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Targeting Ribosome Biogenesis as a Novel Therapeutic Approach to Overcome EMT-related Chemoresistance in Breast Cancer

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Abstract

Epithelial-to-mesenchymal transition (EMT) contributes significantly to chemotherapy resistance and remains a critical challenge in treating advanced breast cancer. The complexity of EMT, involving redundant pro-EMT signaling pathways and its paradox reversal process, mesenchymal-to-epithelial transition (MET), has hindered the development of effective treatments. In this study, we utilized a Tri-PyMT EMT lineage-tracing model and single-cell RNA sequencing (scRNA-seq) to comprehensively analyze the EMT status of tumor cells. Our findings revealed elevated ribosome biogenesis (RiBi) during the transitioning phases of both EMT and MET processes. RiBi and its subsequent nascent protein synthesis mediated by ERK and mTOR signalings are essential for EMT/MET completion. Importantly, inhibiting excessive RiBi genetically or pharmacologically impaired the EMT/MET capability of tumor cells. Combining RiBi inhibition with chemotherapy drugs synergistically reduced metastatic outgrowth of epithelial and mesenchymal tumor cells under chemotherapies. Our study suggests that targeting the RiBi pathway presents a promising strategy for treating patients with advanced breast cancer.

Significance

This study uncovers the crucial involvement of ribosome biogenesis (RiBi) in the regulation of epithelial and mesenchymal state oscillations in breast cancer cells, which plays a major role in the development of chemoresistant metastasis. By proposing a novel therapeutic strategy targeting the RiBi pathway, the study offers significant potential to enhance treatment efficacy and outcomes for patients with advanced breast cancer. This approach could help overcome the limitations of current chemotherapy options and address the complex challenges posed by EMT-mediated chemoresistance.

eLife assessment

This study presents a **valuable** finding that pathways associated with ribosome biogenesis (RiBi) are activated during transition cell states and targeting ribosome biogenesis could be a viable approach to overcome EMT-related chemoresistance in BCs. The evidence supporting the claims of the authors is quite **solid**, although inclusion of additional experimental support that blocking of EMT/MET is necessary for the synergistic effect of standard chemotherapy together with RiBi blockage would have strengthened the study. The work will be of interest to scientists working on breast cancer.

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Introduction

Tumor cells exploit the transdifferentiation program of epithelial-to-mesenchymal transition (EMT) to acquire aggressive properties, including anchorage-independent survival, invasion, and stemness(1). Multiple growth factors (TGFβ, EGF, Wnts, etc), signaling pathways (Smad2/3, PI3K/Akt, ERK1/2, etc), EMT transcription factors (Snail, Twist, Zeb1/2, etc), and hundreds of downstream EMT related genes are involved in the EMT program(1). Such complexity leads to a wide spectrum of EMT phenotypes coexisting at different stages of tumors(2,3). The EMT-endowed features contribute to tumor heterogeneity, metastasis, and therapy resistance, making EMT an attractive therapeutic target.

Current EMT-targeting strategies focus on blocking EMT stimuli, signaling transduction, or mesenchymal features(2,3). However, these approaches may paradoxically promote the reversed process of EMT, mesenchymal to epithelial transition (MET), which also contributes to malignancy development(4,5). Therefore, we proposed that instead of targeting epithelial or mesenchymal phenotype, inhibiting a biological process mediating the transitions of both EMT and MET could effectively overcome the limitations of traditional strategies (2,3).

