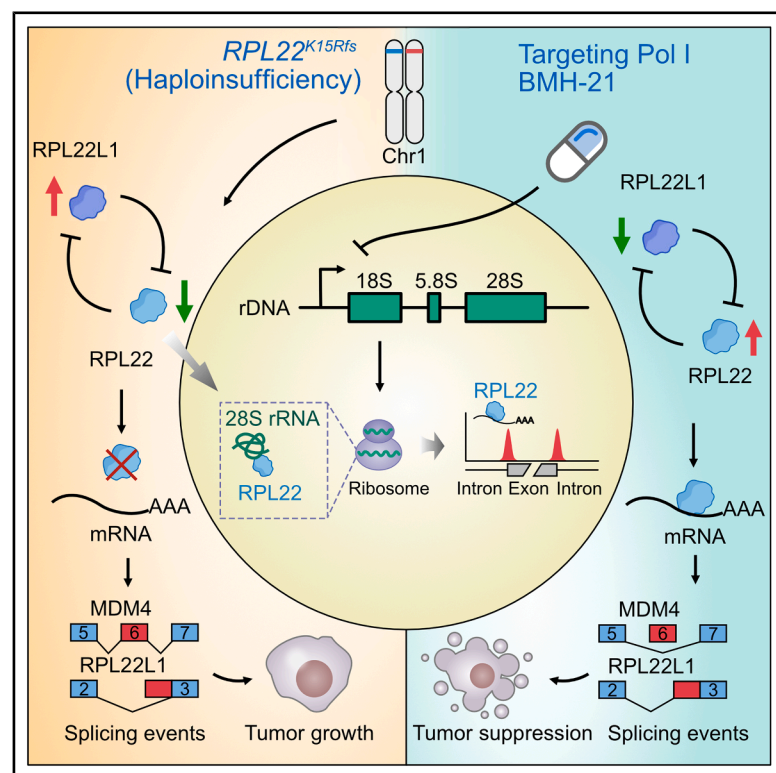


Cell Chemical Biology

Ribosomal RNA transcription regulates splicing through ribosomal protein RPL22

Graphical abstract



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In brief

Fan et al. uncovered a mechanism linking rRNA synthesis to spliceosomal regulation. Using large-scale cancer cell line screens with specific RNA polymerase I inhibitors, they identified a non-canonical ribotoxic stress pathway mediated by RPL22, RPL22L1, and MDM4, with RPL22 acting as a master splicing regulator coordinated with rRNA synthesis.

Highlights

- Unbiased screens reveal RPL22's role in regulating a ribotoxic stress pathway
- Sensitivity to Pol I inhibitors is associated with RPL22, RPL22L1, and MDM4
- Pol I inhibition broadly alters splicing, including RPL22L1 and MDM4 splicing
- RPL22 acts as a master regulator coordinating rRNA synthesis with splicing

Article

Ribosomal RNA transcription regulates splicing through ribosomal protein RPL22

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SIGNIFICANCE RNA polymerase I (Pol I) transcription is hyperactivated in cancers by most oncogenic pathways, serving as a key rate-limiting step for ribosome biogenesis and protein translation. Therapeutic targeting of Pol I is promising but requires identifying factors that drive sensitivity. Using highly specific Pol I inhibitors across a large panel of cancer cell lines, we integrated molecular features and genetic alterations to identify RPL22, RPL22L1, and MDM4 as markers linked to inhibitor sensitivity. This unbiased approach revealed not only activation of the canonical ribotoxic stress pathway but also a non-canonical pathway converging on the tumor suppressor p53. Pol I inhibition markedly suppressed oncogenic RPL22L1 and MDM4 expression and altered their splicing. Mechanistic studies showed RPL22 is essential for regulating RPL22L1 and MDM4. Deep RNA interactome analyses demonstrated that RPL22 and RPL22L1 bind hundreds of mRNAs. Furthermore, chemical and genetic Pol I inhibition caused widespread splicing perturbations, revealing a novel link between ribosome biogenesis and alternative splicing, with RPL22 playing a central role. Collectively, these findings establish RPL22 as a dual-function sensor—both a ribosomal component and a non-ribosomal regulator of cellular splicing programs. Given frequent RPL22 alterations in mismatch repair-deficient cancers of the colon, stomach, and uterus, these findings have important therapeutic implications for Pol I inhibitors. Additionally, altered splicing may impact tumor antigenicity and responsiveness to immune checkpoint inhibitors. This study introduces a new concept connecting rRNA synthesis with post-transcriptional and spliceosomal regulation.

SUMMARY

Ribosome biosynthesis is a cancer vulnerability targeted by inhibiting RNA polymerase I (Pol I) transcription. We developed specific Pol I inhibitors that activate a ribotoxic stress pathway to uncover drivers of sensitivity. Integrating multi-omics and drug response data from a large cancer cell panel, we found that RPL22 frameshift mutations confer Pol I inhibitor sensitivity. Mechanistically, RPL22 interacts directly with 28S rRNA and mRNA splice junctions, acting as a splicing regulator. RPL22 deficiency, intensified by 28S rRNA sequestration, promotes splicing of its paralog RPL22L1 and the p53 negative regulator MDM4. Both chemical and genetic inhibition of rRNA synthesis broadly remodel mRNA splicing controlling hundreds of targets. Notably, RPL22-dependent alternative splicing is reversed by Pol I inhibition, revealing a non-canonical ribotoxic stress-initiated tumor suppressive pathway. This study uncovers a robust mechanism linking rRNA synthesis activity to splicing, coordinated by the ribosomal protein RPL22.

INTRODUCTION

Ribosome biogenesis is indispensable for cancer cell growth.^{1–3} The first and rate-limiting step in ribosome biosynthesis is RNA polymerase I (Pol I) transcription, and in most cancers the transcription is pervasively activated. Key oncogenic drivers such as MYC, RAS/ERK, AKT/PKB, and mTOR upregulate Pol I transcription and conversely, the transcription is repressed by tumor suppressors most frequently inactivated in cancers, namely p53, RB1, CDKN2A (ARF), and PTEN.^{1,3–5} Despite this knowledge, Pol I has remained an underexplored target for selective inhibition of cancer cell growth. However, several chemotherapeutic drugs repress Pol I transcription.^{4–7} Most inhibit Pol I indirectly by targeting topoisomerases essential for resolving torsional stress of the heavily transcribed rRNA genes.^{5,6,8} This shortcoming hampers the understanding of the contribution of topoisomerase inhibition versus Pol I inhibition toward anticancer efficacy and compromises the identification of sensitivity drivers. CX-5461, previously nominated as specific Pol I inhibitor,⁹ has unequivocally been identified as a TOP2 targeting agent and conveys its therapeutic activity by this mechanism.^{10–13}

We have identified structurally distinct Pol I-targeting molecules that block three critical steps in Pol I transcription cycle, namely initiation, promoter escape, and elongation.^{14–20} Consequently, the inhibitors activate proteasome-dependent degradation of the enzyme catalytic subunit by SCF^{FBXL14} E3 ligase.^{14,21} Pol I degradation is only observed in transcription-competent cells, linking polymerase stability to transcriptional activity.¹⁷ The activity of the inhibitors, exemplified by tool molecule BMH-21, is genetically dependent on the Pol I enzyme complex in cancer cells and in yeast, confirming Pol I as their direct and specific target.^{17,22,23} Furthermore, genome-wide functional and biochemical analyses show that the inhibitors are selective against Pol I compared to Pol II and Pol III.^{18,19} In contrast to the chemotherapeutic Pol I inhibitors, these direct Pol I inhibitors do not cause DNA damage.^{10,14–16,24–26}

Inhibition of Pol I transcription activates a ribotoxic stress pathway that culminates in the activation of p53.^{2,27} This pathway is orchestrated by ribosomal proteins RPL5 and RPL11 that block the ability of MDM2 to ubiquitinate and degrade p53.^{2,27–29} However, this knowledge has not been therapeutically exploitable given the lack of alterations of RPL5 and RPL11 in cancers. Hallmarks of Pol I transcription stress, namely nucleolar reorganization and RPL5/RPL11 ribotoxic stress response are triggered by the BMH-21 class of inhibitors.^{14,30,31} Here, we report the discovery of an alternative ribotoxic stress mechanism that operates parallel to the RPL5/RPL11 regulatory circuitry. Using cancer cell line screens and by conducting correlative analyses for molecular features associated with Pol I inhibitor sensitivity, we identified an inactivating mutation in a tumor suppressor and ribosomal protein *RPL22*, and high expression of its paralog *RPL22L1* and p53 repressor *MDM4* as markers for sensitivity to the inhibitors. These markers were enriched in microsatellite unstable (MSI) cancer cell lines, and cell lines and tumor models bearing these markers were highly, but not exclusively, responsive to Pol I inhibition. We demonstrate that *RPL22* binds hundreds of mRNAs and alters their splicing. We find that Pol I inhibition regulates the splicing of a multitude of mRNAs and specifically, *RPL22L1* and *MDM4*,

and that this activity is conveyed by *RPL22*. Our data reveal that high rRNA synthesis activity sequesters *RPL22* into the ribosomes and counters the *RPL22*-mediated splicing regulation. Thus, *RPL22* coordinates rRNA synthesis with splicing programs. These findings provide a molecular mechanism that connects rRNA synthesis activity with post-transcriptional and spliceosomal control and reveal the perturbations of this axis in human cancers.

RESULTS

Cancer cell line screens for Pol I inhibition identify non-canonical ribotoxic stress response factors

We assessed the growth inhibition 50 (GI₅₀) of the tool compound BMH-21 and an optimized derivative, BOB-42,¹⁶ in a panel of over 300 cancer cell lines representing 18 lineages (Figure 1A). The responses of cell lines to BOB-42 and BMH-21 were highly similar with Pearson correlation (*r*) over 0.9 (Figure 1B). The most sensitive lineages were leukemia, kidney, colorectal, myeloma, pancreas and ovarian, but exceptional responders were observed in all (Figure 1C; Table S1). Cell lines that were MSI-high due to defects in mismatch repair (dMMR), were significantly more sensitive to the inhibitors than microsatellite stable (MSS) lines although exceptional responders were among those as well (Figure 1D).

Molecular features associated with the cancer cell line responses were interrogated by correlative analyses using DepMap database (Figure 1E).³² Features associated with Pol I inhibitor sensitivity showed several strong and statistically highly significant correlations. Comparison to the Achilles gene essentiality database showed high correlation with CRISPR-Cas9 knockout (KO) of three Pol I complex subunits (Figure 1F). *RPL22*^{K15Rfs} mutation was the top-ranking mutational event (Figure 1G) and *RPL22* mutant lines were significantly more sensitive to BOB-42 (Figure S1A). These were all frameshift mutations affecting almost exclusively *RPL22*^{K15R}, which is a hotspot mutation present in up to 70% MSI cancer cell lines and dMMR tumors.^{33–35} Interrogation of the RNA-seq database for transcript expression identified *RPL22L1* as the top-ranking transcript correlating with sensitivity (Figure 1H). This finding is robust, as we observed a similar correlation with BMH-21 response in an independent NCI60 cancer cell line dataset (Figure S1B).

RPL22 and *RPL22L1* are a synthetic lethal paralog pair. *RPL22* is a negative regulator of *RPL22L1* and consequently, haploinsufficiency or loss of *RPL22* increases *RPL22L1*.^{36–38} Accordingly, we found that lines with *RPL22* mutation had significantly higher expression of *RPL22L1* (Figure S1C). Finally, the top-ranking protein correlate was *MDM4* (Figure 1I). Given the sensitivity of MSI-high cancer cell lines to the inhibitors, we compared the expression of *MDM4* protein in MSS and MSI lines. As shown in Figure S1D, *MDM4* protein was significantly higher in MSI lines. Also, the expression of *MDM4* full-length (FL) splice variant and wild-type *TP53* were more frequent in the MSI lines compared to MSS lines (Figures S1E and S1F).

In contrast, neither *TP53* mutation (*p* 0.252) nor the expression of *RPL5* (*p* 0.290) or *RPL11* (*p* 0.0513) reached statistical significance in these analyses. This is consistent with the observation that while *TP53* wild-type cancer cell lines responded well to the

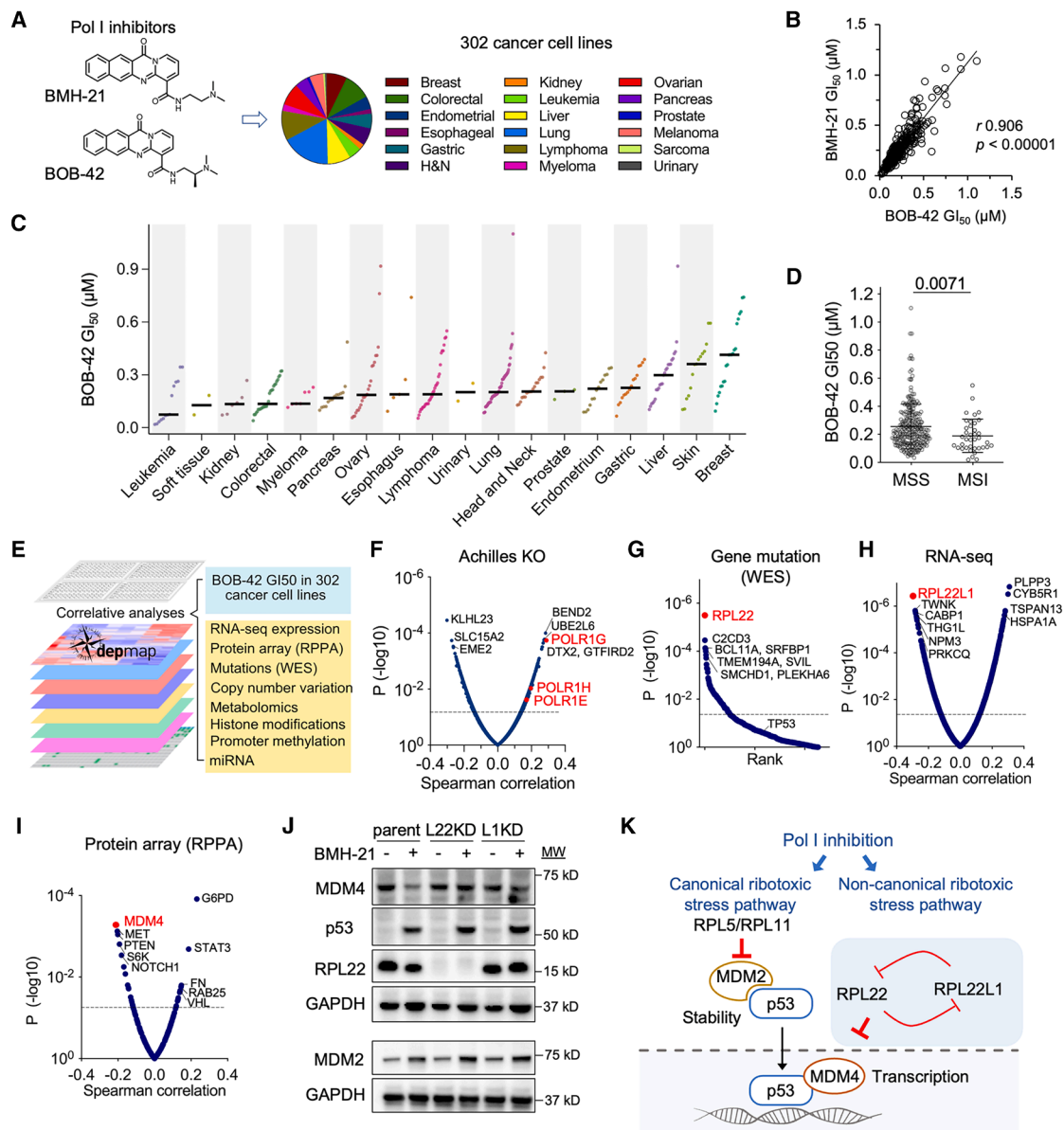


Figure 1. Cancer cell line screens identify markers for sensitivity to Pol I inhibitors

(A) Schematic of cancer cell line screens.
 (B) Pearson correlation analyses for BMH-21 and BOB-42 growth inhibitory dose 50 (GI_{50}) ($n = 302$ cell lines).
 (C) CellTiterGlo viability assay for BOB-42. GI_{50} (μM) is shown.
 (D) Dependency of BOB-42 GI_{50} on microsatellite stable (MSS) and unstable (MSI) status ($n = 291$ cell lines). p , two-tailed Mann-Whitney test.
 (E) Schematic of correlative analyses of BOB-42 cancer cell line response profiles with DepMap features.
 (F) BOB-42 GI_{50} efficacy was correlated to Achilles database. Pol I subunit genes are shown in red ($n = 184$ cell lines). Dashed line, $p = 0.05$.
 (G) Gene mutations ranked based on significance (whole exome sequencing, WES).
 (H and I) Spearman correlations of BOB-42 GI_{50} with (H) gene expression (RNA-seq) ($n = 280$ cell lines), and (I) protein expression (RPPA) ($n = 270$ cell lines).
 (J) Immunoblotting of HCT116 parent, RPL22 (L22KD) and RPL22L1 (L1KD) knockdown cells. BMH-21, 1 μM . Representative experiment of $n = 3$ is shown.
 (K) Schematic of ribotoxic stress pathways.
 See also Figure S1 and Table S1.

