Mature erythrocyte membrane homeostasis is compromised by loss of the GATA1-FOG1 interaction

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GATA1 plays essential roles in erythroid gene expression. The N-terminal finger of GATA1 (GATA1-Nf) is important for association with FOG1. Substitution mutations in GATA1-Nf, such as GATA1V205M that diminish the GATA1-FOG1 association, have been identified in human thrombocytopenia and anemia cases. A mouse model of human thrombocytopenia has been established using a transgenic complementation rescue approach; GATA1-deficient mice were successfully rescued from embryonic lethality by excess expression of GATA1V205G, but rescued adult mice suffered from severe thrombocytopenia. In this study, we examined GATA1-deficient mice rescued with GATA1V205G at a comparable level to endogenous GATA1. Mice rescued with this level of GATA1V205G rarely survive to adulthood. Rescued newborns suffered from severe anemia and jaundice accompanied with anisocytosis and spherocytosis. Expression of Sla4a1, Spna1, and Aqp1 genes (encoding the membrane proteins band-3, α-spectrin, and aquaporin-1, respectively) were strikingly diminished, whereas expression of other canonical GATA1-target genes, such as Alas2, were little affected. Lack of these membrane proteins provoked perturbation of membrane skeleton. Importantly, the red cells exhibited increased reactive oxygen species accumulation. These results thus demonstrate that the loss of the GATA1-FOG1 interaction causes a unique combination of membrane protein deficiency and disturbs the function of GATA1 in maintaining erythrocyte homeostasis. (Blood. 2012;119(11):2615-2623)

Introduction

GATA1 is a founding member of the GATA family of transcription factors. Because GATA1, GATA2, and GATA3 are highly expressed in hematopoietic lineages, these factors are collectively referred to as hematopoietic GATA factors.1 The expression of hematopoietic GATA factor genes are strictly regulated through distinct molecular mechanisms, consequently each GATA molecule shows specificity in its expression profile.1 In particular, GATA1 is expressed in lineage-committed cells, such as erythroid, megakaryocytic, eosinophilic, mast, and dendritic cells.2,3 GATA1 plays key roles in regulating the differentiation and survival of these hematopoietic lineage cells. Targeted knockout of the Gata1 gene leads to in utero lethality of the mutant embryos because of defects in primitive and definitive hematopoiesis.4,5 Conditional Gata1 gene knockout gives rise to red cell aplasia and severe thrombocytopenia.6 In addition, GATA1 dysfunction is predictive of specific leukemias in mice and humans.2

GATA1 contains 3 functional domains, that is an N-terminal transactivation (NT) domain, an N-terminal zinc finger (NF) domain, and a C-terminal zinc finger (cf) domain.7 These 3 domains have been shown to be important for GATA1 function.7 In transgenic complementation rescue analyses, GATA1 lacking either the NF or cf domain poorly rescues GATA1-deficient mice from embryonic lethality, which indicates the importance of both NF and cf domains in GATA1 function in vivo.7 The NF domain interacts with FOG1 (friend of GATA1), and also stabilizes the DNA binding of GATA1.8,9 FOG1 was originally identified as a cofactor of GATA1, but it also retains the potential to associate with GATA2.8,10 In the Nf domain of GATA1, specific amino acid residues responsible for the interaction with FOG1 are located on the opposite surface of the protein to those involved in DNA contact.11,12

Clinical observations further support the importance of the Nf domain. Patients harboring substitution mutations in the amino acid residues, which are important either for the FOG1 interaction, or the DNA interaction, suffer from hereditary thrombocytopenia and dyserythropoiesis.13 A human case with the substitution mutation of valine 205 to methionine in GATA1 (GATA1V205M), which impairs the association of GATA1 with FOG1, has been reported. Patients with this mutation rarely survive until birth without in utero red blood cell transfusion.14 Similarly, mice harboring a substitution mutation at valine 205 to glycine (GATA1V205G) were embryonic lethal because of insufficient erythropoiesis.15 These mutant mouse analyses have also shown that the GATA1-FOG1 interaction is essential for the terminal maturation of megakaryocytes, but it is less important in early megakaryopoiesis as long as the GATA2-FOG1 interaction is conserved.15,16 Instead of the Nf domain, the NT domain of GATA1 is required in early megakaryopoiesis.17 Thus, although the function of GATA1 in erythropoiesis appears to be highly dependent on FOG1 and the importance of the GATA1-FOG1 interaction has been delineated in megakaryopoiesis, it still remains to be clarified how FOG1 is involved in GATA1 function during erythroid differentiation.