To investigate the EMT process in metastatic tumor progression, we previously developed an EMT lineage-tracing model (Tri-PyMT) by combining MMTV-PyMT, Fsp1(S100a4)-Cre, and Rosa26-mTmG transgenic mice (6). This model traces EMT via a permanent RFP-to-GFP fluorescence switch induced by mesenchymal-specific Cre expression. The absence of EMT reporting in metastatic lesions in this model has sparked a lively debate about the proper definition of EMT status in tumor cells and the biological significance of EMT in tumor progression (2,7). Rheenen's group also posited that the Fsp1-Cre mediated EMT lineage tracing model might not accurately capture the majority of EMT events in comparison to an E cadherin-CFP model(8). Although we disagree with this assessment of Fsp1-Cre model's fidelity in tracing EMT, we acknowledge the limitations of relying on a single EMT marker to investigate the EMT contributions in tumor metastasis. Notably, using the refined EMTracer animal models, Li et al. discovered that N-cadherin+ cells, rather than the Vimintin+ cells, were predominantly enriched in lung metastases(9). These findings with different mesenchymal-specific markers underscore the complex nature of the EMT process; metastases formation does not necessarily require the expression of many traditional mesenchymal markers.

The Tri-PyMT model, despite its limitations in comprehensive tracing of metastasis, provides unique opportunities to study EMT's role in tumor progression and chemoresistance. In particular, the fluorescent marker switch of the established Tri-PyMT cell line reliably reports changes in

EMT phenotypes(6,10). Using single-cell RNA sequencing (scRNA-seq) technology, we characterized the differential contributions of EMT tumor cells in tumor progression(10). Importantly, post-EMT(GFP+), mesenchymal tumor cells consistently demonstrated robust chemoresistant features compared to their parental epithelial cells (pre-EMT RFP+) (6), inspiring us to further study chemoresistance using the Tri-PyMT model.

Materials and Methods

Cell lines and cell culture

Tri-PyMT cells were established in our lab from primary tumors of MMTV-PyMT/Fsp1-Cre/Rosa26-mTmG transgenic mice(6). For experiments, we used Tri-PyMT cells from passage 5 (p5) to passage 10 (p10) which contains both RFP+ and GFP+ cells. MDA-MB231-LM2 cells were a gift from Dr. Joan Massague. To facilitate *in vivo* imaging, both cell lines were genetically labeled with luciferase by lenti-Puro-Luc. Cells were cultured in DMEM with 10% FBS, 1% L-Glutamine, and 1% Penicillin Streptomycin. A routine assay for Mycoplasma (Universal Mycoplasma Detection Kit, ATCC, Cat#30-1012K) was performed to avoid contaminations.

Animals and tumor models

All animal works were performed in accordance with IACUC approved protocols at Weill Cornell Medicine. CB-17 SCID mice were obtained from Charles River (Wilmington, MA). For the experimental metastasis model, RFP+ and GFP+ Tri-PyMT cells were sorted from the 5-10th passage cells and re-mixed at a ratio of 1:1. Total cells (1.5 ×10^5 cells) were injected through the tail vein in 100 µL of PBS in 10-week-old females. For animals subjected to chemotherapy, Cyclophosphamide (CTX, 100mg/kg, Signa-aldrich, Cat# C0768) was administered once per week, i.p., for 3-4 weeks. BMH21 (25mg/kg, Sellechehem, Cat#S7718) was administrated 5 times/week, i.p., for 3-4 weeks. The progression of lung metastasis tumors was monitored by bioluminescent imaging (BLI) every 3-5 days on the Xenogen IVIS system coupled with analysis software (Living Image; Xenogen).

Flow cytometry and cell sorting

For cultured cells, single-cell suspensions were prepared by trypsinization and neutralizing with the growth medium containing 10% FBS. For the metastatic lungs, cell suspensions were prepared by digesting tissues with an enzyme cocktail containing collagenase IV (1 mg/mL), hyaluronidase (50 units/mL), and DNase I (0.1 mg/mL) in Hank's Balanced Salt Solution containing calcium (HBSS, Gibco) at 37°C for 20-30 minutes. Cells were filtered through a 40-µm cell strainer (BD Biosciences) and stained with anti-Epcam antibody (G8.8, Biolegend), if needed, following a standard immunostaining protocol. SYTOX Blue (Invitrogen) was added to the staining tube in the last 5 minutes to facilitate the elimination of dead cells.

