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A Role of Miz-1 in Gfi-1-Mediated Transcriptional Repression of *CDKN1A*

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Abstract

Zinc-finger (ZF) transcriptional repressor Gfi-1 plays an important role in hematopoiesis and inner ear development, and also functions as an oncoprotein that cooperates with c-Myc in lymphomagenesis. Gfi-1 represses transcription by directly binding to conserved sequences in the promoters of its target genes. *CDKN1A* encoding $p21^{Cip1}$ has been identified as a Gfi-1 target gene and shown to contain Gfi-1 binding sites in the upstream promoter region. We show here that Gfi-1 represses *CDKN1A* in a manner that is independent of its DNA binding activity. Gfi-1 interacts with POZ-ZF transcription factor Miz-1, originally shown to be a c-Myc interacting partner, and via Miz-1 binds to *CDKN1A* core promoter. Interestingly, Gfi-1 and c-Myc, through Miz-1, form a ternary complex on the *CDKN1A* promoter, and act in collaboration to repress *CDKN1A*. Gfi-1 knockdown results in enhanced levels of $p21^{Cip1}$ and attenuated cell proliferation. Notably, similar to c-Myc, the expression of Gfi-1 is downregulated by TGFβ and the level of Gfi-1 influences the response of cell to the cytostatic effect of TGFβ. Our data reveal an important mechanism by which Gfi-1 regulates cell proliferation and may also have implications for understanding the role of Gfi-1 in lymphomagenesis.

Keywords

Gfi-1; Miz-1; CKD inhibitors; proliferation

Introduction

Growth factor independence 1 (Gfi-1) is a nuclear transcriptional repressor that is expressed in hematopoietic stem cells (HSCs), lymphoid and granulocytic cells as well as nonhematopoietic tissues, including lung, sensory epithelia, neuronal precursors and the developing epithelia of the inner ear (Moroy, 2005). In hematopoietic system, Gfi-1 is required for granulocytic differentiation, and plays an important role in T and B cell development, and in the maintenance of stem cell functional integrity. Gfi-$1^{-/-}$ mice lack mature neutrophils and have reduced numbers of mature T and B cells (Hock *et al.*, 2003;

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Conflict of interests

The authors declare no conflict of interest.

Karsunky *et al.*, 2002b; Zhu *et al.*, 2006). HSCs from Gfi-1$^{-/-}$ mice show elevated proliferation and are functionally impaired in long-term repopulation and serial transplantation assays (Hock *et al.*, 2004; Zeng *et al.*, 2004). In nonhematpoietic tissues, Gfi-1 has been shown to regulate the development of inner ear hair cells, lung neuroendocrine cells and intestinal epithelium (Kazanjian *et al.*, 2004; Shroyer *et al.*, 2005; Wallis *et al.*, 2003).

Increasing evidence indicates that aberrant activation of Gfi-1 may lead to oncogenesis. The *Gfi-1* locus was first identified as a provirus integration site that rendered Moloney murine leukemia virus-induced T cell lymphoma lines independent of interleukin 2 (Gilks *et al.*, 1993). Overexpression of Gfi-1 was later found to inhibit apoptosis and override cell cycle arrest induced by growth factor withdrawal (Grimes *et al.*, 1996b; Karsunky *et al.*, 2002a; Rodel *et al.*, 2000; Zhu *et al.*, 2002). Transgenic mice that overexpress Gfi-1 in T cells are weakly predisposed to lymphoma, and combinatorial activation of Gfi-1 and Myc or Pim-1 leads to accelerated development of lymphoma (Scheijen *et al.*, 1997; Schmidt *et al.*, 1998; Zornig *et al.*, 1996). These results indicate that Gfi-1 is a weak oncoprotein, but cooperates with Myc and Pim-1 in lymphomagenesis. Additionally, Gfi-1 may play a role in the development of lung and prostate cancers (Dwivedi *et al.*, 2005; Kazanjian *et al.*, 2004).

Gfi-1 consists of a 20 amino acid N-terminal SNAG domain, a middle portion and 6 C-terminal zinc fingers (ZFs). The transcriptional repression activity of Gfi-1 is dependent on the integrity of its N-terminal SNAG domain (Grimes *et al.*, 1996a). Gfi-1 represses transcription by directly binding to the consensus DNA sequence AATC through the C-terminal ZFs. An asparagine-to-serine substitution in the fifth ZF of Gfi-1 (N382S), identified in patients with severe congenital neutropenia, abolishes the DNA binding activity and the N382S mutant acts in a dominant negative (DN) manner (Person *et al.*, 2003). Transcriptional repression by Gfi-1 involves recruitment, through its different domains, of corepressors and histone modifying enzymes, including eight-twenty-one (ETO), CoREST, histone demethylase LSD1, histone deacetylases (HDACs) 1 and 2, and the histone lysine methyltransferase G9a (Duan *et al.*, 2005; Grimes *et al.*, 1996a; McGhee *et al.*, 2003; Saleque *et al.*, 2007).

Little is known about how Gfi-1 regulates cell cycle progression and survival. *CDKN1A*, which encodes the cyclin dependent kinase inhibitor (CDKI) p21^{Cip1}, has been identified as a Gfi-1 target gene. Gfi-1 was shown to bind to the upstream sequences in the *CDKN1A* promoter and repress transcription through recruiting HDAC1 and G9a (Duan andHorwitz, 2003; Duan *et al.*, 2005). Indeed, overexpression of Gfi-1 antagonized p21^{Cip1} upregulation by phorbol ester in Jurkat T cells (Karsunky *et al.*, 2002a). Consistent with these observations, the level of p21^{Cip1} is increased in mouse Gfi1$^{-/-}$ T cells (Pargmann *et al.*, 2007). Interestingly, unlike T cells, deficiency of Gfi-1 in mouse HSCs leads to reduced expression of p21^{Cip1} (Hock *et al.*, 2004; Zeng *et al.*, 2004)]. It remains to be determined whether p21^{Cip} downregulation in Gfi1$^{-/-}$ HSCs results directly from Gfi-1 deficiency or rather is an indirect event. Irrespectively, these results indicate that the effects of Gfi-1 on p21^{Cip1} expression are cell context-dependent.

Miz-1 is a POZ domain ZF transcription factor that possesses a potent anti-growth function and was originally identified as a c-Myc interacting protein (Wanzel *et al.*, 2003). Miz-1 has been implicated in c-Myc-mediated repression of *CDKN1A*, *CDKN2B* encoding another CDKI $p15^{INK4B}$, and *Mad4* (Herold *et al.*, 2002; Kime and Wright, 2003; Seoane *et al.*, 2002; Seoane *et al.*, 2001; Staller *et al.*, 2001). Miz-1 binds to the core promoters of these genes and activates their transcription. c-Myc does not directly bind to, but is recruited to them via Miz-1. Transcriptional activation by Miz-1 is abolished upon c-Myc recruitment and the Miz-1/c-Myc complex functions to repress transcription. Significantly, the expression of c-Myc is downregulated by the cytostatic cytokine transforming growth factor β (TGFβ), which represents an important mechanism by which TGFβ activates *CDKN1A* and *CDKN2B*, and exerts its growth-inhibitory effect (Seoane *et al.*, 2002; Seoane *et al.*, 2001). We show here that Gfi-1 is recruited to *CDKN1A* core promoter via Miz-1 and represses *CDKN1A* transcription. Notably, the DNA binding activity of Gfi-1 is dispensable for its repression activity. Our data also indicate that Gfi-1 and c-Myc, via Miz-1, form a ternary complex on the *CDKN1A* promoter and exhibit functional collaboration in the repression of *CDKN1A*. Interestingly, like c-Myc, Gfi-1 is also downregulated by TGFβ and regulates TGFβ sensitivity in hematopoietic cells. These results have implications for understanding the action of Gfi-1 in cell proliferation and its collaboration with Myc in lymphomagenesis.

