Friend of GATA (FOG) Interacts with the Nucleosome Remodeling and Deacetylase Complex (NuRD) to Support Primitive Erythropoiesis in *Xenopus laevis*

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Abstract

Friend of GATA (FOG) plays many diverse roles in adult and embryonic hematopoiesis, however the mechanisms by which it functions and the roles of potential interaction partners are not completely understood. Previous work has shown that overexpression of FOG in *Xenopus laevis* causes loss of blood suggesting that in contrast to its role in mammals, FOG might normally function to repress erythropoiesis in this species. Using loss-of-function analysis, we demonstrate that FOG is essential to support primitive red blood cell (RBC) development in *Xenopus*. Moreover, we show that it is specifically required to prevent excess apoptosis of circulating primitive RBCs and that in the absence of FOG, the pro-apoptotic gene *Bim*-1 is strongly upregulated. To identify domains of FOG that are essential for blood development and, conversely, to begin to understand the mechanism by which overexpressed FOG represses primitive erythropoiesis, we asked whether FOG mutants that are unable to interact with known co-factors retain their ability to rescue blood formation in FOG morphants and whether they repress erythropoiesis when overexpressed in wild type embryos. We find that interaction of FOG with the Nucleosome Remodeling and Deacetylase complex (NuRD), but not with C-terminal Binding Protein, is essential for normal primitive RBC development. In contrast, overexpression of all mutant and wild type constructs causes a comparable repression of primitive erythropoiesis. Together, our data suggest that a requirement for FOG and its interaction with NuRD during primitive erythropoiesis are conserved in *Xenopus* and that loss of blood upon FOG overexpression is due to a dominant-interfering effect.

Introduction

Vertebrate blood development takes place in two waves, referred to as primitive and definitive hematopoiesis. Primitive hematopoiesis is the initial wave of blood development in the embryo in which blood progenitor cells in the mesoderm give rise mainly to primitive red blood cells (RBCs), although some white blood cells are also produced at this time. By comparison, definitive hematopoiesis constitutes the second wave of blood development in which hematopoietic stem cells give rise to blood cells of all lineages (reviewed in [1] and [2]). Definitive hematopoiesis begins later in development and continues throughout adult life. In mammalian embryos primitive hematopoiesis takes place extra-embryonically in the yolk sac blood islands [2]. The analogous structure in *Xenopus* embryos is called the ventral blood island (VBI). The VBI is an intraembryonic structure that forms when distinct populations of cells from the rostral and caudal mesoderm converge during gastrulation and subsequently elongate along the ventral midline [1,3]. As RBCs form during the early tailbud stage, they begin to express the differentiation marker *globin*, which initiates in the anterior aspect of the VBI and extends posteriorly as more hemoglobin is synthesized. During the late tailbud stage (roughly stage 35–36), the heart begins to contract and RBCs begin circulating within the embryo [4].

The GATA family of zinc finger transcription factors plays an essential role during hematopoiesis. There are six vertebrate GATAs; GATA-1, -2 and -3 are required for hematopoiesis, whereas GATA-4, -5, and -6 are required for cardiac, endoderm, gonadal and CNS patterning [5,6] (and reviewed in [7]). Targeted deletion of *GATA-1* causes mice to die early in embryonic development from severe anemia [5,6]. Specifically, *GATA-1* null embryos have defects in primitive erythropoiesis, and RBC progenitor development is arrested at the proerythroblast stage [3]. Similarly, zebrafish embryos in which *GATA-1* is either mutated or depleted with antisense morpholino oligonucleotides (MOs) lack RBCs [3,9]. *GATA-1* has also been shown to support the viability of RBC precursors *in vitro* by suppressing apoptosis [10].

