Hepatocellular carcinomas (HCCs) are characterized by frequent somatic genomic copy number alterations (CNAs), with most of them biologically unexplored. Here, we performed integrative analyses combining CNAs with the transcriptomic data to reveal the cis- and trans-effects of CNAs in HCC. We identified recurrent genomic gains of chromosome 8q, which exhibit strong trans-effects and are broadly associated with ribosome biogenesis activity. Furthermore, 8q gain–driven overexpression of ribosome biogenesis regulator (RRS1) promotes growth of HCC cells in vitro and in vivo. Mechanistically, RRS1 attenuates ribosomal stress through retaining RPL11 in the nucleolus, which, in turn, potentiates MDM2-mediated ubiquitination and degradation of p53. Clinically, higher RRS1 expression levels predict poor clinical outcomes for patients with HCC, especially in those with intact p53. Our findings established that the chromosome 8q oncogene RRS1 promotes HCC development through attenuating the RPL11-MDM2-p53 pathway and provided new potential targets for treatment of this malignancy.

INTRODUCTION
Hepatocellular carcinoma (HCC) is the sixth most common and the second most deadly cancer worldwide. The most important risk factors for HCC remain to be hepatitis B virus and hepatitis C virus infection, aflatoxin B exposure, alcohol consumption, inborn metabolic diseases, and diabetes (1). Although the development of modern medicine and the combined use of various therapeutic strategies have improved the outcomes of patients with HCC, the long-term prognosis of this malignancy remains limited (2).

Sequential accumulation of genetic alterations has been considered as the contributor to HCC initiation and progression (3). Despite progress in characterizing the genomic landscape of molecular aberrations associated with HCC, the targetable biological dependencies remain elusive and poorly characterized. With the exception of the most common mutations (e.g., in the TERT promoter, CTNNB1, and p53), which are not clinically actionable, HCCs display a high degree of intertumor and intratumor heterogeneity at the mutational level (4). The recurrent copy number alterations (CNAs) affecting oncogenes or tumor suppressor genes in cancer genomes potentially represent essential tumor addictions under selection. It has been reported that several cancer driver genes were recurrently affected by CNAs (5), such as CDKN2A deletion and FGF19 amplification in HCCs (6, 7). However, most HCCs display arm-level CNAs, targeting chromosomes 1q, 8p, 8q, and 17p that affect doses of genes, the implications of which are currently unclear.

Chromosome 8q gain is a common feature in many types of tumor. Besides its high occurrence in patients with HCC (4, 8), chromosome 8q gain has also been observed frequently in several other types of cancers, including ovarian (9), primary germ cell tumors (10), melanomas (11), endometrial carcinoma (12), clear cell renal cell carcinoma (13), gastric adenocarcinoma (14), and lung adenocarcinoma (15). Furthermore, gain of 8q is associated with poor survival in clear cell renal cell carcinoma (16) and with tumor recurrence in prostate cancer (17). It has long been known that chromosome 8q harbors important oncogene(s) involved in multiple tumor types, including MTDH for breast cancer (18) and the well-known oncogene MYC (19). However, this chromosomal alteration affects a wide range of genomic regions, which limited the understanding of oncogenic mechanisms involved. The absence of focal amplifications has supported the view that multiple drivers on chromosome 8q may contribute to poor clinical outcomes of patients with cancer. The identification of critical cancer-relevant genes at 8q may provide potential therapeutic targets and provide a rationale for precision therapy for these patients who do not benefit from current treatments.

Here, we performed an integrative omic analysis, taking into account both the genome-wide copy number landscape and transcriptome data in HCCs. We found evidence of widespread trans-effects of arm-level CNAs on general gene expression in HCCs, with chromosome 8q gain as a major contributor. Given the strong association of 8q gain with ribosome biogenesis activity, we identified the ribosome biogenesis regulatory protein, RRS1, as a pivotal oncogene involved in HCC progression. We further dissected the mechanisms underlying RRS1’s oncogenic role in the regulation of the RPL11-MDM2-p53 pathway. These results highlight RRS1 as a functional target of 8q gain in a wide range of cancers, which might be therapeutically targetable.
RESULTS

Genomic gain of chromosome 8q is correlated with an increased ribosome biogenesis activity in HCC

HCC genomes have been shown to be frequently affected by CNAs in certain regions, including gains in chromosomes 1q, 5p, 6q, 7q, 8q, 17q, and 20q and losses in 1p, 4q, 6q, 8p, 9p, 13q, 14q, 16q-p, 17p, 21p-q, and 22q (20–22). Consistent with these findings, we also found relative high-frequency occurrences (>40%) of copy number gains in 1q and 8q and losses in 8p and 17p in HCCs from The Cancer Genome Atlas (TCGA)–liver hepatocellular carcinoma (LIHC) cohort (Materials and Methods; fig. S1, A and B). Genomic CNAs can affect mRNA abundance at the same locus (i.e., cis-effects) or the other loci (i.e., trans-effects). To further explore the contribution of CNAs to transcriptional program, we performed integrative analysis combining the CNAs and the transcriptomic data in HCCs from TCGA. We observed that most of the CNAs exhibit strong cis-effects (Fig. 1A). In addition, strong trans-regulatory effects (i.e., a broader impact on global gene expression) of CNAs were also observed across the genomes, which were mainly centered on chromosomes 1q, 4q, 8q, 17p, and 17q (Fig. 1A and table S1).

Given the universal cis- and trans-effects of arm-level CNAs in HCC, we assessed their contribution to genome alteration. By examining the arm-level CNAs across 32 types of cancer from the TCGA, we observed that the genomes of HCC are moderately affected by arm-level CNAs and have similar pattern with that in breast cancer (fig. S1B). After adjusting for chromosome size per cancer type, we found that the alterations of 1q, 8p, 8q, and 17p have larger weighted proportion of genome alteration (WPGA) scores in HCCs (Materials and Methods; fig. S1C). Notably, the WPGA score of 8q in HCC, with the strongest trans-effects (Fig. 1A), was ranked second across the 32 TCGA cancer types (fig. S1, D and E), suggesting the pivotal contribution of 8q gain to HCC genome alteration.

Next, we focused on identifying the cellular processes correlated with the cis- and trans-effects driven by CNAs in HCC. Through examining the correlation between the genomic alterations and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway signature scores inferred by single-sample gene set variation analysis (GSVA) (23), we identified multiple pathways that were disparate or commonly dysregulated among distinct genomic alterations (Materials and Method; Fig. 1B). For example, loss of chromosome 1q was associated with up-regulation of cell cycle regulation, DNA damage response, and glycosphingolipid biosynthesis and with down-regulation of immune response. Loss of 4q resulted in up-regulation of metabolic pathways and immune response and down-regulation of cell cycle and DNA damage response, the associations of which were similarly enriched by 17p loss (which contains the tumor suppressor p53). 4q was preferentially lost in HCCs containing a p53 mutation and contains genes that can regulate DNA replication in response to DNA damage (24). Loss of 8p resulted in increased immune response and extracellular matrix–modulating pathways, such as transforming growth factor–β and vascular endothelial growth factor signaling. Notably, chromosome 8q gain was strongly associated with up-regulation of ribosome biogenesis. Ribosome biogenesis is the most demanding energetic and metabolic expenditure for cells, with about half of the total cellular transcription in proliferating cells devoted to this process (25). Recently, however, emerging evidence has revealed that hyperactivation of ribosome biogenesis confers competitive advantages to tumorigenesis (26). Collectively, our results indicated that the recurrent chromosome 8q gain may act as a pivotal promoter for ribosome biogenesis activity in the development of HCC.

