The DEAH-box helicase *RHAU* is an essential gene and critical for mouse hematopoiesis

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The DEAH helicase RHAU (alias DHX36, G4R1) is the only helicase shown to have G-quadruplex (G4)–RNA resolvase activity and the major source of G4-DNA resolvase activity. Previous report showed RHAU mRNA expression to be elevated in human lymphoid and CD34+ BM cells, suggesting a potential role in hematopoiesis. Here, we generated a conditional knockout of the *RHAU* gene in mice. Germ line deletion of RHAU led to embryonic lethality. We then targeted the *RHAU* gene specifically in the hematopoiesis system, using a Cre-inducible system in which an optimized variant of Cre recombinase was expressed under the control of the *Vav1* promoter. RHAU deletion in hematopoietic system caused hemolytic anemia and differentiation defect at the proerythroblast stage. The partial differentiation block of proerythroblasts was because of a proliferation defect. Transcriptome analysis of RHAU knockout proerythroblasts showed that a statistically significant portion of the deregulated genes contain G4 motifs in their promoters. This suggests that RHAU may play a role in the regulation of gene expression that relies on its G4 resolvase activity. (*Blood. 2012;119(18):4291-4300*)

**Introduction**

Correct folding of RNA is critical for proper cellular functions such as transcription, splicing, RNA turnover, and translation initiation.1–3 These processes involve dynamic changes in covalent modification and spatial rearrangement between RNA and proteins. RNA chaperones, such as RNA helicases, facilitate the dynamic structural changes and regulate transitional states of RNA. Encoded by all living organisms, RNA helicases use energy released by NTP hydrolysis for unwinding RNA or remodeling protein–RNA complexes.4,5 Down-regulation or overexpression of some mammalian helicases or their homologs has evidenced their critical roles in growth and development.6–12 Deregulated expression of certain RNA helicases may also be linked to some diseases.13 However, the biologic activities of only a small number of mammalian RNA helicases have been studied so far.

*RHAU* (alias DHX36, G4R1), a member of the DEAH family, is evolutionary conserved with true orthologs in almost all animal species.14 Human and mouse RHAU, NP 065916.2 and NP 082412.2, respectively, share ≥ 92% similarity of amino acid sequence. The guanine quadruplex (G4) resolvase activity on both DNA and RNA substrates of RHAU is a unique characteristic among RNA helicases.14,15 RHAU was originally identified as a regulator of urokinase-type plasminogen activator mRNA stability, binding to an AU-rich element in the 3’ untranslated region,16 but further investigation suggested other functions. RHAU protein was found to be localized mainly in the nucleus, colocalized at nuclear speckles with the DEAD-box helicases p68 and p72, suggesting a nuclear function.17 In line with this, RNAi-mediated knockdown of RHAU in HeLa cells was shown to affect the transcription of various genes both positively and negatively.17 Recent reports have shown that RHAU knockdown in plasmacytoid dendritic cells interferes with viral DNA infection responses18 and that RHAU also interacts with the cytoplasmic domain of Toll-like receptor 1,19 suggesting roles in innate immunity. Thus, RHAU appears to be a multifunction protein.

Because RHAU is found only in metazoans, its function(s) appeared later in evolution. Global gene expression analysis of RHAU in humans and mice has shown ubiquitous expression, with predominately elevated levels in lymphoid and BM cells,20 suggesting a role in hematopoiesis. To address the role of RHAU in development and in hematopoiesis, we produced germ line as well as hematopoietic cell–specific conditional knockout mice. Here, we show that conventional RHAU ablation causes embryonic lethality without affecting implantation and that its ablation of this helicase in the hematopoietic system affects development of several lineages, suggesting critical and specific roles for RHAU in these processes.

