Reduced Expression of Ribosomal Proteins Relieves MicroRNA-Mediated Repression

Maja M. Janas,1,3,4 Eric Wang,5,6 Tara Love,1,3,4,8 Abigail S. Harris,7 Kristen Stevenson,2 Karlheinz Semmelmann,7 Jonathan M. Shaffer,7 Po-Hao Chen,1,3,4 John G. Doench,4 Subrahmanyam V.B.K. Yerramilli,7 Donna S. Neuberg,2 Dimitrios Iliopoulos,1,3 David E. Houssman,5,6 Christopher B. Burge,5 and Carl D. Novina1,3,4,*

1Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute
2Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute
3Department of Microbiology and Immunobiology
Harvard Medical School, Boston, MA 02115, USA
4Broad Institute of Harvard and MIT, Cambridge, MA 02141, USA
5Department of Biology
6The Koch Institute
Massachusetts Institute of Technology, Cambridge MA 02139, USA
7Qiagen, Frederick, MD 21703, USA
8Present address: Correlagen Diagnostics, 307 Waverly Oaks Road, Suite 101, Waltham, MA 02452, USA
*Correspondence: carl_novina@dfci.harvard.edu
DOI 10.1016/j.molcel.2012.04.008

SUMMARY

MicroRNAs (miRNAs) regulate physiological and pathological processes by inducing posttranscriptional repression of target messenger RNAs (mRNAs) via incompletely understood mechanisms. To discover factors required for human miRNA activity, we performed an RNAi screen using a reporter cell line of miRNA-mediated repression of translation initiation. We report that reduced expression of ribosomal protein genes (RPGs) dissociated miRNA complexes from target mRNAs, leading to increased polysome association, translation, and stability of miRNA-targeted mRNAs relative to untargeted mRNAs. RNA sequencing of polysomes indicated substantial overlap in sets of genes exhibiting increased or decreased polysomal association after Argonaute or RPG knockdowns, suggesting similarity in affected pathways. miRNA profiling of monosomes and polysomes demonstrated that miRNAs cosediment with ribosomes. RPG knockdowns decreased miRNAs in monosomes and increased their target mRNAs in polysomes. Our data show that most miRNAs repress translation and that the levels of RPGs modulate miRNA-mediated repression of translation initiation.

INTRODUCTION

MicroRNA (miRNA)-mediated translational repression of messenger RNA (mRNA) targets was first described in C. elegans, where lin-4 and let-7 were reported to reduce protein levels without affecting mRNA stability (Olsen and Ambros, 1999; Seggerson et al., 2002). In mammals, polysome profiling studies led to contradictory models of miRNA-mediated translational repression. Although miRNA-targeted mRNAs had reduced polysomal association compared to unrepressed mRNAs in some studies, suggesting repression of initiation (Bhattacharyya et al., 2006a; Pillai et al., 2005), miRNAs and their mRNA targets had robust polysomal association in other studies, suggesting repression after initiation (Maroney et al., 2006; Nelson et al., 2004; Nottrott et al., 2006; Petersen et al., 2006). Additionally, treatment with translation elongation inhibitors shifted miRNAs and miRNA-targeted mRNAs from polysomes toward monosomes, indicating that miRNAs target actively translating mRNAs (Maroney et al., 2006; Nottrott et al., 2006).

Subsequent studies demonstrated that miRNAs could also reduce mRNA stability. In C. elegans, lin-4 and let-7 decreased target mRNA levels (Bagga et al., 2005). In both C. elegans and mammals, miRNAs were shown to promote deadenylation, leading to reduced target mRNA stability (Behm-Ansmant et al., 2006; Wu and Belasco, 2005; Wu et al., 2006). In mammals, transfected miRNAs globally reduced levels of target mRNAs (Lim et al., 2005), and ribosome footprinting determined that miRNA targeting resulted in a decrease in ribosome density that was only modestly (~15%) greater than the decrease in mRNA level, suggesting that miRNAs act primarily to destabilize mRNAs (Guo et al., 2010). More recent ribosome footprinting analysis at earlier time points demonstrated that repression of translation initiation precedes deadenylation for the majority of miR-430-targeted mRNAs in zebrafish and that mRNA decay is likely a consequence of these earlier activities (Bazzini et al., 2012).

Here we report that ~70% of all miRNA species expressed in HeLa cells are detected in monosomal and polysomal fractions, implying that the majority of miRNA species can repress translation. We also demonstrate that ribosomal protein genes...
Ribosomal Proteins Modulate miRNA Activity

RNAi screens for RNAi pathway genes have been used effectively in simpler eukaryotes (Drorner et al., 2006; Eulalio et al., 2007; Kim et al., 2005; Parry et al., 2007; Saleh et al., 2006; Ulvila et al., 2006), leading to identification of many effectors of small RNA function, such as RNA binding proteins, cytoskeletal proteins, and one RPG in C. elegans (Parry et al., 2007), as well as several RPGs and proteasomal components in D. melanogaster (Zhou et al., 2008). However, similar screens have not yet been reported in mammals. Therefore, we used D8 to perform an RNAi screen for effectors and regulators of the human miRNA pathway (Figure 2A). In all, ~7,000 genes were targeted with four individual siRNAs per gene from the Druggable Genome Library of siRNAs (QIAGEN), and effects of knockdowns were assessed with the dual luciferase assay. Using stringent criteria (at least two siRNAs per gene causing derepression of FL ≥ elf6 knockdown), 314 genes (4.3%) scored as hits (Table S1, part A). RPGs were enriched more than 13-fold in this screen: seven out of 12 RPGs (58%) scored as hits. Closer inspection of the screening data showed that all 12 RPG knockdowns derepressed FL to some extent. Gene ontology analysis revealed that the ribosomal pathway was one of the most significantly enriched pathways in the screen (Figure 2B and Table S1, part B). Thus, we hypothesized that as a class RPGs might regulate miRNA repression.

