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Transcriptional Repression of the Neurofibromatosis-1 Tumor Suppressor by the t(8;21) Fusion Protein

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Von Recklinghausen's disease is a relatively common familial genetic disorder characterized by inactivating mutations of the *Neurofibromatosis-1* (*NF1*) gene that predisposes these patients to malignancies, including an increased risk for juvenile myelomonocytic leukemia. However, *NF1* mutations are not common in acute myeloid leukemia (AML). Given that the RUNX1 transcription factor is the most common target for chromosomal translocations in acute leukemia, we asked if *NF1* might be regulated by RUNX1. In reporter assays, RUNX1 activated the *NF1* promoter and cooperated with C/EBPα and ETS2 to activate the *NF1* promoter over 80-fold. Moreover, the t(8;21) fusion protein RUNX1-MTG8 (R/M), which represses RUNX1-regulated genes, actively repressed the *NF1* promoter. R/M associated with the *NF1* promoter in vivo and repressed endogenous *NF1* gene expression. In addition, similar to loss of *NF1*, R/M expression enhanced the sensitivity of primary myeloid progenitor cells to granulocyte-macrophage colony-stimulating factor. Our results indicate that the *NF1* tumor suppressor gene is a direct transcriptional target of RUNX1 and the t(8;21) fusion protein, suggesting that suppression of *NF1* expression contributes to the molecular pathogenesis of AML.

Von Recklinghausen's disease, or neurofibromatosis type I, is a common inherited tumor predisposition syndrome with an overall incidence of approximately 1 in 3,000 worldwide. Affected individuals are prone to the development of benign tumors, such as neurofibromas, and malignant cancers, including glioma, malignant peripheral nerve sheath tumors, and leukemia (28, 59). The disease is characterized by inactivating mutations of the *Neurofibromatosis-1* (*NF1*) gene (11, 17, 61), which indicates that *NF1* is a tumor suppressor gene. Patients with mutant *NF1* are at increased risk for clonal myeloproliferative diseases, including juvenile monomyelocytic leukemia (JMML) (59, 60). In contrast, *NF1* mutation has rarely been detected in acute myeloid leukemia (AML) (29, 42, 55, 56).

Relatively small reductions in the expression of *NF1* produce phenotypic changes in cell proliferation. For example, $Nf1^{+/-}$ astrocytes exhibit increased cell proliferation and motility (3). Similarly, loss of only one *NF1* allele is sufficient to partially complement defects in coat color and mast cells in mice containing mutations in the c-Kit receptor tyrosine kinase (W^{41} mice) that attenuate Ras-dependent signaling (26). In addition, repression of *NF1* by human immunodeficiency virus Tax predisposes transgenic mice expressing Tax to neurofibromas, indicating that loss of *NF1* expression by transcriptional regulation, rather than *NF1* mutational inactivation, is sufficient to promote tumorigenesis (16). These effects are due in part to the role of the *NF1* gene product, neurofibromin, as a negative regulator of RAS and other small GTP binding proteins, acting to accelerate GTP hydrolysis (5, 11, 25). Loss of *NF1* results in increased RAS-mediated signaling in response to multiple stimuli, including granulocyte-macrophage colony-stimulating factor (GM-CSF), leading to excessive proliferation and increased cell survival of lymphoid and myeloid progenitor cells (26, 35, 67).

RUNX1 (runt-related 1, also known as AML 1 or AML1) is a DNA binding transcription factor that acts as a molecular switch to activate or repress transcription. Although RUNX1 can associate with coactivators, it appears to activate transcription through composite sites in which the RUNX1 DNA binding site is adjacent to sites for factors that RUNX1 can physically associate with, such as C/EBPα, PU.1, and ETS1, to form an active transcriptional complex (22, 31, 54, 57, 64, 66). For example, when expressed alone, RUNX1, C/EBPα, and PU.1 are poor activators of the M-CSF promoter. However, when coexpressed, RUNX1 synergizes with C/EBPα or PU.1 to potently activate transcription (54). Conversely, when expressed in some cell types, RUNX1 is capable of repressing transcription through the recruitment of mSin3 and Groucho corepressors and histone deacetylases (HDACs) (13, 38, 46).

The function of RUNX1 is perhaps disrupted more than any other transcription factor in acute leukemia. Chromosomal translocations that affect *RUNX1* include t(12;21), which is present in 20 to 25% of pediatric acute B-cell lymphoblastic

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leukemias, and t(8;21), which is the most frequent chromosomal translocation associated with AML, accounting for 10 to 15% of the cases (58). In addition, inv(16), which may be the second most frequent chromosomal abnormality in AML (58), fuses the RUNX1-interacting factor, core binding factor β (CBFβ), to a smooth-muscle myosin heavy-chain gene (40). The inv(16) fusion protein dominantly inactivates RUNX1-dependent transactivation and stimulates RUNX1-dependent repression (1, 13, 30, 44). t(8;21) creates a fusion protein that acts as a transcriptional repressor by fusing the N-terminal DNA binding domain of RUNX1 to a putative transcriptional corepressor known as MTG8 (myeloid translocation gene on chromosome 8, also known as eight-twenty-one or ETO). MTG8 contacts both the mSin3 and N-CoR families of corepressors, as well as HDAC1, HDAC2, and HDAC3 (2, 21). Thus, repression of RUNX1-regulated genes may contribute to leukemogenesis in over 20% of acute leukemias.

Given that the levels of expression of *NF1* in AML may be epigenetically altered or transcriptionally regulated, rather than the locus mutated (41, 42), we asked whether the master regulatory factors C/EBPα and RUNX1, which are mutated in AML, might regulate *NF1*. We found multiple RUNX1, ETS, and C/EBPα DNA binding sites, some of which were clustered as in other genes regulated by these factors. RUNX1 and C/EBPα independently activated the *NF1* promoter and cooperated to dramatically activate *NF1*. By contrast, the t(8;21) fusion protein, which disrupts RUNX1 and represses C/EBPα (52), repressed the *NF1* promoter in reporter assays and repressed expression of the endogenous *NF1* gene. Like inactivation of *NF1* in individuals with neurofibromatosis, RUNX1-MTG8 expression sensitized myeloid progenitor cells to proliferation in response to GM-CSF, but not interleukin-3 (IL-3). These data suggest that repression of *NF1* may contribute to t(8;21)-mediated leukemogenesis.

MATERIALS AND METHODS

Cell culture, reporter assays, and plasmids. HEL, Kasumi-1, HeLa, COS7-L (Invitrogen), and GP2-293 cells were cultured as previously described (2, 39). The *NF1* promoter fragment was subcloned into pGL2-basic (Promega) and the promoter deletions created by restriction digest and religation at the indicated positions relative to the transcriptional start site (18). Expression plasmids for RUNX1, C/EBPα, ETS2, PU.1, and RUNX1-MTG8 and its mutants were described previously (6, 34, 39, 65). Transfection was performed in 6-well or 12-well plates with 1 μg of *NF1*-luciferase plasmid unless otherwise indicated using Polyfect (QIAGEN). Firefly luciferase activity was measured using the Luciferase Assay kit (Promega) and corrected for transfection efficiency by including plasmids expressing *Renilla* luciferase or secreted alkaline phosphatase (45).

ChIP. Chromatin immunoprecipitation (ChIP) was performed according to the Upstate Biotechnologies ChIP assay protocol with Kasumi-1 or HeLa cells and 4.0 μg of each antibody (see Fig. 3 and Fig. 6). After immunoprecipitation overnight at 4°C, DNA was purified by QIAquick PCR Purification kit (QIAGEN) and 5% of the purified products were used for PCR templates. Promoter sequences were detected with the following PCR primers for *NF1*: a (forward, −2313 to −2291; reverse, −2133 to −2159), 5′-GGCCTGAAGTTTGGGTGT CTTA-3′ and 5′-TGTAGGGAAGAAGATCAGGGAGATAG-3′; b (forward, −1337 to −1318; reverse, −1181 to −1202), 5′-GCACTCCACTGCACAGAG TCA-3′ and 5′-CCCACCTCAGCCTCCCAAAGT-3′; *p16*^{INK4a}, 5′-AGAAAGA GGAGGGGCTGGCTGGTCA-3′ and 5′-CAGCCGTCAGCCGAAGGCTCC A-3′. PCR was performed using HotStarTaq (QIAGEN) or a QuantiTect SYBR Green PCR kit (QIAGEN) on an i-Cycler (Bio-Rad).

