Constitutively Active SHP2 Cooperates with HoxA10 Overexpression to Induce Acute Myeloid Leukemia*

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The homeodomain transcription factor HoxA10 is maximally expressed in myeloid progenitor cells. Sustained HoxA10 expression during differentiation has been described in poor prognosis human acute myeloid leukemia (AML). Consistent with this, engineered overexpression of HoxA10 in murine bone marrow induces a myeloproliferative disorder that progresses to AML over time. This murine model suggests that HoxA10 overexpression is sufficient for myeloproliferation but that differentiation block, and therefore AML, requires acquisition of additional mutations. In myeloid progenitor cells, HoxA10 represses transcription of genes that encode phagocyte effector proteins such as gp91 Phox and p67 Phox. Tyrosine phosphorylation of HoxA10 during myelopoiesis decreases binding to these target genes. In immature myeloid cells, HoxA10 also activates transcription of the DUSP4 gene that encodes Mkp2, an anti-apoptotic protein. HoxA10 binding to the DUSP4 promoter decreases during myelopoiesis. Therefore, both myeloid-specific gene repression and DUSP4 activation by HoxA10 decrease during myelopoiesis. This results in phenotypic differentiation and facilitates apoptosis as differentiation proceeds. HoxA10 is de-phosphorylated by SHP2 protein-tyrosine phosphatase in myeloid progenitors. This mechanism maintains HoxA10 in a nonphosphorylated state in immature, but not differentiating, myeloid cells. Constitutively active SHP2 mutants have been described in human AML, which dephosphorylate HoxA10 throughout myelopoiesis. In this study, we hypothesize that constitutive SHP2 activation synergizes with HoxA10 overexpression to accelerate progression to AML. Because both HoxA10 overexpression and constitutive SHP2 activation are found in poor prognosis human AML, these studies contribute to understanding biochemical aspects of disease progression in myeloid malignancy.

The HOX genes, which encode highly conserved homeodomain (HD) transcription factors, are organized into four paralog groups on four chromosomes in mouse and man (1–3). During definitive hematopoiesis, HOX gene transcription proceeds 3’ to 5’ through each locus. This results in expression of the 3’-most genes (Hox1–4) in hematopoietic stem cells (HSC) and expression of the “Abd” HOX genes (Hox7–11) in granulocyte/megakaryocyte (myeloid) progenitors. During normal myelopoiesis, HOX7–11 transcription decreases with the CD34+ to CD34− transition that accompanies maturation from myeloid progenitor to functional phagocyte (3, 4). However, sustained transcription of ABD HOXA genes during myelopoiesis has been described in human subjects with poor prognosis acute myeloid leukemia (AML). Leukemias that fall into this category include, but are not limited to, AML with translocation or partial duplications involving the MLL (mixed lineage leukemia) gene (4–6).

Several murine models were developed to investigate the impact of Abd HoxA proteins on myelopoiesis and leukemogenesis. In one model, overexpression of Abd HoxA proteins was induced by expressing leukemia-associated MLL fusion proteins in murine bone marrow. Mice transplanted with such bone marrow developed a myeloproliferative disorder (MPD), which was characterized by neutrophilia and progressed to AML over a number of months (5, 7–10).

Overexpression of individual Abd HoxA proteins was also studied in murine transplantation experiments. For example, overexpression of HoxA9 in murine bone marrow expanded the HSC population in vitro, but it also facilitated myeloid differentiation. In vivo, mice that were transplanted with HoxA9-overexpressing bone marrow developed an MPD with mature neutrophils. This MPD progressed to AML only if Meis1 was co-overexpressed or after a very long latency (11–13). In contrast, overexpression of HoxA10 in murine bone marrow expanded a myeloid progenitor population in vitro (14). In vivo, mice that were transplanted with HoxA10-overexpressing bone marrow developed an MPD with mature neutrophils that

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§ The abbreviations used are: HD, homeodomain; AML, acute myeloid leukemia; HSC, hematopoietic stem cell; Jnk, c-Jun N-terminal kinase; Mkp2, mitogen-activated kinase phosphatase 2; MLL, mixed lineage leukemia; MPD, myeloproliferative disorder; MSCV, murine stem cell retrovirus; PMN, polymorphonuclear leukocyte (neutrophil); PTP, protein-tyrosine phosphatase; ICSBP, interferon consensus sequence-binding protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; WT, wild type; IL, interleukin; DME, Dulbecco’s modified Eagle’s; IFN, interferon; G-CSF, granulocyte colony-stimulating factor.
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progressed to AML over 4–6 months (15). These studies indicated that overexpression of Abd HoxA proteins is sufficient for myeloproliferation, but additional genetic lesions are necessary for differentiation block and therefore AML.

Biochemical studies of the role of Abd HoxA proteins in leukemogenesis have been impaired by the paucity of known HoxA target genes. We previously found that HoxA10 represses transcription of genes encoding the NADPH-oxidase proteins gp91PHOX and p67PHOX in myeloid progenitor cells (the CYBB and NCF2 genes, respectively) (16–18). Transcription of these phagocyte-specific genes is rate-limiting for respiratory burst competence during myelopoiesis. Genes that encode secondary and tertiary granule proteins have homologous repressor elements in their promoter regions, suggesting that HoxA10 inhibits functional myeloid differentiation (19, 20).

We previously identified two conserved tyrosine residues in the HoxA10 HD (Tyr-326 and Tyr-343) that are phosphorylated during cytokine-induced differentiation of myeloid cell lines or primary murine myeloid progenitors. Phosphorylation of these HD-Y residues decreases HoxA10-binding affinity for negative cis elements in myeloid-specific genes (19). Consistent with this, overexpressed HoxA10 does not impair CYBB or NCF2 transcription in differentiating myeloid cell lines or gp91PHOX or p67PHOX expression in ex vivo differentiating primary murine progenitors (18, 21). However, overexpression of a form of HoxA10 with mutation of the HD-Y residues blocks CYBB and NCF2 transcription during differentiation of myeloid cell line transfectants and gp91PHOX and p67PHOX expression in differentiating murine myeloid progenitors (18, 21).

These studies suggest that HoxA10 activity is regulated by both abundance and post-translational modification of the protein during myelopoiesis. Therefore, we hypothesized that lesions in pathways that regulate HoxA10 tyrosine phosphorylation might cooperate with HoxA10 overexpression for differentiation block and AML. We also hypothesized that HoxA10 tyrosine phosphorylation state might be less important for regulation of target genes involved in myeloproliferation.

We previously found that HoxA10 is a substrate for SHP1 or SHP2–protein-tyrosine-phosphatase (PTP) in myeloid progenitor cells (18, 21). SHP1 and SHP2 are highly conserved nonreceptor PTPs. Residues in HoxA10 that are substrate for SHP1 or SHP2 include (but are not limited to) the HD tyrosines (18, 21). However, neither SHP1 nor SHP2 retains the ability to dephosphorylate HoxA10 in differentiating myeloid cells (18, 21). This decrease in activity is not because of decreased SHP1 or SHP2 expression and is observed with overexpressed proteins. Therefore, unknown mechanisms regulate SHP1 or SHP2–PTP activity during myelopoiesis.

Recent studies identified leukemia-associated mutations of the gene encoding SHP2 (the PTPN11 gene) (23–27). These mutations induce conformational changes in the SHP2 protein that unmask the PTP domain, resulting in constitutive activation (23). Expression of such activated SHP2 mutants results in cytokine hypersensitivity of myeloid progenitor cells in vitro and in vivo (23–27). We found that expression of constitutively active SHP2 blocks HoxA10–tyrosine phosphorylation during differentiation of myeloid cell lines or primary murine myeloid progenitor cells (21). We also found that co-overexpression of HoxA10 and an activated SHP2 mutant blocks CYBB or NCF2 transcription in myeloid cell lines and gp91PHOX or p67PHOX expression in ex vivo differentiating primary murine myeloid progenitor cells (21).

Based on these results, we hypothesized that constitutive SHP2 activation might cooperate in vivo with HoxA10 overexpression to induce rapid differentiation block and AML. This was of interest because activated SHP2 mutants have been described in myeloid leukemias with translocations involving the MLL gene (28). Because such leukemias also overexpress HoxA10, these two mutations are likely to co-exist in at least some cases of human AML.

In other studies, we identified DUSP4, the gene that encodes Mkp2 (mitogen-activated kinase phosphatase 2), as a HoxA10 target gene. In hematopoietic cells, Mkp2 antagonizes apoptosis by dephosphorylating c-Jun N-terminal kinases (Jnk). We found that HoxA10 activates DUSP4 transcription efficiently in immature myeloid cells (22). We also found that HoxA10 overexpression decreases Jnk phosphorylation and antagonizes apoptosis in an Mkp2-dependent manner (22). During myelopoiesis, the affinity of HoxA10 for the DUSP4 promoter decreases by an unknown mechanism, thereby decreasing Mkp2 expression in differentiating cells (22). These studies suggested that overexpressed HoxA10 might influence myeloproliferation by antagonizing apoptosis. The role of tyrosine phosphorylation in this process was unknown.

The first goal of this study is to determine whether overexpressing a form of HoxA10 with mutation of the HD-Y residues leads to rapid differentiation block and AML in vivo, and if co-overexpressing HoxA10 and a constitutively active SHP2 mutant has the same effect. The second goal of this study is to determine whether HD-Y residues are involved in regulating transcription of genes that impact survival or proliferation of myeloid progenitor cells, such as DUSP4. If not, studies with constitutively active SHP2 may suggest the existence of additional Tyr residues that regulate HoxA10 target genes involved in these functions.

