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Rheb1 loss leads to increased hematopoietic stem cell proliferation and myeloid-biased differentiation *in vivo*

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ABSTRACT

Hematopoietic stem cells constitute a unique subpopulation of blood cells that can give rise to all types of mature cells in response to physiological demands. However, the intrinsic molecular machinery that regulates this transformative property remains elusive. In this paper, we demonstrate that small GTPase Rheb1 is a critical regulator of proliferation and differentiation of hematopoietic stem cells *in vivo*. Rheb1 deletion led to increased phenotypic hematopoietic stem cell/hematopoietic progenitor cell proliferation under a steady state condition. Over-proliferating Rheb1-deficient hematopoietic stem cells were severely impaired in functional repopulation assays, and they failed to regenerate the blood system when challenged with hematopoietic ablation by sublethal irradiation. In addition, it was discovered that Rheb1 loss resulted in a lack of maturation of neutrophils / caused neutrophil immaturation by reducing mTORC1 activity, and that activation of the mTORC1 signaling pathway by mTOR activator 3BDO partially restored the maturation of Rheb1-deficient neutrophils. Rheb1 deficiency led to a progressive enlargement of the hematopoietic stem cell population and an eventual excessive myeloproliferation *in vivo*, including an overproduction of peripheral neutrophils and an excessive expansion of extramedullary hematopoiesis. Moreover, low RHEB expression was correlated with poor survival in acute myeloid leukemia patients with normal karyotype. Our results, therefore, demonstrate a critical and unique role for Rheb1 in maintaining proper hematopoiesis and myeloid differentiation.

Introduction

Hematopoietic stem cell (HSC) proliferation and differentiation are regulated by the networks of signaling pathways.[1] Under stress conditions, HSCs quickly respond to a variety of proliferative stimuli, exit the quiescent phase, and undergo a period of self-renewal and differentiation to restore hematopoietic homeostasis.[2] Fine-tuning is required to adequately control cell growth and quiescence during this process, and multiple signaling pathways are involved in maintaining the correct balance. Interestingly, studies have suggested that common or similar mechanisms regulate stem cell properties in both HSCs and leukemia stem cells (LSCs), suggesting that LSCs may originate from HSCs. The mammalian target of the rapamycin (mTOR) signaling pathway is a key node in these pathways, and plays an essential role in regulating normal and malignant hematopoiesis.[3,4]

Ras homolog enriched in brain (Rheb), a small GTPase, is known to directly acti-

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vate mTORC1.[5,6] Rheb binds to mTORC1 at the lysosome and activates it, while TSC1-TSC2-TBC1D7, a GTPase-activating protein, inhibits mTORC1 activity by reducing GTP-bound Rheb.[7-9] mTORC1 is a serine/threonine (Ser/Thr)-protein kinase complex that responds to multiple signals, regulates cell biological activities, and maintains homeostasis.[10] Dysregulation of the mTORC1 signaling pathway results in myeloproliferative neoplasms (MPN) and, in some cases, acute leukemia, making mTORC an important target for therapeutic treatments.[10] Perturbation of mTORC1 in adult mouse HSCs by Raptor deletion results in the depletion of the long-term reconstitution ability of HSCs.[11] Deletion of TSC1, an upstream negative regulator of mTORC1, causes defective HSC cycling and adversely affects HSC function in mice due to enhanced mTORC1 activity,[12] indicating that the level of mTORC1 activity needs to be precisely regulated. Changes in mTORC1 activity in the hematopoietic system under various conditions alters HSC function and homeostasis.

Rheb1 can also regulate cell proliferation and apoptosis *via* interaction with many other signaling pathways, such as B-Raf, Notch, small Rho GTPase and Akt signaling pathways. Knockdown of Rheb1 in a TSC2-null, angiomyolipoma-derived cell line decreased Notch activity, suggesting that Notch is a downstream target of Rheb1. However, Notch activation could not be blocked by rapamycin, the mTORC1 inhibitor.[13] Rheb1 was also reported to directly interact with the B-Raf kinase in a rapamycin-resistant manner and inhibit its function, resulting in interference with H-Ras-induced transformation in NIH3T3 cells.[14] In addition, Rheb1 interacts with FKBP38 and regulates apoptosis in a rapamycin-insensitive, but amino acid- and serum-sensitive manner.[15] We have previously reported that Rheb1 plays a crucial role in myeloid development. The expression of Rheb1 is high in myeloid progenitor, and is down-regulated during granulocyte differentiation. Rheb1 deletion interferes with myeloid progenitor progression and gene expression.[16] However, ongoing studies have not directly addressed the specific regulatory role of Rheb1 in hematopoietic stem cells.

In this study, we observed that Rheb1 is an essential regulator of hematopoietic development. Rheb1-deficient mice showed increased phenotypic HSCs, immature neutrophils in bone marrow (BM), and splenomegaly. These phenotypes are reminiscent of the hematopoiesis seen in MPNs. Rheb1-deficient HSCs were defective in their ability to reconstitute the blood tissue and differentiate into normal neutrophils. Interestingly, low Rheb expression was associated with poor survival in acute myeloid leukemia (AML) patients. Thus, our data indicate that Rheb is critical for HSC function and may be involved in the initiation of myeloid proliferation-related diseases or MPN-like disorders.

Methods

Mice and genotyping

Rheb1$^{fl/fl}$ mice were kindly provided by Dr. Bo Xiao.[17] Transgenic mice expressing Cre recombinase under the control of the Vav1 promoter (Vav1 Cre) were purchased from the Jackson Lab. The *Rheb1*$^{fl/fl}$ mice were crossed with Vav1-Cre mice to generate specific deletion of Rheb1 in the hematopoietic system. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC), the Institute of Hematology, and Blood Diseases Hospital (CAMS/PUMC). All surgery was performed under sodium pentobarbital anesthesia, and every effort was made to minimize mouse suffering.

Flow cytometry analysis

Peripheral blood (PB) was obtained from either the tail veins or retro-orbital bleeding of mice. Red blood cells (RBCs) were lysed by ammonium chloride-potassium bicarbonate buffer before staining. BM cells were flushed out from tibias, femurs, and ilia by a 25-gauge needle with PBS supplemented with 2% fetal bovine serum (FBS) and 20 mM EDTA (abbreviated as PBE). Cells were stained with antibodies purchased from either eBioscience or BD Bioscience. To analyze intracellular proteins, $3x10^6$ BM cells were labeled with surface antibodies, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X100, then washed 2 times with 1 mL cold PBE. Finally, the cells were resuspended with cold PBS supplemented with 25% FBS, and intracellularly stained with antibodies: p-S6 (Ser24/244), p-4EBP1 (Thr37/46). Cells were analyzed by BD Canto II flow cytometer. FlowJo software was used to analyze the results.

LKS transplant and analysis

Whole BM cells (WBMCs) were obtained and Lin$^-$ cells were sorted using mouse lineage cell depletion kit (Miltenyi Biotec) according to the manufacturer's instruction. LKSs were stained as mentioned above and sorted by BD Influx flow cytometer; 200 LKSs (CD45.1) together with $5x10^5$ whole BM cells (WBMCs) (CD45.2) were injected intravenously into lethally irradiated recipient mice (CD45.2). The reconstitution of PB cells was analyzed every four weeks post transplantation. The recipient mice were sacrificed at four months after transplantation. The self-renewal and differentiation capacities of donor-derived HSCs derived from BM were then analyzed.

Competitive bone marrow transplantation and analysis

Whole BM cells were isolated from the tibias, femurs and ilia of 8-week old *Rheb1*$^{fl/fl}$ (CD45.1) or *Rheb1*$^{\Delta/\Delta}$ mice (CD45.1). $5x10^5$ *Rheb1*$^{\Delta/\Delta}$ WBMCs (CD45.1) together with $5x10^5$ WBMCs (CD45.2) were intravenously injected into the lethally irradiated recipient mice (CD45.2). Then, the reconstituted PB cells were analyzed every four weeks after transplantation.