**Methods**

**Generation of RHAU-targeted mice**

The detailed procedures are described in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Briefly, we used standard procedures for transgenic mouse generation and targeted the exon 8 of the mouse *DHX36* gene. Different conditional RHAU knockout mice were obtained by crossing to Meox2Cre, Mx-Cre, or Vav-iCre (optimized variant of Cre recombinase) lines. Animal experimentation was performed according to regulations effective in the canton of Basel-Stadt, Switzerland. Standard PCR and Western blot analysis were used for detection of RHAU knockout in tissues.
Whole blood counting

Blood was collected by tail bleeding into EDTA-coated BD Microtainer K2E tubes (BD PharMingen). Complete blood cell counting was performed in an Advia 120 Hematology Analyzer, and the results were analyzed by Multispecies Version 2.3.01-MS software (Bayer).

FACS analysis

Eight-week-old mice were used for all analysis. BM cells from femur and tibia were flushed with 3% FCS PBS (FACS buffer). Splenocytes and thymocytes were collected from whole spleen and thymus by pressing with a plunger through a 2-mL syringe into the FACS buffer. Spleen and BM cells were treated with red blood cell (RBC) lysis buffer for 7 minutes on ice and stained for different cell markers. A list of the reagents, target cells, and suppliers is given in supplemental Table 1.

Quantitation of erythropoietin plasma concentrations

Blood samples were collected by tail bleeding into 1.5-mL centrifuge tubes and allowed to clot for 16 hours at 4°C. Clotted blood was centrifuged at 2000 g for 20 minutes. The supernatant fluid was diluted with Calibrator Diluent RD62 provided together with the Mouse/Rat Epo (erythropoietin) Immunoassay kit (R&D Systems). The concentration of Epo in the samples was determined according to the instructions provided by the manufacturer.

BM transplantation

BM cells were collected from femora and tibiae of 8-week-old mice. Cells (3 x 10^6) in 200 μL of HBSS were injected into the tail vein of lethally irradiated (1100 cGy in 2 doses separated by 3 hours) 7- to 10-week-old mice. C57BL/6SJL-PpcrePep3/BoyJ (B6.CD45.1) mice and green fluorescent protein (GFP)-expressing C57BL/6-Tg(UBC-GFP)30Scha/J [Tg(GFP)] (UBC-GFP) mice were used as recipients. Five weeks after BM transplantation, recipient mice were treated with polyinosine-polycytidylic acid (polyI:C). Chimerism of recipient mice was analyzed with anti-CD45.2 peridinin chlorophyll protein complex with cyanine 5.5 (BD PharMingen). Efficiency of RHAU knockout was ∼98% in BM and ∼47% in the spleen as determined by genotyping of individual clones formed in a colony-forming assay.

BrdU incorporation assay

Each 8-week-old mouse was injected with 1 mg of BrdU in sterile solution. Eighteen hours after injection, BM cells were isolated and depleted of RBCs as for FACS analysis. One million cells were stained with anti-CD71–PE and anti–Ter-119–allophycocyanin Abs. Preparation of cells for BrdU detection was achieved with the use of the APC BrdU Flow Kit (BD PharMingen), according to the manufacturer’s instructions.

CFSE staining of splenocytes and cell cycle kinetic analysis

Freshly isolated, RBC-depleted splenocytes were stained with anti-CD71–PE and anti–Ter-119–allophycocyanin Abs. Dead cells were excluded with 4′,6-diamidino-2-phenylindole staining. Sorted proerythroblasts (ProEs; n = 20,000) were incubated with 1 μM CFSE (Sigma-Aldrich) in PBS with 5% FCS at room temperature for 5 minutes. Then cells were washed with IMDM containing 15% FCS, 1% BSA, 200 μg/mL holo-transferrin (Sigma-Aldrich), 10 μg/mL insulin (Sigma-Aldrich), 2mM l-glutamine, 0.4mM 2-mercaptoethanol (Gibco), and 2 U/mL human recombinant Epo (Sigma-Aldrich) for 72 hours before submitted for FACS analysis.