Samples were analyzed using the BD LSRFortessa™ Flow Cytometer coupled with FlowJo_v10 software (FlowJo, LLC). GFP$^+$ and RFP$^+$ cells were detected by their endogenous fluorescence. Flow cytometry analysis was performed using a variety of controls including isotype antibodies, and unstained and single-color stained samples for determining appropriate gates, voltages, and compensations required in multivariate flow cytometry.

For fluorescence-activated cell sorting for *in vitro* culture or tail vein injection, we used the Aria II cell sorter coupled with FACS Diva software (BD Biosciences). The preparation of cells throughout sorting procedures was under sterile conditions. The purity of subpopulations after sorting was confirmed by analyzing post-sort samples in the sorter again.

RNA-sequencing analysis

Total RNA was extracted from sorted RFP+, GFP+, and Double+ Tri-PyMT cells with the RNeasy Plus Kit (Qiagen). RNA-Seq libraries were constructed and sequenced following standard protocols (Illumina) at the Genomics and Epigenetics Core Facility of WCM. RNA-seq data were analyzed with customized Partek Flow software (Partek Inc). In brief, the RNA-seq data were aligned to the mouse transcriptome reference (mm10) by STAR after pre-alignment QA/QC control. Quantification of gene expression was performed with the annotation model (PartekE/M) and normalized to counts per million (CPM). Differential gene expression was performed with Gene Specific Analysis (GSA) algorithm, which applied multiple statistical models to each gene to account for its varying response to different experimental factors and different data distribution.

For heatmap visualizations, Z scores were calculated based on normalized per-gene counts. Algorisms for the biological interpretation of differential expressions between samples such as Gene Set Enrichment Analysis (GSEA) and Over-representation assay, are also integrated in Partek Flow platform. Gene sets of interests were downloaded from the Molecular Signatures Database (MSigDB, *https://www.gsea-msigdb.org/gsea/msigdb*.

Single-cell RNA-sequencing (scRNA-seq) analysis

Single-cell suspensions were prepared following protocols from 10X Genomics. RFP+, GFP+, and Double^{+} Tri-PyMT cells were sorted by flow cytometry, and cells with >90% viability were submitted for sc-RNAseq at the Genomics and Epigenetics Core Facility at WCM. Single-cell libraries were generated using 10X Genomics Chromium Single-cell 3' Library RNA-Seq Assays protocols targeting 8,000 cells from each fraction were sequenced on the NovaSeq sequencer (Illumina). The scRNA-seq data were analyzed with the Partek Flow software (Partek Inc), an integrated user-friendly platform for NGS analysis based on the Seurat R package(11). The raw sequencing data were aligned to the modified mouse transcriptome reference (mm10) containing RFP, GFP, PyMT, and Cre genes by STAR. The deduplication of UMIs, filtering of noise signals, and quantification of cell barcodes were performed to generate the single-cell count data. Single-cell QA/QC was then controlled by the total counts of UMIs, detected features, and the percentage of mitochondria genes according to each sample (**Supplementary Fig. S3A**, **S6A**). The top 20 principal components (PC) were used for tSNE visualization. Differential gene expression analysis, GSEA, Geneset Overrepresentation assay, and Cell Trajectory analysis (Monocle 2 model) are also integrated into the Partek Flow platform (Partek Inc).

To highlight the overall expression of feature genes in a pathway, such as ribosome biogenesis or EMT status, we calculated the AUCell values for the gene list. AUCell calculates a value for each cell by ranking all genes based on their expression in the cell and identifying the proportion of the gene list that falls within the top 5% of all genes. For the epithelial or mesenchymal gene lists, we selected genes based on their overall expression levels in Tri-PyMT cells, ensuring consistency with their reported associations to epithelial or mesenchymal phenotypes in the literature (**Supplementary Fig. S4A**). For RiBi genes, we used the ribosome biogenesis pathway gene list from the GOBP_Ribosome_Biogenesis (GO:0042254) in MSigDB.

Tissue processing, Immunofluorescence, and Microscopy

Lungs with metastases were fixed in 4% paraformaldehyde overnight, followed by immersion in 30% sucrose for two days. They were then embedded in Tissue-Tek O.C.T. compound (Electron Microscopy Sciences). Serial sections (10-20 μm, at least 10 sections) were prepared for immunofluorescent staining.

