A novel human fetal liver-derived model reveals that MLL-AF4 drives a distinct fetal gene expression program in infant ALL

Siobhan Rice¹, Thomas Jackson², Nicholas T Crump¹, Nicholas Fordham¹, Natalina Elliott², Sorcha O’Byrne², Sarah Inglott³, Dariusz Ladon³, Gary Wright³, Jack Bartram³, Philip Ancliff³, Adam J Mead¹, Christina Halsey⁴,⁵, Irene Roberts¹,², Thomas A Milne*¹, Anindita Roy*¹,²,

Affiliations:

¹ MRC Molecular Haematology Unit, MRC Weatherall Institute of Molecular Medicine, NIHR Oxford Biomedical Research Centre Haematology Theme, Radcliffe Department of Medicine, University of Oxford, Oxford, UK

² Department of Paediatrics and NIHR Oxford Biomedical Research Centre Haematology Theme, University of Oxford, Oxford, UK

³ Department of Haematology, Great Ormond Street Hospital for Children, London, UK

⁴ Wolfson Wohl Cancer Research Centre, Institute of Cancer Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom

⁵ Department of Paediatric Haematology, Royal Hospital for Children, Glasgow, UK
* Co-corresponding authors:

Dr Anindita Roy  
University of Oxford Department of Paediatrics  
Level 2, Children's Hospital, John Radcliffe  
Oxford OX3 9DU  
Phone: +44(0)1865 222 419  
Email: anindita.roy@paediatrics.ox.ac.uk

Dr Thomas A Milne  
MRC Molecular Haematology Unit  
MRC Weatherall Institute of Molecular Medicine, John Radcliffe Hospital  
Radcliffe Dept Medicine  
University of Oxford  
Oxford OX3 9DS, UK  
Telephone: +44 1865 222626  
Email: thomas.milne@imm.ox.ac.uk
ABSTRACT

Although 90% of children with acute lymphoblastic leukemia (ALL) are now cured\(^1\), the prognosis of infant-ALL (diagnosis within the first year of life) remains dismal\(^2\). Infant-ALL is usually caused by a single genetic hit that arises in utero: rearrangement of the \textit{MLL/KMT2A} gene (\textit{MLL-\textit{r}}). This is sufficient to give rise to a uniquely aggressive and treatment-refractory leukemia compared to older children with the same \textit{MLL-\textit{r}}\(^3\)-\(^5\). The reasons for disparate outcomes in patients of different ages with identical driver mutations are unknown. This paper addresses the hypothesis that fetal-specific gene expression programs co-operate with MLL-AF4 to initiate and maintain infant-ALL. Using direct comparison of fetal and adult HSC and progenitor transcriptomes we identify fetal-specific gene expression programs in primary human cells. We show that \textit{MLL-AF4}-driven infant-ALL, but not \textit{MLL-AF4} childhood-ALL, displays expression of fetal-specific genes. In a direct test of this observation, we find that CRISPR-Cas9 gene editing of primary human fetal liver cells to produce a \textit{t(4;11)/MLL-AF4} translocation replicates the clinical features of infant-ALL and drives infant-ALL-specific and fetal-specific gene expression programs. These data strongly support the hypothesis that fetal-specific gene expression programs co-operate with MLL-AF4 to initiate and maintain the distinct biology of infant-ALL.

MAIN

In >70\% of infant-ALL cases, the main driver mutation is a chromosomal translocation that leads to rearrangement of the \textit{Mixed Lineage Leukemia (MLL/KMT2A)} gene (\textit{MLL-\textit{r}}\(^2\)-\(^6\)-\(^7\)) producing MLL fusion proteins such as MLL-AF4\(^8\). MLL-AF4 binds directly to gene targets where it aberrantly upregulates gene expression, partly by increasing histone-3-lysine-79 dimethylation (H3K79me2) through DOT1L recruitment\(^9\). The prevalence of \textit{MLL-\textit{r}} in infant ALL contrasts with what is observed in childhood-ALL, where \textit{MLL-\textit{r}} accounts for only 2-5\% of cases\(^3\)-\(^10\). Intriguingly, \textit{MLL-\textit{r}} childhood-ALL has an event-free survival (EFS) of 50-59\%\(^3\)-\(^4\)-\(^11\)
compared to 19-45% in MLL-r infant-ALL \(^4,5\). This inferior outcome for MLL-r infant-ALL does not appear to be due to age-related differences in drug metabolism and/or toxicity since MLL wild-type (MLLwt) infant-ALL has excellent EFS (74-93%) \(^7,12\). This suggests there may be intrinsic biological differences between MLL-r infant-ALL and MLL-r childhood-ALL blasts. In support of this, the MLL breakpoint region tends to differ in MLL-r infant-ALL \(^13\) compared to MLL-r childhood-ALL, and infant-ALL is associated with a high frequency of the poor prognosis HOXA\(^b\)/IRX\(^e\) MLL-r molecular profile \(^14\). However, very little is known about the underlying reasons for these age-related differences.

A characteristic and baffling feature of MLL-r infant-ALL is the fact that this single hit before birth seems to be sufficient to induce a rapidly-proliferating therapy-resistant leukemia without the need for additional mutations \(^15\), unlike many cases of childhood-ALL which also originate in utero but only develop into full-blown leukemia after a second post-natal hit \(^15\). One reason for this could be that the specific fetal progenitors in which the translocation arises provide the permissive cellular context necessary to cooperate with MLL-r to induce infant-ALL \(^16-18\).

To investigate this, we used the most common MLL-r infant-ALL, MLL-AF4, as a disease model \(^8\). Using a previously published patient bulk RNA-seq dataset containing both MLL-AF4 infant-ALL (n=19) and MLL-AF4 childhood-ALL (n=5) samples \(^15\), we found that MLL-AF4 childhood-ALL clustered separately from MLL-AF4 infant-ALL (Fig. 1a). In addition, we observed 2 sub-clusters of MLL-AF4 infant-ALL, representing HOXA\(^b\)/IRX\(^e\) and HOXA\(^b\)/IRX\(^e\) infant-ALL subsets, which have previously been characterized (Fig. 1a, Supplementary Figs. 1a and 1b) \(^14\). Differential gene expression analysis between MLL-AF4 infant-ALL and MLL-AF4 childhood-ALL identified 617 significantly differentially expressed genes (FDR < 0.05), 193 of which were upregulated in MLL-AF4 infant-ALL and therefore represented an infant-ALL-specific gene expression profile (Fig. 1b, Supplementary Fig. 1c, Supplementary Table 1). The two most significantly upregulated genes in MLL-AF4 infant-ALL were HOXB4 and HOXB3 (Fig. 1c, Supplementary Fig. 1d). These HOXB genes were effective as a marker of infant-ALL regardless of the HOXA/IRX status of the infant-ALL patients (Fig. 1d).
We next sought to determine the extent to which normal fetal gene expression programs contribute to the distinct molecular profile of *MLL-AF4* infant-ALL. We compared bulk RNA-seq for sorted human fetal liver (FL) hematopoietic stem and progenitor cell (HSPC) subpopulations previously generated in our lab to a human adult bone marrow (ABM) HSPC RNA-seq dataset. We carried out differential gene expression analysis between comparable subpopulations of FL and ABM HSPCs along the B lineage differentiation pathway (Fig. 1e). The hematopoietic stem cell (HSC) subpopulation shows the greatest number of differentially expressed genes between FL and ABM (3,787 genes), reducing at each subsequent stage of B lineage differentiation (1,509 genes differentially expressed between FL committed B progenitors (CBP) and ABM common lymphoid progenitors (CLP)) (Fig. 1e). A total of 5,709 genes were differentially expressed between FL and ABM in at least one HSPC subpopulation when we combined all differentially expressed gene lists (Fig. 1e, Supplementary Table 2).