Results

DNA binding activity of Gfi-1 is not required for repressing TGFβ-induced activation of *CDKN1A*

Gfi-1 has been shown to repress *CDKN1A*, which contains Gfi-1 binding sites approximately 1.4 kb and 2.8 kb upstream of the transcription initiation site (Duan and Horwitz, 2003; Duan *et al.*, 2005). We addressed whether direct DNA binding is required for repression of *CDKN1A* by Gfi-1. Hela cells were transfected with the luciferase reporter constructs containing the 2.4-kb or 111-bp fragment of the *CDKN1A* promoter (Datto *et al.*, 1995) along with the expression constructs for Gfi-1 and the N382S mutant. Notably, the 111-bp promoter fragment is devoid of a consensus Gfi-1 binding site, and the N382S mutant of Gfi-1 is defective in DNA binding and acts in a DN manner (Person *et al.*, 2003; Zhuang *et al.*, 2006). Cells were subsequently stimulated with TGFβ, which has been shown to induce the expression of *CDKN1A* in epithelial cells (Letterio, 2005; Lin *et al.*, 2005). As shown in Fig. 1, both *CDKN1A* promoter fragments were activated by TGFβ, but were completely inhibited by Gfi-1. The N382S mutant also effectively repressed the two C*DKN1A* promoter fragments. Together, these data indicate that the DNA binding ability is not essential for the inhibitory effect of Gfi-1 on TGFβ-induced activation of *CDKN1A*.

Miz-1 recruits Gfi-1 to the *CDKN1A* promoter

As repression of *CDKN1A* by Gfi-1 is independent of its DNA binding activity, we speculated that Gfi-1 may be recruited to the *CDKN1A* promoter by a DNA binding protein. We recently identified Miz-1 as a Gfi-1-interacting protein that recruits Gfi-1 to the *CDKN2B* promoter and is involved in Gfi-1-mediated repression of *CDKN2B* (Basu *et al.*, 2009). Notably, Miz-1 has been shown to recruit c-Myc to the *CDKN1A* promoter and is required for repression of *CDKN1A* by c-Myc (Seoane *et al.*, 2002). Oligonucleotide

precipitation assays were conducted to investigate whether Gfi-1 bound to the *CDKN1A* promoter via Miz-1. 293T cells were transiently transfected with Miz-1, Gfi-1 or both. Whole cell extracts were incubated with the biotinylated double-stranded oligonucleotide spanning −49 bp to + 16 bp of the *CDKN1A* promoter, which lacks a consensus Gfi-1 binding site and was used by others to specifically pull down Miz-1 (Seoane *et al.*, 2002; Wu *et al.*, 2003). Proteins that bound to the oligonucleotide were precipitated using the streptavidin-coated beads. As expected, Miz-1 was pulled down by the oligonucleotide (Fig. 2A). Notably, Gfi-1 was pulled down only when Miz-1 was present in the whole cell extracts. The N382S mutant was also recruited to the *CDKN1A* core promoter by Miz-1 (Fig. 2B), in agreement with its ability to repress *CDKN1A*

ChIP assays were then conducted on 293T cells ectopically expressing Gfi-1 and Miz-1 to evaluate whether Gfi-1 binding to the *CDKN1A* promoter *in vivo* was also dependent on Miz-1. As shown in Fig. 2C, Gfi-1 barely bound to the *CDKN1A* promoter in 293T cells transfected with Gfi-1 only. Expression of Miz-1 in 293T cells markedly increased Gfi-1 occupancy of the *CDKN1A* core promoter, but not of the upstream and downstream regions. Consistent with this, Miz-1 bound to the proximal promoter region, but not the upstream and downstream regions of *CDKN1A*. ChIP assays on HL-60 and Jurkat cells demonstrated that endogenous Gfi-1 also bound to the *CDKN1A* core promoter *in vivo* (Fig. 2D). Interestingly, although the *CDKN1A* promoter has been shown to contain two Gfi-1 binding sites located approximately 1.4 kb and 2.8 kb upstream of the transcription initiation sites (Duan and Horwitz, 2003; Duan *et al.*, 2005), PCR using primers spanning the two sites failed to demonstrate significant Gfi-1 binding, suggesting that Gfi-1 may not occupy the two sites *in vivo*. To further demonstrate that Gfi-1 occupancy of the *CDKN1A* promoter was dependent on Miz-1, the expression of Miz-1 was knocked down in HL-60 cells through lentivirus-mediated delivery of the Miz-1 shRNA. As shown in Fig. 2E, Miz-1 knockdown significantly reduced Gfi-1 occupancy of the *CDKN1A* promoter. Together, these data indicate that Gfi-1 bound to the *CDKN1A* core promoter via Miz-1.

The C-terminal ZFs of Gfi-1 are involved in interaction with Miz-1

To define the Gfi-1 region implicated in Miz-1 interaction, a series of Gfi-1 mutants (Fig. 3A) were generated and expressed in 293T cells along with Myc-tagged Miz-1. Coimmunoprecipitation assays demonstrated that truncation of the N-terminal SNAG domain (Gfi-1-dN) had no noticeable effect on the ability of Gfi-1 to interact with Miz-1 (Fig. 3B). However, deletion of the C-terminal 3 (Gfi-1-dZF3) or 6 (Gfi-1dZF6) ZFs of Gfi-1 abolished Miz-1 interaction, indicating that the C-terminal ZFs 4–6 are required for Miz-1 interaction. Accordingly, Gfi-1-dN, but not Gfi-1-dZF6, was recruited to the *CDKN1A* promoter by Miz-1 (Fig. 3C).

Gfi-1 represses the activation of *CDKN1A* by Miz-1

As Gfi-1 was recruited to *CDKN1A* via Miz-1, we examined the role of Gfi-1 in Miz-1-mediated activation of the *CDKN1A* promoter using the luciferase reporter assay. As shown in Fig. 4A, the 2.4-kb promoter fragment of *CDKN1A* was activated by Miz-1 in Hela cells, but was repressed by Gfi-1 in a dose dependent manner. Notably, Gfi-1 also repressed Miz-1-mediated activation of the 111-bp core promoter fragment of *CDKN1A* (Fig. 4B). The

activity of the 111-bp promoter fragment was also inhibited by the N382S mutant (Fig. 4C). Thus, analogous to its effect on TGFβ-induced activation of *CDKN1A*, repression of Miz-1-activated *CDKN1A* by Gfi-1 is also independent of direct DNA binding.

To provide evidence that Gfi-1 repressed endogenous *CDKN1A*, we assessed the effect of Gfi-1 knockdown on the level of $p21^{Cip1}$ in myeloid leukemic HL-60 and TF-1 cells, and pro-B Ba/F3 cells. Lentiviral delivery of *Gfi-1* shRNAs, but not the empty vector, significantly reduced the expression of Gfi-1, which was associated with increased levels of $p21^{Cip}$ in the cells (Fig. 4D). We also examined the effect of Gfi-1 knockdown on cell proliferation. As compared with cells infected with the empty lentiviral vector or shRNAs that failed to knock down Gfi-1, cells in which Gfi-1 was knocked down initially grew extremely slowly. The growth-inhibitory effect of Gfi-1 knockdown became less apparent after prolonged culture. Sufficient amounts of cells could be collected for evaluation of Gfi-1 expression and cell proliferation approximately 6 weeks after lentiviral delivery of the Gfi-1 shRNA. At that time, Gfi-1 knockdown exhibited moderate inhibitory effects on cell proliferation (Fig. 5).

Gfi-1 and c-Myc collaboratively repress *CDKN1A*

Because Gfi-1 and c-Myc both interact with Miz-1 and repress transcription activated by Miz-1, we addressed the possibility that Gfi-1 may functionally collaborate with c-Myc in repressing *CDKN1A*. Luciferase reporter assays using the 2.4-kb fragment of the *CDKN1A* promoter were performed in Hela cells transiently transfected with Gfi-1 and c-Myc. As expected, expression of either Gfi-1 or c-Myc inhibited Miz-1-stimulated activity of the *CDKN1A* promoter (Fig. 6A). Notably, coexpression of Gfi-1 and c-Myc resulted in further inhibition of the *CDKN1A* promoter activity.

