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Gfi-1 Attaches to the Nuclear Matrix, Associates With ETO (MTG8) and Histone Deacetylase Proteins, and Represses Transcription Using a TSA-Sensitive Mechanism

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Abstract Gfi-1 and Gfi-1B can repress transcription and play important roles in hematopoietic cell survival and differentiation. Although these proteins are known to bind DNA through a C-terminal zinc-finger domain and may require an N-terminal SNAG domain (SNAIL/Gfi-1) to repress transcription, the mechanism by which Gfi-1 and Gfi-1B act is unknown. A first step towards understanding the mechanism by which these proteins repress transcription is to identify interacting proteins that could contribute to transcriptional repression. ETO (also termed MTG8), was first identified through its involvement in the (8;21) translocation associated with acute myelogenous leukemia. It attaches to the nuclear matrix and associates with histone deacetylases and the co-repressors N-CoR, SMRT, and mSin3A, and may act as a co-repressor for site-specific transcriptions factors. In this report we demonstrate that Gfi-1 interacts with ETO and related proteins both in vitro and in vivo and with histone deacetylase proteins in vivo. We observed that a portion of Gfi-1 and Gfi-1B associated with the nuclear matrix, as is the case with ETO. Moreover, Gfi-1 and ETO co-localize to punctate subnuclear structures. When co-expressed in mammalian cells, Gfi-1 associates with histone deacetylse-1 (HDAC-1), HDAC-2, and HDAC-3. These data identify ETO as a partner for Gfi-1 and Gfi-1B, and suggest that Gfi-1 proteins repress transcription through recruitment of histone deacetylase-containing complexes. J. Cell. Biochem. 89: 1005–1018, 2003.

Key words: ETO (MTG8); Gfi-1; Gfi-1B; nuclear matrix; leukemia; transcription; histone deacetylases

The *gfi-1* (growth factor independence 1) gene was first identified as an integration site for Moloney murine leukemia virus (MoMuLV) in a screen designed to identify genes contributing to the progression of IL-2-dependent T-cell lymphoma lines to IL-2-independent growth [Gilks et al., 1993] and is a common site for proviral integration in T-cell tumors induced by MoMuLV [Liao et al., 1995]. Mouse transgenic models demonstrate that *gfi-1* co-operates with *L-Myc* and *Pim-1* to promote lymphomagenesis [Gilks et al., 1993; Schmidt et al., 1996; Zornig et al., 1996]. Thus, Gfi-1 acts as an oncogene that co-operates with the Myc transcription factor and the Pim-1 serine/threonine kinase to transform lymphoid cells.

Gfi-1 is highly expressed in the thymus, spleen, bone marrow, and testis, and there is evidence to suggest Gfi-1 regulates hematopoietic differentiation and survival. For example, enforced expression of Gfi-1 in IL-2-dependent cell lines allows an escape from G1 arrest and protects against apoptosis following IL-2 withdrawl [Grimes et al., 1996a]. Moreover, enforced

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expression inhibits phorbol-ester-induced G1 arrest in Jurkat T-cells [Karsunky et al., 2002]. Additionally, both Pim-1 and Gfi-1, working in opposition, participate in the regulation of β-selection-associated pre-T-cell differentiation [Schmidt et al., 1998].

Gfi-1B was identified through low-stringency hybridization strategies using a probe derived from the Gfi-1 C-terminus [Gilks et al., 1993; Tong et al., 1998]. It also plays a role in hematopoiesis. Studies performed in the myelomonocytic cell line M1 demonstrate that interleukin 6 (IL-6)-induced differentiation was associated with down-regulation of endogenous Gfi-1B, and enforced expression of Gfi-1B abrogated the differentiation-associated G1 arrest and induction of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip-1}$ [Tong et al., 1998]. Targeted disruption of the *gfi-1b* gene in mice demonstrated that it is required for development of both erythroid and megakaryocytic cell lineages [Saleque et al., 2002]. Together, these studies demonstrate a vital role for the Gfi-1 and Gfi-1B proteins in regulating cell death and cell cycle in hematopoietic cells.

Gfi1 and *Gfi-1b* encode site-specific zinc-finger transcription repressors that bind the consensus DNA sequence TAAATCAC(A/T)GCA via the C-terminal zinc-finger motif [Zweidler-Mckay et al., 1996]. Transcriptional repression may be mediated by an amino-terminal, 20 amino acid SNAG domain (SNAIL/Gfi-1) also conserved in the vertebrate members of the SNAIL/SLUG family [Valerius et al., 1995; Hemavathy et al., 2000a,b]. Potential Gfi-1 binding sites are found in the promoters of human genes encoding interleukin 1a (IL-1a), interleukin 1b (IL-1b), interleukin 4 (IL-4), colony stimulating factor-1 (CSF-1), granulocyte-colony stimulating factor (G-CSF), $p21^{Waf1/CIP-1}$, BAX, and c-Myc [Zweidler-Mckay et al., 1996]. It has been reported that Gfi-1 represses *BAX* promoter activity [Grimes et al., 1996b], and Gfi-1B represses $p21^{Waf1/CIP-1}$ promoter activity [Tong et al., 1998].

Although Gfi-1 and Gfi-1B are important regulators of hematopoiesis, the mechanism by which they repress transcription is unknown. We investigated whether repression was via interactions with histone deacetylase complexes, which act to modify chromatin. Here we demonstrate that Gfi-1 and Gfi-1B interact with the co-repressor ETO (MTG8), first identified as a partner in the t(8;21) translocation associated with acute myelogenous leukemia [Miyoshi et al., 1991; Erickson et al., 1992], and with ETO family members such as MTG16 [Gamou et al., 1998]. Using an in vitro capture assay we show that ETO domains I and II each directly interact with Gfi-1. Co-immunoprecipitation assays suggested ETO and Gfi-1 form complexes in vivo. Moreover, Gfi-1 associated with histone deacetylase-1 (HDAC-1), HDAC-2, and HDAC-3 when co-expressed in mammalian cells. These enzymes direct deacetylation of histones resulting in closed chromatin conformations [Cress and Seto, 2000]. This interaction with HDAC proteins likely plays an important role in Gfi-1's function as Gfi-1-mediated transcription repression is abrogated by the histone deacetylase inhibitor trichostatin A (TSA). Both in situ and biochemical analyses showed that like ETO, a portion of Gfi-1 and Gfi-1B, is associated with the nuclear matrix. These results demonstrate that Gfi-1 and Gfi-1B can associate with components of histone deacetylase complexes and suggests a mechanism of repression involving chromatin modifications.

