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Supplemental Information

A Dominant-Negative Isoform of IKAROS Expands Primitive Normal Human Hematopoietic Cells

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Supplemental Methods

Protocol for lentiviral gene transfer

For lentiviral transduction of mouse BM cells, sorted LSK cells were resuspended in serum-free medium (SFM = IMDM containing BIT^{TM} plus 40 μg/mL low-density lipoproteins from STEMCELL Technologies and 10^{-4} M 2-mercaptoethanol from Sigma) and then exposed to concentrated lentivirus for 4 hours in the presence of added mouse SF (STEMCELL) and IL-11 (Genetics Institute) at final concentrations of 300 ng/ml and 20 ng/ml, respectively.

For lentiviral transduction of human CB cells, purified $CD34^+$ cells or subsets thereof were stimulated overnight (~16 hours) at 1 to 10 $x10^5$ cells/ml in SFM containing 100 ng/ml recombinant human FL (Immunex Corp.) and SF (STEMCELL), plus 20 ng/ml IL-3 (Novartis), IL-6 (Cangene) and G-CSF (STEMCELL) and then exposed to lentivirus for another 8 hours in the same medium.

Intracellular flow cytometric analyses

For detection of intracellular proteins (IKAROS, FOS or CCNB1), cells were fixed with 1.6% paraformaldehyde, detergent permeabilized (eBioscience), incubated with blocking reagent (PBS with 2% fetal bovine serum, 5% human AB serum and anti-human CD32 antibody clone IV.3) and then stained with surface and intracellular antibodies (rabbit anti-IKAROS, FOS or CCNB1; Cell Signaling Technologies) followed by AlexaFluor-594-labelled anti-rabbit secondary (Invitrogen). For detection of intracellular signaling molecules, cells were washed twice in IMDM and then incubated for 3 hours at 37^{o}C without serum or growth factors. Cells were then stimulated (or not) for 10 minutes at 37^{o}C, fixed with 1.6% paraformaldehyde, washed twice, incubated on ice with

blocking reagent, incubated with antibodies against surface antigens, washed twice, then resuspended in 80% ethanol at -80^{o}C, followed immediately by 2 washes. Cells from different conditions were differentially labeled with combinations of amine reactive dyes (Pacific Blue and eFluor 506 and 780, eBioscience), washed twice and combined for staining on ice with intracellular antibodies (AKT pS473, ERK1/2 pT202/pY204, P38 MAPK pT180/pY182, STAT1 pY701, STAT3 pY705 and STAT5 pY694, BD; CREB pS133 and JAK2 pY1008, Cell Signaling Technologies), followed by washing and staining with appropriate secondary antibodies. Cells harvested from mice were first incubated in NH_4Cl, then processed as above without an initial incubation in the absence of serum or growth factors and with the addition of anti-mouse CD16/CD32 clone 2.4G2 to the blocking reagent. All flow cytometry analyses were performed on a Fortessa (BD) and data analyzed using FlowJo software (Tree Star). GFP^+ and YFP^+ cells stained with surface antibodies and labeled with amine reactive dyes (where used) but not with intracellular antibodies were used as negative (unstained) controls.

Gene expression analysis

Global transcriptome data was obtained from 3 biological replicates of purified $CD34^+CD38^-$ cells isolated from 3 mice transplanted 10 weeks previously. RNA quality was assessed using an Agilent 2100 bioanalyzer (acceptable RIN value $\geq$8.0), labeled using the Agilent One-Color Microarray-Based Exon Analysis Low Input Quick Amp WT Labeling v1.0 and then hybridized to Agilent Human GE 8x60K arrays. Data were acquired using an Agilent DNA Microarray Scanner at a 3 mm scan resolution, and processed with Agilent Feature Extraction 11.0.1.1. Probes were retained if detectable in all control or all IK6 samples and had an EntrezGene identifier. Following trimming, data were analyzed using R software. Raw signals were quantile normalized and

differential expression tested per-probe (lmFit, limma) or within gene sets (romer, limma with 10^6 rotations using floormean) using the 'KEGG' and 'transcription factor targets (TRANSFAC)' gene sets from MSigDB version 4, and specific gene lists as indicated, with correction for multiple testing (qvalue). STAT5 targets were derived from the human HEL cell line by intersecting STAT5 ChIP-Seq data with gene expression profiling with and without JAK2 inhibition ((Dawson et al., 2012), courtesy of Bertie Göttgens and Mark Dawson). The NCBI Gene Expression Omnibus accession number for the gene expression array data reported in this paper is GSE60957.