We performed the oligonucleotide precipitation assays to investigate whether Gfi-1 and c-Myc were both recruited to the *CDKN1A* core promoter via Miz-1 in 293T cells transfected with Gfi-1 and c-Myc with or without Miz-1. As shown in Fig. 6B, Gfi-1 and c-Myc were pulled down by the *CDKN1A* promoter oligonucleotide in the presence of Miz-1, but not in its absence. Re-ChIP assays were performed to further evaluate whether Gfi-1, Miz-1 and c-Myc occupied the *CDKN1A* promoter as a ternary complex in 293T cells. Soluble chromatin from the cells was immunoprecipitated with the anti Gfi-1 antibody, eluted from beads and re-immunoprecipitated with the anti c-Myc antibody prior to analysis of the sequences surrounding the *CDKN1A* promoter by semi-quantitative PCR. The *CDKN1A* core promoter sequence, but not the upstream and downstream regions, was immunoprecipitated with the anti Gfi-1 and anti c-Myc antibodies only if Miz-1 was expressed in the cells (Fig. 6C). The *CDKN1A* core promoter sequence was not detected when the irrelevant species-matched antibody was used for immunoprecipitation in the Re-ChIP experiments (Fig. 6D). Together, the results demonstrated that Gfi-1, Miz-1 and c-Myc bind to the *CDKN1A* core promoter as a ternary complex.

TGFβ treatment inhibits the expression of Gfi-1 in hematopoietic cells

It has been shown that TGFβ upregulates $p21^{Cip1}$ at least in part through inhibiting the expression of c-Myc, and enforced expression of c-Myc confers cells resistance to the

cytostatic effect of TGFβ (Kim and Letterio, 2003; Letterio, 2005; Lin *et al.*, 2005). To investigate the effect of TGFβ treatment on the expression of Gfi-1, HL-60, TF-1 and U937 cells were incubated with TGFβ for different times and examined for Gfi-1 expression by semi-quantitative PCR and Western blot analyses. TGFβ treatment dramatically reduced the levels of Gfi-1 mRNA and protein (Fig. 7). Essentially identical results were obtained in another myeloid cell line Mo7e (data not shown). Thus, similar to c-Myc, the expression of Gfi-1 is also downregulated by TGFβ treatment.

Gfi-1 attenuates the cytostatic effect of TGFβ

TGFβ has been shown to exert a cytostatic effect on early hematopoietic progenitors and stem cells whereas leukemia-derived cells are resistant to this effect (Letterio, 2005; Lin *et al.*, 2005). We addressed whether the expression level of Gfi-1 affected cellular response to TGFβ. Murine Pro-B Ba/F3 cells, which were sensitive to the cytostatic effect of TGFβ (Luo and Lodish 1996), were transiently transfected with the retroviral expression construct Gfi-1-RV and sorted based on GFP expression. Transfection of Ba/F3 cells with Gfi-1 moderately counteracted the cytostatic effect of TGFβ (Fig. 8A). We further examined the effect of Gfi-1 knockdown on the TGFβ response of Ba/F3 cells. Consistent with the results obtained in HL-60 and TF-1 cells, Gfi-1 knockdown through lentivirus-mediated delivery of the Gfi-1 shRNA inhibited the proliferation of Ba/F3 cells (Fig. 8B). Significantly, the Gfi-1 knocked-down Ba/F3 cells exhibited markedly increased sensitivity to the cytostatic effect of TGFβ. These data implicate Gfi-1 as an important regulator of cell sensitivity to TGFβ.

Discussion

In this paper, we have demonstrated that Gfi-1 interacts with Miz-1 through its C-terminal ZF domains and, via Miz-1, is recruited to the *CDKN1A* promoter, resulting in transcriptional repression. Consistent with this, repression of *CDKN1A* by Gfi-1 is independent of its DNA binding activity, as evident from the facts that Gfi-1 represses the 111-bp *CDKN1A* promoter fragment which is devoid of a Gfi-1 binding site and the DNA-binding defective N382S mutant of Gfi-1 is fully capable of repressing *CDKN1A* promoter activity. We recently showed that Gfi-1 represses *CDKN2B* encoding p15INK4B through interaction with Miz-1 (Basu *et al.*, 2009). The data presented here add *CDKN1A* to the list of genes that are regulated by Gfi-1 through Miz-1.

CDKN1A has been shown to contain two Gfi-1 binding sites located at approximately −1.4 kb and −2.8 kb in the *CDKN1A* promoter (Duan and Horwitz, 2003; Duan *et al.*, 2005). Gfi-1 occupancy of the *CDKN1A* promoter was confirmed in HL-60 cells by ChIP assays in which the *CDKN1A* promoter fragment spanning from approximately −390 bp to −250 bp was amplified by PCR (Duan and Horwitz, 2003). However, we were unable to demonstrate significant Gfi-1 binding using PCR primers that flank the two sites in our ChIP assays although Gfi-1 binding to the *CDKN1A* core promoter was consistently detected in HL60 and Jurkat cells, suggesting that Gfi-1 may not bind to the upstream regions of the *CDKN1A* promoter *in vivo*. Notably, Miz-1 knockdown significantly reduced Gfi-1 binding *in vivo* to the *CDKN1A* promoter. These data suggest that Miz-1 may be required for Gfi-1 binding to the *CDKN1A* promoter.

Miz-1 has been implicated in c-Myc-mediated repression of *CDKN1A*, *CDKN2B* and *Mad4* (Herold *et al.*, 2002; Kime and Wright, 2003; Seoane *et al.*, 2002; Seoane *et al.*, 2001; Staller *et al.*, 2001). Like Gfi-1, c-Myc does not bind to, but is recruited to the *CDKN1A* core promoter via Miz-1. Notably, the C-terminal ZFs 1–12 of Miz-1 are involved in interaction with Gfi-1 whereas the regions flanking ZFs 1–12 are required for binding to Myc (Basu *et al.*, 2009; Peukert *et al.*, 1997). Thus, Miz-1 may interact simultaneously with Gfi-1 and Myc through distinct regions. Our data indicate that Gfi-1 and c-Myc form a ternary complex with Miz-1 on the *CDKN1A* promoter and exhibit functional cooperation in repressing *CDKN1A*. We have recently demonstrated that Gfi-1 cooperates with c-Myc in repressing *CDKN2B* (Basu *et al.*, 2009). Taken together, these results support a model in which Miz-1 recruits Gfi-1 and c-Myc to form the Gfi-1/Miz-1/c-Myc ternary complex on the core promoters of *CDKN1A*, *CDKN2B* and probably other Miz-1 target genes, leading to cooperative repression of these genes by Gfi-1 and c-Myc. Interestingly, Gfi-1 has been shown to collaborate with c-Myc in lymphomagenesis (Scheijen *et al.*, 1997; Schmidt *et al.*, 1998; Zornig *et al.*, 1996). Our data, while not directly addressing, offer a potential explanation for the role of Gfi-1 in Myc-induced lymphomagenesis (Scheijen *et al.*, 1997; Schmidt *et al.*, 1998; Zornig *et al.*, 1996).

TGFβ inhibits the proliferation of epithelial and early hematopoietic cells, and impaired TGFβ signaling has been implicated in hematopoietic and epithelial malignancies (Letterio, 2005; Lin *et al.*, 2005). The cytostatic effect of TGFβ is dependent on its ability to induce the expression of $p21^{Cip1}$ and $p15^{INK4B}$ at least in part through rapid downregulation of c-Myc (Chen *et al.*, 2002; Frederick *et al.*, 2004; Yagi *et al.*, 2002). Our data indicate that Gfi-1 also suppresses TGFβ-induced activation of *CDKN1A* and counteracts the cytostatic effect of TGFβ. Significantly, like c-Myc, the expression of Gfi-1 is downregulated by TGFβ at both mRNA and protein levels, consistent with a recent study (Zhu *et al.*, 2009). Thus, TGFβ downregulates both Gfi-1 and c-Myc, which may release Miz-1 from the Gfi-1/Miz-1/c-Myc complex to activate *CDKN1A*, *CDKN2B* and other possible target genes involved in the negative regulation of cell proliferation (Fig. 9). It is possible that suppression of TGFβ-induced activation of *CDKN1A* and other Miz-1 target genes may contribute to the oncogenic potential of Gfi-1.