Pol I inhibitors, p53 is neither required nor the sole driver of sensitivity to BMH-21.¹⁴ We then tested whether RPL22 and RPL22L1 function in the classical ribotoxic stress pathway and stabilize p53. We used HCT116 MSI colorectal cancer cell line, which harbors the *RPL22*^{K15Rfs} mutation, and generated stable knock-

down lines for RPL22 and RPL22L1 (Figures S1G–S1I). Strikingly, their depletion altered the expression of neither MDM2 protein nor its transcript or affected the abundance of p53 (Figures 1J and S1K–S1M). However, p53 was robustly stabilized following treatment with the Pol I inhibitor and MDM2 was

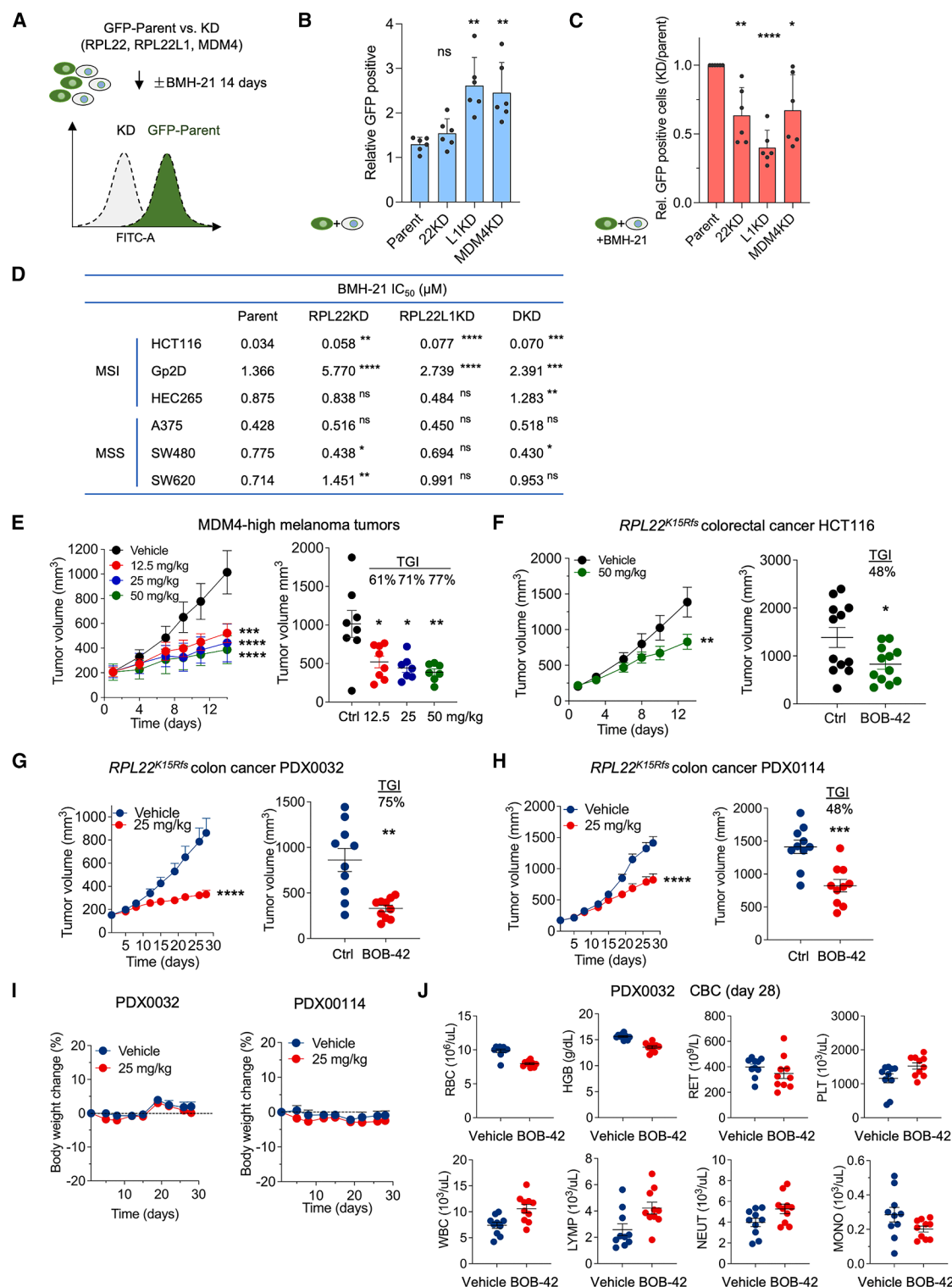


Figure 2. BOB-42 in vivo efficacy in tumor models expressing the sensitivity markers

(A) Schematic outline of GFP-competition growth assay.

(B and C) Competition assay of GFP-labelled parent cells mixed with unlabeled MDM4^{KD}, RPL22^{KD}, and RPL22L1^{KD} cells followed by flow cytometry. (B) The fraction of GFP-positive parent cells is shown. (C) Cells were treated with BMH-21 (0.25 μM) for 14 days. The relative fold change of GFP-positive cells was normalized to the parent cells set as 1. $n = 6$ independent biological replicates, mean $\pm$ SD. p values, Student's two-sided t test. ns, non-significant, * < 0.05, ** < 0.01, **** < 0.0001.

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induced in the knockdown cells (Figures 1J and S1K–S1M). Similar results were obtained in another MSI line, HEC265 (Figures S1N and S1O) as well as in two microsatellite stable lines, A375 and G361 (Figures S1P–S1S). These data show that neither RPL22 nor RPL22L1 function in the known ribotoxic stress response pathway that stabilizes p53 through MDM2. This is consistent with observations of their roles in mouse developmental models.^{39,40} These findings identify that RPL22, RPL22L1, and MDM4 correlate with sensitivity to Pol I inhibition and plausibly converge to a distinct ribotoxic stress pathway that operates in parallel to the canonical RPL5/RPL11-mediated stress response (Figure 1K).

Pol I inhibitor is highly effective in MDM4-high and RPL22-mutant preclinical mouse models

We used a GFP-competition assay to examine the marker-dependent sensitivity to BMH-21. GFP-labelled parent HCT116 cells were mixed at 1:1 ratio with either unlabeled parent, RPL22^{KD}, RPL22L1^{KD}, or MDM4^{KD} cells followed by treatment with BMH-21 and analysis by flow cytometry (Figure 2A). RPL22L1^{KD} and MDM4^{KD} caused a significant growth delay, suggesting that they promote cancer cell growth (Figure 2B). The knockdown of RPL22, MDM4, and RPL22L1 each led to significant loss of sensitivity to Pol I inhibition (Figure 2C). To expand this finding, we then used three MSI lines (HCT116, Gp2D, and HEC265) and three MSS lines (SW480, SW620, and A375) to further analyze the dependency of inhibitor-sensitivity of RPL22 and RPL22L1. For this purpose, we generated RPL22 and RPL22L1 lentiviral shRNA knockdown and double knockdown lines for each cell line. As compared to the parent lines the MSI double knockdown lines were less sensitive to Pol I inhibition (Figure 2D).

Our preclinical studies *in vivo* have shown effective targeting of cancers such as melanoma, colon and prostate by BMH-21.^{14,22} BOB-42 is an advanced molecule with optimal drug-like properties and pharmacokinetics suitable for preclinical studies (Figure S2A).¹⁶ We conducted *in vivo* efficacy studies in models expressing the identified markers. First, BOB-42 dose-dependent efficacy was tested in a MDM4-high A375 melanoma xenograft model. As shown in Figure 2E, BOB-42 effectively inhibited tumor growth by up to 77%. The treatment was well tolerated without mouse weight loss or alterations in clinical chemistry (Figure S2B; Table S2). Complete blood count differential analysis showed that while there was no change in white blood cells or platelets, the treatment caused a dose-dependent decrease in reticulocytes (Table S3). This was not considered rate-limiting for therapeutics. A further dose-response study using alternative dosing schedules showed the highest efficacy (tumor growth in-

hibition 90%) at BOB-42 100 mg/kg (Figure S2C). BOB-42 tumor uptake was excellent (30- to 100-fold as compared to plasma levels) and strongly correlated with tumor growth control (Figure S2D). Furthermore, the exposure strikingly correlated with a reduction in Pol I transcription demonstrating inhibition of the target, as shown by quantitative *in situ* hybridization for 45S precursor rRNA (Figure S2E). Analysis of BOB-42 efficacy in HCT116 tumor xenografts showed significant reduction in tumor growth (Figure 2F).

We then tested the efficacy of BOB-42 in two human MSI colon carcinoma patient-derived xenografts (PDX) wild type for *TP53* and with *RPL22*^{K15Rfs} mutation. There was up to 75% tumor growth control (Figures 2G and 2H). No toxicity signals were detected. There was no effect of the daily 4-week BOB-42 treatment on mouse well-being, weight, or complete blood count (Figures 2I and 2J).

Pol I inhibition decreases the expression of RPL22L1 and regulates its splicing

High expression of RPL22L1 correlated with drug sensitivity (Figure 1H). We hypothesized that the expression of RPL22L1 is regulated by Pol I inhibition. We observed a dose-dependent decrease in RPL22L1 by BMH-21 in both MSI and MSS lines (Figures 3A and S3A). Testing RPL22L1 expression in a kinetic experiment showed that it was repressed by 80% by BMH-21 at 16 h (Figure 3B). We then treated *TP53* proficient (p53^{+/+}) and deficient (p53^{-/-}) HCT116 isogenic cells with BMH-21 and found that the treatment profoundly decreased RPL22L1 expression in both lines (Figure 3C). We also tested the effect of the inhibitor treatment in the HCT116 xenograft tumors and observed a significant decrease in RPL22L1 (Figure 3D). However, BMH-21 caused only a minor 20% decrease of RPL22 mRNA and protein in the parent cells (Figures 3E–3G).

Given that RPL22 and RPL22L1 are a synthetic lethal paralog pair,³⁷ we also tested whether the expression of RPL22 is under the control of RPL22L1. RPL22L1 was effectively knocked out using sgRNAs (Figure S1J). RPL22 expression and protein abundance increased by 2-fold in the RPL22L1^{KO} cells, and this increase was unaffected by BMH-21 (Figures 3E–3G).

RPL22 is a haploinsufficient tumor suppressor gene.^{41,42} We analyzed RPL22 expression in a panel of colon, prostate, and endometrial cancer cell lines that were either wild type or *RPL22*^{K15Rfs} mutant. Notably, all except one *RPL22* mutant line maintained RPL22 protein expression consistent with heterozygosity (Figure 3H). RPL22 negatively regulates RPL22L1.³⁶ We hence tested whether the regulation of RPL22L1 by Pol I inhibition depends on RPL22. BMH-21 significantly decreased RPL22L1 in each line, irrespective of *RPL22* or *TP53* mutation (Figure 3I). Given

(D) CellTiterBlue viability assay for BMH-21. IC₅₀ (μM) is shown. *p* values, ordinary one-way ANOVA.

(E) Tumor growth curves and volumes of MDM4-high A375 melanoma xenografts following treatments with BOB-42. *n* = 8 mice/group. TGI, tumor growth inhibition.

(F) Tumor growth curves and volumes of HCT116 colon cancer xenografts following treatment with BOB-42 (50 mg/kg). *n* = 12 mice/group.

(G and H) Tumor growth curves and volumes of human *RPL22*^{K15Rfs} *TP53* wild-type MSI-high colon carcinoma patient-derived xenografts (PDX) following treatment with BOB-42 (25 mg/kg). (G) PDX0032. (H) PDX0114. *n* = 10 mice/group.

(I) Mouse body weights in PDX tumors PDX0032 (G) and PDX0114 (H).

(J) Complete blood counts in (G).

Whiskers and bars in E – J represent mean ± SEM. *p* values for tumor growth curves in E, F, G and H, ordinary two-Way ANOVA and for volumes, unpaired two-sided t-test with Welch's correction.

See also Figure S2, Tables S2 and S3.

