Reduced ribosomal protein gene dosage and p53 activation in low risk myelodysplastic syndrome

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Abstract

Reduced gene dosage of ribosomal protein subunits has been implicated in 5q- myelodysplastic syndrome and Diamond Blackfan anemia, but the cellular and pathophysiologic defects associated with these conditions are enigmatic. Using conditional inactivation of the Ribosomal protein S6 gene in laboratory mice, we find that reduced ribosomal protein gene dosage recapitulates cardinal features of the 5q- syndrome, including macrocytic anemia, erythroid hypoplasia and megakaryocytic dysplasia with thrombocytosis, and that p53 plays a critical role in manifestation of these phenotypes. The blood cell abnormalities are accompanied by a reduction in the number of hematopoietic stem cells, a specific defect in late erythrocyte development, and suggest a disease-specific ontogenetic pathway for megakaryocyte development. Further studies of highly purified normal and MDS patient hematopoietic stem cells link reduced expression of ribosomal protein genes to decreased red blood cell maturation, and suggest an underlying and common pathophysiologic pathway for additional subtypes of myelodysplastic syndrome.
**Introduction**

Myelodysplastic syndrome (MDS) is a heterogeneous group of blood cell disorders characterized by defective hematopoiesis, increased susceptibility to leukemia and thought to involve abnormalities in hematopoietic stem cells. Approximately 50% of affected individuals have blood cell cytogenetic abnormalities, of which deletions of chromosome 5q are the most common and portend a favorable prognosis. Identification of causal genes in 5q- and other MDS subtypes has been challenging, but recent advances in genetics and genomics have enhanced our understanding of how specific chromosomal alterations and their molecular consequences contribute to the pathogenesis of MDS.

Based on a large-scale RNAi screen, Ebert and colleagues identified *RPS14* as a critical gene on 5q whose hemizygosity in bone marrow cells recapitulates many of the features in 5q- MDS. Intriguingly, erythroid abnormalities in 5q- MDS are similar to those in Diamond Blackfan anemia (DBA), a dominantly inherited disorder in which germline mutations in one allele of either a 40S (encoded by *RPS* genes) or a 60S (encoded by the *RPL* genes) ribosomal protein gene have been identified. However, there are also important differences between the blood cell phenotypes of the two conditions; thrombocytosis and megalakaryocytic dysplasia are cardinal features of 5q- MDS, but not of DBA. Furthermore, recent work from Starczynowski et al. suggests that two microRNAs are critical mediators of the 5q- phenotype, because knockdown of these genes in immature hematopoietic cells leads to megakaryocytic dysplasia and thrombocytosis after transplantation in mice. Increased understanding of how ribosomal protein mutations cause disease might provide additional insight into the pathogenesis of MDS and DBA.

Spontaneous and induced ribosomal protein mutations have been studied in many model organisms, including yeast, flies, plants, fish and mice. Studies based on these models indicate that reduced dosage of ribosomal protein genes compromises ribosome biogenesis, protein synthesis, cell proliferation and cell survival. While these processes are likely to account for impaired organismal and cellular growth, the mechanisms responsible for tissue-specific phenotypes, including anemia and thrombocytosis, remain unresolved.

Additional insight into the pathogenesis of ribosomal protein-mediated bone marrow failure has come from recent work in our laboratory: mice with ribosomal protein mutations develop epidermal melanocytosis and dark skin in which activation of the transcription factor p53 is a critical event. Here, we ask whether these observations can be applied to understand the pathogenesis of MDS, based on conditional and bone marrow-specific ablation of a ribosomal protein subunit, Rps6. We find that activation of p53 is necessary to elicit a bone marrow failure phenotype in mice whose features recapitulate essential aspects of 5q- MDS, and which suggests specific roles for p53 activation in both hematopoietic stem cell dysfunction and defects in red cell maturation. In addition, we find that ribosomal protein dysregulation occurs in non-5q- MDS in human patients, suggesting a shared pathogenic mechanism among different MDS subtypes.
Materials and Methods

Generation of Ribosomal Protein Mutant Mice and Animal Experiments

We obtained mice carrying Tg.MxCre from Jackson Laboratory, Rps6\textsuperscript{lox}\textsuperscript{−} from S. Volarevic and G. Thomas\textsuperscript{12}, Tg.K5Cre from S. Artandi and J. Jorcano\textsuperscript{17} and Trp53\textsuperscript{ko} from T. Jacks\textsuperscript{18}. Rps19\textsuperscript{Dsk3+/−} (C3HeB/FeJ), Tg.K5Cre (C3HeB/FeJ), Trp53\textsuperscript{ko} (129/SvJ) and Tg.MxCre (C57BL/6J) are maintained on an isogenic background. Mdm2\textsuperscript{puro/Δ7-9} and +/+ controls are (129/SvJ x C57BL/6J)\textsuperscript{F1} littermates.

Rps6\textsuperscript{lox+/+};Tg.MxCre/+ were generated by crossing Rps6\textsuperscript{lox+/+} (129/SvJ x C57BL/6J F1) with Tg.MxCre/+. 7-9 week old animals were given 250\(\mu\)g polyI:C intraperitoneally (Invivogen) on days 1, 3 and 5. All experiments using Rps6\textsuperscript{lox+/+};Tg.MxCre/+ animals were performed at least 16 weeks following polyI:C treatment.

eADA was evaluated as previously described\textsuperscript{20}. All experiments were carried out under protocol approved by the Stanford Administrative Panel on Laboratory Animal Care.

Immunofluorescence, Histology and Cytology

Adult skin or femurs were fixed in 4% paraformaldehyde. Femurs were decalcified in 500mM EDTA, pH7.2. Immunofluorescence was carried out with p53 (Novocastra) or von Willebrand factor (Dako) antisera after antigen retrieval using 0.01M citrate buffer, pH 6 in a pressure cooker. Skin sections were incubated with goat anti-rabbit Cy3 antisera (Jackson ImmunoResearch) and ProLong antifade reagent with DAPI (Invitrogen). Femur sections were incubated with goat anti-rabbit biotinylated antisera (Jackson ImmunoResearch), Vectastain Elite Avidin-Biotin complex reagent (Vector Labs) and Tyramide-Cy3 amplification reagent (PerkinElmer). Ter119 antisera (BD Pharmingen), mouse anti-rat biotinylated antisera (BD Pharmingen), avidin-biotin complex reagent and Tyramide-Fluorescein amplification reagent (PerkinElmer) were used for double immunofluorescence staining on femur sections after a second treatment with 0.01M citrate buffer, pH 6 in a pressure cooker. Histologic sections were stained with hematoxylin and eosin. Bone marrow cytopsins and peripheral blood smears were stained with Wright Giemsa reagents.