FOG-1 (Friend of GATA 1) functions as a critical transcriptional cofactor for both GATA-1 and GATA-2 during hematopoiesis. FOG is a large multi-domain protein that includes nine conserved zinc fingers, four of which mediate GATA binding [11,12]. In the mouse, targeted deletion of FOG-1 blocks primitive erythropoiesis at the pro-erythroblast stage, phenocopying the RBC defect seen in *GATA-1* null mice [13]. This function appears
FOG Function in *Xenopus* hematopoiesis

In the current study, we have used a loss of function approach to address discrepancies in the literature regarding FOG function during primitive hematopoiesis in *Xenopus*. We find that xFOG and its interaction with the NuRD complex are required for primitive erythropoiesis, but that direct interaction with CtBP is dispensable. While we find that overexpression of xFOG also disrupts normal blood development, we show that it does so independent of its ability to interact with other cofactors known to be required for blood. This phenomenon is thus more consistent with a dominant-negative effect of xFOG overexpression rather than a reflection of its endogenous function. Finally, we show that loss of xFOG leads to upregulation of the pro-apoptotic gene *Bim-1* and excessive apoptosis of RBCs. Taken together, these studies show that FOG function is in fact conserved in *Xenopus* and is important for primitive RBC survival.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Oregon Health and Science University (Protocol number A719).

Embryo culture and manipulation

Ovulation was induced in adult *Xenopus laevis* females by injection of 50 IU of human chorionic gonadotropin (Sigma) into the dorsal lymph sac to induce spawning the night before egg collection. Embryos were staged according to Nieuwkoop and Faber [32,33]. Capped synthetic mRNA was synthesized by *in vitro* transcription of linearized template cDNA using a MegaScript kit (Ambion) and injected into the two vegetal blastomeres on the ventral side of eight-cell embryos to target prospective blood forming cells, as described previously [33].

Morpholinos and cDNA constructs

Sequence encoding the N-terminus of *Xenopus* FOG was obtained by 5’ RACE using oligonucleotide primers complementary to sequence near the 5’ end of the published partial length xFOG cDNA (accession no. AF241228). RNA was isolated from *Xenopus* embryos at stage 34, reverse transcribed and used as a template for PCR-mediated amplification of an xFOG cDNA (GenBank accession number GU384581) that encodes the entire open reading frame by PCR-mediated amplification of an *Xenopus* cDNA. Silent mutations to prevent morpholino annealing were engineered using a QuickChange XL (Stratagene) mutagenesis kit to generate a FOG rescue construct (xFOGr) with the following sequence at the 5’ end of the open reading frame, as determined by alignment with FOG from other species (Figure S1). A closely related cDNA that most likely represents a duplicate copy of the xFOG gene was identified by searching The Gene Index Project database (Accession number TC411807). Morpholino antisense oligonucleotides (MOs) complimentary to both alleles of xFOG: 5’-ATGTGCTCT-GTTTCTTCTTGGAACATG-3’ and xFOGbs: GCTGGAGGA-CAAGGCGAGGATCGAC were purchased from Gene Tools, LLC (Philomath, OR). Equal amounts of the two FOG MOs were mixed and the dose was titrated to 40 ng per embryo. Sequence encoding a MYC epitope tag was appended to the 5’ end of the xFOG open reading frame by PCR-mediated amplification. Silent mutations to prevent morpholino annealing were engineered using a QuikChange XL (Stratagene) mutagenesis kit to generate a FOG rescue construct (xFOGr) with the following sequence at the 5’ end: 5’ATG GAA CAA AAA CTT ATT TCT GAA GAA GAT CTG TCT AGA CGG AAG CAG AGT AAT CTCC AGA CAG. MYC tag is in bold, morpholino 2 target sequence is underlined, translation start site is italicized and silent mutations are bold and

to be conserved in zebrafish, as RBCs from embryos in which FOG has been depleted are properly specified but fail to mature [14]. Interestingly, point mutations in humans that disrupt GATA-1/FOG-1 interaction are associated with familial dyserythropoietic anemia and thrombocytopenia [15], and a point mutation in GATA-1 that inhibits FOG-1 binding cannot rescue erythroid differentiation in a GATA-1 deficient cell line. This defect, however, is rescued by co-expression of FOG-1 that bears a reciprocal mutation that restores binding [16]. The similarity in the FOG-1 and GATA-1 loss-of-function phenotypes together with in vitro mutant rescue analysis strongly suggest that they function in concert to promote RBC development.

