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A Forward Genetic Screen Targeting the Endothelium Reveals a Regulatory Role for the Lipid Kinase Pi4ka in Myelo- and Erythropoiesis.

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Authors
Ziyad, Safiyyah
Riordan, Jesse D
Cavanaugh, Ann M
et al.

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A Forward Genetic Screen Targeting the Endothelium Reveals a Regulatory Role for the Lipid Kinase Pi4ka in Myelo- and Erythropoiesis

Graphical Abstract

Highlights
- Initiation of mutagenesis in the hemogenic endothelium yields hematopoietic malignancy
- Pi4ka is expressed in hematopoietic stem progenitor cells
- Pi4ka has a regulatory role in myelo- and erythropoiesis
- PI4KAP2 is a protein-coding negative regulator of Pi4ka signaling

Authors
Safiyyah Ziyad, Jesse D. Riordan, Ann M. Cavanaugh, ..., Jau-Nian Chen, Adam J. Dupuy, M. Luisa Iruela-Arispe

Correspondence
arispe@mcdb.ucla.edu

In Brief
Using transposon mutagenesis that targets the endothelium, Ziyad et al. identify Pi4ka as an important regulator of hematopoiesis. Loss of Pi4ka inhibits myeloid and erythroid cell differentiation. Previously considered a pseudogene in humans, PI4KAP2 is shown to be protein-coding and a negative regulator of PI4KA signaling.

Data and Software Availability
GSE108355
A Forward Genetic Screen Targeting the Endothelium Reveals a Regulatory Role for the Lipid Kinase Pi4ka in Myelo- and Erythropoiesis

Safiyyah Ziyad, Jesse D. Riordan, Ann M. Cavanaugh, Trent Su, Gloria E. Hernandez, Georg Hilfenhaus, Marco Morselli, Kristine Huynh, Kevin Wang, Jau-Nian Chen, Adam J. Dupuy, and M. Luisa Iruela-Arispe

INTRODUCTION

The hematopoietic lineage emerges during a narrow developmental window from a specialized subset of endothelial cells: the hemogenic endothelium (HemEnd) (Dzierzak and de Pater, 2016). Hematopoietic stem progenitor cells (HSPCs) enter the circulation from HemEnd sites to first seed and expand the fetal liver and later occupy the bone marrow.

The specification of the HemEnd requires Hedgehog and Etv2 (Clements and Traver, 2013; Eliades et al., 2016). In recent years, the field has come to appreciate the diversity, repopulation capacity, and plasticity of single hematopoietic stem cells (HSCs) at the HemEnd stage, meaning before their seeding in hematopoietic organs (Guibentif et al., 2017). For example, VE-Cadherin-expressing HemEnd gives rise to myeloid-erythroid biased HSPCs (Chen et al., 2011), while TGF-β and BMP signaling differentially activate myeloid versus lymphoid-biased HSCs (Challen et al., 2010; Crisan et al., 2015). It is well known that lineage-specific transcription factors that drive specification of blood cells (CEBPa, Ikaros, MLL, SCL, Etv6, etc.) can lead to leukemia when deregulated (Orkin and Zon, 2008). Similarly, genes involved in the regulatory process that control budding of HSCs from the HemEnd might also promote neoplastic transformation when disrupted.

SUMMARY

Given its role as the source of definitive hematopoietic cells, we sought to determine whether mutations initiated in the hemogenic endothelium would yield hematopoietic abnormalities or malignancies. Here, we find that endothelium-specific transposon mutagenesis in mice promotes hematopoietic pathologies that are both myeloid and lymphoid in nature. Frequently mutated genes included previously recognized cancer drivers and additional candidates, such as Pi4ka, a lipid kinase whose mutation was found to promote myeloid and erythroid dysfunction. Subsequent validation experiments showed that targeted inactivation of the Pi4ka catalytic domain or reduction in mRNA expression inhibited myeloid and erythroid cell differentiation in vitro and promoted anemia in vivo through a mechanism involving deregulation of AKT, MAPK, SRC, and JAK-STAT signaling. Finally, we provide evidence linking Pi4ka, previously considered a pseudogene, to human myeloid and erythroid leukemias.
hematopoiesis and demonstrated its link to Akt and Erk signaling, the former classically known to regulate hematopoietic differentiation. Furthermore, we identified the human PI4KA “pseudogene,” PI4KAP2, as a dominant-negative inhibitor of the PI4KA signaling pathway.

RESULTS

HemEnd Mutagenesis Promotes Hematopoietic Malignancies

We targeted mutagenesis to the endothelium using a conditional SB transposon strategy (Dupuy et al., 2009) (Figures 1A and 1B). VE-Cadherin-Cre (VEC-Cre) recombinase (Alva et al., 2006) was used to drive expression of the transposase enzyme specifically in endothelial cells, where it could cut and paste transposons randomly into TA dinucleotides distributed throughout the genome (Riordan et al., 2014). VEC-Cre is first expressed in the HemEnd by embryonic day (E) 9.5 in a salt-and-pepper manner with progressive penetration and homogeneous expression by E12.5 (Alva et al., 2006). Due to this mosaic expression pattern in the HemEnd (transient phase lasting from E10.5–E12.5) by E10.5, some cells were targeted by mutagenesis while others were not, creating a competitive mixture of mutated and non-mutated populations.