Immunophenotyping, Purification of and Assay for Rsp6 Protein from Mouse Bone Marrow

Total bone marrow cells were c-kit enriched using magnetic beads (Miltenyi) and stained with fluorochrome-conjugated antibodies (Table S3) and propidium iodide (Molecular Probes). Cell populations were fractionated based on Pronk et al.\textsuperscript{21} or Socolovsky et al.\textsuperscript{22} (Table S4). Quantitative measurements presented in Figures 5A, 5C and 5D are based on 1 million cells from each of 2-3 animals per genotype, with values from Rps6\textsuperscript{lox−/−};Tg.MxCre/+ animals divided by a factor of 2.13 (or 1.18 for Rps6\textsuperscript{lox−/−};Tg.MxCre/+;Trp53\textsuperscript{ko/ko}) to account for the reduction in bone marrow cellularity (Table 2); comparisons between genotypes therefore correspond to absolute differences on a per-animal basis. Cells were sorted or immunophenotyped with FACS Aria II cell sorters (Becton Dickinson) and data was analyzed with FlowJo software (Tree Star).

Rps6 protein levels were evaluated from bone marrow cells after staining for surface antigens, fixation with 1.6% paraformaldehyde, permeabilization with cold methanol and staining with
fluorescently-conjugated mouse Rps6 antisera (Cell Signaling Technology). Mean fluorescence intensity for a population of cells is a surrogate for Rps6 protein levels.
Results

RPS6 HEMIZYGOSITY CAUSES ROBUST P53 ACTIVATION IN SKIN AND BONE MARROW

We previously reported mutations of Rps19 and Rps20 as the cause of two dominantly inherited dark skin mutants, Dark skin 3 (Dsk3) and Dark skin 4 (Dsk4), respectively, that were identified during the course of a forward genetic screen for new pigmentary abnormalities. These animals exhibit increased accumulation of epidermal melanocytes in the footpads, ears and tail that becomes apparent shortly after weaning and persists throughout adult life. We observed a similar phenotype in animals with keratinocyte-specific hemizygosity for a third ribosomal protein, Rps6 (Rps6lox/+;Tg.K5Cre/+). Additional studies showed that activation of p53 is both necessary and sufficient for the pigmentation phenotype. Thus, mutations in three different components of the 40S ribosome trigger a common pathophysiologic pathway that causes accumulation of p53, epidermal melanocytosis and dark skin.

Rps19Dsk3/+ animals develop a mild blood cell phenotype (a 5-10% reduction in red blood cell counts) and Rps20Dsk4/+ animals have no blood cell abnormalities. However, we also noticed that Rps19Dsk3/+ animals exhibited skin color darkening that was intermediate between non-mutant and Rps6lox/+;Tg.K5Cre/+ animals (Figure 1A), which suggested that the level of skin darkening might serve as a proxy for the extent of p53 activation. We investigated this observation more carefully using tail skin sections processed and quantified in parallel. Immunofluorescence for p53 is undetectable in the skin of non-mutant animals, whereas Rps19Dsk3/+ and Rps6lox/+;Tg.K5Cre/+ animals have a 15-fold and 94-fold increase in staining, respectively, compared to non-mutant animals (Figure 1B, 1C). Thus, hemizygosity for Rps6 has a stronger effect on p53 activation than the Rps19Dsk3 mutation.

To investigate how the aforementioned difference would manifest itself in the bone marrow, we crossed Rps6lox/+ mice to animals carrying an interferon-inducible Cre driver that has been used previously to modify genes in the adult bone marrow, Tg.MxCre. At 7-9 weeks of age, non-mutant (+/+, Rps6lox/+ or Tg.MxCre/) and mutant (Rps6lox/+;Tg.MxCre/) animals were injected with polyinosinic:polycytidylic acid (polyI:C) to activate the Mx promoter, and were evaluated 16 – 75 weeks later.

The effect of Cre-mediated recombination on Rps6 mRNA levels was assessed using quantitative RT-PCR on peripheral blood and sorted bone marrow cells from non-mutant and Rps6lox/+;Tg.MxCre/+ animals. Overall, and in all cell populations examined (peripheral blood, CD71+ erythrocytes (Table S4), and megakaryocyte-erythrocyte progenitors), Rps6 expression is significantly reduced (P=0.001, 0.042 and 0.006, respectively) in Rps6lox/+;Tg.MxCre/+ animals compared to non-mutant controls (Figure S1A-E). The levels of reduction are variable, presumably due to differences in Cre-mediated excision (Figure S1A-E), yet the overall decrement in Rps6 expression persists over time (Figure S1D, S1E). Notably, the degree of Rps6 mRNA reduction correlates with alterations in peripheral blood cell counts (Figure S1C, S1E). We also measured Rps6 protein expression in mutant and non-mutant bone marrow cells using a FACS-based approach, and observed an ~35% reduction (Figure S1F, S1G).

To assess the effects of ribosomal protein mutations on p53 in the bone marrow, we developed a protocol for immunohistochemistry of femur sections. Although different transgenic strategies were used for Rps6 in the skin (Tg.K5Cre) and bone marrow (Tg.MxCre followed by polyI:C injection), the level of p53 immunostaining in the bone marrow still correlated with the extent of
skin darkening (Figure 1B-E), consistent with previous reports that hemizygosity for Rps6 is generally more severe than hemizygosity for Rps19\textsuperscript{10,12}.

**HEMATOLOGIC CHARACTERIZATION OF RPS6 MUTANTS**

We first examined peripheral blood and bone marrow from non-mutant (+/+; Rps6\textsuperscript{lox/+} and Tg.MxCre/+), and Rps6\textsuperscript{lox/+}; Tg.MxCre/+ mutant animals. The MxCre transgene by itself had no effect on blood cell counts (Table S2); however, Rps6\textsuperscript{lox/+}; Tg.MxCre/+ animals exhibit a robust macrocytic anemia that worsens over time (Figure 2, Table 1, Figure S2 and Table S2). At 16-weeks after induction of Cre recombinase, mutant animals have significant alterations in red blood cell count (36% reduction, P<0.001) and mean corpuscular volume (MCV, 25% increase, P<0.001), and a modest reduction in reticulocyte count (Table S2). Mutant mice also exhibit marked leukopenia (and associated lymphopenia) and a progressive thrombocytosis (Figure 2, Table 1, Figure S2 and Table S2). In addition, a spectrophotometric assay for erythrocyte adenosine deaminase (eADA) function in peripheral blood (a biomarker for disease activity in DBA patients\textsuperscript{20}) shows a 2-fold increase in enzyme activity in mutant compared to non-mutant control animals (Table 1).

