The N-terminal domain of human GATA1 prevents dyserythropoietic anemia and megakaryocyte dysplasia *in vivo*

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Key Points

1. Novel germline mutation within the 5'-UTR of *GATA1* disrupts splicing of full-length GATA1, causing congenital dyserythropoiesis and megakaryocyte dysplasia
2. Human full-length GATA1 isoform is produced exclusively in erythroblasts and megakaryocytes to orchestrate RBC and platelet production

Abstract

Inherited mutations in the X-linked hematopoietic transcription factor *GATA1* lead to a wide range of blood disorders, from Diamond-Blackfan anemia to myelodysplastic syndrome. Alternative splicing produces two functionally distinct isoform of GATA1: full-length GATA1 and short GATA1 (GATA1s), which lacks the N-terminal activity-regulating module. The *in vivo* role of the N-terminal GATA1 domain in human hematopoiesis is not fully understood. Here, we describe the first congenital disease-causing mutation within the 5'UTR of human *GATA1* (*GATA1*$^{c.21A>G}$). We show that loss of full-length GATA1 expression due to disruption of consensus splice site by the *GATA1*$^{c.21A>G}$ mutation results in a constellation of dyserythropoietic anemia; thrombocytosis; functional platelet dysfunction and megakaryocyte dysplasia. We employed a novel immunohistochemistry-based approach to demonstrate that expression of the GATA1 N-terminus is restricted to erythroblasts and megakaryocytes in normal human bone marrow, consistent with impaired erythropoiesis and megakaryopoiesis seen in the *GATA1*$^{c.21A>G}$ patient. Our findings provide novel insights into the clinically relevant *in vivo* function of the N-terminal domain of GATA1 in human hematopoiesis.

Introduction

The X-linked transcription factor GATA1 (globin transcription factor 1) orchestrates the production of red blood cells (RBCs) and platelets (1-3). Alternative RNA splicing produces two GATA1 isoforms (4). Shortened GATA1 (GATA1s) differs from full-length GATA1 (flGATA1) by the lack of the 83 amino acid-long N-terminus that activates GATA1-driven erythropoiesis program (4, 5) and recruits flGATA1 to a subset of megakaryocyte and erythroblast genes (6). Thus, the GATA1 isoforms control partially overlapping but not identical transcriptional modules of erythroblast and megakaryocyte maturation.

Germline *GATA1* mutations occur in a spectrum of blood disorders (7). Mutations affecting the DNA/cofactor-binding zinc fingers of GATA1 cause X-linked macrothrombocytopenia (8, 9) and erythrodysplasia (10). The clinical importance of the N-terminal GATA1 domain was realized upon the discovery of disease-associated mutations disrupting flGATA1 and promoting synthesis of the truncated GATA1s isoform (known as "*GATA1s* mutations" (6)). Germline *GATA1s* mutations are the only non-ribosomal genetic defects implicated in Diamond-Blackfan anemia (DBA) (11, 12), a syndrome of RBC aplasia and otherwise normal hematopoiesis (13). Ribosomopathy in DBA selectively impairs GATA1 translation (14) and GATA1s binds erythroid promoters less efficiently than flGATA1 (5); thus, reduced net GATA1 activity may contribute to the RBC aplasia in DBA. The GATA1s function is not fully conserved between species as baseline postnatal hematopoiesis in *Gata1s* mice appears normal (15), although inhibition of the interferon cascade unblocked megakaryocyte hyper-proliferation in *Gata1s* mice (16), suggesting a persistent signaling defect in postnatal *Gata1s* megakaryocytes.

Since some *GATA1s* patients develop only an isolated RBC aplasia (11, 12), human flGATA1 may be dispensable for megakaryopoiesis. However, all the affected members of one *GATA1s* family developed platelet abnormalities and dyserythropoiesis instead of isolated erythroblast aplasia (17), and an unrelated *GATA1s* patient suffered from a childhood myelodysplastic syndrome (MDS) (18). Thus, the clinical impact of *GATA1s* mutations on non-

erythroid hematopoiesis needs clarification. Here, we identified the first pathogenic mutation within the 5'UTR of human *GATA1* and explored its impact on transcript splicing and hematopoiesis.