To address GATA1 function in vivo, we used a transgenic complementation rescue approach. In this approach, wild-type...
(WT) or mutant GATA1 molecules are expressed in transgenic mice under the regulatory influence of the GATA1 hematopoietic regulatory domain (G1HRD). Transgenic expression of WT GATA1 under the regulatory influence of G1HRD nicely rescues GATA1-deficient mice from embryonic lethality, and the rescued mice show normal hematologic indices. In contrast, transgenic expression of specific GATA1 mutants results in hematologic abnormalities. One important advantage of this approach is that transgenic expression of distinct levels of GATA1 is possible. A dyserythropoietic phenotype can be rescued to a certain extent by the expression of excess amounts of mutant GATA1. Consequently, rescued mice with an extended life span enable us to analyze later defects. Indeed, we previously demonstrated that GATA1-deficient pups rescued with transgenic expression of GATA1V205G at a level comparable with endogenous GATA1 rarely survived to adulthood, whereas excess amounts of GATA1V205G created a mouse model that provided a phenocopy of inherited thrombocytopenia in humans.

In this study, we found that the GATA1-FOG1 interaction was especially important for the expression of genes involved in erythrocyte homeostasis. Transgenic expression of the GATA1V205G mutant with decreased ability to interact with FOG1 rescued mice from embryonic lethality. However, the mice were prone to die from acute hemolytic events triggered by delivery at birth because of membrane fragility. This phenotype could be partially avoided by more abundant expression of the GATA1V205G mutant. Consequently, mice occasionally escaped from neonatal death and survived until adulthood with abnormal red cell membrane function. In these rescued mice, expression levels of genes related to membrane integrity were decreased, whereas expression levels of genes for heme biosynthetic enzymes and globins were relatively preserved. Interestingly, we also noted increased accumulation of intracellular reactive oxygen species (ROS). The analysis of these mice provides a novel insight into the causes of the hemolytic anemia associated with hereditary hematopoietic disease.

Methods

Hematologic and scanning electron microscopy analyses

Experimental procedures were approved by the Institutional Animal Experiment Committee of the Tohoku University, and experiments were carried out in accordance with the Regulation for Animal Experiments in Tohoku University. Adult mice were bred from the retro-orbital plexus. Peripheral blood was also collected from perinatal pups euthanized by decapitation, followed by 6-times dilution with saline. Hematopoietic indices were determined using a hemocytometer (Nihon Koden). Reticulocytes were counted after staining blood with new methylene blue using staining capillary tubes (VC-RO75 NM; Terumo). Red cell distribution width (RDW) was calculated by the following formula using the parameters provided by red-cell flow cytometry: RDW (%) = 100 × (SD of red cell volume)/(mean cell volume). For the analysis of drug-induced hemolysis, mice from the age of 6 to 12 months were treated with phenylhydrazine at a dose of 3.75 mg/kg body weight for 2 consecutive days. A small amount of blood sample was collected from the tail vein into microhematocrit tube. Plasma concentrations of total and direct bilirubin were measured using Fuji Dry-Chem system 7000V according to the manufacturer’s protocol (Fuji Film). Indirect-bilirubin was determined by subtracting the concentration of direct bilirubin from the total bilirubin concentration. Plasma concentration of erythropoietin was determined using Quantikine Mouse/Rat Erythropoietin ELISA kit (RD Systems). For the scanning electron microscopy analysis, peripheral blood was fixed in 0.75% glutaraldehyde solution in 0.1M phosphate buffer (pH 7.4). Imaging of samples was conducted using JEOL 5600LV scanning electron microscope (JEOL).

Spleens were fixed in 3.7% formaldehyde solution in 0.02M phosphate buffer (pH 7.4). After embedding in paraffin, the tissues were sectioned and stained with H&E solution. Microscope images were captured by a Leica DMIRE (Leica) and recorded using a DP71 camera (Olympus) and DP Controller software (Olympus). Acquired images were processed with Photoshop Elements 4.0 software (Adobe).