There are two FOG homologs in the mouse, mFOG-1 and mFOG-2. Although they appear to be functionally redundant (e.g. forced expression of FOG-2 can rescue erythroid maturation in a FOG-1−/− cell line [17]) their non-overlapping patterns of expression enforce interaction with different GATA subfamilies, and result in distinct in vivo functions. Specifically, FOG-1 interacts with GATA-1, -2 and -3 and is involved in hematopoiesis, while FOG-2 interacts primarily with GATA-4, -5 and -6 (reviewed in [18]) to regulate development of other organ systems. FOG is both a transcriptional co-activator and co-repressor of GATA target genes [19,20,21,22], but the mechanisms by which it carries out each of these functions is unclear. In addition to its interaction with GATA, FOG also has been shown to recruit C-terminal binding protein (CtBP) and members of the Nucleosome Remodeling and Deacetylase (NuRD) complex. Binding domains for CtBP and NuRD are highly conserved across species in the majority of FOG isoforms [23,24,25]. Both are ubiquitously expressed during early *Xenopus* development [26,27,28], and would thus be spatially and temporally poised to function in primitive erythropoiesis.

While an essential role for FOG-1 during hematopoiesis has been well documented in mice and fish, the role of Xenopus FOG (xFOG) in this process is less clear. Only one FOG homolog has been identified in *Xenopus laevis* (Figure S1 and [29]). It is most similar to mFOG-1 but is co-expressed with, and thus predicted to interact with all six GATA factors [29]. The current model of FOG function in frogs is based primarily on overexpression of either a truncated *Xenopus* transcript or mouse FOG-2 (mFOG-2) in *Xenopus* embryos, both of which led to defects in primitive erythropoiesis [29]. Additionally these studies demonstrated that overexpression of an mFOG-2 mutant that was unable to bind CtBP did not inhibit blood formation and in fact led to greater hematopoietic activity. These findings suggest that in frogs, xFOG is acting solely as a transcriptional repressor through recruitment of CtBP, and that its normal function is to inhibit blood development [29]. This interpretation would suggest that FOG function in *Xenopus* is significantly different from its role in mice and fish.

Mice that harbor a targeted knock-in allele of FOG that lacks CtBP binding capacity show no defects in hematopoiesis, suggesting that this domain may be dispensable for normal function [23]. By contrast, mice that harbor a targeted knock-in allele of FOG that lacks NuRD binding capacity exhibit defects in fetal hepatic and adult marrow-derived definitive erythropoiesis and megakaryopoiesis [30,31]. A subset of these mutant embryos have pale yolk sacs at E10.5–12.5 suggesting that primitive RBC survival is thus still open.

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overnight at 4°C. Anti-MYC (1:500) and anti-actin (Sigma, 1:10,000) antibodies were detected by chemiluminescence (Pierce). Anti-HA (3F10; Roche, 1:1000), anti-MYC (71D10; Cell Signaling, 1:1000) and anti-actin (Sigma, 1:10,000) antibodies and detected by chemiluminescence (Pierce).

Collection and analysis of peripheral blood samples

Tails were severed from tadpoles and blood was collected into medium containing 0.7X PBS, 0.5% BSA and 10 IU/ml of heparin. Cells were concentrated onto slides using a cytopsin and stained with the Hema 3 stain set (Fisher Diagnostics). A minimum of 20 embryos were bled per experimental group and for each embryo the number of apoptotic and total cells present in four random fields was counted at 20X magnification. Each experiment was repeated a minimum of three times and results were pooled.

Immunoprecipitation and Western blot analysis

xFOG and xFOGr mutant expression vectors were generated by subcloning full-length Xenopus FOG into pCS2+. To verify that FOG MO targets xFOG but not xFOGr, 0.5 μg of each construct was injected into Xenopus embryos at the 2–4 cell stage. Embryos were cultured to stage 15 and protein was harvested from the xFOG substitution into the xFOGΔNt construct using restriction enzymes. xFOG4ZM was generated by using a QuickChange XL mutagenesis kit (Stratagene). xFOG4ZM harbors a single tyrosine to alanine substitution in each of the four GATA-binding zinc fingers (fingers 1, 5, 6 and 9). Introduction of analogous point mutations into mouse FOG-1 has been shown to be sufficient to inhibit GATA-1 binding [35]. All cDNAs were subcloned into pCS2+ for RNA transcripción and transient transfection of mammalian cells.