RRS1 acts as a potential functional target at chromosome 8q gain

To identify the potential contributors within chromosome 8q locus, we ranked all 8q genes based on the correlation between their mRNA levels and ribosome activity and highlighted the ones with CNA cis-effects (Fig. 1C). As expected, the genes mapping to 8q exhibited stronger positive correlation with ribosome pathway activity than did the genes located outside 8q [false discovery rate (FDR) q < 0.05; Materials and Methods]. That positive correlation was even stronger for 8q genes with CNA-mRNA correlation than for those 8q genes without cis-effects (FDR q < 0.05). Collectively, these findings highlighted a role for CNA-driven overexpression of 8q genes in promoting ribosome biogenesis activity. Furthermore, the top-ranked genes were shown to contain four ribosome protein (RP)–coding genes RPL30, RPS20, RPL7, and RPL8 and a known ribosome biogenesis regulator RRS1. It was well known that disruption of ribosome biogenesis can cause ribosomal stress that activates tumor suppressor p53 signaling (27). As expected, the p53 pathway activity was significantly negatively correlated with the ribosome activity (r = −0.85, P < 2.2 × 10−16; Fig. 1D). Again, the genes mapping to 8q displayed stronger negative correlation with p53 pathway activity than did the other genes located outside 8q (FDR q < 0.05; Fig. 1C; Materials and Methods). A more strengthened correlation with p53 pathway activity was further observed for the 8q genes with cis-effects than those without (FDR q < 0.05; Fig. 1C). Together, these findings supported the possibility that the CNA-driven genes encoded on chromosome 8q promote ribosome biogenesis activity, leading to reduction of p53 pathway activation.

Among these five ribosome biogenesis–associated genes, RRS1 exhibited the most marked up-regulation in HCC tissues compared to adjacent nontumor liver tissues from 12 publicly available HCC patient cohorts (fig. S1F and table S2). In addition, two datasets (GSE67680 and GSE93392) (28) of diethylnitrosamine (DEN)–induced murine HCC models consistently exhibited increased Rrs1 expression in liver tumor tissues relative to nontumor liver tissues (fig. S1G). RRS1 is a conserved protein in eukaryotes, and together with Rpf2, it promotes the maturation of 60S ribosome subunit (29, 30). Despite extensive studies on the oncogenic role of RRS1 in several types of cancer (31–34), the role and the fundamental mechanism of RRS1 in HCC remain largely unclear. Notably, the expression levels of RRS1 showed significantly negative correlation with the p53 pathway activity (r = −0.36, P = 2.0 × 10−12; Fig. 1E) and the expression levels of p53 target genes, including the cell cycle repressor CDKN1A (r = −0.27, P = 3.3 × 10−7) and the apoptotic protein TNRFSF10B (r = −0.22, P = 4.21 × 10−6; Fig. 1F). Thus, we hypothesized that the RRS1 encoded on chromosome 8q acts as a pivotal contributor to ribosome biogenesis–driven hepatocarcinogenesis and merits further investigation.

Higher expression levels of RRS1 predict poor clinical outcomes of patients with HCC

We next assessed whether RRS1 expression levels are correlated with the progression of patients with HCC. Copy number gain of 8q has been reported in several types of solid tumor, including HCC (21, 35). Consistently, genomic CNA analyses in specimens from the validation cohort (designated as VALI; n = 144; table S3) showed frequent copy number gains of RRS1 in HCCs (~44%; Fig. 2A).
Immunohistochemistry (IHC) analyses in specimens from the VALI cohort showed that RRS1 protein locates in both the nucleus and cytoplasm of cells and the expression levels of RRS1 protein in nucleus were significantly higher in tumor tissues compared with nontumor liver tissues ($P = 0.0032$; Fig. 2B). Higher expression of RRS1 is observed in HCC tumors with genomic gains of RRS1 compared to those without gains (Fig. 2C). Furthermore, we found that the patients with higher RRS1 expression levels exhibit higher serum alanine transaminase levels than the others ($P = 0.015$; table S4). Kaplan-Meier analyses revealed that higher RRS1 levels in HCC tissues

**Fig. 1. Genomic gain of chromosome 8q correlates with increased ribosome biogenesis activity in HCC.** (A) The effects of the genomic CNAs on mRNA expression levels. Top: The correlations of CNAs with mRNA expression levels. Positive and negative correlations are indicated in red and blue, respectively. Diagonal lines indicate the cis-effects of CNAs on mRNA expression levels. Bottom: The frequencies of the significant correlations. (B) Hierarchical clustering of the correlations between the chromosomal CNAs (vertical) and activity scores of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (horizontal). The pathway activity scores were inferred by single-sample gene set variation analysis (GSVA). TGF-β, transforming growth factor–β; VEGF, vascular endothelial growth factor; ECM, extracellular matrix. (C) Identification of the critical ribosome biogenesis modulators encoded on 8q. Top: All the quantifiable genes in the transcriptome and CNAs are ranked on the basis of the correlations between the mRNA levels and ribosome pathway activity. Middle: The correlations between the mRNA levels and p53 pathway activity. Bottom, from top to bottom: The 8q genes, 8q genes with CNA cis-effects, and 8q genes involved in ribosome biogenesis with CNA cis-effects are highlighted, respectively. FDR, false discovery rate. (D) The ribosome pathway activity shows negative correlation with the p53 pathway activity. (E) The ribosome pathway activity shows negative correlation with the mRNA levels of RRS1. (F) RRS1 mRNA expression levels show negative correlation (Spearman’s correlation analysis) with the mRNA levels of two p53 downstream targets (CDKN1A and TNFRSF10B).
are markedly correlated with decreased overall survival (OS) and disease-free survival (DFS) rates ($P = 0.015$ and 0.083, respectively; Fig. 2D).

We also evaluated the RRS1 expression levels in patients with HCC from TCGA-LIHC ($n = 359$) and the International Cancer Genome Consortium Liver Cancer–RIKEN Japan (ICGC-LIRI-JP; $n = 212$) cohorts. Again, higher mRNA expression levels of RRS1 are significantly associated with adverse OS ($P = 0.023$) and DFS ($P = 0.038$) in patients with HCC from TCGA-LIHC (Fig. 2E) and adverse OS in patients from ICGC-LIRI-JP ($P = 0.035$; Fig. 2F) cohorts.

Furthermore, we extended our analyses to the other types of cancer from TCGA pan-cancer cohorts. We observed that the genomic gain of RRS1 is a common feature in many other types of cancer, with a relative high frequency in solid tumors (>40%), such as uveal melanoma (UVM), uterine carcinosarcoma (UCS), and testicular germ cell tumor (fig. S2A). Furthermore, markedly higher mRNA expression levels of RRS1 were observed in most types of cancer by comparing the expression levels in tumor tissues with those in matched nontumor tissues (fig. S2B). Accordingly, higher protein expression levels of RRS1 were observed in multiple types of cancer indexed in the Human Protein Atlas database (fig. S2C). Again, survival analyses in TCGA pan-cancer cohorts showed that higher RRS1 mRNA expression levels predict poor survival in patients with several other types of cancer, including adrenocortical carcinoma, head and neck squamous cell carcinoma, kidney renal papillary cell carcinoma, and mesothelioma (fig. S2D). However, the expression levels of the other four RP-coding genes (RPL30, RPS20, RPL7, and RPL8) exhibited no significant prognostic relevance for patients with HCC (fig. S2E). Together, these findings supported the potential of RRS1 as a promising prognosis marker for HCC and several other types of cancer, which increases the candidacy of RRS1’s oncogenic potential.

