The Transcriptional Repressor GFI-1 Antagonizes PU.1 Activity through Protein-Protein Interaction

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Mice lacking the zinc finger transcriptional repressor protein GFI-1 are neutropenic. These mice generate abnormal immature myeloid cells exhibiting characteristics of both macrophages and granulocytes. Furthermore, Gfi-1−/− mice are highly susceptible to bacterial infection. Interestingly, Gfi-1−/− myeloid cells overexpress target genes of the PU.1 transcription factor such as the macrophage colony-stimulating factor receptor and PL1 itself. We therefore determined whether GFI-1 modulates the transcriptional activity of PU.1. Our data demonstrate that GFI-1 physically interacts with PU.1, repressing PU.1-dependent transcription. This repression is functionally significant, as GFI-1 blocked PU.1-induced macrophage differentiation of a multipotential hematopoietic progenitor cell line. Retroviral expression of GFI-1 in primary murine hematopoietic progenitors increased granulocyte differentiation at the expense of macrophage differentiation. We interbred Gfi-1−/− and PL1−/− mice and observed that heterozygosity at the PL1 locus partially rescued the Gfi-1−/− mixed myeloid lineage phenotype, but failed to restore granulocyte differentiation. Our data demonstrate that GFI-1 represses PU.1 activity and that lack of this repression in Gfi-1−/− myeloid cells contributes to the observed mixed lineage phenotype.

GFI-1 (growth factor independence-1) is a zinc finger transcriptional repressor containing an N-terminal SNAG domain necessary for transcriptional repression and six C-terminal zinc fingers critical for DNA binding (1). GFI-1 was first characterized as a T cell oncogene (2–5). It is expressed in the common lymphoid progenitor and developing T and B lymphocytes; however, during normal development, levels decrease as these cells mature (6, 7). GFI-1 is also expressed in non-lymphoid hematopoietic cells, including the hematopoietic stem cell and the granulocyte-macrophage progenitor (6). In mature hematopoietic cells, its expression is limited to granulocytes; however, GFI-1 can be induced in mature T cells and macrophages by mitogenic stimuli and lipopolysaccharide, respectively (1, 8–10).

Gfi-1 is required for granulocyte development. Human GFI-1 mutations were isolated from neutropenic patients, and these mutations hinder the ability of GFI-1 to repress transcription (11). Additionally, two independent Gfi-1−/− mouse strains that exhibit neutropenia were engineered (9, 10). An abnormal population of CD11b+Gr-1+ cells sharing characteristics of granulocytes and macrophages develops in Gfi-1−/− mice (10). These cells lack cytoplasmic granules and do not express RNA for secondary granule proteins. Bone marrow from Gfi-1−/− mice exhibits higher levels of macrophage colony-stimulating factor receptor (M-CSFR) and PL1 RNAs compared with bone marrow from wild-type mice. Because both of these genes are transcriptional targets of the Ets family transcription factor PU.1, we hypothesized that some of the defects observed in mutants may be due to the lack of antagonism of PU.1 activity in granulocytes by GFI-1.

Mice lacking PU.1 do not make mature macrophages, granulocytes, or B cells (12, 13). Interestingly, PU.1 concentration is critical for the specification of cell fates in the hematopoietic system. High levels of PU.1 via retroviral expression direct macrophage development of PL1−/− fetal liver hematopoietic progenitors; however, low levels promote B cell differentiation instead (14). We have observed that PU.1 levels are involved in determining whether myeloid progenitors develop into macrophages or granulocytes (15). PU.1 interacts with several important transcription factors involved in the specification of cell fates: GATA-1, CCAAT/enhancer-binding protein-α (C/EBPα), and B cell-specific activator protein (16–21). All of these factors inhibit the ability of PU.1 to activate the transcription of specific promoters. For GATA-1 and C/EBPα, it has been proposed that the ratios of PU.1 to these factors in...
uncommitted hematopoietic progenitors are important in determining cell fate decisions (15, 19, 20).

Here, we show that GFI-1 physically interacts with PU.1 and represses its transactivation capability. GFI-1 specifically blocks PU.1-induced macrophage differentiation of a myeloid cell line, and exogenous GFI-1 expression in primary hematopoietic progenitors increases granulocyte differentiation. Finally, we examined bone marrow from Gfi-1−/− PU.1+/+ mice and observed that decreasing PU.1 dosage reduced the mixed myeloid lineage phenotype of PU.1-deficient cells. Our data suggest that GFI-1 antagonism of PU.1 is critical for down-regulation of macrophage gene expression in developing granulocytes.

**EXPERIMENTAL PROCEDURES**

**Reporter Constructs and Expression Plasmids** — The human M-CSFR promoter was isolated by PCR using K562 DNA as a template. The −416/+124 human M-CSFR promoter was amplified with a previously described primer set (22), and the resultant product was subcloned into pGL3 (Promega Corp.). The pcDNA3-PU.1, MigR1-C/EBPα, MigR1-PU.1, (Gal4)3-luciferase, and Gal4-PU.1 fusion plasmids have been described previously (15, 23). The retroviral (rv) green fluorescent protein (GFP), rv-GFI-1, and rv-GFI-1-Pro-to-Ala (GFI-1-P/A) constructs (24) were provided by Dr. Jinfang Zhu (National Institutes of Health). Plasmid pSport-GFI-1 was obtained from American Type Culture Collection (IMAGE clone 5585794).

