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Transcriptional Regulation of GATA-3 by an Intronic Regulatory Region and Fetal Liver Zinc Finger Protein 1

Eun Sook Hwang,* Andrew Choi,* and I-Cheng Ho2*†

GATA-3 is a T cell-specific transcription factor and is essential for the development of the T cell lineage. The transcriptional regulation of GATA-3, however, remains elusive. In this study, we report the identification of a regulatory region located within the first intron of the murine GATA-3 gene. The intronic regulatory region contains both a positive and a negative cis-acting element but, as a whole, serves as a potent T cell-specific enhancer and is essential for the promoter activity in vitro. By using yeast one-hybrid screening, we discovered that fetal liver zinc finger protein 1 (Fliz1) could bind specifically to the negative cis-acting element, the sequence of which is conserved between the mouse and human GATA-3 genes. More importantly, overexpression of Fliz1 repressed the expression of GATA-3 in vivo and in vitro. Our data suggest that the expression of GATA-3 might be partly regulated by the intronic regulatory region and Fliz1 in a developmental stage-specific fashion.


While the function of GATA-3 in regulating the development of T cell lineage and the differentiation of Th2 cells are well characterized (1–4), the molecular mechanisms mediating its T or Th2 cell-specific expression remain unclear. In adult animals, GATA-3 is mainly expressed in the T cell lineage (5, 6). The expression of GATA-3 can be readily detected in various stages during thymic ontogeny (2). However, the level of GATA-3 transcripts is relatively low, if not detectable at all, in naive peripheral Th cells, which quickly up-regulate the expression of GATA-3 upon encountering Ags under the conditions favoring the development of type 2, but not type 1, Th cells (3, 4, 7). In addition to the T cell lineage, the expression of GATA-3 can be detected in nonlymphoid organs, such as nervous tissue and kidney, during embryogenesis (8). Taken together, these results indicate that the expression of GATA-3 is regulated by a complex mechanism that is subjected to temporal and spatial modification.

The murine GATA-3 locus contains several T cell-specific DNase I hypersensitivity sites. An ∼2.5-kb genomic fragment encompassing several of the DNase I hypersensitivity sites is sufficient to support the expression of a reporter gene in a T cell-specific manner in vitro (8, 9). A recent report showed that the T cell specificity of the human GATA-3 gene might be regulated by an upstream silencer and a positive cis-acting element, which is located in the 3′ end of the first intron, however, neither of the regulatory elements was cell-type specific (10, 11). Even more elusive are the identities of the transcription factors that dictate the expression of the GATA-3 gene. Mice rendered deficient in NF-κB p50−/− or mel-18, a polycomb protein, have defects in inducing Th2 immune responses, and their Th cells express lower levels of GATA-3 (12, 13). It remains to be determined, however, whether the subnormal GATA-3 level is the cause or the result of impaired Th2 responses in these mice. Although it was reported that E-box binding proteins could bind to the upstream silencer of the human GATA-3 gene (10), the in vivo functions of E-box binding proteins in regulating the expression of GATA-3 remain unclear.

Fetal liver zinc finger protein 1 (Fliz1)3 is a recently cloned CCCH-type zinc finger protein (14). Thus far, only a handful of CCCH-type zinc finger proteins have been identified, nearly all of which function as RNA binding proteins (15–17). For example, TIS11/Nup475/TTP is a critical protein that regulates the stability and levels of TNF-α transcripts by specifically binding to an AU-rich element in the 3′ untranslated region of the transcripts (15–17). During embryogenesis, Fliz1 is expressed nearly exclusively by hemopoietic progenitors in fetal livers (14). Its unique tissue distribution suggests that it might play a critical role in regulating the development and maturation of hemopoietic cells.

In this study, we report that the 3′ end of the first intron of the murine GATA-3 gene contains a very potent regulatory region, which is composed of both a negative and a positive cis-acting element. As a whole, the intronic regulatory region (IRR) serves as a T cell-specific enhancer and is essential for the activity of the GATA-3 promoter. By using yeast one-hybrid screening, we uncovered that Fliz1 could bind to the negative cis-acting element in a sequence-specific fashion. Overexpression of Fliz1 in vitro and in vivo substantially repressed the activities of exogenous and endogenous GATA-3 promoters. Taken together, our data demonstrate that the transcriptional regulation of GATA-3 might be partly regulated by IRR and Fliz1.

