Cdx4 dysregulates *Hox* gene expression and generates acute myeloid leukemia alone and in cooperation with Meis1a in a murine model

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***HOX* genes have emerged as critical effectors of leukemogenesis, but the mechanisms that regulate their expression in leukemia are not well understood. Recent data suggest that the caudal homeobox transcription factors CDX1, CDX2, and CDX4, developmental regulators of *HOX* gene expression, may contribute to HOX gene dysregulation in leukemia. We report here that *CDX4* is expressed normally in early hematopoietic progenitors and is expressed aberrantly in ≈25% of acute myeloid leukemia (AML) patient samples. Cdx4 regulates *Hox* gene expression in the adult murine hematopoietic system and dysregulates *Hox* genes that are implicated in leukemogenesis. Furthermore, bone marrow progenitors that are retrovirally engineered to express Cdx4 serially replate in methylcellulose cultures, grow in liquid culture, and generate a partially penetrant, long-latency AML in bone marrow transplant recipients. Coexpression of the Hox cofactor Meis1a accelerates the Cdx4 AML phenotype and renders it fully penetrant. Structure–function analysis demonstrates that leukemic transformation requires intact Cdx4 transactivation and DNA-binding domains but not the putative Pbx cofactor interaction motif. Together, these data indicate that Cdx4 regulates *Hox* gene expression in adult hematopoiesis and may serve as an upstream regulator of *Hox* gene expression in the induction of acute leukemia. Inasmuch as many human leukemias show dysregulated expression of a spectrum of HOX family members, these collective findings also suggest a central role for CDX4 expression in the genesis of acute leukemia.**

transcriptional regulation | Hox regulation | leukemogenesis | self-renewal

The CDX homeobox transcription factors are homologues of the *Drosophila Caudal* gene (1) and are thought to function as master regulators of *HOX* gene expression. Consensus binding sites for the three Cdx homologues Cdx1, Cdx2, and Cdx4 are present in the promoters of multiple *Hox* genes (2), and several lines of evidence highlight the importance of CDX family members as upstream regulators of *HOX* gene expression. These include a key role for CDX2 in regulation of *HOX* gene expression in the epithelium of the gastrointestinal tract (3), and similar homeotic transformations in *Cdx1*-, *Cdx2*-, and *Cdx4*-deficient mice and zebrafish to those observed in *Hox*-deficient animals (4, 5).

CDX family members are also important in hematopoietic development. Loss of function of Cdx4 in zebrafish results in a bloodless phenotype, associated with homeotic transformation, and marked abnormalities in *Hox* gene expression pattern during development (5, 6). These phenotypes can be rescued, in part, by expression of Hox family members, further supporting a role for Cdx4 as an upstream positive regulator of *Hox* gene expression. Additional data support the role of CDX4 in hematopoietic development and suggest an epistatic relationship between CDX4 and MLL. For example, overexpression of Cdx4 in ES cells increases hematopoietic colony formation from embryoid bodies, upregulates a *Hox* gene expression program, and rescues Mll-deficient hematopoiesis *in vitro* (5, 7, 8). *CDX* genes also have been implicated in acute myeloid leukemia (AML). For example, an acquired t(12;13) in AML results in overexpression of *CDX2* as a consequence of juxtaposition of *CDX2* to the *ETV6* promoter (9), and overexpression of Cdx2 results in AML in a murine bone marrow (BM) transplantation model (10).

Hox genes are expressed in hematopoietic precursors, with preferential expression in self-renewing hematopoietic stem cells (HSC) and are down-regulated during differentiation (11). Their importance in normal blood formation and maintenance has been demonstrated in gene-targeting studies in which abnormalities of multiple hematopoietic lineages have been observed in mice deficient in individual *Hox* genes (12). Moreover, overexpression of specific *Hox* genes, such as *HOXB4* or *HOXA9*, is associated with expansion of the HSC compartment *in vitro* and *in vivo* and results in enhanced competitive repopulating activity in murine transplantation experiments (13, 14).

Substantial evidence also has linked aberrant expression of *HOX* genes to the pathogenesis of acute leukemia. For example, fusions of the *HOXA9* or *HOXD13* genes with *NUP98*, a gene that encodes a component of the nuclear pore complex, have been described in AML, and expression of NUP98–HOXA9 or NUP98–HOXD13 results in AML in murine models of disease (15, 16). In addition, overexpression of individual HOX family members, including HOXB3 (17), HOXB8 (18), or HOXA10 (19), by retroviral expression or retroviral insertional mutagenesis also generates AML in murine models. Although these findings show the relevance of dysregulated expression of a single HOX family member in AML, translocations involving individual *HOX* family members are rare. Moreover, in the majority of cases of AML and some cases of acute lymphoblastic leukemia (ALL) there is evidence for widespread dysregulated expression of multiple HOX family members, a phenotype associated with a poor prognosis (20, 21). This finding suggests that dysregulation of *HOX* gene expression in acute leukemias may involve upstream master regulators. In ≈5–10% of cases, dysregulation can be explained by the expression of fusion proteins involving the mixed-lineage leukemia (MLL) gene that have been shown to mediate their effects in part through up-

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Abbreviations: AML, acute myeloid leukemia; MLL, mixed-lineage leukemia; qRT-PCR, quantitative RT-PCR; BM, bone marrow.