For staining cultured or sorted cells, 2×10^3 cells/well were seeded in 8-well chamber slides (Nunc Lab-Tek, Thermo Fisher) and cultured overnight in growth medium. Standard immunostaining protocols were followed using fluorescent-conjugated primary antibodies. Fluorescent images were captured using a Zeiss fluorescent microscope (Axio Observer) with Zen 3.0 software (Carl Zeiss Inc.).

Western blot analysis

Cells were homogenized in 1x RIPA lysis buffer (Millipore) containing protease and phosphatase inhibitors (Roche Applied Science). The samples were then boiled in 1x Laemmli buffer with 10% β-mercaptoethanol and loaded onto 12% gradient Tris-Glycine gels (Bio-Rad). Western blotting was performed using the antibodies listed in the antibody table. Quantification of the Western blots was carried out using ImageJ. The relative intensity of each band was normalized to that of β-actin or tubulin, serving as loading controls for the same blot.

Antibodies used in the experiments

Species	Antigen	Cat#, Company	Dilution	Application
Mouse	E-Cadherin	144725, Cell Signaling	1:40	WB
Mouse	EpCAM	G8.8 Biolegend	1:100	FC, WB,IF
Mouse	Vim	5741T, Cell Signaling	1:500	WB
Mouse	β-actin	Ab8227, Abcam	1:2000	WB
Mouse	Fibrillalin	Ab5821, Abcam	1:100	IF
Mouse	p-ERK	4370, Cell Signaling	1:1000	WB
Mouse	ERK	4695, Cell Signaling	1:1000	WB
Mouse	p-mTORC1	3895s, Cell Signaling	1:1000	WB
Mouse	mTORC1	2983T, Cell Signaling	1:1000	WB
Mouse	p-rps6	4858T, Cell Signaling	1:1000	WB
Mouse	Tublin	11224-1-AP, Proteintech	1:1000	WB
Mouse	Snail	3895, Cell Signaling	1:500	WB

Cell viability assays

To determine the viability of Tri-PyMT cells under chemotherapy, cells (2×10^3 cells/well) were seeded in 96-well adherent black-walled plates, and treated with a serial concentration of 5-FU, Cisplatin, 4-Hydroperoxy Cyclophosphamide, Doxorubicin, Gemcitabine, and Paclitaxel together with BMH21, for 72 hours. After treatment, cell viability was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

To quantify the cytotoxic effect and potential synergic effects of drug combinations, we used SynergyFinder 3.0(12) for data analysis. Basically, normalized cell viability data were formatted and analyzed with online server at SunergyFinder (*https://synergyfinder.fimm.fi*). The LL4 and ZIP methods were chosen for curve fitting and synergy calculation, respectively.

Cell proliferation, transcription activity and nascent protein translation assays

To characterize cellular activities in nascent DNA, RNA, and protein synthesis, we applied EdU (5-ethynyl-2'-deoxyuridine), EU (5-ethynyl uridine), and OPP (O-propargyl-puromycin) incorporation assays, respectively. Cells (3×10^5 cells/well) were seeded in a 6-well plate and treated (or left untreated) according to experimental settings. Cells were labeled with EdU (10 μM, for 30 min), EU (1 mM, for 1 hour), or OPP (10 μM, for 30 min) in the incubator. After labeling, cells were harvested by trypsinization, fixed with 3.7% formaldehyde in PBS, and permeabilized with 0.5% Triton-X100 in PBS. Labeling was detected using the Click-&-Go® detection kit (Vector Laboratories) following the standardized protocol and analyzed by flow cytometry.

RT-PCR analysis

Total RNA was extracted from sorted RFP+, GFP+, and Doub+ Tri-PyMT cells using the RNeasy Plus Kit (Qiagen). For cDNA synthesis, 100 ng of RNA was used with the qScript cDNA SuperMix (Quanta Bio). Q-PCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) with target gene specific primers (as shown in the table) and Gapdh as the housekeeping control. The PCR protocol included an initial denaturation at 98°C for 2 minutes, followed by 40 cycles of 98°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, with signal readings at the end of each cycle. This was followed by a final extension at 72°C for 5 minutes and melt curve analysis on the CFX96 Real-Time System (Bio-Rad).