Intracellular ROS accumulation

For the intracellular ROS measurement, erythrocytes were stained with 10μM 2’,7’-dichlorodihydrofluorescein diacetate (Invitrogen Life Technologies) for 30 minutes at 37°C with or without 50μM hydrogen peroxide. Stained cells were analyzed by flow cytometry using FACS-Calibur (Becton Dickinson).

Analysis of erythroid membrane proteins

Peripheral blood was collected in ethylenediaminetetraacetic acid (EDTA) and washed with PBS and then lysed with 1 mL of 1mM hypotonic phosphate buffer (pH 7.4). After centrifugation at 16 000g at 4°C, pellets were resuspended in Laemmli buffer and sonicated. Samples were denatured in the presence of 2-mercaptoethanol and separated by sodium dodecyl sulfate (SDS) 8% polyacrylamide gel electrophoresis (PAGE). To visualize the red cell membrane proteins, the gel was fixed and stained with 0.25% Coomassie brilliant blue-R250 dissolved in 5% acetic acid and 50% methanol solution, subsequently decolorized by 5% methanol and 7% acetic acid solution. For immunoblotting, proteins in SDS-PAGE gel were transferred on to Immobilon-P membrane (Millipore). The membrane was blocked for 1 hour in a solution containing 0.1% triton X-100, 4% nonfat milk, and 1% BSA. Blotted proteins were sequentially probed by primary antibodies and hors eradish peroxidase-conjugated secondary antibodies (Invitrogen-Life Technologies). Immunoreactive signals were detected by enhanced chemiluminescence plus Western blotting detection reagents (GE Healthcare). Antibodies against α-spectrin, β-spectrin, ankyrin, protein 4.1R, and protein 4.2 were as previously described. Antibodies against band-3 (AE1; Millipore), aquaporin-1 (H-55), and actin (AB5; BD Pharmingen) were purchased.

Osmotic fragility test

Peripheral blood was collected in 0.1 mg/mL heparin-sodium solution and subsequently diluted with PBS to obtain a final hematocrit value of 10%. Red cells were suspended in a series of solutions with NaCl ranging from 0.2% to 0.6% as final concentration. After incubation for 30 minutes at room temperature, samples were centrifuged at 700g at 4°C and the supernatant was collected. Hemoglobin concentration in the supernatant was measured from the absorbance at 540 nm with a Spectra-MAX microplate reader (Molecular Devices).

Flow cytometry

Mononuclear cells were prepared from embryonic day 18.5 (E18.5) livers and neonatal spleens, and stained with FITC-conjugated anti-CD71, PE-conjugated anti-TER119, and allopurinol-conjugated anti-c–Kit antibodies (all purchased from BD Pharmingen), for 30 minutes on ice. FITC, PE, and allopurinol-conjugated rat IgG were used as isotype matched controls. Dead cells were excluded by the addition of 2 μg/mL propidium iodide.

Cell sorting

Mononuclear cells prepared from E18.5 livers were treated with Biotin-conjugated anti-TER119 antibody (BD Pharmingen) for 30 minutes on ice. After washing with cold PBS, cells were treated with streptavidin-conjugated Magnet Beads (QIAGEN) for 30 minutes on ice and then resuspended in PBS with 2% FBS. TER119-positive cells were collected by using a magnet stand.

Semi-quantitative and quantitative RT-PCR

Total mRNA from spleen or sorted cells was prepared by using Isogen (Nippon Gene). First strand cDNA was synthesized with Super Script II
reverse transcriptase (Invitrogen Life Technologies). Polymerase chain reaction (PCR) amplification was performed with Ex-Taq DNA polymerase (Takara Bio). The amount of cDNA was adjusted by dilution to give rise to the equivalent amount of Hprt expression. Primer sequences are described in supplemental Table 1 (available on the Blood Web site; see the Supplemental Materials link at the top of the online article). Quantitative reverse transcription (RT)–PCR was carried out using qPCR-Mastermix Plus for SYBR Green I (Eurogentec) with ABI7300 Real-Time PCR system (Applied Biosystems-Life Technologies). Expression levels were normalized to Gapdh. Primer sequences are described in supplemental Table 2.

