The ITIM-containing receptor LAIR1 is essential for acute myeloid leukaemia development

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Conventional strategies are not particularly successful in the treatment of leukaemia, and identification of signalling pathways crucial to the activity of leukaemia stem cells will provide targets for the development of new therapies. Here we report that certain receptors containing the immunoreceptor tyrosine-based inhibition motif (ITIM) are crucial for the development of acute myeloid leukaemia (AML). Inhibition of expression of the ITIM-containing receptor LAIR1 does not affect normal haematopoiesis but abolishes leukaemia development. LAIR1 induces activation of SHP-1, which acts as a phosphatase-independent signalling adaptor to recruit CAMK1 for activation of downstream CREB in AML cells. The LAIR1–SHP-1–CAMK1–CREB pathway sustains the survival and self-renewal of AML stem cells. Intervention in the signalling initiated by ITIM-containing receptors such as LAIR1 may result in successful treatment of AML.

Leukaemias are malignant blood diseases characterized by uncontrolled overproduction of haematopoietic progenitors or terminally differentiated leukocytes. AML is the most common adult acute leukaemia. Acute lymphoblastic leukaemia (ALL) is the most common malignancy in children and is also diagnosed in adults. Current chemotherapies are not particularly successful in treating AML and some ALL. For example, despite continuous treatment, most AML patients relapse within 5 years. It has been suggested that leukaemia stem cells, a small population of stem-like cancer cells that have the capacity for indefinite self-renewal, are responsible for initiation and relapse. To effectively inhibit the activity of leukaemia stem cells and treat acute leukaemia, new molecular targets and therapeutic approaches need to be identified.

It is hypothesized that leukaemia stem cells reside in a bone marrow (BM) microenvironment or niche and play an important role in the regulation of initiation, differentiation, migration and chemoresistance of leukaemia. In addition, systematic inflammatory and oxidative factors are critical extrinsic factors for leukaemia development. Specific surface receptors on leukaemia cells presumably interact with the extrinsic environment and regulate the fates of leukaemia cells through unique signalling pathways. These include tyrosine kinase receptors, cytokine receptors, chemokine receptors, adhesion molecules and integrins (such as CD44, CD49d, integrin beta 3, CD47, CD96, CD33; refs 11–16), Notch, Wnt receptors, Smoothened receptors for TGF-beta family, and other surface molecules. Some of these receptors mediate signalling that differs in leukaemia cells from that in normal haematopoietic cells, which should enable the development of new anti-leukaemia strategies.

In our attempt to identify stem cell and leukaemia-related surface receptors, we isolated human leukocyte immunoglobulin (lg)-like receptor B2 (LILRB2) and mouse paired lg-like receptor (PirB) as receptors for angiopoietin-like proteins (Angptls). These receptors contain ITIM motifs in their intracellular domains and are classified as inhibitory receptors because ITIM motifs can recruit phosphatases such as SHP-1, SHP-2 and SHIP to negatively regulate cell activation. We showed that PirB is expressed on AML cells and required for AML development in mouse leukaemia models. Nevertheless, it is unknown whether ITIM receptors have direct effects on leukaemia cells.
Here we demonstrated that some ITIM receptors are expressed on leukaemia cells and directly support leukaemia development. We further discovered a signalling pathway initiated from LAIR1, a representative ITIM receptor. This identified ITIM-receptor signalling pathway may represent an ideal target for AML treatment. Our demonstration that some ITIM receptors are not ‘inhibitory’ but supportive of leukaemia development will alter the current understanding of the mechanisms of cancer pathogenesis, cell signalling and therapeutic approaches.

RESULTS

The expression of some ITIM receptors inversely correlates with AML development

To identify potential surface receptor genes that support leukaemia development, we performed an in silico analysis of the relationship between gene expression and the overall survival of AML patients. The expression of 2 out of 58 ITIM receptors positively correlated with the overall survival of AML patients. In contrast, 20 of these receptors had a negative correlation between expression and survival (Supplementary Fig. 1a and Supplementary Table 1). To determine the functions of these ITIM receptors, we inhibited expression of these receptors individually in human leukaemia cell lines using lentivirus-encoded small hairpin RNAs (shRNAs) and found that cell growth was blocked when expression of certain receptors was silenced (Fig. 1a and Supplementary Fig. 1b). These results suggest that some ITIM receptors directly support human leukaemia cell growth.

LAIR1 is essential for the growth of human acute leukaemia cells

On the basis of the shRNA knockdown screening, we selected LAIR1 as a representative ITIM receptor that does not affect normal haematopoiesis29 but is essential for leukaemia development (Fig. 1a and Supplementary Fig. 4) for further mechanistic study. LAIR1 is a type 1 transmembrane glycoprotein containing one extracellular Ig-like domain that binds collagens or surfactant protein D (ref. 30) and two intracellular ITIMs that recruit SHP-1 and SHP-2. LAIR1 is known to be expressed on various lineages of haematopoietic cells and haematopoietic progenitor CD34+ cells31. Our study suggests that collagens, but not the surfactant protein D, are major ligands for LAIR1 in the haematopoietic system (Supplementary Fig. 1e–g). Crosslinking of LAIR1 using antibodies delivers an inhibitory signal to some immune cells in vitro29,31,32; however, as lair1-null mice have no overt defects in haematopoiesis, the biological function of LAIR1 in vivo remains unresolved29. Flow cytometry analysis indicated that LAIR1 is highly expressed on a number of human acute leukaemia cell lines, including MV4-11 (AML), THP-1 (AML), U937 (AML), 697 (B-ALL), Kasumi2 (B-ALL) and RCH-ACV (B-ALL), but not on K562 (Fig. 1b).

To study the potential function of LAIR1 in human leukaemia, we silenced the expression of lair1 by introducing lentivirus-encoded shRNAs into human leukaemia lines. All three shRNAs tested efficiently decreased total and surface expression of LAIR1 (Fig. 1c,d) and essentially blocked the in vitro growth of each of those leukaemia lines that express surface LAIR1 (Fig. 1e,i,j and Supplementary Fig. 2a). In contrast, knockdown of lair1 did not influence the growth of K562 cells (Supplementary Fig. 2b). We further validated shRNA-226 as a lair1-specific shRNA (Fig. 1f,g) and used it in subsequent experiments.

To determine the underlying mechanism by which LAIR1 supports leukaemia cell growth, we found that LAIR1 knockdown did not alter the cell cycle status but markedly increased apoptosis of leukaemia cells (Fig. 1h and Supplementary Fig. 2c).

Next we investigated whether inhibition of lair1 expression alters in vivo engraftment of MV4-11 and 697 leukaemia cells in transplanted NOD/SCID-IL2RG (NSG) mice. Transplantation with cells deficient in lair1 diminished leukaemia development (Fig. 2a–e and Supplementary Figs 2d,e and 3). Very few GFP+ cells, which mark the lentivirus-infected human cells, were observed in mice transplanted with cells that expressed the shRNA targeting lair1 compared with controls. Critically, the observed GFP+ cells in the groups transplanted with cells treated with shRNA targeting lair1 did express surface LAIR1 (Fig. 2a,b), suggesting that cells in which the shRNA was lost but GFP retained might have been selected. A further sorting of different cell populations followed by real-time PCR indicates that GFP+ LAIR1high cells in the LAIR1 shRNA-226 knockdown samples isolated from the transplanted NSG mice contain much less LAIR1 shRNA than the original GFP+ LAIR1low counterparts before transplantation (Supplementary Fig. 2d). Therefore, LAIR1-expressing leukaemia cells successfully engrafted mice; but if there was no surface expression of LAIR1, the cells failed to engraft. Similar results were obtained in B-ALL 697 cells (Fig. 2c,d). The effects of lair1 silencing on the in vitro growth and in vivo development of leukaemia cells are summarized in Fig. 1k. These results clearly indicate that LAIR1 is essential for growth of these human AML and ALL leukaemia cell lines.

