FAM122A Inhibits Erythroid Differentiation through GATA1

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SUMMARY

FAM122A is a highly conserved housekeeping gene, but its physiological and pathophysiological roles remain greatly elusive. Based on the fact that FAM122A is highly expressed in human CD71+ early erythroid cells, herein we report that FAM122A is downregulated during erythroid differentiation, while its overexpression significantly inhibits erythroid differentiation in primary human hematopoietic progenitor cells and erythroleukemia cells. Mechanistically, FAM122A directly interacts with the C-terminal zinc finger domain of GATA1, a critical transcriptional factor for erythropoiesis, and reduces GATA1 chromatin occupancy on the promoters of its target genes, thus resulting in the decrease of GATA1 transcriptional activity. The public datasets show that FAM122A is abnormally upregulated in patients with β-thalassemia. Collectively, our results demonstrate that FAM122A plays an inhibitory role in the regulation of erythroid differentiation, and it would be a potentially therapeutic target for GATA1-related dyserythropoiesis or an important regulator for amplifying erythroid cells ex vivo.

INTRODUCTION

Erythropoiesis, a stepwise process of differentiation by which red blood cells (RBCs) are generated from hematopoietic stem and progenitor cells (HSPCs), is finely controlled by master transcription factors that tightly regulate erythroid-specific gene expression networks (Alvarez-Dominguez et al., 2017; Li et al., 2019; Merryweather-Clarke et al., 2011; Nandakumar et al., 2016; Perreault and Venters, 2018). The core erythroid network of transcription factors is comprised of DNA-binding GATA1, TAL1, and KLF1, as well as non-DNA-binding LDB1 and LMO2 (Nandakumar et al., 2016; Xu et al., 2012). For example, it is well known that erythroid differentiation depends upon GATA-1 in a dose-dependent manner, which is also important for the survival and cell-cycle regulation of erythroid progenitors by erythropoietin (EPO) signaling (Fujiwara et al., 1996; Gutierrez et al., 2020; Xu et al., 2012). Accordingly, GATA1 deficiency arrests erythropoiesis at a proerythroblast stage and induces apoptosis (Fujiwara et al., 1996; Gutierrez et al., 2020).

As a housekeeping gene, FAM122A (also known as C9orf42) is highly conserved among a variety of mammalian species (Eisenberg and Levanon, 2013). Previously, we reported that FAM122A inhibits the phosphatase activity of protein phosphatases of the type 2A family (PP2A) by interacting with its Aα scaffold and B2 regulatory subunits (Fan et al., 2016), a major fraction of cellular Ser/Thr phosphatase activity in any given human tissue, to play important roles in germ cell maturation, embryonic development, metabolic regulation, tumor suppression, and homeostasis of many adult organs (Reynhout and Janssens, 2019). We also demonstrated that FAM122A is critical for maintaining the growth of hepatocellular carcinoma cells and acute myeloid leukemia (AML) cells in a PP2A activity-independent or -dependent manner (Liu et al., 2020; Zhou et al., 2020). However, the biological functions of FAM122A protein are poorly understood to date. Based on the fact that FAM122A is highly expressed in human CD71+ early erythroid cells, here we report that FAM122A significantly inhibits erythrocyte differentiation in primary human erythroid cells and erythroleukemia cells through interacting with and inhibiting the transcriptional activity of GATA1.

RESULTS

Downregulation of FAM122A Expression during Erythroid Differentiation

During erythropoiesis, CD34, CD71, GATA1, and hemoglobin are expressed closely in relation to early and late erythroid progenitors, such as erythroid burst-forming unit (BFU-E) and colony-forming unit (CFU-E) and the early stages of erythroid terminal differentiation. In brief, CD34 is a marker of progenitor cells, such as early erythropoietic progenitors, including BFU-E and CFU-E but is lost with differentiation, while CD71 is expressed in late erythroid progenitors and during the early stages of terminal erythroid differentiation (Chen et al., 2009; Hu et al., 2013; Li et al., 2014). In search of the human FAM122A gene expression in BioGPS datasets (http://biogps.org/dataset/GSE1133/geneatlas-u133a-gcrma/) (Su et al., 2004), we found that FAM122A is highly expressed in

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human CD71+ erythroid cells among all tissues and cells (Figure 1A). Thus, we asked whether FAM122A is involved in erythroid development, during which GATA1 is expressed in both early and late erythroid progenitors and during the early stages of terminal differentiation, but is at peak expression level in late erythroid progenitors at the same time as CD71 expression (Kobayashi and Yamamoto, 2007), and hemoglobin (Hb) is expressed during terminal differentiation. Also, erythroid cells at successive terminal stages of human erythropoiesis were also identified by using the combination of glycophorin A (GPA) and Band 3 (Auffray et al., 2001; Hu et al., 2013). Toward this end, we used hemin at 50 μM to treat human erythroleukemic K562 cells as a cellular model for erythroid differentiation induction, as indicated by benzidine staining-positive (DAB+) cells (an indicator for Hb production) and accumulation of HbG (Rutherford et al., 1979; Wang et al., 2018). More intriguingly, our results demonstrated that FAM122A mRNA and protein levels were gradually decreased upon hemin-induced erythroid differentiation (Figures S1A, 1B, and 1C). We also in vitro expanded primary CD34+ HSPCs from human umbilical cord blood by 100 ng/mL stem cell factor (SCF), 10 ng/mL interleukin-3 (IL-3), and 1 U/mL EPO for 6 days, followed by 3 U/mL EPO for an additional 6 days, as depicted in Figure 1D. Consistent with the previous report (Sun et al., 2015), the treatment effectively induced CD34+ cells to undergo erythroid maturation, as assessed by morphological features and HbA/HbG expression (Figures S1B and 1E). As expected, both FAM122A protein and mRNA were reduced in terminal erythroid differentiation after EPO treatment for 12 days (Figures 1E and 1F). In line, human RBCs are

