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Published in final edited form as:
Nature. 2008 January 17; 451(7176): 335–339. doi:10.1038/nature06494.

Identification of RPS14 as a 5q- syndrome gene by RNA interference screen

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Abstract

Somatic chromosomal deletions in cancer are thought to indicate the location of tumor suppressor genes, whereby complete loss of gene function occurs through biallelic deletion, point mutation, or epigenetic silencing, thus fulfilling Knudson's two-hit hypothesis.[1] In many recurrent deletions, however, such biallelic inactivation has not been found. One prominent example is the 5q- syndrome, a subtype of myelodysplastic syndrome (MDS) characterized by a defect in erythroid differentiation.[2] Here, we describe an RNA interference (RNAi)-based approach to discovery of the 5q- disease gene. We find that partial loss of function of the ribosomal protein RPS14 phenocopies the disease in normal hematopoietic progenitor cells, and moreover that forced expression of RPS14 rescues the disease phenotype in patient-derived bone marrow cells. In addition, we identified a block in the processing of pre-rRNA in RPS14 deficient cells that is highly analogous to the functional defect in Diamond Blackfan Anemia, linking the molecular pathophysiology of the 5q- syndrome to a congenital bone marrow failure syndrome. These results indicate that the 5q- syndrome is caused by a defect in ribosomal protein function, and suggests that RNAi screening is an effective strategy for identifying causal haploinsufficiency disease genes.

The 5q- syndrome was reported in 1974 as the first chromosomal deletion in cancer associated with a distinct clinical phenotype.[2] Patients have a severe macrocytic anemia, normal or elevated platelet counts, normal or reduced neutrophil counts, erythroid hypoplasia in the bone marrow, and hypolobated micromegakaryocytes.[3] These patients also have a propensity to progress to acute myeloid leukemia, albeit at a rate lower than other

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Author contributions: B.L.E., J.P., J.B., C.Y.C., P.T. and S.R.E. performed experiments and analyzed data. D.E.R. provided essential reagents. N.G., A.R., and E.A. provided patient samples. B.L.E. and T.R.G. wrote the manuscript.

The authors declare no other competing financial interests.

forms of MDS.[4] A major cause of morbidity and mortality for these patients is the erythroid defect that often requires ongoing red blood cell transfusions resulting in iron overload and subsequent organ dysfunction.[4] The 5q- syndrome is also unique because this subtype of MDS shows a remarkable response to treatment with the thalidomide analog lenalidomide, although the mechanism of lenalidomide action remains unknown.[5]

Over the past 30 years, physical mapping methods have been used to narrow the region of recurrent somatic deletion on 5q to a 1.5 megabase common deleted region (CDR) containing 40 genes.[6] Importantly, no 5q- syndrome patients have been reported to have biallelic deletions within the CDR, and no point mutations have been reported in the remaining allele of any of the 40 genes in the region. This observation led us to speculate that the 5q- syndrome may be caused by haploinsufficiency, suggesting that an alternate approach would likely be required to identify the causal gene. We therefore asked whether the principle hallmarks of the disease (an erythroid maturation block with preservation of megakaryocyte differentiation) could be experimentally recapitulated with shRNAs targeting each of the genes within the CDR.

We designed multiple lentivirally expressed shRNAs for each of the candidate genes in order to control for possible off-target effects of any individual shRNA. The shRNAs were introduced into normal CD34+ human hematopoietic progenitor cells, and the cells were induced to differentiate for 10 days along the erythroid and megakaryocytic lineages. The effect of each shRNA was assessed by FACS analysis using erythroidand megakaryocyte-specific cell surface markers. The shRNAs targeting one gene, RPS14, recapitulated the 5q- syndrome phenotype: a severe decrease in the production of erythroid cells with relative preservation of megakaryocytic cells (Figure 1). Furthermore, using the sequential expression of CD71 and glycophorin A during erythroid differentiation (Figure S1), we found that shRNAs targeting RPS14 blocked the production of terminally differentiated erythroid cells, also consistent with the 5q- syndrome disease phenotype (Figure S2). In a statistical analysis that groups all shRNAs targeting each gene into a single set, RPS14 is the only gene that significantly alters differentiation (Figure S3). Based on these results, we focused our attention on RPS14 as a candidate disease gene.

