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# Klf5 controls bone marrow homing of stem cells and progenitors through Rab5-mediated β1/β2-integrin trafficking

**E. Taniguchi Ishikawa**[1,2,6], **K.H. Chang**[1,2,6], **R. Nayak**[2], **H.A Olsson**[3], **A. Ficker**[2], **S.K. Dunn**[1], **M. Madhu**[2], **A. Sengupta**[2], **J.A. Whitsett**[4], **H.L. Grimes**[3,5], and **J.A. Cancelas**[1,2,*]

[1]Hoxworth Blood Center, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0055, USA

[2]Division of Experimental Hematology and Cancer Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229-3039, USA

[3]Division of Immunobiology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229-3039, USA

[4]Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229-3039, USA

[5]Department of Pathology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229-3039, USA

## Abstract

Kruppel-like factor 5 (Klf5) regulates pluripotent stem cell self-renewal but its role in somatic stem cells is unknown. Here we show that Klf5 deficient haematopoietic stem cells and progenitors (HSC/P) fail to engraft after transplantation. This HSC/P 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 β1/β2-integrins. *In vivo* inducible gain-of-function of Klf5 in HSCs increases HSC/P adhesion. The expression of Rab5 family members, mediators of β1/β2-integrin recycling in the early endosome, is decreased in *Klf5*$^{\Delta/\Delta}$ HSC/Ps. Klf5 binds directly to the promoter of *Rab5a/b* and overexpression of Rab5b rescues the expression of activated β1/β2-integrins, adhesion and bone marrow homing of *Klf5*$^{\Delta/\Delta}$ HSC/Ps. Altogether, these data indicate that Klf5 is indispensable for adhesion, homing, lodging and retention of HSC/Ps in the bone marrow through Rab5-dependent post-translational regulation of β1/β2 integrins.



*Correspondence: Jose A Cancelas, Division of Experimental Hematology & Cancer Biology, 3333 Burnet Ave., and Hoxworth Blood Center, University of Cincinnati, 3130 Highland Ave., Cincinnati, OH 45267-0055, USA. jose.cancelas@uc.edu.

[6]These authors contributed equally to this manuscript.

E.T-H, 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-H., KH. C. and J.A.C. wrote the manuscript. E.T-H, 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 methods and any associated references are available in Supplementary Information.

**Competing financial interests**

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## Introduction

Kruppel-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 cultures[1]. Mice null for the Klf5 alleles are embryonic lethal and die before embryonic day (E) 8.5[2]. Klf5 works as a downstream activator of transcriptional programs associated with pluripotent stem cell activity and differentiation[2], and its deficiency results in impaired blastocyst formation and implantation[3]. Using in vivo models, Klf5 has been shown to have a role in biological processes such as embryonic development, cardiovascular remodeling, adipogenesis, inflammatory stress responses, intestinal and lung development[4]. However, our understanding of the specific roles of Klf5 in adult stem cells from high-turnover tissues is minimal.

Hematopoietic stem cells (HSC) located in the bone marrow (BM) represent an example of self-renewing cells which need to replenish billions of cells a day. BM HSC are located within specialized BM niches where they recognize specific adhesion ligands present on cells and on the extracellular matrix. Modulation of HSC adhesion influences essential cellular processes 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 which act together to bind specific ligands. At present, eight α and 18 β integrin subunits have been described. Out of them, the β1-subunit, a component in 11 receptors with distinct chains[5], 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 signaling events[8]. 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 cycle[9]. Recycling of heterodimers containing β1-integrins has been shown to be crucial to the control of cell adhesion[10] by controlling the levels of active and inactive β1-integrin on the cell membrane[11]. 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 recycling [12]. Aberrant expression of the Rab GTPases has been found in many human diseases including cancer[13]. The Rab5 subfamily members, Rab5a and Rab5b, are mainly localized at the cytosolic face of plasma membrane, early endosomes, and clathrin-coated vesicles, and have been shown to be key regulators of intracellular vesicle traffic from the

plasma membrane to early endosomes[14] and its activity is required for maturation of the β1-integrin-containing early endosomes[15].

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 β1-integrin and β2-integrin in HSC/P, but not defective in primary or stressed hematopoiesis. The reduced expression of membrane β1/ β2 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*$^{flox/flox}$ mice[4] and *Mx-Cre* transgenic[16] mice to generate *Mx1-Cre;Klf5*$^{flox/flox}$ mice 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*$^{+/+}$ (WT) mice (Figure 1a-c) or *Mx1-Cre;Klf5*$^{flox/flox}$ without pl:pC injection as controls (Supplementary Figure 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 Figure 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 signaling[17,18], *Klf5*$^{\Delta/\Delta}$ mice showed a modest decrease in the myeloid cell count, without T-cell or B-cell lymphopenia (Supplementary Figure S2b), thrombocytopenia or reticulocytopenia (day 0 data in Supplementary Figures 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 Figure S2c).

Klf5 regulates cell proliferation and survival in other tissues, such as the basal layer of the esophagus or the intestinal crypts[19,20]. Klf5-deficiency had no effect on proliferation or survival of HSC/P as assayed by in vivo bromodeoxyuridine (BrdU) incorporation or caspase-3 assay, respectively (Supplementary Figures 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 (5-FU), a sublethal cytostatic drug that only spares quiescent HSC and induces stress recovery hematopoiesis of 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 Figure S3c), platelets (Supplementary Figure S3d) or reticulocytes (Supplementary Figure S3e), supporting the concept that Klf5 is dispensable for the regulation of HSC/P-dependent stress induced hematopoiesis.

Competitive repopulation is the golden standard assay for HSC regenerative activity where a few HSC are to repopulate a full animal in competition with normal HSC. 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 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*$^{\Delta/\Delta}$ HSC/P into lethally irradiated recipients revealed abnormal competitive engraftment and hematopoiesis at each test interval for peripheral blood (Figure 1d) and BM (Figure 1e) suggesting that all *Klf5*$^{\Delta/\Delta}$ 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 (Figures 1f-g) indicating that the repopulation ability of the long-term 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 and secondary recipients (Supplementary Figures 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*$^{\Delta/\Delta}$ HSC/P have decreased adhesion and lodging in BM

Since proliferation and survival of *Klf5*$^{\Delta/\Delta}$ HSC/P was not impaired, we next examined whether the impaired competitive repopulation ability of *Klf5*$^{\Delta/\Delta}$ HSC/P was accompanied by changes in their homing to the BM. An equal number of WT or *Klf5*$^{\Delta/\Delta}$ BM cells were injected into non-irradiated WT littermates. Quantitative phenotypic and functional analysis of homing of HSC/P was performed using flow cytometry analysis of carboxyfluorescein (CFSE)-labeled BM cells or a clonogenic progenitor assay (colony-forming-unit-cells, CFU-C). After 16 hours of transplantation, femoral BM cells from recipient mice were harvested and assayed. The homing of different populations (long-term HSC, short-term HSC, multipotential progenitors and CFU-C) of *Klf5*$^{\Delta/\Delta}$ HSC/P was significantly impaired at similar levels (~50-70% reduction, Figures 2a-d & Supplementary Figure S5). Specific locations within the BM cavity are associated with enrichment of HSC niches[21,22]. To evaluate homing to HSC-niche enriched BM areas, we analyzed the HSC/P lodging in serial femoral longitudinal sections of mice transplanted with CFSE-labeled WT or Klf5-deficient Lin$^-$/c-kit$^+$/Sca-1$^+$ (LSK) cells, as previously described[23, 24]. Lodging of LSK cells (Figures 2e-f) within the putative microanatomical endosteal space of the BM (Figure 2e) was significantly impaired. About 45% of WT HSC/P lodged in the endosteal region, while only 9% of the Klf5-deficient HSC/P that homed to the BM were found inside the endosteal area ($p<0.05$) (Figure 2g). The deficient BM homing/lodging of transplanted HSC/P was also associated with a ~3.5-fold increase in circulating HSC/P (Figure 2h), while the number of HSC/P in the BM was the same in both WT and Klf5-deficient mice (Supplementary Figure S2c). Together, these data indicate that the retention of *Klf5*$^{\Delta/\Delta}$ 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 have 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 integrins[27] and laminin binds to heterodimers containing β1 and β3 integrin chains[25]. We found that the adhesion of *Klf5*$^{\Delta/\Delta}$ HSC/P to CH-296, a C-terminus fibronectin peptide, was

