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Klf5 controls bone marrow homing of stem cells and progenitors through Rab5-mediated $\beta 1/\beta 2$ -integrin trafficking

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Krüppel-like factor 5 regulates pluripotent stem cell self-renewal, but its role in somatic stem cells is unknown. Here we show that Krüppel-like factor 5-deficient haematopoietic stem cells and progenitors fail to engraft after transplantation. This haematopoietic stem cell and progenitor defect is associated with impaired bone marrow homing and lodging and decreased retention in bone marrow, and with decreased adhesion to fibronectin and expression of membrane-bound $\beta 1/\beta 2$ -integrins. *In vivo*-inducible gain-of-function of Krüppel-like factor 5 in haematopoietic stem cells increases haematopoietic stem cell and progenitor adhesion. The expression of Rab5 family members, mediators of $\beta 1/\beta 2$ -integrin recycling in the early endosome, is decreased in *Klf5* ^{Δ/Δ} haematopoietic stem cells and progenitors. Krüppel-like factor 5 binds directly to the promoter of *Rab5a/b*, and overexpression of *Rab5b* rescues the expression of activated $\beta 1/\beta 2$ -integrins, adhesion and bone marrow homing of *Klf5* ^{Δ/Δ} haematopoietic stem cells and progenitors. Altogether, these data indicate that Krüppel-like factor 5 is indispensable for adhesion, homing, lodging and retention of haematopoietic stem cells and progenitors in the bone marrow through Rab5-dependent post-translational regulation of $\beta 1/\beta 2$ integrins.

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Krüppel-like factor 5 (Klf5) is a member of the Klf family of transcription factors. Klf5 is expressed in highly proliferative epithelial cell types during embryogenesis and in the adult including the skin, gut, prostate, lung, mammary gland and bladder, as well as in immortalized epithelial cell lines and proliferating primary cultures¹. Mice null for the Klf5 alleles are embryonic lethal and die before embryonic day 8.5 (ref. 2). Klf5 works as a downstream activator of transcriptional programs associated with pluripotent stem cell activity and differentiation², and its deficiency results in impaired blastocyst formation and implantation³. Using *in vivo* models, Klf5 has been shown to have a role in biological processes such as embryonic development, cardiovascular remodelling, adipogenesis, inflammatory stress responses, and intestinal and lung development⁴. However, our understanding of the specific roles of Klf5 in adult stem cells from high-turnover tissues is minimal.

Haematopoietic stem cells (HSCs) located in the bone marrow (BM) represent an example of self-renewing cells, which need to replenish billions of cells a day. BM HSCs are located within specialized BM niches where they recognize specific adhesion ligands that are present on cells and on the extracellular matrix. Modulation of HSC adhesion influences essential cellular processes which are required for homing and engraftment, a crucial process that allows HSC to be transplanted in the clinical setting. Major molecular mediators of these effects are the integrins, heterodimeric transmembrane glycoproteins that link extracellular matrix components to the intracellular actin cytoskeleton. Integrins consist of α - and β -subunits that act together to bind specific ligands. At present, 8 α - and 18 β -integrin subunits have been described. Of these, the β 1-subunit, a component in 11 receptors with distinct chains⁵, has been shown to be crucial in the function of HSC^{6,7}. Upon ligand binding, the short cytoplasmic sequence of all integrin variants regulates several types of transmembrane signalling events⁸. Biochemical recycling assays have clearly demonstrated that certain integrin heterodimers are continually internalized from the plasma membrane into endosomal compartments and then recycled back to the cell surface, thus completing an endo-exocytic cycle⁹. Recycling of heterodimers containing β 1-integrins has been shown to be crucial to the control of cell adhesion¹⁰ by controlling the levels of active and inactive β 1-integrin on the cell membrane¹¹. Active endosome trafficking ensures spatio-temporal regulation of the turnover of adhesion complexes at both the trailing and leading edges of a polarized migrating cell. This involves enhanced internalization within early endosomes and recycling at the leading and trailing edges coupled with localized Rac guanosine triphosphatase (GTPase)-dependent organization of the actin cytoskeleton, and increased focal adhesion assembly/disassembly rates.

The Rab family of small GTPases regulates the membrane trafficking and intracellular vesicle transport, including receptor-mediated endocytosis, exocytosis and receptor recycling¹². Aberrant expression of the Rab GTPases has been found in many human diseases including cancer¹³. The Rab5 subfamily members, Rab5a and Rab5b, are mainly located at the cytosolic face of the plasma membrane, early endosomes, and clathrin-coated vesicles. They have been shown to be key regulators of intracellular vesicle traffic from the plasma membrane to early endosomes¹⁴, and their activity is required for maturation of the β 1-integrin-containing early endosomes¹⁵.

In this report, we demonstrate that Klf5 is required for the adhesion, homing and engraftment of HSC. Loss of Klf5 results in decreased membrane expression of active membrane β 1- and β 2-integrin in HSC and progenitor (HSC/P) cells, but does not result in defective primary or stressed hematopoiesis. The reduced expression of membrane β 1/ β 2-integrins on the membrane of HSC/P depends upon Klf5-mediated expression of Rab5.

Results

Klf5 is required for hematopoietic repopulation. To understand the role of Klf5 in HSC/P activity, we crossed *Klf5^{lox/lox}* mice⁴ and *Mx1-Cre* transgenic¹⁶ mice to generate *Mx1-Cre;Klf5^{lox/lox}* mice, and then administered polyinositide:polycytidine (pI:pC) to induce *Mx1-Cre* expression and *in vivo* deletion of floxed Klf5 alleles. Peripheral blood genotyping and expression of Klf5 mRNA and protein in HSC/P demonstrated efficient gene deletion when compared with *Mx1-Cre;Klf5^{+/+}* wild type (WT) mice (Fig. 1a–c) or *Mx1-Cre;Klf5^{lox/lox}* without pI:pC injection as controls (Supplementary Fig. S1). To examine whether the specific loss of Klf5 expression in HSC/P impairs the blood-formation ability of Klf5-deficient mice, peripheral blood was collected at different time points after the last pI:pC injection (Supplementary Fig. S2a), and the numbers of circulating myeloid, T cells and B cells in peripheral blood at 15 and 44 days post pI:pC administration were counted. In agreement with the described role of Klf5 in granulocyte-macrophage differentiation in response to G-CSF signalling^{17,18}, *Klf5^{Δ/Δ}* mice showed a modest decrease in the myeloid cell count, without T- or B-cell lymphopenia (Supplementary Fig. S2b), thrombocytopenia or reticulocytopenia (Day 0 data in Supplementary Fig. S3d,e). BM HSC/P content was also normal in Klf5-deficient mice as analyzed for as long as 6 weeks after the last pI:pC injection (Supplementary Fig. S2c).

Klf5 regulates cell proliferation and survival in other tissues, such as the basal layer of the oesophagus or the intestinal crypts^{19,20}. Klf5 deficiency had no effect on proliferation or survival of HSC/P as assayed by *in vivo* bromodeoxyuridine incorporation or caspase-3 assay, respectively (Supplementary Fig. S3a,b). To assess whether the loss of Klf5 altered the hematopoietic ability of HSC/P function dependent on Klf5 activity under stress conditions, Klf5-deficient and WT mice were injected with 5-fluorouracil, a sublethal cytostatic drug that only spares quiescent HSC and induces stress recovery hematopoiesis in surviving HSC that are forced to enter the cell cycle. Interestingly, we observed no significant recovery impairment in the peripheral blood counts of neutrophils (Supplementary Fig. S3c), platelets (Supplementary Fig. S3d), or reticulocytes (Supplementary Fig. S3e), supporting the concept that Klf5 is dispensable in the regulation of HSC/P-dependent stress-induced hematopoiesis.

Competitive repopulation is the gold standard assay for HSC regenerative activity where a small population of HSC compete against normal HSC to repopulate a full animal. In this assay, intravenously transplanted HSC compete for lodging sites within the BM niches, and fit HSC engraft in a complex process that involves cellular and extracellular matrix adhesion and migration towards chemo-attractant gradients. The contribution of the different HSC/P populations to engraftment is monitored by analyzing the hematopoietic chimera populations in the peripheral blood and BM at different time points. Serial transplantation of competitively transplanted animals provides specific information on long-term engrafting HSC populations. Transplantation of *Klf5^{Δ/Δ}* HSC/P into lethally irradiated recipients revealed abnormal competitive engraftment and hematopoiesis at each test interval for peripheral blood (Fig. 1d) and BM (Fig. 1e), suggesting that all *Klf5^{Δ/Δ}* HSC/P populations were similarly impaired in their ability to contribute to repopulation *in vivo*. This phenotype was also reproduced in serially transplanted secondary recipient mice (Fig. 1f,g), indicating that the repopulation ability of the long-term HSC (LT-HSC) compartment was impaired. The impaired engraftment of Klf5-deficient HSC did not induce significant bias or skew in multi-lineage repopulation. Myeloid, B and T cells behaved similarly to the overall leukocyte engraftment in primary

