Cdx4 Is Dispensable for Murine Adult Hematopoietic Stem Cells but Promotes MLL-AF9-Mediated Leukemogenesis

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters.

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Published Version</td>
<td>doi:10.3324/haematol.2010.023168</td>
</tr>
<tr>
<td>Accessed</td>
<td>June 20, 2017 10:16:29 AM EDT</td>
</tr>
<tr>
<td>Citable Link</td>
<td><a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:13041334">http://nrs.harvard.edu/urn-3:HUL.InstRepos:13041334</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>

(Article begins on next page)
Cdx4 is dispensable for murine adult hematopoietic stem cells but promotes MLL-AF9-mediated leukemogenesis

Sumin Koo, 1 Brian J. Huntly, 1, 2 Yuan Wang, 2, 4 Jing Chen, 1, 6 Kristina Brumme, 3 Brian Ball, 3 Shannon L. McKinney-Freeman, 3 Akiko Yabuuchi, 2 Claudia Scholl, 1, 6 Dimple Bansal, 1 Leonard I. Zon, 3, 7 Stefan Fröhling, 1, 6 George Q. Daley, 3 D. Gary Gilliland, 1 and Thomas Mercher 1, 8

1Division of Hematology, Department of Medicine, Brigham & Women’s Hospital, Harvard Medical School, Boston, Massachusetts, USA; 2Department of Hematology, University of Cambridge, Cambridge, UK; 3Division of Hematology/Oncology, Children’s Hospital Boston, Harvard Stem Cell Institute, Boston, USA; 4Institute of Biomedical Research, East China Normal University, Shanghai, China; 5Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University of Medicine, Atlanta, USA; 6Department of Internal Medicine III, University Hospital of Ulm, Germany; 7Division of Hematology/Oncology, Dana Farber Cancer Institute, Boston, USA, and 8INSERM U985, Institut Gustave Roussy, Université Paris XI, France

ABSTRACT

Background
Cdx4 is a homeobox gene essential for normal blood formation during embryonic development in the zebrafish, through activation of posterior Hox genes. However, its role in adult mammalian hematopoiesis has not been extensively studied and its requirement in leukemia associated with Hox gene expression alteration is unclear.

Design and Methods
We inactivated Cdx4 in mice through either a germline or conditional knockout approach and analyzed requirement for Cdx4 in both normal adult hematopoiesis and leukemogenesis initiated by the MLL-AF9 fusion oncogene.

Results
Here, we report that loss of Cdx4 had a minimal effect on adult hematopoiesis. Indeed, although an increase in white blood cell counts was observed, no significant differences in the distribution of mature blood cells, progenitors, or stem cells were observed in Cdx4-deficient animals. In addition, long-term repopulating activity in competitive transplantation assays was not significantly altered. In vitro, B-cell progenitor clonogenic potential was reduced in Cdx4-deficient animals but no significant alteration of mature B cells was detected in vivo. Finally, induction of acute myeloid leukemia in mice by MLL-AF9 was significantly delayed in the absence of Cdx4 in a retroviral transduction/bone marrow transplant model.

Conclusions
These observations indicate that Cdx4 is dispensable for the establishment and maintenance of normal hematopoiesis in adult mammals. These results, therefore, outline substantial differences in the Cdx-Hox axis between mammals and zebrafish and support the hypothesis that Cdx factors are functionally redundant during mammalian hematopoietic development under homeostatic conditions. In addition, our results suggest that Cdx4 participates in MLL-AF9-mediated leukemogenesis supporting a role for Cdx factors in the pathogenesis of myeloid leukemia.

Key words: Cdx4, homeobox, leukemia, MLL-AF9, hematopoietic stem cell, Hox.


©2010 Ferrata Storti Foundation. This is an open-access paper.
**Introduction**

The *Cdx* genes are the mammalian homolog of the *Drosophila caudal* gene and encode homebox transcription factors that regulate axial elongation and anterior-posterior patterning during embryogenesis through modulation of *Hox* gene expression. The *Cdx* gene family consists of three members: *Cdx1*, *Cdx2*, and *Cdx4*. Several studies have indicated a role for *Cdx* genes in normal hematopoiesis during embryonic development. In the zebrafish embryo, *cdx1* and *cdx4* are important for blood formation through activation of posterior hematopoiesis, which participates in the control of normal embryonic hematopoiesis, likely through a regulatory network involving *Hox* genes.