LAIR1 supports mouse AML development during serial transplantation

To gain a deeper understanding of the mechanism by which LAIR1 supports AML development, we analysed AML development in lair1-null mice29. These mice have normal haematopoiesis and normal Lin−Sca-1+Kit+ percentages in the BM (ref. 29). There was no difference in repopulation or homing between lair1-null and wild-type (WT) haematopoietic stem cells (Supplementary Fig. 4a–e).

We used several retrovirus transplantation models including MLL-AF9 (AML; refs 25,33,34), AML1-ETO9a (AML; ref. 35) and N-Myc (B-ALL; ref. 36) to study the role of LAIR1 in leukaemia development. Whereas there was no significant difference in AML development on primary transplantation of 100,000 infected WT and lair1-null cells (Supplementary Fig. 4a,b), the mice transplanted with MCV-MLL-AF9-IREs-YFP-transduced lair1-null cells developed AML much more slowly than controls in secondary transplantation (Fig. 3a–e). The gradual decrease of YFP+ lair1-null cells in most mice and the occasional spontaneous increase in numbers of YFP+ cells in a few mice (Fig. 3c) suggest that the lair1-null AML cells indeed successfully engrafted into these recipient mice on transplantation. We performed this experiment independently seven times by using different and pooled BM samples (Supplementary Fig. 5c,d), and all experiments gave similar results.

Consistent with data from the MLL-AF9 model, the deficiency of lair1 in the AML1-ETO9a AML model35 and in the N-Myc B-ALL model36 also largely eliminated the disease during secondary transplantation.
Figure 1. LAIR1, a representative ITIM receptor, is essential for the growth of human leukaemia cell lines. (a) Effects of shRNA-mediated silencing of the expression of the indicated ITIM receptors on the growth of MV4-11 and NB4 AML cells. The cells were counted on day 6 post-infection, and normalized to cells treated with scrambled shRNAs (mean ± s.e.m., Student’s t-test; n = 3 samples, LAIR1 shRNA ***P < 0.0001; KIR3DL1 shRNA ***P = 0.0051; PECAM1 shRNA ***P = 0.0061; SIGLEC11 shRNA ***P = 0.0089; SIGLEC6 shRNA ***P = 0.0326; LILRB1 shRNA ***P = 0.0412; LILRB2 shRNA ***P = 0.0059; LILRB3 shRNA ***P < 0.0001; LILRB4 shRNA ***P < 0.0001; KIR2DL2 shRNA *P = 0.0182; KIRG1 shRNA ***P = 0.0096). (b) Flow cytometry analysis showing that LAIR1 is highly expressed on the cell surfaces of RCH-ACV, 697, Kasumi2, MV4-11, U937 and THP1 human leukaemia cells but not on K562 cells. The x axis indicates LAIR1 expression, and the y axis indicates cell numbers. The bar graph summarizes mean fluorescence intensities (MFIs) of cell-surface LAIR1 (relative fold changes to isotype control). (c) Endogenous LAIR1 expression was inhibited by lentivirus vector-based expression of different shRNAs (226, 277, 593) in MV4-11 cells as determined by western blotting at 48 h after lentiviral infection. (d) Cell-surface LAIR1 expression was inhibited by shRNAs in MV4-11 cells as determined by flow cytometry at 48 h after lentiviral infection. MFIs (obtained after subtraction of the background MFI of the unstained control) indicate LAIR1 expression levels; GFP expression indicates infection. Data are from a single experiment, representative of 3 independent experiments. (e) Treatment with shRNA targeting lair1 inhibited the growth of MV4-11 cells. (f) Expression from Flag–lair1-6m is not silenced by shRNA-226 as determined by western blotting. (g) Rescue of lair1-knockdown phenotype. RFP-tagged mutant lair1 (6m)-infected MV4-11 cells are resistant to the shRNA-226-induced growth inhibition. (h) Apoptosis is increased in MV4-11 cells after treatment with shRNA-226 to inhibit lair1 expression (mean ± s.e.m., Student’s t-test; n = 3 samples, day 2 *P = 0.0112; day 4 ***P < 0.0001; day 6 ***P < 0.0001; day 9 ***P < 0.0001). KD, knockdown. (i) Treatment with each shRNA targeting lair1 inhibited the growth of 697 cells. (j) LAIR1 knockdown inhibited the growth of THP1, U937, RCH-ACV and Kasumi2 cells as measured on day 6. In b, e, i, j, data from one experiment with n = 3 technical replicate samples are shown. Error bars represent s.e.m. The experiment was repeated 3 times with similar results. Unprocessed original scans of blots are shown in Supplementary Fig. 9.

LAIR1 deficiency exhausts mouse AML stem cells

Further analysis of the mouse leukaemia models enabled a deeper mechanistic investigation of the function of LAIR1. There were
Figure 2 Knockdown of lair1 blocks xenograft of human leukaemia cell lines. (a) MV4-11 cells (1 × 10^6 cells) were infected with virus designed to express GFP and either scrambled shRNA or shRNA-226. GFP⁺ cells were collected and transplanted into NSG mice (n = 7 mice) one day post-infection. Left panels show representative flow cytometry plots indicating decreased BM engraftment of MV4-11 cells treated with shRNA targeting lair1. Staining with anti-human CD45 and anti-LAIR1 antibodies confirmed engraftment was from transplanted human leukaemia cells. The percentages of each population are indicated in red numbers, and the median fluorescent intensity of LAIR1 expression is indicated in black numbers. Shown on the right are percentages of GFP⁺ cells in BM, spleen, liver and PB at 1 month after transplantation (mean ± s.e.m., Student’s t-test; n = 7 samples, BM **P < 0.0001; spleen **P = 0.0004; liver *P = 0.0233; PB *P = 0.01471). (b) Comparison of the sizes of spleens of the mice transplanted with control MV4-11 leukaemia cells or cells expressing shRNA targeting lair1 (mean ± s.e.m., Student’s t-test; n = 7 samples, *P = 0.0028). (c) GFP⁺ 697 cells infected by scrambled shRNA or shRNA-226 virus were collected, and 5 × 10⁶ cells were transplanted into NSG mice (n = 5 mice for Scramble, n = 7 mice for lair1 null). Left are representative flow cytometry plots showing the decreased BM engraftment of lair1-knockdown 697 cells. Human CD45 and LAIR1 antibody staining confirmed engraftment was from transplanted human leukaemia cells. The percentages of each population are indicated in red numbers, and the median fluorescent intensity of LAIR1 expression is indicated in black numbers. Shown on the right are percentages of GFP⁺ cells in BM, spleen, liver and PB at 1 month after transplantation (mean ± s.e.m., Student’s t-test; n = 5 mice for Scramble, n = 7 mice for lair1 null, BM **P = 0.0004; spleen **P = 0.0002; liver ***P < 0.0001; PB *P = 0.0057). (d) Comparison of the sizes of livers of the mice transplanted with control or lair1-knockdown 697 leukaemia cells (mean ± s.e.m., Student’s t-test; n = 5 mice for Scramble, n = 7 mice for lair1 null; *P = 0.0048). (e) Summary of the effects of lair1 silencing in the indicated human leukaemia cell lines on inhibition of cell growth in vitro and xenograftment in NSG mice.

significantly more differentiated B220⁺ and CD3⁺ cells in secondarily transplanted mice that received lair1-null cells than in those given control WT cells (Fig. 4a–d). These differentiated haematopoietic cells increased in numbers from day 18 to day 28 post-transplantation (Supplementary Fig. 4g–i).