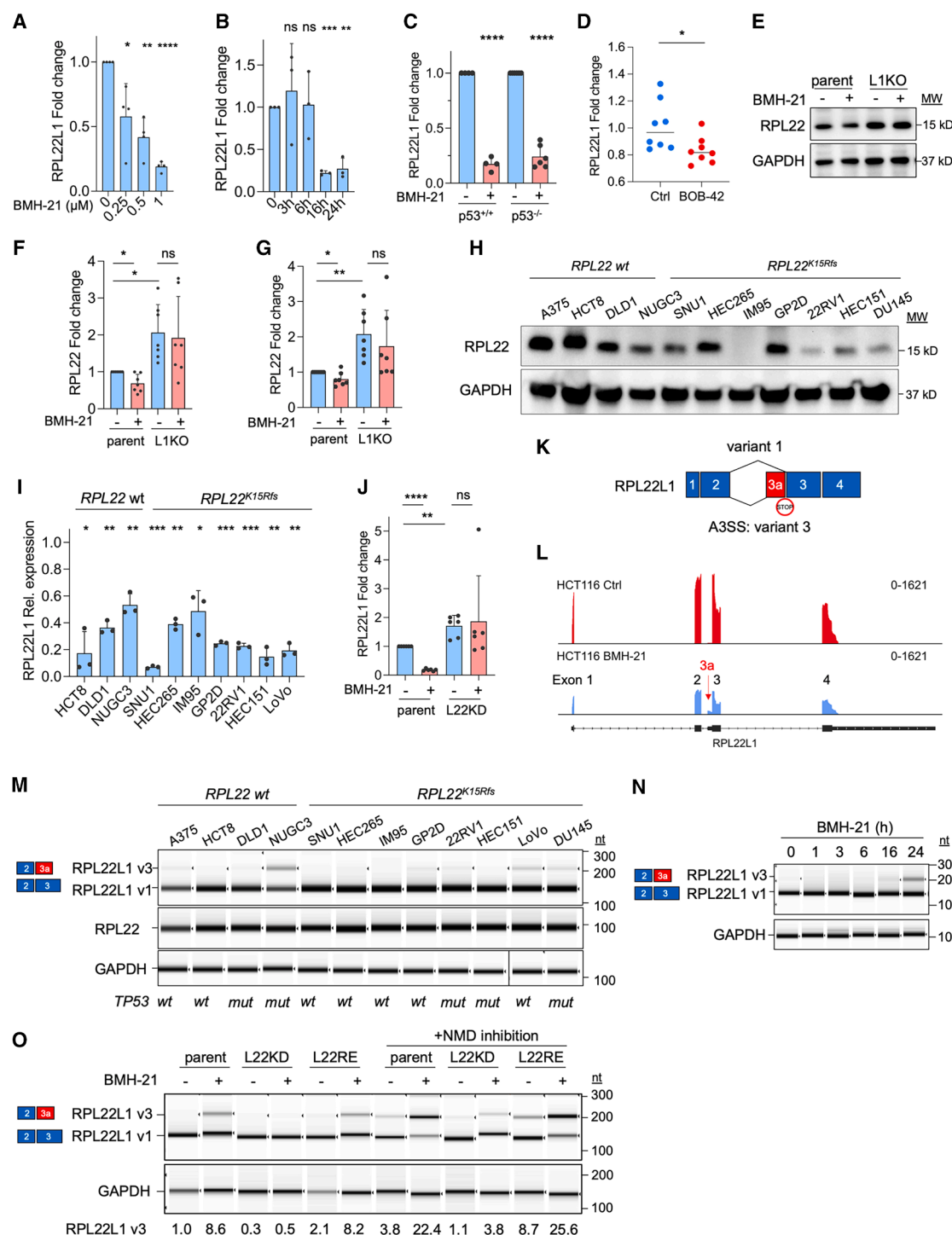


Figure 3. Pol I inhibition decreases the expression of RPL22L1

(A) qPCR for RPL22L1 in HCT116 p53^{+/+} cells treated with the indicated doses of BMH-21. (B) qPCR for RPL22L1 in HCT116 p53^{+/+} cells treated with BMH-21 (1 μM) for the indicated times. (C) qPCR for RPL22L1 in HCT116 TP53 isogenic cells treated with BMH-21 (1 μM). (D) qPCR for RPL22L1 in HCT116 xenograft tumors in Figure 2F. (E and F) Immunoblotting and quantification of RPL22. BMH-21, 1 μM. (G) qPCR for RPL22 in parent and RPL22L1^{KO} cells. BMH-21, 1 μM. (H) Immunoblotting for RPL22 in cancer cell lines wild type (wt) or mutant for RPL22. TP53 mutant cell lines are indicated. (I) qPCR for RPL22L1 in cancer cell lines wild type (wt) or mutant for RPL22. BMH-21, 1 μM.

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that the MSI lines maintained substantial RPL22 expression, we used stable RPL22 knockdown (RPL22^{KD}) cells to assess this dependency. RPL22^{KD} led to a moderate but significant compensatory increase in RPL22L1 (Figure 3J). Remarkably, the decrease in RPL22L1 by BMH-21 was abrogated by RPL22 knockdown (Figure 3J). Similar results were observed in another MSI line, HEC265, and two MSS lines, A375 and G361 (Figure S3B).

RPL22L1 undergoes alternative splicing.^{43–45} Splicing of an alternative 3' splice site in intron 2 leads to a frameshift and expression of a putative 64 amino acid product with an early stop codon that lacks the RPL22L1 RNA interaction domain and is termed as variant 3 here (Figure 3K). Variant 3 has been detected in glioblastoma cells.⁴³ For unbiased analysis of RPL22L1 splicing and its regulation by Pol I inhibition, we first conducted RNA-seq in both MSI and MSS cells and visualized the reads using Integrative Genomics Viewer. These showed increased number of reads on variant 3 (Figures 3L and S3C). We then analyzed the main isoform (called variant 1) and variant 3 using splicing specific primers by semiquantitative PCR (sqPCR) in cancer cell lines and identified constitutive, albeit low expression of variant 3 in several lines independent of RPL22 mutation (Figure 3M). We then analyzed RPL22L1 isoform regulation by Pol I inhibition and observed increased variant 3 expression following 16-h BMH-21 treatment (Figure 3N). We then asked whether the splicing is dependent on RPL22. RPL22 knockdown led to the complete abrogation of RPL22L1 splicing by Pol I inhibition, whereas the reconstitution of RPL22 in the RPL22^{K15Rfs} mutant cells increased the expression of variant 3 by BMH-21 (Figure 3O). Variant 3 expression was further stabilized by inhibiting nonsense-mediated decay (NMD) by blocking SMG1 or by using anisomycin, a less selective NMD inhibitor (Figures 3O and S3D).^{46,47} Together, these findings demonstrated that RPL22L1 variant 1 and 3 expression is oppositely regulated by Pol I inhibition in a RPL22-dependent manner and showed that Pol I inhibition predominantly affects RPL22L1 but not RPL22 abundance.

Repression of rRNA synthesis decreases MDM4 by altering its splicing

Ribotoxic stress stabilizes p53 by inhibition of MDM2 by RPL5 and RPL11.² Previous studies showed that MDM4 protein stability was decreased by ribotoxic stress.^{48,49} We first analyzed changes in MDM4 protein abundance by using BMH-21. MDM4 protein robustly decreased and became undetectable by 16 h in both HCT116 p53 isogenic cell lines (Figure 4A). In parallel, p53 was stabilized and p53 target genes CDKN1A and MDM2 were induced in p53^{+/+} but not in p53^{-/-} cells indicating transcriptional activation of p53 (Figures 4A, S4A, and S4B).

Earlier studies linked RPL22, RPL22L1, and MDM4 with sensitivity to MDM2 inhibitor Nutlin-3a.^{35,44} However, these relation-

ships were not mechanistically investigated. Alternative splicing of MDM4 generates MDM4 full-length (FL) and short (S) isoforms by inclusion or skipping of exon 6, respectively (Figure 4B).^{50–53} MDM4-FL represses p53, whereas MDM4-S transcript undergoes NMD.⁵² We hypothesized that Pol I stress could impact MDM4 splicing. We analyzed MDM4 splicing by using sqPCR and qPCR using splicing specific primers in p53^{+/+} and p53^{-/-} cells. As shown in Figures 4C–4E, BMH-21 robustly decreased the expression of MDM4-FL by 80% in both lines. This response was dose-dependent in both MSI and MSS lines (Figure S4C). MDM4-S transcript increased by over 20-fold, but only in p53 proficient cells (Figure 4E). This showed that MDM4-FL splicing was independent of p53, while that of MDM4-S was not. MDM4-FL/S ratio, which robustly predicts the expression of MDM4 protein,⁵² was profoundly repressed in both p53 isogenic lines (Figure 4F).

We then tested MDM4 splicing in ten cancer cell lines that were either RPL22 wild-type or mutant. First, analysis of these lines by sqPCR showed that they highly expressed MDM4-FL, and that TP53 mutant lines also highly expressed MDM4-S consistent with the earlier observation that TP53 mutation was associated with MDM4 splicing change (Figure 4G).³⁵ Second, BMH-21 decreased the expression of MDM4-FL in all MSI and two MSS lines irrespective of RPL22 or TP53 genotype (Figures 4H, S4D, and S4E). However, the decrease in MDM4-FL strongly and significantly correlated with the decrease in RPL22L1 (Pearson r 0.864, p 0.00128) shown in Figure 3I highlighting their coordinate regulation by Pol I inhibition (Figure 4I).

To further explore the regulation of RPL22L1 and MDM4-FL we used a broad-spectrum Pol I inhibitor actinomycin D and CLK-kinase inhibitor TG003 that inhibits SRS-splicing factor phosphorylation,⁵⁴ and compared these to MDM2 inhibitor Nutlin-3a. Temporal analysis of these chemical perturbations showed that actinomycin D reduced both RPL22L1 and MDM4-FL, whereas Nutlin-3a was largely without effect on either MDM4 isoform or RPL22L1 (Figure S4F). TG003 robustly increased the expression of MDM4-S consistent with its ability to inhibit MDM4-regulating splicing factors (Figure S4F).⁵¹ These data underscore that Pol I inhibitors possess an exceptional capacity to inhibit both MDM4 and RPL22L1.

MDM4 splicing is dependent on RPL22

Earlier studies in developmental models showed that RPL22 causes exon skipping and RPL22L1 exon inclusion of Smad2.⁵⁵ Having ascertained that Pol I activity regulates MDM4 isoform expression, we asked whether RPL22 or RPL22L1 regulate MDM4 splicing. For unbiased assessment of splicing, we conducted RNA-seq followed by the analysis of splicing alterations using rMATS⁵⁶ in HCT116 parent cells, cells reconstituted with

(J) qPCR for RPL22L1 in parent and RPL22^{KD} cells. BMH-21, 1 μ M.

(K) Schematic of RPL22L1 splicing.

(L) IGV plots of RNA-seq reads on RPL22L1 gene in HCT116 p53^{+/+} cells treated with BMH-21 (1 μ M) for 24 h

(M) RPL22L1 isoforms in cancer cell lines. RPL22 genotypes and TP53 mutant lines are indicated. Representative sqPCR is shown.

(N) RPL22L1 isoforms in p53^{+/+} cells. BMH-21, 1 μ M. Representative sqPCR of $n = 2$ is shown.

(O) RPL22L1 isoforms in RPL22^{KD} and RPL22^{RE} cells treated with BMH-21 (1 μ M) and SMG1 inhibitor 11e (0.2 μ M). Representative sqPCR of $n = 2$ is shown. Data are representative of at least three independent experiments (A–C, E–G, I, J) and mean $\pm$ SD are shown. p values, Student's two-sided t test. ns, non-significant, * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

See also Figure S3.

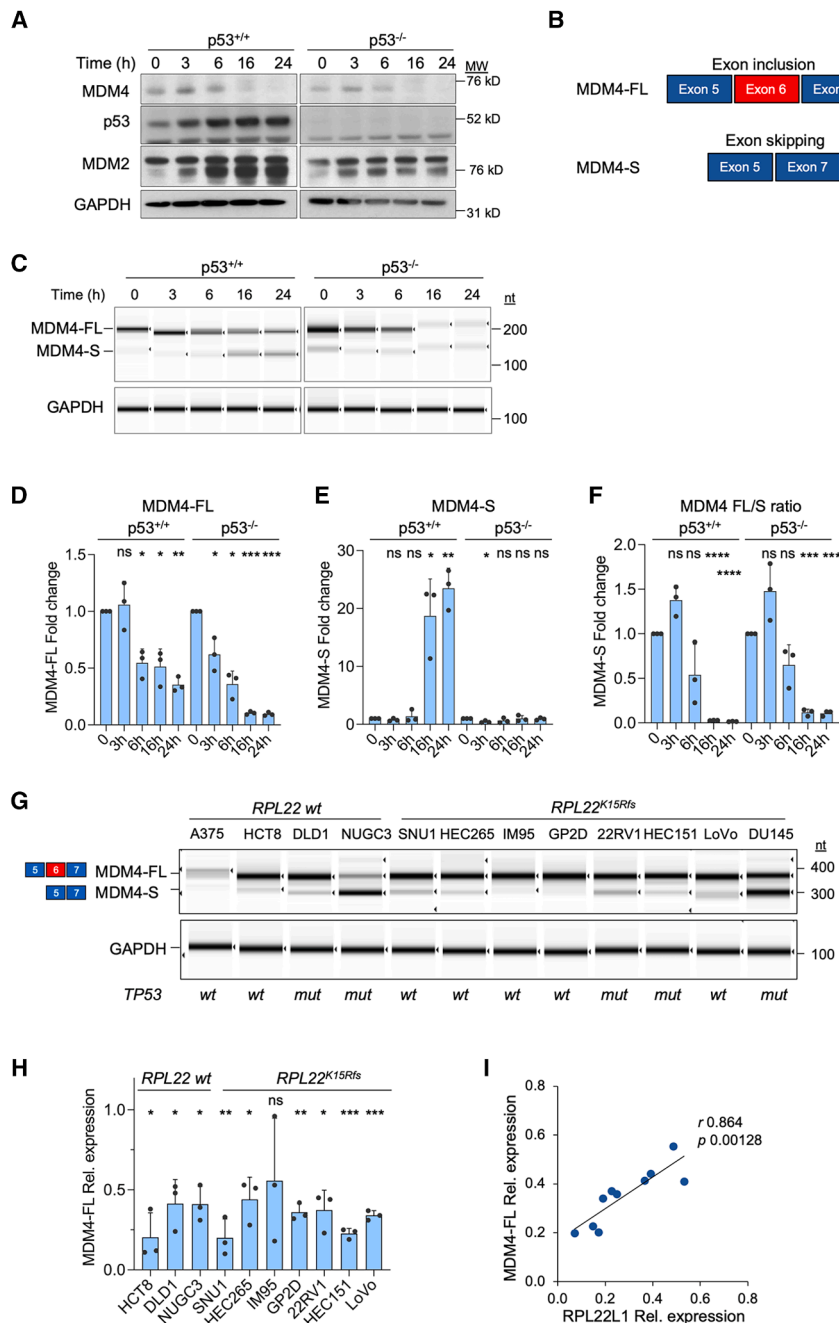


Figure 4. Pol I inhibitors alter MDM4 splicing and repress MDM4-FL

(A) Immunoblotting of HCT116 isogenic cells. BMH-21, 1 μ M. (B) Schematic diagram of MDM4 splicing. (C) SqPCR analysis for MDM4-FL and MDM4-S isoforms in p53 isogenic cells. BMH-21, 1 μ M. (D–F) p53^{+/+} and p53^{-/-} cells were treated with BMH-21 (1 μ M) for the indicated times. (D) MDM4-FL and (E) MDM4-S isoforms were measured by qPCR and their ratios are shown in (F). BMH-21, 1 μ M. (G) MDM4 isoforms in cancer cell lines. Representative sqPCR of $n = 2$ is shown. (H) qPCR for MDM4-FL in cancer cell lines. *RPL22* genotypes and *TP53* mutant lines are indicated. BMH-21, 1 μ M. (I) Correlation of regulation of MDM4-FL and RPL22L1 by BMH-21. *Inset*, Pearson r and statistical significance. Data are representative of at least three independent experiments (A, C, D–F, H, I) and mean $\pm$ SD are shown. p values, Student's two-sided t test. ns, non-significant, * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001. See also Figure S4.