**Design and Methods**

**Generation of Cdx4 knockout mice**

To create the conditional *Cdx4* mouse strain, a targeting vector containing loxP sites flanking the first exon, S’ untranslated region and the proximal promoter region of the *Cdx4* gene was generated (Figure 1B). This exon encodes the major portion of the coding sequence of *CDX4* (165 out of 282 amino acids including the homeodomain) and its excision would preclude expression of a stable, functional protein. This construct was transfected into mESC, and ESC clones were selected with hygromycin and screened by polymerase chain reaction (PCR) and Southern blot analyses. Correctly targeted ESC (i.e. clone 9 shown in Figure 1) were injected into Balb/C blastocysts to obtain chimeric animals that were crossed with C57/B6 wild-type mice to obtain germline transmission of the floxed *Cdx4* allele (termed *Cdx4fl*). The *Cdx4* allele was back-crossed for at least six generations with C77/B6 wild-type animals (Stock # 000664, The Jackson Laboratory).

**Real-time quantitative reverse transcription polymerase chain reaction**

Total RNA from sorted cells or hematopoietic tissues was isolated using the Trizol reagent (Invitrogen). RNA samples were reverse-transcribed with the Superscript II kit (Invitrogen). Real-time quantitative PCR assays for *Cdx4* and *β-actin* (reference gene) were obtained from Applied Biosystems (Mm01205647_g1 and Mm01205647_g1, respectively). PCR were performed on an ABI-7000 sequence detection system. The ΔΔCt method was used to calculate expression of *Cdx4* relative to *β-actin*. *Hox* genes were quantified as described previously. All reactions were performed on an ABI-7000 sequence detection system using SYBR Green PCR Master Mix or Taqman Universal PCR Master Mix reagents (Applied Biosystems).

**Flow cytometry and cell sorting analyses**

Single-cell suspensions were prepared from bone marrow cells after red blood cell lysis (Puregene). Cells were stained following standard procedures using antibodies purchased from BD-Pharmingen. Briefly, Lin-Sca1+cKit+ (LSK) cells and myeloid progenitors, including common myeloid progenitors (CMP), granulo-
cyte-macrophage progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP), were purified as follows. Total bone marrow cells from 6- to 8-week old wild-type C57/B6 animals were obtained after flushing the femora, tibiae and humeri and red blood cells were lysed (Puregene RBC lysis buffer, Qiagen). Cells were then stained with a cocktail of rat-anti-mouse antibodies against mature cells (lineage-positive cells), including Ter119, B220, CD3, CD4, CD8, IL7-R, CD19, and Gr1. After incubation with sheep anti-rat antibody coated magnetic beads (Dynabeads M-450, Dynal, Invitrogen), lineage-positive cells were physically depleted using a magnet (Invitrogen). Cells were then incubated with goat-anti rat PE-Cy5.5 conjugated antibody. After washing, cells were blocked with rat IgG prior to incubation with anti-CD34 FITC conjugated antibody, c-Kit APC conjugated antibody, Sca-1 PE-Cy7 conjugated antibody (BD Pharmingen) and FcγR/IIIb PE conjugated antibody (Abcam). Cells were flow sorted using a double laser (488 nm/550 nm Enterprise II and 647 nm Pecr) FACs (FACSaria, BD Biosciences). For T-cell populations, thymocytes were obtained from 6- to 8-week old wild-type C57/B6 animals. Cells were stained with anti-CD4, anti-CD8 and anti-CD3 antibodies (BD Pharmingen) and populations were purified as indicated in Figure 1A. For B-cell populations, total bone marrow cells from 6- to 8-week old wild-type C57/B6 animals were stained with anti-B220, anti-CD19, anti-IgM and anti-CD43 and were purified using the gates exemplified in Figure 2B. Cells were flow sorted using a double laser (488 nm/550 nm Enterprise II and 647 nm Pecr) FACs (FACSaria, BD Biosciences) and analyses were carried out on a four-color FACS Calibur (Becton Dickinson). Raw data were analyzed using FlowJo software.

