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Transcriptional cofactors of the FOG family interact with GATA proteins by means of multiple zinc fingers

Archa H.Fox, Chu Liew, Melissa Holmes[1], Kasper Kowalski, Joel Mackay and Merlin Crossley[2]

Department of Biochemistry, G08, University of Sydney, NSW, 2006 and [1]Centre for Thrombosis and Vascular Research, School of Pathology, University of New South Wales, NSW, 2052, Australia

[2]Corresponding author
e-mail: M.Crossley@biochem.usyd.edu.au

Friend of GATA-1 (FOG-1) is a zinc finger protein that has been shown to interact physically with the erythroid DNA-binding protein GATA-1 and modulate its transcriptional activity. Recently, two new members of the FOG family have been identified: a mammalian protein, FOG-2, that also associates with GATA-1 and other mammalian GATA factors; and U-shaped, a *Drosophila* protein that interacts with the *Drosophila* GATA protein Pannier. FOG proteins contain multiple zinc fingers and it has been shown previously that the sixth finger of FOG-1 interacts specifically with the N-finger but not the C-finger of GATA-1. Here we show that fingers 1, 5 and 9 of FOG-1 also interact with the N-finger of GATA-1 and that FOG-2 and U-shaped also contain multiple GATA-interacting fingers. We define the key contact residues and show that these residues are highly conserved in GATA-interacting fingers. We examine the effect of selectively mutating the four interacting fingers of FOG-1 and show that each contributes to FOG-1's ability to modulate GATA-1 activity. Finally, we show that FOG-1 can repress GATA-1-mediated activation and present evidence that this ability involves the recently described CtBP co-repressor proteins that recognize all known FOG proteins.

Keywords: FOG/GATA-1/gene expression/transcription/zinc finger

Introduction

GATA family proteins are zinc finger transcription factors that recognize (A/T)GATA(A/G) motifs in DNA. The defining feature of this family is the presence of one or two Cys-X_2-Cys-X_{17}-Cys-X_2-Cys zinc fingers. The proteins occur in organisms from yeast to man and co-ordinate a variety of different developmental programmes. The founding member of the family, GATA-1, plays a central role in red blood cell differentiation and it has been suggested that it is involved in regulating the expression of most, if not all, genes that are expressed specifically in the erythroid lineage (Pevny *et al*., 1991; Simon *et al*., 1992; Weiss *et al*., 1994; Fujiwara *et al*., 1996). Additional mammalian GATA proteins play other important roles: GATA-2 and -3 are also involved in hematopoietic development (Tsai *et al*., 1994; Pandolfi *et al*., 1995; Ting *et al*., 1996), whilst GATA-4, -5 and -6 are active in different tissues (Laverriere *et al*., 1994; Jiang and Evans, 1996; Huggon *et al*., 1997).

In an effort to understand the molecular mechanisms through which GATA proteins regulate gene expression, researchers have sought to characterize the functional domains of the proteins and the cofactors with which GATA proteins interact. Studies on GATA-1 have shown that the protein consists of an N-terminal activation domain (Martin and Orkin, 1990) and two zinc fingers: the C-terminal finger binds DNA and the N-finger stabilizes DNA binding at certain complex double GATA sites (Trainor *et al*., 1996). In addition to contacting DNA, the N-finger is also involved in mediating contact with cofactor proteins. Most notably, it has been shown that the N-finger interacts with a recently identified transcriptional cofactor, Friend of GATA-1 (FOG-1; Tsang *et al*., 1997).

FOG-1 is a large protein that contains nine zinc fingers: four of these appear to be classical TFIIIA-like Cys–Cys:His–His fingers and five have a more unusual Cys–Cys:His–Cys configuration. The expression of FOG-1 is largely (but not exclusively) confined to hematopoietic tissues and the liver. FOG-1 and GATA-1 cooperate to drive erythroid and megakaryocytic differentiation and have been shown synergistically to activate a hematopoietic promoter (the p45 NF-E2 promoter) in cellular assays (Tsang *et al*., 1997). Knockout studies have shown that like GATA-1, FOG-1 is essential for the development of primitive and definitive erythrocytes and that FOG-1 also plays critical roles in megakaryopoiesis (Tsang *et al*., 1998). Shortly after the isolation of FOG-1, a related *Drosophila* protein, U-shaped, was isolated and it was demonstrated that it physically interacted with the *Drosophila* GATA factor, Pannier (Haenlin *et al*., 1997). Genetic experiments have confirmed that the two proteins functionally interact *in vivo* to co-ordinate bristle cell differentiation and influence neural cell fates (Cubadda *et al*., 1997). More recently, a new mammalian FOG family member, human FOG-2, has been cloned (M.Holmes, J.Turner, A.Fox, O.Chisholm, M.Crossley and B.Chong, submitted). Unlike FOG-1, FOG-2 is broadly expressed and may modulate the activities of GATA proteins in tissues in which FOG-1 is not expressed. The three FOG proteins are diverse in sequence but their individual zinc finger regions share considerable homology.

Although zinc fingers are noted for their roles in binding DNA, it is apparent that they also play important roles in mediating protein–protein interactions: examples include Ikaros/Aiolos (Sun *et al*., 1996; Morgan *et al*., 1997), YY1/CREB (Zhou *et al*., 1995), GATA-1/EKLF (Merika and Orkin, 1995), TFIIIA (Del Rio and Setzer, 1993) and Roaz/Olf-1 (Tsai and Reed, 1998). Of most relevance here, the sixth zinc finger of FOG-1 physically interacts

with the N-terminal zinc finger of GATA-1. The key contact residues in the GATA-1 N-finger have been defined and are also found in the N-fingers of GATA-2 and GATA-3 (Fox *et al*., 1998). Accordingly, it has been shown that FOG-1 can also interact with these proteins (Tsang *et al.*, 1997). The residues are also highly conserved in GATA-4, -5 and -6, but contact with these proteins has not yet been reported. In contrast, the key residues are absent from all GATA C-terminal fingers, a result that may explain the specificity of FOG-1 for N- rather than C-terminal GATA fingers (Fox *et al*., 1998).

Here we have investigated the FOG family proteins and have first sought to identify which fingers can bind GATA proteins. We show that in addition to finger 6, fingers 1, 5 and 9 of FOG-1 physically interact with the GATA-1 N-finger but not the C-finger. We have also examined the fingers of FOG-2 and U-shaped, shown that these proteins also contain multiple GATA-interacting fingers and identified the common features which allow these fingers to bind to GATA-1. We have tested the functional role of the different GATA-interacting fingers in FOG-1 and present evidence that each finger contributes to FOG-1's function *in vivo*.

Whilst it has been demonstrated previously that FOG-1 can cooperate with GATA-1 to activate transcription, we show that on certain promoters FOG-1 can also inhibit GATA-1-mediated activation. We have investigated the mechanism by which FOG-1 inhibits transcription and show that the protein contains a potent repression domain that interacts with the transcriptional co-repressor mCtBP2. The motif to which mCtBP2 binds is conserved in FOG-2 and in U-shaped, suggesting that CtBP family proteins may also mediate the activity of these proteins.

