THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 19, pp. 16832–16845, May 13, 2011
Printed in the U.S.A.

HoxA10 Influences Protein Ubiquitination by Activating Transcription of *ARIH2*, the Gene Encoding Triad1*

Received for publication, December 18, 2010, and in revised form, March 7, 2011 Published, JBC Papers in Press, March 28, 2011, DOI 10.1074/jbc.M110.213975

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HoxA10 is a homeodomain transcription factor that is maximally expressed in myeloid progenitor cells. An increase in HoxA10 expression correlates with poor prognosis in human acute myeloid leukemia (AML). Consistent with this scenario, HoxA10 overexpression in murine bone marrow induces a myeloproliferative neoplasm that advances AML over time. Despite the importance of HoxA10 for leukemogenesis, few genuine HoxA10 target genes have been identified. The current study identified *ARIH2*, the gene encoding Triad1, as a HoxA10 target gene. We identified two distinct HoxA10-binding cis elements in the *ARIH2* promoter and determined that HoxA10 activates these cis elements in myeloid cells. Triad1 has E3 ubiquitin ligase activity, and we found that HoxA10-overexpressing myeloid cells exhibited a Triad1-dependent increase in protein ubiquitination. Therefore, these studies have identified the regulation of protein ubiquitination as a novel function of Hox transcription factors. Forced overexpression of Triad1 has been show previously to inhibit colony formation by myeloid progenitor cells. In contrast, HoxA10-overexpressing myeloid progenitor cells exhibited increased proliferation in response to low doses of various cytokines. We found that Triad1 knockdown further increased cytokine-induced proliferation in HoxA10-overexpressing cells. Therefore, these studies have identified a HoxA10 target gene that antagonizes the overall influence of overexpressed HoxA10 on myeloproliferation. This result suggests that the consequences of HoxA10 overexpression reflect a balance between the target genes that facilitate and antagonize proliferation. These results have implications for understanding the mechanisms of leukemogenesis in AML with Hox overexpression.

HOX genes encode a set of highly conserved homeodomain transcription factors that are involved in the regulation of embryogenesis and definitive hematopoiesis. *HOX* genes are arranged in four groups (A–D) on four different chromosomes in mouse and man (1). Each group includes between 9 and 11 genes, numbered according to the homology between groups (1). *HOX* gene transcription is tightly regulated during hematopoiesis, with 5′ *HOX* genes (*HOX1–4*) actively transcribed in hematopoietic stem cells and 3′-genes (*HOX7–13*) activated in committed progenitors (2). Decreased *HOX* gene transcription is also important for the normal progression of hematopoiesis (2).

Increased expression of a specific group of *HOX* genes (HoxB3, -B4, -A7, -A9, and -A10) is associated with poor prognosis in human AML[2] (3–5). In AML, Hox expression is both increased and prolonged in differentiating hematopoietic cells. Several murine models support the functional significance of aberrant Hox expression for myeloid leukemogenesis (6–12). For example, overexpression of HoxB3 or HoxB4 in murine bone marrow expands the hematopoietic stem cell population *in vitro* and *in vivo*. Also, overexpression of HoxA9 or HoxA10 expands the common granulocyte/monocyte progenitor population *in vitro* and results in the development of a myeloproliferative neoplasm *in vivo* (8–12). The myeloproliferative neoplasm in HoxA10-overexpressing mice progresses to AML over time, suggesting that dysregulated HoxA10 expression predisposes to the accumulation of additional mutations required for the progression to AML. However, the mechanisms by which Hox proteins influence these cellular events are largely unknown because few genuine Hox target genes have been identified.

The goal of our studies was to identify HoxA10 target genes that are functionally significant for leukemogenesis. Using various approaches, we identified a number of HoxA10 target genes that may contribute to the pathogenesis of AML. For example, we found that HoxA10 represses the transcription of a number of phagocyte effector genes in myeloid progenitor cells (13–15). HoxA10-induced repression of these genes decreases as myelopoiesis proceeds, permitting differentiation stage-specific transcription (14, 15). We also identified genes involved in regulating the proliferation and survival of hematopoietic progenitor cells as HoxA10 target genes. This included genes encoding Mkp2, β3-integrin, and Tgfβ2 (16, 17). These target gene products contribute to the expansion of HoxA10-overexpressing myeloid progenitor cells (16, 17). In the current study, we report that HoxA10 activates *ARIH2* transcription. This gene encodes Triad1, an E3 ubiquitin ligase (also referred to as ariadne-like 2) (18, 19).

Triad1 contains two RING (really interesting new gene) domains that bind substrate proteins and E2 ubiquitin ligases. E2 (or ubiquitin-conjugating) proteins bring ubiquitin to the E3

* This work was supported, in whole or in part, by National Institutes of Health Grant R01-HL87717. This work was also supported by a Veterans Affairs Merit Review award (to E. A. E.).

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[2] The abbreviations used are: AML, acute myeloid leukemia; RA, retinoic acid; G-CSF, granulocyte-colony-stimulating factor; SCF, stem cell factor; MSCV, murine stem cell virus.

ligase-substrate complex. The E3 ligase attaches ubiquitin to the substrate, thereby tagging the substrate for proteasome-mediated degradation. Previous studies determined that Triad1 interacts functionally with several E2 ligases, including Ubch7 and Ubc13 (19, 20). Co-overexpression of Triad1 and UbcH7 increases total protein ubiquitination in myeloid progenitor cells or myeloid cell lines, but genuine Triad1/UbcH7 substrate proteins have not been identified (20). Although Triad1 is ubiquitously expressed, expression increases during myelopoiesis and is greatest in mature neutrophils (19). Other investigators found that engineered overexpression of Triad1 in myeloid progenitor cells results in decreased colony formation (19). This suggests the possibility that HoxA10-dependent Triad1 expression antagonizes the pro-proliferative consequences of dysregulated expression of other HoxA10 target genes in HoxA10-overexpressing cells.

In these studies, we investigated the hypothesis that HoxA10 regulates the expression of Triad1, thereby influencing protein ubiquitination. The impact of increased Triad1 expression on myeloproliferation has implications for understanding leukemogenesis in Hox-overexpressing AML.

MATERIALS AND METHODS

Plasmids

Protein Expression Vectors—The cDNA for human HoxA10 was obtained from C. Largman (University of California, San Francisco) (21). This cDNA sequence, which represents the major transcript in mammalian hematopoietic cells, encodes a 393-amino acid, 55-kDa protein (22). The Triad1 cDNA was obtained from Dr. Bert A. van der Reijden (Radboud University, Netherlands). Both cDNA sequences were subcloned into the pSRα or pcDNAamp vector for expression in mammalian cell lines and the MSCV vector for the generation of retrovirus (per the manufacturer's instructions (Stratagene, La Jolla, CA)).

shRNA Expression Vectors—HoxA10- and Triad1-specific shRNA and scrambled control sequences were designed with the assistance of the Promega Web site. Double-stranded oligonucleotides representing the complementary sequences separated by a hairpin loop were subcloned into the pLKO.1puro vector (a gift from Dr. Kathy Rundell, Northwestern University, Chicago). Several sequences were tested, and the most efficient of these were used in combination for HoxA10 or Triad1 suppression. Scrambled shRNA sequences were used as negative controls in all studies.

ARIH2 Reporter Vectors—Various fragments of the *ARIH2* 5′-flank were amplified by genomic PCR from U937 chromatin and sequenced to ensure identity with the published sequence (from the Ensembl database). *ARIH2* 5′-flank sequences were subcloned into the pGL3-basic reporter vector (Promega, Madison, WI). Additional constructs were generated with three copies of the −22 to −48-bp (proximal) or −174 to −198-bp (distal) HoxA10-binding sequences from the *ARIH2* promoter in the pGL3-promoter vector. Constructs were also generated with the −22 to −48-bp (proximal) or −174 to −198-bp (distal) *ARIH2* sequences with mutation in the Hox-binding consensus (see below).

Oligonucleotides

Oligonucleotides were custom synthesized by MWG Biotech (Piedmont, NC). Double-stranded oligonucleotides used in electrophoretic mobility shift assays or to generate reporter constructs represented −22 to −48-bp (proximal, wild type 5′-TTAAAAATATAAATATAATTCTTTTCA-3′; Hox-binding mutant, 5′-TTAAAAAT**C**T**CC**ATA**G**AATTCTTTTCA-3′) or −174 to −198-bp (distal, wild type 5′-TCTTGTCAATATAATTATATCATGGA-3′; Hox-binding mutant, 5′-TCTTGTCAA**GCG**AA**GC**ATATCATGGA-3′) sequences from the *ARIH2* promoter. Bold letters represent mutated bases.

Myeloid Cell Lines and Culture

The human myelomonocytic leukemia cell line U937 (24) was obtained from Andrew Kraft (Hollings Cancer Center, Medical University of South Carolina, Charleston). Cells were maintained as described (25). U937 cells were treated for 48 h with retinoic acid (RA) plus dimethyl formamide for granulocyte differentiation.

Primary Murine Bone Marrow Studies

Animal studies were performed according to a protocol approved by the Animal Care and Use Committees of Northwestern University and the Jesse Brown Veterans Affairs Medical Center.

Bone Marrow Harvest and Culture—Bone marrow mononuclear cells were obtained from the femurs of WT, HoxA10$^{+/-}$, or HoxA10$^{-/-}$ C57/BL6 mice (26). Sca1+ cells were separated using the Miltenyi magnetic bead system (Miltenyi Biotec, Inc., Auburn, CA). Bipotential myeloid progenitor cells were cultured (at a concentration of 2×10^5 cells/ml) for 48 h in DME supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, 10 ng/ml murine GM-CSF (R&D Systems Inc., Minneapolis, MN), and 10 ng/ml murine recombinant IL-3 (R&D Systems). Cells were either maintained in GM-CSF + IL-3 for 48 h or differentiated over a 48-h time span in 10 ng/ml granulocyte-CSF (G-CSF).

Bone Marrow Retroviral Transduction—Retrovirus was generated with the HoxA10/MSCV plasmid or control MSCV plasmid using the Phoenix cell packaging line according to the manufacturer's instructions (Stratagene). The average concentration of producer cell supernatants was 10^7 plaque-forming units/ml.

Bone marrow mononuclear cells were cultured for 24 h in 10 ng/ml IL-3, 10 ng/ml GM-CSF, and 100 ng/ml SCF. Cells were transduced by incubation with retroviral supernatant in the presence of Polybrene (6 μg/ml) as described previously (27). Transduced cells were selected for 48 h in puromycin, with or without G-CSF-induced differentiation, and used for gene expression studies. Transgene expression was confirmed by real-time PCR. Transduction studies were repeated at least three times with at least two different batches of retroviruses.