Methods

Patient history: A non-dysmorphic anemic male was referred to Bone Marrow Failure Clinic at Riley Hospital for Children. An informed consent was obtained from his family per protocol approved by Indiana University IRB. His folate and vitamin B12 levels were normal. Normal chromosome-breakage test and lymphocyte telomeres excluded Fanconi anemia and dyskeratosis congenita. Normal cytogenetics, MDS-FISH and blast count excluded MDS. Past medical history revealed pyloric stenosis and undescended testis; family history revealed "anemia" on paternal side. *GATA1* sequencing was performed by Prevention Genetics (Marshfield, WI) and DBA sequencing panel (*RPL11*, *RPL35a*, *RPL5*, *RPS10*, *RPS17*, *RPS19*, *RPS24*, *RPS26*, and *RPS7*) by Ambry Genetics (Aliso Viejo, CA).

GATA1 antibodies: The polyclonal GATA1 antibody (Abcam, ab173816) was used to detect both GATA1 isoforms via immunohistochemistry and Western. Novel monoclonal rabbit antibody against first 20 GATA1 residues (Abcam, ab76121) was employed for immunohistochemistry of the GATA1 N-terminus.

Microscopy. Images were acquired on a Zeiss Axiolab-A1 microscope with an Axiocam-105 color camera and processed with ZEN (Carl-Zeiss, Jena) and Imaris (Bitplane, Zurich) software.

Bioinformatics, immunohistochemistry, Westerns and RT-PCR are detailed in **Supplementary Methods**.

Results and Discussion

A 4-year old male developed fatigue and pallor secondary to anemia (hemoglobin: 4.4 g/dL). His growth and development were normal (**Supplementary Figure 1**). DBA was suspected due to progressive macrocytic anemia with low reticulocytes and persistent fetal hemoglobin first noted at 3 months of age (13). Additionally, past blood counts revealed chronic thrombocytosis (platelets: 387-947,000/mm^3) and occasional neutropenia (lowest absolute neutrophil count: 495/mm^3) (**Figure 1A; Supplementary Table 1**).

Bone marrow analysis showed paucity of RBC precursors (**Figure 1B-C**), dyserythropoiesis (**Supplementary Figure 2**) and prominent megakaryocytosis (**Figure 1B-C**) with megakaryocyte dysplasia (**Figure 1B; Supplementary Figure 3**), which is not seen in classic DBA (13). Since the patient's nine DBA-associated ribosome genes were mutation-free, we asked whether his anemia mimicking DBA (11, 12) but associated with megakaryocyte dysplasia (17) reflected a germline *GATA1* defect. Indeed, Sanger sequencing revealed a novel mutation within the 5' untranslated region (UTR) of *GATA1* (*c.21A>G*) at position 48,791,089 on the X chromosome (GRCh38.p2 primary assembly) (**Figure 1D**). Consistent with an X-linked recessive inheritance, mother was an asymptomatic carrier, and all healthy male siblings had wild-type *GATA1* (**Figure 1D**).

We hypothesized the $GATA1^{c.21A>G}$ transcript splicing may be abnormal as our *in silico* analysis (19) suggested disruption of the 5'UTR consensus splice site. Accordingly, the $GATA1^{c.21A>G}$ mutation almost entirely abolished *in vivo* flGATA1 expression and elevated GATA1s production (**Figure 1E-F**), recapitulating the pattern reported in *GATA1s* patients with different mutations (11, 12, 17, 18). Our patient's anemia improved on oral corticosteroids similar to other *GATA1s* patients (11, 12, 18). To our knowledge, the $GATA1^{c.21A>G}$ mutation is the first known human genetic defect producing the *GATA1s* phenotype through destroying splice site within the 5'UTR of *GATA1* (**Figure 1G**).

Full-length GATA1 is a megakaryocyte tumor suppressor in Down syndrome (20-22), but the role of flGATA1 in non-trisomy-21 human megakaryopoiesis *in vivo* is unclear. Several lines of evidence implicate flGATA1 in megakaryopoiesis. First, *GATA1s* patients experience quantitative platelet abnormalities, from thrombocytopenia (17) to thrombocytosis [(12); this work]. Second, *GATA1s* platelets are functionally defective. We found that the *GATA1*$^{c.21A>G}$ platelets display subclinical aggregation deficiencies consistent with a granule defect (**Supplementary Figure 4**). This finding is in agreement with ultrastructural platelet α-granule/dense body abnormalities reported in *GATA1*$^{c.220G>C}$ patients (17), likely secondary to disrupted cytoskeletal remodeling in *flGATA1*-deficient megakaryocytes (23). Third, dysplastic megakaryocytosis in the *GATA1*$^{c.21A>G}$ marrow (**Figure 1**) may reflect increased cell-cycle progression due to the E2F cell-cycle regulator being unleashed from the repression mediated by the N-terminus of GATA1 (24). Our observations validate elegant *ex vivo* studies showing that hematopoiesis in *GATA1s* patient-derived pluripotent stem cells is skewed towards generation of abnormal megakaryocytes at the cost of erythropoiesis (6). However, some *GATA1s* patients were diagnosed with isolated RBC aplasia (11, 12). Thus, we systematically analyzed GATA1 splicing in bone marrow, hypothesizing that flGATA1 expression correlates with the role of flGATA1 in distinct hematopoietic lineages.