We next asked whether Trp53 was required for the hematologic changes caused by bone marrow specific reduction of Rps6 gene dosage. Rps6\textsuperscript{lox/+}; Tg.MxCre/+ animals were crossed with those carrying a Trp53 knockout allele, Trp53\textsuperscript{ko}\textsuperscript{18}, and assessed for peripheral blood cell counts and eADA levels. In Rps6\textsuperscript{lox/+}; Tg.MxCre/+ animals, deficiency for p53 (Trp53\textsuperscript{ko/ko}) completely mitigated the alterations in white blood cell count, red blood cell count, MCV, platelet count and eADA activity (Table 1). Heterozygosity for Trp53 yielded an intermediate phenotype with partial rescue of the blood cell counts and parameters.

To investigate the origins of the blood cell abnormalities, we examined bone marrow sections stained with hematoxylin and eosin, and observed a prominent megakaryocytosis (a 2.7-fold increase in megakaryocyte number) and marked hypocellularity in Rps6\textsuperscript{lox/+}; Tg.MxCre/+ compared to control animals (Figure 3A, 3B). Immunofluorescence for von Willebrand factor, an immunohistochemical marker for megakaryocytes in normal bone marrow and MDS\textsuperscript{24}, reveals a 2.3-fold increase in megakaryocytes in ribosomal protein mutant marrow (Figure 3C, 3D). To evaluate the degree of hypocellularity and to assess the role of p53 in this process, we flushed marrow from the femurs and tibias of non-mutant, Rps6\textsuperscript{lox/+}; Tg.MxCre/+ and Rps6\textsuperscript{lox/+}; Tg.MxCre/+; Trp53\textsuperscript{ko/ko} animals, and counted the number of cells. Mutant animals (Rps6\textsuperscript{lox/+}; Tg.MxCre/+ ) exhibited a 50% reduction in cellularity that was abrogated completely by loss of p53 (Rps6\textsuperscript{lox/+}; Tg.MxCre/+; Trp53\textsuperscript{ko/ko} ) (Table 2).

We evaluated lineage specific blood cell precursors based on bone marrow cytology. From cytopsin preparations of bone marrow samples flushed from the lower extremities, we observed a marked reduction in the number of lymphoid cells in Rps6 mutant animals, while immature and mature granulocytic and monocytic populations were unaffected (Table 2). The number of lymphocytes in Rps6\textsuperscript{lox/+}; Tg.MxCre/+ bone marrow was significantly reduced (by 56%, P=0.006), and partially rescued in the absence of p53 (Rps6\textsuperscript{lox/+}; Tg.MxCre/+; Trp53\textsuperscript{ko/ko} ).

We examined the cytology of red blood cell precursors from Tg.MxCre/+ and Rps6\textsuperscript{lox/+}; Tg.MxCre/+ and Rps6\textsuperscript{lox/+}; Tg.MxCre/+; Trp53\textsuperscript{ko/ko} bone marrow based on recent work by Pronk\textsuperscript{21} and Socolovsky\textsuperscript{22} in which the morphology of specific, sorted cell populations from the mouse myeloerythroid hierarchy was carefully assessed. We scored cells as either immature (PreCFU-E and CFU-E, Table S4) or mature erythroid precursors (basophilic,
polychromatophilic and orthochromatic erythroblasts, Table S4) and observed a significant relative increase in the number of immature erythroid precursors (P=0.001, Figure 3E, Table 2, Table S4), and a significant decrease in mature erythroid cells (P=0.016, Figure 3E, Table 2, Table S4). Both of these changes were dependent on Trp53 (Table 2).

As a complementary means of evaluating the erythroid lineage in Rps6 mutant animals, we carried out immunofluorescence staining on sections from non-mutant and Rps6\textsuperscript{lox/+};Tg.MxCre/+ bone marrow using Ter119 (a marker for mature red blood cells\textsuperscript{22} and p53. Akin to our previous findings (Figure 1D, 1E), we observed a significant (P=0.013) increase in p53-positive cells in the bone marrow of mutant animals (Figure S3A, S3B). Ter119 staining of nucleated cells (dapi-positive), however, was significantly reduced (P=0.016) in the Rps6 mutant compared to non-mutant animal (Figure S3A, S3C). We also found that while a fraction of the nucleated, Ter119-positive cells also stained with p53, most p53-positive cells were not mature red blood cells (Ter119-positive), suggesting that p53 acts at multiple stages during hematopoiesis.

Megakaryocytes were significantly increased and showed distinct cytologic changes, with frequent, small dysplastic, monolobated megakaryocytes in bone marrow from Rps6\textsuperscript{lox/+};Tg.MxCre/+ mice (Figure 3F, 3G). Thus, from multiple perspectives, bone marrow-specific hemizygosity for Rps6 causes defects that recapitulate key clinical features of 5q-MDS\textsuperscript{25}, including defects in red cell maturation and megakaryocyte dysplasia accompanied by thrombocytosis.

P53 ACTIVATION MIMICS RIBOSOMAL PROTEIN DEFICIENCY IN THE BONE MARROW

Except for the lymphoid abnormalities, the hematologic alterations we observed depend completely on the presence of Trp53. To ask whether activation of p53, was sufficient to trigger these abnormalities, we took two approaches to elicit moderate levels of p53 activation, since robust activation of p53 in developing bone marrow causes a failure to initiate primitive erythropoiesis and embryonic death\textsuperscript{26}.

In one approach, we took advantage of Mdm2\textsuperscript{puro/Δ7-9} mice in which expression of Mdm2, a negative regulator of p53 stability and activity, is reduced ~70% relative to wild type mice\textsuperscript{26}. We measured p53 expression in the bone marrow of Mdm2\textsuperscript{puro/Δ7-9} and non-mutant (+/+) controls. While p53 expression in non-mutant marrow is negligible, immunofluorescent staining in Mdm2\textsuperscript{puro/Δ7-9} animals is increased by 5.2-fold compared to control animals (Figure 4A, 4B). To extend the initial results on the lymphocyte and red cell lineages described by Mendrysa et al.\textsuperscript{26}, we counted megakaryocytes in bone marrow sections, and observed a significant megakaryocytosis (P<0.001) in Mdm2\textsuperscript{puro/Δ7-9} compared to non-mutant (+/+ ) controls (Figure 4C, 4D). In addition, immunofluorescence for von Willebrand factor was significantly increased in bone marrow sections from Mdm2 mutant animals (Figure 4E).

As a complementary approach, we administered the chemotherapeutic agent, 5-fluorouracil, whose ability to cause apoptosis and growth arrest in cells has been attributed to its ability to induce p53\textsuperscript{27}, and which has been widely utilized in mice to investigate the biology of stress hematopoiesis. Based on a regimen from the work of Agosti et al.\textsuperscript{28}, we administered a single dose of 150 mg/kg to wild type control mice, and observed an ~20% decrease in red blood cell count and an ~60% increase in platelet count eleven days later (Figure 4F, 4G). These values returned to near pretreatment levels by day 25. In contrast, the response to 5-fluorouracil in Trp53\textsuperscript{ko/ko} animals was markedly attenuated; at day 11, both red blood cell and platelet counts were significantly different (P=0.007 and P=0.0075, respectively) from non-mutant animals.
Thus, both genetic and pharmacologic approaches in vivo suggest that p53 activation induces erythrocyte and megakaryocyte abnormalities similar to those caused by Rps6 hemizygosity.