**Colonies assays**

Myeloid and pre-B colony-forming assays were performed by plating 20,000 and 50,000 bone marrow cells per dish in duplicate into methylcellulose medium M3434 and M3630 (Stem Cell Technologies, Vancouver, BC, Canada), respectively. Colonies were counted on day 7 and 10, respectively. Bone marrow cells from leukemic mice were plated in methylcellulose medium M3434 at 10,000 cells per dish in duplicate. Colonies were counted on day 7 and serially re-plated every 7 days at a density of 10,000 cells per dish.

**Retroviral production, bone marrow transduction and transplantation assays**

Retroviral supernatant production and bone marrow transplants were performed as previously described using MSCV-MLL-AF9-GFP and MSCV-GFP. Briefly, 8-week old Cdx4-/- or wild-type littermate donor mice were injected with 5-fluorouracil (5-FU: 150 mg/kg) 5 days prior to bone marrow collection. On day 0, primary bone marrow cells were obtained from femora and tibiae by flushing with PBS 1x supplemented with 2% FBS; red blood cells were lysed (Puregene, Qiagen) and cells were then cultured overnight in RPMI 1640 supplemented with 10% FBS and IL3 (10 ng/mL), IL6 (20 ng/mL), SCF (10 ng/mL). Cells were mixed with similar titer viral supernatants twice on day 1 and day 2, centrifuged for 90 min (20 ng/mL), spinfected and returned to the incubator for 2-3 hours. After the second spinfection, cells were washed and re-suspended in Hank’s balanced salt solution 1x (HBSS 1x) and 1x10⁶ cells were injected into the tail veins of each lethally irradiated recipient. Animals were monitored daily for disease development and sacrificed according to institutional guidelines.

Non-competitive and competitive transplants were carried out with two sets of donor mice in two independent experiments, with five recipient mice per group in each experiment. CD45.2 bone marrow cells from Cdx4-/-Flox and control donor mice were injected into the lateral tail veins of lethally irradiated CD45.1+ C57/B6.SJF recipient mice, either alone for non-competitive transplants, or mixed with competitor bone marrow cells from wild-type F1 C57/B6 mice (CD45.1/2) for competitive transplants (Figure 3c). Recipient mice were bled from the eyes every 4 weeks up to 16 weeks after transplantation for analysis of CD45 in the peripheral blood. Bone marrow cells were obtained from recipient mice 16 weeks after transplantation for further analysis of the CD45.2 donor contribution.

**Histopathology and microscopy**

Peripheral blood was collected through non-lethal eye-bleeds under anesthesia with isoflurane in accordance with institutional guidelines. Complete blood counts were determined using a Hemavet 950 cell counter (Drew Scientific, Oxford, CT, USA). Paraffin-embedded tissue sections were prepared at the Dana

---

**Figure 1. Cdx4 expression and conditional inactivation strategy.** (A) Quantitative real-time RT-PCR was used to measure Cdx4 mRNA expression levels relative to β-actin in normal hematopoietic tissues and flow sorted cells. BM: bone marrow; PB: peripheral blood; LSK, enriched for hematopoietic stem cells; CMP common myeloid progenitors; GMP, granulocyte-monocyte progenitors; MEP, megakaryocyte-erythroid progenitors. (B) Homologous recombination targeting strategy to obtain Cdx4+/- mice. (C) Southern blot analysis using the 3' probe indicated in Panel B. WT: wild-type Cdx4 locus, Rec: Cdx4 locus target by the conditional knock-out construct. Clone 9 showed homologous recombination and was used to generate the conditional knock-out mouse line. Clone 8 was not correctly targeted and demonstrates the germline configuration. (D) PCR to assess Cdx4 excision 5 weeks after plpC treatment was performed on DNA from the indicated organs of Cdx4Flox Mx1Cre/+ or Cdx4Flox Mx1Cre/- animals using the three primers shown in Panel B (WT-F, WT-R, Exc-1R). Flox: unexcised allele, Exc: Excised allele. (E) LSK cells and myeloid progenitor populations from Cdx4Flox Mx1Cre/+ were flow sorted 6 weeks after plpC treatment and DNA was extracted to assess excision efficiency as described in (D). DNA from untreated Cdx4Flox Mx1Cre/+ total bone marrow cells was used as a control.
Farber/Harvard Cancer Center Specialized Histopathology Services Core and stained with hematoxylin and eosin. Images were obtained using a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) and a SPOT RT color digital camera (Diagnostic Instruments, Sterling Heights, MI, USA).

