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Early hemopoietic lineage restrictions directed by Ikaros

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Abstract

Ikaros is expressed in early hematopoietic progenitors and is required for lymphoid differentiation. Analysis of Ikaros null populations revealed a lack of defining markers for early fate-restricted progenitors, but it was difficult to discern whether Ikaros was required for formation of these populations, or for expression of these markers. Here we use a GFP reporter based on Ikaros regulatory elements to identify the HSC and separate early progenitors in both wild-type and Ikaros-null mice. The presence of lympho-myeloid progenitors is revealed in Ikaros-null mice, which lack the defining factor Flt3 and are capable of myeloid, but not lymphoid differentiation. In contrast, lack of Ikaros in the common myeloid progenitor results in increased formation of erythro-megakaryocyte at the expense of myeloid progenitors and influences their subsequent differentiation. By this approach, pivotal roles for Ikaros in distinct fate decisions in the early hematopoietic hierarchy are revealed.

Keywords

Ikaros-reporter; hematopoiesis; progenitors; cell fate

The long-term hematopoietic stem cell (HSC), capable of self-renewal and differentiation into a number of distinct lineages, is responsible for the lifelong generation of all blood and immune cell types¹⁻³. Prospective isolation of HSCs and progenitor populations with conventional cell surface markers has identified rare, multipotent cells with defined lineage activities that in turn have been used to infer prevailing models of lineage restriction⁴⁻⁶. For example, the isolation of a common myeloid progenitor (CMP) and a common lymphoid progenitor (CLP), considered to be the respective roots of the erythro-myeloid and lymphoid lineages, has lent support to an early and strict separation of the lymphoid from the erythro-myeloid pathways.

The HSC compartment is operationally defined within the Lin⁻Sca-1^{hi}c-Kit^{hi} (LSK) population that constitutes 0.1% of the adult bone marrow (BM) cells and contains both long-term (LT) and short-term (ST) HSC—also known as multipotent progenitors (MPP)^{7, 8}. Use of additional markers, including CD34 and Flt3, has separated LT-HSCs (Lin⁻Sca-1^{hi}c-Kit^{hi}CD34⁻Flt3^{neg-lo}) from ST-HSCs (Lin⁻Sca-1^{hi}c-Kit^{hi}CD34⁺Flt3^{neg-lo}) and more short-lived lymphoid-primed progenitors (Lin⁻Sca-1^{hi}c-Kit^{hi}CD34⁺Flt3⁺)⁹⁻¹².

Restricted erythro-myeloid progenitors are present within the more abundant Lin⁻Sca-1⁻c-Kit^{hi} (LK) population (0.6–1% of the BM) that can be further subdivided into a common myeloid progenitor (CMP, CD34⁺FcγR^{lo}) and its more restricted progeny of megakaryo-erythrocyte (MEP, CD34⁺FcγR^{lo}) and granulo-monocyte (GMP, CD34⁺FcγR^{hi}) progenitors. A restricted common lymphoid progenitor (CLP) capable of B, T and natural killer (NK)

cell differentiation was purified as a Lin⁻Sca-1^{lo}c-kit^{lo} interleukin receptor 7 α (IL-7R α ⁺) population⁴. Its potential predecessor, the early lymphoid progenitor (ELP), was revealed in low numbers within the LSK compartment by a reporter under the control of the *Rag1* locus that is expressed early in lymphocyte development¹³. Other studies on T cell ontogeny revealed a progenitor with a predominant T cell potential, that unlike the CLP, is readily detected in the circulation, thus challenging the CLP as the root of all lymphoid lineages^{14, 15}. In addition, subdivision of HSC according to Flt3 expression has revealed the existence of lympho-myeloid progenitors (LMPP) with the ability to generate lymphoid and myeloid but not erythroid progeny and cast doubt on the strict separation of the erythro-myeloid from the lymphoid lineages at an earlier step of the pathway¹⁶. However, the physiological contributions of these alternative pathways and their underlying genetic controls remain elusive.

Nuclear regulators expressed in early progenitors control cell fate decisions in development presumably by modulating expression of lineage-specific genes either in a stochastic manner or in response to environmental cues^{17–21}. Of these, the Ikaros protein family of Krüppel-type zinc finger DNA binding factors is essential for normal lymphocyte development and homeostasis^{22–26}. Ikaros null mice lack all B, NK and fetal T cells. The defect in lymphocyte development occurs very early in hematopoietic ontogeny, possibly prior to the generation of CLPs^{14, 24}. Interestingly, and in contrast to the persistent block in B and NK cell development, a small number of early T cell progenitors are detected in the Ikaros-deficient thymi and mature T cells are exported to the periphery²⁴. Myeloid differentiation is not impaired in Ikaros null mice, as mixed-myeloid, granulocyte-macrophage (GM) and macrophage (M) progenitors and their mature progeny are present in normal to elevated numbers as compared to wild-type littermates²⁷.

HSCs in Ikaros null mice lack the Flt3 tyrosine kinase, which is normally upregulated during the transition from ST-HSCs to LMPPs^{16, 27}. Flt3 and its cognate ligand regulate HSCs' ability to repopulate bone marrow under competitive repopulation conditions as well as their ability to differentiate into lymphoid progenitors, properties that are both impaired in the absence of Ikaros^{27–29}. Thus, Ikaros, which is normally present in hematopoietic progenitors, can regulate their developmental restriction possibly by modulating expression of lineage regulators like Flt3. Ikaros and its family members are thought to regulate expression of lineage-specific genes by targeting chromatin remodeling activities in their vicinity. This is in part supported by the stable association of Ikaros proteins with components of the nucleosome remodeling and deacetylation (NuRD) and of the SWI/SNF chromatin remodeling complex and in part by their association with the local chromatin of lineage-specific genes^{30–32}.

Hematopoietic cell expression of *Zfp141*, which encodes Ikaros, is controlled by a complex network of regulatory regions that is composed of elements with both common and distinct activities in the lymphoid, myeloid and erythroid lineages. The minimal combination of a promoter and its downstream intronic region predominantly active in B cells together with one of the Ikaros enhancers can reliably recapitulate Ikaros expression in differentiating and mature lymphoid and myeloid cells³³.

Further investigation of the activity of the Ikaros expression cassette in the HSC and progenitor compartment provided us with a reliable separation of HSC and lympho-myeloid progenitor populations in the absence of their defining markers. This allowed us to evaluate the effects of the Ikaros mutation in the early hematopoietic hierarchy revealing new and unexpected roles for this factor in this developmental process.

