Derepression of the Iroquois Homeodomain Transcription Factor Gene IRX3 Confers Differentiation Block in Acute Leukemia

Highlights

- IRX3 is derepressed in ~30% of AML, ~50% of T-ALL, and 20% of B-ALL
- IRX3 suppresses a mature myelomonocytic differentiation program in AML
- IRX3 impairs T-lineage differentiation, expanding an early T-progenitor population
- IRX3 contributes to the differentiation block that is characteristic of the disease

Data and Software Availability

GSE97450
Derepression of the Iroquois Homeodomain Transcription Factor Gene IRX3 Confers Differentiation Block in Acute Leukemia

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SUMMARY

The Iroquois homeodomain transcription factor gene IRX3 is expressed in the developing nervous system, limb buds, and heart, and transcript levels specify obesity risk in humans. We now report a functional role for IRX3 in human acute leukemia. Although transcript levels are very low in normal human bone marrow cells, high IRX3 expression is found in ~30% of patients with acute myeloid leukemia (AML), ~50% with T-acute lymphoblastic leukemia, and ~20% with B-acute lymphoblastic leukemia, frequently in association with high-level HOXA gene expression. Expression of IRX3 alone was sufficient to immortalize hematopoietic stem and progenitor cells (HSPCs) in myeloid culture and induce lymphoid leukemias in vivo. IRX3 knockdown induced terminal differentiation of AML cells. Combined IRX3 and HOXA9 expression in murine HSPCs impeded normal T-progenitor differentiation in lymphoid culture and substantially enhanced the morphologic and phenotypic differentiation block of AML in myeloid leukemia transplantation experiments through suppression of a terminal myelomonocytic program. Likewise, in cases of primary human AML, high IRX3 expression is strongly associated with reduced myelomonocytic differentiation. Thus, tissue-inappropriate derepression of IRX3 contributes significantly to the block in differentiation, which is the pathognomonic feature of human acute leukemias.

INTRODUCTION

The cardinal pathologic feature of the acute leukemias is a block to normal blood cell differentiation that results in an accumulation in the bone marrow (BM) of incompletely differentiated blast cells and failure of normal hematopoiesis (Wiseman et al., 2014). Although the spectrum of mutations associated with these diseases is now well established, the biologic basis of how mutations interact with one another to establish the pathognomonic differentiation block is less well understood. We recently reported that the Forkhead box transcription factor FOXC1 is misexpressed in approximately 20% of patients with acute myeloid leukemia (AML), in particular in those cases exhibiting high HOXA/B gene expression (Somerville et al., 2015). FOXC1 is neither required for nor expressed in normal hematopoietic cells but is essential for normal development of mesenchymal tissues such as the skeleton, heart, and eye and for the normal function of BM niche cells (Omatsu et al., 2014). Its misexpression in leukemic hematopoiesis contributes to a block in differentiation along both monocytic and B cell lineages and is associated with inferior survival. Given the lack of one-to-one correlation with any specific mutation, tissue-inappropriate derepression of FOXC1 is paradigmatic for a non-mutational mechanism contributing to cellular transformation in myeloid malignancy.

Whether tissue-inappropriate misexpression of other transcription factors contributes to the differentiation block of leukemia is not known. One candidate is the Iroquois homeobox transcription factor gene IRX3, which, like FOXC1, is expressed in a significant proportion of patients with AML (Somerville et al., 2015) but minimally expressed in both normal hematopoietic stem and progenitor cells (HSPCs) and mature blood cells (ENCODE data; Zhou et al., 2011). IRX3 is a member of the three-amino-acid-loop-extension (TALE) superfamily of homeodomain transcription factors, which also includes MEIS1 and PBX1 (Mukherjee and Burglin, 2007). In embryogenesis, it is strongly expressed in the developing nervous system, as well as in mesoderm-derived tissues such as the limb buds, kidney, and heart (Houweling et al., 2001). Of note, the developmental expression pattern of the Lnx3 paralog Lnx5, which sits in the same 2Mb topologically associating domain, is strikingly similar (Cohen et al., 2000; Claussnitzer et al., 2015). These genes exhibit functional redundancy because although Lnx3-null and Lnx5-null mice are viable and fertile, mice lacking both genes die in utero because of severe cardiac and skeletal defects (Zhang et al., 2011; Gaborit et al., 2012; Li et al., 2014; Smemo et al., 2014). Interestingly, non-coding variation in an enhancer...
region 500 kb downstream of IRX3 provides the strongest genetic association with risk for human obesity. Pertinent to this, adult Irx3-null mice display a 25%-30% reduction in body weight due to loss of fat mass, increased basal metabolic rate, and browning of white adipose tissue, attributable to loss of hypothalamic (Smemo et al., 2014) or preadipocyte (Clausnitzer et al., 2015) Ir3x expression. The rs1421085 single-nucleotide variant present in the obesity risk region dictates the extent of local recruitment of ARID5B to the IRX3 enhancer, with consequent regulation of IRX3 expression (Clausnitzer et al., 2015).

Whether IRX3 has a role in human malignancy is unclear. One study reported that IRX3 is strongly expressed in colorectal adenomas in comparison with normal mucosa and negatively regulates TGF-β signaling in colorectal cancer cell lines (Mortell et al., 2014). However, little else is known. Given this, and the observation that IRX3 is highly expressed in a subset of AML patients, we evaluated whether IRX3 has a functional role in acute leukemia.