The gradual disappearance of lair1-null AML after secondary transplantation suggests that LAIR1 supports the activity of AML stem cells (AML-SCs). AML-SCs are enriched in YFP⁺Mac-1⁺Kit⁺ cells in the MLL-AF9-ires-YFP model. There were approximately 90% fewer YFP⁺Mac-1⁺Kit⁺ lair1-null cells at 18 days post-transplantation than in BM of mice transplanted with control AML cells during the secondary transplantation (Fig. 4b; 38% control versus 4% null). As AML-SCs have not yet been purified to homogeneity, we further relied on functional measures to evaluate the activity of
Figure 3 LAIR1 enhances development of MLL-AF9 mouse AML during serial transplantation. (a) Survival curves of mice receiving 3,000 pooled YFP<sup>+</sup> BM cells that were collected from primary recipients transplanted with WT or lair1-null MLL-AF9 AML cells (n=10 mice; P = 0.00002, log-rank test). (b) Comparison of the spleen sizes of mice that were transplanted with WT or lair1-null MLL-AF9 AML cells collected from secondary recipients at 18 days or 28 days after transplantation (mean ± s.e.m., Student’s t-test; n=5 mice, 18 days *P = 0.0245; 28 days **P = 0.0006). (c) Summary of the percentages of YFP<sup>+</sup> WT and lair1-null MLL-AF9 leukaemia cells in PB (open squares or open diamonds) or BM (red squares or red diamonds) of secondarily transplanted mice over time. Mice that were moribund and euthanized are indicated by red symbols, in which the percentages of YFP<sup>+</sup> cells in BM (instead of PB) were used in this analysis (n=10 mice). (d) Summary of percentages of YFP<sup>+</sup> AML cells in PB, BM and spleen of secondary recipient mice transplanted with the WT or lair1-null MLL-AF9 AML cells at day 18 (upper panel) and at day 28 (lower panel) post-transplant (mean ± s.e.m., Student’s t-test; n=5 mice, 18 days PB *P = 0.0124; BM *P = 0.0243; spleen *P = 0.0332; 28 days PB **P = 0.00035; BM ***P < 0.0001; spleen **P = 0.0014). (e) Histological analysis of AML infiltration in the livers, spleens and PB of mice after secondary transplant of WT and lair1-null AML cells at 4 weeks and 20 weeks (haematoxylin/eosin staining for livers and spleens, HEMA 3 staining for cytospin-prepared PB samples). Shown are representative images from at least three similar images. (f) Survival curves of mice transplanted with 1 × 10<sup>5</sup> pooled GFP<sup>+</sup> BM cells that were collected from primary recipients transplanted with WT or lair1-null AML1-ETO9a cells (n=10 mice; P = 0.00012, log-rank test). (g) Percentages of AML1-ETO9a GFP<sup>+</sup> leukaemia cells in BM at 1 month after secondary transplantation (mean ± s.e.m., Student’s t-test; n=10 mice; ***P < 0.0001).
leukaemia stem cells using serial colony-forming unit (CFU) replating and serial transplantation assays. The lair1-null AML cells had an approximately 30% and 90% decrease in CFUs on secondary and tertiary plating respectively (Fig. 4e). The WT YFP⁺Mac-1⁺Kit⁺ AML cells from the secondary transplanted mice formed large, compact colonies, whereas lair1-null cells tended to form smaller and more diffuse colonies indicating decreased potency and higher differentiation potential (Fig. 4f). Similarly, when we isolated WT and

Figure 4 LAIR1 deficiency exhausts tumour-initiating cells by apoptosis. (a) Representative flow cytometry plots showing that secondarily transplanted mice receiving lair1-null AML cells had markedly decreased percentages of Mac1⁺Kit⁺ and increased differentiated Gr-1⁺ cells and increased differentiated B220⁺ cells and increased differentiated B220⁺ cells in YFP⁺ compartments compared with mice receiving WT AML cells. Three pairs of mice were used for the comparison. (b-d) Percentages of YFP⁺Mac-1⁺Kit⁺, YFP⁺Mac-1⁺Gr-1⁺, YFP⁺B220⁺ and YFP⁺CD3⁺ cells in BM (b), PB (c) and spleen (d) of secondary recipient mice transplanted with the WT or lair1-null MLL-AF9 AML cells at day 18 post-transplant (mean ± s.e.m., Student’s t-test; n = 5 mice; (b) Mac1⁺/Gr1⁺; (c) Mac1⁺/Kit⁺; (d) Mac1⁺/Gr1⁺; (e) Mac1⁺/Kit⁺; (f) Mac1⁺/Gr1⁺). (g) Flow cytometry analysis of apoptosis in mouse MLL-AF9 YFP⁺ (AML) cells and YFP⁻ (normal) cells. Early apoptosis was detected as Annexin V-positive/PE-negative/7-AAD-negative staining and late apoptosis was detected as Annexin V-positive/PE-positive/7-AAD-positive staining (mean ± s.e.m., Student’s t-test; n = 3 samples, ***P < 0.0001). (h) Limiting dilution assays comparing the frequencies of AML stem cells in WT and lair1-null MLL-AF9⁺ AML. The indicated YFP⁺ WT and lair1-null MLL-AF9⁺ BM cells that were collected from primary recipients were co-transplanted with 2 × 10⁵ BM competitor cells into lethally irradiated recipients. The competitive repopulating units (CRUs) were calculated by L-Calc software.
lair1-null YFP+ Mac-1+ Kit+ cells from the secondarily transplanted mice for the tertiary transplantation, all mice that received the lair1-null AML-SCs survived whereas those transplanted with WT AML-SCs died within 40 days (Fig. 4g). Concordant with observation of human cells treated with lair1-targeting shRNA, deletion of lair1 did not significantly change cell-cycle status but markedly increased both early and late apoptosis in AML cells in the secondarily transplanted mice (Fig. 4h).

To quantify how LAIR deficiency affects the AML-SC frequency, we performed transplantations with limiting dilutions of sorted YFP+ WT and lair1-null MLL-AF9 BM cells that were collected from primary recipients. Leukaemia development, as measured by survival ratio and latency days, is summarized in Fig. 4i. Strikingly, the frequency of functional AML-SCs in lair1-null primary MLL-AF9 AML model mice was only 1/53 (=116/6109) of that in control WT AML mice. Together, our results indicate that LAIR1 sustains the stemness of AML cells.

**SHP-1 signalling is essential for the long-term survival of AML stem cells**

As LAIR1 recruits the tyrosine phosphatases SHP-1 and SHP-2 for downstream signalling, we sought to determine whether SHP-1 and SHP-2 mediate LAIR1 activity in AML-SCs. Although there was no significant change of total or phospho-SHP-2 levels in WT or lair1-null AML cells, both total and phospho-SHP-1 levels were significantly decreased in lair1-null AML cells, especially in those samples collected from the secondary transplantation (Fig. 5a).

We introduced retroviruses encoding shp-1 or shp-2 into lair1-null AML cells that were collected from previously transplanted mice in rescue experiments to determine which phosphatase mediates the effects of LAIR1 in AML-SCs. The ectopic expression of SHP-1, but not SHP-2, was capable of rescuing the defective phenotype of lair1-null AML cells in CFU assays and in transplantation (Fig. 5b–d and Supplementary Fig. 6a).