**RRS1 promotes HCC cell growth**

We then assessed whether deregulation of RRS1 affects HCC progression. We first investigated the effects of RRS1 on liver cell lines. Knockdown of RRS1 in HCC cell lines HepG2 and MHCC97L markedly decreased the cell growth rates using cell counting assays (Fig. 3A). Consistently, bromodeoxyuridine/propidium iodide (BrdU/PI) assays showed that knockdown of RRS1 decreased the number of cells that incorporated with BrdU (Fig. 3B), indicating that HCC cells exhibit decreased DNA synthesis. Concordantly, RRS1 depletion also decreased the number of cell colonies, as determined by either plate or soft agar colony formation assays (Fig. 3C and D). On the contrary, enforced expression of RRS1 in either normal liver cell line L-02 or HCC cell lines HepG2 and Bel-7402 cells notably increased the growth ability of HCC cells (Fig. 3, E to H, and fig. S3A). Furthermore, the cell growth–inhibiting effects of RRS1 knockdown in HepG2 cells can be completely rescued by overexpression of RRS1 (fig. S3D). We also observed that knockdown of RRS1 in HepG2 cells leads to a significant reduction in the growth of subcutaneous xenograft tumors in nude mice (fig. S3, E and F). The same result was observed in the MHCC97L-derived orthotopic xenograft tumors in nude mice (Fig. 3, I and J). Conversely, overexpression of RRS1 in HepG2 cells significantly increased the orthotopic xenograft tumor growth (Fig. 3, K and L). The pro-proliferative effects...
Fig. 3. RRS1 plays an oncogenic role in HCC cells. (A to D) Knockdown of RRS1 in HCC cells reduces the cell growth determined by cell counting (A), BrdU incorporation (B), plate colony formation (C), and soft agar colony formation assays (D). Scale bars, 2 mm. GAPDH, glyceraldehyde phosphate dehydrogenase. (E to H) Overexpression of RRS1 in HCC cells promotes the cell growth determined by cell counting (E), BrdU incorporation (F), plate colony formation (G), and soft agar colony formation assays (H). Scale bars, 2 mm. (I and J) Knockdown of RRS1 in MHCC97L cells reduces the orthotopic tumor growth. (I) Representative bioluminescent images on day 64 (left) and the growth curves of orthotopic tumors (right; n = 6). (J) Tumor weights were measured at end point. (K and L) Overexpression of RRS1 in HepG2 cells promotes the orthotopic tumor growth. (K) Representative bioluminescent images on day 64 (left) and growth curves of the orthotopic tumors (right; n = 6). (L) Tumor weights were measured at end point. (M) Knockdown of RRS1 promotes the G0-G1 arrest and H2O2-induced apoptosis in HCC cells. (N) Overexpression of RRS1 inhibits the G0-G1 arrest and H2O2-induced apoptosis in HCC cells. Error bars, means ± SD, n = 3. *P < 0.05, **P < 0.01, and ***P < 0.001; n.s., not significant (Student's t-test).
of RRS1 were indicated by Ki-67 expression measured by IHC assays (fig. S3, G to I). Collectively, these in vitro and in vivo findings supported that RRS1 is a previously unidentified oncogene and promotes HCC cell growth.

Furthermore, flow cytometry analyses showed that knockdown of RRS1 displays enhanced G0-G1 arrest and H2O2-induced apoptosis in HepG2 and MHCC97L cells (Fig. 3M). Conversely, enforced overexpression of RRS1 attenuated G0-G1 arrest and apoptosis in HepG2, Bel-7402, and L-02 cells (Fig. 3N and fig. S3J). Together, these observations suggested that RRS1 could promote HCC cell growth by inhibiting G0-G1 arrest and apoptosis.

**RRS1 attenuates ribosomal stress by potentiating ribosomal RNA maturation and ribosome subunit assembly in HCC cells**

RRS1 plays a critical role in 25S ribosomal RNA (rRNA) maturation and 60S ribosomal subunit assembly in yeast (36). Consistently, by examining the codependencies of RRS1 in the Broad Institute genome-wide CRISPR-Cas9 screening database across 769 cancer cell lines, we found that the genes with dependencies correlated with RRS1 (FDR < 0.05, n = 254) are enriched in rRNA processing and modification (Fig. 4, A and B). Gene set enrichment analysis (GSEA) based on preranked genes according to the correlation coefficients revealed similar results (Fig. 4, C and D). We then sought to explore the possible role of RRS1 dysregulation in ribosome biogenesis in HCC cells. Immunofluorescence assays showed that either the endogenous or exogenous RRS1 is highly expressed in the nucleolus and colocalized with the nucleophosmin (NPM), a marker of the nucleoli (fig. S4A). The primers that can distinguish the precursor and mature human rRNAs (fig. S4B) were then used to quantify the mature rRNAs as previously described (37). In HepG2 cells, after knockdown of RRS1, the levels of mature 28S and 18S rRNAs were significantly reduced compared with the controls (Fig. 4E). These findings were further validated by Northern blotting assays, which displayed reduced mature 28S and 18S rRNAs in RRS1-depleted HepG2 cells (Fig. 4F). In addition, polyribosome profiling assays showed that knockdown of RRS1 leads to reduction in monosome and polysome levels (Fig. 4G). Conversely, overexpression of RRS1 in HepG2 cells led to increased levels of processed 28S and 18S rRNAs (Fig. 4, E and F) and ribosome subunits (Fig. 4G). Together, these findings suggested that RRS1 could attenuate ribosome biogenesis stress by promoting rRNA maturation and ribosome subunit assembly in HCC cells.

**RRS1 reduces p53 signaling in HCC cells**

Perturbations of ribosome biogenesis have been shown to contribute to nucleolar stresses that are able to trigger the p53 pathway (27). Genome-wide RNA interference screen has also identified RRS1 as a candidate for p53 synthetic interaction (38). These previous findings encouraged us to investigate whether p53 serves as a sentinel for the dysregulation of RRS1. To this end, we first performed gene expression profile analyses in HepG2 cells upon knockdown of RRS1. GSEA showed that the p53–down-regulated genes (39) are negatively enriched, while the p53–up-regulated genes or p53 direct targets (40) are positively enriched in RRS1-depleted HepG2 cells (Fig. 5, A and B and table S5). We then measured the effect of RRS1 on the protein levels of p53. We found that knockdown of RRS1 induces accumulation of p53 protein in HepG2 cells (Fig. 5C), whereas enforced overexpression of RRS1 led to a decrease of p53 protein levels (fig. S5A). The downstream targets of p53, i.e., the cell cycle arrest–modulating protein p21 and proapoptosis protein p53 upregulated modulator of apoptosis (PUMA), were also suppressed by RRS1 (Fig. 5C and fig. S5A). Consistently, the inhibitory effects of RRS1 on p53 levels were also confirmed in the mouse orthotopic tumor tissues (fig. S5B).

We then examined whether RRS1 plays the oncogenic role in a p53-dependent manner using p53+/− (which contains functional p53) and p53−/− (p53-defective) HCT116 cells. As expected, we observed no colony formation inhibition in p53−/− HCT116 cells but a significant decrease in p53+/− HCT116 cells upon RRS1 depletion (Fig. 5D). Furthermore, knockdown of RRS1 enhanced the G0-G1 arrest and apoptosis in p53+/− HCT116 cells but not in p53−/− HCT116 cells (Fig. 5E and fig. S5C). In line with this, p53+/− HCT116 cells with overexpression of RRS1 exhibited increased colony formation and reduced G0-G1 arrest and apoptosis, which were not observed in p53−/− HCT116 cells (fig. S5, D to F). In addition, the p53-dependent oncogenic role of RRS1 was confirmed in the p53-deficient HCC cell lines Huh7 and Hep3B (Fig. 5, F and G, and fig. S5, G and H). Clinically, some HCCs with low p53 activity lack p53 mutations. Here, we observed that both 8q gain and high expression of RRS1 exhibit stronger anticorrelations with the p53 activity than with the p53 mutation status (Fig. 5H), suggesting an alternative mechanism underlying p53 inactivation driven by RRS1 in HCCs with intact p53. Notably, when we divided the HCC samples from the VALI cohort into two groups based on the mutation status of p53, patients with high expression levels of RRS1 exhibited worse OS and DFS in p53 wild-type (WT) group (P = 0.034 and 0.044, respectively) but not in the p53 mutant group (Materials and Methods; Fig. S1). In agreement with this, stratified survival analyses in the datasets from the TCGA-LIHC cohort displayed similar results (for HCCs with WT p53: P = 0.032 for OS and P = 0.011 for DFS, respectively; Fig. 5I). Together, these findings suggested that RRS1 is involved in modulating ribosome biogenesis–p53 stress response pathway in HCC.