Results

FOG family members each contain several GATA-interacting Cys–Cys:His–Cys zinc fingers

As shown in Figure 1A, FOG-1 contains four conventional Cys–Cys:His–His zinc fingers and five atypical Cys–Cys:His–Cys fingers. One of these unusual fingers, finger 6, previously has been shown to interact with GATA-1 (Tsang *et al*., 1997; Fox *et al*., 1998). We have now investigated whether additional FOG fingers can also interact with GATA-1. Fragments of FOG-1 were tested for their interaction with the N-finger of GATA-1 in the yeast two-hybrid system. Fragments containing fingers 1 and 6 were positive for interaction with GATA-1, a fragment containing finger 5 was weakly positive, while a fragment containing fingers 2, 3 and 4, and a fragment containing fingers 7 and 8 were negative. In addition, a fragment containing fingers 7–9 was found to be positive for the interaction, suggesting that finger 9 might also be capable of interacting with GATA-1. Selective mutation of finger 9, in the context of the finger 7–9 fragment, eliminated the interaction and provided additional evidence that finger 9 could interact with GATA-1, but unexpectedly finger 9 alone was negative for the interaction in the yeast two-hybrid assay (Figure 1A). In order to clarify the situation and investigate whether the failure of finger 9 alone to show interaction in this assay was an artefact of the yeast system, we carried out additional experiments with purified protein using the glutathione *S*-transferase (GST) pull-down assay. This experiment indicated that GST finger 9 could retain *in vitro* translated GATA-1 efficiently. Additional pull-down experiments confirmed that fingers 1, 6 and 9 interacted strongly with GATA-1, finger 5 interacted weakly with GATA-1 and fingers 2, 3, 4, 7 and 8 did not associate with GATA-1 (data not shown).

We have shown previously that the Cys–Cys:His–Cys configuration of FOG-1 finger 6 is critical for its interaction with GATA-1 (Fox *et al*., 1998) and, interestingly, the four fingers of FOG-1 that interact with GATA-1 are all Cys–Cys:His–Cys fingers. We noted that the recently isolated FOG family members, FOG-2 and U-shaped, also contain several Cys–Cys:His–Cys zinc fingers. We therefore investigated whether any of these fingers could interact with GATA-1. The results of yeast two-hybrid and GST pull-down assays are summarized in Figure 1B and C. Fingers 1 and 6 of FOG-2 were positive for interaction with GATA-1, whilst fingers 5 and 8 interacted weakly. Fingers 1 and 9 of U-shaped were strongly positive, whilst finger 5 was weakly positive.

A signature motif is required for contact with GATA-1

All of these interacting fingers have the Cys–Cys:His–Cys configuration, whereas fingers with the conventional Cys–Cys:His–His arrangement (such as FOG-1 fingers 2, 3, 4 and 8, and FOG-2 fingers 2–4) are not able to interact with GATA-1. Nevertheless, several Cys–Cys:His–Cys fingers are also unable to interact with GATA-1 (i.e. FOG-1 finger 7, FOG-2 finger 7 and U-shaped finger 4), suggesting that additional residues are required for the interaction. In order to investigate the additional sequence requirements for binding to GATA-1, we carried out alanine scanning mutagenesis on FOG-1 finger 1.

All residues within and immediately flanking the finger were replaced individually by alanine, with the exception of the zinc co-ordinating residues (Cys257, Cys260, His273 and Cys278), the large hydrophobic residues Tyr264 and Leu270 that are likely to be critical to the packing of the finger core and residues that were already alanines (Ala272 and Ala279). Each mutant was tested for its ability to interact with GATA-1 in the yeast two-hybrid system and in GST pull-downs (Figure 2A and B). The majority of the mutations did not interfere with the interaction with GATA-1. However, several residues appeared to be either essential (such as Ile262, Asn269 and Tyr277, shown as black in Table I) or important (Phe255, Arg265 and Tyr276, boxed in Table I) to the interaction.

Since the mutations could interfere with the interaction by disrupting either key intermolecular contacts or the entire zinc finger structure, we tested whether any of the mutations that interfered with the interaction prevented normal folding of FOG-1 finger 1. Using circular dichroism (CD) spectropolarimetry, it was found that none of these mutations significantly altered the folding of the domain (data not shown). This result suggests that the residues identified are specific GATA-1 contact residues.

Several residues implicated in GATA-1 binding are conserved in known interacting fingers

Table II shows an alignment of the FOG-type Cys–Cys:His–Cys fingers that we have tested to date. In addition

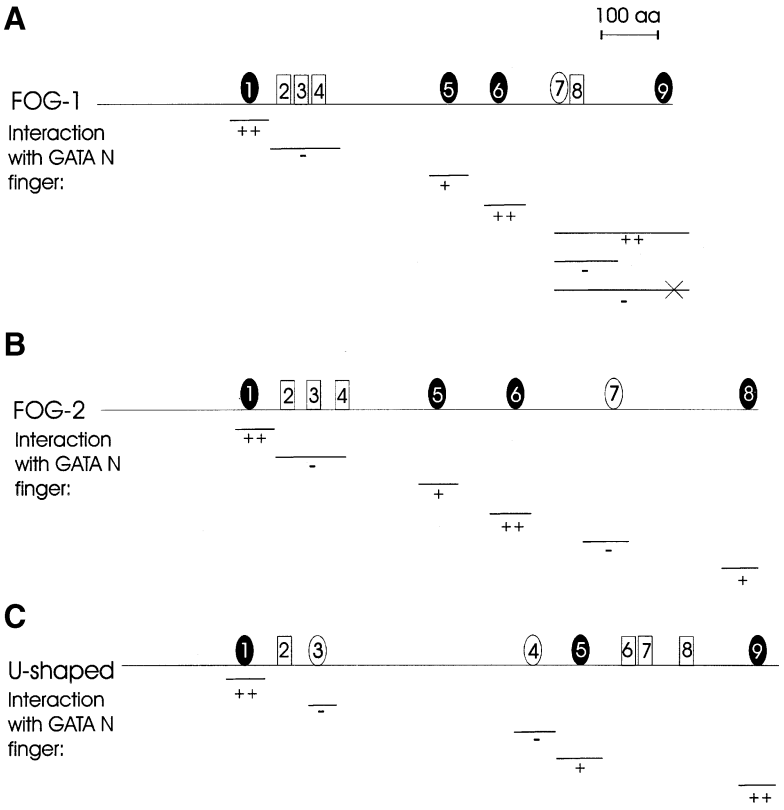

Fig. 1. FOG family members contain several fingers capable of interacting with the N-finger of GATA-1. Schematics of the distribution of zinc fingers within FOG-1 (**A**), FOG-2 (**B**) and U-shaped (**C**) are shown, with black ovals representing Cys–Cys:His–Cys zinc fingers able to interact with the N-finger of GATA-1, clear ovals representing Cys–Cys:His–Cys zinc fingers unable to interact with the N-finger of GATA-1 and clear boxes representing Cys–Cys:His–His zinc fingers. The short bars below the schematics represent regions of each protein which were tested for their ability to interact with the N-finger of GATA-1 (residues 200–254) using the yeast two-hybrid assay. The mutation represented by a cross is Cys986 to His which converts the Cys–Cys:His–Cys finger to a Cys–Cys:His–His finger, and the analogous mutation in FOG-1 finger 6 has been shown previously to abolish the interaction with GATA-1 (Fox *et al*., 1998). Yeast strain HF7c was co-transformed with GATA-1 derivatives harboured in pGBT9, and FOG family derivatives in pGAD10. Transformants were selected on Leu^- Trp^- plates and patched onto Leu^- Trp^- His^- plates. ++ indicates clear growth on Leu^- Trp^- His^- plates when incubated at 29°C for 48 h, + indicates some growth on Leu^- Trp^- His^- plates when incubated at 29°C for 60 h, – indicates no growth (see Figure 2A for an example of actual yeast growth).

