Deletion of ribosomal protein genes is a common vulnerability in human cancer, especially in concert with TP53 mutations

Citation

Published Version
doi:10.15252/emmm.201606660

Permanent link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:32630491

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA

Share Your Story
The Harvard community has made this article openly available. Please share how this access benefits you. Submit a story.

Accessibility
Deletion of ribosomal protein genes is a common vulnerability in human cancer, especially in concert with TP53 mutations

Ram Ajore1,†, David Raiser2,†, Marie McConkey2, Magnus Jöud1, Bernd Boidol2, Brenton Mar2, Gordon Saksena3, David M Weinstock4, Scott Armstrong5, Steven R Ellis6, Benjamin L Ebert2,3,*,† & Björn Nilsson1,3,‡,**

Abstract

Heterozygous inactivating mutations in ribosomal protein genes (RPGs) are associated with hematopoietic and developmental abnormalities, activation of p53, and altered risk of cancer in humans and model organisms. Here we performed a large-scale analysis of cancer genome data to examine the frequency and selective pressure of RPG lesions across human cancers. We found that hemizygous RPG deletions are common, occurring in about 43% of 10,744 cancer specimens and cell lines. Consistent with p53-dependent negative selection, such lesions are underrepresented in TP53-intact tumors (P < 10^{-10}), and shRNA-mediated knockdown of RPGs activated p53 in TP53-wild-type cells. In contrast, we did not see negative selection of RPG deletions in TP53-mutant tumors. RPGs are conserved with respect to homozygous deletions, and shRNA screening data from 174 cell lines demonstrate that further suppression of hemizygously deleted RPGs inhibits cell growth. Our results establish RPG haploinsufficiency as a strikingly common vulnerability of human cancers that associates with TP53 mutations and could be targetable therapeutically.

Keywords cancer; ribosomal gene haploinsufficiency; ribosome function

Subject Categories Cancer; Genetics, Gene Therapy & Genetic Disease

Introduction

The human ribosome consists of an rRNA scaffold and about 80 proteins, divided into two subunits. Ribosomal protein genes (RPGs) are denoted RPS1, RPS2, etc. for the small (40S) subunit; RPL1, RPL2, etc. for the large (60S) subunit.

Several recent lines of evidence hold that mutation of RPGs leads to specific clinical and cellular phenotypes. Germline heterozygous inactivating mutations or deletions in RPS19 and at least eight other RPGs cause Diamond-Blackfan anemia (DBA), a disorder characterized by macrocytic anemia and cancer predisposition, and the founding member of a class of disorders known as ribosomopathies (Drapchinskaia et al, 1999; Gazda et al, 2006, 2008, 2012; Farrar et al, 2008; Narla & Ebert, 2010; Vlachos et al, 2012; Raiser et al, 2014). Acquired somatic mutations in specific RPGs associate with certain malignancies, including the 5q- subtype of myelodysplastic syndrome (hemizygous deletions of RPS14; Ebert et al, 2008), T-cell acute lymphoblastic leukemia (mutations in RPL5, RPL10, and RPL22; Rao et al, 2012; De Keersmaecker et al, 2013), and microsatellite-unstable endometrial and gastric cancer (mutations in RPL22; Nagarajan et al, 2012; Novetsky et al, 2013). Eleven RPGs are tumor suppressor genes in zebrafish, where hemizygous inactivation of these genes causes malignant peripheral nerve sheath tumors with high penetrance (Amsterdam et al, 2004; MacInnes et al, 2008; Lai et al, 2009). In mouse models, RPG haploinsufficiency alters stem cell quiescence (Signer et al, 2014), homeobox gene translation, and tissue patterning (Kondrashov et al, 2011; Xue et al, 2015).