**RRS1 promotes the MDM2-mediated p53 ubiquitination and degradation**

We then investigated how RRS1 reduces the p53 protein levels. Neither knockdown nor overexpression of RRS1 affected p53 mRNA levels (Fig. 6A), suggesting that the inhibitory effect of RRS1 on p53 expression is not due to transcriptional regulation. Through cycloheximide (CHX) studies, we observed that p53 protein half-life is prolonged in RRS1–knocked down HepG2 cells (Fig. 6B), while shortened in RRS1-overexpressed HepG2 cells (Fig. 6C). Furthermore, treatment with the proteasomal inhibitor MG132 reduced the degradation of p53 induced by RRS1 (Fig. 6D and fig. S5D), suggesting that RRS1 decreases the protein stability of p53 via proteasome pathway. Several E3 ligases have been found to target p53 and degrade it through the ubiquitin (Ub)–proteasome pathway in cancer cells, such as MDM2 (41), HECT, UBA and WWE domain-containing E3 ubiquitin protein ligase 1 (HUBE1) (42), Ring finger and WD domain 2 (RFWD2) (43), and RING finger and CHY zinc finger domain-containing protein 1 (RCHY1) (44). We thus examined the E3 ligase(s), which is (are) responsible for RRS1-mediated p53 degradation. The results showed that the RRS1 depletion–induced accumulation of p53 is dependent on MDM2 but minimally influenced by knockdown of other three types of E3 ligase (Fig. 6F). Consistently, the inhibitory effect of RRS1 overexpression on p53 protein levels was abolished by MDM2 inhibition by small interfering RNAs (siRNAs) (Fig. 6G). We then examined whether RRS1 affects the MDM2-mediated p53
Figure 4. RRS1 is required for rRNA maturation and ribosomal subunit assembly. (A to D) Genome-wide analysis reveals that the genes (n = 254) with dependencies (measured by Project Achilles CRISPR-Cas9 gene scores) correlated with RRS1 are enriched in rRNA processing. Spearman's coefficient (r) versus −log10(FDR) is plotted for each gene, and the significant correlations are highlighted with green (FDR < 5%). (D) The representative GSEA plots. (E) Effects of RRS1 knockdown or overexpression on rRNA maturation in HepG2 cells determined by detecting 5′ external transcribed spacer (ETS)–18S rRNA and internal transcribed spacer (ITS)–28S rRNA using the quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays. (F) Effects of RRS1 knockdown or overexpression on rRNA maturation in HepG2 cells determined by detecting 5′ external transcribed spacer (ETS)–18S rRNA using the quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays. (G) Effects of RRS1 knockdown or overexpression on ribosome subunit assembly in HepG2 cells determined by polyribosome fractionation assays. Error bars, means ± SD, n = 3. **P < 0.01 and ***P < 0.001 (Student's t test). OD, optical density.
RRS1 reduces the MDM2-mediated p53 pathway activation. (A) GSEA showed that knockdown of RRS1 in HepG2 cells results in activation of p53 signaling. NES, normalized enrichment score. GSEA was performed in the shRRS1 versus shCtrl HepG2 cells based on the Molecular Signatures Database (MSigDB) gene set collection (v6.0). (B) The expression heatmap of “GO: p53 binding” genes in RRS1-depleted HepG2 cells. (C) Knockdown of RRS1 in HepG2 cells increases the protein levels of p53 and its downstream targets (p21 and PUMA). (D and E) Knockdown of RRS1 reduces the plate colony formation (D) and increases the G1 arrest and H2O2-induced apoptosis (E) in HCT116 p53+/− cells, but not in HCT116 p53−/− cells. (F and G) Knockdown of RRS1 does not affect the plate colony formation (F) and the cell cycle transition and H2O2-induced apoptosis (G) in p53-deficient HCC cells with either p53 deletion (Hep3B) or p53 mutation (Huh7). (H) 8q gain and high expression of RRS1 are anticorrelated with the p53 activity. (I and J) Kaplan-Meier curves showing the OS and DFS rates with respect to the RRS1 expression levels and p53 mutation status in HCC tumors from the VLI (I) or TCGA-LIHC (J) cohort. Log-rank test, P < 0.05. Error bars, means ± SD, n = 3. *P < 0.05, **P < 0.01, and ***P < 0.001 (Student’s t test).
Fig. 6. RRS1 reduces the p53 protein levels via MDM2-mediated p53 ubiquitination. (A) Neither knockdown (by shRRS1-1) nor overexpression of RRS1 affects p53 mRNA levels in HepG2 cells. Error bars, means ± SD, n = 3. *P < 0.05 and **P < 0.01 (Student's t test). (B) Half-life of p53 protein in RRS1-depleted (by shRRS1-1) HepG2 cells was prolonged. CHX, 100 mg/ml. (C) Half-life of p53 protein in HepG2 cells overexpressing RRS1 was shortened. CHX, 100 mg/ml. (D and E) MG132 (20 μM) abolishes the promoting effect of RRS1 depletion (D) and the inhibitory effect of RRS1 overexpression (E) on p53 protein levels in HepG2 cells. (F and G) Knockdown of MDM2 abolishes the promoting effect of RRS1 knockdown (F) and the inhibitory effect of RRS1 overexpression (G) on p53 protein levels in HepG2 cells. (H and I) Effects of RRS1 knockdown (H) or overexpression (I) on the MDM2-mediated p53 ubiquitination in HepG2 cells. HepG2 cells transfected with the indicated siRNAs targeting RRS1 or plasmids were pretreated with MG132 (20 mM). Lysates were immunoprecipitated (IP) and immunoblotted (IB) with antibodies against Flag and HA-tagged ubiquitin (Ub), respectively. (J) Knockdown of MDM2 abolishes the promoting effect of RRS1 overexpression on p53 ubiquitination levels in HepG2 cells. Lysates were immunoprecipitated and immunoblotted with antibodies against p53 and Ub, respectively.
ubiquitination. As expected, coexpression of MDM2 and p53 produced a ladder ofUb-p53 products in HepG2 cells (Fig. 6H). However, the ladder was less intense after knockdown of RRS1 (Fig. 6H). When we overexpressed RRS1 in these MDM2- and p53-expressing HepG2 cells, the ladder was more intense (Fig. 6I). Notably, when we inhibited MDM2 by siRNAs in HepG2 cells, the RRS1-promoted ubiquitination of endogenous p53 was abolished (Fig. 6J and fig. S6). Together, these results suggested that RRS1 promotes the MDM2-mediated p53 ubiquitination and degradation.