**Statistical analysis**

Statistical significance of differences between the results was assessed by a two-tailed unpaired Student’s t-test using Prism software.

**Results**

**Cdx4 expression in normal murine hematopoiesis**

We first determined the expression pattern of Cdx4 during normal hematopoietic differentiation using quantitative reverse transcription PCR on RNA from wild-type C57/B6 murine hematopoietic tissues and flow-sorted progenitors. Cdx4 mRNA was preferentially expressed in myeloid progenitor cells (Figure 1A) and during B-cell differentiation.

**Generation of conditional Cdx4 knockout mice**

To establish the role of Cdx4 in normal hematopoiesis, we engineered two mouse models of Cdx4 inactivation, through either straight knockout (named the Cdx4 allele) or conditional knockout (named the Cdx4 allele) based on inducible deletion of the entire exon 1 of the Cdx4 gene (Figure 1B). This was achieved through homologous recombination in ESC; Southern blot analysis was performed to select clones correctly targeted at the endogenous Cdx4 locus on chromosome X (i.e. clone 9 but not clone 8 in Figure 1C and Online Supplementary Figure S1). After germline transmission of the Cdx4 and Cdx4 alleles, crosses were performed to obtain homozygous animals. Both Cdx4-/- and Cdx4 F/F knockout animals were born at Mendelian ratios, appeared normal, had a weight similar to their wild-type, age-matched littermates, and were fertile. Cdx4 F/F mice were next crossed with Mx1-Cre transgenic animals to allow for inducible Cre recombinase expression. Cdx4 excision in Cdx4 F/F-Mx1-Cre (Cdx4F/F-Cre+ or F/F-Cre+) mice was induced in 4- to 6-week old animals by pIpC treatment. Subsequent analyses were performed 4-6 weeks after pIpC treatment.

![Graphs and images](image-url)

**Figure 2.** Analysis of the hematopoietic compartment in Cdx4 F/F-Cre+ mice. Flow cytometric analysis of the myeloid lineage (A) and B-cell lineage (B) analyzed on bone marrow cells (n=4). Except where otherwise indicated, analyses were gated on total bone marrow cells. (C) Histogram representation of results presented in Panel B (n=4). (D) Myeloid colony-forming potential of total bone marrow cells in SCF, IL3, IL6 and EPO-supplemented methylcellulose cultures (M3434, StemCells). Colonies were scored 7 days after plating and the mean ± SD is shown (n=4). GM, granulocyte macrophage; G/M, granulocyte or monocyte; GEMM, granulocyte erythroid macrophage megakaryocyte; BFU-E, burst-forming unit-erythroid. (E) B-cell colony-forming potential of total bone marrow cells in IL7-supplemented methylcellulose cultures (M3630, StemCells). Colonies were scored 10 days after plating and mean ± SD is shown (n=4).
unless otherwise indicated. Full Cdx4 excision was observed in whole bone marrow (Figure 1D) as well as in purified hematopoietic stem and progenitor-enriched populations (Figure 1E). Full excision of Cdx4 in the bone marrow was also demonstrated at 6 and 12 months (data not shown), indicating that under homeostatic conditions, there was no selective advantage for rare “escaper” cells in which excision had not occurred.