To explore GATA1 splicing at single-cell level, we developed an immunohistochemistry assay specific for the GATA1 N-terminus (**Figure 2A**). Full-length GATA1 was absent from the *GATA1*$^{c.21A>G}$ marrow, confirming that the mutation confers *GATA1s* phenotype (**Figure 2B-C**). Double-immunohistochemistry detected flGATA1 in control megakaryocytes and erythroblasts, but not in other hematopoietic lineages (**Figure 2C**), consistent with the role of flGATA1 in erythropoiesis and megakaryopoiesis. Upon further validation, this assay may provide a rapid screening tool for suspected *GATA1s* syndrome.

Our data support *GATA1* sequencing (including non-coding regions) in males with congenital multilineage dysplasia (this work; (17)) and DBA-like phenotype (11, 12). Megakaryocyte and platelet abnormalities (this work; (10)) provide subtle clinical clues to differentiate *GATA1s* patients from DBA caused by ribosomopathy (13). The *GATA1*$^{c.21A>G}$ patient's disease manifestations fall within the broad spectrum of reported *GATA1s*

phenotypes, from DBA (11, 12) to erythroblast and megakaryocyte dysplasia (10) and overt MDS (18) (**Figure 2D**). Potential genotype-phenotype correlations will remain unknown until more *GATA1s* patients are identified, especially since the same *GATA1*$^{c.220G>C}$ mutation caused DBA in one family (12) and multilineage dysplasia in another (10). Interestingly, MDS-associated spliceosome mutations globally alter expression of multiple hematopoietic regulators, including *GATA1* (25). Given the potential risk of MDS in *GATA1s* patients (18), hematopoiesis should be closely monitored in individuals with the *GATA1s* syndrome.

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Authorship

G.N. and J.Z. directed patient's diagnostics and clinical management; J.Z. designed and performed Western blotting and RT-PCR experiments; C.T. and M.C. designed, performed and analyzed immunohistochemistry experiments; G.N. acquired microscopy images and analyzed data; G.N. wrote and all authors edited the paper.

Conflict of interest disclosure

The authors declare no competing financial interests.

Figure legends

Figure 1. Hypoplastic anemia, megakaryocyte dysplasia and thrombocytosis due to mutation within the 5'UTR of GATA1. (A) Chronic anemia and macrocytosis over the course of 5 years. (B) Decreased erythropoiesis and megakaryocyte dysplasia seen on bone marrow aspirate. (C) Immunohistochemistry with CD71 (erythroblast marker) and CD61 (megakaryocyte marker) reveals decreased erythropoiesis and accumulation of megakaryocytes in the patient's bone marrow compared to a healthy individual. Right panel confirms accumulation of dysplastic megakaryocytes (black arrows) in the patient's marrow (Wright-Giemsa stain). (D) *GATA1* sequencing reveals a novel mutation in the affected child. RT-PCR and Western blotting demonstrate loss of full-length GATA1 transcript (E) and protein (F) in the patient. (G) Schematic representation of GATA1 alternative splicing (only first three exons are shown for simplicity). The $GATA1^{c.21A>G}$ mutation produces *GATA1s* phenotype by disrupting full-length GATA1 splicing.

Figure 2. Full-length GATA1 expression is restricted to erythrocyte and megakaryocyte precursors during hematopoiesis. (A) Antibodies used for immunohistochemistry. Antibody against the N-terminus of GATA1 recognizes only full-length GATA1, while C-terminal antibody recognizes both GATA1 isoforms (flGATA1 and GATA1s). (B) Loss of flGATA1 expression in the $GATA1^{c.21A>G}$ patient's bone marrow. Note that (a) flGATA1 is not expressed in all hematopoietic cells of a healthy individual, and (b) GATA1s production is not affected by the $GATA1^{c.21A>G}$ mutation. (C) Expression of N-terminal GATA1 domain (flGATA1; blue) is restricted to erythroblasts and megakaryocytes during human hematopoiesis. Appropriate hematopoietic lineage markers (brown) were co-stained as shown. (D) *GATA1s* mutations cause a range of phenotypes from Diamond-Blackfan anemia (red) to multilineage hematopoietic dysplasia (blue). Novel mutation described in this work is marked with asterisk.

