Hum Genet (2003) 112 : 42–49
DOI 10.1007/s00439-002-0832-1

ORIGINAL INVESTIGATION

Kathleen Freson · Chantal Thys
Christine Wittewrongel · Jos Vermylen
Marc F. Hoylaerts · Chris Van Geet

Molecular cloning and characterization of the GATA1 cofactor human FOG1 and assessment of its binding to GATA1 proteins carrying D218 substitutions

Received: 26 June 2002 / Accepted: 29 July 2002 / Published online: 16 October 2002

Abstract Erythroid and megakaryocytic lineage differentiation and maturation are regulated via cooperation between transcription factor GATA1 and its essential cofactor friend-of-GATA1 (FOG1). The interaction between these two murine proteins is well studied in vitro and depends on the binding of Fog1 to the N-terminal zinc finger (N-finger) of Gata1. We identified the human *FOG1* gene on chromosome 16q24 and found expression mainly in hematopoietic cells and also in several other tissues. Sequencing of *FOG1* cDNA revealed a 1006 amino-acid protein that contained nine zinc fingers, highly homologous to murine Fog1 fingers. The amino acid sequence and the GATA1-binding capacity of the human and murine finger 5 are however different. Ex vivo binding studies demonstrated that FOG1 interacts with both GATA1 and GATA2. We and others have described patients with mutations in the GATA1 N-finger (V205 M, D218G, D218Y, or G208S), who suffer from macrothrombocytopenia and erythrocyte abnormalities. We now show ex vivo that the interaction between GATA1 and FOG1 is indeed disturbed in platelets and erythrocytes of those patients carrying D218 GATA1 mutations. The identification of the human *FOG1* gene will enable the genetic screening of patients with non X-linked thrombocytopenia and dyserythropoiesis.

The nucleotide sequence for the human friend-of-GATA1 gene has been submitted to the Genbank database under GeneBank Accession no. AF488691

K. Freson · C. Thys · C. Wittewrongel · J. Vermylen
M.F. Hoylaerts · C. Van Geet (✉)
Center for Molecular and Vascular Biology,
University of Leuven,
UZ-Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium
e-mail: Christel.vangeet@uz.kuleuven.ac.be,
Tel.: +32-16-345775, Fax: +32-16-345990

C. Van Geet
Department of Pediatrics, University of Leuven,
UZ-Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium

Introduction

The GATA family of transcription factors binds to (T/A)GATA(A/G) motifs in the promoter and enhancer regions of certain target genes via two highly conserved C4-type zinc fingers (Orkin 1992). Six GATA factors (GATA1 through GATA6) were identified in vertebrates, each displaying a unique temporal and spatial pattern of expression, thereby serving distinct roles in the development of several lineages/tissues (Pevny et al. 1991; Laverriere et al. 1994). GATA1/2/3 are highly expressed in selected hematopoietic cell lines, as confirmed by gene-targeting studies (Pevny et al. 1991; Tsai et al. 1994; Ting et al. 1996; Pandolfi et al. 1995). In contrast, GATA4/5/6 are expressed outside the hematopoietic system, mainly in the heart, gut, and brain (Laverriere et al. 1994; Arceci et al. 1993; Morrisey et al. 1997).

Whereas GATA3 expression is restricted to T-lymphoid cells, GATA2 is highly expressed in hematopoietic progenitors, early erythroid cells, mast cells, and megakaryocytes (Visvader and Adams 1993; Mouthon et al. 1993; Leonard et al. 1993). GATA2 (MIM 137295) is present prior to the formation of the blood islands of the mammalian yolk sac and plays a role in the maintenance of pluripotent stem cells in the hematopoietic system (Tsai et al. 1994; Tsai and Orkin 1997). Slightly later, GATA1 (MIM 305371) is expressed in a highly restricted pattern and regulates the differentiation and maturation of erythrocytes and megakaryocytes (Pevny et al. 1991; Shivdasani 2001).

The transcriptional activator GATA1 does not require a trans-activation domain, but the GATA1 N-finger is absolutely required for terminal erythroid maturation, not through direct DNA binding but via interaction with a cell-restricted nuclear cofactor (Visvader et al. 1995; Weiss et al. 1997). Such a cofactor would link DNA-bound GATA1 (through the C-terminal zinc finger) to other transcription components and thereby provide a combinatorial signal for cell-specific gene expression and differentiation. A yeast two-hybrid protein-interaction screen resulted

in the isolation of such a transcriptional cofactor in the mouse, called friend-of-Gata1 (Fog1; MIM 601950; Tsang et al. 1997). Fog1 is highly expressed in erythroid and megakaryocytic cell lines and in the spleen, liver, and testis (Tsang et al. 1997). The targeted disruption of *Fog1* in mice leads to embryonic lethality, with mice dying of severe anemia between embryonic days 10.5 and 12.5 (Tsang et al. 1998). *Fog1* knockout mice had both impaired primitive and definitive erythropoiesis, with a marked but partial arrest at the pro-erythroblast stage, reminiscent of Gata1-negative erythroid precursors. In contrast to Gata1 deficiency with late megakaryocyte differentiation problems, the loss of Fog1 leads to specific ablation of the megakaryocytic lineage. These findings suggest that Fog1 acts as a cofactor for Gata1 in erythroid cells, but furthermore, that it has a Gata1-independent role in the early stages of megakaryocyte development.

Recently, several patients have been described with an X-linked abnormal hematopoiesis attributable to missense mutations in the N-finger of GATA1 (MIM 300367). The GATA1 mutation V205 M leads to both severe macrothrombocytopenia and dyserythropoietic anemia, mutation D218G to macrothrombocytopenia and mild dyserythropoiesis without anemia, mutation G208S to macrothrombocytopenia, and finally mutation D218Y to deep macrothrombocytopenia, marked anemia, and early mortality (Nichols et al. 2000; Freson et al. 2001, 2002Mehaffey et al. 2001). In vitro binding studies showed that all four GATA1 mutants display a disturbed binding to their essential transcription cofactor Fog1, modeled in these studies by using the murine protein. These observations are compatible with a role of FOG1 as a GATA1 cofactor in late megakaryocyte development. An exact extrapolation of these study results to the human FOG1/GATA1 interaction was however impossible, since the human *FOG1* gene sequence has previously been unavailable.