Results

Targeting of the RHAU gene in mice

To delete the RHAU gene, we designed a targeting vector in which 2 Cre recombinase recognition (loxP) sites flanked exon 8 of the gene as well as a neomycin-resistant gene cassette inserted downstream of exon 8, which itself was flanked by 2 Flippase recognition target sites. Deletion of this exon introduces a frameshift to yield a prematured termination codon that leads to the total decay of the message through a nonsense-mediated mRNA decay mechanism. In the targeted allele, recombination with CRE deletes both exon 8 and the neomycin cassette, whereas FLP deletes only the neomycin cassette (supplemental Figure 1A). The linearized vector was transfected into embryonic stem cells, and correctly targeted cells (clones 2 and 42) with the genotype RHAUfl,neo/fl were detected by Southern blot analysis (supplemental Figure 1B). PCR analysis of these clones with the use of the primer pairs mod4f7 and mod2f8 yielded the predicted products (supplemental Figure 1C). Corrected targeted embryonic stem cells were submitted for chimeric mice generation. Primer pair KO3f and KO3rev was used to distinguish RHAUfl/fl, RHAUfl,neo/− alleles (supplemental Figure 1D). RHAUfl/fl mice were derived by crossing RHAUfl,neo/+ with Meox-CRE mice, whereas RHAUfl/fl mice were obtained by first deleting the neo cassette from RHAUfl,neo/+ by crossing with ROSA26Fipo mice and then interbreeding with RHAUfl/fl mice as described in supplemental Methods, Experimental procedures.

Conventional RHAU knockout in mouse is embryonically lethal

Inbreeding of RHAUfl/fl mice produced either RHAUfl/fl or RHAUfl/fl but no RHAUfl/fl offspring, indicating that RHAU ablation causes embryonic lethality. RHAUfl+/fl pups had a wild-type phenotype and developed normally. RHAUfl/fl × RHAUfl+/− crosses yielded degenerated fetuses at 11.5 days postcoitum (dpc), but genotyping was not possible because of poor recovery of material. At 8.5 dpc, DNA samples of degenerated fetuses were recovered and genotyped as RHAUfl/fl (supplemental Table 2). Structural differences between RHAUfl/fl and RHAUfl/fl embryos were observed already at 7.5 dpc (supplemental Figure 2). However, at 6.5 dpc, structurally degenerate embryos were not observed, although some embryos were RHAUfl/fl (supplemental Table 2). Therefore, RHAUfl/fl embryos died at ∼7.0 dpc.

RHAU gene ablation in the hematopoietic system results in anemia

Given the early embryonic lethality of RHAUfl/fl embryos, the role of RHAU in the hematopoietic system was studied by conditional knockout in vav-iCre mice. These mice show specific Cre recombinase activity in all hematopoietic lineages, beginning with the hematopoietic stem cells. In contrast to RHAUfl/fl pups, RHAUfl/fl,vav-iCre pups were viable with normal Mendelian ratio (RHAUfl/fl,vav-iCre/RHAUfl/fl is 1:1 ratio; χ^2 value = 32; P < .01). RHAUfl/fl,vav-iCre pups were born with pale color, suggesting that the mutant mice are anemic (Figure 1A). Western blot analysis confirmed the efficient and specific ablation of the RHAU protein in RHAUfl/fl,vav-iCre mice in hematopoietic organs (Figure 1B). The RHAU exon 8 was shown to be deleted in Lin IL7Rα−c-Kit−Sca-1−Thy1.1+ HSCs that had undergone FACS (Figure 1B). Adult RHAUfl/fl,vav-iCre mice developed splenomegaly and hepatomegaly (Figure 1C–D). Accordingly, the livers and kidneys of RHAUfl/fl,vav-iCre mice were relatively pale in color. In addition, RHAUfl/fl,vav-iCre mice developed thymic hypoplasia (Figure 1C–D). Furthermore, in standard H&E staining (supplemental Methods), distinct structures of red and white pulp were absent in spleens (Figure 1E), and in contrast there were no obvious structural changes in the thymus (Figure 1E).
RHAU ablation causes decrease in RBCs

To verify the anemic appearance observed in RHAU-ablated mice, peripheral blood samples from 8-week-old RHAU^{fl/fl}, RHAU^{fl/+}:vav-iCre, and RHAU^{fl/fl:vav-iCre} mice were analyzed. As expected, RHAU^{fl/fl:vav-iCre} mice exhibited anemia that was evidenced by typical symptoms, such as lowered RBC count, decreased hemoglobin (Hb) level, and increased mean corpuscular volume (MCV), among others (Figure 2A; supplemental Table 3 group A). Because the phenotypes of these mice did not distinguish whether the anemic phenotype in RHAU^{fl/+:vav-iCre} was because of an intrinsic or extrinsic effect, we generated an IFN-inducible RHAU knockout mouse model to address this issue by BM transplantation experiments.