Fig.1 Zucker et al.

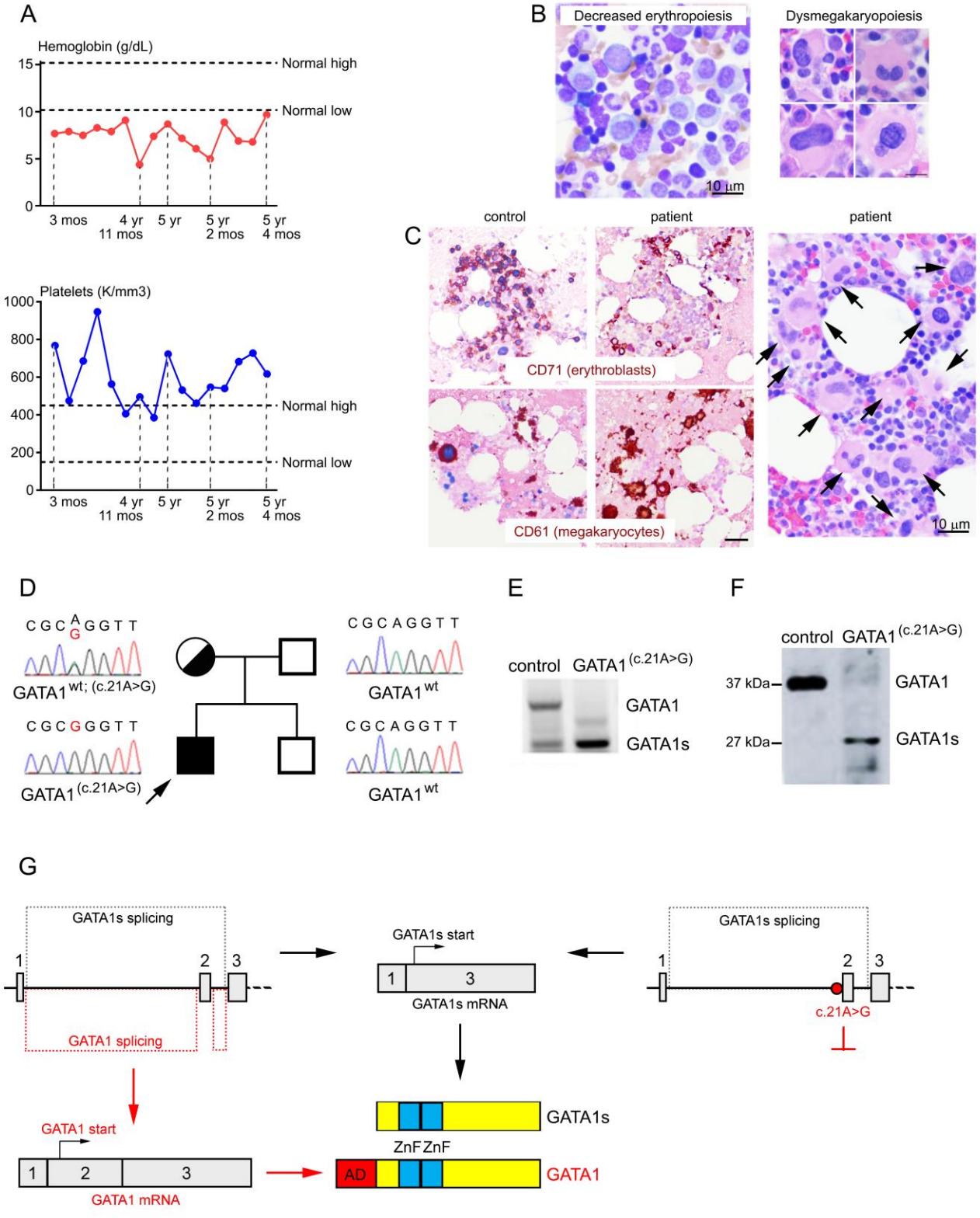