EFFECTS OF RPS6 HEMIZYGOSITY ON HEMATOPOIESIS

Recent work by Pronk et al.\textsuperscript{21} demonstrated that specific cell populations in the mouse myeloerythroid hierarchy can be identified and prospectively separated via differential expression of the cell surface markers endoglin (Eng/CD105), CD150 (Slamf1) and CD41 (Itga2b). Multipotent progenitors (MPP) can be distinguished from hematopoietic stem cells, granulocyte-monocyte populations distinguished from megakaryocyte (MkP) and erythrocyte precursors, and erythroid progenitors can be divided into a maturation hierarchy: PreMegE (megakaryocyte-erythroid precursor), Pre CFU-E and CFU-E/ProEry populations.

To explore the ontogeny of the erythroid and megakaryocytic defects caused by bone marrow-specific Rps6 hemizygosity in the mouse, we analyzed specific precursor populations from the bone marrow as described in Pronk et al.\textsuperscript{21} (HSC and MPP populations were identified on the basis of differential expression of CD34, MPP - CD34+ and HSC - CD34-.) We measured and compared 6 separate types of cells between Rps6\textsuperscript{lox/+};Tg.MxCre/+ mutant and non-mutant (Tg.MxCre/+ controls, and observed two salient features. First, mutant animals exhibited a significant paucity in the absolute number of HSCs (P=0.027), accompanied by significant increases of most downstream populations (MPP, P=0.002; MkP, P<0.001; PreMegE, P=0.004; Pre CFU-E, P<0.001) (Figure 5A-C). Second, mutant animals also exhibited a significant (P=0.017) reduction of more mature erythroid precursors (CFU-E/ProEry) (Figure 5B, 5C).

Additional fractionation of the erythroid lineage based on expression of CD71 and Ter119\textsuperscript{22} supports the above observations: the number of mature erythroid cells (Ter119+CD71+, Figure 5D, S4) is reduced in mutant (Rps6\textsuperscript{lox/+};Tg.MxCre/+ compared to non-mutant (Tg.MxCre/+ bone marrow. This abnormality is also dependent on Trp53 (Figure 5D, S4).

To further investigate how Rps6 hemizygosity (and p53 activation) might affect lineage commitment during blood cell development, we carried out a clonal analysis for HSC differentiation. Single HSCs from Rps6\textsuperscript{lox/+};Tg.MxCre/+ mutant and non-mutant (Tg.MxCre/) control bone marrow were sorted into individual liquid culture wells of a 96-well plate, and the extent of megakaryocyte vs. non-megakaryocyte differentiation in each well was assessed visually after 3 and 5 days of culture. (The nature of this assay does not allow non-megakaryocyte cell types to be subdivided by light microscopy, but megakaryocyte-like cells can be easily distinguished based on their size and morphology, Figure 6A). In non-mutant animals, the proportion of wells that give rise to megakaryocyte-only colonies is very low (generally 1–2 wells per 96-well plate). However, in mutant animals, there is a 20- to 30-fold increase in the proportion of wells that give rise to megakaryocyte-only colonies (Figure 6A).

We performed a similar experiment sorting for MPP, PreMegE, or MkP populations, placing 10 cells per well in a 96-well plate, and then counting the number of megakaryocytes on each of the following 3 days. Similar to results observed with the single cell assay for HSCs (Figure 6A), the number of megakaryocytes recovered from MPP cells (measured as mean number of megakaryocytes per well of a 96-well plate) was significantly higher (P=0.01) in mutant compared to non-mutant animals (Figure 6B). However, no differences in megakaryocyte number were detected between mutant and control cultures established from PreMegE and MkP populations (Figure 6C, 6D), which is surprising given the conventional view of the
megakaryocyte lineage in which there is a stepwise progression from HSC to MPP to PreMegE to MkP (Figure 7B).

We considered whether this apparent paradox—increased numbers of MPP-derived megakaryocytes without a concomitant increase in PreMegE- or MkP-derived megakaryocytes—might be explained by differences in plating efficiency or cell survival. We measured plating efficiency by counting total cell number (without regard to morphology) after 1 day of culture, and observed no differences between mutant and non-mutant HSC, MPP, PreMegE, or MkP cultures (Figure S5). We did, however, observe that cell growth over the next several days was reduced in all mutant cultures, consistent with a cell-autonomous process that causes general growth impairment.

In summary, hemizygosity for \( Rps6 \) in the bone marrow causes not only a profound reduction in overall cellularity, but also affects the relative distribution of hematopoietic subpopulations, with a relative decrease in the number of HSCs, CFU-E/ProEry, CD71+ and CD71- cells, accompanied by a relative increase in all other subpopulations. In culture, growth of each subpopulation is impaired, except for MPP-derived megakaryocytes, which are increased in mutant compared to non-mutant cultures.

**EFFECT OF REDUCED RIBOSOMAL PROTEIN GENE DOSAGE IN HUMAN BONE MARROW CELLS**

To investigate how hemizygosity for \( RPS6 \) would affect human blood cell development, we studied the effects of \( RPS6 \) shRNAs in culture. Cord blood samples were sorted (Lin-CD34+CD38-CD90+CD45RA-) to enrich for hematopoietic stem cells\(^29\), infected with a lentivirus containing short hairpin RNA sequences targeting \( RPS6 \) (or \( RPS14 \) for control), and then cultured under conditions to allow myeloid and erythroid differentiation\(^2\). After 10 days, cultures were analyzed for cell surface markers of myeloid (CD11b+), mature erythroid (GPA+) and immature erythroid (CD71+GPA-) differentiation.

In all cases, shRNA constructs targeting \( RPS6 \) or \( RPS14 \) caused a decrease in the erythroid/myeloid ratio (Figure 5E), the extent which correlated with the efficiency of knockdown as assessed by qRT-PCR (Figure S6A). In addition, \( RPS6 \) knockdown led to a block in erythroid differentiation as assessed by presence of the erythroid lineage cell surface marker GPA (Figure 5F, 5G), similar to observations in bone marrow-specific \( Rps6 \) mutant mice (Figure 5B-D), and consistent with the 5q- MDS phenotype.