significantly decreased compared to WT HSC/P (Figure 2i). In contrast, the adhesion ability of *Klf5*$^{\Delta/\Delta}$ HSC/P to laminin (Figure 2j) or collagen type I (Figure 2k), which binds multiple partner receptors including different β-integrin chains[28], were not significantly impaired. The chemokine Cxcl12 is a crucial molecule which mediates the retention and homing of HSC/P[34, 35]. Similarly, the migration of Klf5-deficient HSC/P towards a Cxcl12 gradient (Figure 2l) was not significantly impaired, regardless the tested concentration (50-100 ng/mL). 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 leukemia* (*Scl*) promoter-driven tetracycline transactivator (*Scl-tTA*) transgenic mice [29] with transgenic animals expressing Klf5 driven by a *Tetracycline responsive element* (*TRE*)[30] to generate *Scl-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 Scl [16]. Serial protein expression analysis from *Scl-tTA/TRE-Klf5* mice demonstrated a peak of Klf5 expression in LSK BM cells by day +5 after doxycycline withdrawal (Figure 3a), which was confirmed by immunoblotting of sorted BM LSK cells (Supplementary Figure S1). Quantitative RT-PCR (Q-RT-PCR) of LSK BM cells showed a 5-fold increase in the levels of *Klf5* mRNA by the same day (Figure 3b). Overexpression of Klf5 was associated with increased (~50%) adhesion of LSK BM cells to fibronectin (Figure 3c), while their migratory ability towards Cxcl12 was not significantly changed (Figure 3d). The homing of Klf5-overexpressing HSC was not significantly improved (Figure 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.

## Klf5 regulates the localization of β1/β2-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-integrins function. To understand whether the expression of β integrins was affected by Klf5 expression, we analyzed the cell membrane expression of β1-integrin, β2-integrin, β3-integrin, β7-integrin, and two major alpha chain partners in HSC/P, α4-integrin and α5-integrin, and the homing cell adhesion molecule (H-CAM), which also binds to the fibronectin fragment CH-294. β1-integrin has been reported to be essential for HSC/P homing through binding to fibronectin [31,32] while β2-integrin expression plays a minimal role in HSC homing [33]. β3 and β7 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 β1- and β2-integrins was significantly decreased in Klf5-deficient LSK BM cells compared to their WT counterparts (Figure 4a, p=0.025, student t-test). The expression levels of membrane α4-, α5-, β3- or β7- integrins were, however, not significantly changed (Supplementary Figures S6a-b). Interestingly, HSC/P whole cell lysate immunoblots showed upregulation of the overall cellular level of β1- or β2-integrin protein expression (Figure 4b). *ScltTA/TRE-Klf5* transgenic expression in BM

HSC/P resulted in the opposite phenotype of *Klf5*$^{\Delta/\Delta}$ HSC/P and was associated with increased membrane expression of β1-and β2-integrin, but not other integrin chains (Figure 4c and Supplementary Figure S6c), indicating the existence of a positive correlation between the expression level of Klf5, membrane β1- and β2-integrin and adhesion to fibronectin. Consistent with the post-translational nature of the changes in β1/ β2-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 Figure 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 since its levels Klf5-deficient or ScltTA/Tre-Klf5 HSC/P were comparable to those ones in WT HSC/P (Supplementary Figures S6e-f). These results suggest that Klf5 specifically controls the localization of β1- and β2-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 β1- and β2-integrin on the cell membrane, we performed gene expression profiling using cDNA from RNA isolated from WT or *Klf5*$^{\Delta/\Delta}$ HSC/P (GEO accession number GSE43806). Of the 1146 genes that showed significant differential expression, gene ontology (GO) analysis identified 14 major functional subgroups (Supplementary Figure 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 involved in the traffic of endocytosis vesicles, control the endosomal traffic of β-integrins[34] and control cell adhesion[35]. While Rab3 and Rab19 are associated to the process of general trans-golgi or post-golgi transport[36], Rab5 and Rab17 play a specific role in the control of formation of early endosomes and therefore, endosomal traffic [37,38], which is essential for β-integrin recycling[39]. We hypothesized that HSC/P Klf5 could transcriptionally regulate the expression of Rab5 and/or Rab17, therefore regulating β1- and β2-integrin trafficking from the cell membrane. Q-RT-PCR analysis showed that Klf5 regulates *Rab5a/b* expression in both *Klf5*$^{\Delta/\Delta}$ (~50% reduction) and *Scl-tTA/TRE-Klf5* transgenic HSC/P (Figures 5a-b, Supplementary Figure S7b) but Q-RT-PCR did not confirm downregulated expression of *Rab17* mRNA expression in Klf5-deficient HSC/P (Supplementary Figure S7c). Decreased Rab5a/b mRNA expression in Klf5-deficient HSC/P associated decreased Rab5a/b protein expression, which also decreased ~50% (Figure 5c). We identified the putative Klf5 binding sites (CACCC- and GC-rich motif) in the promoter region of *Rab5a* and *Rab5b*. Chromatin immunoprecipitation (ChIP) analysis revealed that Klf5 binds to upstream sequences near the transcription start site of both *Rab5a* and *Rab5b* loci in BM HSC/P (Figure 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 β1- and β 2-integrin on the membrane and the deficient homing of *Klf5*$^{\Delta/\Delta}$ HSC/P was a result of their inability to express active β1-integrin on the membrane, suitable-to-bind fibronectin. If true, the exogenous expression of Rab5b in *Klf5*$^{\Delta/\Delta}$ HSC/P should rescue the expression of both membrane-bound active β1- and β2-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*$^{flox/flox}$ mice where *Vav1*-promoter driven *Cre* expression restricts the deficiency of Klf5 to hematopoietic cells[42]. These mice are viable, have a normal lifespan and their HSC/P completely lack Klf5 protein expression (Hematopoietic *Klf5*$^{\Delta/\Delta}$, H- *Klf5*$^{\Delta/\Delta}$, Figure 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 Figures S7d-e). Immunofluorescence analysis by both flow cytometry and confocal microscopy analysis of β1-integrin expression on the membrane of EGFP$^{+}$ HSC/P showed that exogenous expression of Rab5b rescued total membrane β1-integrin expression (Figures 6b-c) and especially membrane-bound active β1-integrin expression (Figures 6d-g), but did not significantly modify the levels of inactive membrane β1-integrin (Supplementary Figures S8a-d). Similarly, membrane β2-integrin expression was also rescued by exogeneous expression of Rab5b (Figure 7a-d) suggesting that restoration of membrane expression of active β1-integrin and β2-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 (Figure 3c), the overexpression of Rab5b did not increase the adhesion of WT HSC/P to fibronectin (Figure 8a). Finally, the overexpression of Rab5b in H-*Klf5*$^{\Delta/\Delta}$ HSC/P resulted in restoration of their ability to home BM to the same level as WT HSC/P (Figure 8b). These results indicate that HSC/P BM homing activity is dependent on direct Klf5 transcriptional control of *Rab5* expression, which mediates active β1-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 play redundant roles. For instance, Klf1 and Klf2-dependent globin expression regulation have been shown to compensate each other in primitive erythtopoiesis but Klf1 has a distinct role in the expression of β-globin in definitive erythropoiesis[43]. Klf2, Klf4 and Klf5 have been shown to play redundant roles in a circuitry controlling embryonic stem cell pluripotency [44]. Klf5, one of the members of the Klf family, is highly expressed in HSC and in differentiated myeloid cells [45]. Klf5 expression, controlled by Klf4, appears to control the granulocyte-macrophage differentiation of committed myeloid progenitors/precursors in response to granulocyte colony-stimulating factor- signaling [17,18] and to regulate monocyte/macrophage differentiation and activation [46,47]. In embryonic stem cells, the Klf family members Klf2,