Quantitative Real-time PCR

RNA was isolated using the TRIzol reagent (Invitrogen) and tested for integrity by denaturing gel electrophoresis. Primers

were designed with Applied Biosystems software, and real-time PCR was performed using SYBR Green according to the "standard curve" method. Results were normalized to 18 S (for mRNA determination) or input chromatin (for chromatin immunoprecipitation studies).

Chromatin Co-immunoprecipitation and CpG Island Screening

U937 cells were incubated briefly in medium supplemented with formaldehyde to generate DNA-protein cross-links. For CpG island microarray screening, cell lysates were sonicated to generate chromatin fragments with an average size of 2.0 kb (28). Lysates underwent two rounds of immunoprecipitation with an antibody custom-generated to a HoxA10-specific peptide as described previously (16). Precipitated chromatin was recovered as described (28). Several batches of immunoprecipitated chromatin were combined for each experiment and amplified by PCR. Chromatin was labeled and used to screen a CpG island microarray as described (16).

The identified genes were confirmed by independent chromatin immunoprecipitation experiments. For these studies, chromatin was co-immunoprecipitated from U937 lysates with antibody to HoxA10 or preimmune serum. In initial studies, lysates were sonicated to generate chromatin fragments of 1000 bp. This precipitated chromatin was analyzed by PCR using primer sets to amplify various 1000-bp sequences in the 5′-flank or first exon (as a negative control). PCR products were separated by agarose gel electrophoresis. In other studies, cell lysates were sonicated to generate chromatin fragments of ~100 bp. This chromatin was used in quantitative real-time PCR experiments.

Myeloid Cell Line Transfections and Assays

Stable Transfectants—U937 cells were transfected by electroporation with equal amounts of a HoxA10 or Triad1 expression vector or with empty vector control (using pcDNAamp) plus a vector with a neomycin phosphotransferase cassette (pSRα) (30 μg each). Stable pools of transfected cells were selected in G418 (0.5 mg/ml), and aliquots of cells were tested for HoxA10 and Triad1 expression by Western blot.

Other cells were transfected by electroporation with a lentiviral construct (pLKO.1puro vector) for expression of HoxA10- or Triad1-specific shRNAs (or scrambled control shRNAs). Stable pools of transfected cells were selected in puromycin (1.2 μg/ml) and tested for HoxA10 or Triad1 expression by Western blot. In some experiments, U937 stable transfectants with either a HoxA10 expression vector or empty vector control were co-transfected with a vector to express either a Triad1-specific shRNAs or scrambled control. Co-transfectants were selected in both G418 and puromycin.

ARIH2 Reporter Assays—To identify the *ARIH2* promoter cis element that is activated by HoxA10, U937 cells were co-transfected with a construct containing various *ARIH2* 5′-flank sequences linked to a luciferase reporter (629-, 333-, 198-, 167-, 109-, 48-, and 29-bp *ARIH2*-pGL3-b or pGL3-b control) (30 μg) and a vector to overexpress HoxA10 (or empty vector control) (50 μg). In other experiments, cells were co-transfected with a luciferase reporter vector containing a minimal promoter and the −22 to −48-bp (proximal) or −174 to −198-bp (distal) HoxA10-binding cis elements from the *ARIH2* promoter (or empty p-GL3-p vector control). Reporter assays were performed with or without 48 h of treatment with retinoic acid (48 h) with dimethyl formamide (24 h). Cells were also transfected with a β-galactosidase reporter vector to control for transfection efficiency (CMV/β-gal).

Western Blots

U937 or murine bone marrow cells were lysed by boiling in 2× SDS sample buffer. Lysate proteins (50 μg) were separated by SDS-PAGE (8% acrylamide) and transferred to nitrocellulose. Western blots were serially probed with antibodies to HoxA10, Triad1, and GAPDH (to control for loading). Each experiment was repeated at least three times with different batches of lysate proteins. Representative blots are shown. For some studies, cells were pretreated with the proteasome inhibitor MG132 for 5 h (5 μM) to stabilize ubiquitinated proteins.

Proliferation Assays

U937 stable transfectants with a vector to overexpress HoxA10 or empty vector control plus Triad1-specific shRNAs or scrambled shRNA control were deprived of fetal calf serum for 24 h and treated with a dose titration of fetal calf serum (0.01–10%) for 24 h. Some cells were also treated with retinoic acid. Cell proliferation was determined by the incorporation of [^{3}H]thymidine (for the last 16 h of incubation) according to standard techniques.

In Vitro DNA Binding Assays

Isolation of Nuclear Proteins—Nuclear proteins were extracted from U937 cells by the method of Dignam (29) with protease inhibitors as described (25).

Electrophoretic Mobility Shift Assays (EMSA)—Oligonucleotides probes were prepared, and EMSA were performed as described (16, 17). For binding reactions that included a HoxA10 antibody, disruption of the complex (not supershift) was anticipated. This is because the HoxA10 peptide used to generate the antibody is in close proximity to the DNA-binding homeodomain.

For all experiments, at least three different batches of nuclear proteins were tested in at least two independent experiments. The integrity of the nuclear proteins and equality of protein loading was determined in control EMSA with a probe representing a classical CCAAT-box from the α-globin gene promoter. Antiserum to HoxA10 (not cross-reactive with other Hox proteins) was obtained from Covance Research Products (Richmond, CA) and Santa Cruz Biotechnology (Santa Cruz, CA).

Genomic Sequence Analysis

Conserved genomic sequences and consensus sequences for Hox protein DNA binding were identified using VISTA software (Genomics Division of the Lawrence Berkeley National Laboratory (Berkeley, CA) (30–32).

Statistical Analysis

Statistical significance was determined by Student's *t* test and analysis of variance methods using SigmaPlot and SigmaStat

TABLE 1
HoxA10 target genes (CpG island array screen)
*, published previously.

HoxA10 Target Genes (CpG island array screen)		
DNA-binding	**Cytokines/ receptors**	**Apoptosis**
IREB2	*TGFB2**	*DUSP4**
PBX1	*TMEM8*	*NME4*
ZNF222	*EML5*	*COX6B2*
ZNF452		*GLUD1*
ZBTB8	**Signal transduction**	**Other**
HIST1H1B	*PPP2R5D*	*EIF4E*
Ubiquitination	*TBXAS1*	*CLAST4*
UBE2S	*AGXT2L1*	
ARIH2		

software. Graphs are presented with error bars representing standard error calculations.

RESULTS

Identifying ARIH2 as a HoxA10 Target Gene—To identify HoxA10 target genes, we coupled chromatin immunoprecipitation with microarray screening as reported previously (16). In these studies, chromatin that co-precipitated with a HoxA10 antibody (or preimmune serum) was used to screen a CpG island microarray. CpG islands that specifically co-precipitated with HoxA10 were identified. Because 70% of all promoters are adjacent to CpG islands, this approach should permit identification of the majority of HoxA10 target genes.

We used U937 cells for these screening studies (24). U937 cells are a myeloid leukemia line that is similar to bipotential granulocyte/monocyte progenitors. U937 cells are able to undergo granulocytic differentiation with RA or dimethyl sulfoxide or monocytoid differentiation with interferon γ or tumor necrosis factor α. Differentiating U937 cells exhibit proliferation arrest and increased sensitivity to apoptosis and also develop phagocyte functional competence (24). Therefore, these cells represent a reasonable model of myelopoiesis.

Using this approach, we identified a number of potential HoxA10 target genes, some of which were reported previously (16). We found increased representation of genes encoding cytokines, cytokine receptors, and signaling intermediates involved in cell proliferation and/or survival (Table 1). This was consistent with the influence of HoxA10 on progenitor expansion. A number of target genes encoding other transcription factors were also identified, consistent with previous observations regarding cross-regulation between homeodomain (HD) proteins.

Interestingly, we also identified HoxA10 target genes, including *ARIH2*, that were involved in protein ubiquitination (18, 19). This suggested a previously unidentified sphere of influence for Hox proteins, that of regulating protein activity by post-translational modification.

We used computer algorithms to analyze the *ARIH2* 5′-flank for conserved DNA-binding consensus sequences for Hox proteins (30–32). The *ARIH2* CpG island that was identified by screening encompassed −320 to −490 bp of the 5′-flank (relative to the transcription start site (+1)). Because chromatin was sonicated to ~2.0 kb for these experiments, we investigated the proximal 2.5 kb of the *ARIH2* 5′-flank for potential Hox-binding sites. We compared the human and murine genes and identified consensus sequences for Hox binding to DNA alone or as a heterodimer with Pbx or Meis proteins (frequent Hox partners). The proximal 1.0 kb of the *ARIH2* 5′-flank was highly conserved between mouse and human (Fig. 1*A*), and we identified eight Hox/Pbx-binding consensus sequences within the proximal 200 bp (Table 2).

To confirm the microarray screening results, independent chromatin co-immunoprecipitation studies were performed. In these experiments, HoxA10-co-precipitating chromatin was amplified by PCR using primers representing various *ARIH2* sequences (Fig. 1*B*, *last five lanes*). Preimmune serum was a negative control in these experiments (Fig. 1*B*, *middle five lanes*), and input nonprecipitated chromatin was a positive control (Fig. 1*B*, *first five lanes*). Chromatin was sheared to an average size of ~1.5 kb for this study.

In these studies, we found that the proximal 1.4 kb of the *ARIH2* 5′-flank specifically co-precipitated with HoxA10. In contrast, chromatin representing the −1.4 to −2.4-kb sequence (Fig. 1*B*, *last lane*) or the first intron (+1.5 to +2.5 kb; not shown) was not enriched in HoxA10-co-precipitating chromatin. These results identified a HoxA10-binding site within the proximal 1.0 kb of the *ARIH2* 5′-flank.

HoxA10 Activated two ARIH2 Cis Elements—We next investigated the influence of HoxA10 on *ARIH2* promoter activity. For these studies, U937 cells were transfected with a series of reporter constructs containing truncations of the proximal *ARIH2* 5′-flank (or empty reporter vector) and a HoxA10 expression vector (or empty expression vector), and reporter activity was determined (Fig. 2*A*). Based on the locations of the Hox-binding consensus sequences, constructs were generated with 629, 333, 198, 167, 109, 48, 29 bp of the *ARIH2* 5′-flank (indicated by *arrows* in Fig. 1*A*). Because previous studies had indicated a higher level of Triad1 expression in neutrophils in comparison with myeloid progenitors, transfectants were analyzed with or without RA-induced differentiation (experiments with RA are represented by *hash-marked bars* in Fig. 2*A*).

The 629-, 333-, 198-, 167-, 109-, and 48-bp constructs all exhibited promoter activity in these transfection experiments (Fig. 2*A*). In contrast, the smallest (29 bp) construct did not have promoter activity under any of the assay conditions (Fig. 2*A*, *bars 1–4*). The activities of the 629-, 333-, 198-, 167-, 109-, and 48-bp constructs were significantly greater in RA-treated transfectants in comparison with untreated transfectants (Fig. 2*A*, $p = 0.002$, $n = 6$; compare *open bars* with *open hash-marked bars*). The increase in reporter activity due to RA treatment was not significantly different for any of the constructs in this group ($p = 0.2$, $n = 6$).