Furthermore, in the MLL-AF9 model, when SHP-1 was conditionally deleted from leukaemia cells by Cre-mediated recombination, the colony-forming ability and the engrafment of leukaemia cells into the mice greatly decreased and mice survived significantly longer (Fig. 5e–g). In agreement with the previous finding that SHP-1 is a negative regulator for normal haematopoietic progenitors (refs 37–39), shp-1 deletion increased normal myeloid CFUs (Fig. 5h and Supplementary Fig. 6b). In addition, in contrast to a decreased SHP-1 protein expression in lair1-null BM AML cells, we found that the SHP-1 protein levels in BM cells of WT and lair1-null mice were similar as determined by western blotting (Fig. 5i). Collectively, our results indicate that SHP-1 is a negative regulator in normal myeloid progenitors but acts as a key positive signalling mediator of LAIR1 in AML-SCs and prevents exhaustion of AML-SCs.

**SHP-1 as an adaptor to recruit CAMK1 in LAIR1-mediated signalling**

To further confirm the role of SHP-1 in LAIR1-mediated signalling, we treated mouse and human AML cells with several protein tyrosine phosphatase (PTPase) inhibitors, sodium stibogluconate, NSC-87877 and PHPS1. To our surprise, the treatment did not significantly suppress AML growth (Supplementary Fig. 6c,d). On the basis of this unexpected finding, we compared the rescue effects of WT SHP-1 with the PTPase-inactive SHP-1 (C453S; ref. 40), a tyrosine-mutant SHP-1 4YF (Grb2-binding-independent mutant41), or the carboxy-terminal PTPase domain only (PTPs) SHP-1 on lair1-null AML cells. Like WT SHP-1, the PTPase-defective SHP-1 and the Grb2-binding-independent SHP-1 rescued the lair1-null phenotype. In contrast, the truncated form of SHP-1 containing only the PTPase domain failed to rescue (Fig. 6a–c and Supplementary Fig. 6e). These assays indicated that the role of SHP-1 in LAIR1 signalling is independent of its phosphatase activity.

To identify potential downstream effectors of LAIR1 and SHP-1 in AML cells, we sought to identify signalling pathways that are defective in lair1-null AML cells. We found no apparent differences in the CAMK1 expression in normal BM samples of WT and lair1-null mice (Fig. 5i); however, the total protein level of CAMK1 was decreased in the lair1-null AML cells (Fig. 6d). This result is in agreement with our previous finding that CAMKs are induced by LILRB2, another ITIM-containing receptor that can also recruit SHP-1/SHP-2 (ref. 25). We thus tested whether CAMK1 is a component of LAIR1–SHP-1 signalling. To this end, we introduced retrovirus infections CAMK1 into WT and lair1-null defective AML cells. Although ectopic expression of CAMK1 did not affect WT AML cell function, it did functionally rescue the defective phenotype of lair1-null AML cells (Fig. 6e–g and Supplementary Fig. 6f).

To examine the relationship of LAIR1, SHP-1 and CAMK1, we performed co-immunoprecipitation assays using mouse MLL-AF9 AML cells. The bidirectional pulldown experiments showed that the endogenous SHP-1 and CAMK1 interact with each other (Fig. 6h). We also confirmed that LAIR1 interacted with SHP-1 (Supplementary Fig. 6g), concordant with a previous result42. These results suggest that LAIR1–SHP-1–CAMK1 form a complex in primary AML cells. Importantly, the phosphatase-inactive SHP-1 C453S mutant bound CAMK1, but the phosphatase domain of SHP-1 alone failed to bind to CAMK1 (Fig. 6i). Our data support the conclusion that SHP-1 plays a phosphatase-independent role as an adaptor to recruit CAMK1 during LAIR1-mediated signalling in AML cells.

**CREB is necessary for LAIR1-mediated signalling**

The AML onco-gene cAMP response element-binding protein (CREB) is a downstream transcription factor induced by CAMKs (ref. 44). We sought to determine whether CREB is a part of the LAIR1–SHP-1–CAMK1 signalling pathway. Phosphorylation of CREB was abundant in WT but not lair1-null AML cells (Fig. 7a). WT CREB robustly rescued the defective phenotype of lair1-null AML cells in vitro and in vivo; however, none of the CREB mutants (transactivation mutants S129A, S133A, or double mutant S129/133A; refs 43, 45) had effects on the phenotype of lair1-null AML cells (Fig. 7b–d and Supplementary Fig. 7a). A CREB inhibitor 666-15 (refs 46, 47; a derivative of naphthol AS-E; ref. 47) significantly reduced the colony-forming ability of AML cells (Fig. 7e). The transactivation-inactive CREB resulted in decreased SHP-1 levels in AML mice in vivo (Supplementary Fig. 6d), suggesting that the lower CREB activation decreases the feedback loop involving SHP-1. Overall, these experiments indicate that CREB is a transcription factor downstream of LAIR1–SHP-1–CAMK1 signalling in AML cells.
LAIR1–SHP-1–CAMK1–CREB signalling is critical for certain human AML development

Our in silico analysis indicated that lair1 was expressed at significantly higher levels by primary human AML BM and peripheral blood (PB) mononuclear cells than by normal counterparts, and its level negatively correlates with the overall survival of human AML patients (Fig. 8a). Normal human haematopoietic cells contain both LAIR1+ and LAIR− compartments (Fig. 8b, cord blood); however, 86% of primary human AML specimens contained mostly LAIR1+ cells (Fig. 8b, AML type 2), and only 14% of AML samples had significant populations of both LAIR1+ and LAIR− cells (Fig. 8b, AML type 1). The separation of LAIR1+ and LAIR− leukaemia cells followed by CFU assay demonstrated that LAIR1+ AML cells had a significantly higher ability than LAIR1− AML cells to form colonies in both

