L-leucine increases translation of *RPS14* and *LARP1* in erythroblasts from del(5q) myelodysplastic syndrome patients

Deletion of the long arm of chromosome 5 [del(5q)] is the most common cytogenetic abnormality found in the myelodysplastic syndromes (MDS).[1] Patients with the 5q- syndrome have macrocytic anemia and the del(5q) as the sole karyotypic abnormality.[1] Haploinsufficiency of the ribosomal protein gene *RPS14*, mapping to the commonly deleted region (CDR) on chromosome 5q,[2] underlies the erythroid defect found in the 5q- syndrome,[3] and is associated with p53 activation,[4-6] a block in the processing of pre-ribosomal RNA,[3] and deregulation of ribosomal- and translation-related genes.[7] Defective mRNA translation represents a potential therapeutic target in the 5q- syndrome and other ribosomopathies, such as Diamond-Blackfan anemia (DBA).[8]

Evidence suggests that the translation enhancer L-leucine may have some efficacy in the treatment of the 5q- syndrome and DBA.[8] A DBA patient treated with L-leucine showed a marked improvement in anemia and achieved transfusion independence.[8] Studies using zebrafish models of the 5q- syndrome and DBA[9,10] and mouse models of DBA[11] found that L-leucine treatment improved hemoglobinization and red cell counts. L-leucine treatment of erythroblasts with *RPS14* knockdown and erythroblasts derived from 2 patients with 5q- syndrome patients increased cell proliferation, erythroid differentiation, and mRNA translation.[6]

In this study, we investigated the mechanism of L-leucine-mediated enhancement of erythropoiesis in the 5q- syndrome and MDS with del(5q). Cultured erythroblasts obtained from bone marrow mononuclear cells of 8 MDS patients with del(5q) (*Online Supplementary Table S1*) and 8 healthy controls were treated with either L-leucine or the inactive isomer D-leucine. A significantly higher percentage of non-erythroid $CD36^-/CD235a^-$ (Figure 1A) and $CD71^-/CD235a^-$ cell populations (Figure 1B), and a lower percentage of $CD36^+/CD235a^+$ (Figure 1A) and $CD71^+/CD235a^+$ intermediate erythroid cell populations (Figure 1B) was observed in erythroblast cultures from del(5q) MDS patients compared to erythroblast cultures from healthy controls treated with D-leucine. L-leucine treatment of erythroblast cultures from patients with del(5q) MDS resulted in a significant increase in the percentage of intermediate erythroid cell populations ($CD36^+/CD235a^+$ and $CD71^+/CD235a^+$) (Figure 1A and B) and a significant decrease in the percentage of non-erythroid $CD36^-/CD235a^-$ (Figure 1A) and $CD71^-/CD235a^-$ (Figure 1B) cell populations, with no significant changes in the erythroblast cultures from healthy controls (Figure 1A and B). Cells positive for α-globin (Figure 1C) and β-globin (Figure 1D) were significantly reduced in del(5q) MDS patient erythroblasts compared to erythroblasts from healthy controls. This finding is consistent with the impaired erythroid differentiation found in erythroblasts from del(5q) MDS patients. Importantly, L-leucine treatment significantly increased the percentage of both α- and β-globin positive cells in del(5q) MDS patient erythroblast cultures, reaching the percentages of cells observed in erythroblast cultures from healthy controls (Figure 1C and D). L-leucine treatment did not alter globin levels in erythroblasts from healthy controls (Figure 1C and D).

Evidence suggests that L-leucine activates the mammalian target of rapamycin (mTOR) signaling pathway, which controls mRNA translation and cell growth.[8] The activated mTOR signaling protein mTOR complex 1 (mTORC1) regulates mRNA translation through phosphorylation of its key downstream targets S6K1 and 4EBP1.[8] We have previously demonstrated that RPS14-deficient erythroblasts and cultured erythroblasts from del(5q) MDS patients treated with L-leucine show increased phosphorylation of S6K1 and 4EBP1.[12] In this study, we investigated the S6K1 target RPS6, which interacts directly with the 40S ribosomal subunit.[8] L-leucine treatment significantly increased the phosphorylation of RPS6, consistent with L-leucine activation of mTORC1 (Figure 1E). Phosphorylation of RPS6 by S6K1 promotes ribosome assembly and mRNA translation elongation.[8]

In accordance with the role of mTOR in regulation of mRNA translation, there is evidence showing that L-leucine treatment leads to an increase in protein production in RPS14-deficient erythroblasts[6] and in a zebrafish model of DBA.[10] However, the mechanism underlying the increased protein production, and which transcripts may be translated more efficiently following L-leucine treatment in del(5q) MDS, are not known.

