Supplementary materials and methods

Animals

C57Bl/6, BoyJ and NOD/SCIDγC$^{-/-}$ (NSG) were obtained from The Jackson Laboratory (Bar Harbor, ME). *Fanca*$^{-/-}$ and *Fanca*$^{+/+}$ mice were generated by interbreeding the heterozygous *Fanca*$^{+/-}$ mice.[1] Animals were maintained in the animal barrier facility at Cincinnati Children's Hospital Medical Center. Immunodeficient mice were maintained on chow supplemented with doxycyclin for one week before and after transplantation. All animal procedures were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center prior to study initiation (IACUC protocol # 2013-0159).

Chemical agents

CASIN was obtained from Chembridge Corporation, and purified to greater than 99% by high-performance liquid chromatography. For *in vitro* studies, CASIN (containing 0.02%-0.2% DMSO) was used at 5-20 μM in IMDM. For *in vivo* experiments, CASIN was dissolved in ddH_2O with 15% ethanol, and administered by intraperitoneal (IP) injection (2.4 mg/kg, twice, 24 hours and 2 hours before transplantation). The regimen of CASIN use was adapted from previous mouse studies and may not be optimized.[2] Fludarabine was obtained from SIGMA, dissolved in DMSO, and administered by IP injection (75 mg/kg, three times, three days, two days and one day before transplantation). For G-CSF conditioning, G-CSF (100 μg/kg, Amgen InC) was administrated daily for 5 days by IP injection followed by bone marrow transplantation (BMT) on day 5.[3]

CFU determination of HSPC mobilization

Human xenografted mice or *Fanca*$^{+/+}$ and *Fanca*$^{-/-}$ mice were treated with or without CASIN (2.4 mg/kg, IP), and progenitor cell numbers in PB were determined at 2 hours post-CASIN injection by adding 200 μl of PB to 4 ml of cytokine-containing methylcellulose (Stemcell Technologies) and plated in triplicate in 35 mm-gridded dishes. Colonies were enumerated 10 days (human) or 7 days (mouse) post-plating.

Engraftment assay

Primary recipients (*Mx:Cre; Cdc42*$^{flox/flox}$ v.s. *Mx:Cre; Cdc42*$^{+/+}$) were transplanted with either 1×10^7 congenic BM cells (CD45.1) four times one day after each of the Poly I:C (InvivoGen) injections and 2 days after the last Poly I:C injection, or 2×10^7 congenic BM cells (CD45.1) once one day after the last Poly I:C injection. BM cells from the primary recipients three months after the primary transplantation were used as donor cells for the secondary transplantation. Each donor was transplanted into 4 lethally irradiated recipients. Separately, BoyJ mice (CD45.1) were transplanted with 5×10^6 congenic BM cells (CD45.2) after being preconditioned with CASIN (one day and 2 hour before transplantation, 1.2 mg/kg, IP) vs. vehicle. Chimerisms of donor-derived cells were followed up till 4 months post transplantation by FCAS analysis.

Transplantations

Human cord blood (HCB) xenograft mouse model was established by intrafemoral or intravenous (i.v.) transplantation of 200,000 CD34$^+$ HCB cells into each of NSG recipient mice.[4] The mice typically contain a low chimerism (<5%) at the time of use for mobilization. Engraftment of HCB was monitored by flow cytometry analysis of both BM and PB. The collection and isolation of human cord blood samples was approved by the IRB of Cincinnati Children's Hospital Medical Center (Protocol # 2011-3023). For competitive transplantation, CASIN (0.5 mM, 200 µl/20 g mouse weight) was administered intravenously into C57Bl/6 donor mice. BM cells were collected at 2 and 24 hours post injections in parallel to controls. 3×10^6 BM cells from each of the donor mice along with equal number of competitor cells from BoyJ mice were transplanted into lethally irradiated BoyJ recipient mice. Chimerism of donor derived cells (CD45.2$^+$) were followed up at 10 months post transplantation.

For BM transplantation with gene-corrected cells, approximately 1×10^5 *Fanca*$^{+/+}$ or *Fanca*$^{-/-}$ Lin$^-$ cells transduced with MIEG3-eGFP or MIEG3-FANCA retrovirus were transplanted into each of the pre-conditioned WT or *Fanca*$^{-/-}$ recipient mice, following intraperitoneal injections with Vehicle or CASIN (2.4

mg/kg; 2 hours and 24 hours prior to BMT). Engraftment was analyzed 4 months later for EGFP-expressing donor-derived cells.

