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Blockade of IL-22 signaling reverses erythroid dysfunction in stress-induced anemias

Mahesh Raundhal[1,2,3,*], **Shrestha Ghosh**[1,2,3], **Samuel A. Myers**[4], **Michael S. Cuoco**[5], **Meromit Singer**[2,4,6], **Steven A. Carr**[4], **Sushrut S. Waikar**[7,8], **Joseph V. Bonventre**[7], **Jerome Ritz**[9], **Richard M. Stone**[9], **David P. Steensma**[9], **Aviv Regev**[5,10,11], **Laurie H. Glimcher**[1,2,3,*]

[1]Department of Cancer Immunology and Virology, Dana-Farber Cancer Institute, Boston, MA 02215, USA.

[2]Department of Immunology, Harvard Medical School, Boston, MA, 02215, USA.

[3]Department of Medicine, Brigham and Women's Hospital, Boston, MA 02215, USA.

[4]Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA.

[5]Klarman Cell Observatory, Broad Institute of Harvard and MIT, Cambridge, MA, USA.

[6]Department of Data Sciences, Dana-Farber Cancer Institute, Boston, MA, USA.

[*]Corresponding authors: Laurie H. Glimcher, M.D., President and CEO, Dana-Farber Cancer Institute, Richard and Susan Smith Professor of Medicine, Harvard Medical School, 450 Brookline Avenue, Dana 1628, Boston, MA 02215, Laurie_Glimcher@dfci.harvard.edu; Mahesh Raundhal, Ph.D., Dana-Farber Cancer Institute, SM746, 1 Jimmy Fund Way, Boston, MA 02215, Mahesh_Raundhal@dfci.harvard.edu.

Author Contributions

M.R. and L.H.G conceived the study, designed experiments, analyzed the data and wrote the manuscript; M.R. performed the experiments; S.G. assisted with *in vitro* experiments, S.A.M. performed proteomic profiling and data analysis, M.S.C. performed RNA-Seq, M.S. processed RNA-Seq data, S.A.C. analyzed proteomic profiling data, S.S.W. and J.V.B. collected CKD patient samples and related clinical information and analyzed the data, J.R., R.M.S, and D.P.S. collected MDS patient samples and related clinical information and analyzed the data. A.R. supervised RNA-Seq experiment and analyzed data.

Competing Interests statement

An invention disclosure has been filed based on the data generated in this study.

From August 4th, 2020, Meromit Singer is an employee of Guardant Health.

S.S.W. has served on the steering committee of GSK trial on an oral hypoxia-inducible factor prolyl hydroxylase inhibitor, as a potential treatment for anemia associated with chronic kidney disease; S.S.W. has also received consulting fees from Public Health Advocacy Institute, CVS, Roth Capital Partners, Kantum Pharma, Mallinckrodt, Wolters Kluewer, GE Health Care, Allena Pharmaceuticals, Mass Medical International, JNJ, Venbio, Strataka, Takeda, Cerus, and Pfizer. D.P.S. has served on independent data safety monitoring committees for clinical trials supported by Takeda, Astex, Janssen and Onconova; has consulted for Celgene and Daiichi Sankyo; and has received research support (to the institution) for clinical trials sponsored by Aprea, H3 Biosciences, Syros and Astra Zeneca. J.R. reports research funding from Amgen, Equillium, and Kite Pharma; and consulting income from Aleta Biotherapeutics, Avrobio, Celgene, Falcon Therapeutics, LifeVault Bio, Rheos Medicines, Talaris Therapeutics and TScan Therapeutics.

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A.R. is a SAB member of ThermoFisher Scientific, Neogene Therapeutics, Asimov and Syros Pharmaceuticals. A.R. is a cofounder of and equity holder in Celsius Therapeutics and an equity holder in Immunitas. From August 1, 2020, AR is an employee of Genentech.

L.H.G. is a former Director of Bristol-Myers Squibb and the Waters Corporation and is currently on the board of directors of and holds equity in GlaxoSmithKline Pharmaceuticals and Analog Devices, Inc. She also serves on the scientific advisory boards of Repare Therapeutics, Abpro Therapeutics and Kaleido Therapeutics. All other authors declare no relevant competing interest.

[7]Renal Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA.

[8]Renal Section, Boston University Medical Center, Boston, MA 02118, USA.

[9]Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02215, USA.

[10]Howard Hughes Medical Institute, Chevy Chase, MD, USA.

[11]Koch Institute for Integrative Cancer Research, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA.

Abstract

Patients with myelodysplastic syndromes (MDS) display severe anemia but the mechanisms underlying this phenotype are incompletely understood. Right open-reading-frame kinase 2 (*RIOK2*) encodes a protein kinase located at 5q15, a region frequently lost in MDS del(5q) patients. Here, we show that hematopoietic cell-specific haploinsufficient deletion of *Riok2* (*Riok2*$^{f/+}$ *Vav1*^{cre}) led to reduced erythroid precursor frequency leading to anemia. Proteomic analysis of *Riok2*$^{f/+}$ *Vav1*^{cre} erythroid precursors suggested immune system activation and transcriptomic analysis revealed an increase in p53-dependent interleukin-22 (IL-22) in *Riok2*$^{f/+}$ *Vav1*^{cre} CD4^{+} T cells (T_H22). Further, we discovered that the IL-22 receptor, IL-22RA1, was unexpectedly present on erythroid precursors. Blockade of IL-22 signaling alleviated anemia not only in *Riok2*$^{f/+}$ *Vav1*^{cre} mice but also in wild-type mice. Serum concentrations of IL-22 were increased in the subset of del(5q) MDS patients as well as patients with anemia secondary to chronic kidney disease (CKD). This work reveals a possible therapeutic opportunity for reversing many stress-induced anemias by targeting IL-22 signaling.

Keywords

IL-22; del(5q) MDS; anemia; myeloproliferation; *Riok2*; p53

Introduction

Myelodysplastic syndromes (MDS) are a group of cancers characterized by failure of blood cells in the bone marrow to mature. About 7 out of 100,000 people are affected and the typical survival time following diagnosis is less than three years. While a sizable percentage of MDS cases progress to acute myelogenous leukemia (AML), most of the morbidity and mortality associated with MDS results not from transformation to AML but rather from hematological cytopenias.

Anemia is the most common hematologic manifestation of MDS, particularly in the subset of patients with del(5q) MDS. Del(5q), either isolated or accompanied by additional cytogenetic abnormalities, is the most commonly detected chromosomal abnormality in MDS, reported in 10–15% of patients[1-4]. The severe anemia in del(5q) MDS patients has been linked to haploinsufficiency of ribosomal proteins such as RPS14 and RPS19[5; 6]. Previous studies using mice with haploinsufficient 5q gene deletions revealed diminished

erythroid progenitor frequency[7-10] but the mechanisms underlying this phenotype are incompletely understood. Right open-reading-frame kinase 2 (RIOK2) encodes an atypical serine-threonine protein kinase with an indispensable function as a component of the pre-40S ribosome subunit[16].

There is growing evidence for the role of activated innate immunity and inflammation as well as immune dysregulation in the pathogenesis of MDS[17-20]. Abnormal expression of numerous cytokines has been reported in MDS[21-24]. Chronic immune stimulation in both hematopoietic stem and progenitor cells (HSPCs) and the bone marrow (BM) microenvironment[25] was suggested to be central to the pathogenesis of MDS. In patients with chronic inflammation, cytokines in the BM have been associated with inhibition of erythropoiesis[26]. Despite growing evidence for a link between the immune system and MDS pathogenesis, no study has identified the mechanism by which the immune microenvironment may initiate or contribute to the MDS phenotype. Further it remains unclear how ribosomal protein haploinsufficiency is connected with the immune system in MDS.

Results

Riok2 haploinsufficiency leads to anemia

We previously noted that *Riok2* expression was reduced in T cells lacking the endoplasmic reticulum stress transcription factor Xbp1[27]. RIOK2 is a little-studied atypical serine-threonine protein kinase[11] encoded by *RIOK2* at 5q15 in the human genome (Extended Data Fig. 1a), adjacent to the 5q commonly deleted regions in MDS and frequently lost in MDS and acute myeloid leukemia[12-15].Gene expression commons (GEXC)[28] analysis revealed that in mouse BM, *Riok2* expression is highest in primitive colony-forming-unit erythroid (pCFU-E) cells, suggesting that RIOK2 may be involved in maintaining red blood cell (RBC) output (Extended Data Fig. 1d). To further study the role of RIOK2 in hematopoiesis, we generated *Vav1-Cre* transgenic floxed *Riok2* (*Riok2*$^{f/+}$ *Vav1*^{cre}*)* mice in which the Cre recombinase is under the control of the hematopoietic cell–specific *Vav1* promoter. *Riok2* floxed mice were generated with exons 5 and 6 flanked by *loxP* sites (Extended Data Fig. 1b, c). Interestingly, no Vav1-Cre floxed *Riok2* homozygous-deficient mice (*Riok2*$^{f/f}$ *Vav1*^{cre}*)* were recovered (Extended Data Fig. 1f), indicating embryonic lethality from complete hematopoietic deletion of *Riok2*. However, heterozygous *Riok2*$^{f/+}$ *Vav1*^{cre} mice were viable with approximately 50% *Riok2* mRNA expression in hematopoietic cells compared to that of *Vav1*^{Cre} controls (Extended Data Fig. 1e). As seen with other ribosomal protein haploinsufficiency mouse models[10], BM cells from *Riok2*$^{f/+}$ *Vav1*^{cre} mice showed reduced nascent protein synthesis *in vivo* compared to *Vav1*^{Cre} controls (Extended Data Fig. 1g), consistent with a RIOK2 role in maturation of the pre-40S ribosome. A recent study[29] showed that ribosomal protein deficiency-mediated reduced protein synthesis predominantly affects erythropoiesis over myelopoiesis.

Consistent with the high expression of *Riok2* in pCFU-e cells in the BM, aged (>60 wks) mice with heterozygous deletion of *Riok2* in hematopoietic cells (*Riok2*$^{f/+}$ *Vav1*^{cre}) displayed anemia with reduced peripheral blood (PB) RBC numbers, hemoglobin (Hb), and hematocrit (HCT) (Fig. 1a). We next determined whether *Riok2* haploinsufficiency-mediated

anemia was secondary to a defect in erythroid development in the BM, the major site of erythropoiesis. We characterized the stages (referred to here as RI, RII, RIII and RIV) of erythropoiesis by flow cytometry using the expression of Ter119 and CD71 (Extended Data Fig. 2a). *Riok2*$^{f/+}$ *Vav1*^{cre} mice had impaired erythropoiesis in the BM (Fig. 1b, Extended Data Fig. 2b). Moreover, *Riok2* haploinsufficiency led to increased apoptosis in erythroid precursors compared to controls (Fig. 1c). Additionally, *Riok2*$^{f/+}$ *Vav1*^{cre} erythroid precursors showed a decrease in cell quiescence with cell cycle block at the G1 phase (Extended Data Fig. 2c). A block in cell cycle is driven by a group of proteins known as cyclin-dependent kinase inhibitors (CKI). The expression of p21 (a CKI encoded by *Cdkn1a*) was increased in erythroid precursors from *Riok2*$^{f/+}$ *Vav1*^{cre} mice compared to *Riok2*$^{+/+}$ *Vav1*^{cre} controls (Extended Data Fig. 2d).

We next examined the effect of *Riok2* haploinsufficiency on stress-induced erythropoiesis using 8-12 wk old mice in which hemolysis was induced by non-lethal phenylhydrazine treatment (25 mg/kg on days 0 and 1). After acute hemolytic stress, *Riok2*$^{f/+}$ *Vav1*^{cre} mice developed more severe anemia and had a delayed RBC recovery response, as compared to *Riok2*$^{+/+}$ *Vav1*^{cre} control mice (Fig. 1d) and succumbed faster to a lethal dose of phenylhydrazine (35 mg/kg on days 0 and 1) compared to *Vav1*^{cre} controls (Extended Data Fig. 2e). The anemia in young phenylhydrazine-administered *Riok2*$^{f/+}$ *Vav1*^{cre} mice seen on day 7 was preceded by a reduction in BM RIII and RIV erythroid precursor frequency on day 6, highlighting an erythroid differentiation defect in *Riok2* haploinsufficient mice (Fig. 1e, Extended Data Fig. 2f). In line with a role for RIOK2 in driving erythroid differentiation, fewer CFU-e colonies were observed in erythropoietin-containing MethoCult cultures from *Riok2* haploinsufficient Lin^{-}c-kit^{+}CD71^{+} cells compared to *Riok2* sufficient cells (Fig. 1f). To determine whether *Riok2* haploinsufficiency in BM cells drives anemia, we generated BM chimeras. Wild-type (WT) mice transplanted with *Riok2*$^{f/+}$ *Vav1*^{cre} whole BM developed anemia compared to *Riok2*$^{+/+}$ *Vav1*^{cre} BM transplanted wt mice (Extended Data Fig. 2g). In addition, tamoxifen-inducible deletion of *Riok2* in *Riok2*$^{f/+}$*Ert2*^{cre} mice led to reduction in PB RBCs, Hb, and HCT compared to *Riok2*$^{+/+}$*Ert2*^{cre} controls (Extended Data Fig. 2h). Taken together, these data show that *Riok2* haploinsufficiency leads to anemia owing to defective bone marrow erythroid differentiation.

Riok2 haploinsufficiency increases myelopoiesis

In addition to the reduction in RBC numbers in PB from aged *Riok2*$^{f/+}$ *Vav1*^{cre} mice, we also observed an increased percentage of monocytes (monocytosis) and decreased percentage of neutrophils (neutropenia) compared to controls (Fig. 1g, Extended Data Fig. 2i). Granulocyte macrophage progenitors (GMPs) in the BM give rise to PB myeloid cells. The percentage of proliferating (Ki67^{+}) GMPs in the BM was increased in *Riok2*$^{f/+}$ *Vav1*^{cre} mice compared to *Riok2*$^{+/+}$ *Vav1*^{cre} controls (Fig. 1h, Extended Data Fig. 2j). To analyze the effect of *Riok2* haploinsufficiency on myelopoiesis in the absence of *in vivo* compensatory mechanisms, we cultured LSK (lineage^{-}Sca-1^{+}Kit^{+}) cells from the BM of *Riok2*$^{f/+}$ *Vav1*^{cre} and *Riok2*$^{+/+}$ *Vav1*^{cre} controls in a MethoCult assay supplemented with growth factors (interleukin 6 (IL-6), IL-3, stem cell factor (SCF) but devoid of erythropoietin). LSKs from *Riok2*$^{f/+}$ *Vav1*^{cre} mice gave rise to an increased percentage of CD11b^{+} myeloid cells (Fig. 1i,

Extended Data Fig. 2k), suggesting a cell-intrinsic myeloproliferative effect due to *Riok2* haploinsufficiency consistent with a myelodysplasia phenotype.

