FOG-1 recruits the NuRD repressor complex to mediate transcriptional repression by GATA-1

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Transcription factor GATA-1 and its cofactor FOG-1 coordinate erythroid cell maturation by activating erythroid-specific genes and repressing genes associated with the undifferentiated state. Here we show that FOG-1 binds to the NuRD corepressor complex *in vitro* and *in vivo*. The interaction is mediated by a small conserved domain at the extreme N-terminus of FOG-1 that is necessary and sufficient for NuRD binding. This domain defines a novel repression module found in diverse transcriptional repressors. NuRD is present at GATA-1/FOG-1-repressed genes in erythroid cells *in vivo*. Point mutations near the N-terminus of FOG-1 that abrogate NuRD binding block gene repression by FOG-1. Finally, the ability of GATA-1 to repress transcription was impaired in erythroid cells expressing mutant forms of FOG-1 that are defective for NuRD binding. Together, these studies show that FOG-1 and likely other FOG-like proteins are corepressors that link GATA factors to histone deacetylation and nucleosome remodeling.

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Introduction

GATA factors physically interact with FOG proteins to regulate the formation of diverse tissues in metazoans. Both GATA-1 and FOG-1 (Zfp1) are essential for the normal development of erythroid cells (Pevny *et al*, 1991; Fujiwara *et al*, 1996; Tsang *et al*, 1998). Mice lacking GATA-1 or FOG-1 die of anemia during early embryogenesis. FOG-1 contains nine zinc-fingers four of which can bind to a defined domain within the first of the two GATA-1 zinc-fingers (Tsang *et al*, 1997; Fox *et al*, 1999). To date, direct DNA binding by FOG proteins has not been detected. The importance of the physical interaction between GATA-1 and FOG-1 is high-

lighted by the observation that patients and mice with a point mutation at valine 205 in the N-terminal zinc-finger of GATA-1 that impairs FOG-1 binding suffer from severe anemia (Nichols *et al*, 2000; Chang *et al*, 2002). It has been considered that this mutation might disrupt the interaction with additional proteins known to bind GATA-1. However, a compensatory mutation in FOG-1 (FOG-1(S706R)) that restores binding to mutant GATA-1 can rescue erythroid maturation in an erythroid cell line, which argues against this possibility (Crispino *et al*, 1999). Valine 205 resides in the N-terminal zinc-finger of GATA-1 at a site opposite to that involved in DNA binding. Therefore, mutations of this residue do not affect DNA binding by GATA-1 to DNA *in vitro* (Crispino *et al*, 1999; Kowalski *et al*, 2002).

In an effort to study GATA-1 in its natural environment, a GATA-1-deficient erythroid cell line, G1E, has been generated that proliferates in an undifferentiated state but matures upon re-expression of GATA-1 (Weiss *et al*, 1997). GATA-1-induced differentiation is accompanied by morphological signs of maturation, production of hemoglobin and cell cycle arrest. This system faithfully recapitulates erythroid differentiation, which is supported by the observation that restoring GATA-1 activity in G1E cells induces all of the known GATA-1 target genes (Welch *et al*, 2004). Activation of many but not all of these genes requires FOG-1 binding by GATA-1. For example, GATA-1 bearing a valine to glycine substitution mutation at residue 205 (V205G) fails to induce globin gene transcription but is still able to induce the expression of the erythroid Kruppel-like transcription factor EKLF (Crispino *et al*, 1999). The determinants within a regulatory region that specify the FOG-1 requirement are unknown.

While GATA-1 has been studied mostly as transcriptional activator, recent evidence suggests an equally prominent role as transcriptional repressor. Time-course microarray analyses of genes regulated by GATA-1 in G1E cells revealed that the number of repressed genes is comparable to that of induced ones (Welch *et al*, 2004). The genes repressed by GATA-1, which include GATA-2, c-myc and c-kit (Crispino *et al*, 1999; Grass *et al*, 2003; Rylski *et al*, 2003; R Kapur and M Weiss, unpublished; this report), are associated with the immature, proliferative state. Chromatin immunoprecipitation (ChIP) experiments showed that GATA-1 associates with these genes *in vivo*, indicating that GATA-1 directly inhibits their expression (Grass *et al*, 2003; Rylski *et al*, 2003; Letting *et al*, 2004). Notably, forced expression of c-myc (Rylski *et al*, 2003) or c-kit (R Kapur and M Weiss, unpublished) in G1E cells inhibited GATA-1-induced cell cycle arrest, demonstrating that both are relevant GATA-1 targets. The importance of understanding the mechanism by which GATA-1 represses proliferation-associated genes is highlighted by the observation that mutations in GATA-1 are found in patients with megakaryoblastic leukemia (Wechsler *et al*, 2002).

Failure of GATA-1(V205G) to repress c-myc and GATA-2 expression (Crispino *et al*, 1999; Letting *et al*, 2004) suggests

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that FOG-1 is required for repression by GATA-1. A repressive role for FOG-1 is further suggested by transfection studies showing that FOG-1 can inhibit the activity of GATA-1 (Fox *et al*, 1999). Recent studies have shed light on the mechanisms by which FOG-1 cooperates with GATA-1. ChIP studies in G1E cells expressing GATA-1V205M revealed diminished GATA-1 occupancy at certain regulatory sites *in vivo* (Letting *et al*, 2004). Moreover, GATA-1 is impaired for binding to its targets in a hematopoietic cell line lacking FOG-1 (Pal *et al*, 2004). Therefore, one function of FOG-1 might be facilitating GATA-1 access to its targets in the context of cellular chromatin.