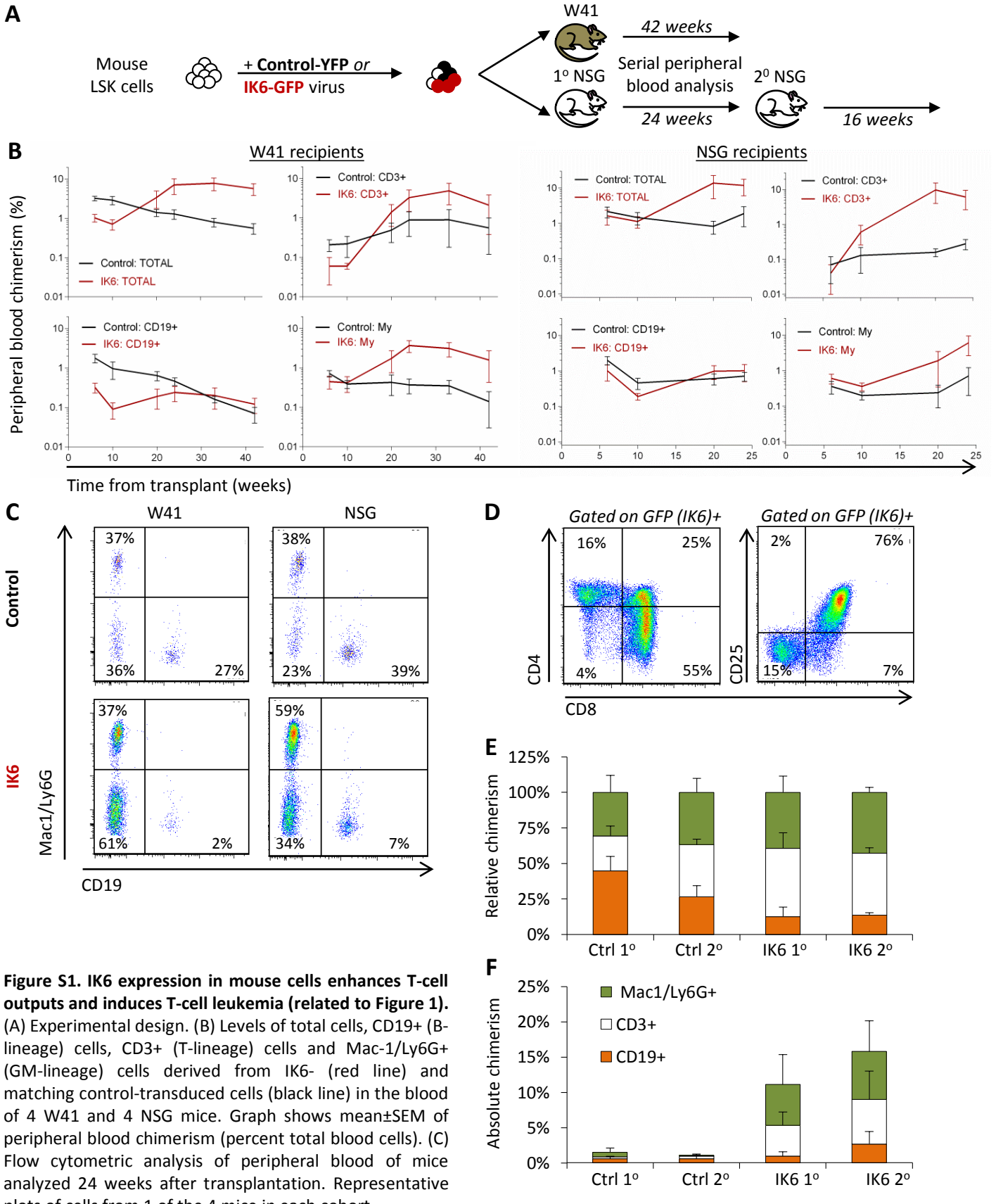
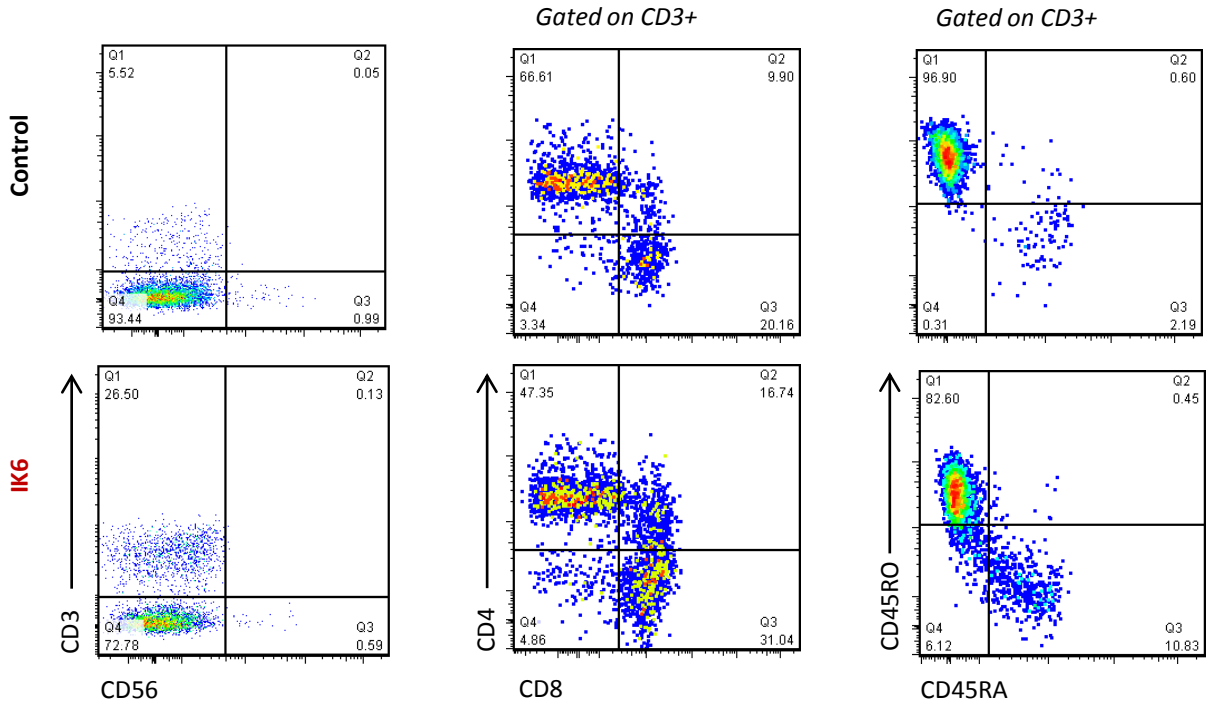