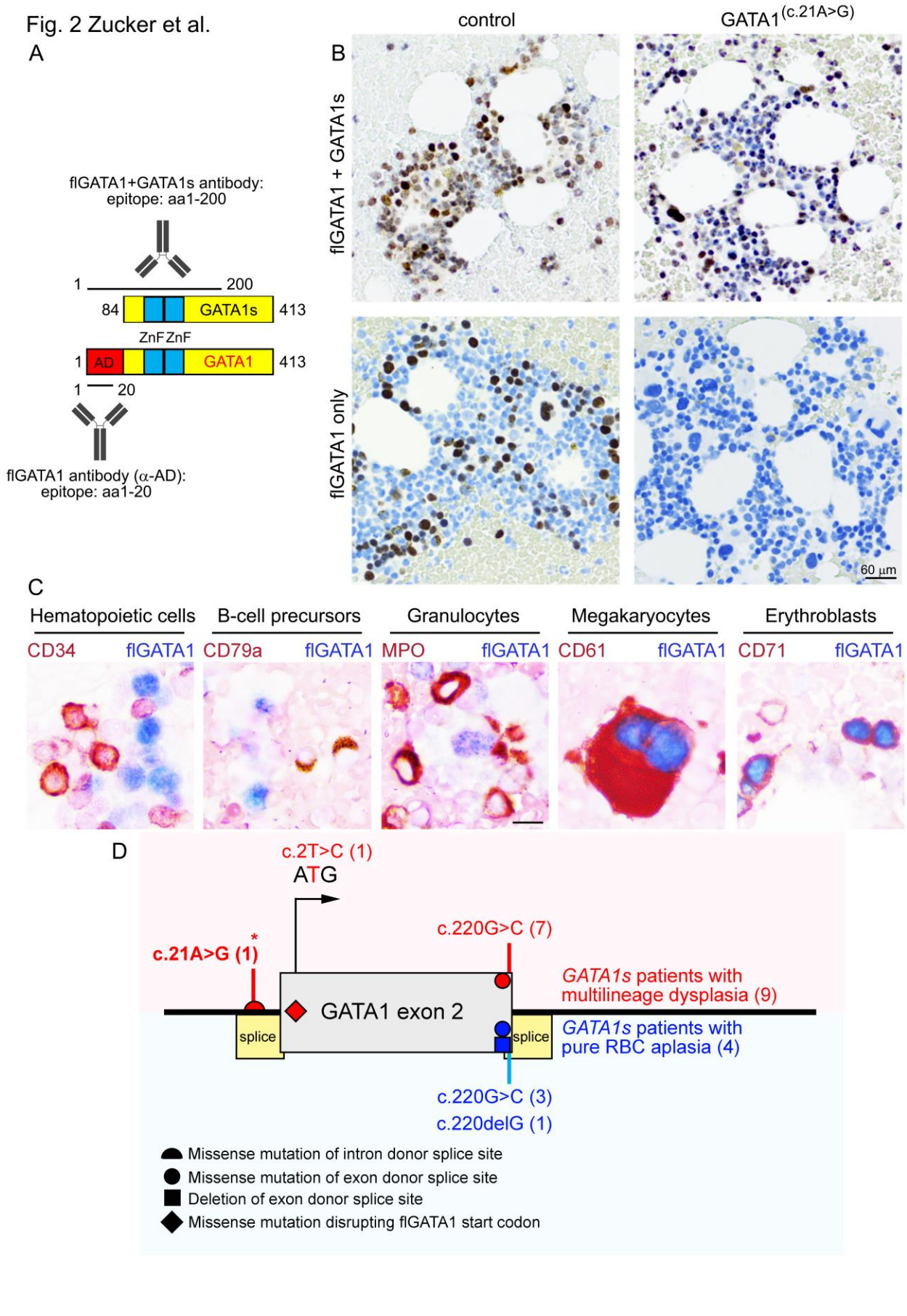


