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# FOG acts as a repressor of red blood cell development in *Xenopus*

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## SUMMARY

**Members of the GATA family of zinc-finger transcription factors have critical roles in a variety of cell types. GATA-1, GATA-2 and GATA-3 are required for proliferation and differentiation of several hematopoietic lineages, whereas GATA-4, GATA-5 and GATA-6 activate cardiac and endoderm gene expression programs. Two GATA cofactors have recently been identified. Friend of GATA-1 (FOG-1) interacts with GATA-1 and is expressed principally in hematopoietic lineages, whereas FOG-2 is expressed predominantly in heart and brain. Although gene targeting experiments are consistent with an essential role for FOG-1 as an activator of GATA-1 function, reporter assays in transfected cells indicate that FOG-1 and FOG-2 can act as repressors. We have cloned a *Xenopus laevis* homologue of FOG that is structurally most similar to FOG-1, but is expressed predominantly in heart and brain, as well as the ventral blood island and adult spleen. Ectopic expression and explant assays demonstrate that FOG proteins can act as repressors in vivo, in part through interaction with the transcriptional co-repressor, C-terminal Binding Protein (CtBP). FOG may regulate the differentiation of red blood cells by modulating expression and activity of GATA-1 and GATA-2. We propose that the FOG proteins participate in the switch from progenitor proliferation to red blood cell maturation and differentiation.**

Key words: GATA, Transcription factor, Hematopoiesis, FOG, *Xenopus*, Co-repressor, C-terminal Binding Protein (CtBP)

## INTRODUCTION

Transcription factors of the GATA family perform conserved functions in the development of several lineages/tissues (Orkin, 1992). Six vertebrate members have been described. Each binds to a consensus WGATAR motif in the promoter and enhancer regions of a broad set of target genes through two homologous zinc fingers. GATA-2 is expressed prior to the formation of the blood islands of the mammalian yolk sac and has an essential role in the maintenance of pluripotent stem cells in the hematopoietic system (Tsai et al., 1994; Tsai and Orkin, 1997). GATA-1 is expressed slightly later within a more restricted pattern and plays a pivotal role in the differentiation and maturation of erythrocytes and megakaryocytes (Pevny et al., 1991; Fujiwara et al., 1996). GATA-3 is essential for the genesis of the T-cell lineage (Ting et al., 1996). GATA-4, GATA-5 and GATA-6 are expressed predominantly in heart, gut and other endoderm-derived tissues, where they play important roles (Arceci et al., 1993; Laverriere et al., 1994; Kuo et al., 1997; Molkentin et al., 1997; Gao et al., 1998).

From various functional studies of the zinc-finger domains (Visvader et al., 1995; Blobel et al., 1995; Weiss et al., 1997), it became apparent that GATA-1 requires a co-factor to regulate transcription of a subset of target genes during erythroid and megakaryocyte maturation. FOG (Friend Of GATA) was isolated using a yeast two-hybrid protein interaction screen with GATA-1 as the bait. FOG interacts specifically with the N-finger of GATA-1, and is co-expressed with GATA-1 in fetal liver, embryonic erythroblasts, mast cells and megakaryocytes as well as adult spleen (Tsang et al., 1997; Fox et al., 1998). Gene targeting studies have shown that FOG plays a crucial role in erythroid and megakaryocytic cell differentiation. Absence of FOG results in a block in erythropoiesis similar to that resulting from loss of GATA-1, but the megakaryocytic defect is more extreme (Tsang et al., 1998). FOG is capable of interacting with any of the GATA factors, shown by forced co-expression in various mammalian cell systems and in yeast (Tsang et al., 1997). FOG appears to cooperate with GATA in erythroid and megakaryocytic cell differentiation in vivo (Tsang et al., 1997, 1998; Crispino et al., 1999), but reporter assays suggest that it also represses transcription (Fox et al., 1999; A. P. Tsang and S. H. O., unpublished data). A FOG homologue in *Drosophila*, u-shaped, represses the action of a GATA-like factor, pannier (Cubadda et al., 1997; Haenlin et al., 1997). A second mammalian FOG factor, FOG-2, has recently been identified, which is expressed predominantly in heart and brain (Tevosian et al., 1999; Lu et al., 1999; Svensson et al., 1999). Functional data indicate that FOG-2 is capable of acting as an activator or a repressor of transcription, depending on what assay system is employed (Tevosian et al., 1999; Lu et al., 1999).

We have used *Xenopus laevis* to further our understanding of FOG function. We cloned a *Xenopus* homologue of FOG (xFOG), which is structurally closely related to FOG-1 but has a broader expression pattern; it encompasses most of the

expression sites of the *Xenopus Gata* genes, suggesting that there may be only a single FOG in *Xenopus* which modulates the activity of multiple GATAs. The xFOG sequence features a short peptide motif (PIDLSK) that is highly conserved among the FOG proteins and mediates interaction with the transcriptional co-repressor CtBP (Poortinga et al., 1998; Nibu et al., 1998; Turner and Crossley, 1998; Schaeper et al., 1998; Sewalt et al., 1999). Ectopic expression of mouse FOG-2 and xFOG in whole embryos results in inhibition of red blood cell formation and maturation. In contrast, injection of a mutant Fog-2 RNA that lacks the binding motif for the co-repressor CtBP (FogΔCtBP) results in stimulation of red blood cell formation. In addition, specific GATA-1 mutants impaired for interaction with FOG-1 (Crispino et al., 1999) also increase globin expression in animal cap assays. In ventral marginal zone and animal cap explants, injection of Fog-1 or Fog-2 inhibits expression of *xGata*-1 and *globin*, but does not affect expression of *xGata*-2.

The data affirm that FOGs interact with GATA-1 and GATA-2 to regulate the differentiation of red blood cells. In *Xenopus*, FOGs can repress xGATA-1/xGATA-2 functions, and repress transcription of xGATA-1 during red blood cell development. This repression appears to be dependent on interaction with the co-repressor CtBP. During erythropoiesis, proliferating progenitors commit to erythroid cell maturation with concomitant upregulation of GATA-1 and downregulation of GATA-2 expression. Our data suggest that FOGs participate in this switch.

## MATERIALS AND METHODS

### Cloning of xFog

Degenerate PCR primers were designed using codon usage data for *Xenopus* (Wada et al., 1990) and were derived from the zinc-finger sequence motifs of FOG-1. Adult *Xenopus* spleen RNA was reverse transcribed and cDNAs were amplified using the forward 5′-TG(C/T) CC(A/T) TT(T/C) CC(A/T) CA(A/G) TG-3′ (coding for CPFPQC, zinc finger 2) and the reverse 5′-TG(G/A) TT(A/G/T) GT(A/C) AC(A/C) AG(A/G) TG-3′ (coding for HLVTNH, zinc finger 4) oligonucleotides. PCR conditions were 94°C for 2 minutes, 44°C for 2 minutes and 72°C for 2 minutes, followed by 30 cycles at those temperatures for 1 minute each. PCR products were subcloned into pGEM-T (Promega), and 220 bp clones, positive by Southern analysis using Fog-1 DNA as a probe, were sequenced. A cDNA encoding zinc fingers 2, 3 and 4 of *Xenopus* FOG was used to screen a *Xenopus* adult spleen library. The library was made from poly(A)+ mRNA isolated from adult spleen tissue using the Stratagene ZAP Express cDNA synthesis and Gigapack cloning kits. One million phage clones were screened and seven positive clones were rescreened prior to phage excision. The clone with the largest insert was selected for sequence analysis from both strands, and contains a single open reading frame that encodes a novel FOG family member. The accession number for *xFog* is AF241228. The *xFog* clone lacks a methionine start codon, and was therefore cloned into the pcs2 expression plasmid along with a start codon and HA tag sequence: 5′-CT CGA G(*Xho*I site)CC ACC ATG (start) TAC CCA TAC GAC GTA CCA GAC TAC GCG (HA tag) TTA GAA TTC GGC (*xFog*)-3′.