Figure 5 SHP-1 rescues the lair1-null AML phenotype. (a) Phospho-SHP-1 and total SHP-1 levels in lair1-null and control MLL-AF9+ BM cells from both primarily and secondarily transplanted mice were determined by western blotting. The red box indicates the positions of p-SHP-1 and SHP-1 in null AML cells in secondarily transplanted mice. The experiment was repeated 5 times, which gave similar results. (b) Retrovirally expressed SHP-1 increased CFU numbers of lair1-null AML cells compared with cells that were infected with control or SHP-2-expressing viruses in colony-forming assays. Retrovirally expressed SHP-1 and SHP-2 had similar levels to endogenous proteins in WT controls. (c,d) Retrovirally expressed SHP-1 rescued the lair1-null phenotype on tertiary transplantation. MLL-AF9+ WT or lair1-null BM cells in secondarily transplanted mice were collected at 28 days and were infected by SHP-1-encoding or control virus. (e) Survival curves of mice transplanted with 3,000 GFP+ SHP-1fl/fl+Cre or SHP-1fl/fl+Cre MLL-AF9 AML cells (n=10; P<0.0001, log-rank test). (f) Comparison of percentages of GFP+ AML cells in the PB from mice injected with SHP-1fl/fl+Cre and SHP-1fl/fl+Cre MLL-AF9 AML cells (mean±s.e.m., Student’s t-test; n=10 mice; **P=0.0067). (g) Comparison of colony-forming abilities of SHP-1fl/fl+Cre and SHP-1fl/fl+Cre MLL-AF9 AML cells. (h) BM cells isolated from 5-fluorouracil-pretreated SHP-1fl/fl mice were transduced by infection with empty vector or Cre-expressing vector to generate control (SHP-1fl/fl+Con) or SHP-1-deficient (SHP-1fl/fl+Cre) AML cells. (i) Total SHP-1 and CAMK1 levels in WT and lair1-null BM cells were determined by western blotting. In b,h,i, data from one experiment with n=3 technical replicate samples are shown. The experiment was repeated 3 times with similar results. Unprocessed original scans of blots are shown in Supplementary Fig. 9.
primary and secondary plating (Supplementary Fig. 8a, bars B1 and B2). In contrast, there was no significant difference in colony-forming potential between the LAIR\(^+\) and LAIR\(^-\) populations of human cord blood mononuclear cells (Supplementary Fig. 8a, bar A). More importantly, when AML cells with high LAIR1 expression (LAIR1\(^{\text{high}}\)) or those with low LAIR1 expression (LAIR1\(^{\text{low}}\)) were transplanted
Figure 7 CREB is a transcription factor for LAIR1 signalling in AML cells. (a) Phospho-CREB and total CREB levels in lair1-/-null and control MLL-AF9 BM cells from both primarily and secondarily transplanted mice were determined by western blotting. (b) Retrovirally expressed CREB WT, but not CREB S129A, S133A or S129/133A, increased CFU numbers of lair1-null AML cells. Data from one experiment with n=3 technical replicate samples are shown. The experiment was repeated 3 times with similar results. (c,d) Retrovirally expressed CREB WT, but not CREB mutants, rescued lair1-null phenotype on transplantation. The red dotted line shows that CREB WT virus-infected leukaemia BM cells reversed lair1-null MLL-AF9+ leukaemia development, whereas the black, blue, brown and green dotted lines show that control, CREB S129A, CREB S133A and CREB S129/133A virus-infected leukaemia BM cells were unable to do so (n=5 mice; P<0.0001, log-rank test; d) ***P<0.0001, mean ± s.e.m., Student’s t-test). (e) Treatment with a CREB inhibitor 666-15 reduced CREB phosphorylation and the colony-forming ability of WT AML cells. The molecule structure of the CREB inhibitor 666-15 is shown. Data from one experiment with n=3 technical replicate samples are shown. The experiment was repeated 3 times with similar results. Unprocessed original scans of blots are shown in Supplementary Fig. 9.

Effective elimination of all cancer cells, including cancer stem cells, remains a major challenge in oncology. Our finding that a representative ITIM-receptor LAIR1 supports the survival and self-renewal of leukaemia cells identified a potential angle to combat haematopoietic malignancies. We showed that the expression of many known ITIM receptors inversely correlates with the overall survival of AML patients and that certain of these receptors are crucial for the growth of human acute leukaemia cells. We conducted a detailed investigation of LAIR1, which does not affect normal haematopoiesis but is essential for acute leukaemia development. Deletion of lair1 from either mouse or human AML cells induced apoptosis, inhibited growth and abrogated AML development in transplantation models. Importantly, our data indicate that SHP-1, but not SHP-2, mediates LAIR1 signalling in AML cells and prevents exhaustion of AML-SCs in vivo. Furthermore, we demonstrated that, although SHP-1 is a negative signalling molecule for normal myeloid differentiation, it acts as a phosphatase-independent adaptor to recruit CAMK1 for activation of the downstream transcription factor CREB in AML cells. The LAIR1–SHP-1–CAMK1 axis may represent a target for treating AML.
Figure 8 LAIR1 signalling is essential for human leukaemia development. (a) Expression of lair1 mRNA differs significantly in BM and PB of AML patients (n = 7 BM or 19 PB respectively) from those in normal samples (n = 10 BM or 9 PB; mean ± s.e.m., Student’s t-test; BM: *P = 0.0329; PB: *P = 0.0421). (b) Representative flow cytometry plots showing different LAIR1 expression patterns in normal mononuclear cells (cord blood) and primary AML cells (type 1 and type 2). A representative plot of a flow cytometry analysis (from at least 3 similar images) of a type 1 sample shows that cells are present that express both high and low levels of LAIR1. Type 2 cytometry analysis (from at least 3 similar images) of a type 1 sample shows primary AML cells (type 1 and type 2). Representative flow cytometry plots showing different LAIR1 expression patterns in normal mononuclear cells (cord blood) and PB (n = 100,000, 150,000 50,000). Data on these primary AML samples were measured in Supplementary Table 3. (c) Summary of percentages of hCD45+ cells (%). Data from one experiment with n = 3 technical replicate samples are shown. The experiment was repeated 3 times with similar results. Unprocessed original scans of blots are shown in Supplementary Fig. 9. Error bars represent s.e.m.
The tumour-supportive role of SHP-1 explains why some immune inhibitory receptors such as LAIR1 directly support cancer development. SHP-1 is capable of binding to Grb2 in a phosphatase-independent manner41; however, the CAMK1 recruitment by SHP-1 represents a different phosphatase-independent mechanism. A role for SHP-1 in AML development is also supported by evidence from other studies showing that SHP-1 suppresses differentiation18 and inhibits apoptosis in some leukaemia cells49. Nevertheless, it is possible that SHP-1 inhibits the growth of certain tumours and certain immortalized cell lines other than the ones we studied here. Moreover, the signalling through LAIR1 observed in leukaemia cells is unlikely to exist in normal cells. In contrast to the role of SHP-1 as an adaptor or scaffolding protein in AML development, the phosphatase activity of SHP-1 is necessary in normal haematopoietic functions. For example, the myeloproliferative phenotype of me/me mice is caused by inactivation of the phosphatase activity of SHP-1 (refs 38,50). In addition, although the LAIR1 deficiency greatly reduced the level of SHP-1 and CAMK1 in leukaemia samples, these protein levels were not affected by lair1 deficiency in normal BM cells (Fig. 5i).

Although CAMK1 is predominantly cytoplasmic31, it can be transported into the nucleus with the existence of a nuclear translocation sequence52. Therefore, the activation of CREB by CAMK1 is possibly direct. Additional downstream targets of CAMK1 other than CREB probably also exist. We speculate that LAIR1–SHP-1–CAMK1 may form a complex that is necessary for the activation or even stability of SHP-1 and CAMK1. A LAIR1 deficiency may initially result in a slightly decreased activation of SHP-1, CAMK1 and CREB. This decrease should become more severe over time (compare our SHP-1 levels in primary and secondarily transplanted AML mice in Fig. 5a) because of a possible feedback loop involving CREB and SHP-1: less activation of CREB will lead to more decreased SHP-1 over time in lair1-null AML cells, eventually resulting in the exhaustion of the signalling system (Supplementary Fig. 7d).

The supportive role of LAIR1 in AML is unlikely to be an exceptional case in cancer biology. As we showed, the lair1 deficiency has a similar effect to the B-ALL model, suggesting that LAIR1 also supports certain ALL. In addition, our study suggests that a number of other ITIM-receptors support AML development, and thus may also activate a similar downstream signalling pathway as LAIR1 in AML cells. As there are numerous types of ITIM receptor, different receptors may have different expression patterns in different types or subtypes of leukaemia and other cancers. It will be important to investigate the mechanisms by which other ITIM receptors support cancer progression.