**MATERIALS AND METHODS**

**Plasmids and Site-directed Mutagenesis**—The human HoxA10 cDNA was obtained from Dr. C. Largman (University of California, San Francisco) and subcloned into the pMSCV-puro vector for retroviral production and the pSRα vector for transfection into cell lines (Stratagene, La Jolla, CA) (21). The cDNA for SHP2 was obtained from Dr. Stuart Frank (University of Alabama, Birmingham) and subcloned into the pMSCV-neo retrovector and the pcDNAAmp vector for transfection into cell lines. HoxA10 with mutation of conserved homeodomain tyrosine residues (Y326/343F HoxA10; referred to as HD-Y-mut HoxA10) and a leukemia-associated, activating mutation in SHP2 (E76K–SHP2) were generated by site-directed mutagenesis, as described (18, 29).

Artificial promoter/reporter constructs were generated as described previously, in the minimal promoter/reporter vector, p-TATAACAT (19) (obtained from Dr. A. Kraft, Hollings Cancer Center at the Medical University of South Carolina, Charleston). Constructs were generated with four copies (in the for-
ward direction) of the −1174 to −1136-bp sequence from the DUSP4 promoter (p-DUSP4-TATACAT) or four copies of the −94 to −134-bp sequence from the CYBB promoter (p-CYBB-TATACAT) (22).

**Oligonucleotides**—Oligonucleotides were synthesized by MEG Biotec (Piedmont, NC). Primers for quantitative real time PCR were designed with the software from Integrated DNA Technologies.

**Culture and Retroviral Transudation of Primary Murine Bone Marrow**—High titer murine stem cell retroviral supernatants were produced using the murine stem cell retroviral vector (MASCO) and PT67 cell line, per the manufacturer’s instructions (Stratagene). Filtered retroviral supernatants were used immediately or stored at −80 °C. Sca1+ bone marrow cells were obtained from the femurs of C57/BL6 mice using the Miltenyi magnetic bead system (Miltenyi Biotechnology, Auburn, CA).

Cells that were to be used for transplantation experiments were cultured for 24 h in DME media with fetal bovine serum (10%), IL6 (10 ng/ml), IL3 (10 ng/ml), SCOFF (100 ng/ml), and penicillin/streptomycin (1%) at 37 °C. Cells were transduced by “spinoculation.” Briefly, 4.0 × 10^6 cells were suspended in 3 ml of DME media with cytokines. An equal volume of retroviral supernatant (−10^8 plaque-forming units of retrovirus) was added and Polybrene to 6 μg/ml. Cells were centrifuged at 1,500 g for 4 h at 15 °C, diluted with media, and incubated overnight. The procedure was repeated.

For ex vivo studies, transduced cells were cultured in DME supplemented with fetal bovine serum (10%), GM-CSF (10 ng/ml), IL3 (10 ng/ml), SCF (100 ng/ml), penicillin/streptomycin (1%), and puromycin (1.2 ng/ml) to select for transduced cells. Proliferation and apoptosis assays were performed on these cells after 48 h of antibiotic selection.

In other studies, nontransduced murine bone marrow cells were cultured under various cytokine conditions, and specific progenitor populations were isolated. Some cells were Sca1+ separated, as described above, and cultured in IL3 (10 ng/ml), IL6 (10 ng/ml), and SCF (100 ng/ml). Cells were harvested after 48 h. Other cells were cultured in GM-CSF (10 ng/ml) and IL3 (10 ng/ml) for 48 h. Some of these cells were harvested and CD34+-selected using the Miltenyi magnetic bead system. Other cells were washed and cultured in G-CSF (20 ng/ml) for 48 h and CD38+-selected using the Miltenyi magnetic bead system. These cells were analyzed by flow cytometry for expression of Sca1, CD34, CD38, Mac1 (CD11b), or Gr1 or by real time PCR.

**Murine Bone Marrow Transplantation**—All murine experiments were performed with approval of the Northwestern University Animal Care and Use Committee. Syngenic C57/BL6 mice were irradiated (560 rads), and transduced bone marrow cells (0.5 × 10^6) were infused by retro-orbital injection. Mice were examined weekly, and protocol stipulated that any animal with >10% weight loss or lethargy was sacrificed. In fact, no mice in these studies met this criteria for sacrifice. Blood counts were performed every 4 weeks by tail bleed. Differential white blood cell counts were performed on Wright-Giesma-stained blood smears by counting 200 cells on duplicate slides. Cohorts of mice (at least six) were sacrificed at 16 and 24 weeks. Viable cells were recovered by a magnetic bead separation technique (Miltenyi Biotechnology), and cells from each mouse were stored and analyzed separately for gene expression by real time PCR.

Peripheral blood leukocyte counts were used to characterize the mice as having normal myelopoiesis, an MPD or AML. Normal myelopoiesis was defined as a normal total leukocyte count with a normal distribution of lymphocytes and neutrophils (7,000–10,000 cells/mm^3 with 70% lymphocytes and 30% neutrophils). For these studies, myeloproliferative disorder was defined as an increase in the total leukocyte count with an increase in the absolute and relative numbers of neutrophils. Acute myeloid leukemia was defined as >20% myeloid blasts in the circulation.

**Proliferation**—Cells were harvested and plated at 10^5 cells per 200 μl in a 96-well dish in DME media with fetal bovine serum (10%) and IL3 (10 ng/ml). After 48 h, media were supplemented with a serial dilution of GM-CSF for 24 h at 37 °C, 5% CO_2 (from 10 to 0.01 ng/ml). [3H]Thymidine was added for the last 8 h. Cells were harvested with an automated cell harvester and 3H incorporation was determined by scintillation counting. Assays were performed in triplicate with cells from at least two independent transductions.

**Apoptosis**—Apoptosis was determined by annexin V/propidium iodide double staining according to manufacturer’s instructions (Beckman Coulter, Miami, FL). Cells were adjusted to a concentration of 1 × 10^6/ml and incubated with annexin-V/fluorescein isothiocyanate solution (2.5 μg/ml) and propidium iodide (12.5 μg/ml) on ice for 15 min and analyzed on a FACScan flow cytometer (BD Biosciences). Assays were performed in triplicate with cells from at least three independent transductions.

**Western Blots**—Murine bone marrow cells were lysed by boiling in 2× SDS sample buffer. Lysate proteins (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose according to standard techniques. Western blots were serially probed with antibodies to HoxA10, SHP2, and glyceraldehyde-3-phosphate dehydrogenase (to control for loading). For immunoprecipitation studies, lysates were immunoprecipitated under denaturing conditions with a mouse monoclonal antibody to phosphotyrosine or an irrelevant murine monoclonal antibody (to glutathione S-transferase) as described (29). Immunoprecipitates were analyzed by probing Western blots with an antibody to HoxA10. All of these studies were performed at least three times with cells from independent transduction experiments. Representative blots are shown.

**Real Time PCR**—RNA was isolated from bone marrow samples from individual mice at 16 or 24 weeks post-transplantation using the TRIzol reagent (Invitrogen). RNA integrity was verified by denaturing gel electrophoresis. Real time PCR was performed using SYBR green according to the “standard curve” method. Result were normalized to 18 S. Experiments were performed in triplicate. Bone marrow from each animal was analyzed individually.

**Myeloid Cell Line Culture**—The human myelomonocytic cell line U937 (30) was obtained from Andrew Kraft (Hollings Cancer Center at the Medical University of South Carolina, Charleston). Cells were maintained and differentiated as described (16, 17, 22). U937 cells were differentiated by treat-
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ment with 500 units/ml human recombinant IFNγ for 48 h (Hoffmann-La Roche).

Transfection and Reporter Gene Assays—Cells were transfected by electroporation as described (16, 17, 22). U937 cells (32 × 10⁶ per sample) were transfected with 50 μg of DUSP4-TATACAT, CYBB-TATACAT, or p-TATACAT (control); 50 μg of HoxA10/pSRα, Y326/343F HoxA10/pSRα (i.e. HD-Y-mut HoxA10), or control pSRα; and 15 μg of p-CMVβ-gal (to normalize for transfection efficiency). In other experiments U937 cells were co-transfected with the same reporter vectors; 50 μg of HoxA10/pSRα or control pSRα; and 50 μg of SHP2/pcDNA, E76K SHP2/pcDNA or control pcDNA; and 15 μg of p-CMVβ-gal. Transfectants were incubated for 48 h at 37 °C, 5% CO₂, with or without IFNγ (500 units/ml). Preparation of cell extracts, β-galactosidase, and chloramphenicol acetyltransferase assays were performed as described (16, 17, 22).

Statistics—Significance of differences between two groups was determined by Student’s t test. Statistical comparisons between groups of three or more were determined by analysis of variance.

RESULTS

HoxA10 Overexpression in Myeloid Cells Induced Cytokine Hypersensitivity—In previous studies, overexpression of HoxA10 in murine bone marrow cells expanded a myeloid progenitor population in vitro and induced an MPD in vivo (14, 15). However, these previous studies did not determine whether the mechanism for this effect of overexpressed HoxA10 was increased proliferation, decreased apoptosis, or both. To begin to address these mechanisms, we investigated whether HoxA10 overexpression in primary murine myeloid progenitors increased the proliferative response to GM-CSF. If so, this would suggest that HoxA10 regulates target genes involved in cytokine-induced proliferation. We also investigated whether HoxA10 overexpression protected primary murine progenitors from apoptosis during IL3 withdrawal. This result would suggest the existence of HoxA10 target genes involved in anti-apoptosis pathways. Because phosphorylation of tyrosine residues in the HoxA10 HD is crucial for regulation of target genes involved in phagocyte effector functions, we also investigated whether these residues played a role in regulating proliferation or apoptosis.