The ΔCtBP mutation was previously described and analyzed by Schaeper et al. (1995); the PIDLSK sequence motif was mutated to PIASSK (ΔDL).

### Embryo culture and manipulation for ectopic overexpression studies and explant assays

*Xenopus laevis* embryos were obtained by in vitro fertilization and dejellied in 3% cysteine (pH 7.6), washed and maintained in 0.5× Marc's modified Ringer's solution (MMR), 3% Ficoll (Peng, 1991). Embryos were routinely cultured at 18°C and staged according to Nieuwkoop and Faber (1994). Embryos were injected either at the 1-cell stage in the animal pole region for animal cap assays, at the 2-cell stage in the marginal zone region of both blastomeres for marginal zone assays, or at the 4-cell stage, when one to four blastomeres were injected on separate experiments. RNA for microinjection was prepared using the mMessage mMachine kit from Ambion, using SP6 polymerase for transcription from pCS2+ plasmids. RNA was quantified by spectrophotometry, and 500 pg was injected per blastomere unless indicated otherwise. All microdissections were performed in 0.5× MMR, 1× penicillin/streptomycin (Gibco BRL) on a bed of bone black stained agarose, using watchmaker's forceps and hair knives. Animal cap explants were dissected at stage 8, and marginal zones were taken from gastrulae at stage 10+. Explants were transferred to fresh 0.5× MMR, 1× penicillin/streptomycin in 24-well plates and cultured until the desired stage as assessed by undissected siblings. When appropriate, animal cap explants were cultured in the presence of 50 ng/ml bFGF (Gibco BRL). Between 8 and 14 explants were dissected for each RNA injection, and each experiment was repeated at least three times, with at least two different batches of RNA for microinjection.

### RNA in situ hybridization and benzidine staining

Embryos were fixed and dehydrated for whole-mount in situ hybridization, which was performed as described by Harland (1991) using BM Purple AP Substrate (Roche). Four different in situ probes were used, obtained by subcloning various restriction digest fragments of the *xFog* clone. All probes resulted in similar staining patterns; a 1 kb fragment that encodes the 5′-end of xFOG yielded the cleanest staining pattern, and is the one represented in Fig. 3. Following in situ hybridization, embryos were photographed above a blue optic lens. Representative embryos were dehydrated and embedded in paraffin for 5-8 μm thick sections. O-dianisidine staining was performed as previously described (Zon et al., 1991; Huber et al., 1998). Embryos were cleared as previously described (Mead et al., 1998a).

### Analysis of molecular markers by RT-PCR

Total RNA was isolated from required stages of explants or whole embryos by proteinase K treatment in SDS lysis buffer followed by two phenol/chloroform extractions and ethanol precipitation. Nucleic acids were collected and RNA was precipitated in 4 M lithium chloride overnight at –20°C, washed extensively and resuspended in water for first strand synthesis. Primer sequences were designed using the DNA Star program to minimize the formation of primer dimers and nonspecific amplification in the PCR reactions. The PCR primer sequences were as follows: forward *xFog* 5′-TAT GCC CAG AAG TTA CAG GAA-3′; reverse *xFog* 5′-CAC CTC CTT TTT GTG CCA GTG-3′; *ODC*, *αT3 globin*, *SCL*, *Gata*-1, *Gata*-2, *Gata*-4, *Gata*-5, *Gata*-6, *N-tubulin*, *NCAM* as previously published (Mead et al., 1996, 1998a) or as supplied on the XMMR PCR primer data website. Standard PCR conditions were altered as required in terms of the annealing temperatures for the individual primers, and in terms of number of cycles for amplification to be in the linear range (usually 20-23 cycles).

## RESULTS

### *Xenopus* FOG is most similar to murine FOG-1

First-strand cDNA was reverse transcribed from *Xenopus* spleen RNA and PCR with degenerate oligonucleotides resulted in the amplification of a 220 bp *Xenopus Fog* cDNA fragment. A *Xenopus* spleen cDNA library was generated (see Materials and Methods) and screened using the 220 bp