In order to investigate the effects of L-leucine treatment on global mRNA translation in MDS with del(5q), we used polysome profiling and RNA sequencing techniques. Total RNA and ribosome bound-RNA (RBR) were extracted from erythroblasts from del(5q) MDS patients and healthy controls, treated with either L-leucine or D-leucine, and sequenced (*Online Supplementary Appendix*). Firstly we investigated differences between del(5q) MDS and healthy control erythroblasts. GSAASeqSP analysis of gene expression using total RNA sequencing data from erythroblasts from del(5q) MDS patients compared to erythroblasts from healthy controls, both treated with the inactive isomer D-leucine, identified ribosomal and

Table 1. Top 20 differentially translated known 5'TOP mRNAs in L-leucine treated erythroblasts from del(5q) myelodysplastic syndrome patients.

Genes	LogFC of TE in patients	z score patients
RPS15	3.55	2.46
RPS27A	3.48	2.40
RPS25	3.47	2.39
RPS20	3.43	2.35
RPL12	3.35	2.29
PABPC4	3.01	2.01
RPS24	2.97	1.98
RPS3	2.95	1.96
EEF2	2.83	1.86
RPS18	2.76	1.80
RPS26	2.75	1.79
RPS5	2.69	1.74
RPS21	2.64	1.70
RPS9	2.54	1.62
EIF3E	2.53	1.61
RPS14	2.52	1.60
EEF1E1	2.52	1.60
RPS19	2.49	1.57
RPS16	2.48	1.57
TPT1	2.46	1.55

For each gene, the log fold change (FC) of the translation efficiency (TE) in L-leucine-treated compared to D-leucine-treated patient erythroblasts is shown as well as the z score.