For these studies, Sca1⁺ cells were isolated from murine bone marrow and cultured in GM-CSF, IL3, and SCF (21). This population was studied because previous investigations suggest that the leukemia stem cells in Abd HoxA-overexpressing murine models represent committed myeloid progenitors, not a more primitive HSC population (11, 15). Cultured cells were transduced with a retroviral vector to express HoxA10, a form of HoxA10 with mutation of HD tyrosine residues 326 and 343 to phenylalanine (HD-Y mutant HoxA10), or with empty control vector (murine stem cell retroviral vector or MSCV).

We determined the effect of HoxA10 overexpression on the proliferative response to a dose titration of GM-CSF (as in Refs. 29, 31). We found significantly more proliferation at low GM-CSF doses in cells that were overexpressing either wild type (WT) or HD-Y mutant HoxA10 in comparison with control MSCV vector-transduced cells (Fig. 1A). However, there was no significant difference in GM-CSF-induced proliferation in cells overexpressing WT HoxA10 versus HD-Y-mut HoxA10 at any GM-CSF dose. These results suggested that HoxA10 overexpression induced GM-CSF hypersensitivity in a manner not regulated by phosphorylation of HD-Y residues.

Aliquots of transduced cells were also analyzed for the effect of HoxA10 overexpression on cell survival in response to a dose titration of IL3. For these studies, apoptosis was determined by annexin V staining and flow cytometry. At low IL3 doses, we found significantly less apoptosis in cells that were overexpressing WT or HD-Y-mut HoxA10 in comparison with control MSCV vector-transduced cells (Fig. 1B). There was no significant difference in apoptosis in cells overexpressing WT HoxA10 versus HD-Y-mut HoxA10 at any IL3 dose. These results suggested that HoxA10 overexpression induced apoptosis resistance in a manner that did not require phosphorylation of HD-Y residues.

In control experiments, we verified equivalent overexpression of WT and HD-Y-mut HoxA10 by Western blots of lysate proteins from transduced cells, as in previous studies (Fig. 1C) (21). Aliquots of cell lysates were also analyzed for HoxA10 tyrosine phosphorylation by immunoprecipitation and Western blot (Fig. 1D). For these studies, cell lysates were immunoprecipitated with either an anti-phosphotyrosine or irrelevant control antibody. Immunoprecipitates were analyzed by probing Western blots with an antibody to HoxA10. Total tyrosine phosphorylation of overexpressed HoxA10 was not significantly different for WT versus HD-Y mutant HoxA10. This was not unexpected, because these two proteins only differ in two Tyr residues (Tyr-9 versus -7). The studies in this section were performed using samples from three independent transduction experiments, and representative blots are shown.

Overexpression of HD Tyrosine Mutant HoxA10 Rapidly Induced AML in Vivo—We next extended our observations to an in vivo murine bone marrow transplantation model. Based on the results in the previous section, the first hypothesis of these studies was that overexpression of WT or HD-Y mutant HoxA10 would induce equivalent myeloproliferation in vivo. However, because overexpressed HD-Y mutant HoxA10 repressed myeloid-specific gene transcription in differentiating cells more efficiently than WT (18, 21), we hypothesized that overexpression of HD-Y mutant HoxA10 would lead to more rapid differentiation block and AML in vivo. To investigate these hypotheses, irradiated mice were transplanted with murine bone marrow that had been transduced with retroviral vectors to overexpress WT or HD-Y mutant HoxA10 or empty MSCV control vector. Peripheral blood counts and leukocyte differential counts were determined every 4 weeks post-transplantation.

Mice that were transplanted with control MSCV vector-transduced bone marrow exhibited normal peripheral blood leukocyte counts throughout the observation period with normal proportions of lymphocytes versus neutrophils (2/3 lymphocytes and 1/3 neutrophils (PMN)) (Fig. 2A). In contrast, mice transplanted with bone marrow that had been transduced with a vector to overexpress either WT HoxA10 (Fig. 2B) or HD-Y mutant HoxA10 (Fig. 2C) exhibited leukocytosis by 4
weeks. The number of circulating leukocytes in these mice was significantly greater than in the control mice at all time points ($p = 0.01, n = 9$). Circulating myeloid and lymphoid cells were approximately equivalent in number in mice transplanted with bone marrow that was overexpressing either WT or HD-Y mutant HoxA10. Therefore, both groups of HoxA10-overexpressing bone marrow exhibited an MPD characterized by an increase in circulating myeloid cells. Although there was also a slight increase in lymphocytes, this did not reach statistical significance. Consistent with our hypothesis, there was no significant difference in total leukocyte counts in mice transplanted with bone marrow that was overexpressing WT versus HD-Y mutant HoxA10 ($p > 0.2, n = 9$).

In previous studies of mice transplanted with HoxA10-overexpressing bone marrow, a distinction was not made between death because of overwhelming MPD versus progression to AML (15). In this study, we were interested in the rate of development of differentiation block and AML in vivo, and the impact of phosphorylation of the HD-Y residues on this process. Therefore, we analyzed transplanted mice for circulating immature myeloid cells as an indication of progression from MPD to AML. For these studies, we employed criteria that are equivalent in mice transplanted with bone marrow that was transduced with either a WT or a HD-Y mutant HoxA10 expression vector. Myeloid blasts from these two groups were morphologically similar (Fig. 2E).

These studies suggested that mutation of the two conserved HD-Y residues eliminated the latency for development of AML in mice overexpressing HoxA10. This was consistent with our hypothesis regarding the role of these HoxA10 residues in transcription of target genes that confer the mature myeloid phenotype.

In control experiments, we investigated HoxA10 expression in the bone marrow of the mice, post-transplant. Quantitative real time PCR was used to evaluate HoxA10 mRNA expression in total bone marrow mononuclear cells at 16 or 24 weeks post-transplantation. At both time points, HoxA10 expression was significantly greater in mice transplanted with bone marrow transduced with WT or HD-Y mutant HoxA10 expression vector in comparison with empty MSCV vector-transduced bone marrow (Fig. 2F). Expression of WT versus HD-Y mutant HoxA10 was not significantly different at either time point ($p = 0.2, n = 9$).

HoxA10 overexpression increased significantly between 16 and 24 weeks in mice transplanted with bone marrow that had used to assess progression to AML in human MPD, greater than 20% blasts in the circulation (32).

As anticipated, no immature myeloid cells were observed in the circulation of mice transplanted with control MSCV vector-transduced bone marrow. In contrast, we observed increasing numbers of immature cells in the circulation of mice transplanted with either WT or HD-Y mutant HoxA10-overexpressing bone marrow. Surface marker expression and histology determined that these were myeloid blasts, consistent with previous studies (15). We found that mice transplanted with HD-Y mutant HoxA10-overexpressing bone marrow exhibited significantly more circulating myeloid blasts at earlier time points in comparison with mice transplanted with WT HoxA10-overexpressing bone marrow ($p < 0.001, n = 10$).

Specifically, we found that 50% of mice transplanted with HD-Y-mut HoxA10-overexpressing bone marrow recovered from the transplant with AML, and 100% had AML by 12 weeks (Fig. 2D). However, it took 16 weeks for 50% of mice transplanted with WT HoxA10-overexpressing bone marrow to exhibit AML. By 24 weeks, the number of circulating myeloid blasts was significantly greater than in the control mice at all time points ($p = 0.01, n = 9$). Circulating myeloid and lymphoid cells were approximately equivalent in number in mice transplanted with bone marrow that was overexpressing either WT or HD-Y mutant HoxA10. Therefore, both groups of HoxA10-overexpressing bone marrow exhibited an MPD characterized by an increase in circulating myeloid cells. Although there was also a slight increase in lymphocytes, this did not reach statistical significance. Consistent with our hypothesis, there was no significant difference in total leukocyte counts in mice transplanted with bone marrow that was overexpressing WT versus HD-Y mutant HoxA10 ($p > 0.2, n = 9$).

In previous studies of mice transplanted with HoxA10-overexpressing bone marrow, a distinction was not made between death because of overwhelming MPD versus progression to AML (15). In this study, we were interested in the rate of development of differentiation block and AML in vivo, and the impact of phosphorylation of the HD-Y residues on this process. Therefore, we analyzed transplanted mice for circulating immature myeloid cells as an indication of progression from MPD to AML. For these studies, we employed criteria that are equivalent in mice transplanted with bone marrow that was transduced with either a WT or a HD-Y mutant HoxA10 expression vector. Myeloid blasts from these two groups were morphologically similar (Fig. 2E).

These studies suggested that mutation of the two conserved HD-Y residues eliminated the latency for development of AML in mice overexpressing HoxA10. This was consistent with our hypothesis regarding the role of these HoxA10 residues in transcription of target genes that confer the mature myeloid phenotype.
been transduced with either HoxA10 expression vector \( (p < 0.01, n = 9) \). These studies suggested that overexpression of HoxA10 conferred an advantage to murine bone marrow cells, resulting in expansion of cells with the highest HoxA10 expression levels. These studies also suggested that this effect was not influenced by the HD-Y residues in HoxA10.

