Defects in Hemopoietic Stem Cell Activity in *Ikaros* Mutant Mice

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Summary

Here we provide evidence that the Ikaros family of DNA binding factors is critical for the activity of hemopoietic stem cells (HSCs) in the mouse. Mice homozygous for an *Ikaros* null mutation display a >30-fold reduction in long-term repopulation units, whereas mice homozygous for an *Ikaros* dominant negative mutation have no measurable activity. The defect in HSC activity is also illustrated by the ability of wild-type marrow to repopulate unconditioned *Ikaros* mutants. A progressive reduction in multipotent CFU-S_{14} (colony-forming unit-spleen) progenitors and the earliest erythroid-restricted precursors (BFU-E [burst-forming unit-erythroid]) is also detected in the *Ikaros* mutant strains consistent with the reduction in HSCs. Nonetheless, the more mature clonogenic erythroid and myeloid precursors are less affected, indicating either the action of a compensatory mechanism to provide more progeny or a negative role of *Ikaros* at later stages of erythromyeloid differentiation. In *Ikaros* mutant mice, a decrease in expression of the tyrosine kinase receptors flk-2 and c-kit is observed in the lineage-depleted c-$kit^+$$Sca\text{-}1^+$ population that is normally enriched for HSCs and may in part contribute to the early hemopoietic phenotypes manifested in the absence of *Ikaros*.

Key words: transcription factors • hemopoiesis • flk2 • c-kit regulation

The hemopoietic system is derived from the mesodermal germ layer early in embryogenesis (1–3). Hemopoietic commitment of the elusive hemangioblast, a mesodermally derived progenitor that gives rise to vascular endothelium and hemopoietic cells (4), occurs over a narrow window of time during embryogenesis. Once established, the hemopoietic system supplies the organism with at least 10 different lineages in a highly regulated manner. As the half-life of mature hemopoietic cells varies from several hours to years, a continuous production of end-stage cells from hemopoietic stem cells (HSCs)[1] is required throughout the life span of the organism. Accordingly, HSCs must be capable of self-renewal to maintain the HSC pool and its more mature progeny. Differentiation from the HSC proceeds through a series of progressive restrictions that give rise to multipotent progenitors with short-term repopulating ability, lineage-restricted precursors, and, finally, terminally differentiated cells (5). To increase the number of mature progeny, proliferative expansions take place along various stages in these pathways. The molecular control of hemopoiesis has been the focus of intense study. The role of membrane receptors, underlying signaling pathways, and potential nuclear effectors has been studied in both vertebrates and invertebrates (6–9).

Ikaros is a hemopoietic-specific member of a family of zinc finger transcription factors that is essential for specification in lymphoid lineages (10, 11). *Ikaros* generates by means of alternate splicing a number of protein isoforms (12, 13) that share a common COOH-terminal zinc finger domain that mediates interactions with self and other family members (14, 15). A subset of the *Ikaros* isoforms (Ik-1, Ik-2, Ik-3, Ik-4) has a second zinc finger domain located at the NH_2-terminal half that mediates sequence-specific DNA interactions (12). The remaining *Ikaros* isoforms, Ik-5, Ik-6, and Ik-7, do not have a DNA-binding domain yet can negatively regulate the activity of the DNA-binding Ikaros proteins (12, 14). Although functionally distinct, both Ikaros zinc finger domains are essential for high-affinity interactions with DNA and are indispensable for transcriptional activity (reference 14 and Koipally, J., and K. Georgopoulos, unpublished results). The relative expression levels of Ikaros splicing variants do not change significantly during development; the DNA-binding isoforms Ik-1, Ik-2, and

[1]*Abbreviations used in this paper:* AGM, aorta-gonad-mesonephros regions; BM, bone marrow; CFC, colony-forming cell; DN, dominant negative; G, granulocytes; HSCs, hemopoietic stem cells; LTR, long-term repopulation; M, macrophages; RT, reverse transcriptase; YS, yolk sac.

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Ik-4 are the predominant species in the hemopoietic populations analyzed thus far (12, 15).

Two distinct deletions have been targeted independently in the *Ikaros* locus in mice (10, 11). Mice homozygous for a deletion in the last translated exon that encodes the COOH-terminal half of the Ikaros proteins are null for any Ikaros protein (11). Ikaros null mice lack both fetal and adult mature B cells as well as the earliest defined B lineage precursors. Fetally derived T cells and their precursors are also absent; however, postnatally, a severely reduced number (10–30-fold reduction) of T cell precursors appears in the thymus (16). These T cell precursors undergo abnormal differentiation along the CD4 α/β T cell pathway, are unable to differentiate along the NK lineage, and produce severely reduced numbers of γ/δ T cells and thymic dendritic APCs (11, 17). A distinct *Ikaros* mutation that deletes part of the NH_2-terminal zinc finger domain abolishes the DNA binding capability of Ikaros proteins but leaves the COOH-terminal zinc finger interaction domain intact. Proteins are stably produced by this Ikaros mutant allele, which can interfere with the activity of the DNA-binding Ikaros isoforms and with other Ikaros family members (14, 15, 18). Consistent with these in vitro observations, mice homozygous for the *Ikaros* dominant negative (DN) mutation display more severe lymphoid defects relative to *Ikaros* null mice. *Ikaros* $DN^{-/-}$ mice lack all cells of lymphoid origin, including NK cells and thymic and splenic APCs (10, 17). In addition, mice heterozygous for the *Ikaros* DN mutation develop T cell leukemias and lymphomas with 100% penetrance within the first 4 mo of their lives (19). The more severe effects on development and homeostasis of the lymphoid lineage caused by the *Ikaros* DN mutation suggest that Ikaros DN proteins interfere with the activity of other family members during lymphopoiesis. One of the *Ikaros* homologues, *Aiolos*, is expressed in lymphoid precursors and mature lymphocytes and interacts both physically and functionally with Ikaros (reference 15 and Cortez, M., and K. Georgopoulos, unpublished data). Two additional family members, *Helios* and *Dedalos*, also form complexes with Ikaros and colocalize within higher order nuclear structures (18, 20, and Morgan, B., manuscript in preparation). Both *Helios* and *Dedalos* are expressed in populations enriched for HSCs and in early thymic T cell precursors (reference 18 and Morgan, B., manuscript in preparation). We have recently identified Ikaros and family members in two distinct higher order chromatin remodeling complexes having nuclear compartmentalization and gene targeting that relies on the DNA binding activity shared by Ikaros and its family members (21). Thus, the more severe phenotypes manifested in *Ikaros* DN mutants may be due to interference with the Aiolos, Helios, and Dedalos protein activity and inappropriate targeting of the Ikaros-associated chromatin remodeling complexes.

In this study, we delineate the effects on HSC activity manifested by the *Ikaros* null and DN mutations. The effects of the *Ikaros* mutations are apparent in long-term repopulation (LTR) activity, which is reduced by 20–40-fold in *Ikaros* null mutants and is undetectable in *Ikaros* DN homozygotes. The more committed multipotent CFU-S_{14} (colony-forming unit-spleen) progenitors and the earliest erythroid-restricted precursors (BFU-E [burst-forming unit-erythroid]) are also reduced in *Ikaros* mutant mice to an extent that correlates with the reduction in HSC activity. Nevertheless, the more mature clonogenic precursors, including CFU-E, are less affected. *Ikaros* null HSCs and $DN^{-/-}$ hemopoietic progenitors lack expression of the tyrosine kinase receptor flk-2 at the mRNA level and express progressively reduced levels of the tyrosine kinase receptor c-kit at the cell surface.

Materials and Methods

Mice. *Ikaros* null and DN mutant mice as well as wild-type control littermates on a mixed C57BL/6J × 129SV background were bred and maintained under sterile conditions in a pathogen-free animal facility at Massachusetts General Hospital. Due to the high morbidity of the *Ikaros* DN mutant mice, all animals were kept on oral antibiotics. Mice used for the different studies were between 2 and 5 wk old. The genotypes of mice were determined by PCR analysis of the *Ikaros* locus with primers and conditions described previously (10). C57BL/6J–Ly5a-Pep^{3b}–congenic mice used as transplant recipients or donors were obtained from The Jackson Laboratory and bred in the animal facility.

Hemopoietic Tissue Preparation. Bone marrow (BM) was prepared by crushing femora and tibiae with a mortar and pestle and then passing the suspension through a 70-μm cell strainer to remove bone debris. Spleen cell suspensions and day 14 fetal liver cells were obtained by disrupting the tissue in PBS (plus 5% dialysed FBS) and passing it through a 70-μm cell strainer. The aorta-gonad-mesonephros regions (AGM) and yolk sacs (YS) from 11 d postcoitum embryos were prepared as described previously (22). In brief, the AGM and YS were dissected in PBS/5% FCS and digested for 1 h at 37°C in 0.125% collagenase (Sigma Chemical Co.). Viable cell counts were based on trypan blue exclusion.

mAbs. The mAbs used for immunofluorescent labeling and the fluorochromes employed are specified elsewhere (17). In brief, three-color flow cytometry was performed using anti-Ly5b (AL1-4A2) to identify donor-derived cells and antibodies against B220 (RA3-6B2), CD4 (RM4-5), CD8 (53-6.7), TCR-α/β (H57-597), Mac-1 (M1/70), Gr-1 (RB6-8C5), and TER-119. All antibodies were purchased from PharMingen.