A role for reduced ribosomal protein gene dosage in both DBA and 5q- MDS suggested to us that other forms of MDS might also involve altered expression of ribosomal protein genes. To explore this idea, we interrogated the results of Affymetrix microarray measurements carried out on 8 MDS patient hematopoietic stem cell samples (7 low-risk and 1 intermediate-risk, none of whom carried 5q-, Table S1)\(^30\), compared to 11 age-matched healthy controls. All microarray data are available in the Gene Expression Omnibus (GEO) under accession number GSE30201. Among 54,676 probes on the array, mRNA levels for 3672 are decreased and 1159 are increased in the MDS patient samples at a false discovery rate of <0.01%. However, ribosomal protein genes are disproportionately represented among the differentially expressed genes: 0.7% of the differentially expressed probes represent ribosomal protein genes whereas 0.4% of all the probes on the array represent ribosomal protein genes (p=0.005, two-tailed z-statistic, CI 95%, z=2.821). More strikingly, of 74 ribosomal protein genes represented on the array, mRNA levels for 21 are decreased, and only 1 is increased (Figure 7A). Thus, ribosomal protein gene
expression is dysregulated -- and largely repressed -- in hematopoietic stem cells from a group of non-5q- MDS patients.
Discussion

5q- MDS is characterized by abnormal erythrocyte maturation with macrocytic anemia, thrombocytosis and a predisposition to leukemia. Intriguingly, DBA, a congenital bone marrow failure syndrome, exhibits erythroid defects similar to 5q- syndrome, and recent studies suggest that both conditions are caused by reduced dosage of one or more ribosomal protein genes. To date, the underlying cellular pathophysiology and potential approaches to treatment for these diseases have been difficult to unravel.

During earlier studies of pigmentary mutations in mice, we realized that reduced dosage of the ribosomal protein genes, Rps6, Rps19, and Rps20, led to activation of p53 in which the differential and specific response of individual cell types in the skin and blood gave rise to a pleiotropic syndrome that was likely relevant to 5q- MDS and DBA. We have now tested this hypothesis more directly, and find that hemizygosity for Rps6 in mouse bone marrow and reduced dosage of ribosomal protein genes in human cells recapitulate cardinal features of the human condition. In addition, studies based on genome wide expression analysis reveal that abnormal expression – and specifically downregulation – of ribosomal protein genes characterizes bone marrow stem cells from patients with low risk, non-5q- MDS. These results connect genotype to phenotype for a relevant animal model of MDS, and suggest that ribosomal protein gene dysregulation may play a role in the pathogenesis of a broad group of bone marrow failure syndromes.

Potential involvement of ribosomal protein genes in 5q- MDS is based on work of Ebert and colleagues in which large-scale siRNA knockdown of candidate genes in cultured CD34+ cells implicates RPS14. Recent work from Starczynowski et al., however, suggests that two microRNAs, miR-145 and miR-146a, are critical mediators of the 5q- phenotype. Our work does not exclude a potential role for microRNAs as an alternative and/or secondary component of 5q-MDS, but demonstrates that reduced dosage of a ribosomal protein gene within bone marrow cells is sufficient to accurately model many aspects of the human condition. From this perspective, it is interesting to compare our results with those of Barlow et al., who engineered mice with a hematopoietic stem cell-specific ~500 kb deletion that removed 8 genes including Rps14 (though not miR-145 or miR-146a). Hematologic abnormalities in the mouse model described by Barlow et al. share many features with the bone marrow-specific Rps6 mice described here, but exhibit thrombocytopenia rather than thrombocytosis, and a reduction rather than increase in megakaryocyte-erythroid progenitor subpopulations. Additional studies of miR-145 and miR146a expression and/or manipulation in vivo are likely to resolve the relative contributions of ribosomal protein and microRNA genes to 5q- MDS.

Our work uses Rps6 as a tool to model 5q- MDS (and to inform our understanding of DBA), yet human mutations of RPS6 have not been identified in either condition. However, germline mutations for RPS6 may be lethal (as they are in mice). Our results confirm that loss-of-function for Rps6 is more severe than loss-of-function for Rps19 or Rps20, because keratinocyte-specific hemizygosity for Rps6 has a more dramatic effect on p53 induction compared to germline mutations in Rps19 or Rps20, and because germline mutations in Rps19 or Rps20 have a very mild effect on bone marrow. We note that Rps6 phosphorylation serves as an indicator of physiological stress and is downstream of the mTOR pathway, and from that perspective, Rps6 is not “just another ribosomal protein”, which may help to explain why lymphopenia develops in our bone marrow-specific Rps6 mutants but not in other models of 5q- MDS (or...
DBA). However, our results also suggest that the effect of \textit{Rps6} mutation-induced bone marrow disease in other bone marrow lineages is similar to that caused by mutations in other ribosomal protein genes, at least with regard to dependence on p53\textsuperscript{31, 33}. We also note that extensive ribosomal protein gene resequencing has not yet been reported for MDS; indeed, our results suggest that some patients with low-risk MDS and normal cytogenetics may have somatic mutations of ribosomal protein genes other than \textit{RPS14}, including \textit{RPS6}.

Similar to the work of Barlow et al.\textsuperscript{31}, we find that many of the bone marrow phenotypes in \textit{Rps6} mutant animals depend on the presence of p53. We find that activation of p53 is necessary for peripheral blood cell abnormalities (leukopenia, macrocytic anemia, thrombocytosis and elevated eADA activity), bone marrow hypocellularity and erythrocyte maturation in mutant bone marrow. We note that parallel mechanisms are likely to exist in other contexts. Activation of p53 during ischemia contributes to cell death, tissue injury and organ failure\textsuperscript{34, 35}, and recent studies of animal models of Fanconi anemia and Treacher Collins syndrome suggest that stabilization of p53 during embryogenesis induces characteristic developmental abnormalities\textsuperscript{36, 37}. These observations support an emerging paradigm in which activation of p53 function can give rise to a variety of developmental or disease-related phenotypes depending on the amount and tissue-specific context in which activation occurs\textsuperscript{38}.

Our studies of hematopoietic subpopulations suggest that reduced ribosomal protein dosage and consequent p53 activation can perturb multiple and independent steps in blood cell development. Impairment of hematopoietic stem cell growth is consistent with both a general role for ribosome function, and/or a pro-apoptotic effect of p53 activation on hematopoietic stem cells\textsuperscript{3, 39, 40}. In this context, the relative increase of downstream progenitors (MPP, PreMegE, Pre CFU-E, MkP) we observed is likely to reflect homeostatic regulation within the bone marrow\textsuperscript{41}. However, the reduction of more mature erythroid progenitors (CFU-E/ProEry, CD71+ and CD71-) points to a specific block in erythroid maturation, and may therefore account for the observation that 5q-MDS and DBA frequently present with a selective reduction in red blood cell development. Finally, akin to observations by Adolfsson and colleagues\textsuperscript{42}, our studies of megakaryocyte-like growth from cultured hematopoietic cell subpopulations suggest that reduced ribosomal protein gene dosage unmarks an alternative pathway for megakaryocyte development directly from HSC and/or MPP rather than via PreMegE and MkP subpopulations.

