Supplementary Methods

PCR primers for Pak2 deletion. PCR genotyping was performed with a primer set to amplify wild-type (306 bp PCR product) or floxed (391 bp PCR product) or null allele (562 bp PCR product): PAK2 alleles: P1, 5'ATCTTCCCAGGCTCTGACT 3'; P2, 5'TGAAGCTGCATCAATCTATTCTG 3'; P3, 5'GAGATGGCTCAGTTATG 3'.

Retroviral vector constructs Retrovirus supernatant was generated from vectors: MIEG2, Cre-GFP 3, PID-GFP4, hCD4 empty vector5, hCD4-ca-MEK15, hCD4-ca-AKT5 in the phoenix-gp cells as previously described2.

Antibodies

Fluorescent labeled antibodies used for flow cytometric analysis included: CD45.1-PE, CD45.1-PE Cy7, CD45.2-FITC, CD45.2-PE Cy5.5, and hCD4-PE Cy7, CD11B-PE, CD3-APC, TER119-PE, B220-APC, and CXCR4-PE (all from BD Biosciences, San Jose, CA).

Isolation and transduction of murine lineage negative lin^-Sca-1^c-Kit^ (LSK) cells

BM cells were isolated from the femora, tibiae, and iliac crests of sacrificed mice by crushing in Iscove's Modified Dulbecco's Media (IMDM) using a mortar and pestle. Low-density mononuclear (LDMN) BM cells were derived using Histopaque-1083 (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s instructions. Lineage positive cells were then depleted using lineage specific biotin-conjugated antibodies and a Dynal MPC-L magnet (Invitrogen, Carlsbad, CA) according to our previously published methods6. The resulting lineage depleted
cells were stained with Streptavidin-PE, Sca-1-FITC, and CD117-APC (all eBioscience, San Diego, CA), in PBS containing 2% (v/v) FCS. Cells corresponding to the lin-Sca-1<sup>-</sup>c-Kit<sup>+</sup> immunophenotype were then isolated by flow sorting (FACS Aria, BD Biosciences, San Jose, CA).

For LSK transduction, freshly isolated LSK cells were pre-stimulated in IMDM supplemented with 10% (v/v) FCS, along with growth cytokines: 100 ng/ml recombinant mouse stem cell factor (rmSCF), 100 ng/ml recombinant human megakaryocyte growth and development factor (rhMGDF) and 100ng/ml recombinant human granulocyte colony stimulating factor (rhG-CSF) (all Peprotech, Rocky Hill, NJ) for 48 hrs at 37°C, 5% CO<sub>2</sub>. Retroviral transduction was performed in non-tissue culture coated multi-well plates (BD Biosciences, San Jose, CA) which had been pre-coated with fibronectin CH296 fragment (Takara Bio Inc., Otsu, Japan) at 4 µg/cm<sup>2</sup>. Thirty-six hrs after the final exposure to viral particles, cells were harvested then re-suspended in PBS, 2% FCS, 5 µg/ml 7-Amino-actinomycin D (7-AAD) (Invitrogen, Carlsbad, CA), and viable GFP<sup>+</sup> cells were isolated by flow sorting (FACS Aria). 2.0x10<sup>6</sup> cells/mL cells were transduced with an MOI to obtain transduction efficiencies of ~65-70% per experiment.