The 628-, 333-, and 198-bp constructs had activities that were similar to each other in untreated transfectants or in RA-differentiated transfectants (Fig. 2*A*; for untreated transfectants, compare *bars 24*, *21*, and *28*, $p = 0.6$ $n = 6$; for RA-differentiated transfectants, compare *bars 19*, *23*, and *27*, $p = 0.4$, $n = 6$). The activities of the 167-, 109-, and 48-bp constructs were also similar to each other in untreated transfectants or RA-differentiated transfectants (Fig. 2*A*; for untreated transfec-

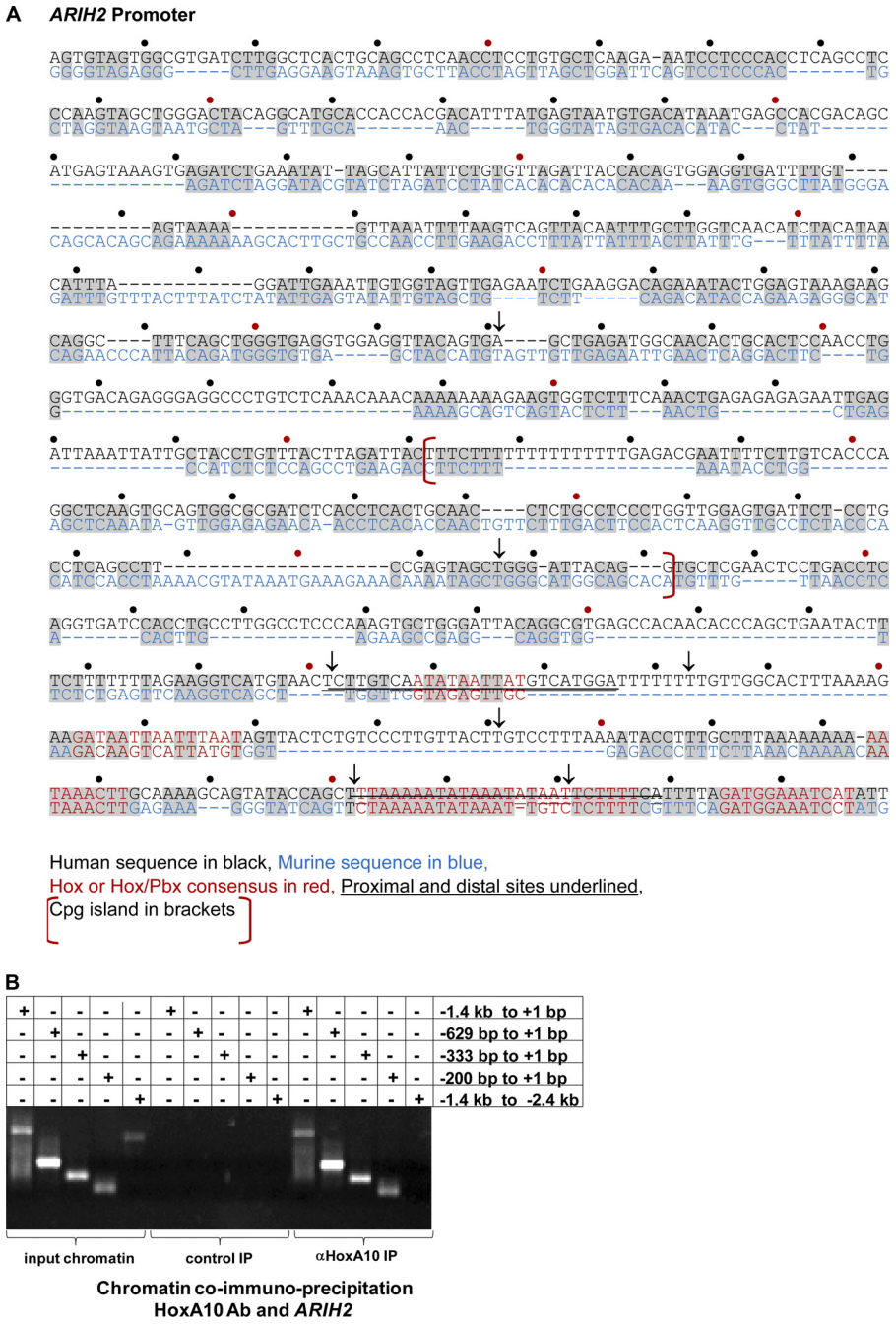

FIGURE 1. **Identification of *ARIH2* as a HoxA10 target gene in myeloid cells.** *A*, the *ARIH2* 5′-flank includes conserved consensus sequences for Hox DNA binding. The 5′-flanks of the human (*black*) and murine (*blue*) *ARIH2* genes were compared for conserved sequences (*gray*). Conserved Hox DNA-binding consensus sequences were identified (*red*). The location of the CpG island identified by chromatin immunoprecipitation is indicated by the *red brackets*. Sequences representing HoxA10-binding cis elements are *underlined*. *Arrows* indicate the location of truncations used in reporter gene assays. *B*, proximal 5′-flank co-immunoprecipitated with HoxA10 from U937 cells. Chromatin co-immunoprecipitation was performed with U937 cells and an antibody to HoxA10 or preimmune serum (as a negative control). Chromatin was amplified by PCR using primers flanking various sequences in the *ARIH2* 5′-flank as indicated. Nonprecipitated (input) chromatin was a positive control.

tants, compare *bars 8*, *12*, and *15*, $p = 0.8$ $n = 6$; for differentiated transfectants, compare *bars 7*, *11*, and *15*, $p = 0.7$, $n = 6$). However, the activities of the 628-, 333-, and 198-bp constructs were significantly greater than the activities of the 167-, 109-, and 48-bp constructs ($p = 0.006$, $n = 8$ for undifferentiated transfectants; $p = 0.0002$, $n = 6$ for RA-treated transfectants).

Overexpression of HoxA10 significantly increased the activity of the 629-, 333-, 198-, 167-, 109-, and 48-bp constructs (Fig. 2*A*; for untreated transfectants, compare the *open bars* and the *gray bars* for this group, $p < 0.01$ $n = 8$; for RA-treated transfectants, compare the *open hash-marked bars* and the *gray hash-marked bars*, $p < 0.01$ $n = 8$). The 628-, 333-, and 198-bp constructs had significantly more reporter activity in HoxA10-overexpressing transfectants in comparison with the activity of the 165-, 109-, and 48-bp constructs in these transfectants (Fig. 2*A*, compare *bars 19*, *23*, and *27* with *bars 7*, *11*, and *15*, $p \leq 0.0002$, $n = 6$). Also, HoxA10 overexpression induced a greater increase in the activity of the 628-, 333-, or 198-bp constructs in

comparison with the increase in the activity of the 165-, 109-, and 48-bp constructs (199.7 ± 7.4% increase *versus* 91.0 ± 0.8% increase, respectively; $p < 0.01$, $n = 8$).

In these experiments, the empty reporter vector had minimal activity, which was not influenced by RA treatment or HoxA10 overexpression. This activity was subtracted as background.

These results suggested that the proximal 629 bp of the *ARIH2* promoter included two cis elements that were activated by HoxA10: −28 and −48 bp and between −165 and −198 bp. Examination of the *ARIH2* promoter revealed three overlapping Hox-binding consensus sequences between −22 and −48 bp (referred to as proximal) and one Hox-binding consensus between −174 and −198 bp (referred to as distal). Therefore, we generated reporter constructs with three copies of the proximal or distal sequence linked to a minimal promoter.

These constructs (or control minimal promoter/reporter vector) were co-transfected into U937 cells with a vector to overexpress HoxA10 (or empty vector control) (Fig. 2*B*). Reporter gene assays were performed with or without RA-induced differentiation. We found that differentiation significantly increased the activity of either of these *ARIH2* sequence containing constructs in these transfectants (Fig. 2*B*, compare *bars 2* and *4* for the distal cis element and *bars 6* and *8* for the proximal cis element, $p < 0.01$, $n = 9$).

HoxA10 overexpression also significantly increased the activity of both of the *ARIH2* cis elements in U937 transfectants (Fig. 2*B*; for untreated transfectants, compare *bars 3* and *4* for the distal cis element and *bars 7* and *8* for the proximal cis element, $p < 0.001$, $n = 9$; for RA-differentiated transfectants, compare *bars 1* and *3* for the distal cis element and *bars 5* and *7* for the proximal cis element, $p < 0.01$, $n = 9$). The proximal cis element was significantly less active than the distal cis element under all conditions tested (Fig. 2*B*; $p < 0.001$, $n = 9$). The activity of the control minimal promoter/reporter vector was less than 10% of the activity of either *ARIH2* cis element-containing construct, was not increased by differentiation or HoxA10 overexpression, and was subtracted as background.

To verity that HoxA10 was acting through the Hox-binding consensus sequences in these constructs, additional reporter constructs were analyzed. The −22 to −48-bp construct includes three sequences that meet the Hox/Pbx-binding consensus. We introduced a mutation in one of these potential binding sites (−32 to −41 bp) and generated a construct with three copies of the mutant sequence linked to a minimal promoter and reporter. A construct with three copies of the distal cis element with a mutation in the single Hox consensus-binding site (−182 to −191 bp) was also generated. These mutant constructs were assayed in U937 transfection experiments as described above. We found that the activity of these mutant constructs was not significantly different from the activity of

TABLE 2
Hox/Pbx consensus sequences

Sequence	*ARIH2* promoter location
ATGATTNATN	Consensus
ATGATTTCCATC	−4 to −14
AAGAATTATATT	−25 to −36
ATATTTATAT	−32 to −41
ATATTTTTTAA	−38 to −48
AAGTTTATT	−68 to −76
ATTAAATTAATT	−132 to −142
ATTAATTAG	−137 to −142
ATAATTATAT	−182 to −191