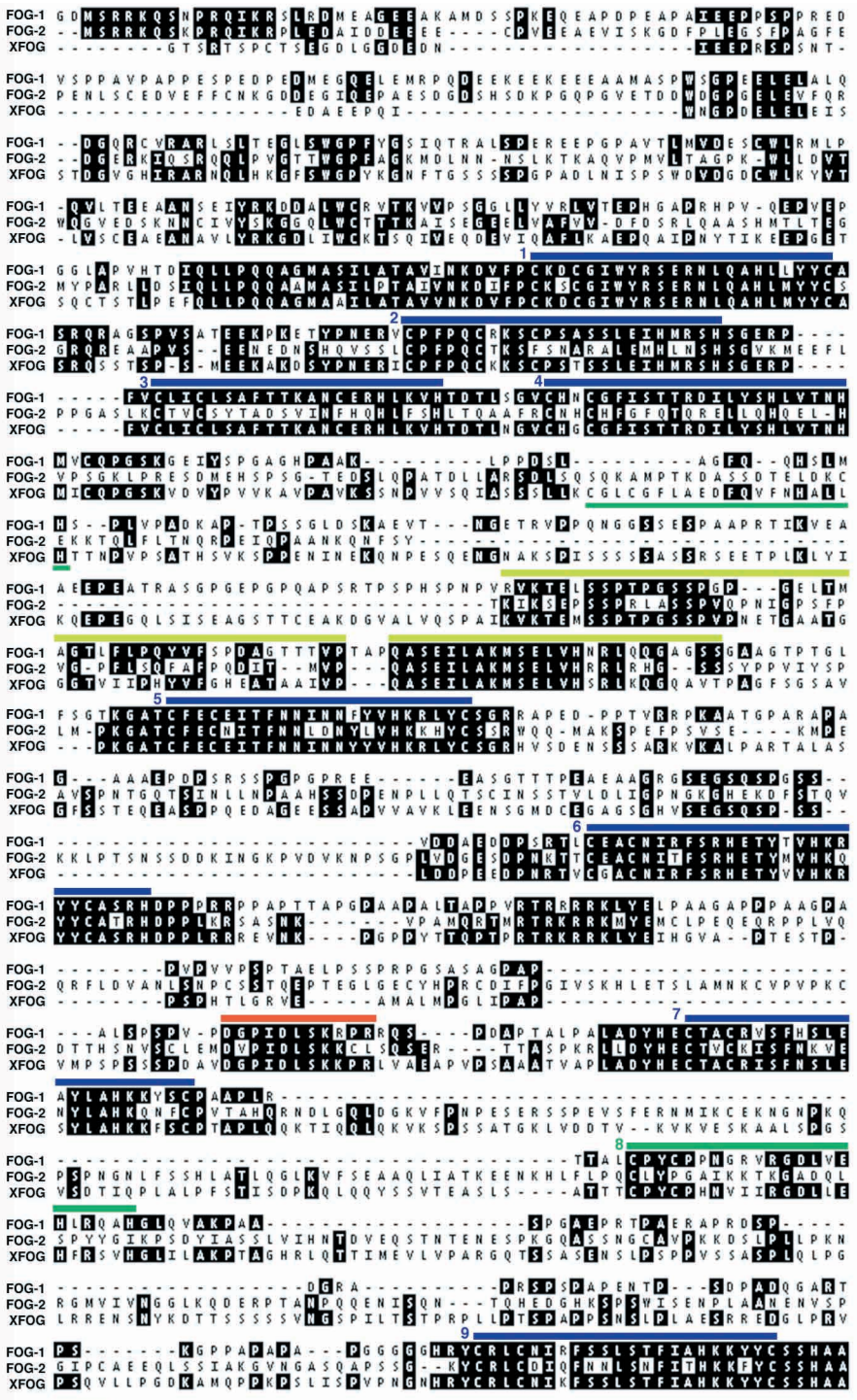


**Fig. 1.** Deduced amino acid sequence alignment between xFOG and the murine FOG proteins, FOG-1 and FOG-2. The nine zinc-finger motifs are indicated by numbered bars (blue). Zinc finger 8, absent in FOG-2 (green bar), is the least conserved. xFOG has an additional zinc-finger motif between fingers 4 and 5, underlined in green, which may have been originally present in FOG-1, as indicated by the remaining histidine residues. The CtBP binding motif 'PIDLSK' (red bar), which is also present in *Drosophila* u-shaped, is highly conserved among the three FOG proteins. The basic region preceding the CtBP binding motif, possibly a nuclear localization signal, is also conserved among the three family members. A region of striking homology but unknown function is indicated with the light green bar.

fragment as a probe. Several clones were isolated, including one near full-length cDNA corresponding to a novel *Xenopus* gene (*xFog*). The predicted amino acid sequence of xFOG is shown in Fig. 1. FOG-1 has nine zinc finger motifs; fingers 1, 5, 6 and 9 have been shown to cooperate in GATA-1 binding (Fox et al., 1999). xFOG also has nine zinc fingers with few amino acid changes in the four GATA-binding fingers (Fig. 1). The most divergent finger, finger 8, is absent in mouse FOG-2 (Tevosian et al., 1999). xFOG has an additional putative zinc-finger domain, although it is unclear whether this is a functional motif; it contains only eleven rather than twelve amino acids between the two cysteine residues and the two histidine residues. Interestingly, the amino acid sequence alignment program paired FOG-1 with xFOG at the two histidine residues of this domain. The two cysteine residues are missing in FOG-1, suggesting that the zinc-finger motif may have been lost during evolution. The nine conserved zinc fingers of xFOG are spaced and ordered similarly to those in FOG-1, and since the gene is expressed in spleen, the hematopoietic organ in frogs, xFOG is more likely a priori to be the homologue of FOG-1 rather than FOG-2. Two additional regions are highly conserved between xFOG, FOG-1 and FOG-2. The first is a short peptide motif that mediates interaction with C-terminal binding protein (CtBP), a co-repressor (Poortinga et al., 1998; Nibu et al., 1998; Turner and Crossley, 1998; Schaeper et al., 1998; Sewalt et al., 1999). The second, a conserved region lying between fingers 4 and 5, is unique to FOG proteins although its functional relevance is unknown.

## The expression pattern of *xFog* encompasses that of murine *Fog-1* and *Fog-2*

RNA was isolated from adult *Xenopus* tissues for northern analysis. The 6 kb *xFog* transcript is expressed in liver, testis and spleen (the major hematopoietic organ in *Xenopus*), as expected for a *Fog*-1 homologue (data not shown). It is also expressed in eyes as well as brain and heart, the latter reminiscent of murine *Fog*-2 expression.

To determine the onset of *xFog* expression, primers were designed to the 3′ coding region and used in RT-PCR analysis for a range of developmental stages (Fig. 2). xFog transcripts are present at early cleavage stages. *xGata-1* is not expressed early during embryogenesis, but *xGata*-2

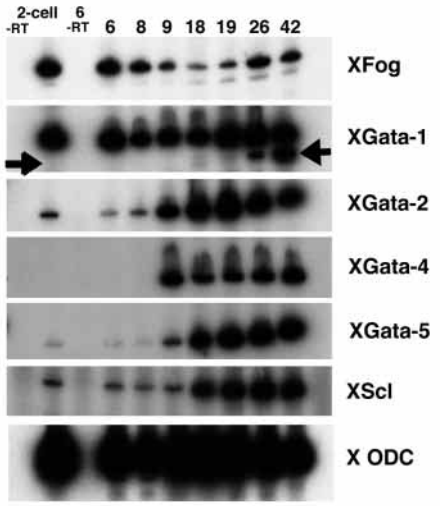


**Fig. 2.** Analysis of *xFog* expression pattern. Semiquantitative RT-PCR analysis, for a range of developmental stages, shows that the *xFog* transcript is maternally derived and continues to be expressed throughout development. *xGata*-2 and *xGata*-5 transcripts are also maternally derived, whereas *xGata*-1 (indicated by arrow) and *xGata*-4 expression is initiated after or around the time of gastrulation. ODC (ornithine decarboxylase) is a control for RNA recovery at the various stages.

and *xGata*-5 are also maternally derived transcripts (Fig. 2; Jiang and Evans, 1996), suggesting that xFOG may act as a co-factor for both xGATA-2 and xGATA-5 during early development.

Whole-mount in situ hybridization of digoxigenin-labeled *xFog* riboprobes was used to examine the embryonic expression pattern in developmentally staged *Xenopus* embryos. The pattern of expression was compared to that of the six *Xenopus Gata* genes, and many regions of co-expression were observed (Fig. 3). The earliest expression detected by this method is in early neurulae, mostly at the anterior end on the ventral side (Fig. 3A), similar to *Gata*-2, *Gata*-6 and *BMP*-4 (not shown; Walmsley et al., 1994; Kelley et al., 1994; Gove et al., 1997; Nakayama et al., 1998). Expression extends to the presumptive pronephros, where *Gata*-3 is expressed (not shown). Both *Gata*-2 and *Gata*-3 are expressed in ventral non-neuronal ectoderm (Kelley et al., 1994). In early tailbud embryos, expression is observed in the heart primordia (Fig. 3B), similar to *Gata*-6 at that stage (stage 23; Fig. 3F). Around stage 27, expression is abundant in the heart tube and in the ventral blood island (Fig. 3C), at a time when *Gata*-2 is still present (Fig. 3G), although *Gata*-1 expression is increasing (not shown, Kelley et al., 1994). Expression can also be detected in midbrain and in the otic vesicle (Fig. 3C,D), similar to *Gata*-3 (Fig. 3H). At tadpole stages, *xFog* is expressed in midbrain, heart, ventral blood island, otic vesicle, eyes, olfactory placode, pronephros and gut (Fig. 3D,E,J), encompassing many of the expression sites of the *Gata* family. Sections of a digoxigenin-stained tadpole (stage 29) show that *xFog* is expressed in the mesodermal layer of the ventral blood island (Fig. 3K,L). This pattern of expression is concordant with the speculation that xFOG may