Transduction and sorting of CD34$^+$ human cord blood (HCB) cells with shRNAs

Preselected CD34$^+$ HCB cells were thawed fresh for shRNA transduction. Lentiviral vectors expressing shRNA targeting Cdc42 or scrambled control shRNA were obtained from Sigma (St. Louise, MO) and produced by transfection of 293T cells. The CD34$^+$ HCB cells transduction was performed as described previously.[5] Transduced cells were sorted for effector pulldown and functional assays.

Western blotting and Cdc42/Rac1 GTPase activity assays

Levels of active GTP-bound Rac1 and Cdc42 were determined by effector pull-down assays as previously described.[6] Briefly, cells (murine *Fanca*$^{+/+}$ or *Fanca*$^{-/-}$ LDBM, or CD34$^+$ HCB) were starved for two overnights and treated with CASIN (2-20 μM) for 2 hours. Cells were then stimulated with cytokines for 10 minutes, lysed, and incubated with the GST-P21-binding domain of PAK1. Both bound- and unbound-forms of the Rho GTPases were probed by immunoblotting with antibodies specific for Rac1 (BD Biosciences) or Cdc42 (BD Biosciences). Immunoblotting was carried out in HCB cells using antibodies specific for p-Wasp, p-PAK and p-aPKC (Cell signaling).

F-actin polymerization assay

Studies of F-actin dynamics were performed using CD34$^+$ HCB cells. Cells were serum-starved two overnights. CASIN was then added to the cells for 2 hours, stimulated with SDF1α (100 ng/ml) for 15, 30, or 60 seconds, and fixed using 4% paraformaldehyde. Cells were stained with rhodamine-conjugated phalloidin for flow cytometry (F-actin mean intensity).

Adhesion/migration assay

For adhesion experiments of $CD34^+$ HCB, cells were treated with CASIN (5 μM) for 2 hours, and plated in a 48-well plate pre-coated with fibronectin (CH296) for 2 hours. Adherent cells were then dissociated and enumerated.

For migration experiments of $CD34^+$ HCB cells, CASIN-treated (5 μM, for 2 hours) cells (5×10^5) were suspended in 100 μl serum-free medium and loaded into the upper chamber of a transwell plate (5 μm pore-size filter) (Costar, Cambridge, MA), whereas the lower chamber contained 0.6 ml chemotaxis buffer (IMDM with 0.5% BSA and 100 ng/ml SDF-1α). After 4 hours of incubation, the cells were harvested and enumerated. Separately, for CFU-migration experiments of LDBM cells from *Fanca*$^{+/+}$ and *Fanca*$^{-/-}$ mice, migrated cells were further added to 4 ml methycellulose containing rmSCF (50ng/ml), rmIL-3 (10 ng/ml), rhIL-6 (10 ng/ml), and rhEpo (4 IU/ml) (Stemcell Technologies), and plated in triplicate in 35 mm-gridded dishes for progenitor activity.

Retroviral expression vector and viral transduction

The full-length cDNA encoding human FANCA was amplified by polymerase chain reaction (PCR), using Pfu DNA polymerase (Stratagene, La Jolla, CA). The resulting PCR fragments were subcloned into the *Not*I site of an improved bicistronic murine stem cell virus (MSCV)-based vector MIEG3 with enhanced green fluorescent protein (eGFP) expression to create MIEG3-FANCA, as described previously.[7] Retroviruses were prepared by the Vector Core of Cincinnati Children's Research Foundation (Cincinnati Children's Hospital Medical Center, Cincinnati, OH). Retroviral supernatant was collected at 36, 48, and 72 h, respectively, after transfection.

For transduction, freshly isolated WT (*Fanca*$^{+/+}$) and *Fanca*$^{-/-}$ Lin^- BM cells were cultured for 24 h in IMEM containing 20% FBS and a cytokine cocktail of 100 ng/mL stem cell factor (SCF), 20 ng/mL interleukin-6 (IL-6), and 50 ng/mL Flt-3 ligand (Flt-3). Transduction was carried out at MOI (multiplicities of infection) 10 in 6-well non-tissue culture plates pre-coated with Retronectin^{TM} in the

presence of 2 μg/mL polybrene (Sigma-Aldrich), and the infection mixes were incubated in a CO_2 incubator at 37 °C for 12 hrs. Transduction was repeated one more time the next day. The transduced cells were expanded in StemSpan medium (Stem Cell Technologies) supplemented with SCF (100 ng/mL) and TPO (50 ng/mL) for 3 days. Transduction efficiencies were determined by flow cytometry.