We also evaluated whether *Riok2* haploinsufficiency affects early hematopoietic progenitors. Frequency and numbers of early hematopoietic progenitors were comparable between young *Riok2*$^{f/+}$ *Vav1*^{cre} and *Riok2*$^{+/+}$ *Vav1*^{cre} mice (Extended Data Fig. 3a), however, long-term hematopoietic stem cells (LT-HSCs) were increased in the BM of aged *Riok2*$^{f/+}$ *Vav1*^{cre} mice (Extended Data Fig. 3a). To further corroborate this data, we analyzed the capacity of *Riok2* haploinsufficient cells in a competitive transplantation assay. Starting at 8 weeks after tamoxifen treatment to induce *Riok2* deletion, *Riok2* haploinsufficient cells out-competed CD45.1^{+} competitor cells, while *Ert2*^{cre} control cells had no competitive advantage (Extended Data Fig. 3b). Similar to non-transplanted mice (Extended Data Fig. 3a), in competitive transplant experiments the frequency of *Riok2*-haploinsufficient LT-HSCs was significantly higher than *Riok2*-sufficient LT-HSCs in relation to competitor CD45.1^{+} cells (Extended Data Fig. 3c). Thus, in addition to its effect on erythroid differentiation, *Riok2* haploinsufficiency increases myelopoiesis and affects early hematopoietic progenitor differentiation.

Reduced Riok2 induces alarmins in erythroid precursors

To elucidate a mechanism for the erythroid differentiation defect observed in *Riok2*$^{f/+}$ *Vav1*^{cre} mice, we performed quantitative proteomic analysis of purified erythroid precursors using mass spectrometry[30]. *Riok2* haploinsufficiency led to upregulation of 564 distinct proteins (adjusted p-value < 0.05) in erythroid precursors compared to those from *Vav1*^{cre} controls (Fig. 2a). Interestingly, *Riok2* haploinsufficiency resulted in down-regulation of other ribosomal proteins, loss of some of which (RPS5, PRL11) has been implicated in driving anemias (Extended Data Fig. 4a). The alarmins including S100A8, S100A9, CAMP, NGP, and others were the most highly upregulated proteins in our dataset and interestingly, correlated significantly with those observed upon haploinsufficiency of *Rps14*[10], another component of the 40S ribosomal complex (Fig. 2b). Using the 26 upregulated proteins in the *Rps14* haploinsufficient dataset as an ‘Rps14 signature’ (Supplementary Table 1), gene set enrichment analysis (GSEA) revealed a marked enrichment for the Rps14 signature in the *Riok2* haploinsufficient dataset, suggesting a shared proteomic signature upon deletion of distinct ribosomal proteins (Fig. 2c). The increased expression of S100A8 and S100A9 in *Riok2*$^{f/+}$ *Vav1*^{cre} mice was confirmed by flow cytometry and qRT-PCR (Extended Data Fig. 4b-e).

In *Riok2*$^{f/+}$ *Vav1*^{cre} erythroid precursors cells, the upregulated proteins with the highest fold-change (S100A8, S100A9, CAMP, NGP) are proteins with known immune functions such as antimicrobial defense. GSEA analysis of the proteomics data indicated a possible role for the immune system in driving the proteomic changes seen in *Riok2* haploinsufficient erythroid precursors (Fig. 2d). An independent analysis of the Riok2 proteomics dataset using MetaCore pathway analysis software showed immune response as the top differentially regulated pathway in *Riok2*$^{f/+}$ *Vav1*^{cre} mice (Fig. 2e). To assess if *Riok2* haploinsufficiency leads to changes in immune cell function, we subjected naïve T cells from *Riok2*$^{f/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls to *in vitro* polarization towards

known CD4$^+$ T helper cell lineages (T_H1, T_H2, T_H17, T_H22) and regulatory T cells (T_{reg}). Secretion of interferon-γ (IFN-γ), IL-2, IL-4, IL-5, IL-13, IL-17A, and the frequency of Foxp3$^+$ T_{reg} cells was similar between *Riok2*$^{f/+}$ *Vav1*^{cre} and *Riok2*$^{+/+}$ *Vav1*^{cre} T cells (Extended Data Fig. 5a - g). Strikingly, however, we observed an exclusive increase in IL-22 secretion from *Riok2*$^{f/+}$ *Vav1*^{cre} naïve T cells polarized towards the T_H22 lineage (Fig. 3a). The frequency of IL-22$^+$CD4$^+$ T cells was higher in *Riok2*$^{f/+}$ *Vav1*^{cre} T_H22 cultures compared to *Vav1*^{cre} control T_H22 cultures (Fig. 3b). The concentration of IL-22 in the serum and BM fluid (BMF) of aged *Riok2*$^{f/+}$ *Vav1*^{cre} mice was also significantly higher as compared to age-matched *Vav1*^{cre} controls (Fig. 3c). Using known IL-22 target genes from the literature, we curated an 'IL-22 signature' (Supplementary Table 1) gene set, which showed a statistically significant enrichment in the Riok2-haploinsufficient proteomics dataset using GSEA (Fig. 2f), further suggesting that IL-22-induced inflammation is a contributing factor for *Riok2* haploinsufficiency-mediated ineffective erythropoiesis and anemia. Increased numbers of splenic IL-22$^+$ CD4$^+$ T, natural killer T (NKT), and innate lymphoid cells (ILCs) were observed in aged *Riok2*$^{f/+}$ *Vav1*^{cre} mice compared to *Riok2*$^{+/+}$ *Vav1*^{cre} mice (Fig. 3d, Extended Data Fig. 5h, i). Interestingly, mild anemia was observed in mice lacking *Riok2* only in T cells (Extended Data Fig. 5k). Expression of IL-23, required for IL-22 production, was enhanced in *Riok2*-haploinsufficient dendritic cells (Extended Data Fig. 5j). *Rps14* haploinsufficient T_H22 cells also secreted elevated concentrations of IL-22 compared to *Vav1*^{cre} control T_H22 cells (Extended Data Fig. 5l). Mutation(s) in the gene adenomatosis polyposis coli (*Apc*), also found on human chromosome 5q, lead to anemia in addition to adenomas[31]. *In vitro* generated T_H22 cells from *Apc*^{Min} mice secreted elevated IL-22 compared to littermate controls (Extended Data Fig. 5m). In total, our analysis of three distinct heterozygous deletions of genes found on human chromosome 5q suggests that increased IL-22 is a generalized phenomenon observed upon heterozygous loss of genes found on chromosome 5q leading to anemia.

p53 upregulation drives increased IL-22 secretion upon *Riok2* loss

To identify cell-intrinsic molecular mechanism(s) driving the increase in IL-22 secretion upon *Riok2* haploinsufficiency, we performed RNA-sequencing (RNA-Seq) on *in vitro* polarized IL-22$^+$ (T_H22) cells purified by flow cytometry from *Riok2*$^{+/+}$ *Il22*$^{tdtomato/+}$ *Vav1*^{cre} and *Riok2*$^{f/+}$ *Il22*$^{tdtomato/+}$ *Vav1*^{cre} mice (Fig. 3e). GSEA analysis of the RNA-Seq dataset identified activation of the p53 pathway in *Riok2*$^{f/+}$ *Vav1*^{cre} mice (Fig. 3f, g). p53 increase in T_H22 cells from *Riok2*$^{f/+}$ *Vav1*^{cre} mice was confirmed by flow cytometry (Fig. 3h, i). p53 upregulation was also observed in *Riok2*$^{f/+}$ *Vav1*^{cre} erythroid precursors (Extended Data Fig. 3f, g). The p53 pathway is activated by decreased expression of ribosomal protein genes[5; 10], however, its involvement in IL-22 regulation has not been known.

p53 is a transcription factor with well-defined consensus binding sites. To assess whether p53 drives *Il22* transcription, we analyzed the *Il22* promoter for potential p53 binding sites using LASAGNA algorithm and found putative p53 consensus binding sequences in the *Il22* promoter (Fig. 3j). Chromatin immunoprecipitation (ChIP) confirmed the presence of p53 on the *Il22* promoter (Fig. 3k). In line with the ChIP data, p53 inhibition by pifithrin-α, p-nitro decreased IL-22 concentrations while p53 activation by nutlin-3 increased IL-22 from *in vitro* polarized wild-type T_H22 cells (Fig. 3l, m). Treatment with either pifithrin-α, p-nitro

or nutlin-3 did not decrease cell viability (Extended Data Fig. 5n). Accordingly, genetic deletion of *Trp53* blunted the increase in IL-22 secretion observed upon *Riok2* haploinsufficiency (Fig. 3n). A significant decrease in IL-22 secretion was also observed upon *Trp53* deletion in Riok2-sufficient cells further suggesting a homeostatic role for p53 in controlling IL-22 production (Fig. 3n). Taken together, these data show that *Riok2* haploinsufficiency-mediated p53 upregulation drives increased IL-22 secretion in *Riok2*$^{f/+}$ *Vav1*^{cre} mice.

IL-22 neutralization alleviates stress-induced anemia

Mice with compound genetic deletion of *Il22* on the *Riok2* haploinsufficient background (*Riok2*$^{f/+}$ *Il22*$^{+/-}$ *Vav1*^{cre}) exhibited increased numbers of PB RBCs compared to IL-22 sufficient *Riok2* haploinsufficient mice on day 7 after two treatments with 25 mg/kg phenylhydrazine treatment (Fig. 4a). Interestingly, we also saw an increase in PB RBCs in *Riok2* sufficient mice heterozygous for *Il22* deletion (*Riok2*$^{+/+}$ *Il22*$^{+/-}$ *Vav1*^{cre}) compared to *Riok2* sufficient IL-22 sufficient mice (*Riok2*$^{+/+}$ *Il22*$^{+/+}$ *Vav1*^{cre}). PB Hb and HCT also were increased in *Il22* haploinsufficient mice, regardless of *Riok2* background, however, this difference did not reach statistical significance (Fig. 4a). Next, we assessed whether the increase in PB RBCs in *Riok2*$^{f/+}$ *Il22*$^{+/-}$ *Vav1*^{cre} mice was due to increased erythropoiesis in the BM of these mice. We observed an increase in RII and RIV erythroid precursors in *Riok2*$^{f/+}$ *Il22*$^{+/-}$ *Vav1*^{cre} compared to *Riok2*$^{f/+}$ *Il22*$^{+/+}$ *Vav1*^{cre} mice (Fig. 4b, Extended Data Fig. 6a). Treatment of mice with a neutralizing IL-22 antibody *in vivo*, also reversed phenylhydrazine-induced anemia as evidenced by increase in PB RBCs and HCT in *Riok2*$^{f/+}$ *Vav1*^{cre} mice as well as *Riok2* sufficient (*Riok2*$^{+/+}$ *Vav1*^{cre}) mice (Fig. 4c). Unexpectedly, IL-22 neutralization also reduced the frequency of apoptotic erythroid precursors in both *Riok2* sufficient as well as *Riok2*$^{f/+}$ *Vav1*^{cre} mice (Fig. 4d). These data indicate that IL-22 neutralization reverses anemia, at least in part, by reducing apoptosis of erythroid precursors. Recently, dampening IL-22 signaling in intestinal epithelial stem cells was shown to reduce apoptosis[32]. The effect of IL-22 deficiency (genetic as well as antibody-mediated) in alleviating anemia in genetically wild-type mice indicated a role for IL-22 in reversing anemia regardless of ribosomal haploinsufficiency. Accordingly, treatment of C57BL/6J mice undergoing phenylhydrazine-induced anemia with anti-IL-22 significantly increased PB RBCs, Hb, and HCT compared to isotype antibody-treated mice (Extended Data Fig. 7b). This increase in PB RBCs could be attributed to the increased frequency of RIII and RIV erythroid precursors in the BM of mice treated with anti-IL-22 compared to isotype-administered controls (Extended Data Fig. 7c). Of note, PB RBCs, Hb, and HCT did not differ in healthy, non-anemic wild-type mice injected with anti-IL-22 versus isotype-matched antibody (Extended Data Fig. 7a). Thus, we show here that IL-22 neutralization, either by genetic deletion or antibody blockade, alleviates stress-induced anemia in *Riok2*$^{f/+}$ *Vav1*^{cre} as well as wild-type mice.

IL-22 worsens stress-induced anemia in wild-type mice

Phenylhydrazine administration to wild-type C57BL/6J mice treated intraperitoneally with recombinant IL-22 (rIL-22) led to decreased PB RBCs, Hb, and HCT owing to decreased BM erythroid precursor cell frequency and number (Fig. 5a, c, Extended Data Fig. 6b). rIL-22 treatment led to increased apoptosis of erythroid precursors (Fig. 5d). This treatment

also led to an increase in PB reticulocytes, an indication of increased erythropoiesis under stress (Fig. 5b). Recombinant IL-22 also dose-dependently decreased terminal erythropoiesis in an *in vitro* erythropoiesis assay (Fig. 5e, f). Importantly, IL-22-mediated inhibition of *in vitro* erythropoiesis led to induction of p53 suggesting a feedback loop between IL-22 and p53 in driving dyserythropoiesis (Fig. 5g). Overall, these data show that exogenous recombinant IL-22 exacerbates stress-induced anemia in wild-type mice.

Erythroid precursors express IL-22RA1 receptors

IL-22 signals through a cell surface heterodimeric receptor composed of IL-10Rβ and IL-22RA1 (encoded by *Il22ra1*)[33]. IL-22RA1 expression has been reported to be restricted to cells of non-hematopoietic origin (e.g., epithelial cells and mesenchymal cells). We discovered, however, that erythroid precursors in the BM also express IL-22RA1 (Fig. 6a, Extended Data Fig. 8a). Moreover, we observed that among BM hematopoietic progenitors, IL-22RA1-expressing cells were exclusively of the erythroid lineage (Extended Data Fig. 8b). Using a second IL-22RA1-specific antibody (targeting a different epitope than the antibody used in Fig. 4A), we confirmed the presence of IL-22RA1 on erythroid precursors (Extended Data Fig. 8c). *Il22ra1* mRNA expression was detected exclusively in erythroid precursors among all lineage-negative cells in the BM (Extended Data Fig. 8d).

Deletion of *Il22ra1* in *Riok2* haploinsufficient mice ($Riok2^{f/+}Il22ra1^{f/f}Vav1^{cre}$) led to improvement in PB RBCs and HCT as compared to IL-22RA1 sufficient *Riok2* haploinsufficient mice ($Riok2^{f/+}Il22ra1^{+/+}Vav1^{cre}$) (Fig. 6b). This improvement could be attributed to the increase in RIII and RIV erythroid precursors in the BM of $Riok2^{f/+}Il22ra1^{f/f}Vav1^{cre}$ mice (Fig. 6c, Extended Data Fig. 6c).