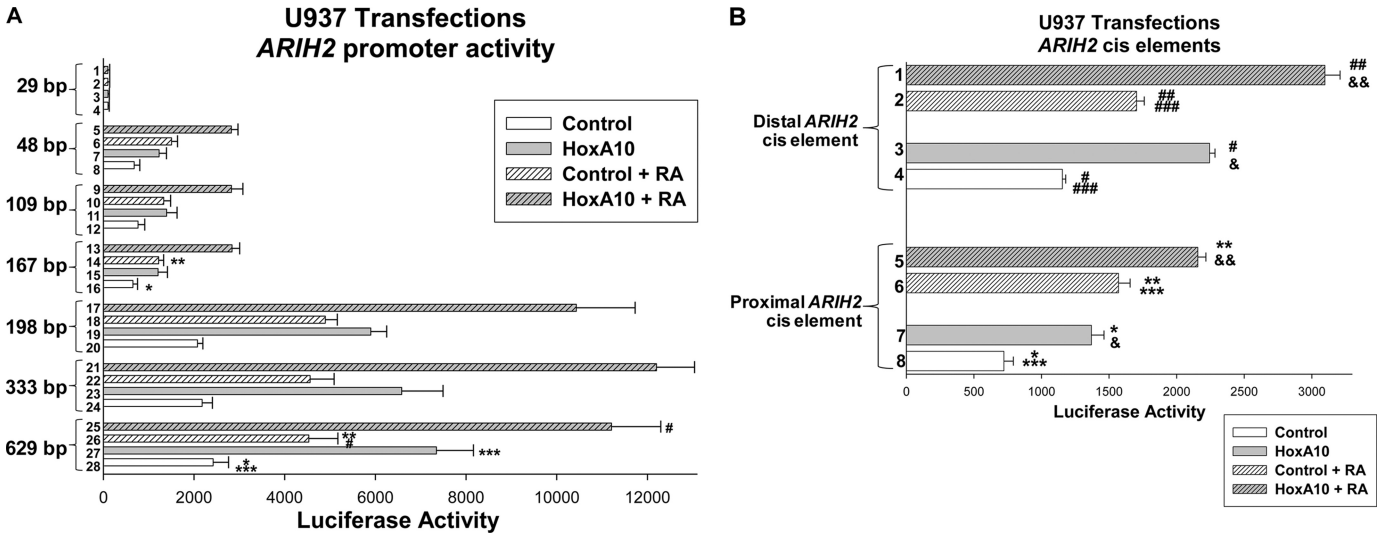

FIGURE 2. **HoxA10 activated two cis elements in the *ARIH2* promoter.** *A*, overexpressed HoxA10 activated cis elements between −198 and −26 bp in the *ARIH2* promoter in myeloid cell line transfection experiments. U937 cells were co-transfected with a series of reporter constructs (629, 333, 198, 167, 109, 48, and 29 bp) representing the *ARIH2* 5′-flank (or empty reporter vector) and a vector to overexpress HoxA10 (or empty expression vector). *, indicates statistically significant differences in reporter gene activity of various constructs; **, indicates significant difference in reporter activity in HoxA10-overexpressing transfectants; *** and #, indicate statistically significant differences in HoxA10-overexpressing *versus* control transfectants. *Bars* are numbered *1–28* and grouped according to construct. *B*, HoxA10 activated two cis elements between −198 and −26 bp in the *ARIH2* promoter. U937 cells were co-transfected with a construct with three copies of the −22 to −48 bp (proximal, *bars 5–8*) or −174 to −198 (distal, *bars 1–4*) sequence from the *ARIH2* 5′-flank linked to a minimal promoter and reporter (or minimal promoter/reporter control vector) and a vector to overexpression HoxA10 (or empty expression vector). Transfectants were analyzed for reporter activity with or without RA-induced differentiation. *, **, #, and ##, indicate statistically significant increase in reporter expression with HoxA10 overexpression; *** and ###, indicate significant increase in reporter expression with differentiation *versus* without differentiation for the proximal and distal cis element-containing constructs, respectively; & and &&, indicate statistically significant differences in reporter activity between the proximal and distal reporter constructs for undifferentiated and RA-treated HoxA10-overexpressing transfectants, respectively.

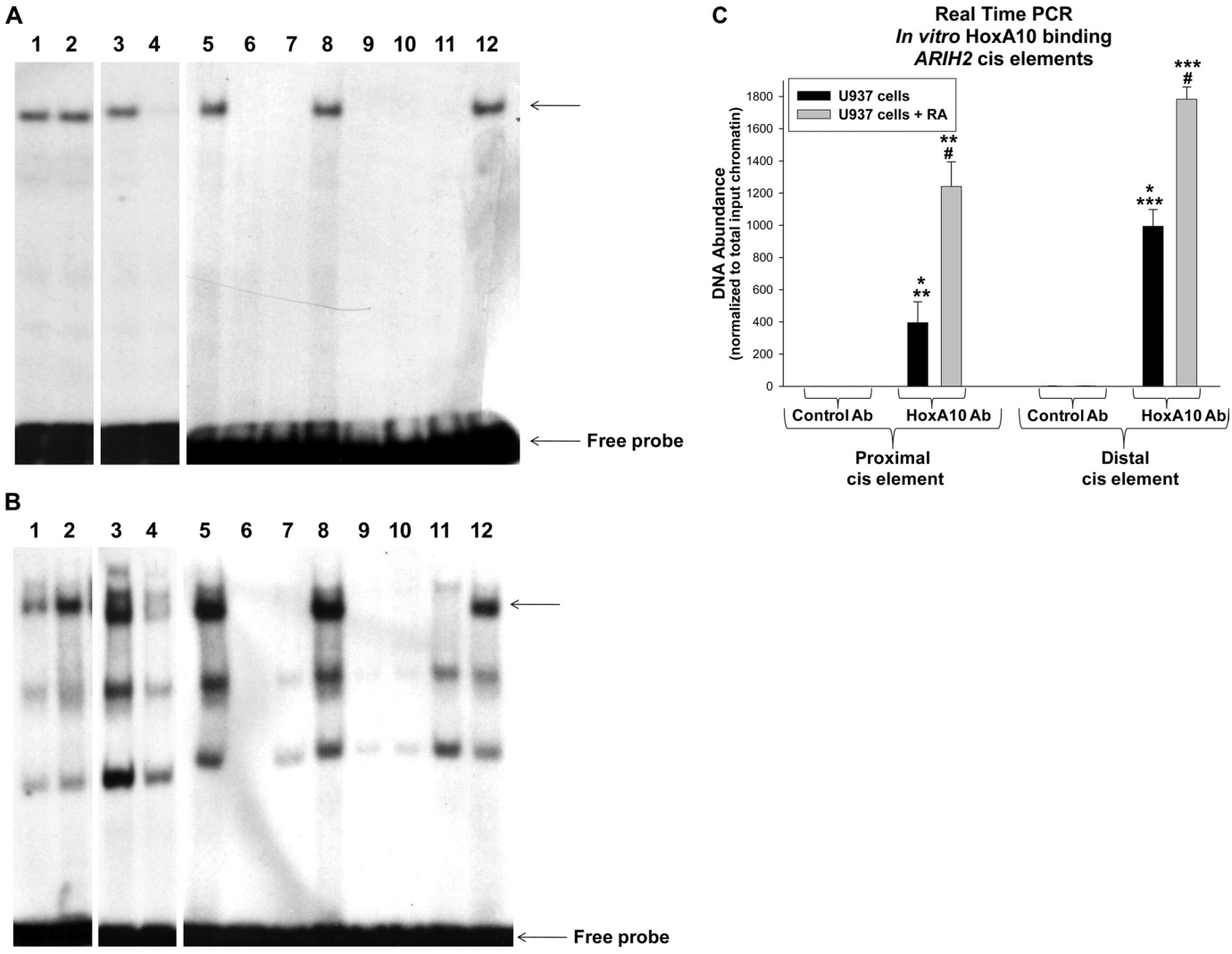

FIGURE 3. **HoxA10 bound to two *ARIH2* promoter cis elements.** *A*, HoxA10 bound to the proximal ARIH2 cis element *in vitro*. EMSA were performed with nuclear proteins from U937 cells and a double-stranded, synthetic oligonucleotide probe representing the proximal (−22 to −48 bp) *ARIH2* cis element. Binding assays were performed with nuclear proteins from untreated U937 cells (*lanes 1* and *2–12*) or from RA-differentiated U937 cells (*lane 2*). Some binding reactions were incubated with control preimmune serum (*lane 3*) or an antibody to HoxA10 (*lane 4*). Other binding reactions were incubated with excess, unlabeled, double-stranded oligonucleotide competitors as follows: no competitor (*lane 5*), the homologous *ARIH2* sequence (*lane 6*), a HoxA10-binding cis element from the *DUSP4* promoter (*lane 7*), the distal or proximal HoxA10-binding cis elements from the *CYBB* promoter (*lanes 8* and *9*, respectively) or an unrelated *CYBB* cis element (*lane 10*), and the distal *ARIH2* cis element (*lane 11*) or a form of the proximal *ARIH2* sequence with a Hox-binding site mutation (*lane 12*). The HoxA10-containing complex is indicated by the *upper arrow* and the free probe by the *lower arrow*. *B*, HoxA10 bound to the distal *ARIH2* cis element *in vitro*. EMSA were also performed with nuclear proteins from U937 cells and a double-stranded synthetic oligonucleotide probe representing the distal (−174 to −198 bp) *ARIH2* cis element. Binding assays were performed with nuclear proteins from untreated U937 cells (*lanes 1* and *2–12*) or from RA-differentiated U937 cells (*lane 2*). Some binding reactions were incubated with control preimmune serum (*lane 3*) or an antibody to HoxA10 (*lane 4*). Other binding reactions were incubated with excess, unlabeled, double-stranded oligonucleotide competitors as follows: no competitor (*lane 5*), the homologous *ARIH2* sequence (*lane 6*), a HoxA10-binding cis element from the *DUSP4* promoter (*lane 7*), the distal or proximal HoxA10-binding cis elements from the *CYBB* promoter (*lanes 8* and *9*, respectively), an unrelated *CYBB* cis element (*lane 10*), the distal *ARIH2* cis element (*lane 11*), and a form of the proximal *ARIH2* sequence with a Hox-binding site mutation (*lane 12*). The HoxA10-containing complex is indicated by the *upper arrow* and the free probe by the *lower arrow*. *C*, HoxA10 bound to two *ARIH2* cis elements *in vivo*. Chromatin co-immunoprecipitation was performed with U937 cells (with and without RA-induced differentiation) and an antibody to HoxA10 (or irrelevant control antibody). Cell lysates were sonicated to generate chromatin fragments of ~100 bp prior to immunoprecipitation. Co-precipitating chromatin fragments were analyzed by real-time PCR using primer sets flanking the −22 to −48 bp (proximal) or −174 to −198 bp (distal) sequence from the *ARIH2* promoter. Results were normalized to total input (nonprecipitated) chromatin. * and #, indicate statistically significant differences in binding to the proximal *versus* distal cis elements; ** and ***, indicate statistically significant differences in HoxA10 binding in untreated *versus* differentiated U937 cells.

the minimal promoter control vector and was not altered by differentiation or HoxA10 overexpression (not shown).