Retroviral infection of murine myeloid progenitor cells. To determine the fetal genotype, once the liver was removed, the remainder of the embryonic tissue was used for preparing genomic DNA as previously described (5). Primers were employed to distinguish disrupted and wild-type alleles using PCR (28).

Recombinant retroviral plasmids were transfected into a GP+E 86 packaging cell line as previously described (20). Stable populations of packaging cells expressing the retrovirus were selected by serially separating green fluorescent protein-positive (GFP$^+$) cells by fluorescence-activated cell sorter (FACS) until more than 95% of the cells were GFP$^+$. The transduction protocol has been previously described (19). Briefly, embryonic day 13.5 fetal liver cells were prestimulated for 48 h in liquid cultures of IMDM (Invitrogen Corporation, Grand Island, NY) containing 20% fetal bovine serum (BioWhittaker, Walkersville, MD) supplemented with 100 ng/ml stem cell factor (SCF; PeproTech, Rocky Hill, NJ) and 200 U/ml IL-6 (PeproTech, Rocky Hill, NJ). Cells were subsequently transduced with viral supernatants on RetroNectin-treated non-tissue culture plates (TaKaRa Bio, Inc., Madison, WI) for 48 h in the presence of SCF and IL-6. Mast cell lines were then established from these transduced progenitors for immunoblot analysis (2).

For methylcellulose colony assays, transduced fetal liver cells were incubated with1 μg of phycoerythrin-conjugated c-Kit antibody/10^6 cells (PharMingen, San Diego, CA) for 20 min at 4°C. Cells were then washed twice with 0.5% bovine serum albumin in phosphate-buffered saline. GFP$^+$ c-Kit$^+$ cells were then separated by FACS. This enriched hematopoietic progenitor population of cells was plated at a concentration of 5,000 cells/ml in triplicate methylcellulose cultures (Stem Cell Tech, Inc., Vancouver, British Columbia, Canada) containing increasing concentrations of GM-CSF (0.01 to 2 ng/ml; PeproTech, Rocky Hill, NJ) or IL-3 (0.1 to 10 U/ml; PeproTech, Rocky Hill, NJ), and granulocyte macrophage CFU were scored on day 7 of culture.

Protein and RNA analysis. Viral supernatants were prepared using vesicular stomatitis virus G glycoprotein-pseudotyped retrovirus from the GP2-293 packaging cell line (Clontech). Infected HeLa cells were selected in puromycin before protein or RNA analysis. COS7-L cells were transfected using Lipofectamine 2000 (Invitrogen). Antibodies directed to human NF1 (10), human p14^{ARF} (Ab-1; Neomarkers), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab8245-100; Norus Biologicals), the MTG8 zinc finger domain (α-ETO/MTG8 ZnF Ab-1; CalBiochem/EMD Biosciences), and RUNX1 (α-N-RUNX1 and α-RHD-RUNX1; CalBiochem/EMD Biosciences) were used for immunoblot analysis. RNA was isolated using an RNeasy Mini kit (QIAGEN), and 10 μg was run on a 1% formaldehyde gel and probed with a radiolabeled *NF1* cDNA fragment encompassing exon 33, a 400-bp fragment of *p14*^{ARF} encompassing exon 1β, or the full-length c-Myc and GAPDH cDNAs.

***NF1* expression in primary AML samples.** Total RNA was extracted from bone marrow samples of 49 AML patients obtained at the time of initial diagnosis. Written consent was obtained from all patients. The presence or absence of RUNX1-MTG8 was analyzed using standard reverse transcription (RT)-PCR procedures and/or cytogenetics. Each sample was analyzed for *NF1* or porphobilinogen deaminase gene (*PBGD*) expression two times by real-time quantitative RT-PCR (QRT-PCR) with specific primers and fluorescence-labeled probes (TaqMan assay). To avoid amplification of homologous sequences and possible *NF1* pseudogenes, we used primers from exons 37 and 38, which are separated by an intron of 3 kb to eliminate priming from genomic DNA (*NF1* forward, 5′-GGACACTGCTCAATATCGCATTAC-3′; reverse, 5′-AGGTACAAGTTA AGGCAC ACAGAAGA-3′; probe, 5′-FAM-GACCGTAAACTCGGGTCAG AACTGCCTA-3′). To test whether the PCR would amplify DNA sequences, 0 to 50 ng of human genomic DNA was used as the template with no resulting product. When analyzed by gel electrophoresis, the QRT-PCR product obtained from patient samples was a single band of the expected size (96 bp). The RNA isolation, cDNA synthesis and input, cycling conditions, and *PBGD* expression measured for normalization were as described previously (39). From each sample, the quantity of *NF1* and *PBGD* mRNAs was measured in duplicate using serial dilutions of cell line RNA as a calibration curve. The *PBGD* mRNA primers used were as follows: forward, 5′-GGCAATGCGGCTGCAA-3′; reverse, 5′-GGGTACCCACGCGAATCAC-3′. They were used in combination with a VIC-labeled probe (5′-VIC-CATCTTTGGGCTGTTTTCTTCCGCC-3′). Total RNA was isolated using RNAzol (Campro Scientific). For cDNA synthesis, 1 μg total RNA was reverse transcribed using random hexanucleotide primers. PCR was performed using universal mastermix (Applied Biosystems). Samples were heated for 2 min at 50°C and 10 min at 95°C and amplified for 45 cycles of 15 s at 95°C and 60 s at 60°C on an ABI/Prism 7700 sequence detector (Applied Biosystems).

Statistical analysis was performed using Minitab release 12 (Minitab, Inc.), and *P* values and confidence intervals determined using the nonparametric Mann-Whitney rank sum test. For example, in comparing AML to AML-M2 samples, for C1, $n = 34$ and median = 0.388 and for C2, $n = 15$ and median = 0.100; the point estimate for ETA1-ETA2 is 0.204, the 95.0% confidence interval for ETA1 − ETA2 is 0.073 to 0.365, W = 974.5, and the test of ETA1 = ETA2 versus ETA1 not = ETA2 is significant ($P = 0.0071$; adjusted for ties).

RESULTS

RUNX1, C/EBPα, and ETS factors cooperate to regulate *NF1*. Patients with *NF1* mutations are at increased risk for the formation of JMML (59, 60), but mutations in *NF1* have not been reported in sporadic AML, leaving open the possibility that this tumor suppressor is regulated transcriptionally. Given the high frequency of disruption of RUNX1 in acute leukemia, we asked if RUNX1 regulates *NF1*. We identified 13 consensus RUNX1 binding sites in the human *NF1* promoter, with 7 of these sites matching the narrower consensus sequence TGT/cGGT (47) (Fig. 1A). Because RUNX1 cooperates with C/EBPα and mutations in C/EBPα are associated with M2 AML (53), we also inspected the *NF1* promoter for C/EBPα binding sites. Three of the C/EBPα binding sites were clustered near RUNX1 sites, similar to other genes that are coregulated by these factors (22, 31, 54, 57, 66). This raised the possibility that *NF1* is a transcriptional target of RUNX1 and C/EBPα. We confirmed that RUNX1 and C/EBPα bind these sites in gel mobility shift assays (data not shown). In reporter assays, RUNX1 activated the *NF1* promoter and the level of activation was enhanced by coexpression of the RUNX1 cofactor CBFβ, which increases RUNX1 DNA binding and stability (23, 24) (Fig. 1B and data not shown). In addition, C/EBPα activated *NF1* approximately sevenfold, but this activation was lost when higher levels of C/EBPα-expressing plasmid were used (Fig. 1B and data not shown). When C/EBPα was coexpressed with RUNX1, these factors dramatically cooperated to activate the *NF1* promoter over 80-fold (Fig. 1B).