RESULTS

Subdivision of LSK progenitors using an Ikaros-based reporter

Given that Ikaros contributes to cell fate decisions during early hematopoiesis we sought to determine the regulatory elements responsible for its expression in HSCs and their immediate progeny. We have previously described a transgenic mouse strain that expresses the green fluorescent protein (GFP) under the control of an Ikaros promoter-enhancer along the lymphoid and myeloid pathways³³. Using these mice, we further investigated the activity of these Ikaros regulatory elements in HSC- and progenitor-enriched BM populations.

The LSK population containing LT- and ST-HSCs exhibited a bimodal distribution of Ikaros-GFP (-GFP) expression (Fig. 1a). A statistical measure of reporter distribution within the LSKs was obtained by analyzing a cohort of mice (Supplementary Fig. 1 online). Two thirds of LSKs were -GFP^{neg-lo} and the remaining third was -GFP⁺. An examination of endogenous Ikaros isoform expression in the LSK subsets revealed a similar trend of upregulation from -GFP^{neg-lo} to -GFP⁺ cells (Fig. 1b).

To determine whether the LSK subsets were functionally different, we first analyzed them for expression of lineage-specific genes that are promiscuously expressed in HSCs and MPPs^{13, 34, 35} (Fig. 1c). The LSK -GFP^{neg-lo} population had low or no expression of the early myeloid lineage-promoting factors *PU.1*, *Gfi-1*, *Csf2ra*, *Csf3r*, *Cebpa*, *Mpo* and *Csf1r*. Higher expression of the erythroid lineage-promoting factors *Gata1*, *Gata2*, *Nfe2* and *Mpl* was observed in the LSK -GFP^{neg-lo} population, but *Epor* (erythropoietin receptor) and *Hbb-b1* (beta-major globin), which are present in more differentiated erythroid cell types, were not detected (Fig. 1c). The LSK -GFP⁺ population exhibited a reciprocal pattern of gene expression with significant upregulation of myeloid-specific and downregulation of erythroid-specific genes (Fig. 1c). Expression of the lymphoid-lineage promoting factor *Flt3* was highest in the LSK -GFP⁺ subset that also expressed the early lymphoid-specific genes *Rag1*, *Zfpn1a3* and *Il7r* but not *Pax5* or *Ebfl* (Fig. 1c and Supplementary Fig. 2b online). In contrast, the LSK GFP^{neg-lo} subset expressed lower levels of Flt3 and had no detectable expression of lymphoid genes (Fig. 1c). The gene expression profiles of these two subsets indicates that the LSK -GFP^{neg-lo} subset is more primitive in nature compared to the LSK -GFP⁺, which has upregulated lymphoid- and myeloid-specific genes and downregulated HSC-specific genes promoting erythroid differentiation.

To determine whether these differences in gene expression also translated into functional distinctions, we evaluated the LSK -GFP subsets for their differentiation potential. Low numbers of LSK cells were cultured under mixed cytokine conditions—stem cell factor (SCF), IL-3, IL-6, IL-11, erythropoietin (Epo), thrombopoietin (Tpo), and granulocyte-macrophage colony stimulating factor (GM-CSF)—that promote differentiation into multiple myeloid and erythroid lineages. Both the LSK GFP^{neg-lo} and LSK -GFP⁺ subsets produced colonies with a remarkably high proliferative potential as has been previously reported for HSCs and MPPs (Fig. 1d-e)^{36, 37}. The majority (66%) of the LSK -GFP^{neg-lo} colonies were of mixed-myeloid origin that included erythrocytes and megakaryocytes (Fig. 1d, e left panels). Microscopic examination of mixed colonies derived from single LSK -GFP^{neg-lo} cells revealed the presence of both GFP⁻ and GFP⁺ clusters of cells with erythroid and myeloid lineage morphology, respectively (Fig. 1e, upper left). The identity of these cells was further verified by May-Giemsa staining (Fig. 1e, upper right). In sharp contrast to the LSK -GFP^{neg-lo}, the majority of the LSK -GFP⁺ colonies were composed of GFP⁺ granulocytes and macrophages (GM and M), and very few (4%) were of mixed-myeloid lineage (Fig. 1d, e lower panels). The LSK -GFP subsets were also tested in cultures supplemented only with Epo, which solely promotes differentiation of committed

erythroid progenitors. No substantial CFU-E activity was detected in either subset (Fig. 1d) consistent with the primitive nature of the LSK -GFP^{neg-lo} population, the loss of erythroid potential in the LSK -GFP⁺ (Fig. 1d) and the lack of *Epor* expression in both LSK subsets (Fig. 1c). These studies indicate that the Ikaros-GFP reporter effectively separates the HSC compartment into multipotent progenitors with myelo-erythroid potential and more committed progenitors with myeloid but not erythroid potential.

We also tested the lymphoid potential of the LSK subsets under conditions that support B or T cell development³⁸. T cell development can be recapitulated *in vitro* from a number of progenitor types co-cultured with OP9 stromal cells expressing the Notch ligand Delta-like 1 (OP9-DL1)³⁸. Under these conditions, both the LSK -GFP^{neg-lo} and LSK -GFP⁺ subpopulations displayed robust B and T cell differentiation *in vitro*, in which the kinetics of lymphocyte specification indicated a progenitor-progeny relationship (Fig. 1f and Supplementary Fig. 3 online). By day 20 of OP9-DL1 culture, both LSK -GFP^{neg-lo} and -GFP⁺ had progressed to the double negative 4 (DN4) stage but higher DN2 to DN3 and DN3 to DN4 ratios for -GFP^{neg-lo} compared to -GFP⁺ indicated that the latter population was developmentally more advanced (Fig. 1f middle). Double positive (DP) thymocytes were also readily seen in the LSK -GFP⁺ but not in the -GFP^{neg-lo} cultures (Fig. 1f bottom). A similar trend in the kinetics with which the LSK subsets progressed through B cell ontogeny was observed. While by day 8, both subpopulations gave rise to B220⁺ cells (Supplementary Fig. 3b online), by day 12 the majority of B220⁺ cells in the LSK -GFP⁺ cultures were CD19⁺ while in the -GFP^{neg-lo} cultures were CD19⁻ (Fig. 1f top). Nonetheless, by day 16, a similar ratio of B220⁺CD19⁺ to CD19⁻ B220⁺ cells was observed in both cultures (Supplementary Fig. 3b online). Thus, the two LSK -GFP subsets have both B and T cell differentiation potential with kinetics of differentiation indicating a progenitor-progeny relationship.