MDM4-S increased in RPL22L1^{KO} cells (Figure 5G). The strong repression of MDM4-FL by BMH-21 was not rescued. However, RPL22 knockdown, but not its reconstitution, completely abrogated the BMH-21-mediated decrease of MDM4-FL (Figure 5H). These findings were corroborated by analyses of the MDM4 splice variants by using qPCR and showed that MDM4 splicing by BMH-21 depended on RPL22 but was independent on RPL22L1 (Figures 5I and 5J). However, as RPL22 has no splicing function it was ambiguous how this dependency was conveyed.

Pol I inhibition perturbs splicing programs

These findings raised the possibility that Pol I activity relates to splicing programs. To assess global transcriptional response to Pol I inhibition stress, we

RPL22 expression (RPL22^{RE}), and RPL22L1^{KO} cells (Figures 5A and S5A). We uncovered substantial alterations in RNA transcript expression and broad changes in splicing in RPL22^{RE} and RPL22L1^{KO} cells (Figures S5B and S5D). Over 1,500 splicing events were detected in each line, and these were predominantly exon-skipping events (Figures 5B and 5C; Table S4). Splicing of MDM4 was a highly significant event in both RPL22L1^{KO} and RPL22^{RE} cells, and Sashimi plot analysis showed an increase in exon 6 skipping (Figures 5D–5F). We then used these cells to analyze basal and BMH-21-regulated splicing of MDM4. Analysis of the splicing variants using sqPCR showed that the basal expression of MDM4-FL was reduced, and the expression of

conducted RNA-seq analysis in p53^{+/+} and p53^{-/-} cells and A375 melanoma cells (wild-type for both *TP53* and *RPL22*) following treatment with BMH-21 (Figure S6A). The alterations of Pol II transcripts were broad and robust, and as expected, GSEA pathway analyses showed prominent activation of p53 pathway in the p53 proficient cells, and increased expression of p53 target genes (Figure S6B). We then conducted rMATS analyses of the RNA-seq data and found 300–600 splicing events in each line using stringent criteria (FDR < 0.05, minimum 10 reads for each event) (Figures 6A–6C and S6C; Table S5). Spliceosome was the most significant pathway identified (Figures 6A–6C). This finding was striking, as the splicing factors undergo

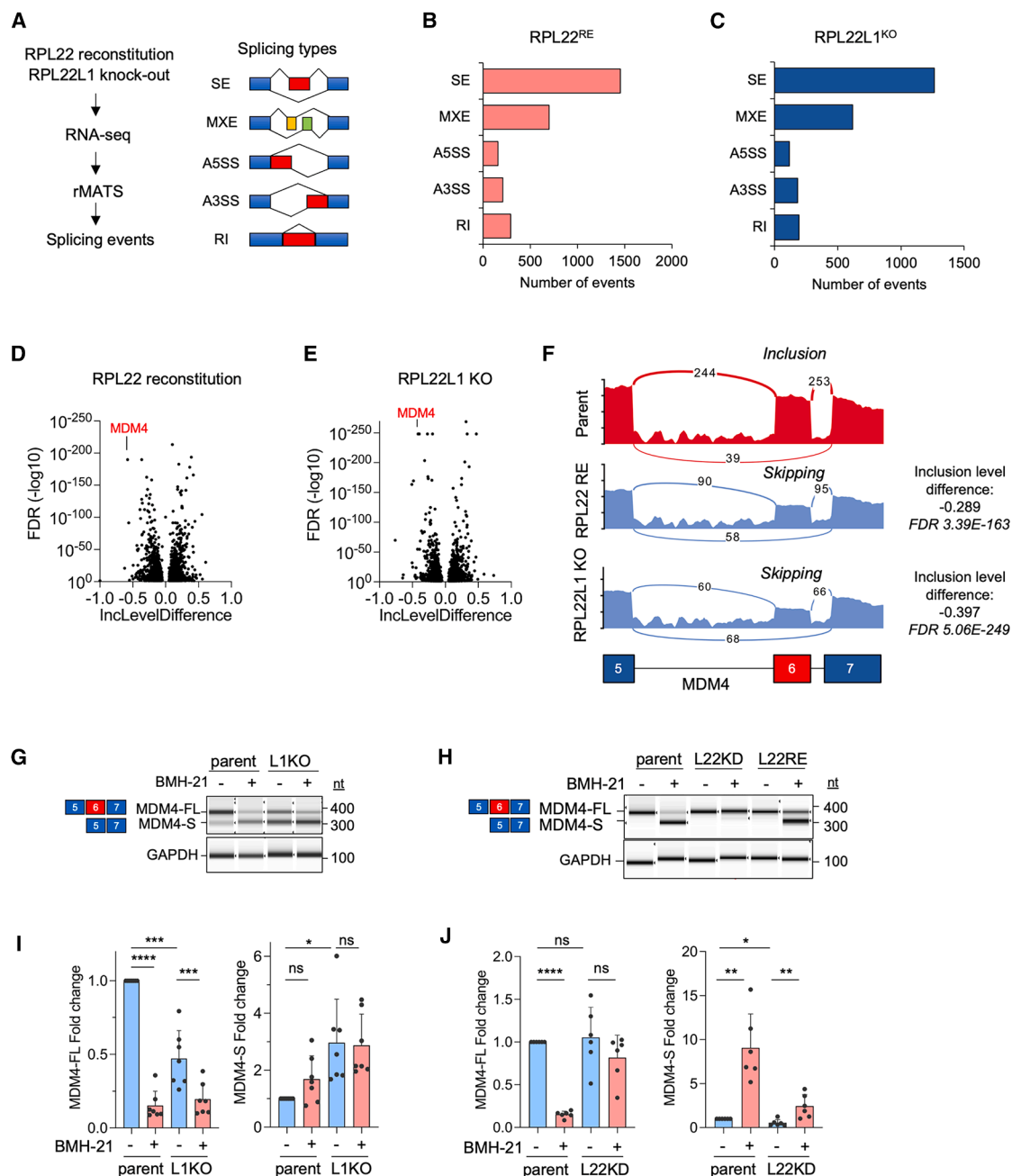


Figure 5. RPL22 is essential for regulation of MDM4 splicing by Pol I inhibition

(A) Schematic diagram of workflow and splicing events. SE, skipped exon; MXE, mutually exclusive exon; A5SS, alternative 5' splice site; A3SS, alternative 3' splice site; RI, retained intron.
(B) Splicing events in RPL22^{RE} cells.
(C) Splicing events in RPL22L1^{KO} cells.
(D and E) Volcano plots of splicing events in RPL22^{RE} and RPL22L1^{KO} cells.
(F) Sashimi plot of MDM4 splicing in RPL22^{RE} and RPL22L1^{KO} cells.
(G) MDM4 isoforms in RPL22L1^{KO} cells. BMH-21, 1 μ M.
(H) MDM4 isoforms in RPL22^{RE} and RPL22^{KD} cells. BMH-21, 1 μ M. A representative sqPCR of $n = 2$ biological replicates is shown.
(I) qPCR for MDM4 isoforms in parent and RPL22L1^{KO} cells. BMH-21, 1 μ M.
(J) Same as in I, RPL22^{KD} cells. Data are representative of at least three independent experiments (G, I, J) and mean $\pm$ SD are shown. p values, Student's two-sided t test. ns, non-significant, * < 0.05 , ** < 0.01 , *** < 0.001 , **** < 0.0001 .
See also Figure S5 and Table S4.

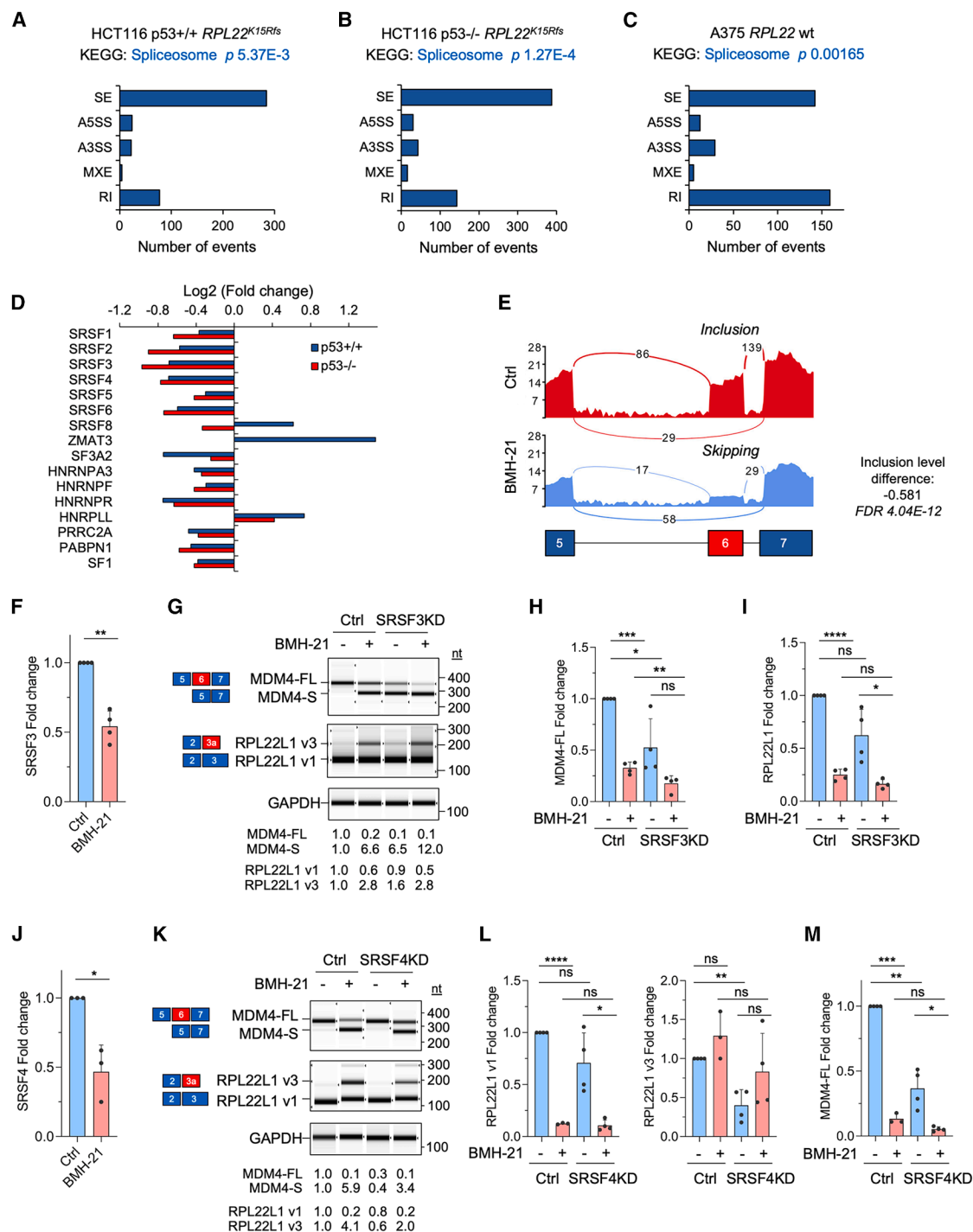


Figure 6. Pol I inhibition perturbs splicing programs

(A–C) rMATs and pathway analyses in BMH-21 treated cells. (A) p53^{+/+} cells, (B) p53^{-/-} cells and (C) A375 melanoma cells. BMH-21, 1 μ M. (D) RNA-seq data of splicing factors. BMH-21, 1 μ M. *n* = 3 biological replicates. Statistics, *q*-values <0.05. (E) Sashimi plot of MDM4 splicing following BMH-21 treatment in A375 cells. (F) qPCR for SRSF3 following BMH-21 (1 μ M) treatment. (G) MDM4 and RPL22L1 isoforms following SRSF3 knockdown. Representative sqPCR of *n* = 3 is shown. (H) qPCR for MDM4 isoforms in parent and SRSF3^{KD} cells. (I) qPCR for RPL22L1 in parent and SRSF3^{KD} cells. (J) qPCR for SRSF4 following BMH-21 (1 μ M) treatment.

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autoregulation.⁵⁷ By analyzing shared splicing factor alterations in the p53^{+/+} and p53^{-/-} cells we saw that transcripts for many splicing factors were repressed following Pol I stress (Figure 6D). These unbiased analyses demonstrated that Pol I inhibition stress perturbs splicing and splicing factor expression.

We then focused on identification of splicing factors involved in the splicing of MDM4 and RPL22L1. Isoform analysis showed that BMH-21 decreased the expression of MDM4-FL in all cell lines (Figure S6D). We visualized MDM4 splicing using Sashimi plot, which showed that BMH-21-treatment significantly increased exon 6 skipping (Figure 6E). The previously identified MDM4 splicing factors are serine-arginine rich splicing factor 3 (SRSF3), protein arginine methyltransferase 5 (PRMT5), and p53 target gene ZMAT3.^{51,52,58,59} SRSF3 and PRMT5 promote MDM4 exon 6 inclusion and ZMAT3 exon 6 skipping. ZMAT3 increases MDM4-S expression in a p53-dependent manner.⁵⁹ ZMAT3 was induced by BMH-21 but only in p53-proficient cells (Figure 6D). Therefore, ZMAT3 is unlikely to be responsible for the p53-independent regulation of MDM4. Instead, SRSF3 was repressed in all cell lines (Figure 6D). We further analyzed the effect of BMH-21 on SRSF3 by using qPCR and found that SRSF3 transcript was downregulated by BMH-21 by 50%, and its protein abundance by 40% (Figures 6F and S6E). We then used sqPCR and qPCR to detect MDM4 splicing isoforms and saw that SRSF3 knockdown decreased basal MDM4-FL, which was further exacerbated by BMH-21 (Figures 6G, 6H, and S6F). These approaches confirm that SRSF3 regulates MDM4 exon 6 inclusion in a Pol I activity-dependent manner. However, SRSF3 did not alter RPL22L1 splicing or affect that by BMH-21 (Figures 6G and 6I).

SRSF4 has been implicated earlier to alter RPL22L1 splicing.⁴³ As expected based on RNA-seq, BMH-21 decreased SRSF4 (Figure 6J). We then used SRSF4 KD and observed that it significantly decreased RPL22L1 variant 3 expression (Figures 6K, 6L, and S6G). Interestingly, the knockdown also decreased MDM4-FL (Figure 6M). We conclude that multiple SRSF splicing factors are regulated by Pol I inhibition and alter the splicing of MDM4 and RPL22L1.

RPL22 connects rRNA synthesis activity with splicing

RPL22 is a constituent member of the ribosome and binds 28S rRNA. This interaction is well resolved in several ribosome cryo-EM structures and mediated by RPL22 codons identified critical for RNA binding.^{60–63} RPL22 also interacts with cellular and viral RNAs that have a small hairpin stem loop with a loosely defined sequence.^{64,65} In zebrafish, RPL22 negatively regulates RPL22L1 by binding to a stem loop on exon 2.³⁶ We identified two potential stem loops in human RPL22L1 intron 2 (Figure S7A). Strikingly, these sequences overlapped the RPL22L1 variant 3 intronic splice site. Given this we hypothesized that the ability of RPL22 to bind to RPL22L1 pre-mRNA mediates the repression of RPL22L1. To test whether the intronic sequences are essential for RPL22L1 repression, we expressed RPL22L1 from a cDNA vector. As shown in Figure S7B, BMH-

21 prominently decreased the endogenous but not the ectopically expressed RPL22L1 transcript.

