transcriptional activity of *G6PD*. Increased binding of HDAC6 specifically in response to HDACi, is consistent with its known role in promoting transcription through its direct association with Pol II on the bodies of active genes.[12]

The generic TF Sp1 was known to be an important regulator of basal G6PD expression[24]; we have now shown that it is also important in terms of response to HDACi. Although Sp1 binding levels at the core promoters of GPPP genes were similar in basal conditions, after treatment with HDACi, they increased only in the promoter of *G6PD*. We identified core promoter Sp1 binding motifs (Motifs 1 through 5) that largely determine the ability of HDACi to induce transcriptional upregulation of *G6PD*. Motif 1 in particular, was also highly enriched in non-GPPP genes that were upregulated in erythroid precursor cells treated with NaBu, suggesting that transcriptional upregulation of *G6PD* in response to HDACi requires, at least in part, the presence of this Sp1-binding motif in the core promoter. Because Sp1 regulates transcription both directly and indirectly through other interactions,[32] we propose the following temporal sequence of changes at the *G6PD* promoter in response to HDACi: histone hyperacetylation, enhanced recruitment of HAT and HDAC and resetting of their dynamic equilibrium, higher promoter chromatin accessibility that allows increased levels of Sp1 binding to the core promoter Motifs 1 through 5, and increased Pol II recruitment resulting in increased rate of transcription.

The clinical features of G6PD deficiency—outside of the neonatal period when it may be associated with neonatal jaundice—are nearly always manifested in red cells. Mature red cells, having lost their nucleus, are unable to replenish G6PD,[14] which physiologically declines as red cells age,[33,34] making them uniquely vulnerable to oxidative stress and ultimately resulting in hemolysis. Nearly all genetic variants of *G6PD* have decreased stability compared with the

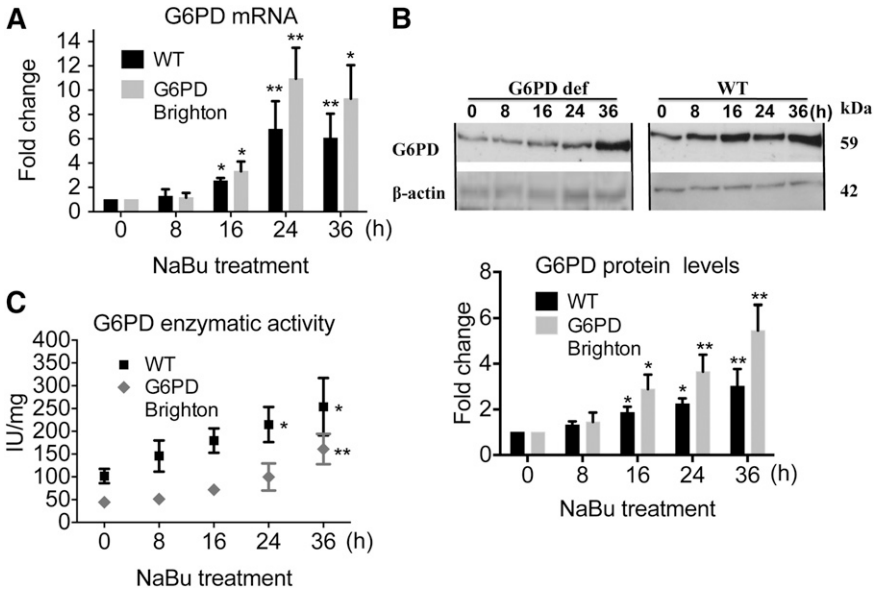

Figure 5. *G6PD* expression and enzymatic activity in cells from patients with G6PD deficiency (def) in response to HDACi. (A) *G6PD* mRNA levels in WT and G6PD-Brighton B-cell lines after treatment with 3 mM NaBu over 36 hours. (B) G6PD protein levels assessed by western blotting (top; representative of 1 of 3 independent experiments is shown) and quantified (n = 3; bottom). (C) G6PD enzymatic activity assessment at the corresponding time points.

WT protein; in many cases, this is due to decreased formation of homodimers and homotetramers, the enzymatically active forms of G6PD.[35,36] From the point of view of ameliorating G6PD deficiency, it is clear that HDACi, by enabling the production of more monomers, would increase the formation of homodimers and homotetramers and therefore of enzyme activity.