Fig. 2 Zucker et al.



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SUPPLEMENTAL MATERIALS & METHODS

Bioinformatics. *GATA1* splice site analysis and identification of the ctcgcaggttaatcc consensus sequence affected by the novel *GATA1*$^{c.21A>G}$ mutation was performed using the neural network prediction method (2) (http://www.fruitfly.org/seq_tools/splice.html) and validated with a SplicePort analysis tool (3) (http://spliceport.cbcb.umd.edu/SplicingAnalyser.html).

Immunohistochemistry: Formalin-fixed paraffin-embedded tissue blocks were cut in 4-mm sections and picked up onto positively charged slides. The sections were then deparaffinized and rehydrated. The antigen was retrieved in Dako's 'PT Module' with their high pH-Target Retrieval Solution. Endogenous peroxidase was blocked with 3% H2O2 for 5 min. Antibodies against GATA1 (Abcam ab76121, 1:100) were then applied to sections from each case for 30 min. Detection was performed using Dako's Flex+Rabbit avidin–biotin system, which were incubated 30 min each. Horseradish peroxidase (hrp) conjugated to the final reagent developed the brown diaminobenzidine chromogen (DAB, 10 min incubation) and the samples were counterstained with hematoxylin. All washes between steps were in TBS (DAKO K8000). The immunostains were then qualitatively reviewed and assessed for the presence or absence of staining.

Double stains of GATA1 with ready-to-use (RTU), mouse monoclonal antibodies, CD34 (DKO IR632), CD61 (Cell Marque 161M-18), CD79a (DAKO IR621), and p53 (DAKO IR616) and 1:100 CD71 (Invitrogen 13-6890) were performed similarly. Briefly, after retrieval, cocktails of GATA1 and the other antibody of interest placed on the samples and incubate for 30 min. This

was followed by 30 min with Biocare's Mach 2/double stain 2 (mouse-hrp and rabbit-AP, MRCT525L). Then hrp was developed with DAB for 10min followed by alkaline phosphatase (AP) development with Ferengi Blue (Biocare FB813S) for 10 min and the sections were counterstained with neutral fast red.

The one double stain with two rabbit antibodies, GATA1 and myeloperoxidase (MPO, DAKO IR511) was performed with a 7-step technique. First, RTU MPO was incubated for 10 min followed by Envision Flex-hrp (DAKO K8010) and DAB for 10 minutes each. Then samples were incubated with a double stain block (DAKO K5361) for 3 min followed by GATA1 (1:100, 45 min) and swine-anti-rabbit-AP (1:100, 30 min, DAKO D0306). This was developed with Ferengi Blue for 20 min.

Western blotting: Whole blood was obtained from the proband and from normal controls. Protein was isolated by diluting 1 mL whole blood 1:20 with 1X RBC Lysis Buffer (QIAGEN). Samples were spun down at 350 x g for 10'. Supernant was decanted and the resulting pellet was resuspended in an additional mL of 1X RBC Lysis Buffer and spun again. Pellets were washed in phosphate buffered solution x 1. Cells were then incubated on ice for 1 hour in xTractor Cell Lysis buffer (Clontech, Takara Bio) with added 1X cOmplete Protease Inhibitor Cocktail Tablets (Roche). Samples were sonicated at high-power 3 cycles of 30" on and 30" off. Cellular debris was pelleted at 15,000 x rpm at 4^0C for 10 minutes. The resulting supernatant was quantified by Bradford protein assay. 20 µg of sample protein was supplemented with PBS and combined with the appropriate volume of 4X SDS sample buffer for a final volume of 20 µL. Samples were incubated at 80^0C for 10 minutes and spun again at 15,000 x rpm for 5' to

remove insoluble protein. Samples were resolved on NuPAGE Novex 4-12% Bis-Tris Protein Gels (lifeTechnologies, Thermo Fisher Scientific) running with 1X MOPS buffer. Protein was transferred to activated PDVF membranes overnight at 4^0C. Membranes were blocked in 5% milk overnight at 4^0 with gentle rocking. Membranes were incubated with rabbit polyclonal anti-GATA1 antibody (ab173816, Abcam) diluted 1:500 in 5% milk at 4^0C overnight. Membranes were washed in 1X PBST/0.1%Tween for 15 minutes X 4 with gentle rocking at room temperature. The membrane was then incubated with anti-rabbit HRP antibody diluted 1:5000 in 2% milk at RT for 2 hours. A final wash of membranes in 1X PBST/0.1% Tween was performed 15 minutes X 4 with gentle rocking at RT. Blots were developed per manufacturer instruction with SuperSignal West Pico Chemiluminescent Substrate (Pierce,ThermoScientific)

RT-PCR. Total RNA was extracted from peripheral blood of the proband and control with TRIzol Reagent (Life Technologies, Thermo Fisher Scientific Inc.). RT-PCR was performed using on freshly isolated RNA using the OneStep RT-PCR Kit (QIAGEN). RT-PCR primers were designed as previously described (4) for GATA1 exon 1 forward, 5'-ACACTGAGCTTGCCACATC-3' and GATA1 exon 3 reverse, 5'-CACAGTTGAGGCAGGG-TAGAG-3'. RT-PCR conditions were 50^0C for 30 seconds for reverse transcription; 95^0 denaturation for 15 seconds, (95^0C-45 seconds, 57^0C-45 seconds, 72^0C-1 minute) runs for 40 cycles, 72^0C-5' for amplification. PCR products were resolved on a 1.5% agarose gel by electrophoresis, visualized with ethidium bromide and shown on Figure 1E.