To determine whether depletion of every RPG inhibits miRNA-mediated repression, we targeted all 79 RPGs with four individual siRNAs per gene in D8. Knockdown of virtually all 40S (Figure 2C) and 60S (Figure 2D) RPGs with at least one siRNA led to derepression of FL that was greater than derepression after elf6 knockdown. After three days (the point of strongest FL derepression), most RPG knockdowns reduced cell numbers by 20%–50%. The Y chromosome-expressed S4Y1 and S4Y2 provide negative controls in female cells and did not score in our HeLa cell validation screen (Figure 2C). The consistent FL derepression after depletion of almost every 40S and 60S RPG suggests that altered ribosomal integrity or altered ribosomal subunit stoichiometry and not extraribosomal functions of RPGs led to decreased miRNA activity. Supporting this conclusion, knockdown of factors involved in either 40S (Bms1 and Tsr1) or 60S (Bop1 and Nip7) subunit biogenesis also led to derepression of miRNA-targeted mRNAs (Figure S2A), and double knockdown of 40S and 60S RPGs led to greater derepression of miRNA-targeted mRNAs compared to single RPG knockdowns (Figure S2B).

To validate the screening results, we chose five representative 40S (S5, S11, S12, S15, and S18) and 60S (L5, L11, L12, L35A, and L15A) RPGs for detailed biochemical analysis. Knockdown of each RPG in D8 (Figure S2C) derepressed FL mostly at the protein levels, although proportional increases in FL mRNA levels were also observed (Figure S2D), consistent with knockdowns of known miRNA pathway genes (Figure 1C). In the luciferase screens, which were not normalized to total protein, depletion of RPGs increased FL levels and decreased RL levels (Figure S2E). These trends were affected by reduced cell numbers with, on average, half the number of cells after 72 hr
Figure 1. D8 Is a Highly Sensitive Reporter of miR-21-Mediated Repression of Translation Initiation

(A) Schematic representation of CMV-driven luciferase reporters stably expressed in D8. Firefly Luciferase (FL) possesses six imperfect miR-21 binding sites in its 3’ UTR, while Renilla Luciferase (RL) lacks miRNA binding sites.

(B) D8 reports on miR-21-mediated repression. A miR-21-specific antagomir was transfected into D8 at indicated concentrations, and FL and RL protein and mRNA levels were assessed after 24 hr by the dual luciferase assay and RT-qPCR, respectively. Fold derepression was calculated as (FLtest/RLtest)/(FLcontrol/RLcontrol). The antagomir reduced miR-21 levels in a dose-dependent fashion as assessed by northern blotting.

(C) D8 reports on miRNA effector function. D8 was transfected with Scrambled (Scr), Ago1-, Ago2-, or eIF6-specific siRNAs, and FL and RL protein and mRNA levels were assessed after 72 hr. Knockdowns of Ago1, Ago2, and eIF6 were confirmed by western blotting.

(D) Polysome profiling demonstrates that knockdown of Ago1 or eIF6 shifts FL mRNA from monosomes to polysomes. Lysates from D8 transfected either with Scr or with Ago1- or eIF6-specific siRNAs were fractionated while absorbance was monitored at 254 nm (A254). FL and RL mRNAs were assessed by northern blotting, and 28S and 18S rRNAs were assessed by ethidium bromide staining.

(E) Quantification of northern blots with ImageQuant. FL and RL mRNA detected in each fraction is represented as the percent of the total mRNA detected in all fractions across the gradient. Bar graphs show the mean ± SD from three independent experiments. See also Figure S1.
Figure 2. RNAi Screening for Effectors and Regulators of the miRNA Pathway Identifies RPGs

(A) Schematic representation of the RNAi screen. D8 was transfected with siRNAs against ~7,000 genes, and FL and RL protein levels were assessed by the dual luciferase assay after 72 hr.

(B) Ribosomal pathway was significantly enriched among hits from the screen as assessed by gene ontology analysis. The p values were calculated with Fisher exact test analysis and corrected p values were calculated with the Bonferroni method.
of RPG knockdown, presumably because RPG depletion reduced cellular proliferation rates as previously reported (Flygare et al., 2005; Kirn-Safran et al., 2007; Miller et al., 2003; Oliver et al., 2004; Cristian et al., 2009; Panić et al., 2007; Volarevic et al., 2000). When normalized to total protein by western blotting, we observed increased FL and unchanged RL levels (Figure S2F). To further validate these findings and to control for possible siRNA off-target effects, we expressed five siRNA-resistant isoforms of RPGs (S5, S12, S15, L12, and LP2) in D8 cells knocking down these RPGs. In all cases, transfection of constructs expressing RPGs with silent mutations in regions complementary to siRNAs partially rescued the FL derepression (Figure S2G), demonstrating that RPG depletion directly led to miRNA-targeted mRNA derepression.

