Lessard, J., and Sauvageau, G. (2003). Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. Nature *423*, 255-260.
Li, Y., He, X., Schembri-King, J., Jakes, S., and Hayashi, J. (2000). Cloning and characterization of human Lnk, an adaptor protein with pleckstrin homology and Src homology 2 domains that can inhibit T cell activation. J Immunol *164*, 5199-5206.
McManus, M. T., and Sharp, P. A. (2002). Gene silencing in mammals by small interfering RNAs. Nat Rev Genet *3*, 737-747.
Micklem, H. S., Ford, C. E., Evans, E. P., Ogden, D. A., and Papworth, D. S. (1972). Competitive in vivo proliferation of foetal and adult haematopoietic cells in lethally irradiated mice. J Cell Physiol *79*, 293-298.
Mikkola, H. K., Klintman, J., Yang, H., Hock, H., Schlaeger, T. M., Fujiwara, Y., and Orkin, S. H. (2003). Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. Nature *421*, 547-551.
Miller, C. L., Rebel, V. I., Lemieux, M. E., Helgason, C. D., Lansdorp, P. M., and Eaves, C. J. (1996). Studies of W mutant mice provide evidence for alternate mechanisms capable of activating hematopoietic stem cells. Exp Hematol *24*, 185-194.
Nakano, T., Kodama, H., and Honjo, T. (1996). In vitro development of primitive and definitive erythrocytes from different precursors. Science *272*, 722-724.
Ogden, D. A., and Micklem, H. S. (1976). The fate of serially transplanted bone marrow cell populations from young and old donors. Transplantation *22*, 287-293.
Ohta, H., Sawada, A., Kim, J. Y., Tokimasa, S., Nishiguchi, S., Humphries, R. K., Hara, J., and Takihara, Y. (2002). Polycomb group gene rae28 is required for sustaining activity of hematopoietic stem cells. J Exp Med *195*, 759-770.
Okuda, T., Takeda, K., Fujita, Y., Nishimura, M., Yagyu, S., Yoshida, M., Akira, S., Downing, J. R., and Abe, T. (2000). Biological characteristics of the leukemia-associated transcriptional factor AML1 disclosed by hematopoietic rescue of AML1-deficient embryonic stem cells by using a knock-in strategy. Mol Cell Biol *20*, 319-328.
Osawa, M., Hanada, K.-i., Hamada, H., and Nakauchi, H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science *273*, 242-245.
Osawa, M., Miyoshi, S., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Hiroyama, T., Motohashi, T., Nakamura, Y., Iwama, A., and Nakauchi, H. (2000). Characterization of the mouse interleukin-13 receptor alpha1 gene. Immunogenetics *51*, 974-981.
Park, I. K., Qian, D., Kiel, M., Becker, M. W., Pihalja, M., Weissman, I. L., Morrison, S. J., and Clarke, M. F. (2003). Bmi-1 is required for maintenance of adult self-renewing

haematopoietic stem cells. Nature *423*, 302-305.
Pawliuk, R., Eaves, C., and Humphries, R. K. (1996). Evidence of both ontogeny and transplant dose-regulated expansion of hematopoietic stem cells in vivo. Blood *88*, 2852-2858.
Reya, T., Duncan, A. W., Ailles, L., Domen, J., Scherer, D. C., Willert, K., Hintz, L., Nusse, R., and Weissman, I. L. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature *423*, 409-414.
Rosendaal, M., Hodgson, G. S., and Bradley, T. R. (1979). Organization of haemopoietic stem cells: the generation-age hypothesis. Cell Tissue Kinet *12*, 17-29.
Sauvageau, G., Thorsteinsdottir, U., Eaves, C. J., Lawrence, H. J., Largman, C., Lansdorp, P. M., and Humphries, R. K. (1995). Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. Genes Dev *9*, 1753-1765.
Sminovitch, L., Till, J. E., and McCulloch, E. A. (1964). Decline in colony-forming ability of marrow cells subjected to serial transplantation into irradiated mice. J Cell Comp Physiol *64*, 23-32.
Sudo, K., Ema, H., Morita, Y., and Nakauchi, H. (2000). Age-associated characteristics of murine hematopoietic stem cells. J Exp Med *192*, 1273-1280.
Szilvassy, S. J., Humphries, R. K., Lansdorp, P. M., Eaves, A. C., and Eaves, C. J. (1990). Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. Proc Natl Acad Sci U S A *87*, 8736-8740.
Takaki, S., Morita, H., Tezuka, Y., and Takatsu, K. (2002). Enhanced hematopoiesis by hematopoietic progenitor cells lacking intracellular adaptor protein, Lnk. J Exp Med *195*, 151-160.
Takaki, S., Sauer, K., Iritani, B. M., Chien, S., Ebihara, Y., Tsuji, K., Takatsu, K., and Perlmutter, R. M. (2000). Control of B cell production by the adaptor protein lnk. Definition of a conserved family of signal-modulating proteins. Immunity *13*, 599-609.
Takaki, S., Watts, J. D., Forbush, K. A., Nguyen, N. T., Hayashi, J., Alberola-Ila, J., Aebersold, R., and Perlmutter, R. M. (1997). Characterization of Lnk. An adaptor protein expressed in lymphocytes. J Biol Chem *272*, 14562-14570.
Takano, H., Ema, H., Sudo, K., and Nakauchi, H. (2004). Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J Exp Med *199*, 295-302.
TeKippe, M., Harrison, D. E., and Chen, J. (2003). Expansion of hematopoietic stem cell phenotype and activity in Trp53-null mice. Exp Hematol *31*, 521-527.
Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W., and