Whereas a previous SB screen targeting HSCs using Vav-Cre (Bergquam-Vrieze et al., 2011) yielded only lymphoid leukemia, the VEC-Cre screen generated both myeloid and lymphoid malignancies. A total of 76 Cre+ and 15 Cre− (non-mutagenized) mice were evaluated, with 59 Cre+ and 0 Cre− mice presenting with pathology. From this cohort, 55.3% (n = 42) developed hematopoietic abnormalities alone, 9.2% (n = 7) developed vascular anomalies, and 13.2% (n = 10) developed a combination of both (Figure 1Ci). Mice with hematopoietic abnormalities were further categorized into those with an enlarged spleen (65.4%, n = 34), an enlarged thymus (13.5%, n = 7), or both (21.2%, n = 11) (Figure 1Ci). Overall, mutagenized mice had a mean survival of 179 days (Figure 1D). An enlarged thymus (13.5%, n = 7), or both (21.2%, n = 11) (Figure 1Ci). Overall, mutagenized mice had a mean survival of 179 days (Figure 1D). Other recurrently mutated genes included Rasgrp1, Akt1, and Akt2, with insertions often occurring concurrently in the same lesion (Figures 2D and 2E). Other recurrently mutated genes included Fli1, Epo, and Runx2 (Figure 2E). Interestingly, several genes were commonly found together in a single spleen, suggestive of a cooperative function in transformation, and included: Eras, Er, and Epo; Er and Ets1; and Fli1 and Runx2 (Figure 2F). These genes have been implicated in pathological myelo-erythropoiesis and hematopoiesis in general, providing strong validation to the screen (Athanasiou et al., 2000; Huang et al., 2009; Zochodne et al., 2009). Interestingly, malignancies resulting from HemEnd-initiated mutagenesis frequently contained mutations in genes like Pi4ka, Erg, and Fli1, which are more highly expressed in HSCs compared with other hematopoietic progenitor cells (Figures 2G–2I) (Chacon et al., 2014). Most splenomegaly associated mutations were also correlated with abnormal blast-like cells in the blood and reduction in polymorphonuclear cells in the bone marrow (Figures S2A and S2B).

Spleen and Thymus Malignancies Have Distinct Mutation Signatures

The SB mutagenesis system allows precise genomic coordinates of transposon-induced mutations to be determined using linker-mediated PCR and Illumina next-generation sequencing (Brett et al., 2011). Subsequent statistical analyses identified recurrently mutated regions containing clonally expanded transposon insertions at a higher rate than would be predicted in the absence of selective pressure. Because these analyses assumed a random transposition pattern, we first confirmed the unbiased distribution of insertions across all chromosomes in affected spleen and thymus DNA samples (Figures S1A–S1C). Indeed, our data were consistent with the well-established unbiased nature of SB screens in general (Bard-Chapeau et al., 2014; Dupuy et al., 2009; Keng et al., 2009; Riordan et al., 2014). We next used gene-centric common insertion site (gCIS) analysis to identify clusters of clonally expanded insertions enriched near protein coding regions. Interestingly, the number of gCISs associated with the thymus phenotype was double that of the spleen phenotype, despite the same average number of total insertions per sample (Figures S1D and S1E), supporting the concept that the cell of origin influences gCIS mutations, which in turn influences malignancy (Bergquam-Vrieze et al., 2011).

Our screen activated transposon-mediated mutagenesis in the HemEnd and thus early definitive HSCs. A comparison of the gCIS list with recurrently mutated genes identified in blood cancers arising from global (non-tissue specific) and Vav-Cre- (HSPC)-driven mouse mutagenesis screens revealed substantial overlap, highlighting the ability of this approach to identify genes relevant to lymphoid malignancies (Figure 2A; Tables S1 and S2; Figure S1F). Of note, previous HSPC-targeting screens were not also able to generate myeloid malignancies without mutant JAK sensitization.

The most commonly mutated genes for the enlarged thymus phenotype were Rasgrp1, Akt1, and Akt2, with insertions often occurring concurrently in the same lesion (Figures 2B and 2C). Additional frequently mutated genes included Notch1 and Myc (Figure 2B). Many of the genes identified in this cohort have been previously associated with T cell malignancy (Manabe et al., 2006; Oki et al., 2011; Rasmussen et al., 2009).

Frequently mutated genes in enlarged spleens were Eras, Erg, and Ets1, which occurred in ~40% of samples (Figures 2D and 2E). Other recurrently mutated genes included Fli1, Epo, and Runx2 (Figure 2E). Interestingly, several genes were commonly found together in a single spleen, suggestive of a cooperative function in transformation, and included: Eras, Erg, and Epo; Erg and Ets1; and Fli1 and Runx2 (Figure 2F). These genes have been implicated in pathological myelo-erythropoiesis and hematopoiesis in general, providing strong validation to the screen (Athanasiou et al., 2000; Huang et al., 2009; Zochodne et al., 2009). Interestingly, malignancies resulting from HemEnd-initiated mutagenesis frequently contained mutations in genes like Pi4ka, Erg, and Fli1, which are more highly expressed in HSCs compared with other hematopoietic progenitor cells (Figures 2G–2I) (Chacon et al., 2014). Most splenomegaly associated mutations were also correlated with abnormal blast-like cells in the blood and reduction in polymorphonuclear cells in the bone marrow (Figures S2A and S2B).