Lineage$^-$ cell homing assay

Whole BM cells were obtained, and LKS$^+$ cells (CD45.1) were sorted by flow cytometry. LKS$^+$ cells were cultured with CFSE at 37°C for 8 minutes (min). The reaction was then terminated with 10% FBS at 4°C for 2 min and washed two times with cold PBS. LKS$^+$ cells ($2x10^6$) were intravenously injected into lethally irradiated (9.5 Gy) recipient mice (CD45.2). The recipient mice were sacrificed at 17 hours (h) or 24 h after transplantation. CFSE$^+$ cells in BM of recipient mice were analyzed by FACS.

Histological and pathological analysis

To examine the histology of the BM neutrophils, the neutrophils were sorted with CD11b and Ly-6G from BM, then cytospun and stained with Wright-Giemsa solution. For pathological analysis, BM, spleen, liver or lung were fixed with 4% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

In vitro bacterial killing assay

Escherichia coli (strain 19138; ATCC, Manassas, VA, USA) were cultured overnight, suspended in PBS at an OD_{600} of 0.10, and

opsonized with 10% mouse serum for 30 min at 37°C. Neutrophils and bacteria were then incubated together at a 1:5 ratio for 30 and 120 min at 37°C with intermittent shaking, and then 100 g/mL gentamicin was added for an additional 30 min to eliminate extracellular bacteria. The cells were then lysed by adding distilled water, and subsequently diluted aliquots were spread on LB agar plates. The CFU was counted after incubating the plates overnight at 37°C. A bacterial suspension without any cells was used as an input control.

Mouse colony-forming cell assay and 3BDO treatment

3BDO was dissolved in DMSO at 60 mM. WBMCs were treated with 3BDO (60 nM) or DMSO for 30 min.[18] Then, GMP/CMPs ($2x10^4$ cells) were sorted and cultured in MethoCult® media (Catalog #03231) in the presence of SCF (50 ng/mL), IL3 (10 ng/mL), and GM-CSF (25 ng/mL). Colonies were counted after 5-7 days.

Serial colony-forming cell assay

Whole BM cells were treated with 3BDO (60 nM) or DMSO for 30 min, then cells ($1x10^5$) were isolated and cultured in MethoCult® media (Catalog #03434). Colonies were counted seven days after plating, and serially replating. Colonies are defined as colony forming unit monocyte (CFU-M), granulocyte-macrophage (CFU-GM), and granulocyte-eythroid-macrophage (CFU-GEM).

Cobble stone area-forming cell assay

Mesenchymal stem cells (MSC) were obtained from mouse BM cells, as previously described.[19] MSCs were cultured in long-term culture medium M5300 (Stem Cell Technologies, Vancouver, BC, Canada) for two weeks, and half of the medium was changed every 3-4 days. Cells were trypsinized, irradiated with 15 Gray, and plated at 5000 cells/well in 96-well plates. LKS^+ cells (500 cells/well) were inoculated on the irradiated cell layers and incubated at 33°C. Colonies (Cobblestone areas) were counted five weeks after plating.

Gene expression profiling and patient data analysis

LKS^+ cells were sorted from WBMCs by BD Influx flow cytometer. The sorted cells were treated with Trizol, and then sent to the Shanghai Biotechnology Corporation for microarray analysis (Agilent, mouse 4*44K). Analysis of gene expression data from *Rheb1*$^{\Delta/\Delta}$ and *Rheb1*$^{fl/fl}$ LKSs revealed 2515 differentially expressed genes, with 922 up-regulated and 1593 down-regulated genes in Rheb1-deficient LKSs. The differentially expressed genes were filtered as $P<0.05$ and fold change greater than 3. The gene-set enrichment data were analyzed using GSEA and GO (*FDR <0.25, P<0.05, -Lg p = -Log10 p*). The microarray data were deposited with the GEO under the accession number GSE79538. For the GSEA analysis of patient data, we examined AML-related data using Molecular Signatures Database (MSigDB) (*http://software.broadinstitute.org/gsea/msigdb/search.jsp*).

Quantitative real-time PCR (qRT-PCR)

Total RNA from BM samples was extracted using the RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized with an oligo-(dT) primer according to the manufacturer's instructions. The mRNA expression was determined by qRT-PCR (Stepone Fast Real-Time PCR system; Applied Biosystems) using FastStart Universal SYBR Green PCR Master mix (Roche). GAPDH was used as endogenous controls for gene expression assays.

Statistical analyses

Experimental results were analyzed using Student *t*-test. $P<0.05$ was considered significant for all tests. Kaplan-Meier survival curves were created using GraphPad Prism 5. All data are presented as mean±Standard Error of Mean (SEM); n ≥3.

Results

Rheb1 deletion induced hematopoietic stem cell / hematopoietic progenitor cells expansion in steady state condition

To clarify the role of Rheb1 in hematopoiesis, *Vav1-cre;Rheb1*$^{fl/fl}$ (*Rheb1*$^{\Delta/\Delta}$) mice were generated and the cellular composition of the peripheral blood (PB) and BM was first analyzed in *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ mice. There was no significant difference in normalized values of white blood cells (WBC), RBCs, hemoglobin (Hgb), hematocrit (Hct) and platelets (PLT) in the PB between *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ mice, while absolute numbers of neutrophils were substantially higher in *Rheb1*$^{\Delta/\Delta}$ mice than those in *Rheb1*$^{fl/fl}$ mice (*Online Supplementary Figure S1A*). The absolute numbers of BM cells did not alter between *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ mice (*Online Supplementary Figure S1B*). The percentage and absolute numbers of LKS^+ and LKS^- cells were increased in *Rheb1*$^{\Delta/\Delta}$ mice (*Online Supplementary Figure S1C* and *D*). In the HSC-enriched LKS^+ subsets, Rheb1 loss significantly increased the absolute numbers of $CD150^+CD48^-LKS^+$ cells (Figure 1A and *Online Supplementary Figure S1E*), while in the hematopoietic progenitor cells (HPC)-enriched LKS^- subsets, Rheb1 loss resulted in an increase in the absolute number of $CD16/32^-CD34^+LKS^-$ cells and $CD16/32^-CD34^+LKS^-$ cells (GMP and CMP) (Figure 1B and C, and *Online Supplementary Figure S1F*). More *Rheb1*$^{\Delta/\Delta}$ LKS^+ cells were in G2/S/M phase of cell cycle than that of the control (Figure 1D and *Online Supplementary Figure S1G*), although the percentage of Annexin V-positive cells was not altered in *Rheb1*$^{\Delta/\Delta}$ LKS^+ (*Online Supplementary Figure S1H*). Thus, Rheb1 deletion led to an expansion of murine HSC/HPCs.

Rheb1 deletion caused neutrophil immaturity in steady condition

Interestingly, the absolute numbers and percentages of myeloid cells ($CD11b^+$) were increased in BM of *Rheb1*$^{\Delta/\Delta}$ mice (*Online Supplementary Figure S2A* and *B*), while no significant changes were found in B ($B220^+$) and T ($CD3^+$) cells in the BM of *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ mice (*Online Supplementary Figure S2C*). We then analyzed neutrophils by flow cytometry (FACS) with CD11b and Ly-6G antibodies that have been used as neutrophil subpopulation markers for the identification of myelocytes/promyelocytes, as well as immature and mature neutrophils. The $CD11b^+Ly\text{-}6G^+$ subpopulation of *Rheb1*$^{\Delta/\Delta}$ neutrophils shifted to the left in the PB and BM (Figure 1E). We divided neutrophils into three distinct subpopulations, indicated as the $CD11b^{low}Ly\text{-}6G^{low}$ population (P1), the $CD11b^{high}$ $Ly\text{-}6G^{low}$ population (P2), and the $CD11b^+$ $Ly\text{-}6G^{high}$ population (P3). We found that the percentages of the P1 and P2 subpopulations were increased, while the percentage of the P3 subpopulation was decreased both in the PB and BM of *Rheb1*$^{\Delta/\Delta}$ mice (Figure 1F and G). Morphological analysis of *Rheb1*$^{fl/fl}$ neutrophils from BM showed that the P1 subpopulation was comprised mainly of myeloblasts with oval-shaped nuclei and a wider, less basophilic cytoplasm. The segmentation of the nuclei became gradually evident in the P2 subpopulation while the P3 subpopulation was mainly composed of cells with doughnut-shaped