Despite these observations, the occurrence of RPG lesions in human cancers has not been investigated systematically. We therefore carried out a large-scale analysis of cancer genome data to...
determine the frequency and selective pressure of RPG lesions across human cancers. We first looked for chromosomal deletions and amplifications and point mutations in RPGs using pre-existing DNA copy number microarray and whole-exome sequencing data from a total of 10,744 cancer specimens and cell lines. Because recent studies have shown that RPG haploinsufficiency activates p53 in ribosomopathies (Narla & Ebert, 2010; Raiser et al., 2014) and the pathobiology of ribosomopathies can be alleviated by p53 inhibition (Volarevic et al., 2000; Sulic et al., 2005; Fumagalli et al., 2009; Barlow et al., 2010; Dutt et al., 2011; Jaako et al., 2011), we hypothesized that inactivation of RPGs could lead to negative selection unless the cells have mutated TP53, and, accordingly, looked for associations between inactivating RPG lesions and TP53 mutation. While we observed few point mutations and homozygous deletions in RPGs, we detected hemizygous RPG deletions in a large proportion (43%) of samples. Consistent with negative selection, further analyses revealed an underrepresentation of RPG deletions in TP53-intact tumors, whereas we did not see any signs of negative selection in TP53-mutant tumors. Furthermore, functional experiments showed that deficiency of frequently deleted RPGs increases p53 activity in TP53-intact cell lines and perturbs rRNA maturation both in cell lines cultured ex vivo and in primary acute leukemia cells with specific RPG deletions and expanded in vivo in xenograft models. Finally, consistent with the low frequency of homozygous deletion, analysis of genomewide shRNA screening data showed that further suppression of hemizygously deleted RPGs inhibits the growth of RPG-haploinsufficient cancer cells. We conclude that RPG haploinsufficiency as a common feature of human cancers that associates with TP53 mutations and could be targetable therapeutically.

Results

Haploinsufficiency for RPGs on the basis of hemizygous regional deletions is a common feature of cancer genomes

We first analyzed DNA copy number profiles of 7,225 cancer specimens belonging to 24 tumor types using data from The Cancer Genome Atlas, TCGA (Cancer Genome Atlas Research Network et al., 2013). Using log2 copy number ratio thresholds between −0.3 and −0.7 (median −0.5), we detected deletions affecting RPGs in 67–22% of specimens (median 43%), and in all tumor types analyzed (Fig 1A and Table 1). In 58–12% (median 32%) of tumors, we detected multiple RPG deletions (Fig 1C). Consistent with negative selection, we observed lower deletion frequencies for RPG than for other genes (Fig 1C and D). Validation in two independent sets of copy number profiles of 2,476 specimens belonging to 13 tumor types from Tumorscape (Beroukhim et al., 2010) and 1,043 cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE; Barretina et al., 2012) identified the same RPGs as frequently deleted (Figs 1B and EV1, and Table 1). In all three data sets, almost all (97–100%) of RPG deletions had copy numbers consistent with hemizygous loss (Fig 2A), and few RPGs showed recurrent homozygous deletions (Table EV1). Throughout, RPGs were hit by regional deletions covering multiple genes, and all frequently deleted RPGs were co-deleted with well-known tumor suppressor genes, including RPL26 on chromosome 17p, which is always co-deleted with TP53, and RPS6 on chromosome 9p, which is always co-deleted with CDKN2A. In addition to copy number changes, we examined the spectrum of point mutations in RPGs using whole-exome sequencing data from 4,655 TCGA samples. In contrast to the high frequency of hemizygous deletions, we found a low frequency of point mutations in RPGs, although we noted recurrent mutations in RPL5, RPL10, and RPL22 in several tumor types as reported previously (Nagarajan et al., 2012; Rao et al., 2012; De Keersmaecker et al., 2013; Novetsky et al., 2013; Table EV2). In summary, these data show that RPG haploinsufficiency on the basis of regional deletions occurs in a large proportion of human cancers.

Deletion of RPGs in cancer cells is restricted by p53-dependent negative selection

Recently, studies aimed at understanding the molecular mechanisms of ribosomopathies have identified p53 as a central mediator of the clinical features of these diseases (Narla & Ebert, 2010; Raiser et al., 2014). In both DBA and the 5q- syndrome, the hematopoietic phenotype is at least partly linked to p53 activation, and animal models have confirmed p53 as a sensor of ribosome dysfunction (Volarevic et al., 2000; Sulic et al., 2005; Fumagalli et al., 2009; Barlow et al., 2010; Dutt et al., 2011; Jaako et al., 2011). In normal cells, the expression of RPGs is tightly coordinated. In DBA and the 5q- syndrome, however, the RPG haploinsufficiency is thought to perturb the stoichiometry of ribosomal proteins, leading to inefficient ribosome assembly and increased concentrations of free ribosomal proteins, some of which (RPL5 and RPL11) regulate key components of the 5S ribonucleoprotein particle (Macias et al., 2010; Donati et al., 2013; Sloan et al., 2013; Goudarzi & Lindstrom, 2016). When ribosome biogenesis is blocked, the 5S SNP pre-ribosomal complex is re-directed from assembly into 60S ribosomes to MDM2 E3 ubiquitin ligase inhibition, thereby inhibiting the ability of MDM2 to target p53 for proteasomal degradation (Fumagalli et al., 2009; Zhang & Lu, 2009; Deisenroth & Zhang, 2010; Miliani de Marval & Zhang, 2011; Zhou et al., 2013; Goudarzi & Lindstrom, 2016). Moreover, in vitro and in vivo studies support that the phenotypic effects of RPG haploinsufficiency can be alleviated by genetic or pharmacological inhibition of p53 (Volarevic et al., 2000; Sulic et al., 2005; Fumagalli et al., 2009; Barlow et al., 2010; Dutt et al., 2011; Jaako et al., 2011).