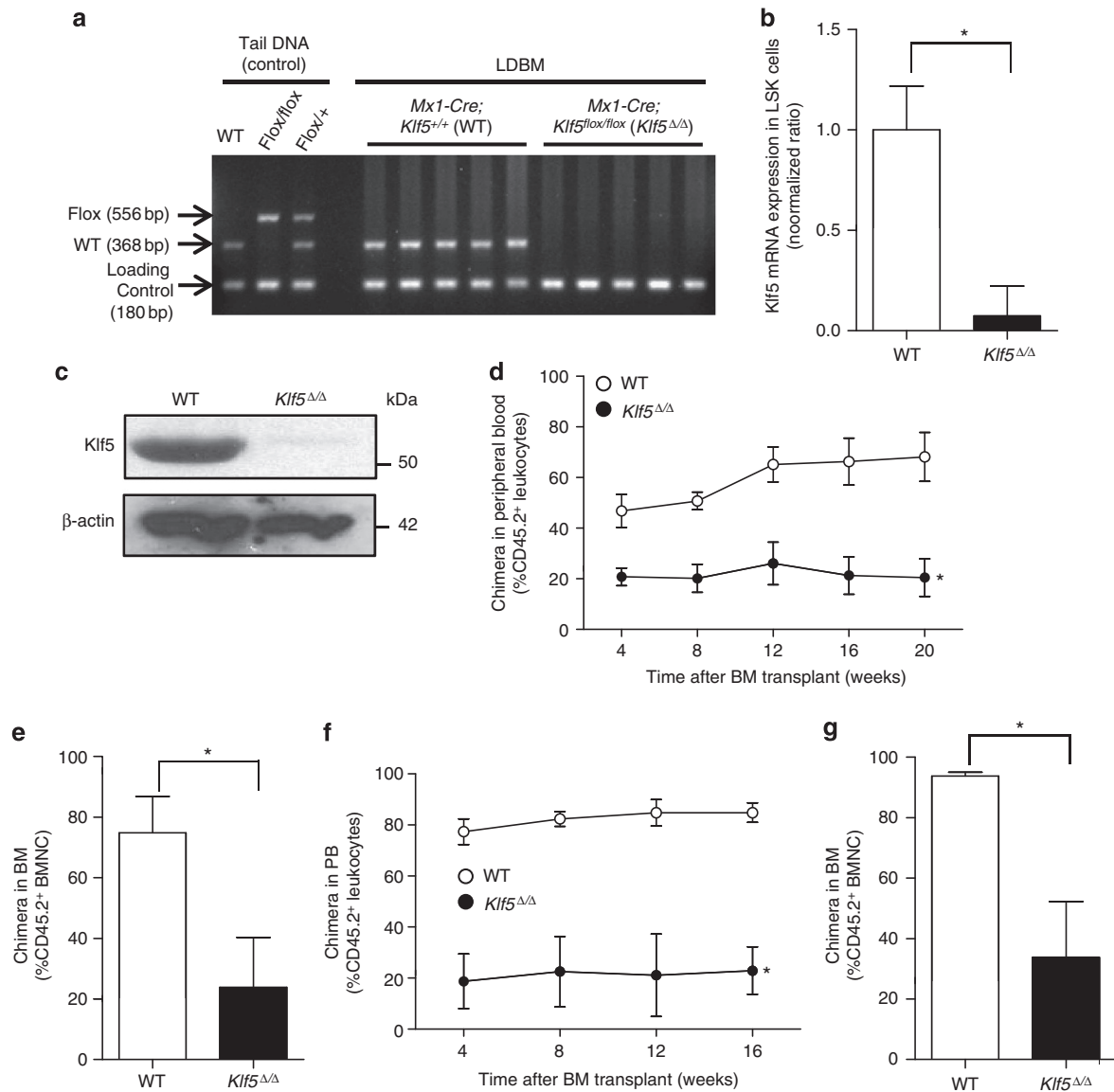


Figure 1 | *Klf5* deficiency impairs HSC competitive repopulation capacity. (a) PCR genotyping of WT, floxed and *Klf5*^{Δ/Δ} alleles from tail and low-density BM (LDBM) cell genomic DNA isolated from the BM of *Mx1-Cre*; WT or *Mx1-Cre*; *Klf5*^{Δ/Δ} mice after five pl:pC injections administered every other day. (b) mRNA expression (Q-RT-PCR) analysis of *Klf5* in LSK cells isolated from pl:pC-treated *Mx1-Cre*, WT or *Mx1-Cre* and *Klf5*^{Δ/Δ} mice. Data are presented as mean \pm s.d., **P* = 0.0002, Student *t*-test. (c) Immunoblot for *Klf5* protein expression in Lin/c-kit-/Sca-1- (LSK) BM cells from pl:pC-treated *Mx1-Cre*; WT or *Mx1-Cre*; *Klf5*^{Δ/Δ} mice. (d) Competitive repopulation assay to determine the role of *Klf5* in HSC engraftment. Lethally irradiated primary recipient CD45.1⁺ mice were transplanted with a mixture of 3×10^6 *Mx1-Cre*; *Klf5*^{Δ/Δ} BM cells (CD45.2⁺) and 3×10^6 WT BM CD45.1⁺ cells (solid circles). The control group was transplanted with a mixture of 3×10^6 *Mx1-Cre*; WT BM cells (CD45.2⁺) and 3×10^6 WT BM CD45.1⁺ cells (open circles). Data are presented as mean \pm s.d., **P* = 0.019, Student *t*-test. (e) Chimera analysis of the BM of primary recipients at 20 weeks after transplantation. Data are presented as mean \pm s.d., **P* < 0.0001, Student *t*-test. (f) Peripheral blood (PB) chimera of secondary recipients transplanted with 10×10^6 BM cells obtained from primary mice (in d). Data are presented as mean \pm s.d., **P* = 0.008, analysis of variance (ANOVA) test. (g) BM chimera in secondary recipients after 4 months of transplantation. **P* = 0.001, Student *t*-test. (b,d-g) Eight to sixteen mice per group were transplanted and analyzed in two independent experiments. Data are presented as mean \pm s.d. BMNC, BM nucleated cell.

and secondary recipients (Supplementary Fig. S4a–f). These data indicate that *Klf5* in HSC/P is dispensable for basal and stressed hematopoietic activity, but indispensable for competitive engraftment of HSC/P.

***Klf5*^{Δ/Δ} HSC/P have decreased adhesion and lodging in BM.** Because proliferation and survival of *Klf5*^{Δ/Δ} HSC/P was not impaired, we next examined whether the impaired competitive repopulation ability of *Klf5*^{Δ/Δ} HSC/P was accompanied

by changes in their homing to the BM. An equal number of WT or *Klf5*^{Δ/Δ} BM cells were injected into non-irradiated WT littermates. Quantitative phenotypic and functional analysis of the homing of HSC/P was performed using flow-cytometry analysis of 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled BM cells or a clonogenic progenitor assay (colony-forming unit cells (CFU-C)). At 16 h after transplantation, femoral BM cells from recipient mice were collected and assayed. The homing of different populations (LT-HSC, short-term HSC (ST-HSC), multipotential progenitors (MPP) and