We repeated the clustering analysis of the patient dataset based on these 5,709 genes and found that they were capable of separating *MLL-AF4* infant-ALL from *MLL-AF4* childhood-ALL (Fig. 1f, Supplementary Fig. 2a). Comparing differentially expressed genes in both the normal and leukemic setting, we found 72 genes that were significantly upregulated in both normal FL HSPCs and *MLL-AF4* infant-ALL (~40% of all genes upregulated in *MLL-AF4* infant-ALL compared to *MLL-AF4* childhood-ALL) (Supplementary Fig. 2b, Supplementary Table 2), including *IGF2BP1*, a member of the fetal-specific *LIN28B* gene expression pathway, which has previously been reported to positively regulate *HOXB4* expression (Supplementary Fig. 2c). Together, these data suggest that the molecular profile of the human fetal HSPCs that form the target cells for leukemic transformation plays a role in determining the distinct gene expression profile of *MLL-AF4* infant-ALL.

To test the hypothesis that to accurately model *MLL-AF4* infant-ALL, the *MLL-AF4* translocation should be expressed in human fetal HSPCs, we directly induced the most common t(4;11)/*MLL-AF4* translocation in infant-ALL (with the *MLL* breakpoint in intron 11) in 13-15 post-conception week (pcw) human FL CD34+ cells by CRISPR-Cas9 genome
Edited samples (n=3) and biologically-matched mock-edited controls (n=3) were transferred to MS-5 co-cultures to facilitate expansion of successfully edited cells along the B lineage (designated CRISPR-MLL-AF4+).

By week 3 of co-culture, CD19+ B cell numbers were >900-fold higher in CRISPR-MLL-AF4+ cultures compared to controls (p<0.005), suggesting that the translocation had successfully transformed the cells (Fig. 2a). RT-qPCR confirmed expression of both MLL-AF4 and AF4-MLL in CRISPR-MLL-AF4+ cells but not controls (Fig. 2b). Virtually all human cells generated from CRISPR-MLL-AF4+ cultures (Supplementary Fig. 3b) were CD19+ B cells, compared to <20% in control cultures (Fig. 2c and 2d). Although there were fewer residual CD34+ cells in CRISPR-MLL-AF4+ cultures, the majority of these were CD19+ B progenitors, unlike control CD34+ cells, suggesting that MLL-AF4-driven B lineage specification occurs at a progenitor stage (Fig. 2d, right). More detailed immunophenotyping showed that the majority of CRISPR-MLL-AF4+ cells were CD34-CD19+CD10+IgM/IgD- preB cells, of which ~10% aberrantly expressed the leukemia-associated marker CD133, a direct gene target of MLL-AF423 (Figs. 2c and 2d). By week 7 of co-culture, when control cultures no longer produced any detectable human cells, the number of human cells in CRISPR-MLL-AF4+ cultures began to decline (Fig. 2a), suggesting MS-5 stroma may not be optimal for long-term maintenance of FL-derived CRISPR-MLL-AF4+ cells.

To test whether CRISPR-MLL-AF4+ cells could generate leukemia in vivo, human FL CD34+ cells (13 pcw, n=4) were edited as before and transplanted into sub-lethally irradiated NSG mice (CRISPR-MLL-AF4+, n=3; control, n=5). By 12 weeks post-transplant, human CD45+ cells were detected in peripheral blood (PB) (Supplementary Fig. 4a) and RT-qPCR showed that both MLL-AF4 and AF4-MLL fusion transcripts were clearly detectable in human CD45+ cells from CRISPR-MLL-AF4+ mice (Supplementary Fig. 4b). B-ALL rapidly developed in all three CRISPR-MLL-AF4+ mice with median latency of 18 weeks, whereas no control mice (0/5) developed any form of leukemia (Fig. 3a). FISH analysis (Fig 3b) and Sanger sequencing
Supplementary Fig.4c) confirmed the presence of a heterozygous MLL-AF4 translocation in CRISPR MLL-AF4+ cells. The B-ALL in CRISPR MLL-AF4+ mice recapitulated key phenotypic features of infant-ALL, including circulating blasts in the PB (Supplementary Fig. 4d), and blast infiltration into spleen and liver (Fig. 3c, Supplementary Fig. 4e)\textsuperscript{24-26}. CRISPR MLL-AF4+ mice also had central nervous system (CNS) disease, with extensive parameningeal blast cell infiltration (Fig. 3d); a key clinical feature of infant-ALL that has not been previously reported in MLL-AF4 mouse models\textsuperscript{27-29}. Although the clinico-pathological features were the same in all leukemic mice (Supplementary Table 3), 2/3 CRISPR MLL-AF4+ mice had a CD19+CD10-CD20-IgM/IgD-CD34+/− proB ALL immunophenotype (Fig. 3e, Supplementary Fig. 4f), while the remaining mouse had a preB ALL immunophenotype, with majority of the cells being CD19+CD10+CD20-IgM/IgD-CD34+/− (Supplementary Fig. 4f and 4g). Further characterization of CRISPR MLL-AF4+ proB ALL revealed that it recapitulated the immunophenotype of MLL-AF4 infant-ALL, including heterogeneous expression of CD133\textsuperscript{23}, NG2\textsuperscript{30} and CD24 (Fig. 3e, Supplementary Fig. 4h). Sequencing of the IgH locus showed that CRISPR MLL-AF4+ ALL was clonal (Supplementary Table 3).

Secondary (n=4) and tertiary (n=3) recipient mice all developed B-ALL with significantly reduced latency compared to primary recipients (median survival 11.5 weeks in secondary (p<0.02) and 8 weeks in tertiary (p<0.03)) (Fig. 3a). The clinico-pathological and immunophenotypic features of primary CRISPR MLL-AF4+ ALL were maintained in secondary recipients, including CNS disease (Supplementary Table 3). Together these data show that CRISPR-Cas9 induced MLL-AF4 translocation in human FL is sufficient to promote a rapidly progressive, fatal, transplantable B-ALL that recapitulates key features of infant-ALL.