**Transient Transfections** — U937 cells were electroporated as described previously (25) using 25 µg of luciferase reporter plasmid, 50 µg of rv-GFI-1 plasmid, and 5 µg of pRL-tk (thymidine kinase promoter-Renilla luciferase) plasmid. The total amount of plasmid DNA was kept constant with rv-GFP. 293T cells were transfected using the BD Biosciences CalPhos mammalian transfection kit. For M-CSFR promoter assays, cells were transfected with 2 ng of Gal4-regulated promoter assays, cells were transfected with 5 µg of (Gal4)3-luciferase; 2.5 µg of Gal4 DNA-binding domain, Gal4-full-length PU.1, or Gal4-PU.1 transactivation domain; 4 µg of rv-GFI-1 or rv-GFI-1P/A; and 25 ng of pRL-tk. For Gal4-regulated promoter assays, cells were transfected with 5 µg of (Gal4)3-luciferase; 2.5 µg of Gal4 DNA-binding domain, Gal4-full-length PU.1, or Gal4-PU.1 transactivation domain; 4 µg of rv-GFI-1 or rv-GFI-1P/A; and 25 ng of pRL-tk. The resultant product was subcloned into pGL3 (Promega Corp.). Plasmids containing PU.1 deletion mutants have been described by resuspension in retroviral supernatant containing Poly-C/EBPα, MigR1-PU.1, (Gal4)3-luciferase, and Gal4-PU.1 fusion plasmids have been described previously (15, 23). The retroviral (rv) green fluorescent protein (GFP), rv-GFI-1, and rv-GFI-1-Pro-to-Ala (GFI-1-P/A) constructs (24) were provided by Dr. Jinfang Zhu (National Institutes of Health). Plasmid pSport-GFI-1 was obtained from American Type Culture Collection (IMAGE clone 5585794).

**In Vitro Binding Assays** — Glutathione S-transferase (GST) fusion proteins were prepared as described (23). Bacterial cells expressing GST-fused full-length GFI-1 (amino acids 1–423), N-terminal GFI-1 (amino acids 1–258), and C-terminal GFI-1 (amino acids 193–243) were obtained from Dr. H. L. Grimes (University of Cincinnati). GST-Zn3–5 and GST-Zn3–6 were constructed by PCR with specific primers and by subcloning of the PCR product into pGEX-4T3. GST-GFI-1 fusion proteins bound to glutathione-agarose were incubated in NETN buffer (0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0), and 200 mM NaCl) with in vitro translated PU.1 proteins prepared by the TnT reticulocyte lysate system (Promega Corp.). Plasmids containing PU.1 deletion mutants have been described and were provided by Dr. M. L. Atchison (University of Pennsylvania) (26). After a 2-h incubation, bound complexes were washed four times with NETN buffer. Bound complexes were eluted in sample buffer and separated by SDS-PAGE. Bound proteins were visualized by autoradiography.

**Co-immunoprecipitations** — 293T cells were transfected with 5 µg of pcDNA3-PU.1 and/or pSport-GFI-1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Forty-eight hours post-transfection, whole cell lysates were prepared with cell lysis buffer (50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1 mM ZnSO4, 5% glycerol, 1% Nonidet P-40, and 1% sodium deoxycholate). Lysates were incubated overnight at 4 °C with 2 µl of anti-PU.1 monoclonal antibody (Pharmerica) or 2 µl of anti-GFI-1 antibody (clone N-20, catalog no. sc-8558, Santa Cruz Biotechnology, Inc.) and protein A-agarose (Invitrogen). Agarose beads and captured protein complexes were washed five times with cell lysis buffer. Protein lysates were eluted in SDS-PAGE sample buffer, separated by SDS-PAGE, and immunoblotted. For U937 immunoprecipitations, 1 × 107 cells were lysed. Lysates were incubated with either anti-PU.1 or anti-tubulin antibody. Immunoprecipitates were then analyzed as described above for transfected 293T cells.

**PUER Cell Differentiation** — The generation of PUER progenitor cells and their differentiation into macrophages and granulocytes have been described in detail (15, 27). Cells were maintained in Iscove’s modified Eagle’s medium, 10% fetal calf serum (HyClone), 1 unit/ml penicillin/streptomycin, 2 mM l-glutamine, and 50 µM β-mercaptoethanol. Medium contained either 5 µg/ml interleukin (IL)-3 (R&D Systems) or 10 ng/ml granulocyte colony-stimulating factor (G-CSF; R&D Systems). Cells were differentiated by the addition of 100 nM hydroxytamoxifen (OHT) to the medium. For morphological analysis of PUER cells, 2.5 × 105 cells were cytocentrifuged onto glass slides, fixed for 30 s in methanol, and stained with Wright stain.

**Isolation of Murine Hematopoietic Progenitors** — The procedure was performed as described previously (14, 28) with minor modifications. Briefly, bone marrow cells were isolated from the tibias and femurs of 8-week-old mice. Mature erythroid cells were removed by ammonium chloride lysis. For hematopoietic colony assays, 2.5 × 104 nucleated bone marrow cells were plated onto methylcellulose medium containing hematopoietic cytokines (MethoCult GF M3434, StemCell Technologies Inc.). Colonies were counted after 7 days of incubation. For retroviral transduction, nucleated cells were lineage-depleted with a StemSep murine hematopoietic progenitor enrichment kit (StemCell Technologies Inc.) according to the manufacturer’s instructions.