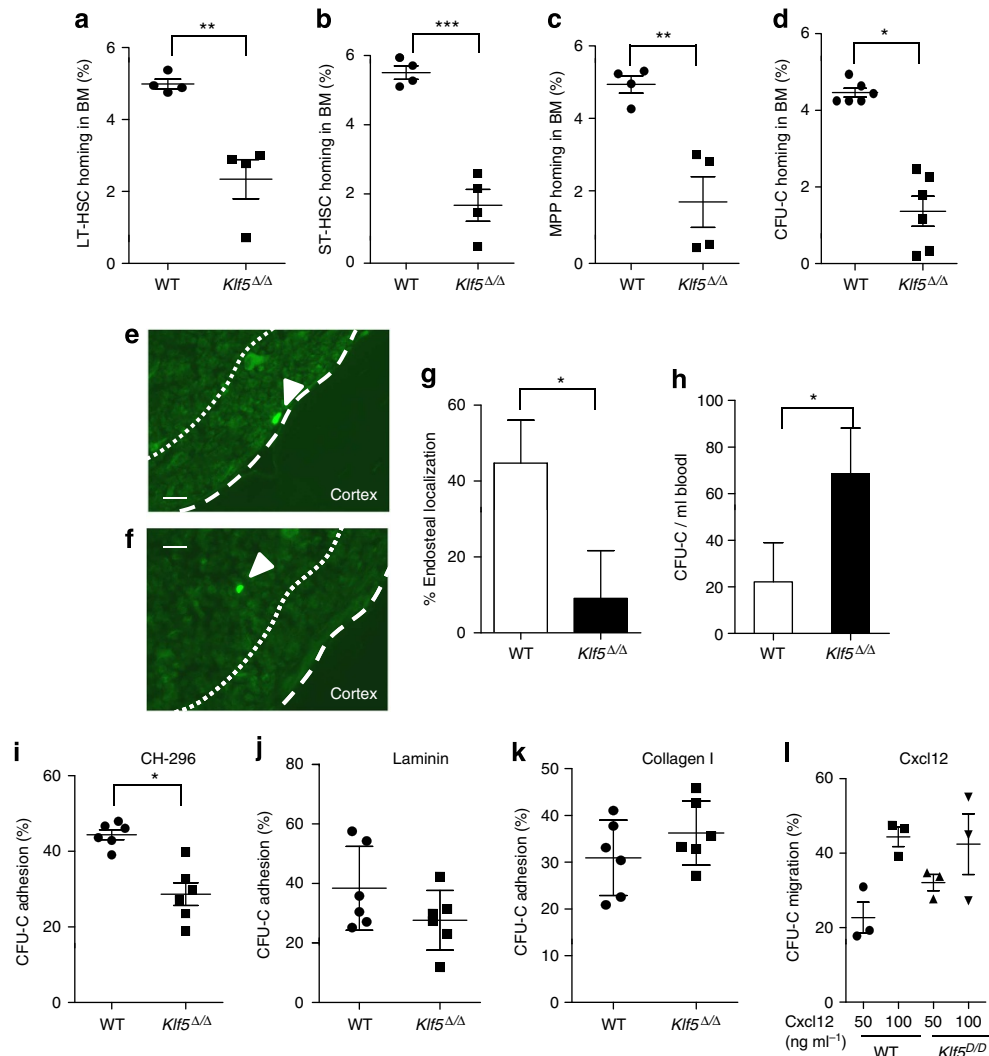


Figure 2 | *Klf5*^{Δ/Δ} HSC/P are impaired in their lodging and adhesion to fibronectin. (a–d) Homing of HSC/P in the BM of WT or *Klf5*^{Δ/Δ} mice at 16 h post transplantation. *n* = 4 to 6 mice per group. (a) Homing of CFSE-labelled LT-HSC (defined as Lin⁻/c-kit⁺/Sca1⁺/CD34⁻/CD135⁻ BM cells), ***P* = 0.0033, Student *t*-test. (b) Homing of CFSE-labelled ST-HSC (defined as Lin⁻/c-kit⁺/Sca1⁺/CD34⁺/CD135⁻ BM cells). ****P* = 0.0003, Student *t*-test. (c) Homing of CFSE-labelled multipotential progenitors (MPPs, defined as Lin⁻/c-kit⁺/Sca1⁺/CD34⁺/CD135⁺ BM cells). ***P* = 0.0047, Student *t*-test. (e,f) Illustration in longitudinal femoral sections of how endosteal localization (e) and central localization (f) were defined for location of transplanted CFSE⁺ LSK BM cells. Short arrows define specific fluorescent cells. Dashed lines indicate the endosteum layer that separates the bone cortex from the BM. Endosteal area was defined as the area covered by 12 cell diameters perpendicular to the endosteum (bidirectional arrow, space between dashed line and dotted line). Central area was defined as the one beyond the endosteal area. Scale bar, 20 μm. (g) Percentage of CFSE⁺ LSK BM WT versus *Klf5*^{Δ/Δ} found in the endosteal area. **P* = 0.04, Student *t*-test, *n* = 56 per group. (h) PB count of CFU-C in primary WT and *Klf5*^{Δ/Δ} mice. **P* = 0.013, Student *t*-test, *n* = 9 per group. (i–k) Adhesion of WT and *Klf5*^{Δ/Δ} BM CFU-C to the recombinant fibronectin fragment CH-296, **P* = 0.007, Student *t*-test, *n* = 6 per group (i), laminin (j) and collagen I (k). (l) Chemotaxis of WT and *Klf5*^{Δ/Δ} BM CFU-C to Cxcl12, *n* = 3 per group.

CFU-C) of *Klf5*^{Δ/Δ} HSC/P was significantly impaired at similar levels (~50–70% reduction, Fig. 2a–d, Supplementary Fig. S5). Specific locations within the BM cavity are associated with enrichment of HSC niches^{21,22}. To evaluate homing to these HSC-niche enriched BM areas, we analyzed the HSC/P lodging in serial femoral longitudinal sections of mice transplanted with CFSE-labelled WT or *Klf5*-deficient Lin⁻/c-kit⁺/Sca-1⁺ (LSK) cells, as previously described^{23,24}. Lodging of LSK cells (Fig. 2e,f) within the putative microanatomical endosteal space of the BM (Fig. 2e) was significantly impaired. About 45% of WT HSC/P lodged in the endosteal region, whereas only 9% of the *Klf5*-deficient HSC/P that homed to the BM were found inside the endosteal area (*P* < 0.05) (Fig. 2g). The deficient BM homing/lodging of transplanted HSC/P was also associated with a ~3.5-

fold increase in circulating HSC/P (Fig. 2h), while the number of HSC/P in the BM was the same in both WT and *Klf5*-deficient mice (Supplementary Fig. S2c). Together, these data indicate that the retention of *Klf5*^{Δ/Δ} HSC/P in BM is decreased.

HSC/P adhesion and retention within the BM largely depend on their binding ability to key proteins, components of the extracellular matrix. Among them, binding to fibronectin and laminin has been postulated to be crucial in the control of HSC adhesion to BM niches^{25,26}. During HSC engraftment, fibronectin binds to heterodimers formed by β1-integrins²⁷, and laminin binds to heterodimers containing β1- and β3-integrin chains²⁵. We found that the adhesion of *Klf5*^{Δ/Δ} HSC/P to CH-296, a C-terminus fibronectin peptide, was significantly decreased compared with WT HSC/P (Fig. 2i). In contrast, the adhesion

ability of *Klf5*^{Δ/Δ} HSC/P to laminin (Fig. 2j) or collagen type I (Fig. 2k), which bind to multiple partner receptors including different β -integrin chains²⁸, was not significantly impaired. The chemokine Cxcl12 is a crucial molecule that mediates the retention and homing of HSC/P^{29,30}. The migration of *Klf5*-deficient HSC/P towards a Cxcl12 gradient (Fig. 2l) was not significantly impaired, regardless the tested concentration (50–100 ng ml⁻¹). These findings indicate that the deletion of *Klf5* in HSC/P impairs their ability to engraft BM and is associated with defects in BM homing/lodging and fibronectin adhesion.

***Klf5* gain-of-function increases adhesion to fibronectin.** To delineate whether *Klf5* expression regulates the adhesion of HSC/P, we crossed stem cell leukaemia (*Scf*) promoter-driven tetracycline transactivator (*Scf-tTA*) transgenic mice³¹ with transgenic animals expressing *Klf5* driven by a tetracycline responsive element (*TRE*)³² to generate *Scf-tTA/TRE-Klf5* mice. In this murine

model, *Klf5* overexpression is induced after doxycycline withdrawal and is restricted to HSC and early progenitors expressing the transcriptional factor *Scf*¹⁶. Serial protein-expression analysis from *Scf-tTA/TRE-Klf5* mice demonstrated a peak of *Klf5* expression in LSK BM cells by day +5 after doxycycline withdrawal (tet-off) (Fig. 3a), which was confirmed by immunoblotting of sorted BM LSK cells (Supplementary Fig. S1). Quantitative PCR with reverse transcription (Q-RT-PCR) of LSK BM cells showed a fivefold increase in the levels of *Klf5* mRNA by the same day (Fig. 3b). Overexpression of *Klf5* was associated with increased (~50%) adhesion of LSK BM cells to fibronectin (Fig. 3c), whereas their migratory ability towards Cxcl12 was not significantly changed (Fig. 3d). The homing of *Klf5*-overexpressing HSC was not significantly improved (Fig. 3e), suggesting functional saturation of *in vivo* homing. Altogether, the results of loss-of-function and gain-of-function of *Klf5* expression indicated that *Klf5* regulates the adhesion of HSC/P to fibronectin.