Flow cytometry analysis

To determine the engraftment of HCB cells in the NSG mice, PB and BM were collected and labeled with markers for human CD45, CD34, CD19, and CD33 (CD45-FITC, CD34-APC; or CD45-FITC, CD19-APC, CD33-PE). To determine the apoptosis of the $CD34^+$ HCB cells, CD34-APC was used in combination with Annexin V-PE and 7-AAD. To determine the murine chimera after transplantation, CD45.1-FITC was used in combination with CD45.2-PE and 7AAD. For BM transplantation with gene corrected cells, donor-derived cells were analyzed for eGFP expression. To determine lineage differentiation in the recipient mice, PB and BM were collected and labeled with cell surface markers for mouse CD45.1, CD45.2, Gr1, CD11b, Ter119, CD3 or B220 followed by flow cytometry analysis.

Statistical analyses

Paired or unpaired student's *t*-test was used for two-group comparison, and one-way ANOVA for more than two-group comparison. Values of *p* less than 0.05 were considered statistically significant. Results are presented as mean ± SD. * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$.

Supplemental reference:

1. Wong JC, Alon N, Mckerlie C, Huang JR, Meyn MS, Buchwald M. Targeted disruption of exons 1 to 6 of the Fanconi Anemia group A gene leads to growth retardation, strain-specific

microphthalmia, meiotic defects and primordial germ cell hypoplasia. *Hum Mol Genet* 2003; **12(16):** 2063-2076.

2. Liu W, Du W, Shang X, Wang L, Evelyn C, Florian MC *et al.* Rational identification of a Cdc42 inhibitor presents a new regimen for long-term hematopoietic stem cell mobilization. *Stem Cell Reports* Under review.
3. Lord BI, Woolford LB, Wood LM, Czaplewski LG, McCourt M, Hunter MG *et al.* Mobilization of early hematopoietic progenitor cells with BB-10010: a genetically engineered variant of human macrophage inflammatory protein-1 alpha. *Blood* 1995; **85(12):** 3412-3415.
4. Wagner JE, Eapen M, MacMillan ML, Harris RE, Pasquini R, Boulad F *et al.* Unrelated donor bone marrow transplantation for the treatment of Fanconi anemia. *Blood* 2007; **109:** 2256-2262.
5. Wunderlich M, Mulloy JC. Model systems for examining effects of leukemia associated oncogenes in primary human $CD34^+$ cells via retroviral transduction. *Methods Mol Biol* 2009; **538:** 263-285.
6. Shang X, Moon SY, Zheng Y. p200 RhoGAP promotes cell proliferation by mediating cross-talk between Ras and Rho signaling pathways. *J Biol Chem* 2007; **282:** 8801-8811.
7. Du W, Li J, Sipple J, Chen J, Pang Q. A cytoplasmic FANCA-FANCC complex interacts and stabilizes the leukemic NPMc protein. *J Biol Chem* 2010; **285(48):** 37436-37444.

SUPPLEMENTAL FIGURE LEGEND:

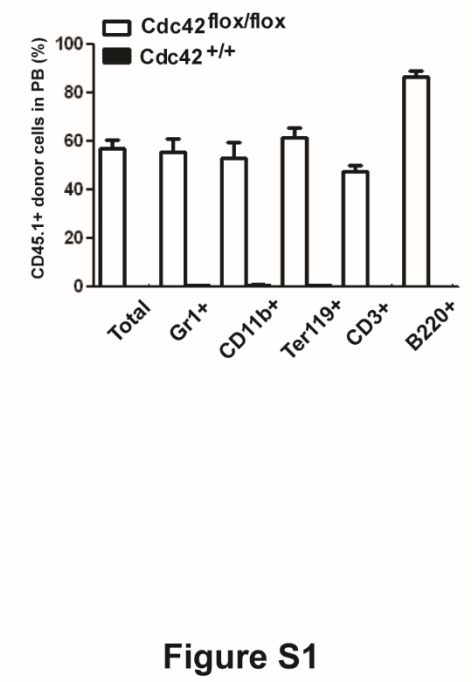

Figure S1

Fig S1. Analysis of donor lineage differentiation in secondary recipients. Percentage of donor derived overall, $CD3^+$, $B220^+$, $Ter119^+$, $Gr1^+$ and $CD11b^+$ PB in lethally irradiated secondary recipients 4 months after the secondary transplantation.