We first confirmed that all five RPS14 shRNAs that scored in the screen in fact knocked down RPS14 protein expression, and that the level of protein expression was on the order of half of the luciferase control cells, consistent with a model of RPS14 haploinsufficiency (Figure 2A). Each of the RPS14 shRNAs decreased erythroid differentiation relative to megakaryocytic differentiation (Figure 2B), and also caused a mild defect in erythroid vs. myeloid differentiation (Figure 2C) precisely as seen in patients with the clinical syndrome. RPS14 knock-down also caused an increase in the ratio of immature-to-mature erythroid cells (Figure 2D), as well as increased apoptosis of differentiating erythroid cells (Figures 2E), consistent with the well-described apoptotic phenotype of MDS.[7] Given the possibility that multiple genes in the CDR might act in collaboration,[8] we tested whether other effective shRNAs might increase the effect of RPS14 knock-down. None of the combinations were more effective than RPS14 shRNAs alone, suggesting that RPS14 is the critical gene in the region that explains the hematopoietic differentiation defect associated with 5q- Syndrome (Figure S4).

To confirm that RPS14 deficiency truly affects the erythroid differentiation program (rather than simply modulating the expression of specific FACS markers), we performed genome-wide expression profiling of cells infected with control or RPS14 shRNAs. We used *Gene Set Enrichment Analysis* (GSEA)[9] to assess the effect of RPS14 knock-down on experimentally-derived signatures of erythroid and megakaryocytic differentiation (Figure 2F). As expected, the gene expression pattern of RPS14 knock-down showed a significant

abrogation of the erythroid differentiation signature ($p < .001$, Figure 2G), in the setting of increased signature of neutrophil and platelet differentiation ($p < .001$ for both) (Figures 2H, 2I). In addition, RPS14 shRNAs induced a signature of sensitivity to Lenalidomide, the only drug approved by the Food and Drug Administration specifically for MDS patients with 5q deletions[5] (Figure S5). Importantly, the RPS14 shRNAs knocked down RPS14 expression on average ~ 60% in these samples, consistent with haploinsufficiency as the cause of these phenotypes. To further exclude the possibility of biallelic inactivation of RPS14, we sequenced the RPS14 gene in 32 MDS patient samples and subjected a subset of these samples to high density SNP-based copy number analysis and gene expression profiling. In no cases did we detect RPS14 point mutations, cryptic biallelic deletions, or loss of expression (e.g. by aberrant methylation, see Figure S6). Taken together, these experiments demonstrate that RPS14 partial loss of function recapitulates the phenotype of the 5q- syndrome.

RPS14 is a component of the 40S ribosomal subunit, but the function of RPS14 in human cells has not been defined. To determine the effect of partial RPS14 loss of function on pre-rRNA processing, we performed Northern blotting of rRNA transcripts and sucrose gradient analysis of intact polysomes. Decreased expression of RPS14 resulted in an accumulation of the 30S pre-rRNA species with a concomitant decrease in levels of 18S/18SE rRNA levels (Figures 3A, 3B), consistent with reports in *S. cerevesiae* that RPS14 is required for 18S pre-rRNA processing.[10] Specifically, a 4-to-9 fold increase in the 30S/18SE ratio was observed in cells expressing RPS14 shRNAs. In addition, RPS14 knock-down abrogated formation of the 40S subunit (Figure 3C and Figure S7). The increased 30S/18SE ratio in RPS14-deficient cells was not simply a consequence of cell death: the ribosomal processing defect occurred prior to the onset of significant apoptosis, and pharmacologically induced apoptosis failed to generate the characteristic 30S/18S defect (Figures S8 and S9). These results indicate that the block in pre-rRNA processing is a specific consequence of RPS14 deficiency.