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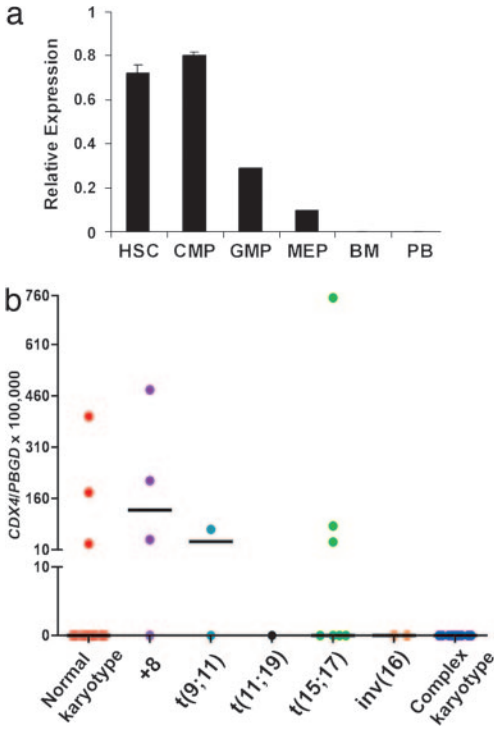

Fig. 1. *Cdx4*/*CDX4* is expressed during murine hematopoiesis and in human acute leukemias. (*a*) The expression pattern of *Cdx4* in flow-sorted stem and progenitor cells and in unfractionated BM and peripheral blood (PB) is shown (relative to GAPDH). HSC, hematopoietic stem cells; CMP, common myeloid progenitor; GMP, granulocyte–monocyte progenitor; MEP, megakaryocyte–erythroid progenitor. (*b*) Dot plot shows expression level of *CDX4* in primary samples isolated from AML patients with diverse karyotypes.

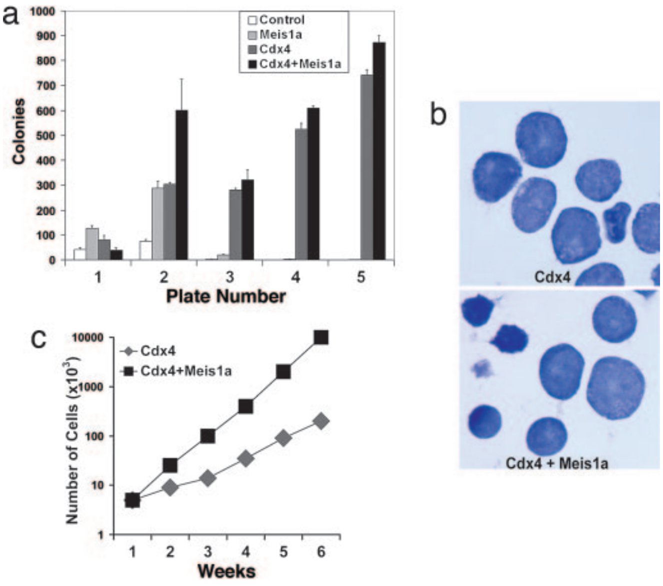

Fig. 2. *In vitro* analyses demonstrate that Cdx4 can confer properties of self-renewal to hematopoietic progenitor cells. (*a*) Bar chart showing results of serial replating experiments. In contrast to primary BM cells transduced with empty *Neo* vector (control) or with Meis1a alone, cells transduced with either Cdx4 alone or Cdx4+Meis1a demonstrated serial replating ability up to the fifth plating. (*b*) Immature myelomonocytic morphology of cells derived from colonies of the fifth round of plating for Cdx4- and Cdx4+Meis1a-transduced BM cells. (*c*) Line graph demonstrating the sustained growth and proliferation of both Cdx4- and Cdx4+Meis1a-transduced BM cells for over 6 weeks in IL3-supplemented media.

regulation of several *HOX* genes (20, 22). In cases of acute lymphoblastic leukemia, dysregulation of *HOX* genes also is associated with expression of *CALM–AF10* or fusions of TCRB and genes from the *HOXA* cluster (23). However, the upstream effectors of dysregulated *HOX* gene expression in the majority of acute leukemias are not known.

During development, Cdx family members are thought to play an important role in *Hox* gene regulation. *Hox* gene expression is tightly controlled in a spatiotemporal manner to confer positional information to the axial and paraxial tissues as they emerge from the posterior portion of the vertebrate embryo (24). This process is not fully understood at the molecular level, but morphogens such as fgf, retinoic acid (RA), and Wnt signals are thought to be involved in the initiation of expression, with establishment of the *Hox* expression domains occurring through the actions of Cdx proteins along gradients of these morphogens. Maintenance of *Hox* gene expression subsequently occurs via complex interactions of Trithorax group proteins, such as Mll, and Polycomb group proteins.