Comparative genome-wide RNA-binding by RPL22 and RPL22L1 has not been analyzed previously. To assess this, we generated cell lines that stably express RPL22 and RPL22L1 HaloTag fusion proteins, whereas cells expressing HaloTag served as control (Figure S7C). We used GoldCLIP-seq⁶⁶ for RNA-pulldowns that enable highly effective RNA-interactome analyses by the virtue of covalent coupling to HaloTag resins and the precision on binding site analysis gained by using RNase digestion (Figure 7A). RPL22 and RPL22L1 interactomes included several RNA species, most prominently rRNA and mRNAs but also a large number non-coding RNAs such as long intergenic non-coding RNAs, miRNAs, small nucleolar RNAs, and RNAs involved in major and minor site splicing (Figure S7D; Table S6). RPL22-HaloTag showed strong preferential binding to 28S rRNA sequence in region III identified in ribosome structural studies as the RPL22 interface (Figures 7B and S7E).^{61–63} RPL22L1-HaloTag was also bound to this site, albeit at greatly lower frequency, likely reflecting RPL22 and RPL22L1 stoichiometry as ribosome constituents. This 28S rRNA sequence has a long stem structure with two mirrored loops but lacks the small stem loops (Figure 7B). We did not detect binding of either protein to 28S rRNA sequences previously identified by SELEX analysis.⁶⁵

The distribution of RPL22 and RPL22L1 on coding genes was striking as both were enriched on introns (Figure S7F). Metagenome analysis on read coverage showed their enrichment at 5' and 3' splice site junctions (Figure 7C). Motif analyses using HOMER identified several significant motifs most of which were distinct for RPL22 and RPL22L1 (Figure S7G). Strikingly, RPL22-HaloTag was prominently and exclusively enriched on MDM4 intron 6 as two distinct peaks that overlapped the predicted SRSF3 and ZMAT3-binding sites (Figure 7D).^{52,59} Instead, RPL22L1 had low read coverage on MDM4 pre-mRNA (Figure 7D). RPL22, but not RPL22L1-HaloTag was enriched on RPL22L1 intron 2 overlapping the splice site for variant 3 (Figure 7E). Interestingly, RPL22L1-HaloTag was enriched on its own exons, suggesting that it could regulate the stability of its transcript (Figure 7E).

We conducted an intersection analysis to ask whether RPL22 or RPL22L1 binding peaks overlapped with the splicing alterations detected in RPL22^{RE} and RPL22L1^{KO} cells. We identified approximately 1,000 genes each for RPL22 and RPL22L1 where splicing events overlapped their binding sites (Figure S7H). Of these, nearly 300 genes were shared, such as MDM4, CD44, MLH1, and MLH3 (Figure S7H). Despite substantial overlap of their binding sites, hundreds of genes were unique suggesting independent regulatory activities by the paralogs (Figure S7H). For RPL22-bound transcripts, the majority were represented by exon skipping and mixed exon splicing events (Figure S7I).

To further assess the dependency of the splicing events on rRNA synthesis activity we used genetic inhibition of Pol I. We knocked down Pol I enzyme catalytic subunits RPA1, RPA2

(K) MDM4 and RPL22L1 isoforms in SRSF4^{KD} cells. Representative sqPCR of $n = 2$ is shown.

(L) qPCR for RPL22L1 variants in parent and SRSF4^{KD} cells.

(M) qPCR for MDM4-FL in parent and SRSF4^{KD} cells. Data are representative of at least three independent experiments (F, G, H, I, J, L, M) and mean $\pm$ SD are shown. p values, Student's two-sided t test. ns, non-significant, * <0.05, ** <0.01, *** <0.001, **** <0.0001. See also Figure S6 and Table S5.

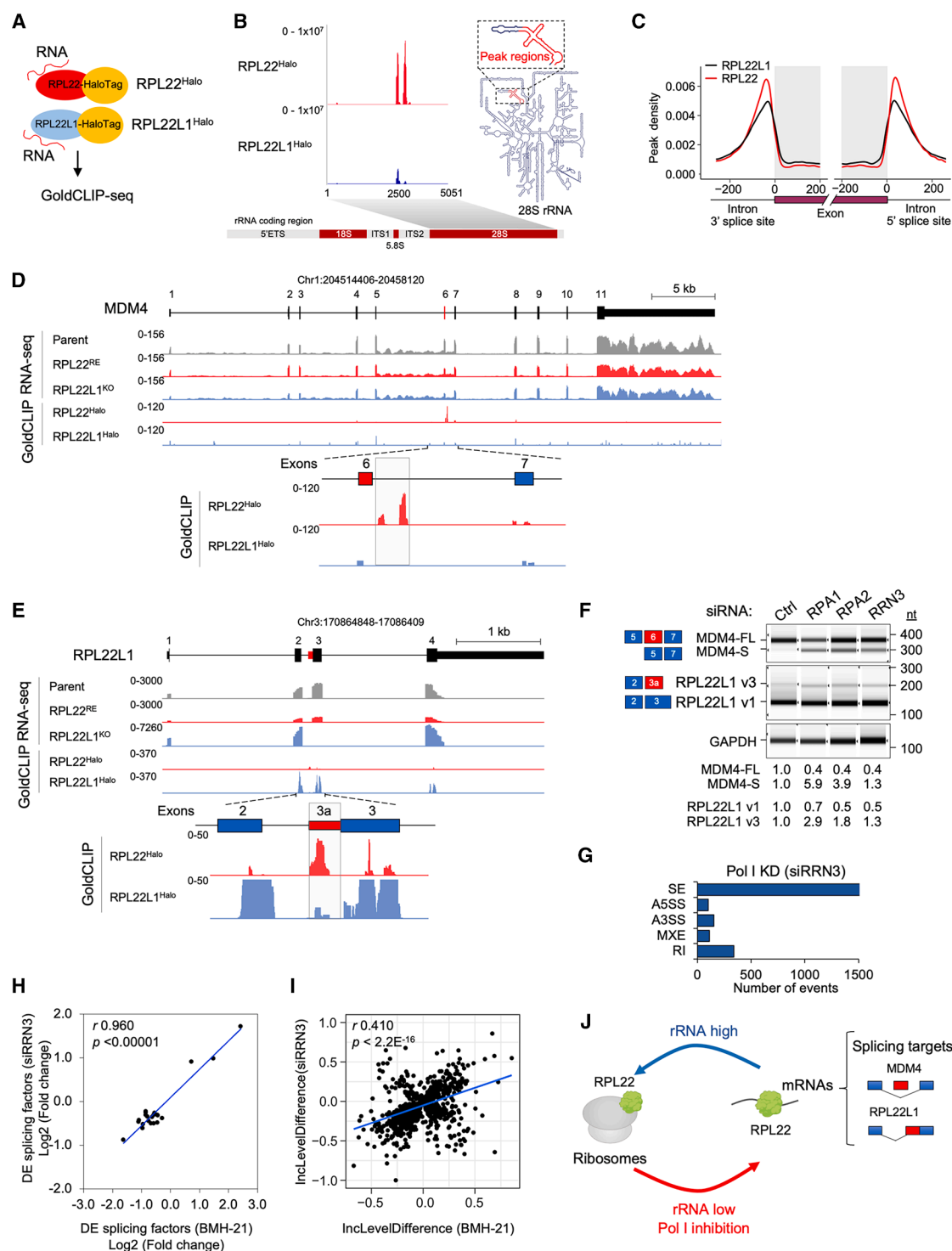


Figure 7. RPL22 coordinates splicing with rRNA synthesis

(A) Schematic of GoldCLIP-seq.
(B) RPL22^{Halo} and RPL22L1^{Halo} binding to 28S rRNA.
(C) Metagene plots of RNA binding peaks in RPL22^{Halo} and RPL22L1^{Halo} cells.
(D) MDM4 expression tracks and HaloTag-fusion protein binding peaks of RPL22 and RPL22L1. Bottom tracks visualize binding peaks in intron 6.
(E) RPL22L1 expression tracks and HaloTag-fusion protein binding peaks of RPL22 and RPL22L1. Bottom tracks visualize binding peaks in intron 2/exon 3a.
(F) RPL22L1 and MDM4 isoforms of Pol I knockdown cells. Representative sqPCR of $n = 3$ and quantification are shown.

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and the essential preinitiation factor RRN3. Each knockdown decreased Pol I transcription by half (Figures S7J and S7K). The splicing of both MDM4 and RPL22L1 substantially and significantly altered (Figures 7F, S7L, and S7M). MDM4-FL was decreased by 40% and MDM4-S was increased by 5-fold by Pol I knockdown (Figure S7M). Furthermore, RPL22L1 variant 1 decreased whereas RPL22 was unaffected (Figures S7L and S7N). Consistent with the drug effect, genetic inhibition of Pol I also significantly decreased SRSF3 expression (Figure S7O). For unbiased assessment whether Pol I genetic inhibition alters splicing factor expression similarly to the chemical inhibition, we conducted RNA-seq in the Pol I KD cells followed by rMATS analysis. rMATS analysis showed extensive alterations in splicing events dominated by exon skipping (Figure 7G). Alterations in the expression of splicing factors were highly similar by the genetic and chemical inhibition (Figure 7H). Comparison of the splicing events by genetic and chemical inhibition of Pol I showed strong and significant correlation (Figure 7I). We conclude that both chemical and genetic inhibition of Pol I robustly perturb expression of splicing factors and splicing and specifically those of MDM4 and RPL22L1.

These data are consistent with a model that RPL22 negatively regulates the splicing and expression of RPL22L1 and MDM4 and that this depends on rRNA synthesis activity (Figure 7J). Accordingly, RPL22 coordinates rRNA synthesis and splicing.

DISCUSSION

Here, we describe a non-canonical ribotoxic stress mechanism that connects rRNA synthesis to cellular splicing by ribosomal protein RPL22. We find that cancer-haploinsufficient RPL22, a constitutive member of the 60S ribosome and 28S rRNA interacting ribosomal protein, binds to hundreds of mRNAs affecting their splicing and is essential for the expression and splicing of its paralog RPL22L1 and MDM4. Strikingly, the ability of RPL22 to repress the expression of RPL22L1 and MDM4 is dependent on rRNA synthesis activity. We present a model (Figure 7J) of how the rRNA synthesis activity defines the distribution of RPL22 between the ribosomes and the target pre-mRNAs controlling the expression of transcripts such as RPL22L1 and the p53 repressive isoform MDM4-FL. Whereas high rRNA synthesis activity sequesters RPL22 into the ribosomes, therapeutic inhibition of Pol I restores RPL22 interaction with its mRNA targets. Given that RPL22 binds mRNAs at exon-intron junctions and changes the splicing of hundreds of genes, it suggests that the impact of these regulatory events in *RPL22*-altered cancers is broad. These findings reveal an unexpected coordination of cellular splicing with rRNA synthesis mediated by RPL22.

Cancer cell line vulnerability screens identified that the sensitivity to Pol I inhibition is linked with *RPL22* mutation and high expression of RPL22L1 and MDM4. MSI cancer cell lines were highly, but not exclusively sensitive to the inhibitors. Cell-based

competition models validated that RPL22, RPL22L1, and MDM4 each conveyed sensitivity to the Pol I inhibitors. Earlier studies had recognized RPL22 and RPL22L1 synthetic paralog relationship and high frequency of *RPL22* mutations in MSI cancers and to highly express RPL22L1 and MDM4-FL.^{35–37} These markers were proposed to mediate the sensitivity to MDM2 inhibitor Nutlin-3a, but this notion was not further explored.^{35,43} We dissected the identified sensitivities and their mechanistic underpinnings and found that pharmacological and genetic inhibition of Pol I decreased the expression of RPL22L1 and MDM4, and that this decrease coupled with their splicing alterations in a RPL22-dependent manner. Nutlin-3a lacked these activities. This mechanism is distinct from, but parallel to the transcription stress-induced reorganization of the nucleolus and inhibition of MDM2 by ribosomal proteins RPL5 and RPL11. Both mechanisms uniquely converge on p53 (Figure 1K). MDM2 inactivation stabilizes p53 while MDM4 splicing change abrogates p53 transcriptional repression. In response to Pol I inhibition, both pathways are activated and convey robust activation of p53. Intriguingly, the RPL5/MDM2 complex also incorporates SRSF1, which is strengthened by Pol I inhibition to activate p53.⁶⁷ However, MDM4 also has p53-independent oncogenic activities, and RPL22L1 is a putative oncogene, suggesting that their repression is therapeutically valuable independent of p53.^{41,68–70} As we have demonstrated earlier, and show here, *TP53* is nonessential for Pol I inhibition.¹⁴

Earlier studies found that Pol I stress regulates the stability of MDM4.^{48,49} However, here we uncover a mechanism how Pol I inhibition switches MDM4 splicing by increasing exon 6 skipping leading to the expression of an NMD-targeted MDM4-S isoform and loss of protein expression. The splicing change may involve several splicing factors including SRSF3 and ZMAT3 previously identified to increase exon 6 inclusion and skipping, respectively.^{52,59} RPL22 is a tumor suppressor, but it is not known how it conveys this function.^{41,42} *TP53* mutations are strikingly underrepresented in *RPL22* mutant tumors. The highly frequent *RPL22* mutations and haploinsufficiency in MSI are suggestive that these alterations predispose to not only increased expression of MDM4-FL and repression of p53, but elevated RPL22L1. A decrease in RPL22L1 expression decreases colon cancer cell growth as shown here and by Rao et al.³⁸ and leads to large-scale changes in gene expression and splicing. The expression of RPL22L1 variant 3 was previously detected in glioblastoma neurospheres and its splicing was promoted by low pH, hypoxia and SR-splicing factors.⁴³ As variant 3 lacks the RNA interaction domain of RPL22L1, it is unclear whether it retains RNA-binding function. Notably, acidosis and hypoxia compromise Pol I transcription⁷¹ suggesting that the mechanism identified here could lead to altered RPL22L1 splicing.

Effects of RPL22 and RPL22L1 on splicing in cancers have not been previously detailed. By residing at the splicing junctions, they may fine-tune the coordinated activity of several splicing factors. The comprehensive RPL22 and RPL22L1 expression,

(G) Splicing events in Pol I KD (siRRN3) cells. SE, skipped exon; MXE, mutually exclusive exon; A5SS, alternative 5' splice site; A3SS, alternative 3' splice site; RI, retained intron.

(H) Correlation of differential expression (DE) of splicing factors by Pol I KD and BMH-21. *Inset*, Pearson *r* and statistical significance.

(I) Correlation of splicing events by Pol I KD and BMH-21. *Inset*, Pearson *r* and statistical significance.