Importantly, an increase of the 30S/18SE ratio was observed in 5q- syndrome bone marrow cells compared to normal marrow (Figure 3D), suggesting that a pre-rRNA processing defect indeed occurs in patient cells. We note that the patient samples contain a mixture of normal and 5q- disease cells, likely explaining why the 30S/18SE ratio is less perturbed than that seen in the experimental setting. The essentiality of RPS14 in ribosome biogenesis also likely explains why complete loss of RPS14 (e.g. through biallelic deletions) is never seen in 5q- syndrome patient cells. Complete loss of RPS14 is likely incompatible with cell survival as it is in yeast.[10]

To further establish that RPS14 deficiency accounts for the hematopoietic defect characteristic of 5q- syndrome, we attempted to rescue the erythroid differentiation defect in patient-derived bone marrow cells using an RPS14 expression construct. CD34+ cells from viably frozen bone marrow mononuclear cells obtained from MDS patients with and without 5q deletions (Table S4) were induced to undergo differentiation *in vitro*. FACS analysis showed that compared to control, lentiviral expression of RPS14 increased erythroid differentiation in 5q- syndrome patients, but failed to do so in patients lacking 5q deletions ($p = 0.004$ for erythroid relative to megakaryocytic differentiation, $p = 0.0003$ for erythroid relative to myeloid; Figure 4 and Figure S10). Furthermore, gene expression profiling coupled with GSEA showed that ectopic expression of RPS14 induced the gene expression signature of erythroid differentiation in 5q- syndrome patient samples ($p < 0.001$, Figure S11). These data demonstrate that overexpression of RPS14 rescues the erythroid differentiation defect seen in patients with 5q- syndrome, and establishes RPS14 as the likely disease-causing gene.

Loss of function of a ribosomal protein might at first seem like an unlikely explanation for a disease with such a distinct hematopoietic phenotype. However, germline heterozygous mutations in two other ribosomal proteins – RPS19 and RPS24 – have recently been described in the congenital disorder known as Diamond Blackfan Anemia (DBA).[11,12] The phenotype of DBA is strikingly similar to the 5q- syndrome: patients have a severe anemia, macrocytosis, relative preservation of the platelet and neutrophil counts, erythroid hypoplasia in the bone marrow, and increased risk of leukemia. Analogous to our results demonstrating RPS14 function in 18S pre-rRNA processing and 40S polysome formation, a similar requirement of RPS19 in ribosomal biogenesis has recently been shown.[13] Beyond DBA, the genes implicated in other pediatric bone marrow failure syndromes including Schwachman-Diamond Syndrome, dyskeratosis congenita and cartilage-hair hypoplasia, are also involved in ribosomal biogenesis.[14] Our findings thus establish an unexpected, but logical link between the 5q- syndrome, caused by somatic deletion of one allele of RPS14, and congenital bone marrow failure syndromes, caused by heritable mutation of other ribosome-associated proteins.

The erythroid specificity of acquired or inherited defects in RPS14, RPS19 or RPS24 expression is noteworthy. While these ribosomal proteins and the ribosomal subunits they constitute are thought to be ubiquitous, the erythroid lineage is under particularly high biosynthetic demand. Erythroid progenitor cells proliferate extraordinarily rapidly (yielding 2×10^{11} new red cells per day in an adult human),[15] and contain extremely high concentrations of globin proteins – all resulting in a high demand for ribosomal biogenesis. Furthermore, erythroid cells must balance the production of heme and the translation of globin proteins precisely, else the cells undergo apoptosis.[16] It is therefore possible that partial loss of ribosomal function in other lineages may not result in an obvious phenotype. We also note that in an unbiased screen in zebrafish for genes that cause tumors after loss of a single allele, 92% of the tumor-prone fish lines had hemizygous mutations in ribosomal protein-encoding genes.[17] These observations suggest that RPS14 loss of function in the 5q- syndrome may explain not only the erythroid differentiation defect seen in affected patients, but also their propensity to progress to acute leukemia. The mechanism by which ribosomal dysfunction is tumorigenic in fish has yet to be determined.

The experiments described in this report establish RPS14 as a causal gene for the 5q- syndrome. It is conceivable, however, that other genes (on 5q or elsewhere) collaborate with RPS14 to cause the disease phenotype. We speculate that whereas RPS14 loss of function may be sufficient for the erythroid differentiation defect, additional mutations may be required for RPS14-deficient cells to reach clonal dominance and to progress to malignant transformation to AML. In that regard, the 5q- syndrome region on chromosome 5 should be distinguished from a more centromeric locus on 5q that has been associated with therapy-related and aggressive subtypes of MDS as well as AML, and for which two candidate genes have been recently reported.[18-20] In most patients, a large portion of 5q is deleted, encompassing both critical regions, so it is possible that loss of both RPS14 and a second collaborating gene is achieved in a single genetic event.