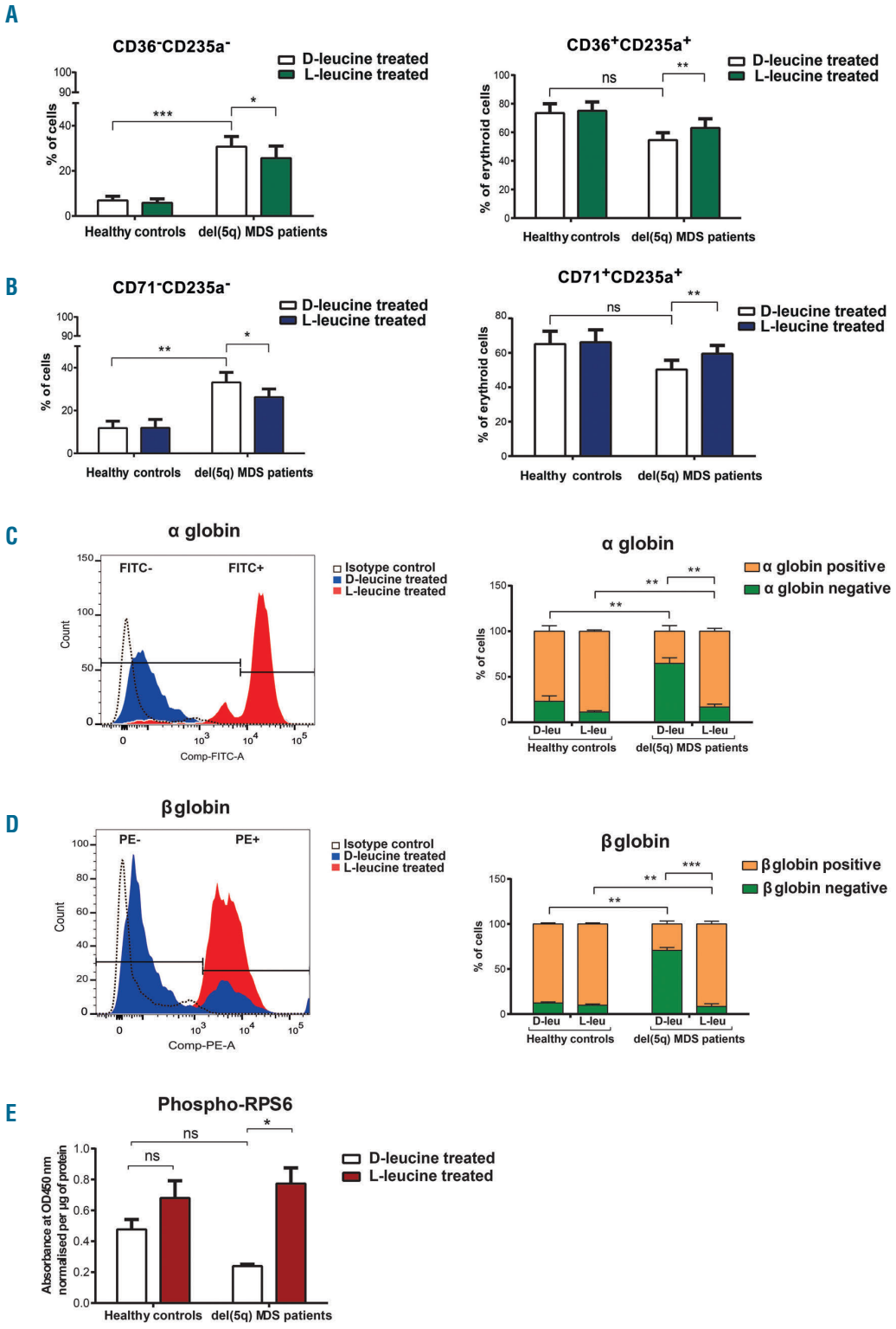

Figure 1. Effects of L-leucine on erythroid differentiation, α- and β-globin and RPS6 phosphorylation in erythroblasts from del(5q) myelodysplastic syndrome (MDS) patients and from healthy controls. (A) Bar graphs showing the percentage of CD36, CD235a double-negative (left) and double-positive (right) cells in healthy controls and del(5q) MDS patient samples, treated with either D-leucine (white) or L-leucine (green). (B) Bar graphs showing the percentage of CD71, CD235a double-negative (left) and double-positive (right) cells in healthy controls and del(5q) MDS patient samples, treated with either D-leucine (white) or L-leucine (blue). (C) (Left) Representative histogram showing α-globin-positive and α-globin-negative cells in a D-leucine and L-leucine treated patient. α-globin was stained with an FITC conjugated antibody. Isotype control is also included. (Right) Bar graph showing the percentage of α-globin-positive (yellow) and α-globin-negative (green) cells in healthy controls and del(5q) MDS patients, treated with either D-leucine or L-leucine measured by intracellular staining and flow cytometry. $^{**}P<0.01$. Error bars represent the Standard Error of Mean (SEM) of 3 biological replicates. (D) (Left) Representative histogram showing β-globin-positive and negative cells in a D-leucine and L-leucine treated patient. β-globin was stained with a PE conjugated antibody. Isotype control is also included. (Right) Bar graph showing the percentage of β-globin-positive (yellow) and β-globin-negative (green) cells in healthy controls and del(5q) MDS patients, treated with either D-leucine or L-leucine measured by intracellular staining and flow cytometry. $^{**}P<0.01$; $^{***}P<0.001$. Error bars represent the SEM of 3 biological replicates. (E) Normalized levels of phosphorylated RPS6, in healthy controls and del(5q) MDS patient samples treated with D-Leucine (white) or with L -leucine (colored), measured by sandwich ELISA. $^{*}P<0.05$; ns: not significant. Error bars represent the SEM of 3 biological replicates.