While Gfi-1 has been implicated in the pathogenesis of lymphomas, little is known about its role in leukemogenesis. It has been shown recently that Gfi-1 may suppress leukemogenesis through repression of *HoxA9*, *Pbx1*, and *Meis1*, which are required for immortalization and expansion of hematopoietic stem and progenitor cells (Horman *et al.*, 2009). However, our results indicate that shRNA-mediated knockdown of Gfi-1 in myeloid leukemic cells led to attenuated cell proliferation. The growth-inhibitory effect of Gfi-1 knockdown appeared to be dramatic at early stages of Gfi-1 knockdown, but became less significant upon prolonged culture, suggesting that leukemic cells may develop compensatory mechanisms. Further studies are needed to investigate the role of Gfi-1 in leukemogenesis.

Materials and Methods

Cells

293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 10 % heat inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) solution. The human myeloid leukemic cell lines HL-60, TF-1 and U937 as well as leukemic T cell line Jurkat were maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics. Murine Pro-B Ba/F3 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 10% WEHI-3B cell conditioned media as a crude source of murine interleukin-3. The cells were grown in humidified incubator at 37 °C with 5% CO2.

Reagents

Human TGFβ-1 was purchased form PeproTech (Rocky Hill, NJ). Antibodies against Gfi-1 (N-20), Miz-1 (N17), ExactaCruz™ D kit used in coimmunoprecipitation experiments, and protein A/G beads were purchased from Santa Cruz Biotechnolgy (Santa Cruz, CA). Monoclonal anti Miz-1 antibody 10E2 was kindly provided by Dr. M. Eilers (University of Marburg, Germany) and used in Western blot analysis. Anti β-actin antibody was from Sigma (Saint Louis, MO). The pLKO.1 lentiviral constructs containing short hairpin RNAs (shRNAs) against Gfi-1 and Miz-1 were purchased from Open Biosystems (Huntsville, AL). Lipofectamine 2000 and *Trans*IT®-LT1 Transfection Reagent were purchased from Invitrogen (Carlsbad, California) and Mirus (Madison, WI), respectively. Luciferase assay reagents were from Promega.

Construction of vectors

The pcDNA3.1/Myc-His expression constructs for Miz-1 and Myc-tagged Miz-1, and the retroviral expression constructs for Gfi-1 (Gfi-1-RV) and the N382S mutant (N382S-RV) containing an internal ribosomal entry sequences (IRES) and humanized GFP cDNA have been described (Basu *et al.*, 2009; Zhuang *et al.*, 2006). Rat Gfi-1 cDNA was kindly provided by Dr. P. N. Tsichlis (Fox Chase Cancer Center, Philadelphia) and cloned into the pcDNA3.1/Myc-His plasmid. The Myc tag sequence of the pcDNA3.1/Myc-His plasmid was subsequently replaced with the sequence encoding the Flag tag. The Gfi-1 dN, dZF3 and dZF6 mutants were generated using the PCR-based strategy. The *CDKN1A* luciferase reporter constructs containing 2.4-kb and 111-bp fragments of human *CDKN1A* promoter (Datto *et al.*, 1995) were kindly provided by Dr. X. F. Wang (Duke University, Durham).

Immunoprecipitation and Western blot analysis

293T cells transiently transfected with Myc-tagged Miz-1 and Flag-tagged Gfi-1 or mutants were lysed in lysis buffer (1% Triton X-100, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 20 mM NaF and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Whole cell extracts were subjected to immunoprecipitation using the anti-Flag antibody. Immunocomplexes were recovered with protein A/G beads and washed 5 times with lysis buffer. Samples were boiled in sodium dodecyl sulfate (SDS) sample buffer and resolved by SDS-polyacrylamide gel electrophoresis prior to transfer to Immobilon membranes. The membranes were

incubated with the appropriate antibodies and the reactive proteins were visualized by enhanced chemiluminescence.

Oligonucleotide precipitation analysis

293T cells were transfected with Gfi-1, Miz-1 or their mutants, and lysed 48 hours post transfection in HKMG buffer (10 mM Hepes [pH 7.9], 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol and 1% NP40). Whole cell lysates were incubated overnight at 4° C with the biotinylated double-stranded oligonucleotide spanning from −49 bp to + 16 bp of the *CDKN1A* promoter in the presence of 25 fold excess of poly(dI-dC). Bound proteins were collected with streptavidin-agarose beads. The beads were washed three times in HKMG buffer prior to Western blot analysis.

Chromatin immunoprecipitation assay (ChIP) and Re-ChIP

Cells were fixed with 1% formaldehyde for 10 min at 37 °C and lysed in hypotonic buffer (5 mM Tris-HCl [pH 7.5], 85 mM KCl and 0.5% NP40). After centrifugation at 6000 rpm for 5 min, nuclei were lysed in ChIP lysis buffer (1% SDS, 10 mM EDTA, and 50mM Tris-HCl [pH 7.5]) and sonicated to shear chromatin DNA to about 500 bp fragments. Nuclear lysates were diluted 8 times with ChIP dilution buffer (1.1% Triton X-100, 0.01% SDS, 1.2 mM EDTA, 16.7 mM Tri-HCl [pH 8] and 167 mM NaCl), precleared with protein A/G-agarose beads and rabbit normal IgG for 1 hour, and subjected to immunoprecipitation with the anti Miz-1, anti Gfi-1 or a species-matched irrelevant antibody. Precipitated chromatin DNA was amplified by PCR using primers spanning the different regions of the *CDKN1A* promoter. For Re-ChIP assays, DNA-protein complexes immunoprecipitated with the anti Gfi-1 antibody in primary ChIP were eluted with 25 μl of 10 mM DTT for 30 min at 37 °C and diluted 20 times with Re-ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl [pH 7.5]). The complexes were then subjected to reimmunoprecipitation with the anti c-Myc antibody prior to analysis of the precipitated chromatin DNA by semi-quantitative PCR.

RNA interference

293T cells were transfected with the shRNA lentiviral constructs along with the packaging plasmids using lipofectamine 2000. Supernatants containing the viruses were harvested 48 and 72 hours post transfection. HL-60, TF-1 and Ba/F3 cells were infected with the viral supernatants in the presence of 8 μg/ml of polybrene and selected with 2 μg/ml puromycin 48 hours later. Cells that survived puromycin selection were examined for expression of Gfi-1 and Miz-1 by Western blot analysis. Alternatively, lentivirus-infected cells were subcloned by limiting dilution and individual clones were examined for Gfi-1 expression.

Luciferase reporter assay

HeLa cells were transfected using lipofactamine 2000. Forty eight hours after transfection, cells were harvested with or without pretreatment with TGFβ (5 ng/ml) for 6 hrs. Luciferase activities were measured using a Molecular Devices Lmax luminometer (Sunnyvale, CA) and normalized on the basis of the co-transfected β-galactosidase activity.

[^{3}H]thymidine incorporation assay

Ba/F3 cells (10^4) were incubated in triplicate in 100 μl of complete culture medium in the absence or presence of TGFβ at different concentrations in 96-well plates. After 24 hours of incubation, 1 μCi of [^{3}H]thymidine (International Chemical and Nuclear, Irvine, CA) was added to each well. The cells were harvested 12 hours later using a Micro Cell Harvester (Skatron Instruments, Sterling, VA) and radioactivity was measured by liquid scintillation counting in an LKB 1205 Betaplate counter. The results were collected as mean counts per minute (cpm) of triplicate wells.