Acquired deletions are a hallmark of cancer and pre-cancerous states. In general, such deletions flag the existence of a tumor suppressor gene conforming to Knudson's two-hit hypothesis, wherein one allele is often deleted, and the other allele is inactivated either by deletion, mutation, or epigenetic modification. In multiple tumor types (e.g. 1p deletions in neuroblastoma, 3p deletions in lung cancer, and 7q deletions in myeloid malignancies), however, the search for the key tumor suppressor gene has been elusive. A possible explanation for the failure to identify these classic tumor suppressor genes is that oncogenesis is caused by allelic insufficiency.[21] The recent discovery of monoallelic deletions or mutations in *PAX5* in acute lymphoblastic leukemia supports this hypothesis.[22]

Our RNA interference-based discovery of the 5q- syndrome gene suggests that haploinsufficient disease genes can be identified using this approach. It is possible that the systematic application of RNAi might similarly identify the genes responsible for other diseases caused by allelic insufficiency.

Methods summary

Culture of hematopoietic progenitor cells

Primary normal human bone marrow or umbilical cord blood CD34+ cells were differentiated in vitro using a two phase liquid culture system using combinations of cytokines that support erythroid, myeloid, and megakaryocytic differentiation.[23] Viable cells from bone marrow aspirates from MDS patients were collected under an IRB approved protocol at Massachusetts General Hospital.

Lentiviral vectors

Multiple shRNA lentiviruses targeting each gene in the CDR for the 5q- syndrome were produced as described previously.[24] The target sequence of each shRNA is listed in Table S2.

Flow cytometry

Hematopoietic differentiation was assessed by flow cytometry using antibodies specific to terminally differentiated erythroid cells (GlyA), immature erythroid cells (CD71), megakaryocytes (CD41), and myeloid cells (CD11b).

Microarrays using gene set enrichment analysis

Linear amplification of RNA was performed using the Ovation Kit (Nugen) and labeled cDNA was applied to oligonucleotide microarrays (Affymetrix). Gene set enrichment analysis was performed as described previously.[9] Microarray experiments and gene sets are listed in Tables S3 and S4, respectively.

Ribosomal RNA processing and polysome profiles

The effect of RPS14 knock-down on pre-rRNA processing was performed by Northern blot analysis. Polysome fractionation on a sucrose gradient and spectrophotometric detection were performed as described previously.[13]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Broad Institute RNAi and Genetic analysis platforms for advice, SNP analysis, and reagents. This work was supported by grants R01 HL82945-02 (T.R.G.), K08 HL078818-02 (B.L.E.), and R01 HL079583 (S.R.E.). T.R.G is an investigator of the Howard Hughes Medical Institute.

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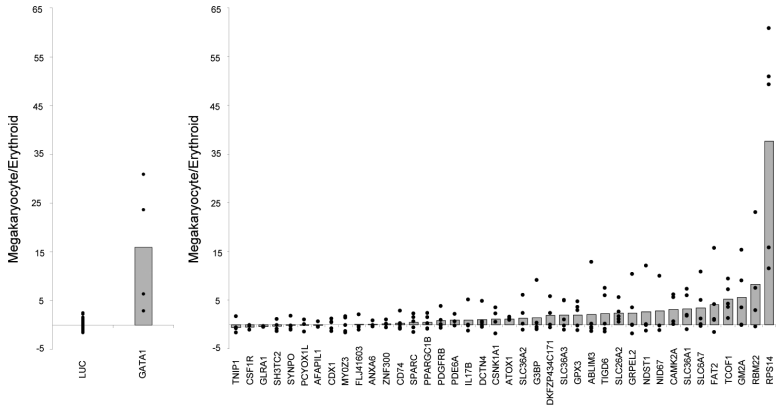

Figure 1.