Figure 1. FAM122A Is Downregulated during Erythroid Differentiation in Human K562 and CD34+ Cells
(A) The expression of FAM122A gene was assessed in a variety of human tissues and cells from the gene annotation BioGPS database. (B and C) K562 cells were treated with 50 μM hemin for the indicated times. The expression levels of FAM122A protein and mRNA were, respectively, examined by western blot (n = 3) (B) and qPCR (n = 3) (C). mRNA level data indicate the means with bar ± SD in an independent experiment (C). (D) Schematics indicated the process of CD34+ cell expansion and differentiation induction by EPO. (E–G) FAM122A levels were examined by western blot (E and G) (n = 3) and qPCR (F) (n = 3), in which the cells were treated as indicated in (D). GAPDH was a protein loading control, and HbG or HbA were used as indicators of erythroid differentiation. FAM122A proteins were quantified according to the densitometric value and the relative protein levels against control cells are shown as means ± SD from three independent experiments bottom panels (B and E) and right panel (G).
Figure 2. The Effects of FAM122A Modulation on EPO-Induced Erythroid Differentiation in CD34+ Cells

CD34+ cells were infected with lentivirus carrying shFAM122A or negative control shRNA (shNC) (A–F), as well as Flag-FAM122A or empty vectors (G–L) for 48 h, followed by induction with EPO at 3 U/mL for 4 days. Lentivirus-infected CD34+ cells with EPO treatment were

(legend continued on next page)
absent of FAM122A protein (Figure 1G). All these results indicate that FAM122A is downregulated during erythroid differentiation.

Inhibition of Terminal Erythroid Differentiation by FAM122A

Next, we attempted to explore the potential roles of FAM122A in erythropoiesis by small hairpin RNA (shRNA)-mediated knockdown in human CD34+ HSPCs from human umbilical cord blood. For this, CD34+ cells were expanded for 4 days and followed by lentivirus infection with specific shRNA against FAM122A (shFAM122A) or a negative control shRNA (shNC). Two days post-infection, a significant silencing effect was confirmed in shFAM122A-expressing CD34+ cells (Figure S1C). By utilizing the flow cytometry-based strategy for isolating human BFU-E and CFU-E (Li et al., 2014), we found that FAM122A knockdown did not impact the amounts of BFU-E (IL-3R+ GPA-CD34+CD36-) and CFU-E (IL-3R+ GPA+CD34+CD36-) populations (Figure S1D), and also failed to affect their colony-forming abilities (Figure S1E). Thus, we examined whether FAM122A knockdown impacts terminal erythroid differentiation in CD34+ cells induced by EPO. Intriguingly, the results showed that FAM122A knockdown significantly increased various globin protein and/or gene expressions (Figures 2A and 2B), and enhanced the percentages of CD71+/GPA+ and GPA+/Band3+ cells (Figures 2C and 2D) and DAB+ cells (Figure 2E). The morphological observation also showed that, under EPO induction for 4 days, the percentages of orthochromatric erythroblasts and reticulocytes were significantly increased in FAM122A knockout (Figures S2J–S2L). Cumulatively, our results indicate that FAM122A is a negative regulator for erythroid differentiation.

Contribution of GATA1 to FAM122A-Regulated Erythroid Differentiation

To identify the possible proteins interacting with FAM122A, we incubated nuclear extracts of K562 cells with the in-vitro-translated GST-FAM122A with GST as a control, followed by GST pull-down. The precipitates were fractionated by SDS-PAGE and stained with Coomassie brilliant blue. The separated proteins by electrophoresis from GST-FAM122A- and GST-bound lysates were excised and further identified by liquid chromatography-tandem mass spectrometry analysis (Table S1). In total, we identified 142 FAM122A-interacting proteins, including GATA1 (Figures 3A and 3B), the latter being further confirmed by western blot (Figure S3). On the other hand, we also performed RNA sequencing to examine the global gene expression profiling of K562 cells with (FAM122A KO no. 1) and without (NC) FAM122A knockout (Figures 3C and 3D). A comparison of the transcriptomes using a statistical cutoff of p < 0.01 and a fold change >1.5 revealed that FAM122A knockout significantly altered the transcriptome of K562 cells with 133 increasing transcripts and 129 decreasing ones (Table S2). By gene ontology analysis, many upregulated genes were closely associated with the components and functions of hemoglobin for molecular functions, cellular components, or biological processes (Figures 3C and 3D; Table S2). Among these upregulated genes, some of which were confirmed by qPCR (Figure 3E), globins (HbA1, HbA2, HbB, HbG1, and HbG2), ALAS2 (a critical enzyme analyzed for the indicated protein expression with western blot (A and G) (n = 3), various globin gene levels with qPCR (B and H) (n = 3), the percentages of CD71+/GPA+ (C and I) (n = 3) and GPA+/Band3+ cells (D and J) (n = 3) with FACS, the percentages of DAB+ cells (E and K) (n = 3), together with morphological observation with Giemsa staining (F and L) (n = 3). The qPCR data were analyzed by normalizing against the corresponding shNC or empty treated with EPO for 4 days (B and H). The related quantitative data are shown on the right panels (A, C, D, E, F, G, I, J, K, and L). The percentages of distinct stages during erythroid differentiation were calculated and shown, in which solid black arrows indicate reticulocytes, dotted blue arrows indicate orthochromatric erythroblasts, dotted red arrows indicate polychromatric erythroblasts, and dotted black arrows indicate proerythroblasts or basophilic erythroblasts. ProE + Baso, proerythroblasts and basophilic erythroblasts; Poly, polychromatric erythroblasts; Ortho + Reti, orthochromatric erythroblasts and reticulocytes (F and L), right panels.
of heme synthesis) (Kaneko et al., 2014), carbonic anhydrase 1 (CA1) (an enzyme for catalyzing the reversible hydration of CO$_2$), and membrane skeleton-related genes SLC4A1 and AQP1 are specific target genes of GATA1 (An et al., 2014).