In the present study, we describe the isolation and characterization of the human *FOG1* gene on chromosome 16q24.3. Human FOG1, like mouse Fog1, contains nine zinc fingers. FOG1 is expressed in hematopoietic cell lines but also in other tissues and, thus, is potentially available for other GATA proteins, such as GATA2. We have tested the importance of FOG1 as a GATA1 cofactor in our patients carrying a mutation in the GATA1 N-finger and have confirmed ex vivo that the association between human FOG1 and GATA1 is indeed disturbed in the platelets and erythrocytes of these patients.

Materials and methods

Nomenclature

Gene symbols used in this article follow the recommendations of the HUGO Gene Nomenclature Committee (Povey et al. 2001).

Cell lines and growth conditions

The human cell lines MEG-01 and K-562 were cultured in RPMI 1640 medium (Life Technologies, Invitrogen, San Diego, Calif.) supplemented with 10% fetal bovine serum (FBS), streptomycin, penicillin, L-glutamine, sodium pyruvate, and non-essential amino acids. Chinese hamster ovarian (CHO) cells were grown in AlphaMEM medium with 10% FBS.

Computer sequence analysis and *FOG1* cDNA cloning

Basic local alignment search tool (BLAST) searches using mouse *Fog1* mRNA sequences were performed against the human high throughput genomic sequences of the National Center for Biotechnology Information to search for homologous human *FOG1* sequences. A human genomic clone (Genbank accession no. AC026467 and NT_024759.3) matched various regions of the mouse *Fog1* sequence.

Oligonucleotides designed from this bacterial artificial chromosome (BAC) clone sequence served as primers for the polymerase chain reaction (PCR) amplification of *FOG1* cDNA in four overlapping fragments from platelet or MEG-01 total RNA. The primer sequences were as follows: FOGF1 (5'-GGGAGGGCGCGCGGCGCCGGAGAC-3'), FOGR4 (5'-AGTAGATCTCACCCTTGGAGCC-3'), FOGF13 (5'-TCCACCACAAGGGACATCCTC-3'), FOGR6 (5'-CAGCTCGGACATCTTGGCCAG-3'), FOGF5 (5'-CTGGCCAAGATGTCCGAGCTG-3'), FOGR7 (5'-CTCGTGGTAGTCGGCCAGCGC-3'), FOGF9 (5'-CAGCCGCACGCTGTGCGAGGC-3'), and FOGR8 (5'-GCTCACTTCACGTGCTCGGCG-3'). After treatment with DnaseI, amplification grade (1 U/µg RNA, Life Technologies), 1 µg total RNA was used for oligo (dT)-primed first-strand cDNA synthesis with M-MLV reverse transcriptase (Life Technologies, Invitrogen). Subsequently, 4 µl of the reaction mixture was PCR-amplified by the gene-specific primers, generating four fragments (FOGF1/FOGR4, FOGF13/FOGR6, FOGF5/FOGR7, and FOGF9/FOGR8). PCR was performed with 0.2 µM each primer, 1× PCRx amplification buffer, 1.5 mM $MgSO_4$, 0× (for fragment FOGF1/FOGR4) – 2× (for fragments FOGF13/FogR6, FOGF5/FOGR7, and FOGF9/FOGR8) PCR×Enhancer solution, 200 mM dNTP, 2.5 U Platinium *Taq* PCR×DNA polymerase (Life Technologies, Invitrogen). PCR was performed in a PTC-100 programmable thermal controller (MJ Research, Watertown, Mass.) under the following conditions: 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 62°C for 1 min, and 72°C for 1.5 min, and a final extension at 72°C for 10 min.

To isolate the full-length cDNA, we performed rapid amplification of cDNA ends (RACE) PCR with the Smart RACE cDNA amplification kit (Clontech, Palo Alto, Calif.) according to the manufacturer's instructions. Gene-specific primer sequences were as follows: FOGR2 (5'-GGCGCAGTAGTAGAGCAGGTG-3'), FOGF11 (5'-GAGCTTCCACAGCCTCGAGGC-3'), and FOGF15 (5'-GTGCGCACGCGCAGACGCCGCAAGC-3').

DNA sequencing

All PCR fragments were cloned into the TOPO-TA cloning vector (Invitrogen, San Diego, Calif.), and automated sequencing by BigDye terminator chemistry (Perkin-Elmer Cetus, Norwalk, Conn.) on an ABI 310 (Perkin-Elmer) sequencer was performed with both vector- and gene-specific oligonucleotide primers (see above). For sequence assembly and editing, we used both the AutoAssembler version 1.45 (PE Biosystems, Norwalk, Conn.) and the DNA strider 1.2 software programs (Marck 1988).

Northern blot and RNA dot blot analysis

Total RNA was isolated from approximately 10^7 cells of the hematopoietic cell lines MEG-01 and K-562 by using TRIzol reagent (Life Technologies, Invitrogen). Total RNA (15 µg) was separated by electrophoresis through formaldehyde-agarose gels. These RNAs were then transferred to Genescreen nylon membrane (NEN Life Science Products, Boston, Mass.). A ^{32}P-labeled *FOG1*-specific PCR fragment (FOGF1/FOGR4) was used as a probe, and hybridization

was performed under high stringency conditions with ExpressHyb (Clontech). This 1200-bp *FOG1* PCR fragment (FOGF1/FOGR4) or a complete *GATA1* PCR fragment were also used to probe a commercial filter, the human Multiple-Tissue Expression Array (MTE, Clontech).

Preparation of cell extracts

Platelet-rich plasma (PRP) from donor blood, anticoagulated with 3.8% (wt/vol) trisodium citrate (9:1), was obtained by centrifugation (15 min at 150 *g*), and platelets were pelleted by recentrifugation of PRP (15 min at 1000 *g*). Erythrocytes and leukocytes were isolated from anticoagulated blood by density gradient centrifugation (Accuspin system-histopaque1077; Sigma). Cell pellets (CHO, MEG-01, K562, erythrocytes, leukocytes, and platelets) were resuspended and lysed in ice-cold PBS containing 1% igepal CA-630 (Sigma) and one complete protease inhibitor cocktail tablet per 50 ml (Complete; Roche), by four cycles of freezing (–80ºC) and thawing (37ºC). Lysates were cleared of insoluble debris by centrifugation at 14,000 *g* for 20 min at 4°C. The total protein concentration was determined via Bradford analysis.