Based on these collective observations, we hypothesized that CDX4 might be a critical upstream regulator of *HOX* gene expression in the hematopoietic system, and that overexpression of CDX4 might result in widely dysregulated *HOX* gene expression and a leukemia phenotype. We tested this hypothesis in cell culture and murine BM transplantation assays and assayed *CDX4* expression in BM samples isolated from a heterogeneous cohort of patients with AML.

Results

Cdx4 Is Expressed During Adult Hematopoiesis and in AML Patient Samples. *Cdx2* is not expressed in adult BM (9), but the expression pattern of *Cdx4* in adult hematopoiesis is unknown. We performed real-time quantitative RT-PCR (qRT-PCR) by using *Cdx4*-specific primers in murine BM, peripheral blood, and flow-sorted hematopoietic progenitor cells. *Cdx4* is preferentially expressed in primitive stem and progenitor cells with expression down-regulated in more differentiated cell types (Fig. 1*a*). In addition, *CDX4* transcripts were detectable in BM or peripheral blood mononuclear cells from 10 of 44 patients (23%) with AML (Fig. 1*b*): 3 of 16 patients with normal cytogenetics, 3 of 4 patients with trisomy 8 as the sole cytogenetic abnormality, 1 of 2 patients with t(9;11)(p22;q23), and 3 of 7 patients with t(15;17)(q22;q11∼21). The median expression level for the *CDX4*-positive samples was 130 (range 28–747). No *CDX4* expression was detectable in 1 patient with t(11;19)(q23;p13), 2 patients with inv(16)(p13q22), and 12 patients with complex karyotypes, defined as three or more cytogenetic abnormalities in the absence of t(8;21)(q22;q22), inv(16), t(15;17), or t(11q23).

Expression of Cdx4 Confers Serial Replating Activity to Murine BM Cells and Generates AML in a Mouse Model. We next cloned *Cdx4* into retroviral expression vectors and demonstrated that transient transfection of *Cdx4* in 293T cells resulted in expression of the expected 32-kDa protein as assessed by Western blot analysis (Fig. 6*a*, which is published as supporting information on the PNAS web site). Myc-tagged Cdx4 was demonstrated to be localized to the nucleus, consistent with its function as a transcription factor (Fig. 6*b*). Transduction of primary murine BM cells with Cdx4 conferred serial replating activity in the absence of stroma (Fig. 2*a*), a surrogate assay for transformation/self-renewal potential (25). In control experiments, nontransduced cells did not replate. Cytospins of single colonies derived from the fifth round of replating of *Cdx4*-transduced BM demonstrated primitive myelomonocytic morphology (Fig. 2*b*). These cells were capable of growth in liquid culture in the presence of IL-3 (Fig. 2*c*).

We next tested the leukemogenic potential of Cdx4 *in vivo*. BM cells derived from 5-fluorouracil-treated BALB/c mice were trans-