RESULTS

IRX3 Is Frequently Co-expressed with HOX Genes in Human AML

To ascertain the frequency and extent of IRX3 expression in AML and in flow-sorted normal human BM cell populations, we performed both qPCR and analyses of published datasets. In a Dutch cohort of AML patients treated intensively with anthracycline-based chemotherapy on the Hemato-Oncologie voor Volwassenen Nederland (HOVON) protocols, IRX3 transcripts were detected at high level (i.e., with a probeset [229638_at] value of log2 > 7.1, approximating to a value among the top 25% of array probeset values) in 159 of 461 bulk presentation samples (34%) (Wouters et al., 2009) (Figure 1A). Likewise, in The Cancer Genome Atlas Research Network series, 49 of 163 cases (30%) expressed IRX3 at high level (Cerami et al., 2012; Ley et al., 2013) (Figure S1A). In flow-sorted populations of AML cells with immature immunophenotypes, IRX3 was highly expressed (i.e., among the top 25% of array probeset values) in 33% (Saito et al., 2010) (Figure S1B), 58% (Kikushige et al., 2010) (Figure S1C), and 19% (Goardon et al., 2011) (Figure S1D) of samples. Concomitant microarray profiling of normal human immunophenotypic HSPCs suggested low or absent IRX3 expression (Figures S1B–S1D). In keeping with this, our qPCR analyses revealed very low levels of IRX3 transcripts in all normal BM populations tested, but in 10 of 29 AML samples (34%), IRX3 expression was increased at least 250-fold over levels observed in the lowest expressing AML sample (Figure 1B; Table S1). IRX3 transcript levels were higher in normal human CD45− BM stromal cells than in normal BM cell populations (Figure 1B) but not as high as those observed in many AML samples. Given that expression of IRX3 and IRX5 are co-regulated in development, we also performed qPCR for IRX5 transcripts. We did not detect IRX5 expression in normal human BM cell populations (data not shown) but did detect low-level expression in 11 of 28 AML samples (39%), typically in cases with high IRX3 expression (Figure S1E). In the Dutch AML cohort, 44 of 461 cases (10%) expressed IRX5 at high level (i.e., probeset [210239_at] value of log2 > 7.1), and in every case there was also high IRX3 expression (data not shown). Thus, the set of IRX5high AML cases is a subset of the group of IRX3high cases.

To confirm that IRX3 protein was also expressed in AML, we performed immunohistochemical staining of a trephine biopsy tissue array. The array included 58 samples from patients with AML and 9 samples from patients with non-malignant BM conditions (Table S2). Immune staining was H-scored by blind evaluation. Strong or moderate nuclear immune staining (i.e., H score ≥ 80) was observed in 20 of 58 cases (34%). Weaker or absent immune staining was observed in 38 of 58 AML cases (66%) and in all non-malignant cases (Figures 1C and 1D).

High IRX3 expression in AML was strongly and positively associated with the presence of an NPM1 mutation or a FLT3 internal tandem duplication (Tables S3 and S4) (Wouters et al., 2009; Ley et al., 2013). A strong positive association with acute promyelocytic leukemia (APML) was also noted (Table S3). There were weaker positive associations with intermediate cytogenetic risk, normal karyotype and the presence of an IDH1 mutation, t(6;9), or an MLL gene rearrangement. High IRX3 expression was negatively associated with the presence of chromosome 5 or 7(9) loss, the presence of t(8;21) or inv(16), the presence of mutations in NRAS, TP53, or RUNX1, or a double CEBPA mutation and the presence of high MECOM expression (Tables S3 and S4). Although detailed genotyping of the tissue array samples was not performed, there was nevertheless a significant association of normal karyotype with strong or moderate IRX3 expression (14 of 17 available karyotypes were normal where strong or moderate nuclear immune staining was present versus 17 of 34 with weak or absent staining, p = 0.035, Fisher’s exact test) (Table S2).

To identify genes co-expressed with IRX3 in human AML, we next compared IRX3high AMLs (probeset 229638_at value of log2 > 7.1) with IRX3low AMLs (probeset 229638_at value of log2 < 6.1) (Wouters et al., 2009) and found HOXA9 and HOXAX5 to be the most differentially expressed transcription factor genes in the IRX3high group whether (data not shown) or not (Figure 1E) cases of APML (which do not express HOX genes) were included. Of the non-APML cases, 133 of 138 IRX3high samples (96%) exhibited high HOX9A expression (i.e., probeset value of log2 > 7.1), and of the HOXAX9high samples, 133 of 319 (42%) expressed high levels of IRX3 (Figure 1F). Of the five IRX3high HOXAX9low samples, three expressed one or more alternative HOX genes at significant level (i.e., log2 > 7.1), indicating that overall, 98.6% of IRX3high non-APML samples exhibited HOX gene co-expression. Similar results were observed in analyses of The Cancer Genome Atlas (TCGA) dataset (Figure S1F) (Ley et al., 2013) and qPCR analysis of our own samples (Figure 1G). Taken together, these data demonstrate that in human AML, the Iroquois homeodomain transcription factor gene IRX3, which is minimally expressed in normal hematopoiesis, is often misexpressed in conjunction with high HOX gene expression, as well as in APML. The former association suggests an explanation for the statistically significant associations of IRX3 expression with the presence of an NPM1 mutation, MLL gene rearrangements, and a t(6;9) because these molecular subtypes are associated with high HOX gene expression.
Figure 1. *IRX3* Expression in Human AML

(A) *IRX3* expression in bulk human AML samples. (B) Relative *IRX3* expression in bulk primary human AML samples (n = 29) and normal human BM cell populations (n = 4 separate individuals per cell type; error bars = SEM). BM, bone marrow; CMP, common myeloid progenitor; Eosin, eosinophils; EryB, erythroblast; GMP, granulocyte-macrophage progenitor; HSC, hematopoietic stem cell (CD34+38<sup>-</sup>CD90<sup>-</sup>CD45RA<sup>-</sup>/Lin<sup>-</sup>); MEP, megakaryocyte-erythrocyte progenitor; Mono, monocytes; MPP, multipotent progenitor; Neut, neutrophils. AML sample numbers refer to Biobank identifier.

(C) Representative images of IRX3 immune staining of human BM trephine biopsies. (D) H scores for IRX3 immune staining. Dashed line indicates cutoff value for moderate/strong immune staining.

(E) Heatmap shows *IRX3<sup>high</sup>* versus *IRX3<sup>low</sup>* AML cases (excluding APML) (Wouters et al., 2009) and the most differentially expressed transcription factor genes with mean fold change.

(F and G) Scatterplots show *IRX3* versus *HOXA9* expression in primary AML samples as determined by (F) array values or (G) qPCR. For scatterplots, percentages in blue text indicate proportion of *IRX3<sup>high</sup>* samples exhibiting high *HOXA9* expression. Percentages in red text indicate the proportion of *HOXA9<sup>high</sup>* samples (in the red boxes) additionally exhibiting high *IRX3* expression (above the dotted gray line).