In accordance with the upregulation of p53 upon *in vitro* IL-22 stimulation in an *in vitro* erythropoiesis assay (Fig. 5g) and p53 upregulation in erythroid precursors upon *Riok2* haploinsufficiency (Extended Data Fig. 4f, g), we observed a synergistic effect of *Riok2* haploinsufficiency in IL-22-responsive ($IL\text{-}22RA1^{+}$) erythroid precursors in $Riok2^{f/+}Vav1^{cre}$ mice (Fig. 6d, e). p53 target genes such as *Gadd45a* and *Cdkn1a1* were also increased in $IL\text{-}22RA1^{+}$ *Riok2* haploinsufficient erythroid precursors compared to $IL\text{-}22RA1^{+}$ *Riok2* sufficient erythroid precursors (Fig. 6f). Given that rIL-22 induced apoptosis in erythroid precursors *in vivo* (Fig. 5d), we sought to determine if *Riok2* haploinsufficiency-mediated p53 upregulation played an independent role in apoptosis induction. In an IL-22-free *in vitro* erythropoiesis assay, p53 inhibition by pifithrin-α, p-nitro inhibited the apoptosis induced by *Riok2* haploinsufficiency (Fig. 6g).

Erythroid cell-specific deletion of IL-22RA1 (using cre recombinase driven by the erythropoietin receptor (*Epor*) promoter) also increased numbers of PB RBCs and HCT (Fig. 7a) due to the increase in RIII and RIV erythroid precursors in the BM (Fig. 7b, Extended Data Fig. 6d). Additionally, rIL-22 failed to exacerbate phenylhydrazine-induced anemia in $Il22ra1^{f/f}Epor^{cre}$ mice lacking the IL-22 receptor only on erythroid cells (Fig. 7c). These data reinforce our view that IL-22 signaling plays an important role in regulating erythroid development regardless of ribosomal haploinsufficiency. Thus, using three different approaches to neutralize IL-22 signaling, we demonstrate that IL-22 plays a critical role in controlling RBC production by directly regulating early stages of erythropoiesis.

IL-22 is increased in del(5q) MDS patients

Next, we assessed whether IL-22 expression is increased in human disorders that display dyserythropoiesis due to ribosomal protein haploinsufficiencies. Given the localization of *RIOK2* on human chromosome 5, we focused on MDS with 5q deletion and compared it to MDS without 5q deletion and healthy controls. We observed a significant increase in IL-22 levels in the BM fluid (BMF) of del(5q) MDS patients compared to BMF from healthy controls and non-del(5q) MDS patients (Fig. 8a). Interestingly, a strong negative correlation between cellular *RIOK2* mRNA expression and BMF IL-22 concentration was evident in the del(5q) MDS cohort (Fig. 8b) indicating that a decrease in *RIOK2* expression is associated with increased IL-22 expression. In our MDS cohort, S100A8 concentrations were found to be higher than healthy controls regardless of del(5q) status (Fig. 8c). However, IL-22 positively correlated with S100A8 concentrations only in the del(5q) MDS group (Fig. 8d). Of note, S100A8 concentrations were higher in BMF from non-del(5q) MDS patients compared to MDS patients with del(5q) (Fig. 8c). These data suggest that the regulation of S100A8 expression may be IL-22-mediated in del(5q) MDS patients, but IL-22-independent in other subtypes of MDS. In a second cohort of MDS patients, the frequency of $CD4^+$ T cells producing IL-22 (T_H22 cells) among freshly isolated peripheral blood mononuclear cells (PBMCs) was significantly higher in MDS patients with 5q deletion compared to healthy controls (Fig. 8e - cumulative data of representative flow plots shown in Extended Data Fig. 9a). Our independent analysis of a large-scale microarray sequencing dataset of $CD34^+$ cells from normal, del(5q) MDS, and non-del(5q) MDS subjects showed that *RIOK2* mRNA was significantly decreased in the del(5q) MDS cohort (78% (37/47)). Additionally, expression of known IL-22 target genes such as *S100A10*, *S100A11*, *PTGS2*, *RAB7A*, and *LCN2* was specifically increased in the del(5q) MDS cohort compared to both the healthy control and non-del(5q) groups (Extended Data Fig. 9b). Using differentially expressed proteins (adjusted p-value < 0.01) from the *Riok2* haploinsufficient proteomics dataset as a reference set, GSEA analyses of the $CD34^+$ microarray dataset revealed significant enrichment scores (Extended Data Fig. 10a, b) further suggesting that the mouse model of *Riok2* haploinsufficiency faithfully recapitulates the molecular changes seen in patients with del(5q) MDS.

High IL-22 in anemic chronic kidney disease patients

Anemia is frequently observed in CKD patients and is associated with poor outcomes. Anemia of CKD is resistant to erythropoiesis-stimulating agents (ESAs) in 10-20% of patients[34], suggesting that pathogenic mechanisms other than erythropoietin deficiency are at play. We found a significant increase in IL-22 concentration in the plasma of CKD patients with secondary anemia compared to healthy controls and to CKD patients without anemia (Fig. 8f). Plasma IL-22 concentration negatively correlated with hemoglobin in CKD patients (Fig. 8g), suggesting a function for IL-22 in driving anemia in some patients with CKD.

Discussion

The overall conclusion of this study is that IL-22 signaling directly controls bone marrow erythroid differentiation and that its neutralization is a potential therapeutic approach for

anemias and MDS. By exploring the function of a little-studied atypical kinase Riok2 in mammalian biology, we identified the erythroid precursors as a novel target for IL-22 action via the IL-22RA1. We further identify IL-22 as a disease biomarker for the del(5q) subtype of MDS and lastly, identify IL-22 signaling blockade as a potential therapeutic for stress-induced anemias irrespective of the genetic background. Interestingly, we also detect elevated levels of IL-22 in patients with anemia secondary to chronic kidney disease (CKD) suggesting that IL-22 signaling blockade may be therapeutic in reversing anemia in a much wider patient population.

Del(5q), either isolated or accompanied by additional cytogenetic abnormalities, is the most commonly detected chromosomal abnormality in MDS, reported in 10-15% of patients and enriched in therapy-related MDS. The severe anemia in MDS patients with isolated del(5q) has been linked to haploinsufficiency of ribosomal proteins such as RPS14 [10] and RPS19 [5]. While much research has focused on the effect of such gene deletions or mutations in hematopoietic stem cells and lineage-committed progenitors, the immunobiology underlying this MDS subtype has remained largely unexplored, thus impeding the development of immune-targeted therapies. With the exception of the TNF-α inhibitor etanercept[35] which proved to be ineffective, the only other therapy against immune cell-derived cytokines, is luspatercept[36], a recombinant fusion protein derived from human activin receptor type IIb[37] which has just been approved for use in anemia in lower risk MDS patients. Here, we identify two critical and independent functions of an understudied atypical kinase, RIOK2, that synergize to induce dyserythropoiesis and anemia. One effect of *Riok2* loss in erythroid precursors is an intrinsic block in erythroid differentiation owing to its indispensable role in the maturation of the pre-40S ribosomal complex, leading to increased apoptosis and cell cycle arrest. The second effect of *Riok2* loss is the induction of the erythropoiesis-suppressive cytokine IL-22 in T cells which then directly acts on the IL-22RA1 on erythroid precursors (Extended Data Fig. 10c). While IL-22RA1 is known to be widely expressed on epithelial cells and hepatocytes, its expression has also been recently reported on specialized cells such as retinal Müller glial cells[38] and now described here, on erythroid precursors. Our data reveal a novel molecular link between haploinsufficiency of a ribosomal protein and induction of erythropoiesis-suppressive cytokine IL-22. While IL-22 has been shown to modulate RBC production by controlling the expression of iron-chelating proteins such as hepcidin[39] and haptoglobin[40], we have uncovered a novel role for IL-22 in directly binding to the previously unknown IL-22R on erythroid precursors leading to their apoptosis. Diminished expression of ribosomal proteins has been shown to increase p53 levels. We show here that *Riok2* haploinsufficiency leads to p53 upregulation in T cells which drives the increase in IL-22 secretion. Additionally, we also show that IL-22-responsive erythroid precursors express elevated p53 further suggesting a role for p53 downstream of IL-22 signaling in driving dyserythropoiesis. Using banked and fresh del(5q) MDS and CKD patient samples, we also show that IL-22 is elevated in these human diseases.

The role of inflammatory cytokines in directly regulating various aspects of BM hematopoiesis in steady-state and diseased conditions is increasingly being recognized[41-43]. IL-22 is known to play a pathogenic role in some autoimmune diseases[44; 45]. Interestingly, autoimmune diseases such as colitis, Behçet's disease, and arthritis are common in MDS patients, with features of autoimmunity observed in up to 10% of patients[46]. It is intriguing

to hypothesize that IL-22 may account both for the onset of MDS and autoimmunity in this subset of patients. Studies have reported that in patients with co-existence of MDS and autoimmunity, treatment for one can alleviate the symptoms of the other[47; 48]. Low-level exposure to benzene, a hydrocarbon, has been associated with an increased risk of MDS[49]. Hydrocarbons are known ligands for aryl hydrocarbon receptor (AHR), the transcription factor that controls IL-22 production in T cells. Stemregenin 1, an AHR antagonist, was shown to promote the *ex vivo* expansion of human HSCs, with the highest fold expansion seen in the erythroid lineage[50]. Overall, these observations suggest that inhibition of the AHR-IL-22 axis may be an attractive approach for treating red blood cell disorders that arise from dyserythropoiesis.

Further, we provide evidence that neutralization of IL-22 signaling may be effective not only in the treatment of MDS and other stress-induced anemias, but also in the anemia of chronic diseases such as CKD, which are very much in need of new therapeutic approaches. With currently approved MDS therapies (lenalidomide and other hypomethylating agents, erythropoiesis-stimulating agents), the survival time of MDS patients after diagnosis is only 2.5-3 yrs. Patients also develop resistance to these therapies thus intensifying the need for additional therapeutic modalities. IL-22-based therapies could be used in conjunction with already existing therapeutics or after first-line therapies have failed due to acquisition of resistance.

METHODS

Human samples and processing

MDS and CKD patient samples were collected under IRB-approved protocols at Dana-Farber Cancer Institute (DFCI) and Brigham and Women's Hospital (BWH), respectively. All samples were de-identified at the time of inclusion in the study. All patients provided informed consent and the data collection was performed in accordance with the Declaration of Helsinki.

Peripheral blood mononuclear cells (PBMCs) from EDTA-treated whole blood were isolated using density gradient centrifugation. PBMCs were then incubated in RPMI with 10% FBS and Cell Activation Cocktail (Tonbo Biosciences) for 4 h and then processed for flow cytometry as described below. Relevant clinical information of MDS samples is provided in Supplemental Table Adult CKD plasma samples were stored at −80 °C until further use. Relevant clinical information of CKD samples is provided in Supplemental Table 3.

Generation of *Riok2* floxed mice

Riok2$^{f/f}$ mice were generated using frozen sperm obtained from Mutant Mouse Resource and Research Centers (MMRRC) (*Riok2*$^{tm1a(KOMP)Wtsi}$)[51]. In brief, a floxed *Riok2* allele was created by inserting an FRT-flanked IRES-LacZ-*neo*^{r} cassette into intron 4 of the *Riok2* gene. *Lox*P sites were inserted to flank exons 5 and 6. After germline transmission, the FRT cassette was removed by crossing to FLPe deleter mice[52] and resulting floxed mice were bred with individual *cre* driver strains to create conditional *Riok2*-deleted mice (Extended

Data Fig. 1a). Genotyping (Extended Data Fig. 1c) was carried out using the following primers:

Forward primer: 5′ GCATCAGTGATTTACAGACTAAAATGCC 3′

Reverse primer 1: 5′ GCTCTTACCCACTGAGTCATCTCACC 3′

Reverse primer 2: 5′ CCCAGACTCCTTCTTGAAGTTCTGC 3′

Mice

Wild-type C57BL/6J mice (Stock no. 000664), *Vav*-icre mice (Stock no. 008610), R26-CreErt2 mice (Stock no. 008463), *Il22ra1*-floxed (Stock no. 031003), CD45.1 C57BL/6J mice (Stock no. 002014), *Trp53*−/− (Stock no. 002101), *Cd4*-cre (Stock no. 022071), and Apc^{Min} (Stock no. 002020) mice were purchased from The Jackson Laboratory. $Il22^{-/-}$ mice were provided by R. Caspi (National Institutes of Health, Bethesda, MD) with permission from Genentech (San Francisco, CA). *Epor*-cre[53] mice were a gift from U. Klingmüller (Deutsches Krebsforschungszentrum (DFKZ), Germany). *Il22*-tdtomato (Catch-22)[54] mice were a gift from R. Locksley (University of California at San Francisco, CA). *Rps14*-floxed mice[55] were a gift from B. Ebert (Dana-Farber Cancer Institute, Boston, MA). Mice were housed in the Animal Research Facility (ARF) at DFCI under ambient temperature and humidity with 12 h light/12 h dark cycle. Animal procedures and treatments were in compliance with the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) at DFCI. Age- and gender- matched mice were used within experiments.

Competitive bone marrow (BM) transplantation

2×10^6 freshly isolated BM cells from $CD45.2^+$ $Riok2^{f/+}Ert2^{cre}$ or $Riok^{+/+}Ert2^{cre}$ mice were transplanted in competition with 2×10^6 freshly isolated $CD45.1^+$ wild-type (WT) BM cells via retro-orbital injection into lethally irradiated 8-10 week old $CD45.1^+$ WT recipient mice. The donor cell chimerism was determined in the peripheral blood four weeks after transplantation before the excision of *Riok2* was induced by tamoxifen injection as well as every four to eight weeks as indicated. Tamoxifen (75 mg/kg) (Cayman Chemical, Cat # 13258) was administered for five consecutive days.

Flow cytometry and cell isolation

Whole bone marrow (BM) cells were isolated by crushing hind leg bones (femur and tibia) with mortar and pestle in staining buffer (PBS (Corning) supplemented with 2% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals) and EDTA (GIBCO). Whole BM was lysed with 1X PharmLyse (BD Biosciences) for 90 s, and the reaction was terminated by adding an excess of staining buffer. Cells were labeled with fluorochrome-conjugated antibodies in staining buffer for 30 min at 4 °C. For flow cytometric analysis, cells were incubated with combinations of fluorochrome-conjugated antibodies to the following cell surface markers: CD3 (17A2, 1:500), CD5 (53–7.3, 1:500), CD11b (M1/70, 1:500), Gr1 (RB6–8C5, 1:500), B220 (RA3–6B2, 1:500), Ter119 (TER119, 1:500), CD71 (C2, 1:500), c-kit (2B8, 1:500), Sca-1 (D7, 1:500), CD16/32 (93, 1:500), CD150 (TC15–12F12.2, 1:150),

CD48 (HM48-1, 1:500). For sorting of lineage-negative cells, lineage markers included CD3, CD5, CD11b, Gr1 and Ter119. For sorting erythroid progenitor cells, the lineage cocktail did not include Ter119. All reagents were acquired from BD Biosciences, Thermo Fisher Scientific, Novus Biologicals, Tonbo Biosciences, or BioLegend. Identification of apoptotic cells was carried out using the Annexin V Apoptosis Detection Kit (BioLegend). Intracytoplasmic and intranuclear staining was performed using Foxp3/Transcription Factor Staining Kit (Thermo Fisher Scientific) or 0.1% saponin in PBS supplemented with 3% FBS. For staining performed with AF647 p53 antibody (Cell Signaling Technology, 1:50), cells were permeabilized with 90% ice-cold methanol. To increase the sorting efficiency, whole BM samples were lineage-depleted using magnetic microbeads (Miltenyi Biotec) and autoMACS Pro magnetic separator (Miltenyi Biotec). Cell sorting was performed on a FACS Aria flow cytometer (BD Biosciences), data acquisition was performed on a BD Fortessa X-20 instrument equipped with 5 lasers (BD Biosciences) employing FACSDiva software. Data were analyzed by FlowJo (Tree Star) version 9 software. Flow analyses were performed on viable cells by exclusion of dead cells using either DAPI or a fixable viability dye (Tonbo Biosciences). Gating for early and committed hematopoietic progenitors was performed as described elsewhere[56]. ILCs and NKT cells were identified as Lin $^{-}$CD45^{+}CD90^{+}CD12^{+} and CD3ε^{+} NK1.1^{+}, respectively.