Analysis of RNA

Total RNA was isolated and Northern blots were hybridized with antisense riboprobes as described previously [36,37]. Bands were visualized with a phosphoimager and quantified using the NIH ImageJ software. Embryos were processed for in situ hybridization according to the protocol outlined in [38]. For Quantitative Real Time PCR (qPCR), total RNA was isolated using Trizol reagent per the manufacturer’s protocol (Invitrogen) and purified using an RNeasy spin column (QIAGEN). For each sample, 2.5 μg of purified RNA was used as template for first strand cDNA synthesis with random oligo dT primers and SuperScript III reverse transcriptase (Invitrogen) per the manufacturer’s protocol. Transcript levels were evaluated by qRT-PCR using the SYBR Green reagent (Invitrogen) on an ABI 7900HT with the following reaction conditions: 95°C, 10 minutes, followed by 95°C, 15 seconds and 60°C for 1 minute for 40 cycles. Expression levels were calculated based on standard curves generated from serial dilutions of cDNA from each sample. All samples were analyzed in triplicate and normalized to ODC. Primers used are as follows: ODC: F 5'-TGC AGA GCC TGG GAG ATA CT-3', R 5'-CAT TGG CAG CAT CTT CCT CA-3'; GATA-1: F 5'-GGA GAC GGA ATG CAA GTG GAG AC-3', R 5'-CCT GCT GCT GAC CTT TCG GGT C-3' BinIt: F 5'-CAG AAC TGT GGA TAG CAC AGG AAC-3', R 5'-CAG ACA AGC TGA GTC ACT TCT CG-3'

Results and Discussion

Fog is required for primitive red blood cell development in Xenopus

To assess whether FOG is required for primitive erythropoiesis in Xenopus, we isolated a full-length Xenopus FOG cDNA (for cloning details, see material and methods and figure S1). We then used this sequence to design anti-sense MOs (illustrated in figure 1A) capable of blocking translation of endogenous xFOG RNA. RNA encoding MYC-epitope tagged xFOG (xFOG-MYC) was injected alone, or together with FOG MO into Xenopus embryos at the two-cell stage, and expression of xFOG was examined by Western analysis of neurula stage (stage 15) embryonic extracts using antibodies specific for the MYC tag. Levels of xFOG-MYC protein translated from wild type RNA were reduced in the presence of the MO, whereas there was no effect on translation of a rescue RNA (xFOGr-MYC) containing silent mutations that prevent MO annealing (Figure 1B), demonstrating that the MO specifically targets wild type xFOG but not the xFOGr rescue RNA. FOG MO was then injected into both ventral vegetal blastomeres of eight-cell embryos (illustrated in figure 1C) in order to target cells that are fated to give rise to the majority of blood-forming mesoderm [3,42,43]. Following injection, embryos were cultured to the tailbud stage (stage 34–36) and examined for changes in expression of the RBC differentiation marker globin, by whole-mount in situ hybridization. MO-mediated knockdown of xFOG resulted in a dose-dependent reduction in globin expression in the ventral blood island (VBI) (Figure 1D), which was further confirmed by Northern blot analysis (Figure 1E).

Globin staining was preserved in the extreme anterior portion of the VBI in most embryos (Figure 1D, arrowheads), consistent with the fact that the anterior VBI is derived from cells originating on the dorsal side of the embryo, which were not targeted in our
injections. We observed a significant reduction in globin expression with both 40 ng and 60 ng of FOG MO (Figure 1D, E), and all subsequent experiments were performed using the lower dose. Expression of the erythroid marker, GATA-1, was also reduced in FOG morphants (data not shown).

To demonstrate the specificity of our FOG MO for its target, we attempted to rescue expression of globin in FOG morphants by coinjection of xFOGr RNA, which does not anneal to the MO. Note that FOG MOs inhibit the translation of xFOG-MYC RNA but do not affect translation of xFOGr-MYC RNA. Actin is shown as a loading control. (n = 3) (C) Schematic of experimental design. MO and/or RNA is targeted to the ventral marginal zone (VMZ) of an eight-cell embryo. Injected embryos are cultured to the tailbud stage and assayed for globin expression by whole mount in situ hybridization or Northern analysis. (D) In situ hybridization analysis of globin expression (purple stain) in embryos injected with increasing doses of FOG MO (n = 4). Arrowheads indicate the anterior-most aspect of the VBI, derived from dorsal cells not targeted by injections. (E) Northern analysis of globin expression in embryos injected with increasing doses of FOG MO (n = 3). (F) Northern analysis of globin expression in embryos injected with FOG MO (40 ng), xFOGr RNA (200 pg), or both (n = 4). Levels of globin expression are normalized to expression of the housekeeping gene ODC and reported as a percentage of control in the graphs in panel E and F. Error bars reflect S.D. Paired t test results are as follows: **, p ≤ 0.005; ***, p ≤ 0.0005.