ETS family members also cooperate with RUNX1 to activate transcription, and ETS factor sites are also adjacent to the RUNX1 binding sites in the *NF1* promoter (Fig. 1A). Therefore, we tested whether the *NF1* promoter can be regulated by RUNX1 and the ETS family members ETS2 and PU.1 in reporter assays. Similarly, ETS2 or PU.1 only modestly activated the *NF1* promoter. However, when RUNX1 was coexpressed with ETS2, these factors cooperated to activate the *NF1* promoter over 90-fold (Fig. 1B). Thus, RUNX1 cooperates with C/EBPα and ETS2 to regulate *NF1*.

The presence of multiple RUNX1, ETS, and C/EBPα binding sites in the *NF1* promoter prompted us to attempt to identify the RUNX1 binding sites that are required for regulation of *NF1*. Because of the large number of binding sites in this promoter, we did not attempt to mutate each site individually but performed deletion analysis of the promoter (Fig. 1A). Deletion of the sequences from −2732 to −1285 reduced the ability of these factors to cooperatively activate *NF1* (Fig. 1C, left side). Further deletion to nucleotide −969 dramatically impaired the cooperative activation, and deletion to −548, which removes the last RUNX1 binding site, yielded only additive, not cooperative, transactivation (Fig. 1C, right side). Thus, the transcription factor binding sites clustered between the AvrII and Tth111I sites are most clearly required for the cooperative action of RUNX1, ETS2, and C/EBPα, although other sites such as the cluster of sites approximately 2.3 kbp upstream of the transcriptional start may also contribute to the regulation of *NF1*.

To demonstrate specificity for the cooperative regulation of *NF1*, we used the −969 promoter fragment that contains a lone consensus RUNX1 binding site but retains some cooperative transactivation. We mutated the proximal RUNX1 consensus sequence CGTGGT at −758 to CGCGAG (Fig. 1D, left side) and tested the ability of RUNX1 and ETS2 to cooperatively activate this promoter. Compared to the minimal promoter (−59) that lacks all of the RUNX1 sites, RUNX1 and ETS2 once again cooperated to activate the −969 promoter (Fig. 1D, right side). Although RUNX1 still activated the mutant promoter, probably through a putative weak RUNX1 binding site at −661, only additive, not cooperative, effects were observed when RUNX1 was coexpressed with ETS2 with the mutant promoter (Fig. 1D, right side).

The t(8;21) fusion protein represses *NF1* gene expression. RUNX1-MTG8 (also known as AML1-ETO) represses genes that contain RUNX1 binding sites (43). Therefore, we tested whether RUNX1-MTG8 could also regulate the *NF1* promoter in reporter assays (Fig. 2A and B). RUNX1-MTG8 repressed *NF1* transcription, whereas a point mutant (L148D) that fails to bind to DNA (37) or a C-terminal deletion mutant (Δ469) that truncates the fusion protein prior to the oligomerization domain, and therefore lacks association motifs for mSin3, N-CoR/SMRT, and HDACs (2, 37), failed to repress the *NF1* promoter (Fig. 2B). CBFβ stimulates the DNA binding functions of the fusion protein, and as expected, the addition of this cofactor increased the ability of wild-type RUNX1-MTG8, but not the mutants, to repress *NF1* (Fig. 2B).

Based on our analysis of RUNX1-mediated transactivation, we used a subset of our deletion mutants of the *NF1* promoter to determine the sequences required for RUNX1-MTG8-mediated repression. Deletion of the sequences from −3363 to −2732 had little effect on both the basal activity of the promoter and the ability of RUNX1-MTG8 to repress the promoter. Further deletion to −1285 reduced the basal activity of the promoter by 70%, but the ability of RUNX1-MTG8 to repress the promoter remained until deletion to −548 that removes all of the consensus RUNX1 binding sites (Fig. 2C). This result prompted us to test the −969 promoter fragment with and without the RUNX1 consensus binding site (Fig. 1D) to determine whether this site is sufficient to mediate RUNX1-MTG8-dependent repression. Although the level of repression by the fusion protein was somewhat lower in this assay, mutation of the RUNX1 binding site impaired RUNX1-MTG8-mediated repression (Fig. 2D).

RUNX1-MTG8 binds the *NF1* promoter in vivo. Given the results of the promoter analysis, we asked whether RUNX1-MTG8 associates with *NF1* in the context of native chromatin. Kasumi-1 cells that contain t(8;21) were cross-linked with formaldehyde, and the DNA associated with RUNX1-MTG8 was isolated using ChIP. The RUNX1-MTG8-associated chromatin was then interrogated for the presence of *NF1* promoter sequences using PCR. Primers flanking the clusters of RUNX1 binding sites labeled a and b in Fig. 3A were selected because the shearing of genomic DNA to 200 to 800 bp in size allows these primers to detect RUNX1-MTG8 bound to several of the perfect and consensus RUNX1 binding sites. Using antibodies directed to RUNX1, we were able to detect both the a and b clusters of RUNX1 binding sites in *NF1* associated with RUNX1-MTG8 in Kasumi-1 cells (Fig. 3B, anti-N-RUNX1, upper and middle parts). As a control, the *NF1* promoter failed to copurify with antibodies directed to RUNX2 and RUNX3, which are not expressed at detectable levels in Kasumi-1 cells