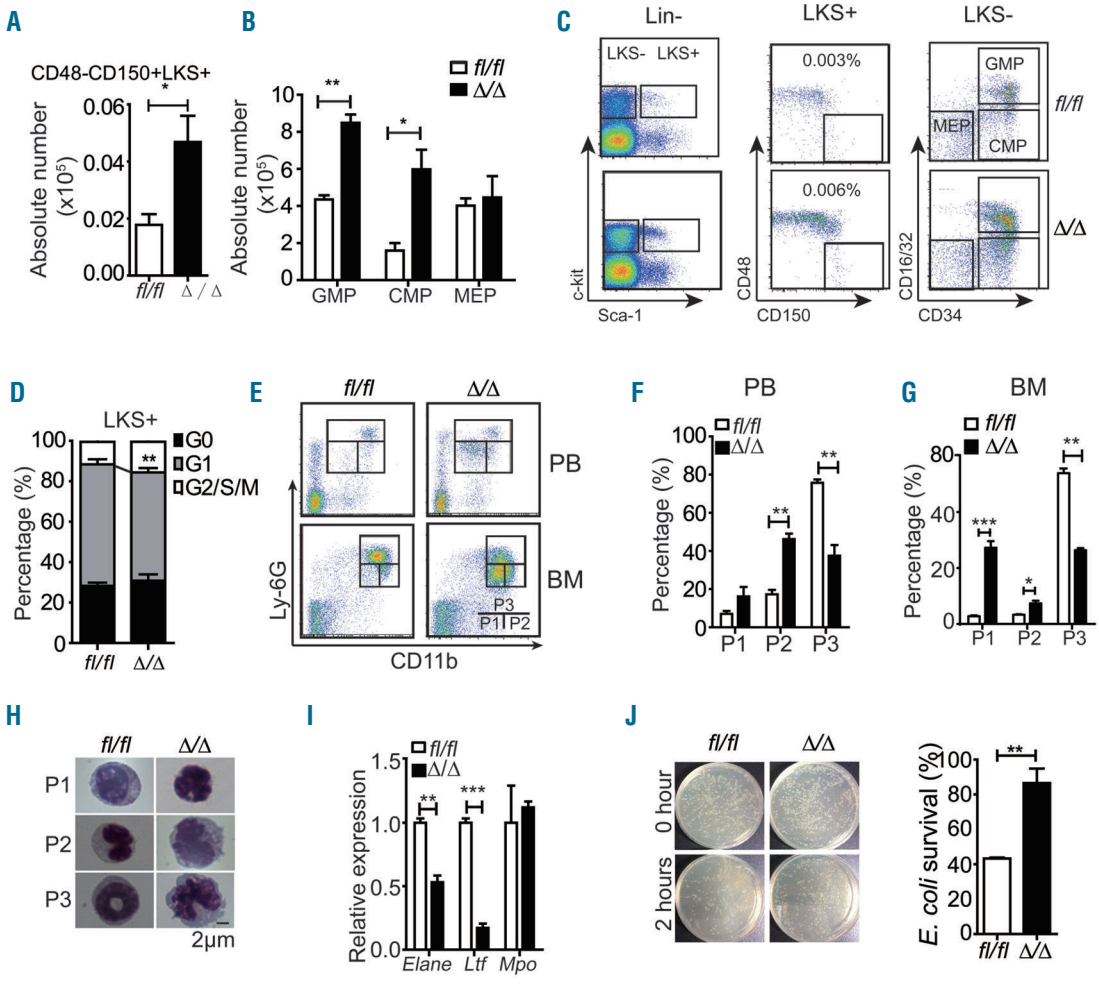

Figure 1. Rheb1 deletion increases the number of hematopoietic stem cell/hematopoietic progenitor cells and immature neutrophils in bone marrow (BM) of *Rheb1*$^{\Delta/\Delta}$ mice. Whole BM cells were isolated from BM of tibias, femurs and ilia of 8-week old mice. (A) The absolute number of CD48$^-$CD150$^+$LKS$^+$ population in the BM of *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ mice; n=3. (B) The absolute cell number of GMP, CMP and MEP populations in the BM of *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ mice, n=3. (C) FACS analysis of LKS$^+$ cells and LKS$^-$ in the BM; n=3. (D) Cell cycle status of *Rheb1*$^{fl/fl}$ or *Rheb1*$^{\Delta/\Delta}$ LKS$^+$; n=3. (E) FACS analysis of neutrophils in *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ PB and BM, n=3. (F and G) The percentages of neutrophil subpopulations in *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ PB and BM; n=3. (H) Representative images of Wright-Giemsa staining of the sorted P1-P3 subpopulations of neutrophils from BM. (I) qRT-PCR analysis for the mRNA expression of granule proteins in neutrophils. Elane: neutrophil elastase; Ltf: lactotransferrin precursor; Mpo: myeloperoxidase; n=3. (J) The *E. coli* survival rates after co-culturing with neutrophils; n=3. Data are presented as mean±Standard Error of Mean. *P<0.05; **P<0.01; ***P<0.001.

nuclei. Interestingly, the P3 subpopulation of *Rheb1*$^{\Delta/\Delta}$ neutrophils was composed of cells with butterfly-shaped nuclei (Figure 1H and *Online Supplementary Figure S2D*). The expression levels of Ltf and Elane (encoding granule proteins) were greatly reduced in *Rheb1*$^{\Delta/\Delta}$ neutrophils from BM (Figure 1I), further indicating the reduced maturity of *Rheb1*$^{\Delta/\Delta}$ neutrophils. The bacterial survival assay showed that *Rheb1*$^{\Delta/\Delta}$ neutrophils killed less than 10% of the bacteria, while *Rheb1*$^{fl/fl}$ neutrophils killed more than 60% of the bacteria (Figure 1J). These results suggest that Rheb1 deficiency caused neutrophil immaturity.

Rheb1 deletion induced extramedullary hematopoiesis in the spleen

Physical examinations revealed that *Rheb1*$^{\Delta/\Delta}$ mice displayed splenomegaly as demonstrated by the increase of their spleen size and weight (Figure 2A and B). Histopathological examination showed evident extramedullary hematopoiesis, including clustered megakaryocytes (Figure 2C, arrowheads) and hematopoietic islands (Figure 2C, arrows) in *Rheb1*$^{\Delta/\Delta}$ mice. However, liver and lung examination showed no significant differences between *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ mice (*Online Supplementary Figure S2E* and *F*). FACS analysis confirmed splenic extramedullary hematopoiesis, characterized by an increase in the percentages of LKS$^+$ (HSCs) and LKS$^-$ (HPCs) cells (Figure 2D and E) and a marked increase in the CD34$^+$LKS$^-$ (GMP/CMPs) populations (Figure 2F). The proportion of myeloid cells was also increased, while those of T and B cells were reduced in *Rheb1*$^{\Delta/\Delta}$ spleens when compared to those of the control spleens (Figure 2G). Moreover, FACS showed an increase in the percentage of neutrophils in *Rheb1*$^{\Delta/\Delta}$ spleens (Figure 2H), in agreement with granulocytic expansion in the PB in comparison with the controls. Collectively, our results demonstrated that Rheb1 deficiency induces extramedullary hematopoiesis in the spleen to compensate the hematopoiesis defect in the BM.