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Abbreviations

ZF	zinc finger
DN	dominant negative
TGFβ	transforming growth factor-β
CDKI	cyclin dependent kinase inhibitor
BM	bone marrow
HSCs	hematopoietic stem cells

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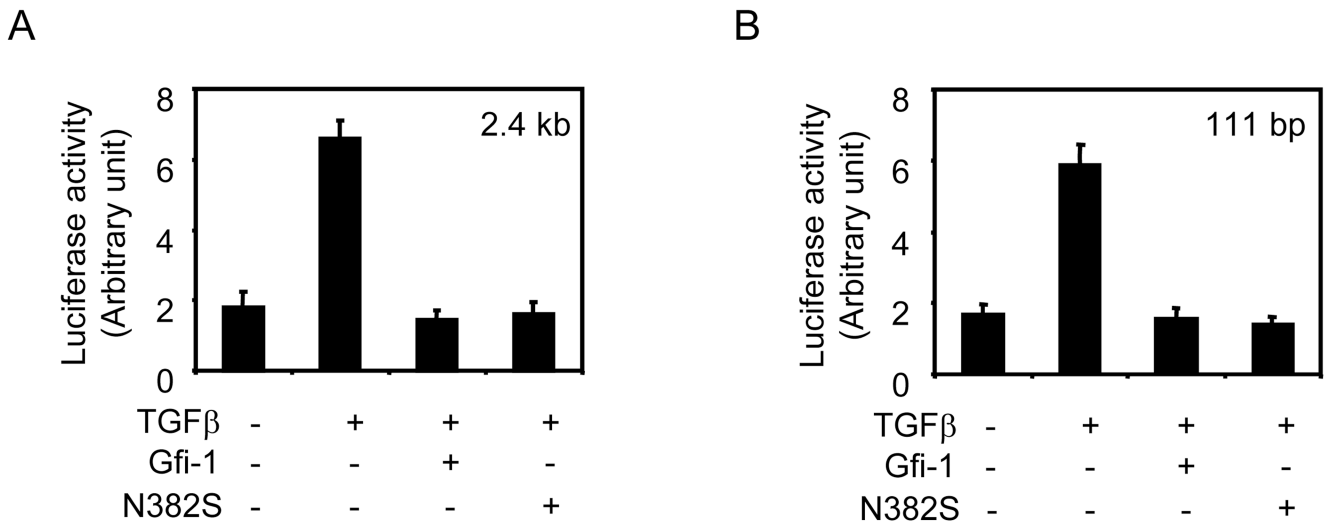

Fig. 1.

Gfi-1 represses TGFβ-induced activation of the *CDKN1A* promoter. Hela cells were transfected with the luciferase reporter constructs containing the 2.4-kb (**A**) or 111-bp (**B**) promoter fragment of *CDKN1A*, along with Gfi-1 or the N382S mutant. Cells were subsequently incubated in the absence or presence of TGFβ (5 ng/ml) for 6 hrs. Luciferase activities were measured and normalized for the co-transfected SV40 β-gal activities. Data are shown as mean ±SD.

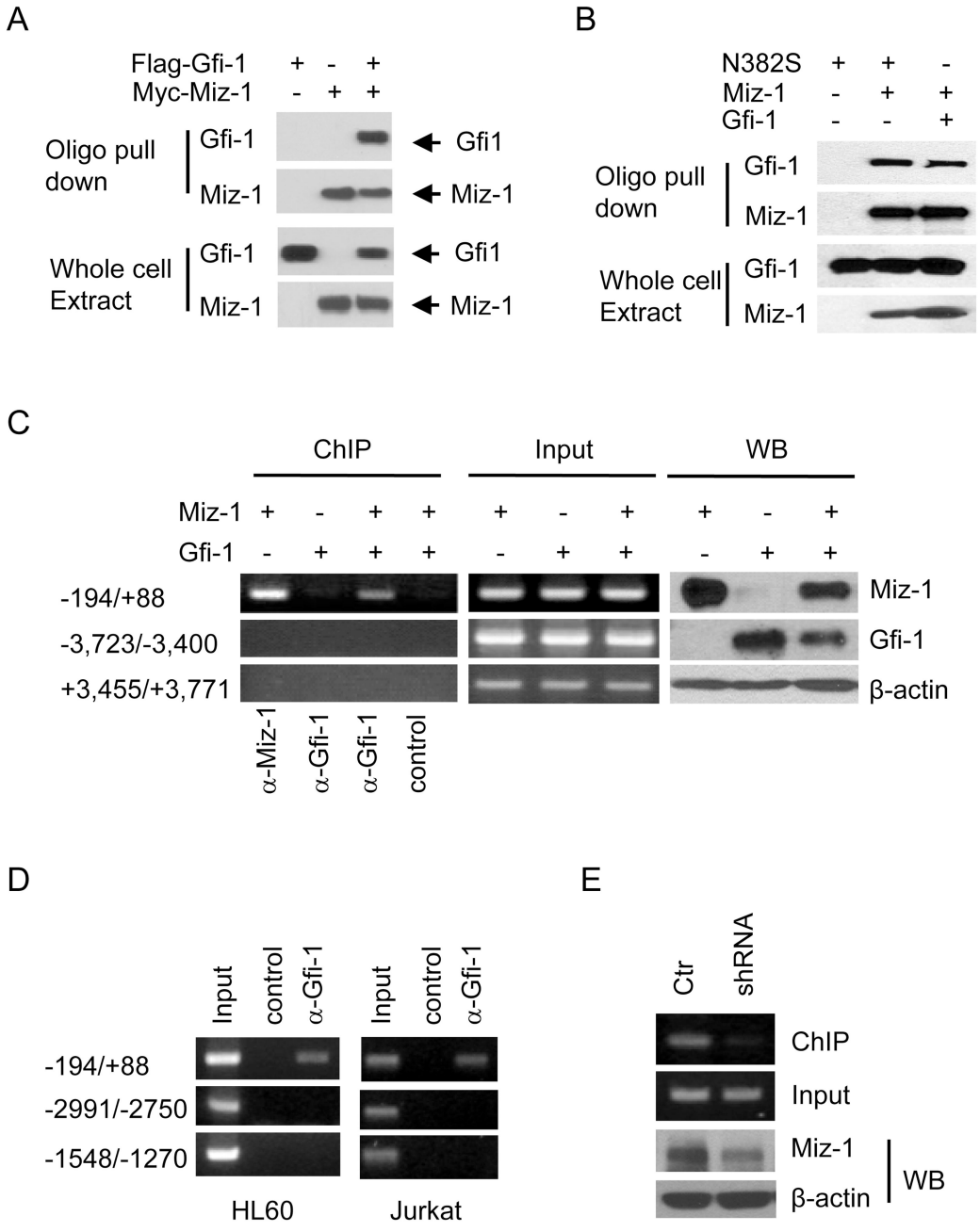

Fig. 2.

Miz-1 recruits Gfi-1 to the core promoter of *CDKN1A*. (**A** and **B**) 293T cells were transfected with Gfi-1 or the N382S mutant either alone or together with Miz-1. Whole cell extracts were subjected to precipitation using the *CDKN1A* promoter oligonucleotide and examined for the indicated proteins by Western blot analysis. (**C**) 293T cells were transfected with Gfi-1 and/or Miz-1. Chromatin DNA from the cells was immunoprecipitated using the antibodies against Gfi-1, Miz-1 or an irrelevant species-matched antibody (control). The indicated regions of *CDKN1A* promoter were amplified by

semi-quantitative PCR. (**D**) ChIP assays were performed on HL-60 and Jurkat cells. The indicated promoter regions of *CDKN1A* were amplified by semi-quantitative PCR. (**E**) HL-60 cells were infected with the empty (Ctr) or Miz-1 shRNA lentiviral construct. ChIP assays were performed using the anti Gfi-1 antibody, followed by PCR amplification of the *CDKN1A* core promoter region. Expression of Miz-1 was examined by Western blot analysis.