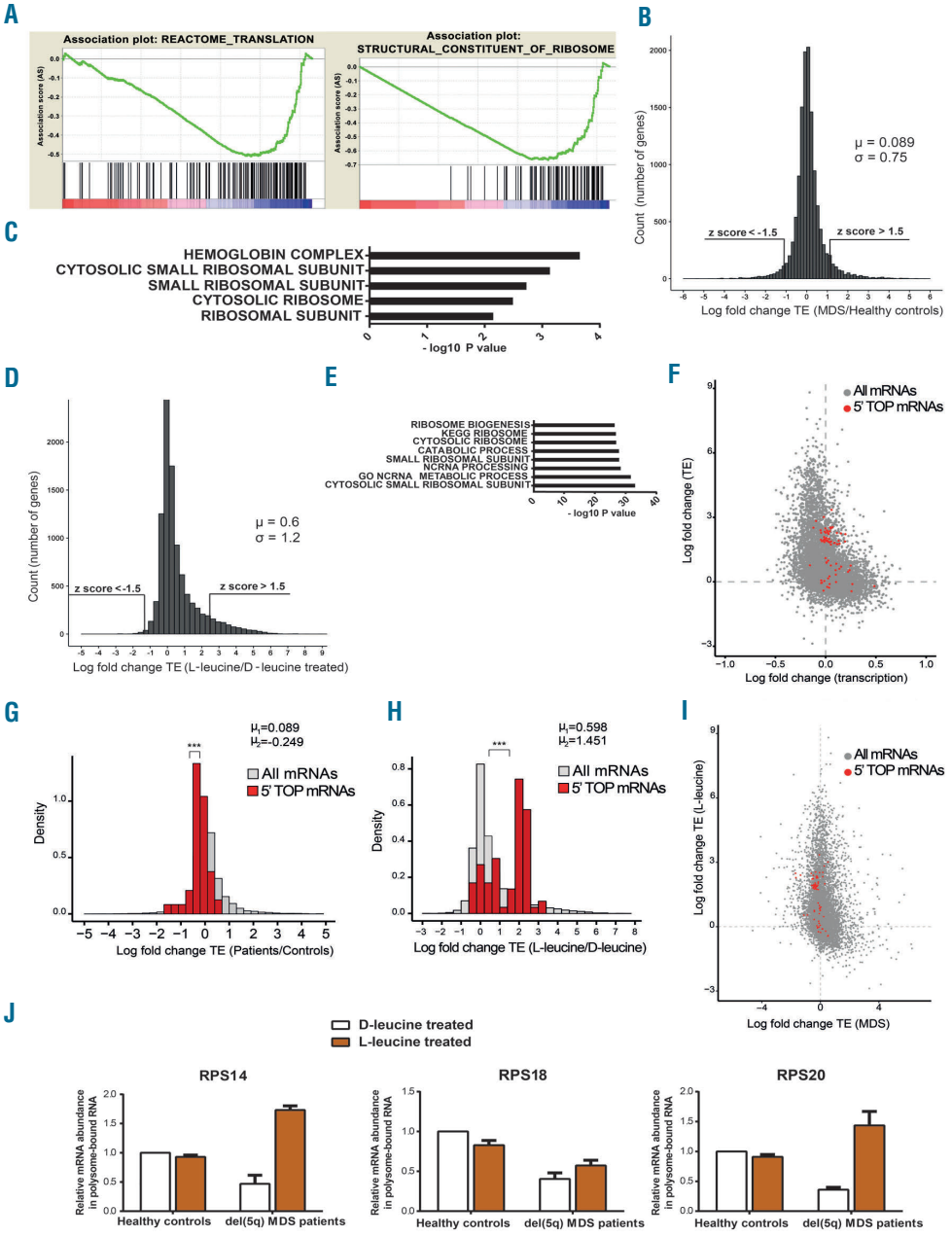

Figure 2. Effects of L-leucine on mRNA translation in erythroblasts from 3 del(5q) myelodysplastic syndrome (MDS) patients and 3 healthy controls, using polysome profiling and RNA sequencing. (A) Enrichment plots generated by GSAASeqSP analysis of RNA-seq data from D-leucine-treated erythroblasts from del(5q) MDS patients compared to D-leucine-treated erythroblasts from healthy controls. Two of the gene sets identified are shown: the "REACTOME_TRANSLATION" gene set (left, q score <0.04) and the "STRUCTURAL CONSTITUENT OF RIBOSOME" gene set (right, q score <0.04). (B) Histogram showing the log2 fold-change (FC) in translational efficiency (TE) of genes in MDS del(5q) patient erythroblasts (treated with D-leucine) compared to (D-leucine treated) healthy controls. The y-axis represents the number of genes for a given log2 FC value. Log2 FC between -6 and 6 are shown. Extreme log2 FC values are not shown for visualization purposes. Transcripts with a z score < -1.5 (corresponding to a LogFC of -1.03) or > 1.5 (corresponding to a LogFC of 1.21) were considered differentially translated. μ represents the mean; σ represents the standard deviation. (C) GO analysis of transcripts showing a significant decrease (z score < -1.5) in TE in erythroblasts derived from del(5q) MDS patients. (D) Histograms showing the log2 fold-change in TE of every mRNA in L-leucine compared to D-leucine-treated del(5q) MDS patient samples. The y-axis represents the number of genes for a given log2 fold-change value. Log2 FC between -10 and 10 are shown. Extreme log2FC values were not shown for visualization purposes. Transcripts with a z score <-1.5 (corresponding to a LogFC of -1.2) or > 1.5 (corresponding to a LogFC of 2.39) were considered differentially translated. μ represents the mean; σ represents the standard deviation. (E) GO analysis of transcripts showing a significant increase (Z score> 1.5) in TE following L-leucine treatment in MDS del(5q) patient samples. (F) Scatter plot showing the log2 FC in transcription (x-axis) and the log2 FC in TE (y-axis) of all mRNAs following L-leucine treatment in patient samples. Red: 5'TOP and 5'TOP-like mRNAs; gray: all other mRNAs. (G) Histograms showing the log2 fold-change in TE of 83 known 5'TOP and 5'TOP-like mRNAs (red) in del(5q) MDS patient erythroblasts compared to healthy controls and all mRNAs measured (gray). Mann-Whitney U test was performed. μ_1 represents the mean of the logFC in TE of all mRNAs; μ_2 represents the mean logFC in TE of 5'TOP mRNAs. ***P<0.001. (H) Histogram showing the $\log_2$ FC in TE of 83 known 5'TOP and 5'TOP-like mRNAs (red) in D-leucine- over L-leucine-treated