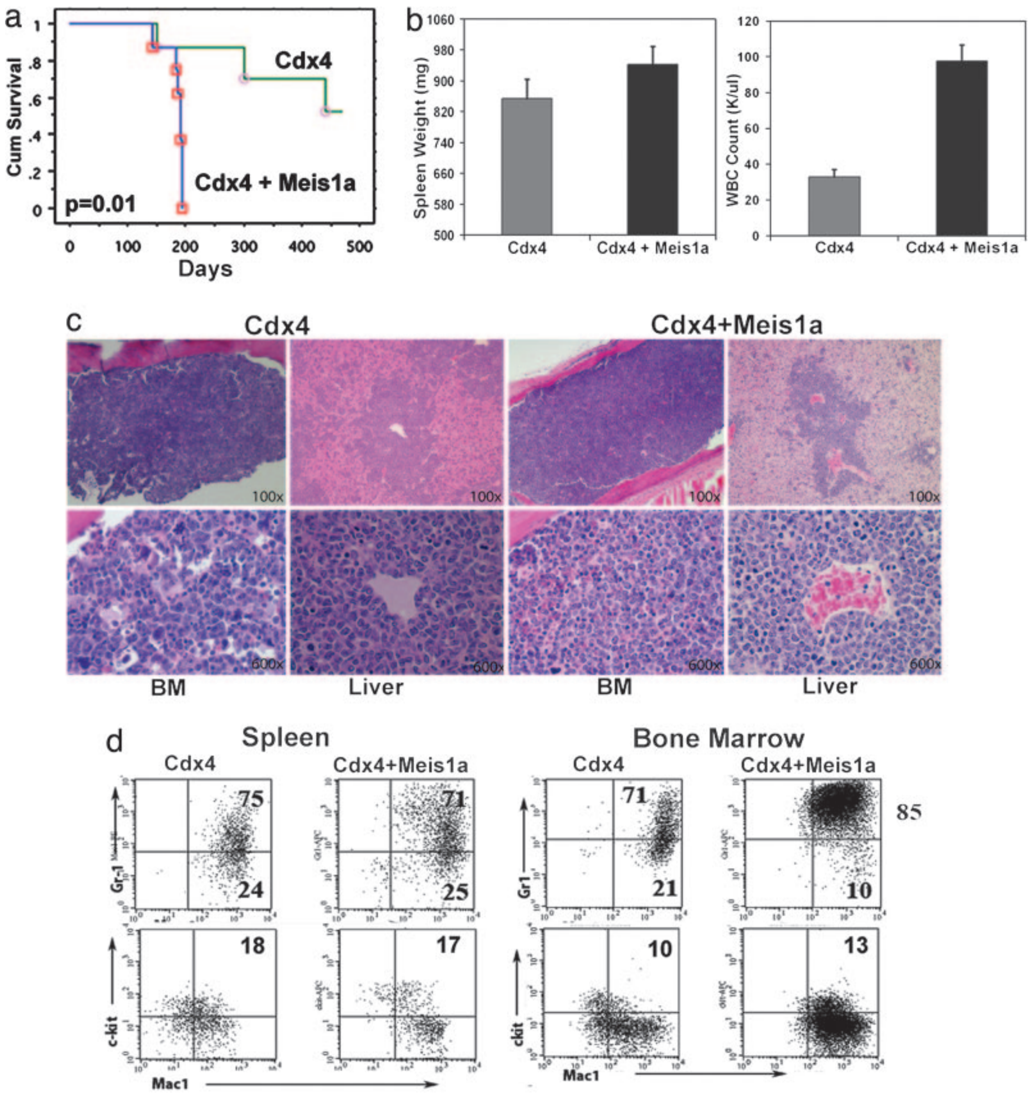

Fig. 3. Overexpression of Cdx4 generates AML in a mouse model. (*a*) Survival curve of mice transplanted with BM cells transduced with either Cdx4 or Cdx4+Meis1a (n = 10 for each). The mice transplanted with cells transduced with Cdx4+Meis1a showed increased penetrance and a significant reduction in the disease latency (P = 0.01 by log-rank analysis). (*b*) Analysis of white blood cell (WBC) counts and spleen weight in mice transplanted with BM cells transduced with Cdx4 or Cdx4+Meis1a. Higher white blood counts and spleen weights were observed in mice transplanted with Cdx4+Meis1a-transduced vs. Cdx4-transduced alone BM cells. (*c*) Histopathology of Cdx4- and Cdx4+Meis1a-transduced BM transplanted mice. BM sections displaying marked hypercellularity and effacement of normal marrow architecture by a prominent population of immature myelomonocytic cells and blast forms along with extensive infiltration of the liver are shown and demonstrate similar histology. (Magnification: *Upper*, ×100; *Lower*, ×600.) (*d*) Flow-cytometric analysis of GFP-positive leukemic BM and spleen cells isolated from the transplanted mice. Similar patterns of myeloid expansion were seen for Cdx4- and Cdx4+Meis1a-transplanted mice.

duced with MSCV-*Cdx4*-IRES-GFP and injected intravenously into lethally irradiated syngeneic recipient mice, and the transplanted mice were observed for disease development. Half of the transplant recipients developed AML, with a median latency of ≈300 days posttransplantation (Fig. 3*a*), that was characterized by an elevated (5- to 10-fold) peripheral white blood (WBC) count, the presence of blasts in the peripheral blood, and splenomegaly (average spleen weight of ≈0.9 g; Fig. 3*b*). Histopathological analysis showed extensive infiltration of the BM, liver, and spleen with immature myeloid cells (Fig. 3*c*). Integration of the MSCV-Cdx4-GFP provirus was demonstrated by Southern blotting analysis (Fig. 7*c Left*, which is published as supporting information on the PNAS web site). Immunophenotypic characterization of leukemic BM and spleen in Cdx4 mice showed a predominance of Mac1+ and Gr1+ cells and a reduction of lymphoid cells (data not shown) in the GFP-positive fraction. Both BM and spleen leukemic cells also showed a large fraction (12–20%) of cKit+, Mac1+ early myeloid cells (Fig. 3*d*). The leukemia generated was transplantable to secondary recipients resulting in a similar phenotype 6–8 weeks after transplantation (Fig. 7).

Meis1a Cooperates with Cdx4 to Transform Hematopoietic Cells. Gene expression studies in human AML (21), retroviral insertional mutagenesis screens in mice (26), and retroviral mouse models (27) have demonstrated Meis1a to be a cofactor for leukemic transformation by *Hox* genes. We hypothesized that Meis1a might cooperate with *Hox* genes whose expression was induced by Cdx4 or might cooperate as a cofactor through direct interactions with Cdx4. To test this hypothesis, murine BM cells were cotransduced with MSCV-*Cdx4*-Neo and MSCV-*Meis1a*-Puro, and serial replating was performed after selection in G418 and puromycin (Fig. 2*a*). Colony numbers were statistically significantly increased in cells cotransduced with Cdx4 and Meis1a (Cdx4+Meis1a cells) when compared with cells transduced with Cdx4 alone (P = 0.04). Similarly, in liquid culture, the growth rate of Cdx4+Meis1a cell lines was consistently higher than that of Cdx4 lines (Fig. 2*c*).