Chromatin immunoprecipitation
Fetal liver cells were cross-linked with 0.4% formaldehyde and neutralized with glycine. After lysing the nuclei, samples were sonicated to produce DNA fragments of approximately 300 to 500 bps. Aliquots of each sample were kept as input DNA controls. The remaining samples were preclreated using Dynabeads Protein-A (Invitrogen Life Technologies) in combination with affinity pure rabbit anti–rat IgG (Jackson ImmunoResearch Laboratories), subsequently immunoprecipitated with rat anti-GATA1 N6 antibody (Santa Cruz Biotechnology). Normal rat IgG (Santa Cruz Biotechnology) was used as a negative control for immunoprecipitation. After incubation for 1 hour at 55°C with 1% SDS and 0.1M sodium bicarbonate containing 200 μg/mL RNase, cross-links were reversed by incubation at 65°C with the same solution supplemented with 300 μg/mL proteinase K. PCR was performed using primers described in supplemental Table 3.

Statistical analysis
Statistical analyses were performed with a Student t test. Probability values of \( P < .05 \) were considered statistically significant.

Results
Defects in the GATA1-FOG1 interaction cause fatal dyserythropoiesis during the perinatal period

Expression of GATA1V205G mutant protein under the control of G1HRD at levels higher than the WT mouse level can rescue GATA1-deficient (Gata1G1.05/Y) mice efficiently from embryonic lethality.\(^6\) In contrast, we rarely obtained rescued mice when GATA1V205G mutant protein was expressed at levels comparable with endogenous GATA1.\(^6\) To delineate the importance of the GATA1-FOG1 interaction for embryonic erythropoiesis, we conducted in vivo complementary rescue analyses using 2 transgenic mouse lines expressing GATA1V205G at either levels comparable with (line A) or higher than (line B) the WT GATA1 level. For the sake of simplicity, we collectively refer to these rescued mice as V205GR, and the mice rescued with line-A and line-B transgenic mouse lines as V205GR-A and V205GR-B, respectively.

We first determined the genotypentype of embryos during the late gestation period. Numbers of live V205GR-A and V205GR-B embryos were comparable with those expected by Mendelian genetics (Figure 1A open bars), and they were easily distinguishable from WT littermates by their anemic appearance (supplemental Figure 1), indicating that transgenic expression of GATA1V205G at endogenous levels is sufficient to rescue Gata1G1.05/Y mice from embryonic lethality, but the expression appears insufficient for full-rescue of GATA1 function.

In contrast to the rescued pups at embryonic stage, frequencies of rescued pups dropped dramatically within 3 days after birth (Figure 1A filled bars). The expressions of mutant Gata1 transcripts in V205GR-A and V205GR-B pups were comparable with or approximately 8-fold higher than WT Gata1 mRNA, respectively in age-matched WT pups (Figure 1B). These results indicate that a lethal event caused by the disturbed GATA1-FOG1 interaction occurs in V205GR neonates.

V205GR neonates show anemia with erythroid hyperplasia
To investigate the cause of the neonatal death, we measured indices of red cells from 5 V205GR-A and 3 V205GR-B neonates of 0 to 3 days old. Eight WT neonates were used as controls. Red blood cell count (RBC), hemoglobin concentration (Hb), and hematocrit value (Hct), were significantly reduced in V205GR-A and V205GR-B mice (Figure 2A), in addition to a marked reduction in platelet count (data not shown). Mean corpuscular volume (MCV) was also significantly reduced in the rescued pups, whereas mean cell hemoglobin content (MCH) was similar, which indicates cell dehydration. Increased mean cell hemoglobin concentration (MCHC) of red cells of rescued mice confirmed cell dehydration (Figure 2A).

The size of V205GR neonatal spleens was slightly smaller than that of their littermates (data not shown). Histologic examinations revealed that the splenic architecture was altered by expansion of the red pulp area in V205GR-A neonates. The demarcation lines between the red and white pulps were not well defined (Figure 2B). High magnification views showed that the number of nucleated cells was significantly elevated in the red pulp of V205GR-A neonates compared with that of WT littermates, although a considerable number of unucleated RBCs were also observed in the spleen of V205GR-A neonates (Figure 2C).