Immunoblot analysis

Cell lysate proteins (100 µg cell extracts) were mixed with Laemmli sample buffer (5% SDS reducing buffer), resolved by SDS/polyacrylamide gel electrophoresis (SDS/PAGE) on 7% acrylamide gels and transferred to Hybond ECL-nitro-cellulose membrane (Amersham, Pharmacia Biotech, Uppsala, Sweden). The blots were blocked for 1 h at room temperature in TRIS-buffered saline with Tween-20 (TBS-T; 0.1% Tween-20) supplemented with 5% non-fat dry milk. Incubation with primary (overnight at 4°C) and secondary (2–3 h at room temperature) antibody was performed in TBS-T with 5% non-fat milk. The primary antiFOG1 polyclonal antibody was produced in our laboratory upon injection of rabbits with a recombinant peptide consisting of the first 380 amino acids of human FOG1 and was purified on protein A Sepharose beads (Pharmacia Biotech); it was used at 50 µg/ml. The antiGATA1 and antiGATA2 monoclonal antibodies were commercially available (Santa-Cruz Biotechnology; 1:1000). The secondary antibody was conjugated with horseradish peroxidase and staining was performed with the Western blotting ECL detection reagent (Amersham, Pharmacia Biotech).

Ex vivo GATA/FOG binding assay by immunoprecipitation

Immunoprecipitation was performed by incubating 1 µg of either a commercial antiGATA1 monoclonal antibody or an antiGATA2 monoclonal antibody (Santa-Cruz Biotechnology) with protein A Sepharose beads and 100 µg of cell lysate proteins (cell extracts) in 300 µl binding buffer consisting of 150 mM NaCl, 25 mM TRIS pH 7.5, 0.1% Igepal CA-630 (Sigma), 10 µM $ZnSO_4$, 0.25% BSA, 1 mM β-mercaptoethanol, and a protease inhibitor cocktail tablet per 50 ml, for 1 h at 4°C. After centrifugation and washing the protein A-coupled GATA antibody beads three times in 1 ml binding buffer, the bound FOG1 was determined by Western blotting (as described above). Beads were mixed with Laemmli sample buffer (5% SDS reducing buffer), resolved by SDS/PAGE on 7% acrylamide gels, and electro-transferred.

In vitro GATA1/FOG1 binding assay by the GST pull-down assay

Human FOG1 finger 9 (969–1006), human FOG1 finger 5 (579–600), and mouse Fog1 finger 5 (592–613) were generated by PCR, cloned in the expression vector pGEX-5X-1 (Pharmacia Biotech), and produced in *Escherichia coli*. Complete *GATA1*, *GATA1-D218G*, *GATA1-D218Y*, and *GATA-2* were cloned in pcDNA3.1 (Invitrogen) and ^{35}S-labeled GATA1 (mutant) was produced by in vitro transcription/translation with the TNT system (Promega, Madison, Wis.). Primer sequences are available on request. In vitro binding studies were performed as described previously (Tsang et al. 1997; Fox et al. 1998).

Results

Identification of the human *FOG1* gene and expression pattern

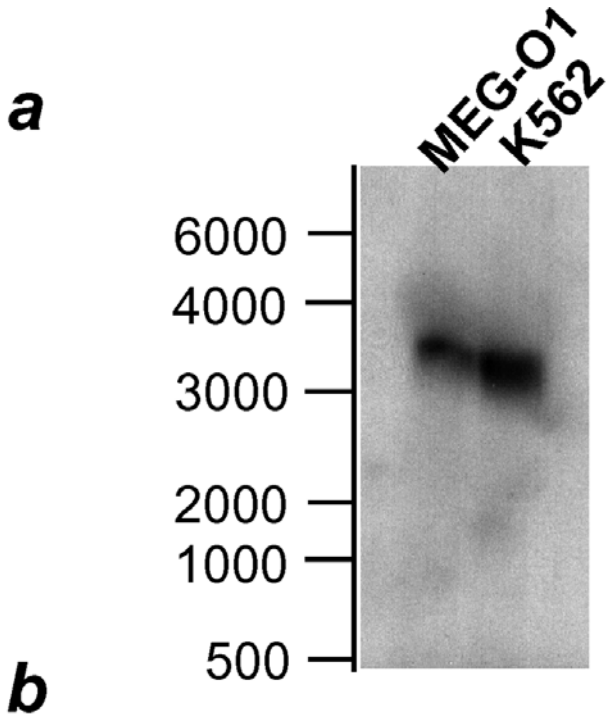
We expected a strong sequence homology between murine Fog1 and human FOG1, primarily restricted to their zinc fingers, as was found earlier between murine Fog1 and human FOG2 and even the *Xenopus laevis* homolog of FOG (xFOG; Holmes et al. 1999; Deconinck et al. 2000). The known murine *Fog1* zinc fingers 1–4 and finger 9 sequences were used as queries in the BLAST sequence-homology search against the human genomic nucleotide-sequence database. A BAC clone (RP11–410M9) showed 86% homology overall with the *Fog1* zinc finger sequences and was located on chromosome 16q24.3 (between markers D16S3026 and D16S2621). Based on these sequences, primers were designed to isolate the complete human *FOG1* cDNA by reverse-transcription-PCR and RACE-PCR on RNA isolated from platelets and the megakaryocytic cell line MEG-01. The transcript contained an ORF of 3018 bp potentially encoding a protein of 1006 amino acids.