Gene specific primers used in the experiments

Target Gene	Forward	Reverse
Bop1	GGTCTCGGAGGAAGAGCACC	ACCGCCAAATAGTCCCCTCG
Gapdh	GGTCCTCAGTGTAGCCCAAG	AATGTGTCCGTCGTGGATCT
Gemin4	CCTCACAGGTCCACGAAGGG	TGCCCACATCCATCACCAGA
Its1	TCCATCTGTTCTCCTCTCTCT	ATCGGTATTTCGGGTGTGAG
Its2	CTGCCTCACCAGTCTTTCTC	ACCTCGACCAGAGCAGAT
Ecad	ACACCGATGGTGAGGGTACACAGG	ACACCGATGGTGAGGGTACACAGG
Ncad	AAAGAGCGCCAAGCCAAGCAGC	TGCGGATCGGACTGGGTACTGTG
Nop58	ACAGCAGAAGCATTAGCAGCA	CGACAGCCAGAGGTTCATGG
Npm1	GCGAGATCTCCTGCGACCAT	ACTTCGGTGTGGGAGAAGCC
Occl	TGCTAAGGCAGTTTTGGCTAAGTCT	AAAAACAGTGGTGGGGAACGTG
Polr1a	GGCTCTGCGCTACAAGACTC	AAGGAAATGCCCTGAAGCCG
Rpl29	GCAGTGAGGGAAGCTTTTCCG	CATGTCTGCACGGTAACCCG
Rpl8	ACAGAGCTGTTCATCGCAGC	ACGATCGTACCCTCAGGCAT
Rps24	ACACAGTAACCATCCGGACCA	TTTTGGCCAGCTTTTCCCGA
Rps28	GATATCCAGAACCCCACCAGC	AATGTCAAAGGCCCCGTTCG
Rps9	GTCTCGGGCCTGAGTTCGTA	CCTGCGCAGTAAAGTGTCGT
S100a4	CCTGTCCTGCATTGCCATGAT	CCCACTGGCAAACTACACCC
Setd4	GGAACTGCGCGTCCTTGTG	GTAACAAAACGCCCTCGGCA
Snail	ACTGGTGAGAAGCCATTCTCCT	CTGGCACTGGTATCTCTTCACA
Utp6	AGGGCATTTGGGGAGTAAGGG	TGCCTGGGGTCTGTCTCAGT
Vim	TGACCTCTCTGAGGCTGCCAACC	TTCCATCTCACGCATCTGGCGCTC
Xpo1	GGGAGCTTCAGCATCAGCAA	TTCCTTCCTTGTCCGGGCTT
Zeb1	GATTCCCCAAGTGGCATATACA	TGGAGACTCCTTCTGAGCTAGTG
Zeb2	TGGATCAGATGAGCTTCCTACC	AGCAAGTCTCCCTGAAATCCTT

Statistical Analysis

To determine the sample size for animal experiments, we performed power analysis assuming the (difference in means)/(standard deviation) is >2.5. Consequently, all animal experiments were conducted with ≥ 5 mice per group to ensure adequate power for two-sample t-test comparison or ANOVA. Animals were randomized within each experimental group, and no blinding was applied during the experiments. Results were expressed as mean ± SEM. Data distribution within groups and significance between different treatment groups were analyzed using GraphPad Prism software. P values < 0.05 were considered significant. Error bars represent SEM, unless otherwise indicated.