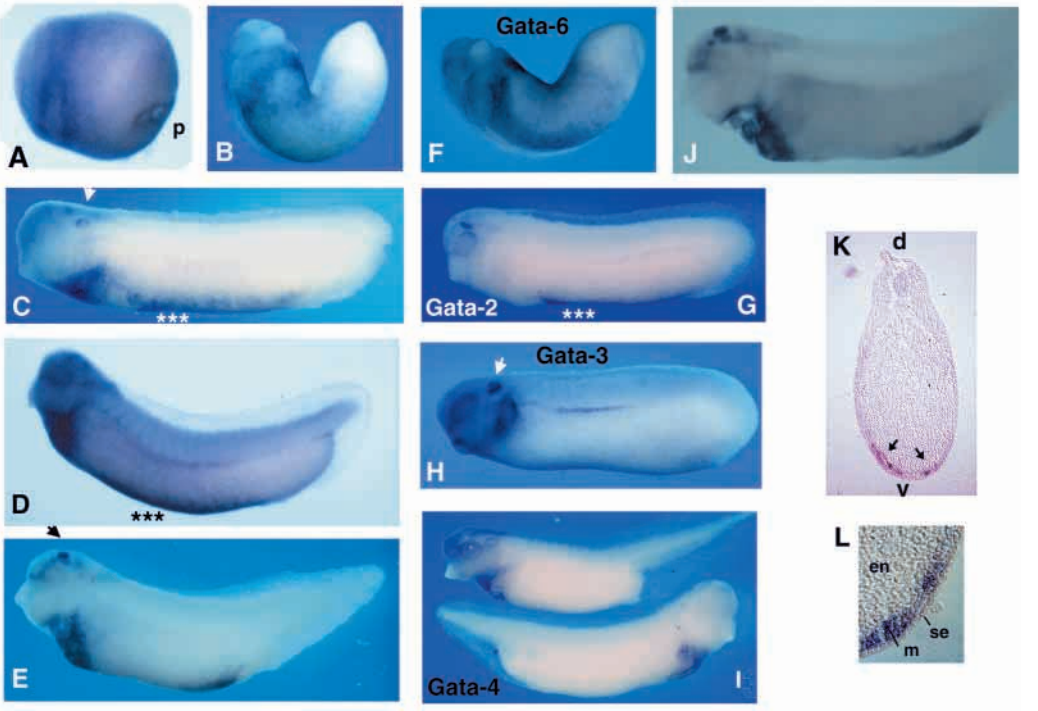


**Fig. 3.** Analysis of *xFog* expression pattern by RNA in situ hybridization on developmentally staged (albino) embryos. The pattern of *xFog* expression (A-E) in developing *Xenopus* embryos is a combination of the *xGata* gene expression patterns (F-I). (A) At stage 15, expression starts in the presumptive heart and the presumptive ventral blood island, possibly extending up into the presumptive pronephric region, but excluded from the presumptive liver. P, posterior. (B) At stage 23, expression is pronounced in heart primordia, similar to *xGata*-6 (F). (C) At stage 27, expression is high in heart tube, and in the ventral blood island (indicated by stars, very similar to *xGata*-2, shown in G). Expression is also seen in midbrain and hindbrain, and in the otic vesicle (indicated by white arrow, similar to *xGata*-3 shown in H). (D) At stage 33, xFog continues to be highly expressed in the heart and ventral blood island. The faint staining in the region around the midline is either the pronephros, similar to *xGata-3* (H) or a blood vessel. Expression is also seen in the eyes and brain (similar to *xGata-3*). (E,J) At stage 38, expression of *xFog* is pronounced in heart and surrounding tissue (similar to *xGata*-4 in I), midbrain and the posterior region of the ventral blood island. (F) *xGata*-6, stage 23; (G) *xGata*-2, stage 28; (H) *xGata*-3, stage 26; (I) *xGata*-4, stages 38 (bottom) and 40 (top); (J) *xFog*, stage 38, cleared embryo. (K,L) Transverse section of a stage 29 embryo. *xFog* is expressed in the mesodermal layer of the ventral blood island (original magnification: (K) ×100, (L) ×400; v, ventral; d, dorsal; se, sensorial ectoderm; en, endodermal yolk mass; m, mesodermal layer.

interact with, and modulate the activity of, all six *Xenopus* GATA factors.

### *xFog* expression is increased by injection of mesoderm-inducing growth factors

To determine which signaling pathways activate *xFog* expression, various growth factors were injected into the animal pole region of fertilized eggs. Animal cap explants were dissected at the late blastula stage and expression of *xFog* was analyzed by RT-PCR at stage 24. *xFog* was not induced by microinjection of activin or culturing in the presence of bFGF. In contrast, Avg, BMP-4 and Xmad each increased *xFog* expression (Fig. 4A). *xGata*-1 was induced only by BMP-4 and Xmad. When animal cap explants were isolated at stage 8 and harvested at a later stage (stage 33), *xGata*-1 and *xFog* were both induced by culturing in bFGF (see later, Fig. 9A).

In whole embryos, *xFog* expression was also increased by injection of *BMP*-4, as assessed by whole-mount in situ hybridization of the ventralized, BMP-4-injected embryos (Fig. 4B). Mix.3, which lies downstream of BMP-4 in the TGFβ signaling cascade (Mead et al., 1998b), also expands *xFog* expression (not shown). These findings are consistent with a role for xFOG in regulating erythropoiesis (Maeno et al., 1996), but suggest that xFOG may be involved in additional signaling pathways of embryonic development.

### Ectopic expression of mFOG-2 blocks erythropoiesis

Increasing amounts of Fog-1 and Fog-2 RNA (100 pg-1 ng RNA per blastomere) were microinjected into embryos at the 2- and 4-cell stage. Injection of Fog-2 RNA caused drastic effects, resulting in axial problems and other phenotypic abnormalities (Fig. 5). To assess whether red blood cell formation was affected, embryos were stained with o-dianisidine (a marker for hemoglobin, the production of which requires the coordinate expression of globin chains and heme biosynthetic enzymes; Zon et al., 1991; Mead et al., 1998a; Huber et al., 1998). Most embryos had reduced or no o-dianisidine staining (Fig. 5), indicating an absence of hemoglobin and mature red blood cells. Thus, ectopic expression of FOG-2 can block red blood cell differentiation. Fog-1 RNA was also microinjected into embryos but toxicity problems prevented phenotypic analysis even at early stages of embryonic development. The highest dose of Fog-1 that did not result in lethality (500 pg) caused no obvious phenotypic changes, apart from a subtle shortening of the body axis. However, o-dianisidine staining was more anterior, diffused and lighter than in control sibling embryos (not shown).