Complete blood count

Mice were bled via the submandibular facial vein to collect blood in EDTA-coated tubes (BD Microtainer ™ Capillary Blood Collector, BD 365974). Complete blood counts were obtained using the HemaVet CBC Analyzer (Drew Scientific) or Advia 120 (Siemens Inc.,) instruments.

In vivo measurement of protein synthesis

100 μL of a 20mM solution of O-Propargyl-Puromycin (OP-Puro; BioMol) was injected intraperitoneally in mice and mice were then rested for 1 h. Mice injected with PBS were used as controls. BM was harvested after 1 h and stained with antibodies against cell surface markers, washed to remove excess unbound antibodies, fixed in 1% paraformaldehyde, and permeabilized in PBS with 3% FBS and 0.1% saponin. The azide-alkyne cyclo-addition was performed using the Click-iT Cell Reaction Buffer Kit (Thermo Fisher Scientific) and azide conjugated to Alexa Fluor 647 (Thermo Fisher Scientific) at 5 μM final concentration for 30 min. Cells were washed twice and analyzed by flow cytometry. ‘Relative rate of protein synthesis’ was calculated by normalizing OP-Puro signals to whole bone marrow after subtracting autofluorescence.

Methylcellulose assay

250 – 500 BM Lin^{-}c-kit^{+} Sca-1^{+} cells were flow sorted and plated in semi-solid methylcellulose culture medium (M3534, StemCell Technologies) and incubated at 37 °C in a humidified atmosphere for 7-10 days. At the end of the incubation period, each well was triturated with staining buffer to collect cells and then processed for flow cytometry as described above. Enumeration of colonies in MethoCult media was performed with StemVision instrument StemCell Technologies).

Phenylhydrazine treatment

Phenylhydrazine (PhZ) was purchased from Sigma and injected intraperitoneally on 2 consecutive days (days 0 and 1) at the dose of 25 mg/kg (sublethal model) or 35 mg/kg (lethal model). Peripheral blood was collected 3-4 days before the start of treatment and at day 4, 7, and 11. PhZ treatment experiments were carried out in 8-12 week old mice.

T cell polarization

Single-cell suspensions of mouse spleens were prepared by pressing tissue through a 70-μm cell strainer followed by red blood cell lysis using PharmLyse. Total splenic $CD4^+$ T cells were isolated using CD4 T cell isolation kit (Miltenyi Biotec). Enriched $CD4^+$ T cells were then incubated with fluorochrome-conjugated antibodies to CD4, CD8, CD25, CD62L, and CD44 to purify naïve $CD4^+$ T cells using fluorescence activated cell sorting (FACS). In the presence of 1 μg/mL plate-bound anti-CD3ε and soluble 1 μg/mL anti-CD28, T cell polarizations were carried out in IMDM supplemented with 10% FBS, 2mM L-glutamine, 100 mg/mL penicillin-streptomycin, HEPES (pH 7.2-7.6) , non-essential amino acids, 100 μM β-mercaptoethanol (BME), and sodium pyruvate as follows – T_H1 (20 ng/mL IL-12, 10 μg/mL anti-IL-4), T_H2 (20 ng/mL IL-4, 10 μg/mL anti-IFN-γ), T_H17 (30 ng/mL IL-6, 20 ng/mL IL-23, 20 ng/mL IL-1β, 10 μg/mL anti-IL-4, 10 μg/mL anti-IFN-γ), T_{reg} cells (1 ng/mL TGF-β, 10 μg/mL anti-IL-4, 10 μg/mL anti-IFN-γ), and T_H22 (30 ng/mL IL-6, 20 ng/mL IL-23, 20 ng/mL IL-1β, 10 μg/mL anti-IL-4, 10 μg/mL anti-IFN-γ, 400 nM FICZ). Pifithrin-α, p-Nitro and Nutlin-3a were purchased from Santa Cruz Biotechnology and Tocris Biosciences, respectively.

IL-22 neutralization and reconstitution

Monoclonal anti-IL-22 (Clone IL22JOP) blocking antibody and isotype control IgG2aκ (Clone eBR2a) were purchased from Thermo Fisher Scientific. Mice were administered anti-IL-22 (50 μg/mouse) or isotype intraperitoneally every 48 h until the conclusion of the experiment. For \recombinant IL-22 treatment, mice were injected with recombinant IL-22 (500 ng/mouse; PeproTech) intraperitoneally every 24 h until the conclusion of the experiment. Mice were administered these reagents at least five times before inducing PhZ-mediated anemia.

Cytokine quantitation

IL-22 in human samples was quantified using either Human IL-22 Quantikine ELISA Kit (D2200, R&D Systems) or SMCTM Human IL-22 High Sensitivity Immunoassay Kit (EMD Millipore, 03-0162-00) according to manufacturers' instructions. The SMC assay was read on a SMC Pro (EMD Millipore) instrument. The lower limit of quantification (LLOQ) of this immunoassay is 0.1 pg/mL. IL-22 in mouse samples was quantified using ELISA MAXTM Deluxe Set Mouse IL-22 (BioLegend, 436304). The LLOQ of this immunoassay is 3.9 pg/mL. S100A8 in human samples was quantified using Human S100A8 DuoSet ELISA (DY4570, R&D Systems).

Concentration of lineage-associated cytokines in cell culture supernatants of polarized T cells were quantified using a custom-made ProCarta Plex assay (Thermo Fisher Scientific) acquired on a Luminex platform. Hepcidin in mouse serum was quantified using a

colorimetric assay from Hepcidin Murine-Compete ™ ELISA Kit from Intrinsic LifeSciences (HMC-001).

mRNA quantitation

Cells were flow sorted directly into the lysis buffer provided with the Cells-to-CT™ 1-Step TaqMan™ Kit (A25605, Thermo Fisher Scientific) and processed according to the manufacturer's instructions. Pre-designed TaqMan gene expression assays were used to quantify mRNA expression by qPCR using QuantStudio 6 (Thermo Fisher Scientific). *Hprt* was used as housekeeping control. Relative expression was calculated using the ΔCt method. Please refer to Supplementary Table 4 for primers details.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed using EZ-ChIP Kit (EMD Millipore) according to manufacturer's instructions. Briefly, cells were fixed and cross-linked with 1% formaldehyde at 25 °C for 10 min and quenched with 125 mM glycine for an additional 10 min. Cells pellet was resuspended in lysis buffer and shearing was carried out Diagenode Bioruptor sonication system for a total of 40 cycles beads. Pre-cleared lysates were incubated with control Mouse IgG or anti-p53 (Santa Cruz Biotechnology) antibodies. *Il22* promoter-specific primer pair was designed using Primer 3.0 Input and were as follows:

Forward Primer: 5′ CCAAACTTAACTTGACCTTGGC 3′

Reverse Primer: 5′ TTCTTCACAGCTCCCA TTGC 3′

In vitro erythroid differentiation

Whole BM cells were labeled with biotin-conjugated lineage antibodies (cocktail of anti-CD3ε, anti-CD11b, anti-CD45R/B220, anti-Gr1, anti-CD5, and anti-TER-119) (BD Pharmingen) and purified using anti-biotin beads and negative selection on the AutoMACS Pro (Miltenyi). Purified cells were then seeded in fibronectin-coated (2 μg/cm^2) tissue-culture treated polystyrene wells (Corning® BioCoat™ Cellware) at a cell density of 10^5/mL. Erythroid differentiation was carried out according to modified published protocols[57]. The erythropoietic medium was IMDM supplemented with erythropoietin at 10 U/mL, 10 ng/mL stem cell factor SCF, PeproTech), 10 μM Dexamethasone (Sigma-Aldrich), 15% FBS, 1% detoxified BSA (StemCell Technologies), 200 μg/mL holotransferrin (Sigma-Aldrich), 10 mg/mL human insulin (Sigma-Aldrich), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, and penicillin-streptomycin. After 48 h, the medium was replaced by IMDM medium containing 20% FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, and penicillin-streptomycin. 50% of the culture media was replaced after 48 h and cell density was maintained at 0.5 × 10^6/mL. Total culture period for the assay was 6 days. Recombinant mouse IL-22 (PeproTech/Cell Signaling) was used where indicated. RII-RIV populations were gated as shown in Extended Data Fig. 2a.

Proteomic profiling

Proteomic profiling of sort-purified erythroid progenitors was performed as described elsewhere[58]. Briefly, cells were captured in collection microreactors and stored at −80 °C.

Cell lysis was performed by adding 10 μL of 8 M urea, 10 mM TCEP and 10 mM iodoacetamide in 50 mM ammonium bicarbonate (ABC) to the cell pellet of 1×10^6 erythroid progenitors and incubatedat room temperature for 30 min, shaking in the dark. 50 mM ABC was used to dilute the urea to less than 2 M and the appropriate amount of trypsin for a 1:100 enzyme to substrate ratio was added and allowed to incubate at 37 °C overnight. Once digestion is completed, the lysate is spun through the glass mesh directly onto a C18 Stage tip (Empore[59]) at 3500 x *g* until the entire digest passes through the C18 resin. 75 μL 0.1% formic acid (FA) is then used to ensure transfer of peptides to the C18 resin from the mesh while washing away the lysis buffer components. C18-bound peptides were immediately subjected to on-column TMT labeling.

On-column TMT Labeling -—Resin was conditioned with 50 μL methanol (MeOH), followed by 50 μL 50% acetonitrile (ACN)/0.1% FA, and equilibrated with 75 μL 0.1% FA twice. The digest was loaded by spinning at 3500 × *g* until the entire digest passed through. One μL of TMT reagent in 100% ACN was added to 100 μL freshly made HEPES, pH 8, and passed over the C18 resin at 350 × *g* until the entire solution passed through. The HEPES and residual TMT was washed away with two applications of 75 μL 0.1% FA and peptides were eluted with 50 μL 50% ACN/0.1% FA followed by a second elution with 50% ACN/20 mM ammonium formate (NH_4HCO_2), pH 10. Peptide concentrations were estimated using an absorbance reading at 280 nm and checking of label efficiency was performed on 1/20th of the elution. After using 1/20th of the elution to test for labeling efficiency, the samples are mixed before fractionation and analysis.

Stage tip bSDB Fractionation -—200 μL pipette tips were packed with two punches of sulfonated divinylbenzene (SDB-RPS, Empore) with a 16-gauge needle. After loading ~20 μg peptides total, a pH switch was performed using 25 μL 20 mM NH_4HCO_2, pH 10, and was considered part of fraction one. Then, step fractionation was performed using ACN concentrations of 5, 7.5, 10, 12.5, 15, 17.5, 20, 25, 42, and 50%. Each fraction was transferred to autosampler vials and dried via vacuum centrifugation and stored at 80 °C until analysis.

Data Acquisition -—Chromatography was performed using a Proxeon UHPLC at a flow rate of 200 nl/min. Peptides were separated at 50 °C using a 75 μm i.d. PicoFrit (New Objective) column packed with 1.9 μm AQ-C18 material (Dr. Maisch, Germany) to 20 cm in length over a 110 min run. The on-ine LC gradient went from 6% B at 1 min to 30% B in 85 mins, followed by an increase to 60% B by minute 94, then to 90% by min 95, and finally to 50% B until the end of the run. Mass spectrometry was performed on a Thermo Scientific Lumos Tribrid mass spectrometer. After a precursor scan from 350 to 1800 *m/z* at 60,000 resolution, the topmost intense multiply charged precursors in a 2 second window were selected for higher energy collisional dissociation (HCD) at a resolution of 50,000. Precursor isolation width was set to 0.7 *m/z* and the maximum MS2 injection time was 110 msecs for an automatic gain control of 6e4. Dynamic exclusion was set to 45 s and only charge states two to six were selected for MS2. Half of each fraction was injected for each data acquisition run.

Data Processing -—Data were searched all together with Spectrum Mill (Agilent) using the Uniprot Mouse database (28 Dec. 2017), containing common laboratory contaminants and 553 smORFs. A fixed modification of carbamidomethylation of cysteine and variable modifications of *N*-terminal protein acetylation, oxidation of methionine, and TMT-11plex labels were searched. The enzyme specificity was set to trypsin and a maximum of three missed cleavages was used for searching. The maximum precursor-ion charge state was set to six. The MS1 and MS2 mass tolerance were set to 20 ppm. Peptide and protein FDRs were calculated to be less than 1% using a reverse, decoy database. Proteins were only reported if they were identified with at least two distinct peptides and a Spectrum Mill score protein level score ~ 20.

TMT11 reporter ion intensities in each MS/MS spectrum were corrected for isotopic impurities by the Spectrum Mill protein/peptide summary module using the afRICA correction method which implements determinant calculations according to Cramer's Rule and general correction factors obtained from the reagent manufacturer's certificate of analysis.

Differential Protein Abundance Analysis -—The median normalized, median absolute deviation- scale data set was subjected to a moderated F-test, followed by Benjamini-Hochberg Procedure correcting for multiple hypothesis testing. We drew an arbitrary cutoff at adj. p val < 0.05.

RNA Sequencing (RNA-Seq)

5000 IL-22 $^{+}$(CD4^{+}IL-22(tdtomato)$^{+}$) cells were FACS sorted directly into TLC Buffer (Qiagen) with 1% β-mercaptoethanol. For the preparation of libraries, cell lysates were thawed and RNA was purified with 2.2x RNAClean SPRI beads (Beckman Coulter Genomics) without final elution[60]. The RNA captured beads were air-dried and processed immediately for RNA secondary structure denaturation (72 °C for 3 min) and cDNA synthesis. SMART-seq2 was performed on the resultant samples following the published protocol[61] with minor modifications in the reverse transcription (RT) step. A 15 μL reaction mix was used for subsequent PCR and performed 10 cycles for cDNA amplification. The amplified cDNA from this reaction was purified with 0.8× Ampure SPRI beads (Beckman Coulter Genomics) and eluted in 21 μL TE buffer. We used 0.2 ng cDNA and one-eighth of the standard Illumina NexteraXT (Illumina FC-131-1096) reaction volume to perform both the tagmentation and PCR indexing steps. Uniquely indexed libraries were pooled and sequenced with NextSeq 500 high output V2 75 cycle kits (Illumina FC-404-2005) and 38 × 38 paired-end reads on the NextSeq 500 instrument. Reads were aligned to the mouse mm10 transcriptome using Bowtie[62], and expression abundance TPM estimates were obtained using RSEM[63].