to the fingers of FOG-1, FOG-2 and U-shaped, we have also tested Cys–Cys:His–Cys fingers from the transcription factors PRD II (Fan and Maniatis, 1990) and EVI-1 (Matsugi *et al*., 1990), but have found that they do not interact with the GATA-1 N-finger. The key residues implicated by the alanine scanning experiments are shaded. As the alignment indicates, three of these residues (Ile11, Tyr25 and Tyr26) are conserved in all fingers that strongly interact with GATA-1. Overall, a consensus sequence emerges which is shown at the bottom of the table. Whilst the exact sequence found in the different fingers varies slightly, a number of residues (shown in bold) are highly conserved; in particular, the presence of a tyrosine immediately prior to the final cysteine appears to be important for contact with the GATA-1 N-finger.

In order to delineate further the contact face of the FOG-like fingers, we sought to map the residues implicated in contacting GATA-1 onto the structure of one of the FOG-like fingers. The only FOG-like finger that has been studied at a structural level is U-shaped finger 1, the structure of which recently has been solved by NMR spectroscopy (C.Liew and J.P.Mackay, unpublished results). We located the putative contact residues on this structure, (Figure 3, shown in black and numbered). Two additional residues, Phe13 and His22, that are also likely to be contact points, but which play important structural roles and were not mutated, are also numbered (Figure 3). As can be seen from Figure 3, the contact points lie on one surface of the zinc finger domain, consistent with the view that these residues are directly involved in FOG–GATA contacts.

Different FOG-like fingers display the same specificity for GATA N-fingers

The observation that the fingers that interact with the GATA-1 N-finger share similar sequence features suggests that they may bind GATA-1 in a similar configuration. We first tested several of the fingers to determine whether, like the original GATA-1-interacting finger (i.e. FOG-finger 6), they interacted exclusively with the GATA-1 N-finger and not the C-finger. We found that in each case tested (FOG-1 fingers 1 and 9, and FOG-2 fingers 1 and 6), the FOG-like fingers interacted only with the GATA N-finger and not with the C-finger (data not shown). We also tested several of these FOG fingers against a set of GATA N-finger substitution mutants that are unable to bind FOG finger 6 (Fox *et al*., 1998). In each case, the new FOG fingers tested showed the same pattern of interaction as originally reported for FOG finger 6. This result suggests that all the FOG fingers that

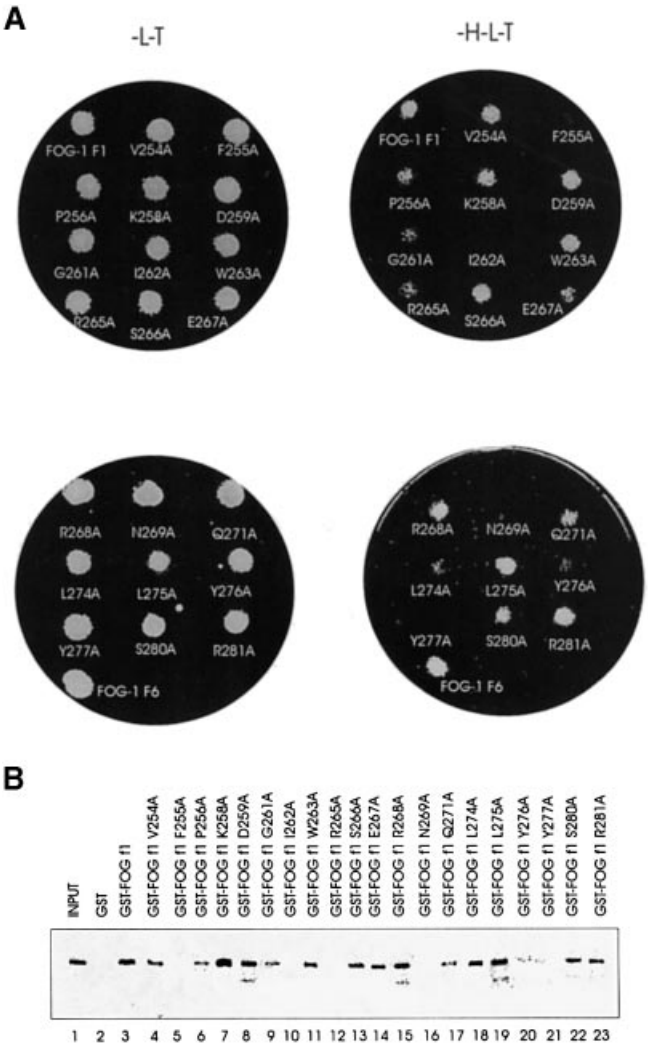

Fig. 2. Interactions between mutant FOG-1 finger 1 and the GATA-1 N-finger. (**A**) HF7c yeast growth after 48 h incubation at 29°C on the indicated minimal media. Each spot contains yeast harbouring pGBT9.GATA-1 N-finger (200–254) and either pGAD10.FOG-1 finger 1 (first spot), various pGAD10.FOG-1 finger 1 mutants or pGAD10.FOG-1 finger 6 (last spot). (**B**) GST pull-down interactions. Lane 1 contains 10% of the input *in vitro* translated ^{35}S-labelled GATA-1. Lane 2 contains GST-coated beads, lane 3 contains GST–FOG finger 1 and lanes 4–23 contain GST–FOG-1 finger 1 mutants as indicated. Each sample was incubated with ^{35}S-labelled GATA-1 and, after extensive washing, the GST or GST–FOG-1 finger 1-coated beads were boiled in loading buffer and subjected to electrophoresis, after which retained GATA-1 was visualized by phosphoimaging.

bind GATA-1 interact with the same face in a comparable configuration.