The identification of ITIM receptors and their downstream signalling as potential therapeutic targets may reshape our views regarding how leukaemia develops, how cancer cells differ from other cells, and how to treat this difficult disease. Our study suggests that some leukaemia cells have unique signalling pathways downstream of ITIM receptors. These inhibitory receptors may enable the leukaemia cells to survive conventional therapies resulting in tumour relapse. As inhibition of the signalling of certain ITIM receptors directly blocks leukaemia growth, and stimulates immunity26,27 that may suppress tumorigenesis but does not disturb normal haematopoiesis52, these ITIM receptors represent ideal targets for treating leukaemia. The blockade of inhibitory receptor signalling in combination with conventional therapies may prove to be an effective strategy for elimination of leukaemia cells. It will be interesting to test whether therapeutic modalities for ITIM receptors may include antibodies against the extracellular domains of these receptors or whether inhibition of the intracellular signalling of ITIM receptors will represent a more powerful means of blocking these targets.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
X.K. performed most of the experiments, analysed data and contributed to writing the paper; Z.L. performed the TCGA and clustering analyses; C.C. performed the CAMK experiments; M.D. performed the LILRB knockdown experiments. Y.F., F.X. and X.X. performed the CREB inhibitor experiments and provided advice; B.D., X.H., R.H.C. and M.I.Y. collected the primary AML samples and provided advice; J.W.T. helped with experiments using leukaemia cell lines and contributed to paper writing; I.E.C. provided LAIR1-deficient mice and contributed to paper writing; C.C.Z. conceived, coordinated and supervised the project, designed experiments, analysed data and wrote the paper.

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METHODS

Mice. C57 BL/6 CD45.2, CD45.1 and NSG mice were purchased from the National Cancer Institute and from the University of Texas Southwestern Medical Center animal breeding core facility. 

Human leukaemia cells. Primary human AML samples were obtained from the tissue bank at the University of Texas MD Anderson Cancer Center. Informed consent was obtained under a protocol reviewed and approved by the Institutional Review Board at the University of Texas MD Anderson Cancer Center. Samples were frozen in fetal bovine serum (FBS) with 10% DMSO and stored in liquid nitrogen. Human leukaemia cell lines were cultured in RPMI supplemented with 10% FBS at 37 °C in 5% CO2 and the normal level of O2. Human leukaemia cell lines were from ATCC.

Flow cytometry. For flow cytometry analyses of mouse AML cells, peripheral blood or BM cells were stained with anti-Gr-1–APC (rat, M1/70, 1:200 dilution), anti-Gr-1–PE (rat, RB6-8C5, 1:200 dilution), anti-CD3–APC (hamster, 145-2C11, 1:200 dilution), anti-B220–PE (rat, RA3-6B2, 1:200 dilution) or anti-Kit–PE (rat, 2B8, 1:200 dilution) monoclonal antibodies (BD Pharmingen). For analysis of apoptosis, GFP- or YFP-positive AML cells were stained at the indicated days with 2B8, 1:200 dilution) monoclonal antibodies (BD Pharmingen). For analysis of signalling domain, we will observe an increase or decrease, respectively, of GFP expression, unless indicated otherwise. Patients were separated into two groups based on whether they had higher (n = 82 patient samples) or lower (n = 83 patient samples) expression than the average expression levels of the analysed gene, with the exception for lair1 analysis in which n = 52 patient samples were used for these two groups. For correlation analysis, scatter plots with regression curves (blue) and 95% prediction intervals (red) indicate the positive correlations among CAMKI, LAIR1 or PTPN6. Correlation coefficient r and P value are shown in Fig. 8k.

Leukaemia characterization. In the retrovirus transplantation model, we isolated Lin– cells from littermate lair1-null and WT mice, and infected them with MSCV-oncogene-IRES-YFP retrovirus followed by transplantation into WT recipient mice to establish leukaemic mice. We monitored the survival, examined the size and histological properties of BM, spleen and liver, and analysed the numbers and infiltration of leukaemia cells in PB, BM, spleen and liver. We also determined the different populations of leukaemia cells using flow cytometry. Serial CFU replating, serial transplantation, and limiting dilution assays were performed as indicated.

Southern blotting. Genomic DNA was extracted from spleens using the Gentra Systems kit (Gentra Systems). For Southern analyses, 10 μg genomic DNA was digested with EcoRI (New England Biolabs) overnight at 37 °C and run on a 1% agarose gel. DNA was transferred overnight to a Genescreen Plus nylon membrane (Perkin Elmer). Membranes were probed with 10 μPm per millilite of [α32P]dCTP (Perkin Elmer)-labelled yellow fluorescent protein (YFP). The YFP probe was derived from the 508 bp fragment of the MLL-AF9-IRES-YFP vector, and was radiolabelled by PCR.

Colony assays. Human or mouse AML cells were diluted to the indicated concentration in IMDM with 2% FBS and were then seeded into methylcellulose medium H4436 or M3534 (StemCell Technologies) for myeloid colony formation analysis as described previously.

Human AML xenograft. Xenografts were performed essentially as we described previously24,25. Briefly, adult NSG mice (6–8 weeks old) were sublethally irradiated with 230 Gy total body irradiation before transplantation. shRNA-infected cells were resuspended in 200 μl PBS containing 1% FBS at a final concentration of 0.5–1 × 106 human CD45+ viable cells per mouse for retro-orbital injection. One to four months after transplantation, the PB, BM, spleen and liver were assessed for the engraftment.
Western blotting. Cell lysates (100 µg samples) were prepared by Transmembrane Protein Extraction Reagent (FIVEphoton Biochemicals, TmPER-200) or RIPA buffer, and were separated by electrophoresis on 4–15% SDS–polyacrylamide gels, and the proteins were electroblotted onto a nitrocellulose membrane. The membrane was probed with the indicated primary antibody for 1 h at room temperature and then incubated with horseradish peroxidase-conjugated secondary antibody, which was detected with the Luminata Crescendo Western HRP Substrate (Millipore). The antibodies used in this paper included: surfactant protein D (ab17781), SHP-1 (ab2020, 1:2,000 dilution), CAMK1 (ab68234, 1:2,000 dilution), mLAIR1 (ab171239, 1:500 dilution), CAMKIV (ab3557, 1:4,000 dilution), CREB (ab5803, 1:2,000 dilution), p-SHP-1 (Y536, ab51171, 1:1,000 dilution) from Abcam; SHP-2 (3397s, 1:1,000 dilution), p-SHP-2 (Tyr580, 3703, 1:1,000 dilution), p-CREB (S133, 9198, 1:2,000 dilution) from Cell Signaling; anti-Flag M2 (F1804, sigma, 1:10,000 dilution), actin (A5228, 1:10,000 dilution) and human LAIR1 (LS-C44463, LifeSpan Biosciences, 1:2,000 dilution). A representative image was shown from at least 3 similar images.