We compared bulk RNA-seq from control and CRISPR MLL-AF4+ bone marrow (Supplementary Fig. 5a) to two independent patient datasets\textsuperscript{15,19} and found that, on a transcriptome-wide level, CRISPR MLL-AF4+ ALL more closely resembled MLL-AF4 ALL patients compared to MLLwt ALL patients (Fig. 4a\textsuperscript{19} and 4b, Supplementary Fig. 5b\textsuperscript{15}). Moreover, CRISPR MLL-AF4+ ALL
resembled \textit{HOXA}^{\text{lo}}/\textit{IRX}^{\text{hi}} \text{MLL-AF4} infant-ALL (Supplementary Fig. 5c). By ChIP-seq, we observed a clear genome-wide correlation between the MLL-AF4 binding profile in \textit{CRISPR} MLL-AF4+ ALL, the MLL-AF4 B-ALL SEM cell line (Supplementary Fig. 5d) and a primary MLL-AF4 ALL patient sample (Supplementary Fig. 5e) and a substantial overlap in MLL-AF4 target genes (2,323 genes bound by MLL-AF4 in all 3 datasets) (Fig. 4c, Supplementary Table 4), with strikingly similar binding profiles, for example at \textit{RUNX1} (Fig 4d).

Finally, we wanted to ask whether inducing an MLL-AF4 translocation in human FL gave rise to a model that specifically recapitulated the molecular profile of MLL-AF4 infant-ALL. The only humanized mouse model of MLL-AF4 ALL that has previously been published introduced a chimeric MLL-Af4 fusion gene into human neonatal (cord blood (CB)) HSPC (hereafter referred to as CB MLL-Af4+ ALL)\textsuperscript{27}. We hypothesized that it may recapitulate MLL-AF4 childhood-ALL, and could be used as a comparison to \textit{CRISPR} MLL-AF4+ ALL.

To examine the fetal and post-natal gene expression programs that are key to determining the age-related differences between MLL-AF4 ALLs, we used the 139 genes up- or downregulated in both FL (compared to ABM) and MLL-AF4 infant-ALL (compared to MLL-AF4 childhood-ALL) (Supplementary Table 2). Clustering analysis based on this core gene list showed that, while \textit{CRISPR} MLL-AF4+ ALL was similar to MLL-AF4 infant-ALL patients, CB MLL-Af4+ ALL clustered away from MLL-AF4 infant-ALL and closer to MLL-AF4 childhood-ALL patients (Fig. 5a). To explore this in more detail, we carried out differential gene expression analysis between \textit{CRISPR} MLL-AF4+ ALL and CB MLL-Af4+ ALL, followed by Gene Set Enrichment Analysis (GSEA). We found that \textit{CRISPR} MLL-AF4+ ALL was significantly enriched for genes upregulated in both FL HSPCs and MLL-AF4 infant-ALL compared to CB MLL-Af4+ ALL (Fig. 5b).

Comparing MLL-AF4 binding at promoters genome-wide in both models, we found that MLL-AF4 in \textit{CRISPR} MLL-AF4+ ALL showed greater enrichment (normalized ChIP-seq reads/bp) at the promoters of infant-ALL- and FL-specific genes compared to MLL-Af4 in CB MLL-Af4+ ALL (Fig. 5c). However, at all other genes, MLL-AF4/MLL-Af4 enrichment was comparable
(Fig. 5c). At iALL- and FL-specific genes IGF2BP1 (Fig. 5d) and HOXB4 (Fig. 5e), we observed an MLL-AF4 peak in CRISPR MLL-AF4+ ALL but not in CB MLL-Af4+ ALL. These data suggest that MLL-AF4 may play an active role in maintaining fetal gene expression programs in infant-ALL. Increased levels of H3K79me2 are a commonly used marker of MLL-AF4 activity\textsuperscript{23,28}. Therefore, using one of the unique features of our model, we carried out H3K79me2 ChIP-seq for the first time in identical primary human FL HSPC before and after leukemic transformation. We observed increased levels of H3K79me2 at MLL-AF4 peaks in FL- and iALL-specific genes such as IGF2BP1 (Fig. 5d) and HOXB4 (Fig. 5e) in CRISPR MLL-AF4+ ALL, further suggesting that MLL-AF4 actively maintains the expression of these fetal-specific genes in MLL-AF4 infant-ALL.

The mechanisms by which the same MLL-r driver mutation could cause more aggressive disease and worse outcomes in infant-ALL compared to childhood-ALL have always been unclear. We hypothesized that there must be intrinsic biological differences between infant-ALL and childhood-ALL blasts, unrelated to the driver mutation, that underlie these age-related differences. Here, we identify the unique molecular profile of MLL-AF4 infant-ALL using primary patient data. Reasoning that this profile drives the distinct phenotype of infant-ALL, we set out to identify factors that could explain it. We find that maintenance of fetal-specific gene expression programs account for a large proportion (~40\%) of the unique molecular profile of MLL-AF4 infant-ALL, suggesting that it is the specific fetal target cell(s) in which it arises that provide the permissive cellular context for aggressive infant-ALL.

Human fetal HSPCs are more proliferative than ABM HSPCs\textsuperscript{32,33}, and they differentiate down distinct developmental pathways\textsuperscript{34,35}, some of which are virtually absent in adult life. Therefore, maintenance of fetal HSPC characteristics provides a possible explanation for the highly-proliferative, therapy-resistant nature of infant-ALL. One of the biggest challenges to understanding the biology of infant-ALL and developing novel, more effective therapies has been the lack of pre-clinical models\textsuperscript{36} that capture the unique characteristics and aggressive nature of the disease. By targeting a t(4;11)/MLL-AF4 translocation to primary human FL
HSPCs, we have created the first bona fide MLL-AF4 infant-ALL model. Our results finally confirm that a human fetal cell context is permissive, and indeed probably required; to give rise to an ALL that recapitulates key phenotypic and molecular features of poor prognosis MLL-AF4 infant-ALL.