Klf4 and Klf5 play redundant roles in self-renewal regulation,[44] and Klf5 is required to prevent differentiation [48]. However, the distinct 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. While Klf5 is not essential for HSC self-renewal, Klf5 is an essential transcriptional factor controlling BM HSC/P homing, lodging in the endosteal space, engraftment, and BM retention. Using gene expression profiling validated by Q-RT-PCR in WT or *Klf5*$^{\Delta/\Delta}$ HSC/P, we identified several members of the Rab GTPase family, including *Rab5a, Rab5b* and *Rab17* as downstream targets of Klf5. Out of them, transcriptional regulation of Rab5 was demonstrated and protein levels and chromosome immunoprecipitation confirmed that Klf5 binds Rab5a and Rab5 proximal promoters in the cellular context of HSC/P. Klf5 downstream targets in other stem cells like embryonic stem cells do not include Rab5 genes [49], 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 recycling [39]. Decreased expression of Rab5 proteins interfere membrane localization of heterodimers containing β1- and β2-integrins (but not α integrin chains or CD44) which resulted in defective adhesion of HSC/P to fibronectin. Inducible overexpression of Klf5 in HSC results in an opposite effect by increasing the membrane expression of β1- and β2-integrins and the adhesion to the relevant extracellular matrix protein fibronectin [26]. However, Klf5 overexpression did 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 β1- and β2-integrins, the HSC/P adhesion to fibronectin, and in vivo BM homing, which associates to 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 Rab5-dependent integrin recycling. While 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 β1- and β2-integrins containing heterodimers in their binding to functionally active HSC niches within the BM[6], such as those that require fibronectin adhesion[27]. 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 α4β1, α5β1 integrins, whose counterreceptors are either fibronectin or the cell surface ligand, VCAM-1. In fact, α4 integrin expression is essential for HSC/P adhesion and different in BM HSC/P compared with circulating HSC/P [50]. A deficiency of one or several β-chains can be compensated by the formation of heterodimers with alternative β-chains like β3-integrins, which are also expressed by HSC [51]. The conformation of integrins, and thus their ability to bind extracellular matrix proteins is modulated by the interaction of their cytoplasmic domain with intracellular signaling 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 state[52]. A general feature of activated integrin traffic is that, rather than degradation, they undergo internalization and are subsequently recycled back to the cell surface [9]. Up to 50% of activated integrins on the cell surface get 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 β1 integrin heterodimers, which mediates interaction with the early endosomal Rab proteins Rab5 and Rab17[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/juxtanuclear recycling compartment using the clathrin/Rab5/Rab11 endosome system. As the earliest endosome-interacting Rab, early endocytic Rab5 protein plays key roles in cell adhesion and motility through the regulation of β1-integrin recycling [53]. As little as a reduction of 50% in the levels of Rab5 proteins has been shown to suffice to abrogate cellular endocytosis [54]. While Rab5-GTP is the active form, the phenotype induced by Klf5 deficiency seems to respond to a decrease in the expression of Rab5 genes since overexpression of Rab5b rescues the integrin expression on the membrane, adhesion to fibronectin and BM homing of HSC/P. Mechanistically, Rab5 activity has been shown to be independent of phophoinositide 3' kinase (PI3K) and the small GTPases, Ras, Rho and Cdc42 [55] and to be dependent on Rac1 activation downstream [56] which is required for HSC homing [24]. Our data indicate that Rab5 deficiency induced by loss of the transcriptional factor Klf5 results in loss of membrane-bound expression of β1- and β2-integrins, including the active form of β1-integrin, resulting in decreased adhesion to the extracellular matrix and homing and lodging in the BM endosteal space.

Altogether, our data indicate that Klf5 plays an essential role in the transcriptional regulation of the Rab5 expression, which in turn controls β1- and β2-integrin availability and localization within the membrane, 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 mice[16] were crossed with Klf5$^{flox/flox}$ mice[4](*Klf5*$^{\Delta/\Delta}$). Klf5 deletion was induced by intraperitoneal administration of double-stranded RNA polyinositide;polycytidine (pI:pC) (Amersham Pharmacia Biotech, Germany) (200 μg) every other day for a total of five times. Experiments were performed after a minimum of seven days after the last pI:pC injection. For constitutive Klf5 gene inactivation, Vav1-Cre mice[42] were crossed with Klf5$^{flox/flox}$ mice. For inducible Klf5 overexpression in HSC/P, ScltTA transgenic mice [29] were crossed with TRE-Klf5 mice[30]. 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; separated by 3 hours, and previously demonstrated to eliminate all endogenous BM CFU-C)[24]. For competitive repopulation experiments, $3\times10^6$ CD45.2$^+$ BM nucleated cells (BMNC) were mixed with $3\times10^6$ CD45.1$^+$ BMNC and were transplanted into lethally irradiatedCD45.1+ B6.SJLPtprca Pep3b/BoyJ recipient mice. Short- and long-term 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*$^{\Delta/\Delta}$ animals were sacrificed and single cell suspensions of the BM from the lower limbs and pelvis were prepared and used for transplantation. $2\times10^4$ BM cells were plated in methylcellulose culture media (MethoCult, Stem Cell Technologies, Vancouver, BC) 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 sacrificed and the BM cells of the lower limbs and pelvis were harvested and cultured in triplicate for CFU-C assay. Homing of immunophenotypically identified HSC/P was performed as previously published[57], Briefly, $20\times10^6$ low-density WT or Klf5-deficient BM cells were labeled with 5μM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Life Technologies, Grand Island, NY). Cells were labeled 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 MPP, defined as Lin$^-$/c-kit$^+$/Sca1$^+$/CD34$^+$/CD135$^+$ BM cells, before and and 16-hours after infusion into congenic myeloablated animals.

For homing of transduced HSC/P, $1\times10^3$ sorted LSK cells (WT + Mock, WT + Rab5b, *Klf5*$^{\Delta/\Delta}$ + Mock or *Klf5*$^{\Delta/\Delta}$ + Rab5b) were plated in methylcellulose culture media for CFU-C assay to quantify the initial content of hematopoietic progenitors. Sorted LSK donor cells ($1\times10^4$) were injected through the tail vein into lethally irradiated C57Bl/6 recipient mice. Sixteen hours after transplant, the recipient mice were sacrificed and the BM cells of the lower limbs and pelvis were harvested 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 BM[58].