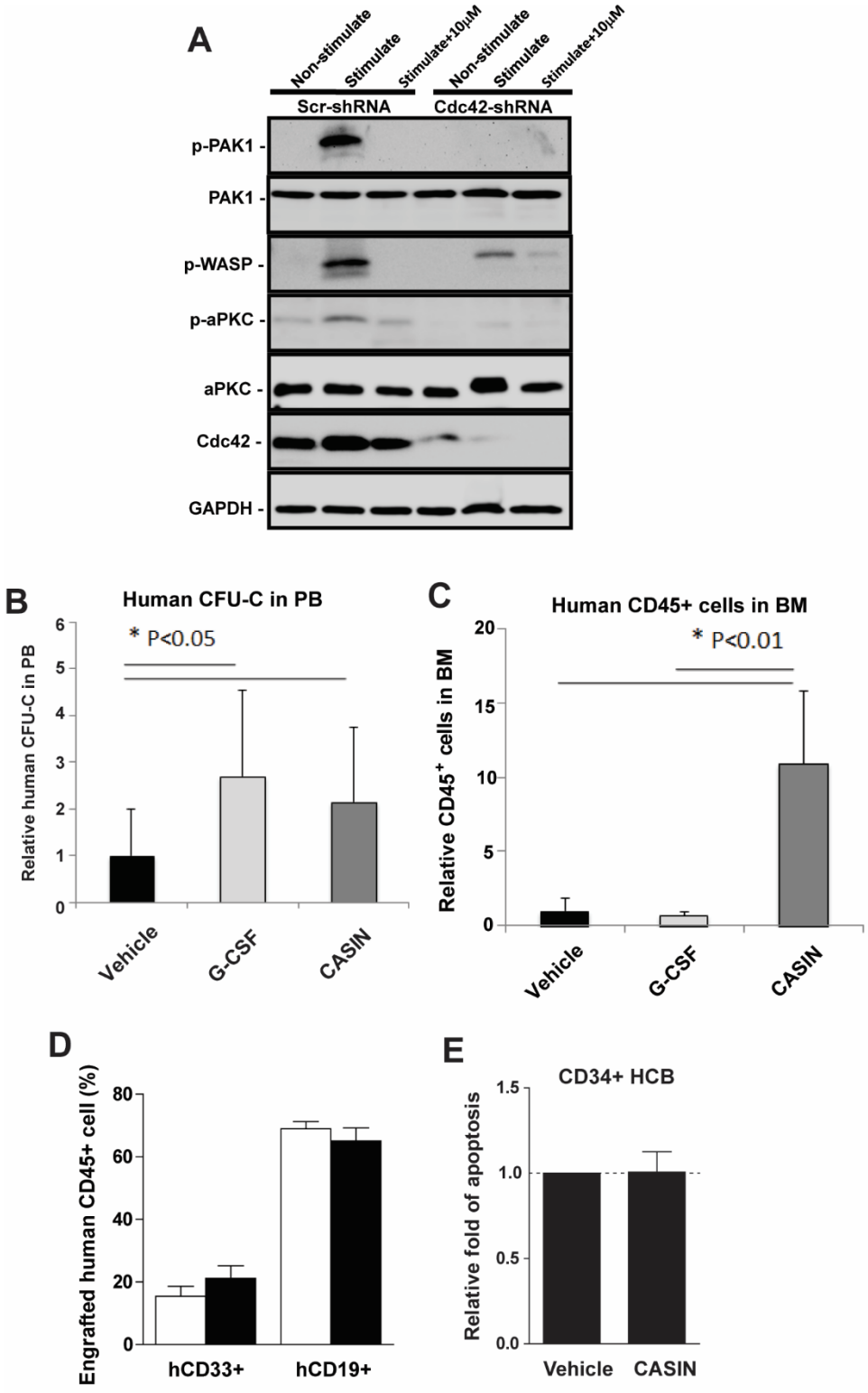
A
Non-stimulate
Stimulate
Stimulate+10μM
Non-stimulate
Stimulate
Stimulate+10μM
Scr-shRNA
Cdc42-shRNA
p-PAK1
PAK1
p-WASP
p-aPKC
aPKC
Cdc42
GAPDH
B
Human CFU-C in PB
* P<0.05
Relative human CFU-C in PB
Vehicle
G-CSF
CASIN
C
Human CD45+ cells in BM
* P<0.01
Relative CD45+ cells in BM
Vehicle
G-CSF
CASIN
D
Engrafted human CD45+ cell (%)
hCD33+
hCD19+
E
CD34+ HCB
Relative fold of apoptosis
Vehicle
CASIN

