Vector constructs. The full-length murine *Hhex* coding sequence was cloned into the pUHD10-3 IRES-GFP vector, which was kindly provided by Dr. Owen Witte (University of California, Los Angeles, Los Angeles, CA). The pUHD10-3 IRES-GFP vector contains an internal ribosomal entry site (IRES) and a green florescent protein (GFP) gene downstream of the tTA responsive promoter, PhCMV*-1. The pUHD-HEX-IRES-GFP vector can then be used in combination with pCAG 20-1, containing the Tet-transactivator, to drive *Hhex* expression.

Transfection. pUHD-HEX-IRES-GFP construct integrity was tested by transfecting 293T cells with pCAG 20-1 and pUHD-HEX-IRES-GFP using the FuGene Transfection Reagent (Roche). At time of transfection, cells were placed with or without Tet for 24hrs. 293T cells were viewed under a fluorescent microscope for GFP expression and assayed for HHEX expression via Western blot.

Generation of Hhex re-expression ES line. The parental *Hhex−/−* Jet ES line underwent two successive rounds of electroporation and selection. First, *Hhex−/−* ES cells were electroporated with pCAG 20-1 and pUHD10-3 Puro to create the tTAHEX ES line. When the Tet-regulatory system is working, the expression of the puromyocin resistance gene is suppressed in the presence of Tetracyline (Tet), and the cells will die in puromyocin. 24hrs after electroporation, clones were selected with 0.75ug/mL puromyocin. Clones were then tested for the presence of the Tet-regulatory system, by placing the cells in 1ug/ml TET and 0.75ug/mL puromyocin. tTAHEX clone A1 then underwent a second round of electroporation with the pUHD-HEX-IRES-GFP vector and a hygromyocin resistance gene containing plasmid, pcDNA3.1 Hygro (+) (Invitrogen). Hex-inducible ES clones (A1HEXJET4) containing the Tetracycline (Tet) inducible system were isolated in the presence of 1ug/ml Tet and 100ug/ml Hygromyocin. Clones were analyzed for eGFP expression at 24 after removal of Tet. A1HEXJet4 clone D8 exhibited 38% GFP expression after 24hrs and a normal ES/OP9 differentiation pattern, based on the parental *Hhex−/−* ES line, and thus was used in subsequent experiments.

Induction of Hhex expression during hematopoietic development. A1HEXJet4 ES were co-cultured in the presence of 100ng/ml Tet, starting at day 0. Tet was removed from the media at either day 3 or 5 of co-culture. Co-cultures were harvested from days 6 to 8 in order to assess the affect of *Hhex* re-expression on the differentiating progenitors. On day 5 of co-culture, both differentiating ES cells and OP9 stromal were harvested with 0.25% Trypsin-EDTA. After trypsinization, $4.31x10^4/cm^2 – 5.51x10^4/cm^2$ total cells were re-seeded on to a new layer of confluent, irradiated OP9 cells until the end of the co-culture time period; for a maximum of 9 total days.

Hhex expression time course. On day 0, $1x10^3$ undifferentiated murine E14Tg2a (tTA5-4) ES cells were co-cultured on non-irradiated, confluent OP9 stroma expressing an mStrawberry Lentiviral expression vector. Differentiating ES cells and OP9 stroma were harvested with 0.25% Trypsin-EDTA (Stem Cell Technologies) on days 4 through 8 of co-culture. Total co-cultures were then flow sorted for ES-derived progeny, mStrawberry negative cells, using the using a FACSVantage SE Sorting Flow Cytometer (Becton Dickinson). mRNA was extracted using the RNeasy Mini kit (Qiagen) and then treated with RNase-free DNAse1 (Promega). cDNA synthesis was performed with SuperScript II reverse transcriptase (Invitrogen) according to manufacturer's directions. Semi-quantitative Real Time PCR was performed using the iQ SYBR

Green Supermix (Bio-Rad Laboratories, Inc.) and an iCycler Thermal cycler (Bio-Rad Laboratories, Inc.). The PCR primers used to detect Hhex were: forward; 5′-AGGAGAATCCTCAAAGCAA-3′, reverse; 5′-AGGCACCTTTATTCTGTTCA-3′.

Figure S1. *Hhex* Expression in differentiating ES cells
Time course of differentiation from E14Tg2a ES cells. (A) Time course of Flk-1 and CD45 expression from ES-derived progeny on days 3 to 9 of co-culture. (B) Real time PCR analysis of *Hhex* expression from differentiating E14Tg2a ES. Y-axis represents fold change in *Hhex* expression relative to Day 4 co-culture derived ES cells after normalization for GAPDH expression.