Materials and Methods

Plasmids and constructs

A genomic fragment containing the upstream region (up to −1475 bp), the first exon, the first intron, and the untranslated part of the second exon (up

3 Abbreviations used in this paper: Fliz1, fetal liver zinc finger protein 1; IRR, intronic regulatory region; mFliz1, murine Fliz1; RT, reverse transcription; TCF1, T cell-specific factor 1; TK, thymidine kinase; hFliz1, human Fliz1.
of +901 bp) of murine GATA-3 gene was amplified from genomic DNA prepared from M12 cells. PCR amplification was performed by using the Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA), and the sequences of the genomic fragment thus obtained were confirmed by sequence analysis and were found to be identical to the published sequences of the GATA-3 promoter. This genomic fragment was cloned into the BgIII site of a luciferase reporter plasmid, pBS-luc (a gift of Dr. K. Murphy, Washington University School of Medicine, St. Louis, MO), to form the parental construct 2.5-kb GATA-3-luc. Subsequent deletion mutants as indicated in Fig. 1A were generated by restriction digestion. Sub-fragments of IRR were generated by restriction digestion or PCR amplification and cloned into an enhancer trap vector, thymidine kinase (TK)-luc (a gift of Dr. T. Hoey, Tularik, South San Francisco, CA), upstream to a minimal thymidine kinase promoter. The mutants of D-25-bp TK-luc reporter gene were generated by site-directed mutagenesis according to the manufacturer’s manual (Clontech Laboratories, Palo Alto, CA).

Cell culture and transfection assays
Murine T cell lines (EL4, WEHI7.1, and BW5147) and B cell lines (M12, 18.81, and Bal 17) were maintained and transfected in RPMI 1640 supplemented with 10% FCS (Life Technologies, Grand Island, NY). Transfection of lymphoid cells (5 × 10^6 cells/transfection) was performed by electroporation at 280 V/975 μF with a total 20 μg of DNA. NIH3T3 and Cos7 cells were cultured in DMEM supplemented with 10% FCS and transfected with Effectene reagent according to the manufacturer’s protocol (Qiagen, Valencia, CA). Cell extracts were prepared in reporter lysis buffer (Promega, Madison, WI) and luciferase activity was assayed by using a luminometer. All transfection experiments were performed at least four times and included a pCMVβ (Clontech Laboratories) reporter to serve as an internal control.

EMSA
The truncated cDNA of murine Fliz1 (mFliz1) obtained from the yeast one-hybrid screening was cloned in-frame into the pET29 vector (Novagen, Madison, WI). The resulting plasmid was used to transform BL21 (DE3). The transformed BL21 (DE3) cells were induced with 1 mM isopropyl β-D-thiogalactoside at 37°C for 2 h to produce recombinant truncated mFliz1. The recombinant protein was enriched in inclusion bodies, solubilized with 6 M urea, and refolded in PBS. One microgram of such prepared rmFliz1 was used in each EMSA reaction. Double-stranded oligonucleotides corresponding to the D-25 bp or SP1 binding sequences were labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) in the presence of [γ-32P]ATP (New England Nuclear, Boston, MA), and 10,000 cpm of labeled oligomer was incubated with rmFliz1 protein in binding buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 20 mM KCl, 4 mM MgCl2, 5 mM DTT, 1 μg polyclonal antibody) at room temperature for 20 min. Complexes were resolved on 4% polyacrylamide gels and subjected to autoradiography. For competition assay, excessive unlabeled D-25 bp or SP1 oligonucleotides were preincubated with rmFliz1 protein before incubation with specific probes.

Immunoblot analysis
Whole cell extracts were prepared by NE-PER (Pierce, Rockford, IL), resolved on 10% SDS-PAGE gels, and transferred to nitrocellulose membranes (BioRad, Hercules, CA). The membranes were incubated with mouse monoclonal anti-myc (9E10) Ab (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-GAL4 DB Ab (Santa Cruz Biotechnology) diluted 1/500 or 1/1000, respectively, in PBS containing Tween 20, followed by incubation with HRP-conjugated secondary Abs and detection with ECL kit according to the manufacturer’s instruction (Amersham, Piscataway, NJ).