(J) Schematic model of connection of rRNA synthesis to splicing by RPL22. *p* values, Student's one-sided *t* test. *ns*, non-significant, * <0.05, ** <0.01, *** <0.001, **** <0.0001. See also Figure S7 and Table S6.

splicing, interactome and motif analyses identified mostly distinct regulatory targets. The RNA-interactome analyses showed binding of RPL22 to introns of MDM4 and RPL22L1, and while the RPL22L1 intron 2 had two RPL22-interacting stem loops precisely at the splice site, the MDM4 intron did not. In contrast, RPL22L1 lacked discernible binding to these sites, suggesting that their regulatory roles in cancer cells are distinct. Instead, RPL22L1 is prominently bound to its own exons, implicating autoregulation of the stability of its mRNA. Future studies should explore the scope of their impact on both splicing and regulation of RNA stability.

Genetic and chemical inhibition of Pol I caused broad changes in splicing that was dominated by exon skipping events. These large-scale perturbations likely involve additional regulatory events beyond the alterations in RPL22L1. Interestingly, the expression of several splicing factors, in particular the SRSF splicing factors was regulated by Pol I inhibition, and chemical and genetic inhibition elicited highly similar responses. These included changes in SRSF3 and SRSF4 that are known splicing factors of MDM4 and RPL22L1, respectively. The mechanistic basis for the regulation of multiple SRSF splicing factors is unclear. However, splicing perturbations are known to cause alterations in the splicing factors through multiple complex mechanisms and suggested to represent a compensatory event.^{72,73} We presume similar mechanisms are at play as a consequence of RPL22 and RPL22L1 rearrangements at the splicing sites.

These markers for Pol I inhibitor sensitivity are frequently altered in cancers and facilitate future development of tumor-agnostic therapeutic strategies. In addition to *RPL22* mutations in dMMR cancers of the colon, stomach and endometrium, *RPL22* is mutated in 10% T cell acute lymphoma/leukemia and *RPL22* locus at chromosome 1p36 is frequently deleted in sporadic colorectal cancers leading to the overexpression of RPL22L1 and 5-FU resistance.^{38,41} *RPL22L1* 3p26 locus is amplified in ~30% squamous lung cancers and over 20% ovarian and esophageal cancers (cBioPortal) and MDM4 is highly expressed in melanoma, retinoblastoma and acute myeloid leukemia.^{53,68,69,74} Consequently, we show that Pol I inhibition is highly effective in xenograft, syngeneic and PDX models with alterations in *RPL22* and MDM4. WRN, and more recently ATR, has been identified as a synthetic lethality gene in MSI, and WRN inhibitors such as HRO761 have entered phase 1 clinical trials.^{75–79} However, WRN inhibitors are effective only in MSI cancers with high repeat expansion signatures.⁸⁰ They are thus mechanistically distinct from the Pol I inhibitors, which have a broader vulnerability profile. Immune checkpoint inhibitors have become standard-of-care in many dMMR tumors, and their effectiveness is encouraging although resistance remains a barrier.⁷⁹ A high mutational load is considered to drive the recognition of the tumor by immune cells.^{81,82} Alternative splicing is a source of tumor antigenicity and generates neoepitopes more frequently than by mutation burden and thus contributes to the immune response.^{83–85}

We propose that RPL22 coordinates rRNA transcription and splicing of mRNA genes and that the cancer splicing program is rewired due to *RPL22* genetic alterations. We predict this circuitry is critical during times of transcriptional bursts, such as growth, developmental processes and pathological states such as cancer. In other words, Pol I transcriptional state coordinates systemic post-transcriptional regulation of splicing programs.

Limitations of the study

The cancer cell screening panel used in this study was broad but not comprehensive. For example, it did not encompass lineages for pediatric cancers or the central nervous system. Furthermore, the limitations of cancer cell lines as representatives of their original tumors and acquisition of genetic alterations during their long-term propagation are well known. For these reasons, any cancer cell line screening study has inherent biases. It is plausible that further cancer cell line screens or functional genomics approaches could identify more factors relevant for the sensitivity or resistance to the Pol I inhibitors.

We found that RPL22 directly binds to splicing junctions of mRNAs and mediates alternative splicing, mostly through exon skipping. While we have established the dependency of splicing on RPL22, the detailed mechanisms by which RPL22 regulates splicing remain unclear. To expand our findings, further investigation into the RPL22 interactome with splicing factors and spliceosome structures will be necessary.

Our analysis on RPL22L1 regulation is limited to the detection of RNA transcripts as we are unable to confirm the expression of RPL22L1 variant 1 or variant 3 proteins due to lack of antibodies with sufficient specificity and sensitivity.

This study relies on cancer cell lines and tumor models relevant for the identified alterations in RPL22 and MDM4. Testing of BOB-42 in further models that assess safety and toxicity are required prior clinical trials. Also, given that *RPL22* mutation and haploinsufficiency is highly prevalent in MSI it is reasonable to propose that splicing alterations contribute to altered tumor antigenicity and impart checkpoint inhibitor responsiveness. Future studies should test this possibility and potential combination therapies with the Pol I inhibitors.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Marikki Laiho (mlaiho1@jhmi.edu).

Materials availability

Plasmids, cell lines, and reagents generated in this study are available upon request to the corresponding author.

Data and code availability

- All deep sequencing data have been deposited to the appropriate repositories and will be made public prior to publication: RNA-seq and Gold-CLIP sequencing data have been deposited to NCBI's Gene Expression Omnibus (GEO) and are accessible through the following identifiers GSE267499, GSE267500, GSE267501, GSE267502, GSE267503, GSE282212, GSE282213, GSE282214.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.L.; methodology, W.F., H.L., T.E.D., G.E., W.C.R., B.O., M.A.R., and N.V.R.; investigation, W.F., H.L., G.C.S., A.B., C.E.D., T.D., W.C.R., Q.Z., B.O., and N.V.R.; writing – original draft, M.L.; writing and editing, W.F., H.L., and N.V.R.; writing – review and editing, all authors; supervision, A.M.D., J.C.B., and M.L.; funding acquisition, W.F. and M.L.

DECLARATION OF INTERESTS

W.F., H.L., G.C.S., A.B., T.E.D., N.V.R., J.C.B., and M.L. are inventors on patents and patent applications on RNA polymerase I inhibitors.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.chembiol.2025.05.012>.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
RPL22	Abcam	Cat#ab229458
MDM4	Bethyl Laboratories	Cat#A300-287A; RRID: AB_263407
MDM2	Abcam	Cat#ab16895; RRID: AB_2143534
p53	Thermo Fisher Scientific	Cat#MA512571; RRID: AB_10986581
SRSF3	Thermo Fisher Scientific	Cat#33-4200; RRID: AB_2533119
HaloTag	Promega	Cat#G9211; RRID: AB_2688011
α -Actin	Abcam	Cat#ab5694; RRID: AB_2223021
α -Tubulin	Abcam	Cat#ab7291; RRID: AB_2241126
GAPDH	Abcam	Cat#ab8245; RRID: AB_2107448
Goat anti-rabbit IgG, HRP conjugated	Agilent	Cat#P026002-2
Swine anti-rabbit IgG, HRP conjugated	Agilent	Cat#P021702-2
Bacterial and virus strains		
DH5 α	Invitrogen	Cat#18258012
One Shot™ TOP10	Invitrogen	Cat#C404003
One Shot™ Stbl3	Invitrogen	Cat#C7373-03
Chemicals, peptides, and recombinant proteins		
BMH-21	Colis et al. ¹⁶	N/A
BOB-42	Colis et al. ¹⁶	N/A
Actinomycin D	Sigma-Aldrich	Cat#A1410
Nutlin-3a	Sigma-Aldrich	Cat#SML0580
TG003	MedChemExpress	Cat#HY-15338
SMG1 inhibitor 11e	MedChemExpress	Cat#HY-124760
Puromycin	Thermo Fisher Scientific	Cat#A1113803
SuperScript III Reverse Transcriptase	Invitrogen	Cat#18080093
D1000 sample buffer	Agilent Technologies	Cat#5190-6502
RIPA lysis buffer	Millipore Sigma	Cat#20-188
Protease inhibitor cocktail	Millipore Sigma	Cat#4693132001
Laemmli sample buffer	Bio-Rad	Cat#1610747
DTT	Sigma-Aldrich	Cat#D-9779
TRIzol Reagent	Thermo Fisher Scientific	Cat#15596026
NEB CutSmart Buffer	New England Biolabs	Cat#B6004
Quick CIP	New England Biolabs	Cat#M0525S
DNase I	Sigma-Aldrich	Cat#10104159001
PNK Buffer	New England Biolabs	Cat#M0201S
SDS	Invitrogen	Cat#15553-027
Proteinase K	New England Biolabs	Cat#P8107S
Acid phenol: chloroform: isoamyl alcohol	Invitrogen	Cat#AM9720
Critical commercial assays		
CellTiterGlo2.0	Promega	Cat#G7570
CellTiterBlue	Promega	Cat#G8081
RNeasy Mini Kit	Qiagen	Cat#74104
PowerUp SYBR Green Master Mix	Thermo Fisher Scientific	Cat#A25778
Q5 Hot start 2x high-fidelity PCR master mix	New England Biolabs	Cat#M0494s

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
D1000 screenTape	Agilent Technologies	Cat#5067-5582
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	Cat#PI23225
SuperSignal West Pico PLUS Chemiluminescent Substrate	Thermo Fisher Scientific	Cat#SKU:34579
Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate	Perkin Elmer	Cat#NEL103001EA
Lipofectamine RNAiMax	Invitrogen	Ca#13778075
CRISPRRevolution sgRNA EZ Kit	Synthego	Cat#7841158
HaloTag Mammalian Pull-Down System	Promega	Cat#G6504
RNAscope 2.5 Brown Kit	ACDBio	Cat#322370
GenePrint 10	Promega	Cat#B9510
GeneArt™ Seamless Cloning and Assembly Enzyme Mix	Invitrogen	Cat#A14606
4D-Nucleofector Kit	Lonza	Cat#V4XC-9064

Deposited data

RNA-seq analysis of RNA polymerase I inhibitor in A375 melanoma cells	This paper	GEO: GSE267499
RNA-seq analysis of parent, RPL22L1 knockout and RPL22 reconstituted HCT116 cells	This paper	GEO: GSE267500
RNA-seq analysis of RNA polymerase I inhibitor in p53 proficient HCT116 colon adenocarcinoma cells	This paper	GEO: GSE267501
RNA-seq analysis of RNA polymerase I inhibitor in p53 null HCT116 colon adenocarcinoma cells	This paper	GEO: GSE267502
GoldCLIP-seq analysis of RPL22HaloTag and RPL22L1HaloTag RNA-binding	This paper	GEO: GSE267503
RNA-seq analysis of RNA polymerase I inhibitor in HCT116 cells	This paper	GEO: GSE282212
RNA-seq analysis of RRN3 knockdown in HCT116 cells	This paper	GEO: GSE282213
RNA-seq analysis of RNA polymerase I inhibitor in A375 melanoma cells	This paper	GEO: GSE282214

Experimental models: Cell lines

22RV1	ATCC	RRID: CVCL_1045
A375	ATCC	RRID: CVCL_0132
G361	ATCC	RRID: CVCL_1220
DLD1	F. Bunz	RRID: CVCL_0248
Gp2D	Sigma	RRID: CVCL_2450
HCT-8	ATCC	RRID: CVCL_2478
HCT116	F. Bunz	RRID: CVCL_0291
HEC151	JCRB	RRID: CVCL_2925
HEC265	JCRB	RRID: CVCL_2928
IM95	JCRB	RRID: CVCL_2961
LNCaP	ATCC	RRID: CVCL_0395
LoVo	ATCC	RRID: CVCL_0399
SNU-1	ATCC	RRID: CVCL_0099
NUGC-3	JCRB	RRID: CVCL_1612
HEK293T	ATCC	RRID: CVCL_0063
SW480	ATCC	RRID: CVCL_0546
SW620	ATCC	RRID: CVCL_0547

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Table S7: RT-qPCR and reverse transcription primers	This paper	N/A
Table S8: Sequencing primers	This paper	N/A
Table S9: Primers for semi-quantitative detection of MDM4 and RPL22L1 variants	This paper	N/A
Table S10: Primers for cloning	This paper	N/A
Table S11: sgRNAs	This paper	N/A
Table S12: siRNAs	This paper	N/A
Recombinant DNA		
pCMV6-RPL22L1-myc-DDK	Origene	Cat#RC211790
pLenti-GIII-CMV-RPL22-HA	Applied Biological Materials	Cat#LV291610
pLenti-GIII-CMV-RPL22L1-HA	Applied Biological Materials	Cat#LV802168
pSMART-Rps3-HaloTag7-3xFlag	Heeseon An	
lentiCRISPRv2 puro	Addgene	Cat#98290
Super PiggyBac Transposase Expression Vector	System Biosciences	Cat#PB210PA-1
PB-EF1 α -MCS-IRES-GFP	System Biosciences	Cat#PB530A-2
pLKO.1-puro-CMV-TurboGFP	Sigma-Aldrich	Cat#SHC003
PB-EF1 α -RPL22-HaloTag-P2A-puro	This study	
PB-EF1 α -RPL22L1-HaloTag-P2A-puro	This study	
RPL22 pLKO shRNA vector clone ID: TRCN0000075015	Sigma-Aldrich	Cat#SHCLNG-NM_000983
RPL22L1 pLKO shRNA vector clone ID: TRCN0000247704	Sigma-Aldrich	Cat#SHCLNG-NM_001099645
MDM4 pLKO shRNA vector clone ID: TRCN0000003859	Sigma-Aldrich	Cat#SHCLNG - TRCN0000003859
SRSF4 pLKO shRNA vector clone ID: TRCN0000231448	Sigma-Aldrich	Cat#1011661807
Software and algorithms		
Image Lab Software	Bio-Rad	N/A
Tophat 2	https://ccb.jhu.edu/software/tophat/index.shtml	N/A
Cuffdiff 2.0	https://cole-trapnell-lab.github.io/cufflinks/cuffdiff/	N/A
HISAT2	https://daehwankimlab.github.io/hisat2/	N/A
StringTie	https://ccb.jhu.edu/software/stringtie/	N/A
rMATS turbo v4.1.1	https://github.com/Xinglab/rmats-turbo/releases	N/A
Ultrplex v1.2.10	https://github.com/ulelab/ultraplex	N/A
Cutadapt v4.8	https://cutadapt.readthedocs.io/en/v4.8/	N/A
STAR v2.7.10a	https://github.com/alexdobin/star/releases	N/A
Salmon v1.10.1	https://github.com/COMBINE-lab/salmon/releases/tag/v1.10.1	N/A
DESeq2 v1.46.0	https://bioconductor.org/packages/release/bioc/html/DESeq2.html	N/A
UMI-tools v1.1.5	https://github.com/CGATOxford/UMI-tools	N/A
MACS3 v3.0.0a6	https://github.com/mac3-project/MACS/releases	N/A
samtools v1.19.2	https://github.com/samtools/samtools/releases/	N/A
GenomicDistributions v1.10.0	https://code.databio.org/GenomicDistributions/	N/A
HOMER v4.11	http://homer.ucsd.edu/homer/	N/A
Integrative Genomics Viewer v2.17.4	https://igv.org/doc/desktop/	N/A
GSEA v4.3.2	Kupkova et al. ⁸⁶ , https://www.gsea-msigdb.org/gsea/index.jsp	N/A
g:Profiler	https://biit.cs.ut.ee/gprofiler/gost	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
KEGG	https://www.genome.jp/kegg/	N/A
WinNonlin v5.3	Pharsight Corporation	N/A
Prism v10.3.1	GraphPad	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell lines

Cell lines used in this study are detailed in Methods Table 1 and were maintained as indicated at 37°C and 5% CO₂ in a humidified atmosphere. Authentication of cell lines was conducted using short tandem repeat profiling (STR) at the Johns Hopkins University Genetic Resources Core Facility using GenePrint 10.