Because of these reports, we hypothesized that acquisition of RPG deletions in cancer cells could lead to p53 activation and thereby negative selection, unless the p53 pathway has been inactivated. To test this hypothesis, we compared the copy number distributions for RPGs versus other genes across 4,675 TCGA samples having both copy number microarray and whole-exome sequence data, enabling the detection of RPG deletions as well as deletions and point mutations in TP53. We found an underrepresentation of hemizygous RPG deletions in TP53-wild-type tumors (Wilcoxon rank-sum \( P < 10^{-10} \)), whereas RPGs and other genes showed identical copy number distributions in TP53-mutant tumors (Fig 2A). Similarly, among TP53-wild-type tumors, but not among TP53-mutant tumors, we found fewer cases with hemizygous deletions affecting RPGs than with hemizygous deletions affecting random gene sets of the same size (permutation testing \( P = 0.04 \) with 1,000 random sets of genes located on other autosomes than chromosome 17), whereas no difference was detected in TP53-mutant tumors. Additionally, TP53-mutant tumors showed higher numbers of
RPG deletions in total (Fig 2B), RPG deletions that could be detected in TP53-wild-type tumors were enriched in tumors harboring alternative p53 pathway-inactivating lesions, including MDM2 amplifications and CDKN2A deletions (Fig EV2). Taken together, these results indicate that the p53 pathway restricts the acquisition of RPG deletions in cancer cells.
Negative selection of RPG deletion observed in shRNA knockdown data, provides a possible explanation for the cancerous tissues (Fig EV6D). These data indicate that RPG-CNAs RPG co-expression patterns comparable to those observed in non-tumors (Fig EV6B and C), whereas tumors with few RPG-CNAs showed (Fig EV6A). Quantifying RPG co-expression by calculating correlation between copy number and expression for most individual RPGs and global mRNA-sequencing data. We found a correlation TCGA samples having both DNA copy number microarray data (RPG-CNAs) and altered RPG expression patterns using 4,919 we tested for associations between RPG copy number anomalies et al deleted in cancer cells. Also from decreased expression of the RPGs that are frequently deleted in cancer cells. Since RPG haploinsufficiency is thought to activate p53 by deletion of RPGs leads to loss of coordinated RPG expression and p53 activation

Previous studies aimed at understanding the molecular mechanisms of DBA and the 5q-syndrome have shown that decreased expression of specific RPGs (RPS6, RPS14, and RPS19) leads to p53 activation (Narla & Ebert, 2010; Raiser et al., 2014). However, the RPGs we found to be deleted in human cancers have not been studied functionally in this regard. To examine whether p53 senses decreased expression of the frequently deleted RPGs, we used shRNA-mediated knockdown in the TP53-wild-type lung adenocarcinoma cell line A549 (Fumagalli et al., 2009; Dutt et al., 2011). For all eight RPGs tested, multiple shRNAs caused elevated p53 protein levels and increased P21 transcript levels (Figs 2C and D, and EV3, and Table EV3). Similarly, knockdown of three of the most frequently deleted RPGs in TP53-wild-type MOLM13 leukemia cells caused elevated expression of p53 and four p53 target genes P21, BAX, PUMA, and NOXA (Figs EV4 and EV5). Taken together, these data indicate that p53 activation results not only from decreased expression of the RPGs that are mutated in ribosomopathies, but also from decreased expression of the RPGs that are frequently deleted in cancer cells.