Here we examined the role of FOG-1 during GATA-1-mediated gene repression. At the GATA-2 and c-kit genes, FOG-1 was dispensable for GATA-1 occupancy but was required for gene repression and histone deacetylation. We further report that FOG-1 binds directly to the nucleosome remodeling and histone deacetylase (NuRD) complex. NuRD binding is required for FOG-1's ability to repress transcription. These results provide a mechanism for GATA-1/FOG-1-mediated gene repression and link GATA-1 and FOG-1 to histone deacetylation and nucleosome remodeling.

Results

FOG-1 functions as corepressor at the c-kit and GATA-2 loci

Prior work indicated that FOG-1 assists GATA-1 in associating with select GATA elements *in vivo* (Letting *et al*, 2004; Pal *et al*, 2004). Using quantitative ChIP, we examined whether FOG-1 is required for GATA-1 occupancy and histone deacetylation at two genes that are repressed by GATA-1, namely GATA-2 (Grass *et al*, 2003) and c-kit (R Kapur and M Weiss, unpublished). To this end, we used the GATA-1-deficient cell line G1E stably expressing an estradiol-dependent form of wild-type GATA-1 (GATA-1-ER) or GATA-1-ER with a mutation that impairs FOG-1 binding (GATA-1(V205M)-ER). Upon treatment with estradiol, GATA-1-ER-expressing cells displayed a robust reduction in the expression of both GATA-2 and c-kit mRNA as measured by real-time RT-PCR (Figure 1A). In contrast, cells expressing GATA-1(V205M)-ER were impaired in their ability to inhibit expression of GATA-2 and c-kit in response to estradiol treatment (Figure 1A). Therefore, the repression of both genes by GATA-1 requires FOG-1 binding.

Lack of transcriptional repression by GATA-1(V205M)-ER was accompanied by failure to substantially lower acetylation of histones H3 and H4 at the c-kit and GATA-2 genes (Figure 1B, and data not shown). Quantitative ChIP analysis with anti-GATA-1 antibodies shows that GATA-1-ER binds inducibly to a regulatory element at the GATA-2 gene 2.8 kb upstream of the 1S promoter (Figure 1C), in accord with previous results (Grass *et al*, 2003). Moreover, GATA-1-ER bound well to a region at the c-kit locus 5 kb downstream of the transcription start site that is conserved and contains three GATA consensus binding sites (R Kapur and M Weiss, unpublished) but not to a control region (Figure 1C, and data not shown). These results confirm that both GATA-2 and c-kit are direct GATA-1 targets. Notably, histone deacetylation at the GATA-2 gene occurred also at sites not occupied by GATA-1, such as the 1S promoter (Supplementary Figure 1A),

suggesting that histone deacetylation once initiated might spread across the GATA-2 locus independent of GATA-1.

We next examined whether FOG-1 binding by GATA-1 is required for GATA-1 occupancy at the GATA-2 and c-kit genes. As measured by ChIP, levels of GATA-1(V205M)-ER at the GATA-2 and c-kit loci were comparable if not slightly higher than that observed with GATA-1-ER (Figure 1C), suggesting that FOG-1 is not required for GATA-1 occupancy at these sites. Similar results were obtained with a second independently derived GATA-1(V205M)-ER-expressing cell line (data not shown). As an additional control, GATA-1 occupancy was determined at the β -major globin gene promoter. Consistent with our previous findings (Letting *et al*, 2004), high levels of GATA-1-ER were detected at the β -globin promoter whereas GATA-1(V205M)-ER was virtually absent (Supplementary Figure 1B). ChIP experiments were repeated with antibodies against the ER portion of GATA-ER and yielded essentially the same results (data not shown). We next examined FOG-1 occupancy by ChIP. Treatment of G1E cells expressing GATA-1-ER augmented the amounts of FOG-1 present at the c-kit locus and, as control, at the β -globin gene promoter (Figure 1D). In contrast, in G1E cells expressing GATA-1(V205M)-ER, FOG-1 recruitment to the c-kit and β -globin loci was diminished (Figure 1D).

Together, these results confirm that FOG-1 is essential for GATA-1 occupancy at some sites but appears largely dispensable at others such as the c-kit and GATA-2 loci. Since GATA-1(V205M)-ER can associate with regulatory elements at the GATA-2 and c-kit genes but fails to inhibit their expression, this suggests that at these sites FOG-1 functions by recruiting corepressor molecules. Given the substantial decreases in histone acetylation, these corepressors are expected to harbor one or more histone deacetylases.

The N-terminal repression domain of FOG-1 associates with a histone deacetylase-containing protein complex

Previous work identified the corepressor CtBP-2 as FOG-1 binding partner (Fox *et al*, 1999). Mutations that abrogate CtBP-2 binding reduce FOG-1's inhibitory activity in transfection assays. Moreover, the ability of FOG-2 to inhibit erythropoiesis in *Xenopus* embryos depends on its ability to bind CtBP (Deconinck *et al*, 2000). Therefore, it was surprising that engineered mice bearing a FOG-1 mutant defective for CtBP binding displayed normal erythropoiesis even under conditions of hematopoietic stress (Katz *et al*, 2002). This suggested that other domains in FOG-1 might provide interactions with corepressors, thus compensating for the loss of CtBP binding. The N-terminus of FOG-1 contains a stretch of 14 amino acids that is identical between mouse and human FOG-1 and differs by only one amino acid between FOG-1 and FOG-2 proteins (Svensson *et al*, 2000). Deletion of this domain impaired the ability of FOG-1 to repress GATA-4 activity in transient reporter assays. When fused to the DNA-binding domain of GAL4, the first 45 amino acids of FOG-2 (Svensson *et al*, 2000) and FOG-1 (see below) are potent inhibitors of transcription, indicating that this domain can function as independent repressor module.