HoxA10 Bound to Two ARIH2 Cis Elements—These studies indicated that HoxA10 influenced the activity of the proximal and distal *ARIH2* cis elements. However, these studies did not demonstrate that HoxA10 interacted with the cis elements. To investigate this, we used *in vitro* DNA binding assays. EMSA were performed using U937 nuclear proteins and synthetic double-stranded oligonucleotide probes with one copy of the proximal (−25 to −48 bp) or distal (−174 to −198 bp) *ARIH2* sequence. Because the activity of the *ARIH2* promoter was increased during RA-induced differentiation, nuclear proteins from untreated and RA-differentiated U937 cells were studied.

We found that differentiation with RA did not significantly alter *in vitro* binding to the proximal cis element probe (Fig. 3*A*, compare *lanes 1* and *2*) but increased *in vitro* binding to the

distal cis element probe (Fig. 3*B*, compare *lanes 1* and *2*). Protein binding to the proximal probe was less efficient than to the distal probe; more protein and longer exposure were used in the former studies.

To determine whether HoxA10 was a component of the dominant protein complex that bound to the probes, we preincubated nuclear proteins with a HoxA10 antibody (or preimmune serum) and performed EMSA. We found that the low mobility protein complex that bound to each of the probes was disrupted by an antibody to HoxA10 (Fig. 3, *A* and *B*, compare *lanes 3* and *4* for the proximal and distal probes, respectively).

We also investigated the binding specificity of the low mobility complexes in assays with unlabeled oligonucleotide competitors. We found that excess unlabeled "self"-oligonucleotide competed for the low mobility complex that bound to each probe (Fig. 3, *A* and *B*, compare *lanes 5* and *6*), but an oligonucleotide with mutation of the Hox-binding consensus did not (Fig. 3, *A* and *B*, compare *lanes 5* and *12*). Oligonucleotides with Hox-binding sites from the *DUSP4* or *CYBB* genes also competed for binding of these complexes (Fig. 3, *A* and *B*, compare *lane 5* with *lanes 7*, *9*, and *10*), but several irrelevant oligonucleotide competitors did not (Fig. 3, *A* and *B*, compare *lanes 5* and *8*). Additionally, oligonucleotide with the proximal *ARIH2* sequence competed efficiently for protein binding to the distal *ARIH2* sequence probe and vice versa (Fig. 3, *A* and *B*, compare *lanes 5* and *11*).

At least three independent batches of nuclear proteins were used for these experiments, and representative results are shown. The protein concentrations of various preparations were normalized by performing control EMSA with a synthetic oligonucleotide probe representing the classical CCAAT-box from the α-globin gene (not shown).

In vivo HoxA10 binding to these *ARIH2* cis elements was investigated by chromatin co-immunoprecipitation. These experiments were performed as described above, except that the chromatin was sonicated to ~100-bp fragments. Primers were designed to amplify ~90 bp surrounding the proximal and distal *ARIH2* cis elements, and real-time PCR was used to quantify co-precipitation. Chromatin that co-precipitated with preimmune serum was a negative control, and results were normalized to total input chromatin.

We found specific, *in vivo* HoxA10 binding to both of these regions of the *ARIH2* promoter (Fig. 3*C*). HoxA10 bound to the distal cis element more efficiently than to the proximal cis element (Fig. 3*C*, compare *black bars*), and binding to both cis elements was increased by differentiation (Fig. 3*C*, compare *black* and *gray bars*).

HoxA10 Influenced Triad1 Expression—We next investigated whether expression of Triad1 mRNA and protein was increased in HoxA10-overexpressing cells. For these studies, U937 cells were stably transfected with a vector to overexpress HoxA10 or empty control vector. Other U937 cells were stably transfected with a Triad1 expression vector or co-transfected with a vector to express two Triad1-specific shRNAs (or scrambled control shRNA) and a HoxA10 expression vector (or vector control). The Triad1 and shTriad1 transfectants were used for protein ubiquitin studies as discussed below.

Triad1 mRNA expression was determined by real-time PCR. We found that differentiation with RA significantly increased Triad1 expression in control transfectants (Fig. 4*A*, compare the *black* and *gray bars* in the *first set*), consistent with previous results (19). HoxA10 overexpression also significantly increased Triad1 expression, both with and without RA treatment (Fig. 4*A*, compare the *first pair of bars* with the *second pair*, $p < 0.0001$, $n = 6$).

As anticipated, Triad1 mRNA was increased in U937 cells that were stably transfected with a Triad1 expression vector (Fig. 4*A*, compare the *first pair of bars* with the *third pair*, $p < 0.001$, $n = 6$) and decreased in transfectants with Triad1-specific shRNA expression vectors (Fig. 4*A*, compare the *fourth pair of bars* with the *fifth pair*, $p < 0.001$, $n = 6$). Triad1 mRNA expression in HoxA10-overexpressing U937 cells was significantly decreased by expression of these Triad1-specific shRNAs (Fig. 4*A*, compare the *last two pairs* of *bars*, $p < 0.001$, $n = 6$).

Expression of Triad1 protein in the U937 stable transfectants was determined by Western blot (Fig. 4*B*). These blots confirmed that differentiation of control transfectants with RA increased Triad1 expression (Fig. 4*B*, *top panel*, compare *lanes 1* and *4*). HoxA10 overexpression also increased expression of Triad1 protein (Fig. 4*B*, *top panel*, compare *lanes 1* with *2* and *4* with *5*). As anticipated, Triad1 protein was increased in transfectants with a Triad1 expression vector (Fig. 4*B*, *top panel*, compare *lanes 1* with *3* and *4* with *6*).

Control experiments were performed to document HoxA10 mRNA expression in these transfectants (Fig. 4*C*). In comparison with control cells, HoxA10 mRNA was significantly more abundant in U937 cells stably transfected with a HoxA10 expression vector (Fig. 4*C*, compare the *first pair* of *bars* with the *second pair*, $p < 0.0001$, $n = 6$). HoxA10 mRNA was not altered by overexpression of Triad1 or expression of Triad1-specific shRNAs (Fig. 4*C*). Expression of HoxA10 protein correlated with HoxA10 mRNA in these transfectants (Fig. 4*B*, *middle panels*).

However, U937 cells are a leukemia line with abnormalities in a number of cellular processes. Therefore, we also investigated the influence of HoxA10 on Triad1 expression in primary murine bone marrow cells. These studies were facilitated by the existence of transgenic mice with *HOXA10* gene disruption. HoxA10 knock-out mice are characterized by urogenital abnormalities and superficially normal hematopoiesis (26).

For these experiments, bone marrow was isolated from HoxA10$^{-/-}$, HoxA10$^{+/-}$, or wild type mice (26). Cells were cultured in GM-CSF, IL-3, and SCF, and CD34+ cells were separated as described previously (27). This process produced a population that was best characterized as bipotential granulocyte/monocyte progenitors. Some cultured cells were further differentiated to neutrophils with G-CSF. This produced a population in which more than 80% of the cells were CD34−CD38+Gr1+ as described previously (27). In other experiments, wild type bone marrow cells were transduced with a retroviral vector to overexpress HoxA10 or with control vector (MSCV) (described in detail in Ref. 27).

Triad1 mRNA expression was determined by real-time PCR. We found that differentiation of control cells with G-CSF sig-

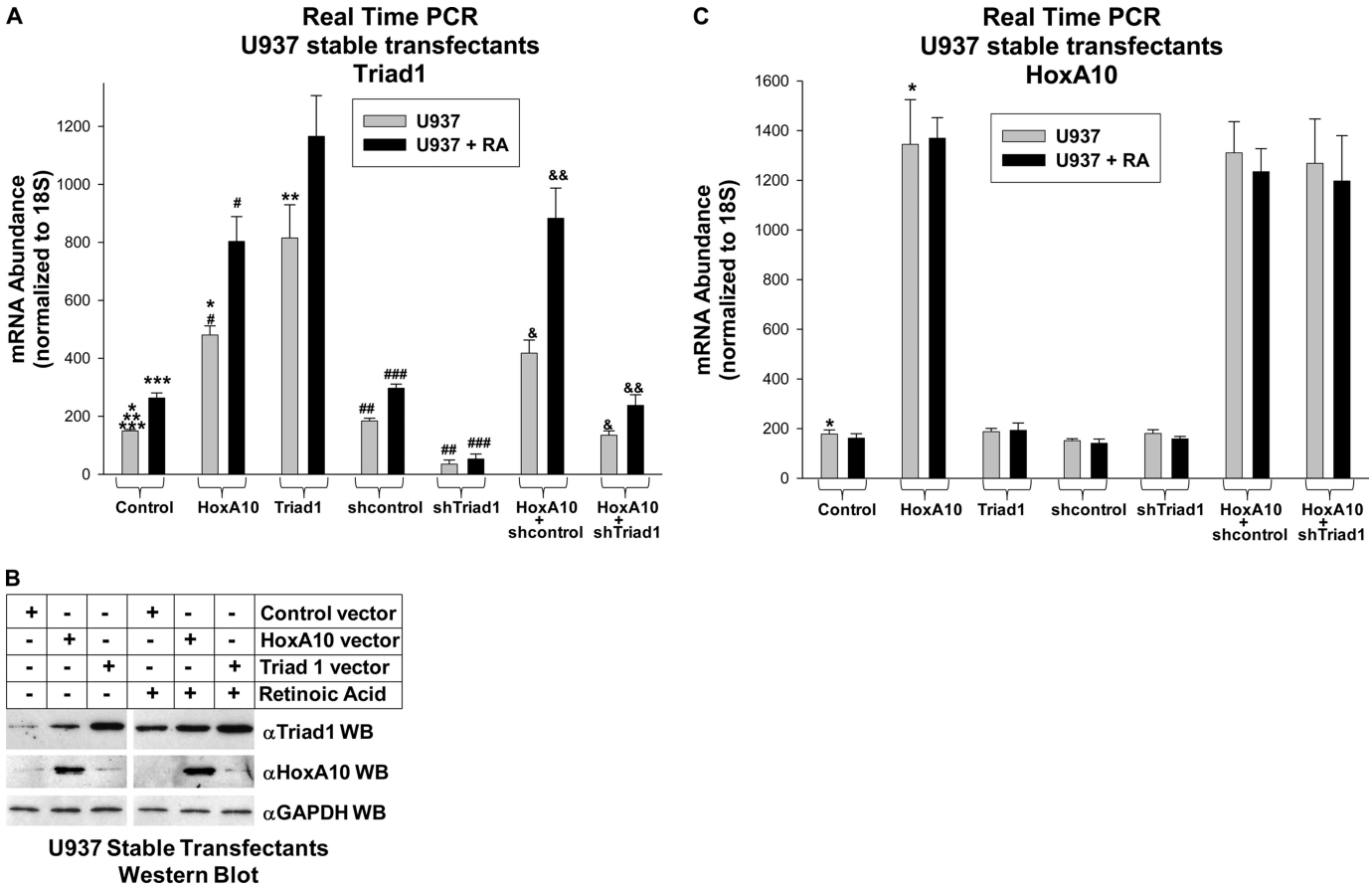

FIGURE 4. **HoxA10 overexpression in U937 myeloid cells increased Triad1 mRNA and protein.** *A*, expression of Triad1 mRNA was increased by overexpression of HoxA10 in U937 cells. U937 cells were stably transfected with vectors to overexpress HoxA10 or Triad1 or with empty expression vector control. Triad1 expression was determined by real-time PCR in untreated and RA-differentiated transfectants. * and **, indicate statistically significant difference in Triad1 expression in HoxA10- or Triad1-overexpressing transfectants, respectively; *** and #, indicate statistically significant increase in Triad1 expression with RA differentiation for control or HoxA10-overexpressing transfectants, respectively; ##, ###, &, and &&, indicate significant decreases in Triad1 expression with expression of Triad1-specific shRNAs. *B*, expression of Triad1 protein was increased by overexpression of HoxA10 in U937 cells. U937 stable transfectants, as described in *A*, were analyzed for protein expression by Western blot. Total cell lysate proteins were separated by SDS-PAGE, and blots were probed serially with antibodies for HoxA10, Triad1, and GAPDH (as a loading control). *C*, expression of HoxA10 mRNA was increased by overexpression HoxA10 in U937 cells but not by Triad1 overexpression or knockdown. U937 cells were stably transfected with vectors to overexpress HoxA10 or Triad1 or with empty expression vector control. HoxA10 expression was determined by real-time PCR in undifferentiated and RA-treated transfectants. *, indicates statistically significant difference in HoxA10 expression in cells transfected with a HoxA10 expression vector.