**RRS1 retains RPL11 in the nucleolus and reduces its competitive binding with MDM2**

Yeast Rrs1 acts as an assembly factor with Rp2, which recruits Rpl5, Rpl11, and 5S rRNA into nascent ribosomes for ribosome biogenesis (29, 30). In response to nucleolar stress, Rpl5 and Rpl11 are released from the nucleolus into the nucleoplasm to bind MDM2 and inhibit MDM2-mediated p53 ubiquitination (45, 46). We therefore explored the roles of these RPs in RRS1-induced p53 response. First, we tested whether these RPs interfere with RRS1-induced p53 degradation. We carried out siRNA-mediated knockdown experiments and found that only the siRNAs against RPL11, but not RPL5, blocked p53 accumulation in HepG2 cells upon RRS1 knockdown (Fig. 7A). Consistently, overexpression of RPL11, but not RPL5, restored p53 accumulation that was impaired by RRS1 overexpression (Fig. S7A). Second, we tested the interaction between RRS1 and RPL11 in HepG2 cells. We expressed exogenous RPL11 and RRS1 in HepG2 cells followed by coimmunoprecipitation (co-IP) and found that RPL11 interacted with RRS1 (Fig. 7B). Furthermore, endogenous RRS1 was also confirmed to bind to RPL11 and ribosome production factor 2 homolog (RPF2), but not RPL5, in untreated HepG2 cells by IP (Fig. 7C). Consistently, glutathione S-transferase (GST) pull-down assays showed that RRS1 binds to RPL11 in vitro (Fig. 7D). More consistent experimental evidence is required to confirm the direct interaction between RRS1 and RPL11. Third, we investigated whether the RRS1-RPL11 binding modulates the expression and/or subcellular localization of RPL11. These results showed that knockdown of RRS1 does not influence the expression of RPL11 (Fig. S7B) but promotes the translocation of RPL11 from the nucleolus into the nucleoplasm determined by cell fractionation assays (Fig. 7E) and confocal microscopy assays (Fig. 7F). In contrast, overexpression of RRS1 retains RPL11 in the nucleolus (Fig. S7C). However, either knockdown or overexpression of RRS1 has little effect on RPL5 translocation (Fig. 7, E and F, and fig. S7C), further suggesting that RRS1 is essential for the nucleolar localization of RPL11, but not RPL5. Fourth, we analyzed the effects of dysregulation of RRS1 on the RPL11-MDM2 binding in HepG2 cells. The co-IP assays showed that knockdown of RRS1 in HepG2 cells enhanced the binding of endogenous MDM2 to RPL11 (Fig. 7G). Conversely, overexpression of RRS1 reduces MDM2-RPL11 binding (fig. S7D). However, dysregulation of RRS1 exhibited no significant effect on MDM2-RPL5 binding (Fig. 7H). Last, functional assays showed that RPL11, but not RPL5, is responsible for promoting effects of RRS1 on colony formation (Fig. 7I) and for inhibitory effects on cell cycle arrest and apoptosis (Fig. 7J) in HepG2 cells.

Collectively, we hypothesize that, when the RRS1 expression increases (e.g., due to genomic gain of 8q), RRS1 binds to RPL11 and prevents it from escaping into the nucleoplasm to bind MDM2, which, in turn, promotes the MDM2-mediated ubiquitination and degradation of p53. Therefore, we demonstrated that RRS1 is a previously unidentified critical regulator for HCC through reducing the RPL11-MDM2-p53 signaling pathway (Fig. 7K).

**DISCUSSION**

Identification of the genomic drivers of tumorigenesis is essential for the design of targeted therapies aimed at the underlying pathways. Our findings established that chromosome 8q genomic gain–driven RRS1 overexpression functions as an oncogenic regulator of the RPL11-MDM2-p53 signaling. Our study therefore indicates that RRS1 might serve as an effective molecular target to block the HCC progression. Concordantly, the expression levels of RRS1 exhibited significant prognostic value for patients with HCC, especially in those with intact p53.

Chromosome 8q cytogenetic gain is one of the most frequent events observed in HCC and several other cancer types, such as UVM, ovarian cancer, UCS, and breast cancer (fig. S1, A and B). However, the large size of the 8q amplification makes it difficult to identify the functional targets. Despite several focal amplifications of important loci within chromosome 8q, including 8q24.3 (MYC) (4) and 8q22 (MTDH) (18), evolution of epithelial tumors may also involve the gain of the entire long arm, in which case, the gain of other driver genes could also be pivotal to the progression of malignancies. Consistent with the strong modulatory effects of 8q gain on genes involved in ribosome biogenesis activity, we here demonstrated that the ribosome biogenesis regulator RRS1 acts as a functional target within 8q gain. Overexpression of RRS1 in conjunction with oncogenic activation is shown to drive tumorigenesis, with significant prognostic value for patients with HCC. This study, therefore, provides sufficient evidence for the functional dissection of 8q gain in tumor biology.

Inactivation of p53, which plays an important role in cell growth, division, and apoptosis by acting as a transcription factor, is the most common feature in most cancer types (47). Although frequent mutations of p53 were found in HCCs, some HCCs with low p53 singling activity lack p53 mutation (Fig. 5H), suggesting that there exist alternative regulatory mechanisms underlying p53 inactivation. Previous studies have exploited the dependency of p53 WT cancers and revealed genomic gains of several negative regulators of p53 exert functions via p53-dependent stress signals, such as 12q13-14 amplification–encompassed MDM2 in soft tissue sarcomas (48) and 1q gain–driven MDM4 in Burkett lymphoma (49). Recently, many studies have provided another insight that perturbations of ribosome biogenesis can trigger a MDM2–p53–dependent checkpoint signaling pathway independent of DNA damage through inhibitory interactions of specific ribosomal components. Here, we presented a proof of principle that 8q gain–driven overexpression of RRS1 decreases the p53 activity through promoting the ribosomal stress–MDM2–mediated destabilization of p53. Inhibition of RRS1 in p53-intact HCC cell lines led to p53 accumulation that promoted cell cycle arrest and apoptosis. Consistent with this model, we found that RRS1 can reduce ribosomal stress–induced p53 accumulation under actinomycin D treatment (fig. S7E). Collectively, our study showed that RRS1 is a previously unidentified repressor of p53 by attenuating the ribosomal stress.

RRS1 was found as a regulatory protein required for ribosome biogenesis in yeast (50) and acts as an assembly factor with Rp2, which recruits 5S ribonucleoprotein (RNP) that formed by rp5, rpl11, and 5S rRNA into nascent ribosomes (30). However, the involvement of RRS1 in regulation of ribosomal stress in human cells remains
Fig. 7. RRS1 regulates MDM2 by binding and retaining RPL11 in the nucleus. (A) Knockdown of RPL11, but not RPL5, abolishes the promoting effect of RRS1 depletion on p53 protein levels in HepG2 cells. (B) Co-IP of exogenous RRS1 with exogenous RPL11 in HepG2 cells using anti-Myc. (C) Co-IP of endogenous RRS1 with endogenous RPL11, RPL5, and RPFL2 in HepG2 cells using anti-RRS1. (D) The in vitro interaction between the RPL11 (GST-tagged) and RRS1 (His-tagged) was evaluated by GST pull-down assays. (E) and (F) Knockdown of RRS1 reduces the retention of RPL11, but not RPL5, in the nucleolus determined by cell fractionation assays (E) and confocal microscopy assays (F). Tubulin, lamin B, and NPM are used as the cytoplasmic, nucleoplasmic, and nucleolar markers, respectively. Scale bars, 20 μm. DAPI, 4′,6-diamidino-2-phenylindole. (G) and (H) Knockdown of RRS1 increases the binding of endogenous MDM2 with RPL11 (G), but not RPL5 (H). (I) and (J) RRS1 promotes the plate colony formation (I) and reduces cell apoptosis and cell cycle arrest (J) in a RPL11-dependent manner in HepG2 cells. Error bars, means ± SD, n = 3. **P < 0.01 and ***P < 0.001 (Student’s t test). (K) A schematic model for the mechanisms of RRS1 in the development of HCC.
unclear. Our work demonstrated that RRS1 acts as a pivotal contributor to ribosome biogenesis by potentiating rRNA maturation and ribosomal subunit assembly in human HCC cells. Previous studies demonstrated that in cells under ribosome biogenesis stress, the excess 5S RNP binds to MDM2, inhibiting its function and leading to p53 accumulation (51). However, we observed that, except RPL11, the other 5S RNP components (RPL5 and 5S rRNA) do not affect the RRS1 depletion–induced p53 response (fig. S7F). Furthermore, it has been reported that inhibition of RPL11 is sufficient to suppress up-regulation of p53 in response to the ribosome biogenesis stress (51). We found that knockdown of RPP2 reduces the RRS1-RPL11 binding affinity, promotes nucleocytoplasmic translocation of RPL11, and subsequently abolishes the RRS1 depletion–induced p53 response in HepG2 cells (fig. S7, F to H). These results suggested that RRS1 (together with RPP2) interacts with RPL11 and anchors it in the nucleolus and reduces the ribosome biogenesis stress–induced p53 accumulation. This observation is consistent with previous findings that some ribosome biogenesis regulators exhibit dual function that reflects a coordinated pathway coupling ribosome biogenesis to RPs-MDM2-p53 signaling in cancer cells (52).