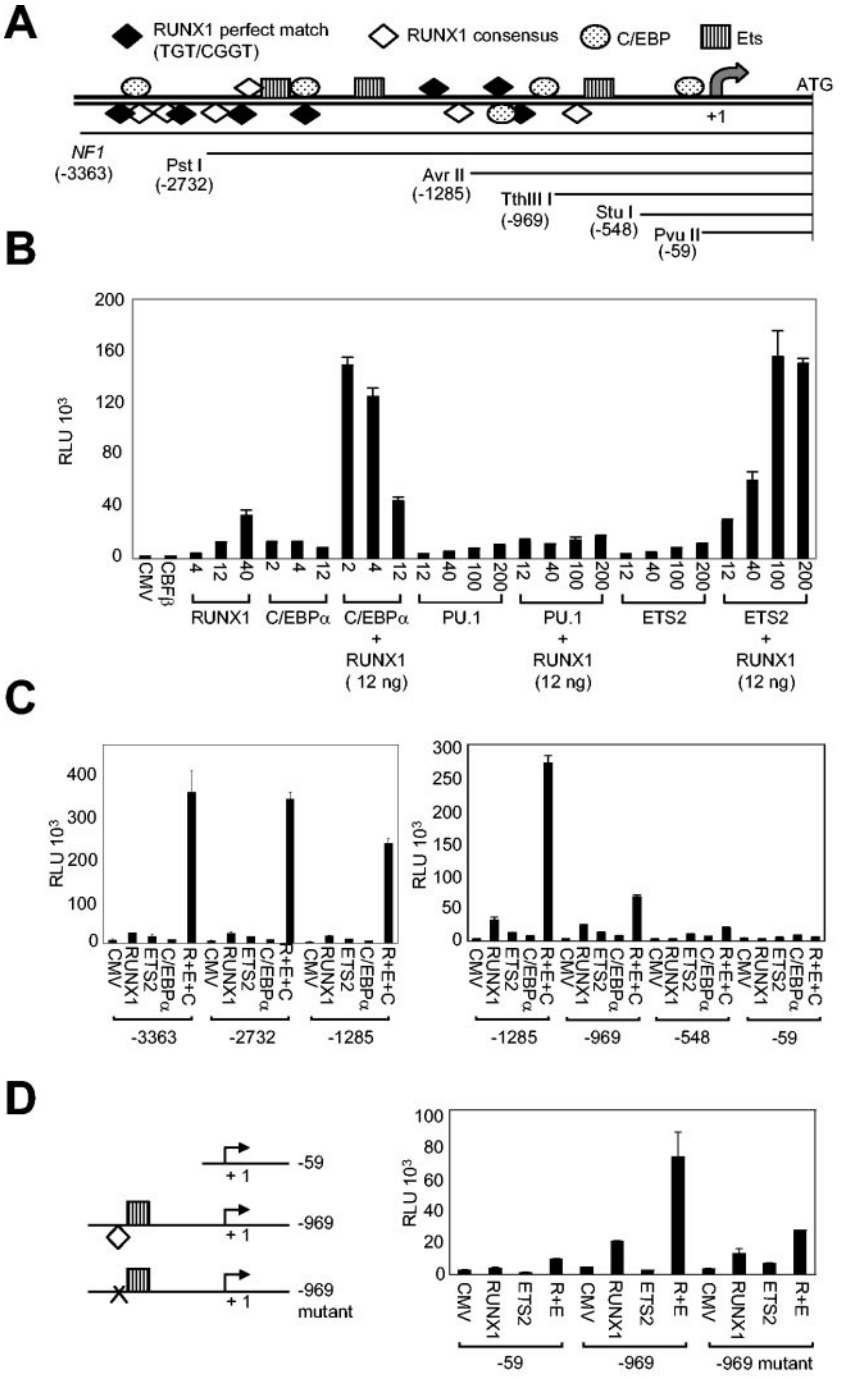

FIG. 1. RUNX1 cooperates with C/EBPα and ETS2 to regulate the *NF1* promoter. (A) Schematic diagram of the *NF1* promoter showing the consensus RUNX1, C/EBPα, and ETS factor binding sites relative to the transcriptional start site (arrow) along with the promoter deletions analyzed in panels C and D. (B) RUNX1 cooperates with C/EBPα and ETS family members to activate *NF1*. HeLa cells were transfected with 0.4 μg of the *NF1*-luciferase plasmid and increasing amounts of plasmids expressing RUNX1, C/EBPα, PU.1, or ETS2 as indicated. pCMV5 was added to keep the total amount of DNA constant, and the values were corrected to account for transfection variation by cotransfecting Rous sarcoma virus-*Renilla* luciferase and adjusting the firefly luciferase values accordingly. Error bars demonstrate the standard deviation. (C) Analysis of the promoter deletions depicted in panel A. Each promoter deletion was tested with RUNX1, ETS2, or C/EBPα alone or in combination (R+E+C). Error bars demonstrate the standard deviation from an experiment performed in triplicate. (D) The synergistic transactivation of the *NF1* promoter by RUNX1 and ETS2 requires a RUNX1 binding site. The proximal RUNX1 consensus site at −758, CGTGGT, was mutated to CGCGAG as shown schematically at the left. The −59 minimal promoter was used as a negative control. Each promoter was tested with RUNX1, ETS2, or both (R+E). Error bars demonstrate the standard deviation. Shown in each part is a representative experiment out of several performed, all of which yielded similar effects upon expression of the indicated proteins. CMV, cytomegalovirus.

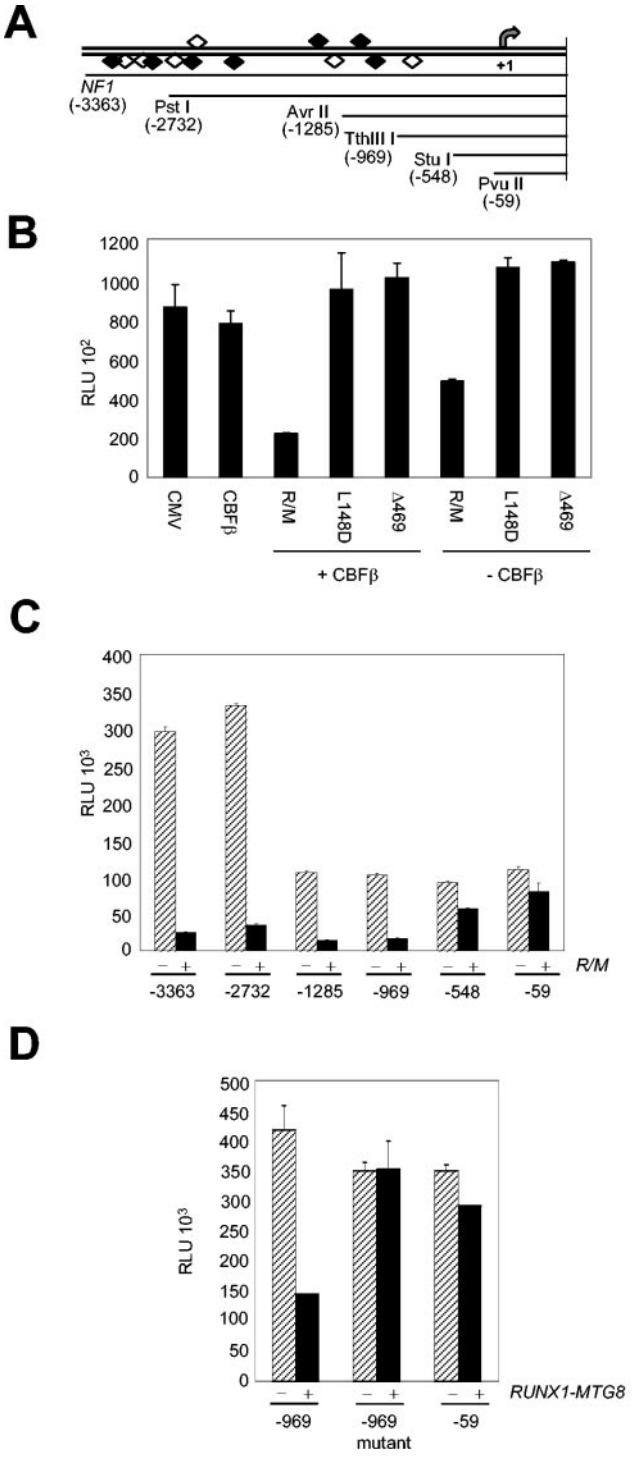

FIG. 2. RUNX1-MTG8 represses the *NF1* promoter. (A) Schematic diagram of the *NF1* promoter and the deletion mutants used in panels C and D. The positions of the restriction sites used to create the promoter fragments are shown relative to the transcriptional start site, which is indicated by the arrow (+1). (B) RUNX1-MTG8-mediated repression of the *NF1* promoter requires DNA binding and corepressor binding domains. HeLa cells were transfected with the *NF1*-luciferase plasmid and increasing amounts of *pCMV5-RUNX1-MTG8* (*R*/*M*) and *pCMV5-RUNX1-MTG8*(*L148D*), which cannot bind DNA, or *pCMV5-RUNX1-MTG8*(Δ*469*), which removes multiple corepressor binding domains (2), with or without pCMV5-CBFβ. pCMV5 was added to keep the total amount of DNA constant, and the values were corrected to account for transfection variation by cotransfecting Rous sarcoma virus-*Renilla* luciferase or cytomegalovirus (CMV)-secreted human placental alkaline phosphatase and adjusting the firefly luciferase values accordingly. Error bars demonstrate the standard deviation of independent analyses. (C) Analysis of the promoter deletions depicted in panel A. HeLa cells were transfected with the indicated *NF1*-luciferase plasmid (shown in panel A) with or without *pCMV5-RUNX1-MTG8*. Error bars demonstrate the standard deviation from an experiment performed in triplicate. (D) RUNX1-MTG8-mediated repression of *NF1* requires the presence of RUNX1 binding sites. HeLa cells were transfected as described for panel B with the same set of luciferase plasmids used in Fig. 1D with or without *pCMV5-RUNX1-MTG8*. Error bars demonstrate the standard deviation. Shown is a representative experiment out of several performed, all of which yielded similar effects upon expression of the fusion protein. RLU, relative light units.

(data not shown), or antibodies directed to the yeast transcription factor GAL4 (Fig. 3B, upper part). Moreover, an antibody directed to the DNA binding domain of RUNX1 (α-RHD) that does not recognize RUNX1 when bound to DNA also was used as a negative control (Fig. 3B).