Cooperation between Meis1a and Cdx4 also was demonstrated *in vivo*. BM cotransduced with Cdx4 and Meis1a generated a leukemia that, in contrast with Cdx4 alone, was fully penetrant with a reduced median survival (≈170 days, P = 0.01; Fig. 3*a*). However, leukemia induced by Cdx4 and Meis1a was indistinguishable by histological and immunophenotypic examination from that induced by Cdx4 alone (Fig. 3 *c* and *d*). This disease also could be transplanted to secondary recipients and generated leukemia more rapidly than Cdx4 alone (Fig. 7). Cooperation between Meis1a and Cdx4 was confirmed by Southern blot analysis showing retroviral integration of each of the respective retroviruses in leukemic cells derived from secondary recipients transplanted at limiting dilution (Fig. 7*c*).

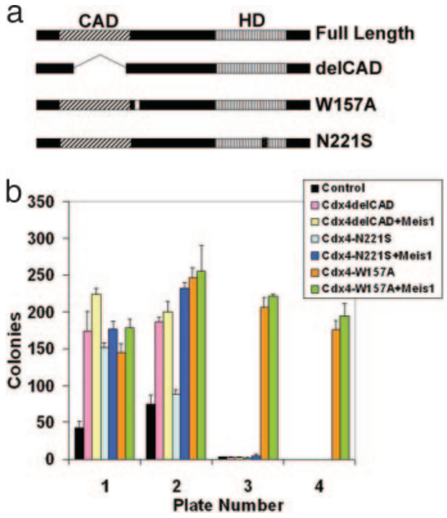

Fig. 4. Structure–function analysis of Cdx4. (*a*) Schematic describing various *Cdx4* deletion and point mutations generated to evaluate the functional requirement of various protein domains in Cdx4-mediated transformation. (*b*) Bar graph showing results of serial replating experiments testing the transformation ability of Cdx4 mutants. Cdx4delCAD and Cdx4-N221S are indispensable for the self-renewal ability of Cdx4. However, BM cells transduced with Cdx4-W157A continue forming colonies up to the fourth plate, suggesting that the Pbx interaction domain is dispensable for the transformation/self-renewal ability of Cdx4.

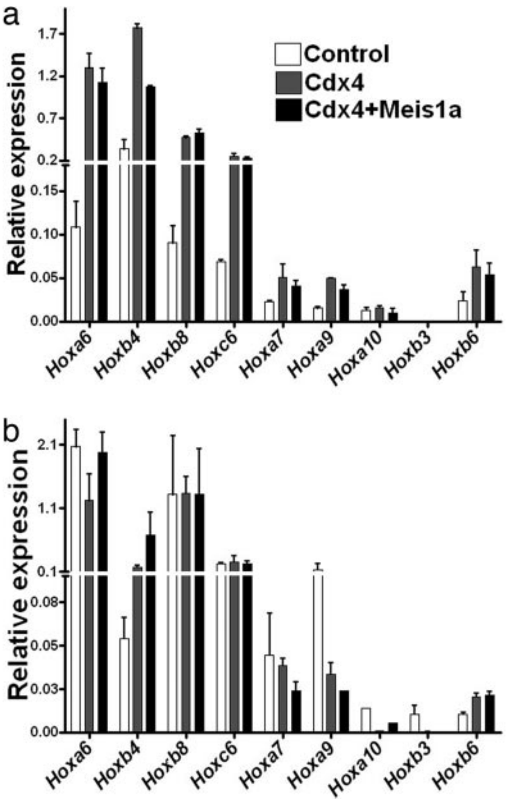

Fig. 5. Expression of Cdx4 generates a *Hox* gene expression program in adult hematopoietic progenitors. Bar graphs showing expression relative to 18S ribosomal RNA of a selected set of *Hox* genes in BM progenitor cells transduced with control (MSCV-Neo), Cdx4, or Cdx4+Meis1a mice (*a*) or BM from control (normal) Cdx4 or Cdx4+Meis1a leukaemic mice (*b*). Represented values are the mean and SD obtained from three individual experiments performed for each sample.