**Loss of Cdx4 results in minimal hematologic abnormalities**

We then performed analyses of the peripheral blood and hematopoietic organs. Both Cdx4fl/fl and Cdx4fl/fl-Cre mice showed a significant increase in the number of lymphocytes compared to their respective wild-type controls, as assessed by an automated cell counter (Table 1, Online Supplementary Table S1). However, there was no consistent difference in myeloid, erythroid and platelets counts, absolute numbers and distribution of bone marrow cells or spleen and liver weights between Cdx4 knockout mice (Cdx4−/− or Cdx4fl/fl-Cre) and their respective littermate controls (Online Supplementary Table S2). Flow cytometric analyses of bone marrow cells from Cdx4 knockout mice identified no significant differences in the major hematopoietic compartments including myeloid, erythroid, B-, and T-cell lineages (Figure 2A-C, Online Supplementary Figures S2A-C and S3). Similar results were observed in a cohort of animals analyzed 8 and 12 months after plpC treatment (Online Supplementary Figure S4A,B and data not shown).

To determine whether loss of Cdx4 affects the clonogenic potential of hematopoietic progenitor populations, we performed in vitro colony-forming unit (CFU) assays. Compared to bone marrow from wild-type littermates, Cdx4 knockout bone marrow produced similar total numbers of myeloid colonies, and no significant differences in the distribution of colony types were observed (Figure 2D, Online Supplementary Figure S2D). We did however, observe a significant decrease in pre-B colony-forming activity in bone marrow cells from Cdx4 knockout mice compared to bone marrow from their wild-type littermate controls in both the conditional as well as the germine knockout model (Figure 2E, Online Supplementary Figure S2E). Of note, we confirmed these observations in older mice that were analyzed 8 and 12 months after plpC treatment. (Online Supplementary Figure S4C,D and data not shown).

**Loss of Cdx4 does not alter the number and repopulating activity of hematopoietic stem and progenitor cells**

Since Cdx4 is expressed in the hematopoietic stem and progenitor compartment, and was reported to be essential for normal hematopoiesis in zebrafish, we next assessed the effect of loss of Cdx4 specifically on hematopoietic stem cell function. We first performed multiparameter flow cytometry analyses on the hematopoietic stem and progenitor compartments. No significant differences in the number of LSK, CMP, GMP, or MEP were observed between both Cdx4 knockout mouse models and their respective wild-type controls (Figure 3A,B and data not shown).

We then performed non-competitive and competitive transplantation assays to assess the repopulating ability of Cdx4-deficient bone marrow cells. CD45.1 CD45.2+ Cdx4fl/fl-Cre or control Cdx4fl/fl-Cre bone marrow cells were transplanted into lethally irradiated CD45.1CD45.2 BM SL- or control mice with or without wild-type CD45.1CD45.2−competitor bone marrow cells (control 3C). The contribution of the Cdx4-deficient and control bone marrow cells to hematopoiesis in the recipients (percentage of CD45.1CD45.2− cells) was assessed in the peripheral blood every 4 weeks over 16 weeks, and in the bone marrow after 16 weeks. We observed a similar contribution of donor-derived CD45.1CD45.2− cells from Cdx4-deficient and littermate control mice in the peripheral blood (Figure 3D) and bone marrow of recipient mice (data not shown). These results show that Cdx4-deficient bone marrow cells are not significantly altered in their normal long-term repopulating ability, indicating that Cdx4 is not essential for hematopoietic stem cell function even under the replicative stress associated with bone marrow reconstitution.

**Cdx4 shortens disease latency but is not essential for MLL-AF9-induced leukemia in mice**

Although Cdx4 does not appear to be essential for homeostatic hematopoiesis, it was previously demonstrated that in vitro differentiation of MLL-ESC into blood cells could be rescued by over-expressing Cdx4, suggesting that Cdx4 might be epistatic to, or a critical downstream effector of MLL. Furthermore, oncogenic fusion proteins involving MLL occur frequently in patients with acute myeloid or lymphoid leukemias. We, therefore, hypothesized that Cdx4 might play a role in the context of MLL-mediated leukemogenesis. To test this idea, we used a murine model in which transplantation of bone marrow cells transduced with MLL-AF9 into lethally irradiated recipients induces a fully penetrant myeloid leukemia (Figure 4A). Recipients of wild-type bone marrow cells transduced with MLL-AF9 developed acute leukemia with a median latency of 49 days. In contrast, recipients of MLL-AF9-transduced Cdx4−/− bone marrow cells developed acute leukemia with a significantly longer latency of 63 days (P=0.0005) (Figure 4B). Histological analysis identified no morphological differences between leukemias derived from wild-type and Cdx4−/− cells (Online Supplementary Figure S5). However, flow cytometric analysis revealed that while leukemias arising in both backgrounds showed a similar expansion of Mac1+ cells in the bone marrow (Figure 4C), leukemic cells generated on the Cdx4−/− background also displayed low levels of expression