**In Vivo Overexpression of WT or HD-Y Mutant HoxA10 Expanded a Bone Marrow Population with Myeloid Progenitor Characteristics**—The development of an MPD in mice transplanted with HoxA10-overexpressing bone marrow implied expansion of a progenitor population. However, the specific population that was expanded in vivo had not been identified nor had the level of differentiation block upon progression to AML. Therefore, the goal of these studies was to determine the molecular characteristics of MPD and AML in mice transplanted with WT or HD-Y mutant HoxA10.
We were interested in characterizing expanded bone marrow populations according to expression of lineage and differentiation stage-specific genes. In mice, Sca1 is expressed in the most primitive populations of progenitor cells. Differentiation to committed myeloid progenitors is characterized by decrease in Sca1 expression and increased expression of CD34 and CD11b (also known as Mac1). As myeloid progenitors differentiate to mature phagocytes, CD34 expression decreases, and expression of CD38, CD11b, and Gr1 increases. For these studies, gene expression was determined by real time PCR of mRNA isolated from murine bone marrow. This permitted us to simultaneously study these genes of interest in each individual mouse in a quantitative manner.

Prior to in vivo studies, we examined ex vivo differentiating primary murine bone marrow cells to verify that expression of Sca1, CD34, CD11b, CD38, or Gr1 protein correlated with mRNA. For these studies, Sca1+ murine bone marrow cells were isolated and cultured under various conditions. To generate immature progenitors, cells were cultured in IL3, IL6, and SCF, and Sca1-selected cells were analyzed. To isolate committed myeloid progenitors, cells were cultured in GM-CSF and IL3 for 72 h, and CD34+ cells were isolated for analysis. For differentiating PMN, cells were cultured in GM-CSF and IL3 for 48 h followed by 24 h with G-CSF and isolation of CD38+ cells. Expression of Sca1, CD34, CD11b, CD38, and Gr1 protein was determined by flow cytometry (Fig. 3, top) and mRNA by real time PCR (Fig. 3, bottom).

Sca1 protein (Fig. 3A) and mRNA (Fig. 3B) expression was significantly greater in cells cultured in IL3, IL6, and SCF (immature progenitor conditions) in comparison with cells cultured in GM-CSF + IL3 (myeloid progenitor conditions) or differentiated with G-CSF (p = 0.02, n = 4 and p = 0.006, n = 4, respectively). We found significantly more CD34 mRNA and protein expression in cells cultured under myeloid progenitor conditions in comparison with immature progenitor conditions (p = 0.01, n = 4 and p = 0.002, n = 4, respectively). CD34 protein and mRNA expression in myeloid progenitor cells decreased significantly during differentiation with G-CSF (p = 0.01, n = 4 and p = 0.04, n = 4, respectively).

In contrast, expression of CD38 mRNA and protein was significantly greater in G-CSF-treated cells in comparison with cells cultured under myeloid progenitor conditions (p = 0.03, n = 4 and p = 0.002, n = 4, respectively). CD38 mRNA and protein expression was also significantly greater in cells cultured in GM-CSF + IL3 in comparison with IL3, IL6, and SCF (p = 0.03, n = 4 and p = 0.02, n = 4, respectively). To identify cells specifically undergoing myeloid differentiation, expression of CD11b and Gr1 was determined. Cells cultured in GM-CSF and IL3 exhibited significantly more CD11b protein and mRNA expression in comparison with cells cultured in IL3, IL6, and SCF (p < 0.0001, n = 4 and p = 0.003, n = 4, respectively). Expression of CD11b protein and mRNA increased significantly upon differentiation with G-CSF (p < 0.001, n = 4 and p = 0.04, n = 4, respectively). Similarly, differentiation of with...
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G-CSF significantly increased expression of Gr1 protein and mRNA \((p < 0.0001, n = 4\) for both).

These results suggested that mRNA and protein expression for these genes correlated at various differentiation stages. Therefore, we used real time PCR to analyze gene expression in total bone marrow mononuclear cells from mice sacrificed 16 or 24 weeks post-transplantation. At 16 weeks, all of the mice transplanted with HD-Y mutant HoxA10-overexpressing bone marrow had developed AML, although the majority of mice transplanted with WT HoxA10-overexpressing bone marrow exhibited an MPD with mature PMN. However, by 24 weeks, the mice in both groups had progressed to AML.

We found no significant difference in bone marrow Sca1 mRNA expression in mice transplanted with HoxA10 or HD-Y-mut HoxA10-overexpressing bone marrow in comparison with mice transplanted with control vector-transduced bone marrow at either 16 or 24 weeks (Fig. 4A). These studies suggested that HoxA10 overexpression did not expand the most primitive progenitor population *in vivo* (consistent with previous *ex vivo* studies) (12).

However, bone marrow CD34 expression was significantly greater in mice transplanted with HoxA10 or HD-Y mutant HoxA10-overexpressing bone marrow in comparison with mice transplanted with control bone marrow at both 16 and 24 weeks \((p \leq 0.02, n = 9\) (Fig. 4B). CD34 expression was not significantly different in the two groups of HoxA10-overexpressing mice at either time point \((p = 0.6, n = 9)\), and it increased significantly between 16 and 24 weeks in both groups \((p \leq 0.05, n = 9)\). These results suggested that both the MPD and subsequent AML that developed in HoxA10-overexpressing mice was characterized by expansion of a CD34-expressing progenitor population.

To determine whether these progenitors were myeloid, we also determined CD11b expression. At 16 weeks post-transplant, we found significantly increased CD11b expression in the bone marrow of mice transplanted with WT or HD-Y mutant HoxA10 in comparison with control MSCV vector-transduced bone marrow \((p < 0.03, n = 9)\) (Fig. 4C). CD11b expression was not significantly different in mice transplanted with WT versus HD-Y mutant HoxA10 expressing bone marrow \((p = 0.7, n = 9)\). Although there was some increase in CD11b expression between 16 and 24 weeks in these two groups of mice, this was not statistically significant \((p = 0.5, n = 9)\). Therefore, in contrast to CD34, expression of CD11b did not significantly increase over time in the mice with WT or HD-Y mutant HoxA10 expression in the bone marrow. This may reflect the fact that CD11b is expressed in myeloid progenitors, but expression also increases during phagocyte differentiation. Therefore, this result might reflect a balance between progenitor expansion on the one hand, and differentiation block on the other.

To specifically investigate the impact of overexpressing WT or HD-Y mutant HoxA10 on myeloid differentiation, we determined expression CD38 and Gr1. At 16 weeks, bone marrow CD38 expression was significantly greater in mice transplanted with control bone marrow in comparison with mice transplanted with HD-Y mutant HoxA10-overexpressing bone marrow \((p = 0.04, n = 9)\) (Fig. 4D). In contrast, bone marrow CD38 expression was significantly less in mice transplanted with control bone marrow in comparison with WT HoxA10-overexpressing bone marrow \((p = 0.03, n = 9)\). This was consistent with differentiation block (less CD38 expression) in mice with HD-Y mutant HoxA10 overexpression, and an MPD with mature neutrophils (increased CD38 expression) in mice with WT HoxA10 overexpression at 16 weeks. By 24 weeks, CD38 expression in mice transplanted with either WT or HD-Y mutant HoxA10-overexpressing bone marrow was significantly less than in control mice \((p < 0.02, n = 9)\). This was consistent with AML and differentiation block in both groups of HoxA10-overexpressing mice at this time.

Similarly, Gr1 expression was significantly less in mice transplanted with control vector-transduced bone marrow in comparison with HoxA10-overexpressing bone marrow at 16 weeks \((p = 0.005, n = 9)\) (Fig. 4E). At this time, expression of Gr1 was significantly less than control in mice transplanted with HD-Y mutant HoxA10-overexpressing bone marrow \((p = 0.04, n = 9)\), consistent with differentiation block in these mice. As for CD38 expression, Gr1 expression at 24 weeks was significantly greater in control mice in comparison with mice transplanted with either WT or HD-Y mutant HoxA10-overexpressing bone marrow.

These results suggested that the MPD in HoxA1-overexpressing mice was characterized by expansion of myeloid progenitors and differentiating phagocytes. As AML developed, there was evidence of molecular differentiation block with decreased expression of genes characteristic of differentiating myeloid cells (CD38 and Gr1).

**Overexpression of HoxA10 Altered Target Gene Expression in Vivo**—We were interested in correlating these results with expression of HoxA10 target gene products in vivo. To investigate impact of HoxA10 overexpression on gp91<sup>PHOX</sup>, we quantitated mRNA expression in total bone marrow mononuclear cells at 16 or 24 weeks post-transplant using real time PCR.

At 16 weeks post-transplant, we found significantly less gp91<sup>PHOX</sup> expression in mice transplanted with HD-Y mutant HoxA10-overexpressing bone marrow in comparison with control or WT HoxA10-overexpressing bone marrow \((p = 0.01, n = 9\) and \(p = 0.03 n = 9\), respectively) (Fig. 5A). However, at 24 weeks, gp91<sup>PHOX</sup> expression in mice transplanted with WT HoxA10-overexpressing bone marrow had decreased significantly \((p = 0.05, n = 9)\). Expression of gp91<sup>PHOX</sup> was not significantly different in the bone marrow of mice overexpressing WT versus HD-Y mutant HoxA10 at this time \((p = 0.4, n = 9)\). These results were consistent with the development of differentiation block and AML by 16 weeks in mice with bone marrow overexpression of HD-Y mutant HoxA10 and by 24 weeks in mice overexpressing WT HoxA10.