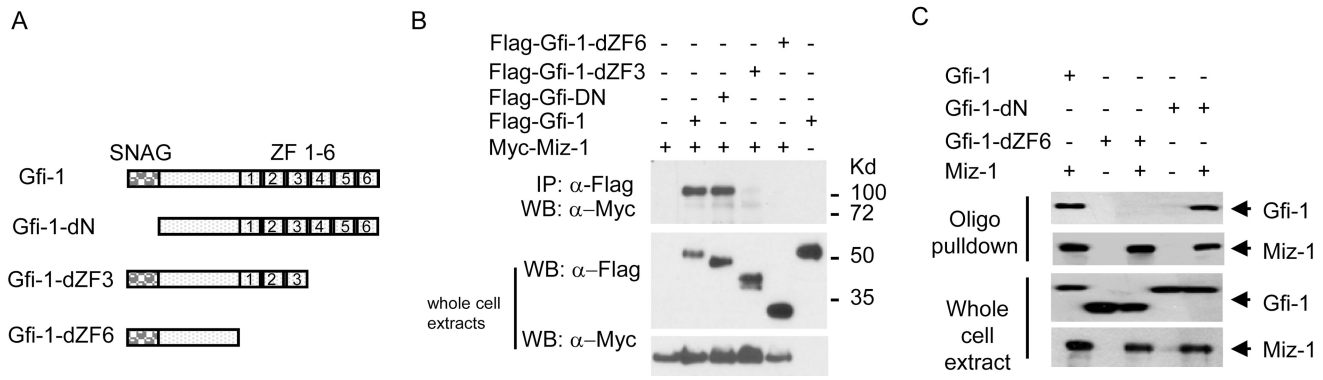

Fig. 3.

Gfi-1 interacts with Miz-1 through its C-terminal ZF domains. (**A**) Schematic diagrams of Gfi-1 and the different mutants. (**B**) 293T cells were transfected with Myc-tagged Miz-1 along with the different forms of Flag-tagged Gfi-1. Whole cell extracts were examined by Western blotting with the anti-Myc and anti-Flag antibodies (middle and bottom panels), or subjected to immunoprecipitation with the anti-Flag antibody prior to Western blotting with the anti-Myc tag antibody (top panel). (**C**) Whole cell extracts from 293T cells transfected with the indicated expression constructs were subjected to precipitation using the *CDKN1A* promoter oligonucleotide. Bound proteins were examined using the anti Gfi-1 and anti Miz-1 antibodies.

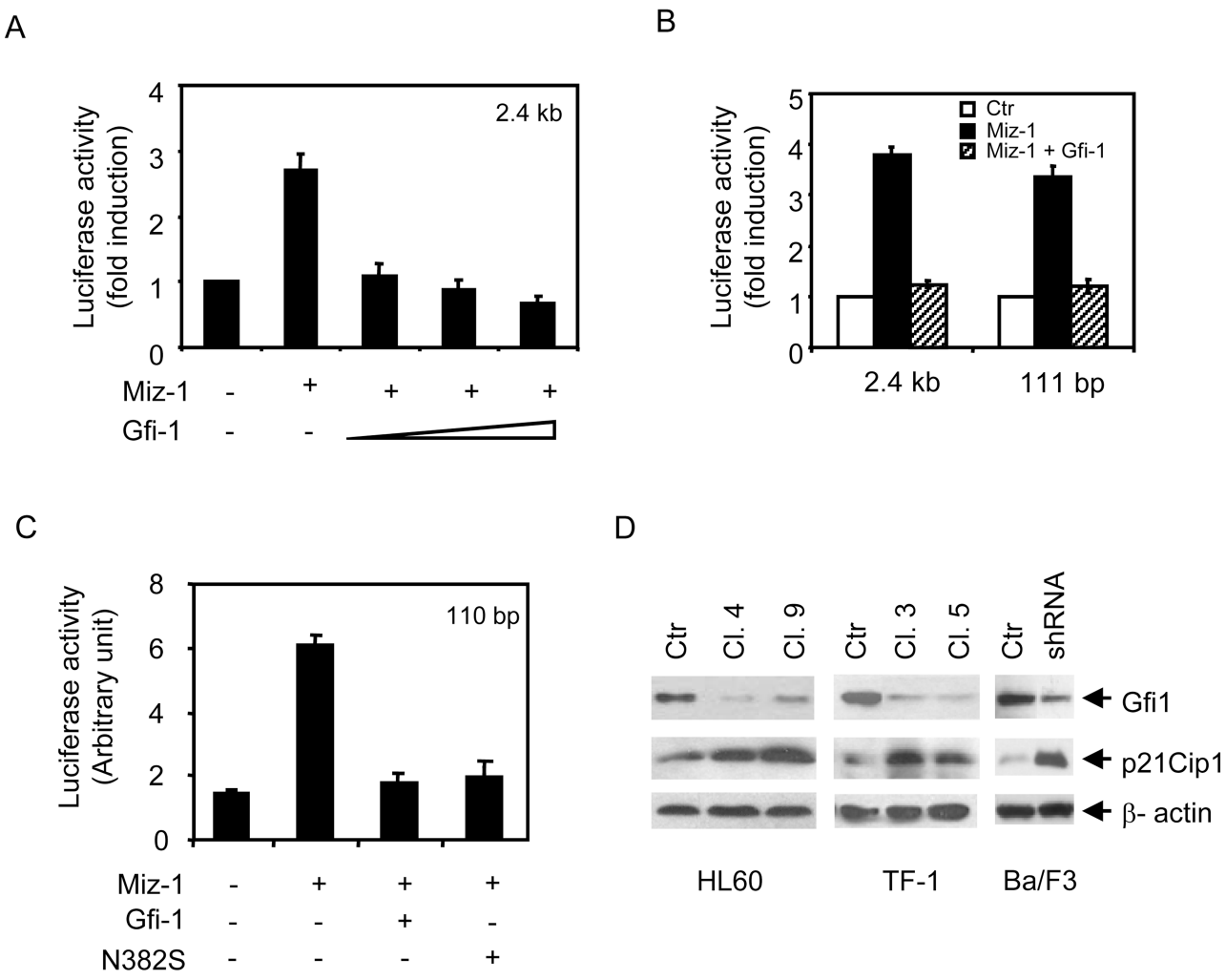

Fig. 4.

Gfi-1 represses Miz-1-mediated activation of the *CDKN1A* promoter. (**A**) Hela cells were transfected with the *CDKN1A* luciferase reporter construct (2.4 kb) and Miz-1 along with increasing amounts of Gfi-1. Luciferase activity was measured 36 hrs later. (**B**) Hela cells were transfected with the *CDKN1A* luciferase reporter constructs (2.4 kb or 111 bp) along with Miz-1 and Gfi-1 prior to assay for luciferase activity. (**C**) Hela cells were transfected with the *CDKN1A* core promoter (111 bp) luciferase reporter construct along with Miz-1 and Gfi-1 or the N382S mutant. Luciferase activities were measured. (**D**) HL-60, TF-1 and Ba/F3 cells were infected with the empty lentiviral construct (Ctr) or the lentiviral construct containing the shRNAs against Gfi-1. The expression of Gfi-1 and p21Cip1 in individual clones of HL-60 and TF-1 cells and in pooled Ba/F3 cells were examined by Western blot analysis.

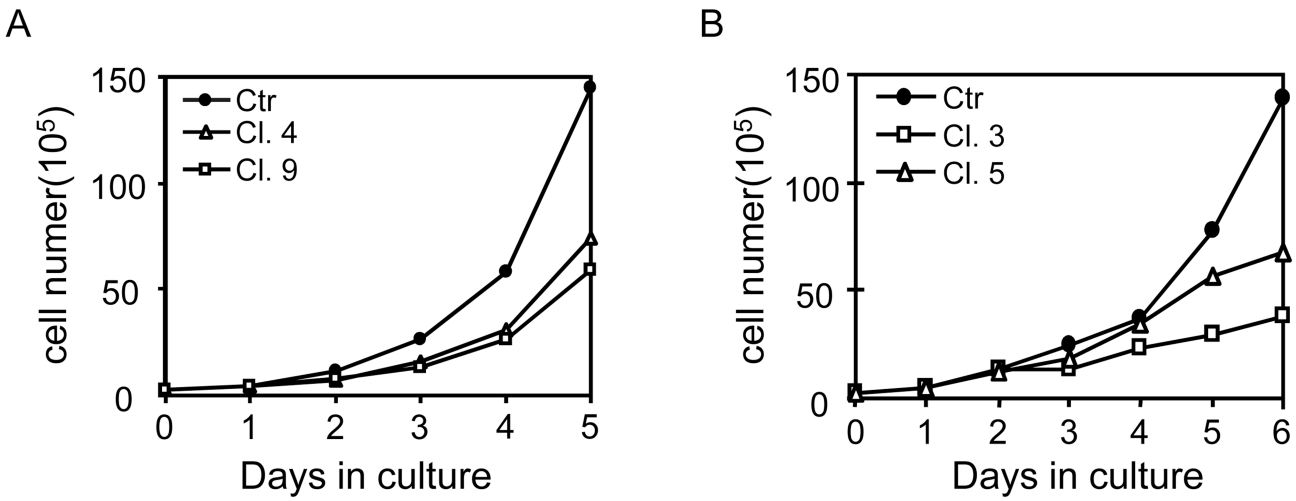

Fig.5.