\textsuperscript{CRISPR}MLL-AF4+ mice represent a previously lacking model in which the function of MLL-AF4 can be investigated in the appropriate human fetal cell context. Moreover, because \textsuperscript{CRISPR}MLL-AF4+ cells were generated by CRISPR-Cas9 genome editing, they express both MLL-AF4 and the reciprocal AF4-MLL at physiological levels. Therefore, \textsuperscript{CRISPR}MLL-AF4+ ALL also provides an opportunity to explore the contribution of the reciprocal fusion protein during leukemogenesis, which has been a topic of debate in the MLL-r ALL field\textsuperscript{37,38}. Finally, the infant-ALL-like features of \textsuperscript{CRISPR}MLL-AF4+ ALL make this an important model for future preclinical testing of novel therapies. To our knowledge, we are the first to report CNS disease in an MLL-AF4 mouse model, which is a common clinical feature of infant-ALL that can lead to CNS relapse\textsuperscript{4}. Therefore, the ability of novel treatments to eradicate blasts from the CNS is an important consideration, and this can now be tested in \textsuperscript{CRISPR}MLL-AF4+ ALL.
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AUTHOR CONTRIBUTIONS

S.R., I.R., A.R., and T.A.M. conceived the experimental design; S.R., N.T.C., N.F., T.J., C.H., D.L., and S.O.B. carried out experiments; S.R., N.T.C, T.J., C.H., and A.R. analyzed and curated the data; S.R., C.H., I.R., A.R., and T.A.M. interpreted the data; S.R., I.R., A.R. and T.A.M. wrote the original manuscript; S.R., N.T.C., T.J., S.I., J.B., A.J.M., C.H., I.R., A.R., T.A.M. contributed to reviewing and editing the manuscript. I.R., A.R., and T.A.M. provided supervision and funding.

DECLARATION OF INTERESTS

T.A.M. is a founding shareholder of OxStem Oncology (OSO), a subsidiary company of OxStem Ltd. The other authors declare no conflicts of interest.
ONLINE METHODS

Fetal Samples

Donated fetal tissue was provided by the Human Developmental Biology Resource (HDBR, [www.hdbr.org](http://www.hdbr.org)), regulated by the UK Human Tissue Authority (HTA, [www.hta.gov.uk](http://www.hta.gov.uk)) and covered under ethics (REC: 18/NE/0290 and 18/LO/0822). FL samples used for CRISPR/Cas9 MLL-AF4 translocation experiments underwent CD34 magnetic bead selection at the time of sample processing and were cryopreserved for future use as described previously39. MLL-AF4 ALL patient samples were obtained from Blood Cancer UK Childhood Leukaemia Cell Bank, UK (REC: 16/SW/0219). Patient samples were anonymized at source, assigned a unique study number and linked.

Animals

All experiments were performed under a project license approved by the UK Home Office under the Animal (Scientific Procedures) Act 1986 and in accordance with the principles of 3Rs (replacement, reduction and refinement) in animal research.

CRISPR-Cas9 MLL-AF4 translocation

CRISPR-Cas9 genome editing was carried out using a previously described protocol40. MLL and AF4 sgRNAs (Synthego) were first tested for editing efficiency individually in FL CD34+ cells. Cryopreserved CD34+ cells from a single primary human FL sample were thawed and placed into suspension culture at a density of 2.5x10^5 cells/ml in StemLine II (Sigma) supplemented with SCF (100ng/ml), FLT3L (100ng/ml) and TPO (100ng/ml) (Peprotech) for 12 hours. Cells were harvested and electroporated with either (i) Cas9 protein (IDT) only or (ii) a Cas9/sgRNA RNP using a Neon™ Transfection System (Thermo Fisher). Electroporated cells were placed into fresh suspension culture media to recover overnight. Cells were harvested and bulk genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen). A ~1kb region of DNA around the target cut site was amplified by PCR and Sanger sequenced (Eurofins). Sanger sequencing traces from samples edited with RNPs were
compared to traces from Cas9 only controls using the ICE Analysis online tool (Synthego, https://ice.synthego.com). Editing efficiency is reported as the percentage of indels detected (Supplementary Fig. 3a).

For each CRISPR-Cas9 MLL-AF4 translocation experiment, cryopreserved CD34+ cells from a single 13-15 pcw primary human FL underwent suspension culture as described. Cells were harvested and electroporated with either (i) Cas9 protein (IDT) only, (ii) Cas9 protein plus MLL-sgRNA only, as biologically matched controls or (iii) a 1:1 mix of Cas9/MLL-sgRNA and Cas9/AF4-sgRNA RNPs using a Neon™ Transfection System (Thermo Fisher). Electroporated cells were placed into fresh suspension culture media to recover overnight before subsequent in vitro culture and in vivo transplantation experiments.

**MS-5 stroma co-culture**

Electroporated FL CD34+ cells (CRISPR MLL-AF4+ and control) were plated onto a confluent layer of MS-5 stromal cells in a 24-well plate at a density of 2,000 cells/well in αMEM (Gibco) supplemented with 10% heat-inactivated batch-tested FBS, 100U/ml Penicillin, 100µg/ml Streptomycin, 2mM L-glutamine, 50µM 2-Mercaptoethanol, 10mM HEPES, SCF (20ng/ml), FLT3L (10ng/ml), IL-2 (10ng/ml) and IL-7 (5ng/ml). Cultures were maintained as previously described\(^{35,39}\). Cells were harvested for flow cytometry analysis once a week beginning at week 2 of culture. MLL-AF4 and AF4-MLL RT-qPCRs were carried out on week 4 of culture.

**Xenograft transplantation**

8-12 week old female NSG mice were sub-lethally irradiated with two doses of 1.25Gy six hours apart (2.5Gy total) and injected via the tail vein with 25,000-35,000 edited FL cells (CRISPR MLL-AF4+, n=3; Cas9 control, n=5; or Cas9 plus MLL-sgRNA control, n=1) plus 30,000 wild-type, unedited, sex-mismatched FL CD34+ carrier cells. Engraftment was monitored by peripheral blood sampling every 3 weeks. Human CD45+ cells were sorted from peripheral blood samples to carry out MLL-AF4 and AF4-MLL RT-qPCR for the detection of successfully edited cells. Animals were monitored regularly using a standardized physical scoring system,
and any mouse found to be in distress was humanely killed. Mice were considered leukemic if they met at least 3 of the following criteria: (i) overt signs of disease (hunching, lack of movement, weight loss, paralysis), (ii) splenomegaly, (iii) PB blast count over 50%, (iv) peripheral organ infiltration, (v) detection of the *MLL-AF4* translocation in both BM and spleen.

**Flow cytometry**

Cells were stained with fluorophore-conjugated monoclonal antibodies in PBS with 2% FBS and 1mM EDTA for 30 minutes and analyzed using BD LSR II or Fortessa X50 instruments. Antibodies used are detailed in Supplementary Table 5. Analysis was performed using FlowJo software where gates were set using unstained and fluorescence minus one controls.

**Histopathology**

On termination, samples of ~0.5-1cm² were taken from the spleen and liver of *CRISPR MLL-AF4*+ and Cas9 control mice and fixed in 10% formaldehyde. After fixation, tissues were processed and paraffin embedded. 4µm paraffin sections were cut onto Superfrost Plus adhesive slides, VWR, Cat No 406/0179/00. Haematoxylin and Eosin (H&E) was performed using the Vector Laboratories H&E kit, Cat No 3502, as per their recommended protocol and mounted using Vectamount, Vector Laboratories, Cat No H5000-60.