We also investigated the impact of HoxA10 overexpression and HD-Y phosphorylation on bone marrow Mkp2 expression at 16 or 24 weeks. We found that Mkp2 expression was significantly greater than control in mice transplanted with HoxA10 or HD-Y mutant HoxA10-overexpressing bone marrow at 16 and 24 weeks \((p < 0.001, n = 9\) and \(p = 0.03, n = 9\), respectively) (Fig. 5B). Mkp2 expression was not significantly different in these two groups at either time point \((p \geq 0.3, n = 9)\).
These results were consistent with equivalent myeloproliferation and apoptosis resistance in bone marrow overexpressing either WT or HD-Y mutant HoxA10. These results also suggested that HoxA10 activation of DUSP4 transcription was not regulated by phosphorylation of the HD-Y residues. This implied that either DUSP4 activation was not regulated by HoxA10 tyrosine phosphorylation, or it was regulated by one of the non-HD tyrosine residues.

Constitutive Activation of SHP2-PTP Increased Cytokine Hypersensitivity in HoxA10-overexpressing Myeloid Cells—PTPN11 gene mutations have been described in association with disease progression in human AML (23–27). These mutations result in constitutive activation of the gene product (SHP2). Such SHP2 mutants conferred hypersensitivity of myeloid cells to a number of cytokines (29, 33). Also, we previously found that expression of constitutively active SHP2 mutants
blocked HoxA10 tyrosine phosphorylation during ex vivo differentiation (21). In this study, we investigated whether constitutive SHP2 activation impacted cytokine hypersensitivity in HoxA10-overexpressing cells.

For these studies, Sca1+ murine bone marrow cells were isolated, cultured in GM-CSF, IL3, and SCF, and transduced with retroviral vectors to overexpress various combinations of HoxA10, an activated SHP2 mutant (E76K), or empty control MSCV vector. Proliferation in response to a dose titration of GM-CSF was determined, as above. Overexpression of either HoxA10 or E76K SHP2 induced GM-CSF hypersensitivity as expected (Fig. 6A) (see Ref. 27). However, proliferation at low GM-CSF doses was significantly greater in cells transduced with both HoxA10 and E76K SHP2 expression vectors in comparison with cells transduced with either vector alone (p ≤ 0.03, n = 4).

Combined with our results above, these studies suggested that E76K SHP2 was not increasing cytokine hypersensitivity in HoxA10-overexpressing cells by dephosphorylating the HD-Y residues. This might reflect dephosphorylation of non-HD-Y residues in HoxA10. Alternatively, the effect of HoxA10 overexpression could be additive with an independent effect of SHP2 activation on non-HoxA10 substrates.

We also determined the impact of combining constitutive SHP2 activation with HoxA10 overexpression on apoptosis during IL3 withdrawal. We found that apoptosis was not significantly different in cells expressing E76K SHP2 in comparison with WT HoxA10 (Fig. 6B) (p ≥ 0.2, n = 3). However, co-overexpression of E76K SHP2 and HoxA10 significantly decreased apoptosis at low IL3 doses in comparison with overexpression of HoxA10 alone (p < 0.01, n = 3).

This result suggested that the effect of constitutive SHP2 activation in HoxA10-overexpressing cells was related to the effect of SHP2 on HoxA10. However, because apoptosis was not significantly different in cells overexpressing WT versus HD-Y mutant HoxA10, this also suggested that the effect of E76K SHP2 was mediated by non-HD-Y residues. Alternatively, this result could be explained if SHP2 activation influenced phosphorylation state of a HoxA10-partner-protein involved in transcriptional regulation of apoptosis-related target genes.

Control Western blots were performed to verify overexpression of HoxA10 and E76K SHP2 in the transduced cells (Fig. 5).
These blots also demonstrated that HoxA10 overexpression did not influence SHP2 expression, and that expressing constitutively active SHP2 did not influence HoxA10 expression. Aliquots of transduced cells were analyzed by immunoprecipitation followed by Western blotting to confirm that E76K SHP2 expression decreased tyrosine phosphorylation of overexpressed HoxA10 (Fig. 6D). Each of these studies was performed with cells from at least two independent transduction experiments.

Constitutive SHP2 Activation Rapidly Induced AML in Vivo in Mice with HoxA10 Overexpression—We previously found that expression of activated SHP2 mutants in HoxA10-overexpressing murine myeloid progenitors blocked myeloid-specific gene transcription during in vitro differentiation (21). Therefore, we hypothesized that combining HoxA10 overexpression and constitutive SHP2 activation would accelerate differentiation block and progression to AML in vivo. Based on the results of the in vitro studies above, we also hypothesized these two leukemia-associated abnormalities might cooperate to increase myeloproliferation in vivo.

To investigate these issues, irradiated mice were transplanted with bone marrow that had been transduced with retroviral vectors to express HoxA10 + E76K SHP2 or HoxA10 + SHP2 (as a control for SHP2 overexpression). Because expres-
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sion of activated SHP2 mutants alone induces myeloproliferation characterized with increased neutrophils (26, 32), additional mice were transplanted with bone marrow that had been transduced with a vector to express WT or E76K SHP2. Peripheral blood counts were determined every 4 weeks as above.

Blood counts in mice transplanted with SHP2 expression vector-transduced bone marrow were not significantly different from control mice (Fig. 7A). Mice transplanted with bone marrow that was transduced with an E76K SHP2 expression vector developed leukocytosis characterized by significantly greater numbers of PMN in comparison with control (p < 0.03, n = 7) (Fig. 7B). Mice transplanted with bone marrow transduced with vectors to co-overexpress HoxA10 + WT SHP2 developed a leukocytosis that was not significantly different from mice transplanted with bone marrow overexpressing HoxA10 alone (p > 0.2, n = 7) (compare Fig. 2B and Fig. 7C).

However, by 8 weeks, mice transplanted with bone marrow that had been transduced with vectors to express both HoxA10 and E76K SHP2 exhibited significantly more leukocytosis in comparison with mice transplanted with bone marrow that was overexpressing HoxA10, E76K SHP2, or HoxA10 + WT SHP2 (p < 0.05, n = 7) (Fig. 7D). The relative leukocytosis was because of an increase in both neutrophils and immature myeloid blasts at this early time point.

At all time points there were significantly more myeloid blasts in the circulation of mice transplanted with HoxA10 + E76K SHP2-overexpressing bone marrow in comparison with mice transplanted with bone marrow that was overexpressing HoxA10 + WT SHP2 (p < 0.02, n = 9). Numbers of circulating myeloid blasts in mice transplanted with HoxA10 + E76K SHP2-overexpressing bone marrow were not significantly different from mice transplanted with HD-Y mutant HoxA10-overexpressing bone marrow (p = 0.3, n = 7).

50% of mice transplanted with bone marrow transduced with HoxA10 + E76K SHP2 expression vectors developed AML by 4 weeks and 100% by 12 weeks (Fig. 7E). This was similar to the rate at which AML developed in mice transplanted with HD-Y mutant HoxA10-overexpressing bone marrow. In contrast, it took 16 weeks for 50% of mice transplanted with HoxA10 + WT SHP2-overexpressing bone marrow to develop AML, similar to mice transplanted with bone marrow overexpressing HoxA10 alone. Myeloid blasts from mice transplanted with HoxA10 + E76K SHP2-transduced bone marrow were morphologically similar to those in mice transplanted with bone marrow overexpressing WT (Fig. 4F) or HD-Y mutant HoxA10 (Fig. 7F).

Importantly, myeloid blasts appeared in the circulation of only rare mice transplanted with E76K SHP2 expression vector-transduced bone marrow and only at late time points (Fig. 7E). This result suggested we were not observing independent, additive effects of HoxA10 overexpression and SHP2 activation but an impact of SHP2 activation on overexpressed HoxA10.

In control experiments, we verified HoxA10 overexpression post bone marrow transplant by real time PCR. At 16 weeks, HoxA10 overexpression was not significantly different in mice transplanted with bone marrow that had been transduced with vectors to express HoxA10 alone, HoxA10 + SHP2, or HoxA10 + E76K SHP2 (p = 0.2, n = 9) (Fig. 2F). This was of interest because only mice transplanted with bone marrow transduced with HoxA10 + E76K SHP2 expression vectors had AML at this time point. This result suggested SHP2 activation was not influencing HoxA10-induced AML by increasing HoxA10 expression.

Between 16 and 24 weeks, HoxA10 overexpression increased significantly in mice transplanted with bone marrow transduced with either HoxA10 + SHP2 or HoxA10 + E76K SHP2 expression vectors. However, significantly more HoxA10 was expressed at 24 weeks in mice transplanted with bone marrow transduced with vectors to express HoxA10 + E76K SHP2 in comparison with HoxA10 alone, HD-Y-mut HoxA10, or HoxA10 + SHP2 (p = 0.02, n = 9). Overexpression of either WT or E76K SHP2 alone did not increase expression of endogenous HoxA10 in the transplanted animals at either 16 or 24 weeks (p ≥ 0.2, n = 9).