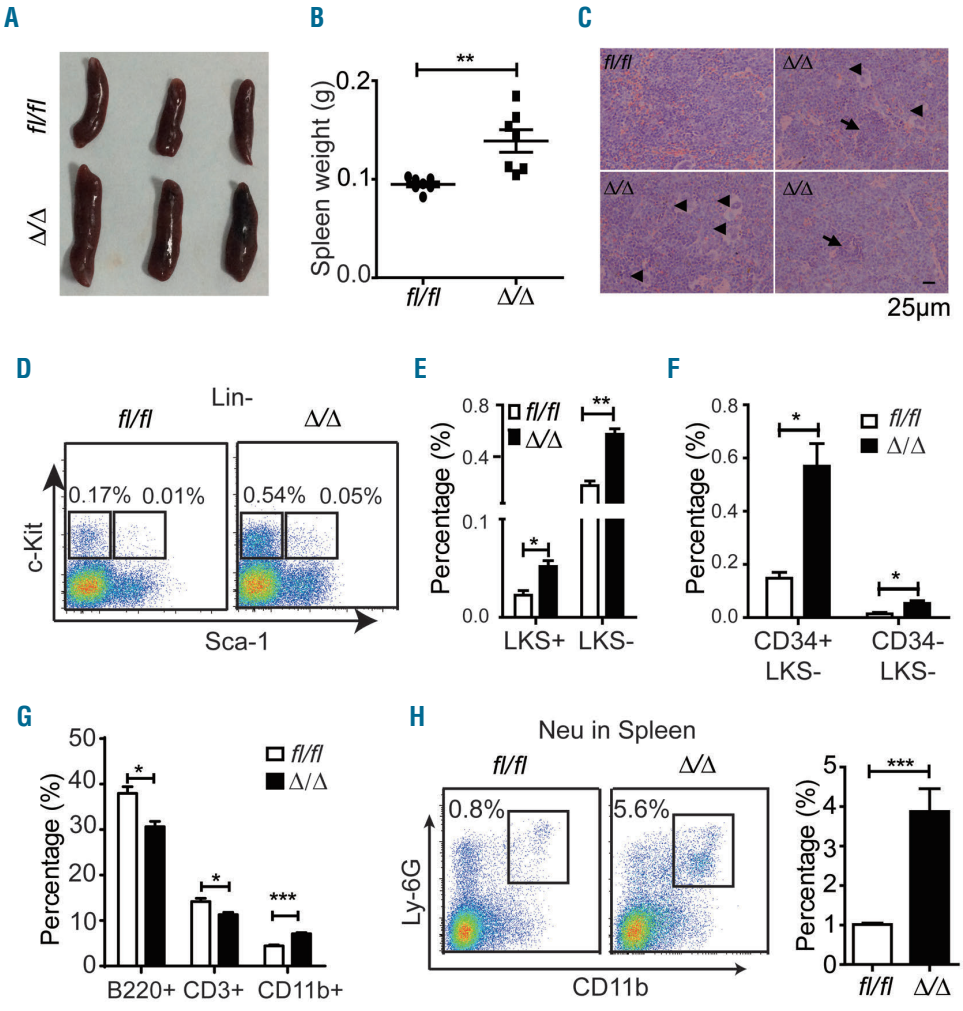

Figure 2. *Rheb1*$^{Δ/Δ}$ mice display extramedullary hematopoiesis in the spleen. (A and B) Size and weight of spleens in 8-week old *Rheb1*$^{fl/fl}$ and *Rheb1*$^{Δ/Δ}$ mice. Data are presented as mean±Standard Error of Mean (SEM); n>6. (C) Hematoxylin & eosin-stained spleen sections of *Rheb1*$^{fl/fl}$ and *Rheb1*$^{Δ/Δ}$ mice. Arrowheads indicate megakaryocytes; arrows point to hematopoietic islands. (D-F) Percentages of LKS$^+$ subsets, LKS$^-$ subsets and corresponding subpopulations in *Rheb1*$^{fl/fl}$ and *Rheb1*$^{Δ/Δ}$ spleens; n=3. (G) Percentages of B cells (B220$^+$), T cells (CD3$^+$) and myeloid cells (CD11b$^+$) in *Rheb1*$^{fl/fl}$ and *Rheb1*$^{Δ/Δ}$ spleens; n=3. (H) Percentages of neutrophils in *Rheb1*$^{fl/fl}$ and *Rheb1*$^{Δ/Δ}$ spleens; n=3. Data are presented as mean±SEM. **P*<0.05; ***P*<0.01; ****P*<0.001.

Rheb1 deletion impaired HSC regeneration ability in transplant assay

To assess the role of Rheb1 in HSC function during hematopoiesis, we transplanted *Rheb1*$^{Δ/Δ}$ BM cells (CD45.1) with competitive cells (CD45.2) into lethally irradiated recipient mice (CD45.2) to examine the role of Rheb1 in adult HSC reconstitution. All lineages derived from *Rheb1*$^{Δ/Δ}$ cells were significantly reduced in the PB and the BM after transplantation (Figure 3A and B, and *Online Supplementary Figure S3A*), while the homing capacity of transplanted *Rheb1*$^{Δ/Δ}$ CFSE$^+$ labeled cells was equivalent to that of *Rheb1*$^{fl/fl}$ cells (Figure 3C).

In addition, *Rheb1*$^{fl/fl}$ and *Rheb1*$^{Δ/Δ}$ mice were subjected to 4 Gy of X-ray irradiation and we examined the effects of Rheb1 deficiency on HSCs under hematopoietic stress. Interestingly, all *Rheb1*$^{fl/fl}$ mice survived while most *Rheb1*$^{Δ/Δ}$ mice died, probably due to impaired recovery of the BM and thymic cellularity (Figure 3D-F) and impaired B lymphopoiesis in the BM and spleen (Figure 3G). The absolute number of *Rheb1*$^{Δ/Δ}$ HS/PCs was reduced compared with *Rheb1*$^{fl/fl}$ HS/PCs at 21 days after 4 Gy irradiation (*Online Supplementary Figure S3B*). Thus, a delicate balance of developmental decisions for HSC homeostasis, including stem cell quiescence, self-renewal and differentiation, was maintained under steady state conditions in *Rheb1*$^{Δ/Δ}$ mice but was broken under hematopoietic stress.

We further examined the self-renewal and differentiation capacities of HSCs using LKS$^+$ transplantation. A total of 200 LKS$^+$ cells isolated from *Rheb1*$^{fl/fl}$ and *Rheb1*$^{Δ/Δ}$ mice (CD45.1) together with 5x10^5 WBMCs (CD45.2) were intravenously injected into lethally irradiated recipient mice. The chimerism in PB was analyzed every four weeks post transplantation. The repopulating capacity of *Rheb1*$^{Δ/Δ}$ LKS$^+$ cells in the PB was significantly lower in recipient mice than the control LKS$^+$ cells (Figure 3H). All lineages derived from *Rheb1*$^{Δ/Δ}$ HSCs were significantly reduced in the PB and the BM after transplantation (*Online Supplementary Figure S3C*). The recipient mice were sacrificed at four months after transplantation and