Screen of common deleted region for the 5q- syndrome. Each gene was targeted by multiple lentivirally expressed shRNAs in CD34+ cells from umbilical cord blood, and the ratio of megakaryocytic to erythroid differentiation was determined by flow cytometry using antibodies against CD41 and GlyA respectively. Controls are shown in the left panel, including an shRNA targeting luciferase, which is not expressed in the primary cells, and multiple shRNAs targeting GATA-1, an erythroid-specific transcription factor. All of the genes in the CDR for the 5q- syndrome are shown in the right panel. The megakaryocytic/erythroid ratio is shown as a z-score using the mean and standard deviation of the control (Luc) replicates. For the control shRNA targeting the luciferase gene, circles represent 30 individual replicates. For all other genes, circles represent the median of three replicates for each individual shRNA. The mean of all shRNAs targeting a given gene is shown with a grey bar.

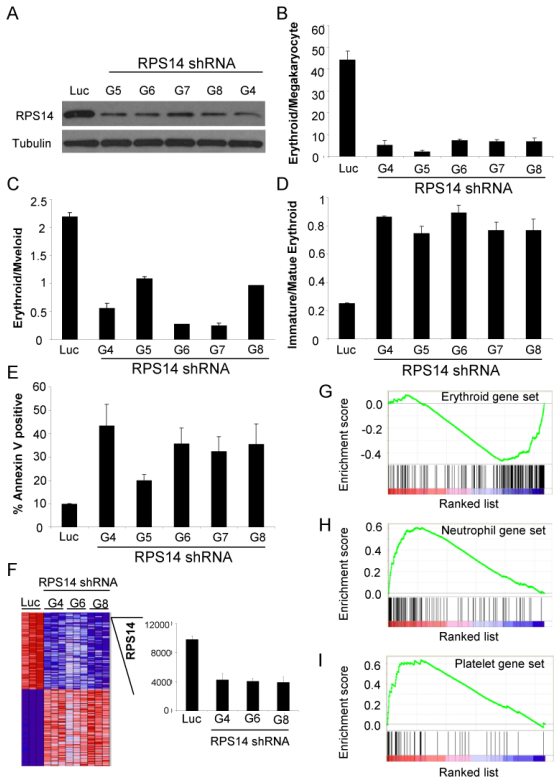

Figure 2.

Multiple shRNAs targeting RPS14 recapitulate the 5q- syndrome in vitro. Western blots demonstrate that five different shRNAs effectively decrease levels of RPS14, as shown by Western blot (panel A). Compared to a control shRNA targeting the luciferase gene (Luc), each of the 5 RPS14 shRNAs block erythroid relative to megakaryocytic differentiation in adult bone marrow CD34+ cells. The ratios of cells from the erythroid and megakaryocytic lineages, indicated on the y-axis, were assessed by flow cytometry using antibodies against GlyA and CD41 respectively (panel B). In addition, RPS14 shRNAs decrease erythroid relative to myeloid differentiation, assessed using antibodies against GlyA and CD11b (panel C); block terminal erythroid differentiation, assessed using antibodies against GlyA and CD71 (panel D); and increase apoptosis, assessed by annexin V expression (panel E). In panels B through E, the effect of RPS14 shRNAs, compared to the luciferase shRNA, was statistically significant ($p < .05$ by Student's two tailed t-test, n=3, mean and S.E.M. shown). Multiple shRNAs targeting RPS14 also alter the transcriptional programs of lineage specific differentiation. The top 100 marker genes that are differentially expressed between cells expressing control versus RPS14 shRNAs, ranked by signal to noise ratio,[26] are shown in panel F. RPS14 is at the top of the list of downregulated genes and is expressed at approximately 40% of the normal level. RPS14 shRNAs significantly decrease expression of an erythroid gene expression signature[27] and increase expression of neutrophil[28] and platelet[29] signatures (panels G – I), as assessed by GSEA. Genes are ranked by signal to noise ratio according to their differential expression between cells expressing RPS14 versus control shRNAs. Genes in the lineage-specific gene sets are marked with vertical bars, and the enrichment score is shown in green.