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Figure 1. FOG is required for primitive erythropoiesis in Xenopus laevis. (A) Alignment of sequence surrounding the translation start site of the two Xenopus FOG alleles. Sequences to which FOG MOs bind are indicated by the black bars and the ATG start codon is in red. (B) Western blot of lysates from embryos injected with MYC epitope tagged wild type xFOG or xFOGr RNA, which harbors silent mutations that prevent MO binding, with either FOG MO or Control MO. Note that FOG MOs inhibit the translation of xFOG-MYC RNA but do not affect translation of xFOGr-MYC RNA. Actin is shown as a loading control. (n = 3) (C) Schematic of experimental design. MO and/or RNA is targeted to the ventral marginal zone (VMZ) of an eight-cell embryo. Injected embryos are cultured to the tailbud stage and assayed for globin expression by whole mount in situ hybridization or Northern analysis. (D) In situ hybridization analysis of globin expression (purple stain) in embryos injected with increasing doses of FOG MO (n = 4). Arrowheads indicate the anterior-most aspect of the VBI, derived from dorsal cells not targeted by injections. (E) Northern analysis of globin expression in embryos injected with increasing doses of FOG MO (n = 3). (F) Northern analysis of globin expression in embryos injected with FOG MO (40 ng), xFOGr RNA (200 pg), or both (n = 4). Levels of globin expression are normalized to expression of the housekeeping gene ODC and reported as a percentage of control in the graphs in panel E and F. Error bars reflect S.D. Paired t test results are as follows: **, p ≤ 0.005; ***, p ≤ 0.0005.
first asked whether overexpression of full-length *Xenopus* FOG also resulted in loss of RBCs. We injected RNA encoding wild type xFOG into the two ventral vegetal blastomeres of eight-cell embryos and analyzed expression of *globin* by Northern blotting (Figure 2A). Injection of 500 pg or more of wild type xFOG (doses comparable to those used in prior studies [29]) inhibited expression of *globin* (Figure 2A). While this was somewhat surprising given that FOG depletion also causes loss of blood in *Xenopus* (Figure 1), there are several explanations for this outcome.

First, overexpression of mFOG-2 that cannot bind CtBP (mFOG-2D:CtBP) in *Xenopus* embryos enhances RBC development [29], raising the question of whether endogenous xFOG might play a paradoxical role in repressing erythropoiesis, possibly by repressing genes required for erythrocyte maturation via CtBP recruitment [29]. Second, more recent studies have shown that downregulation of FOG is essential for commitment to certain non-erythroid hematopoietic lineages (e.g., eosinophil and mast cell lineages), and that prolonged or ectopic expression can disrupt FOG-independent functions of GATA1 genes, thereby preventing differentiation of these cell types [44]. Thus, the loss of RBCs observed upon overexpression of FOG in *Xenopus* may indicate that FOG must be downregulated to allow for differentiation of erythroid cells, similar to what is observed for eosinophils or mast cells. Third, ectopic expression of FOG in inappropriate cell types may interfere with other FOG-independent GATA activities required for blood. Fourth, overexpressed FOG may be prematurely facilitating GATA-1/FOG-1 induced repression at genes required for RBC progenitor expansion such as GATA-2 [45,46] and *Kit* [22], and thereby limiting the number of precursors available to differentiate as RBCs. Finally, it is possible that overexpression-induced loss of blood is a secondary consequence of deregulating the proper balance between FOG and its other binding partners. As such, overexpressed FOG may repress blood by binding and sequestering interaction partners such as GATA-1-2, NuRD and/or CtBP away from endogenous targets. One example of this phenomenon is suggested by the observation that Mta3, a component of the NuRD complex required for primitive erythropoiesis, appears to be recruited to hematopoietic targets through its association with FOG [47].