Previous studies of large-scale gene expression in MDS CD34+ cells have shown that reduced expression of ribosomal protein genes is a hallmark of 5q- MDS\textsuperscript{43, 44}, revealed that pathways related to interferon, thrombopoietin, and Wnt signaling are dysregulated in all MDS subtypes\textsuperscript{3}, and have also identified specific sets of genes that correlate with disease subtype and/or prognosis\textsuperscript{3, 44}. At first glance, some of the results reported by Sridhar et al.\textsuperscript{44} appear opposite to what we find, with elevated levels of ribosomal protein gene expression observed in CD34+ cells in non 5q- MDS. However, a key difference between the work reported here and previous studies\textsuperscript{3, 43-45} is that our expression profiling studies utilize highly-purified HSC (Lin-CD34+CD38-CD90+CD45RA-) from MDS patients. This approach both enriches for hematopoietic stem cells (which constitute \( \sim 1\% \) of total CD34+ cells) and diminishes the presence of multilineage and lineage committed progenitor cells that are also CD34+. This is particularly important for patients with high-risk MDS, in which the CD34+ population is likely to contain a large proportion of actively dividing cells that are metabolically active. In fact, in the work from Sridhar et al.\textsuperscript{44}, expression of ribosomal protein genes in all CD34+ cells is elevated in patients with high-risk (transformed) MDS compared to low-risk (stable) MDS, whereas the
patients studied here all represent low-risk MDS. Nonetheless, additional studies, likely based on large-scale sequencing, will be required to investigate whether the reduction of ribosomal protein gene expression we observe in HSCs from non 5q- MDS represent a critical pathophysiologic event or a secondary consequence of altered growth rate.

Taken together with genetic studies of Diamond-Blackfan anemia, our work suggests that the hematopoietic system is especially sensitive to alterations in ribosomal protein gene dosage. This intersection--of forward genetic approaches in human disease with reverse genetic approaches in mouse models--is reminiscent of an analogous connection between components of the primary cilium and a spectrum of human diseases represented by Bardet-Biedl syndrome, polycystic kidney disease, and heterotaxy\textsuperscript{46}. Additional studies in mouse models will illuminate the extent to which non 5q-MDS and other forms of bone marrow failure represent ribosomopathies.
Acknowledgements

We thank Martin Hrabe de Angelis for Rps19Dsk3/+ mice, G. Thomas and S. Volarevic for Rps6lox mice, S. Artandi and J. Jorcano for Tg.K5Cre mice and T. Jacks for Trp53ko mice. We thank H. Manuel for technical support. K.A.M. and C.Y.P were supported by Mentored Clinical Scientist Development Investigator Awards from the National Institutes of Health. C.Y.P. was also supported by an ARRA award from the National Institutes of Health. W.W.P. is supported by the Stanford Medical Scientist Training Program. I.L.W. is supported by the Ludwig Center at Stanford University, NIH Grant 5U01HL099999-03 and the Leukemia and Lymphoma SCORE grant.
Authorship Contributions and Disclosure

K.A.M. generated the Rps6 mutant mice, analyzed the skin, peripheral blood and bone marrow phenotypes, performed experiments on Mdm2 mutant animals and analyzed the genome wide expression analysis from human bone marrow cells, with help from M.G.P. and C.Y.P.. W.W.P. carried out the siRNA knockdown and genome wide expression analysis in human hematopoietic stem cells, with help from J.V.P. and C.Y.P.. Rps6 protein levels in the bone marrow of mutant mice were evaluated by R.B.. B.E.G. measured eADA activity in the blood of mutant mice, and provided experimental and intellectual advice about DBA. RM and SMM designed experiments and generated the Mdm2 mutant mice, and provided expertise about the role of p53 in the bone marrow. I.L.W. helped design experiments and provided intellectual insight into the role of hematopoietic stem cells in MDS. All experiments were performed under the guidance and leadership of C.Y.P. and G.S.B., who coordinated the project and wrote the manuscript with input from K.A.M..

Conflict of interest disclosure: The authors declare no competing conflict of interest.

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References


Table 1. Effect of Rps6 hemizygosity and Trp53 on blood counts and eADA activity.\(^A\)

<table>
<thead>
<tr>
<th></th>
<th>non-mutant(^B)</th>
<th>Rps6(^{lox/+}); Tg.MxCre/+</th>
<th>Rps6(^{lox/+}); Tg.MxCre/+; Trp53(^{ko/+})</th>
<th>Rps6(^{lox/+}); Tg.MxCre/+; Trp53(^{ko/ko})</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>17</td>
<td>20</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>WBC (x10(^3)/μL)</td>
<td>7.58 +/- 0.47</td>
<td>4.47 +/- 0.35(^{***C})</td>
<td>4.12 +/- 0.53</td>
<td>6.39 +/- 0.75(^{**E,F})</td>
</tr>
<tr>
<td>RBC (x10(^6)/μL)</td>
<td>9.72 +/- 0.13</td>
<td>5.85 +/- 0.19(^{***C})</td>
<td>7.03 +/- 0.11(^{***D})</td>
<td>9.12 +/- 0.83(^{***E,F})</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>47.0 +/- 0.4</td>
<td>59.8 +/- 0.7(^{***C})</td>
<td>55.7 +/- 0.7(^{***D})</td>
<td>48.3 +/- 2.2(^{***E,F})</td>
</tr>
<tr>
<td>Platelet count (x10(^3)/μL)</td>
<td>930 +/- 17</td>
<td>1490 +/- 89(^{***C})</td>
<td>1181 +/- 65(^{**D})</td>
<td>911 +/- 89(^{**E,F})</td>
</tr>
<tr>
<td>No. of animals</td>
<td>6</td>
<td>6</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>e ADA (IU/gram hemoglobin)</td>
<td>1.06 +/- 0.11</td>
<td>2.54 +/- 0.45(^{*C})</td>
<td>2.09 +/- 0.15</td>
<td>1.13 +/- 0.19(^{**E,F})</td>
</tr>
</tbody>
</table>

\(^A\)Blood counts were evaluated 16 weeks after treatment with polyI:C compound. Blood counts given as mean +/- sem with P values based on multiple regression where sex and genotype are factors. A subset of animals was evaluated for eADA activity and values are mean +/- sem with P values based on a two-tailed Student’s t-test, \(^*P<0.05\), \(^{**P<0.01}\), \(^{***P<0.001}\), NS not significant.