MATERIALS AND METHODS

Plasmid Preparation

Production of GFP-ETO and GFP-AML-1/ETO was described previously [Odaka et al., 2000]. The enhanced yellow fluorescent protein cDNA (YFP; Clontech Laboratories, Inc.) joined to the Simian Virus 40 (SV40) Large T antigen (Ag) nuclear localization signal (NLS) was prepared using PCR and the DNA oligomers 5′-TAGGTACCTCGCCACCATGGTGAGCAAGGGCGA-3′ and 5′-TACTCGAGTTAGACCTTTCGCTTCTTCTTCGGCTTGTACAGCTCGTCCAT-3′, sequenced, and then subcloned into pCDNA3 at the *Kpn*-1 and *Xho*I sites. This placed the SV40 Large T antigen NLS C-terminal to YFP. GFP-Gfi-1 was prepared by cloning the rat Gfi-1 cDNA into pEGFP while GFP-Gfi-1B was prepared by PCR using the DNA oligomers TTGGATCCATGCCACGGTCCTTTCTA-3′ and 5′-TTGAATTCTCACTTGAGATTGTGTTG-3′. pGEXGfi-1 and pGEXGfi-1B were made using PCR and the DNA oligomer pairs 5′-CCGAATTCATGCCGCGCTCATTC-3′ and 5′-GGCTCGAGCTCATTTGAGTCCATGC-3′ and 5′-TTGGATCCATGCCACGGTCCTTTCTA-3′ and 5′-TTGAATTCTCACTTGAGATTGTGTTG-3′, respectively. The resultant cDNAs were sequenced and then subcloned into *EcoR*I

and *Xho*I (Gfi-1) or *Bam*HI and *Eco*RI sites of pGex4T-1. pGex-Gfi-1-SNAG containing the N-terminal 20 amino acid SNAG domain was prepared using the DNA oligomer pairs 5′-CCGAATTCATGCCGCGCTCATTC-3′ and 5′-TACTCGAGTCACGGAGAACGCGGCTGGTG-3′. The resultant cDNA was sequenced and then cloned into the *EcoR*I and *Xho*I sites of pGex4T-1. pGexGfi-1-ZF containing the C-terminal zinc-finger region was prepared using the DNA oligomer pairs 5′-TAGAATTCCTACAAATGCATCAACTGC-3′ and 5′-TACTCGAGTCATTTGAGTCCATGCTG-3′. The resultant cDNA was sequenced and then cloned into the *EcoR*I and *Xho*I sites of pGex5X-3. The pCDNA3-Myc-Gfi-1 Δ SNAG was prepared by using PCR and the DNA oligomer pair 5′-TATCTAGACCGAAGAAGCGAAAGGTCGACTACTCCCTGCGCCTG-3′ (containing the SV 40 Large T antigen NLS) and 5′-TACTCGAGTCATTTGAGTCCATCCTG-3′ to form a Gfi-1 protein lacking the SNAG domain and containing a NLS. The resultant PCR product (Gfi-1Δ SNAG) was sequenced and cloned into pJ3M in frame with the Myc-tag. Myc-Gfi-1Δ SNAG was excised and cloned into the *Hind*III and *Xho*I sites of pCDNA3. Large-scale DNA was prepared using an alkaline lysis procedure followed by polyethylene glycol purification. DNA oligomers were purchased from Oligos Etc. DNA sequencing was performed at the Iowa State University Sequencing Facility.

Cell Culture and Transfection

The cell lines C33A, 293, and 293T were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin–streptomycin and 1% L-glutamine. The cells were transfected by using a modified calcium phosphate procedure [Chen and Okayama, 1987].

Cell Preparation and Fractionation

Whole cell and nuclear matrix intermediate filament (NMIF) preparations were prepared by standard stepwise extractions [Fey et al., 1984]. Whole cell preparations were obtained by fixing cells on ice for 10 min in 3.7% formaldehyde in phosphate buffered saline. The NMIF preparations were obtained by extracting the cells twice in cytoskeletal (CSK) buffer (100 mM NaCl, 300 mM Sucrose, 10 mM Pipes pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100) containing 40 μg/ml RNAsin to remove soluble cytoplasmic and nuclear proteins followed by two 30 min extractions in room temperature in digestion buffer (50 mM NaCl, 300 mM sucrose, 10 mM Pipes pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 1.2 mM PMSF, 100 μg/ml DNAse I) containing 50 μg/ml RNAsin to remove chromatin. Preparations were then extracted twice with 250 mM ammonium sulfate to remove any additional CSK proteins. The efficiency of DNA extractions was determined by DAPI staining (5 μg/ml) in PBS + 0.05% triton X-100. In vitro sequential biochemical extraction was performed on harvested and washed cells [Fey et al., 1984]. Cells were extracted in CSK buffer containing 1.2 mM PMSF. Samples were then extracted in RSB-Majik buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris pH 7.4, 1% tween 40, 0.5% deoxycholate, 1.2 mM PMSF). Samples were then extracted for 30 min to remove nucleic acids in nuclease digestion buffer (50 mM NaCl, 300 mM sucrose, 10 mM Pipes pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 1.2 mM PMSF, 100 μg/ml DNAse I, and 50 μg/ml RNAse A; nuclease fraction). Samples were then extracted with 2 M NaCl in CSK buffer to remove loosely associated proteins (NaCl fraction). The resultant core nuclear matrix was solubilized in SDS–PAGE sample buffer (matrix fraction). Cell-equivalent samples from each fraction were loaded on SDS–PAGE gels and transferred to nitrocellulose prior to antibody probing using rabbit polyclonal antiserum directed against full length GFP [Davis et al., 1999]. Control immunoblot analysis was performed using Lamin A/C antibody (SC-6215, Santa Cruz).

Transient Transcription Assays

C33A cells were transiently transfected with a vector containing multimerized Gfi-1 binding sites regulating expression of the reporter gene chloramphenicol acetyltransferase (TKCAT-(B30) ×2) (Grimes et al., 1996a) and pCDNA3-Myc-Gfi-1 or pCDNA3-Myc-Gfi-1Δ SNAG lacking the SNAG domain (amino-terminal 20 amino acids). Transfections also included a Rous sarcoma virus-secreted alkaline phosphatase (RSV-SEAP) plasmid as a control for transfection efficiency. Twenty-four hours post transfection cells were treated with 300 nM TSA (in ethanol) or an equal volume of ethanol. Twenty-four hours post-treatment growth medium and cells were harvested. Cell lysates and growth medium were analyzed for CAT and

SEAP activity, respectively, as previously described [Gorman et al., 1982; Berger et al., 1988].

Direct Fluorescence Microscopy and Image Analysis

Cells grown on slides were transfected with the indicated vectors. The GFP-fluorescence signals were visualized using an Olympus AX-70 epifluorescence microscope equipped with the appropriate filter (41001; Chroma Technology Corp.). Monochrome images were captured using a cooled-CCD camera (NU 200; Princeton Instruments) and processed using IP-lab software (Scanalytics, Inc.). Images of the yellow and cyan GFP-variants were captured using the JP4 dual band filter set (Chroma Technology Corp.). Psuedocolored composite images were produced using IP-Lab software.

Immunoprecipitation and Immunoblot Analysis

To detect in vivo associations between Myc-Gfi-1 and either GFP-ETO or GFP-AML-1/ETO, C33A cells were transfected with the indicated vectors. After 24–36 h cell lysates were prepared and the proteins immunoprecipitated with rabbit polyclonal antiserum directed against GFP [Davis et al., 1999]. Immunoprecipitated proteins were separated by electrophoresis through 10% SDS–PAGE, transferred to nitrocellulose membranes and probed with 9E10 monoclonal antibody (specific for the Myc epitope), followed by alkaline phosphatase-conjugated anti-mouse secondary antibody, and visualized using the Immun-Star kit (Bio-Rad). To detect association between Myc-Gfi-1 and histone deacetylases, C33A cells were transfected with vectors directing expression of Myc-Gfi-1 and FLAG-tagged HDAC-1, HDAC-2, and HDAC-3. Thirty-six hours later cell lysates were prepared and subjected to immunoprecipitation using a rabbit polyclonal antiserum directed against the FLAG epitope (Sigma), and the immunoprecipitated proteins were separated by 10% SDS–PAGE. Immunoblot analysis was then performed using the 9E10 antibody, the appropriate secondary antibody and the Immun-Star detection kit.