**Retroviral Transduction** — Retroviral vectors (rv-GFP, rv-GFI-1, and rv-GFI-1P/A) were cotransfected into 293T cells together with the retroviral packaging vector pCL-Eco (ImageGen Corp.) by calcium phosphate precipitation. Forty-eight hours post-transfection, retroviral supernatants were harvested. PUER and primary progenitor cells were infected by resuspension in retroviral supernatant containing Poly-
precipitates were washed and then eluted with 100 mM NaHCO₃ and 1% SDS. Cross-links were reversed at 65 °C for 4 h, and protein was digested with protease K. Isolated DNA was purified by phenol/chloroform extraction and ethanol precipitation. DNA pellets were resuspended in sterile H₂O. The M-CSFR and glutamate receptor-2 (negative control) promoters were amplified by PCR.

**RNA Analysis**—RNA was isolated from PUER and bone marrow cells using TRIzol (Invitrogen). One microgram of total cellular RNA was used in a 20-μl reverse transcriptase reaction (SuperScript III, Invitrogen). Two microliters of the reverse transcriptase reaction were used in a PCR with gene-specific primers. For real-time PCR, 1 μl of prepared cDNA was used in a 20-μl real-time PCR (TaqMan assay) using gene-specific probes obtained from Applied Biosystems. The results were normalized to the levels of β-actin expression, which was assayed in the same reaction tube. Samples were run in triplicate with a DNA Engine Opticon thermocycler (Bio-Rad). Northern blots were prepared with 15 μg of total RNA for each sample. Blots were sequentially probed with random hexamer-primed 32P-labeled cDNA encoding murine myeloperoxidase and β-tubulin.

**Flow Cytometry**—Single cell suspensions were prepared and stained with monoclonal antibodies obtained from Pharmingen (Mac-3-fluorescein isothiocyanate) and Caltag (Gr-1-phycocerythrin, F4/80-phycocerythrin, and CD11b-biotin). Stained cells were analyzed on a dual-laser cell sorter (FACSCalibur, BD Biosciences). Cell preparations were preincubated with antibody to Fcγ receptor II/III to reduce nonspecific antibody binding and were subjected to propidium iodide uptake to exclude dead cells from the analysis. Fluorescence-activated cell sorter (FACS) data were analyzed using FloJo software (TreeStar Inc.).

**RESULTS**

**GFI-1 Antagonizes PU.1**—Because GFI-1 deficiency results in increased myeloid expression of M-CSFR mRNA, we determined whether GFI-1 represses M-CSFR promoter activity (22). U937 cells were transfected with a 540-bp M-CSFR promoter-luciferase construct in the presence and absence of increasing amounts of the rv-GFI-1 plasmid. The decrease in transcriptional activity induced by GFI-1 was statistically significant (p < 0.0001). B, M-CSFR promoter activity induced in 293T cells with cotransfection of MigR1-PU.1 in the presence or absence of increasing amounts of the rv-GFI-1 plasmid. The decrease in transcriptional activity induced by GFI-1 was statistically significant (p < 0.05). The difference in reporter activity of the M-CSFR reporter transfected in the presence and absence of GFI-1 (no PL1 expression plasmid) was not statistically significant. C, M-CSFR promoter activity induced in 293T cells by cotransfected MigR1-C/EBPα in the presence or absence of the rv-GFI-1 plasmid. The observed increase in transcriptional activity induced by GFI-1 was statistically significant (p < 0.02). D, 293T cells cotransfected with MigR1-PU.1, MigR1-C/EBPα, and rv-GFI-1. GFI-1 repressed transcription mediated by both PU.1 and C/EBPα. This repression was statistically significant (p < 0.002). For A, luciferase activity is reported as relative light units. For B–D, luciferase activity is reported as -fold induction above the activity seen in 293T cells transfected with only the reporter plasmid and rv-GFP. Total DNA content for all transfections was kept constant with the rv-GFP plasmid. All transfections also contained the Renilla luciferase plasmid pRL-tk. Luciferase values were determined 48 h post-transfection and were normalized to Renilla luciferase values. Luciferase values are the means ± S.E. of three independent transfections.

**GFI-1 Inhibits Transactivation from the M-CSFR Promoter**—Because GFI-1 deficiency results in increased myeloid expression of M-CSFR mRNA, we determined whether GFI-1 represses M-CSFR promoter activity (22). U937 cells were transfected with a 540-bp M-CSFR promoter-luciferase construct in the presence and absence of GFI-1 expression plasmid. We observed that GFI-1 repressed M-CSFR promoter activity by >2-fold in this myeloid cell line (Fig. 1A). To determine
GFI-1 Antagonizes PU.1