Platelet aggregometry: Clinically approved platelet aggregation test was performed at IU Health Pathology Laboratories and interpreted by a certified pathologist. The following platelet agonists

were used: arachidonic acid (500 μg/ml); collagen (1.5 μg/ml); ADP (10 μM), and ristocetin (1 mg/ml) at a stir speed of 1200 rpm for 10 minutes. Healthy control platelets were tested in parallel.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Normal weight (a) and height (b) growth curves of the patient.

Supplemental Figure 2. Binucleated erythroblasts (a-c) with nuclear bridges (d), abnormal chromatin condensation (e-h), nuclear budding (i) and micronucleation (j). Scale bar: 2 μm.

Supplemental Figure 3. Micromegakaryocytes (a) with nuclear lobes connected by chromatin bridges (b), abnormal nuclear morphology (c-e) and decreased cytoplasm:nuclear ratio (e). Scale bar: 10 μm.

Supplemental Figure 4. Platelet aggregation test reveals mild platelet aggregation defect in the *GATA1*$^{c.21A>G}$ patient. Platelet aggregation curves in response to indicated agonists (a) are notable for decreased, partially reversible aggregation upon exposure to arachidonic acid (blue curves and asterisks) in comparison to healthy control. Note minor disaggregation of ADP-induced platelet aggregates (red) and high amplitude of ristocetin-induced platelet aggregation curve (green). Maximum platelet aggregation to indicated agonists is shown in (b).