In Vitro GST-Capture Assays

GST-ETO and GST-ETO domain constructs have been previously described [Davis et al., 1999]. The in vitro interactions of Gfi-1 and Gfi-1B with GST-ETO and GST-ETO domain proteins were analyzed using glutathione-S-transferse (GST)-capture assays. ^{35}S-methionine-labeled Gfi-1 and Gfi-1B proteins were prepared in rabbit reticulocyte lysates programmed with the appropriate cDNA. GST and recombinant GST-ETO proteins were produced in *E. coli*, purified on GST-sepharose beads and incubated with 15 μl of the rabbit reticulocyte lysate containing the appropriate labeled protein for 1 h at 4°C. Protein complexes were then pelleted and washed five times in IP buffer [50 mM Tris-HCL (pH 7.5), 150 mM NaCl, 0.4% NP-40, 1 mM EDTA] + 0.1% BSA and four times in IP buffer. Samples were separated by 10% SDS–PAGE, and gels were stained with Coomassie blue to detect GST proteins, and then dried and exposed to Kodak XAR film for 6–16 h at room temperature. The interaction between GFP-ETO and GST-Gfi-1 and portions of Gfi-1 were analyzed essentially as above except that GFP-ETO was expressed in 293T cells and unlabeled cell lysates were incubated with recombinant GST-Gfi-1, GST-Gfi-1-SNAG, GST-Gfi-1-ZF or GST proteins produced in *E. coli*. Protein complexes were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were subjected to immunoblot analysis using an anti-GFP monoclonal antibody (Clontech) and developed using the appropriate secondary antibody and the Immun-Star detection kit.

RESULTS

YFP-Gfi-1 and CFP-ETO Co-Localize in Nuclear Dots

Previously, transfection of cells with green fluorescent protein tagged Gfi-1 (GFP-Gfi-1) resulted in a punctate subnuclear pattern [Rodel et al., 2000]. This localization is strikingly similar to that of ETO, which is partially localized to punctate subnuclear compartments termed ETO nuclear bodies (ENB) that do not co-incide with PML (promyelocytic leukemia protein) [McNeil et al., 1999; Odaka et al., 2000]. To determine whether Gfi-1 and ETO reside in the same or overlapping punctate structures, we transfected 293 cells with a construct able to direct the expression of a fusion protein comprising Gfi-1 and yellow fluorescent protein (YFP-Gfi-1), and with a construct coding for cyano fluorescent protein ETO lacking the zinc-

finger domain (CFP-ETO1-513). CFP-ETO1-513 was used because it exhibits punctate localization in 100% of the transfected cells, while wild-type ETO exhibits a punctate distribution in approximately 30% of the cells within a given field possibly owing to regulated entry into ENB [Odaka et al., 2000]. Transiently transfected live cells were analyzed by epifluorescence microscopy (Fig. 1). Fluorescence images of YFP-Gfi-1 or CFP-ETO1-513 were captured separately using filter sets that distinguish between the emission wavelengths for each protein (Fig. 1, panel A). When YFP-Gfi-1 and CFP-ETO1-513 were co-expressed and their color separated and psuedocolored images merged, the merged image demonstrated a partial co-localization as shown by the yellow color in two closely apposed nuclei (Fig. 1, panel B). Nomarski images are shown in each panel.

GFP-Gfi-1 and GFP-GFI-1B are Nuclear Matrix-Attached

The nuclear matrix is believed to be involved in regulation of gene expression, with several transcription factors identified as matrix attached, including AML-1 [Zeng et al., 1997]. Recently, ETO was shown to be nuclear matrix attached [Le et al., 1998]. To test whether Gfi-1 and Gfi-1B attach to the nuclear matrix we preformed in situ nuclear matrix analysis. 293 Cells on slides were transiently transfected with expression plasmids coding for GFP-Gfi-1, GFP-GFI-1B or GFP alone. Twenty-four hours later, cells were fixed in 3.7% formaldehyde (whole cell preparation) or extracted to remove soluble proteins and chromatin to yield the NMIFs (see methods), and samples stained with DAPI to reveal DNA (Fig. 2). These preparations were analyzed by epifluorescence