**Bone marrow transplantation experiments and in vivo homing assays**

For bone marrow transplantation experiments, 1.0x10<sup>5</sup> transduced and sorted LSK cells were injected into the tail vein of lethally irradiated recipient mice (11.75 Gy, split dose with minimum of 3 hours between doses) along with 5 × 10<sup>5</sup> freshly isolated whole B6.SJL BM competitor cells. The % of GFP+ cells in the peripheral blood was assessed from 3-weeks post-BMT up to 24-weeks following red cell lysis (Pharm Lyse, Becton Dickinson). For Pak-deficient engraftment rescue experiments, LSK cells were co-transduced with PID-GFP or MIEG, and
hCD4, caAKT, or caMEK1, and sorted for GFP+/hCD4-PE Cy7+ double positive cells. 1.5 x 10^5 GFP+/hCD4-PE Cy7+ cells were then co-transplanted with 5.0 x 10^5 B6.SJL WBM cells into lethally irradiated (11.75 Gy, split dose) C57Bl/6J recipient mice. For Pak2^flox/flox experiments, 1.0 x 10^5 Pak2^flox/flox and Pak2^WT/WT LSK cells were transduced with MIEG or Cre-GFP and co-transplanted with 5.0 x 10^5 WBM cells into sublethally irradiated (2.8Gy) NOD/SCID recipient mice (Jackson Labs, Bar Harbor, ME). For Pak1 germline KO experiments, 3 x 10^6 Pak1^-/- or Pak1^WT/WT WBM was mixed with 3.0 x 10^6 WT Het (CD45.1+/CD45.2+) WBM and transplanted into lethally irradiated (11.75 Gy split dose) B6.SJL recipient mice. After red cell lysis (Pharm Lyse, BD Biosciences, San Jose, CA), peripheral blood was assessed for donor chimerism by flow analysis using an LSRII.

For bone marrow homing assays, LSK cells transduced with either PID-GFP or MIEG control from WT CD45.1+ / CD45.2+ heterozygous donor mice were isolated by flow sorting (FACS Aria). 2.5 x 10^5 GFP+ LSK cells were transplanted into lethally irradiated C57Bl6J recipient mice. BM from one leg (iliac crest, tibia, and femur) from each recipient was harvested, stained for viability using Hoechst (Invitrogen, Carlsbad, CA) and the number of GFP+ cells was assessed post-intravenous infusion.

**In vitro chemotaxis and transwell migration assays**

For in vitro time lapse microscopy, approximately 50,000 PID-GFP or MIEG transduced LSK were plated for 1 h on a fibronectin-coated glass coverslip then placed in a Dunn chemotaxis chamber (Hawksley, Lancing, Sussex UK). SDF1α (100 ng/mL) was added as a chemoattractant in the outer well, and cells were imaged for at least one hour at 15-s intervals using an Eclipse Ti microscope and NIS-Elements software (Nikon, Melville, NY). For in vitro transwell assays, 1.0 x 10^5 transduced LSK cells were plated in the upper well of a transwell
chamber with 5-μm pore filters (Corning, Corning, NY) in triplicates. After two hours of incubation at 37°C in 5% CO2, migrated cells in the bottom chamber were enumerated using Countbright Beads (Invitrogen, Carlsbad, CA) according the manufacturer’s instructions and analyzed by FACS (LSR II, BD Biosciences, San Jose, CA). SDF-1α–induced chemokinesis (random migration) was assessed by placing 100 ng/mL SDF-1α in both upper and lower chambers.

F-actin staining and quantification of cell morphological changes in response to chemokine stimulation

XYZ series fluorescence images were captured with a PerkinElmer UltraView Vox Spinning Disc confocal microscope (Nikon Inc., Melville, NY) equipped with a 60x objective lens and ultra view molecular laser, connected to a Hamamatsu C9100-50 camera driven by Velocity software (Perkin Elmer, Waltham, MA). Cell area and perimeter was calculated using ImageJ software (http://rsbweb.nih.gov/ij).

Intracellular phospho-flow

PID-GFP and MIEG transduced LSK were isolated, incubated with cytokines for 3 hours, then starved for 1 hour in 2%BSA+IMDM. Cells were then stimulated for 5 minutes using growth cytokines, rmSCF(100ng/mL), rhG-CSF (100ng/mL), rhMGDF (100ng/mL) (all from Peprotech, Rocky Hill, NJ).
Colony-Forming Cell Assays

1.0 x 10^4 GFP+ cells were isolated and used to perform methylcellulose colony-forming cell (CFC) assays as previously described\textsuperscript{7}.

Supplemental Figure Legends

S1. Efficacy of PID in suppressing Pak activity. NIH-3T3 cells were infected with empty MIEG virus (lanes 2 and 4) or a MIEG virus encoding Gst-PID (lanes 1 and 3). 2 days post-infection, the cells were starved for 4 h in serum-free media, then stimulated with vehicle (lanes 1 and 2) or 5 ng/ml PDGF (lanes 3 and 4) for 5 min and an in-gel kinase assay was performed with MBP as substrate, as described by Ding and Badwey\textsuperscript{1} with myelin basic protein (MBP) as substrate rather than p47\textsuperscript{phox}. Upper panel: anti-(Gst)-PID immunoblot; lower panel, in-gel kinase assay.