Flow cytometry analysis showed that the frequency of c-Kit+ TER119+ erythroid cells was slightly increased in V205GR-A neonatal spleens. Of note, whereas the frequency of erythroblasts in the c-Kit+ CD71+TER119+ fraction, which correspond to orthochromatophilic erythroblasts,\(^19\) was preserved in V205GR-A pups, the TER119 expression in V205GR-A erythroblasts was decreased compared with those in WT littermates (mean intensity; 281 vs 2800; Figure 2D). This observation suggests that erythroid differentiation leading up to the stage of orthochromatophilic erythroblasts may not be disturbed markedly in V205GR-A pups, although they have a decreased assembly of glycophorin.\(^20\)
We next examined expressions of erythroid 5-aminolevulinate synthase (Alas2), erythroid Krüppel-like factor (Eklf), erythropoietin receptor (EpoR), and globin (Hba-a1 and Hbb-b1) genes, which are known to be regulated by GATA1. Expressions of these genes were not changed, whereas Gata2 gene expression was elevated in V205GR-A mouse spleens compared with those in WT mice (supplemental Figure 2). Importantly, expression of glycophorin A (Gypa) was preserved in the V205GR spleen. We hypothesize that discrepancy between expression levels of glycophorin A on developing erythroblast and Gypa transcript in V205GR erythroblasts is probably the result of altered membrane trafficking.

V205GR neonates suffer from spherocytic hemolytic anemia

Through serial neonatal examinations, we noticed that V205GR-A neonates were easily distinguished by their yellowish skin from
their littermates. A representative photo is shown in Figure 3A. This phenotype was strikingly different from that of E18.5 rescued embryos (supplemental Figure 1), which suggests that a dramatic hemolytic event was triggered by the delivery. Plasma color was also markedly yellowish with a high concentration of total and indirect-bilirubin (Figure 3B-C). In addition, many poikilocytes and tear-drop shaped cells were observed in blood smear samples (Figure 3D), although such deformed erythrocytes were not seen in the E18.5 rescued embryos (data not shown). In addition, anisocytic and spherocytic erythrocytes lacking central pallor were abundantly observed in both embryos and neonates (Figure 3D).

Scanning electron microscopic analysis revealed that erythrocytes in V205GR-A mice varied in size with a typical spherocytic morphology, whereas those in WT had a discoid appearance (Figure 3E). Using peripheral blood from V205GR pups we performed osmotic fragility tests. Both V205GR-A and V205GR-B erythrocytes are fragile to hypo-osmotic stress (Figure 3F). We also found that the intracellular ROS concentration was significantly elevated in both erythrocytes (Figure 3G). These findings indicate that V205GR neonates are prone to die because of severe hemolytic crisis with spherocytosis.

Interaction of GATA1 and FOG1 is crucial for membrane protein gene expression

Hereditary spherocytosis (HS) is one of the most common inherited human red cell membrane disorders in humans. HS is characterized by a structural abnormality of the red cell membrane because of quantitative and/or qualitative defects in membrane proteins. To measure amounts of plasma membrane proteins, we performed an SDS-PAGE. We found a cluster of membrane proteins including α-spectrin, β-spectrin, and ankyrin almost disappeared in the V205GR-A mouse erythrocytes, whereas band-3, protein 4.1R, and 4.2 were decreased to a lesser extent (Figure 4A). The reduction of major membrane proteins in V205GR-A erythrocytes was confirmed by immunoblotting analyses (Figure 4B). These results strongly argue that deficiency of multiple membrane structural proteins gave rise to the severe hemolysis in V205GR neonates.

Accumulating lines of evidence demonstrate that GATA1 is one of the key molecules involved in the regulation of membrane protein gene expression. It has been reported that cis-regulatory loci in membrane protein genes are cooccupied by GATA1 and FOG1 in erythroid cells. Because membrane proteins progressively accumulate during erythroid differentiation, we hypothesized that accumulation of such proteins might be modified by an imbalance of erythroid differentiation status and/or ineffective erythropoiesis. Therefore, on examination of membrane protein expression in V205GR erythrocytes we sorted TER119+ cells from the E18.5 livers, as TER119 marks erythroblasts in the mature stages.