For Klf5-overexpressing HSC homing assay, $2.5\times10^8$ BM cells from tet-off, Scl-tTA/WT (WT) or ScltTA/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 sacrificed and the BM cells of the lower limbs and pelvis were harvested. The BM cells (CD45.2$^+$) were then transplanted into lethally irradiated CD45.1$^+$ B6.SJLPtprca Pep3b/BoyJ recipient mice together with $5\times10^5$ CD45.1$^+$ BM cells, and followed by competitive repopulation assay. Short- and long-term 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 5μM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Life Technologies, Grand Island, NY). A total of $4\times10^5$ $CFSE^+$ cells were injected intravenously in non-conditioned C57Bl/6 recipients. After 16 hours, mice were sacrificed by terminal pentobarbital anesthesia (30 mg/kg intraperitoneally), their hearts were cannulated through their left ventricles and a systemic perfusion of phosphate buffered saline (PBS)/2% paraformaldehyde/0.05% glutaraldehyde was initiated at 4 mL/min for 15 min. Subsequently, femorae were removed and after 2-3 weeks incubation in 10% EDTA, pH 7.4 for decalcification, were subsequently dehydrated in graded ethanol and embedded in paraffin. Longitudinal sections (4 μm) of each femur were cut, dewaxed, and rehydrated. Every other longitudinal section (up to 20 sections per femur) was washed in PBS prior to 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, Gottingen, Germany). using a FITC filter for specific signal and a rhodamine filter to discriminate autofluorescence. Analysis was performed by counting 56 sections with a minimum of 3 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 $5\times10^4$ LDBM cells from WT or *Klf5*$^{\Delta/\Delta}$ mice onto a recombinant human fibronectin fragment CH-296 (RetroNectin®, Takara Bio Inc. Japan), laminin (Invitrogen), or collagen type I (Life Technologies) for one hour. 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 ScltTA/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 of $5\times10^4$ LDBM cells derived from WT or *Klf5*$^{\Delta/\Delta}$ animals, on the top chamber of a 24-well transwell plate (Corning Inc., Lowell, MA). Recombinant Cxcl12 (50 or 100 ng/mL; R&D Systems, Inc., Minneapolis, MN) was placed in the bottom wells, and the 24-well plate was incubated at 37°C, 5% $CO_2$. After 4 hours of incubation, cells from the bottom chamber were collected by tripsinization, 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-overexpressing mice, LSK BM cells were counted before and after migration.

### Confocal microscopy immunoflouorescence analysis

For immunofluorescence analysis by confocal microscopy of membrane integrin expression on transduced HSC/P, LSK BM cells from VavCre;Wt and VavCre;Klf5$^{\Delta/\Delta}$ mice were sorted and transduced with mock or Rab5b lentiviral vectors, and then allowed to adhered 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 β1 integrin (9EG7) or inactive form of β1 integrin (MAB13) or with Rat anti mouse β2 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 Apochromat 63X 1.4 NA oil immersion lense. 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 × 512 pixels with a bit depth of 12 bits. Images were acquired using Zen2009 imaging software (Carl Zeiss). To present the β1- or β2-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 β1 integrin, inactive β1 integrin, and β2 integrin on 3D 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 ($1\times10^5$ cells/sample) were cultured in IMDM, 10% FCS, 100 IU penicillin, 0.1 mg/mL streptomycin, 100 ng/mL stem cell factor and 50 ng/mL thrombopoietin, and lentiviral supernatant (two rounds separated 16 hours, MOI=20) for 48 hours at 37°C and either sorted for EGFP expression or analyzed by flow cytometry.

### Statistical analysis

Student’s t test or Anova followed by Bonferroni correction was used for statistical significance test. Level of significance was established at 0.05.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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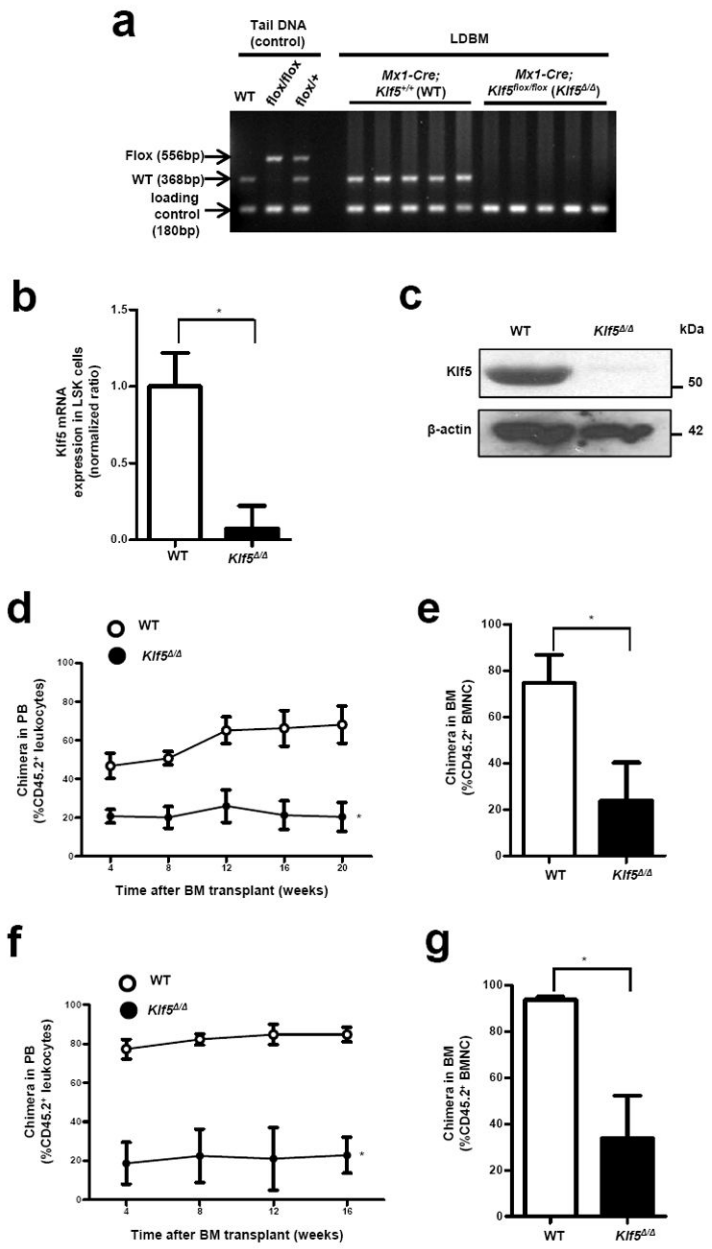


**Figure 1. Klf5 deficiency impairs HSC competitive repopulation capacity**

**(a)** PCR genotyping of wild-type (WT), floxed, and $Klf5^{\Delta/\Delta}$ alleles from tail and low-density bone marrow (LDBM) cell genomic DNA isolated from the BM of Mx1Cre; WT or Mx1Cre; $Klf5^{\Delta/\Delta}$ mice after five poly I:C injections administered every other day. **(b)** mRNA expression (Q-RT-PCR) analysis of *Klf5* in LSK cells isolated from poly I:C treated Mx1Cre; WT or Mx1Cre; $Klf5^{\Delta/\Delta}$ mice. Data are presented as mean ± SD **P* = 0.0002, student t-test. **(c)** Immunoblot for Klf5 protein expression in LSK BM cells from poly I:C treated MxCre;WT or Mx1Cre; $Klf5^{\Delta/\Delta}$ 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 $3\times10^6$ Mx1Cre; $Klf5^{\Delta/\Delta}$ BM cells ($CD45.2^+$) and $3\times10^6$ WT BM $CD45.1^+$ cells (solid circles). The control group was

transplanted with a mixture of $3\times10^6$ Mx1Cre; WT BM cells ($CD45.2^+$) and $3\times10^6$ WT BM $CD45.1^+$ cells (open circles). Data are presented as mean ± SD * $P$= 0.019, student t-test. **(e)** Chimera analysis of the BM of primary recipients at 20 weeks after transplantation. Data are presented as mean ± SD * $P$ <0.0001, student t-test. **(f)** PB chimera of secondary recipients transplanted with $10\times10^6$ BM cells obtained from primary mice (in **d**). **(g)** BM chimera in secondary recipients after 4 months of transplantation. 8-16 mice per group were transplanted and analyzed in two independent experiments. Data are presented as mean ± SD (*$P$ = 0.001, Student t-test).