Consistent with their deficient status, in *G6PD* variant erythroblasts and B cells, basal G6PD activity was 30% to 50% of normal (Table 1) but still higher than in normal mature red cells (see Table 1 and supplemental Table 1). Overall, we obtained a threefold increase from baseline of G6PD activity with NaBu; whether this could translate into a clinically meaningful increase in mature red cells in vivo, in particular for the most unstable variants such as G6PDMed, remains to be formally addressed. We can predict, however, that the decay curve of G6PD protein in mature aging red cells[33] will shift upwards, thus resulting in an increase of overall G6PD activity throughout their life span.

The ability of HDACi to influence transcription by increasing histone acetylation and thus transcriptional output of certain genes provided the rationale for their use as therapeutic agents in sickle cell anemia and thalassemias, aiming to increase transcription of Hbγ1 and Hbγ2 genes required to make Hb F, which is normally silenced in adult life.[37,38] We reasoned that because HDAC binding is required for transcriptional regulation of active genes, HDACi-induced histone hyperacetylation, with consequent increased transcription over and above basal levels, would also be feasible with respect to housekeeping genes such as those of the GPPP. In fact, despite their similar basal epigenetic profile and similar levels of HDAC binding in particular, only transcription of *G6PD* is further enhanced in response to HDACi. Thus, although lack of specificity is an obvious a priori reservation regarding the use of HDACi, analysis of individual genes shows clearly that these agents are not as unspecific as one might have expected. Indeed, our GEP analysis of two independent data sets from two different types of primary cells (erythroid and $CD4^+$ T cells) shows for the first time that active rather than inactive genes are more likely to be upregulated by HDACi. This provides the rationale for the possible use of HDACi in the treatment of a variety of Mendelian diseases caused by noninactivating mutations in genes that encode enzyme proteins (supplemental Table 2).

The high frequency of *G6PD* polymorphic alleles in areas where malaria is endemic poses a veritable challenge to malaria elimination campaigns, because PQ, which will trigger acute hemolytic anemia in G6PD-deficient individuals, is the only drug capable of eradicating *Plasmodium vivax* hypnozoites.[19] It is intriguing to consider whether a pharmacologically induced, substantial increase (likely considerably higher than the threefold increase achieved by a single dose of HDACi) in *G6DP* transcription before administering PQ might be sufficient to make the hemolytic episode no longer a clinical concern.

Table 1. Effect of NaBu on *G6PD* gene expression and enzymatic activity in normal erythroid cells and in erythroid cells with different *G6PD* mutations

G6PD	Mutation(s) in exon(s)	Amino acid change(s)	NaBu-induced *G6PD* mRNA increase (fold; mean ± SEM)	NaBu-induced G6PD protein increase (fold; mean ± SEM)	G6PD activity (IU/mg protein; mean ± SEM)	
					−NaBu	+NaBu
Normal PB (n = 3)	—	—	4.4 ± 1*	3 ± 0.21*	229 ± 28	642 ± 70*
Normal CB (n = 3)	—	—	3.1 ± 0.41*	3 ± 0.81*	270 ± 10.1	599 ± 25.9*
A−	2, 5	M68V, N126D	15	3.6	105	353
Med	5	S188F	2.6	2.6	80	280
Harilaou	7	F216L	10	4.7	91	387
Serres	10	A361V	6.8	5.7	118	297
Brighton	13	K497D	3.3	4	98	306

G6PD mRNA and protein levels and enzymatic activity in CB $CD34^+$-derived, normal PB-derived, and G6PD-deficient peripheral blood mononuclear cell (PBMC)-derived erythroid precursors. Highly purified day 5 erythroid precursors derived in vitro from CB or PBMCs were treated with 1 mM NaBu or medium control for 48 hours.

*$P < .05$.

In conclusion, epigenetic modulation with HDACi can overcome G6PD deficiency in nucleated erythroid cells from patients with G6PD deficiency, a process that is underpinned by coordinate recruitment of both HAT and HDAC. Their ability to increase transcription of a subset of active genes suggests that HDACi may be of therapeutic value for other Mendelian disorders caused by mutations in genes encoding enzymes.