Results

Double+ Tri-PyMT cells mark the EMT transitioning phase

In contrast to their persistence in the epithelial state (RFP+) *in vivo* (6,10), RFP+ Tri-PyMT cells actively transition to GFP+ *in vitro* in growth medium containing 10% FBS (**Supplementary Fig. S1A**). As the fluorescent switch is irreversible, GFP+ cells accumulated over generations until a balanced ratio of RFP+/GFP+ is reached. This phenomenon indicates that the Tri-PyMT actively reports the ongoing EMT process in an EMT-promoting culture condition. Interestingly, we observed a subpopulation of cells, which were double positive for RFP and GFP, constituting approximately 2-5% of total cells (**Fig. 1A**). We posited that these RFP^+/GFP^+ ($Doub^+$) cells represent tumor cells transitioning from an epithelial to a mesenchymal state, since their fluorescent marker cassette has switched to GFP expression induced by Fsp1-Cre, while pre-existing RFP protein lingers due to its tardy degradation. Indeed, immunoblotting analyses confirmed the association of the double positive fluorescence and a hybrid EMT status. $Doub^+$ cells expressed intermediate levels of both epithelial markers (Epcam and E-cadherin) and the mesenchymal marker (Vimentin) (**Fig. 1B**). Further characterization of the Doub+ cells revealed higher percentages of S and G2/M phase cells in the Doub+ population compared to the RFP+ and GFP+ subpopulations (**Supplementary Fig. S1B**).

To further investigate the differential transcriptome in these EMT transitioning cells, we performed bulk RNA sequencing analysis using flow cytometry-sorted RFP^+, $Doub^+$, and GFP^+ Tri-PyMT cells. Consistently, analysis of traditional EMT marker genes revealed that Doub+ cells expressed both epithelial and mesenchymal markers (**Supplementary Fig. S2A**). Differentially expressed genes in Doub+ cells were divided into 4 clusters, Trans_Up, Trans_Down, Epithelial and Mesenchymal markers (**Supplementary Fig. S2B**). Our attention was particularly attracted by upregulated genes within the Trans_Up cluster, as they may represent activated pathways specific in the transitioning phase of EMT. Interestingly, a gene set over-representative assay showed that the KEGG_Ribosome pathway was significantly enriched in the Trans_Up gene list (**Supplementary Fig. S2C**).

Together, these results indicate that Doub+ Tri-PyMT cells represent an active EMT-transitioning phase, as evidenced by well-established EMT markers; specific activations of biological processes in Doub+ cells warranrt further investigation for developing effective strategies to intervene the trasition.

Ribosome biogenesis pathway is enhanced in the EMT transitioning phase

To gain a deeper understanding of transcriptome alterations and ensure adequate representation of EMT-transitioning cells, RFP^+, GFP^+, and $Doub^+$ cells were sorted simultaneously via flow cytometry; equal numbers of each population were remixed and subject for single-cell RNA sequencing (scRNA-seq) analysis.

The *t*-SNE plot demonstrated two major clusters (**Fig. 1C**): one predominantly expressed epithelial genes, while the other displayed overall mesenchymal phenotypes. Doub+ cells were integrated into the two major clusters, suggesting that the overall single-cell transcriptome may not be sensitive enough to identify tumor cells at the EMT-transitioning phase. We therefore performed cell trajectory analysis (Monocle 2) based on all EMT-related genes (EMTome (13)). Five cell states related to EMT status were identified (**Fig. 1D**). All of them aligned well with a specific expression pattern of epithelial and mesenchymal marker genes based on AUC values (**Fig. 1E**), or individual epithelial/mesenchymal pairs, such as Fsp1/Epcam and Vim/Krt18