Since RPG haploinsufficiency is thought to activate p53 by altering ribosomal protein stoichiometry (Fumagalli et al., 2009), we tested for associations between RPG copy number anomalies (RPG-CNAs) and altered RPG expression patterns using 4,919 TCGA samples having both DNA copy number microarray data and global mRNA-sequencing data. We found a correlation between copy number and expression for most individual RPGs (Fig EV6A). Quantifying RPG co-expression by calculating correlation coefficients for all pairs of RPGs, we detected less correlated RPG expression in tumors with a high RPG-CNA burden (Fig EV6B and C), whereas tumors with few RPG-CNAs showed RPG co-expression patterns comparable to those observed in non-cancerous tissues (Fig EV6D). These data indicate that RPG-CNAs lead to uncoordinated RPG expression, which, together with the shRNA knockdown data, provides a possible explanation for the negative selection of RPG deletion observed in TP53-wild-type tumors (Fig 2).

Deletion of RPGs leads to loss of coordinated RPG expression and p53 activation

Deletion of RPGs in cancer cells with intact p53 function

We asked which RPG deletions are permissible in cancer cells with intact p53 function. To this end, we specifically analyzed the 30 cell lines from CCLE that are both TP53-wild-type and sensitive to the p53-activating compound Nutlin-3 (IC50 < 8 μmol; Barretina et al., 2012; Sonkin et al., 2013), where the latter supports that p53 has not been inactivated by alternative mutations in other genes. Interestingly, the most recurrently deleted RPG in these cell lines was RPL22 (Table EV4). Firstly, Rpl22−/− knockout mice have only subtle phenotypes with no significant translation defects, probably because these mice show increased expression of the paralog Rpl22-like1 (Rpl22 l1) which is incorporated in the ribosome instead of Rps22 (O’Leary et al., 2013). Secondly, RPL22 has been identified as a potential tumor suppressor gene that is mutated or deleted in T-ALL and several epithelial tumor types (Rao et al., 2012; Novetsky et al., 2013; Goudarzi & Lindstrom, 2016). Another interesting observation was recurrent deletion of RPS6, which (like in the primary tumors) was always associated with co-deletion of CDKN2A. These data indicate that some RPG deletions are less likely to cause negative selection, either because of gene redundancy, because they do not activate p53, or because they are associated with a pro-proliferative effect that allows the cells to escape the negative effect of p53 activation.

Further suppression of deleted RPGs inhibits the proliferation of cancer cells

The almost complete lack of homozygous RPG deletions in the three copy number data sets supports that such lesions are not tolerated. These observations are consistent with the notion that ribosomal proteins are essential for generation of functional ribosomes and cell survival. To investigate whether RPG haploinsufficiency renders cancer cells vulnerable to further suppression of ribosome function, we analyzed two sets of shRNA screening data from 102 and 72 genomically annotated cancer cell lines (Cheung et al., 2011; Marcotte et al., 2012). These pooled screens targeted 11,194 and 16,056 genes, including 26 and 55 RPGs, respectively. For each targeted gene, the effect of knockdown on proliferation in cell lines

Table 1. Ten most frequently deleted RPGs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr</th>
<th>Start (bp)</th>
<th>End (bp)</th>
<th>TCGA</th>
<th>CCLE</th>
<th>Tumorscape</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>RPL26</td>
<td>17</td>
<td>8,280,833</td>
<td>8,286,565</td>
<td>1,752</td>
<td>24.2</td>
<td>340</td>
</tr>
<tr>
<td>RPL13</td>
<td>16</td>
<td>89,627,064</td>
<td>89,633,237</td>
<td>1,430</td>
<td>19.8</td>
<td>204</td>
</tr>
<tr>
<td>RPS6</td>
<td>9</td>
<td>19,376,253</td>
<td>19,380,235</td>
<td>1,411</td>
<td>19.5</td>
<td>373</td>
</tr>
<tr>
<td>RPL17</td>
<td>18</td>
<td>47,014,850</td>
<td>47,018,935</td>
<td>1,335</td>
<td>18.5</td>
<td>393</td>
</tr>
<tr>
<td>RPL29</td>
<td>3</td>
<td>52,027,643</td>
<td>52,029,958</td>
<td>1,283</td>
<td>17.8</td>
<td>340</td>
</tr>
<tr>
<td>RPL3</td>
<td>22</td>
<td>39,708,886</td>
<td>39,715,670</td>
<td>1,205</td>
<td>16.7</td>
<td>263</td>
</tr>
<tr>
<td>RPL14</td>
<td>3</td>
<td>40,498,782</td>
<td>40,503,863</td>
<td>1,133</td>
<td>15.7</td>
<td>292</td>
</tr>
<tr>
<td>RPS4</td>
<td>3</td>
<td>39,448,203</td>
<td>39,454,032</td>
<td>1,129</td>
<td>15.6</td>
<td>284</td>
</tr>
<tr>
<td>RPL21</td>
<td>13</td>
<td>27,825,691</td>
<td>27,830,702</td>
<td>1,121</td>
<td>15.5</td>
<td>328</td>
</tr>
<tr>
<td>RPS15</td>
<td>19</td>
<td>1,438,362</td>
<td>1,440,492</td>
<td>1,108</td>
<td>15.3</td>
<td>264</td>
</tr>
</tbody>
</table>