Anemic phenotype of RHAU^{fl/fl:vav-iCre} mice is an intrinsic effect

In addition, 2 BM transplantation experiments were performed to test whether the phenotypes observed in RHAU^{fl/fl:vav-iCre} mice were cell autonomous and independent of the hematopoietic microenvironment. First, RHAU^{fl/+:vav-iCre} and RHAU^{fl/fl:vav-iCre} mice were used as donors and UBC-GFP and BL6.CD45.1 mice as recipients. IFN induction of MxCre recombinase activity was achieved by injecting mice with polyI:C after transplantation. Complete blood analysis was performed periodically (Figure 2B; supplemental Table 3 groups A, B, C1, and C2).

When we compared blood of control and targeted mice, we found that mice responded to RHAU ablation consistently in 11 parameters (supplemental Table 3). First, all RHAU-ablated mice were anemic with low RBC count and lowered Hb and hematocrit counts. In addition, anemic mice developed reticulocytosis, as shown by an increase in the proportion of reticulocytes and increased MCV (Figure 2B; supplemental Table 3). All mice had reductions in platelets and white blood cell (WBC) counts, the latter being mainly because of reduced lymphocytes (Figure 2B; supplemental Table 3). Because the phenotypes of mice transplanted with RHAU-ablated BM cells and RHAU^{fl/fl:vav-iCre} mice were similar, the effects of RHAU ablation in RHAU^{fl/fl:3:vav-iCre} mice appeared to be cell autonomous, and these mice were used for further studies of the effects of RHAU ablation on hematopoiesis.
Hemolytic anemia and ineffective erythropoiesis in RHAU\textsuperscript{fl/fl}:vav-iCre mice

RHAU-ablated mice developed anemia accompanied by elevation of the reticulocyte ratios (supplemental Table 3). Such reticulocytosis is induced by Epo signaling, and an increase in Epo is commonly observed in the blood of anemic animals, whereby it compensates for the reduction in erythrocytes.\textsuperscript{25} The plasma Epo level was 800-fold higher in RHAU\textsuperscript{fl/fl}:vav-iCre mice than in control RHAU\textsuperscript{fl/fl} mice (Figure 3A); thus, the reticulocytosis observed here was a secondary effect of RHAU ablation. We also observed spherocytes in the blood of RHAU\textsuperscript{fl/fl}:vav-iCre mice (Figure 3B) and found that the half-life of erythrocytes in RHAU\textsuperscript{fl/fl}:vav-iCre mice was reduced from 20 days to \textsuperscript{10} days (Figure 3C). Furthermore, RHAU\textsuperscript{fl/fl}:vav-iCre mice developed enlarged spleens (Figure 1). These symptoms indicate hemolytic anemia.\textsuperscript{26}