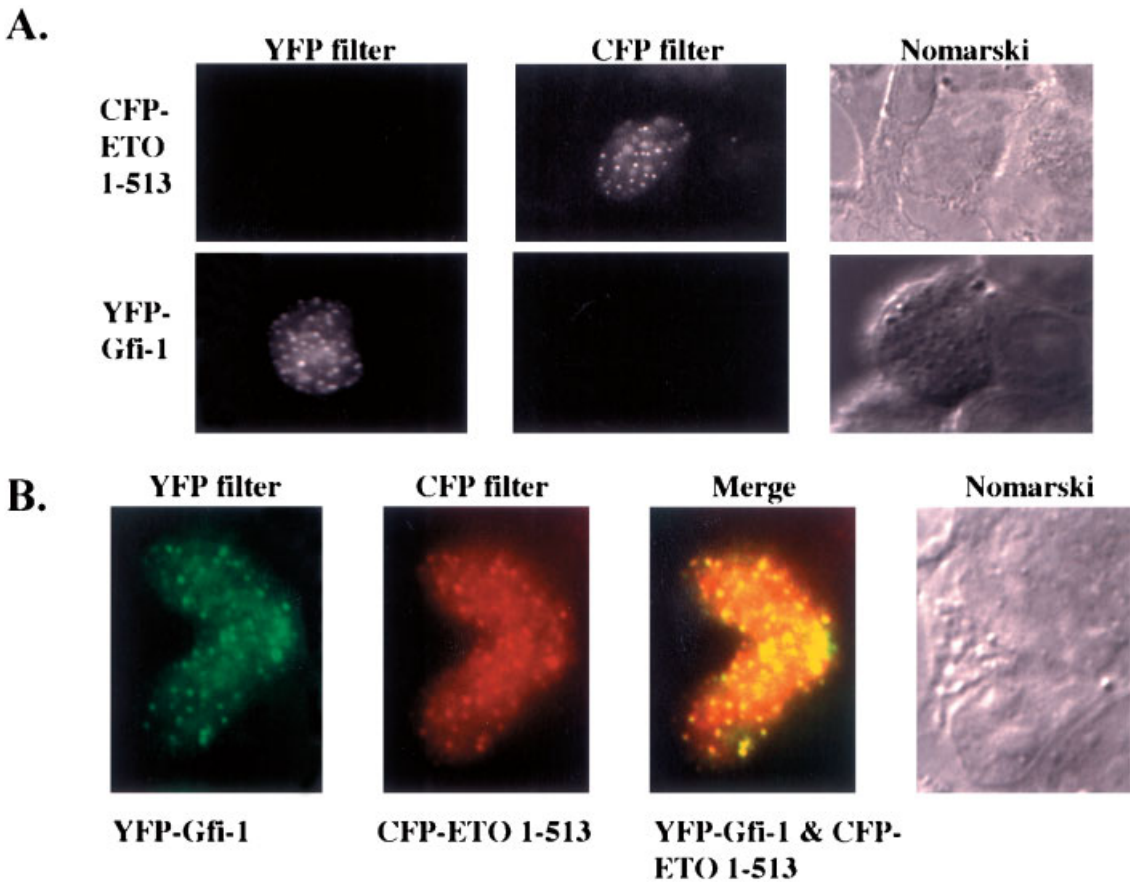


Fig. 1. Gfi-1 co-localizes with ETO 1-513 in the nuclei of live cells. **A**: Fluorescent micrograph of 293 cells transiently transfected with CFP-ETO 1-513 or YFP-Gfi-1, and visualized using YFP- or CFP-specific filters. Nomarski images are shown on the far right of the panel. **B**: Fluorescent micrograph of representative cell nuclei co-expressing YFP-Gfi-1 and CFP-ETO 1-513, captured using YFP or CFP filters (as labeled) and psuedocolored green (YFP) and red (CFP). Co-localization is represented by the yellow color in the merge figure in the far right of **panel B**.

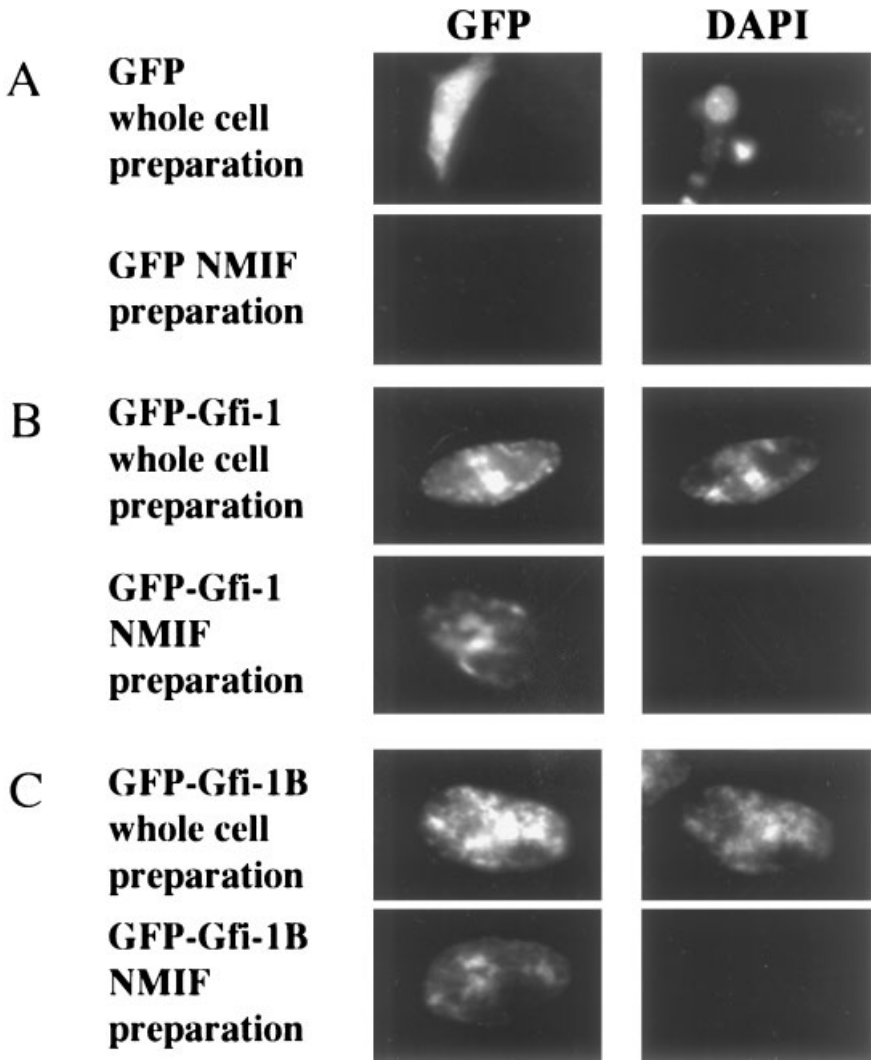


Fig. 2. GFP-Gfi-1 and GFP-Gfi-1B associate with NMIFs in situ. Fluorescent micrographs of whole cell or in situ NMIF preparations. 293 Cells grown on coverslips were transiently transfected with GFP, GFP-Gfi-1 or GFP-Gfi-1B. Micrographs labeled 'whole cell preparation' depict cells fixed with 3.7% formaldehyde, while those labeled 'NMIF preparation' refer to cells from which soluble proteins and chromatin have been extracted. DNA is detected by DAPI staining.

microscopy for retention of expressed proteins on the NMIFs. Whole cell preparations clearly show expression of GFP-Gfi-1, GFP-Gfi-1B, and GFP (Fig. 2, panels A, B, and C; whole cell preparation). GFP-Gfi-1 and GFP-Gfi-1B (Fig. 2, panels B and C; NMIF), but not GFP (panel A; NMIF), were retained on the NMIFs (Fig. 2). DNA is absent in the nuclear matrix in situ preparations, as expected (DAPI) (Fig. 2, panels A, B, and C).

To extend and confirm these results, partitioning of YFP-Gfi-1 was analyzed using a biochemical fractionation procedure that isolates core nuclear matrix. C33A cells were transiently transfected with vectors directing expression of YFP-Gfi-1, GFP-ETO or YFP containing the Simian Virus 40 (SV40) large T antigen NLS (YFP-NLS) to ensure complete nuclear targeting. YFP-NLS was analyzed for subcellular localization in live cells by fluorescent microscopy and found to be nuclear (data not shown). Transfected cells were counted, collected, and sequentially extracted for soluble, chromatin, and nuclear matrix fractions [Fey et al., 1984], and a cell-equivalent amount of each fraction was separated by 10% SDS–PAGE, transferred to a nitrocellulose membrane, and probed with anti-GFP antiserum which also reacts with YFP (Fig. 3). We found that YFP-Gfi-1 (Fig. 3, top panel) was partially

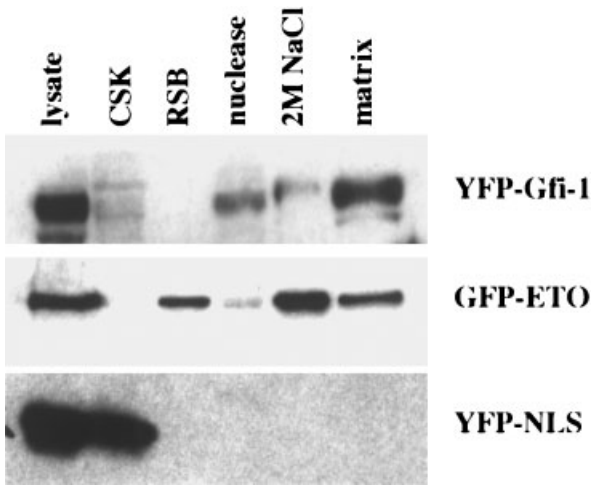


Fig. 3. A portion of the Gfi-1 population associates with the core nuclear matrix. C33A cells expressing YFP-Gfi-1, GFP-ETO or YFP-NLS were solubilized and fractionated, and fractions analyzed by immunoblot using anti-GFP antibody that also detects YFP. Lysate, whole cell lysate; CSK, cytoskeletal soluble fraction; RSB, cytoskeleton fraction; nuclease, proteins released after DNAse I treatment; 2 M NaCl, proteins eluted with high salt wash; and matrix, proteins released only after boiling in SDS–PAGE sample buffer.