© 2017 The Authors
carrying one gene copy, relative to the effect in cell lines carrying at least two gene copies, was scored (Marcotte et al., 2012; Shao et al., 2013). In both data sets, we observed strongly significant enrichments of negative gene scores among RPGs ($P < 1.0 \times 10^{-5}$ and $P = 1.7 \times 10^{-4}$, respectively; Fig 3A and B), indicating that further suppression of RPGs preferentially inhibits the growth of RPG-haploinsufficient cells.

Deletion of RPGs influences rRNA maturation in cancer cells

Previously, in vitro studies have shown that knockdown of RPS6 and other 40S RPGs impairs the processing of pre-ribosomal RNA species into mature rRNA in ribosomopathies (Narla & Ebert, 2010; O’Donohue et al., 2010; Raiser et al., 2014). To explore whether RPG haploinsufficiency leads to rRNA maturation defects also in cancer cells, we decided to analyze rRNA maturation across pediatric acute lymphoblastic leukemia (ALL) samples harboring CDKN2A (9p) deletions with and without concurrent deletion of RPS6. Deletions targeting CDKN2A are present in 20–30% of B-cell ALL and 95% of T-cell ALL. We first genotyped 47 previously banked pediatric ALL samples using copy number microarrays. For two samples (one with homozygous deletion of CDKN2A but intact RPS6 and one with homozygous deletion of CDKN2A and hemizygous deletion of RPS6), we were able to obtain viable cells, which were xenografted into NOD scid gamma immunodeficient mice and expanded in vivo. RNA from sorted tumor cells was assessed by Northern blot to analyze pre-ribosomal RNA processing. Interestingly, the RPS6-haploinsufficient case exhibited rRNA maturation defects similar to

Figure 2. RPG haploinsufficiency associates with TP53 mutation status and p53 activation.

A RPG deletions are underrepresented in TP53-intact tumors, but not in TP53-mutant tumors. Plots show cumulative copy number distributions for RPGs (blue) versus all genes (dashed black) for 4,675 TCGA samples having both copy number microarray and whole-exome sequence data. However, cases without TP53 mutation/deletion show fewer copy numbers consistent with heterozygous loss of RPGs compared to other genes (brackets), whereas cases harboring TP53 mutations/deletions show no corresponding difference. These plots also show that, regardless of TP53 mutation status, homozygous loss of RPGs is extremely rare.

B Number of hemizygous RPG deletions in TP53-mutant and -wild-type tumors. Boxes indicate medians and the first and third quartiles. Whiskers indicate first and third quartiles ±1.5 times the interquartile range. Notches indicate confidence intervals around the median. P-values indicate significance by Wilcoxon rank-sum test.

C, D To obtain further support for p53-negative selection, we used shRNA-mediated knockdown of RPGs in A549 cells, which are TP53-wild type. Panels show p53 protein levels as analyzed by Western blot (β-actin loading control), and P21 transcript levels as analyzed by qPCR (ACTB normalization control) after 4–6 days of expression of two shRNAs per tested RPG. Error bars indicate standard deviation between triplicates. Throughout, we observed increased p53 levels and P21 expression. These data support that RPG deletions lead to p53 activation and negative selection in cancer cells.
those seen previously in shRNA-targeted RPG-deficient cells (cf. Narla & Ebert, 2010; Raiser et al., 2014 and references therein), namely a reduction in 41S species (18S-E, 21S, and 41S) and an accumulation of 30S species (30S and 45S), indicating that heterozygous deletion of \textit{RPS6} impacts on ribosomal RNA biogenesis in ALL cells (Fig 3C and D). The difference in rRNA pattern observed in ALL cells with and without deletion of \textit{RPS6} was comparable to the difference in rRNA pattern observed in \textit{TP53}-wild-type MOLM13 leukemia cells with and without shRNA knockdown of \textit{RPS6} (Fig EV7). Collectively, these observations provide the first evidence indicating that the rRNA maturation defect that is observed in ribosomopathies may also be present in RPG-haploinsufficient cancer cells.