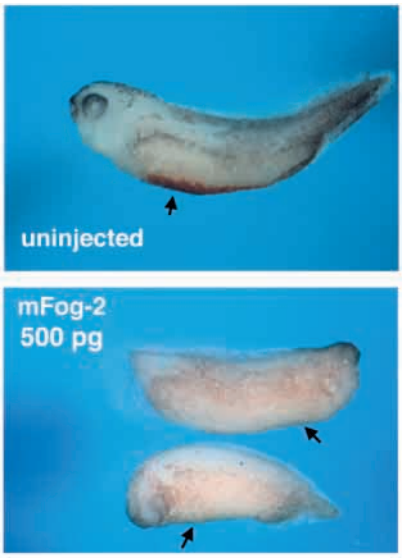


**Fig. 5.** Ectopic expression of FOG-2 in whole embryos can block hemoglobin production. Fertilized eggs were injected with 500 pg Fog-2 RNA per blastomere at the 2- or 4-cell stage. Injected embryos and age-matched control siblings were stained in o-dianisidine to examine the level of hemoglobin in the ventral blood island.

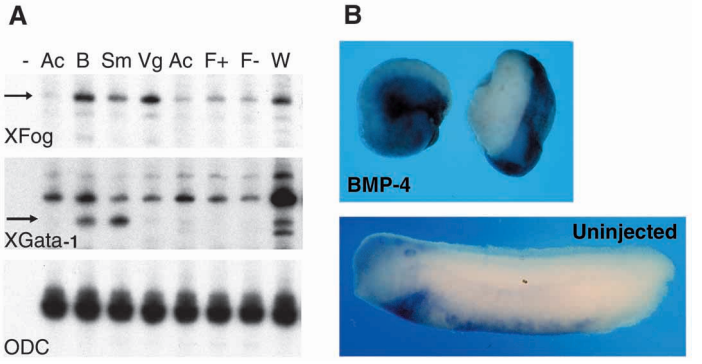


**Fig. 4**. Induction of *xFog* expression by growth factors. (A) Semiquantitative RT-PCR analysis at stage 24, of *xFog* expression after growth factor induction in animal cap assays. Key: –, no reverse transcriptase; Ac, Activin; B, BMP-4; Sm, Xmad; Vg, AVg; F+, bFGF; F–, without growth factor; W, whole embryo. BMP-4, Smad and Avg each induce *xFog*, whereas *xGata*-1 is induced by BMP-4 and Smad, only. ODC is the RNA loading control. (B) In whole embryos, BMP-4 signaling increases the area of *xFog* expression (top panel; ventralized embryos) compared to uninjected sibling controls (bottom panel).

### Red blood cell markers are reduced by both Fog-1 and Fog-2 in explant assays

To determine whether FOG acts on specific transcriptional targets of GATA-1 and GATA-2, marginal zone assays were performed. RNAs were injected into both marginal regions at the 2-cell stage. Ventral and dorsal marginal zone explants (VMZ and DMZ, respectively) were dissected at stage 10+, and cultured until stage 38. RNA was then harvested and used in RT-PCR analysis for red blood cell markers. *xGata*-1 levels were reduced in Fog-2-injected VMZs compared to uninjected VMZ (not shown). To determine whether or not Fog-2 also affects expression of *xGata*-2, marginal zone explants were harvested at an earlier stage (stage 28). Again, *xGata*-1 levels were drastically reduced in VMZs injected with Fog-2. Another red blood cell marker, *xScl*, was slightly reduced in expression, whereas *xGata*-2 was unchanged (Fig. 6A).

To assess whether FOG-1 can also repress expression of late red blood cell markers, animal cap assays were undertaken. Increasing doses of Fog-1 RNA were injected into the animal pole region of fertilized eggs. Animal cap explants were dissected at stage 8.5 and cultured in the presence of bFGF until stage 33, when, in the ventral blood island, *Gata*-2 expression is downregulated whilst *Gata*-1 is upregulated, permitting expression of *globin*. Semiquantitative RT-PCR revealed that an increase in the dose of Fog-1 RNA resulted in a concordant decrease in *globin* expression (Fig. 6B). The inhibition of *globin* expression was abolished by co-injection with Gata-1 (Fig. 6B, +G).

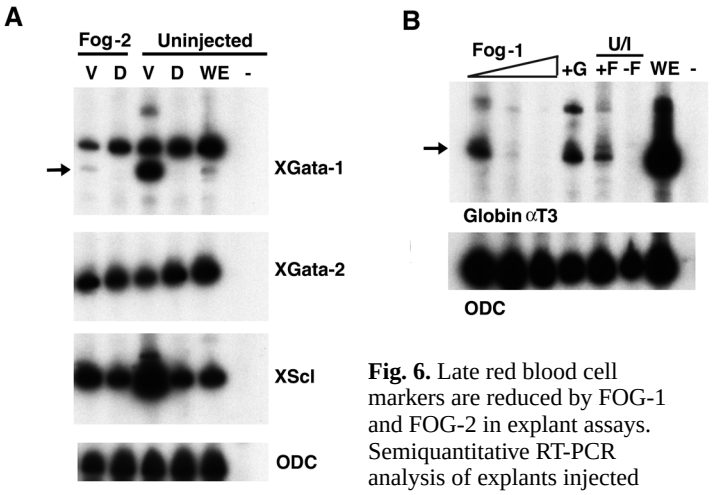


**Fig. 6.** Late red blood cell markers are reduced by FOG-1 and FOG-2 in explant assays. Semiquantitative RT-PCR analysis of explants injected with murine FOG RNAs. ODC is a control for the RNA levels. V, ventral marginal zones; D, dorsal marginal zones; WE, whole embryo; –, control reaction without reverse transcriptase. (A) FOG-2 represses or delays expression of the late red blood cell marker *xGata-1* in ventral marginal zones harvested at stage 28, but *xGata-2*, an early red blood cell marker, is not affected. (B) FOG-1 also inhibits *globin* expression in animal cap assays. *Globin* expression is reduced with increasing doses of Fog-1 (100 pg, 500 pg, 1 ng). Co-injection of Gata-1 (+G: 500 pg Fog-1 + 500 pg Gata-1) counteracts the effect of Fog-1. Uninjected caps in the absence of bFGF do not express *globin* (U/I -F).

## FOG proteins may inhibit red blood cell formation by interaction with the co-repressor CtBP

Red blood cells can be observed in anesthetized tadpoles in the blood vessels in the gills, the tail vein, and most clearly the heart (Fig. 7A). Injection of Fog-2 into a single blastomere of a 4-cell stage embryo (to maximize survival beyond 4-5 days) resulted in tadpoles with hearts that were very pale in comparison to wild-type uninjected siblings (Fig. 7C). Visual inspection revealed a marked decrease in the number of blood cells in circulation. Thus, ectopic FOG interferes with the production of circulating red blood cells. Tadpoles were stained with o-dianisidine to assess levels of hemoglobin as a marker for mature red blood cells. Fog-2-injected tadpoles had a drastic reduction in staining (Fig. 7D) compared to uninjected wild-type sibling controls (Fig. 7B), indicating that FOG delays formation of mature, hemoglobin-positive red blood cells. Some Fog-2-injected tadpoles had abnormal patches of hemoglobin staining, which may be due to aberrant circulation, but may also represent ectopic sites of red blood cell formation.