We next assessed the effect of RPG depletion on various reporter and endogenous miRNA-targeted mRNAs. FL and RL reporters containing six imperfectly complementary binding sites for the artificial CXCR4 miRNA were derepressed in RPG knockdowns only in the presence of the CXCR4 miRNA (Figures S2H and S2I). However, an FL reporter containing one perfectly complementary site for the CXCR4 miRNA was only modestly affected by RPG knockdowns (Figure S2J), indicating that RPGs preferentially regulate miRNA-mediated translational repression and not siRNA-mediated mRNA cleavage. The RL reporter containing HMG2 3’ UTR with seven seed matches to the let-7 miRNA (Mayr and Bartel, 2009) was derepressed upon RPG knockdowns (Figure S2K). Consistent with previous studies showing that repression of HMG2 correlates with its mRNA level (Lee and Dutta, 2007), endogenous HMG2 mRNA levels increased after RPG knockdowns (Figure 2E). Similarly, an RL reporter containing the KRAS 3’ UTR with seven let-7 sites (Johnson et al., 2005) was derepressed in HeLa cells upon RPG knockdowns (Figure S2L), as was endogenous RAS protein (Figure 2F). Other validated endogenous miRNA-targeted mRNAs, such as miR-21-targeted PTEN (Lewis et al., 2003; Talotta et al., 2008), let-7-targeted IMP-1 (Bouyerinas et al., 2008), and miR-21-targeted PDCD4 (Asangani et al., 2008) were all derepressed upon RPG knockdowns (Figure 2F). Although the degree and pattern of derepression varied between different miRNA-targeted mRNAs, as a class RPGs regulated miRNA repression.

**RPG Knockdowns Relieve Repression of Translation Initiation**

To explore the mechanism of derepression of miRNA-targeted mRNAs in RPG knockdown cells, we first examined the biogenesis of any mRNAs tested (Figures S3A and S3B). Additionally, RPG knockdowns did not decrease the levels of Ago1 or Ago2 proteins (Figure S3C). Polysome profiling of D8 cells knocking down S15 (Figures 3A and 3C) or L12 (Figures 3B and 3C) demonstrated a strong shift of FL mRNA but not RL mRNA from monosomes to polysomes, indicating that RPG knockdowns relieve miRNA-mediated repression of translation initiation. Consistent with knockdowns of miRNA pathway genes (Figure 1D), RPG knockdowns increased the combined signal for FL but not RL mRNA across gradients relative to the Scr control, indicating preferential stabilization of FL mRNA. Heavy complexes formed on miRNA-targeted mRNAs after RPG depletion were actively translating polysomes because FL mRNA shifted to lighter fractions after puromycin treatment which dissociates only ribosomes actively translocating on mRNAs (Figures S3D and S3E). Thus, reduced levels of RPGs increased loading of active ribosomes onto miRNA-targeted FL mRNA but not untargeted RL mRNA.

Importantly, the effects of RPG knockdowns on ribosomal subunit levels were similar among all tested 40S and 60S RPGs. Knockdown of 40S RPGs consistently decreased the levels of free 40S subunits and 18S ribosomal RNA (rRNA) and increased the levels of free 60S subunits (Figures 3A, S4A, and S4B). On the other hand, knockdown of 60S RPGs or elf6 consistently decreased the levels of free 60S subunits and 28S rRNA (Figures 3B, S4A, and S4B). These data are consistent with established contributions of 40S and 60S RPGs to biogenesis and/or stability of ribosomal subunits (Lempääläinen and Shore, 2009) and further indicate that defects in ribosome biogenesis rather than extraribosomal functions of RPGs led to reduced miRNA-mediated repression.

To resolve the 40S, 60S, and 80S peaks, polysome profiling was performed with lower-density gradients, higher-speed spins, and longer times, which resulted in reduced resolution of the highest-density complexes (Figures 3A and 3B). These conditions enabled detection of reduced 40S and 60S peaks and thus verified efficient and functional RPG knockdowns. To resolve the highest density polysomes and prevent pelleting of the heaviest complexes, we performed polysome profiling using higher-density gradients, lower-speed spins, and shorter times. Under these conditions, RPG knockdowns led to a consistent shift of FL but not RL mRNAs to polysomes (Figure S4C). Together, these data show that RPG knockdowns inhibited miRNA-mediated repression of translation initiation and that miRNA-targeted mRNA derepression could be a result of perturbed stoichiometry between 40S and 60S ribosomal subunits, either directly through physical interactions with miRNP complexes or indirectly through signaling pathways that sense ribosome biogenesis.
Figure 3. RPG Knockdowns Selectively Increase Translation of miRNA-Targeted mRNAs

(A and B) RPG knockdowns relieve miR-21-mediated repression of translation initiation. Polysome profiles demonstrate that knockdown of S15 (A) and L12 (B) shift miR-21-targeted FL mRNA but not untargeted RL mRNA from monosomes to polysomes.

(C) Quantification of northern blots with ImageQuant. FL and RL mRNA detected in each fraction is represented as the percent of the total mRNA detected in all fractions across the gradient.