**Discussion**

Here we report the novel finding that ribosomal protein genes are routinely deleted across human cancers, particularly in concert with \textit{TP53} mutation. Such a finding could lead to new possibilities for cancer therapy in \textit{TP53}-mutant patients. Previously, mutation of RPGs (without concurrent \textit{TP53} mutation) has primarily been associated with rare ribosomopathies, specific tumor subtypes, and cancer development in zebrafish models.

The analyses in this study are based on a large number of samples from primary samples, belonging to a broad range of different cancer types. The results indicate that RPG deletions are enriched among the samples that have concurrent \textit{TP53} mutation. This finding is in accordance with previous studies on ribosomopathies (particularly...
DBA and the 5q-syndrome) showing that RPG haploinsufficiency leads to activation of the p53 pathway, most likely through the 5S RNP-MDM2 pathway. One of the current main hypotheses is that the ribosomal assembly intermediate 5S ribonucleoprotein particle, containing RPL5, RPL11, and the 5S rRNA, accumulates when ribosome biogenesis is blocked; the excess 5S RNP binds to murine double minute 2 (MDM2), the main p53-suppressor in the cell, inhibiting its function and leading to p53 activation. Our data indicate that targeted degradation of the mRNAs for RPGs that are frequently deleted in cancer leads to p53 activation in A549 and MOLM13 cells, implicating p53 activation and (secondary to that) negative selection as a probable cause of the lower frequencies of RPG deletions seen among T7/P53-wild-type tumors. Moving forward, follow-up studies will be needed to examine whether this activation is mediated through the 5S RNP-MDM2 pathway. Additionally, analyses of clonal heterogeneity (e.g., using single-cell experiments or mathematical modeling of allele ratios) could illuminate whether p53 mutation precedes RPG deletion during oncogenesis.

Further, a central question about the biochemical consequences of RPG haploinsufficiency will be to determine precisely how these alterations affect rRNA maturation in cancer cells. Our data, though limited in sample size, indicate that rRNA patterns in acute lymphoblastic leukemia cells harboring RPS6 deletion are perturbed in the same way as in cell lines with shRPS6 knockdown. These observations motivate further studies with larger numbers of samples to determine how variation in rRNA patterns associates with genetics lesions in RPGs.

Finally, genes that are hemizygously deleted are not necessarily drivers but can confer susceptibility for therapies (Muller et al., 2012; Nijhawan et al., 2012; Liu et al., 2015). Our study represents the first detailed examination of vulnerabilities in a specific cellular component. Our data show that RPG haploinsufficiency is a strikingly common vulnerability that is enriched among p53-deficient tumors, which are often hard to treat. Our results raise the question whether it could be possible to exploit RPG haploinsufficiency to selectively kill cancer cells. A number of drugs that modulate ribosomal function exist, including rapamycin and other inhibitors (“rapa-logs”) of the mammalian target of rapamycin (mTOR; Easton & Houghton, 2006), and compounds that inhibit ribosome biogenesis by inhibiting rRNA synthesis (Drygin et al., 2011; Bywater et al., 2012; Peltonen et al., 2014; Colis et al., 2014). These drugs are active against subsets of human tumors, but their therapeutic scope is unknown and could depend on the presence of ribosomal defects. Future studies will inform how RPG deletions can be used for the treatment of a large subset of human cancer.

Materials and Methods

Cancer genome data sets

Firstly, we obtained normalized, segmented Affymetrix and Agilent DNA copy number data (downloadable.seg files) representing 7,225 primary tumor samples belonging to 24 tumor types from TCGA and with matched whole-exome sequencing data (somatic mutations; 4,675 samples) and RNA-sequencing data (4,919 samples; Cancer Genome Atlas Research Network et al., 2013; http://cancergenome.nih.gov/). Gene-wise copy numbers by averaging the copy number signal across genes (from annotated gene start to gene end) in each sample. Secondly, we obtained corresponding data Affymetrix DNA copy number data representing 2,476 primary tumor samples belonging to 13 tumor types from Tumorscape (Beroukhim et al., 2010; http://www.broadinstitute.org/tumorscape), and for 1,043 cancer cell lines from the CCLE (Barretina et al., 2012; http://www.broadinstitute.org/ccle). To determine whether a gene was deleted, we applied log2 copy number thresholds between −0.3 and −0.7, corresponding to linear scale copy numbers 1.6 and 1.2, respectively. A log2 ratio of −0.7 corresponds to the theoretical copy number amplitude for a hemizygous deletion that present in all tumor cells in a sample with 80% tumor cell fraction (a TCGA inclusion criterion). This threshold is conservative as it does not leave any room for clonal heterogeneity, technical underestimation, or noise. The log2 ratio −0.3 corresponds to the midpoint between the theoretical copy number and the euploid, and is less conservative. To call homozygous deletions, we used a log2 ratio threshold of −2.0, corresponding to copy number 0.5 in linear scale. For completeness, we repeated our analyses with other reasonable thresholds, yielding results in broad agreement with those shown. The file processing and statistical analyses were done with Matlab (http://www.mathworks.com), R (http://www.r-project.org), Ultrasome (Nilsson et al., 2009), RenderCat (Nilsson et al., 2007), and various scripts. The set of RPGs was defined by the 78 annotated genes encoding the known proteins of the small and large ribosomal subunits (Fig 1).