We also injected increasing amounts of xFog RNA (100-1000 pg), to ensure that the mammalian FOGs mimic xFOG when overexpressed in *Xenopus*. Injected embryos displayed similar phenotypic abnormalities as those injected with Fog-1 and Fog-2 RNA, including shortening of the body axis and cardiac oedema (A. E. D., P. E. M. and S. H. O., unpublished data). Furthermore, injection of xFOG into a single blastomere of a 4-cell stage embryo resulted in decreased or absent o-dianisidine staining in the ventral blood island, confirming that overexpression of xFOG also inhibits erythropoiesis (Fig. 7I-J, compared to uninjected sibling control shown in Fig. 7G). Injection of a high dose (1000 pg) of a control xFog RNA did not result in any abnormalities (Fig. 7H).

To determine whether the reduction in red blood cells and apparent block in terminal erythroid differentiation is dependent on the interaction of FOG-2 with the co-repressor CtBP, a Fog-2 RNA (FogΔCtBP) with a mutated CtBP binding motif (A. B. Cantor, S. G. K., S. G. T. and S. H. O., unpublished data) was injected. Tadpoles injected with FogΔCtBP RNA into the ventral marginal zone of 4-cell-stage embryos, had a normal (or perhaps even more pronounced) red coloration in the heart and large blood vessels (Fig. 7E), and normal or elevated levels of hemoglobin (Fig. 7F). This strongly suggests that FOG's repression of erythroid maturation in vivo is dependent, at least in part, on its interaction with the co-repressor protein CtBP.

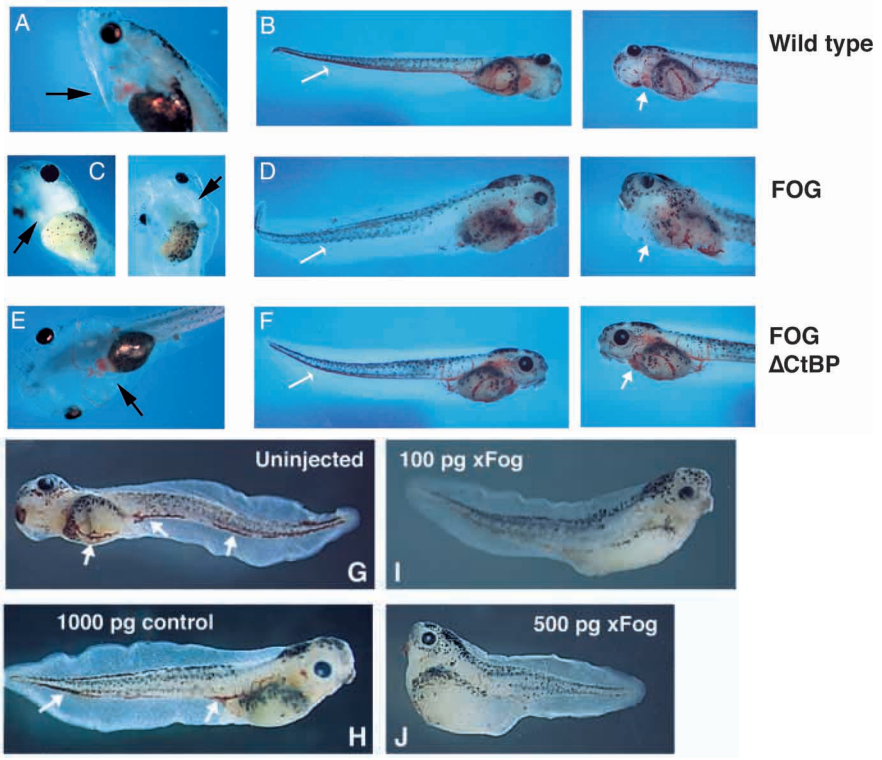


**Fig. 7.** Overexpression of FOG-2 and xFOG inhibits erythropoiesis. The interaction with CtBP appears to be, at least in part, responsible for this inhibition. (A,C,E) Anesthetized tadpoles; (B,D,F,G-J) Tadpoles stained with o-dianisidine. Uninjected (A) tadpoles have beating hearts that pump red blood, whereas Fog-2-injected (C) tadpoles have a beating heart (indicated by black arrow) that pumps very pale blood cells. FogΔCtBP-injected (E) tadpoles have a beating heart that pumps very red blood. Globin-expressing red cells, stained with o-dianisidine, are noticeable along the tail vein and in the heart in uninjected tadpoles (B,G, indicated by arrows), in tadpoles injected with a negative control (3′-5′) xFog RNA (H, white arrows point to staining in major veins), but not in Fog-2 or xFog-injected tadpoles (D,I,J). Tadpoles injected with FogΔCtBP (F) display normal o-dianisidine staining.

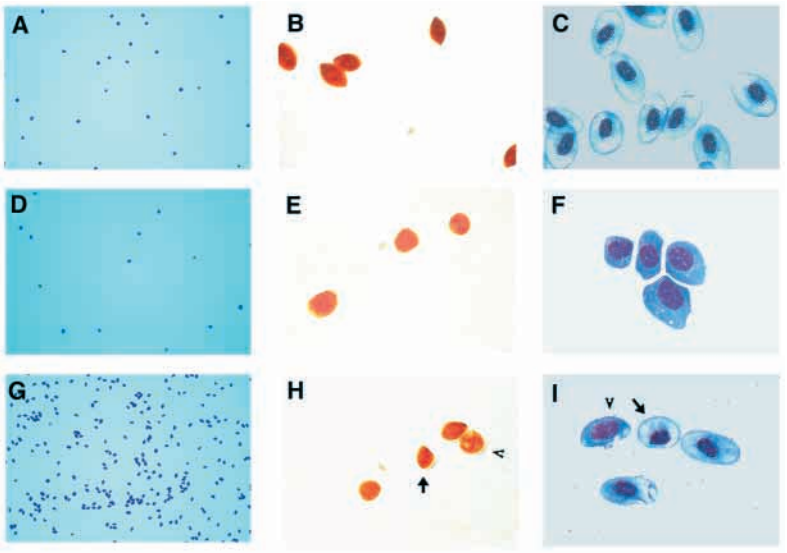


**Fig. 8.** In the absence of CtBP binding, FOG-2 stimulates stem cell proliferation, but maturation and differentiation is not occurring as normal. (A,B,D,E,G,H) Tadpole blood, stained with o-dianisidine and cytospun (original magnification A,D,G, ×100; others, ×400). (C,F,I) May Grünwald Giemsa (MGG) staining of tadpole blood smears. Tadpoles injected with Fog-2 have fewer red blood cells (D), which are paler when stained with o-dianisidine, and rounder (E), compared to uninjected (A,B). Tadpoles injected with FogΔCtBP have a striking increase in number of red blood cells (G), but not all these blood cells are normal in appearance (H, arrowhead) when compared to stage-matched wild-type sibling controls. Red blood cells collected from wild-type tadpoles have compacted nuclei (C), whereas blood cells from tadpoles injected with Fog-2 look developmentally delayed (F). A mixture of normal and developmentally delayed blood cells (arrow and arrowhead, respectively) are found in tadpoles injected with FogΔCtBP (I).