Real-time PCR
Total RNA was prepared and 1 μg of total RNA was used for reverse transcription (RT) reaction and amplification by using a Superscript II RT kit according to the manufacturer’s protocol (Invitrogen). A master mix of TaqMan reagents was prepared and 10 ng of each RT product was used in TaqMan PCR (Applied Biosystems, Foster City, CA). The standard curve method was used to quantitate the amounts of each species of transcripts.

**FIGURE 1.** Identification of a T cell-specific regulatory region in the first intron of murine GATA-3 gene. A and B, 2.5-kb GATA-3-luc or its various deletion mutants (left) was used to transfect the indicated cell lines. The resulting luciferase activities were normalized against the minimal promoter activity (0.3-kb pBS-luc), which was arbitrarily set as 1. E1 and E2 stand for the first and the second exons, respectively. C, The −100-bp fragment from the 3’ end of the first intron and its various deletion mutants were cloned into TK-luc, and 10 μg of each resulting plasmid were used for transfection. The luciferase activities were normalized against that obtained from empty TK-luc, which was arbitrarily set as 1. D, EL4 cells were transfected with wild-type D-25 bp TK-luc (WT) or its various mutants. The luciferase activities were normalized against the wild-type activity, which was set as 100%. The sequences listed are those of wild-type D-25 bp and its various mutants. The boxed regions indicate conserved sequences between human and mouse.
relative to the β-actin in each reaction. Reactions were conducted in 96-well plates by using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The sequences of primers and probes are: GATA-3; 5’-AGAAGCAGGCCTTTATCAA-3’, 5’-AGTTCGCGCAGGATGTTCC-3, 6’-FAM-CACAGCGAAGGCTGTCGGCAG-TAMRA, T cell-specific factor 1 (TCF1); 5’-CGCTGTCATACACCAGATC-3’, 5’-ACCTTGCCCTGCTCCTATTGAG-3’, 5’-AGAACCGGCCCCTTATCAAGAA-3’, 5’-TACGACCAAGGCGATACGGGACAA-3’, 5’-FAM-TGGTGCAGTGGCAGTCA-3’. The sequences of primers and probes are: GATA-3; 5’-ACAAACTGGGACCGATATGGAGAAGA-3’, 5’-AGTTTCGCGCAGGATGTTCC-3’, 6’-FAM-CACAGCGAAGGCTGTCGGCAG-TAMRA. T cell-specific factor 1 (TCF1); 5’-CGCTGTCATACACCAGATC-3’, 5’-ACCTTGCCCTGCTCCTATTGAG-3’, 5’-AGAACCGGCCCCTTATCAAGAA-3’, 5’-TACGACCAAGGCGATACGGGACAA-3’, 5’-FAM-TGGTGCAGTGGCAGTCA-3’. The resulting constructs were separately injected into ICR mouse zygotes. The resulting mutant probes were then radiolabeled and used in EMSA (data not shown). These results suggest that Fliz1 can function as a DNA binding protein and can specifically bind to the AGGTCTC sequence within IRR.

**Molecular cloning of Fliz1, which binds to IRR in a sequence-specific fashion**

To identify trans-acting factors that might interact with IRR, we performed yeast one-hybrid screening by using the D-25-bp fragment as bait to screen a murine Th2 cDNA library, which was described previously (21). cDNA clones thus obtained were further examined by EMSA by using recombinant proteins derived from each clone. One clone thus identified encodes a novel open reading frame that contains three CCCH-type zinc finger domains, the sequences of which are identical to those of the recently cloned mFliz1. The truncated mFliz1 cDNA obtained from the yeast one-hybrid screening lacks 90 amino acid residues in the N terminus which were subsequently obtained by 5’ RACE. In addition, we also obtained human Fliz1 (hFliz1) cDNA from several expressed sequence tag clones (GenBank accession numbers AL530577, AL563786, and AW958306). Sequence analysis revealed that Fliz1 is highly conserved between mouse and human. To determine whether Fliz1 can bind to the D-25-bp fragment in a sequence-specific fashion, we repeated EMSA. As shown in Fig. 2A, rmFliz1 proteins strongly bind to the D-25 bp, but not to a double-stranded oligonucleotide containing a consensus SP1 binding site. In addition, the formation of the Fliz1/D-25-bp complex was completely inhibited by excessive amounts of unlabeled D-25 bp, but not by unlabeled SP1 probe (Fig. 2B). To determine the binding sequences of Fliz1 within the D-25 bp, we introduced a series of single base pair mutations into the D-25-bp probe. The resulting mutant probes were then radiolabeled and used in EMSA reactions. Interestingly, any single base pair mutation within the conserved AGGTCTC sequence dramatically attenuated the binding of Fliz1 protein (Fig. 2C). In contrast, mutations outside the AGGTCTC sequence did not affect the binding of Fliz1. Taken together, these results demonstrate that Fliz1, a CCCH-type zinc finger protein, can function as a DNA binding protein and can specifically bind to the AGGTCTC sequence within IRR.