A

B

C

FIGURE 2. GFI-1 inhibits the activity of a fusion protein between full-length PU.1 and the Gal4 DNA-binding domain. 293T cells were transiently transfected with a reporter construct containing five Gal4 sites upstream of a minimal thymidine kinase promoter driving luciferase expression. A, cells were cotransfected with the Gal4 DNA-binding domain (G4DBD), Gal4-hypoxia-inducible factor-3α activation domain (G4HIF3AD), or Gal4-full-length PU.1 (G4PU.1) expression plasmid in the presence or absence of the rv-GFI-1 plasmid. GFI-1 repression of transcriptional activity induced by Gal4-PU.1 was statistically significant (p < 0.0001). B, cells were cotransfected with the Gal4 DNA-binding domain or Gal4-PU.1 transactivation domain (G4PUAD) expression plasmid in the presence or absence of rv-GFI-1. The slight enhancement of transcription by GFI-1 was statistically significant (p < 0.05). C, Gal4-full-length PU.1 was cotransfected with either the rv-GFI-1 or rv-GFI-1P/A (GFI-1 PtoA) plasmid. rv-GFI-1P/A encodes a form of GFI-1 that contains an inactivating mutation in the SNAG repression domain. The difference in activity observed between GFI-1- and GFI-1P/A-transfected cells was statistically significant (p < 0.001). Total DNA content for all transfections was kept constant with the rv-GFP plasmid. All transfections contained the Renilla luciferase plasmid pRL-tk. Luciferase values were determined 48 h post-transfection and were normalized to Renilla luciferase values. Luciferase activity is the mean ± S.E. of three independent transfections, and luciferase activity is reported as -fold induction above the activity seen in 293T cells transfected with the Gal4 DNA-binding domain expression plasmid.

whether GFI-1 targets PU.1 to repress the M-CSFR promoter, we repeated the reporter assay in 293T cells. The M-CSFR promoter construct is activated in non-hematopoietic cells by cotransfection of the PU.1 and/or C/EBPα transcription factor (30–32). In transfected 293T cells, PU.1 transactivated the M-CSFR promoter construct by 14-fold compared with cells transfected with the reporter plasmid alone. However, if GFI-1 was cotransfected with PU.1, promoter activity was decreased to ~5-fold (Fig. 1B). We did not observe a significant effect on transcription when the M-CSFR promoter construct and GFI-1 were cotransfected in the absence of PU.1. Additionally, GFI-1 did not significantly repress M-CSFR promoter activity induced by C/EBPα (Fig. 1C). Because there was a slight but reproducible transcriptional synergy between GFI-1 and C/EBPα, we tested whether GFI-1 represses M-CSFR promoter activity by both PU.1 and C/EBPα. When PU.1 and C/EBPα activated the M-CSFR promoter, transcription was still repressed by GFI-1 (Fig. 1D). This result indicates that C/EBPα does not abrogate GFI-1 repression of PU.1.

The results from the M-CSFR promoter assays suggested that GFI-1 inhibition is mediated by the repression of PU.1. To confirm these observations, we determined whether GFI-1 affects PU.1 transactivation when PU.1 is tethered to DNA by a heterologous DNA-binding domain. Full-length PU.1 was fused to the Gal4 DNA-binding domain and cotransfected into 293T cells with a luciferase reporter gene regulated by five upstream Gal4-binding sites. The Gal4-PU.1 protein transactivated the reporter by >14-fold above the activity induced by the Gal4 DNA-binding domain alone (Fig. 2A). However, similar to results obtained with the M-CSFR promoter, this activity was reduced by 7-fold in the presence of GFI-1. This effect was specific to PU.1, as GFI-1 had no effect on transcription induced by a fusion protein of Gal4 and the activation domain of the unrelated transcription factor hypoxia-inducible factor-3α. Interestingly, GFI-1 did not repress a fusion between Gal4 and the PU.1 transactivation domain (Fig. 2B). Because GFI-1 inhibited PU.1 activity when PU.1 was tethered to DNA via the Gal4 DNA-binding domain, we concluded that GFI-1 did not block PU.1 activity via inhibition of DNA binding (see also Fig. 4D). Because GFI-1 contains an N-terminal repression domain (SNAG domain), we tested whether this domain is necessary for antagonizing PU.1. Mutating the second amino acid of GFI-1 from proline to alanine abrogates its repression activity (33). When GFI-1P/A was analyzed for its ability to inhibit PU.1 activity, a significant decrease in repression activity compared with wild-type GFI-1 was observed (Fig. 2C). This result demonstrates that the GFI-1 SNAG domain is necessary for inhibiting PU.1 transactivation. The results from the Gal4 and M-CSFR reporter assays demonstrate that GFI-1 inhibits PU.1 activation of two independent promoters in vitro.

GFI-1 Associates with PU.1—Results from the transient transfection assays suggested that GFI-1 represses transcription by directly binding to PU.1. To determine whether GFI-1 inhibition of PU.1 is mediated by protein-protein interaction, we performed co-immunoprecipitation assays. 293T cells were transfected with GFI-1 and/or PU.1 expression vector. Whole cells extracts were prepared and immunoprecipitated with either anti-PU.1 or anti-GFI-1 antibody. Immunoprecipitates
were separated by SDS-PAGE and immunoblotted with anti-
PU.1 or anti-GFI-1 antibody to assay for co-immunoprecipita-
tion. PU.1 was detected in anti-GFI-1 antibody-immunopre-
cipitated complexes (Fig. 3A, upper panel, lane 2) obtained
from cells coexpressing PU.1 and GFI-1. Some nonspecific
precipitation of PU.1 occurred in the absence of PU.1 (lane 3);
however, significantly more PU.1 was immunoprecipitated
in the presence of GFI-1, indicating that these proteins interact in vivo. Similarly, when the same extracts were immunoprecipitated
with anti-PU.1 antibody, GFI-1 was detected in the
immunoprecipitated complexes in cells coexpressing PU.1 and
GFI-1 (Fig. 3A, lower panel).