To identify corepressors that mediate repression by FOG-1, we generated a fusion protein consisting of amino acids 1–45 of FOG-1 fused to GST. GST-FOG-1(45) was bound to glutathione-agarose beads and incubated with nuclear extracts

Figure 1 FOG-1 functions as GATA-1 corepressor. **(A)** Q-RT-PCR measuring c-kit (left) and GATA-2 (right) mRNA levels before and after treatment with estradiol (E2) for 24 h. **(B)** ChIP analysis using anti-acetyl H3 (acH3) antibodies or isotype-matched control antibodies (control) and primer sets for the c-kit gene 0.8 kb upstream of the transcription start site (left), and GATA-2 1S promoter (right). Note the impaired reduction in histone acetylation at both genes in cells expressing GATA-1(V205M)-ER. The mutation at residue V205 of GATA-1 reduces but does not completely abolish FOG-1 binding. Results are averages of two independent experiments. Error bars denote standard deviation. **(C)** ChIP analysis using anti-GATA-1 antibodies or isotype-matched control antibodies (control) and primer sets for the + 5 kb region of c-kit (left) and the -2.8 region of GATA-2 gene (right). Results are averages of three independent experiments. Error bars denote standard deviation. **(D)** ChIP analysis using anti-FOG-1 antibodies or isotype-matched control antibodies (control) and primer sets for the + 5 kb region of c-kit (left) and β -major globin gene promoter (right). Results are averages of four independent experiments. Error bars denote standard deviation.

of the erythroid cell line MEL. Bound proteins were washed extensively and analyzed for histone deacetylase activity by incubation with [3 H]acetate-labeled cellular histone molecules and measuring the release of radioactive acetate. The results show that GST-FOG-1(45) but not GST associates with histone deacetylase activity (Supplementary Figure 2). This activity was inhibited by the addition of the deacetylase inhibitor sodium butyrate, indicating that the reduced levels of histone acetylation were not simply the consequence of protein degradation.

The N-terminal repression domain of FOG-1 binds to the NuRD complex

GST-FOG-1(45)-bound proteins were fractionated by SDS-PAGE and stained with colloidal blue. Several bands were identified that bound to GST-FOG-1(45) but not to GST alone (Figure 2). All proteins were retained following stringent washes with NaCl concentrations up to 650 mM. Bands were excised and proteins identified by tandem mass spectrometry. All proteins identified corresponded to known members of the NuRD complex, including Mi-2 β (also called

Figure 2 The N-terminus of FOG-1 associates with NuRD. Colloidal blue-stained gel of proteins derived from MEL cell nuclear extracts (NE) bound to GST and GST-FOG-1(45). Proteins were identified by tandem mass spectrometry.

CHD4), RbAp46, RbAp48, MTA-1, MTA-2, p66, HDAC1 and HDAC2 (Tong *et al*, 1998; Wade *et al*, 1998; Xue *et al*, 1998; Zhang *et al*, 1998; for review see Bowen *et al*, 2004). Among 33 peptides identified as Mi-2 β , 26 were specific for Mi-2 β and seven were in a region shared between Mi-2 β and Mi-2 α (CHD3). We did not detect any peptides unique to Mi-2 α . However, we cannot entirely rule out the presence of low amounts of Mi-2 α in our purification. Previous reports showed that Mi-2 α and Mi-2 β can coexist within the same complex (Tong *et al*, 1998; Xue *et al*, 1998). While 18 and 13 peptides were found to represent MTA-1 and MTA-2, respectively, only a single peptide corresponding to MTA-3 was identified. This suggests that while MTA-3 is present in small amounts, it is likely substoichiometric with regard to the other NuRD components. In accord with this interpretation, Western blotting with anti-MTA-3 antibodies failed to detect appreciable amounts of MTA-3 (data not shown). One NuRD component, MBD3, was not identified by mass spectroscopy since it comigrates with GST-FOG-1(45), but its presence was confirmed by Western blotting (see below). Components of other deacetylase-containing complexes such as Sin3, N-CoR and SMRT were not detected (data not shown). Moreover, the methylated DNA-binding protein MBD-2 was not detected by Western blot, suggesting that FOG-1 recruits NuRD but not the MeCP-1 complex, which contains MBD2 in addition to NuRD (Ng *et al*, 1999; Zhang *et al*, 1999; Feng and Zhang, 2001). These data show that a single-step high-stringency purification yielded all of the previously described compo-

nents of NuRD. The NuRD component Mi-2 β has ATPase activity that is required for nucleosome remodeling. Together with the presence within NuRD of the histone deacetylases HDAC1 and HDAC2, this suggests that FOG-1 mediates GATA-1-induced gene repression by histone deacetylation and nucleosome remodeling.

FOG-1 and NuRD form a stable complex through multiple steps of protein purification

To determine whether endogenous FOG-1 associates stably with NuRD, we performed conventional protein purification using MEL cell nuclear extracts. The purification strategy and FOG-1 elution profiles are summarized in Figure 3A with details described in Materials and methods. Fractions from the final purification over a Superose6 column were analyzed by Western blot (Figure 3B). Peak levels of FOG-1 were found in fractions 22 and 24, which corresponds to a molecular weight of ~ 1.2 to ~ 1.3 MDa. This size is similar to that described previously for NuRD (Xue *et al*, 1998; Zhang *et al*, 1998). Importantly, FOG-1 and members of the NuRD complex displayed virtually identical elution profiles (Figure 3B), indicating that the FOG-1–NuRD association is stable over multiple steps of purification.