**Fliz1 can function as a transcriptional repressor of the GATA-3 promoter in vitro**

The fact that Fliz1 specifically binds to the AGGTCTC, overlapping with the negative cis-acting element within IRR, suggests that Fliz1 might function as a transcriptional repressor (Fig. 1D). To test this hypothesis, we performed cotransfection experiments. EL4 cells were cotransfected with the 2.5-bp GATA-3 reporter construct along with an expression vector of a myc-tagged hFliz1. As shown in Fig. 3A, overexpression of Fliz1 inhibited the activity of the 2.5-bp GATA-3 promoter in a dose-dependent manner (Fig. 3A). Similar results were obtained when the D-25-bp TK-luc was used (Fig. 3B, left panel). In contrast, overexpression of Fliz1 did not affect the activity of D-25-bp M3 TK-luc, which partially ablates the AGGTCTC sequence (Fig. 1D; Fig. 3B, right panel), or the U-25-bp TK-luc, which does not contain the AGGTCTC sequence (data not shown). These results suggest that Fliz1 can function as a transcriptional repressor of GATA-3 in vitro.
cells by in vitro transfection assays. The expression of the mutants was confirmed by immunoblot analysis (Fig. 4A, lower panel). We found that deletion of the N-terminal 102 amino acid residues resulted in an appreciable reduction in repression (Fig. 4A, Fliz1 103–291), whereas deletion of residues 192–291 (Fliz1 1–191), containing the zinc finger domains, completely abrogated the repression effects of Fliz1. These results indicate that residues 192–291 are essential for the repressor activity. The N-terminal 102 residues (Fliz1 1–102), while required for full repressor activity, are not sufficient to repress the promoter. However, it is possible that Fliz1 1–102, lacking the zinc finger domains, does not contain a DNA binding domain and cannot be recruited to the GATA-3 promoter. To test this hypothesis and to further identify the repression domain in the C terminus of Fliz1, we generated a series of constructs expressing various truncation mutants of Fliz1, which were fused to a GAL4 DNA binding domain. Each of the vectors yielded fusion proteins of expected m.w. as examined by immunoblot analysis (Fig. 4B, lower panel). The expression vectors and a luciferase reporter construct containing GAL4-UAS were used to transfect Cos7 cells. As expected, overexpression of full-length Fliz1 significantly reduced the luciferase activity to 23% (Fig. 4B, Fliz1 1–291), and a substantial repression effect was retained in the mutant Fliz1 192–291. Further removal of residues 192 to 218, containing the first zinc finger, resulted in a complete loss of repressor activity (Fig. 4B, Fliz1 219–291). Interestingly, the N-terminal 102 residues, when fused with GAL4 DB, also conferred moderate repression effects (Fig. 4B, Fliz1 1–102). These results demonstrate that Fliz1 contains at least two independent repression domains. One is located in the N-terminal 102 residues and the other between residues 192–218.