Statistical analyses. Data are expressed as mean ± s.e.m. Data were analysed by Student’s t test and were considered statistically significant if P < 0.05. The survival rates of the two groups were analysed using a log-rank test and were considered statistically significant if P < 0.05. P values are represented as precise values or as ***P < 0.0001.
Supplementary Figure 1 Lair1, a representative ITIM receptor, is essential for the growth of human leukemia cell lines. a. Expression of certain human ITIM receptor mRNAs negatively correlates with the overall survival of AML patients. A total of 58 ITIM receptors were selected based on the criteria that 1) they are plasma membrane receptors and 2) they use ITIM as the main signaling motifs. Data were obtained from the TCGA AML database, and analyzed without normalization (condition 1) or normalized to GADPH expression (condition 2), Affymetrix housekeeping gene expression (condition 3), or total mRNA (condition 4). More information about data analysis can be found in Methods. b) Effects of inhibition of expression of indicated ITIM receptors using shRNAs as determined by real-time RT-PCR. Data are from a single experiment, representative of 3 independent experiments. c) In silico analysis of the correlation between human lair1 mRNA expression and the overall survival of AML patients younger than 65 years old. Data were obtained from the TCGA AML database (n = 52 patient samples for each groups, p = 0.0271, log-rank test). d) An in silico analysis of human lair1 mRNA expression in 43 human AML samples as described previously. e) SP-D is not highly expressed in the bone marrow environment compared with mRNA expression in lung epithelial cells, as determined by real-time RT-PCR. Data are from a single experiment, representative of 3 independent experiments. f) Schematic summary of the LAIR1 chimeric receptor signaling reporter cells system. g) In the LAIR1 chimeric receptor signaling reporter cells system, flow cytometry analysis demonstrated that, while the immobilization collagen 1 (1 μg/ml) or anti-LAIR1 antibody induced LAIR1 activation (as shown by increased GFP induction), the immobilized or soluble SP-D (1 μg/ml) was unable to do so (the upper panels are for control cells, and the lower panels are for the hLAIR1 reporter cells).
Supplementary Figure 2 Depletion of *lair1* suppresses growth of human leukemia cell lines *in vitro* and *in vivo*. a) Representative images (from at least 3 similar images) showing the marked reduction of MV4-11 cell growth upon treatment with shRNAs targeting *lair1* at 4 days after viral infection. Scale bar is 50 μM. b) No effects on 562 cell growth were observed upon treatment with shRNAs targeting *lair1*. Data from one experiment with n=3 technical replicate samples are shown. The experiment was repeated 3 times with similar results. c) No significant cell cycle change was detected between control and *lair1*-deficient MV4-11 cells at 3 and 6 days after infection with virus encoding a control shRNA or virus encoding shRNA 226, respectively. Data from one experiment with n=3 technical replicate samples are shown. The experiment was repeated 3 times with similar results. d) GFP+ LAIR1<sup>high</sup> cells in the LAIR1 shRNA 226 knockdown samples isolated from the transplanted NSG mice contained much less LAIR1 shRNA sequences than the original GFP+ LAIR1<sup>low</sup> counterparts before transplantation. Real-time PCR was performed to quantitate the shRNA sequences from these populations by using the shRNA 226 specific primer (GCTAGTCCATCTGAGTCAG-forward) together with the vector primer (AAGCGAGCTTATCGATACCG-reverse). Data are from a single experiment, representative of 3 independent experiments. e) Flow cytometry analysis showing the decreased engraftment of *lair1*-deficient MV4-11 cells in BM of individual recipient mice (scrambled control vs. *lair1*-shRNA treated samples).
Supplementary Figure 3 Flow cytometry analysis showing the decreased engraftment of lair1-deficient 697 cells in BM of individual recipient mice (scrambled control vs. lair1-shRNA treated samples).
Supplementary Figure 4 LAIR1 deficient mice maintain normal hematopoiesis. a) WT and lair1-null neonatal liver Lin- cells (1 x 10^5 cells) were transplanted together with 1 x 10^5 CD45.1 competitor cells into lethally irradiated (10 Gy) CD45.1 mice (n = 5 mice). Peripheral blood engraftments are shown at 6 and 20 weeks after transplantation. b) Comparison of multilineage contribution between WT and lair1-null cells at 20 weeks after transplantation (n = 5 mice). c) WT and lair1-null BM cells (1 x 10^6 cells) from primary engraftments were transplanted together with 2 x 10^5 CD45.1 competitor cells into lethally irradiated (10 Gy) CD45.1 mice (n = 5 mice). Peripheral blood engraftments are shown at 4 and 16 weeks after transplantation. d) Comparison of multilineage contributions of WT and lair1-null cells at 16 weeks after secondary transplantation (n = 5 mice). e) WT and lair1-null HSCs home similarly to the recipient BM. BM cells from WT or lair1-null mice (n=5 mice) were labeled with carboxyfluorescein succinimidyl ester (CFSE), and 1 x10^7 cells were transplanted into lethally irradiated recipients. After 12 hours, the total percentage of CFSE* cells in the BM, spleen, and liver and LT-HSCs (CFSE*Lin'Sca-1*Kit*Flk2*CD34* cells) in BM were determined by flow cytometry.
**Supplementary Figure 5** LAIR1 enhances leukemia development in several mouse leukemia models during serial transplantation. 