FOG-1 can act as a repressor of transcription

As a first step in determining the functional contribution of each finger to FOG-1's transcriptional activity, we sought to develop cellular assays for monitoring FOG-1 activity. It has been shown previously that FOG-1 can activate the complex p45 NF-E2 promoter, when co-expressed with GATA-1 in transient transfection assays. We wished to examine the behaviour of FOG-1 and GATA-1 at additional GATA-dependent promoters. We first used a simple GATA-dependent reporter containing one GATA site from the mouse α-globin gene promoter upstream of the human growth hormone gene (M1α; Martin and Orkin, 1990). As shown in Figure 4A, lane 2, GATA-1 activates this reporter ~50-fold in transient assays. When we co-transfected increasing amounts of FOG-1 (Figure 4, lanes 3 and 4), we found it repressed the GATA-1 activation in a dose-dependent manner. This repression is dependent on a direct interaction between GATA-1 and FOG-1, as it is abolished when a GATA-1 mutant (HY22/223DP; Fox *et al*., 1998) unable to interact with FOG-1 is used (Figure 4, lanes 5 and 6).

We also tested whether the same effect was observed on a naturally occurring GATA-dependent promoter. It has been shown previously that the EKLF promoter is strongly activated by GATA-1 (Crossley *et al*., 1994). We therefore investigated whether FOG-1 had a direct effect on repressing the activity of GATA-1 at this promoter. As shown in Figure 4B, column 2, GATA-1 strongly activates the EKLF promoter. When increasing amounts of FOG-1 are introduced, a dose-dependent repression of GATA-mediated activation is observed. Thus, while FOG-1 can work together with GATA-1 to activate the p45 NF-E2 promoter (Tsang *et al*., 1997), it can also act to repress GATA-1 activity on different promoters.

The activity of FOG-1 correlates with the number of intact GATA-interacting fingers

We then used our repression assays to determine the contribution of the various GATA-interacting fingers of FOG-1 to its activity as a GATA-1 cofactor protein. We sought to generate mutant FOG-1 proteins which carried subtle mutations that selectively interfered with the activity of individual fingers but did not compromise the general folding and stability of the protein. Using our knowledge of which residues are the most important for GATA binding, we targeted the tyrosine residue at position 26 in each interacting finger and mutated it to alanine. We previously had shown that this mutation significantly interfered with GATA binding but did not measurably alter the folding properties of FOG-like finger domains (Figure 3; data not shown).

FOG-1 constructs were made with single mutant fingers (fingers 1, 5, 6 and 9) as well as double mutants (two fingers mutated), triple mutants (three fingers mutated) and a FOG-1 molecule with four fingers mutated (Figure 5A). These FOG-1 mutants were then co-transfected with GATA-1, and their ability to repress GATA-mediated transactivation at both the M1α and the EKLF promoters was assessed (Figure 5B and C). The data obtained from the two different promoters were essentially the same. The mutants which contained one defective finger only (be it finger 1, 5, 6 or 9) were able to repress activation at a level slightly lower than intact FOG-1. Mutants with two defective fingers were poorer repressors, whilst mutants with three defective fingers could barely repress GATA-mediated activation. The mutant with no GATA-interacting fingers could not repress at all. We also tested a selection of these FOG-1 mutants for their ability synergistically to activate the p45 NF-E2 promoter. Again, the potency of FOG-1 correlated with the number of intact GATA-1-interacting fingers (data not shown). Western analysis confirmed that all mutant FOG-1 proteins were expressed at normal levels (data not shown). It appears, therefore, that the ability of FOG-1 to repress GATA activity or to activate gene expression synergistically

Table I. An alanine scan mutagenesis of FOG finger 1 identifies important residues needed for an interaction with GATA-1

		Interaction with GATA N finger	
		Yeast two hybrid[b]	GST pulldowns[c]
FOG f1 252–282	KDVFPCKDCGIWYRSERNLQAHLLYYCASRQ[a]	++	+
FOG f1 V254→A	--A----------	++	+
FOG f1 F255→A	---A----------	-	-
FOG f1 P256→A	----A----------	++	+
FOG f1 K258→A	------A----------	++	+
FOG f1 D259→A	-------A----------	++	+
FOG f1 G261→A	---------A----------	+	+
FOG f1 I262→A	----------A----------	-	-
FOG f1 W263→A	-----------A----------	++	+
FOG f1 R265→A	-------------A----------	+	-
FOG f1 S266→A	--------------A----------	++	+
FOG f1 E267→A	---------------A----------	+	+
FOG f1 R268→A	----------------A----------	++	+
FOG f1 N269→A	-----------------A----------	-	-
FOG f1 Q271→A	-------------------A----------	++	+
FOG f1 L274→A	----------------------A--------	+	+
FOG f1 L275→A	-----------------------A--------	++	+
FOG f1 Y276→A	------------------------A------	+	-
FOG f1 Y277→A	-------------------------A-----	-	-
FOG f1 S280→A	----------------------------A--	++	+
FOG f1 R281→A	-----------------------------A-	++	+

[a]Black shading indicates an essential residue for GATA interaction, whilst the boxes indicate an important but not critical residue for GATA interaction.
[b]The yeast two-hybrid assay was used to test the interaction between these FOG-1 finger 1 mutants and GATA-1 (residues 200–254); ++, + or – indicate growth on His^- Leu^- Trp^- media as judged from Figure 2A.
[c]Results of the GST pull-down assay, where interaction of GST–FOG-1 finger 1 fusions and *in vitro* translated GATA-1 are indicated by either + signs or – signs reflecting the amount of GATA-1 retained by the bead-bound FOG-1 as judged from Figure 2B.

together with GATA-1 is proportional to the number of intact GATA-1-interacting fingers.

A repression domain within FOG-1 interacts with the co-repressor mCtBP2

Finally, we sought to investigate the mechanism by which FOG-1 acts to repress GATA-mediated transcription. We have noted previously that FOG-1 contains a motif that is bound by the CtBP family of co-repressors (Turner and Crossley, 1998). This site PIDLSKR occurs immediately N-terminal to finger 7. We first used yeast two-hybrid and GST pull-down assays to test whether a small region of FOG-1 (residues 724–834, spanning the CtBP-binding motif) could interact with one family member, mCtBP2. As shown in Figure 6, lane 2, GST–FOG-1(724–834) could retain *in vitro*-translated mCtBP2 efficiently, whereas a mutant FOG-1 containing a mutation in the core region (PIDLSKR to AIAASKR) was unable to retain mCtBP2 (Figure 6, lane 3). Similarly, in the yeast two-hybrid system, FOG-1(724–834) was able to interact with mCtBP2, whereas the mutant could not (data not shown).

To test if this region of FOG-1 could act as a repression domain *in vivo*, we prepared fusions of FOG-1(724–834) (both wild-type and mutant) with the Gal4 DNA-binding domain (DBD) and co-transfected these with a construct harbouring a Gal4-dependent promoter upstream of the human growth hormone reporter gene. As seen in Figure 7, lane 2, Gal4DBD–FOG-1(724–834) represses the basal reporter activity 20-fold. The mutant, however, is unable to repress transcription significantly (lanes 1 and 3). This result indicates that FOG-1 contains a repression domain that can mediate repression by associating with CtBP family proteins.

To determine if CtBP family members are involved in repression by full-length FOG-1, we constructed a FOG-1 molecule containing the PIDL-AIAA mutation. We then tested the effect of this mutation on the ability of FOG-1 to repress GATA-mediated activation of the M1α and EKLF promoters (Figure 8). The mutation reduced the ability of FOG-1 to repress both promoters by ~50% but did not altogether abolish the repression activity of FOG-1 (Figure 8, compare columns 1 and 3 with columns 2 and 4). This result suggests that CtBP proteins are involved in the repression mediated by full-length FOG-1 but that other mechanisms of repression may also operate.