Lentiviral shRNA vectors and infection

Lentiviral shRNAs targeting frequently RPGs in the pLKO.1 or pLKO_TRC005 vector were obtained from the The RNAi Consortium (TRC) at the Broad Institute (Table EV3). Lentivirus was produced in 293TL cells. A549 cells were infected with 1 day after plating in the presence of 8 μg/ml polybrene (Sigma-Aldrich) and selected 24 h later with 2 μg/ml puromycin (Sigma-Aldrich) for at least 48 h before collection and processing for protein lysates or mRNA. A549 cells were maintained at 37°C and 5% CO₂ in F-12K medium (ATCC) supplemented with 10% fetal bovine serum and 1% pen/strep/glutamine (Gibco). MOLM13 cells were transduced by spinfection with 8 μg/ml polybrene (Sigma-Aldrich). The cells were selected at 24 h after transduction with 0.3 μg/ml puromycin for 48 h. Puromycin-selected cells were harvested and divided as per experimental need for processing RNA or protein lysate. MOLM13 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% PEST (Gibco).

Western blot

Cells for protein analysis were lysed with Pierce® IP Lysis Buffer (Thermo Scientific). Western blots were performed using 25–50 μg of protein and primary antibodies against p53 (DO-1; Santa Cruz Biotechnology) at 1:500 dilution and β-actin (C4; Santa Cruz Biotechnology) at 1:3,000 dilution. HRP-conjugated anti-mouse secondary antibody (GE Healthcare) was used at 1:10,000 dilution. Immunoreactive proteins were visualized using SuperSignal® West Pico Chemiluminescence Substrate (Thermo Scientific). MOLM13 cells were lysed with Bioruptor Pico (Diagenode) using 30/30 s on/off for 10-min program in Laemmli sample buffer (Bio-Rad). Blots were probed with primary antibody against p53 (Sc-126, DO-1,
Quantitative real-time PCR

RNA was purified from A549 and MOLM13 cells using TRIzol (Invitrogen) and RNeasy mini kit (cat no 74104), respectively. First-strand cDNA was generated using 100–200 ng of total RNA and oligo(dT) primers with the Superscript III reverse transcription kit (Invitrogen), and Omniscript RT kit (cat no 205113) was used for cDNA synthesis from MOLM13 cells. Quantitative RT–PCR was performed using TaqMan® Gene Expression Master Mix (Applied Biosystems) or SYBR® Green PCR Master Mix (Applied Biosystems) on an ABI Prism 7900HT system and StepOnePlus Real-Time PCR system (Applied Biosystems). We used TaqMan® assays to assess the knockdown effect for RPS6 (Hs01058685_g1), RPL26 (Hs00864008_m1), RPL13 (Hs00744303_s1), RPL21 (Hs00303806_g1), RPL29 (Hs00426490_g1), RPL14 (Hs00304339_g1), RPSA (Hs00347791_s1), and RPS12 (Hs04184906_g1) using ACTB (ABI #401846) as control, and P21 (Hs00355782_m1), NOXA (Hs00560402_m1), PUMA (Hs00248075_m1), BAX (Hs00180269_m1) were assessed using GAPDH (Hs02758991_g1) as endogenous control for MOLM13 cells. Quantitative PCR for P21 and ACTB was performed using SYBR® Green PCR Master Mix (Applied Biosystems) and the following primers: P21 forward 5′-GCTCTGTCGAGGGGACACGC-3′; P21 reverse 5′-GCCGCCGTTTTCGACCCTGA-3′; ACTB forward 5′-GCCGACGAAGGCTCATCATT-3′; and ACTB forward 5′-GCCGACGAAGGCTCATCATT-3′ for A549 cells.