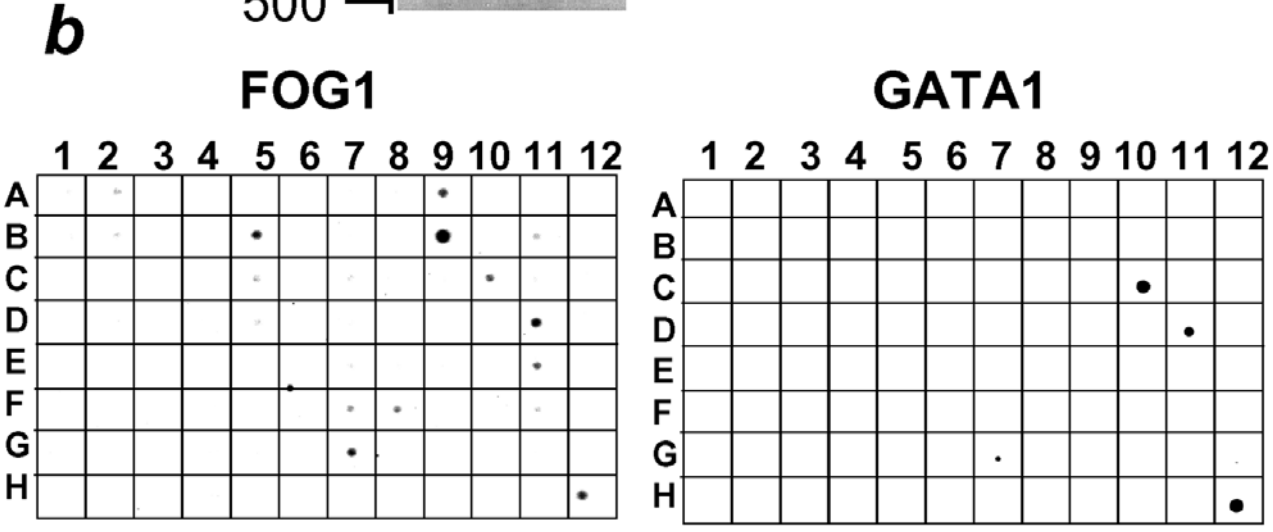
We performed Northern blot analysis on two human cell lines representing an erythroid (K-562) and megakaryocytic (MEG-01) cell line by using a *FOG1*-specific probe (comprising the first 1200 bp, including zinc finger 1). A 3.3-kb *FOG1* transcript of the expected size was detected (Fig. 1a) in both cell lines. We then evaluated the *FOG1* and *GATA1* expression in a dot-blot array of RNAs isolated from 50 human adult and embryonic tissues (Fig. 1a). The *FOG1* mRNA was found not only as expected in bone marrow and K-562 cells but also in other tissues such as adult cerebellum, stomach, testis, lymph node, liver, and pancreas, and fetal heart, liver, and spleen. In contrast, *GATA1* was only found in bone marrow, K-562 cells, and fetal liver.

Primary structure of human FOG1 and in vitro interaction with GATA

The predicted amino acid sequence of human FOG1 and the sequence homology with mouse Fog1 are shown in Fig. 2. Human FOG1 exhibits 76% identity with the murine sequence, sharing highly conserved zinc finger sequences. Of the nine zinc fingers in human FOG1, only numbers 4,

Fig. 1 **a** *FOG1* and *GATA1* expression. Northern blot analysis of ▶ megakaryocytic (*MEG-01*) and erythroid (*K-562*) cell lines with a probe comprising the first 1200 bp of human *FOG1*. A single signal of about 3300 bp was detected. RNA size in basepairs is shown *left*. **b** Multiple-tissue RNA dot blot analysis of a blot containing an array of adult and fetal human tissues, as listed *below*, was performed with either a probe comprising the first 1200 bp of human *FOG1* or the complete human *GATA1*





	1	2	3	4	5	6	7	8	9	10	11	12
A	whole brain	cerebellum left		heart	esophagus	colon transverse	kidney	lung	liver	leukemia HL-60	fetal brain	yeast total RNAc
B	cerebral cortex	cerebellum right	accum-bens nucleus	aorta	stomach	colon desending	skeletal muscle	placenta	pancreas	HeLa S3	fetal heart	yeast tRNA
C	frontal lobe	corpus callosum	thalamus	atrium left	duodenum	rectum	spleen	bladder	adrenal gland	leukemia K-562	fetal kidney	E.coli rRNA
D	parietal lobe	amygdala		atrium right	jejunum		thymus	uterus	thyroid gland	leukemia MOLT-4	fetal liver	E.coli DNA
E	occipital lobe	caudate nucleus		ventricle left	ileum		leukocyte	prostate	salivary gland	Burkitt's lymphoma Raji	fetal spleen	poly r(A)
F	temporal lobe	hippo-campus		ventricle right	ilocecum		lymph node	testis		Burkitt's lymphoma Daudi	fetal thymus	human C_0t-1DNA
G	pg of cerebral cortex	medulla oblongata		inter-ventricular septum	appendix		bone marrow	ovary		colorectal adeno-carcinoma	fetal lung	human DNA 100 ng
H	pons	putamen		appex of heart	colon ascending		trachea			lung carcinoma A549		human DNA 500 ng