patient erythroblasts and the TE of all mRNAs measured (gray). Mann-Whitney U test was performed. μ_1 represents the mean of the logFC in TE of all mRNAs; μ_2 represents the mean logFC in TE of 5'TOP mRNAs. ***P<0.001. (I) Scatter plot showing on the y-axis the log2 fold-change in TE in L-leucine treated patients compared to D-leucine treated patients. x-axis shows the log2 FC in TE in del(5q) MDS patient erythroblasts compared to healthy controls. Red dots represent 5'TOP and TOP-like mRNAs; gray dots represent all other mRNAs. (J) Relative amount of *RPS14, RPS20* and *RPS18* mRNA in the polysome-bound fraction of RNA from 2 healthy controls and 2 MDS del(5q) patient erythroblasts, treated with either D-leucine (white) or L-leucine (orange) measured by RT-qPCR.

translation-related gene sets as being significantly down-regulated in patient erythroblasts (Figure 2A). The translation efficiency (TE) of 9868 transcripts was calculated as the ratio between the read counts for each transcript in the RBR and total RNA fractions of a given sample (*Online Supplementary Appendix*). The log2 fold change in TE of each mRNA was determined in patient erythroblasts compared to erythroblasts from healthy controls, both treated with D-leucine (Figure 2B). Gene ontology (GO) analysis of the mRNAs with significantly reduced TE in patient erythroblasts identified significant gene sets related to the hemoglobin complex and the ribosomal subunits (Figure 2C). No significant GO gene set was identified for transcripts with increased TE in patient erythroblasts. We then determined the effects of L-leucine treatment on mRNA translation in del(5q) MDS and healthy control erythroblasts. L-leucine significantly increased the TE of 440 transcripts in healthy control erythroblasts (*Online Supplementary Figure S1A*) and 910 transcripts in patient erythroblasts (Figure 2D). A significant decrease in TE of 576 transcripts was observed in healthy controls, but only 48 transcripts in patient erythroblasts treated with L-leucine. Thus L-leucine treatment results in an asymmetrical distribution of transcripts showing significant increased or decreased TE in del(5q) MDS patient erythroblasts, with increased TE in more than twice as many transcripts in patients than in healthy controls. The explanation for this difference is not known, but it may relate to the haploinsufficiency of genes mapping to chromosome 5q in del(5q) MDS patients. GO analysis of the 910 transcripts in patient erythroblasts identified enrichment in several ribosome-related gene sets (Figure 2E). L-leucine influenced mRNA translation in patient erythroblasts, while having no significant impact on transcription (Figure 2F).