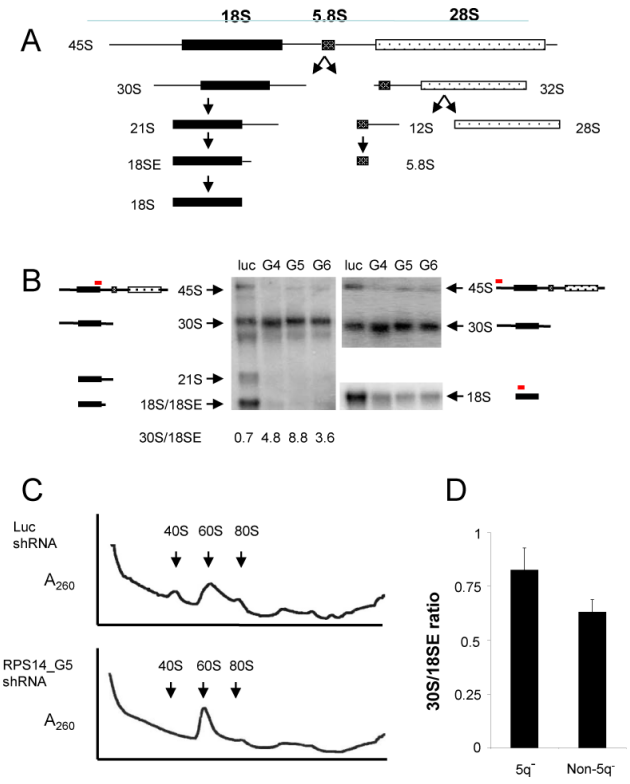

Figure 3.

RPS14 is required for 18S pre-rRNA processing and 40S ribosomal subunit formation. A simplified schematic of pre-rRNA processing is illustrated in panel A. A defect in the 5' processing of 18S pre-rRNA is evident from Northern blots using RNA from TF-1 cells expressing control or RPS14 shRNAs with an accumulation of 30S rRNA and a deficiency of 21S and 18SE pre-rRNAs and mature 18S rRNA (panel B). The Northern blot probes are shown in red. Polysome profiles from TF-1 cells demonstrate that decreased expression of RPS14 results in a 40S subunit deficiency (panel C). The 30S/18SE pre-rRNA ratio is also increased in RNA from bone marrow mononuclear cells from MDS patients with the 5q deletion (n=4) compared to MDS patients without 5q deletions (n=5), as measured by quantification of Northern blots (p=.06, panel D).

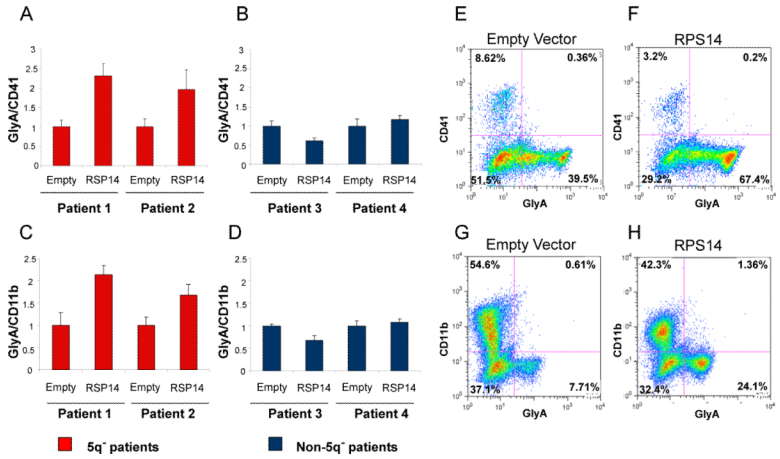

Figure 4.

RPS14 overexpression rescues erythroid differentiation in samples from patients with 5q deletions. CD34+ cells from bone marrow aspirates of patients with the 5q- syndrome (shown in red) and MDS patients without 5q deletions (shown in blue) were infected with a lentivirus expressing the RPS14 cDNA or an empty vector. In patients with 5q deletions, RPS14 overexpression increased erythroid relative to megakaryocytic differentiation (panels A and C) and erythroid relative to myeloid differentiation (panels B and D) shown normalized to the empty vector control. The mean and standard deviation of three independent experiments are shown. Representative flow cytometry plots for patient 1 are shown in panels E to H. Compared to the empty vector control, RPS14 overexpression results in an increase in GlyA expression and a decrease in CD41 and CD11b.