Methods Table 1. Cancer cell lines, genotypes and sources

Cell line	Source#	Lineage	Gender	MSI	TP53	RPL22 ^{K15Rfs}	STR verified	Cell culture medium*
22RV1	ATCC	Prostate	male	unstable	mut	mut	yes	RPMI-1640 + 10% FBS
A375	ATCC	Melanoma	female	stable	wt	wt	yes	DMEM+10% FBS
G361	ATCC	Melanoma	male	stable	wt	wt		DMEM+10% FBS
DLD1	F. Bunz	Colorectal	male	unstable	mut	mut	yes	DMEM+6% FBS
Gp2D	Sigma	Colorectal	female	unstable	wt	mut		DMEM+10% FBS
HCT-8	ATCC	Colorectal	male	unstable	wt	wt	yes	RPMI-1640 + 5% FBS
HCT116	F. Bunz	Colorectal	male	unstable	wt	mut	yes	McCoy's 5A + 10% FBS
HEC151	JCRB	Endometrial	female	unstable	mut	mut	yes	EMEM+15% FCS
HEC265	JCRB	Endometrial	female	unstable	wt	mut		EMEM+15% FCS
IM95	JCRB	Gastric	male	unstable	wt	mut	yes	DMEM+10% FBS
LNCaP	ATCC	Prostate	male	stable	wt	mut	yes	RPMI-1640 + 10% FBS
LoVo	ATCC	Colorectal	male	unstable	wt	mut		F-12K+10% FBS
SNU-1	ATCC	Gastric	male	unstable	wt	mut		RPMI-1640 + 10% FBS
NUGC-3	JCRB	Gastric	male	unstable	mut	wt		RPMI-1640 + 10% FBS
SW480	ATCC	Colorectal	male	stable	mut	wt		RPMI-1640 + 10% FBS
SW620	ATCC	Colorectal	male	stable	mut	wt		RPMI-1640 + 10% FBS
HEK293T	ATCC	Fetal kidney		stable	wt	wt		DMEM+10% FBS

ATCC; American Type Cell Collection, JCRB; Japanese Collection of Research Bioresources Cell Bank * FBS; fetal bovine serum, FCS; fetal calf serum, HS; horse serum.

Animals

Mouse studies were conducted according to the animal experimentation permits of the Animal Care and Use Committee at the Johns Hopkins University (MO15M351, MO18M209, MO15M139, MO18M63). Mice were housed in pathogen-free, controlled conditions in individually ventilated cages and provided an irradiated soy-free diet and autoclaved tap water *ad libitum*. CD-1 mice were used for pharmacokinetic analyses conducted at Pharmaron Inc. Female athymic nu/nu mice, aged 6–8 weeks, were used for patient-derived xenograft (PDX) models. PDX models were generated by subcutaneously implanting xenografted human colorectal tumor tissues (CO-04-0032, passage 5; and CO-04-0114, passage 4) into the right flank of 6–8-week-old female Balb/c nude mice. Once tumors were established (150–200 mm³), mice were randomized (*n* = 10 mice/group) and treated intraperitoneally (i.p.) once daily with either vehicle (0.2 M PBS, pH 6.2) or BOB-42 (25 mg/kg). PDX models were established, and conducted at WuXi AppTec R&D Center, Shanghai, China.

METHOD DETAILS

Chemical synthesis and characterization

Chemical syntheses for BMH-21 and BOB-42 are detailed in Colis et al.¹⁶ and were verified for purity using liquid chromatography/mass spectrometry (LC/MS) and ¹H nuclear magnetic resonance (NMR) spectroscopy. BMH-21 (CID: 3508054) and BOB-42

(CID: 90644360) are deposited to PubChem. BOB-42 was synthesized at Wuxi Apptec Co. Syntheses for BMH-21 and BOB-42 are detailed below.

12-oxo-12H-benzo[g]pyrido[2,1-b]quinazoline-4-carboxylic acid. A mixture of 3-amino-2-naphthoic acid (25 g, 134 mmol), 2-chloronicotinic acid (21 g, 134 mmol) in acetic acid (80 mL) was stirred at 120°C for 12 h. After cooling, the reddish-orange suspension was filtered, concentrated, and dried to give 12-oxo-12H-benzo[g]pyrido[2,1-b]quinazoline-4-carboxylic acid (29.5 g, 76% yield) as a yellow-orange solid. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 9.16 (s, 1 H) 9.03 (dd, *J* = 7.20, 1.64 Hz, 1 H) 8.63 (dd, *J* = 6.95, 1.64 Hz, 1 H) 8.49 (s, 1 H) 8.34 (d, *J* = 8.34 Hz, 1 H) 8.19 (d, *J* = 8.08 Hz, 1 H) 7.76 (t, *J* = 7.07 Hz, 1 H) 7.64 (t, *J* = 6.95 Hz, 1 H) 7.18 (t, *J* = 7.07 Hz, 1 H). MS [*M*+1] = 291.

N-[2-(dimethylamino)ethyl]-12-oxo-12H-benzo[g]pyrido[2,1-b]quinazoline-4-carboxamide (BMH-21). To a solution of 12-oxo-12H-benzo[g]pyrido[2,1-b]quinazoline-4-carboxylic acid (50 mg, 0.17 mmol) and TBTU (82.9 mg, 0.26 mmol) in DMF (1 mL) was added DIPEA (90 μL, 0.52 mmol). After the contents were stirred at room temperature for 15 min, N,N-dimethylethylenediamine (28.4 μL, 0.26 mmol) was added, and stirring continued for 16 h (for convenience). The reaction mixture was added to 100 mL cold water with stirring, and solid was collected by filtration and dried under vacuum to give N-[2-(dimethylamino)ethyl]-12-oxo-12H-benzo[g]pyrido[2,1-b]quinazoline-4-carboxamide (36 mg, 0.10 mmol, 58% yield) as a yellow solid. ¹H NMR (400 MHz, DMSO-*d*₆) δ ppm 11.50 (br. s., 1 H) 9.10 (s, 1 H) 8.91 (d, *J* = 5.81 Hz, 1 H) 8.55 (d, *J* = 5.56 Hz, 1 H) 8.28–8.34 (m, 2 H) 8.12 (d, *J* = 8.34 Hz, 1 H) 7.73 (t, *J* = 7.45 Hz, 1 H) 7.61 (t, *J* = 7.33 Hz, 1 H) 7.05 (t, *J* = 7.07 Hz, 1 H) 3.56 (d, *J* = 5.05 Hz, 2 H) 2.59 (t, *J* = 5.94 Hz, 2 H) 2.40 (s, 6 H). MS [*M*+1] = 361.

N-[(2S)-2-(dimethylamino)propyl]-12-oxo-12H-benzo[g]pyrido[2,1-b]quinazoline-4-carboxamide (BOB-42). To a solution of 12-oxo-12H-benzo[g]pyrido[2,1-b]quinazoline-4-carboxylic acid (15 g, 52 mmol) and TBTU (25 g, 78 mmol) in DMF (450 mL) was added diisopropylethylamine (66 g, 512 mmol) followed by a solution of 2S-2-dimethylaminopropylamine in 50 mL THF dropwise. After the contents were stirred at room temperature for 16 h, the reaction mixture was diluted into 1 L dichloromethane, washed with 3 x 1 L water, dried over sodium sulfate and filtered. Purification by column chromatography (10:1 MeOH:dichloromethane) afforded 14 g (36%) of N-[(2S)-2-dimethylaminopropyl]-12-oxo-12H-benzo[g]pyrido[2,1-b]quinazoline-4-carboxamide. ¹H NMR (400 MHz, CDCl₃) δ ppm 11.71 (br. s., 1 H) 9.11 (s, 1 H) 8.94 (dd, *J* = 7.33, 1.77 Hz, 1 H) 8.73 (dd, *J* = 7.07, 1.77 Hz, 1 H) 8.28 (s, 1 H) 8.12 (d, *J* = 8.08 Hz, 1 H) 8.00 (d, *J* = 8.08 Hz, 1 H) 7.67 (ddd, *J* = 8.27, 6.88, 1.26 Hz, 1 H) 7.56 (ddd, *J* = 8.21, 6.82, 1.14 Hz, 1 H) 6.89 (t, *J* = 7.07 Hz, 1 H) 3.77 (ddd, *J* = 14.02, 6.06, 5.18 Hz, 1 H) 3.45 (ddd, *J* = 13.96, 8.91, 3.41 Hz, 1 H) 2.97–3.06 (m, 1 H) 2.52 (s, 6 H) 1.16 (d, *J* = 6.57 Hz, 3 H). MS [*M*+1] = 375.

N-[(2S)-2-(dimethylamino)propyl]-12-oxo-12H-benzo[g]pyrido[2,1-b]quinazoline-4-carboxamide hydrochloride (BOB-42). To a solution of 14 g (37 mmol) N-[(2S)-2-dimethylaminopropyl]-12-oxo-12H-benzo[g]pyrido[2,1-b]quinazoline-4-carboxamide in 200 mL dichloromethane was slowly added 93 mL of a solution of 4N HCl in ethyl acetate. After stirring the resultant suspension for 10 min, the mixture was filtered and the solid washed with dichloromethane and dried to afford 12 g (78% yield) of N-[(2S)-2-(dimethylamino)propyl]-12-oxo-12H-benzo[g]pyrido[2,1-b]quinazoline-4-carboxamide hydrochloride (BOB-42) as a yellow solid. ¹H NMR (400 MHz, DMSO) δ ppm 11.13 (br. s., 1H), 10.61 (br. s., 1H), 9.11 (s, 1H), 8.96 (d, *J* = 7.2 Hz, 1H), 8.58–8.53 (m, 2H), 8.31 (d, *J* = 8.4 Hz, 1H), 8.12 (d, *J* = 8.5 Hz, 1H), 7.74 (t, *J* = 7.5 Hz, 1H), 7.63–7.59 (m, 1H), 7.10 (t, *J* = 7.1 Hz, 1H), 3.98–3.90 (m, 1H), 3.80–3.69 (m, 2H), 2.83 (t, *J* = 4.7 Hz, 6H), 1.41 (d, *J* = 6.5 Hz, 3H). MS [*M*+1] = 375.

Vectors and vector constructions

Human RPL22 and RPL22L1 cDNAs were amplified from pLenti-GIII-CMV-RPL22-HA and pLenti-GIII-CMV-RPL22L1-HA then cloned into a PB-EF1α-MCS-IRES-GFP PiggyBac expression vector. HaloTag⁸⁷ was amplified from pSMART-Rps3-HaloTag7-3xFlag (gift from Heeseon An), and P2A-puro was amplified from lentiCRISPRv2 puro. The amplified PCR products were cloned using GeneArt Seamless Cloning and Assembly Enzyme Mix according to the manufacturer's protocol to generate PB-EF1α-RPL22-HaloTag-P2A-puro and PB-EF1α-RPL22L1-HaloTag-P2A-puro. Super PiggyBac Transposase Expression Vector was transfected with PB-EF1α-RPL22-HaloTag-P2A-puro and PB-EF1α-RPL22L1-HaloTag-P2A-puro to efficiently integrate the inserts into the genome. For GFP competition assay, pLKO.1-puro-CMV-TurboGFP plasmid was used to generate stable cell lines expressing GFP. Vectors were confirmed by Sanger sequencing.

Cell viability assays

Cells were plated in 96-well plates at a density of 3,000–5,000 cells per well. After 24 h, the cells were treated in triplicate with half-log concentrations of BMH-21 and incubated for 72 h. Viability was assessed using CellTiterBlue Reagent and analyzed on Victor Nivo 3F (PerkinElmer) plate reader. IC₅₀ was calculated using GraphPad Prism software based on non-linear regression analysis.

Cancer cell line analyses

Cancer cell line screening on the Oncosignature panel of lines detailed in [Tables S1](#) and [S2](#) was performed at Horizon Discovery Limited (Cambridge, UK). Cells were thawed from liquid nitrogen, expanded and once dividing at their expected doubling times plated in their respective growth media in black 384-well tissue culture plates at 500 to 1500 cells/well and equilibrated via centrifugation. 24 h after the plating, a “time zero” set of assay plates (V0) was collected and ATP levels were measured using CellTiterGlo2.0 using Envision plate readers (PerkinElmer). The screen was conducted for each drug using 10-point 3-fold dilution series starting at 10 μM in triplicate. Cell viability was measured using CellTiterGlo after 72 h of incubation, and the response curves were used to determine GI₅₀ using a Horizon Discovery proprietary software. GI₅₀ was defined as in Shoemaker⁸⁸ as 100 x [1 - (T - V0)/V-V0], where V0 is the vehicle-treated level at time zero, T the treated level and V the vehicle level at 72 h. Z-factors for each plate were

determined, and assay plates were accepted if the following quality control standards were met: consistent relative luciferase values throughout the entire experiment, Z-factor scores >0.6, and consistent behavior of untreated/vehicle controls on the plate.

Cell line correlative analyses

RNA expression, protein array (RPPA), mutations (WES), copy number variation, metabolomics, histone modifications, promoter methylation and microRNA omics data were obtained from DepMap (19Q3).³² CRISPR gene dependency scores (22Q2) were downloaded from DepMap portal. Batch correlation between BMH-21 and BOB-42 GI₅₀ and -omics data were performed in the R. Spearman method, and statistics were used in the batch correlations. The *p* values were computed and then converted to the negative logarithm of the *p* value for plotting.

qPCR and semiquantitative PCR

RNA was extracted from cells using RNeasy Mini Kit. RNA was reverse transcribed using SuperScript III Reverse Transcriptase. To perform qPCR, the resulting cDNA was mixed with PowerUp SYBR Green Master Mix and the appropriate primer pairs. Analyses were conducted in triplicate using BioRad CFX384 Real-Time System – C1000 Touch Thermal Cycler or the Applied Biosystems QuantStudio 12K Flex Real-Time PCR System. All results were normalized to GAPDH, and RNA levels were quantified using the $\Delta\Delta C_t$ method. All PCR reactions were conducted in triplicates, and the data presented represent a minimum of three independent biological replicates. Bar graphs represent the relative ratios of each gene to GAPDH values and control sample. For semiquantitative PCR, 20 ng cDNA was used in a 10 μ L PCR reaction with Q5 Hot start 2x high-fidelity PCR master mix. After 21 cycles for GAPDH and 26 cycles for other transcripts, 1 μ L was mixed with 3 μ L D1000 sample buffer and run on Agilent 4200 TapeStation platform with D1000 screenTape.