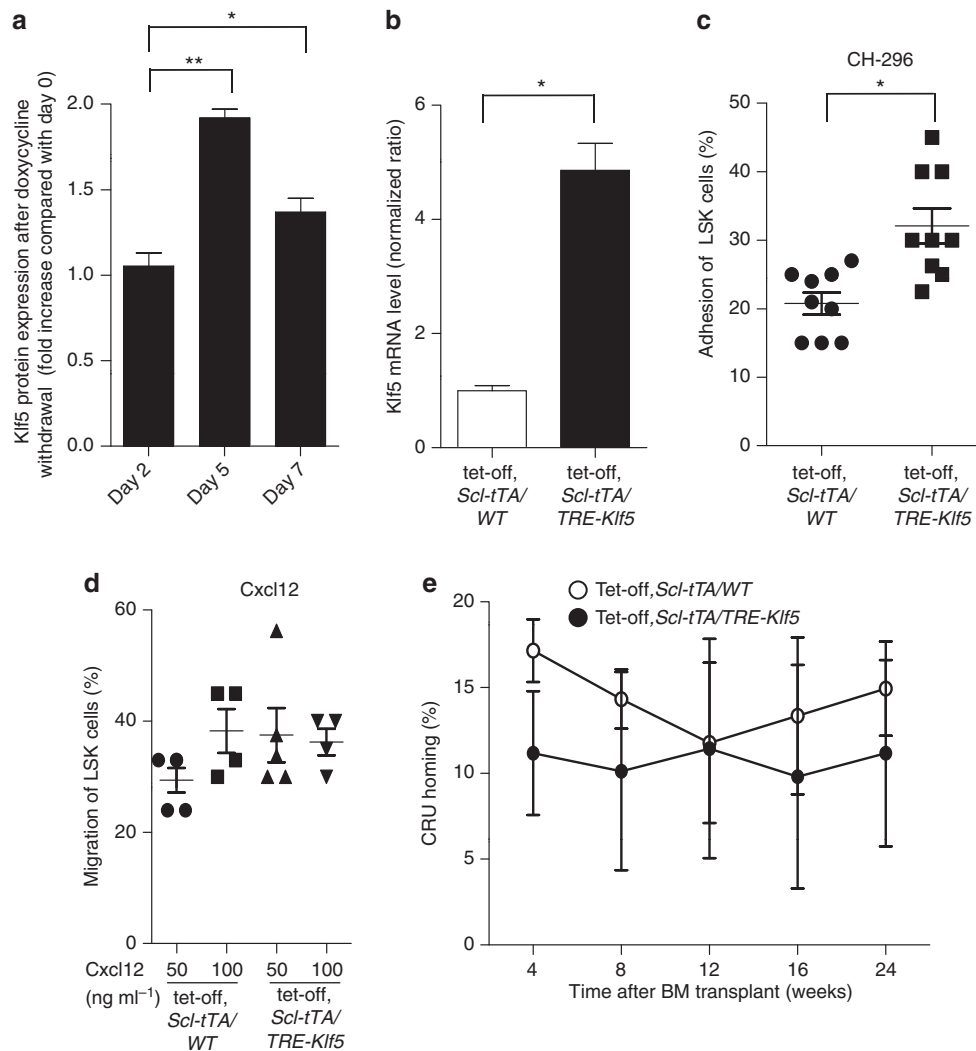


Figure 3 | Inducible expression of *Klf5* *in vivo* induces increased adhesion to fibronectin and normal BM homing. (a,b) *Klf5* expression in LSK BM cells isolated from tet-off, *Scf-tTA/WT* (WT) or *Scf-tTA/TRE-Klf5* mice. (a) Protein was measured by intracellular flow cytometry on BM LSK cells from *Scf-tTA/TRE-Klf5* mice collected on days 2, 5 and 7 post doxycycline withdrawal. $N = 3$ independent animals per group. Data represent mean \pm s.e.m. $**P = 0.001$, $***P = 0.0006$, Student *t*-test. (b) mRNA on sorted BM LSK cells after 5 days post doxycycline withdrawal. $***P = 0.0002$, Student *t*-test. (c) Adhesion of LSK BM cells to fibronectin from tet-off, WT or *Scf-tTA/TRE-Klf5* mice. $*P = 0.019$, Student *t*-test. (d) Chemotaxis of LSK BM cells to CXCL12 from tet-off, WT or *Scf-tTA/TRE-Klf5* mice. Data are presented as mean \pm s.d. (e) Homing of WT and *Klf5*-overexpressing HSC. Graph depicts the homing (%) after 3 h post transplantation of competitive repopulation units (CRU) analyzed as engrafting units after co-transplantation with CD45.1⁺ competitor BM cells into congenic recipients, analyzed at 4, 8, 12, 16 and 24 weeks post transplantation. $N = 8$ to 10 mice per group, from two independent experiments.

Klf5 regulates the localization of β 1-/ β 2-integrins in HSC/P. Based on the data supporting a role for Klf5 in BM HSC/P homing and fibronectin-mediated adhesion, we hypothesized that Klf5 may control β -integrin function. To understand whether the expression of β integrins was affected by Klf5 expression, we analyzed the cell-membrane expression of β 1-, β 2-, β 3-, β 7-integrin and two major alpha chain partners in HSC/P, α 4- and α 5-integrin, and the homing cell-adhesion molecule, which also binds to the fibronectin fragment CH-294. β 1-integrin has been reported to be essential for HSC/P homing through binding to fibronectin^{33,34}, while β 2-integrin expression has a minimal role in HSC homing³⁵. β 3 and β 7 integrin chains, also expressed in HSC/P, are not well characterized functionally in relation to HSC/P homing.

We found that the membrane expression of β 1- and β 2-integrins was significantly decreased in Klf5-deficient LSK BM cells compared with their WT counterparts (Fig. 4a, $P=0.025$, Student *t*-test). The expression levels of membrane α 4-, α 5-, β 3- or β 7-integrins were, however, not significantly changed (Supplementary Fig. S6a,b). Interestingly, HSC/P whole-cell lysate immunoblots showed upregulation of the overall cellular level of β 1- or β 2-integrin protein expression (Fig. 4b). *Scl-tTA/TRE-Klf5* transgenic expression in BM HSC/P resulted in the opposite phenotype of *Klf5* ^{Δ/Δ} HSC/P and was associated with increased membrane expression of β 1- and β 2-integrin, but not other integrin chains (Fig. 4c and Supplementary Fig. S6c), indicating the existence of a positive correlation between the expression level of Klf5, membrane β 1- and β 2-integrin and adhesion to fibronectin. Consistent with the post-translational nature of the changes in β 1-/ β 2-integrin expression, mRNA levels of Klf5-deficient and Klf5-overexpressing mice were not affected by the level of expression of Klf5 compared with their control counterparts (Supplementary Fig. S6d). In agreement, with the absence of difference in Cxcl12-directed migration, we found that the expression level of Cxcl12 receptor, Cxcr4, in HSC/P was not affected by the expression of Klf5. Its levels in Klf5-deficient or *Scl-tTA/TRE-Klf5* HSC/P, were comparable to those in WT HSC/P (Supplementary Fig. S6e,f). These results suggest that Klf5 specifically controls the localization of β 1- and β 2-integrins on the cell membrane.

Klf5 controls the expression of Rab5 mRNA in HSC/P. To identify direct Klf5 targets involved in adhesion activity and expression of β 1- and β 2-integrin on the cell membrane, we performed gene-expression profiling using cDNA from RNA isolated from WT or *Klf5* ^{Δ/Δ} HSC/P (Gene Expression Omnibus

(GEO) accession number GSE43806). Of the 1,146 genes that showed significant differential expression, gene ontology analysis identified 14 major functional subgroups (Supplementary Fig. S7a). The expression of other Klf genes was not significantly modified by Klf5 deficiency (Supplementary Table S1), and Ingenuity analysis identified regulation of two groups of genes responsible for gene processes as statistically significant (Supplementary Table S2). A search for downregulated genes with known activity on integrin transport identified several members of the Rab GTPase family, including *Rab5b*, *Rab17*, *Rab19* and the downstream Rab3 effector *Rph3a*. Rab proteins are small GTPases that are involved in the traffic of endocytosis vesicles, control the endosomal traffic of β -integrins²⁹ and control cell adhesion³⁰. Although Rab3 and Rab19 are associated with the process of general trans-Golgi or post-Golgi transport³⁶, Rab5 and Rab17 have a specific role in controlling the formation of early endosomes and, therefore, endosomal traffic^{37,38}, an essential in β -integrin recycling³⁹. We hypothesized that HSC/P Klf5 could transcriptionally regulate the expression of Rab5 and/or Rab17, thereby regulating β 1- and β 2-integrin trafficking from the cell membrane. Q-RT-PCR analysis showed that Klf5 regulates *Rab5a/b* expression in both *Klf5* ^{Δ/Δ} (~50% reduction) and *Scl-tTA/TRE-Klf5* transgenic HSC/P (Fig. 5a,b, Supplementary Fig. S7b), but Q-RT-PCR did not confirm downregulated expression of *Rab17* mRNA expression in Klf5-deficient HSC/P (Supplementary Fig. S7c). Decreased *Rab5a/b* mRNA expression in Klf5-deficient HSC/P was associated with decreased *Rab5a/b* protein expression, which also decreased ~50% (Fig. 5c). We identified the putative Klf5-binding sites (CACCC- and GC-rich motif) in the promoter region of *Rab5a* and *Rab5b*. Chromatin immunoprecipitation analysis revealed that Klf5 binds to upstream sequences near the transcription start site of both *Rab5a* and *Rab5b* loci in BM HSC/P (Fig. 5d). Taken together, these results suggest that *Rab5a* and *Rab5b* genes are direct transcriptional targets of Klf5.

Overexpressed Rab5b rescues Klf5-dependent HSC/P activity.

Cell homing requires regulated turnover of adhesions, which can be achieved by a combination of integrin activation and inactivation at the cell surface and dynamic cell-surface targeting and endocytic removal of activated integrins from the plasma membrane^{40,41}. We hypothesized that Rab5 controls the levels of active β 1- and β 2-integrin on the membrane, and that the deficient homing of *Klf5* ^{Δ/Δ} HSC/P was a result of their inability to express active β 1-integrin on the membrane, available to bind fibronectin. If true, the exogenous expression of Rab5b in *Klf5* ^{Δ/Δ}