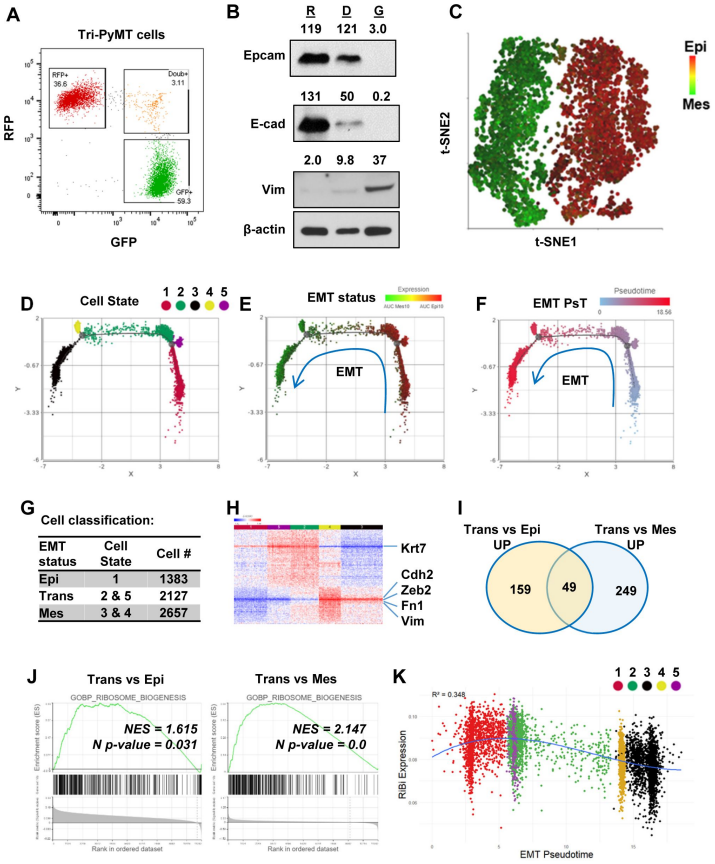

Figure 1.

Activation of ribosome biogenesis pathway during the EMT transitioning phase in Tri-PyMT cells.

A, Representative flow cytometry plot displays the percentage of RFP+, Double+ (Doub+, EMT transitioning cells), and GFP+ Tri-PyMT cells in culture.

B, Western blot of EMT markers with flow sorted RFP+ (R), Doub+ (D), and GFP+ (G) Tri-PyMT cells. The Doub+ cells exhibit an intermediate EMT status with expression of epithelial marker (E-cadherin and Epcam) expression and higher mesenchymal marker (Vimentin) expression as compared with RFP+ cells. The numbers indicate normalized intensity of the band according to the β-actin band of the sample.

C, The t-SNE plot of scRNA-seq analysis of Tri-PyMT cells. Two major cell clusters with differential EMT status are shown with the AUC value of 10 epithelial marker genes (in red) and 10 mesenchymal marker genes (in green). The marker genes are shown in **Supplementary Fig S4A**.

D, E, F, Trajectory analysis using Monocle DDR tree. Cell trajectory analysis was performed with filtered EMT-related genes using Monocle 2 model. Five cell states (D) were identified with differential EMT statuses. From epithelial to mesenchymal phenotype, the Cell States were identified in order of 1, 5, 2, 4, and 3. EMT status (E) of cells was also highlighted by the AUC calculation with epithelial and mesenchymal marker genes. EMT Pseudotime (F) was calculated with Cell State 1 (the most epithelial state) as the root.

G, Classification of cells with EMT states. Cells were classified according to their EMT state as Epi, Trans, and Mes; the cell number in each cluster is indicated in the table.

H, The heatmap of differentially expressed genes in Trans cells compared to Epi and Mes cells. Totally, 313 genes were identified with criteria P <0.01, Fold change > 1.2, and Average expression >=5.

I, The common enriched GO_BP pathways with GSEA when comparing Trans *vs.* Epi and Trans *vs.* Mes. There are 49 common pathways, including 5 pathways related to Ribosome Biogenesis (RiBi) or rRNA processing. Pathway names are shown in **Supplementary Fig S4B**.

J, GSEA plots showing the specific enrichment of Ribosome Biogenesis (RiBi) pathway in EMT transitioning (Trans) cells compared to the Epi or Mes cells.

K, The scatter plot displays the correlation of RiBi activity to EMT pseudotime. The polynomial regression line (order = 3) highlights the elevated RiBi pathway in Trans phase cells, $R^2 = 0.348$, $P < 0.001$.