In summary, our study allows for deconstructing the biological roles of arm-level CNAs and identified 8q gain as a strong contributor to ribosome biogenesis activity in HCC. Furthermore, we demonstrated that RRS1 is a pivotal functional target of 8q gain and acts as an oncogene by facilitating ribosome biogenesis and then attenuating RPL11-MDM2-p53 signaling. Given that 8q gain is one of the most frequent CNAs in cancers, targeting RRS1 to alleviate degradation of p53 might be exploited therapeutically across HCC and other types of cancer with WT p53 harboring 8q gain.

MATERIALS AND METHODS

Human HCC tissue samples

This study included a total of 144 pairs of human HCC tissues and adjacent nontumor liver tissues derived from the patients with HCC of the VALI cohort, who were recruited from the Affiliated Cancer Hospital of Guangxi Medical University (Nanning City, Guangxi Province, China), Jinling Hospital (Nanjing City, Jiangsu Province, China), and Jindu Hospital (Nanjing City, Jiangsu Province, China), respectively, between 2007 and 2016. All these patients with HCC were newly diagnosed, previously untreated (chemotherapy or radiotherapy), pathologically confirmed, and proven not to have other types of cancer. Each tissue specimen was reviewed by a board-certified pathologist to confirm that the frozen section was histologically consistent with the tumor or nontumor liver tissues, and tumor sections had to contain more than 70% tumor cell nuclei and less than 20% necrosis. At recruitment, the informed consent was obtained from all participating patients, and personal information on demographic factors and clinical and histopathologic data was collected from medical records for all the patients. All postoperative patients were closely followed up as outpatients and monitored prospectively for tumor recurrence by a standard protocol. The genomic DNAs from the patients of the VALI cohort were used for RRS1 copy number quantification. The tissue microarrays from the patients with HCC of the VALI cohort were used for IHC assays. There is a subset of samples (n = 79) with p53 mutation statuses available for stratified survival analyses. The p53 mutation statuses were determined by Sanger sequencing, a DNA region spanning exons 5 to 8. This study was performed with the approval of the Medical Ethical Committee of Beijing Institute of Radiation Medicine (Beijing, China). The clinicopathologic information of the patients with HCC was provided in table S3.

Genomic and transcriptomic datasets from the TCGA and ICGC projects

The level 3 seg files inferred from the Affymetrix Genome-Wide Human SNP Array 6.0 profiling of 10,420 tumor samples across 33 types of cancer in TCGA were downloaded from the Genomic Data Commons (GDC) data portal, along with available clinical annotations. Segments were called as genomic copy number gain or loss when their log2 copy ratios were larger than 0.3 or smaller than −0.3, respectively. To further summarize the arm-level copy number change, we used a weighted sum approach (53), in which the segment-level log2 copy ratios for all the segments located in the given arm were added up with the length of each segment being weighted. To estimate the contribution of each arm-level CNA to the genome alteration, we calculated the WPGA score, which is defined as the length-weighted arm-level sum of log2 copy ratios divided by the total length-weighted sum of log2 copy ratios of the genome. We performed hierarchical clustering of the pan-cancer arm-level CNA data using the raw log2 copy ratio data and WPGA data, respectively, to distinguish the chromosomal alteration states across different types of cancer.

The level 3 RNA sequencing profiles of 360 HCC tissues and 49 nontumor liver tissues from the TCGA-LIHC cohort were downloaded from the GDC data portal (https://portal.gdc.cancer.gov/). The level 3 RNA sequencing profiles of 212 HCC tissues and 181 nontumor liver tissues from the Liver Cancer–RIKEN, JP Project from the ICGC-LIRI-JP cohort were downloaded from the ICGC data portal (https://dcc.icgc.org/).

Identification of the cis- and trans-effects of CNAs

The correlations between the genomic copy number and RNA expression levels were performed using Spearman’s rank correlation for ~15,000 quantifiable genes in transcriptomics and CNA data. The cis-effect of CNA were defined as the significant association (FDR < 0.01, Spearman’s rank test) between a given copy number and the gene expression levels from the same genome loci, and the trans-effect of CNA was defined as the significant association (FDR < 0.01, Spearman’s rank test) between a given locus and global gene expression levels.

Inference of activity scores of KEGG pathways and p53 pathway

All activity scores were inferred by single-sample GSEA method from the GSVA R package (23). The pathway gene signature sets are derived from the KEGG database. The p53 pathway activity was inferred from the known p53 transcription targets (MSigDB PEREZ_TP53_TARGETS) (54).

Validation of CNAs by CNVplex assays

The genomic copy number statuses of RRS1 locus in 144 HCC tissues from the VALI cohort were genotyped by CNVplex assays according to the manufacturer’s instructions (Genesky, China) (55). Two individual probes were designed for RRS1 locus (table S6). Four reference genes—POLR2A, POP1, RPP14, and TBX15—were used as internal controls for normalization. The CNVplex assays were performed as previously described (56). Briefly, the ligation reaction was performed in 20 μl for each sample, containing 10× ligase buffer (2 μl), ligase (0.5 μl), probe mix (1 μl), and approximately
150 ng of genomic DNAs. The ligation reaction program was as follows: 4 cycles of 94°C for 1 min and 60°C for 4 hours, 94°C for 2 min, and 70°C forever. Then, the ligation products were subjected to a multiplex fluorescence polymerase chain reaction (PCR) amplification. The PCR reaction was performed in 20 μl for each sample, containing 1x multiplex PCR master mix (10 μl), probe mix (1 μl), and ligation product (1 μl). The PCR program was as follows: 95°C for 2 min; 30 cycles of 94°C for 20 s, 57°C for 40 s, and 72°C for 1.5 min; 60°C for 1 hour; and 4°C forever. PCR products were diluted 20-fold before being run by capillary electrophoresis using an ABI 3130XL genetic analyzer (Carlsbad, USA). Raw data were analyzed using GeneMapper 4.1 (ABI, USA). The copy number of each target segment was determined by the average sample peak height. The sample peak height for each target fragment was first normalized on the basis of four reference genes, respectively. If one of the four normalized sample peak heights deviated more than 25% from the average of the others, then it would be excluded for further analyses.

**Cell lines**

The human normal liver cell line (L-02 cell) and two HCC cell lines (HepG2 and HuH7) were obtained from the China Center for Type Culture Collection (Wuhan City, China). Human HCC cell line Bel-7402 was purchased from the Chinese National Infrastructure of Cell Line Resource (Beijing, China). Human HCC cell lines SMCC-7721, MHCC97L, and MHCC-97H were gifts from S. Sun (The Second Military Medical University, Shanghai, China). Human HCC cell line Hep3B was a gift from C. Tian (Beijing Institute of Lifeomics, Beijing, China). Human colorectal carcinoma cell lines with or without WT p53 (HCT116 p53+/+ and HCT116 p53−/−, respectively) were gifts from Q. Zhan (Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China). All the cell lines were maintained in high-glucose Dulbecco’s modified Eagle’s medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were incubated at 37°C in a humidified incubator containing 5% CO2.