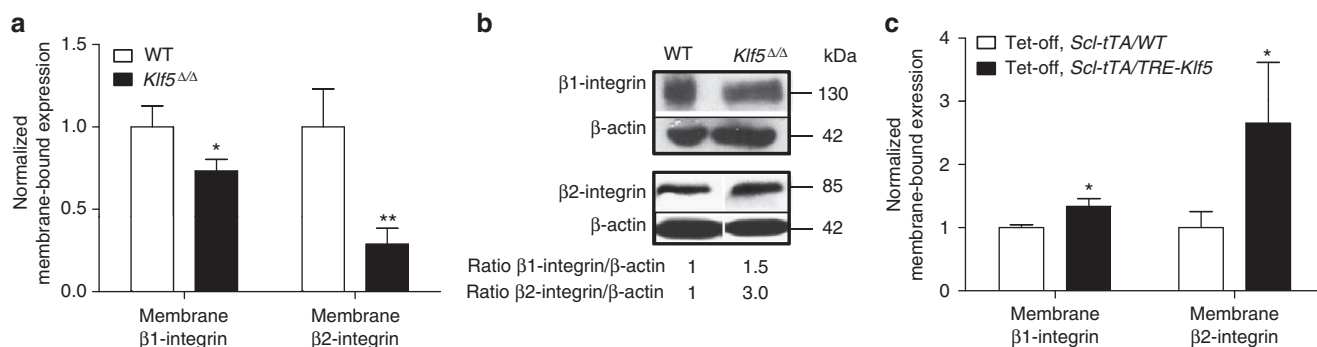


Figure 4 | Defective *Klf5* ^{Δ/Δ} HSC/P homing is attributed to a post-translational decrease in membrane-bound β 1- and β 2-integrins. (a) Expression of membrane β 1- and β 2-integrin in WT (empty bars) and *Klf5* ^{Δ/Δ} (solid bars) LSK BM cells as assessed by flow cytometry (normalized mean fluorescence intensity ratio). * $P=0.03$, ** $P=0.004$, Student *t*-test, $n=3$ to 4 per group. (b) Representative example of immunoblot of β 1- and β 2-integrin expression of whole-cell lysates from WT and *Klf5* ^{Δ/Δ} LSK BM cells. Densitometric ratio analysis was calculated. (c) Expression of membrane β 1- and β 2-integrin in LSK BM cells from tet-off, *Scl-tTA*/WT (empty bars) or *Scl-tTA*/TRE-*Klf5* mice (solid bars). * $P=0.01$, Student *t*-test, $n=4$ per group.

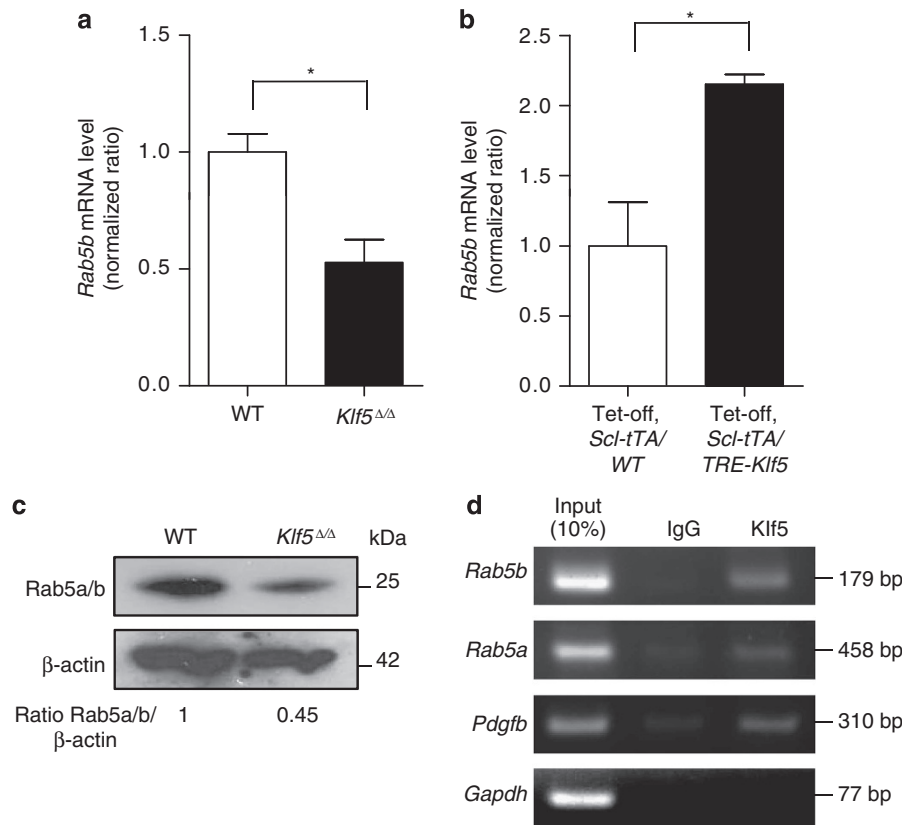


Figure 5 | Klf5 directly regulates the expression of Rab5 family proteins. (a,b) $n = 3$ per group; *Rab5b* expression (Q-RT-PCR) of LSK BM cells from WT and *Klf5* $\Delta\Delta$ mice (a) or tet-off, *Scl-tTA*/WT and *Scl-tTA*/TRE-*Klf5* mice. $*P = 0.028$, Student *t*-test. (b) Data are presented as mean \pm s.d. ($*P = 0.033$, Student *t*-test, $n = 3$ mice per group). (c) Representative example of immunoblotting of Rab5a/b in WT and *Klf5* $\Delta\Delta$ LSK BM cells. $N = 3$ mice per group. (d) Chromatin immunoprecipitation analysis on lysates from Lin $^-$ BM cells. Following the immunoprecipitation using Klf5-specific antibody, PCR was performed using primers designed to encompass the putative Klf5-binding site in the proximal promoter region. Amplification of a *Pdgfb* genomic region serves as positive control. Immunoprecipitation using IgG and amplification of the *Gapdh* gene serve as negative controls.

HSC/P should rescue the expression of both membrane-bound active $\beta 1$ - and $\beta 2$ -integrins, and revert the adhesion and homing defects. For this purpose, we performed rescue experiments of adhesion and homing of HSC/P isolated from *Vav1-Cre/Klf5* $^{lox/lox}$ mice where *Vav1*-promoter-driven *Cre* expression restricts the deficiency of Klf5 to hematopoietic cells⁴². These mice are viable, have a normal lifespan and their HSC/P completely lack Klf5 protein expression (hematopoietic *Klf5* $\Delta\Delta$, H-*Klf5* $\Delta\Delta$, Fig. 6a). H-*Klf5* $\Delta\Delta$ LSK BM cells were lentivirally transduced with bicistronic vectors expressing either Rab5b and enhanced green fluorescent protein (EGFP) (Rab5b) or only EGFP (Mock). Q-RT-PCR confirmed the complete loss of Klf5 transcript expression in H-*Klf5* $\Delta\Delta$ HSC/P and the presence of exogenous Rab5b expression in Rab5b-transduced cells (Supplementary Fig. S7d,e). Immunofluorescence analysis by both flow-cytometry and confocal microscopy analysis of $\beta 1$ -integrin expression on the membrane of EGFP $^+$ HSC/P showed that exogenous expression of Rab5b rescued total membrane $\beta 1$ -integrin expression (Fig. 6b,c), and especially membrane-bound active $\beta 1$ -integrin expression (Fig. 6d–g), but did not significantly modify the levels of inactive membrane $\beta 1$ -integrin (Supplementary Fig. S8a–d). Similarly, membrane $\beta 2$ -integrin expression was also rescued by exogenous expression of Rab5b (Fig. 7a–d), suggesting that restoration of membrane expression of active $\beta 1$ - and $\beta 2$ -integrin depend on Rab5 expression.

Functionally, the exogenous expression of Rab5b rescued the impaired adhesion of H-*Klf5* $\Delta\Delta$ HSC/P to fibronectin, but unlike the overexpression of Klf5 (Fig. 3c), the overexpression of Rab5b

did not increase the adhesion of WT HSC/P to fibronectin (Fig. 8a). Finally, the overexpression of Rab5b in H-*Klf5* $\Delta\Delta$ HSC/P restored their ability to home to the BM to the same level as WT HSC/P (Fig. 8b). These results indicate that HSC/P BM homing activity is dependent on direct Klf5 transcriptional control of *Rab5* expression, which mediates active $\beta 1$ -integrin expression on the cell membrane and subsequent HSC/P adhesion to the BM microenvironment.

Discussion

The Klf transcriptional factor family has both essential and redundant functions. Klf5, similar to the other 16 members of the Klf family, contains three zinc-finger domains that function in DNA binding. In hematopoiesis, Klf proteins have been shown to have redundant roles. For instance, Klf1- and Klf2-dependent globin-expression regulation have been shown to compensate each other in primitive erythropoiesis, and Klf1 has a distinct role in the expression of β -globin in definitive erythropoiesis⁴³. Klf2, Klf4 and Klf5 have redundant roles in a circuitry controlling embryonic stem cell pluripotency⁴⁴. Klf5 is highly expressed in HSCs and differentiated myeloid cells⁴⁵. Klf5 expression, controlled by Klf4, appears to control the granulocyte-macrophage differentiation of committed myeloid progenitors/precursors in response to granulocyte colony-stimulating factor signalling^{17,18} and to regulate monocyte/macrophage differentiation and activation^{46,47}. In embryonic stem cells, the Klf family members Klf2, Klf4 and Klf5 have