Gfi-1 knockdown suppresses the proliferation of HL-60 and TF-1 cells. HL-60 (**A**) and TF-1 (**B**) cells infected with the empty lentiviral construct (Ctr) and two independent clones in which Gfi-1 expression was knocked down were cultured in complete culture medium. The numbers of living cells were determined by exclusion of trypan blue staining on a daily basis.

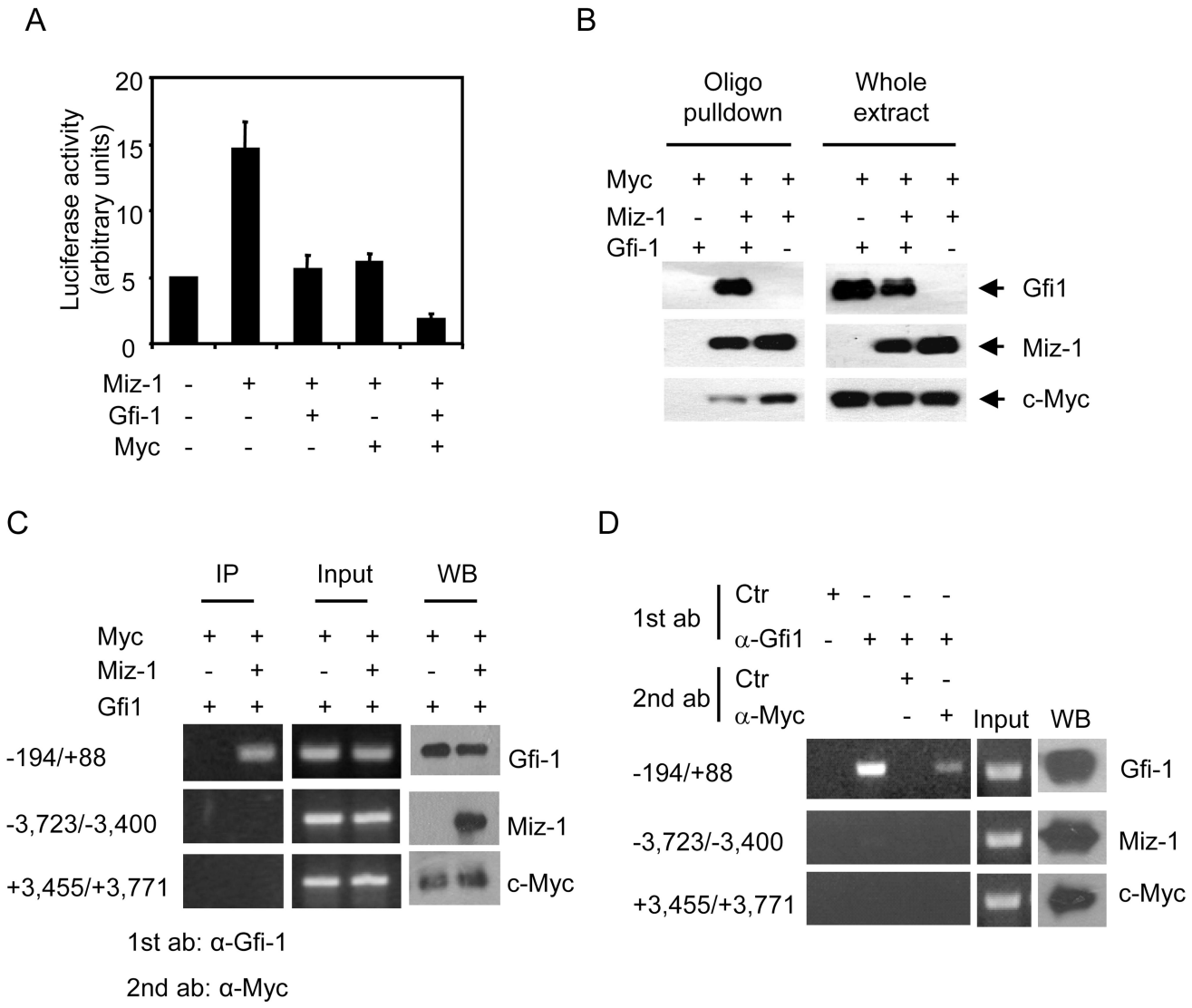

Fig. 6.

Gfi-1 and c-Myc collaborate in the repression of *CDKN1A*. (**A**) Hela cells were transfected with the *CDKN1A* promoter luciferase reporter construct (2.4 kb) and Miz-1 along with Gfi-1 and/or c-Myc prior to assay for luciferase activity. (**B**) Whole cell extracts were prepared from 293T cells transfected with the indicated expression constructs and subjected to precipitation using the *CDKN1A* promoter oligonucleotide. Whole cell extracts and precipitated proteins were examined for the indicated proteins. (**C**) Re-ChIP assays were performed on 293T cells transfected with Gfi-1 and c-Myc without or with Miz-1. Soluble chromatin from the cells was immunoprecipitated with the anti-Gfi-1 antibody, eluted from beads and re-immunoprecipitated with the anti-c-Myc antibody followed by semi-quantitative PCR analysis. (**D**) ChIP and Re-ChIP assays were conducted on 293T cells transfected with Gfi-1, Miz-1 and c-Myc using the irrelevant species-matched antibody (Ctr) or the antibodies against Gfi-1 and c-Myc as indicated.

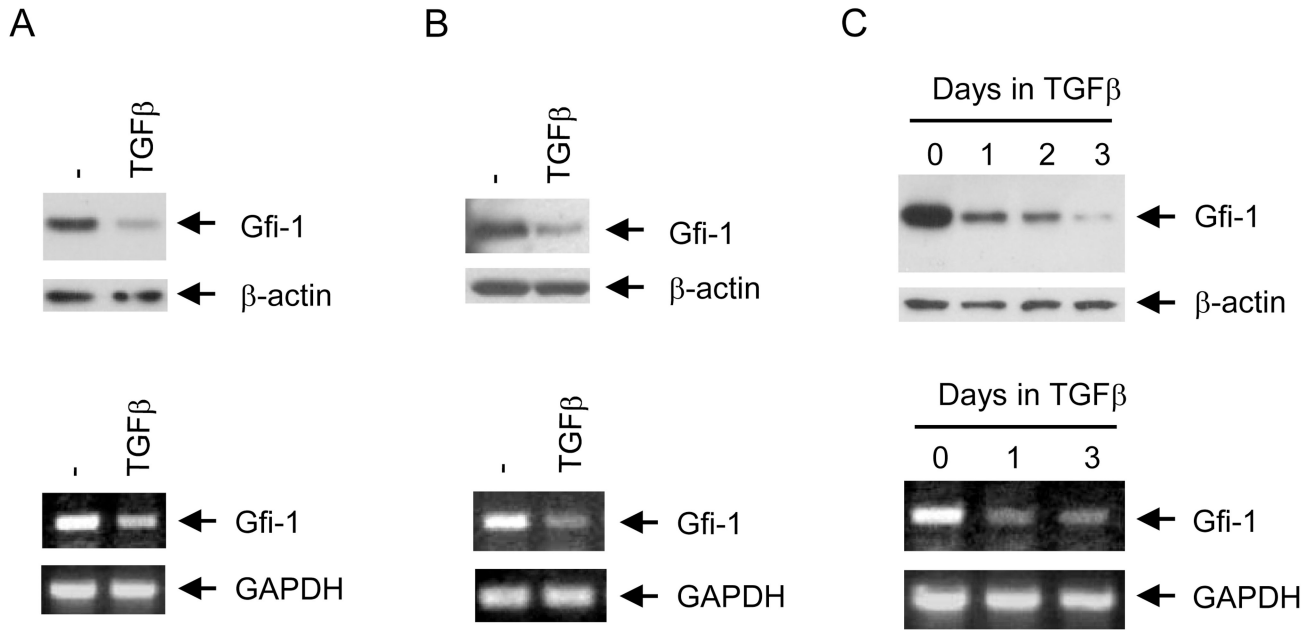

Fig. 7.