nificantly increased Triad1 expression (Fig. 5*A*, compare *black* and *gray bars* in the *first pair*, $p < 0.0001$, $n = 6$). There was significantly less Triad1 mRNA in HoxA10$^{+/-}$ cells in comparison with WT cells, and there was less Triad1 mRNA in HoxA10$^{-/-}$ cells in comparison with HoxA10$^{+/-}$ cells (Fig. 5*A*, WT *versus* HoxA10$^{+/-}$; compare the *first* and *second pairs of bars*, $p < 0.0001$, $n = 9$; +/− *versus* −/−, compare the *second* and *third pair of bars*, $p < 0.00001$, $n = 9$).

Consistent with these results, we found that Triad1 mRNA expression was increased in HoxA10-transduced cells in comparison with cells transduced with control vector (Fig. 5*A*, compare the *two left sets* of *bars*; $p < 0.00001$, $n = 10$ for granulocyte/monocyte progenitors and $p < 0.0001$, $n = 10$ for G-CSF-differentiated cells). Western blots confirmed that Triad1 protein was also increased by differentiation with G-CSF (Fig. 5*B*, compare *lane 1* with *2*), or HoxA10 overexpression (Fig. 5*B*, compare *lane 1* with *3*).

In control experiments, there was significantly less HoxA10 mRNA in HoxA10$^{+/-}$ cells in comparison with WT cells (Fig. 5*C*, compare the *first* and *second pairs* of *bars*, $p < 0.0001$, $n =$ 6). Expression of HoxA10 decreased significantly in both WT and HoxA10$^{+/-}$ cells undergoing differentiation with G-CSF (Fig. 5*C*, compare the *black* and *gray bars* in the *first two pairs*, $p < 0.01$, $n = 6$), consistent with previous results (27). HoxA10 was not expressed in HoxA10$^{-/-}$ cells as anticipated. Conversely, HoxA10 mRNA expression was significantly greater in cells transduced with a HoxA10 expression vector in comparison with control cells (Fig. 5*C*, compare *last two pairs* of *bars*, $p < 0.0001$, $n = 6$).

HoxA10 Influenced Protein Ubiquitination in a Triad1-dependent Manner—Because Triad1 is an E3 ubiquitin ligase, we hypothesized that an increase Triad1 in HoxA10-overexpressing cells would increase protein ubiquitination. Because no specific Triad1 substrates have been identified, we investigated this hypothesis by examining total protein ubiquitination. For these studies, we used U937 cells that were stably transfected with a Triad1 expression vector (or with empty vector) or with vectors to express two Triad1-specific shRNAs (or scrambled control shRNAs). These stable transfectants were discussed above (Fig. 4). Cells were

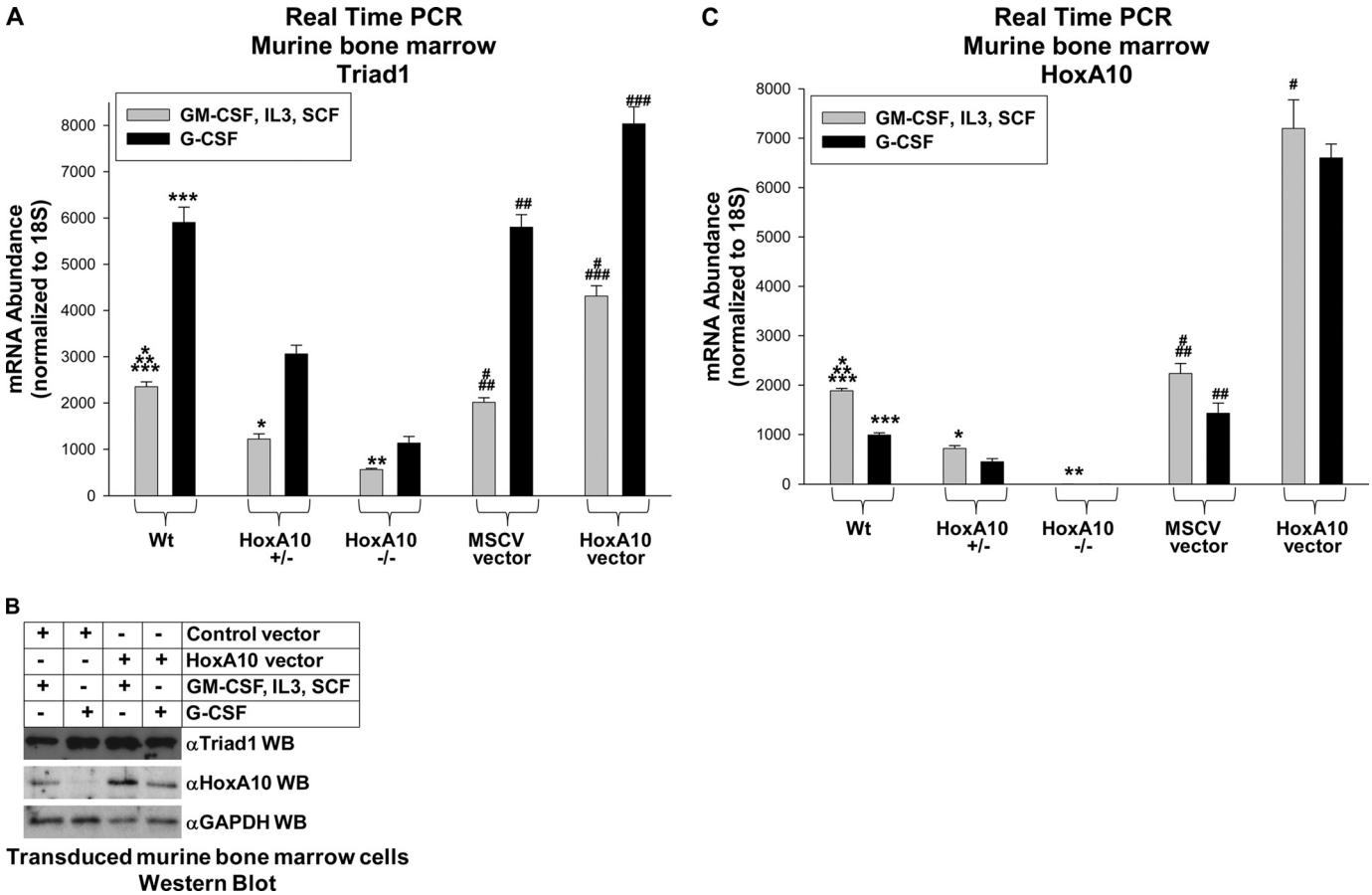

FIGURE 5. **Triad1 mRNA and protein expression in primary murine bone marrow cells was altered by overexpression or knock-out of HoxA10.** *A*, Triad1 mRNA expression in primary myeloid progenitors and differentiating myeloid cells was decreased by HoxA10 knock-out and increased by HoxA10 overexpression. Bone marrow progenitor cells were isolated from WT, HoxA10$^{+/-}$, or HoxA10$^{-/-}$ mice. Some wild type cells were transduced with a retroviral vector to overexpress HoxA10 or with control vector. Cells were cultured in GM-CSF, IL-3, or SCF (granulocyte/monocyte progenitor conditions) or with *ex vivo* differentiation to granulocytes with G-CSF. Triad1 expression was determined by real-time PCR. * and **, indicate statistically significant decrease in Triad1 mRNA expression in HoxA10$^{+/-}$ and HoxA10$^{-/-}$ cells, respectively, in comparison with WT cells; ***, indicates statistically significant increase in Triad1 expression in G-CSF-differentiated cells; #, indicates statistically significant increase in Triad1 expression in HoxA10-overexpressing cells; ## and ###, indicate significant increase in G-CSF-differentiated transduced cells for control vector and HoxA10-overexpressing cells, respectively. *B*, Triad1 protein expression in primary myeloid progenitors and differentiating myeloid cells was increased by HoxA10 overexpression. The transduced primary murine bone marrow cells, as described in *A*, were also analyzed by Western blot (*WB*) for protein expression. Total cell lysate proteins were separated by SDS-PAGE, and blots were probed serially with antibodies for HoxA10, Triad1, or GAPDH (as a loading control). *C*, HoxA10 mRNA expression in primary murine progenitors and differentiating myeloid cells was decreased by HoxA10 knock-out and increased by HoxA10 overexpression. HoxA10 mRNA expression in bone marrow cells, as described in *A*, was determined by real-time PCR. * and **, indicate statistically significant decrease in HoxA10 mRNA expression in HoxA10$^{+/-}$ and HoxA10$^{-/-}$ cells, respectively, in comparison with WT cells; ***, indicates statistically significant decrease in HoxA10 expression in G-CSF-differentiated cells; #, indicates statistically significant increase in HoxA10 expression in HoxA10-overexpressing cells; ##, indicates significant decrease in HoxA10 expression upon G-CSF-induced differentiation of control vector-transduced cells.

treated with MG132 (a proteasome inhibitor) to stabilize ubiquitinated proteins.