Orkin, S. H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature *371*, 221-226.

Varnum-Finney, B., Xu, L., Brashem-Stein, C., Nourigat, C., Flowers, D., Bakkour, S., Pear, W. S., and Bernstein, I. D. (2000). Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. Nat Med *6*, 1278-1281.

Velazquez, L., Cheng, A. M., Fleming, H. E., Furlonger, C., Vesely, S., Bernstein, A., Paige, C. J., and Pawson, T. (2002). Cytokine signaling and hematopoietic homeostasis are disrupted in Lnk-deficient mice. J Exp Med *195*, 1599-1611.

Warren, A. J., Colledge, W. H., Carlton, M. B., Evans, M. J., Smith, A. J., and Rabbitts, T. H. (1994). The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development. Cell *78*, 45-57.

Yokouchi, M., Suzuki, R., Masuhara, M., Komiya, S., Inoue, A., and Yoshimura, A. (1997). Cloning and characterization of APS, an adaptor molecule containing PH and SH2 domains that is tyrosine phosphorylated upon B-cell receptor stimulation. Oncogene *15*, 7-15.

Yuan, Y., Shen, H., Franklin, D. S., Scadden, D. T., and Cheng, T. (2004). In vivo self-renewing divisions of haematopoietic stem cells are increased in the absence of the early G1-phase inhibitor, p18INK4C. Nat Cell Biol *6*, 436-442.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W. G., Ross, J., Haug, J., Johnson, T., Feng, J. Q., *et al.* (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836-841.

Zhou, S., Schuetz, J. D., Bunting, K. D., Colapietro, A. M., Sampath, J., Morris, J. J., Lagutina, I., Grosveld, G. C., Osawa, M., Nakauchi, H., and Sorrentino, B. P. (2001). The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med *7*, 1028-1034.

Figure legends

Figure 1. Experimental design for clonal and quantitative evaluation of self-renewal (A) A mouse is reconstituted with a single HSC. BM cells isolated from this mouse are transplanted into multiple mice. Upon secondary transplantation, a population-type assay (B) and a limiting dilution-type assay (C), both based on competitive repopulation, are performed. The number of RU in defined numbers of BM cells (such as 10^6 cells) can be determined from % chimerism obtained by FACS analysis of peripheral blood cells (B). The number of CRU in the BM can be calculated from the frequency of CRU in defined numbers of BM cells (C). The amount of RU as well as the number of CRU regenerated in BM by 1 CRU can be finally estimated.
Hiro-sensei, this doesn't make sense to me. How is "the amount of RU" different from "the number of RU"? Should this read "The number of RU (and the number of CRU) that 1 CRU regenerates in BM can thus be estimated"?
The MAS, an average RU per CRU, indicates the quality of regenerated CRU. Thus, RU, CRU, and MAS can be used as parameters for measurement of self-renewal activity in an individual HSC. Black circles indicate CRUs. a, cells derived from test cells; b, cells derived from competitor cells; c, cells derived from a host mouse; d, limiting dose of cells.

Figure 2. Increased frequency of HSCs in BM cells of Lnk-deficient mice
Graded numbers of BM cells from wild-type (WT) or Lnk-deficient (Lnk-KO) mice were transplanted together with $2x10^5$ competitor cells. The frequency of CRUs in BM cells of Lnk-deficient or wild-type mice was estimated to be one in 1,900 cells or one in 31,000 cells.