Table II. The Cys–Cys:His–Cys zinc fingers in FOG family proteins that are able to interact with GATA-1 contain a conserved motif

		Interaction with GATA-1 N finger[a]
STRONG INTERACTORS:		
Fog-1 F6	P S R T L C E A C N I R F S R H E T Y T V H K R Y Y C A S R H	++
Fog-1 F1	K D V F P C K D C G I W Y R S E R N L Q A H L L Y Y C A S R Q	++
Fog-1 F9	G G H R Y C R L C N I R F S S L S T F I A H K K Y Y C S S H A	++
Ush F1	P A R F M C L P C G I A F S S P S T L E A H Q A Y Y C S H R I	++
Fog-2 F1	K D I F P C K S C G I W Y R S E R N L Q A H L M Y Y C S G R Q	++
Fog-2 F6	P N K T T C E A C N I T F S R H E T Y M V H K Q Y Y C A T R H	++
WEAK INTERACTORS:		
Fog-1 F5	T K G A T C F E C E I T F N N I N N Y Y V H K R L Y C S G R R	+
Ush F5	Y Q Q L I C A A C G I K Y T S L D N L R A H Q N Y Y C P K G G	+
Ush F9	V M K K Y C S T C D I S F N Y V K T Y L A H K Q F Y C K N K P	+
Fog-2 F5	T K G A T C F E C E I T F N N I N N Y Y V H K R L Y C S G R R	+
Fog-2 F8	T S G K Y C R L C D I Q F N N L S N F I T H K K F Y C S S H A	+
NON-INTERACTORS:		
Prd II	G T M F E C E T C R N R Y R K L E N F E N H K K F Y C S E L H	-
Fog-1 F7	A D Y H E C T A C R V S F H S L E A Y L A H K K Y S C P A A P	-
Fog-2 F7	L D Y H E C T V C K I S F N K V E N Y L A H K Q N F C P V T A	-
Evi-1 F7	R T Q I K C K D C G Q M F S T T S S L N K H R R - F C E G K N	-
CONSENSUS FOR ABILITY TO INTERACT WITH GATA-1 N FINGER:		
	X X X **F** X **C** X X **C** X **I** X X **R/S** X X X **T/N** X X X **H** X X **Y Y C** X X X X	
position	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31	

[a]Interaction judged from yeast two-hybrid assays as detailed in the legend for Figure 1.

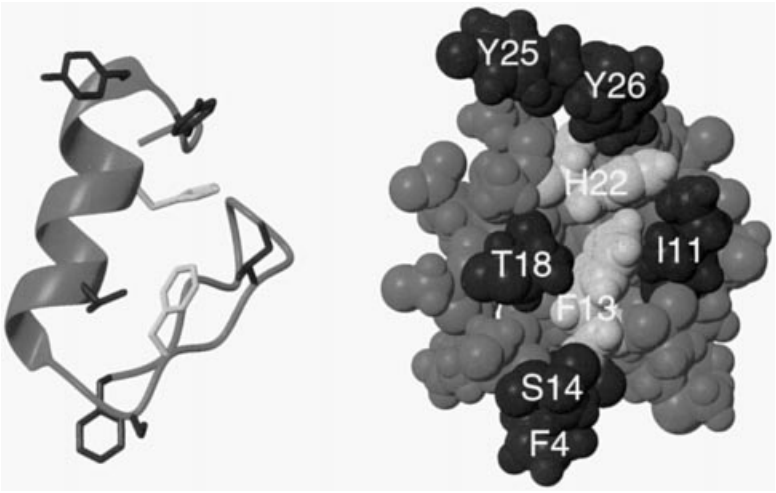

Fig. 3. NMR solution structure of U-shaped finger 1. U-shaped finger 1 is highly homologous to FOG-1 finger 1 and contains the same spacing of zinc-chelating residues. The putative points of contact with GATA-1 (as deduced from the mutagenisis of FOG-1 finger 1 shown in Figure 2) are shown in black and labelled (numbering as in Table II). Two additional residues that are thought to be involved in contacts are also labelled. A ribbon diagram together with key side chains is shown to the left of a space-filling representation on the right.

Discussion

A distinct subset of Cys–Cys:His–Cys fingers interact with GATA-1

In this study, we have examined several members of the FOG family of multi-finger proteins and have demonstrated that each member contains several fingers capable of interacting with GATA-1. These fingers are all variant Cys–Cys:His–Cys fingers, and detailed examination shows that they share a set of key residues (Table II) that are implicated in making physical contact with GATA-1. These key positions all lie on the same face of U-shaped finger 1, the first FOG-like finger whose structure has been solved. Moreover, mutation of these residues interferes with the interaction with GATA-1. We expect that if new FOG-like proteins are discovered in other mammalian tissues or in other organisms, a knowledge of these key residues will be useful in quickly evaluating which fingers are likely to be involved in contacting GATA proteins. We have also examined a number of Cys–Cys:His–Cys

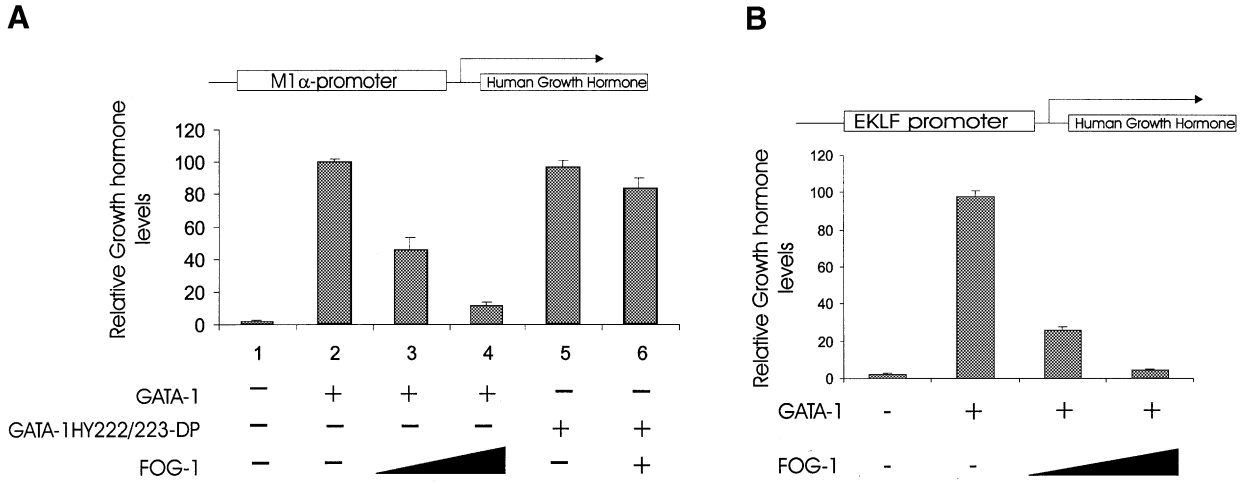

Fig. 4. FOG-1 can repress GATA-1 activation of GATA-dependent promoters. (**A**) NIH 3T3 cells were transfected with 2 μg of M1α reporter alone (column 1) or together with expression plasmids for GATA-1 alone (2 μg) or GATA-1HY222/223-DP and FOG-1 (100 ng column 3, 500 ng columns 4 and 6). (**B**) NIH 3T3 cells were transfected with 2 μg of EKLF promoter reporter alone (column 1) or together with expression plasmids for GATA-1 alone (2 μg) or GATA-1 and FOG-1 (500 ng column 3, 1 μg column 4). Growth hormone levels were assayed after 48 h, normalized to the activity of a co-transfected *lacZ* reporter plasmid and are shown here as relative to a value of 100 for GATA-1 activation. Error bars represent standard deviation of triplicate experiments.