All membrane structure protein genes examined by RT-PCR were abundantly expressed in WT, whereas expressions of band-3 (Slc4a1) and α-spectrin (Spna1) genes were markedly reduced in V205GR-A embryos (Figure 5A). Expression of the aquaporin-1 (Aqp1) gene was also reduced (Figure 5A). In contrast, expressions of other erythroid-membrane proteins, such as β-spectrin (Spnb1), ankyrin 1 (Ank1), protein 4.1R (Epb41), protein 4.2 (Epb42), and glycoporphin (Gypa) were preserved at levels comparable with those in WT littermates. A representative photo is shown in Figure 3A.

We next examined expression levels of globin genes and erythrocyte metabolic enzyme genes. The Hba-a1, Hbb-b1, and pyruvate kinase (Pkrl) genes in the V205GR embryos were expressed at a normal level (Figure 5A). ε-globin was not detectable in V205GR nor in WT littermates even after 32 amplification cycles, excluding the possibilities that globin chain imbalance or persistent expression of embryonic globin influenced the hemolytic event. On the other hand, expression of the glucose-6-phosphatase dehydrogenase (G6pd) gene varied in individuals; 2 of 6 V205GR-A embryos had a reduced expression level, whereas the expression in the remaining 4 was similar to those of WT littermates. Slc4a1 gene expression was reduced in all 6 embryos (data not shown).

We previously reported that the Nf domain of GATA1 was important for gene regulation of heme biosynthesis enzymes in definitive erythroid progenitors. To explore the contribution of GATA1-FOG1 interaction in heme biosynthesis, we also examined expressions of 8 enzymes of the heme biosynthesis pathway. The expressions of these genes were at normal levels except for the Alas2 gene, which encodes the first and rate-limiting enzyme of the heme biosynthesis pathway in erythroid cells. We found that the Alas2 gene expression decreased and magnitude of the decrease varied substantially in embryos (Figure 5A).

GATA1 binding to membrane protein genes

To verify that the Slc4a1 and Aqp1 genes are direct targets of GATA1 in vivo, we performed chromatin immunoprecipitation...
these loci in our ChIP assays (data not shown).

CIATE with the FOG1-defeciency compromised the GATA1 ability to stably associate with the loci in the promoter region of the Slc4a1 and Aqp1 genes, whereas the GATA motifs in the upstream region of the erythroid-specific genes were comparable with the E18.5 V205GR-A and WT littermate livers (Figure 6), which indicates that the FOGL interaction is dispensable for binding of GATA1 to the first intron regions of these genes.

V205GR-B mice suffer from chronic hemolysis and exhibit hemolytic crisis on exposure to drugs

Although approximately one-half of the V205GR-B pups died during the first 5 days, V205GR-B pups that escaped from neonatal death matured to adulthood (data not shown). V205GR-A pups rarely survived beyond day 5 after birth. We therefore examined the hematologic indices of adult V205GR-B mice. As shown in Figure 7A, they showed mild anemia with reticulocytosis and high RDW. Scanning electron microscopy revealed spherocytic morphology of the V205GR-B mouse erythrocytes (supplemental Figure 4). In adult V205GR-B mice erythropoietin concentration was significantly increased (Figure 7B), and the spleen was enlarged with erythroid hyperplasia (Figure 7C-E), which indicates that active erythropoiensis compensated for the anemia caused by chronic hemolysis. Although no marked decreases in the expression levels of various membrane proteins was noted, the levels of Slc4a1, Spna1, and Aqp1 transcripts were significantly reduced in V205GR-B mice (supplemental Figure 5). V205GR-B mice developed a more severe hemolytic anemia after low dose exposure to phenylhydrazine (Figures 7F-G). The expression of Alas2 and Hbb-b1 genes were unaltered in the V205GR-B mice (supplemental Figure 5), which suggests that individual mice that were able to maintain near normal expression levels of erythroid genes, including Alas2 and Hbb-b1, are spared from the neonatal death and grew up to adulthood with well-compensated hemolytic anemia.