To begin to distinguish among these possibilities, we generated several mutant forms of xFOG, including xFOGΔNuRD, xFOGΔCtBP, xFOGΔNuRD/ΔCtBP, and xFOG4ZM (as illustrated in Figure 2B and described in Materials and Methods). These mutations have been shown by others to prevent interaction with CtBP [23], the NuRD repressor complex [34], and/or GATA factors [11]. Mutant proteins were expressed at equivalent steady state levels in HEK cells, demonstrating that differences in activity are not likely to be due to differences in expression or stability (Figure S2). Impairment of GATA-2 binding was shown for xFOG4ZM, as loss of this particular interaction had not been previously verified (Figure S3). 500 pg of each xFOG mutant RNA was injected into the VMZ of embryos at the eight-cell stage...
as described above for WT xFOG, and globin expression was analyzed by Northern blotting at the tailbud stage. Surprisingly, overexpression of any of the mutant constructs produced a reduction in globin expression comparable to that seen with WT xFOG (Figure 2C).

Given that xFOG4ZM, which does not interact with GATA-1 or -2, is able to repress blood when overexpressed, it is unlikely that xFOG interferes with endogenous GATA-driven differentiation or progenitor expansion. Furthermore, as overexpression of FOG lacking either the CtBP or NuRD binding domain, or both caused a reduction in globin equivalent to that produced by overexpression of wild type FOG, abnormal repression of erythroid-specific genes is unlikely to account for the loss of blood. Instead, our results are most consistent with the possibility that overexpression of FOG leads to a dominant-negative squelching effect by which limiting amounts of transcriptional binding partners are prevented from accessing their endogenous target promoters. The discrepancy between our results showing that overexpressed xFOGΔCtBP represses erythropoiesis, and those of the previous studies showing that mFOG-2ΔCtBP enhances erythropoiesis might be due to species- and sequence-specific differences between xFOG and mFOG-2, or might be explained by the different methods used to evaluate erythropoiesis in the two studies. The observation that FOG deletion mutants that are unable to bind select co-factors are still able to repress erythropoiesis suggests that sequestering any one FOG binding partner is sufficient to interfere with erythropoiesis. Collectively, these findings suggest that precise regulation of FOG levels in the embryo is crucial for normal blood development.

CTBP binding is dispensable, whereas NuRD binding is required for FOG function during primitive erythropoiesis

To better understand the relevance of FOG interaction with CtBP and NuRD in Xenopus primitive erythropoiesis, we tested the ability of each FOG mutant to rescue loss of blood in FOG morphants. xFOGΔCtBP contains silent mutations that prevent annealing of the FOG MOs and xFOGΔNuRD lacks the MO recognition sequence altogether due to its N-terminal deletion, thus both are able to serve as MO rescue constructs. To ask whether direct binding of CtBP is required for FOG function in primitive hematopoiesis in Xenopus, FOG MO was injected into the two ventral vegetal blastomeres of eight-cell embryos either alone, or together with 200 pg xFOGΔCtBP RNA and expression of globin was analyzed at the tailbud stage (Figure 3A). FOG morphant embryos showed reduced globin expression. However, co-injection of 200 pg of RNA encoding xFOGΔCtBP with the FOG MO significantly restored globin expression (Figure 3A). These studies, together with published studies showing that mFOG-1ΔCtBP knock in mice display no hematopoietic defects, suggest that direct recruitment of CtBP to FOG is not required for

**Figure 3. The NuRD binding domain of xFOG is essential for primitive erythropoiesis whereas the CtBP binding motif is dispensable.** FOG MO (40 ng) and 200 pg of xFOGΔCtBP RNA (A) or xFOGΔNuRD RNA (B) were injected alone or together into the ventral vegetal blastomeres of eight-cell embryos and expression of globin was analyzed by Northern blotting at stage 34 (n = 3 for each). Levels of globin expression are normalized to expression of ODC and reported as a percentage of control. Error bars reflect S.D. Paired t test results are as follows: **, p<0.01; *, p<0.05. (AN=xFOGΔNuRD, ΔC=xFOGΔCtBP).