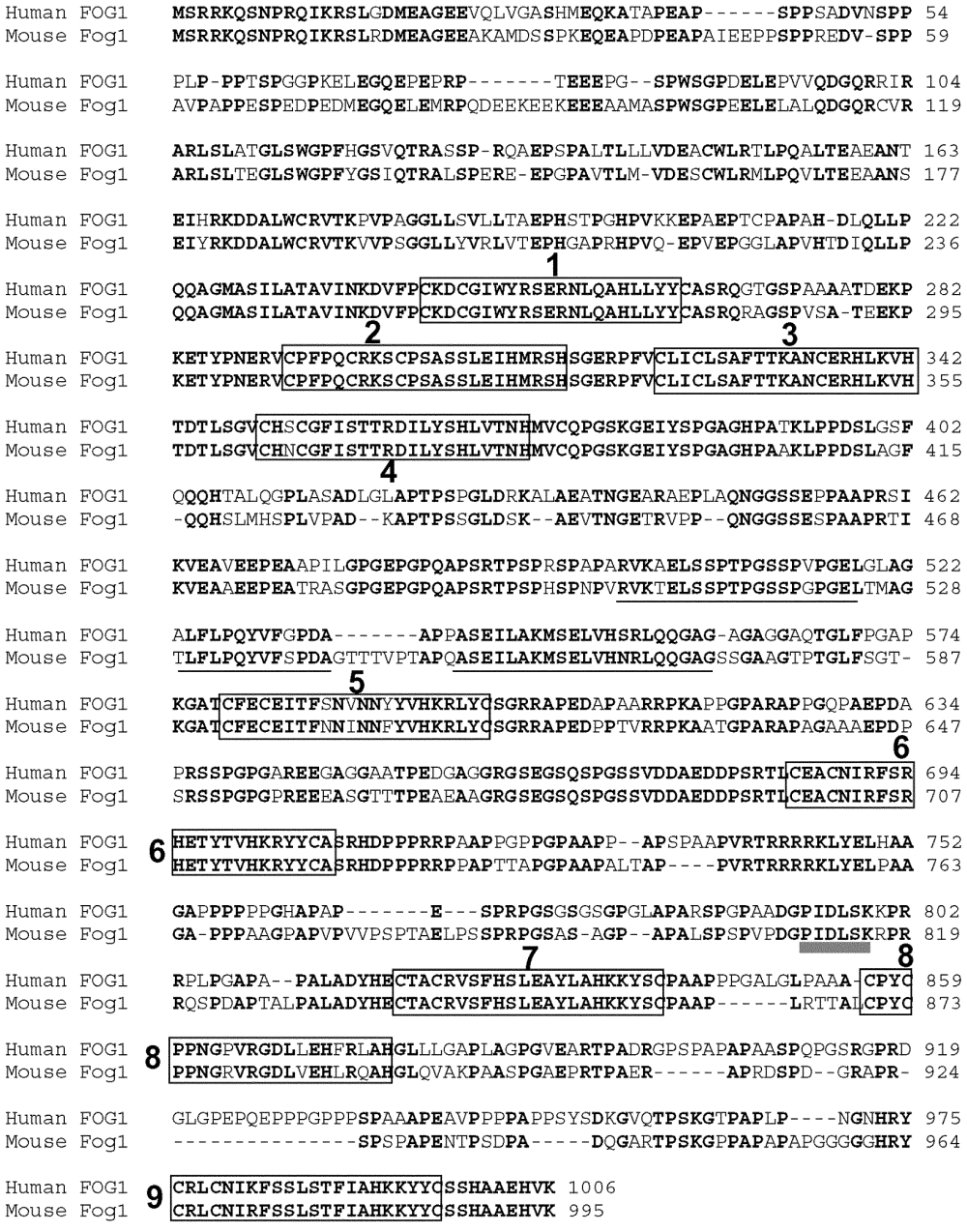


Fig. 2 Deduced amino acid sequence alignment between the human FOG1 and murine Fog1. All amino acid residues common to man and mouse are represented in *bold*. The nine zinc finger motifs (*boxes 1–9*) and the CtBP binding motif 'PIDLSK' (*underlined* by a *gray bar*) are highly conserved. The region with an unknown function between fingers 4 and 5 is also highly conserved (*underlined*)

5, and 8 are not completely conserved, including one of the GATA1-binding fingers (finger 5). Two additional regions, which are conserved between FOG2, Fog1, and xFOG, are also present in human FOG1: a short peptide (PIDLSK) that mediates the interaction with the co-repressor, called C-terminal binding protein (CtBP), and a region with an unknown function located between fingers 4 and 5 (see Fig. 2; Turner et al. 1998).

Mouse Fog1 has been shown to interact with the N-finger of GATA1, mostly through fingers 1, 5, 6, and 9 (Fox et al. 1998), but it can also interact with other GATA family members (GATA2/3; Tsang et al. 1997; Fox et al. 1998). Similarly, mouse Fog2 zinc finger 6 can interact with the N-finger of different GATA family members (GATA1/2/3/4; Tevosian et al. 1999). In vitro binding studies by the generally used GST pull-down experiment were performed as described in various studies (Tsang et al. 1997; Fox et al. 1998). We studied the binding between FOG1 finger 9 (identical between human and mouse), and human and mouse fingers 5 (86% homology) with either complete GATA1, both the naturally occurring GATA1 D218 mutants, or complete GATA2 (Fig. 3). GATA1 and GATA2 have a strong affinity for the various FOG1 fingers, and both GATA1 D218 mutants have a weaker affinity compared with wild-type GATA1. When comparing human FOG1 and mouse Fog1 finger 5, differences in binding capacity were seen: in this semi-quantitative in vitro assay, mouse Fog1 finger 5 interacts more strongly with all GATA proteins than the human homolog. The degree of disturbed interaction of the D218 GATA1 mutants with

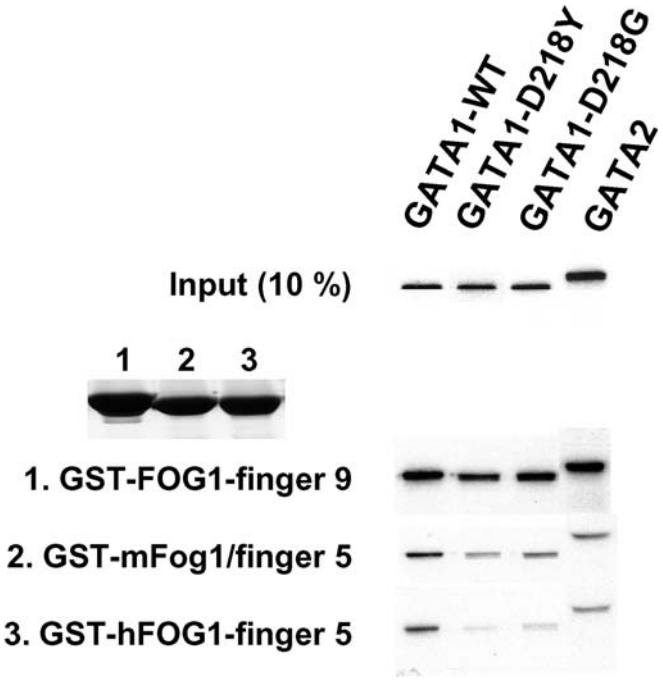


Fig. 3 FOG1/GATA in vitro binding studies. GST pull-down assay to study the interaction between FOG1 finger 9 (*1*.), mouse Fog1 finger 5 (*2*.), and human FOG1 finger 5 (*3*.) as GST fusion proteins and on the other side GATA1-WT, both naturally occurring D218 GATA1 mutants, and GATA2 as in vitro transcription/translated ^{35}S-labeled proteins (*upper series of lanes*). The middle (*numbered*) panel shows the Coomassie blue staining of the gel with the GST fusion proteins: FOG1 finger 9 (*1*), mouse Fog1 finger 5 (*2*), and human FOG1 finger 5 (*3*). The autoradiogram (*other lanes*) shows the amount of input material (10% of the input was loaded on the *upper* series of *lanes*) or retained ^{35}S-labeled GATA1, mutant GATA1 or GATA2 (*lanes 2–4*)