To study blood cell morphology, blood was collected from several tadpoles, and either stained with o-dianisidine prior to cytocentrifugation, or spread onto a glass slide and stained with May Grünwald Giemsa (MGG). The former provides a semiquantitative assay to confirm the observed reduction of circulating red blood cells in the anesthetized tadpoles, whereas the latter, MGG staining, allows a more qualitative study of cellular morphology. There was a reduction in red blood cell number in Fog-injected tadpole blood (Fig. 8D) compared to uninjected tadpoles (Fig. 8A). Furthermore, a dramatic increase in red blood cells in tadpoles accompanied injection of the FogΔCtBP mutant RNA (Fig. 8G). Wild-type red blood cells were dark red and ovoid (Fig. 8B), whereas all the red blood cells from the Fog-injected tadpole were round and lighter in color, indicating a less-differentiated state with less hemoglobin (Fig. 8E). FogΔCtBP RNA injection results in both types of blood cells (Fig. 8H).

The difference in appearance was also evident in MGG-stained blood cells (Fig. 8C). Wild-type blood cells are oval with a clear cytoplasm and a dark, condensed nucleus (98% of cells). In contrast, blood cells from Fog-injected tadpoles were round with a darker cytoplasm and larger, lighter nucleus, again indicating a less advanced stage of erythrocyte maturation (Fig. 8F). Blood cells from tadpoles injected with the FogΔCtBP, however, had a mixture of both types of cells (10%-35% of cells, collected from four tadpoles, looked similar to controls), suggesting that the ΔCtBP mutant stimulates red blood cell formation, but not all the cells terminally differentiate (Fig. 8I). This is the first indication that, in vivo, in the context of a whole animal, FOG can act as a repressor of erythropoiesis by virtue of interaction with CtBP.

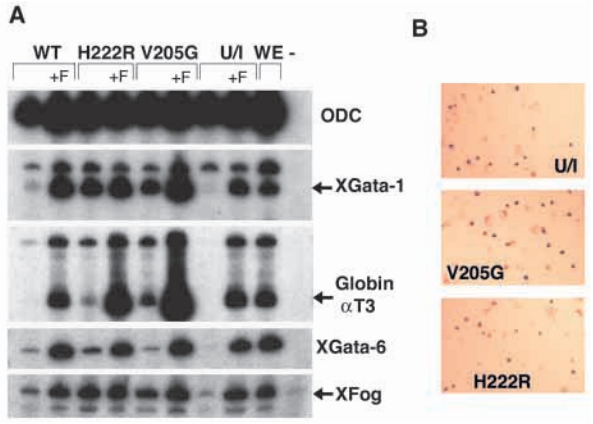


**Fig. 9**. GATA-1 mutants impaired for interaction with FOG (see text) are better inducers of red blood cell differentiation than wild-type GATA-1. (A) Semiquantitative RT-PCR analysis of animal caps cultured until stage 33, in the presence (+F) or absence of bFGF, shows that the GATA-1 binding mutants induce higher levels of late red blood cell markers (*xGata-1* and α*T3 globin)* than wild-type GATA-1. *xGata*-6 is not affected, nor is *xFog* itself, in the presence of bFGF. In the absence of bFGF, however, *xFog* is elevated by injection of the *Gata*-1 binding mutants. WT, wild type; H222R, V205G, specific binding mutants described in Crispino et al. (1999); U/I, uninjected; WE, whole embryo; –, control reaction without reverse transcriptase. ODC is a control for RNA levels. (B) Animal pole regions were injected with *Gata-1* and *Gata*-1 mutants impaired for FOG interaction. Animal cap explants were dissociated, stained with o-dianisidine and cytocentrifuged (original magnification ×200). Expression of the GATA-1 mutants does not affect the heme-biosynthetic pathway or production of hemoglobin.

## GATA-1 mutants impaired for interaction with FOG are better inducers of blood than wild-type GATA-1

Injection of Gata-1 RNA into the animal pole region of fertilized eggs triggers the erythropoietic program in the presence of bFGF. To determine whether FOG is involved in this function of GATA-1, murine GATA-1 mutants impaired for interaction with FOG were expressed in the animal pole region. The mutant H222R has an amino-acid substitution at position 222 (from His to Arg), and the V205G mutant has a substitution at position 205 (from Val to Gly, described by Crispino et al., 1999). Animal cap explants were dissected at stage 8.5 and cultured until stage 33 for RT-PCR analysis. Levels of *xGata*-1 and α*T3-globin* were increased in bFGF-treated caps injected with mutant Gata-1 RNA, compared to bFGF-treated caps injected with wild-type Gata-1 RNA (Fig. 9A).

This indicates that endogenous xFOG may also act as a repressor of red blood cell differentiation in vivo, and that mutant GATA-1 can act independently of FOG, thereby inducing its own (in this case xGata-1) expression. The level of *xGata*-6 expression was unaffected, indicating that the transcriptional repression is specific for *xGata*-1 in mesoderm at this stage. To determine whether the two GATA-1 mutants affect the heme-biosynthetic pathway and production of hemoglobin, animal cap assays were undertaken and analyzed by o-dianisidine staining (as described by Huber et al., 1998). There was no difference between wild-type GATA-1 and mutant GATA-1 (Fig. 9B).

## DISCUSSION

### *Xenopus* FOG homologue

We have cloned a novel *Xenopus* gene, *xFog*, which encodes a transcription factor with multiple zinc-finger motifs. xFOG is highly similar to mouse FOG-1 (Tsang et al., 1997), and is the latest addition to the family of 'Friend of GATA' proteins, which also includes mouse FOG-2 (Svensson et al., 1999; Tevosian et al., 1999; Lu et al., 1999) and *Drosophila* u-shaped (Cubadda et al., 1997; Haenlin et al., 1997). All FOG-like proteins are capable of interacting with all two-finger GATA factors in vitro and, where co-expressed, are likely to do so in vivo. Indeed, the amino acid sequence of the zinc fingers known to be involved in the GATA-factor interaction are the most highly conserved amongst the murine and *Xenopus* FOGs (Tsang et al., 1997; Crispino et al., 1999; Fox et al., 1998).

In addition to the conserved zinc-finger motifs, a region of striking homology that may be functionally important is located between fingers 4 and 5. Likewise, a binding site for the transcriptional repressor CtBP (Turner and Crossley, 1998; Sewalt et al., 1999) is conserved in all FOG-like members. Reporter assays in co-transfected NIH 3T3 cells have suggested that FOG can repress GATA-mediated transcription by associating with CtBP (Fox et al., 1999; Holmes et al., 1999). A more functionally relevant assay suggests that CtBP mediates a transcriptional repressor function of FOG-1 in hematopoietic cells (A. B. Cantor, S. G. K., S. G. T. and S. H. O., unpublished data).