Figure S1. IK6 expression in mouse cells enhances T-cell outputs and induces T-cell leukemia (related to Figure 1). (A) Experimental design. (B) Levels of total cells, CD19+ (B-lineage) cells, CD3+ (T-lineage) cells and Mac-1/Ly6G+ (GM-lineage) cells derived from IK6- (red line) and matching control-transduced cells (black line) in the blood of 4 W41 and 4 NSG mice. Graph shows mean±SEM of peripheral blood chimerism (percent total blood cells). (C) Flow cytometric analysis of peripheral blood of mice analyzed 24 weeks after transplantation. Representative plots of cells from 1 of the 4 mice in each cohort. (D) Representative flow cytometric analysis of peripheral blood of 1 of 3 mice who developed aggressive T-ALL 18-34 weeks after initial transplantation. Transplantation of primary leukemias resulted in rapid (<3 weeks) death of secondary recipients. (E&F) Relative and absolute lineage contribution from control and IK6-transduced cells in 24-week primary (1º) NSG mice and 16-week secondary (2º) NSG mice. Graph shows mean±SEM for 4 primary and 7 secondary mice.

A



B

	CD3+CD4+		CD3+CD8+		CD3+CD45RA+		CD3-CD56+	
	Control	**IK6**	**Control**	**IK6**	**Control**	**IK6**	**Control**	**IK6**
Mean chimerism	1.08%	0.54%	0.14%	0.57%	0.03%	0.12%	0.46%	0.34%
Standard error	0.42%	0.38%	0.08%	0.53%	0.003%	0.09%	0.23%	0.07%
No. of mice	3		3		3		8	
T-test (paried)	0.53		0.51		0.46		0.40	

Figure S2. Effects of IK6 expression on human T- and NK-cell differentiation (related to Figure 2). (A) Representative flow cytometric profiles of IK6- and control-derived NK- and T-cells from the spleens of 10-week transplanted NSG-3GS mice, as shown in Figure 2F. (B) Human IK6- and control-derived T-cells subsets in the spleens of 10-week transplanted NSG-3G3 mice, expressed a percentage of the total number of cells in the spleen.