<table>
<thead>
<tr>
<th>Table 1. Peripheral blood counts of Cdx4fl/fl-Cre and control animals. Blood samples were taken 6-8 weeks after plpC treatment from Cdx4fl/fl-Cre (n=10) and Cdx4fl/fl-Cre (n=10) post-plpC treatment and complete blood counts were obtained with a Hemavet950 cell counter.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>White blood cells (×10³/L)</td>
</tr>
<tr>
<td>Lymphocytes (×10³/L)</td>
</tr>
<tr>
<td>Granulocytes (×10³/L)</td>
</tr>
<tr>
<td>Monocytes (×10³/L)</td>
</tr>
<tr>
<td>Red blood cells (×10¹²/L)</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
</tr>
<tr>
<td>Platelets (×10³/L)</td>
</tr>
</tbody>
</table>
of two lymphoid surface markers, CD3 and B220 (Figure 4D). Together, these studies suggest that Cdx4 is dispensable for leukemia induction by MLL-AF9, but its loss delays disease onset and alters the leukemic phenotype in a bone marrow transplantation model.

**Loss of Cdx4 does not significantly affect Hox gene expression**

Cdx4 has been reported to regulate the expression of Hox genes, including Hoxa9 and Hoxb4.9,11,19 To determine the effect of Cdx4 loss on Hox gene expression in murine hematopoiesis, real-time quantitative RT-PCR was used to measure the expression of several Hox genes in bone marrow cells from wild-type mice and Cdx4-/- mice, as well as in leukemic cells generated from MLL-AF9-transduced wild-type and Cdx4-/- bone marrow cells. This analysis showed that there were no significant differences in the expression of individual Hox genes between Cdx4-/- and wild-type controls (Online Supplementary Figure S6).

**Discussion**

Here we report the first detailed analysis of the role of a caudal gene in adult homeostatic hematopoiesis in mammals and unexpectedly demonstrate that Cdx4 knock-out murine models do not present major hematopoietic defects. Indeed, in both germline and conditional Cdx4 knockout models, hematopoietic stem and progenitor cells as well as mature blood cells were not significantly affected by Cdx4 loss of function. Of note, our conditional Cdx4 knockout model bypasses any compensatory mechanism that might occur during embryonic development in the germline Cdx4 knockout model. These results demonstrate that Cdx4 is not essential for the establishment and maintenance of normal adult hematopoietic stem cell functions in mice. Although these results contrast with the severe hematopoietic defects observed in cdx4 mutant zebrafish,9 it is important to note that mammals have