Transformation by Cdx4 Requires Intact Transactivation and Homeodomains and Dysregulates a *Hox* Gene Expression Program. To assess the structural requirements for Cdx4 transformation, mutations that abrogate three functional motifs of Cdx4 were generated. These motifs included a mutation deleting the caudal activation domain (Cdx4delCAD) and a point mutant (Cdx4N221S) that abrogates homeodomain DNA-binding activity (Fig. 4*a*). Pbx proteins are Hox cofactors that enhance the DNA-binding affinity and specificity of homeobox proteins and bind to clustered and nonclustered homeoproteins through a highly conserved consensus site (28, 29). Cdx4 contains a Pbx consensus binding site, and this putative site was abrogated in the Cdx4W157A point mutant. Protein expression was demonstrated for each mutant in 293T cells (Fig. 6), and the ability to confer serial replating activity to transduced murine BM cells was tested. The Cdx4W157A mutant, but not the Cdx4delCAD or Cdx4N221S mutants, conferred serial replating activity. Based on the evidence that this mutation disrupts a putative Pbx binding site (28, 29), these findings are consistent with the hypothesis that the Pbx interaction motif is dispensable for transformation. Coexpression of Meis1a did not rescue serial replating activity of the Cdx4delCAD or Cdx4N221S mutants (Fig. 4*b*). *In vivo* BM transplantation experiments confirmed that the caudal activation domain and the DNA-binding capacity of the Cdx4 homeodomain, but not the putative Pbx binding site, were required for transformation by Cdx4 (data not shown).

Cdx4 regulates *Hox* gene expression during embryogenesis and developmental hematopoiesis (5). To determine whether leukemic transformation by expression of Cdx4 also involved dysregulation of *Hox* genes, real-time qRT-PCR was used to measure the expression level of a set of *Hox* genes previously implicated in leukemia. 5-Fluorouracil-treated murine BM cells enriched for hematopoietic progenitors were transduced with MSCV-*Cdx4*-Neo, MSCV-*Cdx4*-Neo and MSCV-*Meis-1a*-Puro, or MSCV-Neo constructs. Cells were selected in G418 (Cdx4-Neo, MSCV-Neo) or G418 and puromycin (Cdx4-Neo and Meis1a-Puro) for 1 week. In addition, total BM from leukemic mice transplanted with either Cdx4- or Cdx4+Meis1a-transduced cells also was harvested. Total RNA was isolated from these cellular populations, and cDNA was prepared. In Cdx4- or Cdx4+Meis1a-transduced progenitor cells, a specific subset of *Hox* genes were up-regulated, including *Hoxa6*, *Hoxa7*, *Hoxa9*, *Hoxb8*, *Hoxb4*, and *Hoxc6* (Fig. 5*a*). *Hox* genes also were dysregulated in leukemic BM with up-regulation of *Hoxb4* and *Hoxb6* and down-regulation of *Hoxa7*, *Hoxa9*, and *Hoxa10* (Fig. 5*b*). However, in both the immediately transduced progenitors and the leukemic BM, there was no apparent additive effect of coexpression of Meis1a on the level or pattern of *Hox* gene expression. These findings indicate that Cdx4 regulates *Hox* gene expression in adult as well as developmental hematopoiesis and are consistent with the hypothesis that Cdx4-induced leukemogenesis is mediated, at least in part, through dysregulation of Hox family members.

Discussion

HOX gene expression is frequently dysregulated in human acute leukemias, and the available evidence suggests a causal relationship between expression of certain HOX family members and leukemogenesis. However, the molecular mechanisms that mediate aberrant *HOX* gene expression are known only for a minority of leukemias, namely those involving rearrangement of the *MLL* gene or rearrangement of actual specific *HOX* genes. In this report, we demonstrate that Cdx4, a nonclustered homeobox transcription factor associated with regulation of *Hox* genes during hematopoietic development, can generate AML in a murine model, both alone and in cooperation with the Hox cofactor Meis1a. Cdx4-transforming activity depends on an intact transactivation domain and an intact homeodomain, whereas binding of the cofactor Pbx1 is dispensable for transformation. In addition, we have observed that expression of Cdx4 results in dysregulated expression of a spectrum of *Hox* genes in adult hematopoietic progenitors and leukemia cells and have demonstrated that *CDX4* is expressed in leukemic blasts from patients with AML.

Retroviral transduction of BM with Cdx4 confers serial replating activity in the absence of stroma, IL-3-dependent growth in liquid culture, and AML *in vivo* with incomplete penetrance and long latency. Coexpression of the Hox cofactor Meis1a with Cdx4 enhances *in vitro* proliferation of hematopoietic progenitors as assessed by increased colony number and growth in liquid culture. Furthermore, coexpression of Cdx4 and Meis1a *in vivo* results in AML that is phenotypically indistinguishable from that induced by Cdx4 alone but with complete penetrance and shortened latency. Each of these features are reminiscent of transformation by Hoxa9 (27) the expression of which, along with other *Hox* genes, we have demonstrated to be increased in Cdx4- and Cdx4+Meis1a-expressing hematopoietic progenitors. These similarities suggest that a dysregulated Hox expression program is responsible, at least in part, for the development of the leukemias associated with Cdx4 expression. Downstream interaction between this Hox program and Meis1a may further facilitate leukemia induction.

Cdx4 previously has been shown to regulate *Hox* gene expression during development in zebrafish. Cdx4 also is expressed in murine ES cells during embryoid body formation at days 3 and 4 (5) and rescues Mll-deficient primitive hematopoiesis *in vitro* (7). In addition, overexpression of Cdx4 promotes definitive hematopoiesis from ES cells *in vivo* (8). Each of these developmental processes is enabled, in part, through up-regulation of a specific *Hox* gene program that is thought to enhance mesodermal commitment and competence for hematopoietic specification. We demonstrate here that endogenous *Cdx4* also is expressed in the adult hematopoietic compartment, with preferential expression in primitive stem and progenitor cells and down-regulation in more differentiated cell types. This expression pattern is similar to that described for *Hox* genes (11) and further suggests that CDX4 may regulate *HOX* gene expression in adult hematopoiesis.