fingers that occur in other hematopoietic transcription factors and find that, consistent with the absence of the key residues in these fingers, they do not interact with GATA-1.

The GATA-interacting fingers all recognize the GATA N-finger

Mammalian GATA proteins contain two zinc fingers, the C-finger that is sufficient for DNA binding and the N-finger that stabilizes binding to DNA and is involved in a number of protein–protein interactions (Tsang *et al.*, 1997; Mackay *et al.*, 1998). Although the fingers are highly related, they differ in a number of key residues and it has been shown previously that these differences account for the specificity of FOG-1 finger 6 for GATA N-fingers. It is interesting to note here that all of the GATA-interacting fingers we have examined also share this specificity for the GATA N-finger. In no case did we encounter a FOG finger that could interact with the GATA C-finger. These results are consistent with the observation that all the GATA-interacting fingers share common features and suggests that they may have evolved by duplication of an original finger that had specificity for a GATA N-finger. Presumably, related fingers that interact exclusively with C-fingers may also exist, but such fingers have not yet been identified. In this context, it is interesting that in many organisms (most notably in fungi), GATA proteins typically contain only one finger; these fingers bind DNA and are generally regarded as equivalents to the C-finger of mammalian proteins (Arst *et al*., 1989). It will be interesting to determine whether FOG-like cofactors also operate in lower eukaryotes (such as fungi) or whether the evolution of FOG proteins specific for N-fingers is a late evolutionary event that has followed the duplication of the GATA finger domain in higher organisms. Interestingly, there are a number of Cys–Cys: His–His Kruppel-like fingers, found in proteins such as EKLF and Sp1, that can interact with both the C- and the N-finger of GATA-1 (Merika and Orkin, 1995). Since, unlike FOG fingers, these fingers do not appear to discriminate between C- and N-fingers, it is to be expected that these Kruppel-like fingers will interact with the GATA fingers in a manner different from FOG-like fingers (i.e. it is likely that these fingers may identify a conserved face on N- and C-fingers, rather than contacting the few residues that differ between N- and C-fingers). This issue, however, has not yet been investigated in detail.

Each GATA-interacting finger contributes to the ability of FOG-1 to modulate GATA-1's activity

Our results indicate that FOG-1 contains four zinc fingers that are able to interact with GATA-1 *in vitro*. In order to determine whether these fingers all contributed to FOG-1 activity in cellular assays, we introduced single amino acid mutations to disable the fingers, separately or in combination, and assessed the effect on GATA-mediated transcription. Whilst intact FOG-1 strongly repressed GATA-mediated transcription, FOG mutants with defective fingers were all impaired in their activity: overall, their residual activity correlated with the number of remaining intact GATA-interacting fingers. Thus it appears that a single GATA-interacting finger is sufficient for detectable FOG-1 function but that the presence of multiple fingers augments its activity. This result suggests that the duplication of the finger domains during evolution has enhanced FOG family members' ability to modulate GATA protein activity. Numerous other proteins contain repeated domains, and it is likely that in other cases these repeats contribute to the overall activity of the protein.

The presence of four distinct GATA-interacting domains in a single protein also raises the possibility that FOG proteins may bind multiple GATA proteins and be involved in bridging between GATA proteins bound at distant sites in the control regions of particular genes. Indeed, it has been noted that several promoters contain multiple GATA sites and that in other instances GATA sites are present in both the enhancer and promoter regions of genes (Weiss and Orkin, 1995). It is thus possible that FOG-like proteins are involved in organizing higher order chromatin configurations.

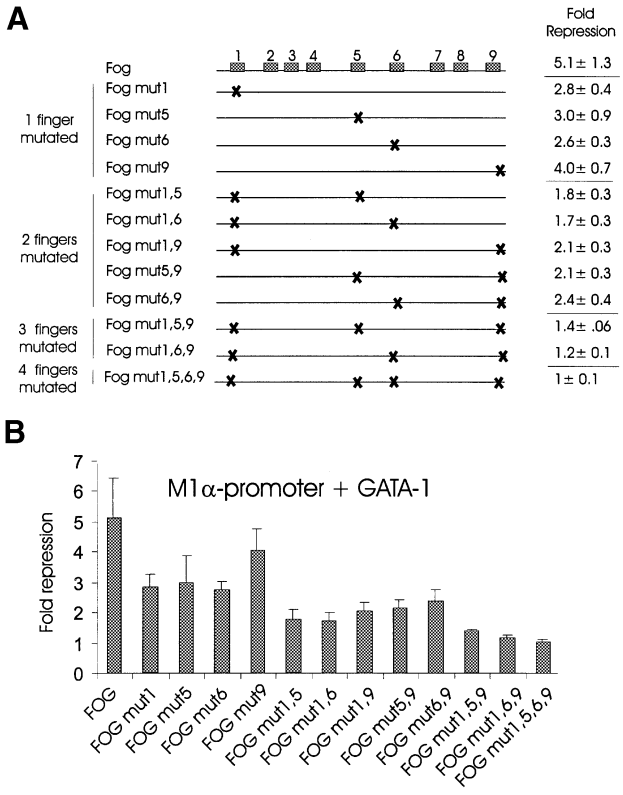

Fig. 5. The activity of FOG-1 depends on the number of intact fingers. (**A**) A schematic of the mutant FOG-1 constructs that were assayed for their ability to repress GATA-1-mediated transcription. The crosses represent substitutions of tyrosine to alanine at position 26 in each finger (see Table II and Figure 3 for numbering). The values for fold repression listed on the right are taken from the graph in (B). (**B**) NIH 3T3 cells were co-transfected with the M1α reporter (2 μg) and GATA-1 (2 μg) as well as 100 ng of FOG-1 or mutants of FOG-1 as indicated. (**C**) NIH 3T3 cells were co-transfected with the EKLF promoter reporter (2 μg) and GATA-1 (2 μg) as well as 100 ng of FOG-1 or mutants of FOG-1 as indicated. Growth hormone levels were assayed as described in the legend for Figure 4.