\(^B\)+/+ or Rps6\(^{lox/+}\)

\(^C\)non-mutant vs Rps6\(^{lox/+}\); Tg.MxCre/+  

\(^D\)Rps6\(^{lox/+}\); Tg.MxCre/+ vs Rps6\(^{lox/+}\); Tg.MxCre/+; Trp53\(^{ko/+}\)  

\(^E\)Rps6\(^{lox/+}\); Tg.MxCre/+ vs Rps6\(^{lox/+}\); Tg.MxCre/+; Trp53\(^{ko/ko}\)

\(^F\)non-mutant vs Rps6\(^{lox/+}\); Tg.MxCre/+; Trp53\(^{ko/ko}\)

WBC, white blood cell count; RBC, red blood cell count; MCV, mean corpuscular volume; eADA, erythrocyte adenosine deaminase activity
Table 2. Effect of Rps6 hemizygosity and Trp53 mutations on bone marrow cell countsA.

<table>
<thead>
<tr>
<th></th>
<th>Tg.MxCre/+</th>
<th>Rps6lox/+:Tg.MxCre/+</th>
<th>Rps6lox/+:Tg.MxCre/+;Trp53ko/ko</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature erythroidA</td>
<td>13.50 +/- 1.66</td>
<td>40.00 +/- 3.24**C</td>
<td>13.67 +/- 3.71**D, NSE</td>
</tr>
<tr>
<td>Mature erythroidA</td>
<td>37.5 +/- 1.26</td>
<td>21.0 +/- 3.72*C</td>
<td>39.0 +/- 4.16*D, NSE</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>70.75 +/- 6.02</td>
<td>29.75 +/- 5.02**C</td>
<td>52.00 +/- 1.73**D, *E</td>
</tr>
<tr>
<td>Myeloblast</td>
<td>2.5 +/- 0.65</td>
<td>3.25 +/- 0.48</td>
<td>4.67 +/- 1.67</td>
</tr>
<tr>
<td>Immature myeloidA</td>
<td>19.75 +/- 0.75</td>
<td>21.00 +/- 1.96</td>
<td>19.67 +/- 3.53</td>
</tr>
<tr>
<td>Mature myeloidA</td>
<td>93.75 +/- 1.89</td>
<td>121.75 +/- 7.22</td>
<td>108.00 +/- 6.93</td>
</tr>
<tr>
<td>Monocyte/macrophage</td>
<td>20 +/- 3.87</td>
<td>22.25 +/- 3.25</td>
<td>20.33 +/- 3.53</td>
</tr>
<tr>
<td>Cellularity (x10^7)B</td>
<td>7.59 +/- 0.42</td>
<td>3.56 +/- 0.28**C</td>
<td>6.39 +/- 0.61*D, NSE</td>
</tr>
</tbody>
</table>

A Bone marrow cytology was evaluated from cytospins from 4 non-mutant, 4 Rps6lox/+:Tg.MxCre/+ and 3 Rps6lox/+:Tg.MxCre/+;Trp53ko/ko animals. 260 cells were evaluated per animal. Immature erythroid cells are Pre CFU-E or CFU-E cells as defined by Pronk et al.,21 (Table S4), mature erythroid cells are either basophilic, polychromatic or orthochromatic erythroblasts22 (Table S4), immature myeloid cells are promyelocytes or myelocytes and mature myeloid cells are band or segmented neutrophils. The number of cells within each population is given as mean +/- sem with P values based on two-tailed Student's t-test, *P<0.05, **P<0.01, ***P<0.001, NS not significant.

B Bone marrow cellularity determined by flushing two femurs and two tibias from 7 non-mutant, 7 Rps6lox/+:Tg.MxCre/+ and 3 Rps6lox/+:Tg.MxCre/+;Trp53ko/ko animals. Values are given as mean +/- sem with P values based on two-tailed Student's t-test, *P<0.05, **P<0.01, ***P<0.001, NS not significant.

C non-mutant vs Rps6lox/+:Tg.MxCre/+;

D Rps6lox/+:Tg.MxCre/+ vs Rps6lox/+:Tg.MxCre/+;Trp53ko/ko

E non-mutant vs Rps6lox/+:Tg.MxCre/+;Trp53ko/ko
Figure Legends

Figure 1. Effects of different ribosomal protein mutations on skin darkness and p53 levels. (A) Tails from non-mutant, Rps19Dsk3/+ and Rps6lox/+/Tg.K5Cre/+ animals. (B, D) Immunofluorescence for p53 in tail (B) and bone marrow (D) sections from the ribosomal protein mutants. White lines (B) mark the dermal-epidermal junction; arrowheads mark p53-positive nuclei. (C, E) Quantitation of p53 immunostaining in the skin (C) and bone marrow (E) of animals of the indicated genotype (n=3-4 mice per group). Values are number of cells per high power field (hpf) +/- sem (6 hpf per animal). P values are based on a two-tailed t-test, *P<0.05, **P<0.01, NS not significant. Scale bars: 0.5 mm (A), 50uM (B, D).

Figure 2. Effects of bone marrow-specific Rps6 hemizygosity on peripheral blood cell counts. Adult (7 – 9 week old) Tg.MxCre/+ (n=12) and Rps6lox/+;Tg.MxCre/+ (n=16) animals were treated with polyI:C and blood cell counts (+/- sem) determined 16 weeks and 38 weeks later. P values refer to a two-tailed paired t-test comparing the 16 and 38 week time points within a genotype, *P<0.05, NS not significant. RBC, red blood cell count; MCV, mean corpuscular volume; WBC, white blood cell count.

Figure 3. Effects of bone marrow-specific Rps6 hemizygosity on bone marrow histology and cytology. (A) Representative histologic sections from animals of the indicated genotype; arrowheads indicate megakaryocytes, quantitated in panel (B) (n=3 animals per genotype, mean number of megakaryocyte per hpf is shown +/- sem, 8 hpf per animal). (C) Immunofluorescence staining for von Willebrand factor (VWF, green) in bone marrow sections counterstained with dapi (blue) from non-mutant and ribosomal protein mutant animals and quantitated in panel (D) (n=3 animals per genotype, mean number of VWF-positive cells with large nuclei per hpf is shown +/- sem, 10 hpf per animal). (E) Cytospin preparations show increased numbers of immature erythroid (IE) cells and decreased numbers of mature erythroid (ME) cells in mutant compared to control samples. (F) Example of normal and abnormal (a monolobated micromegakaryocyte, arrowhead) megakaryocyte morphology in cytospin preparations from non-mutant and mutant bone marrow, quantitated (G) as mean number of monolobated cells among 10 megakaryocytes evaluated per animal +/- sem (n=4-5 animals per genotype). P values are based on a two-tailed t-test, *P<0.05, **P<0.01, NS not significant. Scale bars: 50uM (A), 75uM (C), 20uM (E), 10uM (F).