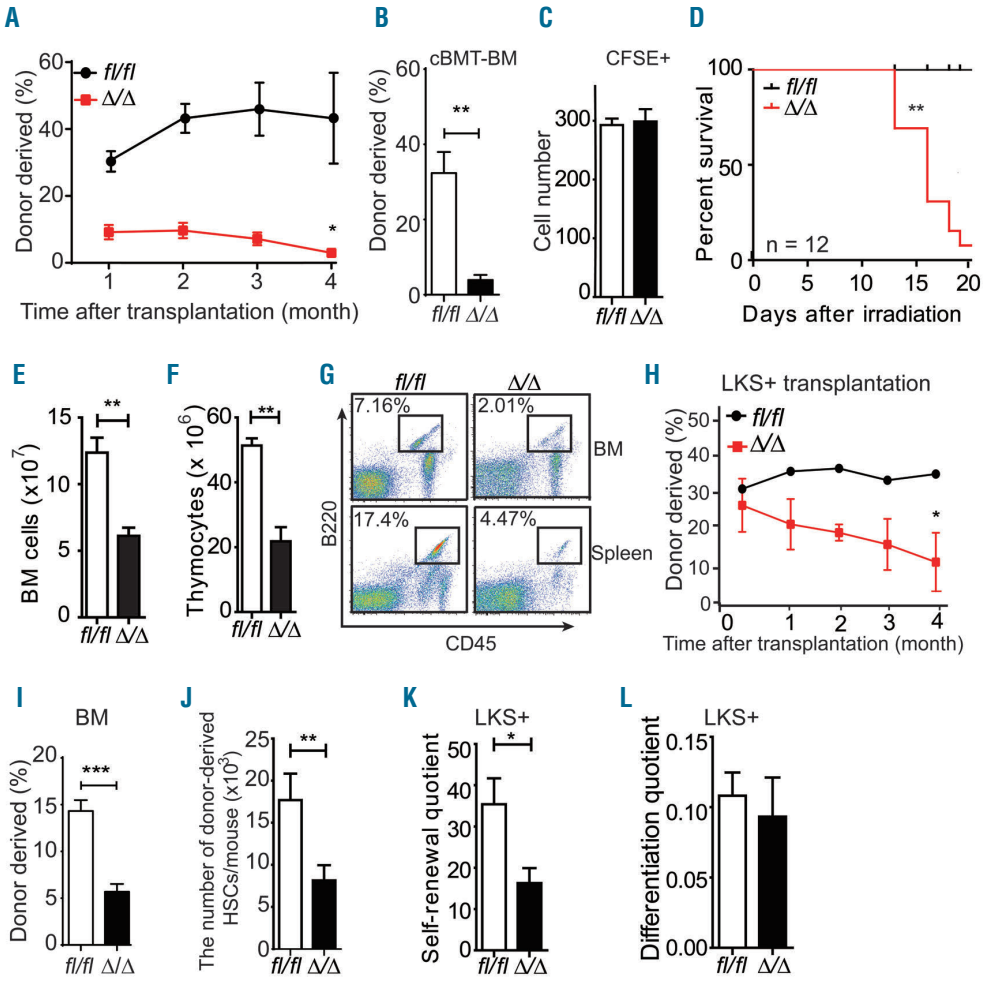

Figure 3. Rheb1 deletion impaired hematopoietic stem cell (HSC) regeneration upon transplantation. (A) Donor-derived-cell chimerism in peripheral blood (PB) of mice transplanted with *Rheb1*$^{fl/fl}$ or *Rheb1*$^{Δ/Δ}$ whole bone marrow cells (WBMC); n=6. (B) Percentage of donor-derived-cells in the BM four months after mice were transplanted with *Rheb1*$^{fl/fl}$ or *Rheb1*$^{Δ/Δ}$ WBMCs; n=6. (C) Homing of CFSE$^+$ 7-AAD- LKS$^+$ cells to the BM 24 hours (h) post transplantation; n=3. (D) Kaplan-Meier survival curve of *Rheb1*$^{fl/fl}$ and *Rheb1*$^{Δ/Δ}$ mice that underwent sublethal irradiation; n=12. (E and F) Absolute number of cells (CD45.1$^+$) in BM and thymus of *Rheb1*$^{fl/fl}$ and *Rheb1*$^{Δ/Δ}$ mice under sublethal irradiation; n=3. (G) Percentage of B cells (B220$^+$) from the spleens and BM of mice under sublethal irradiation; n=3. (H) Donor-derived-cell chimerism in the PB of mice transplanted with *Rheb1*$^{fl/fl}$ or *Rheb1*$^{Δ/Δ}$ LKS$^+$ cells; n=6. (I) Percentage of donor-derived cells in the BM four months after mice were transplanted with *Rheb1*$^{fl/fl}$ and *Rheb1*$^{Δ/Δ}$ LKS$^+$ cells; n=6. (J) Absolute number of donor-derived LKS$^+$ cells in the BM per mouse; n=6. (K and L) Self-renewal and differentiation quotients of donor-derived LKS$^+$ cells; n=6. Data are presented as mean±Standard Error of Mean. *P<0.05; **P<0.01;

their donor-derived HSCs in BM were analyzed. We found the donor-derived *Rheb1*$^{Δ/Δ}$ cells decreased significantly in the BM at four months after transplantation (Figure 3I). To quantify the function of HSCs, we calculated the LKS$^+$ cell amplification and differentiation ability four months after transplantation.[20] We found that the number of *Rheb1*$^{Δ/Δ}$ LKS$^+$-derived HSCs was substantially lower than that of the control (Figure 3J). The self-renewal quotient (the number of donor-derived HSCs recovered at the end of the transplant per original input HSC) was also significantly reduced in mice receiving *Rheb1*$^{Δ/Δ}$ LKS$^+$ cells (Figure 3K), while there was no change in the differentiation quotient (WBC count/μL blood x the percentage test-cell blood chimerism/number of donor-derived HSCs) when compared with the control (Figure 3L). Rheb1 deficiency thus impaired the ability of HSCs to repopulate under hematopoietic stress due to reduced self-renewal capability.

Aged *Rheb1*$^{Δ/Δ}$ mice develop myeloproliferative disorders

Since adult mice lacking Rheb1 have impaired HSC function and splenic extramedullary hematopoiesis, we went on to perform a detailed phenotypic analysis to investigate whether Rheb1 deletion leads to progressive hematopoiesis defects in aged *Rheb1*$^{Δ/Δ}$ mice up to two years of age. We found the survival time of aged *Rheb1*$^{Δ/Δ}$ mice was significantly shorter than that of littermate controls (Figure 4A). Furthermore, *Rheb1*$^{Δ/Δ}$ mice, but not *Rheb1*$^{fl/fl}$ littermates, had evident progressive leukocytosis. Neutrophil count in PB was significantly increased in *Rheb1*$^{Δ/Δ}$ mice (Figure 4B). Peripheral blood analysis revealed that the percentage of neutrophils was also increased in *Rheb1*$^{Δ/Δ}$ mice (*Online Supplementary Figure S4A*). The number of RBC and Hgb was normal in *Rheb1*$^{Δ/Δ}$ mice while the number of PLTs was decreased in *Rheb1*$^{Δ/Δ}$ mice (*Online Supplementary Figure S3B*). Myeloproliferative

disorders were apparent in both BM and PB as defined by myeloid left shift (presence of blasts, promyelocytes, myelocytes, or metamyelocytes) (Figure 4C and D). In addition, Rheb1 deletion led to an expansion of HSCs and myeloid progenitors in BM (Figure 4E-G and *Online Supplementary Figure S4C* and *D*). Furthermore, we isolated BM cells and performed serial colony forming unit assays *in vitro*. We found the number of colonies formed by *Rheb1*$^{\Delta/\Delta}$ BM cells was increased when compared with *Rheb1*$^{fl/fl}$ BM cells both in first plating experiment and second serial replating experiment (Figure 4H and *Online Supplementary Figure S4E*). These data demonstrated that loss of Rheb1 increased proliferation ability of hematopoietic progenitor.

Aged *Rheb1*$^{\Delta/\Delta}$ mice had enlarged spleens in comparison to the littermate controls. Histopathological analysis revealed that *Rheb1*$^{\Delta/\Delta}$ mice developed splenomegaly, consistent with significant myeloproliferation disorder

