# CXCR4 mediated bone marrow progenitor cell maintenance and mobilization are modulated by c-kit activity

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# Online Supplement

## Detailed Methods

### Isolation of BM and PB MNCs

BM cells were collected by flushing femurs and tibias with 5 mM EDTA in PBS (PBS-E), and PB was collected via cardiac puncture with heparin-coated needles, then diluted 2-fold with PBS-E. After collection, the BM and PB were overlayed onto Histopaque-1083 (Sigma-Aldrich) and centrifuged at 2145 RPM for 20 minutes at room temperature. BM or PB MNCs were collected from the buffy-coat and thoroughly washed.

**Flow cytometry**
Cells were blocked with 50% rat serum and mouse Fc blocker (BD Bioscience) for 10 minutes, then stained for 30 minutes with APC-conjugated anti-Lin, PE-Cy7–conjugated anti-c-kit, FITC-conjugated anti-Sca-1, biotin-conjugated anti-CXCR4, PerCP-Cy5.5-conjugated streptavidin, and their corresponding isotype control antibodies (BD Bioscience). Analyses were performed on a multi-parametric flow cytometer (Dako Cyan).

**Transplantation of retrovirally transduced c-kit$^{WT}$ and c-kit$^{D816V}$ BM MNCs**
MIG-HyKIT$^{WT}$ and MIG-HyKIT$^{D816V}$ retroviral vectors were generated by co-transfecting 293FT cells with a packaging plasmid, pIK6.1 MCV.ecopac.UTd, and one of two backbone plasmids: pMSCV-HyKIT$^{WT}$-IRES-eGFP (pMIG-HyKIT$^{WT}$), or pMSCV-HyKIT$^{D816V}$-IRES-eGFP (pMIG- HyKIT$^{D816V}$) [1]; the transfection was performed via the calcium-phosphate method [2], and virus-containing supernatant was collected 48 hours after transfection.

BM MNCs were isolated from donor C57BL/6 mice 2 days after intraperitoneal injection of 5-fluorouracil (150 mg/kg), stimulated for 2 days in IMDM supplemented with 20% FBS, 10 ng/mL SCF, 6 ng/mL IL-3, 50 ng/mL Flt-3 ligand (R&D Systems), and 10 ng/mL thrombopoietin (PreproTech), then infected with the MIG-HyKIT$^{WT}$ or MIG-HyKIT$^{D816V}$ retroviruses. Multiplicities of infection (MIG-HyKIT$^{WT}$: 2.3; MIG-HyKIT$^{D816V}$: 1.5) were based on titration in NIH 3T3 cells, and transfection efficiency was evaluated 3 days later via flow-cytometry analysis of GFP expression. The BM of recipient C57BL/6 mice was ablated via lethal irradiation (12Gy), then $3.4\times10^6$ transfected BM MNCs were injected into the tail vein. The BM of recipient mice was allowed to repopulate for 3 weeks before subsequent analyses were performed.

**Tissue sectioning and immunofluorescent staining**
Mice were subcutaneously injected with AMD3100 (5 mg/kg) or PBS and sacrificed 15 minutes later. Femurs were harvested, fixed in 4% PFA for 5 hours, incubated in 10% EDTA at 4°C under constant agitation for 48-72 hours (the EDTA solution was changed every 24 hours), fixed again in 4% PFA for 30 minutes, incubated in 30% sucrose at 4°C for 24 hours, frozen in OCT compound, and cut into 6-µm sections with a Microm HM550 (Thermo Fisher Scientific Inc.). Subsequent evaluations were performed in H&E-stained sections or in sections stained for immunofluorescence. For immunofluorescent evaluations, sections were incubated in 0.5% Triton for 5 minutes, in 10% horse serum/PBS for 30 minutes, and in 5% horse serum, 2% BSA and 0.1% Tween 20 with rabbit anti-mouse c-kit (1/50) or anti-mouse phospho–c-kit (1:100) antibodies (Santa Cruz) in a humid chamber at 37°C for 2 hours; then the sections were rinsed with PBS and incubated with secondary antibodies (1:250) at room temperature for 1 hour and with DAPI for 10 min. After staining, sections were rinsed in PBS and mounted.