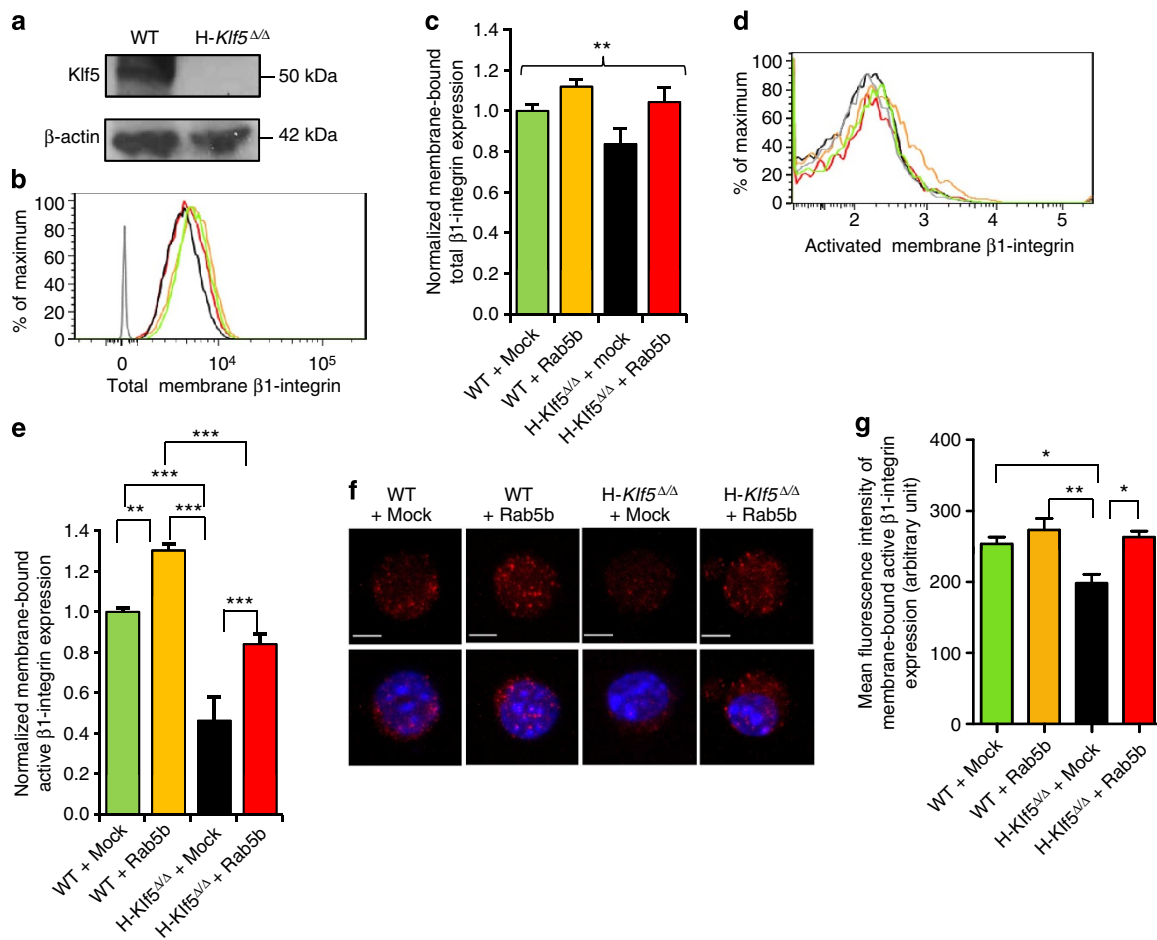


Figure 6 | Lentiviral-vector-mediated Rab5b expression rescues the total and activated fraction of membrane-bound β 1-integrin in $Klf5^{\Delta/\Delta}$ HSC/P.

(a) Immunoblotting of Klf5 in LSK isolated from *Vav1-Cre-WT* (WT) or *Vav1-Cre-Klf5^{fllox/fllox}* mice (*H-Klf5 Δ/Δ*). β -actin shown in figure as a loading control. (b–e) Fluorocytometric analysis of membrane-bound integrin expression of mock- or Rab5b-transduced LSK BM cells from WT or *H-Klf5 Δ/Δ* mice. Sorted LSK BM cells were transduced with a lentiviral vector expressing Rab5b and eGFP or eGFP only (Mock) as a control WT-Mock (green line or bar), WT-Rab5b (orange line or bar), *H-Klf5 Δ/Δ* -Mock (black line or bar) and *H-Klf5 Δ/Δ* -Rab5b (red line or bar) LSK BM cells. (b,c) Membrane expression of total β 1-integrin. (b) Representative example of flow-cytometry analysis; (c) normalized membrane-bound total β 1-integrin expression ($n=3$ replicates per group). $**P=0.0042$, analysis of variance (ANOVA test). (d,e) Membrane expression of activated β 1-integrin. (d) Representative example of flow-cytometry analysis. (e) Normalized membrane-bound activated β 1-integrin expression ($n=3$ replicates per group). (f,g) Confocal microscopy analysis of membrane expression of activated β 1-integrin. (f) Representative picture of membrane-bound active β 1-integrin (red) and 4',6-diamidino-2-phenylindole (DAPI; blue, overlaid in lower row pictures). Scale bar, 5 μ m. (g) Mean fluorescence intensity of membrane-bound active β 1-integrin expression as analyzed by confocal microscopy. A minimum of 50 cells per group was analyzed by confocal microscopy. (e,g) Data are presented as mean \pm s.e.m., $*P<0.05$, $**P<0.01$, $***P<0.001$. ANOVA test with Bonferroni correction.

redundant roles in self-renewal regulation⁴⁴, and Klf5 is required to prevent differentiation⁴⁸. However, the specific roles of Klf5 in postnatal stem cell activity remain to be determined.

We report a previously unknown role for Klf5 in BM adhesion, retention, homing, endosteal lodging and engraftment of HSC/P. Although Klf5 is not essential for HSC self-renewal, Klf5 is an essential transcriptional factor controlling BM homing, lodging in the endosteal space, engraftment and BM retention of HSC/P. Using gene-expression profiling validated by Q-RT-PCR in WT or *Klf5 Δ/Δ* HSC/P, we identified several members of the Rab GTPase family, including *Rab5a*, *Rab5b* and *Rab17* as downstream targets of Klf5. Of these, transcriptional regulation of Rab5 was demonstrated, and protein levels and chromosome immunoprecipitation confirmed that Klf5 binds Rab5a and Rab5 proximal promoters in HSC/P. Klf5-downstream targets in other stem cells such as embryonic stem cells do not include Rab5 genes⁴⁹, strongly suggesting that the cellular context of transcriptional co-activators is crucial in target selection.

Rab5 function is essential for endosomal traffic^{37,38}, which is essential for β -integrin recycling³⁹. Decreased expression of Rab5 proteins interferes with the membrane localization of heterodimers containing β 1- and β 2-integrins (but not α -integrin chains or CD44), which results in defective adhesion of HSC/P to fibronectin. Inducible overexpression of Klf5 in HSC results in the opposite effect by increasing the membrane expression of β 1- and β 2-integrins and adhesion to the relevant extracellular matrix protein fibronectin²⁶. However, Klf5 overexpression does not result in increased BM homing ability over WT HSC/P, suggesting that Klf5-mediated responses are saturated at baseline *in vivo*. Exogenous expression of Rab5b corrects the membrane localization of activated β 1- and β 2-integrins, the HSC/P adhesion to fibronectin, and *in vivo* BM homing, which is associated with decreased adhesion to fibronectin and *in vivo* BM homing of Klf5-deficient HSC/P.

Our work indicates that Klf5 is a positive regulator of the lodging of HSC/P within the BM niches through the regulation of