To our surprise, although spherocytes are usually fragile in the hypo-osmotic condition, erythrocytes from adult V205GR-B mice are resistant to hypo-osmotic stress (supplemental Figure 6). Hemolysis occurred less frequently in V205GR-B erythrocytes (ChIP) assays using E18.5 liver cells. Previous transfection studies revealed that the Slc4a1 gene harbors multiple active GATA-binding motifs in the upstream region of the erythroid-specific promoter, although there is no report of active GATA motifs in the Aqp1 gene. Because GATA motifs often reside in the intronic regions of membrane protein genes, in this study we surveyed the sequence similarity of intronic regions between human and mouse genes. We found the presence of conserved GATA motifs in the first intron of the Slc4a1 and Aqp1 genes. In ChIP assays we identified clear accumulation of GATA1 occupancy in the first intron region of these genes (Figure 6). Consistent with these findings, GATA1 activated the expression of Slc4a1 and Aqp1 enhancer-based reporters, although the activity was abrogated by the incorporation of mutations to the GATA motifs (supplemental Figure 3). We could not detect accumulation of GATA1 occupancy in the upstream promoter region of the Slc4a1 gene (data not shown). Similarly, although it has been reported that GATA1 activated the Spna1 gene expression through GATA motifs in the promoter and the region 3' to the promoter and GATA1 occupancy was identified in these regions, 24,25,28 we could not detect GATA1 occupancy in these loci in our ChIP assays (data not shown).

To examine whether FOGL is required for stabilizing GATA1 binding in vivo, we performed ChIP analyses using E18.5 V205GR-A liver cells. FOGL involvement in the DNA binding of GATA1 has been reported to differ between genes, that is, FOGL-deficiency compromised the GATA1 ability to stably associate with the β-globin and Gata2 genes, but not with the c-Kit gene. Importantly, we found that GATA1 occupancies of the first intron region of the Slc4a1 and Aqp1 genes were comparable with the E18.5 V205GR-A and WT littermate livers (Figure 6), which indicates that the FOGL interaction is dispensable for binding of GATA1 to the first intron regions of these genes.

![Figure 5. Expression of several genes coding for membrane proteins was selectively reduced in V205GR erythrocytes.](image)
Discussion

In this study, we found that transgenic expression of GATA1\textsuperscript{V205G} at comparable levels with endogenous GATA1 appears insufficient to fully rescue GATA1-deficient mice, leading to premature death of newborns because of hemolytic anemia. This premature lethality can be abrogated by more abundant expression of the GATA1\textsuperscript{V205G} mutant protein, but high-level expression of GATA1\textsuperscript{V205G} is still insufficient for the maintenance of erythroid homeostasis, which indicates the presence of a dose-dependent compensatory feature\textsuperscript{35} in the GATA1\textsuperscript{V205G} mutant. A recent genome-wide ChIP study revealed that multiple erythroid transcription factors are coassoci-ated in regions close to membrane protein genes and cooccupancy with GATA1 and FOG1 are frequently observed.\textsuperscript{28} Importantly in this study however, we found that the GATA1\textsuperscript{V205G} mutant protein lacking the FOG1 interaction activates Ank1 and Epb41 genes in vivo to levels comparable with those WT GATA1 activates, which indicates that the GATA1-FOG1 interaction is not essential for the expression of these genes even if GATA and FOG1 are colocalized in ChIP analyses. Our study thus demonstrates that there are 2 types of GATA1 target genes in erythroid cells. One type that heavily depends on the GATA1 association with FOG1 and the other that does not depend on the GATA1-FOG1 interaction.

HS is a common congenital hemolytic disorder caused by quantitative and/or qualitative defects in erythroid membrane proteins.\textsuperscript{36} Phenotypic severities of the diseases are closely linked to the membrane protein level, mutation type, and/or genetic background.\textsuperscript{30,37} Abnormality of band-3 and \( \alpha \)-spectrin is the common cause of HS transmitted in an autosomal dominant fashion. In murine models, heterozygotes bearing either \( Slc4a1 \) or \( Spna1 \) amorphic allele experience mild and fully compensated hemolytic anemia.\textsuperscript{38-40} In contrast, both V205GR-A and V205GR-B mutant mice showed phenotype resembling the HS patients with well-compensated hemolytic anemia with relatively normal levels of expression of erythroid membrane proteins. A plausible explanation for this discrepancy between gene expression levels and phenotype is to assume that the reductions of multiple GATA1 target genes, which are participating in erythrocyte stability, synergistically generate the spherocytic phenotype in V205GR-B pups. Indeed, we previously observed that mice carrying excess amounts of a GATA1 mutant molecule lacking the NF domain showed spherocytosis without the reduction of major membrane structure proteins, including band-3 and \( \alpha \)-spectrin.\textsuperscript{41} Therefore, the hemolytic event might be accelerated by the reduction of other membrane skeletal proteins and/or enzymes related to erythrocyte metabolism caused by the disturbed GATA1-FOG1 interaction.