---

**Figure 3.** Analysis of the hematopoietic stem cell compartment in Cdx4-/-Cre+ mice. (A) Flow cytometric analysis of the LSK (Lin-c-Kit+Sca1+) and myeloid progenitor compartments 4-6 weeks after plpC treatment. Left panels show 10000 cells gated on viable Lin- cells. Right panels show 10000 cells gated on viable Lin- c-Kit+Sca1 cells (B) Histogram representation of results presented in (A). Mean±SD are shown (n=4). (C) Diagram showing non-competitive and competitive bone marrow repopulation assays. (D) Contribution of donor cells (CD45.2-CD45.1+) to the hematopoiesis of lethally irradiated recipients in non-competitive and competitive transplants. The percentages of CD45.2+ and CD45.1- cells in the peripheral blood of recipient mice were measured by flow cytometric analysis at 4, 8, 12 and 16 weeks after transplantations (n=5). Mean±SD of the percentage of CD45.2+ cells are shown.
three Cdx genes (Cdx1, Cdx2, and Cdx4), whereas only two genes have been assigned to the Cdx family in zebrafish, Cdx1 and Cdx4. In addition, although zebrafish cdx4 mutants have a severe hematopoietic defect,10 suggesting a minor degree of redundancy between Cdx1 and Cdx4 during developmental hematopoiesis. It is likely that the critical function of cdx genes in zebrafish developmental hematopoiesis can be extended to the functionally redundant homolog Cdx2. In mammals, Cdx4 deficiency was reported to cause a modest hematopoietic defect during in vitro differentiation of mESC. In addition, although yolk sac hematopoiesis was transiently altered before 9 days post-conception, no significant blood alteration was observed in Cdx4 deficient embryos in the yolk sac 9 days post-conception or in fetal liver. In contrast, Cdx2 deficiency results in a more severe defect, and a combination of Cdx1, Cdx2, and Cdx4 deficiency almost abolished blood formation from mESC. Furthermore, only Cdx2/4 compound mutants, but not Cdx4-deficient or Cdx1/4 double mutants, present axial elongation defects during mouse development. Together, these observations suggest that Cdx4 may have a non-redundant role during very early hematopoietic development but that its function in adult hematopoietic stem cells in vivo is compensated for by redundant mechanisms. This difference could be explained by a lower sensitivity to Cdx gene dosage in definitive compared to primitive hematopoiesis. An alternative possibility is that Cdx function is not essential for adult hematopoiesis. Indeed, it was previously shown that other genes essential for primitive hematopoiesis specification, such as SCL/Tal1, do not play an essential role in definitive adult stem cell function, supporting the existence of two distinct developmental pathways for the generation of embryonic hematopoietic stem cells and maintenance of adult hematopoietic stem cells.

Although Cdx4 deficiency was not associated with a gross hematopoietic phenotype, our results do suggest a previously unappreciated role for Cdx4 in lymphoid development. Indeed, we show that Cdx4 expression is up-regulated in pre-B, pro-B, and B220+ cells. We also observed a reduced capacity of bone marrow cells from Cdx4-deficient animals to form B-cell colonies in vitro. These observations are compatible with a positive role of Cdx4 in lymphopoiesis that would result in a partial block of differentiation during early B-cell development in Cdx4-defi-
phenotype and the absence of a difference in gene expression observed in the majority of AML cases. In addition, Cdx4 can rescue the differentiation of Mll-deficient ESC in vitro and has been shown to interact with menin in the up-regulation of HOX cluster genes during Mll fusion-induced leukemogenesis, suggesting the possibility that oncogenic transformation by Mll fusions may require Cdx4. In support of this hypothesis, we found that although Cdx4 is not absolutely required for leukemia induction by Mll-AF9, its absence significantly prolonged the latency of disease development. Moreover, the phenotype of the resultant disease was altered with increased expression of B- and T-lymphoid markers subsequent to Cdx4 loss. Taken together, this suggests a role for Cdx4 in MLL-induced leukemogenesis. On the other hand, the subtlety of the phenotype and the absence of a difference in hoX gene expression between MLL-AF9-transduced wild-type and Cdx4−/− bone marrow cells, also point to a degree of functional redundancy among Cdx factors in the context of leukemogenesis. In addition, it is conceivable that there are context-specific differences in the requirement for Cdx4 during AML pathogenesis, depending on the underlying mechanism that drives the leukemogenic phenotype. For example, mutant kinases, such as BCR-ABL1, which do not have the potential for activating self-renewal programs in hematopoietic cells, may be more relevant on Cdx4-controlled pathways than alleles such as the MLL fusion genes that can confer self-renewal properties to committed progenitors.

The minimal hematopoietic impairment upon loss of Cdx4 along with its aberrant expression in acute leukemias make CDX proteins, in principle, attractive therapeutic targets. Indeed the therapeutic utility of targeting Cdx2 was suggested by knockdown experiments in AML cell lines. Although targeting transcription factors is very challenging and has not yet been clinically realized, critical protein-protein interactions associated with transcription factor function have recently been successfully targeted with small molecule inhibitors making transcription factors potentially “druggable” targets. In this study, we have shown that loss of Cdx4 significantly prolongs the latency of disease onset in a mouse model of MLL-AF9-induced AML. Although further studies will be necessary to understand the full degree of redundancy between Cdx genes in both normal and malignant hematopoiesis and the precise role of these proteins in the context of other MLL- or HOX-related leukemias, specific drug targeting of CDX factors could be of value in AML.

References