retained in the high-salt resistant biochemical nuclear matrix (matrix), while a small portion was released by high salt elution and following DNAseI treatment (2 M NaCl and nuclease, Fig. 3, top panel). In contrast, YFP-NLS (Fig. 3, bottom panel) is completely soluble and eluted in the cytoplasmic (CSK) fraction. GFP-ETO (Fig. 3, middle panel) was partially retained in the nuclear matrix, while the remainder of the protein was released into the high salt and the RSB fractions. These data are in agreement with a previously published study demonstrating that ETO is nuclear matrix attached [Le et al., 1998]. Control immunoblot analysis using a monoclonal antibody that detects both lamin A and lamin C revealed lamin proteins in the matrix fraction but not in the soluble CSK and RSB fractions, as expected (data not shown).

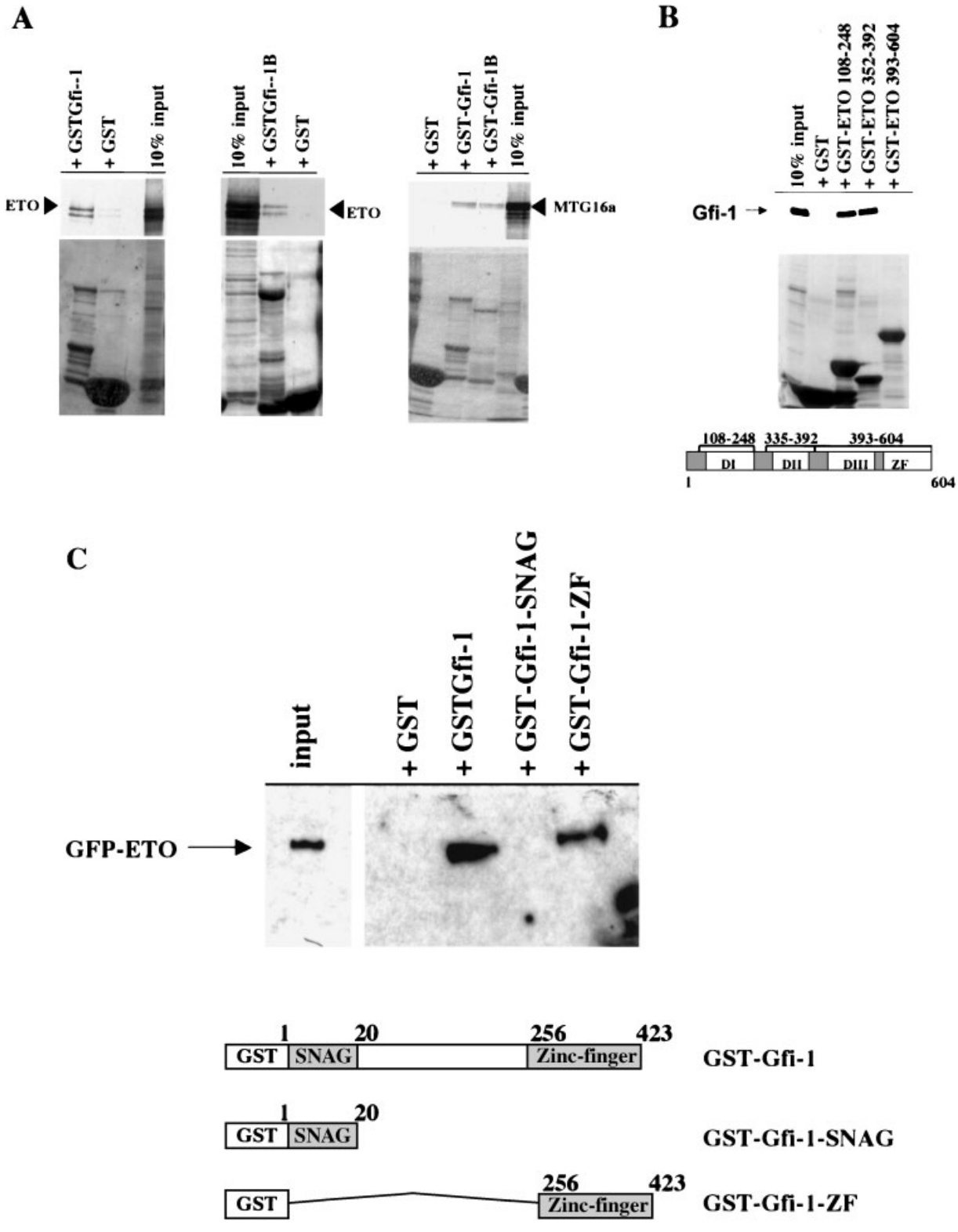
Gfi-1 and Gfi-1B Interact Directly With ETO

Given that Gfi-1 and ETO partially co-localize in subnuclear dots, it was of interest to determine whether they physically interact. To test this, full length GST-Gfi-1, GST-Gfi-1B or GST alone were expressed in bacteria and purified on GST-sepharose beads. The bead-linked GST proteins were incubated with ^{35}S-methionine-labeled ETO produced in rabbit reticulocyte lystes, and the captured proteins separated on SDS–PAGE. Gels were stained with Coomassie blue, dried, and subjected to autoradiography. The results of these in vitro GST capture assays showed that GST-Gfi-1 and GST-Gfi-1B, but not GST, bound ETO (Fig. 4A, left and middle panels).

ETO is a member of a multi-protein family that includes MTG16, a protein expressed in hematopoietic cell types [Gamou et al., 1998; Kitabayashi et al., 1998]. GST-capture assays were used to determine whether MTG16a, the largest splice form of MTG16, interacted with Gfi-1 or Gfi-1B. The results of these assays indicated that GST-Gfi-1 and GST-Gfi-1B, but not GST, bound ^{35}S-methionine-labeled MTG16a (Fig. 4A, right panel).

Based upon the above data, we hypothesized that a conserved ETO family domain mediates binding to Gfi-1 proteins. The ETO proteins are composed of four evolutionarily conserved domains (domains I–III and zinc-finger; also termed nervy homology regions) [for review see Davis et al., 2003]. Domain I contains some homology to human TBP-associated factors. Domain II is a small hydrophic region that mediates protein–protein interactions [Davis et al., 1999]. The zinc-finger region does not appear to bind DNA, but mediates protein interactions [Lutterbach et al., 1998; Wang et al., 1998]. Chimeric proteins consisting of conserved regions of ETO fused to GST [Davis et al., 1999] were attached to GST-Sepharose beads and incubated with ^{35}S-methionine-labeled Gfi-1 produced in vitro. We found that GST-ETO 108-248 (containing domain I) and GST-ETO 335-392 (containing domain II), but not GST-ETO 393-604 (domain III and the zinc-finger region), efficiently bound Gfi-1 (Fig. 4, panel B). The Coomasie blue stained proteins are shown below the GST-capture assays. The illustration below panel B shows the regions of ETO used in the GST-capture assays.