Murine heads were decalcified and processed as previously described⁴¹. Following paraffin wax embedding, 2.5µm sections were cut onto Poly-L-silane coated slides and stained with Gill’s haematoxylin and Putt’s eosin (both made in house). Slides were imaged on a NanoZoomer Digital Pathology (NDP) slide scanner (Hamamatsu) and analyzed with NDP.view 2 software.

**RT-qPCR**

Total RNA was extracted from cells using an RNeasy Micro Kit (Qiagen). cDNA was generated from polyA mRNA using a SuperScript III kit (Invitrogen). qPCR was carried out on cDNA
using SYBRGreen master mix (Thermo Fisher) and a QuantStudio3 Real-Time PCR System (Thermo Fisher). For list of qPCR primers used see Supplementary Table 5.

**RNA-sequencing**

Approximately $3 \times 10^5$ CD45+CD19+ cells were sorted from the bone marrow of 3 primary Crispr 

MLL-AF4$^+$ recipient mice and 3 control primary recipient mice (Cas9 control, n=2; Cas9 plus MLL-sgRNA, n=1). Total RNA was extracted using an RNeasy Mini Kit (Qiagen). Poly(A) purification was conducted using the NEB Poly(A) mRNA magnetic isolation module as per the manufacturer’s protocol. Library preparation was carried out using the Ultra II Directional RNA Library Prep Kit (NEB, E7765). RNA libraries were sequenced by paired-end sequencing using a 150 cycle high output kit on a Nextseq 500 (Illumina). RNA-seq protocols for sorted subpopulations of FL HSPC have been previously described in $^{19}$.

**IgH rearrangement analysis**

Samples were screened for IgH complete (VH-DH-JH) and IgH incomplete (DH-JH) rearrangements using BIOMED-2 protocols to detect clonality. DNA was extracted from cells from the bone marrow of 3 primary Crispr 

MLL-AF4$^+$ recipient mice. IgH rearrangements were analyzed as described in $^{35}$.

**ChIP-sequencing**

The full protocol is described in $^{31}$. In short, up to $5 \times 10^7$ cells were sonicated (Covaris) following the manufacturer's protocol and incubated with antibody overnight. Magnetic protein A and G beads (ThermoFisher Scientific) were used to isolate antibody-chromatin complexes. Antibodies used are detailed in Supplementary Table 5. Beads were washed three times using a solution of 50mM HEPES-KOH (pH7.6), 500mM LiCl, 1mM EDTA, 1% NP40 and 0.7% sodium deoxycholate and once with Tris-EDTA. Samples were eluted and Proteinase K/RNase A-treated. Samples were purified using a ChIP Clean and Concentrator kit (Zymo). DNA libraries were generated using the NEBnext Ultra DNA library preparation kit for Illumina
Libraries were sequenced by paired-end sequencing using a 75 cycle high output kit on a Nextseq 500 (Illumina).

**NGS analysis**

For RNA-seq, following sequencing, QC analysis was conducted using the fastQC package (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Reads were mapped to the human genome assembly using STAR. The featureCounts function from the Subread package was used to quantify gene expression levels using standard parameters. This was used to identify differential gene expression globally using the edgeR package. Differential gene expression was defined by an adjusted p-value (FDR) of less than 0.05. Infant ALL RNA-seq datasets were analyzed as described previously.

To derive a FL vs ABM gene signature, bulk RNA-seq for sorted subpopulations of FL HSPC were compared to matched sorted subpopulations of ABM HSPC (FL HSC vs adult BM HSC, FL MPP vs adult BM MPP, FL LMPP vs adult BM LMPP and FL committed B progenitors (CBP) vs adult BM CLP). Genes that were differentially expressed between FL and ABM in at least one matched HSPC subpopulation were included in the gene signature. Genes that showed a significant change in opposite directions in different HSPC subtypes (e.g. upregulated in FL HSC vs ABM HSC, but downregulated in FL LMPP vs ABM LMPP) or in the normal vs leukemic setting (e.g. upregulated in FL HSPC vs ABM HSPC, but downregulated in MLL-AF4 infant-ALL vs MLL-AF4 childhood-ALL) were filtered out of the gene signature to leave a total of 5,709 genes (Supplementary Table 2).

For ChIP-seq, quality control of FASTQ reads, alignment, PCR duplicate filtering, blacklisted region filtering and UCSC data hub generation was performed using an in-house pipeline (https://www.biorxiv.org/content/10.1101/393413v1) as described. The HOMER tool makeBigWig.pl command was used to generate bigwig files for visualization in UCSC, normalizing tag counts to tags per $1 \times 10^7$. ChIP-seq peaks were called using the HOMER tool.
findPeaks.pl with ChIP input sample used to estimate background signal. Gene profiles were generated using the HOMER tool annotatePeaks.pl.

Statistics

Two-tailed Mann-Whitney, Log-rank (Mantel-Cox) tests and ANOVA followed by multiple comparisons testing were used to compare experimental groups as indicated in the figure legends. Statistical analyses were performed using GraphPad Prism v7.00 or R v4.0.1. Data are expressed as mean ± SEM unless otherwise indicated.

Data availability

Further information and requests for resources and reagents may be directed to and will be fulfilled by the corresponding authors, Dr Anindita Roy (anindita.roy@paediatrics.ox.ac.uk) and Dr Thomas A Milne (thomas.milne@imm.ox.ac.uk).

The accession number for the RNA-seq and ChIP-seq data generated during this study is NCBI GEO: XXXXX

Code availability

ChIP-seq data were analyzed using an in-house pipeline (https://www.biorxiv.org/content/10.1101/393413v1).
**FIGURE LEGENDS**

**Fig. 1** Fetal gene expression programs drive the distinct molecular profile of *MLL-AF4* infant-ALL (iALL)

a. UMAP showing clustering of *MLL-AF4* infant-ALL (iALL (green), n=19) and *MLL-AF4* childhood-ALL (chALL (orange), n=5) from a previously published patient dataset based on the 500 most variable genes.

b. Heatmap showing clustering of *MLL-AF4* infant-ALL (iALL (green), n=19) and *MLL-AF4* childhood-ALL (chALL (orange), n=5) based on 617 significantly differentially expressed genes (FDR<0.05, Supplementary Table 1). Color scale = log2 counts per million (logCPM).

c. Barplot showing significance (-log10(FDR)) for the 10 most significantly upregulated genes in *MLL-AF4* infant-ALL.

d. Expression of *HOXB3, HOXB4, HOXA9* and *IRX1* in *MLL-AF4* infant-ALL (light green = *HOXA/b/IRX/a* infant-ALL (iALL), n=11; dark green = *HOXA/h/IRX/o* infant-ALL (iALL), n=8 (see Supplementary Fig. 1a and 1b)) and *MLL-AF4* childhood-ALL (*HOXA/h/IRX/o* chALL, orange, n=5). Individual values are given as log2 transcripts per million (TPM). Data shown as mean ± SEM.

e. (left) Schematic representation of differential gene expression analysis between FL and ABM. Equivalent HSPC subpopulations were compared and significantly differentially expressed genes (FDR<0.05) for all 4 comparisons were combined into a master list of genes that were differentially expressed in at least 1 HSPC subpopulation (HSC = hematopoietic stem cell, MPP = multipotent progenitor cell, LMPP = lymphoid-primed multipotent progenitor cell, CBP = committed B progenitor, CLP = common lymphoid progenitor). (right) Venn diagram showing overlap of differentially expressed genes for each HSPC subpopulation (see Supplementary Table 2).

f. UMAP showing clustering of *MLL-AF4* infant-ALL (iALL (green), n=19) and *MLL-AF4* childhood-ALL (chALL (orange), n=5) from a previously published patient dataset.
based on 5,709 genes differentially expressed between FL HSPCs and ABM HSPCs (see Supplementary Table 2).