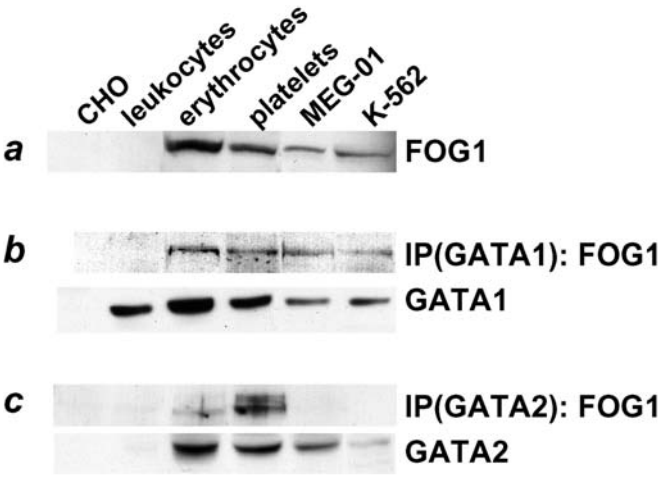


Fig. 4a–c FOG1/GATA ex vivo binding studies. Ex vivo FOG1 co-immunoprecipitation assay. **a** Regular Western blot analysis detecting FOG1 expression in various hematopoietic cells but not in leukocytes or the non-hematopoietic cell line CHO. **b, c** Amount of FOG1 detected after co-immunoprecipitation with either an antiGATA1-specific or antiGATA2-specific antibody. Western blot analysis also shows the amount of GATA1 and GATA2 expressed in the various cells. The same total protein concentration (100 µg) was used in all lanes

human FOG1 correlates with the severity of the clinical phenotype.

FOG1 expression in hematopoietic cells

To analyze for the presence of FOG1, we generated a rabbit polyclonal antiserum reactive with the first 380 amino acids. The antibody recognized a protein with an apparent molecular mass of 155 kDa, which was higher than the predicted molecular mass (115 kDa) probably because of the presence of multiple zinc fingers and the high proline content in FOG1. The FOG1 protein was found in erythrocytes, platelets, and in the hematopoietic cell lines MEG-01 and K-562, whereas leukocytes and CHO cells were negative (Fig. 4a).

Ex vivo interaction of FOG1 with GATA1 and GATA2

We were able to study the real direct binding of human FOG1 to both GATA1 and GATA2 in hematopoietic cells and cell lines by an ex vivo immunoprecipitation assay. FOG1 can be co-immunoprecipitated with an antibody against GATA1 in erythrocytes, platelets, MEG-01, and K-562 cells. Western blot analysis of GATA1 was also performed to visualize its expression level in the various cell extracts (Fig. 4b). When we used the same strategy with an antibody directed against GATA2, we co-precipitated FOG1 in platelets and (weakly) in erythrocytes (Fig. 4c). The expression level of GATA2 in MEG-01 and K562 cells was probably too low to enable co-precipitation of detectable levels of FOG1.

Disturbed ex vivo FOG1/GATA1 interaction in patients with GATA1 (D218) mutations

We have earlier described patients from two families with a missense mutation of residue D218 in the N-finger of GATA1 (D218G and D218Y) leading to a variable degree of macrothrombocytopenia with or without severe abnormalities in the erythrocyte lineage. In vitro binding studies have shown that the binding of these mutant GATA1 N-fingers with mouse Fog1 is reduced, indirectly illustrating the transcriptional deregulation in the platelets and erythrocytes of our patients. Since the mouse Fog1 and human FOG1 fingers 5 behave differently in the in vitro binding assay (Fig. 3), we have searched for more direct evidence that the GATA1/FOG1 binding really is disturbed in these patients. This evidence can now unequivocally be provided by the ex vivo FOG1 co-immunoprecipitation assay, performed as described above, but with patient cell extracts. Platelet and erythrocyte cell extracts of the patients with either the D218G or the D218Y mutation show a strongly reduced binding of mutant GATA1 to FOG1 (Fig. 5).

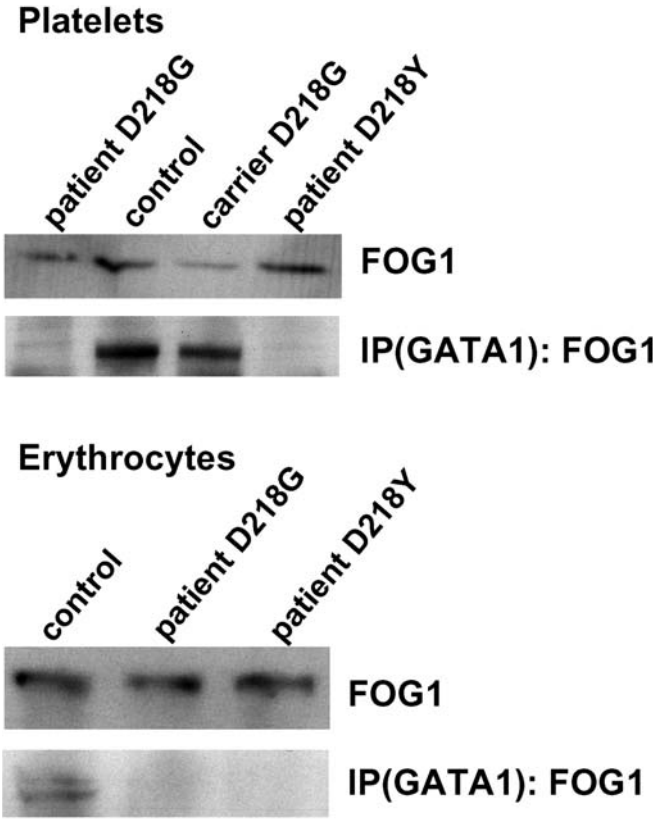


Fig. 5 FOG1/GATA1 binding in patients with a mutation in the N-finger of GATA1 at residue D218. The amount of FOG1 and the GATA1 bound FOG1 were determined by Western blot analysis in platelets and erythrocytes from a control, a patient with GATA1 mutation D218G, an asymptomatic carrier of the GATA1 mutation D218G, and a patient with GATA1 mutation D218Y. In patients with a mutation in the GATA1 N-finger, the GATA1/FOG1 binding was severely affected