Analysis of leukemia samples

We obtained DNA extracted from 47 blood and bone marrow samples taken at diagnosis from patients with pediatric ALL. The samples were collected and banked at the Dane Farber Cancer Institute subject to informed consent (Dana Farber Cancer Institute, institutional review board protocol no. 05-001). The experiments conformed to the principles set out in the WMA Declaration of Helsinki [http://www.wma.net/en/30publications/10policies/b3/] and the NIH Belmont Report [https://www.hhs.gov/ohrp/regulations-and-policy/belmont-report/]. The samples were analyzed on Affymetrix 6.0 arrays (Affymetrix Inc.; data available for download from the ArrayExpress repository accession no. E-MTAB-5450). Copy number changes were delineated using the program Ultrasome (Nilsson et al., 2009). We obtained banked tumor cells and expanded them in vivo in NOD scid gammaimmunodeficient mice for about 6 months. When the mice appeared moribund, they were sacrificed. Cells harvested from the spleens and bone marrow were stained with anti-human CD45 (Miltenyi Biotec) and sorted by fluorescence-activated cell sorting to purify tumor cells, from which RNA was isolated for rRNA maturation analysis by Northern blot (Qiagen kits). For Northern blot analysis, gel-fractionated RNA was transferred to zeta-probe membranes (Bio-Rad). An oligonucleotide probe 5′-CCTGCGTCTCGGGCAGTTTAATGCAT-3′ (complementary to sequences 5520–5547 spanning the boundary between 18S rRNA and ITS1) was labeled with 32P using T4 polynucleotide kinase and hybridized overnight with membrane-bound RNA at 37°C in ULTRAHyb-Oligonucleotide hybridization buffer (Ambion). Membranes were washed at 37°C with 6× SSC and subjected to phosphorimager analysis. MOLM13 RPS6 knockdown RNA samples were electrophoretically fractioned in MOPS-gel running buffer (cat no AM8671, Ambion) and transferred to Biodyne nylon membrane (cat no 77016, Thermo Scientific) using semi-dry blot method. Hybridization and washing steps were followed as recommended in Northern Max kit (AM1940, Thermo Scientific). The biotinylated cDNA probe hybridization was visualized following Chemiluminescent Nucleic Acid Detection Module (cat no 89880, Thermo Scientific).
Analysis of shRNA screening data

To test whether further suppression of hemizygously deleted RPGs inhibits cell growth, we used genomewide, pooled shRNA screening data from the Achilles and shARP studies (Cheung et al., 2011; Marcotte et al., 2012). The Achilles study provided data on 102 cell lines infected with a pool of lentivirally delivered shRNAs, composed of 54,020 shRNAs targeting 11,194 genes. The shARP study provided data on 72 cancer cell lines infected with a pool of 78,432 shRNAs targeting 16,056 genes. Gene scores reflecting the effect on knockdown on cell growth in cell lines carrying one copy versus at least two copies were provided with the original publications (Cheung et al., 2011; Marcotte et al., 2012; Shao et al., 2013). To test for enrichment of negative scores (indicating depletion) among RPGs compared to other genes, we used RenderCat (Nilsson et al., 2007).

References


Expanded View for this article is available online.

Acknowledgements

The project was supported by research grants from the Swedish Foundation for Strategic Research (ICA08-0057), the Marianne and Marcus Wallenberg Foundation (2010.0112), the Swedish Children’s Cancer Fund (PR2012-0084), and a Wallenberg Academy Fellows Award to B.N. (2012.0193). The project was also supported by the National Institute of Health (RO1 HL082945 and PO1 CA108631) and a Leukemia and Lymphoma Society Scholar Award to B.L.E.

Author contributions

BN and BLE designed and supervised the project, performed computational analyses, and wrote the manuscript with input from DMW, SA, and SRE. Experiments were done by DR, RA, MM, BB and BM. Additional computational analyses were done by MJ and GS. All authors contributed to the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

For more information

The Cancer Genome Atlas (http://cancergenome.nih.gov/)
Tumorscape (http://www.broadinstitute.org/tumorscape)
Cancer Cell Line Encyclopaedia (http://www.broadinstitute.org/ccle)
Utrasome (http://www.broadinstitute.org/ultrasome)
Matlab (http://www.mathworks.com)
R project (http://www.r-project.org)


License: This is an open access article under the terms of the Creative Commons Attribution 4.0 License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.