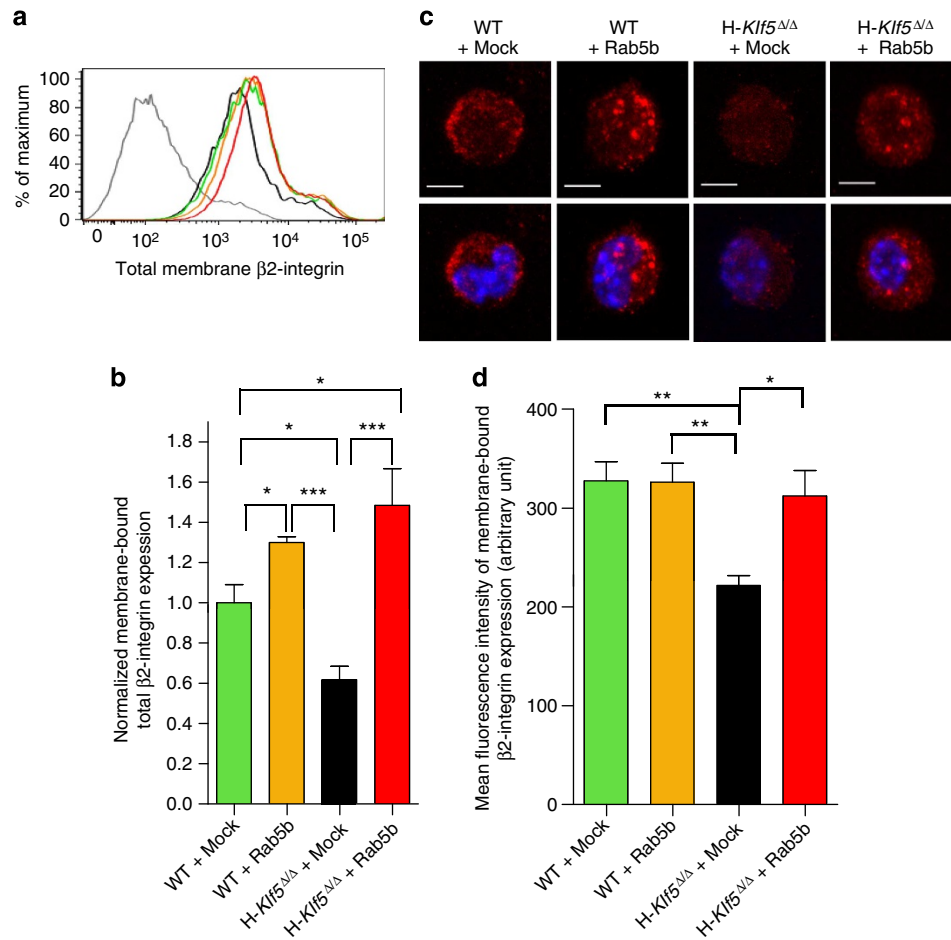


Figure 7 | Lentiviral-vector-mediated Rab5b expression rescues the membrane-bound $\beta 2$ -integrin expression in $Klf5^{\Delta/\Delta}$ HSC/P. (a–d) Membrane expression of $\beta 2$ -integrin. **(a)** Representative example of flow-cytometry analysis. **(b)** Normalized membrane-bound total $\beta 2$ -integrin expression analyzed by flow cytometry. Data are presented as mean \pm s.e.m. ($n = 3$ replicates per group). **(c)** Representative example of membrane expression of membrane $\beta 2$ -integrin (red) analyzed by confocal microscopy. 4',6-diamidino-2-phenylindole (DAPI, blue, overlaid in lower row pictures) was used for nuclear counterstain. Scale bar, 5 μ m. **(d)** Mean fluorescence intensity of membrane-bound $\beta 2$ -integrin expression as analyzed by confocal microscopy. A minimum of 50 cells per group was analyzed by confocal microscopy. Data are presented as mean \pm s.e.m., * $P = 0.05$, ** $P = 0.01$, *** $P = 0.001$, ANOVA test with Bonferroni correction.

Rab5-dependent integrin recycling. Although other *Klf* genes may partly replace *Klf5*, we observed no apparent compensatory transcriptional regulation of other *Klf* genes in HSC/P. Our findings underscore the importance of the binding of $\beta 1$ - and $\beta 2$ -integrins containing heterodimers to functionally active HSC niches within the BM⁶, such as those that require fibronectin adhesion²⁷. Under steady-state conditions, that is, when HSC/P are firmly adherent to the BM stroma, cells express multiple integrins, however, the main integrin determinants of steady-state HSC/P adhesion to the BM stroma are the $\alpha 4\beta 1$ - and $\alpha 5\beta 1$ -integrins, whose counter receptors are either fibronectin or the cell-surface ligand, VCAM-1. In fact, $\alpha 4$ -integrin expression is essential for HSC/P adhesion and is different in BM HSC/P compared with circulating HSC/P⁵⁰. A deficiency of one or more β -chains can be compensated by the formation of heterodimers with alternative β -chains like $\beta 3$ -integrins, which are also expressed by HSC⁵¹. The conformation of integrins, and thus their ability to bind to extracellular matrix proteins, is modulated by the interaction of their cytoplasmic domain with intracellular signalling and cytoskeletal proteins. Outside-in activation of integrins results from a conformational change upon ligand binding. Activation involves severing a salt bridge that tethers the cytoplasmic domains of the α - and β -chains and conformational rearrangement of the A domain of the β -chain to form a high-

affinity matrix-binding pocket, bringing the integrin into the fully active state⁵². A general feature of activated integrin traffic is that, rather than degradation, they undergo internalization and are subsequently recycled back to the cell surface⁹. Up to 50% of activated integrins on the cell surface are recycled back to the membrane. Both internalization and recycling rates are accelerated by Rab proteins. The specificity of integrin-Rab protein binding has been shown in mutational analysis of the cytosolic domain of the $\beta 1$ -integrin heterodimers, which mediates interaction with the early endosomal Rab proteins Rab5 and Rab17 (ref. 38). The process of integrin internalization and recycling ensures spatio-temporal regulation of the activated integrin complex turnover at both the trailing and leading edges of a polarized cell upon adhesion to the extracellular matrix. Activated integrins are continuously transported to a pericentriolar/juxtannuclear recycling compartment using the clathrin/Rab5/Rab11 endosome system. As the earliest endosome-interacting Rab, Rab5 protein has key roles in both cell adhesion and motility through the regulation of $\beta 1$ -integrin recycling⁵³. A reduction of as little as 50% in the levels of Rab5 proteins is sufficient to abrogate cellular endocytosis⁵⁴. Although Rab5-GTP is the active form, the phenotype induced by *Klf5* deficiency seems to respond to a decrease in the expression of Rab5 genes, because overexpression of Rab5b rescues integrin

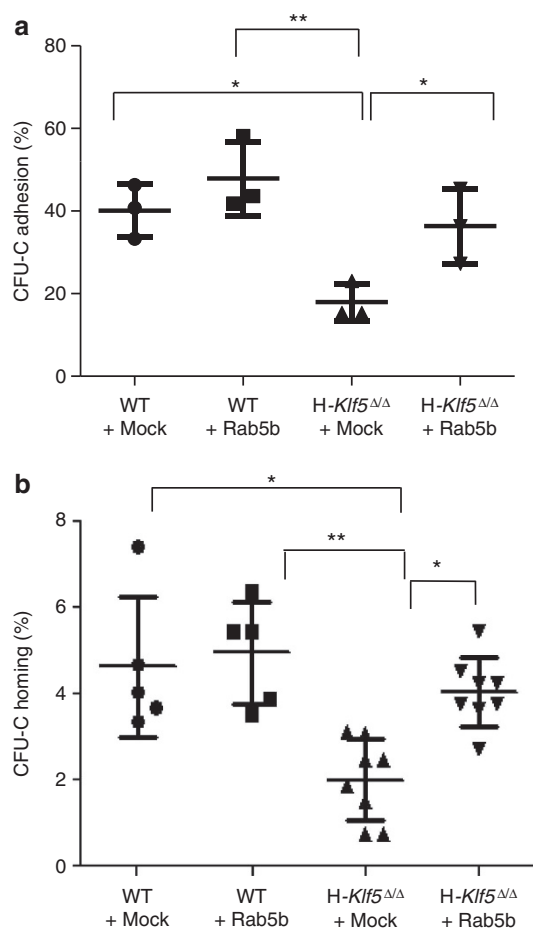


Figure 8 | Forced Rab5b expression rescues the impaired adhesion and homing defect of hematopoietic-specific *Klf5* Δ/Δ HSC/P. (a) Adhesion to fibronectin of *H-Klf5* Δ/Δ LSK cells was rescued by exogenous expression of Rab5b ($n=3$ per group, in each of two independent experiments). (b) CFU-C homing in the BM of *H-Klf5* Δ/Δ mice was rescued by lentivirus transduction of Rab5b. Homing was analyzed at 16 h after transplantation (similar to Fig. 2a–d). Data are presented as mean \pm s.d. $n=5$ to 8 mice per group; * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ANOVA test with Bonferroni correction.

expression on the membrane, adhesion to fibronectin and BM homing of HSC/P. Mechanistically, Rab5 activity has been shown to be independent of phosphoinositide 3-kinase and the small GTPases, Ras, Rho and Cdc42 (ref. 55) and to be dependent on Rac1 activation downstream⁵⁶, which is required for HSC homing²⁴. Our data indicate that Rab5 deficiency induced by loss of the transcriptional factor *Klf5* results in the loss of membrane-bound expression of $\beta 1$ - and $\beta 2$ -integrins, including the active form of $\beta 1$ -integrin, and results in decreased adhesion to the extracellular matrix and homing and lodging in the BM endosteal space.

Altogether, our data indicate that *Klf5* has an essential role in the transcriptional regulation of Rab5 expression, which in turn controls $\beta 1$ - and $\beta 2$ -integrin availability and localization within the membrane, and HSC/P adhesion and homing within specific BM HSC/P niches.

Methods

Animal models. All the animals were backcrossed for a minimum of five generations into C57Bl/6 background. For inducible *Klf5* gene inactivation in the hematopoietic compartment, *Mx1-Cre* mice¹⁶ were crossed with *Klf5*^{flox/flox}

mice⁴ (*Klf5* Δ/Δ). *Klf5* deletion was induced by intraperitoneal administration of double-stranded RNA pI:pC (Amersham Pharmacia Biotech, Germany) (200 μ g) every other day for a total of five times. Experiments were performed after a minimum of 7 days after the last pI:pC injection. For constitutive *Klf5* gene inactivation, *Vav1-Cre* mice⁴² were crossed with *Klf5*^{flox/flox} mice. For inducible *Klf5* overexpression in HSC/P, *Scl-tTA* transgenic mice³¹ were crossed with *TRE-Klf5* mice³². BM was analyzed on day +5 post doxycycline withdrawal.

HSC competitive repopulation assay. Adult recipient mice were lethally irradiated (7 + 4.75 Gy, split doses at a dose rate of 58–63 cGy min⁻¹; separated by 3 h, and previously demonstrated to eliminate all endogenous BM CFU-C)²⁴. For competitive repopulation experiments, 3×10^6 CD45.2⁺ BM nucleated cells were mixed with 3×10^6 CD45.1⁺ BM nucleated cells and were transplanted into lethally irradiated CD45.1⁺ B6.SJLPrca Pep3b/BoyJ recipient mice. ST-HSC and LT-HSC engraftment was analyzed in peripheral blood at 4-week intervals and on week 20 in the BM, to analyze the contribution of CD45.2⁺ leukocyte chimera and lineage differentiation by flow-cytometry analysis.