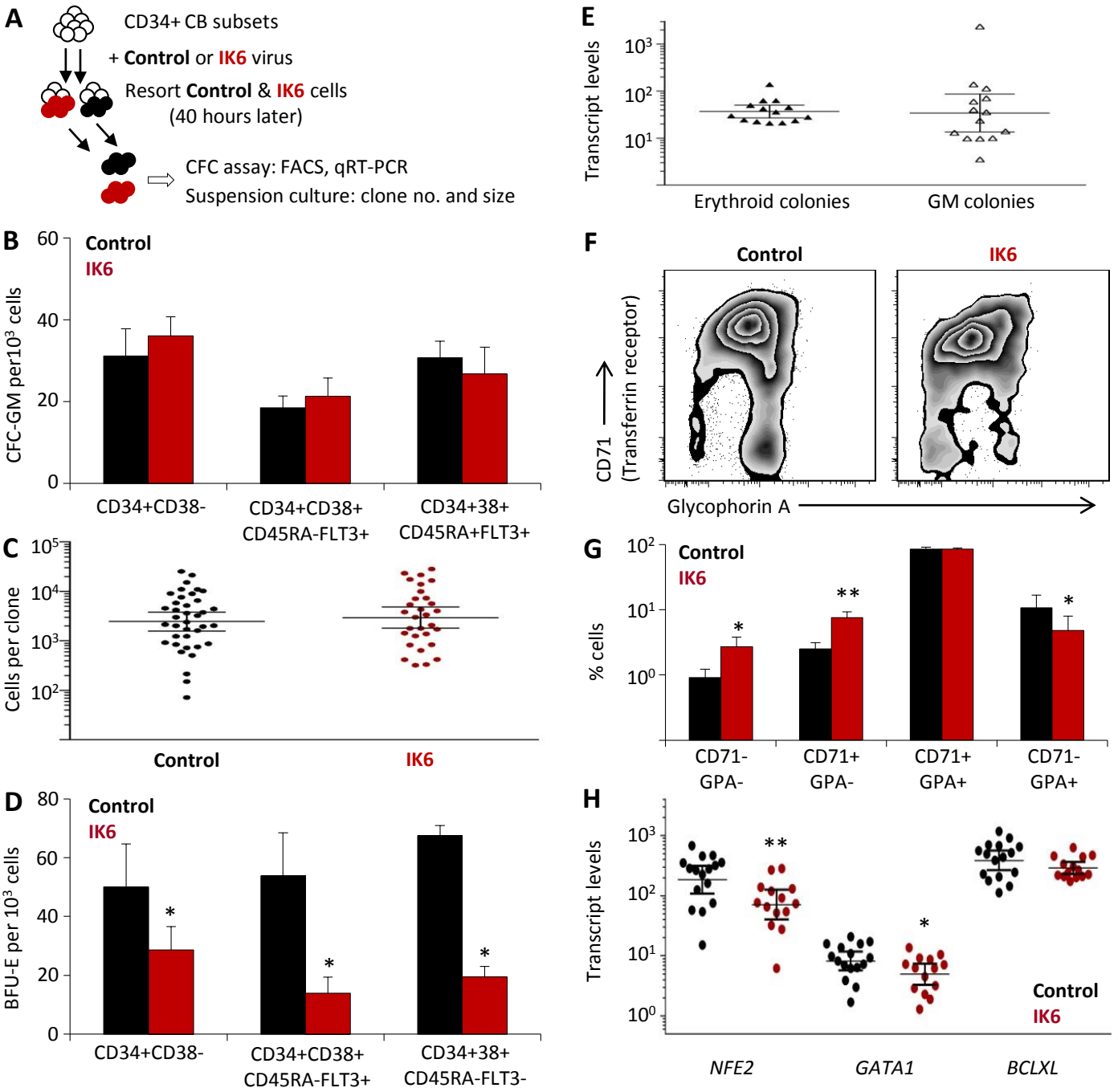
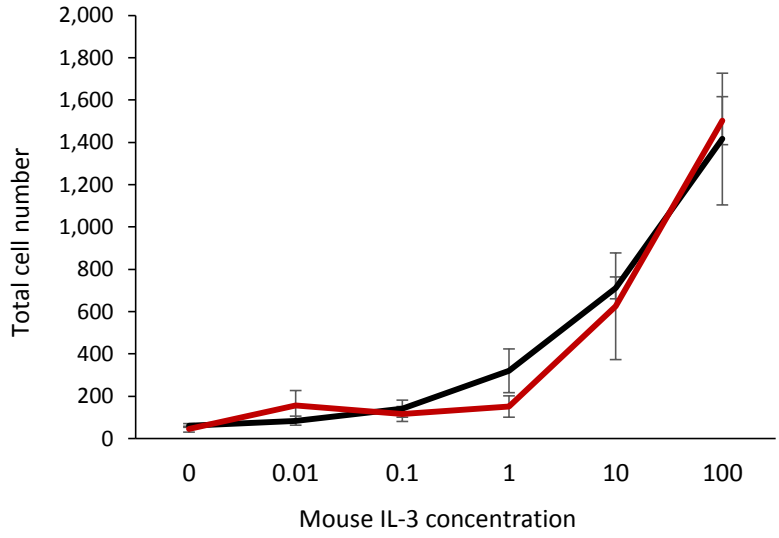