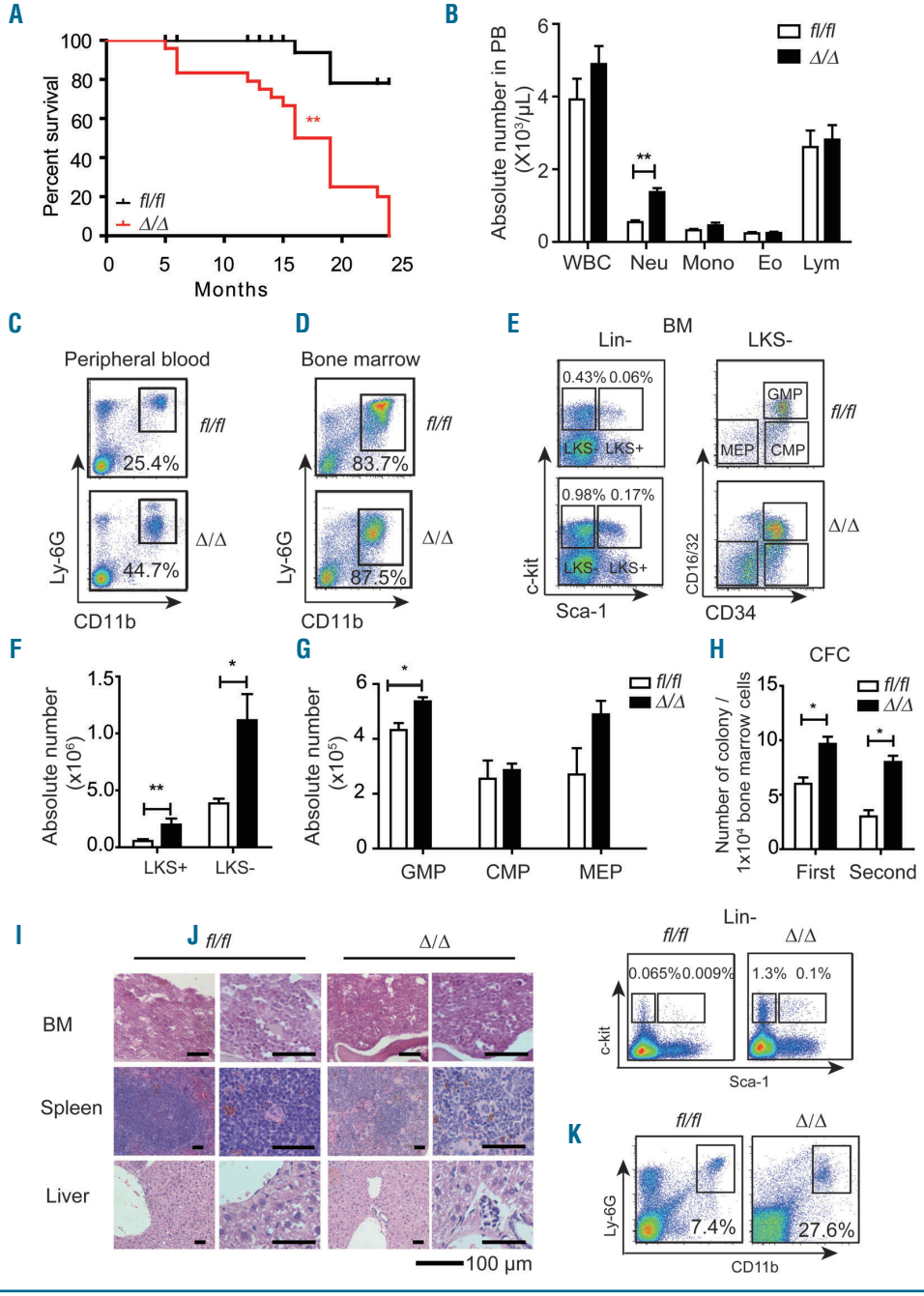

Figure 4. Rheb1 deletion leads to progressive myeloproliferation in bone marrow (BM) and extramedullary hematopoiesis in aged *Rheb1*$^{\Delta/\Delta}$ mice. (A) Survival curves of *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ mice (n=22). (B) The absolute number of different cell populations in peripheral blood (PB) of 2-year old *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ mice; n=3. (C and D) FACS analysis of granulocytes according to the expression levels of CD11b and Ly-6G in PB and bone marrow (BM); n=3. (E) FACS analysis of LKS^{+} cells and LKS^{-} in the BM; n=3. (F) The absolute number of LKS^{+} cells and LKS^{-} in the BM; n=3. (G) The absolute number of GMP, CMP and MEP populations in the BM of 2-year old *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ mice; n=3. (H) Whole BM cells were isolated from 2-year old mice and plated in M3434 methylcellulose. Colonies were counted seven days after plating, and serially replating; n=3. (I) Hematoxylin & eosin-stained BM, spleen and liver sections of 2-year old *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ mice. (J) FACS analysis of LKS^{+} cells and LKS^{-} in the spleen. (K) FACS analysis of granulocytes in the spleen. Data are presented as mean±Standard Error of Mean. *P<0.05; **P<0.01.

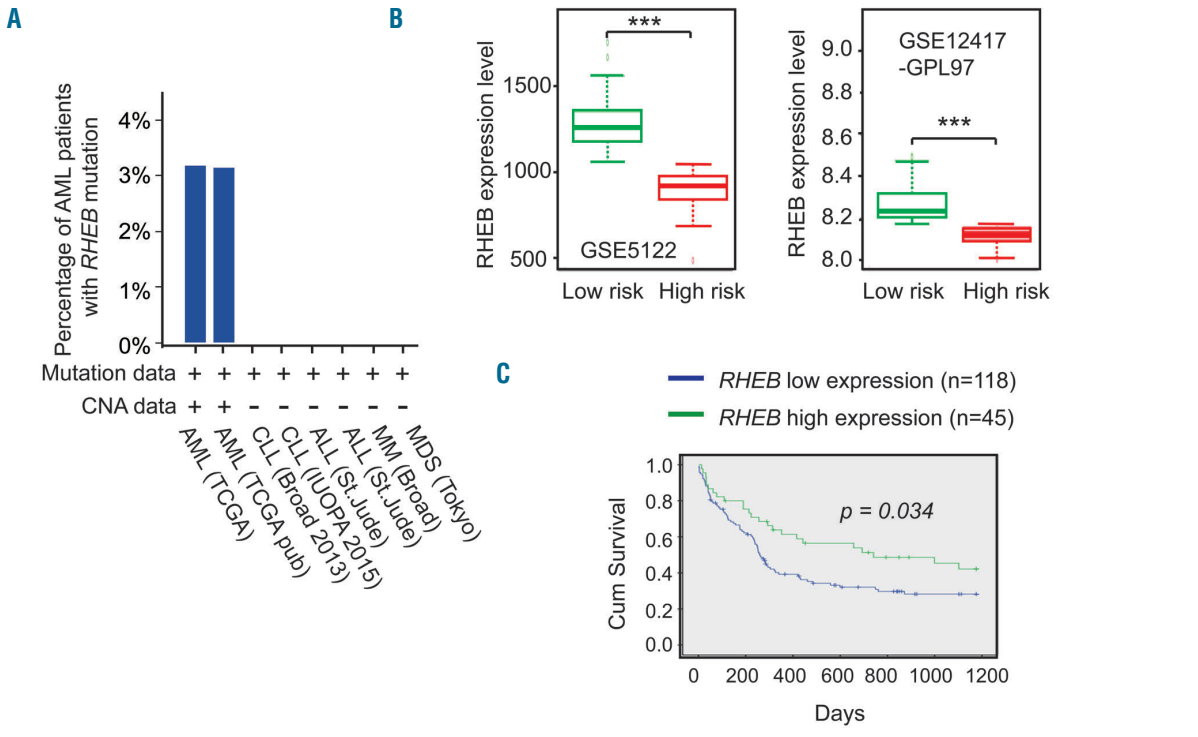

Figure 5. The low expression of RHEB is correlated with poor survival of acute myeloid leukemia (AML) patients with normal karyotype. (A) The percentage of loss-of-function mutations of RHEB in AML patients (*http://www.cbioportal.org/*). (B) The expression of RHEB in two sets of high-risk *versus* low-risk AML patients (*http://bioinformatica.mty.itesm.mx/SurvExpress; GSE5122 and GSE12417-GPL97*). (C) Cumulative (Cum) overall survival of AML patients with different expression levels of RHEB (*http://www.abren.net/PrognoScan-cgi/; GSE12417*). Data are presented as mean±Standard Error of Mean. ***P<0.001.

(*Online Supplementary Figure S4F*). Histopathological analysis revealed that *Rheb1*$^{\Delta/\Delta}$ mice had not only prominent myeloproliferative features including extramedullary hematopoiesis in the spleen, but also infiltration of liver and neutrophilia in BM (Figure 4I). FACS analysis of the spleen revealed a significant enlargement of both the HSC (LKS$^+$) and HPC populations (Figure 4J and *Online Supplementary Figure S4G*), and with granulocytic expansion (Figure 4K). Collectively, these data demonstrate that Rheb1 loss leads to progressive myeloproliferation disorder *in vivo*.