The mTOR pathway preferentially regulates translation of mRNAs with either an established 5' terminal oligopyrimidine tract (5'TOP motif) or a TOP-like motif.[13] We investigated the TE of 5'TOP and TOP-like mRNAs in L-leucine-treated erythroblasts from del(5q) MDS patients. Firstly, we developed a Python script to classify mRNAs as 5'TOP or 5'TOP-like, using the criteria described by Thoreen *et al.*[13] A total of 333 of the 910 transcripts (37%) showing increased TE following L-leucine treatment of erythroblasts from del(5q) MDS patients harbored a 5'TOP (159 transcripts) or 5'TOP-like (174 transcripts) motif. Interestingly, the mRNA binding protein *LARP1* showed a significantly increased TE (log FC= 4.015, z score=2.84) in del(5q) MDS patient erythroblasts treated with L-leucine. *LARP1* maps at 5q33.2, close to the CDR,[2] and reduced *LARP1* mRNA levels have been shown in bone marrow CD34$^+$ cells of patients with 5q- syndrome.[14] LARP1 stabilizes 5'TOP mRNAs in a complex with the 40S ribosome subunit.[14,15] A recent study found that LARP1 is phosphorylated by mTORC1 and acts as a molecular switch for translation initiation of mTOR-regulated mRNAs.[15] Knockdown of *LARP1* in human adult bone marrow CD34$^+$ cells results in a reduction in 5'TOP mRNA levels.[14] Since *LARP1* is a major effector of the promotion of 5'TOP mRNA translation by mTOR activation, we suggest that the observed increase in the TE of *LARP1* by L-leucine plays a role in the mode of action of L-leucine in del(5q) MDS erythroblasts.

Next, we studied a subset of 83 previously confirmed 5'TOP and 5'TOP-like mRNAs.[13] The TE of these 5'TOP and TOP-like mRNAs[13] was significantly decreased in erythroblasts from del(5q) MDS patients (Figure 2G). L-leucine treatment of erythroblasts derived from del(5q) MDS patients (Figure 2H) and healthy controls (*Online Supplementary Figure S1B*) significantly increased the TE of these mRNAs. No significant changes in transcription of these 5'TOP and TOP-like mRNAs were identified in L-leucine-treated patient erythroblasts compared to patient erythroblasts treated with D-leucine (Figure 2F). The translation of 5'TOP mRNAs was down-regulated in erythroblasts from del(5q) MDS patients and increased by L-leucine treatment (Figure 2I), consistent with 5'TOP mRNAs being regulated by mTORC1. 5'TOP mRNAs which showed a significant increase in TE following treatment with L-leucine include ribosomal proteins, such as *RPS15, RPS27A* and *RPS14*, and translation-related genes, such as *EEF2* and *EIF3E* (Table 1). RT-qPCR analysis of three 5'TOP mRNAs (*RPS14, RPS18* and *RPS20*) also confirmed that TE of these transcripts was decreased in patient erythroblasts compared to erythroblasts from healthy controls and was increased by L-leucine in patient erythroblasts, with RPS14 showing a more than 3-fold increase (Figure 2J). Increased TE of *RPS6* (logFC=2.32, z score=1.43) and *S6K1* (logFC=2.65, z score=1.71) mRNAs was also associated with a significant increase in the expression of phosphorylated RPS6 (Figure 1E) and *S6K1* protein (*Online Supplementary Figure S2*) in L-leucine-treated erythroblasts derived from del(5q) MDS patients compared to D-leucine-treated patient samples. CD34$^+$ cells with knockdown of RPS14 exhibit a block in erythroid differentiation and forced expression of *RPS14* rescues the erythroid defect in cultured cells from patients.[3] Thus haploinsufficiency of *RPS14* underlies the anemia in del(5q) MDS.[2,3] We have previously shown that L-leucine treatment of erythroblasts with *RPS14* knockdown to haploinsufficient levels increased erythroid cell growth and differentiation.[6] We suggest that the increase in the TE of 5'TOP mRNAs, and particularly of *RPS14*, plays a critical role in the improved erythroid differentiation observed following L-leucine treatment in del(5q) MDS erythroblasts.

This is the first study using polysome profiling to identify the mRNA transcripts which are more effectively translated in response to L-leucine in erythroblasts from del(5q) MDS patients. We have shown that erythroblasts from del(5q) MDS exhibit decreased translation of 5'TOP and 5'TOP-like mRNAs, a probable consequence of aberrant ribosome biogenesis secondary to haploinsufficiency of *RPS14*.[3,7] L-leucine treatment of patient erythroblasts resulted in increased translation of 5'TOP and other mRNAs, many of which encode ribosomal proteins. Taken together, our data show that L-leucine treatment of erythroblasts from del(5q) MDS patients results in activation of mTOR leading to increased translation of several ribosomal genes and translation factors, including the key targets *RPS14* and *LARP1*, promoting ribosome biogenesis and erythroid differentiation. Our study illuminates the mode of action of L-leucine in del(5q) MDS.

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