Figure 3. Expansion of HSC populations in Lnk-deficient mice
(A) Representative FACS profiles show Sca-1 and c-Kit expression in $CD34^-Lin^-$ BM cells (upper panels) and the SP in Lin^- BM cells (lower panels). The proportions of $CD34^-KSL$ cells or Lin^-SP cells were remarkably increased in Lnk-deficient mice (right panels) as compared with those in wild-type mice (left panels). The percentages of gated cells among total BM cells are shown.
(B) The kinetics of development of $CD34^-KSL$ cells (upper panel) or Lin^-SP cells (lower panel) are shown, along with mouse age.

Figure 4. Multilineage reconstitution by single $CD34^-KSL$ cells from wild-type or Lnk-deficient mice
A single $CD34^-KSL$ cell from wild-type (WT) or Lnk-deficient (Lnk KO) mice was mixed with $2x10^5$ BM cells and transplanted into a lethally irradiated mouse. All myeloid and B- and T-lymphoid lineages were reconstituted with a single test donor cell 4 months after transplantation in 24 of 92 (26%) mice receiving wild-type cells and in 17 of 76 (22%) mice receiving Lnk-deficient cells. (A) The list of RU per cell calculated based on % chimerism for each CRU. The MAS (2.76±3.84, n=17) in Lnk-deficient repopulating cells was significantly higher than that that (0.64±0.80, n=24) in wild-type repopulating cells ($p<0.05$, unpaired t-test with Welch correction). (B) Data are graphically presented with myeloid and B- and T-lymphoid lineage constituents (M-RU, B-RU, and T-RU).

Figure 5. In vitro maintenance of Lnk-deficient HSCs
$CD34^-KSL$ cells (10-cell equivalent) from 8-week-old wild-type or Lnk-deficient mice were transplanted into a group of lethally irradiated mice along with $2x10^5$ competitor cells (indicated as "Before"). Such cells were also co-cultured with OP-9 cells for 7 days, followed by transplantation (indicated as "After"). Recipient mice were analyzed 3 months after transplantation. The mean of RU per 10 $CD34^-KSL$ cells from Lnk-deficient mice (5.04±5.34, n=9) was significantly greater than that from wild-type mice (0.48±0.32, n=9) on Mann-Whitney testing (P=0.0002). 1 of 9 mice that

had received cultured wild-type cells showed reconstitution with test donor cells (0.04 RU/10 cells, n=1). 9 of 9 mice that had received cultured Lnk-deficient cells were reconstituted (3.40±2.55 RU/10 cells, n=9).

Table 1. Increases of RU and CRU in BM of Lnk-deficient mice.

Mice	BM cells ($x10^7$)	RU/10^5 BM cells	CRU/10^5BM cells (95% CI)	MAS (RU/cell)
WT B6	6.4±1.2 (n=8)*	1.0	3.2 (1.8-5.6)	0.3
Lnk KO B6	6.6±1.9 (n=5)	63.3±33.2 (n=5)	52.6 (31.3-83.3)	1.2

Wild-type (WT) and Lnk-deficient (Lnk-KO) B6 mice aged 8 weeks were compared. The numbers of bone marrow (BM) cells in the hindlimbs (two femora and two tibiae) and RU per 10^5 BM cells are expressed as mean±S.D. CRU per 10^5 BM cells is expressed as mean (95% confidence interval). Mean activity per stem cell (MAS) was calculated using the averages of RU and CRU. *, previously published data (Sudo et al., 2000).

Table 2. Self-renewal activity in single HSCs

Clones		Primary transplantation		Secondary transplantation				
Donor mice	No.	RU/cell	BM cells ($x10^6$) /mouse	RU/10^6 cells	RU/BM	CRU/10^6 cells	CRU/BM	MAS (RU/cell)
WT	1	3.26	400	0.16	64	2.44	976	0.07
	2	1.57	245	0.25	61	3.03	742	0.08
	3	0.82	325	0.19	62	2.50	813	0.08
	4	0.69	285	0.18	51	2.17	619	0.08
	5	0.47	280	0.04	11	1.25	350	0.03
	6	0.28	275	0.14	39	1.56	429	0.09
Lnk	1	7.62	385	1.00	385	2.50	963	0.40
KO	2	3.38	545	1.60	872	5.56	3,030	0.29

The number of RU/cell was obtained from analysis of mice that were primary recipients of single $CD34^-KSL$ cells. The numbers of RU and CRU/10^6 BM cells were obtained from analysis of mice that were secondary recipients of BM cells reconstituted with single $CD34^-KSL$ cells (clones) from wild-type or Lnk-deficient mice. The number of BM cells in a primary recipient mouse (BM cells/mouse) was calculated based on the minimal assumption that 20% of all BM cells are present in the femora and tibiae. The total numbers of secondary RU and CRU in BM per mouse (RU/BM and CRU/BM) were obtained by calculation of (BM cells)(RU/10^6 cells)/10^6 and (BM cells)(CRU/10^6)/10^6. The MAS is the number of RU divided by the number of CRU.