Pi4ka Insertion Is Associated with Progenitor Accumulation and Reduced RBCs

We next focused on Pi4ka, which, unlike most of the genes identified by the screen, had not been previously associated with hematopoietic abnormalities or hematopoiesis in general. The three Pi4ka transposon insertions (each from a distinct individual...
Figure 1. Initiating Mutagenesis in the Hemogenic Endothelium Generates Hematopoietic Malignancies

(A and B) Onset of VE-Cadherin-Cre (VEC-Cre) expression, and therefore SB Transposase, at E9.5 in the progeny of (B) SB T2/Onc2; VEC-Cre/Rosa26-LacZ mice.

(C) Frequencies of abnormalities in these mice (i). Relative occurrence of enlarged spleens and thymus (ii).

(D) Overall survival of Cre+ and Cre− mice (number of mice in parentheses).

(E) Kaplan-Meier curve breakdown of animals with indicated maladies.

(F) Cre+ enlarged thymus (i), Cre− normal thymus (ii), Cre+ enlarged spleen and Cre− normal spleen (iii). Scale bar, 5 mm.

(G) White blood cell counts for Cre+ animals with enlarged spleens (S; n = 24), enlarged thymus (T; n = 5), or a combination of both (S+T; n = 10), Cre− littermates (n = 10).

(H) Red blood cell (RBC) concentration, hemoglobin (Hb) concentration, and mean cell volume (MCV) for Cre+ animals with an enlarged spleen (S; n = 24), thymus (T; n = 5), or both (S+T; n = 10) compared with Cre− controls (Cs; n = 10).

(I) Platelet concentrations (PLT) and platelet distribution width (PDW%) in S, T, S+T, and C animals.

(G–I) Data are represented as mean ± SEM, Student’s t test (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001) S, enlarged spleen; T, enlarged thymus; C, Cre negative; VT, vascular tumors; NOA, no obvious abnormalities; Hem, hematopoietic malignancy; HSPC, hematopoietic stem progenitor cell; Hb, hemoglobin; MCV, mean corpuscular volume; PLT, platelet count; PDW%, size distribution of platelet width.
were distributed throughout the gene in both transcriptional orientations (Figure 3A), a pattern suggestive of inactivating mutations (Copeland and Jenkins, 2010). Consistent with this hypothesis, the mutations were associated with decreased Pi4ka mRNA (Figures 3B and 3C), and immunohistochemical analyses indicated a significant decrease of Pi4ka protein compared with controls (Figure 3D). Histological evaluation of affected spleens also revealed expanded red pulp zones compared with Cre− spleens (Figure 3D). May-Grunwald stain of white blood cells from mice with Pi4ka insertions showed a prevalence of myeloid lineage, nucleated erythroid lineage, and blast-like immature cells (Figure 3D, bottom). Strikingly, when the Pi4ka insertion occurred in the absence of additional gCIS mutations (F270), the predominant cell type was blast-like and immature, which was also associated with anemia (Figures 3D [bottom] and 3E).

Pi4ka Insertions Are Associated with Impaired Myelopoiesis and Erythropoiesis

To assess the cellular composition and clonality of spleens with Pi4ka mutations, we first performed transcriptome...
analysis of both mutant and control spleens (Figure 4A). Based on the expression of cell-type-specific markers (Zhu and Emerson, 2002), Pi4ka mutant spleens had less lymphocytic, more HSCs, and more common myeloid progenitor (CMP) features when compared with controls. Animal F270 had the least mature monocyte-macrophage character, both F270 and F262 had increased granulocyte-monocyte progenitor (GMP) features, and both F270 and M267 had decreased mature erythroid hemoglobin membrane (Figure S3A). Pi4ka was also expressed in a subset of CD45+ lineage-cocktail-negative cells in the mouse adult bone marrow, but not in the associated vasculature (Cd5+)(Figures S3B and S3C). To confirm these findings, we performed qPCR for Pi4ka on sorted adult bone marrow cell populations, detecting elevated expression in Lin- Sca1+ cKit+ HSPCs compared with other progenitor populations and the Lin+ cell fraction (Figure S3D). In addition, a microarray data from BloodChIP (Chacon et al., 2014) demonstrates the

scripts. These results led us to hypothesize that Pi4ka might have a role in erythroid and myeloid maturation. Consistently, downregulated genes were enriched for Gene Ontology categories related to mature blood cells markers (Figure 4B), and upregulated genes were enriched for categories related to cell proliferation (Figure 4C). Expression of the genes defined in Figure 4A revealed strong similarity between two independent regions of each affected and control spleen (Pearson correlation; diagonal) (Figure 4D), indicating that the anomalies were clonal. As anticipated, comparisons between individuals showed much lower correlation.

Pi4ka Is Expressed in HSPC Budding from the HemEnd and in Adult Mouse Lineage-Negative Bone Marrow Cells

Next, we sought to evaluate the expression profile of Pi4ka in the hematopoietic compartment. Immunofluorescence staining in E9.5 mouse aortas captured HSPC, budding from HemEnd (CD31+) expressing Pi4ka at the cell membrane (Figure S3A). Pi4ka was also expressed in a subset of CD45+, lineage-cocktail-negative cells in the mouse adult bone marrow, but not in the associated vasculature (Cd5+)(Figures S3B and S3C). To confirm these findings, we performed qPCR for Pi4ka on sorted adult bone marrow cell populations, detecting elevated expression in Lin- Sca1+ cKit+ HSPCs compared with other progenitor populations and the Lin+ cell fraction (Figure S3D). In addition, a microarray data from BloodChIP (Chacon et al., 2014) demonstrates the

Figure 3. Transposon Insertions in the Pi4ka Gene Are Associated with Blast-like Phenotype and Decreased Red Blood Cells

(A) Transposon insertions throughout the Pi4ka gene. 