The N-terminal repression domain of FOG-1 is required for NuRD binding *in vivo*

To examine whether FOG-1 associates with NuRD *in vivo*, MEL cell nuclear extracts were immunoprecipitated with antibodies against the NuRD component MTA-2 followed by Western blotting with antibodies against FOG-1 and other NuRD proteins. Significant amounts of FOG-1 co-precipitated with anti-MTA-2 but not control antibodies (Figure 4A). As expected, other NuRD components but not Sin3A co-precipitated efficiently. Anti-HDAC2 and anti-RbAp48 but not control antibodies precipitated comparable amounts of FOG-1 (Figure 4B), suggesting that FOG-1 associates with the intact NuRD complex. Notably, FOG-1 migrated as a doublet, and it appeared that the upper band preferentially associated with NuRD. At present, it is unclear what accounts for the difference in mobility between these two forms of FOG-1.

To examine whether NuRD binding depends on the N-terminal repression domain of FOG-1, plasmids expressing HA-tagged forms of full-length FOG-1 and FOG-1 lacking the N-terminal 45 amino acids (HA-FOG-1($\Delta 45$)) were transfected into COS cells. Nuclear extracts were immunoprecipitated with anti-HA antibodies followed by Western blotting against endogenous HDAC2. Full-length HA-FOG-1 but not HA-FOG-1($\Delta 45$) immunoprecipitated the NuRD component HDAC2 (Figure 4C, compare lanes 3 and 8, lower panel) although both proteins were expressed at similar levels (lanes 1 and 6). In the converse experiment, the same extracts were precipitated with antibodies against MTA-2. Consistent with the above results, only full-length HA-FOG-1 but not HA-FOG-1($\Delta 45$) associated with MTA-2 (upper panel, lanes 5 and 10). Thus, NuRD binding is dependent on the N-terminus of FOG-1.

Multiple NuRD proteins can bind to FOG-1

The components of NuRD that confer binding to FOG-1 were determined by conducting GST pulldown experiments. GST-FOG-1(45) was incubated with *in vitro*-translated 35 S-labeled NuRD proteins and retained material analyzed by SDS-PAGE and autoradiography. MTA-1 and RbAp48 showed the stron-

Figure 3 Stable association of FOG-1 and NuRD through multiple steps of purification. **(A)** Protein purification scheme. FOG-1 was tracked by Western blot. Numbers indicate molar salt concentrations. **(B)** Western blot with antibodies against indicated proteins of fractions eluted from the Superose6 column.

gest binding to GST-FOG-1(45) relative to the input signal (Figure 4D). In addition, MTA-2 and RbAp46 displayed significant binding albeit at reduced levels when compared to MTA-1 and RbAp48. This indicates that the association between NuRD and FOG-1 can be mediated by at least two classes of molecules. However, it remains possible that other NuRD subunits contribute to the stability of this complex.

Transcriptional repression by FOG-1 requires interaction with NuRD through a novel repression motif

A series of point mutations was generated by substituting conserved, charged amino acids with alanines or glycines up to residue 29 in the context of GST-FOG-1(45) (Figure 5A). GST-FOG-1 proteins were incubated with *in vitro*-translated ³⁵S-labeled MTA-1, and bound protein analyzed by autoradiography. Two mutations (R4G and K5A) substantially impaired MTA-1 binding (Figure 5B). Substitution of residues 3 and 10 showed a moderate reduction in MTA-1 binding, whereas the remaining mutations had no effect. Similar binding profiles were observed for MTA-2, RbAp48 and RbAp46 (Supplementary Figure 3). However, it appeared that binding of RbAp48 and RbAp46 was more sensitive to the R10G mutations of FOG-1. Finally, comparable results were obtained when GST-FOG-1 proteins were incubated with crude MEL cell nuclear extracts and analyzed for NuRD binding by Western blot with antibodies against several NuRD proteins (data not shown).

Together, these results show that NuRD binding by FOG-1 depends on a small, defined motif at the N-terminus of FOG-1.

To determine whether NuRD binding correlates with transcriptional repression by FOG-1, mutants of FOG-1(45) were fused to the DNA-binding domain of GAL4. Transcriptional repression was determined in NIH 3T3 cells by coexpressing these constructs with a luciferase reporter gene driven by the thymidine kinase promoter and five GAL4-binding sites. A *Renilla* luciferase construct was cotransfected to monitor transfection efficiency. Wild-type GAL4-FOG-1(45) repressed transcription in a dose-dependent manner (Figure 5C). In contrast, R4G and K5A substitutions strongly reduced repressive activity (Figure 5C). Mutation at residue 3 partly impaired repression, while all other constructs were unimpaired (Figure 5C). The tight correlation between NuRD binding and transcriptional repression further supports that NuRD serves as a FOG-1 corepressor.

Interrogation of the NCBI database for motifs similar to the N-terminal 16 amino acids of murine FOG-1 identified numerous proteins including EBF4Z, Evi3, Bcl11b, SALL-1 (Figure 5D), and other members of the SALL family (not shown). All of these are transcriptional repressors (Tsai and Reed, 1997; Avram *et al*, 2000; Netzer *et al*, 2001; Kiefer *et al*, 2002) and harbor this domain at their N-terminus. Thus, this work predicts that diverse transcriptional repressors recruit NuRD through a common motif.