Figure S2. Representative immunostaining profiles of wt and *Hhex*−/− co-cultures
CD41 and CD45 analysis gates are indicated and frequencies of each population are labeled. Upper gate (box) represents the CD41+ population analyzed (Ai, Bi). Right-hand gate represesnt the CD45+ population analyzed (Aiii, Biii). Gate represents the c-kit+ population analyzed within either the CD41 or CD45 gate (Aii, Aiv, Bii, Biv).

Figure S3. Normal level of viable *Hhex*−/− CD41+ and CD45+ cells on day 6 to 9 of co-culture
(A) Frequency of 7-AAD+ cells within the CD41+ cell population, as determined by flow cytometry, from wt Jet (○) and *Hhex*−/− (●) co-cultures on days 6 to 9. (B) Frequency of 7-AAD+ cells within the CD45+ cell population from wt Jet (○) and *Hhex*−/− (●) co-cultures.

Figure S4. Cell cycle analysis of early and intermediate progenitor populations
Representative immunostaining profiles of (A) wt and (B) *Hhex*−/− co-cultures for early CD41+CD45– progenitors and intermediate CD41+CD45+ progenitors. The cycling populations were then defined by c-kit (CD41+CD45–c-kit+ and CD41+CD45+c-kit+ cells). Cell cycle was then determined by Hoechst 33342 staining. Upper left gate (box) represents the CD41+CD45- population analyzed (Ai, Bi). The upper right gate represesnt the CD45+CD41- population analyzed (Aii, Bii). The CD41+CD45- and CD45+CD41- populations were then gated on c-kit (Aiii, Aiv, Biii, Biv). Area vs Width profiles were then used to discriminate between single cells and aggregates, single cells were then gated (thick black line) and analyzed for cell cycle parameters (Av, Avi, Bv, Bvi).

Figure S5. Hematopoietic rescue of *Hhex*−/− Jet ES cells
Hhex expression was introduced in A1HEXJET4, cl. D8 ES cells at day 3 (black, square) and day 5(light grey, circle) of differentiation. At day 6 of differentiation, the number of CD45+ cells present in co-culture was the same for control (Hhex−/− cells)(+TET, dark grey, triangle) and Hhex re-expression (-TET, Day 3 and Day 5) co-cultures. Induction of *Hhex* expression at day 3 and day 5 of co-culture resulted in an increase in CD45+ cell numbers on day 7 and 8 of differentiation.

Figure S1.

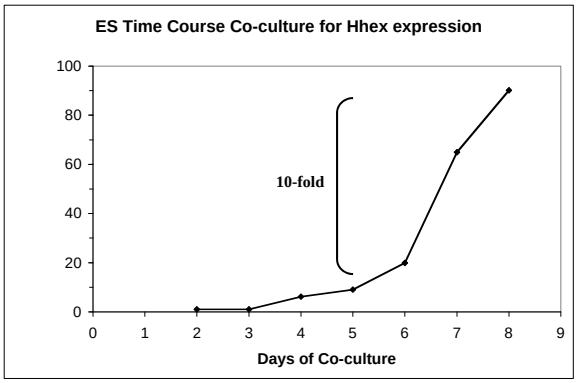

ES Time Course Co-culture for Hhex expression
100
80
60
40
20
0
10-fold
0
1
2
3
4
5
6
7
8
9
Days of Co-culture

Figure S2.