We also observed that ectopic expression of Cdx4 results in dysregulation of a spectrum of Hox family members in the adult hematopoietic compartment, including *Hoxa6*, *Hoxa7*, *Hoxa9*, *Hoxa10*, *Hoxb4*, *Hoxb6*, *Hoxb8*, and *Hoxc6*. These particular Hox family members have been implicated in leukemogenesis in other contexts. For example, it has been proposed by Kersey and colleagues (30) that there is a “Hox code” that is central to leukemogenesis mediated by MLL fusion genes and is minimally defined by the 5′ *HoxA* cluster genes *Hoxa5*–*Hoxa9*. In addition, HOXB4 expression is associated with an increase in the hematopoietic stem-cell compartment size and self-renewal (14) and causes leukemia when coexpressed with Meis1a (31), and mice that overexpress Hoxb8 in the hematopoietic compartment also develop leukemia (18). Similarly, overexpression of HOXB6 in murine BM enhances the self-renewal of myeloid progenitors *in vitro* and generates AML *in vivo* (32). Furthermore, in patients with B or T cell acute lymphoblastic leukemia, *HOXC6* is overexpressed consistently in patients harboring an MLL translocation (20). It also was noted that there were differences between the pattern of *Hox* gene expression in directly transduced progenitors and leukemic cells. This finding may reflect the influence of secondary mutations, extracellular signaling, niche effects, or differential requirements for dysregulation of specific Hox family members during the induction and maintenance of leukemia. Together, our findings demonstrate that expression of Cdx4 dysregulates a *Hox* gene program in hematopoietic tissue, and they are consistent with the hypothesis that this program has the potential to drive leukemogenesis.

Importantly, the long latency of leukemia from BM cells transduced with cdx4 alone implies that cdx4 dysregulation is not sufficient to induce leukemia. This conclusion is strengthened by our observation that coexpression of Cdx4 and Meis1a results in AML with complete penetrance and shortened latency when compared with Cdx4 alone. Meis and Pbx are cofactors for homeobox proteins and participate with them in multiprotein transcriptional complexes (33). Pbx interactions enhance the DNA-binding affinity and specificity of Hox proteins (28, 29). Meis1a itself binds only to 5′ Hox proteins of the Abd-B class (34) but is thought to interact with other Hox family members through association with Pbx proteins (33). Meis1a also is thought to contribute to Hox transcriptional activity both by nuclear localization of Pbx and by directly contributing transactivating activity to the multiprotein transcriptional complex (35). Because Cdx homeodomain proteins contain a putative Pbx interaction domain, the observed cooperation between Cdx4 and Meis1a could be explained by interaction of Meis1a with Cdx4 through Pbx as a cofactor. However, this scenario seems unlikely in that deletion of the Cdx4 Pbx interaction domain had no effect on the ability of Cdx4 to serially replate. Furthermore, coexpression of Cdx4 and Meis1a did not affect the absolute level, or pattern, of expression of the *Hox* family members assayed by qRT-PCR and did not alter the AML phenotype. Therefore, it seems more likely that augmentation of the AML phenotype by coexpression of Meis1a occurs through the cooperative effects of Meis1a on the function of Hox proteins up-regulated by Cdx4.

Together, these data suggest that leukemogenesis mediated by expression of Cdx4 can be attributed to transcriptional targets of Cdx4, in that mutations predicted to ablate DNA-binding or transactivation potential of Cdx4 also abrogate leukemogenic potential. These structural requirements are similar to those observed for transformation mediated by overexpression of Cdx2 (10). In addition, these data also support the hypothesis that cooperativity of Cdx4 and Meis1a is caused by potentiation of Hox transcriptional activity by Meis1a rather than a more direct effect on Cdx4 activity.

We have demonstrated that *Cdx4* is expressed in the adult murine hematopoietic compartment. Furthermore, we also have demonstrated that *CDX4* is expressed in a spectrum of human primary AML samples. The mechanism of expression of CDX4 is not known, but there are several pathways that regulate CDX family member expression during development that also are known to be mutated or dysregulated in human leukemias. These include the canonical Wnt/β-catenin signaling pathway (36), the retinoic acid signaling pathway, (37) and the FGF pathway (38). It will be of interest to determine whether CDX family members are critical effectors of these pathways of transformation.

Materials and Methods

Constructs. Mouse *Cdx4* cDNA was cloned into the MSCV-IRES-GFP and MSCV-pgk-neomycin retroviral vectors. The MSCV-*Meis1a*-YFP and MSCV-*Meis1a*-Puro vectors were a kind gift from Guy Sauvageau (University of Montreal, Montreal, QC, Canada). The deletion mutant of Cdx4 was generated by PCR amplification from the full-length cDNA, and the point mutants were generated by PCR using the Quickchange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers are available on request. All mutants then were cloned in MSCV-IRES-GFP and MSCV-pgk-neomycin vectors, sequenced, and used for the further experiments.