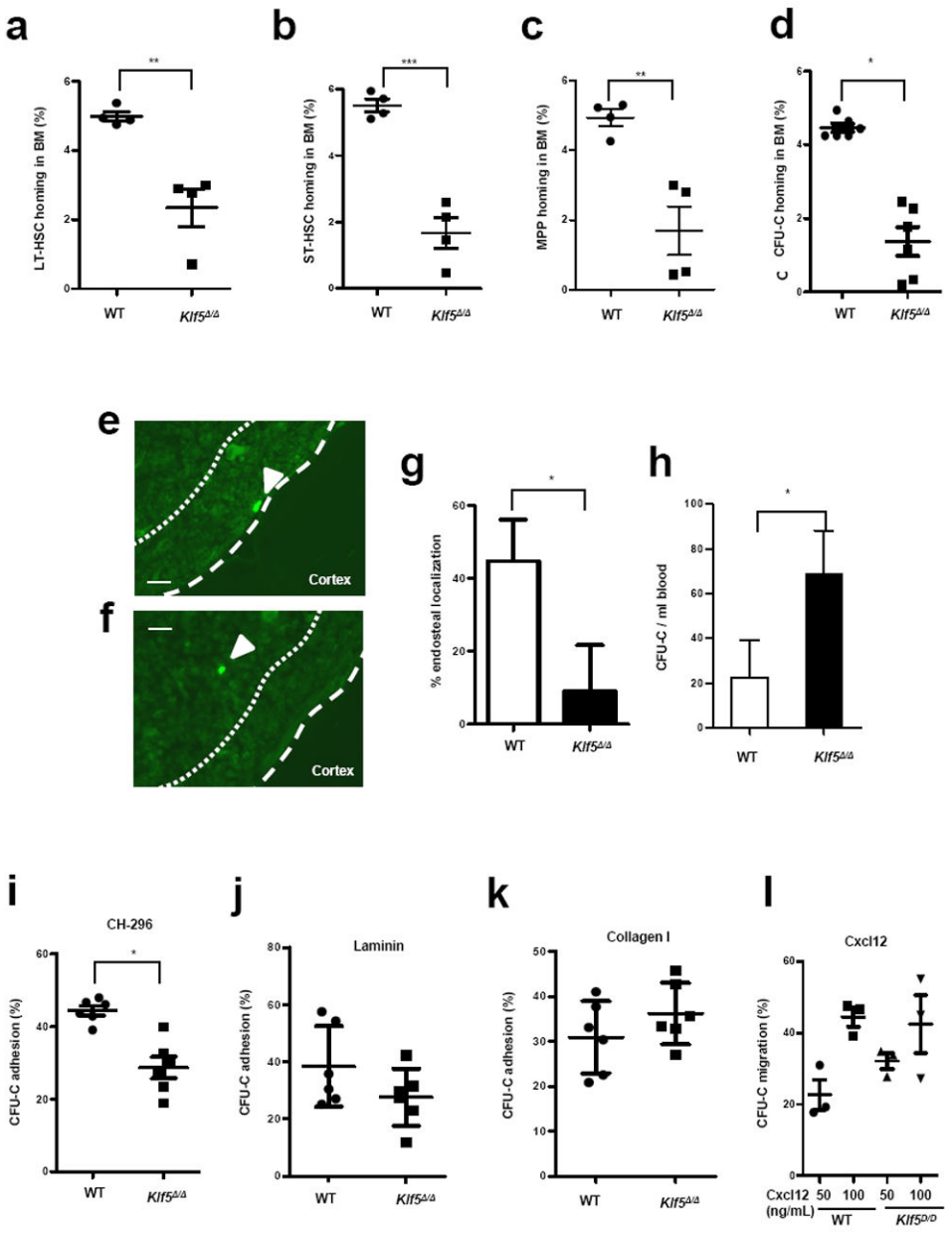


**Figure 2. *Klf5*$^{\Delta/\Delta}$ HSC/P are impaired in their lodging and adhesion to fibronectin**

(**a-d**) Homing of HSC/P in the BM of WT or *Klf5*$^{\Delta/\Delta}$ mice at 16 hours post-transplantation. (**a**) Homing of CFSE-labeled long-term HSC (LT-HSC, defined as Lin$^-$/c-kit$^+$/Sca1$^+$/CD34$^-$/CD135$^-$ BM cells) ** P=0.0033, student t-test. (**b**) Homing of CFSE-labeled short-term HSC (ST-HSC, defined as Lin$^-$/c-kit$^+$/Sca1$^+$/CD34$^+$/CD135$^-$ BM cells). *** P=0.0003, student t-test. (**c**) Homing of CFSE-labeled multipotential progenitors (MPP, 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. Bar = 20 μm. **(g)** Percentage of $CFSE^{+}$ LSK BM WT versus $Klf5^{\Delta/\Delta}$ found in the endosteal area. * P=0.04, student t-test. **(h)** PB count of CFU-C in primary WT and $Klf5^{\Delta/\Delta}$ mice. * P=0.013, student t-test. **(i-k)** Adhesion of WT and $Klf5^{\Delta/\Delta}$ BM CFU-C to the recombinant fibronectin fragment CH-296 **(i)**, laminin * P=0.007, student t-test. **(j)** and collagen I **(k)**. **(l)** Chemotaxis of WT and $Klf5^{\Delta/\Delta}$ BM CFU-C to Cxcl12.