Immunoblotting

Cells were lysed in RIPA lysis buffer supplemented with protease inhibitors. Protein concentrations were determined using Pierce BCA Protein Assay Kit. An equal amount of protein per sample was mixed with Laemmli sample buffer, DTT, boiled and run on an NuPAGE 4–12% Bis-Tris gel (Invitrogen, Cat # NP0322BOX) and transferred to Immobilon PVDF (0.45 μ m) membrane (MilliporeSigma, Cat # IPVH00010). The membrane was blocked with 5% milk followed by primary and secondary antibodies. Primary and secondary antibodies are listed in the [key resources table](#). detected using either SuperSignal West Pico PLUS Chemiluminescent Substrate or Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate. The membranes were imaged using the BioRad Molecular Imager ChemiDoc XRS+ System. Protein densitometry was conducted using Image Lab Software.

Cell counting and competition assays

Isogenic HCT116 cells were transfected with RPA1 siRNAs, incubated for 48 h and treated with or without BMH-21 for 48 h. Cells were then counted using Cellometer AutoT4. For competition assay, stable GFP-expressing HCT116 p53^{+/+} cells were generated, and 0.75 million each of the GFP-parent and stable knockdown cells were mixed and cultured for 2 weeks in the presence of absence of BMH-21 (0.25 μ M). Cells were then trypsinized and analyzed by flow cytometry using Beckman Coulter CytoFLEX S 3-laser flow cytometer.

Gene silencing and knockouts

Cells were transfected using Lipofectamine RNAiMax transfection reagent and incubated for 48 h siRNAs were obtained from Ambion or Thermo Fisher Scientific as listed in Supplemental [Table S12](#). Stable shRNA gene knockdowns were generated as in Pitts et al.²¹ Vectors for shRNAs are listed in the [key resources table](#). RPL22L1 knockout was carried out using Gene Knockout Kit v2. To achieve high knockout efficiencies, two guide sgRNAs targeting RPL22L1 exon 3 were designed and synthesized by Synthego. Cas9 and sgRNA ribonucleoprotein (RNP) complexes were transfected to HCT116 p53^{-/-} cells using nucleofection according to the Synthego protocol. Briefly, sgRNA (30 μ M) and 1 μ L Cas9 were mixed with 18 μ L Nucleofector Solution and incubated for 10 min at room temperature. Cells were then suspended in buffer SE and transfected using 4D-Nucleofector (Lonza) program EN-113. 72 h post nucleofection, single cells were seeded in 96-well plates using limiting dilution. After clonal expansion, single clones were picked followed by genotyping and Sanger sequencing. The knockout of exon 3 was further confirmed by qPCR.

RNA-seq

As indicated, cells were treated with DMSO or BMH-21 (1 μ M) for 6 h. Three biological replicates were prepared for each treatment. RNA was extracted with TRIzol. RNA-seq library for Illumina platform sequencing was prepared using Illumina TruSeq stranded total RNA Sample kit following manufacturer's protocol followed by sequencing on Illumina NextSeq 500 for 2X75 bp paired-end reads. Reads were analyzed using Tophat 2 and Cuffdiff 2.0, respectively for alignment to reference genome and differential expression detection. Pathway analyses were conducted using Gene Set Enrichment Analysis (GSEA). RNA-seq of A375 and HCT116 treated with DMSO or BMH-21 (1 μ M) for 24 h and knockdown of RRN3 in HCT116 cells was conducted on three biological replicates and sequenced using the AVITI sequencer from Element Biosciences. Trimmed reads were aligned to the human reference genome GRCh38 using the STAR aligner. Salmon package was employed to quantify the transcripts and DESeq2 was used to analyze

differential expression. RNA-seq of HCT116 parent, RPL22^{RE} and RPL22L1^{KO} cells was conducted on three biological replicates on Illumina NovaSeq 6000. Paired-end reads were mapped using HISAT2 software and StringTie was used to estimate transcript abundances and differential expression. Significantly differentially expressed transcripts were defined using a *p*-value cutoff <0.05.

Alternative splicing analysis

Alternative splicing analysis was conducted using rMATS 4.1.1⁵⁶ based on triplicate RNA-seq samples. Splicing differences were calculated for five classes of alternative splicing events, skipped exons (SE), alternative 5' splice sites (A5SS), alternative 3' splice sites (A3SS), mutually exclusive exons (MXE), and retained introns (RI). Significant differential alternative splicing events were defined as FDR (false discovery rate) < 0.05 with a requirement of an average read count >10 per event. Data is reported on reads covering exon junctions. Sashimi plot visualization of alternative splicing events was generated by the *rmats2sashimiplot* function in rMATS. Inclusion level differences are reported and represent the average difference between the splicing events calculated from normalized counts.

GoldCLIP-seq

GoldCLIP-seq was performed according to protocol designed by Gu et al.⁶⁶ with modifications. Stable RPL22-HaloTag, RPL22L1-HaloTag, and HaloTag only expressing HCT116 p53^{+/+} cells were generated using PiggyBac transposon system and selected with puromycin (1 μg/mL) for seven days. 1×10^7 cells in ice-cold PBS to cells were irradiated with 120 mJ/cm² at 254 nm using Stratalinker 2400 (Stratagene). Cells were then harvested, and the pulldown of HaloTag proteins was conducted according to HaloTag Mammalian Pull-Down System protocol. Beads were washed thrice with $1 \times$ NEB CutSmart Buffer followed by dephosphorylation by using Quick CIP and DNase I treatment at 37°C for 30 min. Beads were washed with TRIzol once, thrice with Urea Wash Buffer and thrice with PNK Buffer. 3' RNA linker ligation and additional denaturing washes using SDS, and urea buffers were conducted according to Gu et al.⁶⁶ On-bead digestion was performed by adding 100 μL Proteinase K followed by incubation at 37°C for 75 min. Resin-bound RNAs were extracted by acid phenol: chloroform: isoamyl alcohol extraction followed by isopropanol precipitation. RNA pellets were spun down at 12,000 $\times$ g for 15 min at 4°C. The pellets were washed twice in 80% ethanol, air dried, and suspended in 8 μL H₂O. Reverse transcription, gel purification of cDNA and library preparation was as in Gu et al.⁶⁶ RPL22-HaloTag, RPL22L1-HaloTag, and HaloTag only libraries were sequenced on Illumina NovaSeq X Plus.

GoldCLIP-seq data analysis

Read 1 for paired-end reads from GoldCLIP libraries containing indexes were first demultiplexed by Ultraplex (v1.2.10) and the adapter sequences were trimmed by Cutadapt (v4.8). The reads were mapped to customized hg38 genomes with a curated rDNA locus⁸⁹ using STAR (2.7.10a) with parameters: `-genomeLoad NoSharedMemory -readFilesCommand zcat -outBAMcompression 10 -outFilterMultimapNmax 1 -outFilterMultimapScoreRange 1 -outFilterScoreMin 10 -outFilterType BySJout -outReadsUnmapped Fastx -outSAMattrRGline ID:foo.`

`-outSAMattributes All -outSAMmode Full -outSAMtype BAM Unsorted -outSAMunmapped Within -runMode alignReads.` Mapped reads were deduplicated to remove PCR duplicates using UMI-tools (1.1.5) with parameters: `-random-seed 1 -method unique.` Mapped reads were split into two bam files with positive or negative strands for peak calling. MACS3 (v3.0.0a6) was used to call peaks with parameters: `-g hs -B -keep-dup all -nomodel -extsize 30.` Enriched regions were predicted by bdgcmp module using qpois statistic model. Final narrow peak calling was performed using bdgpeakcall function with a cutoff 0.05 for *q*-value. Mapped reads in peak region were counted using samtools (1.19.2) according to RNA biotypes (lncRNA, miRNA, ncRNA, protein coding, rRNA, snoRNA and snRNA). The genome distribution of binding peaks and metagene plot of peaks at the intron-exon boundary were calculated and visualized by R package GenomicDistributions (1.10.0).⁸⁶ Narrow binding peaks of RPL22 and RPL22L1 were used to generate consensus motifs by findMotifsGenome.pl from HOMER (Hypergeometric Optimization of Motif EnRichment) suite (v4.11) (<http://homer.ucsd.edu/homer/>).⁹⁰ Top five motifs (*de novo* and known motifs) with reported *p*-values <10⁻³⁵⁰ are presented. Integrative Genomics Viewer (v2.17.4) was used to visualize the genomic tracks.

Enrichment and ontology analyses

Gene set enrichment analysis was performed using GSEA (v4.3.2).⁹¹ Gene signatures were obtained from the MSigDB "KEGG_MEDICUS subset of CP" collection. Significantly enriched gene sets were ranked and presented using normalized enrichment (NES) score. The gene list from the intersection of RNA-seq and rMATS were subjected to gene enrichment analysis using g:Profiler (<https://biit.cs.ut.ee/gprofiler/gost>).⁹² Top Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.genome.jp/kegg/>) pathway analyses are reported.

45S precursor rRNA chromogenic *in situ* hybridization (CISH)

Tumors were fixed with 10% formalin, embedded in paraffin and cut to 5 μm. *In situ* hybridization for 5'ETS precursor rRNA ACD RNAscope 2.5 Brown Kit was conducted as previously described.⁹³ Image analysis was conducted on a minimum five tumors/treatment on hematoxylin stained and 5'ETS CISH assay slides, scanned at $\times$ 40 using Ventana DP 200 slide scanner (Roche), and deposited to the Proscia digital pathology database. Necrotic areas were excluded. Image analysis was conducted using

HALO v3.0 image analysis platform and Area Quantification module (Indica Labs). The area of brown pixels over the total annotation area (brown+blue) was calculated. The means of these ratios per tumor were used in analyses. All image analyses were conducted in a blinded fashion.

Mouse tumor models and *in vivo* studies

ACUC approval numbers

Mouse studies were conducted according to the animal experimentation permits of the Animal Care and Use Committee at the Johns Hopkins University (MO15M351, MO18M209, MO15M139, MO18M63).

Pharmacokinetic and drug uptake analyses

Pharmacokinetic (PK) studies were conducted using a single 30 or 100 mg/kg i.p. or oral doses at Pharmaron Inc. CD-1 mice ($n = 3$) were sacrificed at various times (5, 15, 45 min, 1.5, 4, 8, 12, 18 and 24 h) and plasma was collected. Plasma samples were analyzed by LC/MS/MS, and PK parameters were determined by non-compartmental analysis of the mean data using WinNonlin version 5.3 (Pharsight Corporation, Mountain View, CA). BOB-42 plasma $T_{1/2}$ was 1.6 h. For tumor uptake of BOB-42, plasma and tumors were collected 4 h after the last dose. The samples were analyzed by LC/MS/MS at the Analytical Pharmacology Facility at Sidney Kimmel Comprehensive Cancer Center.

A375 human melanoma model

A375 cells (ATCC) were cultured in DMEM supplemented with 10% FBS and 4.5 g/L glucose at 37°C in a humidified atmosphere containing 5% CO₂. Cells growing in an exponential growth phase were harvested following trypsin-EDTA treatment and counted for tumor inoculation. A375 cells (3×10^6) were suspended in 0.1 mL of PBS and injected subcutaneously at the right upper flank of 6–8 weeks old female athymic *nu/nu* mice. Mice bearing subcutaneous A375 tumors (200 mm³) were randomized into 4 groups ($n = 8$ mice/group) using two treatment schemes. Mice were administered i.p. daily for 14 days with vehicle (0.2 M PBS, pH 6.2) or BOB-42 at 12.5 mg/kg, 25 mg/kg or 50 mg/kg. Alternatively, they were administered for 11 days with vehicle (daily, 0.2 M PBS, pH 6.2) or BOB-42 at 50 mg/kg (daily), 75 mg/kg (one day on, one day off) or 100 mg/kg (one day on, two days off). Body weights and tumor sizes were measured thrice per week. Mice were euthanized and blood was collected using cardiopuncture. Blood and plasma were analyzed at the Johns Hopkins Mouse Phenotyping and Pathology Core for clinical chemistry and complete blood count (CBC) using ProCyt Dx veterinary CBC hematology analyzer.

Tumor growth was evaluated by measurement of two perpendicular diameters of tumors with a digital caliper. Tumor volume was expressed in mm³ using the formula: $V = (L \times W^2)/2$ where L is the tumor length and W is the tumor width. The tumor size was then used to calculate tumor growth inhibition (TGI) using the formula: $TGI (\%) = [1 - (T_i - T_0)/(V_i - V_0)] \times 100$; T_i is the average tumor volume of a treatment group on a given day, T_0 is the average tumor volume of the treatment group on the first day of treatment, V_i is the average tumor volume of the vehicle control group on the same day with T_i , and V_0 is the average tumor volume of the vehicle group on the first day of treatment.

HCT116 human colorectal cancer model

HCT116 cells (ATCC) were cultured in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO₂. Cells growing in an exponential growth phase were harvested following trypsin-EDTA treatment and counted for tumor inoculation. HCT116 cells (5×10^6) were suspended in 0.1 mL of PBS and injected subcutaneously at the right upper flank of 6–8 weeks old female athymic *nu/nu* mice. Mice bearing subcutaneous tumors (200 mm³) were randomized into 2 groups ($n = 12$ mice/group). Mice were administered i.p. for 13 days with vehicle (0.2 M PBS, pH 6.2) or BOB-42 at 50 mg/kg using scheme six days on, one day off. Body weights and tumor sizes were measured thrice per week.

Human colorectal cancer PDX models

PDX models were established from surgically resected clinical patient samples that were tested for microsatellite repeats and sequenced for *TP53* and *RPL22*. All lines were *TP53* wild-type and *RPL22*^{K15Rfs} mutant. PDX models were generated by implanting xenografted human CO-04-0032 (passage 5) and CO-04-0114 (passage 4) colorectal tumor tissues s.c. into the right flank of 6–8 weeks old female *Balb/c* nude mice. Mice with established tumors (150–200 mm³) were randomized ($n = 10$ mice/group) and treated i.p. once daily with vehicle (0.2 M PBS, pH 6.2) or BOB-42 (25 mg/kg). Body weights and tumor sizes were measured thrice per week. Mice were euthanized and blood was collected using cardiopuncture for complete blood count (CBC) using Siemens Advia 2120i hematology analyzer. The study was conducted at WuXi AppTec R&D Center, Shanghai, China.

QUANTIFICATION AND STATISTICAL ANALYSIS

No data were excluded from the analyses. Data distribution was assumed normal, but this was not formally tested. Mouse treatment groups were randomized throughout the study and the number of animals (n) in each group is shown in the figure legend. There was no randomization of the *in vitro* studies. Data collection and performance were unblinded, unless otherwise stated. Statistical analysis was performed using GraphPad Prism 10. Cell viability assay for IC₅₀ was calculated using nonlinear regression to obtain the best-fit values, correlating inhibitor concentration versus response. Groups in GFP competition and cell viability assays were compared

using a two-tailed Student's *t*-distribution or One-way ANOVA with multiple comparisons (Three or more groups). Data are representative of three independent experiments (*n*) unless otherwise indicated in the figure legend. Mouse experiments for tumor growth were compared using two-way ANOVA with Tukey's multiple comparisons. A *p* value < 0.05 was considered significant. * indicates *p* < 0.05, ** indicates *p* < 0.01, *** indicates *p* < 0.001, and **** indicates *p* < 0.0001. Data are presented as mean ± standard deviation unless otherwise stated in the figure legend. The statistical test methods used are shown in the figure legends.