See also Figure S1 and Tables S1–S4.
Figure 2. IRX3 Enhances Clonogenic Potential in Normal HSPCs
(A) MYC-tagged IRX3 expressed in murine KIT+ BM HSPCs 72 hr after retroviral infection. MTV, empty vector.
(B) Mean ± SEM colony-forming cell (CFC) frequencies during serial replating (n = 3).
(C) Bar chart (left) shows mean ± SEM colony types in round 3 of culture (n = 3). Type 1, tightly packed colonies, contain blast cells only; type 2, contain mixed population of blasts and mature cells; type 3, contain mature cells only. Image (right) shows representative colonies.
(D) Bar chart (left) shows mean ± SEM percentage of the indicated cell types in cytospin preparations after 7 days of culture of retrovirally infected murine KIT+ BM cells in liquid conditions supporting myeloid development (n = 3). Representative images (right) are shown.
(E) Representative image of IRX3-expressing mouse BM cells after 5 weeks in liquid culture.
(F) Representative flow cytometry plots for the indicated cell surface markers after 7 days in liquid culture; red boxes indicate cell sorting gates. Numbers within boxes indicate percentage of cells with the indicated phenotype.
IRX3 Promotes Serial Replating of Normal BM Stem and Progenitor Cells

To evaluate whether misexpressed IRX3 has functional consequences, we performed expression experiments in murine KIT+ BM HSPCs (Figure 2A). Serial replating assays in conditions supporting myeloid lineage differentiation demonstrated that in comparison with control cells, IRX3+ cells exhibited enhanced clonogenic activity (Figures 2A and 2B). In the third round of culture, control cells exclusively formed colonies with type 3 morphology (i.e., diffuse colonies containing mature macrophages), whereas IRX3+ cells in addition formed type 1 (i.e., tightly packed colonies containing blast cells) and type 2 colonies (i.e., mixed colonies containing blast cells and mature cells) (Figure 2C). Reflecting these observations, IRX3+ BM HSPCs cultured in liquid conditions (with interleukin-3 [IL-3], IL-6, granulocyte-macrophage colony-stimulating factor [GM-CSF], and stem cell factor [SCF]) for 7 days following retroviral transduction exhibited significantly impaired morphologic differentiation in comparison with control cells (Figure 2D) and could readily be grown on for at least 5 weeks (Figure 2E). The great majority of day 7 IRX3+ and control cells in liquid culture exhibited a Mac1+Gr1+ immunophenotype, although there was a modest but significant reduction in the percentage of Gr1+ cells in the IRX3 condition (Figure S1G). Flow-sorting analyses confirmed, as expected, that the clonogenic activity of control day 7 cells was near exclusively confined to cells with a Mac1+Gr1+ intermediate immunophenotype. In contrast, IRX3+ cells with a Mac1+Gr1+ intermediate immunophenotype also exhibited strong clonogenic activity (Figures 2F and 2G). qPCR analysis of genes associated with self-renewal in myeloid leukemia (Somervaille et al., 2008) demonstrated significant upregulation of Myc and Myb in IRX3+ populations with significant clonogenic potential (P1 and P2). Furthermore, there was significantly reduced expression in the aberrantly clonogenic IRX3+ P2 population of transcription factor genes such as Gfi1, Irf8, and Klf4, which are associated with myelomonocytic lineage differentiation (Figure 2I). Thus, expression of IRX3 in normal BM HSPCs in conditions supporting myeloid lineage differentiation confers a morphologic and functional differentiation block resulting in sustained clonogenic activity, elevated expression of self-renewal genes Myc and Myb, and reduced expression of myelomonocytic differentiation genes Gfi1, Irf8, and Klf4.

IRX3 Cooperates with HOXA9 to Enhance Differentiation Block in AML

Given the strong association of IRX3 and HOXA9 expression in human AML, we next evaluated the consequences of IRX3 co-expression in Hoxa9-expressing murine BM HSPCs. Cells were infected in pairwise combinations with retroviral vectors expressing Hoxa9, IRX3, or a control vector (to generate Hoxa9/IRX3 and Hoxa9/MTV cells, respectively) and serially replated in semisolid culture. Consistent with IRX3 co-expression conferring an enhanced differentiation block to Hoxa9-immortalized HSPCs, by the fourth round of culture, Hoxa9/IRX3 cells formed significantly more colonies than Hoxa9/MTV cells (Figure 3A), and these were significantly more likely to exhibit type 1 blast-like morphology (Figure 3B). Of note, Hoxa9/IRX3 type 1 colonies were on average 25% smaller in cross-sectional area than Hoxa9/MTV type 1 colonies (Figure 3C); this was explained by there being a significantly reduced proportion of Hoxa9/IRX3 cells in the SG2M phase of the cell cycle (Figure S1H). The immunophenotype of Hoxa9/IRX3 and Hoxa9/MTV cells was similar (Figure S1I).

Transplantation of Hoxa9-expressing murine BM HSPCs into irradiated syngeneic recipients results in short latency AMLs, which exhibit significant myelomonocytic maturation (Kroon et al., 1998; Somerville et al., 2015). To determine whether co-expression of IRX3 influences leukemia cell differentiation in vivo, we transplanted Hoxa9/IRX3 and Hoxa9/MTV double-transduced BM HSPCs into irradiated congenic recipients. Consistent with the cell-cycle status of in vitro transformed Hoxa9/IRX3 cells, analysis of blood at 4, 8, and 12 weeks post-transplantation demonstrated reduced donor:recipient chimerism in blood in Hoxa9/IRX3 versus Hoxa9/MTV recipients (Figure 3D), and Hoxa9/IRX3 recipient mice exhibited delayed onset of donor-derived AML in comparison with Hoxa9/MTV recipients (median 125 versus 270 days) (Figure 3E). Mice from both cohorts presented with substantially elevated blood leucocyte counts (Figure S2A), hepatosplenomegaly (Figure S2B), and effacement of BM due to infiltration by leukemia cells (Figures S2C–S2F).