Because Kasumi-1 cells also express RUNX1, we used anti-MTG8 in ChIP analysis to separate the fusion protein from wild-type RUNX1. *NF1* copurified with anti-MTG8 from Kasumi-1 cells, indicating that the fusion protein was associated with *NF1* in these cells (Fig. 3B). By contrast, the *NF1* promoter was not detected using anti-MTG8 in HEL cells (Fig. 3C), which lack the fusion protein. QRT-PCR demonstrated at least an eightfold (three-cycle) enhancement of *NF1* promoter sequences associated with anti-RUNX1 compared to control antibodies and essentially no association with the $p16^{ink4A}$ promoter, which lacks RUNX1 binding sites (Fig. 3D).

To confirm that RUNX1 can also contact the *NF1* promoter in the absence of the background of the fusion protein, we performed ChIP assays using HEL cells that express easily detectable levels of RUNX1 but fail to express RUNX2 or RUNX3. Only antibodies directed to RUNX1 copurified with the *NF1* promoter sequences (Fig. 3C). Thus, *NF1* is bound by both RUNX1 and RUNX1-MTG8.

RUNX1-MTG8 represses endogenous *NF1* gene expression. RUNX1-MTG8 can repress the *NF1* promoter in reporter assays (Fig. 2) and associates with the *NF1* promoter on native chromatin (Fig. 3). Therefore, we asked whether the fusion protein represses endogenous *NF1*. Because we did not expect complete silencing of *NF1*, a potential obstacle to measuring repression of an endogenous gene is the stability of its mRNA or protein. Therefore, we first determined the stability of *NF1* protein (neurofibromin) and mRNA. HeLa or Cos7-L cells were cultured in the presence of cycloheximide or actinomycin D, and samples removed at various times after addition of the drug for preparation of protein samples or RNA, respectively. Immune and RNA blot analyses were used to determine the half-lives ($t_{1/2}$s) of *NF1* protein and mRNA (Fig. 4A and B). Compared to c-Myc, which has a short $t_{1/2}$, the *NF1* protein and mRNA were long-lived, with a $t_{1/2}$ of NF1 protein of over 10 h and a $t_{1/2}$ of *NF1* mRNA of nearly 8 h.

The stability of the *NF1* mRNA and protein required that we maintain expression of RUNX1-MTG8 for several days. Although counterintuitive, it is well established that the fusion protein inhibits cell cycle progression and its expression is

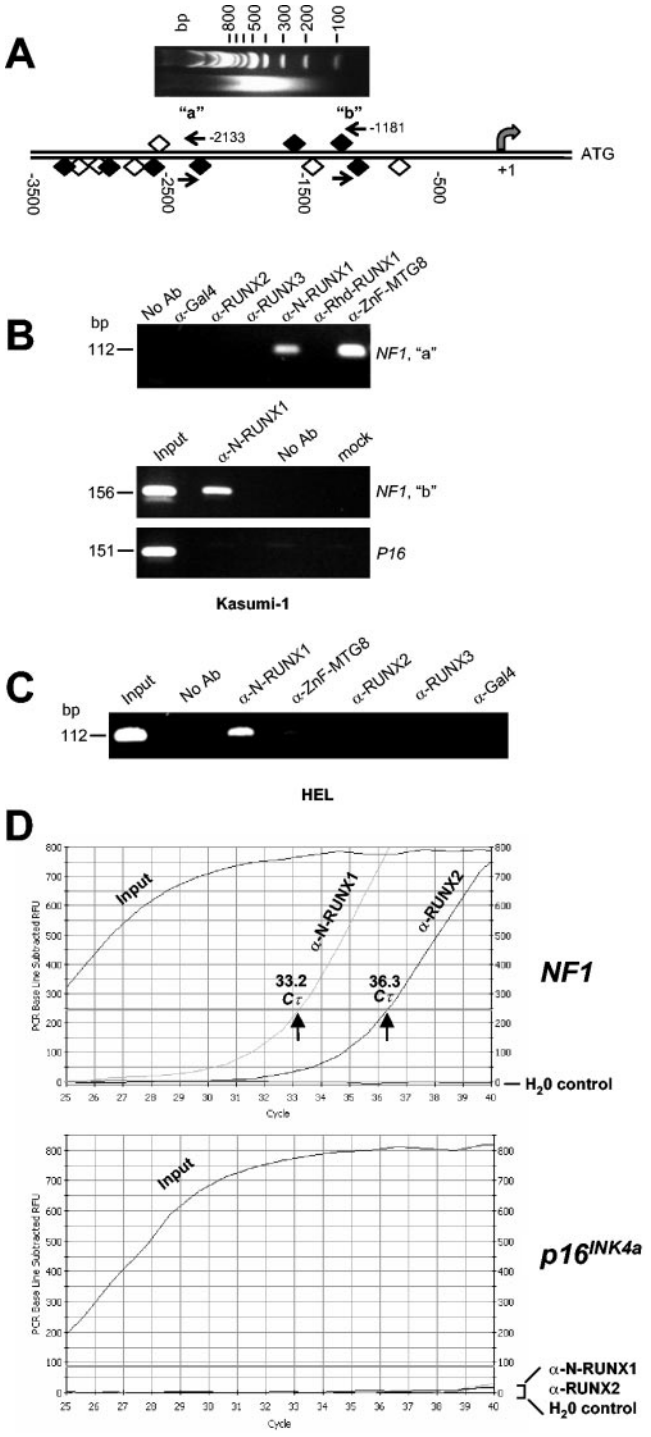

FIG. 3. RUNX1 and RUNX1-MTG8 associate with the endogenous *NF1* promoter. (A) Schematic diagram of the *NF1* promoter showing the locations of the a and b sets of PCR primers (arrows) used in the ChIP analysis. The ethidium bromide-stained gel above the diagram shows a representative analysis of the sheared genomic DNA. (B) ChIP analysis of the *NF1* promoter. The antibodies (Ab) used are as indicated above each lane. α-N-RUNX1, antibody to the N terminus of RUNX1; RHD-RUNX1, a second anti-RUNX1 directed to the DNA binding domain; α-ZnF-MTG8, anti-ETO Ab-1. The a primers were used for the analysis shown in the upper part and the b primers for the middle part. The lower part shows amplification of $p16^{ink4a}$ as a control for experiment b, but similar results were obtained for part a using quantitative PCR. (C) ChIP analysis of the *NF1* promoter from HEL cells, which express RUNX1, but not RUNX1-MTG8. The antibodies used were the same as those described for panel B. (D) SYBR green fluorescence curves from quantitative PCR from a representative experiment using Kasumi-1 cells and the a set of primers. The lower part shows amplification of the $p16^{ink4a}$ promoter as a control. RFU, relative fluorescence units.

difficult to maintain for extended periods of time (2, 8). Therefore, we used retroviral infection of HeLa cells, a cell type that allows RUNX1-MTG8 expression for several days with minimal effects on the cell cycle (39), to express RUNX1-MTG8. Three days after infection, RUNX1-MTG8-expressing cells had reduced levels of *NF1* mRNA (Fig. 4C). The level of NF1 protein was reduced at 5 and 8 days after RUNX1-MTG8 expression, subsequent to the reduction of *NF1* mRNA (Fig. 4C and 4D). As a negative control, the levels of GAPDH were unaffected, whereas the levels of a known direct target of RUNX1-MTG8, $p14^{ARF}$ (39), were also repressed (Fig. 4C and D). To confirm the RUNX1-MTG8-mediated repression of *NF1* in a second cell type, we transfected COS7-L cells, which were selected based on their high transfection efficiency. Using a RUNX1-MTG8-IRES-GFP-expressing plasmid, we confirmed that greater than 95% of the cells expressed GFP as measured by FACS analysis 3 to 5 days after transfection (data not shown). Once again, RUNX1-MTG8, but not a mutant that fails to bind to DNA (L148D) or a mutant that lacks key corepressor recruitment domains (Δ469), repressed *NF1* (Fig. 4E and F). Although the inactive mutants of RUNX1-MTG8 were expressed at high levels, the wild-type protein was expressed at levels comparable to those found in Kasumi-1 cells (Fig. 4F). Thus, *NF1* can be repressed by RUNX1-MTG8.