To evaluate the influence of Triad1 on total protein ubiquitination, Western blots of cell lysates were probed with an anti-ubiquitin antibody (Fig. 6*A*). Transfectants were assayed with or without differentiation by RA. We found that total protein ubiquitination was increased by treating control transfectants with RA (Fig. 6*A*, compare the *first two lanes*). This differentiation-induced increase in ubiquitination was not observed in cells stably expressing Triad1-specific shRNAs (Fig. 6*A*, compare the *first two lanes* with the *third* and *fourth lanes*). Conversely, overexpression of Triad1 increased total protein ubiquitination in untreated and RA-treated transfectants (Fig. 6*A*, compare the *first two lanes* with the *last two lanes*). In control experiments, we documented a decrease in Triad1 protein in U937 cells stably transfected with these Triad1-specific shRNA expression vectors (Fig. 6*B*, *top panel*, compare the *first* and *second lanes*).

We next examined whether HoxA10 overexpression influenced protein ubiquitination in a Triad1-dependent manner. For these studies, we used U937 cells stably transfected with a vector to overexpress HoxA10 (or empty vector control) and a vector to express Triad1-specific shRNAs (or scrambled shRNAs), discussed above (Fig. 4). Western blots of total cell lysates were probed with an anti-ubiquitin antibody.

We found that HoxA10 overexpression increased total protein ubiquitination in U937 transfectants (Fig. 6*C*, *top panel*, for untreated transfectants, compare the *first* and *third lanes*; for RA-differentiated transfectants compare the *second* and *fourth lanes*). The increase in protein ubiquitination in HoxA10-overexpressing cells was abrogated by expression of Triad1-specific shRNAs (Fig. 6*C*, *top panel*, for untreated transfectants com-

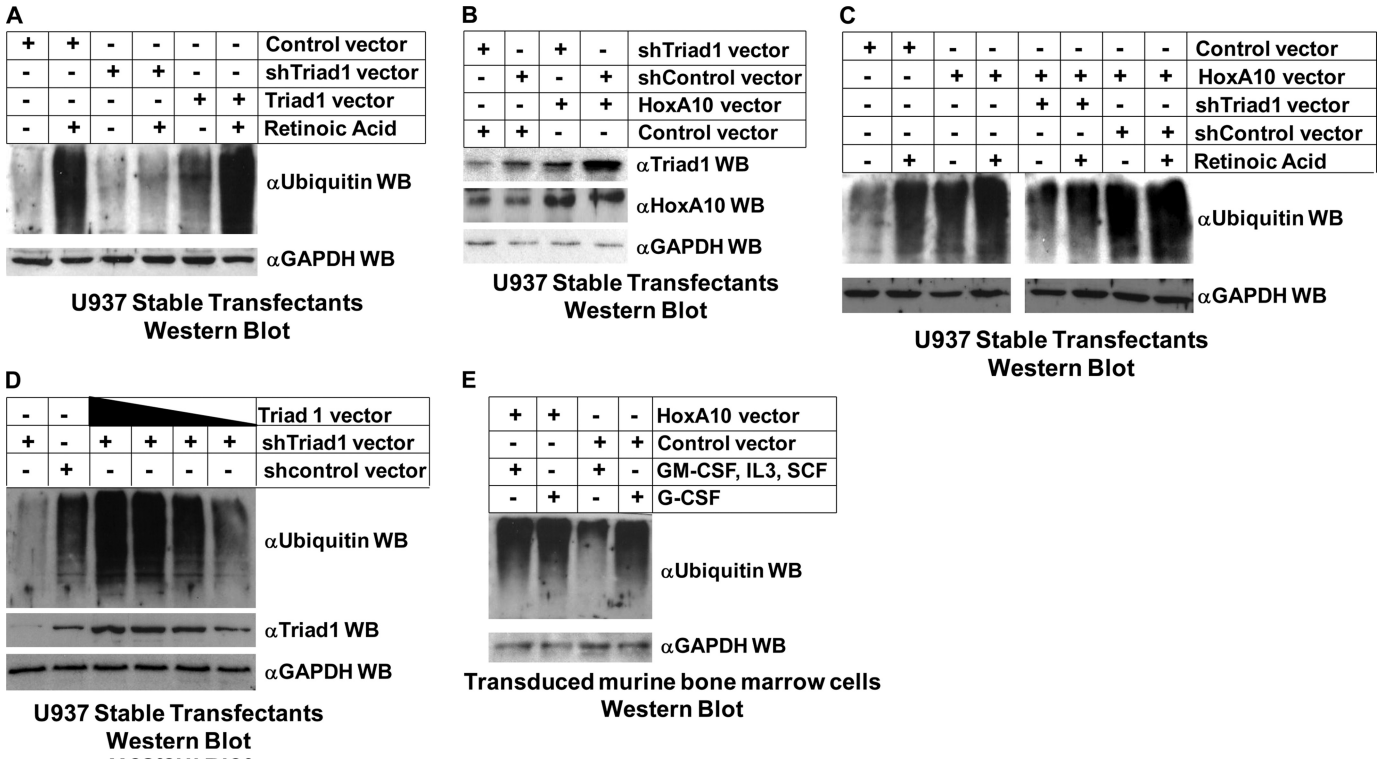

FIGURE 6. **HoxA10 overexpression increased total protein ubiquitination in a Triad1-dependent manner.** *A*, Triad1 overexpression in U937 cells increased total protein ubiquitination. U937 cells were stably transfected with a vector to express Triad1 (or empty vector control) or a vector to express two Triad1-specific shRNAs (or scrambled shRNAs). Untreated and RA-differentiated transfectants were treated with the proteasome inhibitor MG132. Western blots of total cell lysates were probed with an anti-ubiquitin antibody or antibody to GAPDH (as a loading control). *B*, HoxA10 overexpression in U937 cells increased Triad1 expression. U937 cells were stably transfected with a vector to overexpress HoxA10 (or empty expression vector) or a vector to express Triad1-specific shRNAs (or scrambled control shRNAs). Western blots (*WB*) of total cell lysates were probed with antibodies to HoxA10, Triad1, and GAPDH (as a loading control). *C*, HoxA10 overexpression in U937 cells increased total protein ubiquitination in a Triad1-dependent manner. Stable transfectants, as described in *B*, were analyzed by Western blot for total ubiquitinated protein. Blots were also probed with an antibody to GAPDH as a loading control. *D*, re-expression of Triad1 reversed the decrease in total protein ubiquitination in cells with Triad1 knockdown. U937 cells stably transfected with vectors to express two Triad1-specific shRNAs were co-transfected with a dose titration of a Triad1 expression vector (32, 16, 8, and 4 μg; indicated by a *right-pointing triangle*). Western blots of total cell lysates from transfectants that were differentiated with RA were probed with antibodies to ubiquitin, Triad1, and GAPDH (as a loading control). *E*, HoxA10 overexpression in primary murine bone marrow progenitor cells increased total protein ubiquitination. Myeloid progenitor cells were isolated from the bone marrow of WT mice and transduced with a HoxA10 expression vector or empty vector control. Cells were cultured in GM-CSF, IL-3, or SCF or differentiated with G-CSF. Western blots of cell lysates from MG132-treated transfectants were probed with antibodies to ubiquitin and GAPDH (as a loading control).

pare the *fifth* and *seventh lanes*; for RA-treated transfectants compare the sixth and *eighth lanes*). In control experiments, expressing Triad1-specific shRNAs was found to decrease Triad1 protein in HoxA10-overexpressing transfectants (Fig. 6*B*, *top panel*, compare *third* and *fourth lanes*).

Therefore, expression of Triad1-specific shRNAs substantially decreased total protein ubiquitination in RA-differentiated U937 cells. However, these studies did not directly demonstrate that decreased Triad1 protein was the cause of decreased protein ubiquitination in these cells (*i.e.* these studies did not demonstrate the specificity of the effect).

To address this issue, U937 cells were stably transfected with a vector to express Triad1-specific shRNAs plus a dose titration of Triad1 expression vector (32, 16, 8, and 4 μg of vector; indicated by a *right-pointing wedge* in Fig. 6*D*). Total cell lysates from RA-differentiated transfectants were analyzed by Western blot for protein ubiquitination and Triad1 expression (Fig. 6*D*). We found a dose-dependent increase in protein ubiquitination upon re-expression of Triad1 in U937 cells with Triad1 knockdown (Fig. 6*D*, compare *lane 1* with *lanes 3–6*). These studies suggested that specific loss of Triad1 protein decreased protein ubiquitination in cells expressing these Triad1-specific shRNAs.

We also examined the influence of HoxA10 on protein ubiquitination in primary murine bone marrow cells. For these studies, cells were isolated from WT mice and transduced with a retroviral vector to express HoxA10 (or with control vector) as described above. Cells were treated with MG132, and cell lysates were analyzed for total protein ubiquitination by Western blot. We found that differentiation of control cells with G-CSF increased total protein ubiquitination (Fig. 6*E*, *top panel*, compare *third* and *fourth lanes*). Overexpression of HoxA10 also increased total protein ubiquitination with and without differentiation (Fig. 6*E*, compare *first two lanes* with *last two lanes*).

Triad1 Influenced Proliferation of HoxA10-overexpressing Cells—Other investigators found that overexpression of Triad1 in myeloid cell lines or progenitors decreased the colony forming activity of these cells (19). In previous studies, we found that exposure to a given dose of fetal calf serum (FCS) stimulated more proliferation in HoxA10-overexpressing cells in comparison with control cells (*i.e.* that HoxA10-overexpressing cells