In Vitro Colony-forming Cell Assay. Marrow and spleen cells were harvested from neonate and 2–5-wk-old mice. Single-cell suspensions from each tissue were prepared in PBS (plus 5% dialysed FBS), counted, and cultured in IMDM containing 1.2% methyl cellulose, 15% FBS, 0.5% BSA fraction V, transferrin, insulin, lipids, α-thioglycerol, 15 U/ml IL-3, 2 IU/ml erythropoietin, and 50 ng/ml kit ligand. Colonies were scored after 2–3 d for CFU-E and after 7–10 d for all other colony types. Erythroid colonies containing at least two other lineages were attributed to colony-forming cell (CFC)-multi. Pure erythroid colonies on day 7–8 were attributed to BFU-E, and colonies containing at least 500 granulocytes (G) and/or macrophages (M) were attributed to CFC-G/M. Purified murine kit ligand was provided by Genetics Institute.

Spleen Colony-forming Assay (CFU-S_{14}). The CFU-S_{14} content in the BM and spleens of *Ikaros* mutant and wild-type littermates 2–5 wk after birth was determined by injection of 5×10^4 nucleated BM cells or 2.5×10^5 spleen cells into the lateral tail vein of lethally irradiated (9.5 Gy; ^{137}Cs single dose) wild-type recipient

mice. Mice were killed 14 d after the injection, and their spleens were fixed in Bouin's solution for macroscopic examination and weighing. Absolute numbers of CFU-S_{14} per organ were calculated based on the frequency measurement and the cellularity of the spleen and the BM, assuming that one femur and one tibia represent 10 and 5%, respectively, of total BM. To determine the lineage composition of spleen colonies, single colonies were dissected before fixation, erythrocytes were lysed in 0.4 M ammonium chloride buffer, and cells were stained with a subset of antibodies described previously and subjected to FACS™ analysis. For PCR genotyping, colonies were lysed in DNA lysis buffer, and DNA was prepared as previously described (10). Irradiated mice injected with PBS alone were included as controls in all experiments.

Radioprotection and Competitive Repopulation Assays. Congenic C57BL/6J–Ly5a-Pep^{3b} mice were irradiated with a single lethal dose of 9.5 Gy from a ^{137}Cs radiation source (gamma irradiator; J.L. Shepherd) at 0.95 Gy/min. Cell suspensions containing 10^5 wild-type or 6×10^6 mutant BM cells from 2–5-wk-old mice in a final volume of 200 μl PBS were injected intravenously into the lateral tail vein. Recipient mice were maintained on 1.1 g/liter neomycin sulfate (Sigma Chemical Co.) and 10^6 U/liter polymyxin B sulfate (Sigma Chemical Co.) in their drinking water for the duration of the assay. Mice were monitored for survival daily for 35 d. For the competitive assays, lethally irradiated Ly5a mice (9.5 Gy of gamma irradiation) were injected with a fixed amount of 10^5 Ly5a autologous BM cells along with 10^5–10^7 *Ikaros* mutant or wild-type BM cells (Ly5b). Donor-derived (Ly5b) hemopoietic contribution was measured from the peripheral blood at different time points starting on day 19 after transplant and from both peripheral blood and hemopoietic organs at the time mice were killed.

HSC Enrichment. BM cells were collected from femora and tibiae of 2–5-wk-old mice. Cells were layered at 2.5×10^7 cells/ml over 3 ml of sodium metrizoate (Nycodenz; Accurate Scientific) solution (1.077 g/ml) and centrifuged at 1,000 *g* for 20 min. Low density cells were harvested and incubated with mAbs to CD4, CD8, B220, Mac-1, Gr-1, and TER-119 (all antibodies are rat IgG), followed by goat anti–rat IgG coupled to magnetic beads (Miltenyi Biotec). Lin$^+$ cells were depleted by attachment to a magnetic column according to manufacturer's instructions (MACS™; Miltenyi Biotec). For cell sorting and analysis, the lineage-depleted (lin$^-$) population was incubated again with the lineage antibodies followed by goat anti–rat IgG conjugated to allophycocyanin (Caltag Labs.), normal rat IgG (Caltag Labs.), and antibodies to c-kit (PE conjugate; PharMingen) and Sca-1 (fluorescein conjugate; PharMingen). Cells were then analyzed in a dual laser cell sorter (FACScan™; Becton Dickinson). Cells within the blast forward and side light scatter gate that were negative for the lineage markers were gated electronically and analyzed for or sorted according to expression of c-kit and Sca-1.

Reverse Transcriptase–PCR. RNA purification, first-strand cDNA synthesis, and PCR amplification were performed as described previously (15). PCR primers used were as follows: HPRT F, CAC AGG ACT AGA ACA CCT GC; HPRT R, GCT GGT GAA AAG GAC CTC T; FLK1 F, AGA ACA CCA AAA GAG AGG AAC G; FLK1 R, GCA CAC AGG CAG AAA CCA GTA G; FLK2 F, GGA GGA GGG CAG CTA CTT TGA G; FLK2 R, CTG TTA GCC TTT TTA TTC CAA ACT C; GATA1 F, CAT TGG CCC CTT GTG AGG CCA GAG A; GATA1 R, CGG AGA TAA AGT TCG AGG TAG TCC A; GATA2 F, ACA CAC CAC CCG ATA CCC ACC TAT; GATA2 R, CCT AGC CCA TGG CAG TCA CCA TGC; GATA3 F, ACG TCT CAC TCT CGA GGC AGC ATG; GATA3 R, GAA GTC CTC CAG CGC GTC ATG CAC; SCL F, GTC CTC ACA CCA AAG TAG TG; SCL R, GGC ACC TCA AAG CTT GAC TCT CCA; GMCSF F, GAG GTC ACA AGG TCA AGG TG; GMCSF R, GAT TGA CAG TGG CAG GCT TC; PU1 F, GAG TTT GAG AAC TTC CCT GAG; PU1 R, TGG TAG GTC ATC TTC TTG CGG.

Results

Extramedullary Hemopoiesis and Development of Anemia in Ikaros Mutant Mice. In *Ikaros* null and DN$^{-/-}$ mice, the BM cellularity is decreased to 37 and 18% of wild-type levels (Fig. 1 A), exceeding the reduction expected from the lack of B lymphocytes and their precursors. In normal adult mice, the spleen is the secondary hemopoietic organ, with 80–90% of its population comprised of B and T lymphocytes and 10–20% of the cells being of erythromyeloid origin. In *Ikaros* null mice, the cellularity in the spleen is reduced to only 62% of wild-type levels, a much smaller reduction

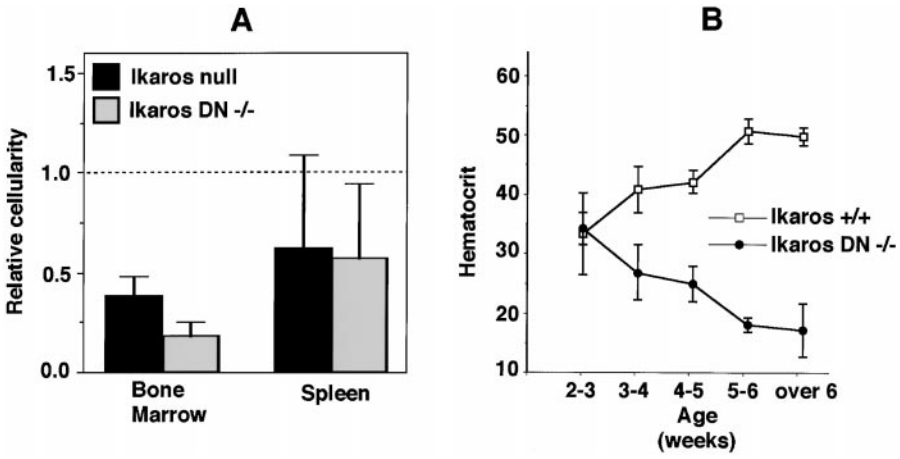

Figure 1. BM hypoplasia, extramedullary hemopoiesis, and progressive anemia in *Ikaros* mutant mice. (A) BM and spleen cellularity of *Ikaros* mutant relative to wild-type mice. BM cellularity is calculated from the cell content of the two hind limbs (30% of total BM). Both BM and spleen cell suspensions were counted after erythrocyte lysis. The cellularity of the wild-type organs is set to 1.0, and mutant values are indicated as a proportion thereof. Numbers of animals included in the analysis were: wild-type, $n = 20$; *Ikaros* null, $n = 16$; and *Ikaros* DN$^{-/-}$, $n = 18$. (B) Hematocrits in wild-type and *Ikaros* DN mutant mice are shown as a function of age. Wild-type mice and mutant littermates were bled at the indicated ages and their hematocrits read in a Readacrit centrifuge. *Ikaros* DN$^{-/-}$ mutant mice (●) initially had hematocrits similar to those of age-matched control littermates (□), but their hematocrits dropped to <50% of wild type by 6 wk of age. Number of mice bled is as follows: +/+, 2–3 wk old, $n = 4$; +/+, 3–4 wk old, $n = 9$; +/+, 4–5 wk old, $n = 4$; +/+, 5–6 wk old, $n = 5$; +/+, over 6 wk old, $n = 4$; −/−, 2–3 wk old, $n = 7$; −/−, 3–4 wk old, $n = 9$; −/−, 4–5 wk old, $n = 5$; −/−, 5–6 wk old, $n = 4$; and −/−, over 6 wk old, $n = 4$.

than that expected given the lack of B cells and impaired T cell development (11, 17). *Ikaros* $DN^{-/-}$ mice, which lack both B and T lymphocytes, also show a reduction to only 57% of the cellularity of the wild-type organ (Fig. 1 A). Taken together, these results reveal a significant increase in myeloerythroid cells in the spleens of *Ikaros* mutants. Such disproportionate changes in hemopoietic populations between BM and spleen are indicative of extramedullary hemopoiesis.