**Fig. 2** A CRISPR-Cas9-induced t(4;11) MLL-AF4 translocation in human FL HSPCs causes a dramatic increase in B cell proliferation *in vitro*

- **a.** Cumulative absolute number of human CD45+CD19+ cells per well over time during CRISPR MLL-AF4+ and control MS-5 co-culture (n=3). *** =p<0.005 (Two-way ANOVA with Sidak correction for multiple comparisons). Data shown as mean ± SEM.
- **b.** RT-qPCR of human CD45+ cells showing expression of MLL-AF4 (n=3) and AF4-MLL (n=2) relative to GAPDH at week 4 of MS-5 co-culture. Data shown as mean ± SEM.
- **c.** Representative flow cytometry plots of viable, single cells from control and CRISPR MLL-AF4+ cultures on week 4 of co-culture. Custom lineage cocktail (Lin) = CD2/CD3/CD14/CD16/CD56/CD235a (see Supplementary Table 5).
- **d.** Quantification of human cell immunophenotypes as a percentage of human CD45+ (hCD45+) cells (left), and progenitor immunophenotypes as a percentage of hCD45+Lin-CD34+ cells (right), in control (n=2-3) and CRISPR MLL-AF4+ (n=2-3) cultures over time. X = data not shown as total number of hCD45+ cells < 50. Data shown as mean ± SEM.

**Fig 3.** CRISPR MLL-AF4+ cells give rise to a B-ALL *in vivo* that recapitulates many key features of iALL

- **a.** Leukemia-free survival for primary (CRISPR MLL-AF4+ n=3; control n=5), secondary (CRISPR MLL-AF4+ n=4; control n=1) and tertiary (CRISPR MLL-AF4+ n=3; control n=2) recipient mice. Mice culled with no signs of leukemia (see Online Methods) are censored (shown as tick above line). Latency significantly reduced for secondary (p<0.02) and tertiary (p<0.03) CRISPR MLL-AF4+ compared to primary CRISPR MLL-AF4+ (Log-Rank (Mantel-Cox) test).
b. Dual FISH (MLL probe = green; AF4 probe = red) showing heterozygous chromosomal translocation in a CRISPR MLL-AF4+ cell isolated from the spleen of a primary recipient mouse. Representative image of 200 cells analyzed.

c. Representative H&E staining of spleen and liver from control and CRISPR MLL-AF4+ primary recipient mice. Scale bar = 50µm.

d. Representative H&E staining of control and CRISPR MLL-AF4+ primary recipient mouse heads (scale bar = 2.5mm; red arrows = regions of concentrated blast cell infiltration). High magnification images (scale bar = 500µm) highlight striking parameningeal blast cell infiltration in CRISPR MLL-AF4+ mice (red arrow) but not control.

e. Representative flow cytometry plots of viable, single cells in control and CRISPR MLL-AF4+ BM at termination (week 17 and 18 respectively).

Fig. 4 CRISPR MLL-AF4+ ALL recapitulates the molecular profile of MLL-AF4 ALL in patients

a. UMAP showing clustering of CD19+ cells from CRISPR MLL-AF4+ and control mice with MLL-AF4 (dark green) and MLLwt (blue) infant-ALL patient samples from a publicly available dataset 19 based on all differentially expressed genes (7,041 genes) between these 4 groups (edgeR generalized linear model (GLM), FDR < 0.05).

b. Gene set enrichment analysis (GSEA) showing CRISPR MLL-AF4+ ALL is more enriched for genes that are upregulated in MLL-AF4 ALL compared to MLLwt ALL (1000 genes) when compared to CD19+ cells from control mice (p<0.01).

c. Venn diagram showing overlap of MLL-AF4-bound genes (genes with an MLL-AF4 peak in the gene body) in CRISPR MLL-AF4+ ALL in a primary recipient mouse, the MLL-AF4+ SEM cell line and a primary MLL-AF4 childhood-ALL (chALL) patient sample. MLL-AF4 peaks = directly overlapping MLL-N and AF4-C peaks.

d. Representative ChIP-seq tracks at the MLL-AF4 target gene, RUNX1 in CRISPR MLL-AF4+ ALL, the SEM cell line and a primary MLL-AF4 childhood-ALL (chALL) patient sample.
Fig. 5 FL-derived CRISPR MLL-AF4+ ALL specifically recapitulates the molecular profile of MLL-AF4 infant-ALL (iALL)

a. UMAP showing clustering of CRISPR MLL-AF4+ ALL with MLL-AF4 infant-ALL and away from MLL-AF4 childhood-ALL (chALL) from a publicly available dataset\(^\text{15}\) and the CB MLL-AF4+ ALL mouse model\(^\text{27}\) based on a set of genes differentially expressed in both FL (compared to ABM) and MLL-AF4 infant-ALL (compared to MLL-AF4 childhood-ALL) (139 genes, see Supplementary Table 2).

b. Gene set enrichment analyses (GSEA) showing CRISPR MLL-AF4+ ALL is significantly more enriched for genes that are upregulated in MLL-AF4 infant-ALL (compared to MLL-AF4 childhood-ALL) (617 genes, \(p<0.03\)) and in FL (compared to ABM) (5,709 genes, \(p<0.001\)) when compared to CB MLL-AF4+ ALL.

c. Bar plots showing MLL-AF4 enrichment (MLL-N ChIP-seq reads/bp normalized to \(10^7\) total reads) is greater in CRISPR MLL-AF4+ ALL than in CB MLL-AF4+ ALL (FLAG ChIP-seq reads/bp normalized to \(10^7\) total reads) at iALL-specific genes (193 genes, Mann-Whitney test, \(p<0.01\)) and FL-specific genes (3,949 genes, Mann-Whitney test, \(p<0.0001\)). Data shown as mean ± SEM.