Figure S3. Opposite effects of IK6 on human CB cell-derived granulopoiesis and erythropoiesis (related to Figure 4). (A) Experimental design. (B) Lack of effect of IK6 on GM colony formation by different subsets of CD34$^+$ CB cells (x-axis) assessed directly post-transduction (mean±SEM, 3 experiments). (C) Lack of effect of IK6 on the size of clones (produced after 14 days in H5100 LTC medium with 20 ng/mL GM-CSF) from transduced CD34$^+$CD38$^-$ cells (mean±SEM). Frequency of clone formation was also similar at 16% (30/188) and 20% (38/188) for IK6- and control-transduced cells, respectively. (D) Colony formation by control- and IK6-transduced BFU-E present in different subsets of CD34$^+$ CB cells (x-axis) assessed immediately after transduction (mean±SEM, same experiments as in panel B). (E) IK6 transcript levels (relative to *B2M* expression) in 14 erythroid and 14 GM colonies generated *in vitro* from IK6-transduced cells (mean±SEM). (F) Representative flow cytometric profiles showing effects of IK6 on transferrin receptor and glycophorin A expression in erythroid cells generated after 14 days from IK6-transduced BFU-Es (1 of 5 such analyses performed on colonies produced in 2 experiments). (G) IK6 alters the proportion of erythroid cells at different stages of maturation (mean±SEM from 5 analyses). (H) IK6 has a slight effect in decreasing *NFE2* and *GATA1* transcripts in erythroid colonies - 14 IK6-derived and 14 controls assessed individually and genotyped by detection (or not) of IK6 transcripts (data not shown). Values are the mean±SEM. CD34$^+$CD38$^-$: multipotent progenitor; CD34$^+$CD38$^+$CD45RA$^-$FLT3$^+$: myloerythroid progenitor; CD34$^+$CD38$^+$CD45RA$^-$FLT3$^-$: erythroid progenitor; CD34$^+$CD38$^+$CD45RA$^+$FLT3$^+$: GM progenitor; CFC-GM: colony-forming cell-granulocyte-macrophage; BFU-E: burst-forming unit-erythroid; GPA: Glycophorin A; *: p=<0.05; **: p=<0.01.



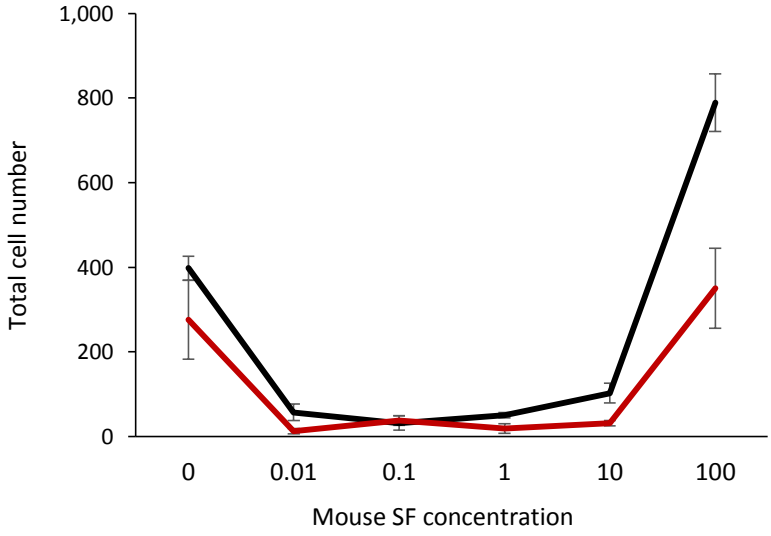


Figure S4. Forced expression of IK6 in primitive mouse hematopoietic cells does not alter their growth factor responsiveness (related to Figure 5). Day 4 cell outputs from IK6- and control-transduced mouse progenitor cells ($Lin^{-}Sca1^{+}CD3^{-}$) cultured in varying concentrations of IL-3 or Steel factor (SF). Figure shows mean±SEM of 3 replicates derived from 1 of 3 independent experiments, all of which showed similar results.