(D) Translation rates of CXCR4 miRNA-targeted FL6X are increased after RPG knockdowns only in the presence of the targeting CXCR4 miRNA. HeLa was transfected with Scr or with S15- or L12-specific siRNAs, and after 48 hr FLAG-tagged FL6X was transfected either with nontargeting Scr (control) or targeting CXCR4 (test) miRNAs. After 24 hr, cells were 35S-pulse-labeled and FL was IP-ed with anti-FLAG agarose. Inputs and precipitates were resolved by PAGE, and the amount of 35S incorporation into FL6X was quantified with ImageQuant and normalized to the Scr transfection.

(E) Overall translation rates are not reduced by depletion of RPGs as assessed by 35S-pulse-metabolic-labeling. HeLa transfected with indicated siRNAs was 35S-pulse-labeled, resolved by PAGE, and either stained with Coomassie as a loading control or visualized with a phosphorimager. As a positive control, pulse labeling was performed in the presence of a known translation inhibitor cycloheximide (CHX).

See also Figures S3 and S4.
To further validate that RPG knockdowns increased translation rates of miRNA-targeted but not untargeted mRNAs, we performed 35S pulse labeling followed by immunoprecipitation (IP) of FLAG-tagged FL protein targeted by the CXCR4 miRNA. RPG knockdowns resulted in up to 3-fold increased incorporation of the 35S label into FL protein in the presence of the targeting CXCR4 miRNA, while RPG knockdowns did not significantly affect 35S incorporation into FL protein in the absence of the CXCR4 miRNA (Figure 3D), demonstrating increased translation rates of a miRNA-targeted but not an untargeted mRNA. Indeed, gross effects on overall translation rates were not observed after RPG knockdowns (Lai et al., 2009; Volarevic et al., 2000), as indicated by unchanged polysome peak heights in A254 absorbance traces (Figures 3A, 3B, and S4A) and pulse 35S labeling of bulk proteins (Figure 3E). Therefore, depletion of individual RPGs specifically increased translation rates of miRNA-targeted mRNAs but not untargeted mRNAs.

RPG Knockdowns Reduce Ago Association with miRNA-Targeted mRNAs

To address the mechanism of RPG knockdowns leading to derepression of miRNA-targeted mRNAs, we assessed Ago1 and Ago2 association with miRNAs and target mRNAs before and after RPG depletion. RPG knockdown followed by IP of FLAG-tagged Ago1 or Ago2 did not affect Ago association with miR-21 or let-7a miRNAs but significantly decreased Ago association with miR-21-targeted FL mRNA relative to control cells (Figure 4A). These data indicate that Ago association with miRNAs remains stable and that intact miRNPs dissociate from miRNA-targeted mRNAs. As observed for FL mRNA in D8, RPG knockdowns reduced association of Ago1 and Ago2 with endogenous miRNA-targeted mRNAs (KRAS and PTEN) while increasing their stability (Figure S4D). Supporting
Figure 5. Reduced RPG Expression Upregulates Polysomal Association of a Consistent Set of Genes that Is Enriched in miRNA-Targeted mRNAs

(A) Genes whose polysome/input ratios change after knockdown of Ago, RPGs, or eIF6 are shared. The numbers of genes whose polysome/input ratios are increased or decreased more than 2-fold in pairs of knockdowns were counted and are displayed in matrix format. The numbers of genes expected to be regulated in the same manner by chance are shown in parentheses. The observed/expected ratio for each comparison is displayed in color.
Ribosomal Proteins Modulate miRNA Activity

the model of miRNP dissociation from miRNA-targeted mRNAs, RPG depletions reduced Ago1 and Ago2 association with ribosomes (Figure 4B). Together, these data demonstrate that RPG knockdown dissociates miRNPs from miRNA-targeted mRNAs and does not affect miRNA assembly or stability.

**RPG, eIF6, and Ago Knockdowns Alter Polysome Association of Common mRNAs**

To globally assess effects on translation of endogenous mRNAs, we conducted RNA sequencing (RNA-Seq) analysis of polysomal fractions and total RNA (“input” to the gradient) before and after knockdowns of RPGs, Agos, or eIF6. A remarkably large number of common genes demonstrated a 2-fold or greater increase in polysome association (polysome/input ratio) after knockdown of RPGs, Agos, or eIF6 (Figure 5A). Many common genes also demonstrated a 2-fold or greater decrease in polysome association for many pairs of knockdowns, particularly between knockdowns of different Agos. These observations suggested the surprising conclusion that large and small subunit RPGs, Agos, and eIF6 have related effects on translational regulation globally, perhaps through common regulatory pathways. This idea was further supported by analysis of mRNAs impacted in three or more of these knockdowns. Instead of decreasing rapidly to zero, as expected if the knockdowns affected independent sets of genes, the numbers of mRNAs with at least 2-fold increased polysome association remained in the several hundred range, even as the number of compared knockdowns increased from three to eight, with 479 mRNAs exhibiting an increase in all eight knockdowns (Figure 5B and Table S2, part A). A smaller but still highly significant set of 57 mRNAs with consistently decreased polysome association in all knockdowns was also observed (Figure 5B and Table S2, part B). Thus, RPGs, Agos, and eIF6 inhibited polysome association of a large common cohort of mRNAs, and promoted polysome association of a smaller common cohort of mRNAs.