(B) Pi4ka RNA sequencing counts per million for affected and C spleens.

(C) Pi4ka expression by qPCR of the individual spleens from affected mice compared to Cre+ spleens (n = 6).

(D) H&E of Cre+ spleens and those with Pi4ka insertions (scale bar, 600 µm). Pi4ka immunohistochemistry (IHC) in spleens of C and affected mice (top: scale bar, 600 µm; middle: scale bar, 60 µm). Bottom: Cytospins of RBC-lysed blood from C and affected animals (scale bar, 15 µm)

(E) CBC analysis in C and Pi4ka-affected animals. For Cs (Cre+), data are represented as mean ± SEM of n = 10 animals.

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highest *PI4KA* expression in human HSC, multipotential progenitor (MPP), and megakaryocyte/erythrocyte progenitor (MEP) populations in addition to acute myeloid leukemia (AML) (Figure S3E).

**Loss of pi4kaa Function in Zebrafish Inhibits Erythroid Differentiation**

To further explore the biological relevance of Pi4ka in an independent system, we evaluated the role of zebrafish homolog *pi4kaa* in...
hematopoiesis. A splice inhibitory morpholino targeting the catalytic domain prevented splicing of \textit{pi4kaa} exons 49 and 50 in a dose-dependent manner (Figure S4A). O-Dianisidine staining indicated lower hemoglobin content in the 48 hr post-fertilization (hpf) morphant embryos compared with controls (Figure S4B). To quantify differences in erythroid lineage cells, flow cytometry was performed on control and morpholino-treated \textit{gata1}:DsRED (erythroid cells); \textit{fli1}:GFP (endothelial and hematopoietic progenitor cells); or \textit{lcr}:GFP (erythroid cells) embryos (100 embryos per biological replicate) at 48 hpf. Morphant (MO) animals were treated with an additional p53 morpholino to enhance embryo viability, whereas control (C) animals were treated with just the p53 morpholino. Due to its long half-life, DsRED measurement was equivalent to total erythroid lineage cells. \textit{Pi4kaa} inhibition resulted in significantly less erythroid lineage cells in both fish lines (Figures 5A and 5B). \textit{fli1}:GFP; \textit{gata1}:DsRED fish were then used to assess the differentiation of erythroid lineage cells in MOs. In C embryos, a prominent \textit{fli1}:GFP;\textit{gata1}:DsRED double-positive population was observed at 24 hpf, characteristic of immature hematopoietic cells (Figure S4C). As development proceeded and cells differentiated, this population was decreased. By 48 hpf, \textit{gata1}:DsRED cells had lost \textit{fli1}:GFP expression in C embryos (Figure S4D). In contrast, the \textit{fli1}:GFP;\textit{gata1}:DsRED double-positive population was partially retained in \textit{pi4kaa} MOs (~3.5 higher median \textit{fli1}:GFP expression) (Figure 5C; Figure S4E).

We performed qPCR on whole \textit{pi4kaa} MO and C embryos to assess markers of stem and mature blood cells. Indicators of treated with an additional p53 morpholino to enhance embryo viability, whereas control (C) animals were treated with just the p53 morpholino. Due to its long half-life, DsRED measurement was equivalent to total erythroid lineage cells. \textit{Pi4kaa} inhibition resulted in significantly less erythroid lineage cells in both fish lines (Figures 5A and 5B). \textit{fli1}:GFP; \textit{gata1}:DsRED fish were then used to assess the differentiation of erythroid lineage cells in MOs. In C embryos, a prominent \textit{fli1}:GFP;\textit{gata1}:DsRED double-positive population was observed at 24 hpf, characteristic of immature hematopoietic cells (Figure S4C). As development proceeded and cells differentiated, this population was decreased. By 48 hpf, \textit{gata1}:DsRED cells had lost \textit{fli1}:GFP expression in C embryos (Figure S4D). In contrast, the \textit{fli1}:GFP;\textit{gata1}:DsRED double-positive population was partially retained in \textit{pi4kaa} MOs (~3.5 higher median \textit{fli1}:GFP expression) (Figure 5C; Figure S4E).
hematopoiesis gata1 (erythroid progenitor marker), lmo2 (erythroid-myeloid progenitor marker), and pu.1 (myeloid progenitor marker) were higher in MOs at 24 hpf (Figure 5D). While beta-globin was slightly lower than in Cs, the mature myeloid markers mpx and l-plastin were not affected in pi4kaa MOs (Figure 5E).