Low expression of RHEB was correlated with poor survival in AML patients with normal karyotype

The phenotypes observed in Rheb1 loss and progressive myeloproliferative disorder prompted us to investigate if RHEB also plays a role in human leukemia or myeloproliferative diseases. We used a curated database (*http://www.cbioportal.org/*), which provides large-scale cancer genomics data sets, to analyze the mutations and copy number alteration (CNA) of RHEB in leukemia patients. It was found that deep deleted mutations in RHEB were about 3% (6 of 188) in an AML patient cohort (Figure 5A). We also analyzed the mutation types in ICGA (international cancer genome consortium; *http://dcc.icgc.org/*) and found that the percentage of loss-of-function mutations in RHEB was 1.7% (2 of 117) in AML patients and 0.92% (2 of 218) in CLL patients (*Online Supplementary Figure S5A*). We then compared the expression of RHEB in AML patients with high-risk *versus* AML patients with low risk in SurvExpress (*http://bioinformatica.mty.itesm.mx/SurvExpress*). We found the expression of RHEB was significantly lower in AML patients with high risk than that of the low risk in two GSE set analyses (GSE5122 and GSE12417-GPL97; P<0.0001) (Figure 5B). We then collected prognostic values for 163 AML patients with normal karyotype in PrognoScan database (*http://www.abren.net/PrognoScan-cgi/, GSE12417*), and divided these patients into two groups for analysis: patients with RHEB expression above median and patients with RHEB expression below median (*Online Supplementary Figure S5B*). The survival curve showed that lower RHEB expression was associated with poor survival in AML patients with normal karyotype (log-rank test; P=0.034) (Figure 5C). These results indicated that loss of RHEB was correlated with AML progression.

3BDO partially rescued the defect of *Rheb1*$^{\Delta/\Delta}$ neutrophils

Intrigued by the underlying phenotypic plasticities observed in *Rheb1*$^{\Delta/\Delta}$ HSCs, we further explored the potential transcriptional changes associated with the phenotypes by microarray analysis. Differentially expressed genes between *Rheb1*$^{\Delta/\Delta}$ and *Rheb1*$^{fl/fl}$ HSCs were significantly enriched in pathways involved in cell adhesion and cell development (Figure 6A, and *Online Supplementary Tables S1* and *S2*), suggesting that complete loss of Rheb1 in hematopoietic stem cells affected the expression of genes involved in HSCs engraftment and differentiation. Gene set enrichment analysis (GSEA) of pairwise comparisons revealed that *Rheb1*$^{\Delta/\Delta}$ HSCs had a significant increase in the expression of genes associated with AML incidence

and development (Figure 6B). The analysis also suggests that *Rheb1*$^{\Delta/\Delta}$ HSCs adopt a transcriptional program similar to AML cells due to loss of Rheb1.

Furthermore, mTORC1 signaling pathway-related genes were also down-regulated due to Rheb1 deletion (Figure 6B). S6 ribosomal protein (Ser240/244) and 4E-BP1 (Thr37/46) are typical downstream targets of mTORC1. We found that p-S6 was reduced significantly in *Rheb1*$^{\Delta/\Delta}$ LKS$^+$ cells while p-4E-BP1 did not change by phosphorylation-flow analysis (Figure 6C). To determine whether reduced p-S6 causes HSCs differentiation to immature neutrophils, we cultured *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ LKS$^+$ cells with mTORC1 activator 3BDO in CFC assays to analyze the myeloid development. *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ BM cells were treated with 3BDO (60 nM) or DMSO for 30 min, then analyzed for phosphorylation level of S6 and sorted for LKS$^+$ cells seeding in M3231 medium. As expected, the p-S6 level in Rheb1-deficient BM cells was lower than that of wild-type (WT) BM cells. However, it was markedly increased with treatment of 3BDO in *Rheb1*$^{\Delta/\Delta}$ BM cells (Figure 6D). p-S6 level was also restored in *Rheb1*$^{\Delta/\Delta}$ Lin$^-$ cells with the treatment of 3BDO measured by phospho-flow analysis (Figure 6E). Interestingly, 3BDO addition in *Rheb1*$^{\Delta/\Delta}$ LKS$^+$ cell culture resulted in a decrease in the percentage of immature neutrophils when compared with non-treatment controls (Figure 6F and G). These data sug-

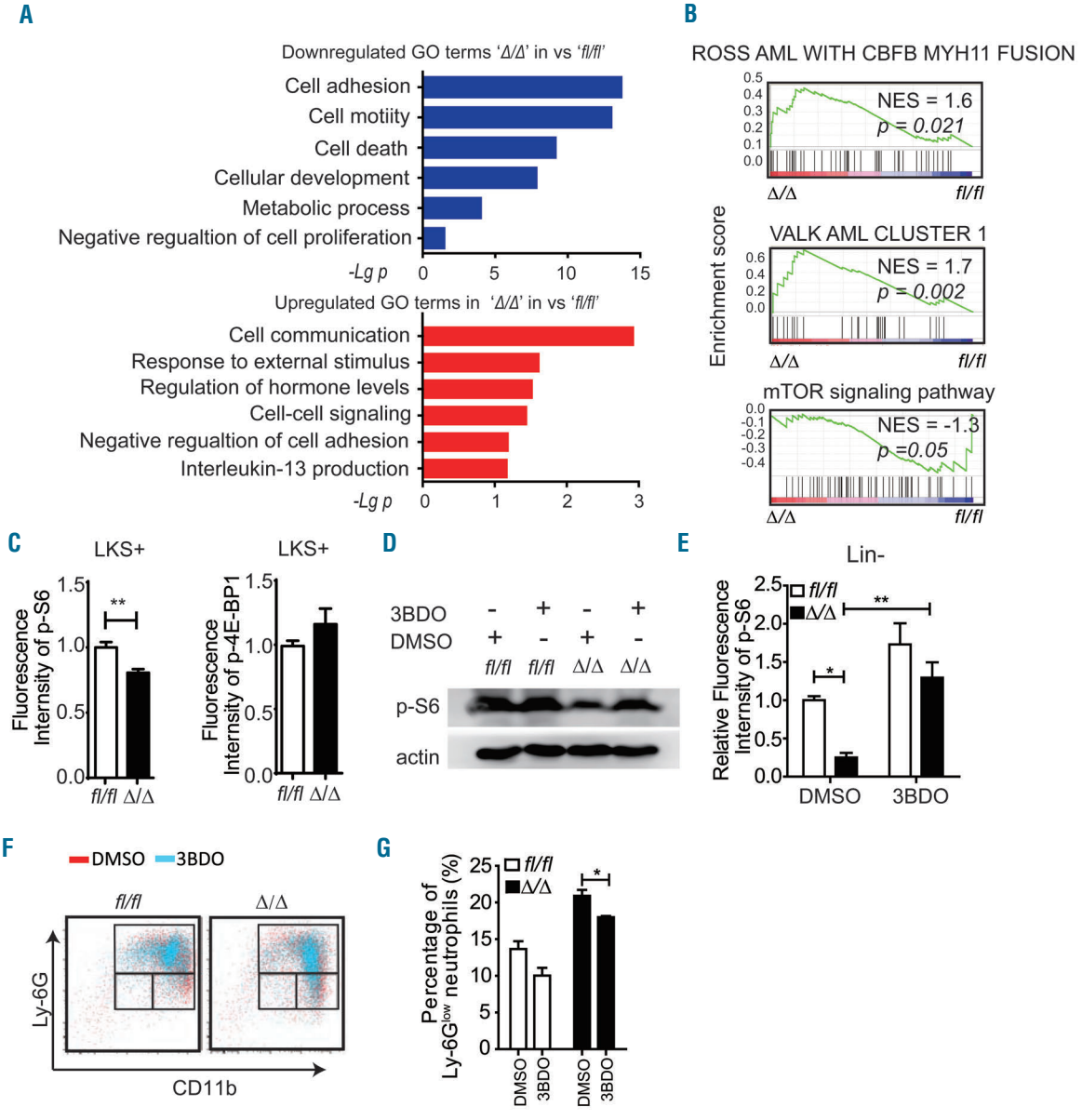

Figure 6. 3BDO restores the maturation of *Rheb1*$^{\Delta/\Delta}$ neutrophils. (A) Gene ontology (GO) enrichment analysis of genes down-regulated and up-regulated in the absence of Rheb1. (B) Enrichment plots of selected gene sets using Gene Set Enrichment analysis. (C) Relative fluorescence intensity of p-S6 and p-4E-BP1 in *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ LKS$^+$; n=3. (D) Protein level of p-S6 in *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ BM cells after 3BDO (60 nM) or DMSO treatment for 30 minutes (min). (E) Relative fluorescence intensity of p-S6 in *Rheb1*$^{fl/fl}$ and *Rheb1*$^{\Delta/\Delta}$ Lin$^-$ cells after 3BDO (60 nM) or DMSO treatment for 30 min; n=3. (F and G) FACS analysis of neutrophils and percentage of immature neutrophils in CFC assay after 3BDO (60 nM) or DMSO treatment for six days; n=3. Data are presented as mean±Standard Error of Mean. *P<0.05; **P<0.01.

gest that Rheb1 may regulate neutrophil differentiation partially through mTORC1 signaling pathway.