Supplemental Figure Legends

Supplemental Figure S1 *Lnk* is expressed in various hematopoietic lineages
Normalized cDNAs obtained from various populations in BM, spleen, and thymus of B6 mice underwent PCR analysis for *Lnk* sequences. Expression of *Lnk* was detected in stem and progenitor cell populations represented by $CD34^-KSL$ and $CD34^+KLS$ cells or KSL cells in combination. *Lnk* and *Hprt* were amplified for 35 cycles using *Lnk* primers (5'-TCT CTC AGG CAC CAG GTT C-3' and 5'-ATT CAC ACG TCT GCC TCT CT-3') and *Hprt* primers (5'-GCT GGT GAA AAG GAC CTC T-3' and 5'-CAC AGG ACT AGA ACA CCT GC-3').

Supplemental Figure S2 Cell cycle analysis of $CD34^-KSL$ cells from wild-type or Lnk-deficient mice
(A) Cell cycle analysis was performed on $CD34^-KSL$ cells isolated from wild-type or Lnk-deficient mice at 8 weeks of age after stained with PI. (B) Cell cycle kinetics was analyzed. Mice were fed water containing BrdU for 2 weeks. 10,000 or more $CD34^-KSL$ cells or $CD34^+KSL$ cells were stained with PI and anti-BrdU antibody. At least 5,000 events were collected on FACS analysis. Data from one of the two experiments are shown.

Supplemental Figure S3 Relationship between RU per cell and secondary CRU
Using data in Table 2, the linear regression between RU/cell and secondary CRU was analyzed. RU/cell is positively correlated with secondary CRU ($p<0.05$). The correlation coefficiency is 0.09. Dotted lines show 95% confidence interval.

Supplemental Figure S4 In vitro survival and division of Lnk-deficient $CD34^-KSL$ cells
(A) Single $CD34^-KSL$ cells isolated from wild-type (WT) or Lnk-deficient (Lnk KO) mice were cultured under serum-free conditions at graded concentrations of KL for 5 days. Subsequently, all cells were cultured in the presence of 10% FCS with 100 ng/ml of KL, 10 ng/ml of thrombopoietin, 10 ng/ml of interleukin-3, and 1 U/ml of erythropoietin for 10 days more. Colonies consisting of 50 or more cells were counted. There was no significant difference between the numbers of colonies formed by WT cells and those formed by Lnk-deficient cells at any concentration of KL examined, indicating that the survival rates by day 5 of culture did not differ between WT and Lnk-deficient $CD34^-KSL$ cells. (B) Single $CD34^-KSL$ cells from WT or Lnk-deficient

mice were cultured with 10 ng/ml of KL for 7 days. The number of cells produced by single cells was monitored. Data show clones (black circles) which gave rise to 2 or more cells. The number of cells produced by single cells did not differ significantly between WT and Lnk-deficient mice.