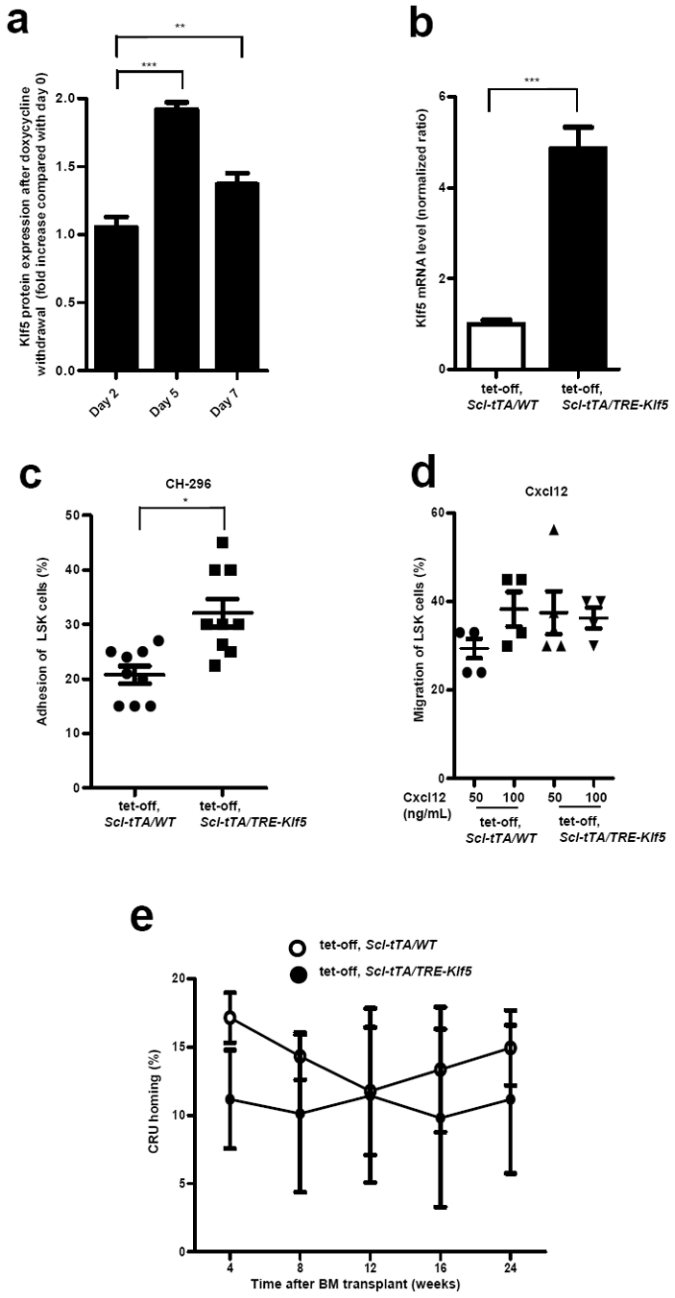


**Figure 3. Inducible expression of Klf5 *in vivo* induces increased adhesion to fibronectin and normal BM homing**

(**a-b**) Klf5 expression in LSK BM cells isolated from tet-off, *Scl-tTA/WT* (WT) or *Scl-tTA/TRE-Klf5* mice. (**a**) Protein was measured by intracellular flow cytometry on BM LSK cells from *Scl-tTA/TRE-Klf5* mice harvested on days 2, 5 and 7 post-doxycycline withdrawal. N=3 independent animals per group. Data represent mean ±SEM. ***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 *ScltTA/Tre-Klf5* mice. **P* = 0.019, student t-test. (**d**) Chemotaxis of LSK BM cells to CXCL12 from tet-off, *WT* or *ScltTA/Tre-Klf5* mice. Data are presented as mean

± SD. **(e)** Homing of WT and Klf5-overexpressing HSC. Graph depicts the homing (%) after 3 hours post-transplantation of competitive repopulation units 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=8-10 mice per group, from two independent experiments.

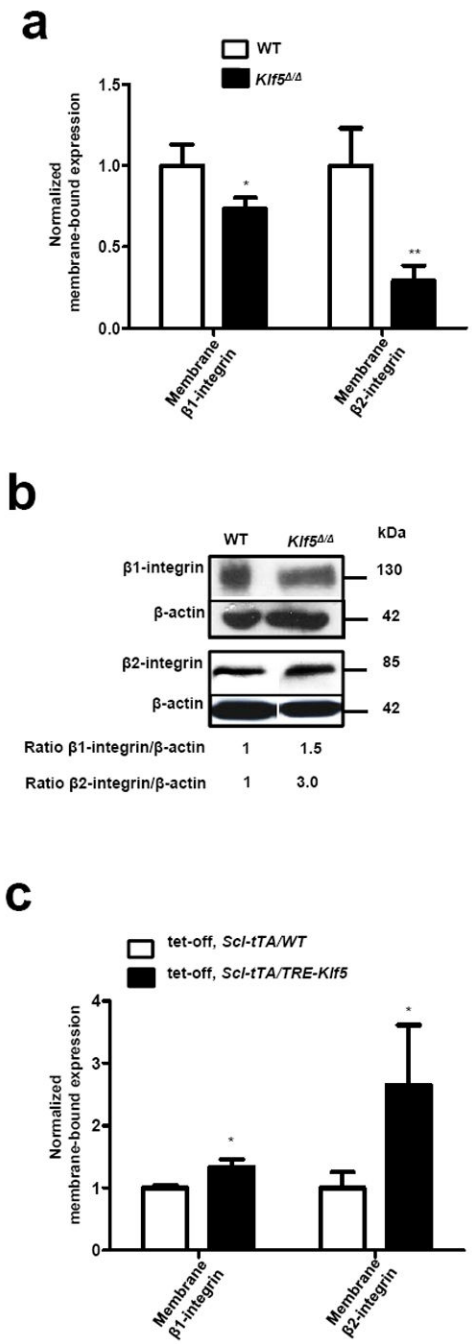


**Figure 4. Defective *Klf5*$^{\Delta/\Delta}$ HSC/P homing is attributed to a post-translational decrease in membrane-bound β1- and β2-integrins**

(**a**) Expression of membrane β1-integrin and β2-integrin in WT (empty bars) and *Klf5*$^{\Delta/\Delta}$ (solid bars) LSK BM cells as assessed by flow cytometry (normalized mean fluorescence intensity ratio). (**b**) Representative example of immunoblot of β1- and β2-integrin expression of whole cell lysates from WT and *Klf5*$^{\Delta/\Delta}$ LSK BM cells. Densitometric ratio analysis was calculated. (**c**) Expression of membrane β1-integrin and β2-integrin in LSK BM cells from tet-off, *Scl-tTA/WT* (empty bars) or *Scl-tTA/TRE-Klf5* mice (solid bars).