**Pi4ka Knockdown in Mouse HSPC Impairs Progression of Mouse Erythro- and Myelopoiesis In Vitro in a Cell-Autonomous Manner**

Given the evidence for a role in zebrafish erythropoiesis, we sought to determine the importance of Pi4ka in murine myeloid and erythropoiesis by evaluating HSPC differentiation in vitro. As the zebrafish experiments involved “whole-body” pi4kaa targeting, we sought to test whether Pi4ka loss functioned in a hematopoietic-cell-autonomous manner. We sorted adult mouse bone marrow HSPCs (Lin- cKit+Sca1+) treated with either lentivirus (lenti)-small hairpin RNA against Pi4ka or scrambled sequence for 24 hr, washed to remove virus, and subsequently co-cultured on an OP9 stromal layer in the presence of cytokines (Figure 5F). After 10 days of culture, flow cytometry revealed significantly less CD71−/C0 (immature erythroid marker), Ter119+ (mature erythroid marker) erythroid lineage cells in cultures derived from shPi4ka (shPi4ka)-treated HSPCs (Figures 5G and 5H). Similarly, an increase in Mac1+ (Mac1 high), F4/80+ (F4/80 high) cells with a complementary decrease in Mac1− (Mac1 low), F4/80− (F4/80 high) cells was observed in the shPi4ka-treated condition (Figures 5I and 5J), reflecting the phenotype seen in mouse F270 from the original screen.

**Akt and Erk Signaling Is Altered Downstream of Pik4a Knockdown In Vitro**

To gain mechanistic clarity on pathways regulated downstream of Pi4ka, we explored the effect of loss of function on a panel of relevant signaling effectors. Pi4ka knockdown by lentiviral shRNA in 32D mouse myeloid lineage cells (Figure 6A, right) significantly increased phospho-ERK (p-Erk) and depressed interleukin-3 (IL-3)-induced p-Akt, as determined by western blot and flow cytometry (Figure 6A; Figure S5A). We validated the signaling effects in another cell type (HEK293), where Pi4ka small interfering RNA (siRNA) enhanced stem cell factor (SCF)-induced p-Erk levels (Figure 6B). Together, these results suggest that Pi4ka is important for regulating the balance between p-Akt and p-Erk signaling downstream of the cytokine receptors interleukin-3 receptor (IL-3R) and cKit.

**Human PI4KAP2 Protein Lacks Kinase Activity and It Is Upregulated in Myelo- and Erythroleukemia Cell Lines**

According to the COSMIC genome database, PI4KA mutations have been found in 363 unique human cancer samples, including somatic frameshift mutation p.T1995fs*4 in three lymphoid neoplasms (COSMIC Study COSU440) and many missense mutations, some occurring in primary and cell line leukemia. Interestingly, the human genome encodes pseudogenes, absent in mice, that could further affect PI4KA function. We hypothesized that human PI4KAP2 (PI4KA pseudogene 2), which encodes an N-terminally truncated, kinase-domain-deleted version of the PI4KA protein (Figures 7A and 7B), could act in a dominant-negative manner. The BloodChIP database indicates that the PI4KA promoter is similarly primed by activation marks in both CD34+ HSPCs and K562 erythroleukemia cells (Figure S6A), while the predicted PI4KAP2 promoter has relatively
Figure 7. The Human PI4KAP2 Gene Yields a Protein Product and Has Higher mRNA Expression Relative to PI4KA in Erythro- and Myelo-Leukemia Cell Lines

(A) Schematic comparing PI4KA and PI4KAP2 proteins. PI4KAP2 lacks the N-terminal domain and has a deletion in the kinase domain (red), PR, proline-rich domain; LKU, lipid kinase unique domain; PH, plekstrin homology domain.

(B) Alignment of PI4KA and PI4KAP2 amino acid sequences shows major homology starting at amino acid 1,258 of PI4KA, except for missing amino acids in the kinase domain of PI4KAP2.

(C) Ratio of mRNA expression of PI4KAP2 and PI4KAP1 compared with PI4KA in a panel of normal cord and peripheral blood cells (black), human lymphoid leukemia (blue), and myeloid leukemia (orange) cell lines.

(legend continued on next page)
higher activation marks in K562 (Figure S6B). Furthermore, K562 cells have both PI4KA and PI4KAP2 promoter binding by the hematopoietic transcription factor Erg, while normal CD34+ HSPCs do not (Figures S6C and S6D).

Given the apparent increased PI4KAP2 promoter accessibility/priming and concomitant hematopoietic transcription factor binding in malignant cells, we assessed differential expression in myeloid leukemia, lymphoid leukemia, normal hematopoietic progenitor cells, and normal blood mononuclear cells (monocytes and lymphocytes) (Figure 7C). We designed primers to distinguish the PI4KA transcript (located in the region deleted in PI4KAP2) from the PI4KAP1 and PI4KAP2 transcripts (reverse primer spanning the region flanking the deleted kinase domain in PI4KAP1/2), although we were unable to distinguish between the two pseudogene transcripts. While PI4KAP1/2 and PI4KA were similarly expressed in CD34+ cord blood cells, peripheral blood mononuclear cells (PBMCs), and a T cell leukemia cell line, the ratio of PI4KAP1/2 to PI4KA expression was generally increased in myeloid- and erythroleukemia cell lines, consistent with the BloodChip data. We confirmed expression of PI4KAP2 protein in the myeloid- and erythroleukemia cell lines using an antibody validated through decreased signal after siRNA knockdown in HEK293 cells (Figure 7D). These results demonstrate that PI4KAP2 codes for an expressed protein and is not a pseudogene, a conclusion further validated by fusing the cDNA sequence to a C-terminal hemagglutinin (HA)-tag. Both the exogenously expressed (HA-tagged) and endogenous protein were detected by western blot with a PI4KAP2 antibody (Figure S7A).