These results suggested that cells with the combination of HoxA10 overexpression and SHP2 activation had a proliferative or survival advantage in comparison with cells overexpressing WT or HD-Y mutant HoxA10 alone. One possible explanation was that the impact of HoxA10 overexpression on cell proliferation/survival was additive with the effect of SHP2 activation on non-HoxA10 substrates. Alternatively, differences between mice transplanted with HD-Y mutant HoxA10-overexpressing bone marrow versus bone marrow transduced with HoxA10 + E76K SHP2 expression vectors might reflect the impact of constitutive SHP2 activation on non HD-Y residues in HoxA10.

We also verified overexpression of SHP2 in the bone marrow by real time PCR. We found equivalent SHP2 overexpression in mice transplanted with bone marrow that was transduced with vectors to express SHP2 (at 16 and 24 weeks), E76K SHP2 (at 16 and 24 weeks), HoxA10 + SHP2 (at 16 and 24 weeks), or HoxA10 + E76K SHP2 (at 16 weeks) (p = 0.8, n = 9) (Fig. 7G). However, significantly more SHP2 was expressed at 24 weeks in mice transplanted with bone marrow transduced with HoxA10 + E76K SHP2 expression vectors in comparison with these other groups (p < 0.04, n = 9). These results were also consistent with a relative advantage for cells overexpressing both HoxA10 and activated SHP2 over time.

Constitutive SHP2 Activation in Mice with HoxA10 Overexpression Expanded a Bone Marrow Population with Myeloid Progenitor Characteristics—As in the studies above, we investigated differentiation stage-specific gene expression in mice transplanted with bone marrow that was transduced with vectors to overexpress HoxA10 with either WT or E76K SHP2, WT or activated SHP2 alone, or control vector. We used real time PCR to investigate expression of Sca1, CD34, CD11b, CD38, and Gr1 in the bone marrow of individual mice 16 or 24 weeks after transplantation.

We found that Sca1 expression was not significantly different at 16 or 24 weeks in mice transplanted with bone marrow that was overexpressing HoxA10 with either WT or E76K SHP2, WT or activated SHP2 alone, or control vector (Fig. 4A). At 16 and 24 weeks, bone marrow CD34 expression was equivalently increased in mice transplanted with HoxA10, HD-Y mutant HoxA10, or HoxA10 + SHP2-overexpressing bone marrow...
FIGURE 7. Expression of constitutively active SHP2 in HoxA10-overexpressing bone marrow increased myeloproliferation and accelerated progression to acute myeloid leukemia in vivo. Mice were transplanted with bone marrow that had been transduced with a vectors to overexpress SHP2, E76K SHP2, HoxA10 + SHP2, or HoxA10 + E76K SHP2. Peripheral blood leukocyte counts were determined every 4 weeks post-transplantation. A, peripheral blood counts are normal in mice transplanted with SHP2-overexpressing bone marrow. Peripheral blood counts from mice transplanted with SHP2 expression vector-transduced bone marrow were not significantly different from mice transplanted with MScV control vector-transduced bone marrow. These mice maintained normal white blood cell (WBC) differential throughout the post-transplantation period and did not exhibit immature cells in the circulation. B, mice transplanted with bone marrow expressing constitutively active SHP2 developed mild MPD. Peripheral blood counts from mice transplanted with bone marrow that was transduced with a vector to overexpress a constitutively active form of SHP2 (E76K SHP2) demonstrated mild myeloproliferation characterized by neutrophilia and lymphocytosis, consistent with previous results. C, mice transplanted with bone marrow that was co-overexpressing HoxA10 + WT SHP2 developed MPD and AML that was similar to mice transplanted with bone marrow overexpressing HoxA10 alone. Mice transplanted with bone marrow that was co-overexpressing HoxA10 + WT SHP2 exhibited a myeloproliferative disorder with mature neutrophils. Peripheral blood blasts increased over time. D, mice transplanted with bone marrow that was co-overexpressing HoxA10 + constitutively active SHP2 developed MPD which rapidly evolved to AML. Mice transplanted with bone marrow that had been transduced with vectors to overexpress HoxA10 + E 76K SHP2 in comparison with mice transplanted with HoxA10 + WT SHP2 overexpressing bone marrow are indicated by * or **, respectively. E, AML developed by 4 weeks in 50% of mice transplanted with bone marrow that was co-overexpressing HoxA10 + constitutively active SHP2. The number of circulating blasts in the peripheral blood of the mice post-transplantation was determined to evaluate development of AML. Mice were considered to have AML if they had >20% blasts in the circulation. At least 10 mice were followed in each group until the last time point. F, myeloid blasts from mice transplanted with bone marrow that was overexpressing HoxA10 or HoxA10 + constitutively active SHP2 were morphologically similar. Peripheral blood was obtained from mice that were transplanted with bone marrow transduced with a vector to overexpress either HoxA10 or HoxA10 + E76K SHP2. Wright-Giemsa stains were performed of blood smears. G, SHP2 expression increased over time in mice transplanted with bone marrow that was co-overexpressing HoxA10 + constitutively active SHP2. Expression of SHP2 in the bone marrow post-transplantation was determined by real time PCR. Results were normalized to 18S rRNA abundance. Statistically significant differences in SHP2 expression at 24 weeks in mice transplanted with bone marrow that was transduced with a vector to overexpress HoxA10 + E76K SHP2 is indicated by *.
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(Fig. 4B). At both time points, CD34 expression was significantly greater in mice transplanted with HoxA10 + E76K SHP2-overexpressing bone marrow than these other three groups \( (p < 0.02, n = 9) \). Expression of WT or E76K SHP2 alone in the transplanted bone marrow did not alter CD34 expression in comparison with empty vector. This suggested that increased CD34 expression in mice transplanted with bone marrow co-overexpressing HoxA10 + activated SHP2 was not because of independent effects of these two proteins but rather to the impact of constitutive SHP2 activation on overexpressed HoxA10.

CD11b expression was significantly greater than control in mice transplanted with bone marrow that was co-overexpressing HoxA10 and either WT or constitutively active SHP2 (Fig. 4C). Expression of CD11b in these mice was comparable with mice transplanted with bone marrow overexpressing WT HoxA10 alone or HD-Y mutant HoxA10. Although CD11b is expressed in myeloid progenitors, expression also increases during terminal myeloid differentiation. Therefore, expression of this gene is influenced by both differentiation stage and myeloid progenitor expansion.

At 16 weeks, CD38 expression was significantly less than control in mice transplanted with bone marrow that was co-overexpressing WT HoxA10 + E76K SHP2 \( (p < 0.01, n = 9) \) (Fig. 4D). At this time point, CD38 expression was not significantly different in mice transplanted with bone marrow that was overexpressing HoxA10 + E76K SHP2 versus HD-Y mutant HoxA10. However, CD38 expression was not significantly different from control in mice transplanted with bone marrow overexpressing activated SHP2 alone \( (p > 0.1, n = 9) \). This was consistent with differentiation block in mice co-overexpressing HoxA10 + E76K SHP2 at 16 weeks, but not in mice overexpressing either protein alone. Co-overexpression of HoxA10 + WT SHP2 did not alter CD38 expression relative to overexpression of HoxA10 alone at 16 weeks.

Bone marrow CD38 expression decreased significantly between 16 and 24 weeks in mice transplanted with HoxA10 + SHP2-overexpressing bone marrow. At this time point, there was no significant difference in CD38 expression in mice that had been transplanted with HoxA10, HD-Y-mut HoxA10, HoxA10 + SHP2, or HoxA10 + E76K SHP2-overexpressing bone marrow \( (p = 0.9, n = 9) \). Mice in all of these groups had progressed to differentiation block and AML at 24 weeks. None of the mice transplanted with WT or E76K SHP2 overexpressing or control bone marrow had decreased CD38 expression or AML at this time point.

At 16 weeks, Gr1 expression was significantly greater than control in mice transplanted with HoxA10 + WT SHP2 co-overexpressing bone marrow \( (p < 0.001, n = 9) \) and was not significantly different from mice overexpressing HoxA10 alone \( (p = 0.5, n = 9) \). In comparison with control mice, Gr1 expression was significantly less in mice transplanted with HoxA10 + E76K SHP2 co-overexpressing bone marrow at 16 weeks \( (p < 0.02, n = 9) \), and was not significantly different from mice transplanted with HD-Y mutant HoxA10-overexpressing bone marrow \( (p = 0.5, n = 9) \).

At 24 weeks, Gr1 expression decreased significantly in mice that had been transplanted with HoxA10 + WT SHP2 co-overexpressing bone marrow \( (p < 0.02, n = 9) \). At this time point, there was no significant difference in Gr1 expression in mice with HoxA10, HD-Y mutant HoxA10, HoxA10 + WT SHP2, or HoxA10 + E76K SHP2 overexpression in the bone marrow \( (p = 0.6, n = 9) \). In contrast, overexpression of WT or E76K SHP2 in the bone marrow did not significantly alter Gr1 expression in comparison with control mice at any time point \( (p = 0.2, n = 9) \).

Constitutive SHP2 Activation in Mice with HoxA10 Overexpression Altered Target Gene Expression in Vivo—To investigate impact of HoxA10 overexpression on gp91\(^{ \text{PHOX}} \) in vivo, we quantitated bone marrow mRNA expression in total murine bone marrow at 16 or 24 weeks post-transplant by real time PCR.