Discussion

We describe the identification and characterization of human FOG1, an important co-regulator of the transcriptional activator GATA1, which regulates the expression of certain genes during erythroid and megakaryocytic differentiation. FOG1 is highly homologous to mouse Fog1 and belongs to the friend-of-GATA family, which also includes FOG2 and the *Drosophila* u-shaped family (Tsang et al. 1997; Tevosian et al. 1999; Svensson et al. 1999; Lu et al. 1999; Haenlin et al. 1997). The cooperation between GATA1 and FOG1 has been studied extensively via in vitro binding studies and in the yeast two-hybrid system, resulting in the identification of zinc fingers involved in their interaction (Fox et al. 1998). Naturally occurring missense mutations in the N-finger of GATA1 lead to X-linked macrothrombocytopenia with some degree of erythrocyte abnormalities (Nichols et al. 2000; Freson et al. 2001, 2002; Mehaffey et al. 2001). These reports, via an indirect in vitro assay with mouse Fog1, have shown that the GATA1/Fog1 binding is affected for all four mutant GATA1 N-fingers. In this report, we now present binding studies between GATA1 and the human FOG1. Such experiments seem warranted, since the FOG1 finger 5, which is important for recognition by GATA1, differs in three amino acids between the mouse and human sequence; this results in different binding affinities for GATA. The binding affinities between wild-type versus D218 mutant GATA1 and the human FOG1 or mouse Fog1, however, appear not to be very different. We have also studied FOG1/GATA1 binding by an ex vivo co-immunoprecipitation assay. Platelet and erythrocyte extracts from two patients with either the D218G or D218Y GATA1 N-finger mutation exhibit a severely affected binding of FOG1 to GATA1. Although the clinical severity between the two patients is markedly different, this difference could not readily be illustrated by the ex vivo binding experiment.

FOG-1 may also have a GATA1-independent role, especially in megakaryocytes. Megakaryocytes from lineage-selective GATA1 knockout mice show reduced proliferation and platelet production (Tsang et al. 1998; Shivdasani et al. 1997). FOG1 knockout mice completely lack these two processes, implying a GATA1 independent role for FOG1 in megakaryocytes. Recently, it has been shown in mice that, during early megakaryopoiesis, Fog1 also interacts with Gata2 when Gata1 is absent (Chang et al. 2002). Thus, the role of Fog1 in megakaryocytes seems to be fully GATA-factor dependent. It is not known whether this is also the case for non-hematopoietic tissues. Although we have detected *FOG1/GATA1* co-expression in hematopoietic fetal liver, *FOG1* also is present in fetal spleen and heart, and adult cerebellum, stomach, lymph node, bone marrow, testis, liver, and placenta. The *FOG1* expression pattern therefore differs from the *GATA1* profile that only gives a signal in bone marrow, again implying a GATA1-independent function for FOG1 in non-hematopoietic tissues. The human *GATA2* expression profile also differs from that of *FOG1* for most non-hematopoietic tissues, such as fetal kidney, and adult prostate, kidney, and uterus, except for its co-expression with FOG1 in the lymph node and placenta (data not shown). It will be interesting to ascertain whether other GATA family members are involved in FOG1 binding.

Although FOG1 contains numerous zinc fingers of the type that could interact directly with DNA, such a physiological role has never been attributed to it (Bellefroid et al. 1996). FOG1 probably does not bind directly to DNA, but other transcriptional factors within the GATA family are good candidates for binding to FOG1. We tested this concept in various hematopoietic cells and found that, in platelets (and to a much lesser extent also in erythrocytes), FOG1 not only binds GATA1 but also GATA2. In CD4+ T cells FOG1 has recently shown to repress GATA3 activity (Zhou et al. 2001).

In conclusion, we have identified and characterized the human *FOG1* gene, located on chromosome 16q24, with high homology to mouse *Fog1*. We have confirmed the disturbed interaction between D218 mutants of GATA1 and FOG1 in patients with macrothrombocytopenia and dyserythropoiesis. Further investigation can now be started to study the role of FOG1 in human physiology and pathology.

Acknowledgements This work was supported by research grants G. 0271.00 and KAN2000–1523100 from the FWO-Vlaanderen, OT/00/25 from KULeuven. C. Van Geet is holder of a clinical fundamental research mandate of the FWO.

References

Arceci RJ, King AA, Simon MC, Orkin SH, Wilson DB (1993) Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. Mol Cell Biol 13:2235–2246

Bellefroid EJ, Bourguignon C, Hollemann T, Ma Q, Anderson DJ, Kintner C, Pieler T (1996) X-MyT1, a *Xenopus* C_2HC-type zinc finger protein with a regulatory function in neuronal differentiation. Cell 87:1191–1202

Chang AN, Cantor AB, Fujiwara Y, Lodich MB, Droho S, Crispino JD, Orkin SH (2002) GATA-factor dependence of the multiple zinc-finger protein FOG-1 for its essential role in megakaryopoiesis. Proc Natl Acad Sci USA 99:9237–9242

Deconinck AE, Mead PE, Tevosian SG, Crispino JD, Katz SG, Zon LI, Orkin SH (2000) FOG acts as a repressor of red blood cell development in *Xenopus*. Development 127:2031–2040

Fox AH, Kowalski K, King GF, Mackay JP, Crossley M (1998) Key residues characteristic of GATA N-fingers are recognized by FOG. J Biol Chem 173:33595–33603