The developmental potential of the LSK Ikaros-GFP⁺ subset recalls LMPP progenitors that were identified within the LSK by high Flt3 expression¹⁶. The LSK -GFP subpopulations demonstrated substantial differences in Flt3 expression: LSK -GFP⁺ expressed large amounts of Flt3 whereas the LSK -GFP^{neg-lo} cells expressed much smaller amounts of Flt3 (Fig. 1g and Supplementary Fig. 4 online). Thus, high expression of the Ikaros reporter or Flt3 within the LSK compartment identifies more developmentally restricted progenitors with lymphoid and myeloid but no erythroid potential (Supplementary Fig. 5a online).

Subdivision of erythro-myeloid progenitors with the Ikaros-GFP reporter

Given the differential activity of the Ikaros-GFP reporter in the HSC compartment and its ability to identify functionally distinct progenitors, we examined its activity in more restricted erythro-myeloid progenitors. A bimodal distribution of reporter expression was also observed within the LK population, but here the -GFP^{hi} was the major, while the -GFP⁻ was the minor subset (Fig. 2a and Supplementary Fig. 1 online). In contrast to the LSK, reporter activity in the LK did not recapitulate endogenous Ikaros expression indicating that additional regulatory elements are required for proper expression in these cells (Fig. 2b).

We first examined functional differences between the LK -GFP subsets by analyzing expression of lineage differentiation markers. The LK -GFP⁻ fraction expressed genes specific for the erythro-megakaryocyte (EMk)-lineage (Fig. 2c right panel). It is noteworthy that of the EMk markers analyzed, *Gata1*, *Epor* and *Hbb-b1* were upregulated in the LK -GFP⁻ relative to the LSK -GFP^{neg-lo} population, whereas *Mpl* and *Gata2*^{34, 39}, which are expressed in LT-HSC, were downregulated (Fig. 2c and 1c). In sharp contrast genes associated with GM differentiation, were either not present or detected at very low levels (Fig. 2c). A largely reciprocal expression profile was detected in the LK -GFP^{hi} population. Genes involved in GM differentiation were greatly enriched, whereas EMk genes were

absent or severely reduced (Fig. 2c). Within the LK -GFP^{int} population, a combination of erythroid and myeloid genes was detected either reflecting an incomplete separation of erythroid from myeloid progenitors or the presence of mixed lineage progenitors in this subset (Fig. 2c). Interestingly, low amounts of *Flt3* and the early lymphoid lineage genes *Zfpn1a3* and *Il7r* were also detected in the LK -GFP^{hi} and -GFP^{int} but not in the -GFP⁻ subset (Fig. 2c). This was not due to contamination with either the LSK -GFP⁺ or CLP population (Supplementary Fig. 2 online). A Flt3⁺ subset of LK myeloid progenitors previously shown to contain *in vivo* lymphoid reconstitution activity is likely responsible for this gene expression profile⁴⁰.

We next evaluated LK -GFP subsets for erythro-myeloid differentiation potential. When cultured with Epo, a progressive increase in CFU-E and mature BFU-E (mBFU-E) activities was detected from the LK -GFP^{int} to the -GFP⁻ subset whereas the -GFP^{hi} had no detectable activity (Fig. 2d left). Under mixed-cytokine conditions (Fig. 2d right), the LK -GFP⁻ population produced mostly BFU-E, EMk, megakaryocyte (Mk) and some mast cell, but virtually no GM colonies. In contrast, the majority of colonies produced from the LK -GFP^{hi} population were M and GM. The GM colonies produced by the LK -GFP^{hi} were substantially smaller than those produced by the LSK GFP⁺ population indicating a decreased proliferative potential (Fig. 2e bottom right and Fig. 1d left). The LK -GFP^{int} population produced the greatest variety of colonies including BFU-E, EMk, Mk, M, GM and Mast cell. A small fraction of these colonies contained erythroid and myeloid cells (Mix), indicating the presence of less restricted progenitors with CMP-like multi-lineage potential. It is again noteworthy that the mixed-lineage colonies observed in the LK -GFP^{int} were much smaller than those in the LSK -GFP^{neg-lo}, indicating a limited proliferative capacity.

To provide an independent line of support for the identity of these progenitors we analyzed the LK -GFP subsets for expression of CD34 and FcγRII/III, markers that define distinct myelo-erythroid progenitors. The following pattern was revealed: LK GFP⁻ CD34⁻ FcγR^{lo}, LK GFP^{int} CD34^{int} FcγR^{int} and LK GFP^{hi} CD34⁺ FcγR^{hi} (Supplementary Fig. 4 and Supplementary Fig. 5a online). Our assignment of MEP, CMP and GMP progenitor activities to the LK -GFP⁻, -GFP^{int} and -GFP^{hi} subsets is thus consistent with their previous classification using conventional cell surface markers.

LMPPs in the Ikaros null LSK are revealed by the Ikaros-GFP reporter

While Ikaros-null mice demonstrate major defects in lymphopoiesis, efforts to study these effects in early progenitors has been hampered by the potential deregulation of markers that define these cells. Given the ability of the Ikaros reporter to identify the LMPP in a fashion similar to Flt3 (Fig. 1g), we examined its expression in the Ikaros-null LSK compartment, which lacks appreciable amounts of Flt3 and possibly LMPPs (Fig. 3a). In Ikaros-null LSKs, the Ikaros-GFP reporter was expressed in a bimodal pattern similar to that observed in wild-type LSKs (Fig. 3a and Supplementary Fig. 1 online), indicating the presence of putative LMPPs despite the lack of Flt3 expression.

We first evaluated the potential subdivision of Ikaros-null LSK by the Ikaros-GFP reporter by gene expression. In Ikaros-null as in wild-type cells, expression of GM markers was present predominantly in the LSK -GFP⁺ compartment (Fig. 3b). Of the few lymphoid differentiation markers normally expressed in LSK -GFP⁺, *Zfpn1a3* but not *Flt3*, *Il7r* or *Rag1* were present (Fig. 3b). Markers of EMk differentiation were present predominantly in the Ikaros-null LSK -GFP^{neg-lo} and not in the -GFP⁺ subset, as with their wild type counterparts (Fig. 3b and Fig. 1c). Thus, the Ikaros reporter subdivides the Ikaros-null LSK into subsets with distinct lineage-specific gene profiles that mirror their wild-type counterparts.