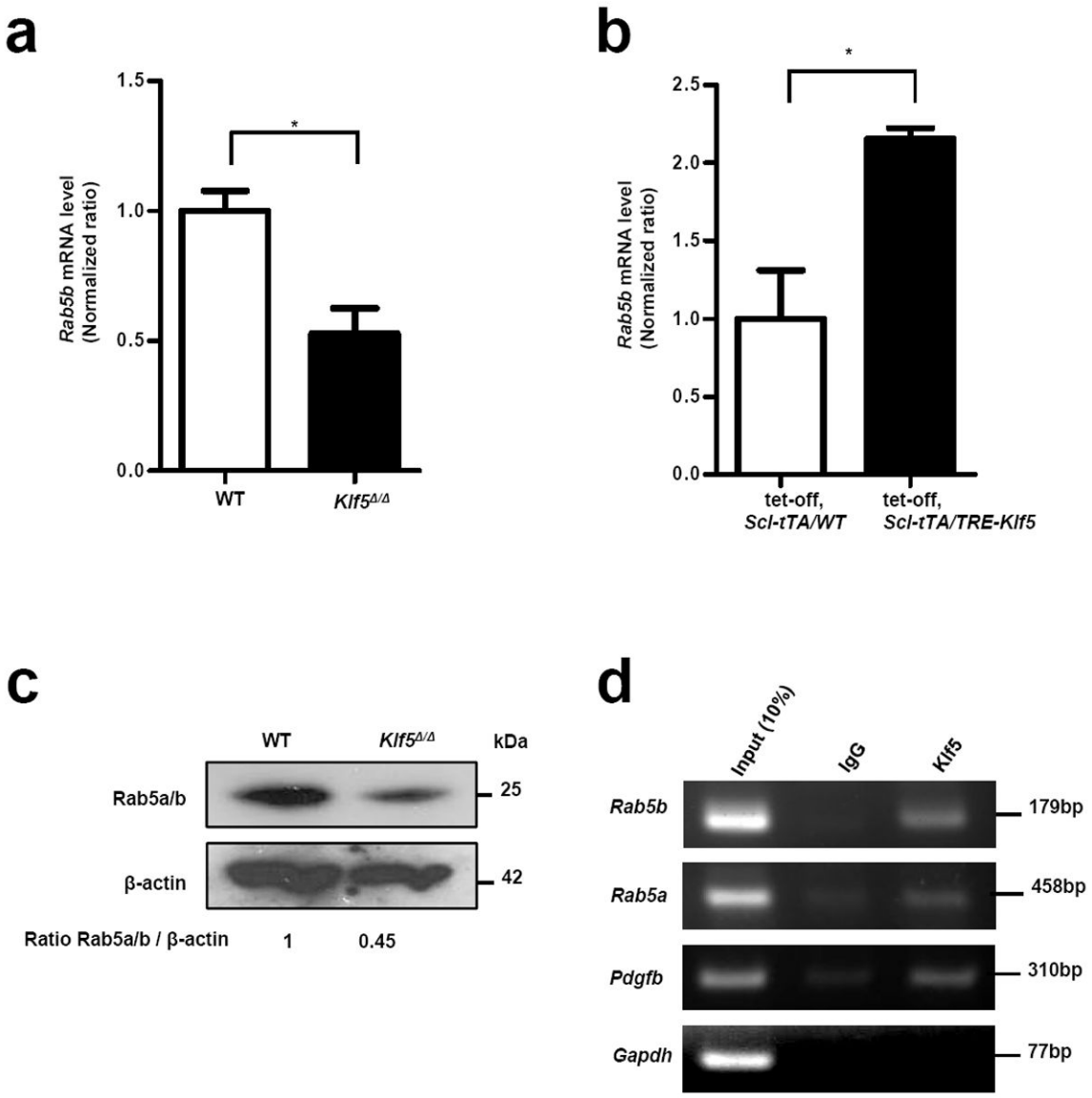


**Figure 5. Klf5 directly regulates the expression of Rab5 family proteins**

(**a-b**) *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 ± SD (**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 lineage negative 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.

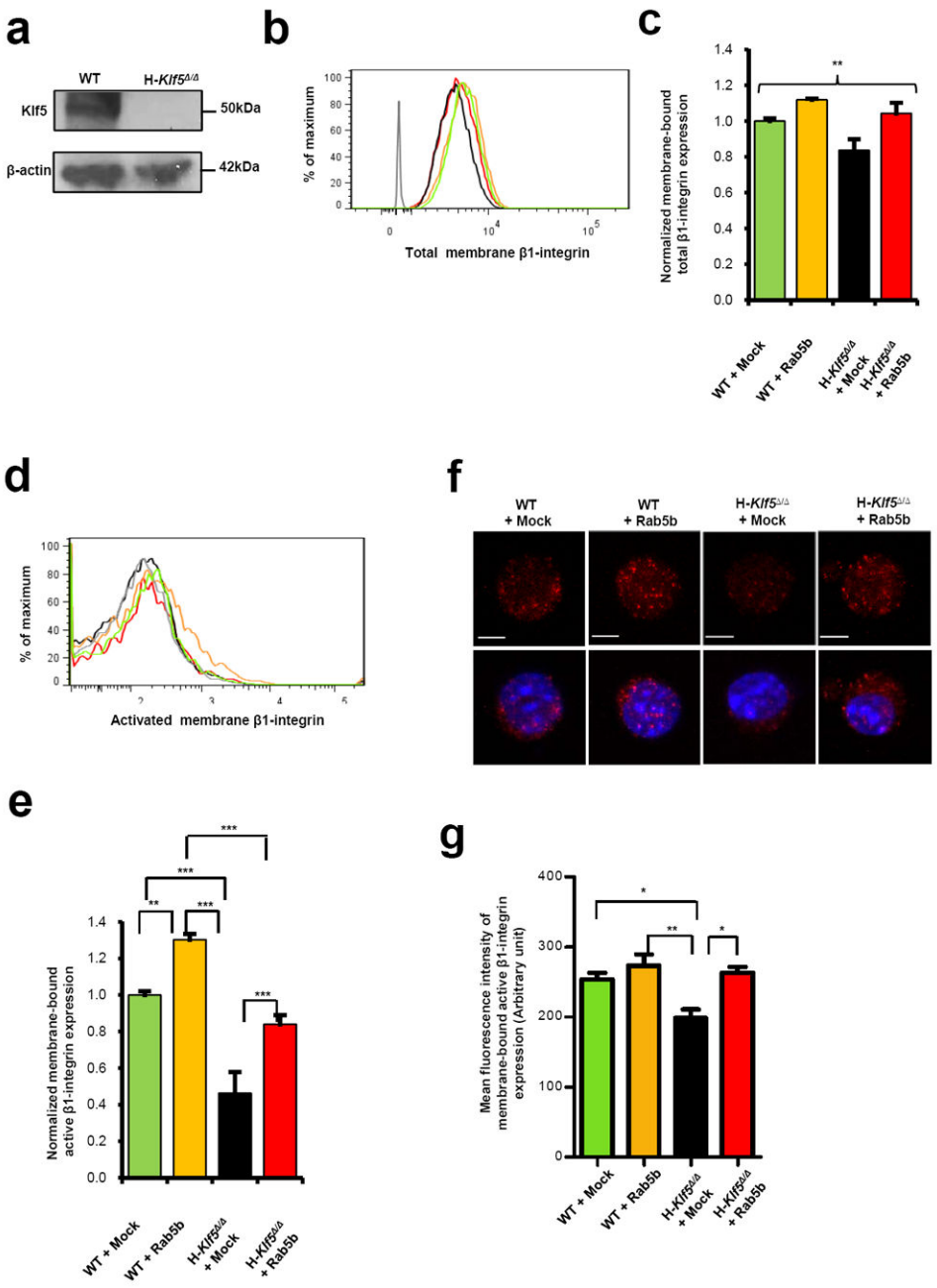


**Figure 6. Lentiviral-vector-mediated Rab5b expression rescues the total and activated fraction of membrane-bound β1-integrin in Klf5$^{\Delta/\Delta}$ HSC/P**

(**a**) Immunoblotting of Klf5 in LSK isolated from *Vav1Cre-WT* (WT) or *Vav1Cre-Klf5$^{flox/flox}$mice*(*H-Klf5$^{\Delta/\Delta}$*). 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 WTor *H-Klf5$^{\Delta/\Delta}$*) mice. Sorted LSK BM cells were transduced with a lentiviral vector expressing Rab5b and enhanced green fluorescent protein (eGFP) or eGFP only (Mock) as a control WT-Mock (green line or bar), WT-Rab5b (orange line or bar), *H-Klf5$^{\Delta/\Delta}$*-Mock (black line or bar) and *H-Klf5$^{\Delta/\Delta}$*-Rab5b (red line or bar) LSK BM cells. (**b-c**) Membrane expression of total β1-integrin (**b**) Representative example of

flow cytometry analysis. (**c**) Normalized membrane-bound total β1-integrin expression (** p=0.0042, ANOVA test). (**d-e**) Membrane expression of activated β1-integrin. (**d**) Representative example of flow cytometry analysis. (**e**) Normalized membrane-bound activated β1-integrin expression. Data are presented for c and e as mean ± SEM (n=3 replicates per group ***p<0.0001, ANOVA test). (**f-g**) Confocal microscopy analysis of membrane expression of activated β1-integrin. (**f**) Representative picture of membrane-bound active β1-integrin. Active β1-integrin (red) and DAPI (blue, overlaid in lower row pictures). Bar=5 μm. (**g**) Mean fluorescence intensity of membrane-bound active β1-integrin expression as analyzed by confocal microscopy. A minimum of 50 cells per group was analyzed by confocal microscopy. Data are presented as mean ± SEM. ** $P < 0.0035$, ANOVA test with Bonferroni correction.