RUNX1-MTG8 expression phenocopies loss of *Nf1* for growth of myeloid cells induced by GM-CSF. Although the *NF1* promoter is not highly conserved between humans and mice, the murine promoter contains four perfect RUNX1 binding sites, two of which are clustered similar to the a and b sites shown in Fig. 3. Therefore, we tested the effects of RUNX1-MTG8 on the expression of *Nf1* in primary myeloid progenitor cells. For comparison, we isolated myeloid progenitor cells from embryonic day 13.5 livers of mice derived from breeding $Nf1^{+/-}$ mice, which yielded wild-type, $Nf1^{+/-}$, and $Nf1^{-/-}$ embryos (fetal liver cells were used due to the mid-gestation lethality observed in $Nf1^{-/-}$ embryos) (7, 28). Fetal liver hematopoietic progenitor cells of each genotype were infected with RUNX1-MTG8-expressing retroviruses, and repression of *Nf1* in the GPF-expressing population was observed using immunoblot analysis after FACS-based cell sorting (Fig. 5A).

One of the effects of loss of *NF1* is to stimulate RAS-dependent signaling due to the loss of negative regulation (5, 12). However, these effects are small and difficult to measure accurately. Nevertheless, these small changes in RAS-GTP levels have dramatic effects on signaling (25, 27, 36). A more robust assay for the loss of NF1 function is the observation that loss of *NF1* sensitizes myeloid progenitor cells to the growth-promoting effects of GM-CSF, but not IL-3 (5, 15, 35). When equal numbers of infected progenitor cells were cultured in methylcellulose containing IL-3, a similar number of colonies were observed in all genotypes in the presence or absence of

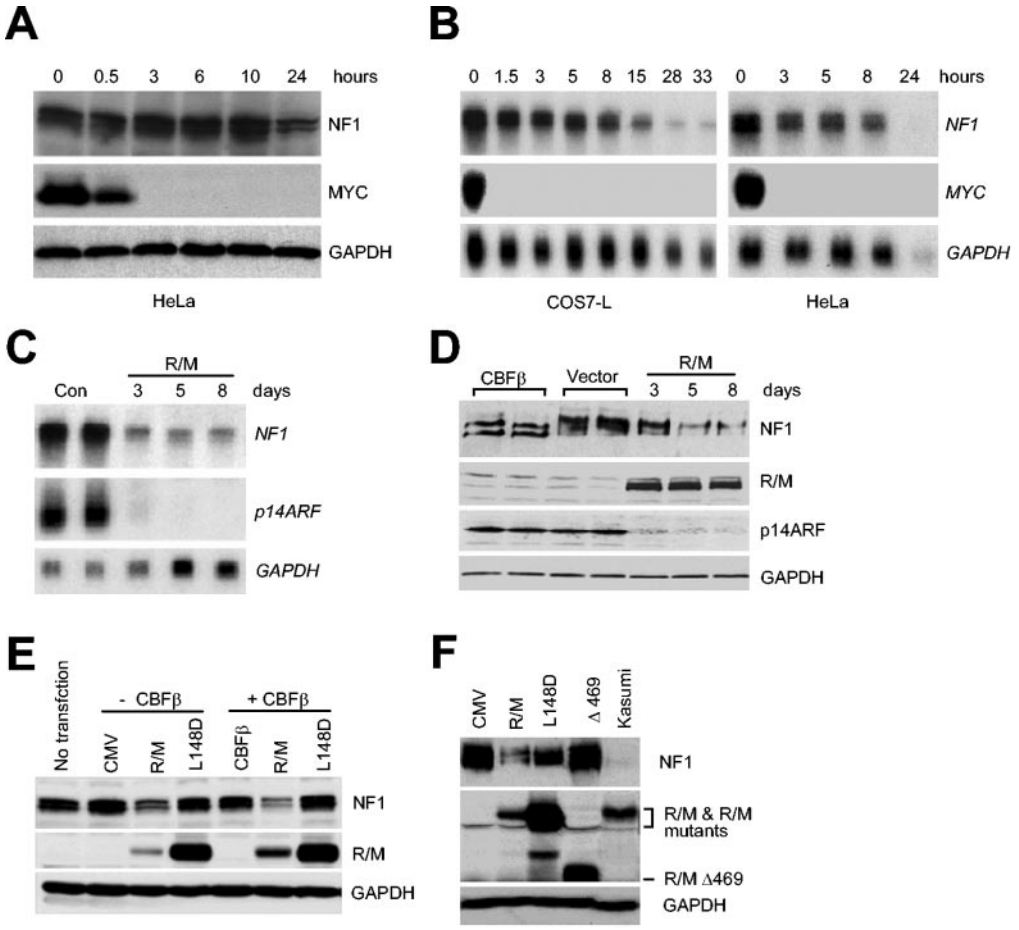

FIG. 4. *NF1* encodes long-lived mRNA and protein. (A) NF1 is a stable protein in HeLa cells. HeLa cells were treated with cycloheximide to block protein synthesis, and NF1 protein levels were determined by immunoblot analysis at the times indicated above the lanes. GAPDH and c-Myc were used as controls (Con) for a stable and an unstable protein, respectively. (B) *NF1* mRNA has a prolonged $t_{1/2}$. COS7-L and HeLa cells were treated with actinomycin D to block transcription, and mRNA extracted for RNA blot analysis at the times indicated above the lanes. *GAPDH* and c-*Myc* were used as controls. (C and D) RUNX1-MTG8 represses endogenous *NF1*. In panel C, cells infected as described for panel A were used for RNA blot analysis to detect the indicated mRNAs at 3, 5, and 8 days postinfection. The bottom part shows the same blot probed with GADPH as a loading control. In panel D, HeLa cells were infected with recombinant retroviruses expressing CBFβ-IRES-GFP and then reinfected with retroviruses expressing RUNX1-MTG8-IRES-puromycin resistance. Immunoblot analysis was used to detect the indicated proteins at 0, 3, 5, and 8 days postinfection. Duplicate samples of cells expressing only CBFβ or the puromycin resistance-encoding empty vector (Vector) and selected in puromycin for 5 days are shown as controls. (E and F) RUNX1-MTG8 represses *NF1* in COS7-L cells. COS7-L cells were transfected with the indicated plasmids, and the levels of the indicated proteins were determined by immunoblotting 5 days later (the cells were split once at day 3). R/M, RUNX1-MTG8; CMV, cytomegalovirus.

the fusion protein, indicating that RUNX1-MTG8 had little or no effect on the growth of myeloid progenitor cells cultured in IL-3 (Fig. 5B). However, when cultured in methylcellulose containing GM-CSF, RUNX1-MTG8 expression dramatically increased the number of colonies formed in all but the highest levels of GM-CSF (Fig. 5C). In fact, the RUNX1-MTG8-expressing cells in a wild-type or heterozygous *Nf1* background grew similarly to *Nf1*-null cells (Fig. 5C). However, RUNX1-MTG8 did not further enhance the number of colonies formed when both alleles of *NF1* were deleted (Fig. 5C, RUNX1-MTG8/*NF1*$^{-/-}$), suggesting that the RUNX1-MTG8-mediated enhancement of growth was due to the repression of *NF1* and was not due to a general enhancement of proliferation.