**Cell adhesion assay**
Six-well tissue culture plates were coated with 10 ug/mL recombinant murine VCAM-1 (R&D Systems) for 30 min and blocked with 10% goat serum, then $6\times10^6$ freshly

isolated BM MNCs, suspended in serum-free HEPES medium, were added to each well and incubated in a 5% $CO_2$ incubator at 37°C for 15 min. Nonadherent and loosely attached cells were removed by tapping each plate and gently washing the wells 3 times with PBS. Anti-α4 integrin PS/2 antibodies (100 ug/mL, purified from cultured hybridoma cells [ATCC] with Montage Antibody Purification kits) [3], AMD3100 (100 ng/mL), recombinant SDF-1 protein (200 ng/mL; R&D systems), SCF (100 ng/mL), or the corresponding delivery vehicles were added to each well, then the cells were incubated at 37°C under 5% (v/v) $CO_2$ for 15 min, and adhesion was evaluated under a light microscope.

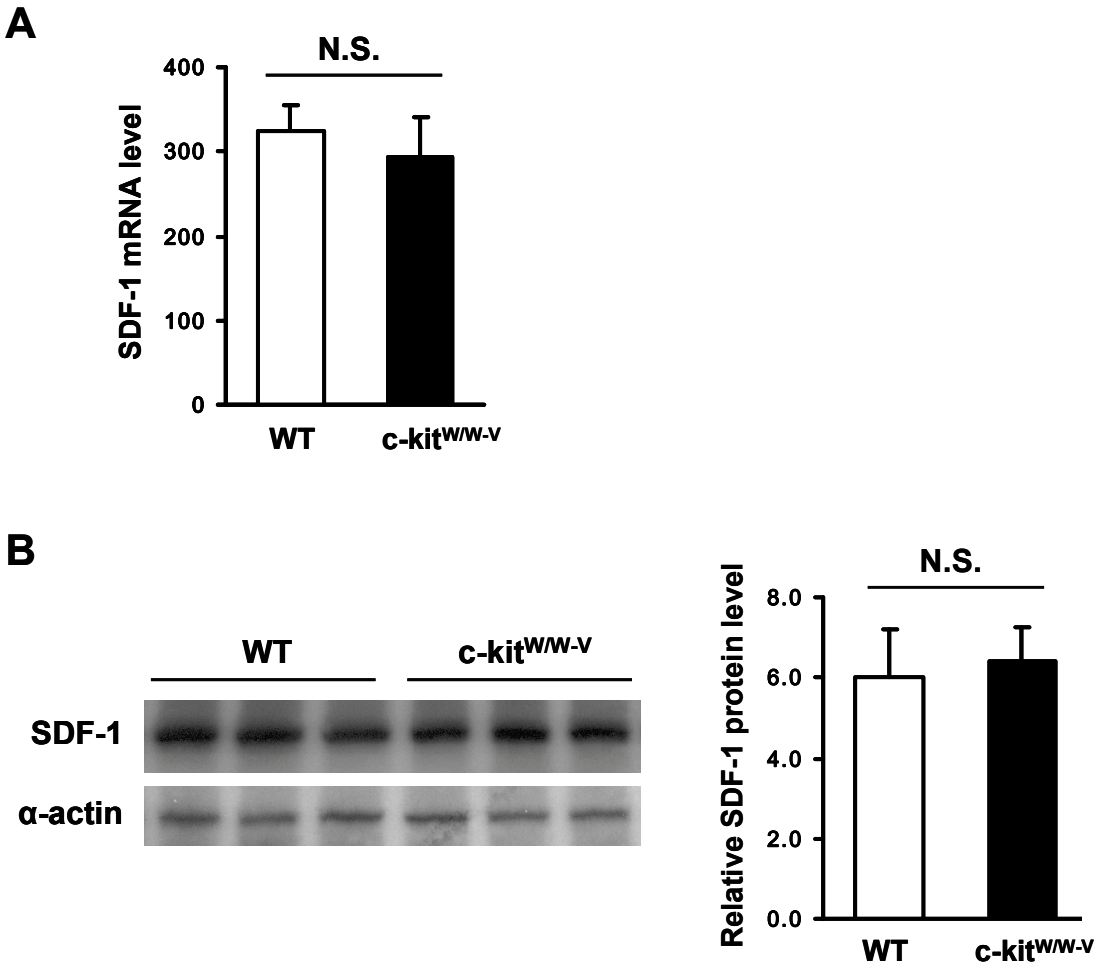
**Western blotting and co-immunoprecipitation**