GST-capture assays also were used to determine which portion of Gfi-1 bound to ETO. To do this, we prepared cell lysates from 293 cells transfected with a vector directing the expression of GFP-ETO and incubated these lysates with recombinant GST-Gfi-1 proteins. We found that GST proteins containing the Gfi-1 C-terminal zinc-finger region (GST-Gfi-1-ZF) bound GFP-ETO in a manner similar to that of full-length Gfi-1. However, GST proteins containing a 20 amino acid SNAG repression


A
+ GSTGfi--1
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+ GST-ETO 393-604
Gfi-1
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+ GST
+ GSTGfi-1
+ GST-Gfi-1-SNAG
+ GST-Gfi-1-ZF
GFP-ETO
1
20
256
423
GST
SNAG
Zinc-finger
GST-Gfi-1
GST-Gfi-1-SNAG
GST-Gfi-1-ZF

Fig. 4.

domain (GST-Gfi-1-SNAG) failed to capture GFP-ETO in this assay (Fig. 4, panel C). These results suggest that co-repressors interact with Gfi-1 outside of the SNAG repression domain. The illustration below panel C shows the regions of Gfi-1 used in the GST-capture assay.

Gfi-1 Associates With ETO, AML-1/ETO, HDAC-1, HDAC-2, and HDAC-3 In Vivo

Since Gfi-1 and Gfi-1B directly bound ETO in GST-capture assays, we tested for association in vivo. In addition, we tested for interactions between the t(8;21) protein AML-1/ETO and Gfi-1. C33A cells were transfected with vectors directing the expression of GFP-ETO and GFP-AML-1/ETO alone or in combination with Myc-Gfi-1. Whole cell extracts were prepared, immunoprecipitated using α-GFP antiserum, and the precipitates separated using 10% SDS–PAGE and immunoblotted using the 9E10 mouse monoclonal antiserum, which is specific for the myc epitope. These experiments clearly showed that GFP-ETO and GFP-AML-1/ETO form in vivo complexes with Gfi-1 (Fig. 5; direct immunoblot analysis of the same cellular extracts is shown in the bottom two panels).

ETO associates with histone deacetylase-1 (HDAC-1), HDAC-2, and HDAC-3 [Amann et al., 2001]. To test whether Gfi-1 formed complexes with histone deacetylase proteins in vivo we transfected 293 cells with vectors directing the expression of Myc-Gfi-1 alone or with FLAG-HDAC-1, FLAG-HDAC-2 or FLAG-HDAC-3-expressing vectors or with GFP-ETO as a positive control. Cell lysates were prepared and an α-FLAG antibody antibody was used to immunoprecipitate FLAG-tagged histone deacetylase proteins, while an α-GFP antibody was used to immunoprecipitate GFP-ETO from control cell extracts. Immunoprecipitates were analyzed by immunoblot using the 9E10 antiserum. Total cell lysate protein was also subjected to immunoblot analysis using 9E10 to verify Myc-Gfi-1 expression or the α-FLAG antibody to determine expression of histone deacetylase proteins. We found that Myc-Gfi-1 was detected in α-FLAG immunoprecipitates from lysates derived from cells doubly transfected with Myc-Gfi-1 and any of the FLAG-HDACs (Fig. 6). Myc-Gfi-1 was also detected in α-GFP immunoprecipitates from lysates derived from cells doubly transfected with Myc-Gfi-1 and GFP-ETO (Fig. 6). In contrast, Myc-Gfi-1 was not detected in immunoblots of α-FLAG- or α-GFP-immunoprecipitated proteins from cells transfected with Myc-Gfi-1 alone (Fig. 6). As a control, lysates were immunoprecipated with a nonimmune antibody and immunoprecipitates were analyzed by immunoblot using the 9E10 antiserum and Myc-Gfi-1 was not detected (data not shown).

Gfi-1 Represses Transcription Using a TSA-Sensitive Mechanism

The physical association of Gfi-1 with ETO and HDAC proteins suggested that Gfi-1 would repress transcription in a histone deacetylase-dependent manner. To test this we assayed the ability of Gfi-1 to repress transcription in the absence or presence of the histone deacetylase inhibitor TSA (Fig. 7). Myc-Gfi-1 repressed transcription of a minimal promoter containing

Fig. 4. ETO and MTG16a interact with Gfi-1 and Gfi-1B in vitro. **A**: GST-Gfi-1 or GST-Gfi-1B and GST were prepared in *E. coli* and purified on glutathione Sepharose beads. Bead-linked proteins were incubated for 1 h with in vitro-produced, ^{35}S-Met-labeled ETO or the ETO-related protein MTG16a, after which beads were collected, washed, re-Suspended in SDS–PAGE loading dye and proteins separated on 10% SDS–PAGE. One microliter of rabbit reticulocyte lysate containing labeled ETO or MTG16a was electrophoresed for comparison (10% input). Gels were dried and autoradiograhed. Coomassie blue-stained gels showing GST protein expression are shown below the autoradiographs. GST-Gfi-1 co-migrates with a contaminating *E. coli* protein visible in the GST lanes. **B**: Identification of ETO regions able to interact with Gfi-1. GST or GST-ETO portions containing domain I (GST-ETO 108-248), domain II (GST-ETO 335–392) or domain III plus the zinc-finger region (GST-ETO 393–604) were prepared in *E. coli*, purified, and incubated with in vitro-produced, ^{35}S-Met-labeled Gfi-1. Captured Gfi-1 protein was analyzed using 10% SDS–PAGE. Dried gels were autoradiographed for 12 h. A Coomassie blue-stained gel showing expression of GST and GST recombinant proteins is shown below the autoradiograph. The schematic illustrates the ETO regions fused to GST and used in the capture assay. ETO family members contain four conserved regions shown as white boxes and labeled DI (TAF homology), DII (hydrophobic heptad repeat), DIII, and ZF (zinc-finger region). Numbers refer to amino acids. These constructs were previously described [Davis et al., 1999]. **C**: The Gfi-1 zinc-finger region binds ETO. GST, GST-Gfi-1 or GST-Gfi-1 portions containing the N-terminal SNAG domain (GST-Gfi-1-SNAG) or the C-terminal zinc-finger region (GST-Gfi-1-ZF) were prepared in *E. coli*, purified, and incubated with 293T cell lystates containing GFP-ETO. Captured GFP-ETO protein was analyzed using 10% SDS–PAGE, transferred to a nitrocellulose membrane and immunoblotted using an anti-GFP monoclonal antibody to detect GFP-ETO. The schematic illustrates the Gfi-1 portions fused to GST and used in the capture assay.