Figure 4 Association of FOG-1 and NuRD occurs *in vivo* and *in vitro* and depends on the N-terminus of FOG-1. (A) MEL cell extracts were immunoprecipitated with anti-MTA-2 antibodies or control (goat IgG) followed by Western blotting with indicated antibodies. (B) Immunoprecipitation of MEL cell extracts with anti-MTA-2, HDAC2 and RbAp48 antibodies or isotype-matched control antibodies followed by Western blotting with anti-FOG-1 antibodies. (C) Immunoprecipitation of extracts from COS cells transfected with HA-FOG-1 and HA-FOG-1(Δ45) with antibodies against HA, MTA-2 or isotype-matched controls. Western blot was performed with antibodies against HA (upper panel) and HDAC2 (lower panel). (D) MTA-1, MTA-2 and RbAp48 bind GST-FOG-1(Δ45) *in vitro*. cDNAs of NuRD proteins were transcribed and translated *in vitro* in the presence of [³⁵S]methionine and incubated with GST or GST-FOG-1(Δ45). Bound proteins were analyzed by SDS-PAGE and autoradiography. In parallel, 5% of *in vitro*-translated product was examined (input).

NuRD associates with GATA-1-repressed genes *in vivo*

To examine whether NuRD is present at GATA-1-repressed genes *in vivo*, ChIP assays were performed with antibodies against the NuRD protein Mi-2β in GATA-1-ER-expressing cells. Treatment with estradiol led to increased association of Mi-2β with the GATA-2 and c-kit genes but not the GAPDH gene (Figure 6A and B). We also observed the presence of MTA-2 and HDAC1 at the GATA-2 locus (Supplementary Figure 4). Notably, MTA-2 and HDAC1 occupied broader regions than GATA-1 and FOG-1 (Supplementary Figure 4), suggesting that NuRD and consequently histone deacetylation might spread along the GATA-2 gene following the initial recruitment by GATA-1 and FOG-1.

To examine the generality of our findings, we examined NuRD occupancy at three additional genes regulated by GATA-1. The c-myc and c-myb genes are repressed by GATA-1 in a FOG-1-dependent manner (Supplementary Figure 5A; Crispino *et al*, 1999). At both genes we observed by ChIP substantial levels of MTA-2 and HDAC1 (Supplementary Figure 5B and C). Signals were specific since they were not detected at control regions located in *cis*. As additional control, we also examined the eosinophil-specific gene MBP, which is controlled by GATA-1 (Du *et al*, 2002) but is not expressed at significant levels in G1E cells before and after differentiation (Welch *et al*, 2004). By ChIP, we detected

only small amounts of MTA-2 and HDAC1 at this gene, the significance of which remains uncertain. While our results generally agree with those reported in the accompanying paper by Rodriguez *et al*, there appear to be differences with regard to the relative levels of NuRD proteins found at the c-myc, c-myb and MBP genes. These differences might reflect the use of antibodies against distinct components of NuRD, or distinct epitope access during gene repression. Nevertheless, together our results demonstrate the presence of NuRD proteins at all four genes that are actively repressed by GATA-1 and FOG-1, suggesting that NuRD is a critical corepressor for GATA-1/FOG-1-mediated gene repression.

NuRD binding by FOG-1 is required for GATA-1-mediated repression *in vivo*

To analyze the activity of FOG-1 mutants without the confounding effects of endogenous FOG-1, we used G1E cells expressing GATA-1(V205M)-ER. Expression of FOG-1(S706R), which can bind to GATA-1(V205M), can partially restore erythroid differentiation in these cells (Crispino *et al*, 1999) (Figure 7A). HA-tagged FOG-1(S706R), a derivative lacking the N-terminal 45 amino acids (HA-FOG-1(S706R)Δ45) and one containing the K5A mutation (FOG-1(S706R)K5A) were introduced into a retroviral vector upstream of an internal ribosomal entry site and GFP. GATA-1(V205M)-ER cells were

Figure 5 NuRD binding is required for transcriptional repression by FOG-1 and is mediated by a protein domain present in various transcriptional repressors. (A) The N-termini of mammalian FOG-1 and FOG-2 are conserved. Point mutations were generated at the indicated residues of GST-FOG-1(45). (B) Upper panel: *In vitro*-generated MTA-1 was examined for binding to GST-FOG-1(45) proteins as described in Figure 4D. Lower panel: Anti-GST Western blot of samples analyzed in parallel revealed the presence of equal amounts of GST fusion proteins. (C) Luciferase assays of NIH 3T3 cells transiently transfected with increasing amounts of intact and mutant GAL4-FOG-1(45) expression constructs (1, 10 and 50 ng) and 0.5 µg of a tk-luciferase reporter gene containing five GAL4-binding sites. Results, which are averages of at least three independent experiments, were normalized for transfection efficiency by measuring *Renilla* luciferase activity of a cotransfected SV40-luciferase vector (20 ng). Error bars denote standard deviations. Anti-GAL4 Western blot shows comparable expression of GAL4 fusion proteins. (D) Protein BLAST search of the NCBI database identified numerous transcriptional repressors that share significant homology with the N-terminus of FOG-1 including those listed here.

infected and sorted for GFP expression by FACS analysis. Pools of cells were analyzed by anti-HA Western blotting to confirm comparable expression levels (Figure 7D). Repressive activity of GATA-1(V205M)-ER was determined by measuring c-kit levels via flow cytometry. In the absence of estradiol, approximately 3% of HA-FOG-1(S706R)-expressing cells had low c-kit levels (Figure 7B and C). Upon estradiol treatment, this number increased to ~13% (Figure 7B and C). This result is significant since only ~10–15% of cells expressing HA-FOG-1(S706R) differentiate as determined by staining for hemoglobin (data not shown), consistent with previous observations (Crispino *et al*, 1999). In contrast, 2.1% of untreated HA-FOG-1(S706R)Δ45 cells displayed low c-kit levels. Estradiol treatment raised this number to ~3.8%, which is comparable to estradiol-treated cells lacking FOG-1(S706R) (data not shown).