Although the shortened half-life of erythrocytes could explain the decreased RBC counts in RHAU\textsuperscript{fl/fl}:vav-iCre mice, the total reticulocyte count was not significantly enhanced in these mice, which strikingly developed an 800-fold elevated Epo level (supplemental Table 3). Because an elevated Epo level is expected to promote erythropoiesis through increasing the progenitor population, the response to Epo of RHAU\textsuperscript{fl/fl}:vav-iCre mice might be impaired.\textsuperscript{27} We therefore examined the different subsets of erythroblasts that correspond to different stages of maturation. ProE is the earliest progenitor that is completely committed to erythropoiesis and undergoes maturation through the following stages: basophilic erythroblast, late basophilic erythroblast, polychromatic erythroblast, orthochromatic erythroblast, and, finally, reticulocyte that is enucleated and released into the blood stream.\textsuperscript{28} Erythroblast subpopulations can be well defined by FACS according to cell size and the expression of Ter-119 and CD71 (Figure 4A).\textsuperscript{28} Ter119med CD71\textsuperscript{hi} cells correspond to the ProE fraction. The high Ter-119 subpopulation was further analyzed by CD71 and forward scatter parameter to identify 3 erythroblast subpopulations, Ery.A, Ery.B, and Ery.C. Previous reports determined that basophilic erythroblasts contribute to Ery.A, late basophilic and polychromatic erythroblasts contribute to Ery.B, whereas orthochromatic erythroblasts contribute to Ery.B, whereas orthochromatic erythroblasts and reticulocytes contribute to Ery.C.\textsuperscript{28} Examination of erythroblasts in BM of RHAU\textsuperscript{fl/fl}:vav-iCre mice found dramatic increases in the proportions of ProE and Ery.A fractions (Figure 4B); in contrast, Ery.B and Ery.C decreased (Figure 4B). In the spleen, the ProE and Ery.A fractions increased, whereas Ery.B did not change and Ery.C decreased (Figure 4B). Thus, the increase in the proportions of ProE and Ery.A in both BM and spleen indicated a partial differentiation block at an early stage of erythropoiesis.

Figure 2. RHAU ablation leads to anemia. (A) Dot plots of total RBC counts, Hb values, and MCV from total blood count analysis from RHAU\textsuperscript{fl/fl} and RHAU\textsuperscript{fl/fl}:vav-iCre mice. (B) Dot plots of RBC, Hb, and MCV data from RHAU\textsuperscript{fl/fl} and RHAU\textsuperscript{fl/fl}:vav-iCre mice and recipient mice of BM transplantation assays. Genotypes of mice are shown in the table. The data points are presented in supplemental Table 3. \textsuperscript{*}P < .05; n = 3. • represents control conditions; and ○, RHAU-ablated condition.
With respect to total erythroblast counts, BM and spleen responded differently (Figure 4C). In the BM, total erythroblasts decreased, whereby ProE increased and Ery.B and Ery.C decreased significantly. In contrast, total erythroblasts increased dramatically 4.7-fold in spleen, with all subgroups contributing to the increase.

**Figure 4**. Differential effects of RHAU ablation on erythroid cell differentiation in the BM and spleen. (A-B) The different developmental stages were identified by FACS with the use of Ter-119 and CD71 markers together with the forward scatter (FSC) parameter. Ter119+ cells are classified into the following 4 subgroups: ProE (Ter119+CD71hi), Ery.A (Ter119+CD71hiFSClo), Ery.B (Ter119+CD71hiFSCm), and Ery.C (Ter119+CD71mFSCm). The mean proportions of each subgroup ± SEs (n = 4) are shown beside the boxes in the dot plots. (C) Effect of RHAU ablation on total numbers of erythroblasts in the BM and spleen. Cell numbers of the 4 subgroups are indicated in the bars by different colors, denoting, from the top, Pro.E, Ery.A, Ery.B, and Ery.C. *P < .05; n = 4.

**Ineffective differentiation of progenitors and stem cells in RHAUfl/fl:vav-iCre mice**

In response to elevated Epo levels in RHAU-ablated mice, ProE cells increased in number by 3.6- and 74-fold in BM and spleen, respectively (Figure 5A). However, RHAU ablation resulted in a progressive decline in the fold change of Ery.A, Ery.B, and Ery.C cells compared with the control in both BM and spleen (Figure 5A). Therefore, RHAU ablation led to progressive differentiation defects in erythropoiesis that are most prominent at the ProE stage.

Hematopoiesis is a stepwise process, and the long-term repopulating HSCs (LT-HSCs) give rise to all different hematopoietic cells through different stages of maturation.29 Because RHAU ablation led to differentiation defects at the ProE stage, we speculated whether lack of RHAU would also affect other progenitors in the hematopoietic system. To examine this we performed FACS analysis of BM using Abs directed against markers specific for early hematopoietic differentiation stages, including megakaryocyte/erythroid (MEPs), granulocyte/macrophage progenitors (GMPs), common myeloid progenitors (CMPs), lymphoid primed multipotent progenitors (LMPPs), short-term repopulating HSCs (ST-HSCs), and LT-HSCs. Indeed, a similar differentiation defect was also seen in the other hematopoietic progenitors in the BM. Figure 6A-B shows the gating of CMPs, MEPs, GMPs, LMPPs, ST-HSCs, and LT-HSCs. According to the percentage of the gated cells, the absolute numbers of different populations were calculated and presented as bar charts. RHAU ablation had no effect on CMP total cell number, either in the BM or spleen, but caused a significant decrease in GMP cell number in the BM and an increase in the MEP cell number in spleen (Figure 6B). For earlier progenitors, both LT-HSC and ST-HSC numbers increased, but LMPPs did not change compared with the control mice (Figure 6D).