Pathway analysis

Gene set enrichment analysis (GSEA) was performed with Broad Institute's GSEA Software[64]. The 'IL-22_Signature' and 'Rps14_Increased' gene sets were created from the literature[55,65] (Fig. 2c,f). Full lists of genes in the individual gene sets can be found in Supplementary Table 1. Other reference gene sets are available from MSigDB[66]. For GSEA

analyses, mouse UniProt IDs were converted to their orthologous human gene symbols using MSigDB 7.1 CHIP file mappings. Pathway enrichment (Fig. 2e) was performed using Clarivate Analytics' MetaCore™ software[67].

Microarray data analysis

Microarray data of $CD34^+$ cells from healthy, del(5q), and non-del(5q) MDS subjects was obtained from a previously published study[68] submitted in Gene Expression Omnibus[69,70] accessible under GSE19429.

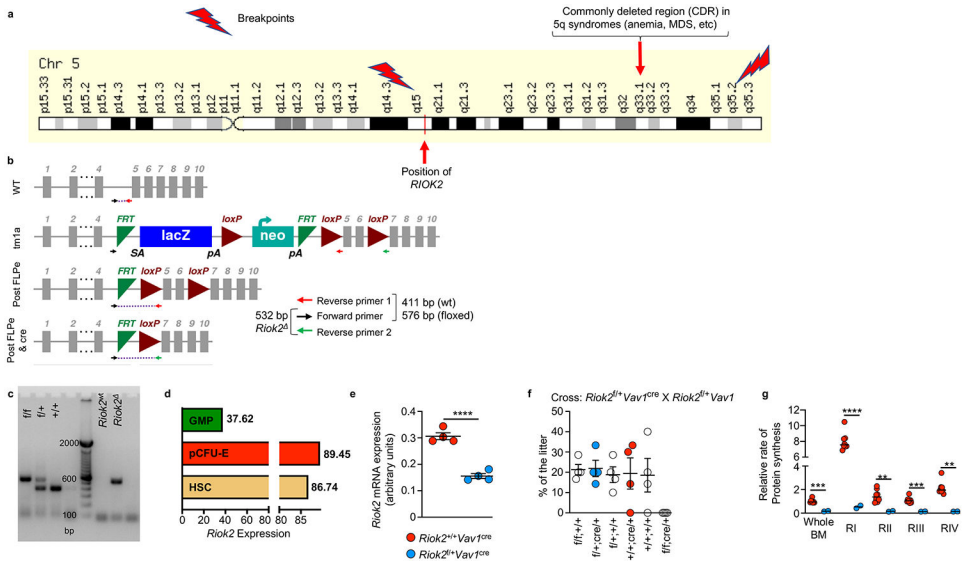
Statistical tests

Data are presented as mean ± s.e.m unless otherwise indicated. Comparison of two groups was performed using paired or unpaired two-tailed *t*-test. For multiple group comparisons, analysis of variance (ANOVA) with Tukey's correction or Kruskal-Wallis test with Dunn's correction was depending on data requirements. Statistical analyses were performed using GraphPad Prism v8.0 (GraphPad Software Inc.). A *P*-value of less than 0.05 was considered significant.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

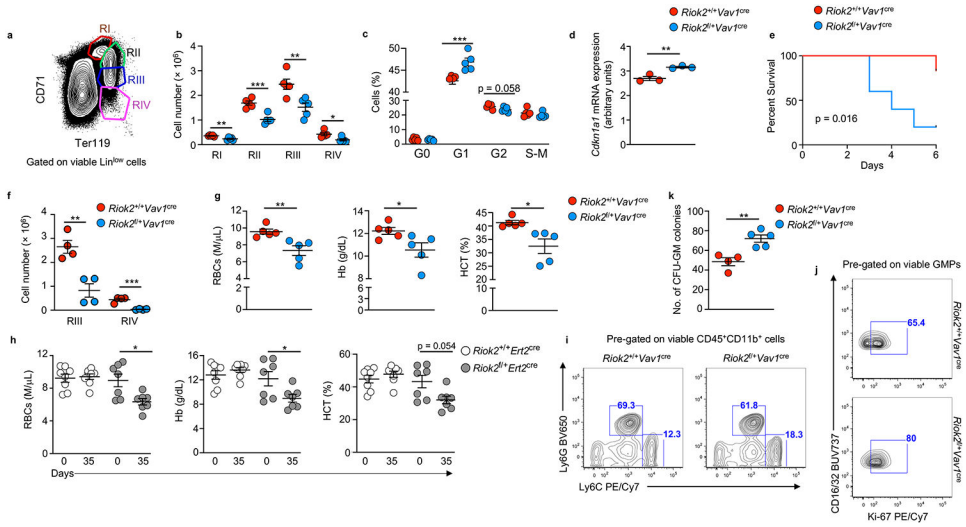
Extended Data



Extended Data Fig. 1. Localization and expression of *Riok2*.

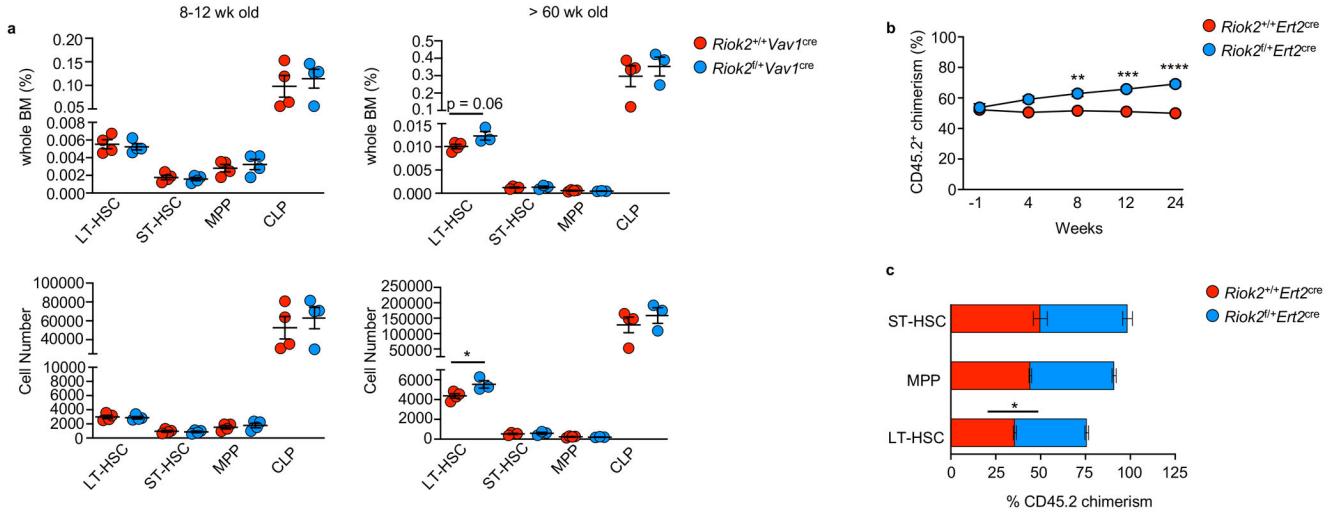
(**a**) Location of *RIOK2* gene on human chromosome 5. (**b**) Schematic representation of the *Riok2*$^{tm1a(KOMP)Wtsi}$ allele and generation of *Riok2* floxed mice. (**c**) Agarose gel showing genotyping of *Riok2* floxed mice. *Riok2*$^{\Delta}$ indicates deletion of *Riok2*. No band expected in the *Riok2*^{wt} lane. (**d**) Expression of *Riok2* mRNA in mouse BM cells. Modified from Gene Expression Commons. Numbers next to bars indicate expression level. (**e**) *Riok2* mRNA

expression by qRT-PCR in BM cells from *Riok2* haploinsufficient mice and *Vav1*^{cre} controls. n=5 mice/group. (**f**) Frequency of the genotypes indicated on the X-axis among 4 litters from 4 different breeding crosses of the genotypes mentioned. (**g**) *In vivo* protein synthesis rates in the indicated cell types from *Riok2* haploinsufficient mice (n=2) and *Vav1*^{cre} controls (n=8). Unpaired two-tailed *t*-test (e), multiple unpaired two-tailed *t*-tests with Holm-Sidak method (g) used to calculate statistical significance. Data are shown as mean ± s.e.m (e,f) or mean ± s.d. (g) and are representative of two (e, g) or four (c, f) independent experiments. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.



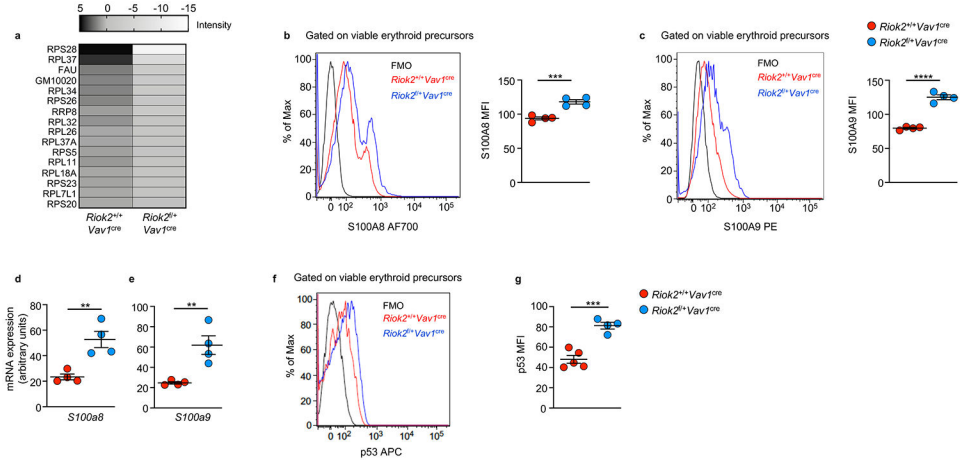
Extended Data Fig. 2. *Riok2* haploinsufficient mice display anemia and myeloproliferation.
(**a**) Gating strategy used for the identification of erythroid progenitor/ precursor cells in the BM. (**b**) Number of erythroid progenitor populations among viable BM cells in *Riok2*$^{f/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls. n=5/group. (**c**) Cell cycle analysis of erythroid progenitor/ precursor cells from *Riok2* haploinsufficient mice in comparison to *Vav1*^{cre} controls. n=5 mice/group. (**d**) *Cdkn1a* mRNA expression by qRT-PCR in erythroid progenitors from *Riok2* haploinsufficient mice and *Vav1*^{cre} controls. n=3 mice/group. (**e**) Kaplan-Meier survival curve for *Riok2* haploinsufficient mice and *Vav1*^{cre} controls subjected to lethal dose of PhZ. (**f**) Number of RIII and RIV erythroid precursor populations among viable BM cells in *Riok2*$^{f/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls day 6 after PhZ treatment. n=4/group. (**g**) PB RBC numbers, Hb, and HCT in mice transplanted with either *Riok2* haploinsufficient mice or *Vav1*^{cre} BM cells. n=5 mice/group. (**h**) PB RBC numbers, Hb, and HCT in mice with tamoxifen-inducible deletion of *Riok2*. Tamoxifen administered on days 3 – 7. n=8 and 7 for *Riok2*$^{+/+}$ *Ert2*^{cre} and *Riok2*$^{f/+}$ *Ert2*^{cre} mice, respectively. (**i**) Representative flow cytometry plots showing frequency of monocytes (CD11b$^+$Ly6G$^-$Ly6C^{hi}) and neutrophils (CD11b$^+$Ly6G$^+$) in the PB of *Riok2*$^{f/+}$ *Vav1*^{cre} and *Riok2*$^{+/+}$ *Vav1*^{cre} mice. (**j**) Representative flow cytometry plots showing Ki-67$^+$ GMPs in the BM of *Riok2*$^{f/+}$ *Vav1*^{cre} and *Riok2*$^{+/+}$ *Vav1*^{cre} mice. (**k**) Number of CFU-GM colonies in MethoCult from Lin$^-$Sca-1$^+$c-kit$^+$ BM cells from *Riok2*$^{f/+}$ *Vav1*^{cre} mice (n=5) and *Riok2*$^{+/+}$ *Vav1*^{cre} controls (n=4) after a 7-day culture period. n=4-5/group. Multiple unpaired two-tailed *t*-tests with Holm-Sidak method (b, c), unpaired two-tailed *t*-test (d, f, g, k), log-

rank test (e), and 2-way ANOVA with Sidak's correction for multiple comparisons (h) used to calculate statistical significance. Data are shown as mean ± s.e.m and are representative of two (b to k) independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001.



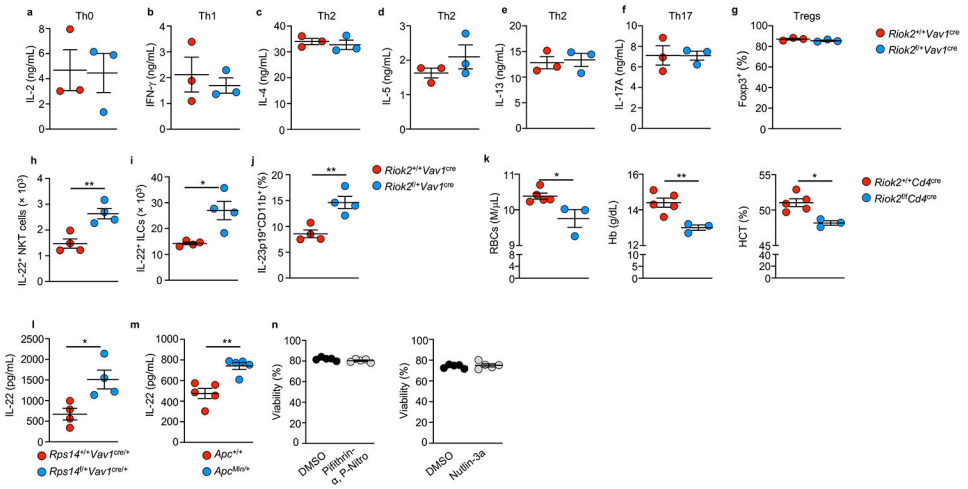
Extended Data Fig. 3. *Riok2* haploinsufficiency alters early hematopoietic progenitors in an age-dependent fashion.

(a) Frequency and number of indicated cell types in the bone marrow of *Riok2*$^{f/+}$ *Vav1*^{cre} and *Riok2*$^{+/+}$ *Vav1*^{cre} mice. n = 4/group. LT-HSC = long term hematopoietic stem cells, ST-HSC = short term hematopoietic stem cells, MPP = multipotent progenitors, CLP = common lymphoid progenitors. (**b**) % CD45.2 (donor) chimerism in PB from competitive BM transplant with CD45.1 recipient cells. Time point '−1' reflects first bleeding 4 weeks after transplantation and one day before tamoxifen induced deletion of *Riok2*. Donor (CD45.2) chimerism of the HSC compartment in the BM of competitive transplantation experiments. n = 5/group. (**c**) Frequency of donor (CD45.2^{+}) early hematopoietic progenitors 24 weeks after tamoxifen treatment in a competitive transplantation assay as described in (b). n=5 and 4 for *Riok2*$^{+/+}$ *Ert2*^{cre} and *Riok2*$^{f/+}$ *Ert2*^{cre} mice, respectively. Unpaired two-tailed *t*-test (a, c) and 2-way ANOVA with Sidak's multiple comparison test (b) used to calculate statistical significance. Data are shown as mean ± s.e.m and are representative of two (a-c) independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.