**mRNA Target Sites Are Enriched in Polysome-Shifted mRNAs**

To explore potential connections to the miRNA pathway, we analyzed the fraction of mRNAs containing conserved 8-mer seed matches to HeLa-expressed miRNAs, a relatively stringent set of putative targets that likely excludes many authentic targets with weaker or less stringently conserved seed matches (Friedman et al., 2009). Significant enrichment of conserved target sites relative to controls was observed in the set of mRNAs with consistently increased polysomal association in all knockdowns (Figure 5C). The extent of target site enrichment increased as the number of intersected knockdowns increased (Figure 5D). The observed enrichment for miRNA target sites in this common set of polysome-increased mRNAs suggested that miRNAs are involved in repression of this gene set via a mechanism involving translation, and that the perturbations introduced by RPG, Ago, and eIF6 knockdowns after ribosome loading onto a cohort of mRNAs that is strongly enriched for miRNA-targeted mRNAs. Mechanisms involving miRNA-directed changes in stability of polysome-associated versus non-polysome-associated mRNAs are also possible. In contrast, the set of genes with consistently decreased polysomal association in the knockdowns was not enriched for conserved miRNA target sites (Figures 5C and 5D), suggesting that this set of mRNAs is less often directly impacted by miRNAs. Although RPG, Ago, and eIF6 knockdowns also altered mRNA levels of a common cohort of genes, these sets of genes were not enriched for conserved 8-mer seed matches to HeLa-expressed miRNAs (Figure S5). Together, these data show that RPGs, Agos, and eIF6 contributed to reducing polysome association of a common subset of mRNAs enriched for miRNA-targeted mRNAs. Because miRNA-targeted mRNAs were not enriched in a common subset of mRNAs exhibiting increased mRNA levels after knockdowns, we observed translational derepression and not stabilization of miRNA-targeted mRNAs.

**miRNAs Predominantly Cosediment with Polysomes**

The preceding studies demonstrated that miRNA-targeted mRNAs associate with monosomes and that RPG knockdowns increase association of miRNA-targeted mRNAs with polysomes without changing miRNA levels or miRNA association with Ago proteins. To gain additional insights into RPG regulation of miRNA-mediated repression, we performed global miRNA profiling of total, monosomal, and polysomal RNA after sucrose gradient fractionation of HeLa cells before and after RPG knockdown. Out of 669 unique miRNA species expressed in HeLa cells, 462 miRNAs (70%) were reliably detected in monosomal and polysomal fractions at steady state (Table S3, part A). The majority of miRNAs were equally abundant in monosomal and polysomal fractions. Specifically, out of 462 miRNAs detected in monosomes and polysomes, 265 miRNAs (57%) had no statistically significant enrichment in either fraction. Thus, the majority of miRNAs are equally distributed across monosomal and polysomal fractions and may contribute to
multiple modes of translational repression, suggesting mechanistic variation for molecules of the same miRNA species. Interestingly, 197 of these miRNAs (43%) were differentially present in monosomal versus polysomal fractions (Figure 6A and Table S3, part B). Strikingly, 195 of those 197 miRNAs (99%) were enriched in polysomal fractions, while only two miRNAs (miR-21 and miR-126*) were enriched in monosomal fractions (Figure 6B and Table S3, part B). These data demonstrate that (1) the majority of HeLa miRNA species cofractionate with ribosomes and thus can repress translation; (2) miR-21 predominantly represses translation initiation, consistent with biochemical characterization of the D8 cell line where miR-21-repressed FL mRNA accumulated in monosomes (Figures 1D, 3A, and 3B); (3) individual miRNA species can be found preferentially in polysomal relative to monosomal fractions, indicating that miRNA or target mRNA identity can affect the mode of translational repression; and (4) most miRNAs preferentially co-sediment with polysomal fractions. Because puromycin treatment did not significantly affect sedimentation of the 195 miRNAs enriched in polysomal fractions (Figure S6A), these miRNAs were either associated with heavy processing body (P body) aggregates that cosedimented with polysomes or with ribosomes stalled on target mRNAs, suggesting a postinitiation block.

RPG Knockdowns Decrease miRNA Association with Monosomes

To assess RPG regulation of miRNA-mediated repression, we performed global miRNA profiling of total, monosomal, and polysomal RNA after S15 knockdown in HeLa cells (Figure 6C and Table S3, part C). S15 knockdown did not globally alter
miRNA levels (Figure S6B), consistent with results from northern blotting experiments (Figure S3B). S15 knockdown also did not affect miRNA abundance in polysomes, where the average mean fold change (0.99 with a range 0.95–1.06) was not statistically significant according to the p values adjusted for multiple comparisons. These data indicate that RPG depletion does not affect miRNA-mediated repression of translation after initiation or the abundance of miRNAs in P bodies.

In contrast, S15 knockdown reduced monosome association of 67 out of 462 (15%) miRNAs detected in monosomes and polysomes, demonstrating that RPGs regulate miRNA-mediated repression of translation initiation (Figure 6C). Importantly, all 67 of these miRNAs were reduced in monosomes after S15 knockdown and none of these miRNAs were increasingly associated with polysomes. These data suggest that miRNPs containing these miRNAs dissociated from target miRNAs, consistent with reduced association of miRNA-targeted miRNAs with Agos after RPG knockdowns (Figure 4A, 4B, and S4D). Notably, multiple let-7 family members were among the 67 miRNAs reduced in monosomes (average 3.5-fold decrease) and was not affected in polysomes (average 1.1-fold increase). However, we note that the decreased association of miR-21 with monosomes was not considered...
statistically significant using strict limits (the raw p value for Scr versus S15 siRNA was 0.03, but the FDR p value [q value] was 0.07).