We have previously shown (11) that *Ikaros* null mice have normal hematocrits throughout their life spans. *Ikaros* $DN^{-/-}$ mice, however, display a drop in hematocrit with age (Fig. 1 B). The hematocrits of 2–3-wk-old *Ikaros* $DN^{-/-}$ mice are similar to those of wild-type littermates but soon after drop and by 6 wk of age reach a value of <50% of wild-type levels. During this period, *Ikaros* $DN^{-/-}$ mice also develop extensive infections from opportunistic microorganisms and die (10). Therefore, the cause of death in these mice cannot be unequivocally ascribed to the lack of an immune system or to hemopoietic failure but rather may be due to both. Thus, in addition to lymphoid defects, *Ikaros* deficiency has other effects on hemopoiesis.

Radioprotection Capabilities of Ikaros Null and $DN^{-/-}$ BM. Mice exposed to high doses of whole body irradiation die within 9–18 d from hemopoietic failure unless they are transplanted with hemopoietic precursors and progenitors that provide radioprotection and short-term reconstitution. The radioprotective quality of *Ikaros* null and $DN^{-/-}$ BM was assayed in strains of mice congenic for the panleukocyte marker Ly5. Both *Ikaros* null and $DN^{-/-}$ donor mice expressed the Ly5b variant, whereas the transplant recipients expressed the Ly5a allele.

Ikaros null and wild type BM cells, when given at a dose of 10^5 cells, radioprotected 100% of the lethally irradiated (900 rads) recipients for at least 30 d after transplant (Fig. 2 A). Animals receiving 10^6 *Ikaros* null BM had almost 100% donor contribution to the myeloid (Mac-1^+) lineage 7 mo after transplant (Fig. 2 B, left). *Ikaros* $DN^{-/-}$ BM cells provided at doses of 1–6 $\times$ 10^6 were capable of only short-term radioprotection at first (Fig. 2 A), with a steady decrease in donor contribution observed between 3.5 and 5 wk after transplant (Fig. 2 B, right). The hematocrits of *Ikaros* $DN^{-/-}$ BM recipients also decreased during this time period. 3 wk after transplant, hematocrits of *Ikaros* $DN^{-/-}$ BM recipients were <50% of wild-type BM recipients, and by day 35 they were down to one-third of wild-type values (Fig. 2 C). All recipients of *Ikaros* $DN^{-/-}$ BM died by day 35 after transplant from severe anemia.

Given the extramedullary hemopoiesis manifested in the spleens of *Ikaros* $DN^{-/-}$ mice, we examined whether hemopoietic progenitors developed in this secondary hemopoietic site. Splenocytes from these mutants were unable to radioprotect even when provided at a dose of 1.2 $\times$ 10^7 cells (data not shown). This observation suggests that there is no shift in the production or expansion of hemopoietic progenitors from the BM to the spleen and that extramedullary hemopoiesis at this site is due to the differentiation of more committed and short-lived erythromyeloid precursors.

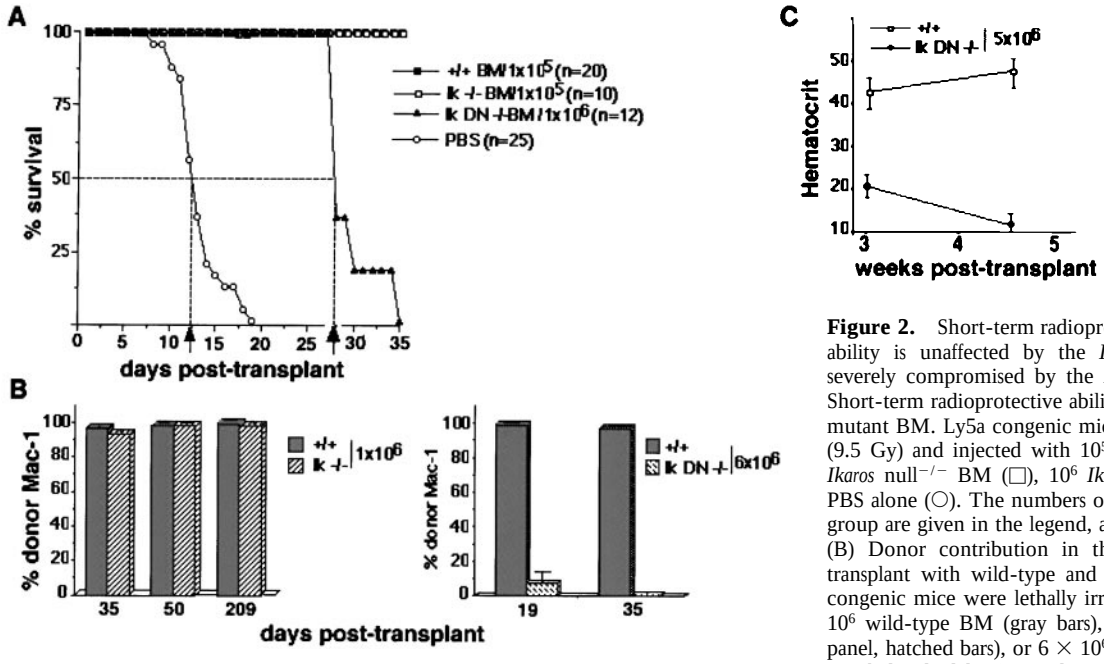

Figure 2. Short-term radioprotection and repopulation ability is unaffected by the *Ikaros* null mutation but severely compromised by the *Ikaros* DN mutation. (A) Short-term radioprotective ability of wild-type and *Ikaros* mutant BM. Ly5a congenic mice were lethally irradiated (9.5 Gy) and injected with 10^5 wild-type BM (■), 10^5 *Ikaros* null$^{-/-}$ BM (□), 10^6 *Ikaros* $DN^{-/-}$ BM (▲), or PBS alone (○). The numbers of mice injected per donor group are given in the legend, and their age was 3–4 wk. (B) Donor contribution in the myeloid lineage after transplant with wild-type and *Ikaros* mutant BM. Ly5a congenic mice were lethally irradiated and injected with 10^6 wild-type BM (gray bars), 10^6 *Ikaros* null BM (left panel, hatched bars), or 6 $\times$ 10^6 *Ikaros* $DN^{-/-}$ BM (right panel, hatched bars). At the indicated time points after transplant, recipients were bled and assayed for donor contribution to the myeloid (Mac-1^+) lineage by FACS™ analysis. A pool of four wild-type or mutant mice was used as a donor population. The experiment was repeated twice, and data shown are the analysis of one representative mouse out of eight recipients. (C) Development of lethal anemia in recipients of *Ikaros* $DN^{-/-}$ BM. 5 $\times$ 10^6 *Ikaros* $DN^{-/-}$ (●) or wild-type (□) BM cells were transplanted into lethally irradiated recipients, and recipient hematocrits were monitored over 5 wk. Recipients of *Ikaros* $DN^{-/-}$ BM (●) eventually died of anemia 5 wk after transplant. Numbers of mice bled were as follows: +/+ recipients, $n = 12$; and *Ikaros* $DN^{-/-}$ recipients, $n = 28$. Donors were 2–4 wk old.

Competitive Repopulating Units Are Reduced among Ikaros Mutant Hemopoietic Cells. The LTR potential of *Ikaros* null and $DN^{-/-}$ BM was analyzed in a competitive repopulation assay (23, 24). *Ikaros* mutant Ly5b BM cells were injected into lethally irradiated Ly5a recipients along with a constant competitor dose of 10^5 Ly5a marrow. When *Ikaros* null BM (10^5) was transplanted with an equal amount of wild-type competitor marrow, it failed to contribute to myeloid (Mac-1^+) cells in the BM or the periphery of recipients (Fig. 3 A, top right panel). In sharp contrast, wild-type BM contributed to 44% of the BM myeloid (Mac-1^+) populations (Fig. 3 A, top left panel). Ikaros null BM injected in 10-fold excess (10^6) over wild-type competitor gave 17% contribution to the myeloid (Mac-1^+) lineage in the BM (Fig. 3 A, center panels). No significant contribution to the T cell lineage was observed in the thymus or the spleen (data not shown). In comparison, wild-type BM coinjected at a similar dose contributed to >99% of the myeloid (Fig. 3 A, center panels) and lymphoid (data not shown) populations in the BM and periphery. When 7.5×10^6 *Ikaros* null BM cells were injected alongside 10^5 competitor BM cells, donor-derived contribution to the myeloid lineage increased to 75.3% (Fig. 3 A, bottom panels). This analysis indicates that there is a 30–40-fold decrease in LTR activity in the *Ikaros* null BM.

In a previous study (17), we reported short-term myeloid lineage contribution by *Ikaros* $DN^{-/-}$ BM when injected at a ratio of 100:1 over competitor BM. At this ratio, *Ikaros* $DN^{-/-}$ BM contributed to 52% of myeloid (Mac-1^+) cells by 19 d after transplant, but by day 27, this contribution declined to 6% and by day 48 to 3% (Fig. 3 B). No donor-derived myeloid contribution was detected 120 and 260 d after transplant (Fig. 3 B). PCR analysis of DNA prepared from the blood, spleens, and BM of recipients also failed to detect donor-derived cells, confirming the lack of LTR activity in *Ikaros* $DN^{-/-}$ BM (data not shown).