As well as containing four GATA-interacting fingers, FOG-1 contains an additional five fingers of unknown function. It is our expectation that these fingers may be involved in either binding DNA or contacting additional proteins. Similarly, other multiple zinc finger proteins such as Ikaros (Sun *et al.*, 1996) and Roaz (Tsai and Reed, 1998) have distinct clusters of fingers devoted either to protein–DNA or protein–protein interactions.

FOG-1 can repress GATA-mediated activation

It has been shown previously that FOG-1 and GATA-1 can synergistically transactivate the NF-E2 p45 promoter

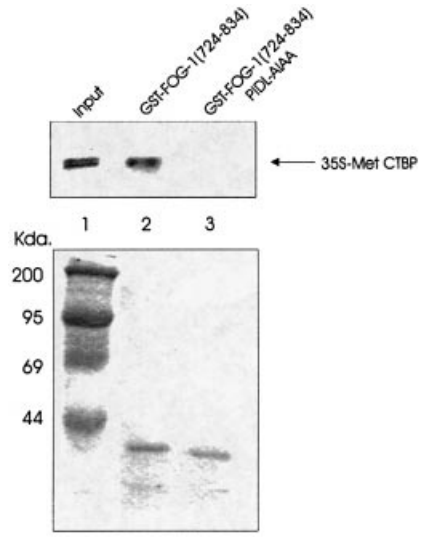

Fig. 6. FOG-1 interacts with the co-repressor mCtBP2. Lane 1 contains 10% of the input *in vitro* translated ^{35}S-labelled CtBP protein. Lane 2 contains GST–FOG-1(724–834) and lane 3 contains GST–FOG-1(724–834) with a mutation of PIDL to AIAA in the CtBP-binding motif. Samples were incubated with radiolabelled mCtBP2 then treated as described in the legend for Figure 2. The amount of mCtBP2 retained by each GST fusion protein is shown in the top panel, and a sample of the GST fusion protein stained with Coomassie Blue in the bottom panel.

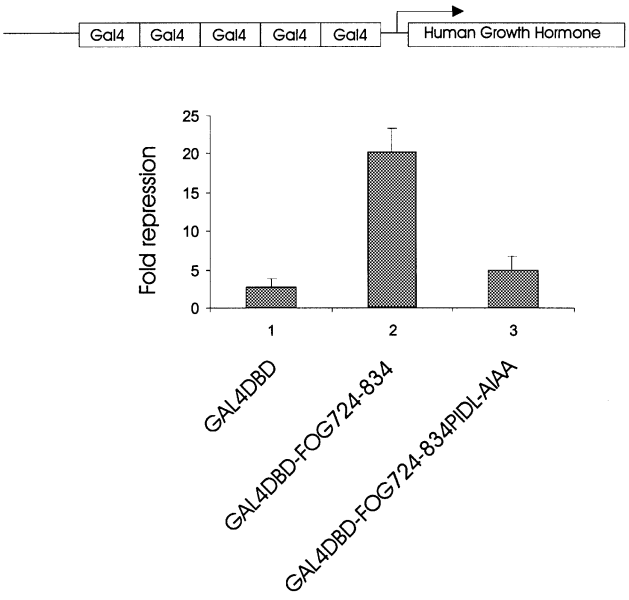

Fig. 7. FOG-1(724–834) is a CtBP-dependent repression domain. NIH 3T3 cells were co-transfected with 5 μg of reporter and 250 ng of either Gal4DBD (column 1), Gal4DBD–FOG-1(724–834) (column 2) or Gal4DBD–FOG-1(724–834) mutant (PIDL-AIAA) (column 3). Values are represented as fold repression of basal reporter gene levels. Growth hormone levels were assayed as described in the legend for Figure 4.

(Tsang *et al.*, 1997). The promoter fragment that responds to FOG-1 and GATA-1 consists of 7 kb of sequence that has not been fully characterized. In contrast to the result obtained using this promoter, we find that other GATA-dependent promoters are strongly repressed by FOG-1 (Figure 4; unpublished results). Moreover, *in vivo* evidence suggests that the *Drosophila* FOG family member, U-shaped, acts as a repressor of GATA-mediated

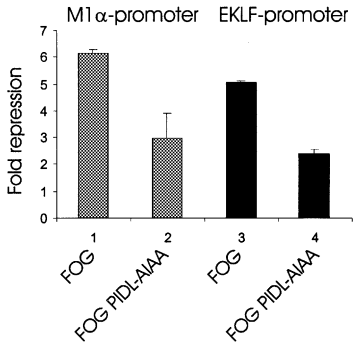

Fig. 8. CtBP proteins are involved in repression by FOG-1. NIH 3T3 cells were co-transfected with 2 μg of M1α reporter (columns 1 and 2) or 2 μg of EKLF promoter reporter (columns 3 and 4) and pXM.GATA-1 (2 μg). In addition, transfections represented by columns 1 and 3 contained FOG-1 (100 ng), whilst columns 2 and 4 represent transfections with FOG-1 (PIDL-AIAA) (100 ng). Values are represented as fold repression of GATA-1 activation. Growth hormone levels were assayed as described in the legend for Figure 4.

activation (Cubadda *et al.*, 1997) and we have therefore explored the possibility that FOG-1 can repress GATA-1-mediated transactivation. We find that FOG-1 effectively represses GATA-mediated activation and that this repression is strictly dependent on physical contact between FOG-1 and GATA-1 (i.e. repression is eliminated when a mutant FOG-1 protein unable to bind GATA-1 or a mutant GATA-1 protein unable to bind FOG-1 is used).

In order to elucidate the mechanism by which FOG-1 might repress GATA-1 activity, we investigated whether FOG-1 could interact physically and functionally with co-repressor proteins. It has been noted previously that recognition sites for the newly characterized transcriptional proteins of the CtBP family occur in FOG-1 (Turner and Crossley, 1998), human FOG-2 (M.Holmes, J.Turner, A.Fox, O.Chisholm, M.Crossley and B.Chong, submitted), murine FOG-2 (Svensson *et al*., 1999; Tevosian *et al*., 1999) and in U-shaped (Turner and Crossley, 1998). Here we have demonstrated that one member of the co-repressor family, mCtBP2, can associate with a repression domain in FOG-1 and that its physical interaction with FOG-1 contributes to repression activity. It should be noted, however, that although a mutant FOG-1 protein, unable to bind CtBP, displayed reduced repression activity, it did retain some ability to repress transcription. This result suggests that additional mechanisms of repression may operate. Interestingly, two other repressors that utilize CtBP, BKLF and Hairy, also contain CtBP-dependent and -independent repression domains (Poortinga *et al*., 1998; Turner and Crossley, 1998; Zhang and Levine, 1999).

The realization that FOG-1 can act to repress transcription raises the question of which genes it operates upon during development. Recent work involving a mutant GATA-1 protein that is unable to interact with FOG-1 demonstrated the derepression of two genes, namely two GATA-2 and Myc (Crispino *et al.*, 1999). Our results on the EKLF promoter support the view that FOG-1 can repress gene expression directly. It should be noted, however, that Crispino *et al*. also identified a large subset of other genes that required FOG-1 for their expression. Thus it is likely that FOG proteins may act as either co-activators or co-repressors depending on promoter context or availability of accessory proteins such as CtBP family members.