Along the differentiation path, LT-HSCs differentiate into ST-HSCs, and ST-HSCs differentiate into MPPs that differentiate into either LMPPs or CMPs.30 Here, we observed a progressive decrease in the proportions of LT-HSCs, ST-HSCs, and LMPPs in RHAU-ablated mice compared with the control mice (Figure 5B). At the beginning, the RHAUfl/fl:vav-iCre mice developed 5.8-fold more LT-HSCs than control mice. However, this increase in number disappears in the later stages of differentiation and finally showed no difference to the control mice at the LMP and CMP stages (Figure 5B). This suggests that RHAU ablation impairs population expansion and reduces the differentiation potential of
HSCs. Competitive BM transplantation experiments were performed to verify that the reduction in population expansion and differentiation potential of HSCs were caused by RHAU ablation in these cells. Lethally irradiated BL6.CD45.1 mice were used as recipients for mixtures of BM cells composed either of UBC-GFP and RHAUfl/fl or UBC-GFP and RHAUfl/fl:MxCre, both in ratios of 1:10. The mice were then divided into 3 groups. Group 1 consisted of recipients transplanted with UBC-GFP and RHAUfl/fl cells and treated with polyI:C, group 2 consisted of recipients transplanted with UBC-GFP and RHAUfl/fl:MxCre cells and treated with polyI:C, and group 3 consisted of recipients transplanted with UBC-GFP and RHAUfl/fl:MxCre cells and treated with polyI:C, group 2 consisted of recipients transplanted with UBC-GFP and RHAUfl/fl:MxCre cells and treated with polyI:C, group 2 consisted of recipients transplanted with UBC-GFP and RHAUfl/fl:MxCre cells and treated with polyI:C. Group 2 recipient mice were transplanted with UBC-GFP and RHAUfl/fl:MxCre cells (n = 3), group 2 recipient mice were transplanted with UBC-GFP and RHAUfl/fl:MxCre cells (n = 4), and group 3 recipient mice were transplanted with UBC-GFP and RHAUfl/fl:MxCre cells (n = 4). Mice from groups 1 and 2 were treated with polyI:C 5 weeks after BM transplantation and mice in group 3 were kept untreated; *P < 0.05.

Reduced proliferation and enhanced cell death can lead to differentiation block in erythropoiesis.31 To test whether the defects of differentiation of ProEs is because of a decreased rate of cell division, we monitored in vivo proliferation rates of ProEs in BM by injecting the DNA analog BrdU in mice (see “BrdU incorporation assay”). At 18 hours after injection, RBC-depleted BM cells were treated according to the protocol from the manufacturer, and BrdU+ cells were analyzed by the gated ProE (Ter119+CD71+). As shown in Figure 7A, most of control RHAUfl/fl ProEs were undergoing proliferation as the peak for the BrdU+ population higher than that of the BrdU− population. In contrast, the proportion of BrdU− and BrdU+ populations were similar in RHAU-ablated ProEs (Figure 7A). The quantification in Figure 7B shows that the percentage of BrdU+ ProEs in RHAUfl/fl:vav-iCre mice was significantly lower than in control mice. This