Figure 4. Effects of Mdm2 gene dosage and 5-fluorouracil on p53 levels and hematologic phenotypes. Example (A) and quantitation (B) of p53 immunostaining in bone marrow sections from animals with reduced Mdm2 activity (Mdm2puroΔ7-9) compared to control (+/+) animals. For panel B, n=5 mice per group, mean number of p53-positive cells per hpf +/- sem is shown, 6 hpf per animal. Example (C) and quantitation (D) of megakaryocyte abundance in bone marrow sections from Mdm2puroΔ7-9 and +/+ animals. For panel D, n=3 animals per genotype, mean number of megakaryocytes per hpf +/- sem is shown, 10 hpf per animal. (E) Quantitation of von Willebrand Factor (VWF)-positive cells in bone marrow sections from non-mutant (+/+) and Mdm2 mutant (Mdm2puroΔ7-9) animals (n=4 animals per genotype, mean number of VWF-positive cells with large nuclei per hpf is shown +/- sem, 10 hpf per animal). (F, G) Red blood cell and platelet counts (per µL) after administration of 5-fluorouracil to control (+/+, n=6) and p53-deficient (Trp53ko/ko, n=4) animals. Values before 5-fluorouracil treatment (white bars), 11
days after treatment (black bars) and 25 days after treatment (gray bars) are shown +/- sem. P values are based on a two-tailed t-test (B, D, E) or multiple regression where sex and genotype are factors (F, G), **P<0.01, ***P<0.001, NS not significant. Scale bars: 75uM (A), 50uM (C).

**Figure 5. Effects of bone marrow-specific Rps6 hemizygosity on selected hematopoietic lineage subpopulations.** (A) Number of HSC and MPP cells from Rps6 mutant and non-mutant bone marrow. (B) Flow cytometric profiles of the erythroid lineage from Tg.MxCre/+ and Rps6lox/lox;Tg.MxCre/+ animals. Immature erythroid precursors (Pre CFU-E) express higher levels of CD150 than mature erythroid precursors (CFU-E/ProEry); mutant animals exhibit a specific block in this step of erythroid maturation. (C) Number of megakaryocytic (MkP) and erythroid (PreMegE, Pre CFU-E, CFU-E/ProEry) cells from Rps6 mutant and non-mutant bone marrow. (D) Number of mature erythroid cells based on expression of Ter119 and CD71 from Tg.MxCre/+; Rps6lox/lox;Tg.MxCre/+ and Rps6lox/lox;Tg.MxCre/+;Trp53ko/ko animals. Ter119+CD71+ cells are the sum of the Ter119+CD71high and Ter119+CD71int populations (Figure S4). For panels A, C and D, open bars represent mean number observed per 1 million bone marrow cells analyzed from non-mutant animals (+/- sem, n=3), black bars represent the corresponding numbers from Rps6lox/lox;Tg.MxCre/+ animals (+/- sem, n=3) after dividing by a factor of 2.13 to account for the reduced number of total bone marrow cells in mutant animals, and gray bars (D) represent the mean number of cells from Rps6lox/lox;Tg.MxCre/+;Trp53ko/ko animals (+/- sem, n=2) after dividing by 1.18 (Table 2). (E, F, G) shRNA constructs targeting RPS6 (KD1, KD2 and KD3) or RPS14 decrease the ratio of mature erythroid (GPA+) to myeloid (CD11b+) cells (E), and also decrease the ratio of mature erythroid to immature erythroid (CD71+GPA-) cells (F, G). Each bar in panels E and G represents mean +/- sem, n=3. P values are based on a two-tailed t-test, *P<0.05, **P<0.01, ***P<0.001, §=0.07, §§=0.08.

**Figure 6. In vitro phenotypes of sorted hematopoietic populations from Rps6 mutant animals.** (A) Photomicrographs (above) of a representative non-megakaryocyte (left) and megakaryocyte (right) colony derived from a single HSC after five days in liquid culture. The percent of wells that differentiate into megakaryocyte colonies at day 3 and 5 is shown for Tg.MxCre/+ and Rps6lox/lox;Tg.MxCre/+ animals (below, n=5 animals per genotype, at least 24 wells were evaluated per animal). (B-D) Photomicrographs (above) and the mean number of megakaryocytes (below) derived from 10 MPP (B), PreMegE (C) and MkP (D) cells sorted into a single well. Colonies were evaluated on days 1, 2 and 3. (n=24 wells per animal with 2 animals of each genotype examined). P values are based on a two-tailed t-test, *P<0.05, ***P<0.001, NS not significant. Scale bars: 50uM (A), 150uM (B-D).

**Figure 7. Ribosomal protein gene dysregulation in human MDS and summary of a mouse model.** (A) Fold-change of mRNA for 8 patients with low risk, non-5q- MDS compared to 11 healthy, age-matched controls; each bar represents a different array probe for ribosomal protein genes that are differentially expressed. As described in the text, of 74 ribosomal protein genes represented on the array, 21 genes exhibit reduced mRNA levels and 1 gene exhibits increased mRNA levels (represented by a total of 34 probes). The figure shows all ribosomal protein gene probes that exhibit significant (<0.01% false discovery rate) differences; genes with multiple probes are indicated with an asterisk (*). (B) Diagram of the hematopoietic lineage and differences observed in bone marrow-specific Rps6 mutant mice. Red arrows summarize alterations in Rps6 mutant animals based on Figures 5A, 5C and 5D, including a reduction in the
size of the HSC population, a concomitant increase in the size of most downstream progenitor populations, and a block in erythroid maturation.
Figure 1

A. non-mutant  $Rps19^{Dsk3/+}$  $Rps6^{lox/+};Tg.K5Cre/+$

B. Tail skin

C. non-mutant  $Rps19^{Dsk3/+}$  $Rps6^{lox/+};Tg.K5Cre/+$

D. Bone marrow

E. non-mutant  $Rps19^{Dsk3/+}$  $Rps6^{lox/+};Tg.MxCre/+$
Figure 2

- **Platelet count**
  - 16 weeks: 3000 (x10^3/ul)
  - 38 weeks: 4500 (x10^3/ul)
- **RBC**
  - 16 weeks: 65 (x10^6/ul)
  - 38 weeks: 60 (x10^6/ul)
- **MCV**
  - 16 weeks: 11 (fl)
  - 38 weeks: 9 (fl)
- **WBC**
  - 16 weeks: 4.5 (x10^3/ul)
  - 38 weeks: 3.5 (x10^3/ul)

- Non-mutant (Tg.MxCre/+)
- Mutant (Rps6^lox/+; Tg.MxCre/+)

* indicates statistical significance.
Figure 4

A.

B. +/+ and Mdm2^puro/Δ7-9

C. +/+ and Mdm2^puro/Δ7-9

D. +/+ and Mdm2^puro/Δ7-9

E. +/+ and Mdm2^puro/Δ7-9

F. day 0, day 11, day 25

G. day 0, day 11, day 25
Reduced ribosomal protein gene dosage and p53 activation in low risk myelodysplastic syndrome

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