To determine whether dissociation of the 67 miRNAs from monosomes after S15 knockdown affected repression, we performed miRNA target prediction analysis using TargetScan on the set of 479 mRNAs that commonly shift toward polysomes after RPG, Ago, or elf6 knockdowns as assessed by RNA-Seq (Figure 5B and Table S2, part A). Only favorable targets, defined as mRNAs with TargetScan context score in the top two quartiles for each miRNA, were considered. We observed a highly significant enrichment of mRNA targets of those 67 miRNAs that shifted to polysomes following RPG knockdowns (Table S3, part D). These data indicate that RPG knockdowns relieve repression of translation initiation by decreasing miRNP association and increasing ribosome association of these miRNA-targeted mRNAs.

**p53 Pathway Activation Relieves miRNA-Mediated Repression**

Global analysis of transcriptome changes (Figure S5) identified robust effects on p53 pathway genes after RPG (S15, L11, and L12) but not Ago knockdowns (Figures S7A and S7B and Table S4). Specifically, gene network analysis of mRNAs commonly dysregulated in RPG knockdowns revealed enrichment for a cell repair network (p value of 6.2 × 10^{-41}; Figure S7B) and a cell-cycle network (p value of 8.1 × 10^{-28}; Figure S7B). Because perturbation of ribosome subunit biogenesis induces nucleolar stress and leads to p53 pathway activation (Deisenroth and Zhang, 2010; Hözel et al., 2010; Panic et al., 2007; Rudra and Warner, 2004; Volarevic et al., 2000), we hypothesized that reduced RPG expression led to p53 pathway activation, which, in turn, reduced repression of translation initiation of miRNA-targeted mRNAs. Notably, the human papillomavirus 16/18 E6 protein degrades endogenous p53 in HeLa cells (Schefnner et al., 1990; Werness et al., 1990). Thus, RPG depletion activates p53 pathway genes even in the absence of the p53 protein, possibly by activating other p53 family members (such as p63 and p73).

To test the hypothesis that reduced RPG expression induces p53 pathways that inhibit miRNA-mediated repression, we used the A549 human lung cancer cell line which expresses wild-type p53. RPG knockdowns upregulated p53 at the protein level (Figures 7A and S7C), suggesting induction of the nucleolar stress response. Importantly, simultaneous knockdown of RPG and p53 reversed derepression of miRNA-targeted mRNAs (Figure 7A), directly implicating the p53 pathway in RPG regulation of miRNA activity.

To test the effects of nucleolar stress on miRNA activity independently of RPG knockdowns, we chemically induced nucleolar stress with low concentrations of Actinomycin D (ActD) or 5,6-dichloro-1-b-D-ribofuranosylbenzimidazole (DRB) (David-Pfeuty et al., 2001; Hözel et al., 2010). Similar to RPG knockdowns, ActD and DRB treatments led to dose-dependent p53 activation, reduced cell numbers, and specifically increased the expression of miRNA-targeted mRNAs without affecting miRNA levels (Figure 7B and S7D). Derepression of CXCR4 miRNA-targeted FL mRNA was observed at 8 hr after treatment (Figure S7E), suggesting that decreased miRNA activity is a direct result of nucleolar stress induction and not an indirect result of altered cell metabolism. Taken together, our data demonstrate that induction of nucleolar stress (triggered by RPG knockdowns or by small molecules) activates the p53 pathway, which leads to decreased miRNA activity (Figure 7C).

**DISCUSSION**

**RPGs Regulate miRNA-Mediated Repression of Translation Initiation**

An RNAi screen for effectors and regulators of miRNA function identified an unexpected role for RPGs in regulating miRNA-mediated repression of translation initiation. Previous reports have implicated RPGs in small RNA pathways in other organisms, suggesting RPG regulation of small RNA pathways may be conserved across phyla. In Drosophila, 11 RPGs scored in a screen of small RNA pathways, constituting 6% of all 177 hits (Zhou et al., 2008). However, RPG knockdown did not affect miRNA-mediated repression but instead increased endo-siRNA-mediated repression. Depending upon the RPG tested, siRNA-mediated repression increased in some cases but decreased in others. In C. elegans, a genome-wide RNAi screen for mRNA pathway genes identified one RPG, though this hit was not confirmed (Parry et al., 2007). In contrast to these model organisms, our data functionally implicates RPGs (as a class) as regulators of miRNA-mediated repression of translation initiation in human cells.