d. (left) Barplot showing expression of IGF2BP1 in MLL-AF4 childhood-ALL (chALL)\(^\text{15}\), CB MLL-AF4+ ALL\(^\text{27}\), MLL-AF4 infant-ALL (iALL)\(^\text{15}\) and CRISPR MLL-AF4+ ALL. Data shown as mean ± SEM. (right) ChIP-seq tracks for MLL-AF4 (FLAG ChIP-seq) in CB MLL-AF4+ ALL (yellow), MLL-N and AF4-C ChIP-seq in CRISPR MLL-AF4+ ALL and H3K79me2 ChIP-seq in CRISPR MLL-AF4+ ALL and the biologically-matched unedited FL samples from which CRISPR MLL-AF4+ ALL was derived, at IGF2BP1. ChIP-seq data shown is normalized to \(10^7\) total reads.

e. (left) Barplot showing expression of HOXB4 in MLL-AF4 childhood-ALL (chALL)\(^\text{15}\), CB MLL-AF4+ ALL\(^\text{27}\), MLL-AF4 infant-ALL (iALL)\(^\text{15}\) and CRISPR MLL-AF4+ ALL. Data shown as mean ± SEM. (right) ChIP-seq tracks for MLL-AF4 (FLAG ChIP-seq) in CB MLL-AF4+ ALL (yellow), MLL-N and AF4-C ChIP-seq in CRISPR MLL-AF4+ ALL and
H3K79me2 ChIP-seq in CRISPR MLL-AF4+ ALL and the biologically-matched unedited FL samples from which CRISPR MLL-AF4+ ALL was derived, at HOXB3/HOXB4. ChIP-seq data shown is normalized to $10^7$ total reads.
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Fig. 1

a

![UMAP scatterplot](UMAP1 vs UMAP2)

- MLL-AF4 iALL
- MLL-AF4 chALL

b

Heatmap showing logCPM values for different gene sets across different cell types.

- MLL-AF4 iALL
- MLL-AF4 chALL

Gene expression analysis:

- **HOXB4**
- **HOXB3**
- **GNG11**
- **IL27RA**
- **VANGL1**
- **SARDH**
- **ST14**
- **RPL22**
- **IL13RA1**
- **HMGA2**

Gene expression levels are plotted against -log10(FDR).

d

- **HOXB3**
- **HOXB4**
- **HOXA9**
- **IRX1**

Expression levels are shown for different cell types.

- MLL-AF4 iALL
- MLL-AF4 chALL

Gene expression analysis:

- **HOXA9**
- **IRX1**

Gene expression levels are plotted against log2 TPM.

e

- **HSC**
- **MPP**
- **LMPP**
- **CBP**
- **CLP**
- **FL**
- **ABM**

Diagram showing cell type relationships.

- HSC (3,787 genes)
- MPP (2,584 genes)
- LMPP (1,844 genes)
- CBP (1,509 genes)

f

UMAP plot showing distribution of MLL-AF4 iALL and MLL-AF4 chALL.

- MLL-AF4 iALL
- MLL-AF4 chALL
Fig. 2

(a) Absolute number of CD19+ cells over time.

(b) Expression relative to GAPDH for MLL-AF4 and AF4-MLL.

(c) Flow cytometry analysis for Control and CRISPRMLL-AF4+ samples.

(d) Percentage of human CD45+ cells and Lin-CD34+ cells over time.
Fig. 3

(a) Graph showing survival rates over weeks for different groups:
- Control primary (n=5)
- CRISPRMLL-AF4+ primary (n=3)
- Control secondary (n=1)
- CRISPRMLL-AF4+ secondary (n=4)
- Control tertiary (n=2)
- CRISPRMLL-AF4+ tertiary (n=3)

(b) Micrograph image of MLL AF4

(c) Micrographs of control and MLL AF4+ tissues:
- Control spleen
- MLL AF4+ spleen
- Control liver
- MLL AF4+ liver

(d) Micrographs of control and MLL AF4+ CNS tissues:
- Control CNS
- MLL AF4+ CNS

(e) Flow cytometry plots for control and MLL AF4+ samples:
- hCD45-AF700
- Lin-PerCP-Cy5.5
- CD34-PE-Cy7
- CD38-BV605
- CD19-APC
- CD10-FITC
- CD133-PE
- CD20-e450

- Control
- CRISPRMLL-AF4+
Fig. 4

(a) UMAP 1 vs UMAP 2 for different cell types: Control, CRISPRMLL-AF4+, MLL-AF4 iALL, and MLLwt iALL.

(b) Enrichment plot for MLL-AF4 vs MLLwt ALL showing a p < 0.01 significance level.

(c) Overlapping circles showing different cell types: SEM cells, MLL-AF4 chALL patient, and CRISPRMLL-AF4+.

(d) Enrichment plots for specific regions of RUNX1 with different cell types.
Supplementary Fig. 1

a. Barplot showing expression of HOXA9 in MLL-AF4 infant-ALL (iALL, n=19) and MLL-AF4 childhood-ALL (chALL, n=5) from a previously published patient dataset\(^1\). Values are shown as transcripts per million (TPM). Data shown as mean ± SEM. Patients were considered to have a \(\text{HOXA}^{\text{lo}}/\text{IRX}^{\text{hi}}\) molecular profile when they showed a HOXA9 expression < 20 TPM. (light green = \(\text{HOXA}^{\text{lo}}/\text{IRX}^{\text{hi}}\) infant-ALL (iALL), n=11; dark green = \(\text{HOXA}^{\text{hi}}/\text{IRX}^{\text{lo}}\) infant-ALL (iALL), n=8; orange = \(\text{HOXA}^{\text{hi}}/\text{IRX}^{\text{lo}}\) childhood-ALL (chALL)). No childhood-ALL patients in this dataset showed a \(\text{HOXA}^{\text{lo}}/\text{IRX}^{\text{hi}}\) molecular profile.

b. UMAP showing clustering of MLL-AF4 infant-ALL (\(\text{HOXA}^{\text{lo}}/\text{IRX}^{\text{hi}}\) iALL = light green, n=11; \(\text{HOXA}^{\text{hi}}/\text{IRX}^{\text{lo}}\) iALL = dark green, n=8) and MLL-AF4 childhood-ALL (chALL = orange, n=5) from a previously published patient dataset\(^1\) based on the 500 most variable genes.

c. Volcano plot showing all differentially expressed genes between MLL-AF4 infant-ALL and MLL-AF4 childhood ALL (dark green = significantly upregulated in MLL-AF4 infant-ALL (FDR,0.05, logFC>0); orange = significantly upregulated in MLL-AF4 childhood-ALL (FDR<0.05, logFC<0), gray = not significantly differentially expressed (FDR>0.05)). A selection of the most differentially expressed genes are labelled.

d. Barplot showing the genes with the greatest logFC in MLL-AF4 infant-ALL (green; top 20) and MLL-AF4 childhood-ALL (orange; top 10).