Cells were lysed in SDS-containing sample buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromphenol blue) or in cell-lysis buffer (50 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 1% w/v Triton X-100, 10 mM Sodium orthovanadate, 10 mM sodium beta-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% v/v β-mercaptoethanol, and protease-inhibitor tablets [one tablet/10 mL, Roche Applied Science], pH 7.5). Immunoblotting was performed via standard techniques [4-5] with anti-phospho-c-kit (Tyr 719), anti-c-kit, anti-phospho-Src (Tyr 416), anti-Src, anti–SDF-1 (Cell Signaling Technology), and control IgG (Santa Cruz Biotechnology, Inc) antibodies. For co-immunoprecipitation, cells were lysed in cell-lysis buffer containing 0.1% w/v Triton X-100 and the cell lysates were incubated overnight at 4°C with the appropriate antibody, then incubated with protein A/G plus-Agarose (Santa Cruz) for 1 hour at 4°C. After washing, the immunoprecipitates were eluted by boiling for 5 min, and extracts were analyzed by immunoblotting as described above. Band intensities were determined densitometrically with Image J software.

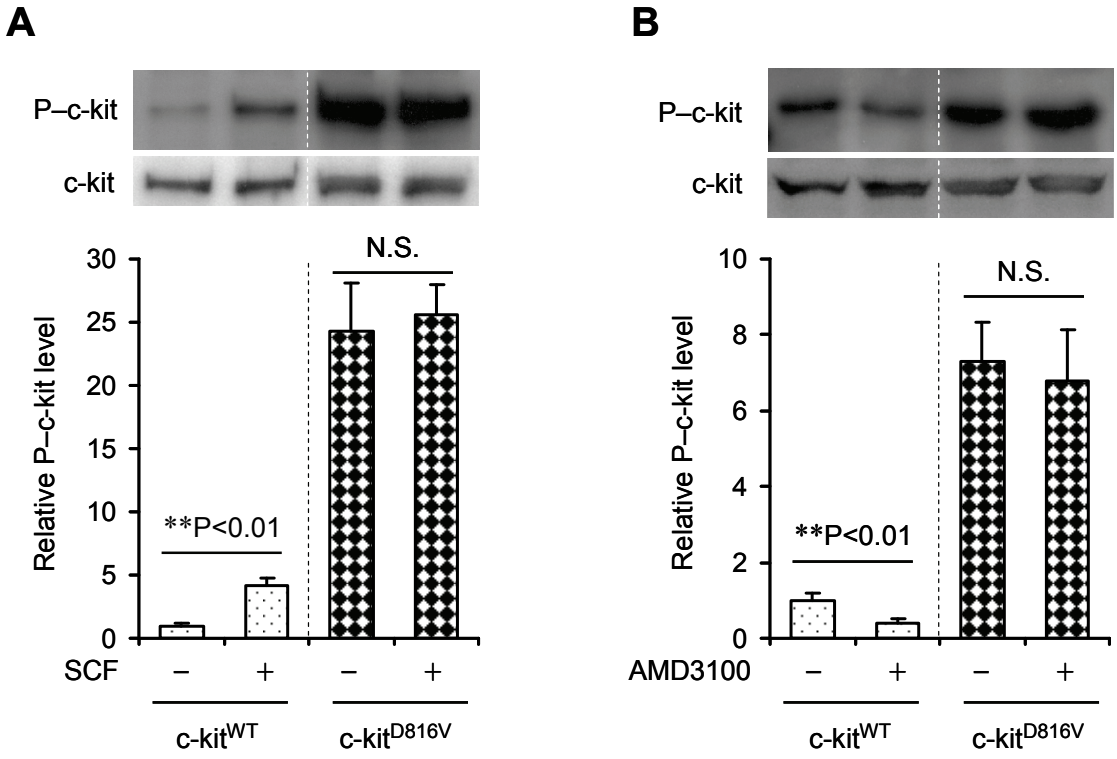