To determine whether endogenous PU.1 and GFI-1 pro-
teins co-immunoprecipitate, we performed immunoprecipita-
tions on lysates obtained from U937 cells. Both PU.1 and
GFI-1 were detected in extracts of this myeloid cell line.5
Lysates were prepared from U937 cells and incubated with

5 R. Dahl, S. R. Iyer, K. S. Owens, D. D. Cuylear, and M. C. Simon, unpublished data.

either anti-PU.1 antibody or nonspecific antibody (anti-tubulin).
Protein complexes were isolated, immunoblotted, and probed with
anti-GFI-1 antibody. Immunoreactive GFI-1 bands were detected in
whole cell extract and in the anti-PU.1 immunoprecipitate, but not in the
lane precipitated with nonspecific antibody (Fig. 3B).

The GFI-1 domain(s) required for mediating the interaction with PU.1
was identified by incubating in vitro translated PU.1 protein with GST
fusions to GFI-1 deletion mutants (Fig. 3C). Consistent with the
immunoprecipitation experiments, full-length GFI-1 associated with
PU.1 in vitro. PU.1 also bound a fusion protein containing all six
GFI-1 zinc fingers, but not a fusion protein containing the N-terminal
portion of GFI-1 lacking all of the zinc fingers. This result suggested
that the zinc fingers are necessary and sufficient to mediate GFI-1
binding to PU.1. To further nar-
row the region of GFI-1 required
for binding to PU.1, we incubated
PU.1 protein with a GST fusion to
the region of GFI-1 containing
Zn3–5 and with a fusion protein
containing GFI-1 truncated after
the second zinc finger (ΔZn3–6).
PU.1 did not bind to GST-GFI-1
deleted of its C-terminal zinc
fingers (ΔZn3–6); however, it did
bind to GST-GFI-1 containing
only Zn3–5. Interestingly, these are the zinc fingers that are
sufficient to mediate DNA binding (1).

To determine which PU.1 domain(s) interacts with GFI-1, in vitro
translated PU.1 deletion mutants were incubated with
GST-GFI-1. Deletion of the N terminus (Δ7–30), C terminus
(Δ255–272), or the PEST domain (Δ118–160) did not affect
PU.1 binding to GFI-1. However, deletion of the transactivation
domain of PU.1 (Δ33–100) or the PU.1 Ets domain (Δ201–272)
greatly diminished binding to GFI-1 (Fig. 3D). Our results dem-
strate that both the transactivation and Ets domains are
required for PU.1 to efficiently associate with the zinc finger
DNA-binding domain of GFI-1.

PU.1-dependent Macrophage Differentiation Is Blocked by
GFI-1—We next determined whether GFI-1 has effects on the
biological activity of PU.1 by expressing GFI-1 in a PU.11/− cell
line expressing a conditional version of PU.1 (PUER) (27).
When the PUER protein is activated by OHT, the conditional
PU.1 protein induces differentiation of the myeloid progenitor
cell line into macrophages and granulocytes depending on the
growth conditions (15). To test what effect GFI-1 has on PU.1-
**GFI-1 Antagonizes PU.1**

**A**

![Image](Image 49x397 to 408x733)

**B**

![Image](Image 372x26 to 400x38)

**C**

![Image](Image 26 to 30x30)

**D**

![Image](Image 30 to 30x30)

**E**

![Image](Image 30 to 30x30)

**F**

![Image](Image 30 to 30x30)

**FIGURE 4.** GFI-1 blocks macrophage differentiation of PUER myeloid progenitor cells. A, shown is an immunoblot of GFI-1, PU.1, and β-actin expression in PUER cells that were superinfected with rv-GFP or rv-GFI-1. Cell lines were either undifferentiated (no OHT) or differentiated (100 nM OHT) for 8 days in the presence of IL-3, 8, the PUER-GFP and PUER-GFI-1 cell lines were induced to differentiate with 100 nM OHT for 8 days and subsequently cytocentrifuged. Cells were stained with Wright stain to evaluate morphology (magnification ×400). B, shown are the results from immunoblot analysis of M-CSFR expression in whole cell lysates obtained from OHT-treated PUER cells infected with either rv-GFP or rv-GFI-1. Whole cell lysates were prepared from PUER cells at 0, 1, 2, 4, and 8 days after OHT addition. C, shown are the results from immunoblot analysis of M-CSFR expression in whole cell lysates obtained from PUER cells using anti-Pu.1 antibody. PUER cell lines were either untreated or treated for 7 days with OHT as indicated. NRS, normal rabbit serum. D, ChIP was performed with the M-CSFR promoter from PUER cells using anti-Pu.1 antibody. PUER cell lines were either untreated or treated with 700 nM OHT for 4 days.

induced differentiation, PUER cells were superinfected with rv-GFI-1 internal ribosome entry site (rv-GFI-1)-GFP or control rv-GFP only. Clonal cell lines were generated by limiting dilution.