We next examined Ikaros-null LSK -GFP subsets for differentiation into various hematopoietic lineages. *In vitro* cultures of the LSK -GFP^{neg-lo} subset were composed of mix and GM colonies as in the wild-type (Fig. 3c, d). However, the -GFP^{neg-lo} subset yielded a substantial increase in the number of EMk, GM and M colonies relative to wild-type, while a corresponding reduction in mix colonies containing erythrocytes was seen (Fig. 3c). Similar to its wild-type counterpart, the mutant LSK -GFP⁺ compartment was predominantly composed of GM progenitors; however, their colony size was smaller indicating a reduced proliferative potential (Fig. 3d bottom left).

To extend insight into the cell-type composition of the Ikaros-null mixed cytokine cultures, we analyzed them for expression of lineage-specific surface markers. In both the Ikaros-null and wild-type LSK -GFP⁺ cultures, the majority of cells were myeloid in origin. However, while in wild-type cultures the majority of these cells were GFP^{hi} in Ikaros-null they were GFP^{int}, potentially indicating a difference in their developmental state. Ikaros-null and wild-type LSK -GFP^{neg-lo} cultures yielded cells (62–79%) that were of myeloid (Mac-1⁺) origin with intermediate to high GFP expression, acquired during *in vitro* differentiation (Fig. 3e). A higher expression of the Mk-lineage marker CD41 was observed in Ikaros-null LSK -GFP^{neg-lo} cultures (Fig. 3e) consistent with the increase in EMk and Mk colonies in this population (Fig 3c).

We next examined the lymphoid differentiation potential of the Ikaros-null LSK subsets. When Ikaros-null LSK subsets were co-cultured with OP9-GFP cells they failed to undergo B cell differentiation (data not shown). Ikaros-null LSK -GFP⁺ cells exhibited limited expansion under T cell differentiation conditions, and their kinetics of differentiation were also delayed when compared to wild-type counterparts. T cell-restricted precursors at the DN2-DN3 stage were apparent by day 20 in the mutant as opposed to day 12–14 in the wild-type cultures (Supplementary Fig. 3a, c online). The subsequent transition of Ikaros-null DN precursors to the DP stage of differentiation was apparent by day 28 as opposed to day 16 in wild-type cultures (Fig. 3f and Supplementary Fig. 3a, c). Limiting dilution analysis of the Ikaros-null LSK -GFP⁺ subset revealed a greater than 20-fold reduction in T cell progenitor activity relative to the wild-type cells (Supplementary Table 1, online).

Thus, analysis of the Ikaros-null LSKs using the Ikaros GFP reporter indicates that LMPPs are generated in the absence of Ikaros and Flt3. These mutant LMPPs, which in the wild-type represent lymphoid-primed progenitors¹⁶, can differentiate normally along myeloid but not lymphoid pathways (Supplementary Fig. 5a online).

Ikaros as a pivotal factor for lymphoid vs. myeloid differentiation at the LMPP

Having identified an LMPP in Ikaros-null BM, we sought to determine whether the output of lymphoid and myeloid progenitors was altered at this stage in development. Thus, we examined the effects of Ikaros deficiency at the single LMPP cell level. The combined lympho-myeloid potential of single LSK -GFP⁺ progenitors from wild-type and Ikaros-null BM was tested after limited *in vitro* expansion. The majority of wild-type LSK -GFP⁺ progenitors (53%), grown initially in the presence of SCF, Flt3L, and IL-7 (Fig. 4a), gave rise to colonies with combined lympho-myeloid potential. Colonies with only lymphoid (27%) or myeloid (20%) potential were also present. When the initial expansion of LSK -GFP⁺ progenitors was performed in the absence of IL-7 (Fig. 4a) most (71%) clones had only myeloid potential. Colonies with combined lympho-myeloid potential were still detected but their number was greatly reduced (29%). Under these conditions, colonies with only lymphoid potential were not obtained.

The Ikaros-null LSK -GFP⁺ progenitors gave a similar number of clonal expansions as in the wild type (Fig. 4a), with, however, very distinct differentiation outcome. Whether grown

in the presence or absence of IL7, the Ikaros mutant progenitors gave rise to mostly myeloid colonies (Fig. 4a). Only a very small fraction of these colonies (4%) retained lympho-myeloid potential under either condition. Furthermore, the Ikaros-null LSK -GFP⁺ lympho-myeloid clones had only T and no B cell potential. A substantial decrease in colonies with T cell potential was detected from the Ikaros-null LMPPs whereas the number of cultures with myeloid potential was greatly increased compared to their wild-type counterparts (Fig. 4b).

Taken together these single-cell assays reveal a change in the differentiation potential of the LMPP in the absence of Ikaros. The LMPP appears to be fairly plastic and can be polarized to differentiate along the lymphoid or myeloid pathways depending on cytokine context. In the absence of Ikaros, the LMPP is stripped of its B cell potential, its T cell potential is severely reduced but its myeloid potential remains intact.

The common myeloid progenitor requires Ikaros for proper myeloid differentiation

Given Ikaros' role in LMPP differentiation we examined its potential effects on the CMP and its progeny. Analysis of the Ikaros-null LK compartment with CD34 and FcγRII/III markers revealed an unexpected change in progenitor distribution. The MEP population was increased whereas the GMP was decreased (Fig. 5a). This data is in contrast to previous²⁷ and current examinations of unfractionated Ikaros-null bone marrow (Fig. 5e) that indicated a normal to elevated number of multi-lineage, GM and Mk progenitors and a decrease in early erythroid (BFU-E and to a smaller degree CFU-E) progenitors.

Given the possibility for Ikaros-mediated deregulation of the CD34 and FcγRII/III markers, we tested Ikaros-GFP expression in the LK compartment. In the mutant LK, and in contrast to LSK, the Ikaros reporter distribution was drastically altered. The LK -GFP^{hi} subset that predominates in wild-type was greatly reduced, whereas the LK -GFP⁻ population was increased (Fig. 5b and Supplementary Fig. 1 online).

To determine whether the Ikaros-GFP reporter subdivided Ikaros-null and wild-type LKs in a similar fashion, these subsets were tested for expression of lineage-specific markers. In Ikaros-null as in wild-type progenitors, expression of EMk markers was detected in the -GFP⁻ but not in the diminished -GFP^{hi} population and expression of GM markers displayed a reciprocal pattern (Fig. 5c). The LK -GFP^{int} subset expressed markers of both erythroid and myeloid differentiation with the exception of *Cebp1a*, which was greatly reduced (Fig. 5c). Thus gene expression profiles from Ikaros-null LK -GFP subsets are similar to wild-type counterparts.