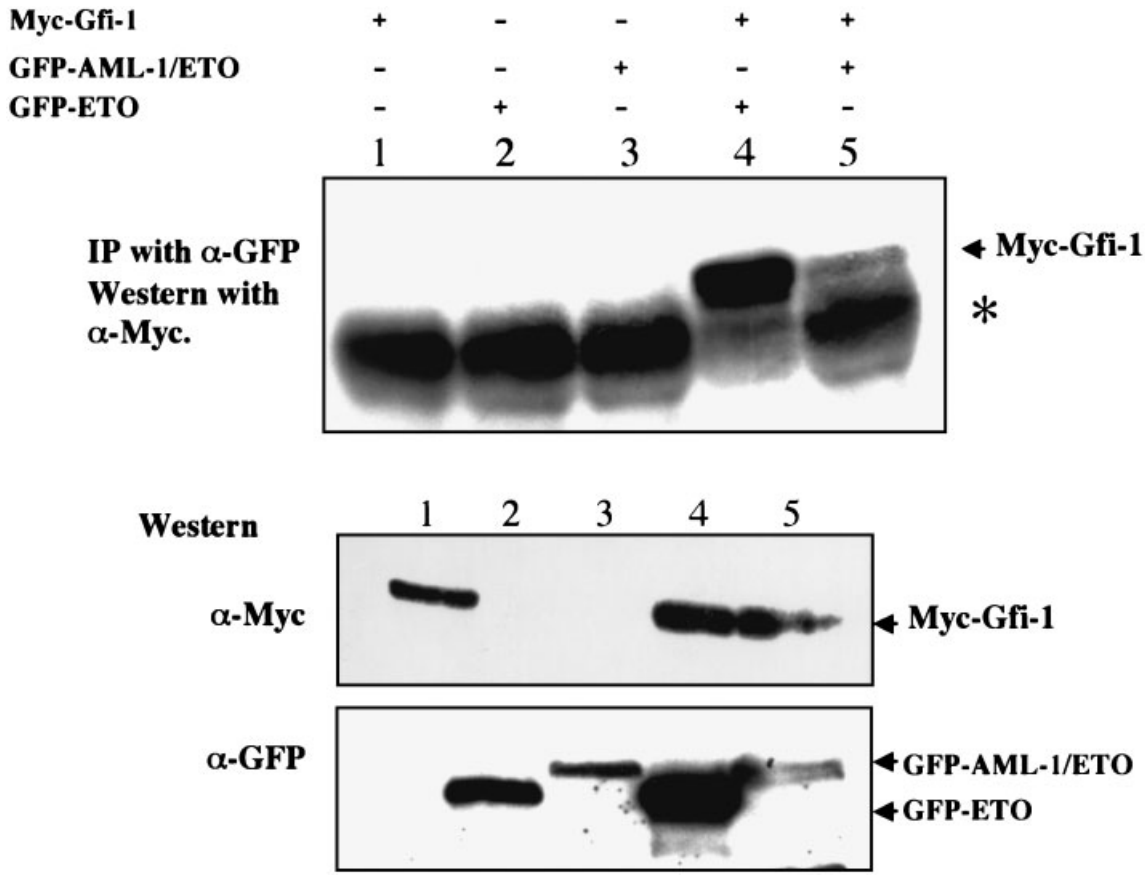

Fig. 5. Gfi-1 associates with ETO and AML-1/ETO in vivo. C33A cells were transfected with the indicated expression constructs, lysates prepared and proteins immunoprecipitated with anti-GFP rabbit polyclonal antiserum. Immunoprecipitated proteins were separated by 10% SDS–PAGE and immunoblotted using the 9E10 monoclonal antibody to detect Myc-Gfi-1 (α-Myc; **top panel**). Immunoblot analysis of total cellular extracts using either 9E10 (α-Myc) or α-GFP antisera is shown below. The asterisk (*) denotes the immunoglobulin heavy chain.

two Gfi-1 binding sites [TKCAT-(B30)*2] approximately 10 fold. Addition of 300 nm TSA, but not ethanol alone, abrogated this repression. In addition, a Gfi-1 protein lacking the SNAG repressor domain (Myc-Gfi-1 ΔSNAG) also repressed transcription of this reporter, albeit at a lower level than wild-type Gfi-1. Interestingly, Myc-Gfi-1 ΔSNAG repression was also abolished by the addition of TSA. Taken together, these data suggest a histone deacetylase-mediated repression that is only partially dependent upon the presence of the SNAG domain.

DISCUSSION

ETO was first described due to its involvement in the t(8;21) associated with acute myelogenous leukemia [Miyoshi et al., 1991; Erickson et al., 1992; Miyoshi et al., 1993]. This translocation produces a fusion protein comprising most of ETO plus the DNA binding domain of AML-1, resulting in a chimeric transcription factor AML-1/ETO. AML-1 is required for normal hematopoiesis, and AML-1/ETO functions, at least in part, to inhibit AML-1-mediated transactivation of target promoters [Frank et al., 1995; Meyers et al., 1995; Okuda et al., 1996]. AML-1/ETO inhibition of AML-1-mediated transactivation requires sequences within ETO [Lenny et al., 1995].

ETO family members contain four highly conserved regions, two of which are implicated in protein–protein interactions. The zinc-finger (MYND) region mediates a direct interaction with the co-repressor nuclear receptor corepressor (N-CoR) [Gelmetti et al., 1998; Lutterbach et al., 1998], and mediates association with histone deacetylases in vivo, while domain II, which contains a hydrophobic heptad repeat, has been implicated in dimerization among ETO family members [Davis et al., 1999] and