**Figure 5. Loss of RHAU leads to progressive reduction in the population expansion ability of hematopoietic cells.** (A) The numbers of ProE, Ery.A, Ery.B, and Ery.C cells in the BM and spleen are shown. The number at the top of each bar indicates the fold increase/ decrease after RHAU ablation. *P<0.05; n = 4. The order of cell differentiation in the erythroid lineage is indicated: ProE → Ery.A → Ery.B → Ery.C. (B) Effect of RHAU ablation on the number of stem cells. The mean numbers ± SEs of different cell types are shown as bar charts. The number at the top of each bar indicates the fold change after RHAU ablation. Arrows indicate the path of early hematopoiesis: LT-HSC → ST-HSC → LMPP → CMP. *P<0.05; n = 6. (C) The progressive reduction of RHAU-ablated hematopoietic cells in population expansion ability was shown in competitive repopulation assays. Two different combinations of BM cells, (i) UBC-GFP and RHAUfl/fl in a 1:10 ratio or (ii) UBC-GFP and RHAUfl/fl:MxCre in a 1:10 ratio, were transplanted into BL6.CD45.1 mice. The recipient mice were divided into 3 groups. Group 1 recipient mice were transplanted with UBC-GFP and RHAUfl/fl:MxCre cells (n = 3), group 2 recipient mice were transplanted with UBC-GFP and RHAUfl/fl:MxCre cells (n = 4), and group 3 recipient mice were transplanted with UBC-GFP and RHAUfl/fl:MxCre cells (n = 4). Mice from groups 1 and 2 were treated with polyI:C 5 weeks after BM transplantation and mice in group 3 were kept untreated; *P < 0.05.
ProEs from BM cells were sorted as Ter119med CD71su population and were immediately stained with CFSE, after which time they were grown for 3 days in the presence of Epo. From the histograms (Figure 7C), the RHAU-ablated ProEs gave a higher CFSE signal compared with the wild-type ProEs. To test whether RHAU ablation leads to enhanced cell death of ProEs, freshly harvested ProEs were stained with annexin V to monitor apoptotic activity. However, RHAU-ablated ProE cells did not show a higher apoptotic rate than wild-type ProEs (Figure 7D). Together, these data show that the partial differentiation block at the ProE stage in RHAU-ablated cells is because of decreased cell cycle kinetics and is independent of apoptosis.

**Preferential deregulation of genes carrying a G4 motif in their promoter in RHAU-ablated ProEs**

Given the reduced expansion of the cell population observed in the erythocyte lineage, we examined the effect of RHAU ablation on gene expression in ProEs, an erythroblast stage associated with extensive population expansion. Transcriptomes of wild-type and RHAU knockout ProEs were obtained by microarray analysis, and a list of deregulated genes was generated with a cutoff of 2-fold change and a *P* value of < .01. Because RHAU is a DNA/RNA-G4 resolvase, we examined whether there is a correlation between the deregulated genes in RHAU-ablated ProEs and the ratio of genes whose promoters contain G4 motifs. According to the Greglist
specific manner. As hematopoiesis involves many discrete steps, the role for this helicase in leukemia development has not yet been confirmed. However, the effects of RHAU-ablation on basophils and eosinophils were not consistent between the animals with and without BM transplantation. The cause of the discrepancy in basophils and eosinophils is unknown; RHAUfl/fl :vav-iCre mice and their control siblings were housed in open cages, whereas the mice that received a transplant were housed in sterilized filter-top cages. Thus, the increase in basophils and eosinophils in the RHAUfl/fl :vav-iCre mice might be a consequence of exposure to airborne pathogens.

The anemic phenotype of the RHAUfl/fl :vav-iCre mice was largely because of the shortened half-life of erythrocytes. In addition, spherocytosis indicated that the enhanced phagocytic activity of RBCs in the spleen could be the primary cause of the hemolytic anemia in the RHAUfl/fl :vav-iCre mice. Therefore, RHAUfl/fl :vav-iCre mice could provide a valuable tool for studying the mechanism of hemolytic anemia associated with splenomegaly.