Given the lack of the kinase domain in PI4KAP2, we explored its potential role as an antagonistic regulator of PI4KA. As expected, PI4KAP2 protein purified from HEK293 cells using immuno-precipitation of the HA-tag displayed no in vitro kinase activity, in contrast to purified PI4KA protein (Figure S7B). We compared the impact of PI4KAP2 gain of function to PI4KA loss of function by probing an antibody microarray with lysates from HEK293 cells transfected with PI4KAP2 expression vector, C vector, siRNA against PI4KA, or C siRNA (Figures S7C and S7D). Protein categories affected by altering PI4KA signaling included cytoskeleton regulation, Src-family kinases, MAPK family, NFkappaB, receptor tyrosine kinases, cell cycle regulators, JAK-STAT signaling molecules, intracellular kinases, adaptors, and lipases (Figure 7E). Although there were some differences in the effects of PI4KAP2 expression and PI4KA knockdown, commonly affected proteins included: LIMK1, FYN, KIT, p38δ MAPK, and STAT5A. The entire array can be found in Table S3.

**DISCUSSION**

Our results are consistent with literature reporting a pleiotropic requirement for PI4ka in normal morphogenesis. In zebrafish, pi4kaa was shown to be necessary for pectoral fin development downstream of fibroblast growth factor receptor (FGFR) signaling through regulation of PI3K-Akt signaling (Ma et al., 2009). In *Drosophila*, it was shown to be required for smoothened activation during imaginal wing disc development (Yavari et al., 2010). Furthermore, global deletion of PI4ka in adult mice uncovered its requirement for gastrointestinal stability (Bojjireddy et al., 2014; Vaillancourt et al., 2012). Our data add to this body of knowledge and implicate PI4ka in hematopoiesis.

We postulate that PI4ka is likely to regulate hematopoiesis in several ways in addition to its effect in Akt signaling. PI4ka is a lipid kinase that phosphorylates the D4 position of the phosphatidyl-inositol-4-phosphate ring (Minogue and Waugh, 2012). The resulting phosphatidylinositol-4-phosphate (PIP4) provides a docking point for other proteins to bind to the inner leaflet of the plasma membrane (Ball et al., 2009). Once docked, additional lipid kinases can phosphorylate the ring at other positions, creating more complex phosphatidyl-inositol that can become substrates for phospholipases. Interestingly, phospholipase gamma 1 has been shown to have a role in primitive zebrafish hematopoiesis (Ma et al., 2007). In fact, we observe defects of the primitive erythroid lineage when we inhibit pi4kaa in our zebrafish model. Furthermore, oxysterol-binding proteins, which insert sterols into the plasma membrane (Villasmi et al., 2012), require PIP4 to dock. Oxysterols themselves can inhibit the proliferation of hematopoietic cell progenitors (Gregorio-King et al., 2002). Plasma membrane fluidity, as well as the specific constituency of lipids, influences cell-surface receptor signaling (Sunshine and Iruela-Arispe, 2017). Indeed, sterols have been shown to affect smoothened activation and hedgehog signaling, which are known to regulate HemEnd - HSCs (Crisan et al., 2016). Finally, there are early reports correlating changes in phosphatidyl-inositol lipid composition and hematopoietic cell proliferation and differentiation (Michell et al., 1990).

PI4KAP2, previously thought to not encode a functional protein, is the result of gene duplication in humans and does not exist in mice (Szentpetery et al., 2011). However, we demonstrated that this gene is not only transcribed, but also translated into a protein that has signaling consequences. Based on its lack of a kinase domain, we hypothesized that the PI4KAP2 protein could act in a dominant-negative fashion (similar to the effects of losing expression of PI4KA). The ratio of PI4KAP2 to PI4KA is higher in myeloid and erythroid cell lines compared with other cell types tested. Strikingly, the ERG transcription factor was documented to bind to the promoter region, specifically in malignant (K562) cells. One could speculate of a scenario in which higher levels of ERG (such as in Down syndrome) could enhance PI4KAP2 expression and deregulate the PI4KA pathway in the context of in utero leukemia development. Our findings indicate that PI4KAP2 overexpression or PI4KA knockdown induces similar alterations in MAPK, Src-family kinases, and JAK-STAT signaling pathways. We also documented the effect of PI4KAP2...
and PI4KA on proteins like FAK, PAK1, STAT5, and KIT, which are known leukemia drivers (Chatterjee et al., 2014). In addition, pi4kaa is known to regulate the PI3K-AKT pathway downstream of FGFR signaling (Ma et al., 2009). Our findings demonstrate similar effects in mouse 32D myeloid progenitor cells. FGFR1 signaling appears to be required for HSC repopulation, and increased expression of FGFR3 has been reported in CD34+ myeloid leukemia cells (de Haan et al., 2003; Dvorak et al., 2003). In this study, PI4KAP2 overexpression altered FGFR3 protein levels as well as findings in the antibody array.

In conclusion, this forward genetic screen supports the concept that mutations initiated at the hemogenic endothelium stage can carry consequences for the hematopoietic lineage. We identified Pi4kaa as an important cell-autonomous regulator of hematopoiesis, which in turn pointed to PI4KAP2, found to be dysregulated in human myeloid and erythroid leukemia cell lines.