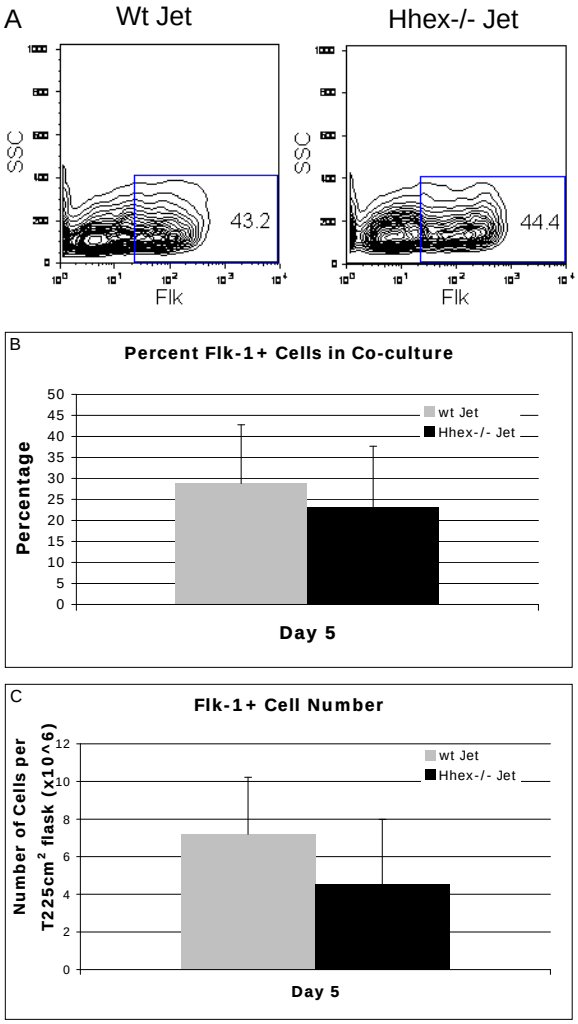

A
Wt Jet
Hhex-/- Jet
SSC
Flk
43.2
44.4
B
Percent Flk-1+ Cells in Co-culture
Percentage
wt Jet
Hhex-/- Jet
Day 5
C
Flk-1+ Cell Number
Number of Cells per T225cm² flask (x10^6)
wt Jet
Hhex-/- Jet
Day 5

Figure S3.

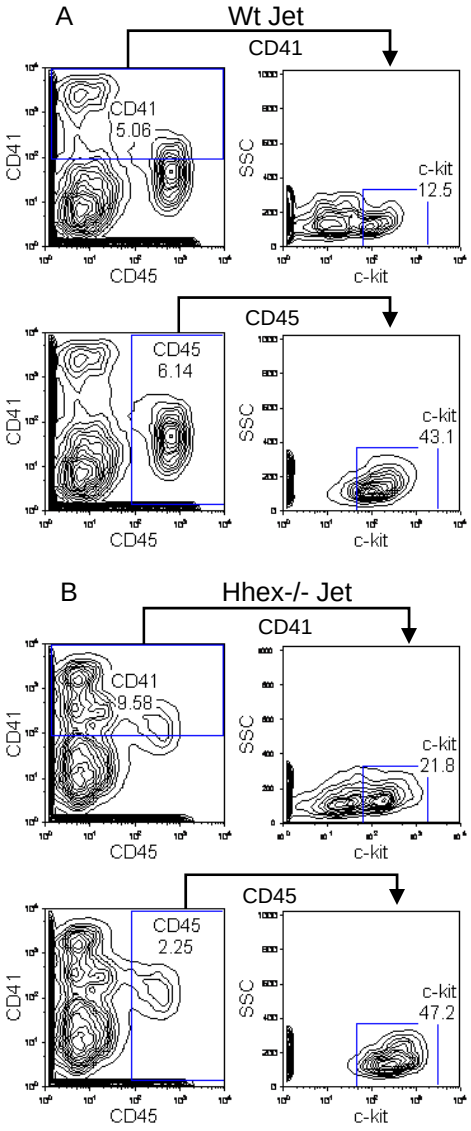
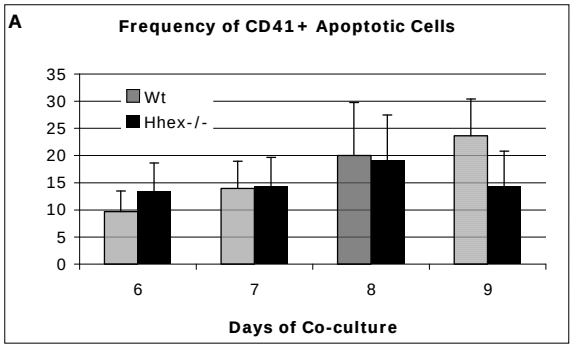

A
Wt Jet
CD41
CD41
5.06
CD45
SSC
c-kit
12.5
c-kit
CD45
CD41
CD45
6.14
CD45
SSC
c-kit
43.1
c-kit
B
Hhex-/- Jet
CD41
CD41
9.58
CD45
SSC
c-kit
21.8
c-kit
CD45
CD41
CD45
2.25
CD45
SSC
c-kit
47.2
c-kit

Figure S4.


A
Frequency of CD41+ Apoptotic Cells
35
30
25
20
15
10
5
0
Wt
Hhex-/-
6
7
8
9
Days of Co-culture