The above-described results led us to extrapolate that GATA1 has a role in FAM122A-modulated erythroid differentiation. To consolidate this, two pairs of GATA1 shRNAs (shGATA1 no. 1 and shGATA1 no. 2) with shcontrol were infected into K562 cells to knockdown GATA1 in either FAM122A knockdown or shNC cells (Figure 4A). Subsequently, these cells were, respectively, treated with or without hemin at 50 μM for 48 h. As shown in Figures 4B–4D, GATA1 knockdown significantly abrogated the effects of FAM122A silencing-increased hemoglobin and globin gene expressions either in the presence or absence of hemin induction. However, knockdown of GATA2 did not affect the effects of FAM122A silencing on differentiation (Figures S4A–S4C). These results suggest that GATA1 mediates FAM122A-regulated erythroid differentiation.

**Direct Interaction of FAM122A with GATA1**

To elucidate how GATA1 works in FAM122A-regulated erythroid differentiation, we found that FAM122A did not affect GATA1 expression in K562 and CD34$^+$ cells (Figure S4D and S4E). As described above, our protein interactomic analysis showed
that FAM122A interacts with GATA1. To confirm this, 293T cells were co-transfected with Flag-tagged FAM122A and GFP-tagged GATA1, followed by co-immunoprecipitation assays. The results showed that Flag-FAM122A could pull-down GATA1, and GFP-GATA1 could reciprocally precipitate FAM122A (Figure 5A). The physical interaction of endogenous FAM122A and GATA1 was further confirmed in K562 cells (Figure 5B). The immunofluorescence assay also revealed intranuclear colocalization of FAM122A and GATA1 proteins in K562 and CD34+ cells (Figure 5C). The in vitro GST pull-down assay further showed that FAM122A interacts directly with GATA1 (Figure 5D). With a series of GATA1-truncated mutants (Figure 5E), we showed that
Inhibition of the Transcriptional Activity of GATA1 by FAM122A

Considering that the C-terminal zinc finger domain of GATA1 for its interaction with FAM122A is also a critical region for the binding of GATA1 to its target DNA (Ferreira et al., 2005; Kaneko et al., 2012), we tested whether FAM122A affects the DNA binding activity of GATA1. For this, a biotin-labeled DNA probe bearing the core canonical GATA DNA sequence (Cantor and Orkin, 2002) was incubated with purified GATA1 and/or FAM122A proteins expressed in *E. coli*. The electrophoretic mobility shift assay (EMSA) showed that a specific shift of the DNA-protein complex was observed only with the incubation of GATA1 protein (lane 3, Figure 6A) but not with the...
FAM122A protein (lane 2, Figure 6A), while this shift band was competitively eliminated when in the presence of excessive amounts of wild-type (WT) probes without biotin labeling (lanes 6 and 7, Figure 6A), but still appeared in the presence of the excessive probe with mutant DNA binding sequence (lane 8, Figure 6A). A super-shift band could be seen when co-incubating the GATA1 antibody with the GATA1 protein (lane 9, Figure 6A), suggesting that GATA1 specifically and efficiently bound to this probe. As expected, the co-incubation of FAM122A together with GATA1 almost eliminated the shift band produced by GATA1 binding activity (lanes 4–5, Figure 6A).
Furthermore, FAM122A also reduced the super-shift band intensity (lane 10, Figure 6A). These results implied that FAM122A can significantly inhibit the DNA binding activity of GATA1.

These facts promoted us to ask whether FAM122A influences the transcriptional activity of GATA1. For this purpose, 293T cells were co-transfected with luciferase reporters containing the promoter regions of ALAS2 or PGK2 (Surinya et al., 1997; Wu et al., 2014) and increasing amounts of GFP-FAM122A plasmids, together with or without Flag-GATA1. The results showed that FAM122A inhibited GATA1-triggered reporter activity in a dose-dependent manner (Figure 6B).

We further assessed whether FAM122A affects the chromatin occupancy of GATA1 in FAM122A KO and NC K562 cells treated with hemin by a chromatin immunoprecipitation (ChIP) assay, and monitored GATA1 recruitment to several promoter and enhancer regions of erythroid-specific genes, including PBGD, AHSP, and AQP1 (Hasegawa et al., 2012; Welch et al., 2004). As shown in Figure 6C, FAM122A KO significantly enhanced GATA1 chromatin occupancy at the promoter regions of these genes (upper, Figure 6C), suggesting that FAM122A deletion also increases the association of GATA1 with the promoter of its target genes in vivo. In addition, FAM122A overexpression reduced GATA1 chromatin occupancy on the promoters of its target genes (bottom, Figure 6C).

To further investigate the potential role of FAM122A in dyserythropoiesis, we analyzed FAM122A mRNA expression levels in the purified early and late erythroblasts from CD34+ cells, isolated from the peripheral blood of six transfusion-dependent patients with β-thalassemia (before transfusion) and six healthy controls (Forster et al., 2015). We found that FAM122A is significantly upregulated in the patient group with delayed erythroid maturation post-induction with EPO for 14 days (Figure S5A). Furthermore, we examined another dataset from a patient (daughter) and carrier (mother) with β-thalassemia, and her mother in one family with inherited β-thalassemia (Taghavifar et al., 2019), and found that FAM122A expression is also abnormally upregulated in the blood of the patient (daughter) and carrier (mother) with β-thalassemia (Figure S5B).

**DISCUSSION**

In this work, we showed that FAM122A is abundant in erythroid progenitor cells and downregulated during terminal differentiation, similar to the expression pattern of CD71 and/or GATA1. Moreover, FAM122A negatively regulates the terminal differentiation of erythrocytes, but does not affect the early process of erythropoiesis, as determined in either human CD34+ or K562 cells with genetically modulated FAM122A expression, suggesting that FAM122A specifically contributes to the process of terminal erythroid differentiation. How FAM122A expression is regulated during terminal erythroid differentiation remains to be further investigated. According to our results that both mRNA and the protein of FAM122A were downregulated during terminal differentiation, we extrapolated that the regulation of FAM122A expression during erythroid differentiation is mainly involved in its transcriptional level.