Thus, in the absence of *Ikaros*, LTR activity as estimated in competitive repopulation assays is reduced by 30–40-fold. However, a >100-fold reduction in LTR activity is observed in mice homozygous for the DN *Ikaros* mutation.

BM Engraftment into Unconditioned Ikaros Mutant Recipients. Lethal irradiation of normal murine recipients before BM

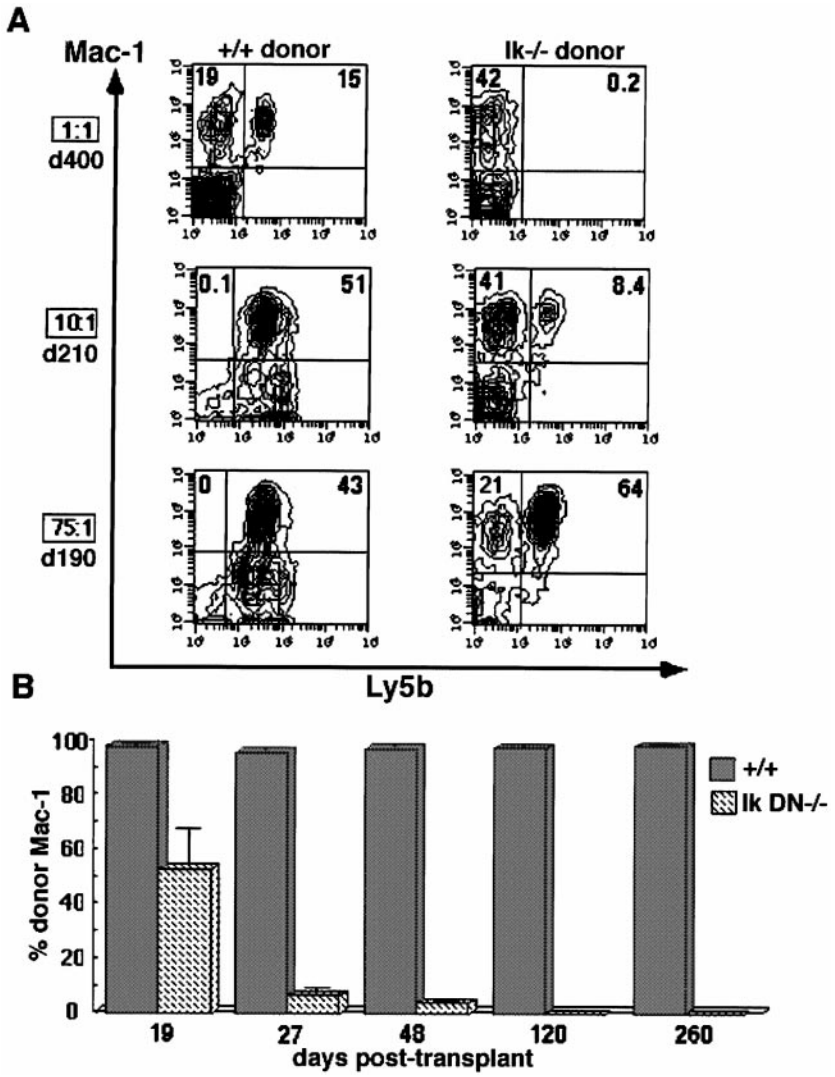

Figure 3. A progressive reduction in competitive repopulation units from *Ikaros* null to *Ikaros* $DN^{-/-}$ hemopoietic populations. (A) Competitive repopulation assay with wild-type and *Ikaros* null mutant BM. Lethally irradiated Ly5a mice were coinjected with a mixture of test (wild-type [+/+] or *Ikaros* null [Ik−/−]) Ly5b BM and a constant dose of 10^5 cells of competitor Ly5a BM at the indicated ratios. At various times (3–12 mo) after transplant, recipient mice were killed and analyzed for the hemopoietic contribution to marrow Mac-1^+ cells from test and competitor populations by FACS™ analysis. One representative out of four recipient mice killed is shown. Donors were 4 wk old. (B) Lack of competitive LTR activity in *Ikaros* $DN^{-/-}$ BM. 5×10^6 *Ikaros* $DN^{-/-}$ Ly5b BM cells were transplanted along with 5×10^4 competitor Ly5a BM cells into lethally irradiated Ly5a recipients. At the indicated time points after transplant, recipient mice were bled and contribution to the Mac-1 lineage from each injected population was determined by FACS™ analysis. The average of six recipients per group is shown for each time point. Donors were 2–3 wk old.

transplant is required to ensure engraftment and detection of donor-derived hemopoietic cells. However, if the hemopoietic system of the recipient is already compromised, then donor-derived hemopoiesis is detectable even in the absence of prior conditioning of the recipient. This phenomenon has been observed with c-kit receptor (*W*) mutant mice (25). Given the depletion in hemopoietic progenitors resulting from the null and DN *Ikaros* mutations, we attempted to repopulate unconditioned *Ikaros* mutants with wild-type BM.

A transplant of 10^6 wild-type Ly5a BM cells into unconditioned *Ikaros* null mice completely repopulated the B cell lineage, consistent with the total lack of B cells and their precursors in *Ikaros* mutants (Fig. 4 A, $Ly5b^-/B220^+$ cells). Donor repopulation of T cells and some myelocytes was also observed, albeit at low levels, possibly due to competition with endogenous precursor populations (Fig. 4 A, $Ly5b^-/TCR\text{-}\alpha/\beta^+$ and $Ly5b^-/Mac\text{-}1^+/Gr\text{-}1^+$).

Ikaros DN homozygous mutants and heterozygous littermates were injected with 10^6 wild-type Ly5a BM cells at 12 d of age and subsequently analyzed at 4, 12, and 22 wk after transplant. No detectable donor contribution in any hemopoietic lineages was observed after transplant of wild-type Ly5a BM into unconditioned Ly5b *Ikaros* DN heterozygotes (Fig. 4 B, top panels, $Ly5a^+/lin^+$ cells). In striking contrast, complete donor repopulation of B and T lymphocytes and myeloid cells was observed in unconditioned *Ikaros* $DN^{-/-}$ mice receiving 10^6 wild-type BM cells (Fig. 4 B, bottom panels, $Ly5a^+/B220^+$, $Ly5a^+/TCR\text{-}\alpha/\beta^+$, and $Ly5a^+/Mac\text{-}1^+/Gr\text{-}1$), which persisted for the full duration of the experiment. To detect potential hemopoietic contribution by endogenous *Ikaros* $DN^{-/-}$ mutant cells at levels that may fall below the detection limit of FACS™, PCR analysis of DNA prepared from various hemopoietic tissues was employed (Fig. 4 C). Given a PCR DNA detection limit of 1:1,000 mutant/wild-type DNA (Fig. 4 C), we conclude that host-derived hemopoietic cells were at least 1,000-fold less frequent in unconditioned *Ikaros* $DN^{-/-}$ recipients after transplant of wild-type BM.

Thus, the hemopoietic system of *Ikaros* null and $DN^{-/-}$ mice is readily repopulated by wild-type HSCs without the need for prior myeloablative conditioning. This repopulation of unconditioned *Ikaros* mutant mice by wild-type HSCs reflects a severe depletion in the endogenous pool.