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normal primitive erythropoiesis in vertebrates. However, it
remains possible that CtBP is indirectly recruited to FOG by
other nucleating factors that can themselves bind FOG. Prece-
dence for this type of cooperative interaction between multiple
binding partners, in which elimination of a single binding site does
not interfere with functional complex assembly in vivo exists, for
example, in the case of the axin destruction complex during Wnt
signal transduction [49]. Chromatin immunoprecipitation to
ascertain whether or not CtBP occupancy changes at blood-
specific genes regulated by FOG-1 and GATA-1 or -2 in CtBP
binding mutants (e.g. in FOG morphant embryos rescued with
xFOGΔCtBP or in the mFOG-1ΔCtBP mutant mouse) relative to
wild type controls would be necessary to definitively address this
question in vivo.

In contrast to the ability of xFOGΔCtBP to substitute for wild
type FOG during Xenopus primitive hematopoiesis, xFOGΔNuRD
was not able to rescue loss of globin in FOG morphants in multiple
experiments (Figure 3B). In some respects, this was unexpected
given previous studies in which mutant forms of FOG lacking large
portions of the N-terminus encompassing and extending beyond
the NuRD binding domain are able to rescue erythroid differen-
tiation in a FOG-1 null cell line [35]. It is likely that
functional differences between cell lines and in vivo differen-
tiation, and the degree of overexpression attained in cell culture account
for this discrepancy. Consistent with our findings, however,
previous studies have shown that mice harboring knock-in alleles
of FOG deficient in NuRD binding exhibit mild defects in
definitive erythropoiesis and may have impaired primitive
erthropoiesis as well [30,31].

Circulating RBCs in FOG morphant embryos are reduced
in number and have abnormal morphology

Our initial studies demonstrate that FOG is essential for
primitive erythropoiesis in Xenopus, consistent with its role in other
vertebrates. However, how it affects the cellular phenotype
during this process is unclear. To begin to address this question,
we first wished to determine whether xFOG was affecting
erythroid development prior to differentiation. Consistent with
previous data in fish showing that FOG is not required for
specification of primitive blood [14], we found that expression of
the RBG specification marker Amyloid Leukemia (Aml) was
unchanged as analyzed by in situ hybridization in stage 15
FOG morphants (not shown). We therefore looked to identify
erythroid defects at later stages. To test whether xFOG affects
primitive RBC differentiation or maintenance later in erythroid
development, we collected total circulating blood cells from
individual embryos at the tadpole stage (stage 42) and examined
them for changes in RBC number and morphology. Compared
to wild type sibling controls, the overall number of RBCs in FOG
MO-injected embryos was drastically reduced (Figure 4A,B). In
addition, FOG morphant RBCs were smaller in size, showed
reduced cytoplasm and condensed nuclei relative to control
RBCs (Figure 4A, black arrows). The nearly complete absence of
circulating RBCs in FOG morphants was striking, given the more
modest reduction in levels of globin at earlier (tailbud, stage 34–36)
stages of development (Figure 1). However, when globin levels
were analyzed by Northern blotting in FOG morphants at the
tadpole stage (stage 42), we observed a more severe reduction in
globin expression that paralleled the dramatic loss of circulating
RBCs (Figure 4C). Together, our data indicate that depletion of
xFOG in mesoderm leads to a loss globin at the tailbud stage.
Moreover, this deficiency becomes progressively worse, and is
reflected in a loss of red blood cells over time, suggesting that
xFOG may be important in supporting erythrocyte survival. In
support of this hypothesis, the morphology of the RBCs isolated
from FOG morphants appeared consistent with that of cells
undergoing programmed cell death, a phenotype that was not
apparent in RBCs from wild-type siblings.