**EXPERIMENTAL PROCEDURES**

**Cells**

OP9 cells (a gift from the Mikkola Laboratory, UCLA) were cultured in alpha minimum essential medium (αMEM) with 2 mM L-glutamine, 1% pen-strep, 20% HyClone (Thermo Fisher Scientific) fetal bovine serum (FBS). For OP9/leukocyte co-cultures, media was supplemented with 5 ng/mL thrombopoietin (TPO), 50 ng/mL SCF, 10 ng/mL FMS-like tyrosine kinase 3 ligand (Flt3L), 50 mmol/L interleukin-6 (IL-6), and 5 ng/mL IL-3 (Peprotech). 32D cells (CRL-11346 American Type Culture Collection [ATCC]) were cultured according to ATCC recommendations with 10 ng/mL IL-3. Lentiviral HEK293 cells (632180 Clonetech) were cultured in DMEM 10% FBS. G1E-ER4 cells (a gift from the Ganz Laboratory, UCLA) were cultured with tamoxifen according to Rylski et al. (2003), BV173, KLC22, and K562 cells (a gift from the Colicelli Laboratory, UCLA), TFIa (ATCC CRL-2451) and HEL 92.1.7 (ATCC TIB-180) were all cultured according to ATCC recommendations.

Human PBMC and CD34+ cord blood cells were purchased from the UCLA Core Facility Research (CFAR) Virology Core Laboratory. Mononuclear cells were isolated using human Miltenyi MACS CD34 MicroBead Kit Ultra Pure and used for RNA. For cord blood, CD34+ (positive selected) and CD34− (negative selected) blood cells were isolated using human Mitenyi MACS CD34 MicroBead Kit Ultra Pure and used for RNA.

**Flow Cytometry and Cell Sorting**

Flow cytometry was performed using BD Fortessa and LSRII machines (BD Biosciences). FACS was performed using BD Aria instruments. For RNA isolation of bone marrow subpopulations, Lin−, HSC (Lin−, cKit+, Sca1−, CD34−), GMP (Lin−, cKit+, Sca1−, CD34−, FcyR−), MEP (Lin−, cKit+, Sca1−, CD34−, FcyR+), and CMP (Lin−, cKit+, Sca1−, CD34−, FcyR+) cells were sorted from RBC-lysed bone marrow. For OP9 cultures, HSC (Lin−, cKit+, Sca1−) cells were sorted after lineage depletion of bone marrow using mouse lineage depletion kit and LS columns (Miltenyi). Mouse hematopoietic cell differentiation on OP9/G1E-ER4 cells was assessed from a single-cell suspension generated from cultured cells using antibodies against CD45, Mac1 (CD11b), F4/80, Ter119, and CD71.

Zebrafish flow cytometry and sorting was based on flt1:GFP, lcr:GFP or gata1:dsRED fluorescent signal (n = 100 per treatment). Dechorionated embryos were digested with 5 μg/mL Liberase-TM (Roche) for 1 hr at 33°C as in Bertrand et al. (2007).

**Molecular Cloning**

The PI4kap2 coding sequence was amplified from pDONR233-PI4KAP2 (23601 Addgene) using primers (Supplemental Experimental Procedures) that introduced an HA-tag at the 5’ end and flanked the fragment with PstI.

**Fish**

Zebrafish lines were maintained in accordance with the UCLA Department of Laboratory Animal Medicine’s Animal Research Committee guidelines. The following lines were used: Tg(gata1:DSRED; flt1:GFP), Tg(lcr:GFP) (Gans et al., 2012), and wild-type AB fish. lcr:GFP fish were purchased from the UCLA Zebrafish Core Facility. All embryos were treated with 1 x 1-phenyl-2-thiourea (PTU) to inhibit pigment formation at 24 hpf. Eight pg or 12 pg of the splice-inhibitory pi4kaa morpholino was injected with 2 pg or 3 pg of p53 morpholino, respectively. Splicing efficiency was examined with previously published primers. See oligonucleotide sequences in Supplemental Experimental Procedures. O-Dianisidine stain was used to stain hemoglobin.

**qPCR Transcriptional Analysis**

RNA was isolated using RNeasy Mini and Micro kits (QIAGEN). Mouse tissue cDNA was made using a Superscript III system (Invitrogen). Zebrafish and human cDNA were generated with an iScript cDNA synthesis kit (BioRad). Twenty whole zebrafish per treatment were used for RNA isolation. Zebrafish, mouse, and human qPCR primers are listed in Supplemental Experimental Procedures. SYBR-Green-based qPCR (BioRad) was performed as previously described (Briot et al., 2014).

**Hematology**

Complete blood count (CBC) analysis was performed using a Hemavet machine (Drew Scientific). After RBC lysis, leukocytes were spun onto slides using a Shandon CytoSpin 4 (Thermo Fisher Scientific). Slides were stained with May-Grumwald and Giemsa stains (Sigma-Aldrich).

**Immunohistochemistry**

Paraffin-embedded tissues were deparaffinized, subjected to heat-mediated antigen retrieval, blocked with normal serum, and stained with antibody against Pi4ka. Biotinylated anti-rabbit secondary antibody was followed by Avidin-Biotin Complex Elite and DAB Peroxidase Kit (Vector Laboratories). See antibodies in Supplemental Experimental Procedures. An Olympus DP73 camera and cellSens software were used to image non-fluorescent stains.