We also found that the expression of the Aqp1 gene is markedly disturbed in the V205GR mouse. Aquaporin-1 is a water-channel abundantly expressed in erythrocytes as well as in kidney. Simple \( Aqp1 \) gene knockout mice died as a result of water depletion with dehydration, but the mice showed no hematopoietic phenotype except for reduced water-permeability of erythrocytes.\textsuperscript{42} A case of human \( AQP1 \) polymorphism trait has been reported showing only a subtle phenotype of reduced red cell surface area and short life span of erythrocytes.\textsuperscript{32} Recently, it was reported that membrane expression of aquaporin-1 was down-regulated during maturation of
reticulocytes into mature erythrocytes and this process is dependent on the osmotic condition of the environment.\textsuperscript{44} We surmise that targeting-deficiency of aquaporin-1 may worsen the hemolysis event in V205GR pups, which may be triggered by the environmental changes during delivery.

The erythrocytes from V205GR neonates appear to be fragile against the hypo-osmotic stress, suggesting that various stresses render the erythrocytes at this stage to the serious hemolytic events. We also found that the intracellular ROS level is elevated in the V205GR erythrocytes. Erythrocytes have an extensive antioxidant defense system because of their fundamental function as oxygen carriers. Although expression of glucose-6–phosphatase dehydrogenase was not significantly changed, ROS detoxification mechanisms might be impaired in V205GR mice. The defect in GATA1-FOG1 interaction may give rise to an antioxidant imbalance in erythrocytes, leading to the destruction of membrane integrity. We surmise that decreased fragility against hypo-osmotic stress in adult V205GR mouse erythrocytes may in part be because of the membrane rigidity caused by membrane protein hyperoxidation, as observed in erythrocytes lacking AMP-activated protein kinase, which is important for maintaining redox homeostasis in erythrocytes.\textsuperscript{45} We propose that the differences between the neonatal and adult V205GR mice in osmotic fragility test and other features are caused by the subtle changes in expression level of GATA1\textsuperscript{V205G} in individuals. V205GR mice with sufficient expression of GATA1\textsuperscript{V205G} to sustain the compensatory erythropoiesis and to overcome acute/chronic hemolytic conditions are selected to develop to adulthood.

In approximately 10% of human HS cases mutations have not been identified.\textsuperscript{46} We envisage at least 2 possibilities as the cause of these unclassified HS cases. One possibility is to assume that mutations reside in regulatory regions of specific membrane protein genes. Indeed, a hypomorphic allele with a mutation in the mouse \textit{Ank} gene promoter has been established and homozygous mutant mice showed an HS-like phenotype resembling the human case.\textsuperscript{47} Together with the finding that the functional binding sites of transcription factors are frequently located in intronic regions,\textsuperscript{28} mutation in such regulatory regions have a latent potential to cause HS. Another possibility is to assume that mutations reside in a molecular critical for regulation of membrane proteins. It has been reported that a single amino acid mutation in EKLF (erythroid Krüppel-like factor) causes spheroctosis in mice because of the impaired DNA binding of this mutant molecule to the dematin gene.\textsuperscript{48} EKLF has also been shown to be important for membrane structural gene expression.\textsuperscript{49} GATA1 dysfunction affects expression of multiple target genes simultaneously and consequently V205GR mice show complicated phenotypes and unexpected alterations in hematologic parameters. For example, the HS-like erythrocytes in V205GR mice are resistant to hypo-osmotic pressure. Based on these results, we propose that the GATA1 gene may be one of the candidates responsible for human unclassified HS.

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Authorship

Contribution: A.H. performed most experiments, and contributed to the project design and preparation of the paper; R.S. and M.Y. contributed to the project design, supervised the study, and wrote the paper; N.M. provided supervised expertise and wrote the paper; and all authors contributed to the integration and discussion of the results.

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