Lineage-specific effect of RHAU ablation on cell differentiation

In the competitive BM transplantation experiments, outgrowth of RHAU-expressing hematopoietic cells over RHAU-ablated cells was observed in the recipient mice. Therefore, RHAU appeared to be essential in the development of both myeloid and lymphoid lineages. This defect can be accounted for by the inefficient population propagation of stem cells and progenitors before lineage commitment. However, analysis of individual lineages showed that RHAU is differentially required for the development of different lineages. The erythroid lineage, RHAU ablation hindered progression of erythroblast cells at the ProE-to-Ery:A stage. Nevertheless, RHAU-ablated erythroblasts still developed to mature RBCs. Beside the erythroid lineage, the lymphoid lineage also requires RHAU for its development because the peripheral lymphocyte counts were dramatically decreased in RHAU-ablated mice. Furthermore, although the numbers of differentiated lymphoid cells and erythrocytes was low, the number of LT-HSCs was enhanced in the absence of RHAU. We speculate that the increase in LT-HSCs might be a response to insufficiently functioning hematopoietic stem cells. This might be similar to the phenomenon of increased ProEs in response to elevated Epo in anemic RHAUfl/fl :vav-iCre mice.

Possible involvement of the G4 motif in RHAU-dependent gene regulation in ProE cells

G4-DNA and G4-RNA are highly stable structures formed in guanine-rich regions of DNA or RNA under physiologic conditions. G4-DNA/RNAs are 4-stranded, thermodynamically highly stable, nucleic acid structures with numerous varieties. G4 motifs appear throughout the genome, across species, and in a functional orientated manner. This suggests that G4 structures are involved in diverse biologic functions. G4 motifs are present in the promoter regions of many proliferation-associated genes, telomeres, repetitive DNA regions, and also in immunoglobulin heavy chain genes. It was suggested that complex secondary structures at the promoter may act as transcription repressors. Removing the G4 structure by substituting the critical guanine residues with adenines was found to enhance basal transcription levels several fold. Similarly, G4 motifs are also present in untranslated regions of some mRNAs and diminish their translation efficiency. Depletion of RHAU protein in HeLa cells, either by treating the cell extracts with a specific monoclonal Ab or by expressing RHAU-specific shRNA in the cell, causes a dramatic reduction in G4-DNA– and G4-RNA–resolving activities in the cell extracts. Previous reports had further shown that increasing quantities of purified recombinant RHAU are directly proportional to the resolution activity of tetramolecular and unimolecular G4 DNA in vitro in an ATP-dependent manner. These results argue for a critical role of RHAU in G4-mediated cellular functions. The observation that RHAU can bind to and resolve both DNA and RNA and also can shuttle between nucleus and cytoplasm suggests that it could...
regulate gene expression at both the DNA and RNA levels.\textsuperscript{17} Although it was shown that RHAU binds and resolves G4-RNA with a higher efficiency than G4-DNA, this does not necessarily imply that RHAU mainly acts on RNA.\textsuperscript{43} Indeed, other RNA helicases that are shuttling between nucleus and cytoplasm, such as RHAU, p68, and p72, are also involved in both RNA and DNA metabolisms.\textsuperscript{44} In fact, the localization and the composition of the ribonucleoprotein complexes define the functions and mechanisms of helicases rather than the helicase by itself. Accordingly, as shown in this study, decreased proliferation and altered transcriptomes in RHAU-ablated ProE cells suggests that G4 motifs in promoter regions play a role in the regulation of genes involved in cell cycle regulation and whose products are localized in the nucleus. The grouping of genes deregulated after RHAU ablation correlated with enrichment in G4-containing promoters. Thus, through a common mechanism that involved G4, RHAU may cooperatively regulate specific groups of genes at the transcription level. It is not yet clear which mechanisms may be involved in RHAU-mediated regulation of embryogenesis and hematopoiesis because RHAU binds to and resolves both G4-DNA and G4-RNA structures.\textsuperscript{42} Analysis of the DNA and RNA sequences bound by RHAU by ChIP-seq and RNA immunoprecipitation will provide important insights on how RHAU regulates gene expression.


Reference


Authorship

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Authorship

Contribution: J.C.L., S.P., D.P, and H.K. designed and performed the research and analyzed data; R.C.S., P.M., and Y.N. designed the research and contributed vital new reagents; and J.C.L., P.M., and Y.N. wrote the paper.

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The DEAH-box helicase RHAU is an essential gene and critical for mouse hematopoiesis

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