Figure S2

Figure S2. CASIN inhibits Cdc42 signaling activity and function in CD34$^+$ HCB cells. (A) CASIN, mimicking effects of shRNA knockdown of Cdc42, blocks Cdc42 downstream signaling. CD34$^+$ HCB cells expressing Cdc42 ShRNA or control scramble shRNA were treated with or without CASIN (10 μM) in the presence of SDF-1 (Stimulate). The whole cell lysates were analyzed by Western blot for p-PAK, p-aPKC and p-WASP. (B) Mobilization and engraftment of human blood progenitor cells in xenograft mice after CASIN or G-CSF treatment. 5×10^4 CD34$^+$ selected human umbilical cord blood cells were injected intravenously into sublethally irradiated (250 rad) NSG mice and engraftment of human CD45$^+$ cells was confirmed by tail vein bleed at week 8. Mice were injected with G-CSF (100 μg/kg once daily for 5 days, ending 18h prior to sampling), or CASIN (1.2 mg/kg 24h and 2h prior to sampling), versus PBS control. PB was obtained by pericardial puncture and 180 μL was plated in triplicate in methylcellulose. Colony counts were performed at day 7-14. (C) Conditioning of the recipient mice with CASIN results in higher engraftment of human progenitor cells. NSG mice were injected with G-CSF (100 μg/kg once daily for 5 days, ending 18 h prior to transplant), or CASIN (1.2 mg/kg 24 h and 2 h prior to transplant) versus PBS control. Human CD34$^+$ umbilical cord blood cells were selected by immunomagnetic beads, then 4×10^4 cells were injected via tail vein. Engraftment was measured by aspiration of right femurs and FACS analysis after staining for murine and human CD45. (D) CASIN does not affect differentiation. PB from the transplanted mice treated with vehicle or CASIN were stained for hCD33 or hCD19 followed by Flow Cytometry analysis. n=5 per group. (E) CASIN does not induce apoptosis. CD34$^+$ HCB from the transplanted mice described in (D) were gated for Annexin V/7AAD analysis. Results are means plus or minus SD of 3 independent experiments (n=8 per group).