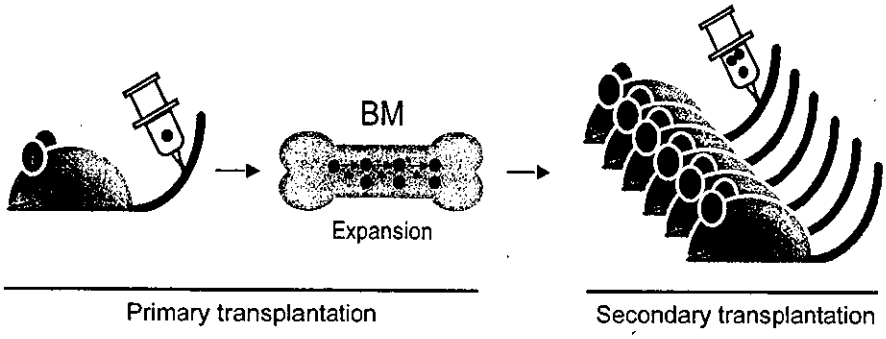
Supplemental Procedures

BrdU labeling and analysis

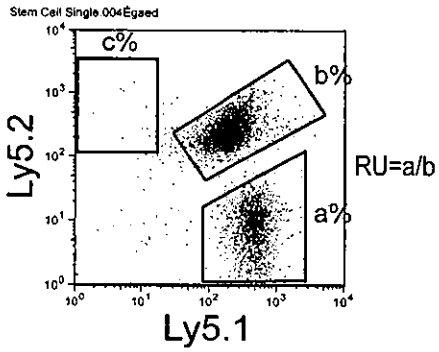
A stock solution of (+)-5-bromo-2'-deoxyuridine (Sigma) was prepared in sterile water at a concentration of 10 mg/ml and stored at -20 ℃. Mice were fed water containing 0.5 mg/ml of BrdU for 2 weeks; the water bottle containing BrdU was protected from light and was changed twice a week. $CD34^+KSL$ cells or $CD34^-KSL$ cells were sorted into an Eppendorf tube containing one ml of 10% FCS in α-MEM. Cells were centrifuged at 5,000 rpm for 3 min. After the supernatant was discarded, cells were resuspended in 150 μl of PBS, followed by addition of 350 μl of ethanol at -20 ℃. Cells were fixed at 4 ℃ overnight. After two washes with PBS, cells were gently vortex-resuspended in 500 μl of 4N HCl and 20% Tween 20 in water and were incubated at room temperature (RT) for 30 min. After being spun down, cells were incubated in borate-buffered saline (pH 9.0) at RT for 5 min. Cells were washed twice with 500 μl of 0.5 % Tween 20 in PBS and allowed to react with a titrated amount of FITC-conjugated anti-BrdU antibody (BD Biosciences) at RT for 30 min. After being washed with PBS, the cells were resuspended in 300 μl of PBS containing 15 μg/ml of RNase A (Sigma) and 5 μg/ml of PI, and were incubated at RT for 30 min. FACSCalibur was used for cell cycle analysis.