The FOG-1(S706R)K5A construct produced an intermediate response, with levels of c-kit-low cells increasing from 3.9 to 7.4% upon estradiol treatment (Figure 7B and C). While

the K5A mutation clearly impairs FOG-1 function, remaining repressive activity is likely due to residual interaction with NuRD. This is consistent with detectable binding of MTA-2 to GST-FOG-K5A (Supplementary Figure 3).

It is important to note that the rescue efficiency of GATA-1(V205M) by FOG-1(S706R) is moderate in part because the affinity between them is not as high as that between wild-type GATA-1 and FOG-1. Moreover, while GATA-1(V205M) is substantially impaired for FOG-1 binding, a residual interaction remains (Nichols *et al*, 2000) that might account for the repressive activity of GATA-1(V205M)-ER after prolonged estradiol treatment (data not shown). Thus, the 'window' for FOG-1(S706R)-mediated rescue is somewhat narrow. Nevertheless, the number of cells showing c-kit repression by HA-FOG-1(S706R) parallels that of globin induction, indicating that gene repression by GATA-1(V205M)-ER was partially restored. Taken together, these findings show that gene repression by GATA-1 requires the FOG-1–NuRD interaction.

Figure 6 *In vivo* association of NuRD with genes repressed by GATA-1. ChIP assays of G1E cells expressing GATA-1-ER in the presence or absence of estradiol (E2) with anti-Mi-2 (filled bars) or isotype-matched control antibodies (open bars). Results are averages of two independent experiments. Error bars denote standard deviations. Primer sets were used for +5 kb region of *c-kit* (A) and the -2.8 region of *GATA-2* gene (B), and, as control, *GAPDH* (C) that is not regulated by GATA-1.

Figure 7 Gene repression by GATA-1 depends on the interaction between FOG-1 and NuRD. (A) Experimental strategy to measure *c-kit* repression in cells expressing GATA-1(V205M)-ER. (B) Representative flow cytometric analysis of GATA-1(V205M)-ER-expressing cells infected with virus containing HA-FOG-1(S706R), HA-FOG-1(S706R) Δ 45 and HA-FOG-1(S706R)K5A and treated with estradiol (E2) for 24 h. (C) Graphic representation of *c-kit*-low/negative cells before and after treatment with E2 for 24 h. Results are averages of three independent experiments. Error bars denote standard deviation. (D) Anti-HA Western analysis of GATA-1(V205M)-ER expressing cells infected with virus containing HA-FOG-1(S706R), HA-FOG-1(S706R) Δ 45 and HA-FOG-1(S706R)K5A. NS, nonspecific band.

Discussion

Here we report that NuRD associates with FOG-1 and is required for GATA-1/FOG-1-mediated transcriptional repression. The interaction between NuRD and FOG-1 resisted high-

- A** (1) Infect G1E cells expressing GATA-1(V205M) with virus expressing FOG-1(S706R) Δ 45 IRES GFP
(2) Flow cytometric analysis for GFP and *c-kit*

salt washes during co-immunoprecipitations and *in vitro* binding studies, was maintained throughout a multistep purification procedure, and was entirely dependent on the N-terminus of FOG-1. Notably, while conventional protein purification showed coelution of FOG-1 and NuRD, GATA-1 was lost during this purification (data not shown), suggesting that the association between FOG-1 and NuRD is more stable than that between GATA-1 and FOG-1.

RbAp48 and MTA-1 and to a somewhat lesser extent MTA-2 and RbAp46 were able to bind FOG-1. These subunits showed a comparable binding pattern to a series of mutant FOG-1 proteins. Substitutions of FOG-1 residues 3, 4 and 5 diminished transcriptional repression, suggesting that all critical interactions with NuRD components were impaired. Therefore, these NuRD proteins appear to contact the same or at least overlapping sets of residues within FOG-1, which is surprising given the lack of overt similarity between MTA and RbAp proteins. The independent association of FOG-1 with MTA-1, MTA-2, RbAp48 and RbAp46 suggests functional overlap between these molecules where any one of them might be able to recruit NuRD to FOG-1 *in vivo*. This might explain our failure to observe a significant loss of FOG-1-mediated transcriptional repression in erythroid cells in which MTA-1 levels had been reduced by siRNA-mediated gene silencing (data not shown).

NuRD interacts with other transcription factors with repressive activities, notably the lymphoid transcription factor Ikaros (Kim *et al*, 1999), the transcriptional corepressor KAP-1 (Schultz *et al*, 2001), the tumor suppressor p53 (Luo *et al*, 2000) and Bcl-6 (Fujita *et al*, 2004). In these cases, the interactions are mediated by Mi-2 α (Ikaros and KAP-1), MTA-2 (p53) and MTA-3 (Bcl-6). Thus, transcriptional regulators utilize distinct NuRD subunits for recruitment. Mutational analysis revealed that transcriptional repression by FOG-1 requires NuRD binding and pinpoints a small N-terminal region that mediates the interaction. This region is conserved among mammalian FOG proteins and is found in various transcriptional repressors, including EBF3, Evi3, Bcl11b (COUP-TF-interacting protein), and members of SALL protein family. As in FOG-1, these repressors bear this motif close to their N-terminus. In the case of SALL1, the N-terminal 76 amino acids associate with HDAC1, HDAC2, RbAp46/48, MTA-1 and MTA-2 (Kiefer *et al*, 2002). However, Mi-2 was not detected in these studies. It is unclear whether this reflects the formation of diverse corepressor complexes harboring distinct NuRD subunits, or whether failure to detect Mi-2 was the result of technical limitations. In either case, it is likely that other nuclear proteins that contain the repression domain defined here associate with NuRD. While this work was under review, analysis of the N-terminus of FOG-2 yielded very similar results regarding the critical amino-acid residues required for transcriptional repression (Lin *et al*, 2004).