In keeping with a model whereby IRX3 misexpression in vivo blocks myeloid lineage differentiation, evaluation of the lineage composition of donor-derived cells in blood prior to development of AML demonstrated a significant proportional reduction in myeloid and a proportional increase in B-lineage differentiation at 4 and 12 weeks post-transplantation in Hoxa9/IRX3 versus Hoxa9/MTV recipients (Figures 3F and S3A). Once mice developed full-fledged AML, this was further confirmed by analysis of blood smear and BM cytosin morphology: Hoxa9/IRX3 recipients developed leukemias exhibiting significantly greater differentiation block in comparison with Hoxa9/MTV controls, as demonstrated by the proportion of blast cells being on average twice as high in the former versus the latter and the proportion of more differentiated leukemia cells being approximately half as much (Figures 3G and 3H). Flow cytometry analysis of leukemic BM cells confirmed donor origin and myeloid lineage (Figures 3I, 3J, and S3B) and also revealed a distinctive immunophenotype: Hoxa9/IRX3 AMLs expressed significantly lower levels of both CD45.1 (Figures 3I and S3C) and the monocye/macrophage differentiation marker F4/80 versus Hoxa9/MTV controls. There were also significantly fewer Mac1+Gr1+ leukemia cells in Hoxa9/IRX3 recipients and, on average, double the percentage of cells positive for the stem and progenitor.
Figure 3. IRX3 Expression Confers an Enhanced Myeloid Differentiation Block in Hoxa9+ AML

(A) Bar chart shows mean ± SEM colony-forming cell (CFC) frequencies during serial replating of murine KIT+ BM cells co-transduced with the indicated retroviral or control (MTV) expression vectors (n = 3). *p < 0.05 by unpaired t test.

(B) Bar chart shows mean ± SEM colony types in round 4 (n = 3). *p < 0.05 by unpaired t test.

(C) Bar chart (left) shows mean ± SEM area of type 1 colonies (n = 26–40) from (B) and representative images (right).

(D–J) Murine CD45.1+ KIT+ BM cells were infected in pairwise combination with retroviral vectors, and 96 hr later 10⁶ drug-resistant cells were transplanted into CD45.2+ irradiated congenic recipients. (D) Line graph shows mean ± SEM percentage donor-derived CD45.1+ cells in blood at the indicated times post-transplantation. (E) Survival curves of transplanted mice (n = 7 per cohort). (F) Line graphs show the mean ± SEM percentage contribution of donor-derived cells in blood to the indicated lineages at the indicated times post-transplantation. (G) Bar chart (left) shows mean ± SEM percentage leucocyte type in blood at death, as determined by morphologic analysis of blood smears (n = 4 or 5 per cohort). Representative images (right) are shown. (H) Bar chart (left) shows mean ± SEM (legend continued on next page)
marker KIT (Figure 3J), although in view of heterogeneous levels of expression, this difference did not reach statistical significance. Hoxa9/IRX3 AML cells induced leukemias in secondarily transplanted recipients (Figure S3D), and high-level IRX3 expression was readily detected in Hoxa9/IRX3 AML BM cells (Figure S3E). Interestingly, in contrast to observations in vitro, cell-cycle analysis of leukemia cells from BM and spleen of leukemic mice demonstrated no difference in the fraction of cycling cells between the cohorts (Figure S3F), although in both cases, the SG2M fraction was substantially lower in the in vivo setting in comparison with the in vitro setting (i.e., 10%–20% versus 40%–50%).

Altogether, our observations demonstrate that co-expression of IRX3 modulates the phenotypic consequences of Hoxa9 expression both in vitro and in vivo, conferring a significantly enhanced myeloid lineage differentiation block.

**Functional Contribution of IRX3 to Differentiation Block in AML Cells**

To further confirm that tissue-inappropriate expression of IRX3 contributes functionally to the differentiation block in AML, we performed knockdown (KD) experiments in human THP1 AML cells, which exhibit the highest levels of IRX3 expression among AML cell lines we tested (Figure S4A), IRX3 KD led to loss of clonogenic potential (Figures 4A–4C), which was due to induction of differentiation, as evidenced by upregulation of the myeloid differentiation markers CD11b, CD14, and CD66 (Figures 4D and 4E) and morphologic analysis (Figure 4F). The proportion of apoptotic cells was unaffected (Figure S4B). To confirm that the observed phenotype was an on-target consequence of IRX3 KD, similar experiments were performed using KD construct #2, which targets the 3′UTR region of IRX3, in a THP1 line engineered to express an IRX3 cDNA that lacks it. Sustained IRX3 expression in THP1 cells infected with lentiviral vectors expressing KD construct #2 was confirmed (Figures 4G and 4H), as was rescue of the differentiation phenotype (i.e., loss of clonogenic potential and upregulation of the differentiation marker CD66) (Figures 4I, S4C, and S4D). Similar experiments in murine MLL-AF9 AML cells, which also express IRX3, gave similar results: KD cells exhibited loss of clonogenic potential and terminal monocyte/macrophage lineage differentiation (Figures S4E–S4G). IRX3 KD in a range of additional human AML cell lines also led to loss of clonogenic potential in many cases, but not all (Figure S4H). Of note, KD construct #2 had no effect on the clonogenic potential of K562 cells, which do not express IRX3 (Figure S4A).

Furthermore, the formation of colony-forming unit granulocyte monocyte (CFU-GM) and colony-forming unit monocyte/macrophage (CFU-M) from normal human CD34+ cells (which express IRX3 at very low levels) was unaffected by IRX3 KD construct #2 (Figures S4I and S4J), although, for unclear reasons, there was a reduction in the formation of erythroid burst-forming units.

Thus, in keeping with in vitro and in vivo murine experiments, misexpressed IRX3 contributes to the differentiation block in AML cells and sustains clonogenic potential.