Reduction in CFU-S_{14} in Ikaros Mutant Mice. The more

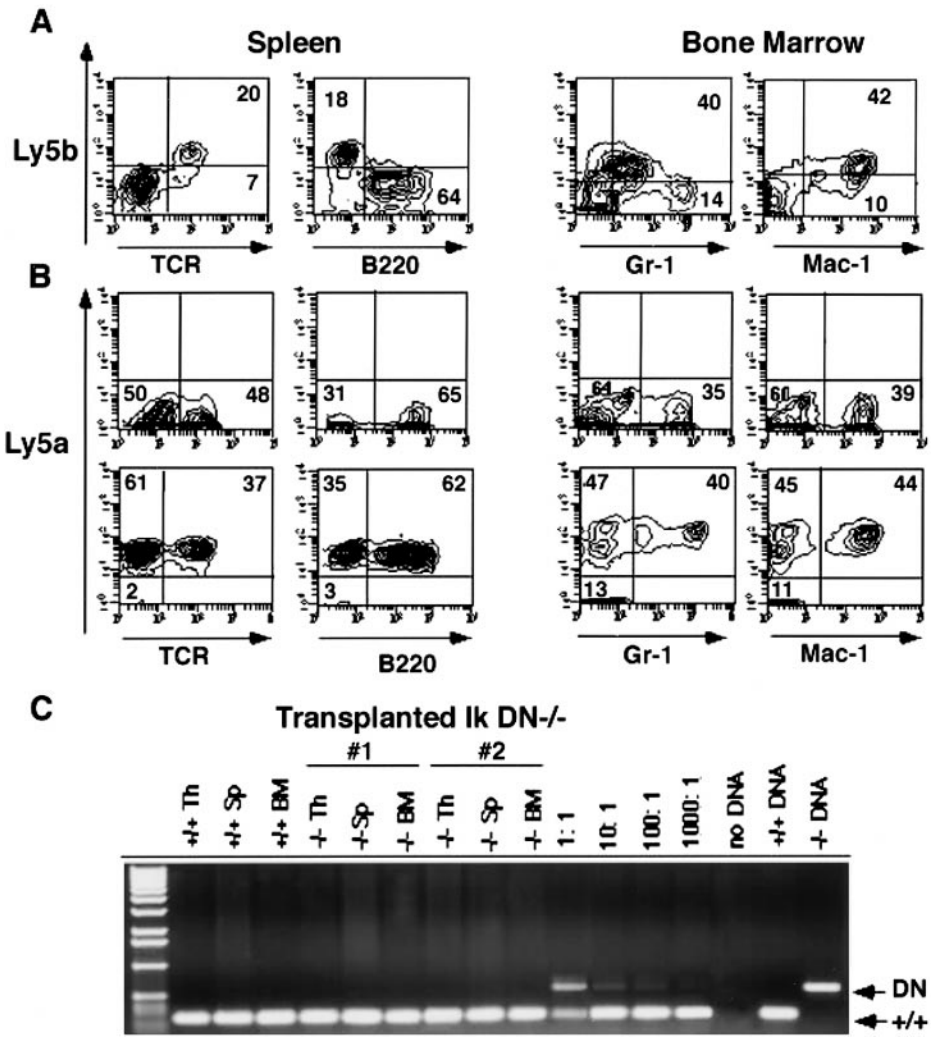

Figure 4. Hemopoietic lineage repopulation in unconditioned *Ikaros* mutant mice points to a severe depletion in hemopoietic progenitors. (A) 10^6 wild-type Ly5a BM cells were injected into 3-wk-old Ly5b *Ikaros* null mice without prior conditioning. At different time points (3–6 mo) after transplant, recipient mice were killed and analyzed for Ly5a contribution by FACS™ analysis. (B) 10^6 wild-type Ly5a BM cells were injected intravenously into 12-d-old *Ikaros* $DN^{+/-}$ or $DN^{-/-}$ Ly5b recipients without prior conditioning. At different time points after transplant, recipient mice were killed and analyzed for donor Ly5a contribution by FACS™ analysis. Analysis of representative recipients 5.5 mo after transplant is shown. Top panels correspond to *Ikaros* $DN^{+/-}$ recipients, and bottom panels correspond to *Ikaros* $DN^{-/-}$ recipients. (C) PCR analysis of the hemopoietic organs of two independent *Ikaros* $DN^{-/-}$ recipients of wild-type BM. Total DNA was extracted from the hemopoietic organs of wild-type control mice and *Ikaros* $DN^{-/-}$ mutant mice that were transplanted with wild-type BM without prior irradiation. No *Ikaros* DN mutant DNA (upper band) is detectable in $DN^{-/-}$ recipients, indicating lack of recipient contribution in any hemopoietic lineage. The DNA titration (lanes 10–13) indicates that the larger mutant band can still be detected in a 1:1,000 dilution into the smaller, and thus more readily amplifiable, wild-type band.

committed myeloid precursor content of *Ikaros* mutant mice was investigated in a spleen colony-forming assay (CFU-S_{14}) (26). CFU-S_{14} levels in *Ikaros* null and $DN^{-/-}$ BM were reduced 9.2- and 81-fold relative to wild type (Fig. 5 A). Splenic CFU-S_{14} was also reduced by three- and fivefold in *Ikaros* null and $DN^{-/-}$ mice relative to wild type (Fig. 5 A). The combined limb BM and splenic CFU-S_{14} contents of *Ikaros* null and $DN^{-/-}$ mice were reduced 7.7- and 34-fold relative to wild type. Although these data assume similar spleen seeding efficiencies by wild-type and *Ikaros* mutant CFU-S, we have not tested this hypothesis.

Qualitative effects of the *Ikaros* mutations on the size and lineage composition of day 14 spleen colonies were also observed. First, spleen colonies derived from *Ikaros* mutant cells were smaller than those derived from wild-type populations (Fig. 5 A, bottom panels, arrows). Second, *Ikaros* mutant colonies contained 20–40% of nucleated erythroid ($TER\text{-}119^{+}$) cells, whereas wild-type colonies consisted mainly (76–91%) of more mature erythroid cells (data not shown). The donor origin of the CFU-S_{14} colonies was confirmed by PCR (data not shown). Interestingly, a small number of endogenous colonies was detected in recipients of *Ikaros* mutant BM and spleen but not in recipients of wild-type populations or in PBS controls. A possible facilitating effect is suggested, perhaps in the form of growth factors produced by the *Ikaros* mutant cells that promote expansion and differentiation of endogenous progenitors.

The CFU-S_{14} content of day 10 YS and AGM and day 14 fetal livers of *Ikaros* $DN^{-/-}$ mutant and wild-type embryos was also measured. The CFU-S_{14} content in these late embryonic and early fetal sites of hemopoiesis was drastically reduced in *Ikaros* $DN^{-/-}$ embryos compared with wild type (Fig. 5 C). Thus, the lack of *Ikaros*, coupled with interference toward the activity of other family members, affects the production of both fetal and adult hemopoietic progenitors.

Effects of the Ikaros Mutations on In Vitro Clonogenic Precursors. BM and splenic hemopoietic populations were analyzed for their content of myeloid-restricted in vitro CFCs. The absolute number of precursors giving rise to multilineage colonies (CFC-multi) and G- and/or M-restricted colonies (CFC-G/M) in *Ikaros* null BM was within the range of

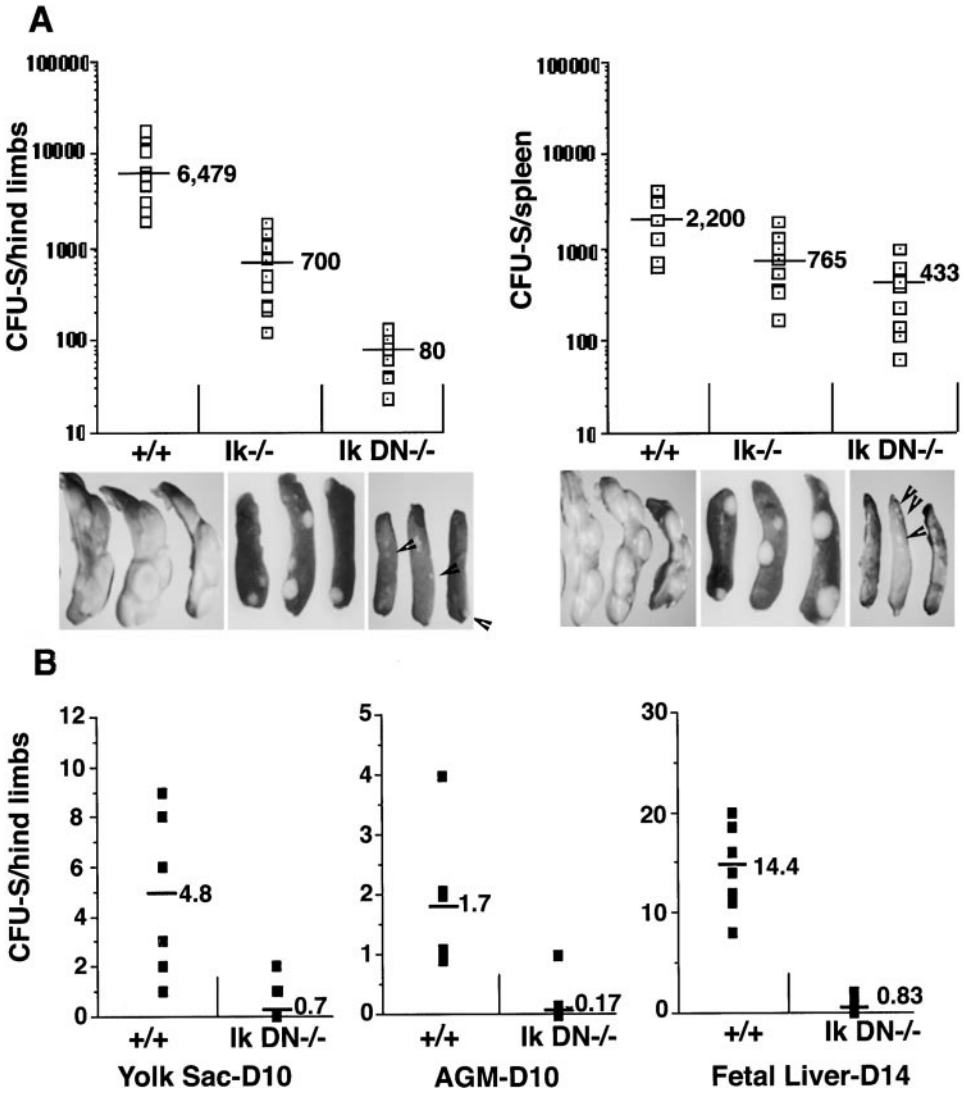

Figure 5. Reduction in hemopoietic progenitors (CFU-S_{14}) caused by the *Ikaros* mutations. (A) Absolute number of CFU-S_{14} in the two hind limbs (left) and spleens (right) of wild-type and *Ikaros* mutant mice. Numbers do not include seeding efficiency conversions. Each square represents the average of four recipients of either 5×10^4 BM or 2.5×10^5 spleen cells from one donor mouse. The number of donor mice is as follows: +/+ BM, $n = 12$; +/+ spleen, $n = 12$; DN BM, $n = 14$; DN spleen, $n = 14$; null −/− BM, $n = 10$; and null −/− spleen, $n = 10$. Donors were 2–4 wk old. Spleen colonies generated upon injection of wild-type and *Ikaros* mutant BM and spleen are shown in the bottom panels. (B) CFU-S14 content of day 10 YS (Yolk Sac-D10), day 10 AGM (AGM-D10), and day 14 fetal liver (Fetal Liver-D14) without seeding factor correction is shown for wild-type (6–7) and *Ikaros* $DN^{-/-}$ embryos (4).

wild-type values (Fig. 6 A). In contrast, the absolute number of mature erythroid-restricted clonogenic precursors (CFU-E) was reduced to 30% of wild-type levels (Fig. 6 A). Furthermore, the most immature erythroid-restricted precursors (BFU-E) were reduced to 5% of wild-type numbers. In the spleens of *Ikaros* null mice, all CFC classes, including CFU-E, BFU-E, CFC-G/M, and CFC-multi, were reduced in absolute number (Fig. 6 A). However, the frequencies of all CFCs, with the exception of BFU-E, were higher than in wild type (Fig. 6 B) due to the observed decrease in spleen (and BM) cellularity in *Ikaros* mutants.