The in vitro protein binding assay accompanied with mass spectrometry (MS) analysis and RNA sequencing data showed that GATA1 might be involved in the effects of FAM122A on erythroid differentiation. Knockdown of GATA1, but not GATA2, can significantly rescue FAM122A silencing-enhanced erythroid gene expression and maturation potential, indicating that GATA1 mediates the effect of FAM122A-regulating erythroid differentiation. GATA1 plays a central role in the development of erythrocytes, especially in terminal erythroid differentiation (Moriguchi and Yamamoto, 2014), and abnormal regulation of GATA1 is associated with dyserythropoietic disorders (Ferreira et al., 2005; Gutierrez et al., 2020; Tremblay et al., 2018).

GATA1 activity can be regulated by transcriptional and/or translational regulation, posttranslational modification, and protein-protein interaction (Ferreira et al., 2005; Moreau et al., 2004). FAM122A modulation does not change the mRNA and protein levels of GATA1, excluding the possibility of the regulation by transcriptional or translational levels. The posttranslational modifications of GATA1, including acetylation, phosphorylation, and sumoylation, have been found to regulate its DNA binding and/or transcriptional activity (Gutierrez et al., 2020; Hernandez-Hernandez et al., 2006; Yu et al., 2010). Considering that FAM122A was previously identified as a PP2A inhibitor, we further found that FAM122A modulation did not alter the phosphorylation of GATA1 at Ser142 and Ser310 (data not shown), the latter site being correlated with the binding and transcriptional activities of GATA1 (Kadri et al., 2005; Zhao et al., 2006).

Mounting evidence shows that GATA1 exerts its function by interacting with a serious of cofactors, either co-activators or co-repressors (Ferreira et al., 2005; Gutierrez et al., 2020; Moreau et al., 2004). FAM122A interacts directly with GATA1 and inhibits its DNA binding and transcriptional activities, supporting the notion that FAM122A may act as a co-repressor of GATA1 to suppress the transcriptional activity, since their interaction reduces the association of GATA1 with the target gene promoters, thus interfering with erythroid differentiation. On the other hand, several lines of evidence showed that GATA1 is acetylated at two conserved lysine-rich motifs localized closer
to its C-terminal zinc finger domain and that this modification promotes its transcriptional activity (Lamonica et al., 2006). FAM122A can interact directly with the C-terminal zinc finger of GATA1, thus we do not exclude the possibility that this interaction may affect the acetylation state of GATA1 and/or influence its transcriptional activity, which deserves to be investigated in future.

Recently, we have demonstrated that FAM122A is abnormally upregulated in AML patients and its expression level is negatively correlated with the overall survival of AML patients. More importantly, FAM122A is found to be essential for the growth of AML cells in vitro and in vivo by modulating PP2A activity and sustaining c-Myc protein levels (Liu et al., 2020), showing an essential role of FAM122A in hematological malignancy. In this study, we found that FAM122A is a negative regulator of normal human erythropoiesis process possibly by acting as a corepressor to interfere with the DNA binding and transcriptional activities of GATA1, pointing to the potential and physiological role of FAM122A as a GATA1 coregulator in erythroid differentiation. The aberrant upregulation of FAM122A in patients with β-thalassemia further implies the important role of FAM122A in the regulation of erythropoiesis.

A deep understanding the mechanisms of erythropoiesis is extremely important and necessary for not only generating massive amounts of erythroid cells in vitro or ex vivo for transplantation and therapeutics (Chang et al., 2011; Zeuner et al., 2012), but also for providing the opportunity to govern stress or pathological dyserythropoiesis (such as blood loss, allogeneic stem cell transplantation, anemia, and β-thalassemia). Our findings propose a novel mechanism for the inhibitory effect of FAM122A on the regulation of human erythropoiesis ex vivo using CD34+ cells, and inhibition of FAM122A may enhance the effect of erythroid differentiation and amplify the bulk products of erythroid cells, which will potentially overcome current hurdles in the fields of bulk RBC production due to the lack of blood donor resources and high costs (Zeuner et al., 2012). During the last decade, efficient procedures or technology to produce RBC ex vivo using primary HSCs, embryonic stem cells, or induced pluripotent stem cells, have become an increasing concern to achieve maximal RBC quality, quantity, and maturation. Our results suggest that limitation of the inhibitory effects by negative regulatory factors, such as FAM122A, may significantly enhance the quantity of matured RBCs similar to TRAIL (Migliaccio et al., 2011; Zeuner et al., 2012).

In summary, our study demonstrates that FAM122A plays an inhibitory role in human erythropoiesis in a GATA1-dependent manner by suppressing the DNA binding and transcriptional activities of GATA1. These findings not only elucidate the new function of FAM122A in the regulation of erythropoiesis, but also propose that FAM122A would be a potentially therapeutic target for GATA1-related dyserythropoietic disorders or an important regulator for amplifying erythroid cells ex vivo.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture Conditions**

293T cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen). Human erythroleukemia cells K562 were cultured in RPMI 1640 medium (Invitrogen). All media were supplemented with 10% FBS (Invitrogen) and 1% penicillin/streptomycin. For erythroid differentiation, K562 cells were induced by addition of 50 μM hemin (Sigma, USA).

**Purification and In Vitro Culture of Human CD34+ Cells**

CD34+ cells were purified from human umbilical cord blood by applying CD34+ magnetic selective beads system (Miltenyi Biotec, Germany) according to the manufacturer’s instructions. Cells were cultured at 10⁵ cells/mL for 5–6 days in Serum-Free Expansion Medium (STEMCELL Technologies) supplemented with 10% FBS (STEMCELL Technologies), 100 ng/mL SCF, 10 ng/mL IL-3, and 1 U/mL EPO (STEMCELL Technologies) at 37°C in 5% CO₂ for cell expansion, and then cultured in the medium with the presence of 30% FBS and 3 U/mL EPO for erythroid differentiation at the indicated days (4 or 6 days). CD34+ cells were derived from human umbilical bloods obtained in the Department of Obstetrics and Gynecology of Ren-Ji hospital. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (SJTU-SM and national) and with the Helsinki Declaration of 1975, as revised in 2013. Informed consents were obtained from all patients included in the study.