Cells were grown in IL-3 and 100 nM OHT for 8 days (macrophage conditions). PUER-GFP cells initially expressed endogenous GFI-1; however, upon OHT treatment, GFI-1 expression was extinguished. In contrast, GFI-1 expression was still detected in OHT-treated PUER-GFI-1 cells (Fig. 4A). GFI-1 expression did not affect PUER protein levels as evaluated by Western blotting. The majority of PUER-GFP cells became adherent during the course of OHT treatment, whereas the majority of PUER-GFI-1 cells remained non-adherent. To determine whether macrophage development is blocked by GFI-1, cell morphology was examined by cytocentrifugation and histochemical staining (Fig. 4B). OHT-treated PUER-GFP cells had the morphology characteristic of macrophages: large cells, vacuolated cytoplasm, and small nucleus. However, PUER-GFI-1 cells looked similar to untreated cells. Additionally, rv-GFI-1-infected cells had reduced expression of the macrophage antigen F4/80 compared with rv-GFP-infected cells (supplemental Fig. S1, A and B).

Because GFI-1 represses M-CSFR promoter activity in vitro, we further examined expression of M-CSFR protein. In GFP-infected cells, M-CSFR was detected by immunoblotting after 4 days of differentiation. However, M-CSFR was barely detected in GFI-1-infected cells after 8 days of differentiation, consistent with the previous in vitro results (Fig. 4C). We also determined whether GFI-1 affects PU.1 binding to the endogenous M-CSFR promoter. GFI-1 did not block PU.1 association with the M-CSFR promoter, as ChIP demonstrated that PU.1 associated with the promoter in the presence or absence of GFI-1 (Fig. 4D). Finally, we examined whether GFI-1 directly associates with the M-CSFR promoter. Using ChIP, we detected the M-CSFR promoter co-immunoprecipitating with GFI-1 in PUER-GFI-1 cells induced to differentiate with OHT (Fig. 4E). As expected, OHT-treated PUER-GFP cells, which do not express detectable levels of GFI-1 protein, did not precipitate the M-CSFR promoter with anti-GFI-1 antibody. ChIP assays with untreated PUER-GFP or PUER-GFI-1 cells did not consistently immunoprecipitate M-CSFR.

The expression of several myeloid genes was examined in PUER-GFP and PUER-GFI-1 cells by reverse transcription (RT)-PCR (Fig. 4F). These genes are direct transcriptional targets of PU.1: CD64, M-CSFR, CD11b, and c-fes (22, 25, 34, 35). GFI-1 efficiently repressed the expression of the CD64 and M-CSFR genes, which are expressed preferentially in macrophages. In contrast, GFI-1 barely repressed the expression of the CD11b and c-fes genes, which are transcribed in both granulocytes and macrophages. The expression levels of the CD11b and M-CSFR genes were confirmed by real-time RT-PCR analysis (supplemental Fig. S1D). These results suggest that GFI-1 preferentially represses PU.1 macrophage target genes, but not PU.1 targets expressed in both macrophages and granulocytes.
Interestingly, the PU.1 target genes c-fes (25) and CD11b (35) were abundantly expressed in the absence of OHT. The CD11b gene has been observed previously in untreated PUER cells (without OHT) because of slight leakiness of the PUER protein (27). When CD11b and c-fes gene expression was examined 24 h post-OHT treatment, we observed a slight induction of both genes above levels detected in untreated cells (data not shown). However, the induction was not greatly affected by GFI-1 expression (data not shown).

**GFI-1 Does Not Block PUER-induced Granulocyte Commitment**—When PUER cells are pretreated with G-CSF before OHT activation of PU.1, they differentiate into granulocytes instead of macrophages upon activation of the PUER protein (15). PUER cells do not fully differentiate into mature granulocytes, as their nuclei do not completely segment; and although they express the secondary granule protein neutrophil gelatinase, we have never detected lactoferrin. PUER-GFP and PUER-GFI-1 cells were differentiated under granulocyte conditions to determine whether enforced GFI-1 expression blocks only macrophage development or all myeloid development. Differentiation was evaluated by examining the morphology of cells after cytocentrifugation and histochemical staining. Both GFP- and GFI-1-infected PUER cells appeared by gross morphology to be neutrophilic granulocytes with characteristic segmented nuclei and were indistinguishable from one another (Fig. 5A). RNA was prepared from GFP- and GFI-1-infected granulocyte cultures, and RT-PCR was performed for the granulocyte genes myeloperoxidase, neutrophil gelatinase, and gp91. Results obtained from representative GFP- and GFI-1-expressing cell lines are shown. Neutrophil gelatinase and gp91 expression was detected in differentiated PUER-GFP cultures, but not in undifferentiated cells (Fig. 5B). Similar results were obtained in GFI-1-expressing cells, indicating that GFI-1 expression is permissive for PU.1-induced granulocyte differentiation. Myeloperoxidase is a primary granule protein, the expression of which is greatly enhanced in Gfi-1−/− hematopoietic cells along with another primary granule protein, neutrophil elastase (ELA2) (10). Consistent with GFI-1 knock-out data, myeloperoxidase expression was reduced in PUER cells by exogenous GFI-1 as determined by Northern blotting (Fig. 5C).