A more severe reduction in the absolute number of all CFCs was detected in *Ikaros* $DN^{-/-}$ mice. BFU-E cells were the most drastically affected in BM and spleen, with absolute numbers <1% of wild type (Fig. 6 A). CFU-E cells were also reduced, but to a lesser extent than BFU-E. A CFU-E reduction to 5% of wild type was noted in the BM and to 25% of wild type in the spleen. In the BM, the absolute number of CFC-G/M cells was reduced to 15% of wild-type levels, reflecting the reduction in BM cellularity. Thus, the CFC-G/M frequency was similar to that of wild-type BM. However, in the *Ikaros* $DN^{-/-}$ spleen, CFC-G/M numbers were greatly increased (greater than twofold), indicating a dramatic increase in granulomonocytic precursors in this normally lymphoid organ (Fig. 6 A). Nonetheless, no increase in splenic erythroid precursors (BFU-E and CFU-E) was detected, suggesting specific effects of the *Ikaros* mutation on the expansion of myeloid versus erythroid precursors (see Fig. 8).

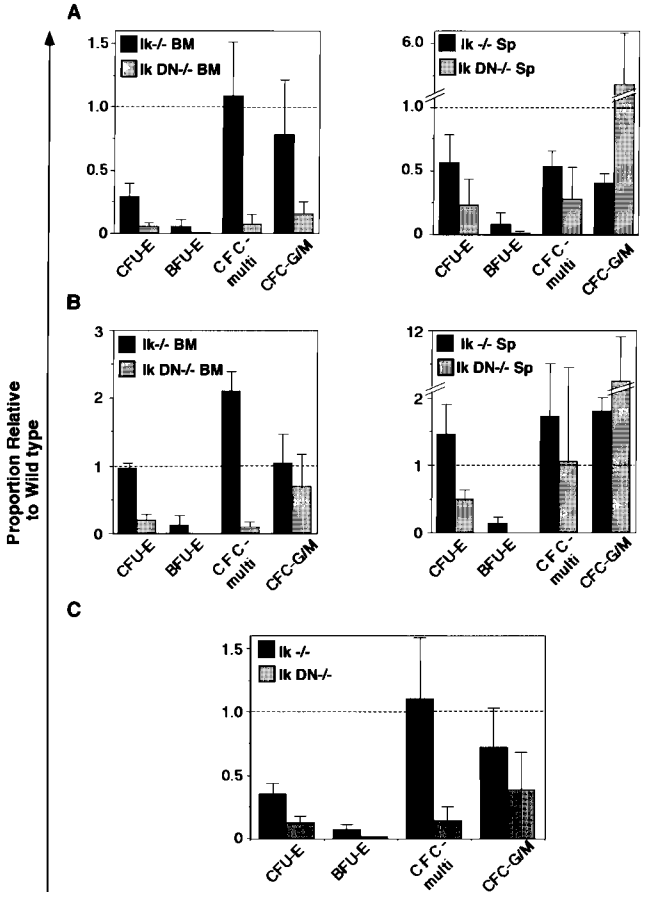

Figure 6. Dynamics in myeloid differentiation in *Ikaros* mutant mice. (A) Proportion of absolute CFCs recovered from BM and spleens (Sp) of *Ikaros* null and $DN^{-/-}$ mutants relative to wild type (dashed line) is shown. The data represent the average ±SEM from a total of eight animals per genotype. Precursor classes are defined in Materials and Methods. (B) Frequency of CFCs (i.e., number per 10^5 cells) in *Ikaros* null and *Ikaros* $DN^{-/-}$ BM and spleens is shown. Data are standardized to wild-type frequencies, represented by the dashed line. (C) The combined CFC content of BM and spleen relative to wild type is shown. CFC content for the entire bodily BM compartment is included in the calculation by assuming that one femur represents 10% of total BM.

Given the BM hypoplasia and abnormal splenic hemopoiesis manifested in both *Ikaros* mutants, the combined total BM and spleen content of hemopoietic precursors was calculated per mouse (Fig. 6 C). BFU-E cells are reduced 10-fold in the *Ikaros* null and 30-fold in *Ikaros* $DN^{-/-}$ mice (Fig. 6 C). These BFU-E reductions are in line with the reductions seen in the most primitive HSC population. However, the total number of the later erythroid precursors (CFU-E) was reduced only three- and sixfold in *Ikaros* null and $DN^{-/-}$ mice, respectively. Furthermore, in both *Ikaros* mutants, CFC-G/M cells were reduced to 40–75% of wild-type levels, a less severe decrease than that observed for other CFC classes.

Although the numbers of myeloid precursors in the spleens of the *Ikaros* null and $DN^{-/-}$ mice (with the exception of CFC-G/M) are decreased, the number of terminally differentiated erythroid ($TER\text{-}119^+$) and myelomonocytic ($Mac\text{-}1^+/Gr\text{-}1^+$) cells is increased in this organ (10, 11). This suggests that the splenic microenvironment is conducive to the rapid differentiation of myeloid precursors to their mature progeny.

Lack of BM Cells with the $Lin^-c\text{-}kit^+Sca\text{-}1^+$ Stem Cell Phenotype in Ikaros $DN^{-/-}$ Mice. Hemopoietic cells that lack expression of mature lineage markers (lin^-) and that coexpress Sca-1/Ly6A and the tyrosine kinase receptor c-kit on their cell surfaces ($lin^-c\text{-}kit^+Sca\text{-}1^+$) are highly enriched in HSC activity in normal mice (27, 28). The HSC-enriched $lin^-c\text{-}kit^+Sca\text{-}1^+$ population was detected in *Ikaros* null BM (Fig. 7 A, center). In sharp contrast, no $c\text{-}kit^+/Sca\text{-}1^+$ cells were present among the lin^- BM cells of *Ikaros* $DN^{-/-}$ mice (Fig. 7 A, right). The lack of BM cells with a $lin^-c\text{-}kit^+Sca\text{-}1^+$ surface phenotype in *Ikaros* $DN^{-/-}$ mice is consistent with the dramatic depletion in HSC activity in these mutant mice.

Analysis of lin^- BM cells from *Ikaros* null and DN mice revealed a progressive decrease in the cell surface expression level of c-kit (Fig. 7 B). Given the importance of the steel factor/c-kit signaling pathway in the expansion and differentiation of hemopoietic progenitors (29), we predict that the decreased expression of c-kit in *Ikaros* mutant hemopoietic cells may underlie some of the progenitor defects.

Molecular Analysis of Ikaros Mutant Hemopoietic Progenitors. To address the molecular basis of the hemolymphoid deficiencies in *Ikaros* mutant mice, we analyzed expression of growth factor receptors, signaling molecules, and transcription factors known to be important in the production, maintenance, and differentiation of hemopoietic cells.