Extended Data Fig. 4. *Riok2* haploinsufficient erythroid precursors express increased S100 proteins.

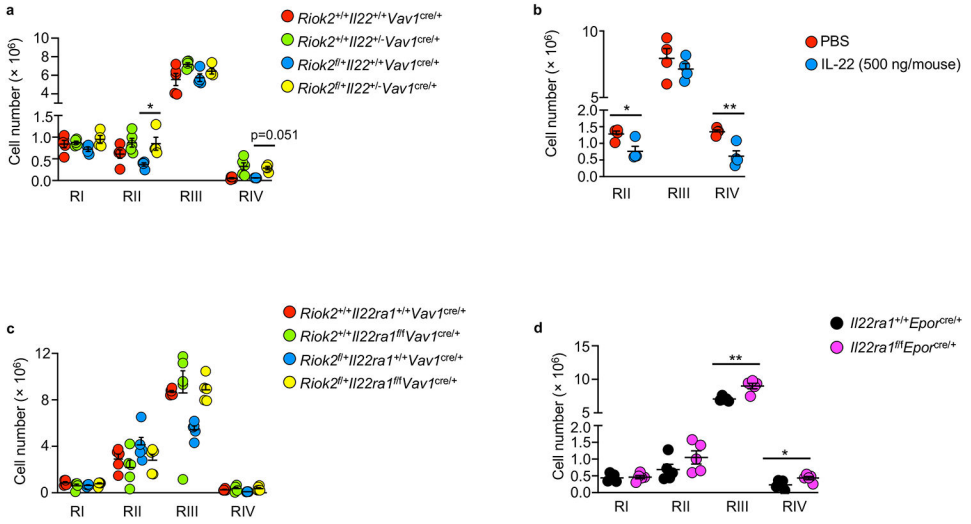
(**a**) Expression of ribosomal proteins quantified by proteomics in *Riok2*$^{f/+}$ *Vav1*^{cre} and *Riok2*$^{+/+}$ *Vav1*^{cre} erythroid precursors. S100A8 (**b**) and S100A9 (**c**) expression assessed by flow cytometry in BM erythroid precursors from *Riok2*$^{f/+}$ *Vav1*^{cre} and *Riok2*$^{+/+}$ *Vav1*^{cre} mice. n=4mice/group. (**d, e**) *S100a8* and *S100a9* mRNA expression in erythroid precursors isolated from *Riok2*$^{f/+}$ *Vav1*^{cre} and *Riok2*$^{+/+}$ *Vav1*^{cre} mice. n=4 mice/group. (**f**) p53 expression assessed by flow cytometry in BM erythroid precursors from *Riok2*$^{f/+}$ *Vav1*^{cre} and *Riok2*$^{+/+}$ *Vav1*^{cre} mice. (**g**) Graphical representation of data shown in (e). n=5 mice/group. Data are shown as mean ± s.e.m and are representative of two (b to g) independent experiments. Unpaired two-tailed *t*-test (b to g) used to calculate statistical significance. ** p < 0.01, *** p < 0.001, **** p < 0.0001.



Extended Data Fig. 5. Expression of lineage-associated T cell cytokines is comparable between *Riok2* haploinsufficient and sufficient T cells.

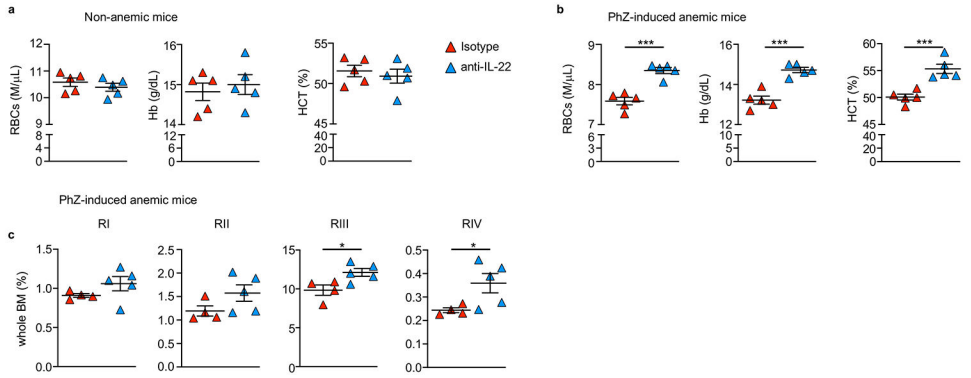
a-g, Concentration of IL-2 (**a**), IFN-γ (**b**), IL-4 (**c**), IL-5 (**d**), IL-13 (**e**), IL-17A (**f**) and frequency of Foxp3^{+} cells (**g**) from *in vitro* polarized T cells of the indicated genotypes. n=3 mice/group. (**h-i**) Number of IL-22^{+} NKT cells (H) and ILCs (I) in the spleens of *Riok2*$^{fl/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls (n=4/group). (**j**) Frequency of

IL_23p19^{+} DCs in *Riok2*$^{f/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls. n=4 mice/group. (**k**) PB RBC numbers, Hb, and HCT in *Riok2*$^{f/f}$ *Cd4*^{cre} mice (n=3) in comparison to *Riok2*$^{+/+}$ *Cd4*^{cre} controls (n=5) . (**l**) Secreted IL-22 from *in vitro* polarized T_H22 cells from *Rps14* haploinsufficient mice and *Vav1*^{cre} controls. n=4 mice/group. (**m**) Secreted IL-22 from *in vitro* polarized T_H22 cells from *Apc*^{Min} mice and littermate controls. n=4 mice/group. (**n**) Viable cells (expressed as percentage of total cells in culture) for the indicated treatments assessed by flow cytometry. n=5 mice/group. Data are shown as mean ± s.e.m and are representative of two (a to n) independent experiments. Unpaired two-tailed *t*-test (a to n) used to calculate statistical significance. * $p < 0.05$, ** $p < 0.01$.



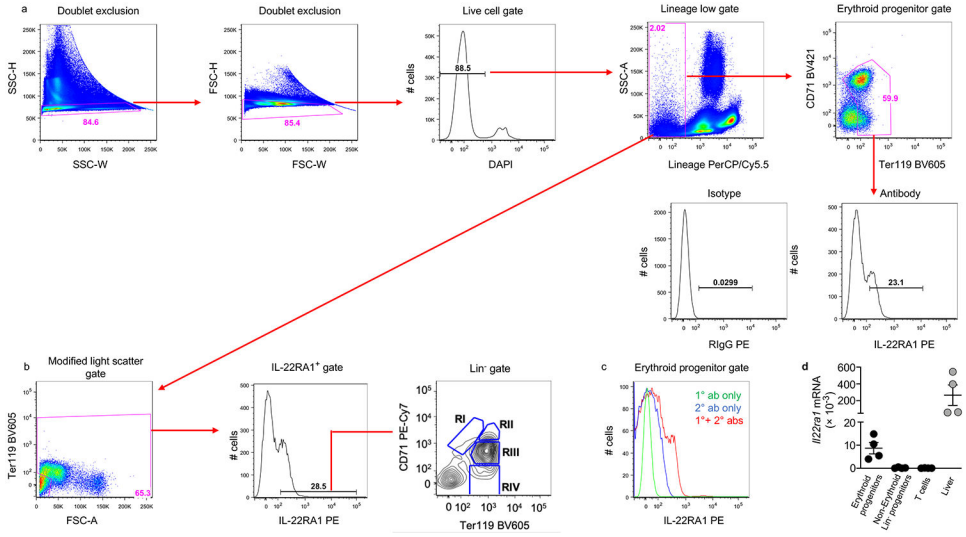
Extended Data Fig. 6. Neutralization of IL-22 signaling increases number of erythroid precursors.

a-d, Number of RI-RIV erythroid populations among viable BM cells in the indicated strains undergoing PhZ-induced stress erythropoiesis. For (a), n=5,5,4, and 4 for *Riok2*$^{+/+}$ *Il22*$^{+/+}$ *Vav1*^{cre}, *Riok2*$^{+/+}$ *Il22*$^{+/-}$ *Vav1*^{cre}, *Riok2*$^{f/+}$ *Il22*$^{+/+}$ *Vav1*^{cre}, *Riok2*$^{f/+}$ *Il22*$^{+/-}$ *Vav1*^{cre}, respectively. For (d), n=5/group. Data are shown as mean ± s.e.m and are representative of three (a, c) or two (b, d) independent experiments. 1-way ANOVA with Tukey's correction (a, c) or unpaired two-tailed *t*-test (b, d) used to calculate statistical significance. * $p < 0.05$, ** $p < 0.01$.



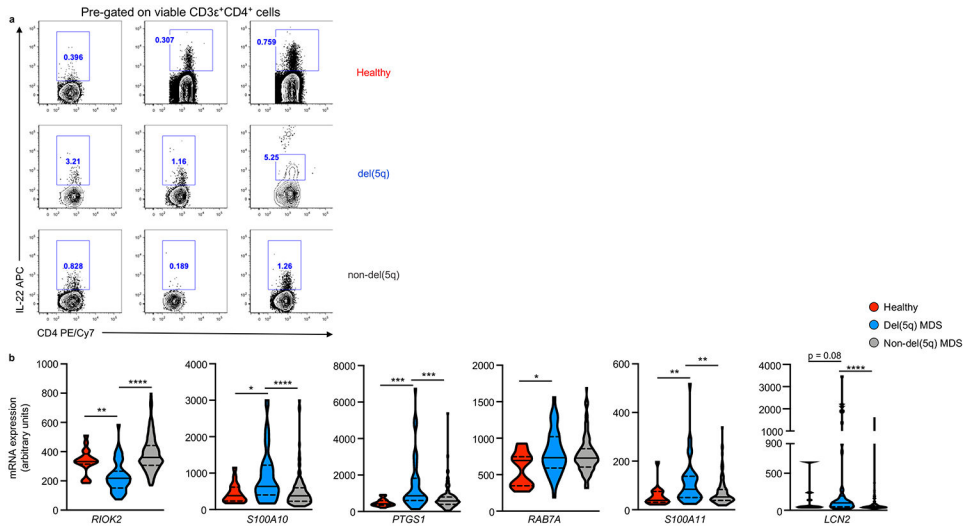
Extended Data Fig. 7. IL-22 neutralization alleviates anemia in wt mice undergoing PhZ-induced stress erythropoiesis.

(**a**) PB RBC numbers, Hb, and HCT in naïve wt C57BL/6J mice treated with isotype control (Rat IgG2aκ, 50 mg/mouse) or anti-IL-22 antibody (50 mg/mouse). n=5 mice/group. (**b**) PB RBC numbers, Hb, and HCT in wt C57BL/6J mice undergoing PhZ-induced stress erythropoiesis treated with isotype control or anti-IL-22 antibody. n=5 mice/group. (**c**) Percentage of RI-RIV erythroid precursors in the BM of mice treated as in (**b**). n=4 and 5 for *Il22ra1*$^{+/+}$*Epor*^{cre} and *Il22ra1*$^{f/f}$*Epor*^{cre} mice, respectively. Data are shown as mean ± s.e.m and are representative of three (a, b) or two (c) independent experiments. Unpaired two-tailed *t*-test (a to c) used to calculate statistical significance. * $p < 0.05$, *** $p < 0.001$.



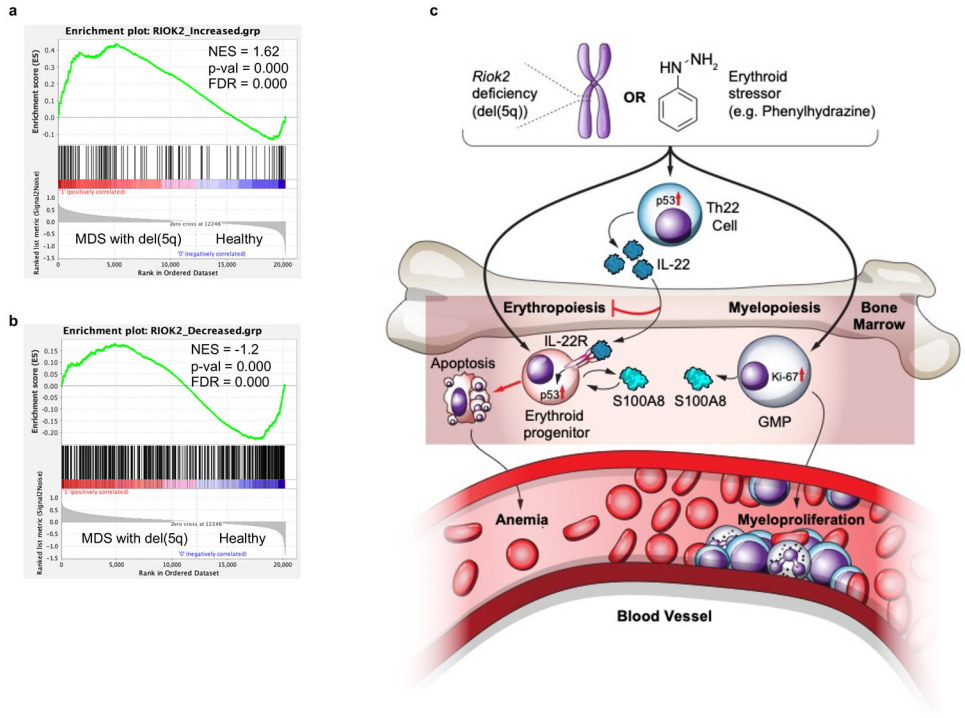
Extended Data Fig. 8. Erythroid precursors express IL-22RA1.

(**a**) Gating strategy employed for assessing IL-22RA1 expression on erythroid precursors. (**b**) Gating strategy to show that majority of IL-22RA1^{+} cells in the mouse BM are erythroid precursors. (**c**) IL-22RA1 expression on erythroid precursors assessed using flow cytometry and a second antibody targeting a different epitope of IL-22RA1. (**d**) *Il22ra1* mRNA expression in the indicated cell types assessed by qRT-PCR. T cells and liver represent negative and positive controls, respectively. n=4 mice/group. Data are shown as mean ± s.e.m (d) and are representative of three (a to c) or two (d) independent experiments.



Extended Data Fig. 9. Increased IL-22 and its signature genes in del(5q) MDS subjects.

(**a**) Representative flow cytometry plots showing frequency of $CD4^+IL\text{-}22^+$ cells among total PBMCs in the peripheral blood of MDS patients and healthy subjects. Pre-gated on viable $CD3\varepsilon^+CD4^+$ cells. Cumulative data shown in Figure 4e. (**b**) Expression of indicated IL-22 signature genes in $CD34^+$ cells from healthy controls and del(5q) and non-del(5q) MDS patients. n = 17, 47, and 136 for healthy, del(5q) MDS, and non-del(5q) MDS, respectively. Kruskal-Wallis test with Dunn's correction for multiple comparisons (b) used to calculate statistical significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Solid lines represent median and dashed lines represent quartiles (b).