**IRX3 Expression in AML Represses a Myelomonocytic Differentiation Program**

To evaluate the consequences for the transcriptome of co-expression of IRX3 with Hoxa9, we performed RNA sequencing of flow-sorted KIT+Gr1+ leukemia cells recovered from the BM of sick mice, three from each cohort. Cells with this immunophenotype are enriched for leukemia-initiating activity in Hoxa9/Meis1 murine leukemias (Gibbs et al., 2012). Analysis of 8,954 protein-coding genes that passed threshold criteria (i.e., expression >0.5 reads per kilobase per million mapped reads [RPKM]) in at least one sample) revealed that 197 were upregulated and 403 down-regulated by at least 2-fold in Hoxa9/IRX3 versus Hoxa9/MTV leukemias (Figure 5A). Gene Ontology analysis revealed significant enrichment within the Hoxa9/IRX3 downregulated gene set of biological process terms such as “immune response,” “leucocyte activation,” and “defense response” (Table S5), suggesting that IRX3-repressed genes are associated with mature myeloid cells. At similar levels of significance, there were no enriched terms among the Hoxa9/IRX3 upregulated gene set.

Gene set enrichment analysis (GSEA) confirmed that co-expression of IRX3 with Hoxa9 led to repression of a mature myeloid lineage program in KIT+Gr1+ leukemia cells, in keeping with morphologic analysis (Figures 3G and 3H). Among genes downregulated in Hoxa9/IRX3 versus Hoxa9/MTV AML cells, there was significant enrichment for genes highly expressed in both mature monocytes and mature neutrophils (i.e., myelomonocytic genes) (Figure 5B; Tables S6 and S7).

GSEA also revealed significant overlap with expression patterns observed in murine models of leukemia associated with high-level expression of Hoxa9 and Meis1. Mixed-lineage leukemia (MLL) fusion oncogenes require Hoxa9 and Meis1 to properly transform HSPCs and to establish a leukemia cell hierarchy. We observed that in Hoxa9/IRX3 versus Hoxa9/MTV AML cells, there was significant enrichment of expression of the transcriptional sub-program that contributes to blocked differentiation and enhanced self-renewal of leukemia stem cells (LSCs) in murine MLL leukemias (LSC maintenance program; Somerville et al., 2009) (Figure 5C; Tables S6 and S7).

Likewise, the set of genes anti-correlated with the LSC maintenance program (i.e., associated with differentiated leukemia cells, downstream of the LSC) was strongly repressed in Hoxa9/IRX3 versus Hoxa9/MTV AML cells (Figure 5C; Tables S6 and S7). Similar positive and negative enrichments were observed for gene sets derived from transformed progenitor cells with sustained (Hoxa9/Meis1 POSITIVELY REGULATED) or withdrawn (Hoxa9/Meis1 NEGATIVELY REGULATED) Hoxa9/Meis1 dual expression (Hess et al., 2006) (Figure S5A). All together, these analyses demonstrate that co-expression of IRX3 with Hoxa9 enhances expression of genes previously associated with LSC...
self-renewal and represses expression of a terminal myeloid line-
age differentiation program.

We next evaluated whether a signature of IRX3 transcriptional
activity could be detected in human AML. We ranked protein-
coding genes in IRX3 compared to IRX3 low human AMLs (Wouters
et al., 2009) using a signal-to-noise ranking metric (Table S6)
and performed GSEA using the set of genes repressed by IRX3
in murine Hoxa9/IRX3 AML cells (Table S5). There was highly
significant negative enrichment of IRX3-repressed genes in human
IRX3 high versus IRX3 low human AMLs whether all AMLs were
considered or just those expressing high levels of HOXA9 (Fig-
ure 5D). Indeed, in leading-edge analyses, there was a highly

Figure 4. IRX3 Sustains the Differentiation Block and Clonogenic Potential of AML Cells

Human THP1 AML cells were infected with lentiviral vectors targeting IRX3 for KD or a non-targeting control vector (NTC).
(A) Bar chart shows mean + SEM relative transcript expression in IRX3 KD versus control cells (n = 3) after 48 hr.
(B) Bar chart shows the mean + SEM colony-forming cell (CFC) frequencies of KD cells relative to control cells enumerated after 10 days in semi-solid culture (n = 4).
(C) Representative images of colonies from (B).
(D) Bar chart shows mean + SEM percentage of cells positive for the indicated cell surface markers, as determined by flow cytometry analysis 6 days following
initiation of KD (n = 4). *p ≤ 0.05 using one-way ANOVA with Fisher’s least significant difference post hoc analysis for KD conditions versus NTC.
(E) Representative flow cytometry plots from (D).
(F) Representative images of cytosplins of cells from (D).
(G) Bar chart shows mean + SEM relative transcript expression in THP1 AML cells expressing either IRX3 or a control retroviral vector (MTV) in IRX3 KD#2 cells relative to control cells (n = 3).
(H) Western blot shows expression of the indicated proteins in the indicated conditions.
(I) Bar chart (left) shows mean + SEM CFC frequencies of THP1 AML cells expressing either IRX3 or a control retroviral vector (MTV) in IRX3 KD#2 cells relative to
control cells. Colonies were enumerated after 10 days in semi-solid culture (n = 4). Image (right) shows representative colonies. *p ≤ 0.05 using one-way ANOVA
with Fisher’s least significant difference post hoc analysis for the indicated comparison. NS, not significant.
See also Figure S4.
significant association of higher IRX3 expression in human HOXA9+ AML with greater repression of IRX3-repressed genes identified in murine leukemias (Figure 5E; Table S7). Remarkably, when the morphologic classification of HOXA9+ AMLs was considered, among cases with high IRX3 expression, there were significantly fewer AMLs exhibiting myelomonocytic differentiation (i.e., French-American-British [FAB] M4 subtype) and significantly more AMLs exhibiting minimal differentiation (i.e., FAB M1 subtype) or maturation toward the granulocytic lineage (i.e., FAB M2 subtype) (Figure 5F). Thus, in primary human AML, as in murine AML, misexpression of IRX3 contributes functionally to blockade of myelomonocytic lineage differentiation.

**IRX3 and FOXC1 Differentially Repress Expression of Myelomonocytic Transcription Factors**

We previously reported that the mesenchymal transcription factor gene FOXC1 is also frequently misexpressed in human AML to confer, in particular, a monocytic lineage differentiation block (Somerville et al., 2015). With regard to the morphologic classification of human AML, FOXC1-expressing leukemias...
IRX3 were significantly less likely to exhibit monocytic lineage differentiation (i.e., FAB-M5) and significantly more likely to exhibit granulocytic lineage differentiation (i.e., FAB-M2) (Somerville et al., 2015). The presence of high FOXC1 expression in AML was also associated with inferior outcome, in contrast to the presence of high IRX3 expression (Figure S5B). Murine Hoxa9/FOXC1 leukemias also exhibited shortened latency versus Hoxa9/MTV leukemias (Somerville et al., 2015), in contrast to the latencies observed for Hoxa9/IRX3 leukemias (Figure 3E).