**Benzidine Staining, Cell Morphology, and Colony Assay**

For benzidine staining, cells were washed twice with ice-cold phosphate-buffered saline. Benzidine dihydrochloride (Sigma, USA) solution was prepared with 0.5 M ethylic acid. One microliter of 30% hydrogen peroxide was prepared and added to a 50-μL benzidine solution immediately before use. Then, 1 μL of fresh whole benzidine solution containing hydrogen peroxide was added in 10 μL cells. The dark blue particles of oxidized benzidine were readily distinguished under a light microscope. Two or three hundred cells (about five fields) were examined in each assay, and the percentages of benzidine-positive cells were calculated. For cell morphology and colony assays, see Supplemental Information.

**Plasmids, RNA Interference, and CRISPR/Cas9**

For details, see Supplemental Information.

**Flow Cytometry Analysis**

To perform flow cytometry analysis of BFU-E and CFU-E cells, 2–5 × 10⁵ CD34+ cells were stained with the following mouse anti-human antibodies: PE-conjugated anti-CD34 (560941, BD
Biosciences), FITC-conjugated anti-CD36 (555454, BD Biosciences), PE-Cy7-conjugated anti-IL-3R (CD123) (4318669, ebioscience), APC-conjugated anti-GPA (551336, BD Biosciences), or appropriate isotype controls (all from BD Biosciences) for 30 min in the dark. To monitor erythroblast differentiation, 2–5 × 10^5 CD34+ cells were labeled with FITC-conjugated anti-CD71 (1930273, ebioscience), FITC-conjugated anti-BAND3 (130-119-780, Miltenyi Biotec), and APC-conjugated anti-GPA (551336, BD Biosciences) for 30 min. Cells were then washed twice with 1 mL phosphate-buffered saline/0.5% BSA. Labeled cells were analyzed for fluorescence emission using a FACSCaliber (BD Biosciences) and Cell Quest Pro software (BD Biosciences/Pharmingen) for acquisition and analysis.

RNA Sequencing and qPCR
See Supplemental Information. RNA sequencing data have been deposited in the Gene Expression Omnibus (GEO).

In Vitro Binding Studies and MS Analysis
GST-FAM122A (10 mg) was incubated with 2 mg of K562 nuclear extracts overnight in buffer containing 20 mM Tris-HCl (pH 7.6); 150 mM NaCl; 1 mM EDTA; 0.5% NP-40; 10% glycerol; 1 mM PMSF; protease inhibitor cocktail. Bound proteins were washed five times in the washing buffer with 50 mM Tris-HCl (pH 7.6); 300 mM NaCl; 1 mM EDTA; 0.5% NP-40; 10% glycerol, separated by SDS-PAGE and visualized by Coomassie brilliant blue staining. The separated proteins by electrophoresis from GST-FAM122A- and GST-bound lysates were excised for identification by liquid chromatography-tandem MS analysis, as described in our previous report (Shen et al., 2018, 2019).

Immunoprecipitation
For detecting the interactions between FAM122A and GATA1 and its domain mapping, 2 × 10^6 cells were harvested with immunoprecipitation lysis buffer (20 mM Tris-HCl [pH 7.6]; 150 mM NaCl; 1 mM EDTA; 0.5% NP-40; 10% glycerol; 1 mM PMSF; protease inhibitor cocktail). After brief sonication, the lysates were centrifuged (1,7000 × g, 15 min) at 4°C. After pre-clearing with normal IgG beads (A0919, Sigma, Germany) at 4°C for 2 h, supernatants were incubated with antibody against GATA1-1 (sc-265, Santa Cruz, CA) or antibody against FAM122A (sc-242677, Santa Cruz), together with protein A/G Plus Agarose gel (A2220, Sigma, St. Louis, MO) or anti-GFP Agarose (D153-8, MBL, CO) overnight at 4°C. After immunoprecipitation, the beads were washed five times with washing buffer (50 mM Tris-HCl [pH 7.6]; 300 mM NaCl; 1 mM EDTA; 0.5% NP-40; 10% glycerol), then the precipitates were analyzed by western blot.

GST Pull-Down
GST alone and GST-tagged FAM122A fusion proteins were expressed in E. coli BL21 by induction with isopropyl β-D-1-thiogalactopyranoside at 28°C for 6 h and purified with GST Bind Resin (Novagen). GATA1 was bacterially expressed as six His-tagged protein, followed by purification using nickel-nitriilotriacetic acid resin (Qiagen). The purified GST or GST-tagged FAM122A proteins were incubated with the purified GATA1 protein for 2 h at room temperature. Then the precipitates were eluted by the SDS sample buffer and followed by western blot.

EMSA
EMSA was carried out using the LightShift Chemiluminescent EMSA Kit (Pierce). In brief, the following double-stranded 5′-biotin-labeled probes or unlabeled cold competitors were synthesized. Oligonucleotides probes harboring two copies of GATA1 binding sequence (sense: 5′-CAGTGGATAACACAAAGT GATAACTCT-3′; antisense: 5′-AGAGTATTACCTTGTTATACA AGTG-3′) and mutant competitor probes (sense: 5′-CAGTGGAC GACACAAAGTAGCGACTCT-3′, antisense: 5′-AGAGTGGCTACT TTGTGTGGCTAAGTG-3′) were synthesized from Sigma. GATA1 and FAM122A protein lysates used in this assay were expressed in vitro by the E. coli BL21, the protein concentration was determined using the BCA protein assay reagent (Pierce). Binding assays were performed in a buffer containing 10 mM Tris (pH 7.6), 50 mM KCl, 1 mM EDTA, 10 mM (NH4)2SO4, 1 mM DTT, 0.2% Tween 20, and 1 μg poly(dI:dC). For the competition assay, 100-200-fold excess of unlabeled WT competitor probes or mutant competitor probes were added to the reaction mixture. Anti-GATA1 antibody (NBp1-47492, Novus) was used for super-shift assay.