Freson K, Devriendt K, Matthijs G, Van Hoof A, De Vos R, Thys C, Minner K, Hoylaerts MF, Vermylen J, Van Geet C (2001) Platelet characteristics in patients with X-linked macrothrombocytopenia due to a novel GATA-1 mutation. Blood 98:85–92

Freson K, Matthijs G, Thys C, Mariën P, Hoylaerts MF, Vermylen J, Van Geet C (2002) Different substitutions at residue 218 of the X-linked transcription factor GATA1 lead to altered clinical severity of macrothrombocytopenia and anemia and are associated with variable skewed X inactivation. Hum Mol Genet 11:147–152

Haenlin M, Cubadda Y, Blondeau F, Heitzler P, Lutz Y, Simpson P, Ramain P (1997) Transcriptional activity of pannier is regulated negatively by heterodimerization on the GATA DNA binding-binding domain with a cofactor encoded by the u-shaped gene of *Drosophila*. Genes Dev 11:3096–3108

Holmes M, Turner J, Fox A, Chisholm O, Crossley M, Chong B (1999) hFOG-2, a novel zinc finger protein, binds the co-repressor mCtBP2 and modulates GATA-mediated activation. J Biol Chem 274:23491–23498

Laverriere AC, MacNeill C, Mueller C, Poelmann RE, Burch JB, Evans T (1994) GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. J Biol Chem 269:23177–23184

Leonard M, Brice M, Engel JD, Papayannopoulou T (1993) Dynamics of GATA transcription factor expression during erythroid differentiation. Blood 82:1071–1079

Lu JR, McKinsey TA, Xu H, Wang DZ, Richardson JA, Olson EN (1999) FOG-2, a heart- and brain-enriched cofactor for GATA transcription factors. Mol Cell Biol 19:4495–4502

Marck C (1988) "DNA Strider": a "C" program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. Nucleic Acids Res 16:1829–1836

Mehaffey MG, Newton AL, Gandhi MJ, Crossley M, Drachman JG (2001) X-linked thrombocytopenia caused by a novel mutation of GATA-1. Blood 98:2681–2688

Morrisey EE, Ip HS, Tang Z, Lu MM, Parmacek MS (1997) GATA-5: a transcriptional activator expressed in a novel temporally- and spatially-restricted pattern during embryonic development. Dev Biol 183:21–36

Mouthon MA, Bernard O, Mitjavila MT, Romeo PH, Vainchenker W, Mathieu-Mahul D (1993) Expression of tal-1 and GATA-binding proteins during human hematopoiesis. Blood 81:647–655

Nichols KE, Crispino JD, Poncz M, White JG, Orkin SH, Maris JM, Weiss MJ (2000) Familial dyserythropoietic anaemia and thrombocytopenia due to an inherited mutation in GATA1. Nat Genet 24:266–270

Orkin SH (1992) GATA-binding transcription factors in hematopoietic cells. Blood 80:575–581

Pandolfi PP, Roth ME, Karis A, Leonard MW, Dzierzak E, Grosveld FG, Engel JD, Lindenbaum MH (1995) Targeted disruption of the GATA-3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis. Nat Genet 11:40–44

Pevny L, Simon MC, Robertson E, Klein WH, Tsai SF, D'Agati V, Orkin SH, Costantini F (1991) Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature 349:257–260

Povey S, Lovering R, Bruford E, Wright M, Lush M, Wain H (2001) The HUGO Gene Nomenclature Committee (HGNC). Hum Genet 109:678–680

Shivdasani RA (2001) Molecular and transcriptional regulation of megakaryocyte differentiation. Stem Cells 19:397–407

Shivdasani RA, Fujiwara Y, McDevitt MA, Orkin SH (1997) A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. EMBO J 16:3965–3973

Svensson EC, Tufts RL, Polk CE, Leiden JM (1999) Molecular cloning of FOG-2: a modulator of transcription factor GATA-4 in cardiomyocytes. Proc Natl Acad Sci USA 96:956–961

Tevosian SG, Deconinck AE, Cantor AB, Rieff HI, Fujiwara Y, Corfas G, Orkin SH (1999) FOG-2: a novel GATA-family cofactor related to multitype zinc-finger proteins friend of GATA-1 and U-shape. Proc Natl Acad Sci USA 96:950–955

Ting CN, Olson MC, Barton KP, Leiden JM (1996) Transcription factor GATA-3 is required for development of the T-cell lineage. Nature 384:474–478

Tsai FY, Orkin SH (1997) Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. Blood 89:3636–3643

Tsai FY, Keller G, Kuo LI, Weiss M, Chen J, Rosenblatt M, Alt FW, Orkin SH (1994) An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature 371:221–226

Tsang AP, Visvader JE, Turner CA, Fujiwara Y, Yu C, Weiss MJ, Crossley M, Orkin SH (1997) FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation. Cell 90:109–119

Tsang AP, Fujiwara Y, Hom DB, Orkin SH (1998) Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA-1 transcriptional cofactor FOG. Genes Dev 12: 1176–1188

Turner J, Crossley M (1998) Cloning and characterization of mCtBP2, a co-repressor that associates with basic Kruppel-like factor and other mammalian transcriptional regulators. EMBO J 17:5129–5140

Visvader JE, Adams JM (1993) Megakaryocytic differentiation induced in 416B myeloid cells by GATA-2 and GATA-3 transgenes or 5-azacytidine is tightly coupled to GATA-1 expression. Blood 82:1493–1501

Visvader JE, Crossley M, Hill J, Orkin SH, Adams JM (1995) The C-terminal zinc finger of GATA-1 or GATA-2 is sufficient to induce megakaryocytic differentiation of an early myeloid cell line. Mol Cell Biol 15:634–641

Weiss MJ, Yu C, Orkin SH (1997) Erythroid cell-specific properties of transcription factor GATA-1 revealed by phenotypic rescue of a gene-targeted cell-line. Mol Cell Biol 17:1642–1651

Zhou M, Ouyang W, Gong Q, Katz SG, White JM, Orkin SH, Murphy KM (2001) Friend of GATA-1 represses GATA-3 dependent activity in CD4+ T cells. J Exp Med 194:1461–1471