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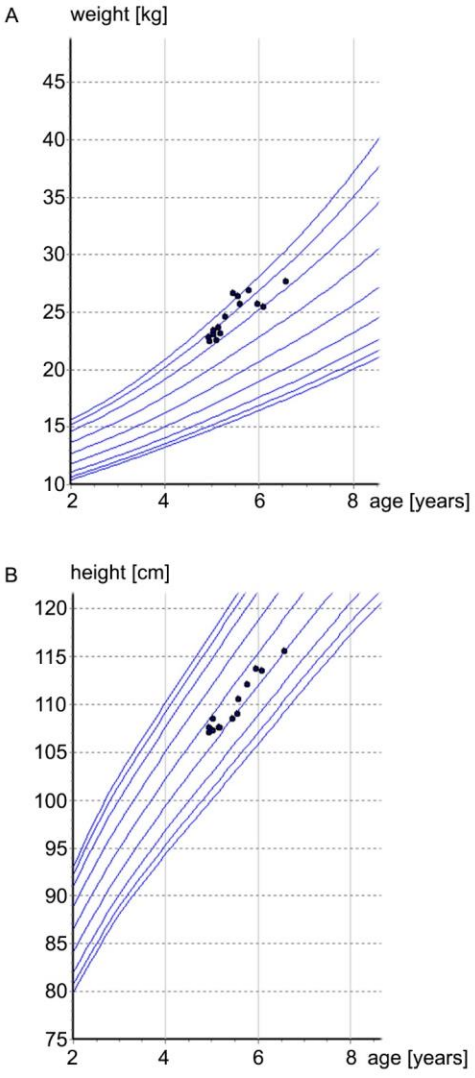
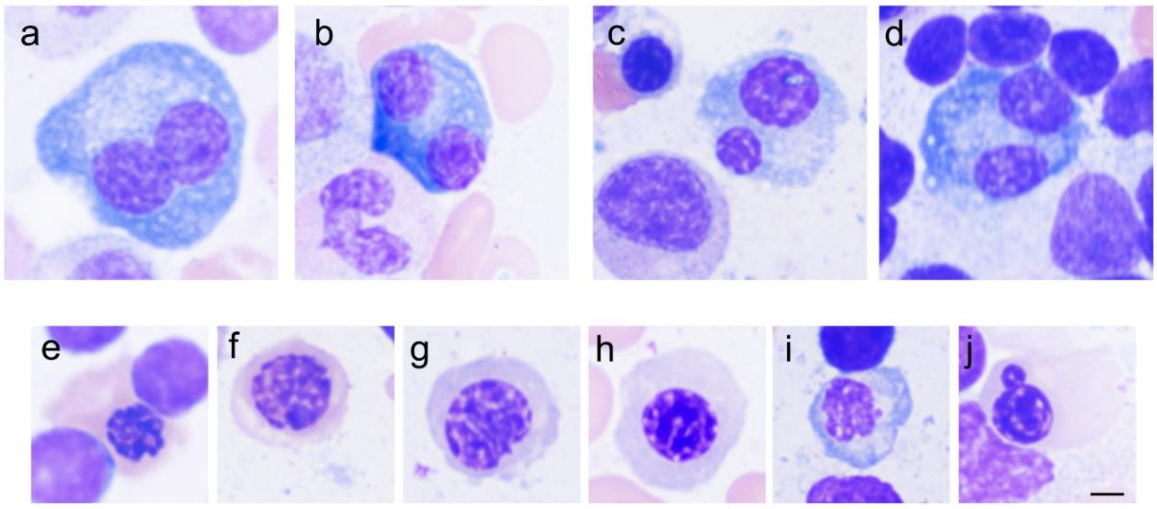
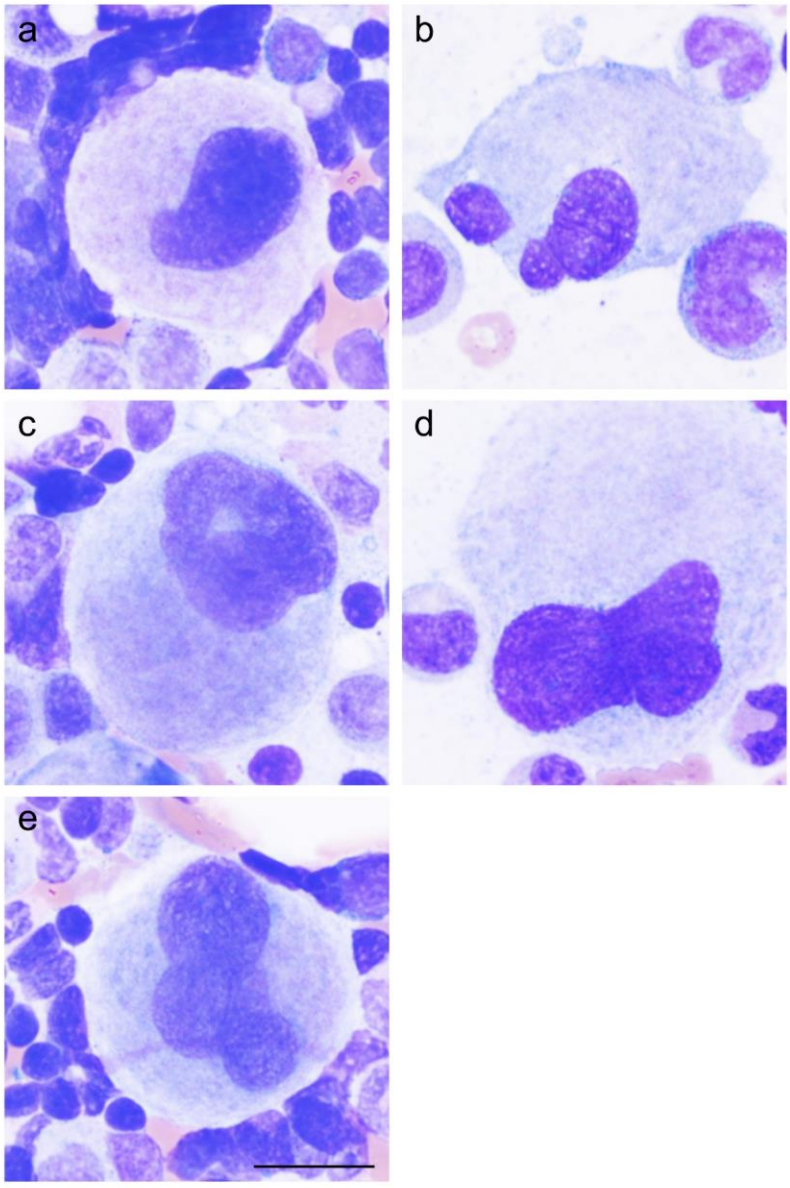
Supplemental Figure 1.



Supplemental Figure 2.



Supplemental Figure 3.



Supplemental Figure 4