We showed that RPG depletion reduced the association of 67 miRNAs with monosomes without increasing miRNA association with polysomes or reducing total miRNA levels. Importantly, RPG depletion specifically increased polysomal association of mRNAs targeted by these miRNAs. Together, these high-throughput data independently confirm the biochemical data and support a model in which RPG depletion inhibits repression of translation initiation mediated by miRNAs. Moreover, we propose that parallel miRNA and mRNA expression profiling from monosomes and polysomes could be an accurate method of target mRNA identification.

miRNAs have been shown to activate translation under certain stress conditions. Steitz and colleagues reported that cells forced into quiescence can switch miRNA activity from translational repression to translational activation (Vasudevan et al., 2007). It is important to note that RPG knockdowns did not activate translation but rather derepressed miRNA-targeted mRNAs. In D8, it was impossible to distinguish derepression from activation because FL was constitutively repressed by endogenous miR-21, and thus the expression level of unpressed FL was unknown. Therefore, we knocked down RPGs in HeLa cells transiently transfected with luciferase reporters with imperfect binding sites to the artificial CXCR4 miRNA (Figures S2H and S2I). The expression of unpressed luciferase (in the absence of CXCR4 miRNA) was consistently higher than the expression of derepressed luciferase (in the presence of CXCR4 miRNA) after RPG knockdowns. These data demonstrate that RPG depletion derepressed but did not activate translation of miRNA-targeted mRNAs.
p53 Pathways Regulate miRNA Function

RPG and elf6 knockdowns led to substantial changes in abundance of ribosomal subunits. The p53 pathway and nucleolar stress have been implicated in sensing perturbed ribosomal subunit stoichiometry, leading to cell-cycle arrest (Bachand et al., 2006; Deisenroth and Zhang, 2010; Hölzel et al., 2010; Panić et al., 2007; Rudra and Warner, 2004; Volarevic et al., 2000). Interestingly, gene ontology analysis of transcriptional changes revealed that cell cycle, DNA replication, and p53 signaling were affected in all RPG knockdowns but not Ago knockdowns (Figures S7A and S7B and Table S4). Although the connection between ribosomal subunit imbalance, nucleolar stress, and the p53 pathway activation was already known, the connection between ribosomal subunit imbalance and global miRNA activity has not been established prior to this study. We propose that global reduction in miRNA-mediated repression may be an adaptive response allowing cells to increase translation of subsets of mRNAs in response to nucleolar stress. miRNA-mediated repression has been linked to oxidative- and endoplasmic reticulum-, and nutrient deprivation-stress responses (Bhattacharyya et al., 2006a, 2006b). In contrast to nucleolar stress, these stress responses do not induce the p53 pathway and are distinguished by their effects on general translation. For instance, amino acid starvation increases eIF2 phosphorylation levels (Bhattacharyya et al., 2006a, 2006b), which we did not observe in RPG knockdowns (Figure S6C). Amino acid starvation also does not affect miRNA activity unless an AU-rich element is present. In contrast, RPG knockdowns derepress both reporter and endogenous miRNA-targeted mRNAs even in the absence of AU-rich elements.

The Majority of miRNA Species Can Repress Translation

Previous studies have identified miRNA-targeted mRNAs cosedimenting with polysomes suggesting repression of translation postinitiation (Kim et al., 2004; Maroney et al., 2006; Nelson et al., 2004; Nottrott et al., 2006; Olsen and Ambros, 1999; Petersen et al., 2006; Seggerson et al., 2002). In agreement with more recent studies (Bhattacharyya et al., 2006a; Pillai et al., 2005), we detected mir-21-targeted FL mRNAs predominantly in monosomes, suggesting repression of translation initiation. There has been much speculation about the causes and implications of these different results (reviewed in Filipowicz et al., 2008). One possible explanation may be the efficiency of repression. Less efficient repression of translation initiation may result in a larger population of miRNA-targeted mRNAs in polysomes, whereas more efficient repression of translation initiation might result in a larger population of miRNA-targeted mRNAs in monosomes. In D8, where FL is repressed by miR-21 via six target sites, strong repression and monosome association was observed.

We report global miRNA expression profiles from HeLa monosomal and polysomal fractions. Detection of all miRNAs in both monosomal and polysomal fractions suggests that the mechanisms of miRNA-mediated translational repression are not uniform. Differential sedimentation of particular miRNAs implies that miRNA and/or mRNA identity may affect the mechanism of translational repression. miRNAs cosedimenting with polysomes did not appear to associate with actively translating ribosomes. Puromycin treatment reduced polysomes (Figure S3D) but did not affect sedimentation of these miRNAs (Figure S6A), suggesting that miRNAs may associate with stalled ribosomes or non-translating multimegadalton complexes (e.g., P bodies).

eIF6 Indirectly Affects the Human miRNA Pathway

Our screen identified elf6, a eukaryotic translation initiation factor that has been implicated in growth and transformation (Gandin et al., 2008). elf6 binds to 60S subunits and functions as an antiassoication factor by preventing 60S subunits from joining with 40S subunits (Ceci et al., 2003). elf6 has also been implicated in miRNA-mediated translational repression in worms and human cells (Chendrimada et al., 2007). Human elf6 has been shown to associate with Agos, miRNAs, and 60S subunits (Chendrimada et al., 2007). In contrast to these observations, we did not detect any interaction between elf6 and Agos, miRNAs, or miRNA-targeted mRNAs (data not shown). Indeed, we did not detect any association of elf6 with Agos even by the highly sensitive Multidimensional Protein Identification Technology analysis (data not shown). Our data suggest that elf6 may affect miRNA-targeted mRNA repression indirectly by altering ribosome subunit stoichiometry. elf6 is required for 18S and 5.8S rRNA maturation which, in turn, is required for generating the 60S ribosomal subunit (Basu et al., 2001). Indeed, depletion of elf6 reduced 60S ribosomal subunits (Figure 1D), the same phenotype as in 60S RPG knockdowns.