S2. Flow cytometric analysis of GFP+ cells from MIEG and PID transduced LSK. GFP+ cells were isolated stained with anti- CXCR4-PE. We found no differences in the surface expression of CXCR4 in LSK transduced with PID or MIEG. n=10 donors per experiment, 2 independent experiments.

S3. Colony forming assays of GFP+ cells from PID or MIEG transduced LSK cells. The number of erythroid (BFU-E), myeloid (CFU-GM) or mixed (CFU-GEMM) colonies were scored 7 days after culture in compete methylcellulose medium. Data represent the mean± SEM of CFU performed in triplicate, two independent experiments. ns=not significant.

S4. Multi-lineage differentiation of MIEG transduced LSK. Six months post-BMT, BM was harvested and lineage analysis was performed. Flow cytometry using lineage specific antibodies gated on the GFP+ donor populations was performed. Recipients transplanted with PID transduced LSK were completely absent for donor cells.
S5. Constitutively active MEK1 (caMEK1) transiently rescues defects in Pak-deficient HSC/P engraftment and proliferation. 1.5x10^5 PID or MIEG (GFP+) cells co-transduced with caMEK, caAKT or hCD4 empty vector control, were co-transplanted with 5.0x10^5 CD45.1 whole bone marrow into lethally irradiated C57Bl/6J (CD45.2) recipient mice. At 6-weeks post transplantation, PID + caMEK double transduced cells were no longer present. Data shows the % of GFP cells in the peripheral blood at 6-weeks.

S6. Verification of Pak2 deletion in Pak2\textsuperscript{fl/fl} cells by DNA PCR using primers that amplify Pak2 Pak2 flox (306 bp), and Pak2 deleted (562bp) sequences.

S7. Colony forming assays of GFP+ cells from Pak2\textsuperscript{WT/WT} and Pak2\textsuperscript{fl/fl} transduced LSK cells with Cre-GFP (grey) or MIEG empty vector control (black). The number of colonies were counted 7 days after plating in complete methylcellulose medium. Data represent the mean± SEM of CFU performed in triplicate from n=10 donor mice per genotype. ns=not significant.

Movie S1. Migration of MIEG transduced LSK cells adhered on a fibronectin-coated coverslip, subjected to an SDF1α gradient in a Dunn chemotaxis chamber and imaged by time-lapse microscopy (3 frames/min for 1 h). Field dimensions are 227.5 × 227.5 μm.

Movie S2. Migration of PID transduced LSK cells adhered on a fibronectin-coated coverslip, subjected to an SDF1α gradient in a Dunn chemotaxis chamber and imaged by time-lapse microscopy (3 frames/min for 1 h). Field dimensions are 227.5 × 227.5 μm.
Supplemental Figure S1.

The figure shows a blot with lanes labeled 1 through 4, with bands indicated for PID and Pak. The PID blot shows a difference in expression levels between the lanes.

Supplemental Figure S2.

The figure depicts a histogram for CXCR4, with different conditions represented by the colored lines. The Y-axis represents the percentage of cells, and the X-axis shows the frequency of cell counts.

Supplemental Figure S3.

The figure illustrates a bar graph showing the number of colonies for different cell types: BFU-E, CFU-GM, and CFU-GEMM. The graph includes error bars indicating variability and ns annotations for non-significant differences.
Supplemental Figure S4.

Supplemental Figure S5.

Supplemental Figure S6.
Supplemental Figure S7.
REFERENCES

1. Ding J, Badwey JA. Stimulation of neutrophils with a chemoattractant activates several novel protein kinases that can catalyze the phosphorylation of peptides derived from the 47-kDa protein component of the phagocyte oxidase and myristoylated alanine-rich C kinase substrate. *J Biol Chem.* 1993;268(23):17326-17333.