Figure 4. FOG morphants show a loss of circulating RBCs and altered RBC morphology. (A) Wright-Giemsa stain of blood cells from wild
type and FOG.MO injected embryos. Black arrows indicate primitive RBCs. (B) Graph showing the number of circulating RBCs in tadpoles injected with
FOG MO at the eight-cell stage as compared to their wild type siblings. The number of RBCs is expressed as a percentage of control (n=4). (C)
Northern analysis of globin expression in stage 42 uninjected control and FOG MO injected embryos. Levels of globin expression are normalized
relative to expression of ODC and reported as a percentage of control (n=6). Error bars reflect S.D. Paired t test results are as follows: *** p<0.0005.
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Circulating RBCs in FOG depleted embryos show an increase in apoptosis

To more stringently test the possibility that loss of xFOG results in excess erythrocyte apoptosis, we performed TUNEL assays on total circulating blood cells from wild type and FOG morphant tadpoles. We observed a significant increase in the fraction of TUNEL positive cells in FOG morphants (Figure 5A and B, quantified in Figure 5C), suggesting that FOG is required for survival of primitive erythrocytes, and that in the absence of FOG, these cells undergo excess apoptosis.

We also examined expression of the hematopoietic specific gene, GATA-1, and the pro-apoptotic gene, Bim1 in tailbud stage embryos. Bim1 is strongly upregulated in FOG morphants as early as the tailbud stage (stage 34), whereas levels of GATA-1 are decreased (Figure 5D). GATA-1 is required for activation of most or all erythroid-specific genes, including those involved in mediating RBC survival [49,50]. Specifically GATA-1 is known to directly activate expression of Lrf [also known as Zeb7a/POKEMON/FBI-1], which in turn represses transcription of the pro-apoptotic gene, Bim [49]. Mice that harbor a targeted deletion of Lrf show increased apoptosis during terminal erythroid differentiation that results in a lethal anemia [49]. In a similar fashion, RBCs in FOG morphant embryos are able to form but then subsequently appear to recede and undergo a disproportionate degree of apoptosis compared to their wild type counterparts. Our data, which show that levels of GATA-1 transcripts are reduced in FOG, morphants may reflect a requirement for FOG in maintenance or upregulation of GATA-1 gene expression as development proceeds. Alternatively, given that regulation of most GATA-1-dependent genes requires binding to FOG, the loss of primitive erythropoiesis in FOG morphants may be due to loss of GATA-1 function, rather than expression, and this leads to upregulation of Bim1 expression and excessive apoptosis of primitive RBCs. In this scenario, the reduction in levels of globin and GATA-1 transcripts would be due to loss of RBCs rather than loss of transcription. Taken together, our data provide evidence for a model in which FOG regulates GATA-1 function and/or expression, which in turn directly or indirectly represses expression of pro-apoptotic genes such as Bim in order to support RBC survival.

Supporting Information

Figure S1 Alignment of Xenopus FOG with human, mouse and fish FOG homologs. Alignment of full-length X. laevis FOG with zebrafish, mouse and human FOG-1 [51]. The NuRD binding domain is highlighted in red, the CtBP binding...
domain in yellow, the conserved zinc fingers in gray and a putative tenth zinc finger conserved in fish and frogs is highlighted in green. (DOC)

**Figure S2** Mutations in xFOG do not affect steady state levels of FOG protein. Western analysis of cell lysates from HeLa cells transiently transfected with MYC epitope tagged wild type and mutant xFOG cDNA constructs. Steady state levels of HeLa cells transiently transfected with MYC epitope tagged wild type and mutant xFOG cDNA constructs. Steady state levels of xFOG, ΔN = xFOGANuRD, ΔC = xFOGΔCtBP, ΔNAC = xFOGANuRD/ΔCtBP, 4ZM = xFOG4ZM. (TIF)

**Figure S3** GATA-2 binding is severely impaired in the xFOG4ZM mutant. Wild type and mutant MYC epitope tagged xFOG constructs were transfected into HeLa cells alone, or together with HA-tagged GATA-2. Western blots (WB) of cell lysates (input) or anti-MYC immunoprecipitates (IP) were probed with an anti-HA antibody to detect interaction with GATA-2-HA. Levels of GATA-2 protein detected by co-IP are normalized to the xFOG-2 input and to the xFOG IP and reported as a percentage of the wild type control below each lane. ΔN = xFOGANuRD, ΔC = xFOGΔCtBP, ΔNAC = xFOGANuRD/ΔCtBP, 4ZM = xFOG4ZM. (TIF)

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**Author Contributions**

Conceived and designed the experiments: MSM JLC. Performed the experiments: MSM. Analyzed the data: MSM. Wrote the paper: MSM JLC.

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