To investigate the function effects of 3BDO on *Rheb1*$^{\Delta/\Delta}$ HSCs, we performed a long-term culture assay *in vitro* and found that the CAFC (cobblestone area) formed by *Rheb1*$^{\Delta/\Delta}$ HSCs was similiar to that of the control in both 3BDO and DMSO groups (*Online Supplementary Figure S6A* and *B*).We further treated *Rheb1*$^{\Delta/\Delta}$ and *Rheb1*$^{fl/fl}$ BM cells with 3BDO or DMSO. Then, we transplanted *Rheb1*$^{\Delta/\Delta}$ and *Rheb1*$^{fl/fl}$ BM cells (CD45.1) together with competitive cells (CD45.2) to lethally irradiated mice. The chimerism was analyzed every four weeks after transplantation. We found that the repopulating capacity of *Rheb1*$^{\Delta/\Delta}$ BM cells in the PB was significantly lower when compared with *Rheb1*$^{fl/fl}$ cells, both in 3BDO and DMSO groups (*Online Supplementary Figure S6C* and *D*). These data indicated that Rheb1 may regulate HSCs engraftment independently of the mTORC1 signaling pathway.

Discussion

Although Rheb1 has been known to regulate TSC1/2 upstream of mTORC1, its role in the regulation of hematopoiesis is still not fully understood. The somatic loss-function mutation in the *RHEB* gene in AML patients also provides genetic evidence that mutational inactivation of *RHEB* might be a pathogenic event in myeloid malignancies (Figure 5). Notably, we showed here that AML patients with low RHEB expression had shorter survival time than AML patients with high RHEB expression, indicating that RHEB could be a prognosticator for leukemia patient survival. In a Rheb1 conditional deletion mouse model, we determined that loss of Rheb1 caused defective HSCs and an increased number of immature neutrophils in BM, accompanied by excessive extramedullary hematopoiesis in the spleen. In addition, Rheb1 loss leads to progressive myeloproliferation in aged *Rheb1*$^{\Delta/\Delta}$ mice. These data suggest that Rheb1 participates in proliferation and differentiation in myeloproliferative disease.[21] Our gene transcriptional expression data also reinforce the idea that Rheb1 loss leads to an increased expression of myeloid leukemia-related gene expression programs (Figure 6A and B). Further studies are needed to functionally dissect the downstream targets of Rheb1 that confer enhanced proliferation ability for stem/progenitor cells or LSCs.

In the steady-state condition, the majority of HSCs are in a quiescent state.[22] However, when mice are under hematopoietic stresses, such as transplantation, HSCs actively proliferate to generate progenitors that enable rapid hematologic regeneration.[23] Successful reconstitution upon transplantation of HSCs depends on multiple parameters, including correct homing to the BM, residing and proliferating successfully in the BM niche, the right cell-cycle status of the transplanted cells, and the adequate rate of apoptosis. Here, although *Rheb1*$^{\Delta/\Delta}$ mice could survive with increased HSCs and HPCs in BM and spleen to compensate for the loss of Rheb1 under steady-state conditions, *Rheb1*$^{\Delta/\Delta}$ HSCs were unable to reconstitute adult BM in transplantation. However, CAFC assay showed the proliferation capacity of *Rheb1*$^{\Delta/\Delta}$ HSCs was similar to that of *Rheb1*$^{fl/fl}$ HSCs *in vitro* (*Online Supplementary Figure S6A* and *B*). Our results thus suggested that the overproliferation of HSCs in *Rheb1*$^{\Delta/\Delta}$ mice may be caused by multiple factors including extrinsic factors such as BM microenvironment. Indeed, our gene transcriptional expression data showed that Rheb1 loss leads to a decrease in expression of adhesion-related gene expression programs (Figure 6A and B). Rheb1 may regulate HSC regeneration through non-canonical signaling pathways rather than being fully dependent on mTORC1 signaling pathway. This is consistant with the findings of Peng *et al.* that the proliferation ability of HSCs was impaired in Raptor-deficient HSCs (Raptor is a component of mTORC1 downstream of Rheb1), but not in Rheb1-deficient HSCs upon transplantation.[24] Interstingly, in Peng *et al.*'s model of TAM injection at eight weeks post transplantation of *Rheb1*$^{fl/fl}$;Rosa-CreERT2 BM cells into wild-type (WT) recipient mice, no significant difference was observed in the regeneration of donor cells when compared to WT competitor cells. This is also consistant with the idea that Rheb1 deficiency does not affect HSC proliferation.[24] It will be important to determine the contribution of Rheb in canonical and non-canonical signaling pathways in future studies.

Neutrophils are specially developed cells to provide a defense against bacterial infection and are essential for host survival. *Rheb1*$^{\Delta/\Delta}$ mice showed severe neutrophilia in PB with an increased percentage of immature neutrophils in the BM. The augmented GMP/CMPs may be the reason for the neutrophilia, as the absolute number of GMP/CMPs significantly increased in the BM and spleen of *Rheb1*$^{\Delta/\Delta}$ mice (Figures 1B and 2F). Although the number of neutrophils was higher in the PB and BM in *Rheb1*$^{\Delta/\Delta}$ mice when compared to the control (*Online Supplementary Figure S1A*), *Rheb1*$^{\Delta/\Delta}$ neutrophils could not kill bacteria effectively *in vitro* (Figure 1J). It has been reported that Raptor deficiency resulted in decreased numbers of Gr-1^{+}Mac-1^{+} (Gr-1^{+}CD11b^{+}) granulocytes.[25] However, our results showed that Rheb1 deletion led to an increase in the number of Ly-6G^{+}CD11b^{+} granulocytes. This discrepancy may be due to Ly-6G marking only a subset of Gr-1 cells, while Ly-6G is used for separating granulocytes from Mac-1^{+} myeloid cells. Furthermore, we found mTORC1 signaling was inhibited in *Rheb1*$^{\Delta/\Delta}$ progenitor cells, as evidenced by a reduced level of p-S6 (Figure 6C and D). A specific mTORC1 activator, 3BDO could only partially rescue Rheb1 deficiency-induced immature neutrophils, indicating Rheb1 regulates neutrophil development at least partially *via* the mTORC1 pathway. It is possible that Rheb1 regulates neutrophil differentiation *via* other signaling pathways. This possibility still needs to be explored in future studies.

We have shown that mutations or reduced expression of RHEB are associated with leukemia. Interestingly, although Rheb1 deficiency leads to an MPN-like disorder in aged *Rheb1*$^{\Delta/\Delta}$ mice, loss of Rheb1 shortens the life of *Rheb1*$^{\Delta/\Delta}$ mice but does not lead to spontaneous leukemia in *Rheb1*$^{\Delta/\Delta}$ mice in our observations (Figures 4 and 5). It is possible that the reduced survival of aged *Rheb1*$^{\Delta/\Delta}$ mice may be due to ineffective myelopoiesis-related inflammation. Although Rheb1-deficiency was not a key factor for leukemogenesis, low expression of Rheb1 does associate with the initiation of myeloid proliferation-related diseases, such as MPN. We predict that additional mutations and/or pro-leukemia development environment factors are needed to initiate leukemogenesis in *Rheb1*$^{\Delta/\Delta}$ hematopoietic cells.

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