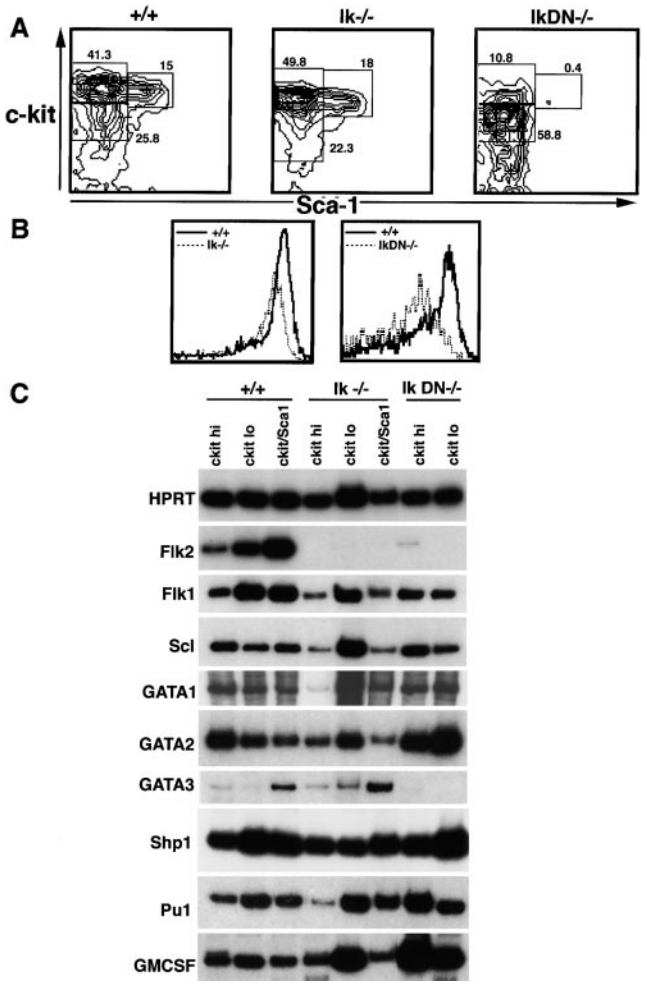

Figure 7. Analysis of lin$^-$ *Ikaros* mutant hemopoietic cells. (A) Total BM cells from 2–4 wk-old wild-type (+/+), *Ikaros* null (Ik −/−), and *Ikaros* (Ik) DN$^{-/-}$ mice were lineage depleted and stained for c-kit and Sca-1 (as described in Materials and Methods). The c-kit^{hi}, c-kit^{lo}, and c-kit$^+$Sca-1$^+$ populations are boxed and percentages are indicated. (B) Histograms of cell surface c-kit expression in *Ikaros* mutant lin$^-$ BM populations (dashed lines) and wild type (solid lines) are shown. The left and right panels contrast *Ikaros* null and *Ikaros* DN$^{-/-}$ cells, respectively, with wild-type cells. (C) RT-PCR analysis of sorted hemopoietic cells from wild-type and *Ikaros* mutant mice. BM cells were lineage depleted and sorted according to expression of c-kit and Sca-1, as indicated in A. cDNA was obtained from each sorted population and PCR amplified for the indicated genes. cDNA levels were normalized to HPRT levels.

Lin$^-$ BM cells from *Ikaros* null and wild-type mice were sorted into c-kit^{hi}, c-kit^{lo}, and c-kit^{hi}Sca-1$^+$ populations, and those from *Ikaros* DN$^{-/-}$ mice were sorted into c-kit^{hi} and c-kit^{lo} populations and subsequently used for reverse transcriptase (RT)-PCR analysis (Fig. 7 C). mRNA expression of the tyrosine kinase receptor flk-2, shown to be required for HSC differentiation along the B cell lineage (30), was undetectable in all lin$^-$ BM populations, including those that are highly enriched for HSC activity, in *Ikaros* null mice (Fig. 7 C, flk-2). Expression of a second related tyrosine kinase receptor, flk-1, required for development of the vascular endothelium from the hemangioblast, was also tested (31). Flk-1 was expressed in the *Ikaros* mutant lin$^-$ populations but at a somewhat reduced level compared with wild type (Fig. 7 C, Flk1). The mRNA level of the tyrosine phosphatase Shp-1, a downstream effector of the c-kit signaling pathway, was also determined and found not to be significantly different between *Ikaros* mutant and wild-type populations (Fig. 7 C, Shp1).

Expression of several transcription factors known to play key roles in the development of the hemopoietic system was also determined. *Ikaros* mutant lin$^-$ cells were analyzed for the presence of SCL, GATA-1, GATA-2, GATA-3, and PU.1 transcripts. A small decrease in SCL levels was seen in the *Ikaros* null c-kit^{hi} and c-kit^{hi}Sca-1$^+$ population (Fig. 7 C, Scl). Small changes in GATA-2 but not GATA-1 levels were also seen among the *Ikaros* null and DN$^{-/-}$ hemopoietic populations, the most dramatic being an increase in GATA-2 levels in the c-kit^{lo} cells in the *Ikaros* DN$^{-/-}$ BM. These possibly reflect changes in hemopoietic progenitor composition in the lin$^-$ compartment. GATA-3 is expressed among the lin$^-$ hemopoietic cells, possibly in progenitors undergoing specification along the T cell lineage. GATA-3 was expressed within lin$^-$ progenitor populations of *Ikaros* null mice, which generate T cell precursors. However, it was not detected in *Ikaros* DN$^{-/-}$lin$^-$ populations lacking T cell differentiation potential, reflecting either lack of the relevant precursor population or lack of expression of the GATA-3 factor in these cells. Finally, levels of PU.1 are reduced in the c-kit^{hi} population of the *Ikaros* null mutants but elevated in the same population in *Ikaros* DN$^{-/-}$ BM. The *Ikaros* mutant lin$^-$ hemopoietic populations were also analyzed for expression of mRNAs encoding hemopoietic growth factors and receptors. In the c-kit^{lo} of *Ikaros* null and in the c-kit$^{lo\text{-}med}$ populations of *Ikaros* DN$^{-/-}$ BM, levels of GM-CSF receptor were significantly elevated relative to wild type, which could be the cause or effect of the increase in GM precursors observed in clonogenic assays.

Discussion

We have previously shown that *Ikaros* is an essential regulator of lymphoid lineage specification in the fetal and adult hemopoietic system. *Ikaros* is, however, expressed early during ontogeny of the hemopoietic system and is detected at high levels in mesodermal progenitors in the splachnopleura of the day 8 embryo and within the blood islands of the YS (reference 18 and Georgopoulos, K., unpublished results). In addition, in adult hemopoietic sites, *Ikaros* is expressed within cell populations that are highly enriched in HSCs (15, 32). A detailed analysis of the entire hemopoietic hierarchy in *Ikaros* mutant mice is presented here, revealing a role for *Ikaros* in the production or activity of the self-renewing HSC. These studies also provide evidence for a functional interplay between *Ikaros* and other nuclear factors, including family members during differentiation of the most primitive of hemopoietic progenitors.

Mice homozygous for an *Ikaros* null or DN mutation show a decrease in BM cellularity that cannot be ascribed solely to the lack of B cell differentiation. In addition, an

increase in erythromyeloid precursors is detected in the spleens of *Ikaros* mutant mice, an observation not made in other strains of mice depleted of lymphocytes. The progressive development of anemia in mice homozygous for the *Ikaros* $DN^{-/-}$ mutation, manifested as a progressive drop in hematocrit, is also indicative of a failure of the hemopoietic system to supply mature erythrocytes at normal levels.

BM progenitors from *Ikaros* null mice provide short- and long-term contribution to most of the hemopoietic lineages, apart from the lymphoid, when transferred alone into lethally irradiated recipients. However, when measured against wild-type BM in a competitive assay, a severe depletion in both short- and long-term repopulating activities is revealed. *Ikaros* null BM is unable to contribute to any hemopoietic lineages when competed against wild-type congenic BM at a ratio below 10:1. Even when present in a >10-fold excess, only limited hemopoietic contribution is observed from *Ikaros* null BM, suggesting a reduced number of HSCs in these mutants. In contrast, wild-type BM, when used at an excess of 10:1 over competitor, repopulates 100% of all hemopoietic lineages tested. Thus, there is a quantitative reduction estimated to be 30–40-fold in the number of HSCs in *Ikaros* null mice (Fig. 8).

A greater reduction in HSC numbers is detected in mice homozygous for a DN *Ikaros* mutation. *Ikaros* $DN^{-/-}$ BM is unable to provide LTR in a competitive assay, even when used at 100-fold excess to wild-type congenic BM, indicating a severe depletion in stem cell activity below assayable levels. A limited short-term hemopoietic contribution from the *Ikaros* $DN^{-/-}$ BM was detected in both competitive and radioprotective assays, suggesting a transient expansion in short-term repopulating cells. Wild-type BM cells sorted according to the absence of cell surface lineage-specific markers and expression of c-kit and Sca-1/Ly6A are enriched for HSC activity (28). This population of cells was absent from the BM of *Ikaros* $DN^{-/-}$ mice, consistent with the depletion in both short- and long-term repopulating activity in these mutants.

The defect in hemopoietic progenitors within *Ikaros* mutants was also illustrated by the ability of wild-type BM to provide hemopoietic reconstitution to these animals without their prior conditioning. Transplant of wild-type BM cells into unirradiated *Ikaros* null mice resulted in chimeric animals in which some hemopoietic lineages, such as T, Mac-1^{+}, and Gr-1^{lo} cells, all of which are unaffected by the *Ikaros* mutation, derive from both wild-type and *Ikaros* mutant precursors. B lineage cells and Gr-1^{hi} cells, neither of which are produced from *Ikaros* mutant progenitors, are derived exclusively from wild-type precursors. In *Ikaros* $DN^{-/-}$ mutants, repopulation by wild-type BM without the need for prior conditioning is observed for all lineages. The ability of normal BM to function in *Ikaros* mutants suggests that the *Ikaros* defects are manifest within hemopoietic populations rather than in nonhemopoietic accessory cells of the BM and spleen.