**IHC assays**

IHC assays of the paraffin-embedded tissue sections were performed as previously described (57). Tissue microarray slides, containing 144 paired HCC tissues and nontumor liver tissues, were initially deparaffinized using xylene, rehydrated with xylene and ethanol, immersed in 3% hydrogen peroxide solution for 10 min, heated with citrate at 95°C for 25 min, and cooled at room temperature for 60 min. The slides were incubated overnight at 4°C with four primary antibodies separately, including rabbit polyclonal anti-RRS1 (1:100; HPA055549, Sigma-Aldrich, USA). The slides were mounted with gum for examination and capture by the Olympus BX51 Microscopic/Digital Camera System for study comparison. The IHC signals were scored as previously described (57). Briefly, a proportion score was assigned, representing the estimated proportion of positive staining tumor cells (0, none; 1, <1/100; 2, 1/100 to <1/10; 3, 1/10 to <1/3; 4, 1/3 to 2/3; and 5, >2/3). Average estimated intensity of staining in positive cells was assigned an intensity score (0, none; 1, +; 2, ++; 3, +++; and 4, ++++). The overall scores (0 or 2 to 9) were obtained by combining these two parameters.

**Knockdown and overexpression studies**

The pCMV-Flag-p53, pCMV-Myc-MDM2, and pCMV-HA–tagged Ub were provided by J. Wang (State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing, China). For overexpression of RRS1, recombinant lentiviruses were constructed by subcloning human full-length RRS1 cDNA into the pLV-EGFP-N vector. Various constructs of RRS1 and RPL11 were generated according to standard molecular techniques. For stable short hairpin RNA (shRNA) expression, shRNAs targeting RRS1 were designed and synthesized by GenePharma (Shanghai, China). Recombinant lentiviruses were constructed by subcloning sequences of shRNAs into the GV248 (GenePharma, China) vector (table S6). All the recombinant constructs were then transiently transfected into human embryonic kidney–293T cells using Lipofectamine 2000 (Invitrogen, USA). After 48 hours, viral supernatants were collected, then clarified by filtration, and concentrated by ultracentrifugation. The concentrated virus was used to infect the L-02, HepG2, Bel-7402, SMMC7721, and MHCC97L cells with polybrene (8 mg/ml). Puromycin (2 μg/liter) was used to select for the stably transduced cells.

**Reverse transcription PCR assays**

Total RNAs were isolated by TRIzol and were converted to cDNAs using the SuperScript III First Strand Synthesis System (Invitrogen, USA). Quantitative PCR was then performed using the Bio-Rad iQ5 System (Bio-Rad). PCR reactions were carried out in 20-μl reactions using SYBR Green PCR master mix (Bio-Rad) and 0.2 μM specific primers. The relative expression levels of mRNAs were calculated using the comparative C_{method normalized to GAPDH.

**Immunoblotting assays**

Protein extraction was done in ice-cold lysis buffer [20 mM Heps, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 5 mM iodoacetate acid, 20 mM okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktail (Roche Diagnostics, Germany)]. Tissue extracts were done in the same buffer with a tissue homogenizer after thawing frozen tissues collected by liquid nitrogen snap freezing. Protein concentrations of the extracts were determined using a Bio-Rad protein assay kit, and equal amounts of protein samples were loaded on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to polyvinylidene difluoride membrane (Millipore, USA), and immunoblotted with a primary antibody, followed by incubation with a secondary antibody. The blots were visualized by an enhanced chemiluminescence system (Santa Cruz Biotechnology, USA). The following antibodies were used: antibodies to RRS1 (1:500; 15329-1-AP, Proteintech, USA), p53 (1:500; 10442-1-AP, Proteintech, USA), MDM2 (1:1000; OP46-100UG, Merck Millipore, France), p21 (1:600; 10355-1-AP, Proteintech, USA), B-cell lymphoma 2 (BCL2) (1:300; 12789-1-AP, Proteintech, USA), PUMA (1:500; 55120-1-AP, Proteintech, USA), RPL5 (1:500; 15430-1-AP, Proteintech, USA), RPL11 (1:500, 16277-1-AP, Proteintech, USA), RFWFD2 (1:500; C15565, Anbo Biotech, USA), HUWE1 (1:200; 19430-1-AP, Proteintech, USA), CHYH1 (1:200; 13820-1-AP, Proteintech, USA), NPM (1:600; 60096-1-lg, Proteintech, USA), lamin (1:1000; 12987-1-AP, Proteintech, USA), tubulin (1:2000; CW0098, CWBIO, China), Ub (1:1000; 102012-2-AP, Proteintech, USA), Myc (1:200; 16286-1-AP, Proteintech, USA), Flag (1:2000; F1804, Sigma, USA), hemagglutinin (HA) (1:3000; sc-57592, Santa Cruz Biotechnology, USA), green fluorescent protein (1:1000; sc-101525, Santa Cruz Biotechnology, USA), glyceraldehyde phosphate dehydrogenase (GAPDH) (1:2000; CW0100, CWBIO, China), and β-actin (1:2000; sc-47778, Santa Cruz Biotechnology, USA).

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Cell proliferation, plate and soft agar colony formation, and cell cycle and apoptosis assays

**Cell counting assays**
Cells (1 x 10^3) were plated in six-well culture dishes in triplicate, and cell numbers were counted every day under the microscope with hemocytometers. Cell number for each time point was determined in triplicate and expressed as means ± SD.

**Cell Counting Kit-8 assays**
Cell viability was evaluated by Cell Counting Kit-8 (CCK-8) assay according to the manufacturer’s instructions (Beyotime Institute of Biotechnology China). Cells (2 x 10^3 cells per well) were plated in 96-well plates in triplicate. CCK-8 was added to each well at a final concentration of 10% at different time points, and incubation continued at 37°C for 50 min. Then, the absorbance of the samples was measured at 450 nm using a Multiskan MK3 microplate reader (Thermo Fisher Scientific, USA) to calculate the numbers of viable cells in each well.

**BrdU incorporation assays**
The BrdU incorporation assays were performed as previously described (56). Cells were grown on six-well culture dishes in triplicate and incubated in 10 μM BrdU [stock of 10 mM in phosphate-buffered saline (PBS); Roche, Germany] for 1 hour. Cells were fixed at −20°C with 70% ethanol for 1 hour, suspended in 2 mol/L HCl/Triton X-100 for 30 min, and then neutralized with 0.1 M Na2B4O7·10H2O. Cells were washed in 0.5% Tween 20/1% bovine serum albumin/PBS, and immunostaining was done with primary anti-BrdU monoclonal antibodies (1:100; 347580, BD Biosciences, USA) and gene-specific primers (table S6). The resultant cDNAs were used to calculate the numbers of viable cells in each well.

**Plate colony formation assays**
Cells (1 x 10^3) were plated onto a 6-cm plate and cultured for 14 days. The surviving colonies (>50 cells per colony) were fixed with 100% methanol and stained with 0.1% crystal violet (Sigma-Aldrich, USA) and counted.

**Soft agar colony formation assays**
Cells (5 x 10^3) were resuspended in 10% FBS in respective culture medium containing 0.7% agarose and plated onto a layer of 1.2% agarose-containing medium in a six-well plate. Colonies consisting of more than 50 cells were counted after 3 weeks.

**Cell cycle assays**
The cell cycle was analyzed by flow cytometry. Cells were harvested by trypsinization, fixed in 70% ethanol, and stained with PI (50 μg of total RNAs was size-separated by electrophoresis on a 1% agarose-formaldehyde gel. For detection of 3 H-labeled RNAs, the gel was washed twice in methanol and then incubated with 1x PBS and lysed in TTrZol (Invitrogen, USA). The total RNAs were extracted, and 5 μg of total RNAs was size-separated by electrophoresis on a 1% agarose-formaldehyde gel. For detection of 3 H-labeled RNAs, the gel was washed twice in methanol and then incubated with 3% 2,5-diphenyloxazole in methanol for at least 3 hours. After incubation in 1.4-bis(5-phenyl-2-oxazolyl)benzene for 30 min, the gel was dried and exposed to film at ~80°C for autoradiography.