Luciferase Assay
For reporter plasmid construction, human ALAS2 gene promoter (−797~ −617 bp) and human proteoglycan 2 gene promoter (−117 ~ −67 bp) were PCR amplified and cloned into pGL3 basic vector (Promega). 293T cells were plated at 5 × 10^5 cells per well in 12-well plates 1 day before being transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 24 h, transfected cells were lysed with passive lysis buffer and lysates were analyzed for both firefly and Renilla luciferase activity using a Dual-Luciferase Reporter Assay Kit (Promega). Luciferase activity was normalized for transfection efficiency using Renilla activity as an internal control (10 ng).

ChIP Assay
ChIP assays were performed using Pierce Agarose ChIP Kit (26156, Pierce Biotechnology, Rockford, IL) according to the manufacturer’s instructions. K562 cells were treated with 50 μM hemin for 48 h, and protein-DNA complexes were crosslinked with 1% formaldehyde for 30 min at room temperature. The reaction was stopped by adding glycine to a final concentration of 125 mM and incubating for 5 min at room temperature. Chromatin solutions were precipitated overnight with rotation at 4°C using GATA1 antibody (NBp1-47492, Novus) or anti-mouse IgG (sc-3877, Santa Cruz) as a negative control. The DNA associated with immunoprecipitates was isolated and used as a template for the PCR to amplify the promoter and enhancer sequences containing the GATA1 binding element. The PCR conditions were as follows: 95°C for 3 min and 38 cycles of 30 s at 94°C, 30 s at 60°C, and 40 s at 72°C, followed by an extension time of 5 min at 72°C. The primer pairs used were as followed: PBGD gene, 5′-TCTAGTCCTACCTGAGGTGCC-3′ and 5′-ACCAAGGCAATGTG CAGTGG-3′, yielding a 231-bp fragment; AHSP gene, 5′-AGGGCT CAGTAAAGTC-3′ and 5′-AGAAGGGAGAGGCTCC-3′, yielding a 186-bp fragment; AQP1 gene, 5′-ATGTCAGGCTGGTATGCC-3′ and 5′-TCTAGTCCTACCTGAGGTGCC-3′, yielding a 231-bp fragment.
CGGCTC-3' and 5'-TGACACCTTATGCATCTGCCTCC-3', yielding a 120-bp fragment. The precipitated DNAs were further analyzed by qPCR. Each sample was detected in triplicate, and the amount of precipitated DNA was calculated as the percentage of input sample.

**Western Blots**

Proteins were fractionated on 10% SDS-PAGE and transferred to the Immobilon PVDF transfer membranes (Millipore, Billerica, MA). The membrane was blocked with 5% nonfat milk or 5% BSA for 1 h and incubated with specific primary antibodies overnight at 4°C. The following primary antibodies were used: anti-GATA1 (3535, Cell Signaling Technology), anti-GATA2 (ab70366, Abcam), anti-hemoglobin a (14537-1-AP, Proteintech), anti-HBG1 (25728-1-AP, Proteintech), anti-HRP-GAPDH (60004, Proteintech), anti-phospho-GATA1 (Ser142) (11041, Signalway Antibody), and anti-phospho-GATA1 (Ser310) (11042, Signalway Antibody). Anti-FAM122A antibody was from ABclonal. The blot was washed three times with PBS-Tween and then incubated with HRP-linked secondary antibodies (7074, Cell Signaling Technology). The signals were detected by chemiluminescence phototope-HRP Kit (Millipore, WBKLS0500) according to the manufacturer's instructions. As necessary, blots were probed with anti-HRP-linked GAPDH (60004, Proteintech) antibody as loading controls.

**Immunofluorescence**

Cells were cytospun on slides and fixed for 10 min at room temperature in 4% formaldehyde and permeabilized in 0.1% Triton X-100 for 15 min at room temperature. Nonspecific sites were blocked by incubation with PBS containing 2.5% BSA for 1 h at room temperature. Cells were then incubated with anti-GATA1 (NBP1-47492, Novus) and anti-FAM122A (NBP2-31646, Novus) antibodies overnight at 4°C. Cells were subsequently washed three times with 1X PBS. Secondary antibodies (Alexa Fluoro secondary 488/595) (z25402/z25407; Invitrogen, Carlsbad, CA) were applied three times with 1X PBS. Cells were subsequently washed three times with 1X PBS. Secondary antibodies (Alexa Fluoro secondary 488/595) (z25402/z25407; Invitrogen, Carlsbad, CA) were applied at 1:200 dilution for 1 h at room temperature. Finally, the cells were incubated in 4,6-diamidino-2-phenylindole for 10 min at room temperature. Stained cells were visualized using a confocal laser scanning microscope Nikon Eclipse Ti (Nikon, Kanagawa, Japan).

**Statistical Analyses**

Data are expressed as mean ± SD and were analyzed by Student’s t test, with p < 0.05 indicating significant difference. All experiments were repeated at least three times.

**Data and Code Availability**

The accession numbers for the RNA-seq data reported in this paper is GEO: GSE141735.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2020.07.010.

**AUTHOR CONTRIBUTIONS**

J.C. designed the research and performed most of the experiments. Q.Z. provided normal human umbilical cord bloods. M.H.L., Y.S.Y., and Y.Q.W. cultured the cells, analyzed the results, and carried out some of the experiments. G.Q.C. and Y.H. designed the research, analyzed and interpreted data, and prepared the manuscript.