Materials and methods

Plasmids and mutagenesis

Several of the plasmids used in this study have been described previously: pGBT9.GATA-1 N-finger, pGBT9.GATA-1 C-finger, pXM.GATA-1 HY222/223-DP (Fox *et al*., 1998), RcCMV.GATA-1 and pØH.EKLF promoter (–77 to +34) (Crossley *et al*., 1994), pXM.GATA-1 (Martin and Orkin, 1990), pcDNA3.mCtBP2 and pØGH.GAL4(BS)$_5$ (Turner and Crossley, 1998). New pGEX2T.FOG-1 and pGAD10 plasmids (F1 amino acids 241–295 and alanine mutants, F2–4 amino acids 285–407, F5 amino acids 573–656, F6 amino acids 677–760, F7–8 amino acids 818–933, F9 amino acids 945–995, FOG RD amino acids 724–834 and mutant RD amino acids 724–834), FOG-2 (F1 amino acids 236–290, F2–4 amino acids 280–407, F5 amino acids 530–617, F6 amino acids 669–757, F7 amino acids 837–917, F8 amino acids 1099–1151) and EVI-1 (F7 amino acids 199–279) were generated by cloning appropriate PCR fragments {amplified from the murine FOG-1 cDNA (Tsang *et al*., 1997), human FOG-2 cDNA (M.Holmes, J.Turner, A.Fox, O.Chisholm, M.Crossley and B.Chong, submitted) or EVI-1 cDNA} into the *Bam*HI and *Eco*RI sites of pGEX2T (Pharmacia) in-frame with the GST gene or similarly into pGAD10 in-frame with the gene encoding the Gal4 activation domain. U-shaped (F1 amino acids 190–270, F3 amino acids 323–390, F4 amino acids 708–790, F5 amino acids 779–860, F9 amino acids 1101–1182) and PRDII-BFI (amino acids 1048–1136) were constructed similarly using PCR fragments generated from *Drosophila* or mouse genomic DNA. Full-length FOG-1 carrying a FLAG tag at the N-terminus was generated by using a 5′ primer encoding the FLAG epitope, together with a 3′ FOG-1 primer, and the product was cloned into the *Bam*HI and *Eco*RI sites of pcDNA3 to create pcDNA3.FlagFOG-1. In a similar manner, full-length FOG-1 with a mutation in the CtBP-binding site was generated by PCR and cloned into the *Bam*HI and *Eco*RI sites of pcDNA3 to create pcDNA3.FOG-1 (PIDL-AIAA). In order to create the Gal4DBD–FOG-1(724–834) fusion and the mutant containing a disrupted CtBP interaction domain, the relevant fragments were amplified and cloned into pGBT9 and then the entire fusion gene was excised with *Hin*dIII and *Eco*RI and cloned into the mammalian expression vector pcDNA3. The generation of the mutated CtBP-binding site and all other site-directed mutagenesis was performed by overlap PCR using *Pfu* or *Pfu turbo* (Stratagene). Sequencing was carried out to verify that the mutagenesis had been successful.

Yeast two-hybrid analysis

Competent HF7c yeast cells were transformed simultaneously with both the appropriate pGBT9.GATA-1 and pGAD10.FOG finger constructs (Clontech Two-hybrid Matchmaker system protocol) and the transformants selected on Leu$^-$ Trp$^-$ minimal media plates after growth at 29°C. Transformants were then patched onto His$^-$ Leu$^-$ Trp$^-$ plates and monitored for growth for up to 3 days.

Western blotting

Whole-cell extracts were prepared from cells transfected with FLAG-tagged pcDNA3.FOG-1 and mutants (Merika and Orkin, 1995) and run on an SDS–polyacrylamide gel. After blotting onto nitrocellulose, Western analysis was performed with m2-FLAG antibody (Kodak), according to the manufacturer's instructions. Secondary antibody was detected using an ECL kit (Amersham).

GST fusion protein binding assays

The expression of both GST fusion proteins and GST alone was performed using the *Escherichia coli* strain DH5α, and purification was carried out as described previously (Smith and Johnson, 1988). ^{35}S-labelled GATA-1 or mCtBP2 was prepared by *in vitro* transcription/translation from RcCMV.GATA-1 or pcDNA3.mCtBP2 using the TNT system according to the manufacturer's instructions (Promega). *In vitro* binding assays were performed in 0.3 ml of buffer [150 mM NaCl, 20 mM Tris–HCl pH 7.5, 0.5% NP-40, 10 μM $ZnSO_4$, 0.25% bovine serum albumin (BSA), 1 mM β-mercaptoethanol and 1.5 mM phenylmethylsulfonyl fluoride (PMSF)] with 1 μg of fusion protein attached to glutathione beads and 2 μl of radiolabelled GATA-1 or mCtBP2. In all cases, levels of the various GST fusion proteins were confirmed by Coomassie staining. Reaction mixtures were incubated for 1 h at 4°C

and the beads were then washed repeatedly with binding buffer. Samples were boiled in loading buffer and subjected to SDS–PAGE. The gel was then dried and the amount of retained radioactively labelled protein monitored using a PhosphorImager (Molecular Dynamics).

Transfections

NIH 3T3 cells were transfected with 2 μg of the reporter M1αGH (Martin and Orkin, 1990) or the reporter pØGH.EKLF promoter, 2 μg of either pXM.GATA-1 or pXM.GATA-1 HY222/223DP and varying amounts of pcDNA3.FOG-1 and pcDNA3.FOG-1 mutants (100 ng, 500 ng and 1 μg) using the calcium phosphate method (Sambrook *et al*., 1989). A titration was carried out with pcDNA3.FOG-1 and mutants, and 100 ng of plasmid was found to be suitable for analysing differences between wild-type FOG-1 and mutants with the Tyr26→Ala or PIDL→AIAA mutations. To examine the FOG-1 repression domain, 5 μg of pØGH.GAL4$(BS)_5$ reporter and 250 ng of either pcDNA3.Gal4DBD, pcDNA3.FOG-1(724–834)Gal4DBD or pcDNA3.mut FOG-1 (724–834)GalDBD were transfected into NIH 3T3 cells using the calcium phosphate method. Growth hormone assays were carried out using Nichols Institute Allegro GH assay kits according to the manufacturer's instructions. All cell culture data are the result of three separate experiments and have been normalized to LacZ levels derived from a co-transfected β-galactosidase-encoding plasmid, EF1α-LacZ.

CD spectropolarimetry

CD spectra were recorded on a Jasco J- 720 spectropolarimeter using a 1 mm pathlength quartz cuvette. FOG finger 1 peptides (20 μM) were dissolved in a buffer containing acetic acid (10 mM), Tris (2-carboxyethyl) phosphine (TCEP, 30 μM) and $ZnSO_4$ (30 μM), pH 5.0. CD spectra (190–260 nm) were recorded at 20°C with a step size of 0.5 nm, a 1 s response time, a 20 nm/min scan rate and a 1 nm bandwidth. Data were acquired as the sum of five separate spectra.

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