**Apoptosis assays**
Apoptosis was detected by flow cytometry using the Annexin V–APC/7-AAD Apoptosis Detection Kit (Keygen, China). Briefly, cells were harvested, washed, resuspended in the staining buffer, and doubly stained with annexin V and 7-amino-actinomycin D (7-AAD). For each experiment, 5 x 10^3 cells were analyzed using FACScalibur and CellQuest software (BD Biosciences, USA). The annexin V–positive cells were regarded as apoptotic cells.

**Animal studies**
All of the BALB/C nude mice used in this study were purchased from Charles River (Beijing, China). All mouse studies were approved and supervised by the Institutional Animal Care and Use Committee of Beijing Institute of Radiation Medicine (Beijing, China). All mice were 4 to 6 weeks of age at the time of injection.
To examine the ubiquitination of exogenous p53 protein, HepG2 cells expressing HA-Ub, Flag-p53, pCMV-MDM2, or shRRS1 in cells (60% confluence, six-well plate) were transfected with the plasmids expressing p53. To confirm the interaction of RPL11 with RRS1, a GST pull-down assay was performed. RRS1 with His tag protein, RPL11 with GST tag protein, and GST tag protein were expressed in Escherichia coli, respectively. Bacteria-expressed GST or GST-RPL11 proteins were immobilized on Glutathione Sepharose 4B beads (GE Healthcare, UK) and washed, and then, the beads were incubated with His-RRS1 protein, which was purified with Ni-nitrilotriacetic agarose beads (Qiagen, Germany) for 3 hours at 4°C and eluted with His elution buffer [20 mM sodium phosphate, 500 mM NaCl, and 500 mM imidazole (pH 7.3)]. Beads were washed with GST binding buffer (100 mM NaCl, 50 mM NaF, 2 mM EDTA, 1% Nonidet P-40, and protease inhibitor cocktail) to remove the unbound proteins. The interacting proteins were eluted by 50 μl of GST elution buffer [10 mM glutathione (pH 8.0)], and the eluted solution was subjected to immunoblotting assays.

**Ub ligase activity assays**

To examine the ubiquitination of endogenous p53 protein, HepG2 cells (60% confluency, six-well plate) were transfected with the plasmids expressing HA-UB, Flag-p53, pCMV-MDM2, or shRRS1 in various combinations. At 48 hours after transfection, cells were treated with 20 μM proteasome inhibitor MG132 (Sigma-Aldrich) for 6 hours. Cells from each plate were harvested and divided into aliquots, one for immunoblotting, and the other for ubiquitination assays. Cell pellets were lysed in Hepes lysis buffer (0.02 M Hepes, 0.05 M NaCl, 1 mM Triton X-100, 1 mM NaF, 1 mM DTT, and 1 mM Na3VO4) and incubated with Flag antibody at 4°C for 2 hours and then incubated with Protein A/G Sepharose (Santa Cruz Biotechnology, USA) for 8 hours. Sepharoses were washed three times with lysis buffer and were analyzed by immunoblotting with indicated antibodies.

**Immunofluorescence assays**

HepG2 cells were seeded on glass coverslips. Cells were fixed with 4% paraformaldehyde for 30 min and permeabilized in 0.2% Triton X-100 (PBS). Cells were incubated with rabbit anti-RPL11 (1:50; ABIN2705200, Abnova, China) for 1 hour. The cells were then washed with PBS and incubated with fluorescent-conjugated secondary antibodies (Alexa Fluor 594, conjugated anti-rabbit, Invitrogen, USA). Nuclei were stained with 4,6-diamidino-2-phenylindole (100 mg/ml). Cells were examined and analyzed by Zeiss confocal microscopy and ZEISS LSM Image Browser software (version 2.80; Carl Zeiss, Germany).

**In vitro GST pull-down assays**

To confirm the interaction of RPL11 with RRS1, a GST pull-down assay was performed. RRS1 with His tag protein, RPL11 with GST tag protein, and GST tag protein were expressed in Escherichia coli, respectively. Bacteria-expressed GST or GST-RPL11 proteins were immobilized on Glutathione Sepharose 4B beads (GE Healthcare, UK) and washed, and then, the beads were incubated with His-RRS1 protein, which was purified with Ni-nitrilotriacetic agarose beads (Qiagen, Germany) for 3 hours at 4°C and eluted with His elution buffer [20 mM sodium phosphate, 500 mM NaCl, and 500 mM imidazole (pH 7.3)]. Beads were washed with GST binding buffer (100 mM NaCl, 50 mM NaF, 2 mM EDTA, 1% Nonidet P-40, and protease inhibitor cocktail) to remove the unbound proteins. The interacting proteins were eluted by 50 μl of GST elution buffer [10 mM glutathione (pH 8.0)], and the eluted solution was subjected to immunoblotting assays.

**Cell fractionation assays**

The cytoplasmic, nuclear, and nucleolar fractionations were performed as previously described with minor modifications (52). Briefly, a total of 1 × 10^9 cells were resuspended in 500 μl of hypotonic buffer [10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, and 0.5 mM DTT]. After centrifugation (500g) for 10 min, the supernatant was recovered to serve as the cytoplasmic fraction. The pellet was resuspended in 300 μl of S1 buffer (0.25 M sucrose and 10 mM MgCl2), overlaid onto 300 μl of S2 buffer (0.35 M sucrose and 0.5 mM MgCl2), and centrifuged (1500 × g) for 5 min. The supernatant was resuspended in 300 μl of S2 buffer to serve as the nuclear fraction. Then, the nuclear fraction was sonicated (30 s of sonication, 60-s intervals for 5 cycles), overlaid onto 300 μl of S3 buffer (0.88 M sucrose and 0.05 mM MgCl2), and centrifuged (3000g) for 10 min. Supernatant was collected as the nucleoplasmic fraction. The pellet was resuspended in RIPA buffer to serve as the nucleolar fraction.

**Gene set enrichment analyses**

GSEA (58) was performed to determine the biological pathways documented in the Molecular Signatures Database (MSigDB, v6.0) that are altered after knockdown of RRS1 [Gene Expression Omnibus (GEO) accession no. GSE109361]. Statistical significance was calculated by permuting the gene set 1000 times with a significance threshold of FDR q value less than 0.05.

**RRS1 codependency analysis**

The Project Achilles CRISPR-Cas9 viability database was analyzed to identify the gene dependencies that are most closely associated with RRS1 dependency. This analysis was performed on the published Achilles cell lines, composed of 18,119 gene dependency z scores across 769 cancer cell lines (39). Gene dependencies were ranked by Spearman’s correlation coefficient with RRS1 dependency across cancer cell lines, and FDR (<0.05) was controlled by the Benjamini and Hochberg method. The prereanked GSEA (58) was performed to identify the pathways positively correlated with RRS1 dependency based on MSigDB (v6.0, c2).

**Statistical analyses**

All results were confirmed in at least three independent experiments, and data from one representative experiment were shown. All
quantitative data are presented as means ± SD. For those datasets that fulfilled Pearson normality test criteria, an unpaired Student’s t test was performed; otherwise, nonparametric Mann-Whitney test was chosen. Fisher’s exact test or chi-square test was used for the analyses of contingency tables depending on the sample sizes. Kaplan-Meier method was used to estimate the survival rate, and the difference between survival curves of two groups was assessed by the log-rank test. The hazard ratio and 95% confidence interval were calculated using the univariate Cox proportional hazards regression analysis. P < 0.05 was considered as statistically significant. All the statistical analyses, except where otherwise noted, were performed using GraphPad Prism (v6) and R (v3.0.0).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/35/eabf4304/DC1

View/request a protocol for this paper from Bio-protocol.

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