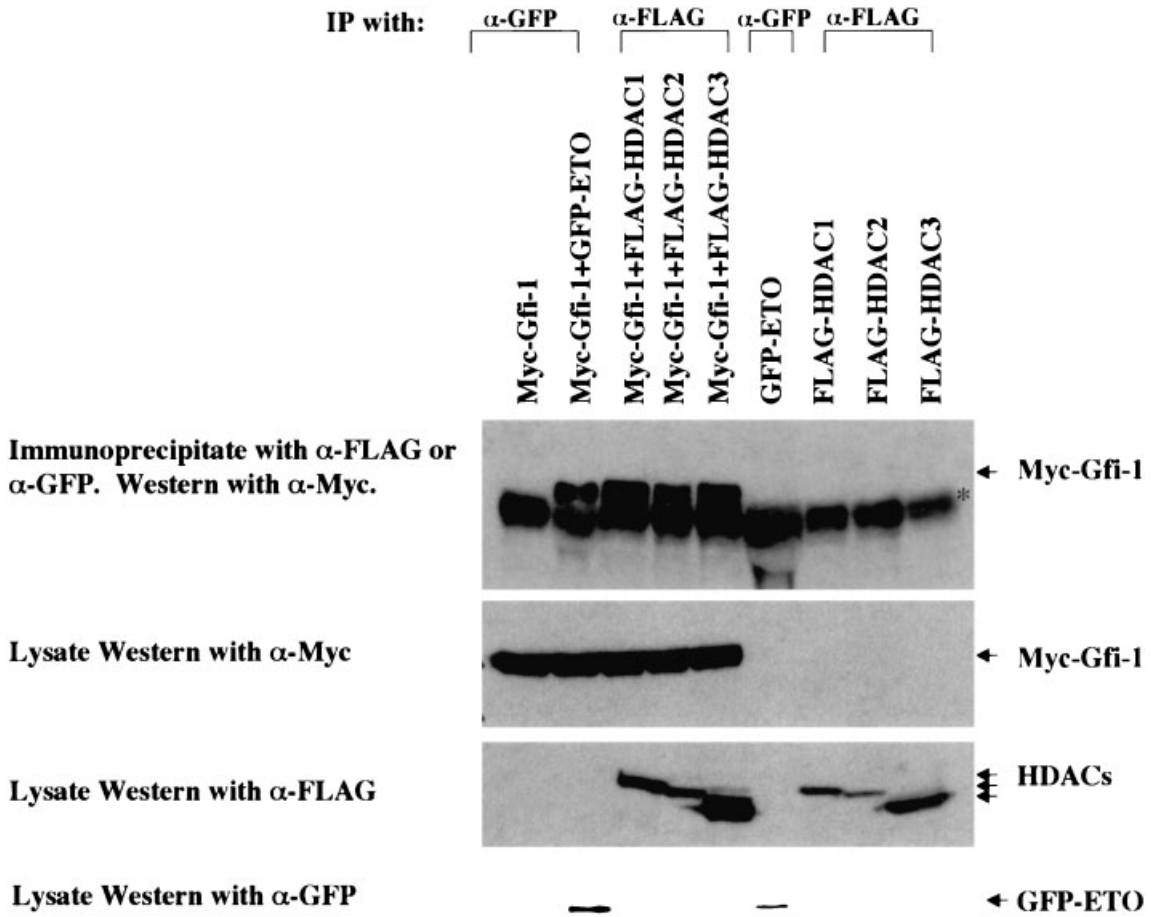

Fig. 6. Gfi-1 associates with ETO, HDAC-1, HDAC-2, and HDAC-3 in vivo. C33A cells were transfected with the indicated expression constructs, lysates were prepared and proteins immunoprecipitated with either anti-FLAG (for HDACs) or anti-GFP (for GFP-ETO) antibodies (first two lanes), and then immunoblotted using anti-Myc antibody to detect Myc-Gfi-1 (**top panel**). Total lysate was also immunoblotted with appropriate antibodies to show Myc-Gfi-1 (**second panel**) or FLAG-HDAC-1, 2 or 3 (**third panel**) expression. GFP-ETO expression in total lysate was determined using anti-GFP antibody (**fourth panel**). The asterisk (*) denotes the immunoglobulin heavy chain.

interaction with mSin3A [Amann et al., 2001; Hildebrand et al., 2001]. Interaction with histone deacetylase containing complexes suggest that ETO might function as a co-repressor, linking sequence specific transcriptional repressors to histone deacetylase-containing complexes. In support of this idea, ETO interacts with the leukemia associated promyelocytic zinc finger protein (PLZF) to repress transcription [Melnick et al., 2000; Wood et al., 2000].

In the current work we identified an interaction between ETO and the transcriptional repressors Gfi-1 and Gfi-1B, both in vitro and in vivo. Moreover, we find that Gfi-1 can bind to the ETO family member MTG16a and, not surprisingly, the chimeric AML-1/ETO which contains most of the ETO protein [Miyoshi et al., 1993; Erickson et al., 1994]. Portions of ETO containing domain I, a region with overlapping homology to human TBP-associated factor 130 (hTAF 130), hTAF 105, and *Drosophila* TAF 110 [Hoey et al., 1993; Mengus et al., 1997], and domain II are each able to interact with Gfi-1 in vitro. These data assign to domain I, a novel protein interaction function, and add to the list of protein partners bound by domain II; namely ETO and ETO family members, mSin3, and Gfi-1. Moreover, our data demonstrates an in vivo association between Gfi-1 and histone deacetylases and suggests that Gfi-1 represses transcription through the recruitment of histone deacetylase-containing complexes. Data demonstrating that Gfi-1-mediated transcriptional repression is abrogated by TSA further strengthens this idea. Interestingly, our data demonstrate repression in the absence of the

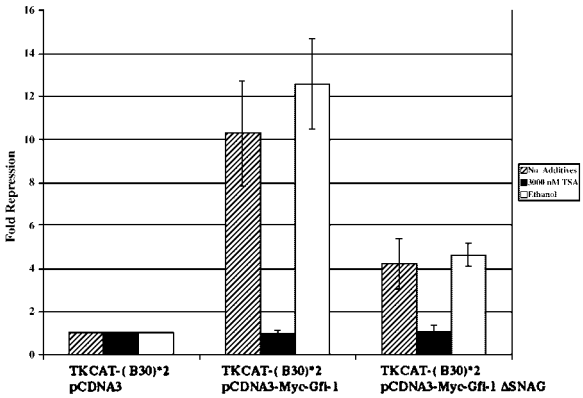

Fig. 7. Gfi-1 and Gfi-1 lacking the SNAG domain repress transcription using a TSA-sensitive mechanism. C33A cells were transfected with 2 μg of pCDNA3, pCDNA3-Myc-Gfi-1, or pCDNA3-Myc-Gfi-1 ΔSNAG, lacking the N-terminal 20 amino acid SNAG repression domain, 2 μg of TKCAT-(B30)*2 reporter containing two Gfi-1 consensus binding sites linked to a minimal promoter and the chloramphenicol acetyltransferase gene (*CAT*) and 5 μg of RSV-SEAP (Rous sarcoma virus long terminal repeat upstream of the secreted alkaline phosphatase gene). Twenty-four hours post-transfection cells were left untreated (no addition) or treated with 300 nM TSA or ethanol. Results show the average fold repression of CAT activity from three different experiments. The values were corrected using secreted alkaline phosphatase. Bars indicate the standard deviation.

SNAG domain, a 20 amino acid region previously implicated in repression [Grimes et al., 1996a]. This is consistent with our data showing that the Gfi-1 zinc-finger domain and not the SNAG domain binds the ETO co-repressor. Thus, Gfi-1 may have two (or more) regions involved in transcriptional repression.

We also found that a portion of Gfi-1 remains nuclear matrix-attached during biochemical fractionation. The nuclear matrix is believed to organize nuclear functions and proper targeting of regulatory factors to specific nuclear regions, and plays a large role in regulation of replication and transcription [Davie, 1995]. A number of leukemia-associated proteins are localized to the nuclear matrix including AML-1 and ETO [Zeng et al., 1997; Le et al., 1998; Meyers and Hiebert, 2000]. Moreover, ETO and Gfi-1 co-localize to distint subnuclear bodies, which we previously termed ETO nuclear bodies [Odaka et al., 2000]. These bodies are distinct from PML oncogenic domains and contain the Atrophin-1 transcriptional repressor and mSin3A in Neuro-2a cells [Odaka et al., 2000; Wood et al., 2000]. Thus, Gfi-1 localizes to nuclear regions containing proteins associated with transcriptional repression. It remains to be seen what role subnuclear targeting plays in the biological function of Gfi-1.

The association of Gfi-1 with ETO and AML-1/ETO suggests that transcriptional pathways directed by these proteins and leading to oncogenesis likely converge. Thus, genes whose expression is regulated both by Gfi-1 and AML-1/ETO could represent essential targets during leukemogenesis.

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