To account for changes in the Ikaros null LK progenitors, we examined their differentiation properties. As with their wild-type counterparts (Fig. 2d) and predicted by their gene expression profile (Fig. 5c), the Ikaros null LK -GFP⁻ contained BFU-E, Mk and EMk progenitors while the Ikaros-null (and diminished) LK -GFP^{hi} contained GM progenitors (Fig. 5d, f). Multi-lineage colonies were detected in the Ikaros-null LK -GFP^{int} as well as colonies of more restricted potential. Here an unexpected increase in Mk differentiation was detected in both mixed-lineage as well as in the more developmentally restricted Mk and EMk colonies (also seen in the LK -GFP⁻ subset). Further analysis of these colonies revealed an increase in the Mk-specific marker CD41 consistent with the increase in Mk differentiation (Fig. 5f). The large decrease in Mac-1⁺ cells also indicated a major defect in myeloid differentiation of the CMP present in this compartment (Fig. 5f, d). Thus the Ikaros reporter can reliably subdivide the Ikaros-null LK into CMP, MEP and GMP containing subsets, revealing a significant decrease in GMP production from the CMP.

These studies indicate that Ikaros is not only required at the LMPP for lymphoid differentiation but it also promotes cell fate decisions at several branchpoints of the myelo-

erythroid pathway (Supplementary Fig. 5 online). At the CMP it directs myeloid differentiation and further downstream at the more restricted MEP erythroid differentiation.

DISCUSSION

Here we address the role of Ikaros in early hematopoietic lineage restrictions. An Ikaros-based reporter that distinguishes early hematopoietic progenitors independent of their characteristic cell surface markings was used to analyze Ikaros-null progenitors. These studies revealed that although Ikaros is not required for an initial segregation of erythro-myeloid and lympho-myeloid progenitors, it is required for their subsequent choice of cell fate. Finally, its depletion in these two cell types causes severe defects along the lymphoid and erythroid lineages respectively.

A GFP reporter driven by Ikaros regulatory elements displayed a bimodal distribution within the hematopoietic stem cell (LSK) compartment that identified two major progenitor populations both with high proliferative potential but distinct differentiation properties. The -GFP^{neg-lo} progenitors were predominantly multipotent with erythro-myeloid and lymphoid potential, while the -GFP⁺ subset was more lineage-restricted with progenitors of lympho-myeloid but no erythroid potential. The gene expression profiles of these subsets also correlated with a characterization based on differentiation properties. A similar separation of LMPPs from the LSK compartment was achieved on the basis of Flt3 expression, which correlated with Ikaros reporter activity and expression of the endogenous Ikaros locus.

Flt3 expression, one defining characteristic of LMPPs, is actually dependent on Ikaros activity in these cells. The LSK population in Ikaros-null mice lacks Flt3. While this could imply absence of the LMPP subset, use of the Ikaros reporter revealed the presence of mutant LMPPs that were generated in the absence of Flt3 activity, and that would otherwise be undetectable. In the absence of Flt3, Ikaros-null LMPPs are unable to undergo normal lymphoid differentiation. In culture systems that promote lymphocyte development, they failed to generate B cells but generated T cells, albeit at a frequency that was reduced by at least one order of magnitude and with delayed kinetics. The Ikaros-null LMPP readily underwent myeloid differentiation even under conditions that normally promote lymphoid development. These conditions involve IL-7R signaling, and the inability of mutant LMPPs to express or induce IL-7R α is a likely cause for this defect. Given that Ikaros-null mice have T cell but no B cell precursors and that they lack CLPs, it is likely that LMPPs are the source of T cell differentiation in these mice. It is also possible that the LMPP gives rise to an impaired CLP (and CLP-2⁴¹) that lacks detectable IL-7R α , a central CLP defining characteristic. A requirement for Ikaros in B cell differentiation also precludes identification of this cell type by functional criteria. Further studies into Ikaros-null progenitor populations utilizing the Ikaros reporter for their dissection will shed more insight into the LMPP and CLP connection.

The Ikaros-GFP reporter expression was also used to analyze the more restricted erythro-myeloid progenitors. A bimodal reporter distribution was again apparent in the wild type population with high expression (LK -GFP^{hi}) revealing myeloid-restricted (GMP) progenitors and lack of expression (LK -GFP⁻) revealing progenitors with erythroid and megakaryocyte (MEP) potential. An intermediate population (LK -GFP^{int}) contained more primitive progenitors with mixed-myeloid and -erythroid activities similar to the CMP. Both the LK mixed-myeloid and GMP-restricted progenitors were of significantly lower proliferative potential compared to progenitors with similar differentiation properties in the LSK compartment. Gene expression analysis of the three LK -GFP subsets provided independent support for their state of differentiation. Analysis of the LK -GFP subsets with CD34 and Fc γ RII/III, previously used to identify distinct populations of erythro-myeloid

progenitors^{5, 42}, revealed an expression pattern that is consistent with their differentiation potential, indicating that the Ikaros reporter can effectively substitute for expression of these markers. Importantly, separation of progenitors with the Ikaros reporter was also achieved on genetic backgrounds that affect expression of these markers.

Unexpectedly, an examination of Ikaros-null erythro-myeloid progenitors revealed a dramatic depletion of GMPs. In the Ikaros mutants, the observed reduction of GMPs derived from CMPs despite the presence of normal myeloid progenitor activity in the whole BM was inconsistent with the classical CLP vs. CMP model of the hematopoietic hierarchy. The apparent absence of an LMPP in Ikaros-null based on *Flt3* expression made this observation difficult to reconcile with the more recent LMPP vs. MEP model of hematopoiesis as well¹⁶. However, the identification of an Ikaros-null LMPP devoid of *Flt3* expression and with increased ability to generate GMPs but not pro-B cells and possibly not CLPs, explains the normal complement of GMPs in the mutant BM. In contrast to the Ikaros-null LMPP, the Ikaros-null CMP produced fewer GMPs and instead generated increased numbers MEPs. Thus, our studies based on two independent genetic approaches centered on Ikaros support a third model by which a major bifurcation downstream of the ST-HSC gives rise to either an erythro-myeloid (CMP) or a lympho-myeloid (LMPP) progenitor as gateways into two independent myeloid pathways as depicted in Supplementary Figure 5. Although we do not address which of the two myeloid progenitors (LMPPs or CMPs) is the major contributor to the myeloid lineage in the presence of Ikaros we demonstrate for the first time that the LMPP is able to generate physiological levels of myeloid cells in an intact animal model in the absence of Ikaros. These studies also reveal that within the EMk pathway, Ikaros is required for restriction of MEPs into the erythroid lineage. Ikaros deficiency results in a large expansion of Mk at the possible expense of erythroid progenitors. This is consistent with the reported increase in mature Mks in the periphery of Ikaros mutant mice^{23, 43}.