a) No significant difference in AML development were observed upon primary transplantation with MLL-AF9-infected WT or lair1-null hematopoietic progenitors in primary transplantation; survival curves are shown (n = 10 mice; p = 0.1121, log-rank test). The experiment was repeated three times with similar results. 
b) Summary of percentages of YFP+ AML cells, YFP+Mac-1+Gr-1+, and YFP+Mac-1+Kit+ cells in BM of primary recipient mice transplanted with the WT or lair1-null MLL-AF9 AML cells (n = 10 mice), the experiment was repeated three times with similar results. 
c) The clonal relationship between the transplanted cells was studied by northern blotting on genomic DNA isolated from 4 pairs of WT and lair1 null MLL-AF9 induced leukemia samples. The result demonstrates that the leukemias were oligoclonal. The southern blotting was performed using a probe for YFP. 
d) Southern blotting analysis of the oligoclonal nature of MLL-AF9 integration in genomes of 3 primarily and 2 secondarily transplanted mice. The secondarily transplanted AML mice contained multiple MLL-AF9 clones from different primary samples. The bands indicated by the red number coordinates between primary and secondary samples. 
e) Survival curves of mice receiving N-Myc infected WT or lair1-null hematopoietic progenitors in primary transplantation (n = 10 mice, p = 0.4204, log-rank test). 
f) Survival curves of mice receiving 3,000 pooled GFP+ BM cells that were collected from primary recipients transplanted with WT or lair1-null N-Myc B-ALL cells (n = 5 mice, p = 0.0031, log-rank test). 
g-i) Leukemia development vanishes upon secondary transplantation of lair1-null MLL-AF9 cells. Summary of percentages of YFP+ AML cells and YFP+Mac-1+Kit+, YFP+Mac-1+Gr-1+, YFP+B220+, and YFP+CD3+ cells in (f) BM, (g) PB, and (h) spleen of secondary recipient mice transplanted with the WT or lair1-null MLL-AF9 AML cells at day 28 post-transplant (mean ± s.e.m., Student’s t-test; n = 5 mice; (g) Mac1+/Gr1+ **p = 0.0015; B220+ **p = 0.0019; CD3+ *** p <0.0001; (h) Mac1+/Gr1+ **p = 0.0031; B220+ **p = 0.0022; CD3+ **p = 0.0017; (i) Mac1+/Gr1+ **p = 0.0002; B220+ **p = 0.0035; CD3+ *** p <0.0001).
Supplementary Figure 6 SHP-1-CAMK1 rescues the lair1-null AML phenotype.
a) Retrovirally-expressed SHP-1 increased CFU numbers of lair1-null AML cells in secondary plating. Data from one experiment with n=3 technical replicate samples are shown. The experiment was repeated 3 times with similar results. b) The expression of endogenous shp-1 was inhibited by Cre virus infection, as determined by Q-PCR at 48 hours after infection. Data are from a single experiment, representative of 3 independent experiments. c-d) SHP-1 inhibitors have little effect on (c) CFU activity of MLL-AF9 AML cells or (d) cell growth capacity of human AML leukemia cell line (MV4-11). Data from one experiment with n=3 technical replicate samples are shown. The experiment was repeated 3 times with similar results. e) Retrovirally-expressed SHP-1 WT, C453S, and 4YF(278, 303, 538, 566), but not SHP-1 PTPc, increased CFU numbers of lair1-null AML cells in secondary plating. Data from one experiment with n=3 technical replicate samples are shown. The experiment was repeated 3 times with similar results. f) Retrovirally-expressed CAMK1 increased CFU numbers of lair1-null AML cells in secondary plating. Data from one experiment with n=3 technical replicate samples are shown. The experiment was repeated 3 times with similar results. g) IP-western assay of MLL-AF9 BM cells of wide-type mouse by precipitating SHP-1, followed by detection of CAMK1 or LAIR1. h) Additional 4 times of western-blot analyses as in Figure 4A, showing SHP-1 protein levels in both primarily and secondarily transplanted WT and LAIR1 null leukemic mice.
Supplementary Figure 7 Transcription factor CREB is necessary for LAIR1-mediated signaling in AML cells. a) Retrovirally-expressed WT CREB, but not CREB S129A, S133A, or the double mutant S129/S133A, increased CFU numbers of Lair1-null AML cells. Data from one experiment with n=3 technical replicate samples are shown. The experiment was repeated 3 times with similar results. b-c) Treatment of (b) 697 or (c) U937 cells with CREB inhibitor XX15 inhibited growth. YFP+ WT AML cells (20,000) were sorted by flow cytometry, plated in 1.55-cm wells, and treated with the indicated concentration of XX15. Cell numbers were determined on days 1, 2, and 3 from triplicate wells. Data from one experiment with n=3 technical replicate samples are shown. The experiment was repeated 3 times with similar results. d) Retrovirally-expressed CREB S219/133A decreased the total SHP-1 levels in the WT AML mouse bone marrow samples as shown by western blotting.
**Supplementary Figure 8** LAIR1/SHP-1/CAMK1 axis supports human AML development. a) LAIR1<sup>high</sup> primary AML cells have greater colony-forming ability difference in both first and second plating (samples B1 and B2), whereas no significant difference in colony-forming ability was detected between LAIR1<sup>high</sup> and LAIR1<sup>low</sup> cord blood mononuclear cells (sample A). Data from one experiment with n=3 technical replicate samples are shown. The experiment was repeated 3 times with similar results. b) Endogenous shp-1 expression was inhibited using shRNAs (201, 698, 768) in MV4-11 cells as determined by Q-PCR at 48 hours after infection. Data are from a single experiment, representative of 3 independent experiments. c) No clear association between shp-2 expression and AML patient survival was observed. Data were obtained from the TCGA AML database (n = 82 patient samples for high or n = 83 patient samples for low, p = 0.9451, log-rank test). d) Endogenous camk1 expression was inhibited using an shRNA in MV4-11 cells as determined by Q-PCR at 48 hours after infection. Data are from a single experiment, representative of 3 independent experiments. e) LAIR1 expression is independent of the selected human AML stem cell phenotypic markers. The expression of LAIR1 and phenotypic markers (CD34/CD38/CD90) were analyzed by flow cytometry in four AML clinical samples. f) LAIR1 knockdown decreased colony-forming ability in all seven tested primary human AML cells as determined by CFU assays. Data from one experiment with n=3 technical replicate samples are shown. The experiment was repeated 3 times with similar results. g) The survival curves of mice receiving control or LAIR1-knockdown primary human patient AML cells (sample# 6). n = 9 mice; p < 0.0001, log-rank test. h) Schematic summary of the novel signaling pathway mediated by the ITIM receptor LAIR1 in leukemia cells.
Supplementary Figure 9 Original uncropped images of western blots.
In *silico* analysis of the correlation between human ITIM domain-containing receptor mRNA expression and the overall survival of AML patients. A total of 58 ITIM receptors were selected based on the criteria that 1) they are plasma membrane receptors and 2) they use ITIM as the main signaling motifs. Data were obtained from the TCGA AML database (https://tcga-data.nci.nih.gov/tcga/; accessed November 5, 2012), and expression was normalized to GAPDH expression, Affymetrix housekeeping gene expression, or total mRNA. For lair1, the overall survival of AML patients younger than 65 years old were analyzed (n=58 samples for higher and n=58 samples for lower). In all other cases, patients were separated into two groups based on whether they had higher (n = 82 samples) or lower (n = 83 samples) than the average expression levels of the indicated genes (n = 165 samples).

Supplementary Table 1
| Gene target | target sequence |
|-------------|-----------------|
| kir2d2      | AAGAGTCTGAGGACACAGAA |
| kir3d1      | AAGACCTGAGGAGGTGACAT |
| klr41       | AAGAGCCTGAGGACACAGAA |
| pecam1      | AAGAGTGAAGTCGTCACCGTG |
| siglec6     | AAGCCTGATGTCTACATCC |
| siglec7     | AAGGAGAAGGCACAGCATCCA |
| lilrb1      | GGACATCGACCCAGGAGAAA |
| lilrb2      | GGCAATCTGGACACAGAAGAAA |
| lilrb3      | GGGAATTCTGGACACAAA |
| lilrb4      | AGGAATCGAGGGAGATA |
| lilrb5      | AGGAATCGAGGGAGATA |
| lair1-226   | GCTAGTCCATCGAGTCAG |
| lair1-277   | GGAAATGCGGCCCTTATC |
| lair1-593   | GCAAGGACGGAGCAGGAG |
| shp-1-201   | AAGTTTCGACTCGACAGG |
| shp-1-696   | AAGCAGGAGTCGAGGAGTACA |
| shp-1-768   | AAGAAATTCGCCACAGGCTCG |

**Supplementary Table 2** shRNA targeting sequences for the indicated ITIM receptors.
supplementary Table 3. Clinical sample information

| Sample | Blast cells% in PB | Blast cells% in BM | Subtype | Prognosis | Survival (M) | Age | Sex |
|--------|-------------------|--------------------|---------|-----------|--------------|-----|-----|
| 1      | 82%               | 95%                | M5b, Therapy related | dead     | 3.2          | 56  | Female |
| 2      | 84%               | 95%                | M5b, Therapy related | dead     | 3.2          | 56  | Female |
| 3      | 90%               | 86%                | M1      | Alive, CR | 91.9         | 21  | Female |
| 4      | 88%               | 94%                | M1      | dead      | 4.6          | 63  | Female |
| 5      | 73%               | 73%                | Acute myelomonocytic leukemia | Alive, CR | 77.9         | 58  | Male |
| 6      | 94%               | 61%                | AML Developing from MDS | dead      | 3.8          | 61  | Male |
| 7      | 80%               | 72%                | AML after Stem cell transplantation | dead      | 1.8          | 47  | Male |
| 8      | 96%               | 56%                | M7; relapse | dead     | 9.6          | 28  | Male |
| 9      | 96%               | 54%                | M7; relapse | dead     | 9.6          | 28  | Male |
| 10     | 80%               | >20%               | Unclassified | dead     | 5.1          | 51  | Female |
| 11     | 90%               | >20%               | Unclassified | dead     | 5.2          | 51  | Female |
| 12     | 88%               | 64%                | M2      | dead      | 12.1         | 70  | Male |
| 13     | 91%               | 94%                | M2; Relapse | dead     | 4.0          | 68  | Female |
| 14     | 72%               | 73%                | M5b     | dead      | 23.4         | 42  | Male |
| 15     | 94%               | 89%                | M1; relapse | dead     | 1.0          | 57  | Female |

Supplementary Table 3 Clinical sample information.
References

1. Lukk, M. et al. A global map of human gene expression. *Nat Biotechnol* **28**, 322-324 (2010).