Retrovirus Production and BM Transplantation. High-titer retroviral supernatants were produced, and BM transduction and transplant experiments were performed as described (39).

Serial Replating and Generation of IL-3-Dependent Primary BM Cell Lines. Serial replating experiments and the generation and counting of cell lines were performed as described (25).

Immunofluorescence Analysis. Ba/F3 cells were transduced with MSCV-*Cdx4*-IRES-GFP retrovirus. After 48 h, transduced cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100, and blocked in 3% BSA plus PBS. Cells were incubated with anti-myc polyclonal antibody (Cell Signaling Technology, Danvers, MA) overnight, washed, and incubated with secondary antibody (Cy3-tagged anti-rabbit; The Jackson Laboratory, Bar Harbor, ME) in the dark for 1 h. Stained cells were analyzed by using an inverted confocal microscope (Bio-Rad, Foster City, CA).

Western Blot Analysis. 293T cells were transiently transfected with MSCV-*Cdx4*-Neo, MSCV-*Cdx4*-Neo mutants, and MSCV-*Meis1a*-Puro with FuGene according to the manufacturer's instructions (Roche Pharmaceuticals, Nutley, NJ). Cells were cultured for 48 h in DMEM plus 10% FBS and then scraped and lysed in lysis buffer (150 mM NaCl/20 mM Tris/5 mM EDTA/1% Triton X-100) plus protease inhibitors (Roche Pharmaceuticals), and protein concentration was estimated by using the Dc protein assay kit (Bio-Rad). Sixty micrograms of total protein lysate was loaded on 10% SDS/PAGE gels (Invitrogen, Carlsbad, CA) and transferred to a PVDF membrane. Antibodies against Cdx4 (polyclonal; Aviva Biosystems, San Diego, CA) and Meis1a (polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect the respective proteins.

Patient Samples. We analyzed mononuclear cells prepared from diagnostic BM or peripheral blood samples from 44 adult patients with AML. All specimens were karyotyped by chromosome banding and fluorescence *in situ* hybridization. The diagnosis of AML was made according to the World Health Organization classification of hematological malignancies. All patients gave informed consent according to the Declaration of Helsinki.

Real-Time qRT-PCR Analysis. Specific murine stem and progenitor populations were flow-sorted as previously described (25) to determine Cdx4 expression, and RNA was isolated by using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. 5-Fluorouracil-primed BM enriched for murine hematopoietic progenitors was transduced with Cdx4 and Cdx4+Meis1a or empty vector and selected for 1 week with G418 (1 mg/ml) alone (Cdx4 and empty vector) or G418 and puromycin (2.5 μg/ml) (for Cdx4+Meis1a) in liquid culture (RPMI medium 1640 plus 20% FBS). Total RNA was isolated from BM cells by using TRIZOL reagent according to the manufacturer's instructions. cDNA was prepared by using random hexamers, expression of *Cdx4* (primers, 5′-CGTGTGGTCTACACAGATCATCAA-3′, 5′-GGCTCTGCGATTCTGAAACC-3′; probe, 5′-CATCAGGAGGAAGTCAGAGCTGGCAGTTA-3′) and various *Hox* genes was assayed by using a *Taq*man qRT-PCR method and primers as previously described (7). For AML primary patient samples, total RNA was isolated by using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and was reverse-transcribed (2 μg of RNA in a reaction volume of 30 μl) by using the *Taq*Man Gold RT-PCR Kit (Applied Biosystems, Foster City, CA). Quantification of *CDX4* expression was performed by real-time qRT-PCR with primers CDX4 RQ-F (5′-CAA GGC CAG TTC CCC CAG-3′) and CDX4 RQ-R (5′-GAA TTC CTT TTC CAG CTC CAA TCT-3′) and a 6-carboxy-fluorescein-labeled probe (5′-FAM-AGG AGC CGC CAC AGC CCC TAT G-3′). For normalization, the porphobilinogen deaminase gene (*PBGD*) was used according to the following formula: *CDX4* copy number ÷ *PBGD* copy number × 10^5. *CDX4* and *PBGD* expression levels were determined by absolute mRNA quantification by using plasmid standard curves. Reactions were run in duplicate with 2.5 μl of cDNA in a total reaction volume of 25 μl by using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

Histopathology. Histopathology was performed and analyzed as described in ref. 25.

Immunophenotypic Analysis of Leukemic Cells. Single-cell suspensions of BM and spleen were prepared and analyzed as described in ref. 25.

Southern Blot Analysis for Proviral Insertion and Clonality. Tumor DNA was prepared, digested, electrophoresed, and hybridized as described in ref. 25. Southern blots were hybridized with probes designed against the *Cdx4* or *Meis1a* MSCV constructs.

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