The contrasting effects of Ikaros on fate choices in distinct progenitor populations (e.g. LMPP and CMP) reveals the complexity of its function and underscores the necessity for evaluating gene expression in highly defined populations. With the ability to isolate these distinct progenitor populations provided by the Ikaros-GFP reporter it is now possible to identify the genes regulated by Ikaros in LMPP, CMP and MEP progenitors to further dissect the genetic controls of lineage choice. Already the demonstrated deregulation of lymphoid promoting factors like *Flt3* and *Il7r* (as well as other early lymphoid-specific genes like *Rag1*) present excellent candidates to mediate Ikaros' ability to promote the lymphoid fate at the LMPP. Positive regulation of myeloid promoting factors like *Ceb1a* and or negative regulation of erythroid and Mk promoting factors may be responsible for the altered myelo-erythroid and megakaryo-erythroid fate choices at the CMP and MEP stages respectively. The use of these Ikaros-based reporters to dissect the distinct roles of the Ikaros gene products in different cell types open many new avenues for future investigation and are rapidly filling in the "Terra Incognita" on the map of hematopoietic differentiation that is the genetic hierarchy of early lineage decisions.

METHODS

Mice

Transgenic (B-p-GFP-C line, C57BL/6 × C3H)³³ and Ikaros null-mice (I74 line, C57BL/6 × 129SV)²⁴ were bred and maintained under pathogen free conditions in the animal facility at Massachusetts General Hospital, Bldg. 149. Mice were 4–12 weeks of age at the time of analysis. All animal experiments were done according to protocols approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital (Charlestown, MA) and in accordance with the guidelines set forth by the National Institutes of Health.

Antibodies

All antibodies were purchased from BD PharMingen or CALTAG. Antibodies and the specific clones used were: CD3 (17A2), CD4 (L3T4), CD5 (53-7.3), CD8 α (53-6.7), CD8 β (H35-17.2), CD19 (1D3), CD25 (PC61), CD44 (IM7), TCR β —(H57—597),— TCR $\gamma\delta$ —(GL3),—Flt3 (A2F10.1), c-Kit (2B8), IL-7R α —(A7R34), Sca-1 (D7 or E13-161.7), Mac-1 (M1/70), Thy1.2 (53-2.1), B220 (RA3-6B2), DX5, Gr-1 (RB6-8C5), CD34 (RAM34), Fc γ RII/III (2.4G2), Ter119, NK1.1 (PK136) and 7/4.

Flow cytometric analysis and cell sorting

BM cells were isolated and immunolabeled as previously described²⁷. Briefly, BM cells were harvested from femurs and tibias and subjected to red blood cell (RBC) lysis using ACK buffer (0.15 M Ammonium Chloride, 10 mM Potassium Bicarbonate, 0.1 mM EDTA). Lineage positive cells were subsequently labeled with antibodies against the lineage markers TER119, B220, Mac-1, Gr-1, 7/4, CD3, CD5, CD8 α , CD8 β , CD19, TC β , TCR $\gamma\delta$, and DX5 and were removed with magnetic beads conjugated to sheep anti-rat IgG (Dyna). The remaining cells were labeled with R-phycoerythrin-Cy5.5 conjugated (PE-Cy5.5)-anti-rat IgG to label any remaining lineage positive cells. Cells were then labeled with allophycocyanin conjugated (APC)-c-Kit and R-phycoerythrin conjugated (PE)-Sca-1 prior to FACS analysis and cell sorting. For additional Flt3 visualization, lineage depleted cells were labeled with biotin-conjugated anti-rat IgG or the magnetic depletion was performed using biotin-conjugated lineage antibodies followed by staining with allophycocyanin-Cy7 conjugated (APC-Cy7)-streptavidin. The lineage negative cells were then labeled with the antibodies APC-c-Kit, PE-Cy5.5-Sca-1 and PE-Flt3 prior to FACS analysis. For CMP, GMP and MEP staining, BM cells were harvested and magnetically depleted as described above using lineage antibodies against Ter119, B220, CD19, IgM, Gr-1, 7/4, CD3, CD4, CD8 α , TCR $\gamma\delta$, and NK1.1. Lineage negative cells were analyzed with APC-c-Kit, PE-Cy5.5-Sca-1, PE-Fc γ RII/III and Fluorescein isothiocyanate (FITC)-CD34. CLPs were isolated as previously described⁴.

Flow cytometric analysis was performed using a two-laser FACSCanto™ (Becton Dickinson), a two-laser FACSCalibur™ (Becton Dickinson) or a three-laser MoFlo® (Cytomation). Cell sorting was performed using a three-laser MoFlo®. The resulting files were uploaded to FlowJo (Tree Star) for further analysis.

RT-PCR assays

Total RNA was prepared from approximately 1000–20000 sorted cells using TriZol reagent (Invitrogen). cDNA was synthesized using the SuperScript™ II First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA product was quantified with real-time PCR using SYBR® Green PCR Master Mix (Applied Biosystems) with *Actb* primer pairs. For subsequent PCR reactions, the amount of cDNA from different populations was normalized based on *Actb* signals. To label reaction products, [α -³²P] dCTP was added into each PCR reaction. Reaction products were visualized with a Phosphorimager screen (Molecular Dynamics) or with Biomax MS film (Kodak). Each reaction contained cDNA from approximately 10–20 cells. Five-fold more template was used in reactions performed to detect lymphoid-specific genes. The primer sequences and expected product sizes are found in Supplementary Table 2 online.