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Supplemental Information

FAM122A Inhibits Erythroid Differentiation through GATA1

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Supplemental Information

Supplemental Figures and Legends

**Figure S1.** Effect of FAM122A modulation on colony formation of CD34\(^+\) cells. Related to Figure 1 and Figure 2. (A-B) K562 and CD34\(^+\) cells were respectively treated with hemin or EPO (performed as in Figure 1D) for differentiation induction. Representative images of benzidine staining were photographed by light microscopy (upper panel, A) and the percentages of DAB\(^+\) cells were statistically analyzed by observing 5 random fields (bottom panel, A) in K562 cells (n=3). Representative morphological changes of erythroid differentiation were observed under microscopy in CD34\(^+\) cells by Giemsa staining (B). The high-magnification images (bottom panels) were from the framed areas with dotted lines as indicated cells (n=3). (C-H) CD34\(^+\) cells were infected with lentivirus carrying shFAM122A or negative control shRNA (shNC) (C-E), as well as Flag-FAM122A or empty vectors (F-H) for 48 hours. The knockdown (C) or overexpression (F) effect of FAM122A in CD34\(^+\) cells was examined by western blot (n=3). The infected cells were analyzed for BFU-E (IL-3R GPA\(^-\)CD34\(^+\)CD36\(^-\)) and CFU-E (IL-3R GPA\(^-\)CD34\(^-\)CD36\(^+\)) populations by FACs in FAM122A knockdown (D) or overexpression (G) (left panels of D and G), and the quantitative data were shown as the mean ± SD from 3 independent experiments (n=3) (right panels of D and G). The sorted BFU-E and CFU-E cells were subsequently performed colony forming assay (E, H) and the statistical analysis of colony numbers was shown (bottom panels of E and H, n=3).
Figure S2. Effect of FAM122A modulation on hemin-induced erythroid differentiation of K562 cells. (A-C) K562 cells were infected with lentiviruses expressing two independent sgRNAs targeting different regions of FAM122A (designated as FAM KO#1 and FAM KO#2) and a negative control gRNA (NC) as the control. The pool knockout cells were generated by puromycin selection and followed by the treatment with 50µM hemin for 48 hours. The specific knockout effect of FAM122A was confirmed by western blot and HBG protein was analyzed with GAPDH as a loading control, the related quantitative data were shown on the bottom panels (n=3) (A). Representative images of benzidine staining in FAM122A KO and control cells with hemin treatment were shown (upper panel of B) and the percentages of DAB+ cells were calculated (bottom panel of B) by observing 5 random fields (n=3), qPCR was performed to show the expression of globin genes in FAM122A KO and control K562 cells (n=3) (C). (D-F and J-L) K562 cells were transduced with the indicated lentivirus constructs with or without hemin induction. FAM122A knockdown and reexpression in FAM122A silencing cells (shFAM122A/Flag-FAM122A, D), and overexpression (J), were confirmed by western blot and relative HBG expression was analyzed to normalize against empty or shNC cells, the related quantitative data were shown on the bottom panels (D and J, n=3). Percentages of DAB+ cells were calculated by observing 5 random fields (E and K, n=3) and various globin genes were analyzed by qPCR in these FAM122A modulated cells (F and L, n=3). mRNA expression was calculated after normalization against GAPDH (internal reference) and further against the control cells (NC, empty or shNC) without hemin treatment. The graph presented the means ± SD in an independent experiment (C, F and L). (G-I) The effect of PIP5K1B knockdown on erythroid differentiation, as determined by HBG expression (n=3) (G) and DAB+ cells (n=3) (I).
Figure S3. GATA1 is possibly interacted with FAM122A. Related to Figure 3. The experimental process is shown in Figure 3A. The candidate proteins associated with FAM122A were further confirmed by western blot (n=3).
Figure S4. GATA2 does not participate in hemin-induced erythroid differentiation. Stable shFAM122A and shNC expressing K562 cells were further infected with two pairs of GATA2 shRNAs or non-specific shRNA (shControl) followed by puromycin selection. (A) Knockdown effect of GATA2 was confirmed with western blot (n=3). (B) The indicated cells were treated with or without 50 μM hemin for 48 hours and the cell lysates were immunoblotted with indicated antibodies. The relative HBG expression was analyzed against shNC without hemin induction (right panel, n=3). (C) Representative images of benzidine staining was captured with light microscopy (upper panel) and the statistical analysis of the percentages of DAB⁺ cells by observing 5 random fields (bottom panel, n=3). (D-E) GATA1 expression is not regulated by FAM122A. The expression levels of GATA1 mRNA and protein were examined in FAM122A knockdown and overexpression CD34⁺ treated with EPO (D) and K562 (E) by qPCR and western blot. Data of mRNA expression were presented after normalization against corresponding control cells with means ± SD from an independent experiment. Relative GATA1 protein expression was calculated after normalization with GAPDH and against the corresponding control cells (n=3).
**Figure S5.** *FAM122A* expression is abnormally increased in β-thalassaemia patients.

(A) Heat map shows the mRNA levels of *FAM122A* and various erythroid differentiation-related genes in the *in vitro* cultured erythroid progenitor cells of both β-thalassaemia patients (n=6) and healthy control people (n=6) from GEO dataset (GSE62431). (B) Heat map shows the mRNA expressions of indicated genes from the blood in healthy people (N), clinically asymptomatic carrier for β-thalassaemia (mother, M) and β-thalassaemia patient (daughter, D), from GEO dataset (GSE96060).
**Supplemental tables**

Table S1. Mass spectrometry analysis of proteins derived from K562 cell nuclear extracts bound to GST and GST-FAM122A, Related to Figure 3 (3A, 3B) and Figure S3.