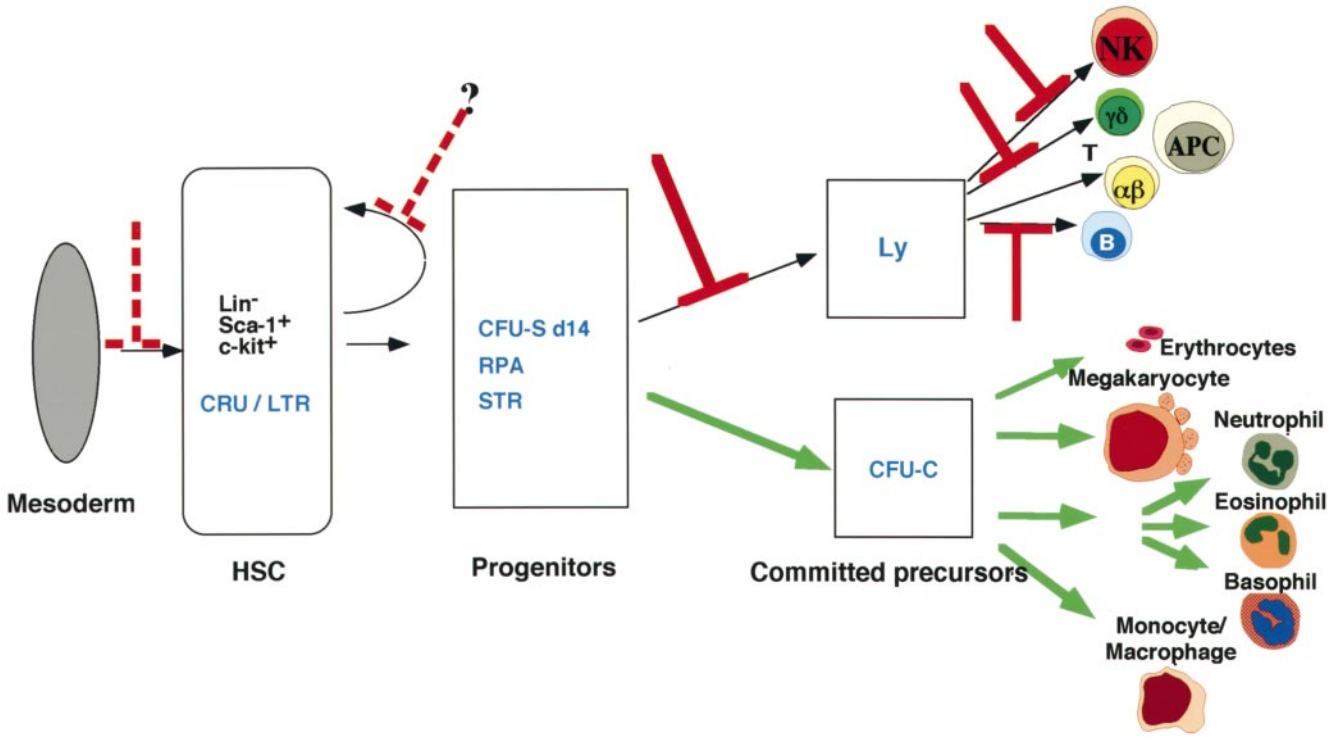

Figure 8. Effects of *Ikaros* deficiency on the hemopoietic progenitor/precursor hierarchy. Commitment of a mesodermal precursor to the HSC fate, self-renewal (curved arrow), and subsequent progression through intermediate steps that involve long- and short-term repopulating progenitors and precursors to terminally differentiated progeny is illustrated. The mesodermal precursor commitment step as a possible target for the *Ikaros* deficiencies and a cause for the reduction in HSCs is indicated as the perpendicular intersection of two broken lines, which also indicates a possible defect in HSC renewal. Complete blocks in the differentiation of *Ikaros*-deficient progenitors toward the B cell and NK lineages are shown as the perpendicular intersection of two solid lines. The increase in the differentiation output of these mutant precursor populations toward the erythromyeloid lineage is depicted with green arrows.

Evaluation of the more committed hemopoietic precursor compartment in *Ikaros* mutant mice reveals amelioration of their phenotypes. CFU-S_{14} clonogenic assays were used as a measure of the multilineage erythromyeloid-restricted progenitors. An eightfold reduction in CFU-S_{14} activity was detected in *Ikaros* null mice, whereas the reduction in *Ikaros* $DN^{-/-}$ mice was 34-fold. In vitro clonogenic assays show that BFU-E, the earliest erythroid-restricted precursor, is reduced by 10- and 30-fold in *Ikaros* null and *Ikaros* $DN^{-/-}$ mice, respectively. This reduction in BFU-E may in part explain the decrease in CFU-S_{14}, particularly as wild-type spleen colonies are comprised mainly of erythroid lineage cells. However, later stage erythroid precursors (i.e., CFU-E) are not as severely compromised, with only three- and sixfold reductions detected in *Ikaros* null and $DN^{-/-}$ mice. Lack of *Ikaros* has an even lesser effect (25 and 50% decrease) on the number of myelomonocytic precursors, indicating their possible preferential expansion relative to BFU-E (Fig. 8). The decrease in CFU-S_{14} and BFU-E activity in *Ikaros* mutant mouse strains is smaller than the estimated 30–100-fold decrease in LTR cells, suggesting the existence of partial compensatory mechanisms after HSC commitment. Alternatively, *Ikaros* may normally function in early hemopoietic progenitors to limit differentiation along the myeloid lineages, a constraint that is removed upon *Ikaros* inactivation (Fig. 8).

To determine if the hemopoietic defects manifested in *Ikaros* mutants are the result of a defect in the production or maintenance of HSCs, we examined the hemopoietic precursor content of embryonic and fetal hemopoietic sites. CFU-S_{14} activities were measured in the AGM and the YS on day 10 and the fetal liver on day 14 of *Ikaros* $DN^{-/-}$ embryos. CFU-S_{14} activity was depleted in all three sites in *Ikaros* $DN^{-/-}$ embryos. The effects of Ikaros mutation on fetal hemopoietic precursors closely match those seen postnatally in the BM and spleen. Depletion of fetal precursors in the *Ikaros* mutant embryos supports the idea that *Ikaros* regulates the production of hemopoietic progenitors during ontogeny of the hemopoietic system.

Molecular analysis of lin^- hemopoietic populations in *Ikaros* mutant mice revealed a severe reduction in expression of two tyrosine kinase receptors important for HSC development. Surface expression of c-kit is progressively reduced from *Ikaros* null to *Ikaros* $DN^{-/-}$ hemopoietic cells, and mRNA expression of flk-2 is missing in both mutant populations. Flk-2 was originally identified as a tyrosine kinase receptor expressed in fetal liver populations enriched for HSCs (33). It has previously been reported that flk-2 null BM has reduced competitive repopulation activity against wild-type BM, suggesting a potential depletion in HSCs (30). In addition, the number of B cell precursors in the flk-2 null mice is reduced. Both flk-2 hemopoietic phenotypes correlate with those observed in *Ikaros* mutants. However, hemopoietic progenitors in *Ikaros* mutant mice appear to be more severely reduced in number, and B cell precursors are absent. It is possible that lack of the flk-2 tyrosine kinase receptor, compounded with a reduction in levels of cell surface c-kit, and possibly other unidentified factors, which are also required at the early stages of B, T, and HSC differentiation, may account for the more severe HSC and B cell phenotypes manifested in *Ikaros* mutant mice. Indeed, flk-2 and c-kit double mutants display a far more severe hemopoietic phenotype than that manifested by either mutations alone, resulting in early lethality (30). It is also significant that the difference in c-kit expression between *Ikaros* null and $DN^{-/-}$ progenitors directly correlates with the severity in HSC defect manifested in these mutant mice. Expression of the T cell–determining transcription factor, GATA-3, is missing from the *Ikaros* $DN^{-/-}$ mice but is present among the *Ikaros* null lin^- hemopoietic populations. GATA-3 expression, or lack thereof, correlates with the T cell differentiation potential of *Ikaros* mutants. Interference from the Ikaros DN isoforms toward the activity of other Ikaros-interacting factors expressed in the early hemopoietic progenitor compartment may impair GATA-3 expression in these cells and block their T cell differentiation potential. Finally, increase in expression of the myeloid-specific GM-CSF receptor within *Ikaros* mutant early hemopoietic progenitors may underlie the apparent expansion of the myeloid precursors and their progeny despite the dramatic reduction in hemopoietic progenitor numbers.

The progressive depletion in long- and short-term hemopoietic progenitors and T cell precursors from the *Ikaros* null to $DN^{-/-}$ mice supports a role for Ikaros and its interacting factors (21) in the development and differentiation of HSCs. Ikaros proteins are all engaged in a higher order complex with family members and components of two distinct chromatin remodeling complexes (21). The proper nuclear compartmentalization and gene targeting of these chromatin remodeling complexes manifested in the presence of Ikaros and its family members are likely to be as critical for differentiation and homeostasis of the hemopoietic system as the Ikaros protein itself. The progressive depletion in long- and short-term hemopoietic progenitors and T cell precursors from the *Ikaros* null to $DN^{-/-}$ mice may reflect progressive mistargeting of these remodeling complexes in the nuclei of HSCs in the absence of Ikaros and through DN interference with the activity of its family members, the expression of which is not affected by the *Ikaros* mutations (data not shown).

Ikaros remodeling complexes may control hemopoietic lineage commitment decisions by potentiating expression of genes that include at least the tyrosine kinase receptors flk-2 and c-kit and possibly the transcription factor GATA-3, required for differentiation along the B and T cell lineages. We have recently shown that Ikaros proteins provide T cell activation thresholds (34), possibly by regulating changes in chromatin structure (21). *Ikaros* may thus control HSC activation by providing thresholds to signaling pathways for c-kit and other receptors. In the absence of *Ikaros*, the signaling thresholds for these pathways may be lowered, allowing HSCs and their progeny to enter the cell cycle more readily and possibly deplete the most primitive quiescent stem cell pool. Further studies on the cycling status of *Ikaros* mutant HSC and the effects of cell cycle–promoting factors on the hemopoietic compartment of *Ikaros* mutant mice will address this central question of self-renewal.

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