Mutational analysis of FOG-1 in G1E cells and in the context of Gal4 fusion experiments revealed a tight correlation between NuRD binding and transcriptional repression. Notably, in G1E cells, a single point mutation in full-length FOG-1 that impaired NuRD binding reduced the repressive activity of GATA-1 by half. Since no proteins other than NuRD were found to bind to the N-terminus of FOG-1 during our affinity purification, this strongly suggests that NuRD is responsible for GATA-1-induced gene repression *in vivo*.

Our results also agree with transient transfection assays showing that deletion of the N-terminal 230 amino acids of FOG-1 abrogated its ability to inhibit GATA-4 activity (Svensson *et al*, 2000). However, mutants of FOG-1 lacking the N-terminal repression domain were previously found to be competent to induce erythroid differentiation and hemoglobin production in a FOG-1-null cell line (Cantor *et al*, 2002). It remains possible that cells expressing the N-terminally deleted FOG-1 contain elevated levels of GATA-2, c-kit, c-myc or c-myb, which might not necessarily interfere with globin gene expression. In support of this, sustained expression of c-myc in G1E cells impaired GATA-1-induced cell cycle arrest but not hemoglobin synthesis (Rylski *et al*, 2003). Finally, forced expression of FOG-1 above normal levels could obscure phenotypic defects that would otherwise be observed upon loss of a functionally important domain.

It is also possible that other FOG-1-interacting molecules might compensate for the lack of NuRD binding in some settings. CtBP-2 appeared especially attractive as mediator of repression function since its binding site is present in all known FOG proteins and mutations at the CtBP-2 binding site altered FOG activity in various assays (Fox *et al*, 1999; Deconinck *et al*, 2000). However, CtBP-2 binding by FOG-1 is dispensable for normal erythroid development in whole animals (Katz *et al*, 2002), which might reflect compensation by NuRD or other corepressors.

In contrast to its role in gene repression, FOG-1 also functions as coactivator and has been detected by ChIP at genes activated by GATA-1 (Wang *et al*, 2002; Pal *et al*, 2004; Figure 1D). This raises the critical question as to how FOG-1 can function as activator despite its strong association with NuRD. Several possibilities exist. First, FOG-1 might associate with NuRD only at genes repressed by GATA-1/FOG-1. At activated genes, NuRD might be displaced by a coactivator complex, analogous to what occurs with nuclear hormone receptors upon ligand binding. However, our affinity purifications of FOG-1 from induced and uninduced erythroid cells and megakaryocytic cells failed to detect associated proteins other than NuRD (data not shown). Alternatively, it is possible that subunits of NuRD play a role during both gene repression and activation. Indeed, a recent study showed that one of the defining subunits, the ATPase Mi-2 β , is present at the active CD4 gene in T-lymphocytes and is required for CD4 transcription and histone acetylation (Williams *et al*, 2004). This result is consistent with flexible functions of NuRD proteins during gene regulation and raises the possibility that some components might act outside the complete NuRD complex as originally defined. Finally, it is possible that post-translational modifications of FOG-1 or of NuRD proteins regulate the recruitment and/or activity of NuRD. For example, methylation of lysine 4 disrupts NuRD binding to histone H3 (Nishioka *et al*, 2002; Zegerman *et al*, 2002), raising the possibility that modifications of FOG-1 might similarly impair binding of NuRD at genes where GATA-1/FOG-1 activates transcription.

Our findings suggest that NuRD recruitment by FOG-1 contributes to histone deacetylation at GATA-1-regulated genes. It has also been reported that HDAC5 can interact directly with GATA-1 and repress its activity (Watanabe *et al*, 2003). Co-immunoprecipitation experiments showed that

while the N-terminal zinc-finger of GATA-1 could interact with HDAC5, the C-terminal zinc-finger appeared to participate in this interaction. It remains an open question to what extent the GATA-1/HDAC5 interaction contributes to GATA-1 function *in vivo* since a mutation in GATA-1 that diminishes FOG-1 binding impairs transcriptional repression by GATA-1. Since repression can be restored by FOG-1 (S706R), the major repressive function of GATA-1 appears to be mediated by a FOG-1-bound complex.

Since NuRD contains several subunits that potentially link the complex to DNA-bound transcriptional regulators, it is possible that additional nuclear factors might function through NuRD during regulation of erythroid gene expression. Indeed, a protein complex containing Ikaros and NuRD was found to be associated with an element upstream of the human δ -globin gene (O'Neill *et al*, 2000). Moreover, isotope-coded affinity tag (ICAT) technique aimed to identify proteins associated with NF-E2p18/mafK identified several NuRD proteins (Brand *et al*, 2004). ChIP studies showed that NuRD was associated with inactive globin genes at elevated levels when compared to active ones (Brand *et al*, 2004). Together with our results, these reports suggest that distinct nuclear proteins might function through NuRD to inhibit globin gene expression at early stages of erythroid differentiation and to repress proliferation-associated genes at later stages of differentiation.