Table S2. Differentially expressed genes upon FAM122A modulation in K562 cells. Related to Figure 3 (3C-3E).
Supplemental Experimental Procedures

RNA interference and lentivirus transduction

FAM122A shRNA (AACTCACCAGCGAAATTC) and GATA1 shRNAs (shGATA1#1: AACTGGAAGCGCCTGATTGTCAGT; shGATA1#2: AACTGGCGCCTGATTGTCA GTAAA) were cloned into shRNA vector pLl3.7-DsRed. The shRNA constructs targeting GATA-2 and PIP5K1B were bought from the DNA library in our school (shGATA2#1: CGGAACCGGAAGATGTCCA; shGATA2#2: AGGCTCGTTCCTGTTCAGA; shPIP5K1B#1: AGGACTTAGATTTCTTGCA; shPIP5K1B#2: GGCCTTATGTCGTGTACAT). The plasmids were transfected into 293T cells along with packaging and envelope plasmids (pMD2G and psPAX2), and viral supernatants were collected after 48 hours to infect target cells. Human CD34+ cells were transduced with lentiviruses and 8μg/ml polybrene by spin-infection at 2000rpm for 2 hours at 37°C on day 5 of the expansion period. For human K562 cells, the cells were infected with lentivirus expressing either shRNAs or Flag-FAM122A together with the corresponding control NC or empty for 48 hours supplemented with 8μg/ml polybrene. The transfection efficiency of the shRNA knockdown or overexpression was examined by western blot and qPCR.

CRISPR/Cas9-mediated knockout of FAM122A

FAM122A was knocked out via CRISPR/Cas9 system in human erythroleukemia cells. Guide RNA (gRNA) sequences (FAM KO#1: ACCGGGGCGCTGTTAGACCTCCTG; FAM KO#2: ACCGACTG AGGCCCTGGATCAGGG) were designed and cloned into a lentiCRISPR vector (pLenti-U6-spgRNA v2.0-CMV-Puro-2F-3Flag-spCas9). 293T cells were cotransfected with lentiCRISPR-gRNA constructs, psPAX2 and pMD2G, and after 48 hour transfection, viral supernatants were collected to infect K562 cells. Adding 1μg/ml puromycin to the infected cells after infection for 48 hours may obtain the selected resistant cells. The pool cells were examined for FAM122A knockout effect by western blot.

Plasmid constructs

The expression plasmids encoding Flag-FAM122A, GFP-FAM122A and GST-tagged FAM122A were constructed previously in our laboratory. Full-length GATA1 and mutant GATA1 plasmids were generated by reverse transcription-polymerase chain reaction (RT-PCR) and cloned the cDNAs into pEGFP-C1. For in vitro protein expression, GATA1 cDNA was sub-cloned into PET-28a vector.

Cell morphology

A total of 2x10^5 cells in 100μl 1×PBS were spun for 3 minutes at 2000 rpm onto glass slides using a cytopin apparatus (Thermo Scientific). After air-drying for 5 minutes, slides were stained with May-Grünwald (Sigma-Aldrich MG500) solution for 5 minutes, rinsed in 40mM Tris buffer (pH 7.2) for 90 seconds, and subsequently stained with 1:20 diluted Giemsa solution (Sigma-Aldrich GS500) for 15 minutes. Stained cells were observed, and images were acquired with an Olympus BX51 microscope and QCapture Pro 6.0 (Tokyo, Japan).

Colony assay

Cells were diluted at a density of 500 cells in 1ml of MethoCult® H4434 classic medium for BFU-E colony assay and 1ml of MethoCult® H4330 medium for CFU-E colony assay (Stem Cell Technologies) and incubated at 37°C in a humidified atmosphere with 5% CO2 in air. CFU-E colonies were counted on day 7 and BFU-E colonies were counted on day 14 after seeding cells into 60 mm dish with the specific medium.

RNA sequencing and analysis
Total RNA was extracted from control and FAM122A knockout K562 cells with two biological replicates for each group. RNA quality was determined with the Bioanalyzer (Agilent2100). cDNA Libraries were prepared using the standard illuminaTruSeq kit and then sequenced using the Illumina 2500 platform. FastQC software was used to check the quality of sequence data and Trimmomatics v0.36 was used to remove adapters and reads with poor quality. The reads were then aligned to the human hg19 reference genome by STAR v2.4.2a. Differential gene expression was determined using EdgeR, a bioconductor package in R and gene annotation enrichment analysis of Gene ontology terms was performed using GOstats, a bioconductor package in R or DAVID Bioinformatics Resources 6.8. And significant differentially expressed genes were chosen according to two criteria: 1) significance level FDR < 0.05, |FC|>=1.5; 2) expression level average FPKM values were larger than 1 in either treatment or control groups.

**Quantitative real-time PCR (qPCR)**

Total RNA was isolated by Trizol kit (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized using the cDNA synthesis kit (Takara, Dalian, Liaoning, China) according to the manufacturer’s instructions. Quantitative PCR was performed using SYBR Green qPCR Master Mixes (Takara, China). Relative expression was determined using GAPDH as the internal control. qPCR was performed in triplicate and standard deviations representing experimental errors were calculated. All data were analyzed using ABI PRISM SDS 2.0 software (Perkin–Elmer). Pairs of PCR primers used to amplify the target genes were shown as followed.

| Gene   | Forward primers               | Reverse primers               |
|--------|-------------------------------|-------------------------------|
| HBA    | CAACCTCAAGCTAAGCCACTGC        | CGGTGCTCAGACAAGGAGCCAG        |
| HBB    | AGGAGAAGTCTGCGCTTACTG         | CCGGACACTTTTCTTGGCATAGA       |
| HBD    | GACTGCTGCAATGCCCTTGT          | AAAGGAACCTAGCACTTTCTT        |
| HBE    | ATGGTGCATTGGTCAAGGAGGGCATG    | GGGGACGACAGGTTCCAAA          |
| HBG    | GAGAAACCCCTGGGAAGGACTTC       | TGTCCTTCATTGGGTT             |
| GAPDH  | CATGAGAATGCTGACACAAGCCTT      | AGTCCTTTCCACGATAACAAATG      |
| FAM122A| AAGATGGAGCTAGACCTGGAG         | CCGGCAAGTGTCGACTGAG          |
| GATA1  | TGGGGACCTCAGACCCCTTG          | GGCTGCATTGGGGAAGTG           |
| PIP5K1B| TGACCCCAAGCACTCAGAC          | GCTCCAGGGTTAGACAGTTT         |