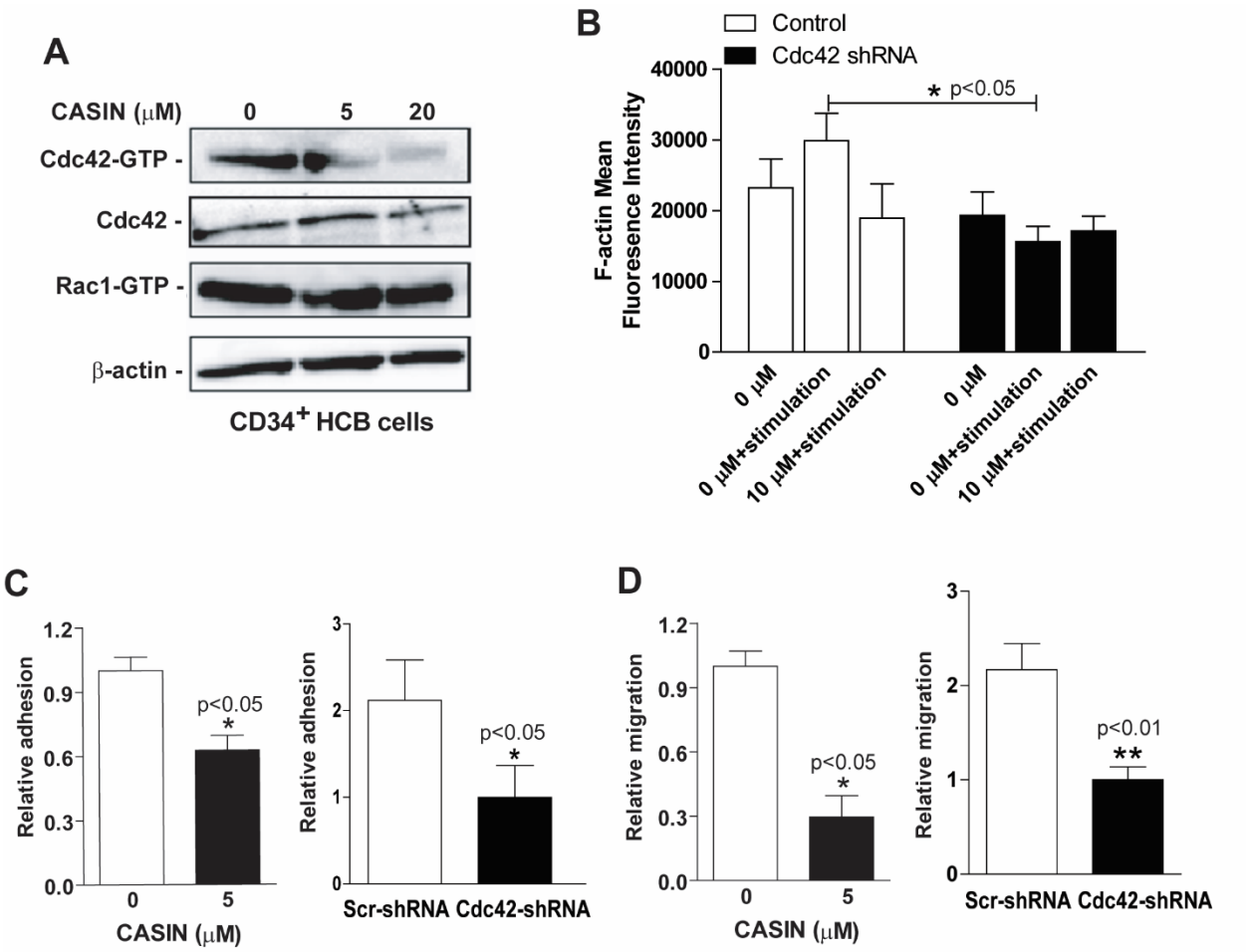

Figure S3

Figure S3. CASIN mobilizes CD34$^+$ HCB cells in xenografted immunodeficient mice. (A) CASIN inhibits Cdc42 activity in CD34$^+$ HCB cells. CD34$^+$ HCB cells were treated with the indicated dose of CASIN followed by protein lysate extraction. Levels of the active GTP-bound Cdc42, Rac, total Cdc42 and Rac were determined by the effector domain (GST-PAK1) pull-down assay. (B) CASIN inhibits F-actin polymerization in WT cells but not in Cdc42 knockdown cells. CD34$^+$ HCB cells expressing Cdc42 shRNA or control scramble shRNA were treated with or without CASIN (10 μM) in the presence of SDF-1 followed

by analysis of F-actin polymerization. Results are means plus or minus SD of 3 independent experiments (n=3 per group). (C, D) CASIN inhibits cell adhesion (C) and migration (D). $CD34^+$ HCB cells were treated with 5 μM CASIN or vehicle followed by determination of cell adhesion to fibronectin CH296 motif (C, left) and migration towards SDF-1α (D, left). Cdc42 knocking down by shRNA led to similar inhibition of adhesion and migration (C & D, right). Results are means plus or minus SD of 3 independent experiments (n=3 per group).