TGFβ downregulates the expression of Gfi-1. HL-60 (**A**), TF-1 (**B**) and U937 (**C**) cells were treated with TGFβ (5 ng/ ml) for 24 hours or days as indicated. Whole cell extracts were prepared and examined for Gfi-1 by Western blot analysis (upper panels). Total RNA was extracted and Gfi-1 transcript was examined by semi-quantitative RT-PCR (lower panels).

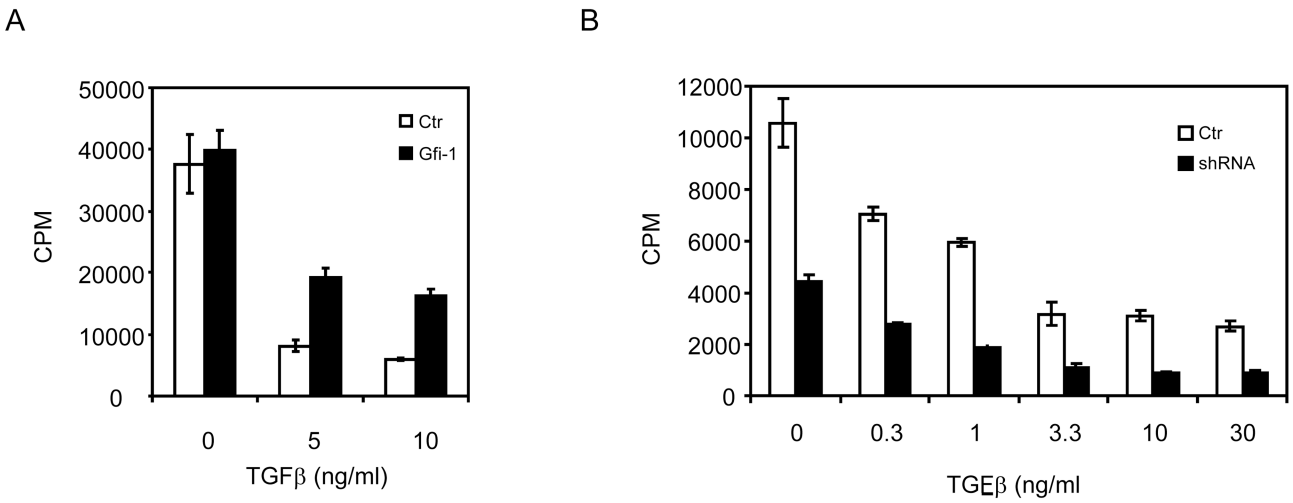

Fig. 8.

Gfi-1 regulates the sensitivity to the cytostatic effect of TGFβ. (**A**) Ba/F3 cells were transfected by electroporation with GFP-RV (Ctr) or Gfi-1-RV, and sorted based on GFP expression 24 hours later. (**B**) Ba/F3 cells were infected with the empty lentiviral construct (Ctr) or the lentiviral construct containing Gfi-1 shRNA. Cells were cultured in the absence or presence of TGFβ at the indicated concentrations and cell proliferation was measured by [^{3}H]thymidine incorporation assay. Data are represented as mean counts per minute (cpm) of triplicate wells.

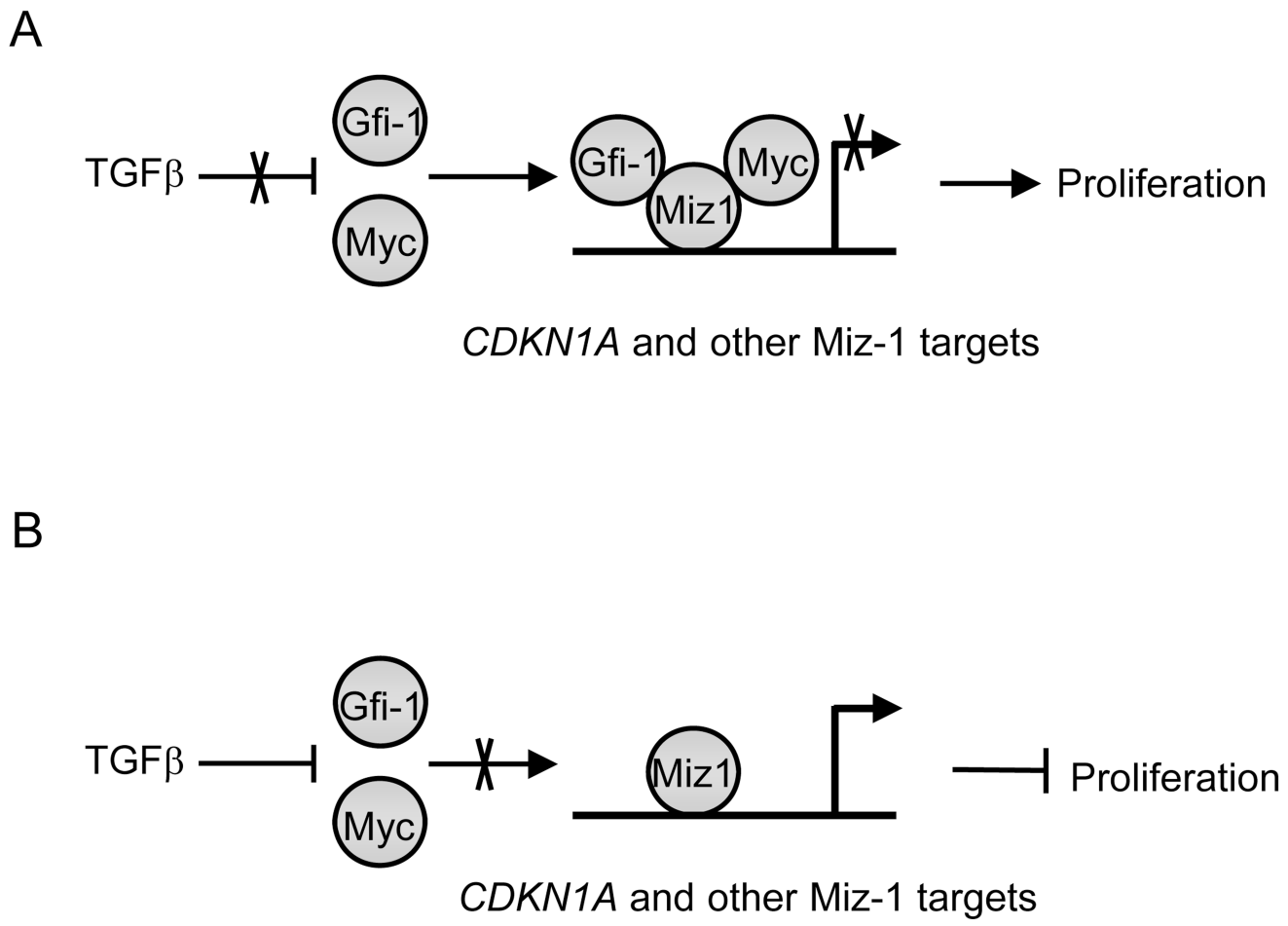

Fig. 9.

A model of Gfi-1-mediated repression of *CDKN1A* and other Miz-1 target genes. (**A**) In the absence of TGFβ or if the TGFβ signaling pathway is defective, Gfi-1 and Myc are expressed and recruited to *CDKN1A* and other Miz-1 target genes via Miz-1, leading to transcriptional repression and augmented cell growth. (**B**) TGFβ downregulates the expression of Gfi-1 and c-Myc. As a consequence, Miz-1 binds *to CDKN1A* and other Miz-1 target genes to activate their expression, causing reduced cell proliferation.