Extended Data Fig. 10. *Riok2* haploinsufficiency recapitulates del(5q) MDS transcriptional changes.

(**a to b**) GSEA enrichment plots comparing proteins up-regulated (**a**) and down-regulated (**b**) upon *Riok2* haploinsufficiency to the transcriptional changes seen in del(5q) MDS. (**c**) Schematic of mechanism underlying *Riok2* haploinsufficiency-induced, IL-22 –induced anemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Data availability

The original mass spectra may be downloaded from MassIVE (http:\\massive.ucsd.edu), MSV000085287. The data are directly accessible via ftp://massive.ucsd.edu/MSV000085287/. Raw RNA-Seq data is accessible via Gene Expression Omnibus (GEO) under the accession code GSE165467.

Source data for all applicable figures (Main and Extended) will be provided with the paper. The remaining data supporting the findings of this study are available from the corresponding authors upon reasonable request. Materials will be provided with material transfer agreements (MTA) as appropriate.

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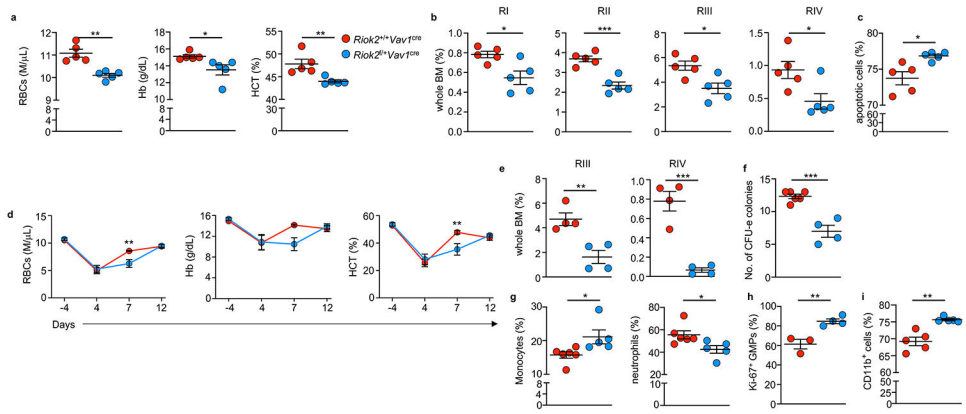
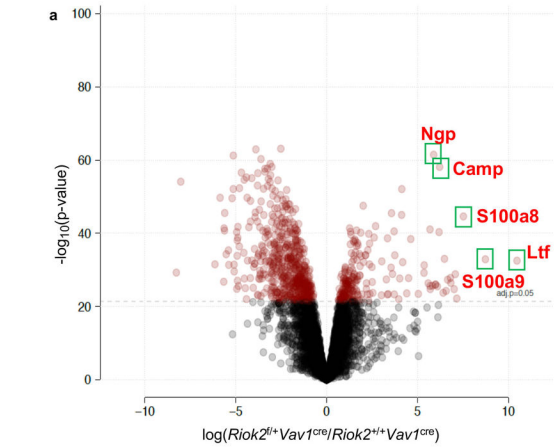


Figure 1. *Riok2* haploinsufficient (*Riok2*$^{f/+}$*Vav1*^{cre}) mice display anemia and myeloproliferation.

(**a**) Peripheral blood (PB) RBC numbers, hemoglobin (Hb), and hematocrit (HCT) in *Riok2*$^{f/+}$ *Vav1*^{cre} mice in comparison to *Riok2*$^{+/+}$ *Vav1*^{cre} controls (n=5/group). (**b**) Frequency of erythroid progenitor/precursor populations among viable bone marrow (BM) cells in *Riok2*$^{f/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls (n=5/group). (**c**) Frequency of apoptotic erythroid precursors among viable BM cells in *Riok2*$^{f/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls (n=5/group). (**d**) PB RBC numbers, Hb, and HCT in *Riok2*$^{f/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls undergoing phenylhydrazine (PhZ)-induced stress erythropoiesis (n=7/group). (**e**) Frequency of RIII and RIV erythroid precursor populations among viable BM cells in *Riok2*$^{f/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls day 6 after PhZ treatment (n=4/group). (**f**) Number of CFU-e colonies in Epo-containing MethoCult assay using Lin^{-}c-kit^{+}CD71^{+} cells from *Riok2*$^{f/+}$ *Vav1*^{cre} mice (n=4) in comparison to *Riok2*$^{+/+}$ *Vav1*^{cre} controls (n=6). (**g**) Percentage of monocytes (CD11b^{+}Ly6G^{-}Ly6C^{hi}) and neutrophils (CD11b^{+}Ly6G^{+}) in the PB of *Riok2*$^{f/+}$ *Vav1*^{cre} mice (n=6) in comparison to *Riok2*$^{+/+}$ *Vav1*^{cre} controls (n=5). (**h**) Percentage of Ki-67^{+} granulocyte-macrophage progenitors (GMPs) in the BM of *Riok2*$^{f/+}$ *Vav1*^{cre} mice (n=3) and *Riok2*$^{+/+}$ *Vav1*^{cre} controls (n=4). (**i**) Percentage of CD11b^{+} cells obtained from Lin^{-}Sca-1^{+}c-kit^{+} BM cells from *Riok2*$^{f/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls cultured in MethoCult for 7 days (n=5/group). Unpaired two-tailed *t*-test (a to c, e to i) and 1-way ANOVA with Tukey's correction for multiple comparisons (d) used to calculate statistical significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are shown as mean ± s.e.m and are representative of two (c, d, f-h) or three (a, b, e, i) independent experiments.



b

	Riok2 haploinsufficiency		*Rps14* haploinsufficiency	
Proteins	Adj. p-val	Log Fold Change	Adj. p-val	Log Fold Change
Cathelicidin (CAMP)	0.002	12.03	0.003	1.37
NGP	0.002	8.73	0.003	1.35
S100A9	0.002	8.57	0.002	1.45
Lactotransferrin (LTF)	0.001	7.38	0.005	1.45
S100A8	0.003	6.29	0.001	2.05

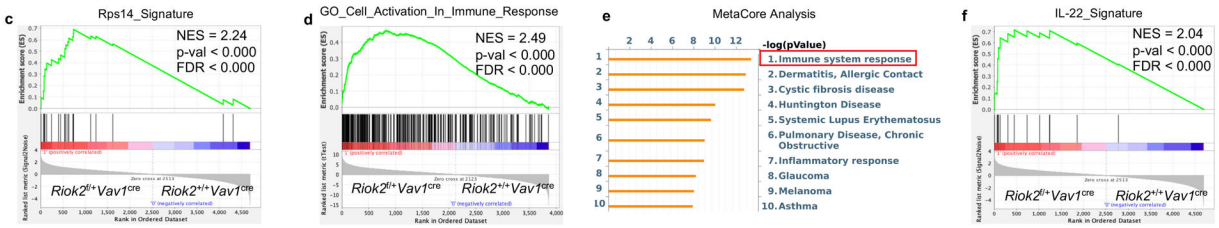


Figure 2. Quantitative proteomics of *Riok2* haploinsufficient erythroid precursors reveals immune activation signatures.

(**a**) Proteomic analysis of changes in protein expression in erythroid progenitors from *Riok2* haploinsufficient mice and *Vav1*^{cre} controls. n = 4-5 mice/group. (**b**) Comparison of upregulated proteins with their respective p-values and log fold-change values in erythroid precursors from *Riok2* haploinsufficient mice and *Rps14* haploinsufficient mice with their respective controls. (**c** to **d, f**) GSEA performed on proteomics data shown in (a) to reveal similarity with *Rps14* haploinsufficient data (c), activation of immune response (d) and enrichment of IL-22 signature genes (f). NES = Normalized enrichment score, FDR = False discovery rate. **(e)** MetaCore analysis of the Riok2 proteomics dataset shown in (a). Two sample moderated *t*-test with multiple hypothesis corrections used to calculate the statistical significance in (b).

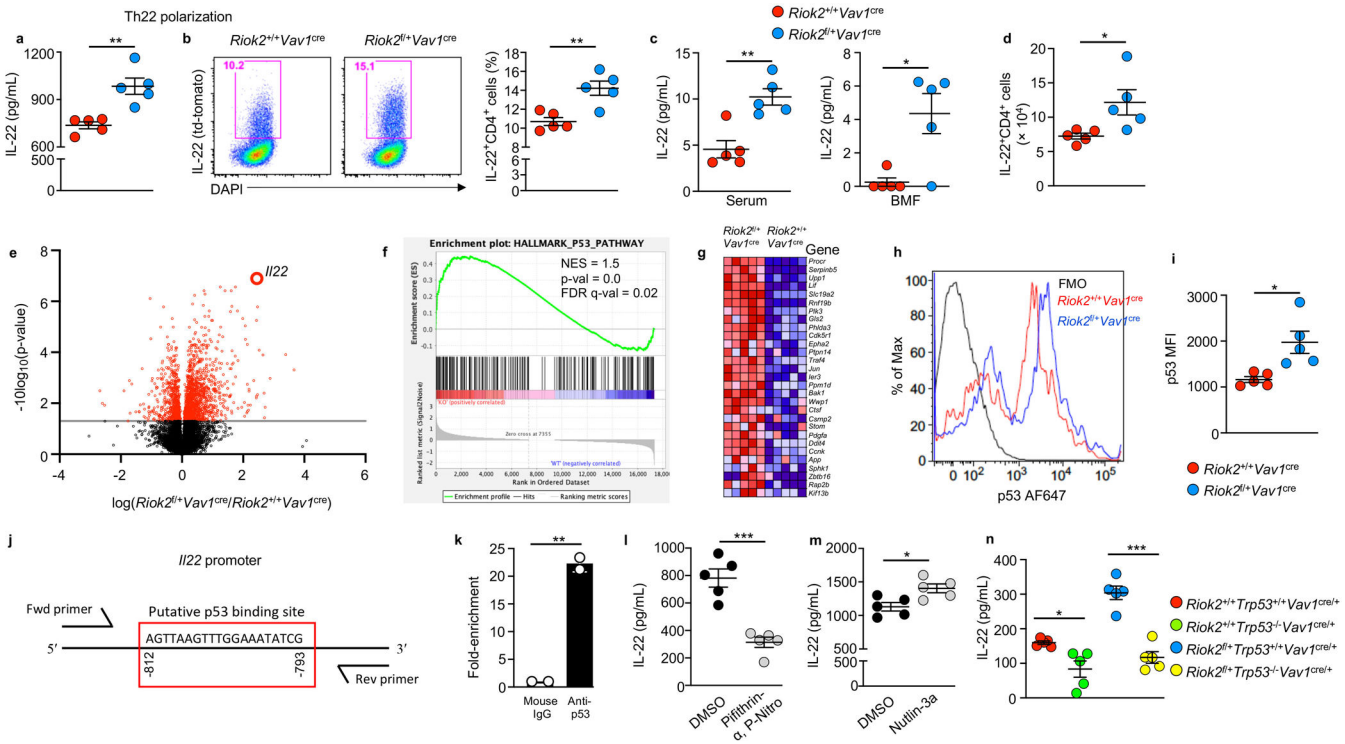


Figure 3. *Riok2* haploinsufficiency-driven p53 upregulation drives increased IL-22.

(a) Secreted IL-22 and (**b**) Percentage of IL-22^{+}CD4^{+} T cells from *in vitro* polarized T$_{H}$22 cells from *Riok2*$^{fl/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls (n=5/group). (**c**) IL-22 levels in the serum (left) and bone marrow fluid (BMF) (right) in *Riok2*$^{fl/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls (n=5/group). (**d**) Number of IL-22^{+}CD4^{+} cells in the spleens of *Riok2*$^{f/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls (n=5/group). (**e**) Volcano plot showing transcriptomic changes in purified IL-22^{+} cells from *Riok2*$^{f/+}$ *Vav1*^{cre} mice in comparison to *Riok2*$^{+/+}$ *Vav1*^{cre} controls. n=5/group. (**f**) GSEA analysis showing activation of the p53 pathway in IL-22^{+} cells from *Riok2*$^{fl/+}$ *Vav1*^{cre} mice in comparison to *Riok2*$^{+/+}$ *Vav1*^{cre} controls. (**g**) Snapshot of differentially expressed genes in the p53 pathway shown in (F) in *Riok2*$^{f/+}$ *Vav1*^{cre} and *Riok2*$^{+/+}$ *Vav1*^{cre} mice. (**h**) Flow cytometry plot showing p53 expression in *in vitro* polarized T$_{H}$22 cells from *Riok2*$^{fl/+}$ *Vav1*^{cre} and *Riok2*$^{+/+}$ *Vav1*^{cre} controls. (**i**) Graphical representation of data shown in (H). n=5/group. (**j**) Predicted p53 binding site in the *Il22* promoter region. (**k**) Chromatin immunoprecipitation showing p53 occupancy at the *Il22* promoter in T cells. n=2 independent experiments. (**l**) Secreted IL-22 from wt T$_{H}$22 cells cultured in the presence or absence of p53 inhibitor, pifithrin-α, p-nitro (1 μM). n=5 mice/group. (**m**) Secreted IL-22 from WT T$_{H}$22 cells cultured in the presence or absence of p53 activator, Nutlin-3 (100 nM). n=4 mice/group. (**n**) Secreted IL-22 from *in vitro* polarized T$_{H}$22 cells from the indicated strains. n=5/group. Unpaired two-tailed *t*-test (a to d, i, k-m) and 1-way ANOVA with Tukey's correction for multiple comparison (n) used to calculate statistical significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are shown as mean ± s.e.m and are representative of two (c - d, h, i, k - n) or three (a,b) independent experiments. Data in (k) is represented as mean ± s.d. and is pooled from two independent experiments.