Ema et al. Fig. 1

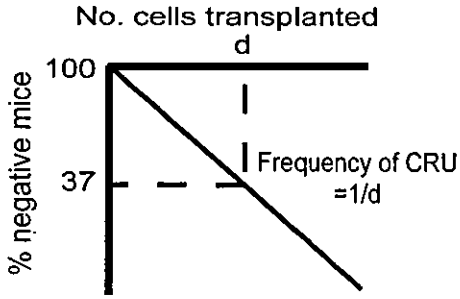
A. Single cell reconstituted mouse



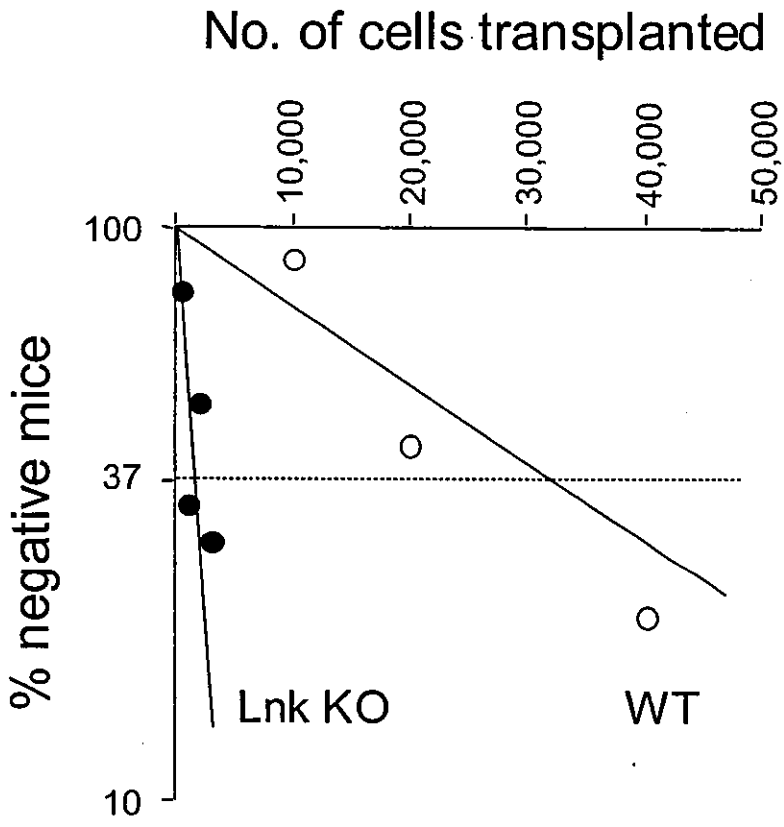
B. Population type CR



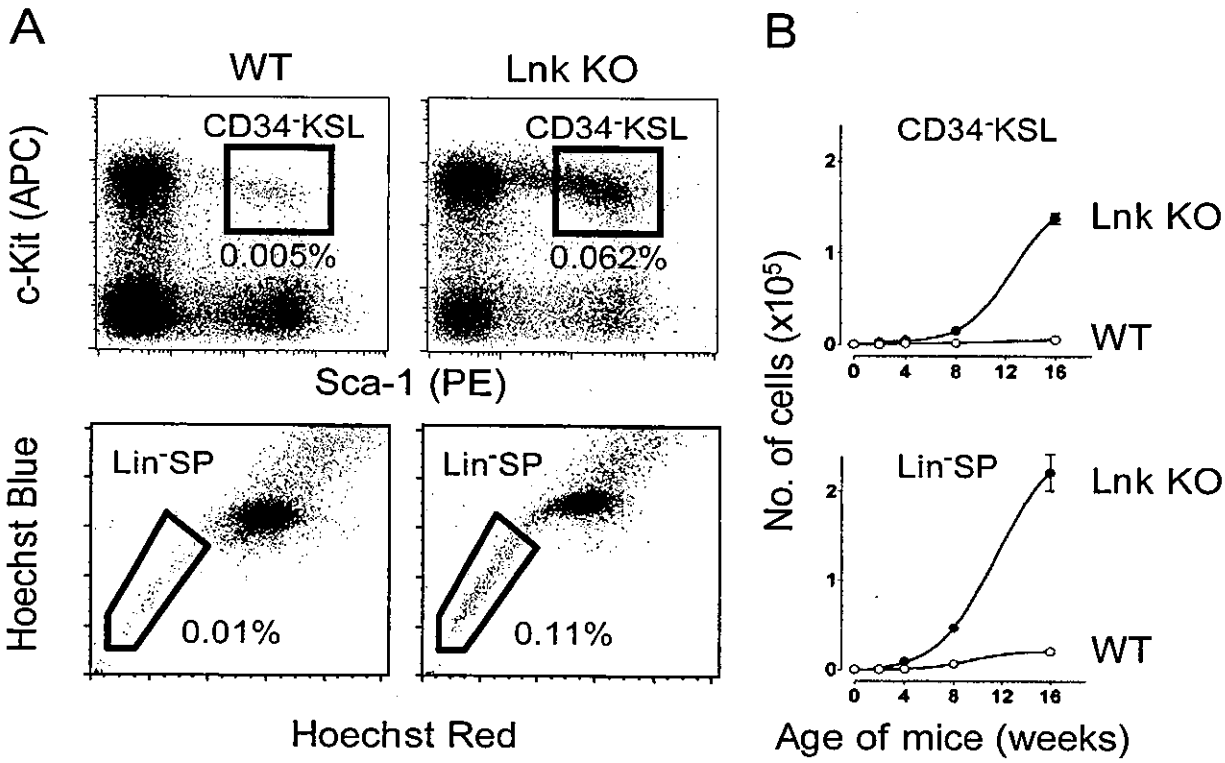
C. Limiting dilution type CR



Ema et al. Fig. 2



Ema et al. Fig. 3



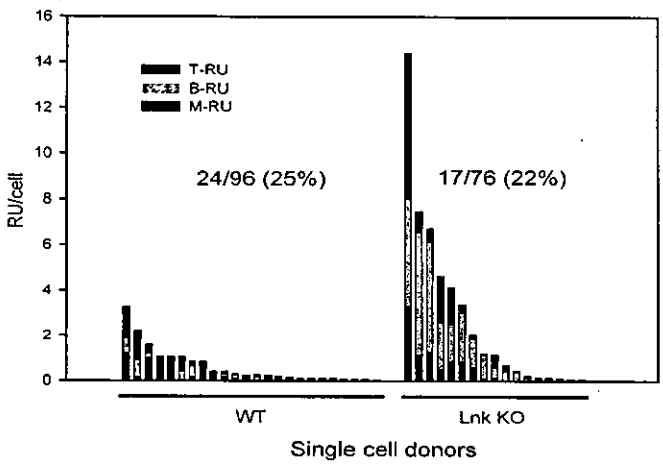
Ema et al. Fig. 4

A

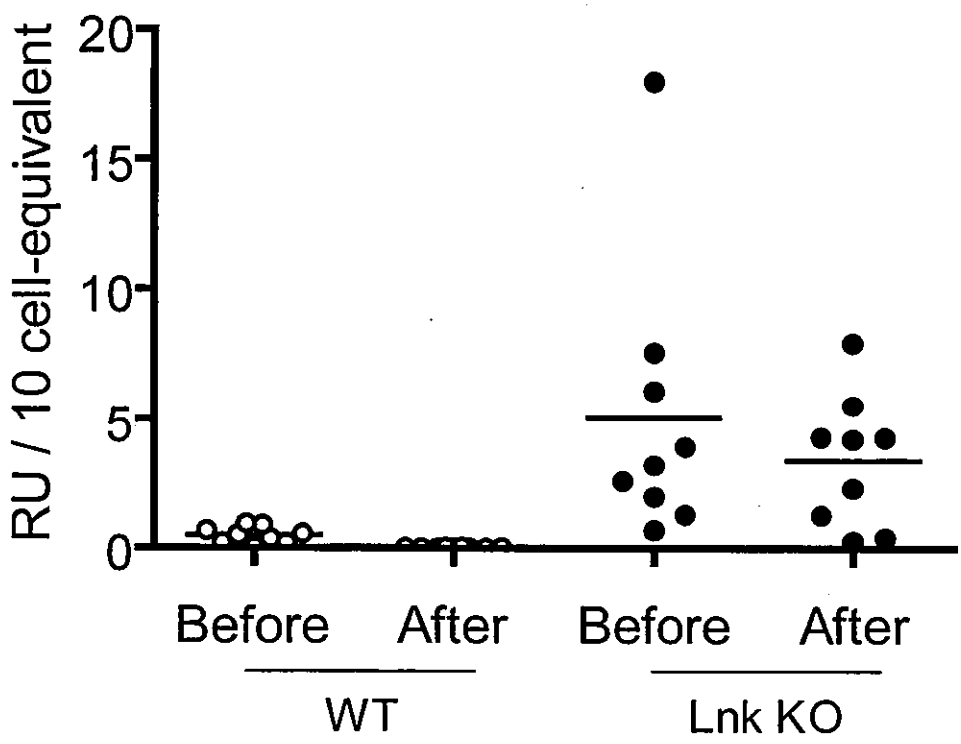
WT		Lnk KO	
% Chimerism	RU/cell	% Chimerism	RU/cell
62.0	3.26	87.8	14.39
52.6	2.22	79.2	7.62
44.0	1.57	76.7	6.58
39.7	1.32	69.8	4.62
37.7	1.21	67.4	4.13
34.3	1.04	62.8	3.38
29.0	0.82	50.4	2.03
25.7	0.69	36.9	1.17
19.0	0.47	36.6	1.15
16.8	0.40	26.6	0.72
12.9	0.30	18.2	0.44
12.4	0.28	10.3	0.23
12.1	0.28	7.1	0.15
10.9	0.24	6.8	0.15
9.4	0.21	4.5	0.09
7.0	0.15	4.5	0.09
6.2	0.13	3.0	0.06
5.9	0.13	MAS	2.76±3.84 (n=17)
5.9	0.13		
5.9	0.12		
5.0	0.11		
3.8	0.08		
3.1	0.06		
2.5	0.05		
MAS	0.64±0.80 (n=24)		