***NF1* mRNA is underrepresented in M2 AML.** Patients with *NF1* mutations are at increased risk for the formation of JMML, but mutations in *NF1* have not been reported in sporadic AML. Therefore, we defined the levels of expression of *NF1* in AML patient samples to determine whether *NF1* is regulated epigenetically. We used QRT-PCR to measure *NF1* mRNA levels in AML (Fig. 6A). *NF1* was differentially expressed over a range of nearly 3 logs. Given that hematopoietic cells containing only one allele of *Nf1* display sensitivity to cytokines, this finding suggests that *NF1* levels may be affected in many forms of AML. Nevertheless, we noted that the median levels of *NF1* mRNA in FAB M2 AML [with and without t(8;21)] were lower than the median of the other AML samples (Fig. 6A; 95% confidence interval, 0.073 to 0.365; *P* value of 0.0071). Because t(8;21) is frequently present in M2 AML, we further subdivided M2 leukemia to compare AML samples with and without t(8;21). Compared to the other subtypes of AML, *NF1* mRNA levels were about 50% lower in the AML M2 samples without t(8;21) (Fig. 6A; 95% confidence interval,

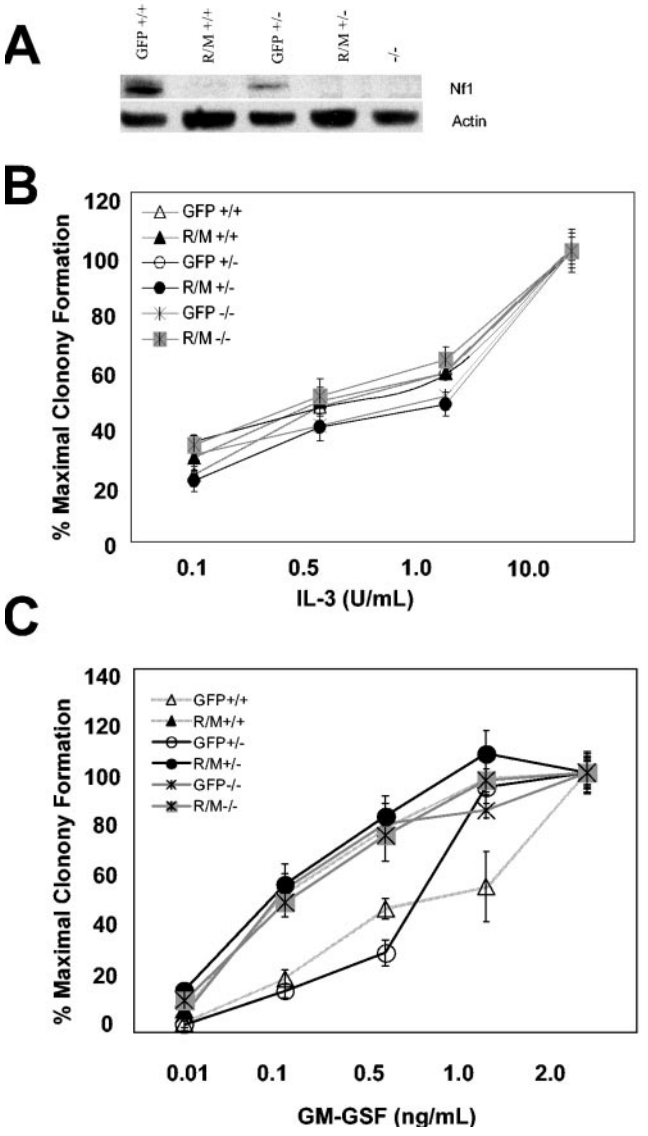

FIG. 5. RUNX1-MTG8-expressing cells are hypersensitive to GM-CSF. (A) RUNX1-MTG8 represses *Nf1* in murine myeloid progenitor cells. Murine myeloid progenitor cells were isolated from the fetal livers of day 13.5 embryos derived from a cross of $Nf1^{+/-}$ mice, which allowed the isolation of cells that contained both alleles ($Nf1^{+/+}$) or one allele ($Nf1^{+/-}$) or lacked *Nf1* ($Nf1^{-/-}$). These cells were then infected with recombinant retroviruses expressing GFP or RUNX1-MTG8-IRES-GFP. GFP^+ c-Kit^+ double-positive cells were isolated by FACS and expanded in vitro, and the levels of Nf1 protein were determined by immunoblot analysis. (B and C) RUNX1-MTG8 stimulates colony formation in response to GM-CSF. RUNX1-MTG8-expressing fetal liver progenitor cells were identified by FACS and plated in methylcellulose containing IL-3 (B) or GM-CSF (C). The number of colonies growing in IL-3 (B) or GM-CSF (C) was plotted as a function of the cytokine concentration. A representative experiment, performed in triplicate, is shown. +/+, ±, and −/− denote *Nf1* wild-type, heterozygous, and null genotypes, respectively. R/M, RUNX1-MTG8; GFP, MSCV-IRES-GFP vector control.

−0.0539 to 0.4321; *P* value of 0.0994) and fourfold lower in the samples containing t(8;21), although one sample appeared to have aberrantly high levels of *NF1* mRNA [Fig. 6A; 95% confidence interval for t(8;21) versus other AML, 0.047 to 0.414; *P* value of 0.0169].

Although the levels of *NF1* mRNA appeared to be lower in patient samples containing t(8;21) (Fig. 6A), the variability of *NF1* expression across various subtypes of AML complicates the interpretation. Therefore, we sought to confirm that RUNX1-MTG8 was associated with the *NF1* promoter in t(8;21)-containing leukemic blasts. We obtained a fresh bone marrow aspirate from a patient who had relapsed t(8;21)-containing AML with a relatively high blast count (66%). Proteins were cross-linked to DNA using formaldehyde and subjected to ChIP analysis using anti-N-terminal RUNX1 coupled with QRT-PCR (Fig. 6B, upper part). In addition, we were able to obtain several vials of marrow from this same patient which had only been frozen for a few days and that retained very high viability (>90%) to perform a second round of ChIP using anti-ZnF-MTG8 (anti-ETO Ab-1, Fig. 6B, middle part). Both anti-N-RUNX1 and anti-ZnF-MTG8 yielded a positive signal compared to an irrelevant control antibody or to the $p16^{ink4a}$ promoter (Fig. 6B, bottom part and data not shown), which places RUNX1-MTG8 at the endogenous *NF1* promoter in leukemic blasts.

DISCUSSION

Mutations in *NF1* represent a common familial cancer syndrome, but de novo mutations of *NF1* in leukemia are rare (29, 42, 55, 56). We have established that *NF1* is a direct transcriptional target of RUNX1 and the t(8;21) fusion protein. Given that t(8;21) is the most frequent chromosomal translocation in AML (58), our results suggest that regulation of *NF1* is a frequent event in AML. In addition, RUNX1 is inactivated by point mutations in M0 AML, and RUNX1-dependent gene regulation is disrupted directly or indirectly by other chromosomal translocations in myeloid and B-cell acute leukemia (43). Therefore, up to 20% of acute leukemias may show misregulation of *NF1* expression through disruption of RUNX1 function alone. In addition, C/EBPα cooperated with RUNX1 to activate *NF1* (Fig. 1), implying that this factor may also regulate *NF1*. Although we did not focus on C/EBPα, mutations in *CEBP*A are associated with the M2 subgroup of AML (49, 53). Thus, *NF1* may also be misregulated in these cases. Because phenotypes such as sensitivity to GM-CSF are associated with haploinsufficiency of *NF1* (35), even a modest reduction in the levels of *NF1* should stimulate proliferation and survival (3, 26) and cooperate with secondary mutations. In this regard, it is notable that c-*KIT*, but not other tyrosine kinase receptors, is mutated at high frequency in t(8;21)-containing AML, and NF1 modulates signals emanating from c-KIT (4, 26, 33, 62).