**Fliz1 attenuates the expression of GATA-3 in vivo**

To further examine whether Fliz1 can serve as a transcriptional repressor of the GATA-3 gene in vivo, we have generated transgenic mice overexpressing Fliz1 in a T cell-specific and tetracycline-inducible fashion. The generation of the mice was described in Materials and Methods. Mice were induced to express a myc-tagged Fliz1 with doxycycline or left uninduced before analyses, and the overexpression of the myc-tagged Fliz1 thymocytes was confirmed by immunoblot analysis (Fig. 5A, top panel). We found that overexpression of Fliz1 in vivo resulted in a substantial reduction (50–75%) in the levels of GATA-3 transcripts in thymocytes as examined by real-time PCR (Fig. 5A). Comparable results were obtained from two independently generated Fliz1 \times \text{rTA} transgenic mouse lines (Fig. 5B). The effect of Fliz1 is specific to the GATA-3 gene because no reduction was detected in the expression of other transcription factors such as TCF-1, LEF-1, T-bet, and c-maf (Fig. 5C; data not shown). In addition, the reduction in the levels of GATA-3 transcripts appears to be dependent on the

**Fliz1 contains at least two repression domains**

To map the functional domains that are required for the repressor activity of Fliz1, we performed structure-function analyses. We first created several truncation mutants of myc-tagged hFliz1 and examined their effects on the 2.5-kb GATA-3 promoter in EL4

**FIGURE 2.** Sequence-specific binding of Fliz1 protein to D-25 bp. A, rmFliz1 protein (1 μg) was used in each EMSA reaction as described in Materials and Methods. Radiolabeled wild-type D-25 bp and a double-stranded oligonucleotide containing a consensus SP1 binding site were used as probes. B, A radiolabeled wild-type D-25-bp probe was incubated with rmFliz1 protein in the absence (–) or presence of competitors. Unlabeled D-25 bp or SP1 probe, in indicated molar excess, was used as specific or nonspecific competitors, respectively. C, Wild-type D-25 bp and its various mutants carrying the indicated point mutations were radiolabeled, and each of the probes was incubated with rFliz1 protein in EMSA reaction. The arrows indicate mFliz1/DNA complexes.

**FIGURE 3.** Repression of GATA-3 promoter activity by Fliz1 in vitro. EL4 cells were cotransfected with 5 μg of 2.5-kb GATA-3-luc (A), D-25-bp TK-luc (B, left panel), or D-25-bp M3 TK-luc (B, right panel) along with a Fliz1 expression vector in indicated amounts. The upper panel in A is an immunoblot analysis of Fliz1 expression. The resulting luciferase activities were normalized against that of empty expression vector transfection, which was set as 100%.

**FIGURE 3.** Repression of GATA-3 promoter activity by Fliz1 in vitro. EL4 cells were cotransfected with 5 μg of 2.5-kb GATA-3-luc (A), D-25-bp TK-luc (B, left panel), or D-25-bp M3 TK-luc (B, right panel) along with a Fliz1 expression vector in indicated amounts. The upper panel in A is an immunoblot analysis of Fliz1 expression. The resulting luciferase activities were normalized against that of empty expression vector transfection, which was set as 100%.
Discussion

Although the function of GATA-3 in regulating the development of T cell lineages and the differentiation of Th2 cells are well characterized, very little is known about the molecular mechanism mediating its T cell-specific expression. Our data demonstrate that the expression of the murine GATA-3 gene might be partly regulated by the T cell-specific IRR, which is also essential for the activity of the GATA-3 promoter. The finding is somewhat different from that of a previous report showing that the 3' end of the first intron of the human GATA-3 gene, also required for promoter activity, did not confer T cell specificity (10). The discrepancy suggests that the transcriptional regulation of the human GATA-3 gene might differ from that of the murine GATA-3 gene. Despite our in vitro results, it needs to be cautiously pointed out that an ~625-kb genomic fragment encompassing the murine GATA-3 locus fails to support the expression of a reporter gene in lymphoid organs in vivo (22). This finding strongly indicates that cis-acting elements outside the 625-kb region are also required for the expression of the GATA-3 gene in T cells. Murine IRR and the upstream silencer of the human GATA-3 gene, however, might contribute more to T cell-specificity than to activity in regulating the expression of the GATA-3 gene. In addition, IRR probably has a very limited role in dictating the expression of GATA-3 in Th2 cells, because IRR is comparably active in both Th1 and Th2 cells in in vitro transfection experiments (data not shown). The real function of IRR can be addressed by studying "knock-in" mice bearing deletion or mutations of IRR via homologous recombination.