Our work suggests that NuRD is an essential corepressor complex for FOG-1 and required to repress GATA-1 target genes during terminal erythroid maturation. FOG-1 also associates with NuRD in megakaryocytic cells (data not shown), and human patients and mice with mutations in GATA-1 that impair FOG-1 binding harbor megakaryocytes with altered differentiation and growth properties (Nichols *et al*, 2000; Chang *et al*, 2002). This suggests that aberrant cell growth results from failure of FOG-1-mediated NuRD recruitment to proliferation-associated genes.

Distinct GATA factors regulate the development of diverse tissues. For example, GATA-3 and GATA-4 play essential roles during lymphocyte and heart development, respectively, and FOG proteins mediate some of their functions. Therefore, we predict that NuRD recruitment by FOG members plays an important role during GATA factor-regulated differentiation of numerous cell types.

Materials and methods

Plasmids

GST-FOG-1(45) and GAL4-FOG-1(45) were generated by PCR and inserted into pGEX-2TK (Amersham Biosciences) and pcDNA3-Gal4 (gift from M Poncz), respectively. pcDNA3-GAL4 contains amino acids 1–147 of GAL4. pGEM7Zf(+)–rMTA1 and pcDNA3CF-mMTA2 were gifts from W-M Yang (Yao and Yang, 2003). pcMV5-MTA-3 and pcDNA3 β -Mi-2 β were gifts from P Wade and Y Zhang, respectively (Feng and Zhang, 2001; Fujita *et al*, 2004). pcDNA3-HDAC1 and CMV-RbAp48 were gifts from M Lazar and N Barlev, respectively. pcDNA3-RbAp46 and pcDNA3-p66 were generated by RT-PCR from murine mRNA and inserted between the *Bam*H1 and *Eco*R1 sites of pcDNA3 (Invitrogen). pBluescript-HDAC2 and pSPORT-MBD3 were from American Type Culture Collection (ATCC). pcDNA3-HA-FOG-1 was generated by inserting the FOG-1 cDNA between the *Bam*H1 and *Eco*R1 sites of pcDNA3 with a PCR-generated HA tag at the N-terminus. pcDNA3-HA-FOG-1(Δ 45) was generated by replacing the 5' *Bam*H1 and *Kpn*I fragment with the corresponding fragment lacking the first 45 amino acids. Reporter vector G5-TK-luciferase was a gift from W-M Yang (Yao and Yang, 2003).

Affinity chromatography and *in vitro* binding studies

A 20 μ g portion GST-FOG-1 was incubated with 3 mg of MEL nuclear extracts overnight in buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.5% Igepal (Sigma), protease inhibitor cocktail (P 8340, Sigma, 1:500) and 1 mM DTT. Bound proteins were washed twice in the above buffer with 350 mM NaCl, three times with NaCl raised to 650 mM, followed by an additional wash in 350 mM NaCl, separated by SDS-PAGE and stained with colloidal blue (Invitrogen). Proteins were identified by tandem mass spectroscopy analysis on an LCQ Deca XP (ThermoFinnigan, CA) mass spectrometer coupled online with a mini-C18 reversed-phase capillary high-performance liquid chromatography column using electrospray ionization (ESI) interface at the Proteomics Core Facility of the Genomics Institute, University of Pennsylvania.

Cell culture, transfections, antibodies and ChIP

G1E cells were maintained as described (Weiss *et al*, 1997). 3T3 or COS cells were transfected with Lipofectamine (Invitrogen). Immunoprecipitations and Western blots were performed with antibodies against Mi-2 β (PHD-P, gift from W Wang), MTA-1 (sc-9445), MTA-2 (sc-9447), RbAp46 (sc-8272), HDAC1 (sc-7872), HDAC2 (sc-7899), MBD3 (sc-9402), FOG-1 (sc-9361) and Gal4-DBD (sc-510) (Santa Cruz Biotechnology Inc.), p66 (07-365) and RbAp48 (05-524) (Upstate Biotechnology Inc.) and MTA-3 (gift from P Wade).

ChIP assays were performed as described (Letting *et al*, 2003). Antibodies were against GATA-1 (sc-265, Santa Cruz), FOG-1 (sc-9361, Santa Cruz), acetyl-H3 and acetyl-H4 (Upstate Biotechnology Inc.). Mi-2 β antibodies were a gift from K Georgopoulos. MTA-2 antibodies used for ChIP were from Santa Cruz (sc-9447). All primers are listed in Supplementary data.

Histone deacetylase assays

3 H-labeled acetylated histones from HeLa cells were incubated with GST-FOG-1(45)-bound proteins in 400 μ l HDAC buffer (150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 10% glycerol). Where indicated, 125 mM sodium butyrate was added 30 min prior to adding 3 H-labeled acetylated histones. Reactions were stopped with 100 μ l 1 M HCl and 0.16 M acetic acid. Liberated [3 H]acetate was extracted with 600 μ l ethyl acetate and measured by scintillation counting.

Virus preparation, cell infection, flow cytometry and FACS sorting

Vectors with HA-FOG-1, HA-FOG-1(S706R), HA-FOG-1 Δ 45 or HA-FOG-1(S706R) Δ 45 were generated by inserting HA-tagged FOG-1 constructs into MSCV MIGR1-GFP (gift from W Pear). Virus was prepared using a transient transfection system (Pear *et al*, 1993). At 48 h after infection, GFP-positive cells were isolated by FACS. A total of 10^6 cells treated with 0.1 μ M estradiol for 24 h were reacted with 0.5 μ l phycoerythrin-conjugated anti-mouse CD117 (c-kit) monoclonal antibodies (553869, BD Biosciences) and analyzed by two-color flow cytometry.

Protein purification

See Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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