At 16 weeks post-transplant, we found significantly less gp91\(^{ \text{PHOX}} \) expression in mice transplanted with bone marrow that was overexpressing HoxA10 + E76K SHP2 in comparison with bone marrow overexpressing HoxA10 + WT SHP2 or control bone marrow \( (p < 0.001, n = 9) \) (Fig. 5A). Expression of gp91\(^{ \text{PHOX}} \) was not significantly different in mice transplanted with HD-Y-mut HoxA10 or HoxA10 + E76K SHP2 \( (p = 0.8, n = 9) \). These results were consistent with the development of differentiation block and AML early after transplant in such mice, but not in mice transplanted with bone marrow that was overexpressing HoxA10 alone or with WT SHP2.

By 24 weeks post-transplant, there was a further decrease in gp91\(^{ \text{PHOX}} \) expression in mice transplanted with bone marrow transduced with vectors to express HoxA10 + WT SHP2. At this point, there was no significant difference in gp91\(^{ \text{PHOX}} \) expression between mice transplanted with HoxA10-, HD-Y-mut HoxA10-, HoxA10 + SHP2-, or HoxA10 + E76K SHP2-overexpressing bone marrow \( (p = 0.9, n = 7) \). This was consistent with development of AML in the majority of mice in all these groups by 24 weeks.

gp91\(^{ \text{PHOX}} \) expression was not significantly different from control at 16 or 24 weeks in mice transplanted with bone marrow overexpressing WT or E76K SHP2 alone \( (p = 0.9, n = 9) \). This suggested that the greater effect of co-overexpression of HoxA10 and activated SHP2 than either alone was not because of additive, independent effects, but the effect of SHP2 activation on overexpressed HoxA10.

We also investigated the impact of co-overexpression of HoxA10 and activated SHP2 on bone marrow Mkp2 expression. Mkp2 expression was significantly greater than control in mice transplanted with HoxA10 + WT SHP2-overexpressing bone marrow at 16 weeks \( (p = 0.03, n = 9) \) and 24 weeks \( (p = 0.007, n = 9) \) (Fig. 5B). Mkp2 expression was not significantly different in mice that were transplanted with bone marrow that was co-overexpressing HoxA10 + WT SHP2 in comparison with overexpressing HoxA10 alone, or HD-Y mutant HoxA10 \( (p = 0.2, n = 9) \) at 16 and 24 weeks. However, Mkp2 expression was significantly greater in mice transplanted with HoxA10 + E76K SHP2-overexpressing bone marrow than these three groups of mice at 16 and 24 weeks \( (p = 0.03, n = 9) \). Mkp2 expression increased significantly between 16 and 24 weeks in all four groups of HoxA10-overexpressing mice \( (p = 0.05, n = 9) \).
In contrast, bone marrow Mkp2 expression was not significantly different in mice transplanted with WT or E76K SHP2-overexpressing bone marrow in comparison with control vector-transduced bone marrow at 16 ($p = 0.4$, $n = 9$) or 24 weeks ($p = 0.2$, $n = 9$) post-transplantation. These results suggested that the increase in Mkp2 expression in mice with co-overexpression of HoxA10 + E76K SHP2 was not related to the independent effects of overexpressing HoxA10 and E76K SHP2. The studies in the previous section suggested that phosphorylation of the conserved HD-Y residues in HoxA10 did not regulate transcription of the gene encoding Mkp2. Therefore, these studies suggested that DUSP4 transcription may be impaired by phosphorylation of non-HD-Y residues that are substrates for constitutively active SHP2.

**Constitutive SHP2 Activation Influences Regulation of CYBB and DUSP4 Transcription by HoxA10**—In previous studies, we found that HoxA10 interacted with a negative cis element in the CYBB promoter and repressed transcription in immature myeloid cells (16, 17, 21). During cytokine-induced differentiation, HoxA10 binding to the CYBB cis element decreased. This decrease could be prevented by mutation of conserved tyrosine residues in the homeodomain or by co-expression of activated SHP2 (16, 17, 21).

These studies implied that activated SHP2 was contributing to sustained HoxA10 repression of CYBB transcription in differentiating myeloid cells de-phosphorylating the HD-Y residues in HoxA10. However, those studies did not exclude the possibility that other actions of constitutively active SHP2 were involved in this effect. For example, activated SHP2 might dephosphorylate additional residues in HoxA10 that were functionally relevant or might impact a partner protein for cis element repression.

We investigated these possibilities in additional transfection experiments. For these studies, we used the U937 myeloid leukemia cell line, as in our prior CYBB promoter investigations (16). Differentiation of these cells can be induced by various cytokines (including IFNγ and tumor necrosis factor-α) or by chemical agents (such as phorbol esters and retinoic acid) (16, 17, 30). During differentiation, U937 cells acquire functional characteristics of mature phagocytes (respiratory burst activity and phagocytosis) and undergo proliferation arrest and eventual apoptosis. Therefore, this cell line represents a reasonable model for myeloid differentiation.

U937 cells were co-transfected with a vector with multiple copies of the CYBB cis element linked to a minimal promoter and a reporter gene (referred to as CYBB-TATACAT) or empty control vector (p-TATACAT) (22), a vector to overexpress HoxA10 or HD-Y mutant HoxA10 or empty vector control, and a vector to overexpress WT or constitutively active SHP2 or vector control. Reporter gene activity was analyzed with or without IFNγ differentiation.

We found that activity of the DUSP4 cis element was increased significantly by HoxA10 overexpression, similar to our previous results ($p < 0.03$, $n = 3$) (Fig. 8B). We also found that activation of the DUSP4 cis element was not significantly different in transfecants overexpressing WT versus HD-Y mutant HoxA10 ($p = 0.6$, $n = 3$). Differentiation of the transfecants with IFNγ decreased the impact of overexpressed WT or HD-Y mutant HoxA10 on DUSP4 cis element activation. Activation by these two forms of HoxA10 was not significantly different in IFNγ-treated transfecants ($p = 0.8$, $n = 3$).

We found that co-overexpression of WT SHP2 did not significantly alter activation of the DUSP4 cis element by overexpressed WT or HD-Y mutant HoxA10 in undifferentiated or IFNγ-differentiated transfecants ($p \geq 0.3$, $n = 9$) (Fig. 8B). However, DUSP4 cis element activity was significantly greater in undifferentiated transfecants co-overexpressing WT or HD-Y mutant HoxA10 alone ($p < 0.001$, $n = 9$). Also, co-overexpression of constitutively active SHP2 with WT or HD-Y mutant HoxA10 prevented the decrease in DUSP4 cis element activity observed during IFNγ differentiation of such transfecants ($p > 0.8$, $n = 9$ with versus without differentiation).

In control experiments, overexpression of WT SHP2 or E76K SHP2 alone did not significantly alter activity of the DUSP4 cis element without or with differentiation ($p \geq 0.1$, $n = 9$). These results suggested that HoxA10 overexpression and constitutive SHP2 activation were not functioning independently to increase DUSP4 transcription during myeloid differentiation. These studies also suggested that constitutively active SHP2 was functioning to dephosphorylate non-HD-Y residues in HoxA10 that regulate activation of the positive DUSP4 cis element.
The results of these transfection experiments were consistent with our in vivo observations of the role of constitutive SHP2 activation on expression of endogenous Mkp2 and gp91phox in mice with HoxA10 overexpression in the bone marrow.

**DISCUSSION**

In previous studies, HoxA10 overexpression in murine bone marrow expanded myeloid progenitor populations in vitro and induced a myeloproliferative disorder characterized by increased circulating neutrophils in vivo (14, 15). The likely mechanism for the myeloproliferative disorder was dysregulation of mostly unidentified HoxA10 target genes involved in cell proliferation and/or survival. Over time, this MPD evolved to AML, characterized by the appearance of immature, myeloid blasts in the peripheral blood. This suggested that HoxA10 overexpression alone was not adequate for differentiation block and AML but required acquisition of additional mutations. Based on our previous studies, we hypothesized that mutations that impair HoxA10 tyrosine phosphorylation would cooperate with HoxA10 overexpression to dysregulate target genes involved in phenotypic and functional differentiation. We also hypothesized that leukemic-associated, activation mutations of the gene encoding SHP2 was a candidate lesion. We investigated this hypothesis using a murine leukemogenesis model.

We previously found that phosphorylation of HoxA10 homeodomain tyrosine residues during myelopoiesis decreased binding to negative cis elements in myeloid-specific genes, such as those encoding gp91phox and p67phox. Therefore, although overexpressed WT HoxA10 did not block myeloid-specific gene transcription during in vitro differentiation, overexpression of HoxA10 with mutation of these HDY residues did. We also identified HoxA10 as a substrate for SHP2 in undifferentiated myeloid cells, an effect that diminished during...
myelopoiesis. However, a constitutively active form of SHP2 dephosphorylated HoxA10 throughout ex vivo myelopoiesis and sustained repression of HoxA10 target genes involved in phagocyte effector functions.

In this study, our main interest was identifying leukemia-associated genetic lesions that might cooperate with HoxA10 overexpression for differentiation block and AML. Therefore, we studied a HoxA10-overexpressing murine model using the clinical criteria that identify development of secondary AML in human subjects with myeloproliferative disorders (34).

We found that mice transplanted with bone marrow that was overexpressing an HD-Y mutant form of HoxA10 developed rapid AML in comparison with mice transplanted with WT HoxA10-overexpressing bone marrow. Co-overexpression of HoxA10 and an activated form of SHP2 had a similar effect on rapid differentiation block and AML. In contrast, overexpression of activated SHP2 alone did not lead to differentiation block and AML within this time frame, consistent with previous reports (33). These studies suggested that SHP2 activation and HoxA10 overexpression were not independently inducing AML in an additive manner, but that SHP2-activation augmented the effect of HoxA10 overexpression for myeloid differentiation block and AML. Therefore, these studies provided the first identification of a genetic lesion that cooperated with HoxA10 overexpression and a mechanism for the cooperative effect.