To evaluate the consequences of misexpressed IRX3 and FOXC1 on expression levels of transcription factors required for normal myelomonocytic lineage differentiation, and to determine why the phenotypic consequences of IRX3 and FOXC1 misexpression differed one from another, we performed qPCR using murine leukemia samples and analyzed expression levels in published human AML datasets. In flow-sorted murine BM KIT*Gr1+ AML cells, expression levels of transcription factor genes such as Ifi8, Ifi5, and Klf4 (which promote monocytic lineage differentiation) were significantly lower in Hoxa9/FOXC1 AMLs in comparison with Hoxa9/IRX3 and Hoxa9/MTV AMLs (Figure 6A). There was no significant difference in expression levels of the myeloid lineage master regulator Sfi1. In the case of Gfi1 (which promotes granulocytic lineage differentiation), there was a variable increase in expression in Hoxa9/IRX3 AMLs compared with the other subtypes, although this did not achieve statistical significance.

In primary human AML samples, a very similar pattern was observed. In comparison with FOXC1low or IRX3low cases, FOXC1high or IRX3high cases respectively exhibited significantly lower level expression of IRF8 and KLF4, and for both genes, the proportionate reduction in expression was greater for FOXC1high cases than for IRX3high cases (Figure 6B). IRF5 expression was significantly lower in FOXC1high versus FOXC1low cases and no different between the IRX3 groups, whereas GFI1 expression was significantly higher in IRX3high versus IRX3low cases and no different between the FOXC1 groups. Expression levels of SPI1 did not differ. Similar analyses using a separate, smaller dataset from TCGA also gave similar results (Figure S5C), in particular with regard to expression levels of GFI1 and IRF8. Together these data demonstrate that although IRX3 and FOXC1 misexpression contribute to the differentiation block observed in human AML, they repress myelomonocytic lineage transcription factor genes in a distinctive way.

See also Figure S5.
Figure 7. IRX3 Misexpression in Lymphoblastic Leukemia
(A) IRX3 expression in bulk human T-ALL samples.
(B) Heatmap shows IRX3high versus IRX3low T-ALL cases and the most differentially expressed transcription factor genes with mean fold change.
(C) IRX3 versus HOXA9 expression in primary human T-ALL (Haferlach et al., 2010). Percentage in red text indicates the proportion of HOXA9high samples (red box) with high IRX3 expression (above the dotted gray line).
(D) Immunophenotypic definition of early T cell progenitor double-negative (DN) populations.
(E) Mean + SEM fold expansion in OP9 DL1 stromal culture of ETP/DN1 cells over input number on day 7 (n = 4–6). MTV, empty vector.
(F) Representative flow cytometry plots.
(G) Immunophenotypic developmental stage of Lin−/−BM HSPCs expressing the indicated gene combinations cultured for 30 days on OP9 DL1 stroma (mean + SEM percentage, n = 6).

(legend continued on next page)
manner: FOXC1 represses monocytic lineage genes more profoundly than does IRX3, whereas the opposite is the case for GF11.

**Polycomb Activity Sustains IRX3 Repression in Normal Human CD34+ Stem and Progenitor Cells**

IRX3 is only minimally expressed in normal CD34+ cells, and its genetic locus is marked by high levels of H3K27 trimethylation (Zhou et al., 2011) (Figure 6C), suggesting that its relative transcriptional silence is maintained by Polycomb-mediated repression. To address this question, normal human CD34+ HSPCs from multiple donors were treated in vitro for 5 days with a dual EZH1 and EZH2 inhibitor (UNC1999; Konze et al., 2013). We observed a significant increase in IRX3 expression but no change in Hoxa9 expression (Figure 6D); the HOXA9 locus is not marked by H3K27 trimethylation in normal CD34+ HSPCs (Figure 6C). Thus, the Polycomb complex contributes to continued repression of IRX3 in normal HSPCs.

**IRX3 Is Frequently Co-expressed with HOX Genes in Human Acute Lymphoblastic Leukemia**

To provide a more comprehensive evaluation of the role of IRX3 in human acute leukemia, we analyzed published expression datasets from patients with acute lymphoblastic leukemia (ALL). In a cohort of T-ALL patients (Microarray Innovations in Leukemia [MILE] study; Haferlach et al., 2010), IRX3 transcripts were detected at high level (i.e., with a probeset [229638_at] value of ≥ 0.42, approximating to a value among the top 25% of array probeset values) in 84 of 174 cases (48%) (Figure 7A). To identify transcription factor genes concordantly expressed with IRX3 in T-ALL, we compared IRX3high (array signal intensity > 0.42, n = 84) with IRX3low (array signal intensity < 0.3, n = 80) cases and observed that only HOXA genes were upregulated by greater than a mean 2-fold change in array signal intensity (and with p < 10−5, by unpaired t test) (Figure 7B). Of the 84 IRX3high cases, 52 (62%) expressed Hoxa9 at high level, 18 (21%) were Hoxa9low but expressed one or more alternate HOX genes, and 8 (14%) were HOXlow but instead expressed the homeodomain transcription factors TLX1 or TLX3 (Figure 7C). Of the HOXA9high T-ALL cases (i.e., 60 of 174), 52 of 60 (87%) were IRX3high (Figure 7C). High HOXA expression is a feature of human T-ALL associated with CALM-AF10 fusions, SETNUP214 fusions, MLL gene rearrangements, or inv(7) or t(7;7) translocations (resulting in apposition of the HOXA locus to TCR-β regulatory elements; Soulier et al., 2005; Van Vlerberghhe et al., 2008).