**Quantitative real-time RT-PCR**

Real time RT-PCR was performed via standard techniques [6]. Briefly, total RNA was extracted with RNA Stat-60 (Tel-Test, Friendswood, TX), and RNA was reverse transcribed with the Taqman Multiscribe RT kit (Applied Biosystems). Real-time PCR was performed in duplicate with cDNA from 10 ng of RNA by using the Lightcycler hybridization Probes Master Mix (Roche); a negative control (lacking a template) was included for each probe set. Relative gene expression was calculated using the Ct method and normalized to GAPDH. The primer sequences for mouse CXCR4 were 5'-CCTCGCCTTCTTCCACTGTT-3' (forward) and 5'-CTGGGCAGAGCTTTTGAACTTG-3' (reverse). The primer sequences for mouse SDF-1 were 5′-CCTCCAAACGCATGCTTCA-3′ (forward) and 5′-CCTTCCATTGCAGCATTGGT-3′ (reverse).

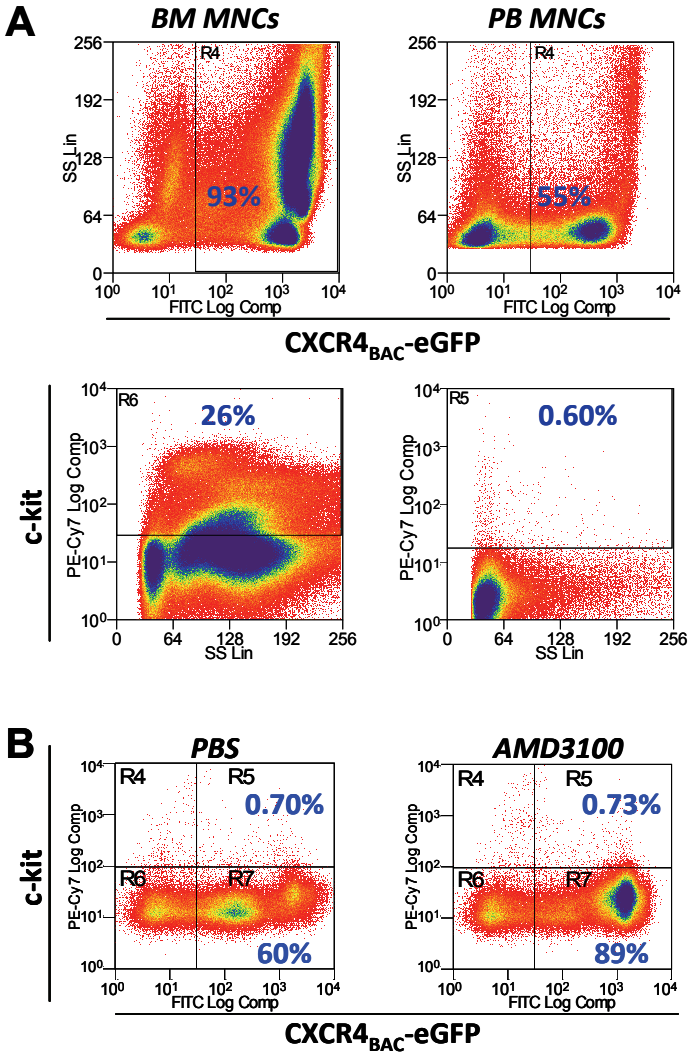
## Supplemental Figures and Figure Legends




**Online Figure I: WT and c-kit$^{W/W-V}$ mice express similar levels of SDF-1 in the BM.** (**A**) SDF-1 mRNA levels were assessed in BM lysates from WT mice and c-kit$^{W/W-V}$ mice via quantitative real-time RT-PCR and normalized to 18S (n=6). (**B**) SDF-1 protein levels were evaluated by Western blotting (*left panel*), quantified densitometrically, and normalized to the levels of α-actin (*right panel*) (n=3). *N.S.*, not significant.