**Infection of Primary Murine Bone Marrow Progenitors with rv-GFI-1 Promotes Granulocyte Differentiation**—Bone marrow was harvested from 8–10-week-old wild-type mice. Nucleated bone marrow cells were depleted of lineage-positive cells (CD5, CD11b, Gr-1, B220, and Terr119) and infected for 2 days with rv-GFI-1 in the presence of the cytokines IL-3, IL-6, and stem cell factor. Cells were grown for an additional 4 days in G-CSF before being analyzed for differentiation by flow cytometry (Fig. 6). We have shown previously that cultured bone marrow cells expressing cell surface CD11b and F4/80 are macrophages and that cells expressing CD11b and Gr-1 are granulocytes (15).

When we gated on cells expressing no or low levels of GFP (corresponding to low levels of GFI-1 because the two proteins are expressed from the same mRNA), ~60% of the cells were granulocytes, and 40% of the cells were macrophages as determined by CD11b and Gr-1 staining (Fig. 6A, upper panels). However, if we gated on the high GFP population (high GFI-1 levels), 80% of the cells were granulocytes, and only 20% were macrophages. Similar results were obtained by evaluating differentiation with CD11b and F4/80 staining (Fig. 6A, lower panels). Because we observed an increased percentage of the CD11b+ cell population becoming Gr-1+ in the high GFI-1 fraction, we conclude that GFI-1 increases granulocyte differentiation at the expense of the macrophage population. Results shown are representative of three independent infections.

This increase in granulocyte differentiation requires an active SNAG repression domain, as the inactivating GFI-1P/A mutant (33) did not increase granulocyte differentiation. Interestingly, the GFI-1P/A mutant induced a mixed lineage phenotype with ~90% of the infected cells (high GFP) coexpressing Gr-1 and F4/80 (Fig. 6B). This result is consistent with knock-in studies showing that when the Pro-to-Ala mutant is knocked into the Gfi-1 locus, it phenocopies the null mutant animals (36). Finally, no differences in myeloid differentiation were observed between low and high GFP populations of bone marrow progenitors infected with control rv-GFP (data not shown).
GFI-1 Antagonizes PU.1

Expression of the M-CSFR, CD11b, and Ela2 genes was examined by real-time RT-PCR with wild-type, PLU.1+/+; Gfi-1−/−, and Gfi-1−/−PLU.1+/+ bone marrow. As observed previously (10), M-CSFR and Ela2 were overexpressed in mutant Gfi-1 bone marrow (Fig. 7B). Interestingly, the levels of M-CSFR (but not the pan-myeloid gene CD11b) were significantly reduced in the compound mutant (Gfi-1−/−PLU.1+/+) bone marrow compared with the Gfi-1−/− bone marrow (p < 0.05). The granulocyte gene Ela2 was slightly increased in the compound mutants compared with the Gfi-1 mutants, but this was not statistically significant. Three mice of each genotype were examined. This decrease in the compound mutant does not appear to be due to a general reduction in PU.1 targets by PLU.1 heterozygosity because we did not observe a significant decrease in PLU.1+/+ mice analyzed. Although PLU.1 heterozygosity rescues the overexpression of the macrophage targets, it did not rescue the expression of the granulocyte genes neutrophil gelatinase and lactoferrin (supplemental Fig. S2).

Finally, Hock et al. (10) observed that GFI-1 mice exhibit increased numbers of mixed lineage Mac-3+Gr-1+ cells in their bone marrow. Using flow cytometry, we examined wild-type, Gfi-1−/−, and Gfi-1−/−PLU.1+/+ bone marrow for the presence of these cells. In four independent experiments, we always observed a decrease in Mac-3+Gr-1+ cells in the Gfi-1−/−PLU.1+/+ bone marrow compared with bone marrow isolated from Gfi-1 mutants. Representative FACS plots from one experiment are shown (Fig. 7C). These results demonstrate that decreased PLU.1 gene dosage lowers the overexpression of macrophage genes in the Gfi-1 mutant.

sequences in vivo, we interbred mutant PLU.1 and Gfi-1 mice. We performed hematopoietic colony assays with bone marrow obtained from wild-type, Gfi-1−/−, and Gfi-1−/−PLU.1+/+ mice. We observed a significant increase in colony-forming unit-macrophage (CFU-M; p = 0.05) and colony-forming unit-granulocyte (CFU-G; p = 0.02) colonies obtained from Gfi-1−/− bone marrow compared with wild-type bone marrow. The Gfi-1−/− and Gfi-1−/−PLU.1+/+ CFU-G colonies did not contain mature cells, as RT-PCR with colony RNA did not detect expression of either neutrophil gelatinase or lactoferrin

FIGURE 6. GFI-1 increases granulocyte differentiation of primary progenitors. Nucleated bone marrow cells were lineage-depleted to enrich for hematopoietic progenitors and infected with rGFI-1 (A) or rGFI-1PA (B) for 2 days in the presence of stem cell factor, IL-6, and IL-3. Cells were cultured for an additional 4 days in G-CSF and analyzed for expression of GFP, CD11b, Gr-1, and F4/80. Cells were gated on two populations: low and high GFP and GFP. Upper panels, expression of CD11b and Gr-1; lower panels, expression of CD11b and F4/80. Sorting of populations has shown previously that CD11b−Gr-1− cell are granulocytes and that CD11b+ F4/80+ cells are macrophages (15).
and reduces the number of mixed lineage cells. This supports the conclusion that an important function for GFI-1 in myelopoiesis is to antagonize PU.1.