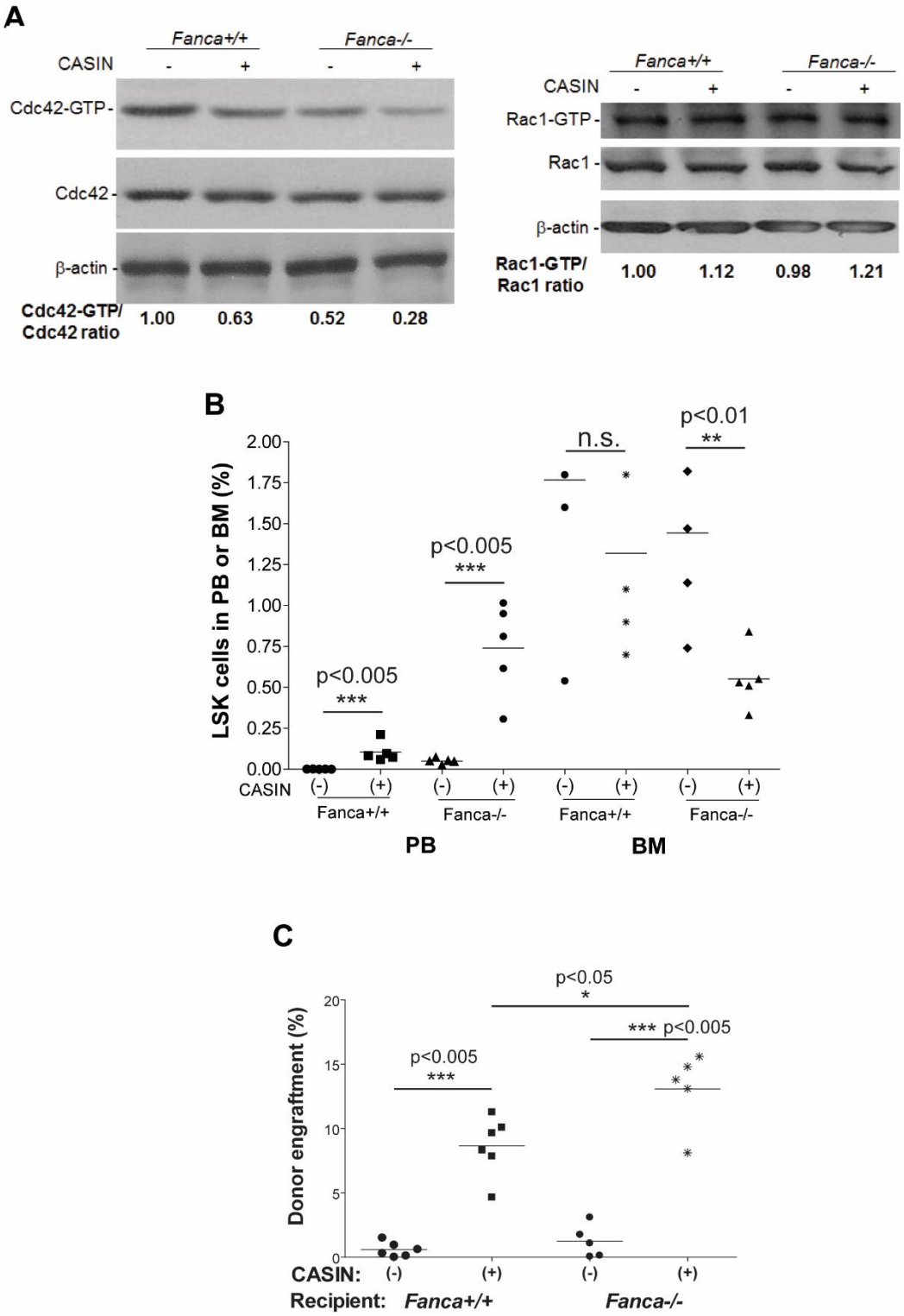
A
Fanca+/+
Fanca-/-
CASIN
- + - +
Cdc42-GTP
Cdc42
β-actin
Cdc42-GTP/ Cdc42 ratio
1.00 0.63 0.52 0.28
Rac1-GTP
Rac1
Rac1-GTP/ Rac1 ratio
1.00 1.12 0.98 1.21
B
LSK cells in PB or BM (%)
p<0.005
***
n.s.
p<0.01
**
(-) (+) (-) (+) (-) (+) (-) (+)
Fanca+/+ Fanca-/- Fanca+/+ Fanca-/-
PB
BM
C
Donor engraftment (%)
p<0.05
*
*** p<0.005
p<0.005
***
CASIN: (-) (+) (-) (+)
Recipient: Fanca+/+ Fanca-/-