Because myeloproliferation develops rapidly in mice transplanted with HoxA10-overexpressing bone marrow, we hypothesized that HoxA10 tyrosine phosphorylation state would have less of an impact on this effect than on differentiation block and AML. We initially determined whether HoxA10 overexpression in primary murine bone marrow progenitor cells induced hypersensitivity to GM-CSF or IL3. We found that overexpressed HoxA10 increased sensitivity to both of these cytokines and that mutation of the conserved HD-Y residues did not alter this effect. Consistent with these results, in vivo overexpression of either WT or HD-Y mutant HoxA10 in murine bone marrow resulted in an equivalent myeloproliferative disorder (equivalent increase in circulating myeloid cells). In contrast, co-overexpression of HoxA10 and activated SHP2 increased hypersensitivity of these cells to both GM-CSF and IL3 in comparison with HoxA10 alone. In vivo, myeloproliferation was also relatively increased by co-overexpression of HoxA10 and activated SHP2 in comparison with HoxA10 alone.

However, in vivo expression of activated SHP2 alone also induced myeloproliferation (characterized by an increase in neutrophils) and GM-CSF hypersensitivity. We found that the impact on GM-CSF hypersensitivity of co-overexpressing HoxA10 and activated SHP2 was approximately the sum of overexpressing either protein alone. Therefore, unlike the studies of differentiation block and AML, it was possible that HoxA10 overexpression and SHP2 activation might be functioning independently rather than cooperatively. This could be due to the influence of SHP2 on non-HoxA10 substrates.

In previous studies, we identified the interferon consensus sequence-binding protein (ICSBP) as such a substrate. We found that constitutive SHP2 activation blocked ICSBP-induced transcription of the gene encoding neurofibromin 1 (29, 33). This increased Ras activation by GM-CSF in cells with ICSBP haploinsufficiency and SHP2 activation (29). Alternatively, our data were also consistent with the possibility that SHP2 activation impacts GM-CSF hypersensitivity through dephosphorylation of non HD-Y residues in HoxA10. Such unidentified residues might regulate unidentified target genes involved in proliferation or both mechanisms might be functionally relevant. Clarification will require identification of such HoxA10 target genes.

The situation was somewhat different with IL3 hypersensitivity. We found that co-overexpression of HoxA10 and activated SHP2 had a significantly greater anti-apoptotic effect during IL3 withdrawal than HoxA10 overexpression alone. However, constitutive SHP2 activation alone did not have any protective effect. This observation suggested that additive, independent effects of HoxA10 overexpression and SHP2 activation were not likely to increase IL3 sensitivity in cells co-overexpressing HoxA10 and activated SHP2. These studies suggested that dephosphorylation of non-HD-Y HoxA10 residues by activated SHP2 might dysregulate target genes involved in apoptosis.

Our previous studies identified a HoxA10 target gene that might be involved in this process. We identified DUSP4 (encoding Mkp2) as a HoxA10 target (22). We found that HoxA10 activated DUSP4 transcription most efficiently in immature myeloid cells (22). In this study, we demonstrate sustained activation of the DUSP4 cis element in differentiating myeloid cells that were co-overexpressing either WT or HD-Y mutant HoxA10 + activated SHP2. In contrast, overexpression of WT HoxA10, HD-Y mutant HoxA10, or activated SHP2 alone did not sustain cis element activation during differentiation.

This suggested the possibility that dephosphorylation of non-HD-Y residues in HoxA10 by constitutively active SHP2 dysregulated DUSP4 transcription. Consistent with this hypothesis, co-overexpression of HoxA10 and activated SHP2 resulted in greater Mkp2 expression in vivo in comparison with overexpression of HoxA10 alone, HD-Y mutant HoxA10, or HoxA10 + WT SHP2. Because in vivo expression of activated SHP2 alone did not increase Mkp2 expression in the bone marrow of mice post-transplantation, this also suggested that HoxA10 overexpression and SHP2 activation were exerting cooperative effects.

We further investigated the characteristics of myeloproliferation and AML in this animal model at the molecular level. For these studies, we investigated expression of five differentiation stage-specific genes as follows: Sca1, CD34, CD38, CD11b, and Gr1. Sca1 expression is characteristic of primitive progenitor cells in murine bone marrow. Myeloid progenitor lineage commitment is characterized by expression of CD34 and CD11b. Progression of myeloid differentiation is characterized by decreased CD34 expression and increased expression of CD38, CD11b, and Gr1. CD38 is expressed in maturing myeloid progenitors, and long term repopulating leukemia stem cells are generally CD38− in murine leukemia models (34).

We determined mRNA expression of these genes in the bone marrow of individual mice post-transplantation. This approach permitted us to examine multiple genes in individual mice.
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simultaneously in a quantitative manner. We previously used this approach in our studies of cooperation between constitutive activation of SHP2 and decreased expression of ICSBP (33).

We compared the differentiation stage of the bone marrow population in mice transplanted with control bone marrow and mice transplanted with WT or HD-Y mutant HoxA10-overexpressing bone marrow. At 16 weeks post-transplant, the majority of mice with HoxA10-overexpressing bone marrow exhibit a myeloproliferative disorder characterized by mature neutrophils. In comparison, the majority of mice transplanted with HD-Y mutant HoxA10-overexpressing bone marrow have AML with circulating myeloid blasts.

At 16 weeks, expression of CD34 and CD11b was significantly increased in mice transplanted with WT or HD-Y mutant HoxA10-overexpressing bone marrow in comparison with control mice. This indicated increase in cells with myeloid progenitor characteristics. In mice with WT HoxA10-overexpressing bone marrow, we also found increased expression of CD38 and Gr1, as anticipated with an increase in differentiating phagocytes. Mice transplanted with HD-Y mutant HoxA10 had decreased CD38 and Gr1 expression in the bone marrow, consistent with differentiation block and AML in these mice at 16 weeks. Between 16 and 24 weeks, the majority of mice transplanted with WT HoxA10-overexpressing bone marrow progressed to AML. During this time, the gene expression profile in the bone marrow of these mice became similar to mice transplanted with HD-Y mutant HoxA10-overexpressing bone marrow.

These results suggested that phosphorylation of HD-Y residues in HoxA10 regulated differentiation but not myeloproliferation. In contrast, we found that co-overexpression of HoxA10 and activated SHP2 influenced both differentiation block and myeloproliferation. We found that the gene expression profile in mice transplanted with HoxA10 + E76K SHP2 exhibited evidence of differentiation block at 16 and 24 weeks, similar to HD-Y mutant HoxA10 (decreased CD38, Gr1, and gp91PHOX). Importantly, expression of these genes was not decreased by overexpression of activated SHP2 alone at any time point. These results suggested a cooperative effect of constitutive SHP2 activation and HoxA10 overexpression rather than independent, additive effects of the two proteins.

It is important to note that the extent of HoxA10 overexpression was equivalent in mice transplanted with bone marrow overexpressing HoxA10, HoxA10 + SHP2, HD-Y mutant HoxA10, or HoxA10 + E76K SHP2 at 16 weeks. This suggested that differentiation block and AML in the latter two groups at this time point was not because of relatively greater abundance of overexpressed HoxA10 in comparison with the former two groups, but it was more likely related to HoxA10 phosphorylation state.

We found that CD34 and Mkp2 expression was increased in mice transplanted with HoxA10 + activated SHP2-overexpressing bone marrow in comparison with mice with HoxA10 or HD-Y mutant HoxA10-overexpressing bone marrow at both 16 and 24 weeks. This correlated with more profound leukocytosis in the former group. Expression of these genes was not significantly different in mice transplanted with activated SHP2-overexpressing bone marrow in comparison with control. These results suggested that SHP2 activation influenced the anti-apoptotic effect of overexpressed HoxA10 on cells with myeloid progenitor characteristics. These data also implied that dephosphorylation of non-HD HoxA10 Tyr residues by SHP2 might mediate the effect.

Our results suggest that the tyrosine phosphorylation state of overexpressed HoxA10 influences both myeloproliferation and differentiation block. However, the influence of phosphorylation on myeloproliferation appears less profound. This may be because phosphorylation of HD-Y residues decreases HoxA10 affinity for negative cis elements in myeloid-specific genes to a greater extent than phosphorylation of unidentified residues decreases HoxA10 affinity for positive cis elements in apoptosis-related genes. Supporting this hypothesis, we found that HoxA10 repression of the CYBB cis element was completely abolished by differentiation, but activation of the DLISP4-cis element was only impaired.

HoxA10 is overexpressed in poor prognosis human AML, including leukemia with translocations involving the MLL gene. Activating mutations of SHP2-PTP have also been described in human subjects with translocations involving the MLL gene (28). These results suggest that the combination of HoxA10 overexpression and constitutive SHP2 activation are likely to be found in AML. In human AML, SHP2 mutations appear in sub-clones, indicating that they are late events in the course of disease (28). These results suggested a possible model where increased ABD HOXA expression was induced by translocations of the MLL gene (or other genetic lesions) as an early event. In such cases, disease progression could be related to acquisition of activating mutations in SHP2. Further studies of human myeloid leukemias will be of interest in terms of validating this mechanism.

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