Acknowledgments

This work was supported by grants from the Medical Research Council, the Sir Halley Stewart Trust, and the National Institute for Health Research Biomedical Research Centre at Imperial College Healthcare National Health Service Trust.

Authorship

Contribution: K.M., V.S.C., J.R.C., D.I., D.R., L.R., and N.B. performed experiments and interpreted data; B.L. performed bioinformatic analysis; D.O., E.T., E.G., D.M.L., and M.P. contributed patient samples; A.K. and I.R. conceived and supervised the work; and A.K., I.R., L.L., and V.S.C. wrote the paper with input from all authors.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Anastasios Karadimitris, Centre for Haematology, Department of Medicine, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK; e-mail: a.karadimitris@imperial.ac.uk.

References

1. Shahbazian MD, Grunstein M. Functions of site-specific histone acetylation and deacetylation. *Annu Rev Biochem.* 2007;76:75-100.
2. Clayton AL, Hazzalin CA, Mahadevan LC. Enhanced histone acetylation and transcription: a dynamic perspective. *Mol Cell.* 2006;23(3): 289-296.
3. Marks PA. Histone deacetylase inhibitors: a chemical genetics approach to understanding cellular functions. *Biochim Biophys Acta.* 2010; 1799(10-12):717-725.
4. Dokmanovic M, Marks PA. Prospects: histone deacetylase inhibitors. *J Cell Biochem.* 2005; 96(2):293-304.
5. Davie JR. Inhibition of histone deacetylase activity by butyrate. *J Nutr.* 2003;133(7 Suppl): 2485S-2493S.
6. Delcuve GP, Rastegar M, Davie JR. Epigenetic control. *J Cell Physiol.* 2009;219(2):243-250.
7. Almeida AM, Murakami Y, Baker A, et al. Targeted therapy for inherited GPI deficiency. *N Engl J Med.* 2007;356(16):1641-1647.
8. Caputo VS, Costa JR, Makarona K, et al. Mechanism of Polycomb recruitment to CpG islands revealed by inherited disease-associated mutation. *Hum Mol Genet.* 2013;22(16): 3187-3194.
9. Gray SG, Qian CN, Furge K, Guo X, Teh BT. Microarray profiling of the effects of histone deacetylase inhibitors on gene expression in cancer cell lines. *Int J Oncol.* 2004;24(4):773-795.
10. Joseph J, Mudduluru G, Antony S, Vashistha S, Ajitkumar P, Somasundaram K. Expression profiling of sodium butyrate (NaB)-treated cells: identification of regulation of genes related to cytokine signaling and cancer metastasis by NaB. *Oncogene.* 2004;23(37):6304-6315.
11. Rada-Iglesias A, Enroth S, Ameur A, et al. Butyrate mediates decrease of histone acetylation centered on transcription start sites and down-regulation of associated genes. *Genome Res.* 2007;17(6):708-719.
12. Wang Z, Zang C, Cui K, et al. Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. *Cell.* 2009; 138(5):1019-1031.
13. Hirono A, Kanno M, Miwa S, Beutler E. Pyruvate kinase deficiency and other enzymopathies of the erythrocyte. In: Scriver CR, Beaudet WS, Valle D, et al, eds. The Metabolic and Molecular Bases of Inherited Disease. Vol. 2. New York, NY: McGraw-Hill; 2001:4637-4664.
14. Luzzatto L, Poggi V. Glucose-6-phosphate dehydrogenase deficiency. In: Orkin SH, Nathan DG, Ginsburg D, Look AT, Fisher DE, Lux SE, eds. *Nathan & Oski's Hematology of Infancy and Chilhood.* 7th ed. Philadelphia, PA: Saunders; 2009:883-907.
15. Luzzatto L, Mehta A, Vulliamy T. Glucose 6-phosphate deficiency. In: Scriver, Beaudet, Valle, et al, eds. The Metabolic and Molecular Bases of Inherited Disease. Vol. 2. McGraw-Hill; 2001:4517-4554.
16. Beutler E. G6PD deficiency. *Blood.* 1994;84(11): 3613-3636.
17. Luzzatto L, Seneca E. G6PD deficiency: a classic example of pharmacogenetics with on-going clinical implications. *Br J Haematol.* 2014;164(4): 469-480.