In vitro methylcellulose colony forming cell assay

For the CFU-E and mature BFU-E assay, 500 cells sorted from the LK GFP subsets, 200 cells from the LSK GFP subsets or 5×10^4 cells from whole bone marrow were cultured in Methocult M3334 (StemCell Technologies). Colonies were scored after 2–4 days of culture

according to the criteria described in the technical manual provided with the assay media (StemCell Technologies). For the multi-colony assay, 250 cells from the LK subsets, 100 cells from the sorted LSK subsets or 5×10^4 cells from whole bone marrow, were cultured in Methocult M3434 (StemCell Technologies) supplemented with recombinant human TPO (50 ng/ml), recombinant human IL-11 (50 ng/ml) and recombinant murine GM-CSF (5 ng/ml). Colonies were scored from days 5–17 as previously described³⁷. The identity of colonies was confirmed by staining individually picked colonies with May-Giemsa (Harleco) followed by examination using a Axiovert 200M Inverted Microscope (Zeiss). Cytospins were performed in a Cytospin 4 (ThermoShandon) at 500–1000g for 4–5min. Cytokines were purchased from StemCell Technologies and R&D systems.

OP9 and OP9-DL1 cultures

OP9-GFP and OP9-DL1 cells were maintained and co-cultured as previously described³⁸. Briefly, 100–500 cells from sorted wild-type and Ikaros-null LSK subpopulations were cultured for 8–28 days on OP9-GFP and OP9-DL1 in the presence of recombinant murine Flt3L and recombinant murine IL-7. The extent of B or T cell differentiation at each time point was determined by antibody labeling of B or T cell specific markers followed by analysis with a FACSCaliburTM (BD) after removal of the stromal OP9-GFP or OP9-DL1 cells. Residual stromal cells were excluded from analysis by electronic gating based on size and granularity. Cytokines were purchased from R&D Systems.

Single cell cultures

Single cells from the LSK GFP⁺ population from wild-type and Ikaros null mice were sorted directly into Terasaki plates containing IMDM, 20% FCS(HyClone), 1% BSA (detoxified from StemCell Technologies) and supplemented with SCF, Flt3L and IL-7. Deposition of single cells was confirmed microscopically and divisions were followed for 5–6 days. At day 6, wells with more than 20 cells were split into three cultures as follows: (a) ST2 stromal cells supplemented with SCF, Flt3L, IL-7, IL-3, G-CSF, GM-CSF and IL-6 (b) IMDM, 20% FCS, 1% BSA, SCF, Flt3L, IL-6, IL-3, GM-CSF and G-CSF (c) OP9-DL1 with Flt3 ligand and IL-7. After 1–2 weeks of culture, cells were harvested and stained for myeloid and lymphoid differentiation markers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Molecular and functional subdivision of the HSC compartment using the Ikaros-GFP reporter. **(a)** Analysis of BM HSCs for Ikaros-GFP expression. Lineage-negative BM cells were examined for expression of Sca-1, c-Kit (LSK-left plot) and Ikaros-GFP (right histogram). The percentage of the LSKs is indicated (left) as well as the percentage of LSK progenitors falling within the two -GFP^{neg-lo} and -GFP⁺ gates (right). Sorted LSK -GFP^{neg-lo} and -GFP⁺ subsets were used for subsequent studies. **(b)** Semi-quantitative RT-PCR analysis of endogenous *Zfpn1a1* (*Ikaros*) expression with primers spanning exons 3–8. Ikaros isoforms are indicated on the right side of the panel. Analysis is representative of two independent studies. **(c)** Semi-quantitative RT-PCR analysis for lineage-specific genes in LSK -GFP subsets. C, normalization and identity controls; GM, granulocyte-macrophage; EMk, erythro-megakaryocyte; Ly, lymphoid. Data is representative of at least four independent studies. **(d)** Myelo-erythroid differentiation potential of 100 sorted cells from LSK -GFP subpopulations revealed by colony assays performed in the presence of Epo or mixed cytokines (Mix). The percentage of colonies obtained from each subset is shown. Colonies were scored from day 2–4 for the Epo cultures, and from day 5–17 for the Mix cultures. CFU-E, colony-forming unit erythroid; BFU-E, burst forming unit erythroid; EMk,

erythroid-megakaryocyte; Mk, megakaryocyte; G, granulocyte; GM, granulocyte-macrophage; M, macrophage; Mc, mast cells; Mix, mixed colonies contain more than three lineages usually G, M, E and Mk. The colony assays shown are from one representative out of more than five independent experiments with similar results. **(e)** Microscopic examination of LSK -GFP colonies. A 50x magnification of a representative mix-lineage colony from the LSK -GFP^{neg-lo} cultures (upper left) and a GM colony from the LSK -GFP⁺ cultures (upper right) visualized by fluorescence microscopy. A 600x magnified image of a mix-colony from the LSK -GFP^{neg-lo} culture (lower left) and a GM colony from the LSK -GFP⁺ culture (lower right) stained by May-Giemsa and visualized by bright field microscopy. **(f)** Lymphoid activities of LSK -GFP^{neg-lo} and -GFP⁺ subsets. 200–500 sorted cells were co-cultured on OP9-GFP (top) and OP9-DL1 (middle and bottom) stromal cells. Differentiating cells were analyzed by expression of lymphoid markers and their percentages are shown in each quadrant. One representative out of three independent studies is shown. **(g)** Flt3 expression in LSK -GFP subsets. The shaded and open histograms represent cells stained by Flt3 and isotype control antibodies respectively. Data shown is representative of two independent studies (and Supplementary Fig. 4c online).

Figure 2.

Molecular and functional subdivision of erythro-myeloid progenitors by the Ikaros-GFP reporter. **(a)** Analysis of Ikaros-GFP expression in erythro-myeloid progenitors. Lineage-depleted BM cells were first analyzed for expression of c-Kit and lack of Sca-1 expression (LK-left) and then for Ikaros-GFP activity (right). Three gates were set on the LK Ikaros-GFP histogram, -GFP⁻, -GFP^{int}, -GFP^{hi}, and the number of cells falling into each gate is shown (and Supplementary Fig. 1). The LK -GFP⁻, -GFP^{int}, and -GFP^{hi} subsets were sorted and used in subsequent studies. **(b)** Semi-quantitative RT-PCR analysis of endogenous *Zfpn1a1* (*Ikaros*) expression as shown in Fig. 1b. **(c)** Semi-quantitative RT-PCR analysis for expression of lineage-specific genes in the LK -GFP⁻, -GFP^{int} and -GFP^{hi} subsets as shown in Fig. 1c. **(d)** The erythro-myeloid differentiation potential of 250 cells sorted from the LK -GFP subsets was determined under Epo and mixed cytokine (Mix) cultures as described in Fig. 1d. The colony nomenclature used is described in Fig. 1d except for m.BFU-E which represents mature BFU-E. Data shown is representative of at least two independent studies performed with LK -GFP subsets. **(e)** Microscopic examination of LK -GFP colonies. A 600x magnified image of an EMk colony from an LK -GFP⁻ culture (**upper left**), a Mix colony from an LK -GFP^{int} culture (**upper right**) and an M colony from an LK -GFP^{hi} culture (**lower left**) all stained by May-Giemsa and visualized by bright field microscopy. A 50x magnified image of an M colony from an LK -GFP^{hi} culture (**lower right**) visualized by fluorescence microscopy.