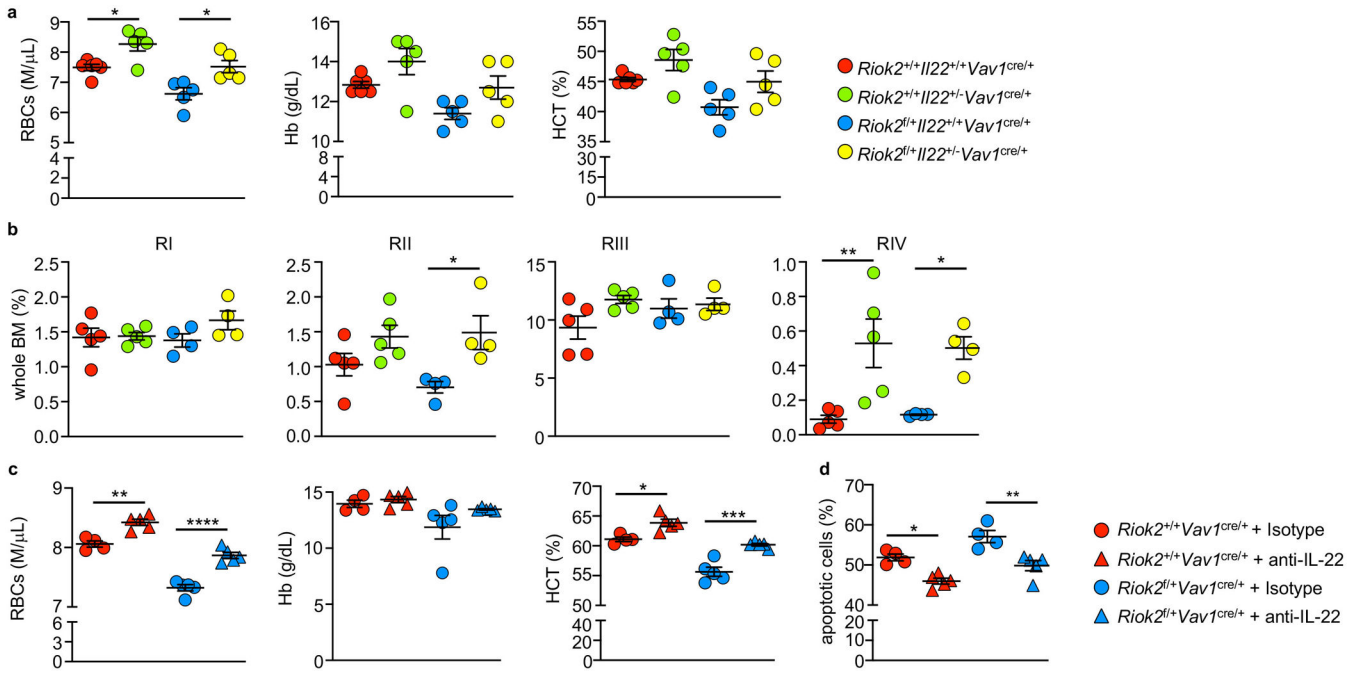


Figure 4. IL-22 neutralization alleviates stress-induced anemia in Riok2 sufficient and haploinsufficient mice.

(**a**) PB RBC numbers, Hb, and HCT in the indicated strains undergoing PhZ-induced stress erythropoiesis. n=6,5,5, and 5 mice for *Riok2*$^{+/+}$ *Il22*$^{+/+}$ *Vav1*^{cre}, *Riok2*$^{+/+}$ *Il22*$^{+/-}$ *Vav1*^{cre}, *Riok2*$^{f/+}$ *Il22*$^{+/+}$ *Vav1*^{cre}, *Riok2*$^{f/+}$ *Il22*$^{+/-}$ *Vav1*^{cre}, respectively. (**b**) Frequency of erythroid progenitor/precursor populations among viable BM cells in the indicated strains undergoing PhZ-induced stress erythropoiesis (n=4-5/group). (**c**) PB RBC numbers, Hb, and HCT in *Riok2*$^{f/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls undergoing PhZ-induced stress erythropoiesis treated with either an isotype control or anti-IL-22 antibody (n=4-5/group). (**d**) Frequency of apoptotic erythroid precursors among viable BM cells in *Riok2*$^{f/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls undergoing PhZ-induced stress erythropoiesis treated with either an isotype control or anti-IL-22 antibody. n=4,5,4, and 5 mice for isotype-treated *Riok2*$^{+/+}$ *Vav1*^{cre}, anti-IL-22-treated *Riok2*$^{+/+}$ *Vav1*^{cre}, isotype-treated *Riok2*$^{f/+}$ *Vav1*^{cre}, and anti-IL-22-treated *Riok2*$^{f/+}$ *Vav1*^{cre} mice, respectively. 1-way ANOVA with Tukey's correction for multiple comparison (a to d) used to calculate statistical significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Data are shown as mean ± s.e.m and are representative of two (c, d) or three (a, b) independent experiments.

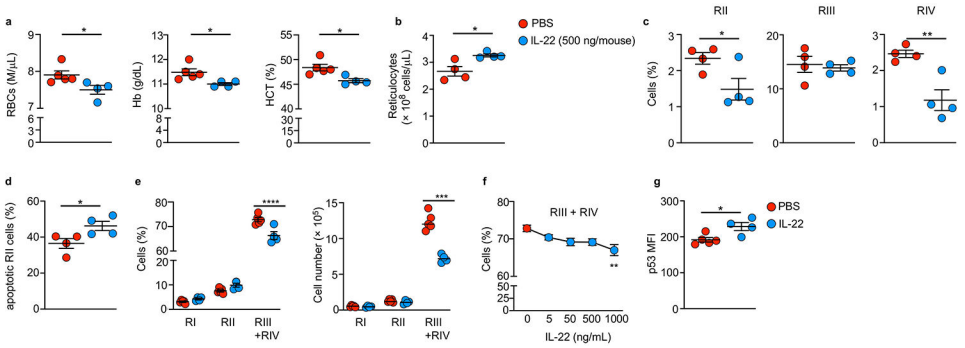


Figure 5. Recombinant IL-22 exacerbates PhZ-induced anemia in wt mice.

(**a**) PB RBC numbers, Hb, and HCT in wt C57BL/6J mice administered PBS (n=5) or rIL-22 (n=4) and subsequently treated with PhZ. n=4-5 mice/group. (**b**) PB reticulocytes in mice treated as in (**a**). n=4 mice/group. (**c**) Percentage of RII-RIV erythroid precursors in the BM of PBS- or rIL-22-treated C57BL/6J mice 7 days after PhZ administration. n=4 mice/group. (**d**) Percentage of apoptotic RII erythroid precursors in mice treated as in (C). n=4 mice/group. (**e**) Effect of recombinant IL-22 (500 ng/mL) on the frequency (left) and cell number (right) in an *in vitro* erythropoiesis assay and (**f**) dose dependent effect of recombinant IL-22. n=5 and 4 for PBS and IL-22 groups, respectively. (**g**) p53 expression in *in in vitro* erythropoiesis culture treated with rIL-22 or PBS. n=5 and 4 mice for PBS and IL-22 groups, respectively. Data are shown as mean ± s.e.m and are representative of three (a, b) or two (c to g) independent experiments. Unpaired two-tailed *t*-test (A to D, G), multiple unpaired two-tailed *t*-tests with Holm-Sidak method (e) and 1-way ANOVA with Tukey's correction for multiple comparisons (f) used to calculate statistical significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

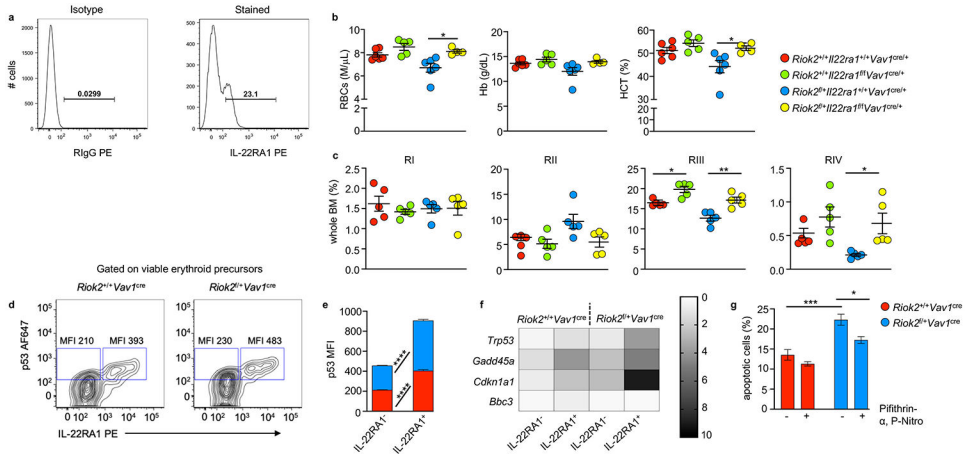


Figure 6. Genetic deletion of *Il22ra1* alleviates anemia in *Riok2* haploinsufficient mice.

(**a**) IL-22RA1 expression on BM erythroid precursors in wild-type (WT) mice assessed by flow cytometry using antibody from Novus Biologicals targeting the extracellular domain of IL-22RA1. (**b**) PB RBC numbers, Hb, and HCT in the indicated strains undergoing PhZ-induced stress erythropoiesis. n=6,5,6, and 4 mice for *Riok2*$^{+/+}$ *Il22r1a*$^{+/+}$ *Vav1*^{cre}, *Riok2*$^{+/+}$ *Il22ra1*$^{f/f}$ *Vav1*^{cre}, *Riok2*$^{f/+}$ *Il22ra1*$^{+/+}$ *Vav1*^{cre}, *Riok2*$^{f/+}$ *Il22ra1*$^{f/f}$ *Vav1*^{cre}, respectively. (**c**) Frequency of erythroid progenitor/ precursor populations among viable BM cells in the indicated strains undergoing PhZ-induced stress erythropoiesis (n=5/group). (**d**) Flow cytometry plots showing p53 expression in IL-22RA1^{+} and IL-22RA1^{-} erythroid precursors in *Riok2*$^{f/+}$ *Vav1*^{cre} mice and *Riok2*$^{+/+}$ *Vav1*^{cre} controls. n=5/group. (**e**) Graphical representation of data shown in (D). (**f**) Gene expression of *Trp53* (p53) and listed p53 target genes in IL-22RA1^{+} and IL-22RA1^{-} erythroid precursors from *Riok2*$^{f/+}$ *Vav1*^{cre} and *Riok2*$^{+/+}$ *Vav1*^{cre} mice assessed by qRT-PCR. n=3/group. (**g**) Frequency of apoptotic cells (assessed by flow cytometry) with (n=4) or without (n=5) p53 inhibitor, pifithrin-α, p-nitro (1 μM), in an in *vitro* erythropoiesis assay using Lin^{-} BM cells from *Riok2*$^{f/+}$ *Vav1*^{cre} and *Riok2*$^{+/+}$ *Vav1*^{cre} mice. 2-way ANOVA with Tukey's correction for multiple comparisons (e, g) and 1-way ANOVA with Tukey's correction for multiple comparison (b, c) used to calculate statistical significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Data are shown as mean ± s.e.m and are representative of two (D-G) or three (a to c) independent experiments.

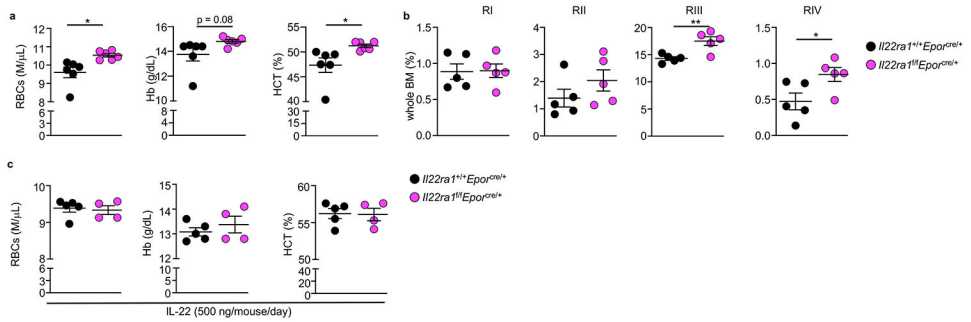


Figure 7. Erythroid-specific deletion of IL-22RA1 alleviates stress-induced anemia.

(**a**) PB RBC numbers, Hb, and HCT in the indicated strains undergoing PhZ-induced stress erythropoiesis (n=6/group). (**b**) Frequency of erythroid progenitor/precursor populations among viable BM cells in the indicated strains undergoing PhZ-induced stress erythropoiesis (n=5/group). (**c**) PB RBC numbers, Hb, and HCT in *Il22ra1*$^{+/+}$*Epor*^{cre} (n=5) and *Il22ra1*$^{f/f}$*Epor*^{cre} (n=4) mice administered rIL-22 and subsequently treated with PhZ. n=4-5 mice/group. Unpaired two-tailed *t*-test (a-c) used to calculate statistical significance. * $p < 0.05$, *** $p < 0.001$. Data are shown as mean ± s.e.m and are representative of two (a-c) independent experiments.

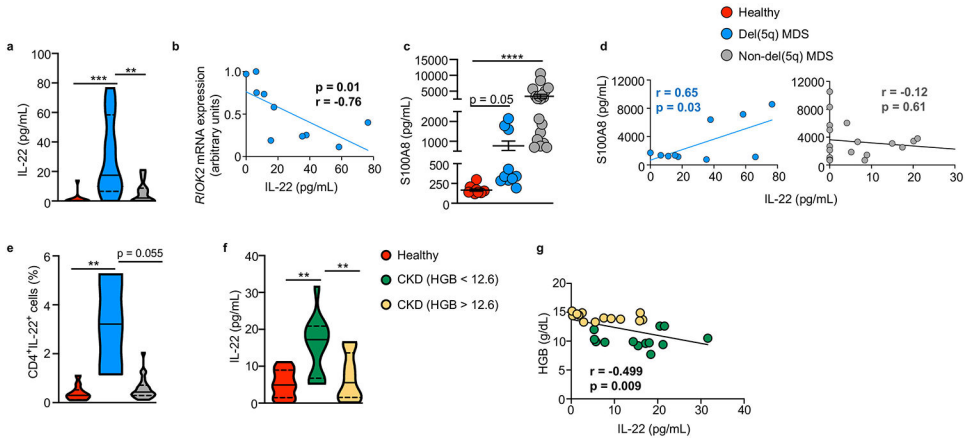


Figure 8. MDS patients exhibit increased IL-22 levels and IL-22 associated signature.

(**a**) IL-22 concentration in the BM fluid of healthy controls (n=12), del(5q) MDS (n=11), and non-del(5q) MDS (n=22) patients. (**b**) Correlation between *RIOK2* mRNA and BM IL-22 concentration in the del(5q) cohort shown in (a), n=10. (**c**) S100A8 concentration in the samples shown in (a). n = 11, 10, and 19 for healthy, del(5q) MDS, and non-del(5q) MDS, respectively. (**d**) Correlation between IL-22 concentration and S100A8 concentration in BM fluid of del(5q) (left, n=10) and non-del(5q) (right, n=19) samples. (**e**) Frequency of IL-22 producing $CD4^+$ T cells in the PB of healthy controls (n=11), del(5q) (n=3) and non-del(5q) (n=24) MDS patients. **(f)** Plasma IL-22 concentration in healthy subjects (n=10) and chronic kidney disease (CKD) patients with (n=13) or without (n=13) secondary anemia. (**g**) Correlation between plasma IL-22 concentration and hemoglobin (HGB) in CKD patients with (n=13) or without (n=13) anemia. Kruskal-Wallis test with Dunn's correction for multiple comparisons (a to c, e), 1-way ANOVA with Tukey's correction for multiple comparisons (f) used to calculate statistical significance. Pearson correlation co-efficient (b, d, g), used to calculate statistical significance and correlation coefficient. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Data are shown as mean ± s.e.m (c). Solid lines represent median and dashed lines represent quartiles (a, e, f).