Supplementary Fig. 2

a. Heatmap showing clustering of MLL-AF4 infant-ALL (iALL (green), n=19) and MLL-AF4 childhood-ALL (chALL (orange), n=5) based on 5,709 significantly differentially expressed genes between FL and ABM HSPCs (FDR<0.05, Supplementary Table 2). Color scale = log2 counts per million (logCPM)

b. Pie charts showing proportion of genes upregulated in MLL-AF4 infant-ALL (compared to MLL-AF4 childhood ALL; dark green) that are also upregulated in FL (compared to ABM; light green), and the proportion of genes downregulated in MLL-AF4 infant-ALL
(compared to MLL-AF4 childhood ALL; orange) that are also downregulated in FL (compared to ABM; yellow) (see Supplementary Table 2). Values shown as number of genes.

c. Barplots showing expression of LIN28B, IGF2BP1 and HOXB4 in FL and ABM HSPC subpopulations (HSC = hematopoietic stem cell, MPP = multipotent progenitor cell, LMPP = lymphoid-primed multipotent progenitor cell, CBP = committed B progenitor, CLP = common lymphoid progenitor), as well as MLL-AF4 infant-ALL (iALL) and MLL-AF4 childhood ALL (chALL). Data shown as mean ± SEM.

Supplementary Fig. 3

a. Synthego ICE Analysis (https://ice.synthego.com/) results for individual sgRNA efficiency tests for MLL-sgRNA and AF4-sgRNA in FL CD34+ cells. (left) Sanger sequencing tracks for edited cells (top) and unedited controls (bottom) around the PAM site. (right) Quantification of indels in edited cells. MLL-sgRNA and AF4-sgRNA both showed an editing efficiency of 77%.

b. Cumulative absolute number of human CD45+ cells per well over time during MS-5 co-culture assay of CRISPR MLL-AF4+ and control CD34+ cells (n=3). * =p<0.02 (Two-way ANOVA with Sidak correction for multiple comparisons). Data shown as mean ± SEM.

Supplementary Fig. 4

a. PB engraftment of human CD45+ (hCD45+) cells over time in primary CRISPR MLL-AF4+ (n=3) and control (n=5) recipient mice. Quantified as a percentage of all CD45+ cells (mouse CD45.1+ and human CD45+). Data shown as mean ± SEM.

b. RT-qPCR showing expression of MLL-AF4 (n=2) and AF4-MLL (n=2) relative to GAPDH in human CD45+ cells isolated from PB at week 12 post-engraftment.

c. Sanger sequencing tracks showing MLL-AF4 and AF4-MLL genomic DNA breakpoints in blasts isolated from the spleen of CRISPR MLL-AF4+ mice. Breakpoint regions were
amplified by PCR before Sanger sequencing in order to examine the translocated allele without contamination by the remaining WT allele. MLL and AF4 portions are labelled below each track.

d. Representative H&E-stained peripheral blood (PB) films for control (left) and CRISPR MLL-AF4+ (right) primary recipient mice. Low magnification images (scale bar = 50µm) show multi-lineage cells in the PB in controls and predominantly circulating blast cells in CRISPR MLL-AF4+ mice. High magnification image (scale bar = 10µm) shows a representative blast cell from CRISPR MLL-AF4+ PB.

e. Representative images of the spleens of control and CRISPR MLL-AF4+ mice.

f. Representative flow cytometry plots of viable, single cells in proB CRISPR MLL-AF4+ (top) and preB CRISPR MLL-AF4+ (bottom) BM at termination. (mCD45.1, mouse CD45; hCD45, human CD45).

g. Representative flow cytometry plots of viable, single cells in control and CRISPR MLL-AF4+ BM at termination (week 17).

h. Representative flow cytometry plots of CD19+ blasts from CRISPR MLL-AF4+ BM at termination (week 18) (left) and an MLL-AF4 iALL patient BM (right). Datapoints are colored in all plots based on surface NG2 and CD24 expression.

Supplementary Fig. 5

a. Heatmap showing significantly differentially expressed (FDR < 0.05) genes between primary control and CRISPR MLL-AF4+ mice. A selection of genes known to be upregulated in MLL-AF4 ALL are labelled.

b. UMAP showing clustering of CRISPR MLL-AF4+ (light green) and control (gray) mice with MLL-AF4 (dark green) and MLLwt (blue) ALL patients from a publicly available dataset\(^1\) based on all differentially expressed genes (5,785 genes) between these 4 groups (edgeR generalized linear model (GLM)).
c. Barplot showing expression of HOXA9 and IRX1 for HOXA\textsuperscript{hi}/IRX\textsuperscript{lo} infant-ALL (iALL), HOXA\textsuperscript{lo}/IRX\textsuperscript{hi} infant-ALL (iALL) and CRISPR\textsuperscript{MLL-AF4+} ALL (designated HOXA\textsuperscript{lo}/IRX\textsuperscript{hi} based on HOXA9 TPM <20). Data shown as mean ± SEM.

d. Heatmap showing MLL-N ChIP-seq enrichment for a 6kb region centered on the promoter (transcriptional start site (TSS)) of all genes in CRISPR\textsuperscript{MLL-AF4+} ALL, the SEM cell line and a primary MLL-AF4 childhood-ALL (chALL) patient sample, sorted by MLL-N ChIP-seq signal in CRISPR\textsuperscript{MLL-AF4+} ALL. Scale = reads/bp/10\textsuperscript{7} total reads.

REFERENCES

1 Andersson, A. K. et al. The landscape of somatic mutations in infant MLL-rearranged acute lymphoblastic leukemias. Nat Genet 47, 330-337, doi:10.1038/ng.3230 (2015).
Supplementary Fig. 1

(a) HOXA9 Expression

(b) UMAP

(c) -log10 FDR

(d) logFC
Supplementary Fig. 3

a

Edited Sample 43 to 108 bp

Control Sample 43 to 108 bp

% of indel mixture

Edited Sample 113 to 178 bp

Control Sample 113 to 178 bp

% of indel mixture

b

Absolute number of hCD45+ cells

- CRISPR MLL-AF4
- Control

Week 0 Week 2 Week 3 Week 4 Week 5 Week 6 Week 7

*
Supplementary Fig. 4

(a) Graph showing the percentage of hCD45+ cells (PB) over time for Control primary (n=5) and CRISPR MLL-AF4+ primary (n=3).

(b) Bar charts showing expression relative to GAPDH for MLL-AF4 and AF4-MLL.

(c) Graph showing mRNA expression in Control and CRISPR MLL-AF4+.

(d) Images of cells stained with anti-CD10, anti-CD20, and anti-CD19 antibodies.

(e) Images showing the number of CD19+ and CD10+ cells.

(f) Flow cytometry plots for CRISPR MLL-AF4+ proB ALL and preB ALL.

(g) Flow cytometry plots for CD19+, CD10+, and IgM/IgD+ states.

(h) Flow cytometry plots for CRISPR MLL-AF4+ proB ALL and MLL-AF4 iALL patient CD19+ cells.