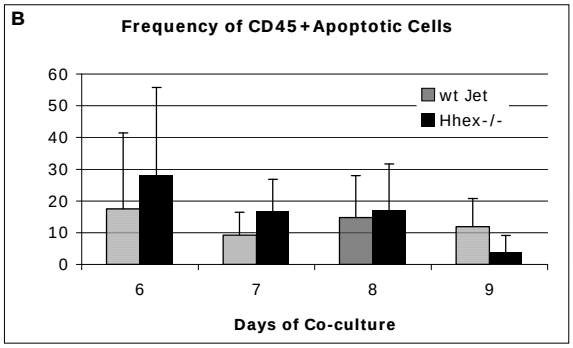
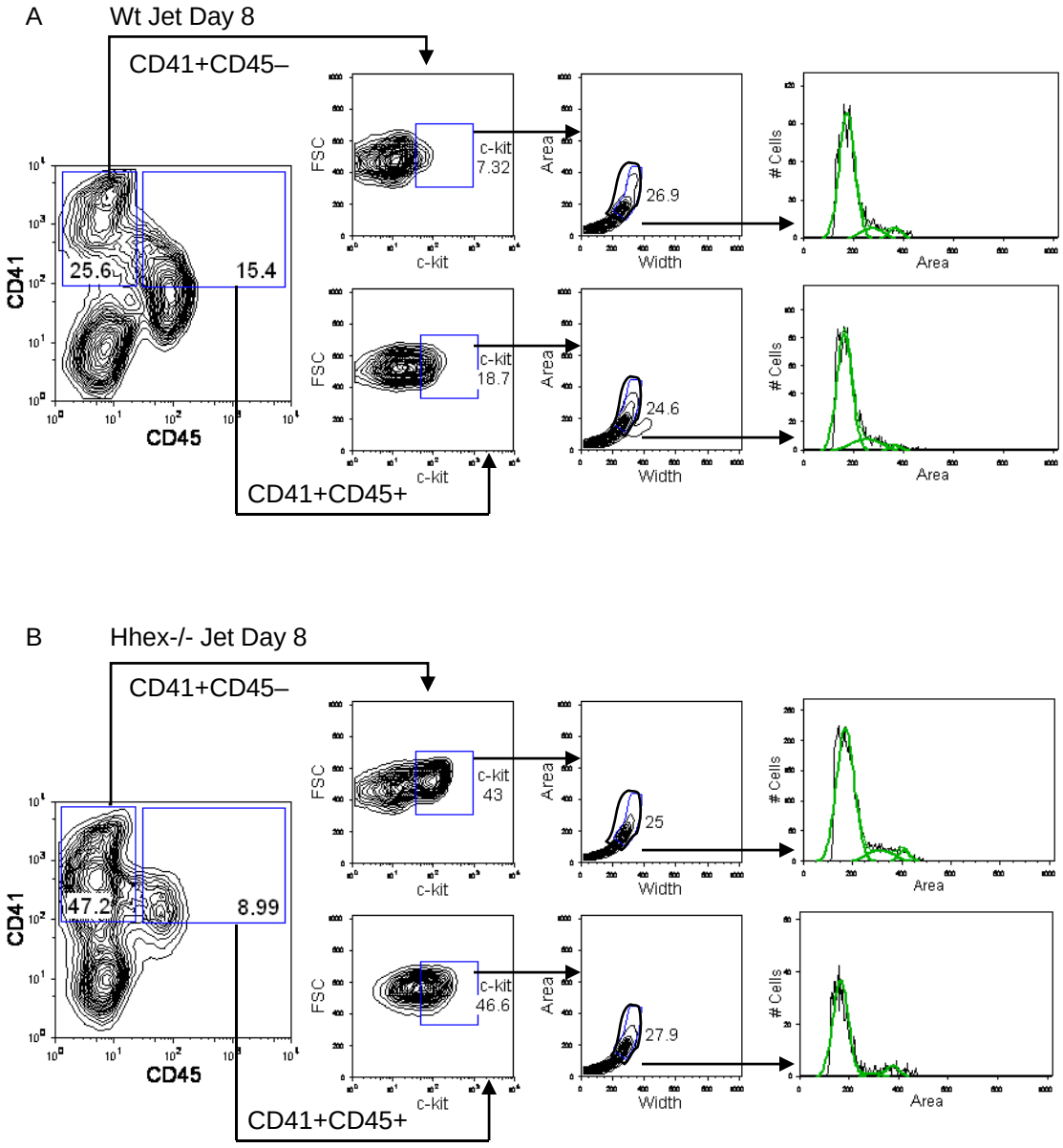

B
Frequency of CD45+Apoptotic Cells
60
50
40
30
20
10
0
wt Jet
Hhex-/-
6
7
8
9
Days of Co-culture

Figure S5.


A
Wt Jet Day 8
CD41+CD45–
CD41
CD45
25.6
15.4
FSC
c-kit
c-kit
7.32
Area
Width
26.9
Cells
Area
c-kit
18.7
24.6
CD41+CD45+
B
Hhex-/- Jet Day 8
CD41+CD45–
47.2
8.99
c-kit
43
25
c-kit
46.6
27.9
CD41+CD45+