Figure 3.

Molecular and functional subdivision of the Ikaros-null LSK with the Ikaros-GFP reporter. (a) Analysis of Ikaros-null BM LSKs (left) for Ikaros-GFP expression (middle) as described in Fig. 1a (Supplementary Fig. 1, online). Expression of Flt3 in wild-type (blue line) and Ikaros-null (red line) LSKs is shown (right) together with an isotype control for staining (dotted line). Flt3 expression is representative of at least two independent experiments. The Ikaros-null LSK -GFP^{neg-lo} and -GFP^{hi} subsets were sorted and used for subsequent studies. (b) Semi-quantitative RT-PCR analysis of lineage-specific genes in Ikaros null LSK -GFP^{neg-lo} and -GFP⁺ subsets (as described in Fig. 1c), is representative of at least two independent experiments. (c) Myeloid differentiation potential of 100 LSK -GFP^{neg-lo} and

-GFP⁺ sorted cells from wild-type (WT) and Ikaros-null (Null) mice cultured under mixed cytokine (Mix) conditions. The percentage of cells yielding colonies and the type of colony are presented. Colonies were scored from day 5–17 as described in Fig. 1d. Data is representative of three independent experiments. **(d)** Microscopic examination of Ikaros-null LSK colonies. A 50x magnification of a representative mix-lineage colony (upper left) from the Ikaros-null LSK -GFP^{neg-lo} exhibiting clusters of GFP⁻ (erythroid) and GFP⁺ (myeloid) cells, and GM colonies (lower left) from the Ikaros-null LSK -GFP⁺ cultures. A 600x magnification of these colonies stained by May-Giemsa is shown under bright field (upper and lower right). **(e)** FACS analysis of mix colonies from wild-type (WT) and Ikaros-null (Null) LSK -GFP^{neg-lo} and -GFP⁺ populations at day 12 of culture. Cells were harvested and stained for Mac-1 (left) revealing myeloid and for CD41 (right) revealing Mk differentiation. Data is representative of two independent studies. **(f)** T lymphoid activity of the Ikaros-null LSK -GFP⁺ subset. 250–500 cells were sorted and co-cultured on OP9-DL1 stromal cells. Cells were labeled with antibodies against CD44 and CD25 or CD4 and CD8 after 28 days of co-culture and subjected to FACS analysis. The percentage of cells going through specific stages of T cell differentiation is indicated in each quadrant. Data is representative of two independent studies.

Figure 4.

Clonal analysis of wild-type and Ikaros-null LMPPs. **(a)** Single wild-type and Ikaros-null LMPPs were initially expanded (6 days) in the presence of SCF, Flt3L and IL-7 (+IL-7) or SCF and Flt3L (-IL-7). A fraction of the expanding clones (18 wild type and 32 mutants) were subsequently examined for their potential under culture conditions that favor B-myeloid and T cell differentiation. The differentiation outcome in the secondary cultures was determined by expression of lymphoid and myeloid surface markers (i.e. B220, CD19 and Mac-1 or Thy-1.2, CD44 and CD25) and by morphology using May-Giemsa staining. The percentage of wild type and Ikaros-null clones undergoing differentiation, lympho-myeloid (MyLy: T+M or B+M or B+T+M-black), lymphoid (Ly: T or B or T+B-medium gray) or myeloid (My-light gray), is shown in a bar graph representing the total number of LMPP clones under investigation. The percent of clones that displayed no growth (NG) after secondary culture is shown in white. **(b)** A total of 216 wild-type and 800 Ikaros-null clones were initially expanded under SCF, Flt3L and IL-7 conditions in three independent experiments. From these clones, 57 wild-type and 107 Ikaros-null were subsequently split and analyzed for lymphoid (T and B) and Myeloid (My) differentiation potential. The average percentage of clones with T or My differentiation potential is shown. Note that clones with multi-lineage potential are included in this score.

Figure 5.

Molecular and functional analysis of Ikaros-null erythro-myeloid progenitors. **(a)** The CMP, GMP and MEP distribution in Ikaros-null BM LKs. Lineage negative BM cells (not transgenic for Ikaros-GFP) from wild-type and Ikaros-null mice were labeled with antibodies against c-Kit, Sca-1, CD34 and FcγRII/III and electronically gated to reveal CMP (CD34^{int}FcγRII/III^{lo}), GMP (CD34⁺FcγRII/III^{hi}), and MEP (CD34⁻FcγRII/III^{lo}) within the LK compartment. The percentage of LK progenitors inside each gate is shown. Data is representative of at least three independent experiments. **(b)** Relative Ikaros-GFP reporter expression in the wild-type and Ikaros-null LK compartment. LK progenitors from wild-type (blue line) or Ikaros-null (red line) BM, transgenic for Ikaros-GFP, were analyzed for reporter expression. The percentage of Ikaros-null LK -GFP subsets is shown. Data is representative of at least three independent experiments. **(c)** Semi-quantitative RT-PCR analysis of LK -GFP⁻, -GFP^{int} and -GFP^{hi} cells from Ikaros-null mice for lineage-specific markers. Experiments were performed as described in Fig. 1c. **(d)** Differentiation potential of Ikaros-null erythro-myeloid progenitors. 250 LK GFP⁻, GFP^{int} and GFP^{hi} cells sorted from wild-type and Ikaros-null BM were assayed under mixed cytokine (Mix) conditions (as described in Fig. 1d). One representative out of three independent experiments with similar results is shown. **(e)** Erythro-myeloid colony assays on unfractionated wild-type and Ikaros-null BM under mixed cytokine conditions. 2.5x10⁴ nucleated cells were plated and colonies were scored from days 5–17. Data is representative of at least two independent experiments. **(f)** Analysis of day 12 mix cultures with wild-type and Ikaros-null LK -GFP^{int} and -GFP^{hi}

cells for expression of lineage-specific markers. Cells were harvested and analyzed for myeloid (Mac-1-left panel) or Mk (CD41-right) markers by FACS.