P<0.05

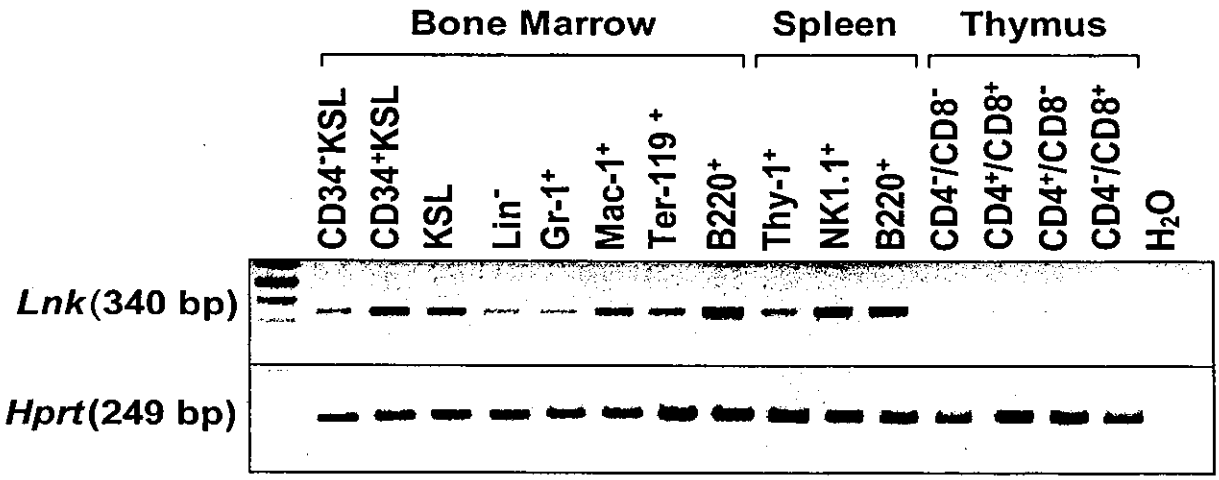
B



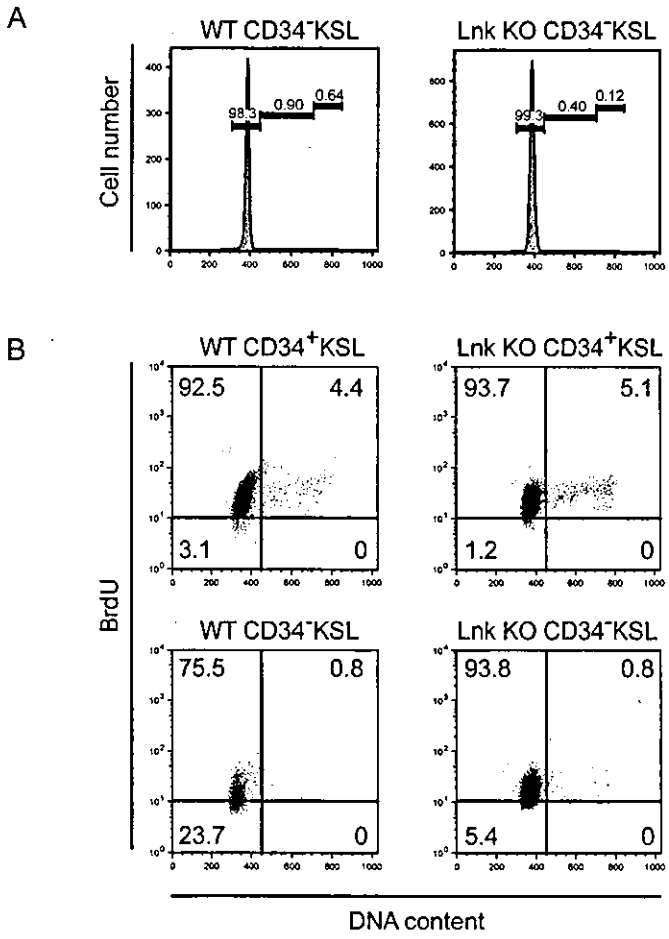
Ema et al. Fig. 5



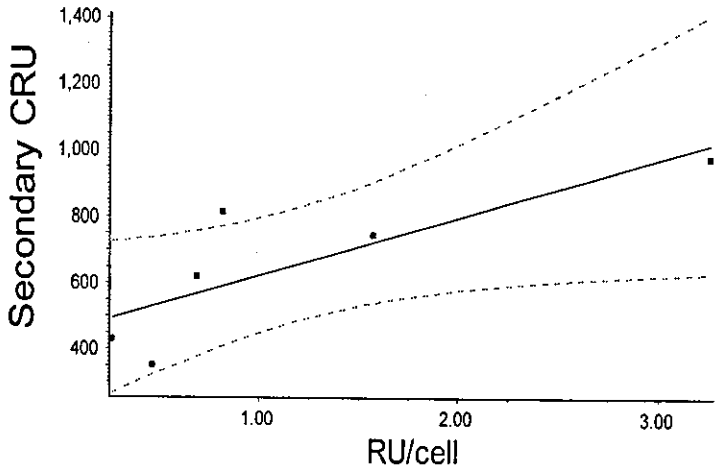
Ema et al. Supplemental Fig. S1



Ema et al. Supplemental Fig. S2



Ema et al. Supplemental Fig. S3



Ema et al. Supplemental Fig. S4