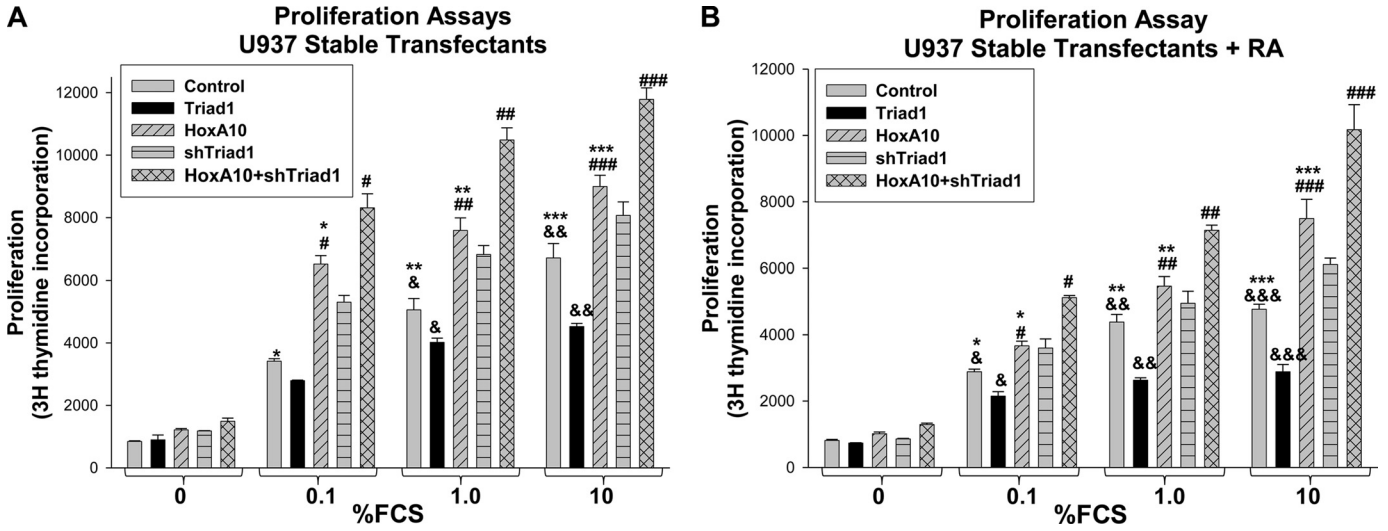

FIGURE 7. **Increased sensitivity of HoxA10-overexpressing myeloid cells to FCS-induced proliferation was influenced by Triad1 expression level.** *A*, Triad1 knockdown increased the sensitivity of HoxA10-overexpressing U937 cells to FCS-induced proliferation. U937 cells were stably transfected with a vector to overexpress HoxA10, Triad1, or empty vector control. Other cells were stably transfected with a vector to express two Triad1-specific shRNAs (or scrambled shRNA control) with or without a HoxA10 expression vector. Cells were deprived of FCS, and proliferation in response to a dose titration of FCS was determined by the incorporation of [^{3}H]thymidine. *, **, and ***, indicate statistically significant differences in [^{3}H]thymidine incorporation in HoxA10-overexpressing transfectants in comparison with vector control cells; #, ##, and ###, indicate statistically significant increase in proliferation in HoxA10-overexpressing transfectants with Triad1 knockdown; & and &&, indicate statistically significant decrease in [^{3}H]thymidine incorporation in cells with Triad1 overexpression. *B*, Triad1 knockdown increased the sensitivity of HoxA10-overexpressing, RA-differentiated U937 cells to FCS-induced proliferation. U937 stable transfectants described in *A* were also assayed for FCS-induced proliferation after RA-induced differentiation. *, **, and ***, indicate statistically significant differences in [^{3}H]thymidine incorporation in HoxA10-overexpressing transfectants in comparison with vector control cells; #, ##, and ###, indicate statistically significant increase in proliferation in HoxA10-overexpressing transfectants with Triad1 knockdown; &, &&, and &&&, indicate statistically significant differences in [^{3}H]thymidine incorporation in Triad1-overexpressing transfectants.

were cytokine-hypersensitive) (33). These results suggested that increased Triad1 expression in HoxA10-overexpressing cells would antagonize, rather than contribute to, the cytokine hypersensitivity of these cells. We performed additional experiments to investigate this hypothesis.

For these studies, we used U937 cells that were stably transfected with a HoxA10 or Triad1 expression vector (or empty vector control), a vector to express Triad1-specific shRNAs (or scrambled shRNA control), or vectors to simultaneously overexpress HoxA10 and knock down Triad1, as described above. These transfectants were deprived of FCS for 24 h followed by stimulation with a FCS dose titration (as in Ref. 33). Cells were analyzed with or without RA-induced differentiation, and proliferation was determined by the incorporation of [^{3}H]thymidine.

We found increased proliferation in HoxA10-overexpressing cells in comparison with control cells at all FCS doses (Fig. 7*A*, compare *gray bars* with *hash-marked gray bars*, $p < 0.001$, $n = 6$) as described previously (33). Although differentiation with RA decreased proliferation of both HoxA10-overexpressing and control cells (compare in Fig. 7, *A* and *B*), proliferation of HoxA10-overexpressing transfectants was still significantly greater than control cells at all FCS doses (Fig. 7*B*, compare *gray bars* and *hash-marked gray bars*, $p < 0.01$, $n = 6$).

Triad1 overexpression significantly decreased proliferation at higher FCS dose in untreated transfectants (Fig. 7*A*, compare *gray bars* and *black bars*, $p < 0.01$, $n = 6$). The effect of Triad1 overexpression was greater in differentiating transfectants, where proliferation was significantly less at all FCS doses in comparison with control cells (Fig. 7*B*, compare *gray bars* and *black bars*, $p < 0.001$, $n = 3$). Conversely, knockdown of Triad1 increased proliferation at most FCS doses in undifferentiated and differentiating transfectants (Fig. 7, compare *gray bars* and *gray bars* with *horizontal lines*, $p < 0.01$, $n = 3$).

Triad1 knock down further increased proliferation in HoxA10-overexpressing transfectants at all FCS doses (Fig. 7, compare *gray hash-marked* and *gray cross-hatched bars*, $p < 0.01$, $n = 3$ and $p < 0.02$, $n = 3$, respectively). Therefore, increased Triad1 expression antagonized the overall pro-proliferative effect of overexpressed HoxA10.

DISCUSSION

HoxA10 is a homeodomain transcription factor that influences proliferation, apoptosis, and differentiation of myeloid progenitor cells. In AML, HoxA10 overexpression is presumed to contribute to leukemogenesis by dysregulating transcription of target genes that impact these cellular activities. In previous studies (13–17, 33), we identified HoxA10 target genes that are involved in progenitor cell proliferation (*TGFB2* and *ITGB3*), apoptosis resistance (*DUSP4*), and phenotypic myeloid differentiation (phagocyte effector genes such as *CYBB* and *NCF2*).

In the current study, we identified *ARIH2* as a HoxA10 target gene. We found that HoxA10 activated transcription of *ARIH2* with a consequent increase in expression of the E3 ubiquitin ligase, Triad1. In contrast to our studies of other target genes, we found that increased Triad1 expression in HoxA10-overexpressing cells antagonized the overall pro-proliferative effect of HoxA10 overexpression. The antiproliferative effect of Triad1 in HoxA10-overexpressing cells was most pronounced during differentiation but was also observed in myeloid progenitor cells. Therefore, the regulation of myelopoiesis by HoxA10 is complex and likely to be influenced by differentiation stage-

specific expression profiles. These studies also identified a novel function for Hox proteins: the regulation of protein ubiquitination.

We found a Triad1-dependent increase in total protein ubiquitination in myeloid progenitor cells undergoing granulocyte differentiation, overexpressing HoxA10, or both. These results suggested two possibilities; either Triad1 was a major E3 ubiquitin ligase in differentiating myeloid cells, or Triad1 exerted a global influence on protein ubiquitination. No substrates have been identified for Triad1 E3 ubiquitin ligase activity, which makes it difficult to investigate these hypotheses. In previous studies, Triad1 was found to interact with Gfi-1, but this interaction did not result in Gfi-1 ubiquitination or degradation (19). Because some Hox proteins are ubiquitinated under various conditions (34, 35), overexpressed HoxA10 might itself be a Triad1 substrate. Studies to investigate this possibility are ongoing in our laboratory.

Triad1 might exert a global influence on protein ubiquitination if it functioned as a Nedd8 E3 ligase. Neddylation was first described for the cullin family of proteins, and additional neddylation substrates have subsequently been identified (reviewed in Ref. 36). Cullins are RING proteins that form a scaffold for ubiquitin ligase assembly. Neddylation increases the activity of cullin-based ubiquitin ligases (36). Therefore neddylation of cullins by Triad1 would influence ubiquitination of all cullin-based ligase substrates. Nedd8 ligase activity has not been described for Triad1, but this intriguing possibility will be explored in future investigations in our laboratory.

In vivo overexpression of HoxA10 in murine bone marrow rapidly induces a myeloproliferative neoplasm that progresses to AML over time (27). These studies suggested that HoxA10 overexpression is not sufficient for AML but that the myeloproliferative neoplasm induced by overexpressed HoxA10 predisposes to the accumulation of mutations leading to AML. Increased proliferation of HoxA10-overexpressing cells could contribute to this predisposition. If so, increased Triad1 expression in HoxA10-overexpressing bone marrow would delay the development of AML.

Therefore, our current studies suggest that interfering with the activation of *ARIH2* transcription by HoxA10 might accelerate disease progression in AML. Mutations that impair the function of the ubiquitin ligase complex might also cooperate with HoxA10 overexpression to accelerate disease progression. This has implications for the clinical use of proteasome inhibitors in HoxA10-overexpressing AML. Expression of Triad1 is variably increased or decreased in various forms of myeloid leukemia (37), further indicating the complexity of this issue.

Our studies employed various chromatin immunoprecipitation-based approaches to identify HoxA10 target genes. The goal was to identify target genes that are functionally significant for myeloid leukemogenesis. We have not emphasized cataloging putative target genes or diagramming hypothetical pathways influenced by HoxA10. Rather the goal has been to perform mechanistic studies of specific, discrete intermediates that may identify the molecular markers of disease prognosis or targets for therapeutic intervention in AML.

We identified a CpG island in the *ARIH2* gene that specifically co-precipitated with HoxA10. Further investigations identified two HoxA10-binding cis elements within the proximal *ARIH2* promoter. Although our investigations suggested that these were the only conserved HoxA10-binding sites in this promoter, additional sites may exist. Our goal in this study was to demonstrate the functional significance of HoxA10 for *ARIH2* transcription and Triad1 expression. Therefore, further search for additional binding sites was not pursued, as it would not contribute to understanding the role of HoxA10 in regulating ARIH2 transcription and protein ubiquitination.

Previous studies identified multiple HoxA10-binding sites in various target genes, including *CYBB*, *NCF2*, *TGFB2*, and *DUSP4*. For each of these genes, multiple HoxA10-binding cis elements were identified within the proximal 1 kb of promoter, generally within 100–200 bp of each other. The activity of some tandem cis elements was cooperative (*i.e.* the *CYBB* and *NCF2* genes), and for some it was additive (the *TGFB2*, *DUSP4*, and *ARIH2* genes).

HoxA10 binding affinity for tandem cis elements was also variable. This variability may contribute to differentiation stage-specific target gene regulation, because both abundance and post-translational modification of HoxA10 are altered during myelopoiesis. For example, we found that tyrosine phosphorylation of HoxA10 increased during myeloid differentiation, resulting in decreased binding to cis elements in the *CYBB*, *NCF2*, and *DUSP4* genes. Studies of the impact of HoxA10 post-translational modification on *ARIH2* transcription are ongoing in our laboratory.

HoxA10 binds to most target genes as part of a multiprotein complex. For the *CYBB* and *NCF2* cis elements, this complex included HoxA10, Pbx1, and HDAC2 (14, 23). For the *DUSP2* and *ITGB3* genes, the complex included Pbx2 and a protein with histone acetyltransferase activity (16, 17). In the current studies, higher mobility bands were observed during EMSA with the distal (high affinity) *ARIH2* cis element probe. These bands were cross-immunoreactive with HoxA10 and had cross-competitive binding specificity with other HoxA10-binding probes. These three complexes may represent HoxA10 binding as a monomer, dimer, and higher order multiprotein complex. Other components of the *ARIH2*-binding complex will be identified in future studies.

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