It is still unclear what transcription factor, if any, binds to the positive cis-acting element within IRR. Various attempts of EMSA have failed to show any binding activity to IRR in endogenous T cell extracts. Repeated yeast one-hybrid screenings were also nonrevealing. Possibly, endogenous IRR-binding proteins, including Fliz1, are present in very low levels or are degraded during the process of preparation. Furthermore, alternative EMSA binding

![FIGURE 4. Identification of repression domains of Fliz1. A. The indicated deletion mutants of myc-tagged Fliz1 were constructed in pCMV vector and, along with 2.5-kb GATA-3-luc, were used to transfect EL4 cells. The lower panel is an immunoblot analysis of Fliz1 by using anti-myc Abs. B. Full-length Fliz1 and the indicated deletion mutants were fused in-frame with a GAL4 DNA binding domain (GAL4 DB) and, along with GAL4-UAS- luc, were used to transfect Cos7 cells. The expression of Fliz1 was detected by using anti-GAL4 DB Ab. The resulting luciferase activities were normalized against that of empty expression vector transfection, which was set as 100%. The left panels in A and B are schematic diagrams of full-length and truncated Fliz1. Zn, zinc finger domains.](http://www.jimmunol.org/)

![FIGURE 5. Attenuation of GATA-3 expression by Fliz1 in vivo. Fliz1 × rtTA (A–C) or Fliz1-ΔN × rtTA (D) double-transgenic mice littermates were fed with control water (−Dox) or doxycycline-containing water (+Dox) for 4 days before sacrifice. The induced expression of Fliz1 and Fliz1-ΔN in thymocytes was confirmed by immunoblot analyses by using anti-myc Abs (A and D, top panels). Total RNA was prepared from thymocytes and the levels of GATA-3 (A, B, and D) and TCF-1 (C) transcripts were measured by real-time PCR as described in Materials and Methods. The data shown are the relative levels of GATA-3 transcripts after normalization against the levels of β-actin transcripts. Data shown in A and D are representatives from #1 Fliz1 × rtTA and the Fliz1-ΔN × rtTA lines, respectively. The error bars were calculated from triplicate real-time PCR readings. Data shown in B and C are cumulative results derived from #1 and #2 Fliz1 × rtTA lines. B, Twenty mice from each experimental group were examined for GATA-3 expression. C, Four mice from each experimental group were examined for TCF1 expression. B and C, The levels of GATA-3 and TCF1 transcripts were further normalized against those of −Dox groups, which were arbitrarily set as 1.](http://www.jimmunol.org/)
conditions might be needed to allow the formation of protein-DNA complexes.

Although our data firmly demonstrate that overexpression of Fliz1 can repress the activities of the GATA-3 promoter in vitro and in vivo, the real function of Fliz1 remains unclear. Of note, during embryogenesis, Fliz1 is exclusively expressed in fetal liver hemopoietic progenitors (14). It is tempting to postulate that Fliz1 might play a critical role in lineage determination by repressing GATA-3 expression and allowing the development of non-T cells. In adult animals, Fliz1 is expressed at low levels in limited numbers of organs, including thymus, and it has been shown that GATA-3 levels vary in thymocytes maturing from the double-negative to the single-positive stage (2, 23). Thus, it is possible that Fliz1 might contribute to the subtle variation of GATA-3 levels during thymic ontogeny. Alternatively, Fliz1 might regulate the expression of other genes, in addition to GATA-3, which have yet been identified. In the inducible Fliz1-transgenic mice, a very low level of Fliz1 was induced in peripheral T cells and precluded analyses on its effects on peripheral T cells (data not shown). How- ever, Fliz1 probably has a very limited role in regulating the Th2 cell-specificity of GATA-3, given the very low expression of endogenous Fliz1 in the peripheral T cells (14). Generation and study of Fliz1-deficient mice will be very informative.

The observation that Fliz1 can function as a sequence-specific DNA binding protein merits further discussion. Thus far, nearly all CCCH-type zinc finger proteins are RNA binding proteins (15–19). To the best of our knowledge, Fliz1 is the first sequence-specific DNA-binding CCCH-type zinc finger protein. It is still very likely, however, that Fliz1 can also bind to RNA with a yet-to-be-defined sequence. Thus, Fliz1 might regulate the levels of GATA-3 by altering the stability of GATA-3 transcripts in addition to repressing the transcription of the GATA-3 gene.

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