**DISCUSSION**

Because PU.1 target genes are overexpressed in myeloid cells obtained from Gfi-1<sup>−/−</sup> mice (9, 10), we examined whether the essential granulocyte transcription factor GFI-1 antagonizes PU.1 activity. We observed that GFI-1 inhibited the transactivation activity of PU.1 and physically interacted with PU.1. Inhibition required an intact N-terminal SNAG repression domain. The interaction between these two factors is functionally significant, as GFI-1 expression blocked PU.1-induced macrophage differentiation of a myeloid cell line. Although GFI-1 blocked PU.1-induced macrophage differentiation of the PUER myeloid progenitor cell line, it did not block granulocyte commitment. We also observed that exogenous GFI-1 expressed in primary murine hematopoietic progenitors increased granulocyte differentiation at the expense of macrophage development. Previously, the related protein GFI-1b was shown to block differentiation of the myelomonocytic M1 cell line (37). However, we report for the first time that GFI-1 inhibits macrophage differentiation of a myeloid cell line and primary hematopoietic cells.

These results suggest that GFI-1 may be important in the granulocyte-macrophage progenitor decision to become a macrophage or granulocyte by antagonizing PU.1 activity similar to what we hypothesized previously for C/EBPα (15). However, in the absence of GFI-1, hematopoietic cells commit to the granulocyte cell fate as CFU-G progenitors are generated in the bone marrow, as shown by our hematopoietic colony assays (Fig. 7A). Notably, we did not observe a significant change in the ratio of CFU-G to CFU-M progenitors between wild-type and mutant GFI-1 animals. We observed an increase in the total number of both CFU-G and CFU-M colonies, which is likely due to the increased percentage of granulocyte-macrophage progenitor cells in the bone marrow of Gfi-1<sup>−/−</sup> mice (6). If GFI-1 played a role in the granulocyte-macrophage cell fate decision, one would expect that its deletion would result in an increase in CFU-M progenitors relative to CFU-G progenitors.

The retroviral expression results may be due to an effect of GFI-1 being misexpressed in common myeloid progenitors. Annexin V staining did not show that GFI-1 expression induced apoptosis of macrophage progenitors (data not shown).

We did not demonstrate a role for GFI-1 in directing cell fate decisions by repressing PU.1. However, we suggest that GFI-1 “locks in” the granulocyte cell fate by repressing macrophage genes regulated by PU.1. This is demonstrated by the reduction in M-CSFR expression when the PUL1 gene dosage was reduced in a Gfi-1-null background, but the expression of the pan-myeloid PU.1 target gene CD11b was unaffected. Additionally, it was shown previously that Mac-3<sup>Gr-1<sup>+</sup></sup> mixed lineage cells are increased in mutant Gfi-1 bone marrow (10), and we showed that there is a reduction in this cell population in Gfi-1<sup>−/−</sup>PUL1<sup>−/−</sup> animals (Fig. 7C).

Interestingly, GFI-1 efficiently repressed PU.1 macrophage-specific target genes (M-CSFR and CD64), but not targets that are expressed in both macrophages and granulocytes (CD11b and c-fes). It is unclear how GFI-1 discriminates between repressing PU.1 macrophage-specific target genes and PU.1 targets expressed in all myeloid cells (Fig. 4F). However, one difference we observed in the expression of these two sets of genes is that PU.1 targets expressed in both macrophages and granulocytes require only low levels of PU.1 for their expression.

Low PU.1 activity present in the PUER cell line in the absence of OHT is enough to induce their expression, whereas it is not enough to activate the expression of the macrophage-specific target genes M-CSFR and CD64. This suggests that PU.1 regulation of pan-myeloid and granulocyte-specific genes is mechanistically distinct from PU.1 regulation of macrophage-specific genes. Optimal regulation of the macrophage-specific genes M-CSFR and macrosialin requires the transcription factor c-Jun as a cofactor for PU.1 (38, 39). In granulocytes, c-Jun expression decreases during granulocyte differentiation, suggesting that its role as a PU.1 cofactor is less important in granulocytes (40). We are currently investigating whether the requirement for c-Jun as a PU.1 coactivator confers specificity for GFI-1 repression of PU.1 target gene subsets.

Targeted mutations in mice and naturally occurring mutations isolated from human neutropenic patients demonstrate that GFI-1 is essential for granulocyte development (9–11). Therefore, an understanding of how GFI-1 functions in granulocyte differentiation is important for the design of future targeted therapies for granulocyte disorders. Here, we have shown that GFI-1 binds PU.1, repressing its ability to activate transcription. Genetic data from interbreeding PU1.1 and Gfi-1 mutants demonstrate that this interaction is important in vivo. The data support a conclusion that a critical function of GFI-1 is to repress PU.1-regulated macrophage-specific genes for proper granulocyte development.

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