Reduced RPGs in Ribosomopathies and Cancers

Cancer pathway genes were significantly enriched in polysomes after RPG, Ago, and elf6 knockdowns (Table S4). Downregulation of RPGs has been identified in precancerous states, cancers in situ, and metastatic cancers (van Riggelen et al., 2010). Additionally, several genetic diseases that predispose patients to cancers (Avondo et al., 2009; Campagnoni et al., 2008; Gazda et al., 2008) are characterized by mutations in or reduced expression of RPGs, including dyskeratosis congenita (DKC1), cartilage-hair hypoplasia (RMRP), Shwachman-Diamond syndrome (SBDS), Turner syndrome (S4X), Noonan syndrome (L6), Camurati-Englemann disease (S18), and, most notably, Diamond-Blackfan anemia (S7, S15, S17, S19, S24, S27A, L5, L11, L35A, and L36). Interestingly, decreased expression of individual RPGs in zebrafish (Amsterdam et al., 2004; Lai et al., 2009; MacInnes et al., 2008) and flies (Stewart and Denell, 1993; Watson et al., 1992) promotes tumorigenesis, suggesting that RPGs may act as haploinsufficient tumor suppressors. This relationship is counterintuitive because rapid growth and proliferation of tumors must require robust translational activity. Our data offer a possible resolution in that reduced levels of RPGs may preferentially derepress protein production from a cohort of miRNA-targeted messages, many of which contribute to cellular proliferation and oncogenesis.

EXPERIMENTAL PROCEDURES

Detailed experimental procedures can be found in the Supplemental Experimental Procedures.
Polysome Profiling
HeLa cells were incubated with 100 μg/ml cycloheximide (CHX) for 5 min at 37°C and washed on ice twice with 5 ml cold PBS containing 100 μg/ml CHX. Cells were scraped in 500 μl lysis buffer (15 mM Tris [pH 7.4], 15 mM MgCl2, 150 mM NaCl, 1% Triton X-100, 100 μg/ml CHX, and 1 mg/ml heparin) and centrifuged at 12,000 g for 5 min at 4°C. Supernatant was loaded onto 12 ml 4.5%–45% sucrose gradients in 15 mM Tris (pH 7.4), 15 mM MgCl2, 150 mM NaCl, and 100 μg/ml CHX. Gradients were centrifuged in SW41Ti rotor at 39,000 rpm for 2.5 hr at 4°C and 1 ml fractions were collected. RNA was extracted with Trizol LS (Invitrogen) or by addition of 750 μl guanidinium hydrochloride and 800 μl isopropanol to 500 μl of a fraction and incubation at –20°C overnight. Samples were centrifuged at 10,000 rpm for 25 min at 4°C, pellets washed with 70% ethanol, and resuspended in 180 μl TE buffer. After addition of 20 μl 3 M sodium acetate (pH 5.2) and 600 μl 100% ethanol, RNA was precipitated at –80°C overnight. Samples were centrifuged at 10,000 rpm for 25 min at 4°C, pellets washed with 70% ethanol, and RNA was resuspended in water.

RNA-Seq Read Mapping and RPKM Calculation
Short reads were mapped to the human genome and a precomputed set of splice junctions using Bowtie (Langmead et al., 2009). Reads per kilobase of exon model per million mapped reads (RPKM) for each Entrez gene was computed by using all reads mapping to constitutive Refseq exons.

Global miRNA Expression Profiling
RNA from unfraccionated HeLa cells and from fractionated monosomes and polysomes was extracted with Trizol LS (Invitrogen). Complementary DNA (cDNA) synthesis, cDNA preamplification, and real-time PCR were performed with the miScript PCR System (QiAGEN). miRNA Ct measures were compared between monosomes and polysomes over all samples and separately for Scr and S5 knockdown groups using a two-sided Welch t test. p values were adjusted with the method of Benjamini and Hochberg and considered significant at the 0.05 level. Supervised hierarchical clustering was performed based on the Euclidean distance function with the complete linkage agglomeration method for the miRNAs found to be differentially expressed between groups. Only comparisons based on Ct were used, rather than ΔCt, because expression of the 5S control varies between monosomes and polysomes, and is altered in S75 knockdown.

SUPPLEMENTAL INFORMATION
Supplemental Information includes four tables, Supplemental Experimental Procedures, and seven figures and can be found with this article online at doi:10.1016/j.molcel.2012.04.008.

ACKNOWLEDGMENTS
We would like to thank Dr. Steffen Schubert and The Institute of Chemistry and Cell Biology (ICCB) Screening Facility at Harvard Medical School for helping with designing and performing the RNAseq. We also thank Dr. Stephen Buratowski, Dr. Danesh Moazed, Dr. Kai Wucherpfennig, and Dustin Griesemer for insightful discussions. This work was supported by a Distinguished Young Scholars Award from the W.M. Keck Foundation to C.D.N.

Received: March 17, 2011
Revised: February 16, 2012
Accepted: April 5, 2012
Published online: April 26, 2012

REFERENCES
Genes Dev. 20, 1885–1898.
Genes Dev. 21, 2558–2570.
ribosomal protein S19 in CD34+ cells generated by siRNA blocks erythroid development and mimics defects seen in Diamond-Blackfan anemia. Blood 105, 4627–4634.