18. Wells TN, Burrows JN, Baird JK. Targeting the hypnozoite reservoir of Plasmodium vivax: the hidden obstacle to malaria elimination. *Trends Parasitol.* 2010;26(3):145-151.
19. Howes RE, Piel FB, Patil AP, et al. G6PD deficiency prevalence and estimates of affected populations in malaria endemic countries: a geostatistical model-based map. *PLoS Med.* 2012;9(11):e1001339.
20. Chaidos A, Caputo V, Goudevenou K, et al. Potent antimyeloma activity of the novel bromodomain inhibitors I-BET151 and I-BET762. *Blood.* 2014;123(5):697-705.
21. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.* 2003;31(4):e15.
22. Bailey TL, Boden M, Buske FA, et al. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res.* 2009;37(Web Server issue): W202-W208.
23. Yang XJ, Seto E. HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. *Oncogene.* 2007;26(37):5310-5318.
24. Philippe M, Larondelle Y, Lemaigre F, et al. Promoter function of the human glucose-6-phosphate dehydrogenase gene depends on two GC boxes that are cell specifically controlled. *Eur J Biochem.* 1994;226(2):377-384.
25. Franzè A, Ferrante MI, Fusco F, et al. Molecular anatomy of the human glucose 6-phosphate dehydrogenase core promoter. *FEBS Lett.* 1998; 437(3):313-318.
26. Blume SW, Snyder RC, Ray R, Thomas S, Koller CA, Miller DM. Mithramycin inhibits SP1 binding and selectively inhibits transcriptional activity of the dihydrofolate reductase gene in vitro and in vivo. *J Clin Invest.* 1991;88(5):1613-1621.
27. McGonigle DP, Lalloz MR, Wild BJ, Layton DM. G6PD-Brighton: A new class I glucose-6-phosphate dehydrogenase variant due to a deletion in exon 13. *Br J Haematol.* 1998; 101(Suppl.1):117.
28. Poggi V, Town M, Foulkes NS, Luzzatto L. Identification of a single base change in a new human mutant glucose-6-phosphate dehydrogenase gene by polymerase-chain-reaction amplification of the entire coding region from genomic DNA. *Biochem J.* 1990;271(1): 157-160.
29. Vulliamy TJ, Kaeda JS, Ait-Chafa D, et al. Clinical and haematological consequences of recurrent G6PD mutations and a single new mutation causing chronic nonspherocytic haemolytic anaemia. *Br J Haematol.* 1998;101(4):670-675.
30. Georgiou E, Layton M, Karadimitris A. Inherited GPI deficiency: a disorder of histone hypoacetylation. *Birth Defects Res C Embryo Today.* 2009;87(4):327-334.
31. Perissi V, Jepsen K, Glass CK, Rosenfeld MG. Deconstructing repression: evolving models of co-repressor action. *Nat Rev Genet.* 2010;11(2): 109-123.
32. Wierstra I. Sp1: emerging roles—beyond constitutive activation of TATA-less housekeeping genes. *Biochem Biophys Res Commun.* 2008; 372(1):1-13.
33. Morelli A, Benatti U, Gaetani GF, De Flora A. Biochemical mechanisms of glucose-6-phosphate dehydrogenase deficiency. *Proc Natl Acad Sci USA.* 1978;75(4):1979-1983.
34. Marks PA, Johnson AB, Hirschberg E. Effect of Age on the Enzyme Activity in Erythrocytes. *Proc Natl Acad Sci USA.* 1958;44(6):529-536.
35. Cancedda R, Ogunmola G, Luzzatto L. Genetic variants of human erythrocyte glucose-6-phosphate dehydrogenase. Discrete conformational states stabilized by NADP + and NADPH. *Eur J Biochem.* 1973;34(1):199-204.
36. Luzzatto L, Testa U. Human erythrocyte glucose 6-phosphate dehydrogenase: structure and function in normal and mutant subjects. *Curr Top Hematol.* 1978;1:1-70.
37. Olivieri NF, Rees DC, Ginder GD, et al. Treatment of thalassaemia major with phenylbutyrate and hydroxyurea. *Lancet.* 1997;350(9076):491-492.
38. Dover GJ, Brusilow S, Charache S. Induction of fetal hemoglobin production in subjects with sickle cell anemia by oral sodium phenylbutyrate. *Blood.* 1994;84(1):339-343.