Homing assays. WT or *Klf5* Δ/Δ animals were killed, and single cell suspensions of the BM from the lower limbs and pelvis were prepared and used for transplantation. BM cells (2×10^4) were plated in methylcellulose culture media (MethoCult; Stem Cell Technologies, Vancouver, BC, Canada) for CFU-C assay to quantify the initial content of hematopoietic progenitors. Ten million BM donor cells were injected through the tail vein into lethally irradiated C57Bl/6 recipient mice. Sixteen hours after transplant, the recipient mice were killed and the BM cells of the lower limbs and pelvis were collected and cultured in triplicate for CFU-C assay. Homing of immunophenotypically identified HSC/P was performed as previously published⁵⁷. Briefly, 20×10^6 low-density WT or *Klf5*-deficient BM cells were labelled with 5 μ M CFSE (Molecular Probes, Life Technologies, Grand Island, NY). Cells were labelled for analysis of LT-HSC, defined as Lin⁻/c-kit⁺/Sca1⁺/CD34⁺(RAM34)⁻/CD135 (4G8)⁻ ST-HSC, defined as Lin⁻/c-kit⁺/Sca1⁺/CD34⁺/CD135⁻ and multipotential progenitor, defined as Lin⁻/c-kit⁺/Sca1⁺/CD34⁺/CD135⁺ BM cells, before and 16 h after infusion into congenic myeloablated animals.

For homing of transduced HSCs/Ps, 1×10^3 sorted LSK cells (WT + Mock, WT + Rab5b, *Klf5* Δ/Δ + Mock or *Klf5* Δ/Δ + Rab5b) were plated in methylcellulose culture media for CFU-C assay to quantify the initial content of hematopoietic progenitors. Sorted LSK donor cells (1×10^4) were injected through the tail vein into lethally irradiated C57Bl/6 recipient mice. Sixteen hours after transplant, the recipient mice were killed and the BM cells of the lower limbs and pelvis were collected and cultured in triplicate for CFU-C assay. To estimate the BM recovery, the femora, tibiae and pelvis were considered to contain 25% of the overall BM⁵⁸.

For *Klf5*-overexpressing HSC homing assay, 2.5×10^8 BM cells from tet-off, *Scl-tTA*/WT (WT) or *Scl-tTA*/TRE-*Klf5* mice were injected through the tail vein into lethally irradiated (7 + 4.75 Gy) C57Bl/6 recipient mice. Three hours after transplant, the recipient mice were killed and the BM cells of the lower limbs and pelvis were collected. The BM cells (CD45.2⁺) were then transplanted into lethally irradiated CD45.1⁺ B6.SJLPrca Pep3b/BoyJ recipient mice together with 5×10^5 CD45.1⁺ BM cells, and followed by competitive repopulation assay. ST-HSC and LT-HSC engraftment was analyzed in peripheral blood drawn at 4, 8, 12, 15 and 24 weeks post transplantation for the contribution of CD45.2⁺ leukocytes. Competitive repopulating units were calculated as previously described^{59,60}.

BM spatial distribution assay. It was performed as previously described^{23,24}. Briefly, sorted LSK cells were stained with 5 μ M CFSE (Molecular Probes, Life Technologies). A total of 4×10^5 CFSE⁺ cells were injected intravenously in non-conditioned C57Bl/6 recipients. After 16 h, mice were killed by terminal pentobarbital anaesthesia (30 mg kg⁻¹ intraperitoneally), their hearts were cannulated through their left ventricles and a systemic perfusion of PBS/2% paraformaldehyde/0.05% glutaraldehyde was initiated at 4 ml min⁻¹ for 15 min. Subsequently, femorae were removed, and after 2–3 weeks of incubation in 10% EDTA, pH 7.4 for decalcification, they were subsequently dehydrated in graded ethanol and embedded in paraffin. Longitudinal sections (4 μ m) of each femur were cut, dewaxed and rehydrated. Every other longitudinal section (up to 20 sections per femur) was washed in PBS before mounting in Pro-Long Gold Antifade Reagent (Invitrogen, Life Technologies) and used for analysis. All sections were analyzed under an epifluorescent microscope (AxioObserver Z1; Zeiss, Göttingen, Germany) using a fluorescein isothiocyanate filter for specific signal and a rhodamine filter to discriminate autofluorescence. Analysis was performed by counting 56 sections, with a minimum of three mice per group. Endosteal area was defined as the area covered by 12 cell diameters perpendicular to the endosteum.

Adhesion and migration assays. Adhesion of HSC/P was performed by incubating 5×10^4 low-density BM (LDBM) cells from WT or *Klf5* Δ/Δ mice onto a recombinant human fibronectin fragment CH-296 (RetroNectin; Takara Bio Inc., Japan), laminin (Invitrogen) or collagen type I (Life Technologies) for 1 h. An aliquot of LDBM cells was set aside for CFU-C assay to calculate the number of input cells. Non-adherent cells were removed carefully by washing in PBS at least twice, and the adherent cells retrieved by trypsinization. The percentage of

adhesion was calculated from the input and output cell assay results. To assay adhesion using *Scl-tTA/TRE-Klf5* mice, input and output cells were stained with LSK markers, and the percentage of the LSK cell population was analyzed by flow cytometry.

Migration of HSC/P was analyzed by incubating 5×10^4 LDBM cells derived from WT or *Klf5 Δ/Δ* animals, on the top chamber of a 24-well transwell plate (Corning Inc., Lowell, MA). Recombinant Cxcl12 (50 or 100 ng ml⁻¹; R&D Systems, Inc., Minneapolis, MN) was placed in the bottom wells, and the 24-well plate was incubated at 37 °C, 5% CO₂. After 4 h of incubation, cells from the bottom chamber were collected by trypsinization, washed in PBS and the number of migrated hematopoietic progenitors was determined by CFU-C assay. The percentage of migrating cells was determined dividing the number of colonies present in the output fraction by the total number of input CFU-C. Input and output CFU-C were analyzed in triplicate. For migration assays using HSC/P *Klf5*-over-expressing mice, LSK BM cells were counted before and after migration.

Confocal microscopy immunofluorescence analysis. For immunofluorescence analysis by confocal microscopy of membrane integrin expression on transduced HSC/P, LSK BM cells from *VavCre;Wt* and *VavCre;Klf5 Δ/Δ* mice were sorted and transduced with mock or Rab5b lentiviral vectors, and then allowed to adhere to the poly-L-lysine-coated slides. The adhered cells were fixed with 4% paraformaldehyde in PBS for 30 min and then blocked with 5% normal mouse serum. Cells were treated with primary antibody against the active form of $\beta 1$ integrin (9EG7) or inactive form of $\beta 1$ integrin (MAB13) or with Rat anti mouse $\beta 2$ integrin antibody (C71/16), followed by treatment with Alexa-Fluor 568-conjugated goat anti-Rat IgG and Topro3 for nuclear staining. Cells were mounted with Vectashield mounting media containing antifading agent (Vector Labs, Burlingame, CA). The stained cells were analyzed by LSM 510 confocal system (Carl Zeiss) equipped with an inverted microscope (Observer Z1; Carl Zeiss) using Plan Achromat 63 \times 1.4 NA oil immersion lens. Argon ion 488, HeNe543 and HeNe633 laser lines were used to excite EGFP, Alexa Fluor568 and Topro3, respectively. The emission signals were collected using band pass filters BP 505–530 for EGFP, BP 560–615 for Alexa Fluor568 and a long pass filter 650LP for Topro3. Confocal images through optical plane along the z axis from top to bottom of cells were acquired at a resolution of 512 \times 512 pixels with a bit depth of 12 bits. Images were acquired using Zen2009 imaging software (Carl Zeiss). To present the $\beta 1$ - or $\beta 2$ -integrin staining throughout the cell surfaces, the composite image of all the optical plane along the z axis of the cell was generated using Zen2009 software. The mean fluorescence intensities of the secondary antibody for active $\beta 1$ -integrin, inactive $\beta 1$ -integrin and $\beta 2$ integrin on three-dimensional reconstituted cells were measured using Imaris Bitplane software. A minimum of 50 cells was analyzed per group and anti-integrin staining.

Lentiviral Rab5b transduction. The lentiviral vector pCDH-MCS-EF1-copGFP carrying the sequence of *Klf5* was used to produce the virus. The empty vector was used as negative control. LSK BM cells (1×10^5 cells per sample) were cultured in IMDM, 10% FCS, 100 IU penicillin, 0.1 mg ml⁻¹ streptomycin, 100 ng ml⁻¹ stem cell factor and 50 ng ml⁻¹ thrombopoietin and lentiviral supernatant (two rounds separated 16 h, multiplicity of infection = 20) for 48 h at 37 °C and either sorted for EGFP expression or analyzed by flow cytometry.

Statistical analysis. Student *t*-test or analysis of variance followed by Bonferroni correction was used for statistical significance test. Level of significance was established at 0.05.

Additional methods and any associated references are available in Supplementary Information.

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Author contributions

E.T.I., K.H.C., R.N., H.A.O. and J.A.C. performed and analyzed experiments. A.F., S.K.D., M.M., A.S. and J.A.W. developed indispensable experimental tools and reagents. E.T.I., K.H.C. and J.A.C. wrote the manuscript. E.T.I., K.H.C., H.L.G. and J.A.C. designed experiments. J.A.W., H.L.G. and J.A.C. critically reviewed the manuscript.

Additional information

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