**Online Figure II: c-kit remains constitutively active in c-kit$^{D816V}$–transduced BM MNCs for at least 3 weeks after transplantation.** MNCs were harvested from WT mice, transduced *ex vivo* with retroviral vectors coding for eGFP-linked WT c-kit (c-kit$^{WT}$) or eGFP-linked constitutively active c-kit (c-kit$^{D816V}$) as described in Figure 2, then transplanted into WT mice after lethally irradiating the endogenous BM. Three weeks after transplantation, BM MNCs were isolated from the recipient mice, and c-kit and phospho–c-kit (P–c-kit) levels were measured in (**A**) cells treated with SCF-1 (100 ng/ml) or Vehicle (–) and in (**B**) cells applied to VCAM-1–coated plates and sequentially treated with SDF-1 and AMD3100 or with SDF-1 and Vehicle as described in Figure 3; each treatment lasted for 15 min. c-kit and P–c-kit levels were analyzed by Western blot and quantified densitometrically. P–c-kit levels were normalized to the total c-kit levels and expressed as the fold-difference from the levels measured in vehicle-treated c-kit$^{WT}$–MNCs. Values are mean ± SEM (**P<0.01, n=10 per group). SCF significantly upregulated c-kit phosphorylation, and AMD3100 significantly downregulated SDF-1–induced c-kit phosphorylation, in c-kit$^{WT}$ MNCs but not in c-kit$^{D816V}$ MNCs. Collectively, these observations confirm that c-kit was constitutively activated (i.e., c-kit activity could be neither upregulated nor downregulated) in MNCs from mice transplanted with c-kit$^{D816V}$–transduced BM cells.




**Online Figure III: AMD3100 treatment does not alter the proportion of PB MNCs that express c-kit.** (**A**) BM and PB MNCs were isolated from CXCR4$_{BAC}$-eGFP mice, then the proportion of cells that expressed CXCR4 (i.e., eGFP$^+$ cells, *upper panels*; n=3) or c-kit (*lower panels*; n=4) were evaluated via flow cytometry. (**B**) CXCR4$_{BAC}$-eGFP mice were subcutaneously injected with PBS or AMD3100; 2 hours later, PB MNCs were isolated and the proportion of cells that expressed CXCR4 (i.e., eGFP$^+$ cells) or co-expressed CXCR4 and c-kit was evaluated via flow cytometry (n=4).

## Supplemental References


1. Xiang Z, Kreisel F, Cain J, Colson A, Tomasson MH. Neoplasia driven by mutant c-kit is mediated by intracellular, not plasma membrane, receptor signaling. *Mol Cell Biol*. 2007;27:267-282

2. Qin G, Takenaka T, Telsch K, Kelley L, Howard T, Levade T, Deans R, Howard BH, Malech HL, Brady RO, Medin JA. Preselective gene therapy for fabry disease. *Proc Natl Acad Sci U S A*. 2001;98:3428-3433

3. Qin G, Ii M, Silver M, Wecker A, Bord E, Ma H, Gavin M, Goukassian DA, Yoon YS, Papayannopoulou T, Asahara T, Kearney M, Thorne T, Curry C, Eaton L, Heyd L, Dinesh D, Kishore R, Zhu Y, Losordo DW. Functional disruption of alpha4 integrin mobilizes bone marrow-derived endothelial progenitors and augments ischemic neovascularization. *J Exp Med*. 2006;203:153-163

4. Qin G, Kishore R, Dolan CM, Silver M, Wecker A, Luedemann CN, Thorne T, Hanley A, Curry C, Heyd L, Dinesh D, Kearney M, Martelli F, Murayama T, Goukassian DA, Zhu Y, Losordo DW. Cell cycle regulator e2f1 modulates angiogenesis via p53-dependent transcriptional control of vegf. *Proc Natl Acad Sci U S A*. 2006;103:11015-11020

5. Zhou J, Zhu Y, Cheng M, Dinesh D, Thorne T, Poh KK, Liu D, Botros C, Tang YL, Reisdorph N, Kishore R, Losordo DW, Qin G. Regulation of vascular contractility and blood pressure by the e2f2 transcription factor. *Circulation*. 2009;120:1213-1221

6. Tang YL, Zhu W, Cheng M, Chen L, Zhang J, Sun T, Kishore R, Phillips MI, Losordo DW, Qin G. Hypoxic preconditioning enhances the benefit of cardiac progenitor cell therapy for treatment of myocardial infarction by inducing cxcr4 expression. *Circ Res*. 2009;104:1209-1216