In B-acute lymphoblastic leukemia (B-ALL), high IRX3 expression was detected in 116 of 563 cases (21%) in the MILE cohort (Figure S6A). There was a particular association of high IRX3 expression with the presence of an MLL gene rearrangement (31 of 70 cases [44%]), or the presence of t(12;21), the cytogenetic hallmark of the ETV6-RUNX1 fusion (44 of 58 cases [76%]) (Figure S6B). To identify transcription factor genes exhibiting concordant expression with IRX3 in B-ALL, we compared IRX3high (array signal intensity > 0.42, n = 116) with IRX3low (array signal intensity < 0.3, n = 398) B-ALLs. Only Hoxa9, MEIS1, and SOX11 were upregulated by greater than a mean 2-fold change in array signal intensity (and with p < 10−5, by unpaired t test) in IRX3high B-ALLs (Figure S6B). Of the 57 MLL rearranged HOXA9high cases, 27 (47%) were IRX3high (Figure S6C). IRX3high cases with a t(12;21) did not express HOX genes at high level (Figure S6B). Together these data demonstrate that IRX3, which is expressed at very low levels in normal hematopoiesis, is frequently highly expressed in human ALL.

**IRX3 Impedes Phenotypic Differentiation of T Cell Precursors and Induces Lymphoid Leukemias**

To evaluate the functional consequences of IRX3 expression in early stage lymphoid development, Lin− murine BM HSPCs were double-transduced with pairwise combinations of IRX3, Hoxa9, or control retroviral vectors and, following drug selection, co-cultured on stromal layers. Where BM cells were co-cultured on OP9 stroma (which supports B-lineage differentiation), we observed no significant difference in upregulation of the B-lineage markers B220 and CD19 or expansion of cell numbers (Figure S6D). In contrast, where cells were co-cultured on OP9 DLL1 stroma (which ectopically expresses the Notch ligand DLL1 and supports T-lineage differentiation), we observed a highly significant block in differentiation of Hoxa9/IRX3 co-expressing cells at the early thymic progenitor (ETP) stage. Differentiating T cell progenitors exhibit sequential expression of CD44 and CD25, which together define developmental stages ETP/DN1 through to DN4 (Figure 7D) (Yui and Rothenberg, 2014). Over the 4 week assay, there was a mean 100-fold greater expansion of cells with an ETP/DN1 immunophenotype in the presence of Hoxa9/IRX3 co-expression, whereas cells expressing Hoxa9 alone or control cells readily progressed to DN2 and DN3 downstream differentiation stages (Figures 7E–7G). Overall expansion of cell numbers was similar (Figure 7H). Of note, in contrast to BM HSPCs cultured in myeloid conditions (Figure 2), IRX3/MTV expressing Lin− cells failed to expand on OP9 DLL1 stroma (n = 4).

In keeping with a role for IRX3 in promoting the development of lymphoid leukemias, we found that irradiated congenic mice transplanted with IRX3-expressing Kit+ BM HSPCs developed lymphoid leukemias with incomplete penetrance. In the 12 weeks after transplantation, although there was significantly reduced donor:recipient chimerism in comparison with animals receiving...
control cells (Figure S6E) (as observed in mice transplanted with IRX3/Hoxa9-expressing cells; Figure 3D), there was no significant proportional difference in myeloid, B-lineage, and T-lineage engraftment (Figures S6E and S6F). Three of six IRX3-expressing HSPC recipients developed donor-derived lymphoid leukemia (Figures 7I and 7J); in two cases, the leukemia was T-lineage, and mice exhibited splenomegaly and near total BM involvement (Figures 7K and S7A). These cases expressed Hoxa genes at comparable levels to KIT BM HSPCs (Figure S7B). In the third case, although the mouse was found dead and detailed autopsy could not be completed, flow cytometry analysis of blood cells performed 8 days before death revealed a CD45.1+B220+CD19+ population accounting for 92% of donor-derived leucocytes (Figure S7C). This population was not present 4 weeks earlier, suggesting that this mouse died of a B-lineage leukemia. Three of six other mice died during the 400 day follow-up period, but all succumbed to recipient-derived hematologic malignancies likely induced by irradiation at transplant conditioning (Figures 7J, 7K, and S7A). At experiment termination, there was no evidence of incipient hematologic neoplasms in remaining mice. Together these data demonstrate that IRX3 expression impedes normal T-progenitor differentiation in vitro and induces T-lineage leukemias in vivo.

DISCUSSION

Our studies demonstrate that tissue-inappropriate misexpression of IRX3 is both frequent and functional in human acute leukemias of multiple lineages. The lack of a major role for IRX3 and its paralog IRX5 in normal hematopoiesis is emphasized by the observation of minimal or absent expression in human BM cell populations and genetic knockout experiments that demonstrate that Irx3−/− and Irx5−/− mice are viable and fertile (Smemo et al., 2014). At least in AML, IRX3 misexpression is found in both bulk and the putative stem cell compartments, suggesting functional contribution throughout the leukemia clone. With regard to co-regulated gene expression within the IRX3 topologically associated domain, our qPCR and published dataset analyses indicate that the set of IRX5-expressing AMLs is a subset of the IRX3-expressing cases, with lower expression levels of IRX5 than IRX3.

Why is IRX3 expressed so extensively in acute leukemia? Our in vitro and in vivo studies demonstrate that misexpression of IRX3 contributes to the cardinal pathologic feature of acute leukemia, the differentiation block. In cultures supporting myeloid lineage differentiation, IRX3 expression alone or in combination with Hoxa9 enhanced clonogenic potential and impeded differentiation of normal HSPCs. In vivo, IRX3 co-expression with Hoxa9 dramatically enhanced the degree of differentiation block in murine AMLs, even though the onset of AML was delayed. Critically, the same transcriptional signature of IRX3-mediated repression of myelomonocytic differentiation was readily identified in human AML, confirming that IRX3 misexpression is both frequent and functional in human acute leukemia. The paradigm that misexpression of IRX3 confers a differentiation block is further supported by our observation that co-expression of IRX3 with Hoxa9 in T-lineage cultures impeded differentiation of ETPs into downstream developmental stages and that IRX3-expressing HSPCs generated lymphoid leukemias in vivo. The expanded ETPs population may serve as a reservoir for acquisition of genetic mutations required for full-fledged leukemia. The observations that HSPCs expressing IRX3 alone were immortalized in myeloid culture, failed to expand significantly in OP9 stromal culture, but generated Hoxa-expressing lymphoid leukemias in vivo reflect the importance of the cellular microenvironment in supporting phenotypic outcome. The outcomes also emphasize the importance of the interaction of misexpressed IRX3 with cell type-specific patterns of chromatin accessibility for transcription factor binding. Cells of different lineages, and of different differentiation states, express different repertoires of transcription factor genes; it is likely that IRX3 binds to chromatin and interferes with gene expression in distinct ways in cells of different lineages.