Figure S4

Figure S4. CASIN further decreases Cdc42 activity, enhances *Fanca*$^{-/-}$ progenitor mobilization and promotes long-term HSC engraftment. (A) CASIN further decreases Cdc42 activity without affecting Rac1 activity in *Fanca*$^{-/-}$ BM cells. Mice were injected with 15% ethonol (Vehicle) or CASIN (1.2mg/kg, i.p). 48 hrs later, low density BM cells were collected and levels of the active, GTP-bound Cdc42 (left) or Rac1 (right) were determined by the GST-PAK1 pulldown assay. The relative levels of active Cdc42 or Rac1 to total Cdc42 or Rac1 are indicated. (B) CASIN mobilizes Lin$^-$Sca$^+$c-Kit$^+$ progenitor cells (LSK) from BM to PB. *Fanca*$^{-/-}$ or WT mice were treated with CASIN (1.2mg/kg, i.p) or Vehicle followed by PB and BM cell harvest 48 hrs later. Samples were then subjected to Flow Cytometry analysis for LSK cells. Results are means plus or minus SD of 3 independent experiments (n=3-5 per group). (C) CASIN promotes engraftment of WT HSCs in *Fanca*$^{-/-}$ recipient mice. WT (*Fanca*$^{+/+}$) and *Fanca*$^{-/-}$ recipient mice were injected by i.p. twice (24h and 2 h prior to BMT) with 15% ethanol (Vehicle) or CASIN (1.2mg/kg), and transplanted with 3-5 million Boy J BM cells. Engraftment was evaluated 4 months later using antibodies against the mouse CD45.1 (donor) and CD45.2 (recipient). Results are means plus or minus SD of 3 independent experiments (n=5-6 per group).

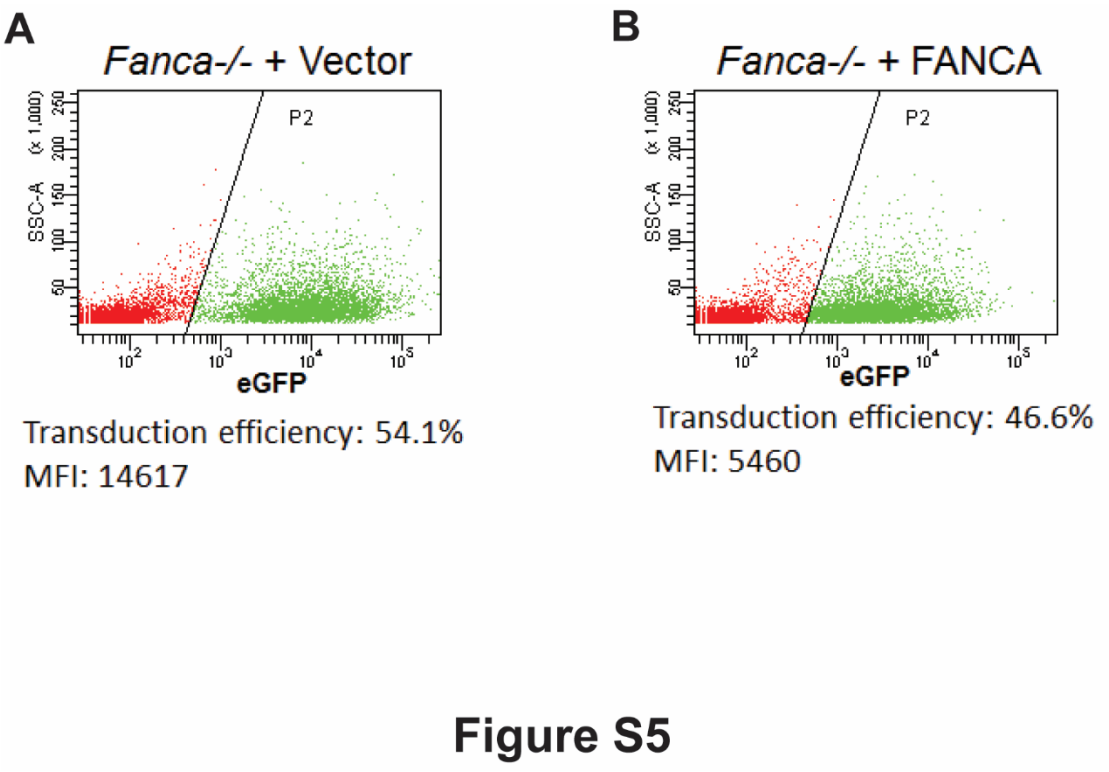

Figure S5

Figure S5. Analysis of transduction efficiency. Lin^- BM cells from *Fanca*$^{-/-}$ mice were transduced with an eGFP vector alone (A) or an eGFP vector expressing the *FANCA* gene (B). The transduction efficiencies were analyzed by flow cytometry for % $eGFP^+$ cells.