Supplemental Data

Supplemental Figures

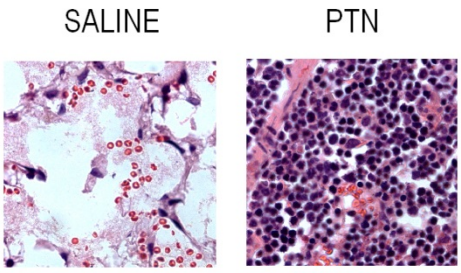

Figure S1. H&E stained femurs (H&E, 63x) are shown from 700 cGy-irradiated mice at day +10, treated every other day with saline or PTN.

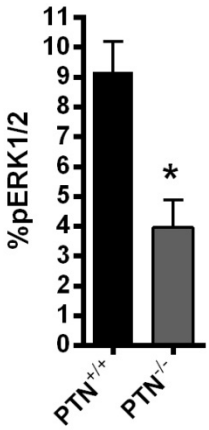

Figure S2. Mean percentage p-ERK1/2 levels are shown in BM KSL cells from $PTN^{+/+}$ and $PTN^{-/-}$ mice (*P=0.005, n=3-5).

Supplemental Methods

Radiation Survival Studies and Hematopoietic Cell Transplantation Studies

Ten to 12 week old female C57Bl6 mice were irradiated with an LD50/30 radiation dose (700 cGy) of TBI using a Cs137 irradiator. The first group of mice received intraperitoneal injection of 2 or 4 μg PTN or saline control in a volume of 100 ul starting at 24 hours post-irradiation. Treatment was administered every other day through day +14. For all other survival studies, mice received PTN subcutaneously (SQ). For the survival studies which compared PTN treatment versus PTN + Tipifarnib, PTN 4 μg SQ and Tipifarnib 1 mg (or vehicle), via gavage, were administered every other day through day +21. For hematopoietic cell transplant studies, 10-12 week old female C57Bl6 mice were irradiated with an LD100/30 dose of 850 cGy. Mice were then transplanted via tail vein with non-irradiated donor C57Bl6 BM cells (1×10^5 cells/mouse). Mice then received PTN or saline beginning at 24 hours post-irradiation. Treatment was administered every other day through day +14.

HSC culture and Ras inhibition studies

BM HSCs were isolated and purified from mice as previously described (22). Lineage$^-$ cells were stained with FITC-conjugated anti-CD34 (eBioscience), PE-conjugated anti-sca-1, and APC-conjugated anti-ckit (Becton Dickinson [BD]), or isotype controls. Sterile cell sorting was conducted on a BD FACS-Aria cytometer. Purified ckit$^+$sca-1$^+$lin$^-$ (KSL) cells or CD34$^-$c-kit$^+$sca-1$^+$lin$^-$ (34$^-$KSL) cells were collected into IMDM + 10% FBS + 1% pcn/strp.

In vitro inhibition of Ras signaling was performed by addition of the farnesyltransferase inhibitor FTI-276 (R&D Systems) at a concentration of 20 uM. In vivo inhibition of Ras signaling was accomplished by treating mice with the Ras inhibitor, Tipifarnib (SelleckChem) at +1 hr following TBI and every other day through day +21. Tipifarnib was dissolved in a 20% (2-Hydroxypropyl)-β-cyclodextrin solution at a concentration of 1 mg/100 μL. Mice were gavaged with 100 uL of Tipifarnib or cyclodextrin vehicle.

Competitive Repopulating Unit (CRU) Assays

BM 34^-KSL cells from donor $CD45.1^+$ B6.SJL mice were isolated. The cells were cultured for 7 days in TSF media (IMDM containing 10%FBS, 1% pen-strep, 20 ng/ml Thrombopoietin, 125 ng/ml SCF, 50 ng/ml Flt-3 ligand, R&D) alone, TSF media + 100 ng/ml PTN, or TSF media + PTN + 20 uM FTI-276. Recipient $CD45.2^+$ C57Bl6 mice received 950 cGy total body irradiation (TBI) using a Cs137 irradiator and were then injected via tail vein with the progeny of 10 BM 34^-KSL cells from the above culture conditions. Non-irradiated host BM MNCs (1×10^5 cells/mouse) were injected as competitor cells. Multi-lineage hematopoietic engraftment was measured in the peripheral blood (PB) by flow cytometry, as previously described (16, 22).

Colony Forming Cell (CFC) Assays

Total CFC assays and colony-forming unit-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM) assays, to measure multipotent hematopoietic progenitor cells, were performed as we have previously described (8). For the studies of irradiated and transplanted mice, 2×10^4 BM cells from each mouse were plated per CFC dish. For the in vitro studies, the progeny of 200 KSL cells cultured for 7 days were plated per CFC dish. Colonies were grown in MethoCult media (StemCell Technologies) for 14 days.

Flow Cytometric Analyses

KSL cells were cultured in the following conditions: IMDM media alone, IMDM + 100 ng/ml PTN (R&D), IMDM + 100 ng/ml PTN (R&D) + 20 uM FTI-276 (R&D). After 6 hours of treatment, cell cycle analysis was performed as previously described (8). After fixation and treatment with Saponin for permeabilization, cells were stained for 30 min with Ki-67 FITC antibody (BD), rinsed again, and then re-suspended in Saponin buffer with 7AAD (BD). At +24, +48, and +72

time points, Annexin V-FITC /7AAD staining was also performed to assess cell death per the manufacturers protocol (BD).

For phospho-flow cytometric analysis of pGrb2 (pS159, Sigma Aldrich SAB4504672), pERK1/2 (pT202/pY204, BD Biosciences 612952), and pALK (Y1604, Cell Signaling Technology 3341), the following methodology was used. KSL cells were stimulated with 100 ng/ml rPTN for 15-30 minutes at 37°C. Cells were fixed with BD Cytofix™ buffer (Catalogue number 554655) for 10 minutes at 37°C and then permeabilized by adding BD™ Phosflow Perm Buffer III (Catalogue number 558050) for 30 minutes on ice. Cells were washed twice and stained with the primary antibody for 1 hour. The cells were then rinsed and stained with the secondary antibodies prior to FACS analysis.

Immunofluorescence and Immunohistochemical Analyses

Hematoxylin and Eosin (H&E) staining was performed on femur sections from mice. Femurs were fixed overnight in 4% paraformaldehyde, decalcified and embedded in OCT medium. Cryostat sections were stained for H&E as previously described (8, 16). pERF (pT526, Abcam ab59178) analysis was performed by treating lineage negative BM cells for 30 min in IMDM or IMDM + 100 ng/ml rPTN. Cells were then spun onto glass slides using a Cytospin centrifuge at a density of 2×10^4 cells/slide. Samples were fixed with 4% paraformaldehyde for 20 minutes, washed with PBS + 0.1% Tween, and blocked with PBS + 0.1% Tween + 20% goat serum. The primary antibody was diluted 1:100 in PBS + 0.1% Tween and added for 1 hour. After several rinses, the secondary antibody (goat anti-rabbit Alexa Fluor 488; Life Technologies) was added and the slides were rinsed and mounted with Vectashield Mounting Media with DAPI. The number of cells in each image which were pERF positive were counted.