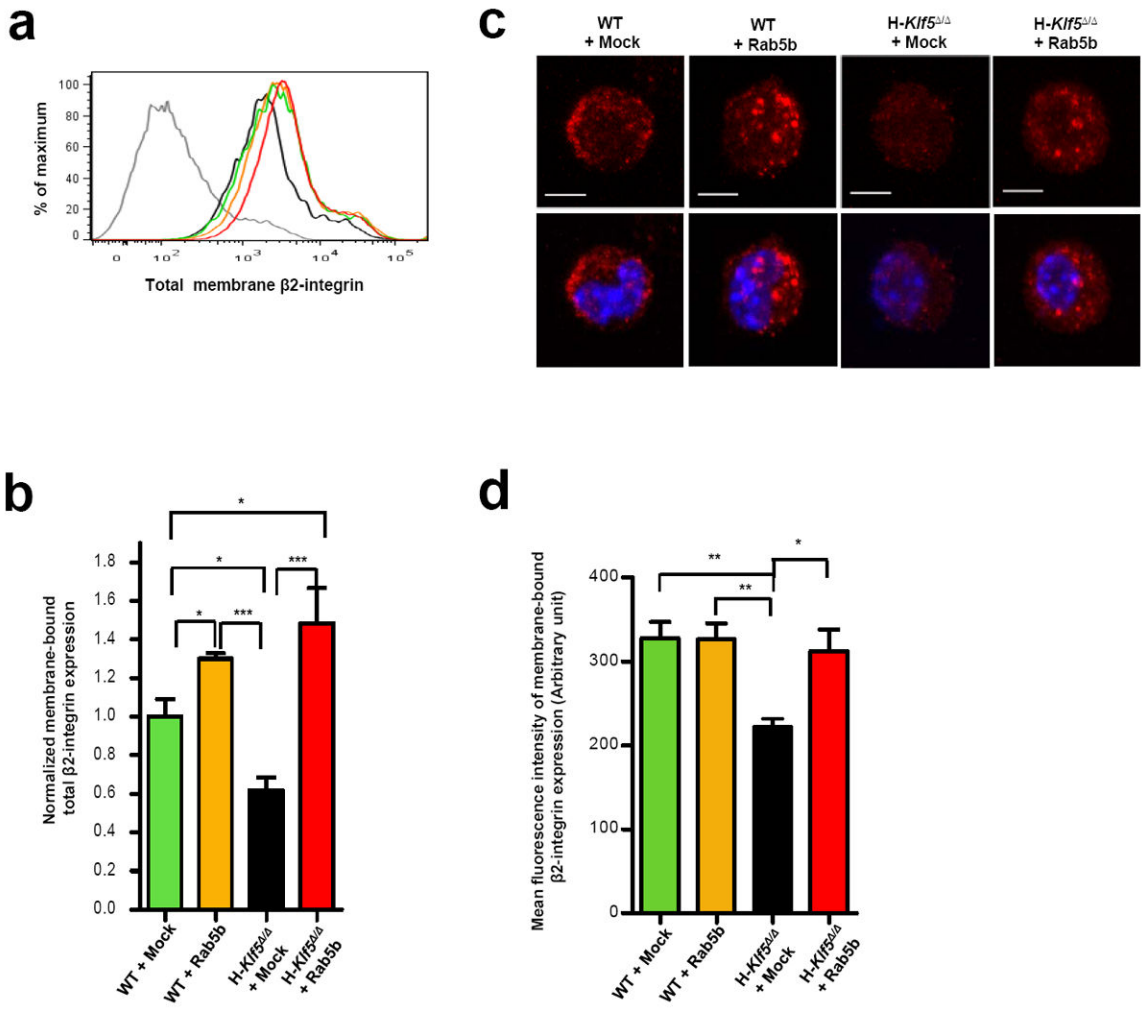


**Figure 7. Lentiviral-vector-mediated Rab5b expression rescues the membrane-bound β2-integrin expression in Klf5$^{\Delta/\Delta}$ HSC/P**

(**a-d**) Membrane expression of β2-integrin. (**a**) Representative example of flow cytometry analysis. (**b**) Normalized membrane-bound total β2-integrin expression analyzed by flow cytometry. Data are presented as mean ± SEM (n=3 replicates per group,** p<0.0001, ANOVA test). (**c**) Representative example of membrane expression of membrane β2-integrin (red) analyzed by confocal microscopy. DAPI (blue, overlaid in lower row pictures) was used for nuclear counterstain. Bar=5 μm. (**d**) Mean fluorescence intensity of membrane-bound β2-integrin expression as analyzed by confocal microscopy. A minimum of 50 cells per group was analyzed by confocal microscopy. Data are presented as mean ± SEM. *** *P* = 0.0009, ANOVA test with Bonferroni correction.

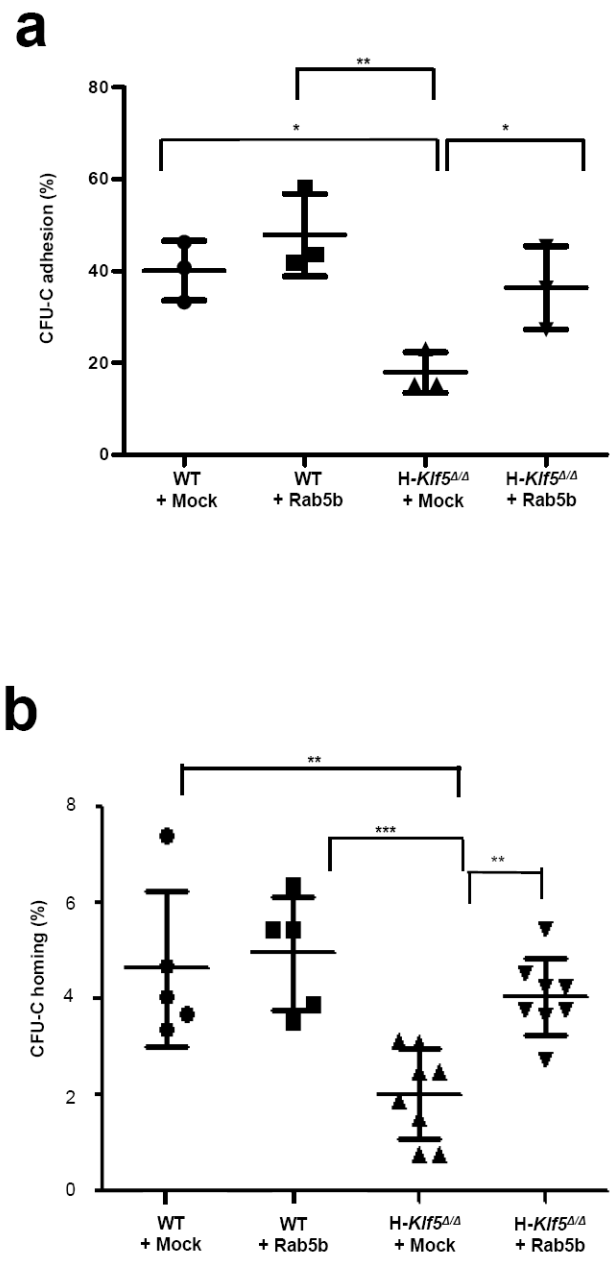


**Figure 8. Forced Rab5b expression rescues the impaired adhesion and homing defect of hematopoietic-specific *Klf5*$^{\Delta/\Delta}$ HSC/P**

(**a**) Adhesion to fibronectin of *H-Klf5*$^{\Delta/\Delta}$ LSK cells was rescued by exogenous expression of Rab5b (n=3 per group, in each of two independent experiments *p=0.007, ANOVA test). **(b)** CFU-C homing in the BM of *H-Klf5*$^{\Delta/\Delta}$ mice was rescued by lentivirus transduction of Rab5b. Homing was analyzed at 16 hours after transplantation (similar to Fig. 2a-d). Data are presented as mean ± SD (*$P$ = 0.03, n=5-8 mice per group; ANOVA test with Bonferroni correction).