How is IRX3 gene expression turned on in hematopoietic cells? Although its expression is positively associated with NPM1 and FLT3 mutations in AML, the link is not absolute; for example, more than a third of NPM1 mutant cases do not express IRX3 (Wouters et al., 2009). The positive association with other genetic lesions that near invariably lead to high-level HOX gene expression (i.e., a t[6;9] or translocations targeting MLL at chromosome 11q23) raises the possibility that IRX3 is positively regulated by HOX transcription factors. In keeping with this, when HOX9 or HOXA10 is expressed in human CD34+ HSPCs, IRX3 is upregulated (Ferrell et al., 2005). Relatedly, in Xenopus development, HOX84 and IRX5 have overlapping patterns of expression, and the latter is a direct target of the former (Theokli et al., 2003). However, high-level HOX gene expression alone in AML is not sufficient to result in IRX3 expression, because only 40% of HOX9A+ cases express IRX3. This suggests that additional factors act combinatorially to induce IRX3. For example, the Wnt signaling pathway, which is active in AML, induces IRX3 in forebrain development (Braun et al., 2003). In addition to these candidate positive regulators, it seems likely that loss of repressor activity makes a significant contribution. Our observation that the IRX3 locus is marked by H3K27me3 in normal CD34+ HSPCs and that treatment of cells with a dual EZH1 and EZH2 inhibitor led to IRX3 upregulation demonstrates that its repression in normal hematopoiesis is dependent on Polycomb.

As well as the strong association of IRX3 with HOX gene expression in acute leukemias of multiple lineages, there was also high-level expression of IRX3 in ~90% of cases of APML and in ~75% of cases of t(12;21) B-ALL, leukemias that do not express HOX genes. It is possible that IRX3 is induced as a direct consequence of PML-RARA or ETV6-RUNX1 fusions, respectively, although the close association of co-expressed SOX11 in the latter case suggests potential collaboration.

The molecular consequences of IRX3 misexpression in the acute leukemias remain unclear. It is known that TALE family transcription factors such as MEIS1 and PBX can form triplex complexes with HOX9A that bind to PBX-HOX9A consensus sequences to regulate gene expression (Shen et al., 1999). We speculate that misexpressed IRX3 might alter the function or stability of HOX transcription factor heterotrimeric complexes, perhaps to prevent downregulation of self-renewal genes or upregulation of transcription factors required for terminal...
determination. Alternatively, it may redirect HOX transcription factors to new binding sites or function on its own to activate or repress key transcription regulators.

Like IRX3, the Forkhead transcription factor gene FOXC1 is also frequently misexpressed in AML, although the phenotypic consequences in mouse models and primary human AMLs are quite distinct. This is likely related to distinct mechanisms of action and sites of genomic binding; for example, Forkhead and Iroquois transcription factors have different consensus binding motifs and will bind different sites in the genome to regulate overlapping but fundamentally distinct gene sets. In particular, FOXC1 seems more effective than IRX3 at suppressing expression of monocytic lineage transcription factor genes such as IRF8, IRF5, and KLF4. In contrast, in comparison with IRX3low AML cases, in IRX3high cases there is increased expression of the granulocytic lineage regulator gene GFI1. In some cases both FOXC1 and IRX3 are misexpressed, and here the resulting cellular phenotype will represent the integrated consequence of the prevailing nuclear transcription factor milieu.

In summary, we demonstrate that the Iroquois homeodomain transcription factor IRX3 is frequently misexpressed in human acute leukemia to contribute to the differentiation block that is the pathognomonic feature of the disease. Future investigations will identify approaches to target these transcription factors for pro-differentiation therapies to improve patient outcomes.

EXPERIMENTAL PROCEDURES

Human Tissue and Ethical Approval
Normal CD34+ HSPCs surplus to requirements were from patients undergoing autologous transplantation for lymphoma. Their use was authorized by the Salford and Trafford Research Ethics Committee and, for samples collected since 2006, following written informed consent from donors. Normal human BM was collected with informed consent from healthy adult male donors, with the ethical approval of the Yorkshire Independent Research Ethics Committee. Primary human AML samples were from Manchester Cancer Research Centre’s Tissue Biobank (approved by the South Manchester Research Ethics Committee). Their use was authorized by the Tissue Biobank’s scientific sub-committee, with the informed consent of donors.

Murine Experiments
Experiments using mice (female, aged 6–12 weeks) were approved by Cancer Research UK Manchester Institute’s Animal Ethics Committee and performed under a project license issued by the United Kingdom Home Office, in keeping with the Home Office Animal Scientific Procedures Act of 1986. C57BL/6 (CD45.2+) mice were from Envigo. B6.SJL-Pgpc3/+/BoyJ (CD45.1+) mice were from Jackson Laboratories and bred in house. Details of transplantation procedures are in the Supplemental Information.

Reagents, Cell Culture, and Flow Cytometry
Details are in the Supplemental Information.

RNA Preparation, qPCR, RNA Sequencing, Bioinformatics, and Statistics
Details are in the Supplemental Information.

Statistical Analyses
Statistical analyses were performed using StatsDirect software version 1.9.7 (StatsDirect), Microsoft Excel, or SPSS for Mac version 22 (IBM). Survival curves were generated using Prism software version 6.0 (GraphPad Software).

DATA AND SOFTWARE AVAILABILITY
The accession number for the RNA sequencing data files reported in this paper is GEO: GSE97450.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.12.063.

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AUTHOR CONTRIBUTIONS
T.D.D.S., F.S., J.A.C., E.L.W., G.J.S., and T.C.P.S. performed experiments and analyzed data. K.B., E.T., and R.J.B. generated the tissue array and performed histopathological stains and analyses. C.W. performed bioinformatics analyses. T.D.D.S. and T.C.P.S. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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