**RNA Sequencing**

RNA was purified using an RNeasy mini kit (QIAGEN), and libraries were prepared with a TrueSeq polyA selection kit using 1 μg of RNA manually or a TruSeq stranded polyadenylation (poly-A) selection kit with 50 ng of RNA using the NeoPrep system (Illumina). Libraries were sequenced on a HiSeq 4000 system (illumina). For the clonal analysis, we sequenced single-end 50 bp. For the cell-subtype expression analysis, we sequenced paired-end 100 bp.
and Agel restriction enzyme sequences. The fragment was ligated into the pJet cloning vector using CloneJET kit instructions (K1231 Thermo Fisher Scientific) and amplified in Stbl3 bacteria (C737303 Thermo Fisher Scientific). The plV-EF1a-MCS-IRE-PRO-uro plasmid and the Pi4kap2Ha-pJet vector were linearized with PstI and Agel. The Pi4kap2-HA fragment was gel purified and ligated into the plV vector using T4 ligase. For the shRNA vector purchased from Origene (TLS10615), the MND-GFP cassette (a gift from the Kohn Laboratory) was used to replace the CMV-GFP reporter.

**Lenti shRNA Transduction**

Pi4ka or scrambled shRNA plasmids along with VSG-G and Δ8.2 packaging plasmids were transfected into 293T cells using Lipofectamine 2000 (LifeTechnologies). Virus was collected and concentrated by centrifugation. For primary HSPCs, high concentration virus was used to infect cells using Retronectin-coated (Clontech) plates (40 μg/mL) after an overnight pre-stimulation in serum-free StemSPAN (StemCell Technologies, Inc.) or StemMACS (Miltenyi) supplemented with four times the cytokine concentration used in OP9 coculture for the course of 24 hr before being washed and moved to OP9 stromal cells. For 32D and G1E-ER4 cells, the same virus was used to infect cells using Retronectin-coated plates as above for 24 hr in the presence of culture medium.

**Cell Transfection**

For siRNA studies, HEK293 cells were treated with 60 pmol non-targeted (4390843 Invitrogen), Pi4ka-targeted siRNA (4392420 ID:s224264, Invitrogen) or Pi4kap2-targeted siRNA (4390771 ID:n30618) in the presence of Lipofectamine RNAi Max (Invitrogen) for two days. Similarly, for overexpression of Pi4kap2, cells were treated with 3 μg of plV-EF1a-Pi4kap2-HA-RFP in the presence of Lipofectamine2000 (Invitrogen) for two days.

**Western Blot**

32D cells and HEK293 cells were lysed in modified radioimmunoprecipitation assay (mRIPA) buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 0.15% NaCl, and 10mM beta-glycerophosphate) in the presence of 200 μM Na3VO4 and protease inhibitor cocktail (11873580001, Sigma). Lysates were run on 4%-20% gradient acrylamide gels (BioRad) and transferred to nitrocellulose membranes. See antibodies in Supplemental Experimental Procedures.

**Antibody Array**

Cell lines expressing Ef1a-Pi4kap2-HA-RFP, Ef1a-RFP empty vector, non-targeted siRNA, or Pi4ka-targeted RNA were lysed in protein lysis buffer (5 mM EDTA, 20 mM EGTA, 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.0, 20 mM NaF, 20 mM Na3P04, 1 mM Na3VO4, 60 mM beta-glycerophosphate, 50 mM phenylarsine oxide, 1% Triton X-100) containing protease inhibitor cocktail and phosphatase inhibitors. Samples were sonicated and then centrifuged for 30 min at 14,000 × g. Protein was quantified and pooled at equal concentrations. Lysates were then probed using the Kinexus KAM-900P array (Kinexus Bioinformatics Corporation), which contains 613 phospho-site-specific antibodies and 265 pan-specific antibodies (targeting 878 cell signaling proteins).

**Statistical Analysis**

For every dataset, it was first determined wither parametric or non-parametric test was used to discern the significance of transcriptional differences between sorted hematopoietic sub-populations with the null assumption that they were the same. Paired Student’s t test was used to assess significance between experimental and control conditions for zebrafish and OP9 co-culture experiments. Unpaired Student’s t test was used for western blot and MFIR calculations. Statistical analyses were performed in Prism 7.0 according to the manufacturer’s recommendations (GraphPad Software) (p ≤ 0.08; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001).

**DATA AND SOFTWARE AVAILABILITY**

The accession number for all datasets reported in this paper is GEO: GSE108355.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.01.017.

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**AUTHOR CONTRIBUTIONS**

S.Z. designed and performed the experiments, analyzed the data, and wrote the paper. J.D.R. designed and performed the experiments, analyzed the data, and assisted in manuscript preparation. A.M.C. designed and performed the experiments and analyzed the data. G.E.H. performed the experiments and analyzed the data. K.H., M.M., T.S., K.C.W., and G.H. performed the experiments. J.-N.C. and A.J.D. provided critical reagents and guidance. L.I.-A. designed the study, analyzed the data, and wrote the paper.

**DECLARATION OF INTERESTS**

We have no conflicts of interest to report.

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