We have analyzed the pattern of expression of *xFog* and show that it is a maternally derived transcript, similar to *Xenopus Gata*-2 and *Gata*-5 (Walmsley et al., 1994; Kelley et al., 1994; Jiang and Evans, 1996). During development, *xFog* is expressed at sites of GATA factor expression (Kelley et al., 1993, 1994; Walmsley et al., 1994; Jiang and Evans, 1996; Gove et al., 1997), suggesting that it may interact with and modulate multiple GATA factors. As yet, we have been unable to isolate additional *Xenopus* FOGs. We therefore suspect that the FOG family has a single representative in *Xenopus*.

*xFog* transcripts are particularly abundant in the ventral blood island (similar to *Gata*-2 and then *Gata*-1), and heart (similar to *Gata*-4, *Gata*-5 and *Gata*-6). *xFog* is also expressed in developing brain and the otic vesicle (ectoderm, similar to *Gata*-2 and *Gata*-3), in pronephros (similar to *Gata*-3), and in endoderm (similar to *Gata*-4 and *Gata*-6). In adult tissues, *xFog* is expressed predominantly in spleen and heart. Interestingly, although the structure of xFOG is more similar to that of FOG-1, the expression pattern of *xFog* is a composite of murine *Fog-1* and *Fog-2*, and more. Consequently, additional mammalian FOG homologues may exist. Taken together, the structure and expression data suggest that xFOG may be the common ancestor for both FOG-1 and FOG-2.

*xFog* is expressed in the mesodermal layer in the ventral blood island and, indeed, *xFog* expression is increased in animal cap assays following injection of mesoderm-inducing growth factors BMP-4 and Xmad. *xFog* is also upregulated by BMP-4 in whole embryos. Mix.3, a protein upstream of GATA factors in the TGFβ signaling cascade, that has been shown to pattern mesoderm and endoderm (Mead et al., 1998b), also induces *xFog* expression. These data are consistent with a role for xFOG during mesodermal differentiation.

### FOG proteins repress red blood cell development in vivo at least in part by interaction with CtBP

In mouse, FOG-1 is required for erythroid differentiation and appears to cooperate with GATA-1. However, in whole *Xenopus* embryos, FOG proteins repress red blood cell formation. In marginal zone explant assays, *xGata*-1 levels are drastically reduced in Fog-2-injected VMZs compared to uninjected VMZs. *SCL* is also reduced in Fog-2-injected VMZs but the difference is less pronounced. It appears that FOG proteins only affect terminal (or near-terminal) differentiation events, such as *xGata*-1 and *globin* expression, leaving earlier red blood cell markers, especially *xGata*-2, mostly unaffected. GATA-1 promotes differentiation of committed erythroid precursors, both by suppressing genes involved in progenitor cell proliferation (such as *Gata-2* and *c-myb*), and by inducing differentiation-specific genes (such as globin and heme biosynthetic enzymes). The reduction in *xGata*-1 levels, caused by overexpression of FOG-2, may be directly responsible for the delay in erythrocyte maturation. How FOG reduces the level of *xGata*-1 transcripts is unclear, but it probably acts by repressing xGATA-1 and xGATA-2, proteins capable of influencing their own, and each others, expression levels (Zon and Orkin, 1992). The decrease in the number of circulating red blood cells may be caused by the interaction between xFOG/FOG-2 with xGATA-2; xGATA-2 is repressed, and thus self-renewal of early erythroid progenitor cells. Indeed, expression of FOGΔCtBP (FOG with an impaired CtBP interaction motif), augments red cell production in whole embryos, presumably because xGATA-2 is not kept in check by FOGΔCtBP. Intriguingly, some blood cells of FogΔCtBP-injected tadpoles are developmentally delayed. Thus, although FogΔCtBP stimulates red blood cell formation, the terminal differentiation into mature red blood cells may not proceed properly. This could be due to a premature excess of red blood cell progenitors with a concomitant lack in endogenous signals that are required for normal terminal differentiation. One such signal that is necessary for terminal differentiation, is downregulation of *xGata*-2 expression (see below). Alternatively, FOG may also be capable of acting as a repressor independent of the CtBP.

### Uniting *Xenopus* with mouse

Our data confirm that the transcriptional consequences of FOG and GATA interaction are highly context-dependent. In *Xenopus* explant assays, GATA-1 mutants impaired for interaction with FOG are better inducers of blood than wild-type GATA-1, indicating that xFOG represses GATA-1

function. The findings presented in this paper are in apparent contradiction with previous functional data.

Firstly, FOG-1 cooperates with GATA-1 to promote differentiation of a GATA-1 null erythroid murine cell line (G1E, Weiss et al., 1994; Tsang et al., 1997; Crispino et al., 1999). In G1E cells, erythroid differentiation is blocked due to the absence of GATA-1. Expression of wild-type GATA-1 restores terminal maturation, whereas GATA-1 proteins impaired for interaction with FOG-1 fail to rescue (Crispino et al., 1999). Thus, direct interaction between the two proteins is required for terminal differentiation to proceed. The level of GATA-2 is high in G1E cells until differentiation begins, at which point it drops precipitously. Downregulation of GATA-2 is a requisite for differentiation to proceed (Briegel et al., 1993, 1996; Persons et al., 1999). A FOG-1/GATA-1 complex may act to repress transcription of GATA-2 in committed erythroblasts. In *Xenopus*, unlike in G1E cells, wild-type GATA-1 is present in addition to the ectopically expressed mutant GATA-1. These GATA-1 mutants can activate transcription of the endogenous, wild-type *Gata*-1, independently of FOG, resulting in increased blood cell differentiation.

Secondly, in FOG-1-deficient mice, the hematopoietic defect is similar to that of GATA-1-deficient mice. This phenotype is at odds with the increase in number of hemoglobinized cells that we would predict upon loss of a repressor. Studies in *Xenopus*, however, emphasize the role of FOG in early developmental events. Thus, although an early role of mouse FOG-1 may be to limit the number of cells with an erythropoietic fate, once cells are committed to the erythroid lineage, FOG-1 may cooperate alongside GATA-1 in erythroblasts. Alternatively, it is possible that, in mouse, FOG-1 acts solely with GATA-1 in erythroid maturation. In this setting, its first function may be to downregulate expression of GATA-2, and other proteins that promote progenitor proliferation versus differentiation, as a critical step towards red blood cell differentiation (Briegel et al., 1996; Persons et al., 1999). In the absence of FOG-1, GATA-1 may be unable to shut off transcription of GATA-2. The erythropoietic program might then be stalled at the blast-phase, similar to what is seen upon deficiency of GATA-1.

In early development, FOG proteins regulate GATA-dependent activities in ventral mesoderm, ectoderm and endoderm. Specifically in blood, their role may be to limit differentiation into erythrocytes to prevent depletion of pluripotent stem cells. We show that, in vivo, this role is at least in part dependent on interaction with the co-repressor protein CtBP. In conclusion, FOG may participate in the switch from stem cell proliferation to differentiation during erythropoiesis.

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