In addition to *NF1*, the $p14^{ARF}$ tumor suppressor is also repressed by RUNX1-MTG8 (Fig. 4) (39). $p14^{ARF}$ is induced by oncogenic activation such as overexpression of c-*Myc* or mutational activation of RAS (14). Once induced, $p14^{ARF}$ binds to and inactivates MDM2, which stabilizes the p53 tumor suppressor, to trigger growth arrest or apoptosis. In addition, $p14^{ARF}$ has p53-independent activity in growth inhibition (63). Loss of $p19^{ARF}$ expression and loss of *NF1* expression cooperate to induce multiple tumor types (32). The repression of *NF1* and $p14^{ARF}$ suggests a model for how RUNX1-MTG8 may set the stage for the development of leukemia. The repression of *NF1* may stimulate RAS activity or sensitize the cells to pro-

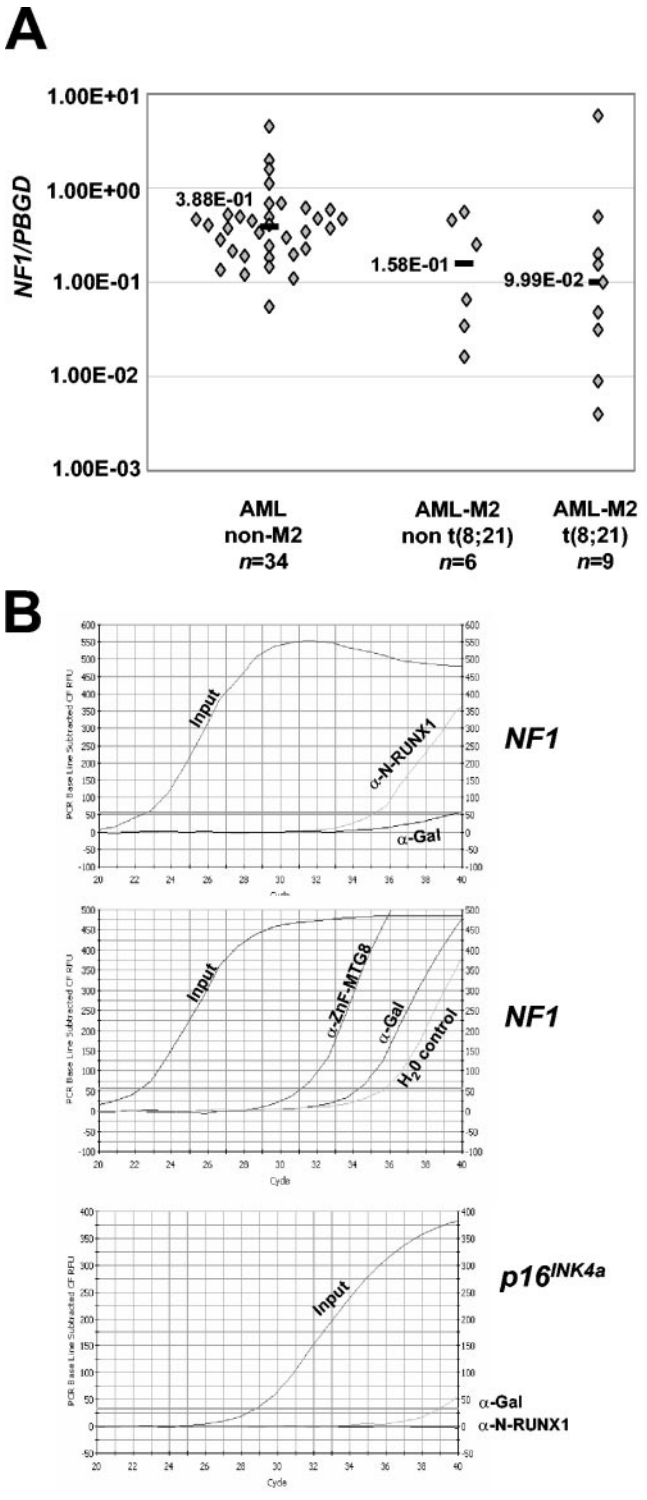

FIG. 6. Levels of *NF1* mRNA in AML samples. *NF1* mRNA levels in 49 AML patient samples were determined using QRT-PCR and normalized using the porphobilinogen deaminase gene (*PBGD*) as an internal control for the amount of mRNA added to each reaction mixture. The level of *NF1* mRNA in each sample was plotted on a logarithmic scale. The median is indicated with a solid bar with the value shown beside it. The samples were subdivided into non-M2 AML, FAB M2 without t(8;21), and t(8;21)-containing samples. (B) ChIP analysis of the *NF1* promoter from a t(8;21)-containing patient sample. The antibodies used are described in more detail in the legend to Fig. 3. The a primers (Fig. 3A) were used for the analysis shown. The lower part shows amplification of the $p16^{ink4a}$ promoter as a control. RFU, relative fluorescence units.

liferation in response to cytokines (e.g., GM-CSF; Fig. 5), while the repression of $p14^{ARF}$ inactivates the oncogene checkpoint to allow unchecked growth. If this is so, it would provide hematopoietic stem cells and myeloid progenitors harboring t(8;21) a selective advantage, perhaps by increasing their survival, and allow further oncogenic mutations to arise.

The targeting of *NF1* and $p14^{ARF}$ by the t(8;21) fusion protein has a corollary in solid tumors as deletion of 9p22, encompassing the $p14^{ARF}/p16^{ink4A}$ locus, is associated with progression to malignancy in neurofibromatosis type I patients (50). One distinction in this case is that the 9p22 deletion also removes the $p16^{ink4A}$ tumor suppressor. While $p16^{ink4A}$ did not appear to be a direct target for repression by RUNX1-MTG8, silencing of $p16^{ink4A}$ may be one of the secondary events that cooperate with repression of *NF1* and $p14^{ARF}$ in leukemogenesis because $p16^{ink4A}$ mRNA levels were also very low in t(8;21)-containing leukemic blasts (39). Nevertheless, in gene ablation studies with mice, inactivation of only $p19^{ARF}$ (the murine homologue of $p14^{ARF}$) cooperated with loss of *NF1* in tumorigenesis (32). Moreover, when only one allele of each tumor suppressor was deleted, a situation that may be more analogous to repression, the latency of tumor formation was shortened (32). Thus, repression of *NF1* and $p14^{ARF}$ would be anticipated to cooperate with other mutations, including epigenetic regulation of $p16^{ink4A}$ or oncogenic activation of tyrosine kinases such as c-KIT, in leukemogenesis.

The analysis of the expression of a given gene in AML samples is extremely difficult because only the endpoint (transformed cells) can be used without the appropriate normal control cell that would match the leukemic blast. This is further complicated by differences in the genetic backgrounds of the individuals, which can greatly affect the basal transcription of genes (9, 48, 51). When these differences are coupled with secondary mutations, and the varied AML subtypes with distinct phenotypes and blocks of differentiation, it is extremely difficult to interpret subtle differences in gene expression. Our cohort of 49 AML samples contained an unusually high number of AML M2 (Fig. 6), which aided our analysis of this subgroup. Even so, the differences in *NF1* mRNA levels were not as distinct as those observed previously for $p14^{ARF}$ or *CEBPA* (39, 52). Given the multitude of data indicating that *NF1* is a direct transcriptional target of RUNX1-MTG8 (Fig. 3 to 6), including detecting the fusion protein bound to the *NF1* promoter in leukemic blasts, this may simply indicate that *NF1* is a more widespread target for gene silencing in AML, thereby making repression by RUNX1-MTG8 less evident. Nevertheless, in our cohort the results were significant at the 95% confidence level.

The observation that RUNX1-MTG8 represses *NF1* and stimulates the growth of isogenic myeloid progenitor cells isolated from inbred mouse strains in response to GM-CSF (Fig. 5) may also have therapeutic implications. Our results suggest that t(8;21)-containing leukemic blasts may be hyperproliferative in response to GM-CSF or SCF and therefore sensitive to

inhibitors of these signaling pathways. However, the varied genetic backgrounds of patients and the presence of additional mutations that are required for cellular transformation (e.g., mutations of N-RAS or FLT3) complicated a similar analysis of patient samples. When we plated t(8;21)-containing leukemic blasts into methylcellulose containing GM-CSF, the majority of the samples did indeed form colonies in response to GM-CSF (R. Delwel, unpublished observations). However, there was not a dramatic difference in the overall response to cytokines compared to randomized AML samples that often form colonies in response to cytokines. Nevertheless, it is possible that the use of inhibitors of the RAS signaling cascade may be advantageous for t(8;21)-containing patients. In addition, the repression of *NF1* by the t(8;21) fusion protein may provide another marker to measure the effectiveness of transcriptional therapies such as HDAC inhibitors that attempt to re-express critical genes that are silenced in leukemogenesis.

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