(**Supplementary Fig. S3B**, **S3C**). Furthermore, we calculated the EMT pseudotime of each cell and designated State 1 (the most epithelial state) as the root (**Fig. 1F**). Cells were then classified into three main categories, Epi (State 1 cells), Trans (State 5&2) and Mes (State 4&3), based on their position within the EMT spectrum (**Fig. 1G**). Differential gene expression analysis confirmed that Trans cells gained expression of mesenchymal markers such as Cdh2, Vim, Fn1 and Zeb2, while retaining expression of epithelial markers such as Krt7 (**Fig. 1H**).

With the differential expression gene list, we analyzed the pathway activation in Trans cells by Gene Set Enrichment Analysis (GSEA) with the biology process (BP) gene sets of the GO term (14). Among the 49 overlapped gene sets that represented significantly upregulated pathways when comparing Trans vs. Epi and Trans vs. Mes ($P < 0.05$), six pathways were related to the ribosome or rRNA processing (**Fig. 1I**, **Supplementary Fig. S4B**). These findings were in line with the previous bulk RNA sequencing analysis that indicated activation of RiBi pathway in EMT-transitioning (Doub+) cells. Consistently, GSEA using scRNAseq confirmed significant enrichment of RiBi genes in Trans cells compared to cells at Epi or Mes phase (**Fig. 1J**). We further mapped the EMT spectrum of cells based on their EMT pseudotimes or cell states, and correlated them with their RiBi activities. A significant trend of RiBi activation was observed in the trasitioning phase of EMT process (**Fig. 1K**, **Supplementary Fig. S3D**). It is worth noting that the RiBi activity is lowest in cells characterized with the latest EMT pseudotime, indicating that the elevation of RiBi activities is transcient during EMT (**Fig. 1K**). RT-PCR analysis of also confirmed the relatively higher expression of RiBi related genes in Doub+ cells compared to RFP+ and GFP+ cells (**Supplementary Fig. S5**).

Ribosome biogenesis pathway was upregulated during MET process

The transient elevation of RiBi during EMT prompted us to ask whether the MET process required the same. Since the fluorescence switch of Tri-PyMT cells is permanent, we tracked MET by monitoring the regain of epithelial marker by post-EMT (GFP+/Epcam-) cells. We sorted GFP^+/$Epcam^-$ Tri-PyMT cells via flowcytometry and injected them into *Scid* mice via tail vein. Approximately 50% of tumor cells expressed EpCam at 4 weeks post-injection, indicating active MET (**Fig. 2A**). We then sorted GFP^+ tumor cells, including both $EpCam^+$ and $EpCam^-$ cells, from the lungs for scRNA-seq analyses.

Similar to our observations with *in vitro* cultured Tri-PyMT cells, GFP+ cells from lungs formed two main clusters in the *t*-SNE plot, exhibiting overall epithelial or mesenchymal phenotypes (**Fig. 2B**). We performed trajectory analysis with EMTome genes and identified nine cell states with different EMT statuses (**Fig. 2C**). The relative expression of epithelial *versus* mesenchymal markers clearly illustrated the MET spectrum of tumor cells (**Fig. 2D**, **Supplementary Fig. S6B**). By designating the most extreme mesenchymal state (State 1) as the root, we calculated the MET pseudotime of individual cells (**Fig. 2E**). Consistent with the analysis for EMT, we found that tumor cells with epithelial phenotypes displayed elevated RiBi activity (**Fig. 2F**, **Supplementary Fig. S6C**), indicating its reactivation during MET process. A significant positive correlation was detected between RiBi gene upregulation and MET pseudotime (**Fig. 2G**). To eliminate the possibility that activation of RiBi pathway was solo related to the proliferation of cells, we project the S phase score to the scatter plot of Ribi activity/MET pseudotime. Indeed, cells in the far mesenchymal state show low S phase score, while the proliferating cells were mostly detected in the transitioning phase and epithelial phase (**Supplementary Figure S6D**). Together, these results suggested that upregulated RiBi pathway is equally needed for mesenchymal tumor cells to undergo MET during their outgrowth in the lungs.

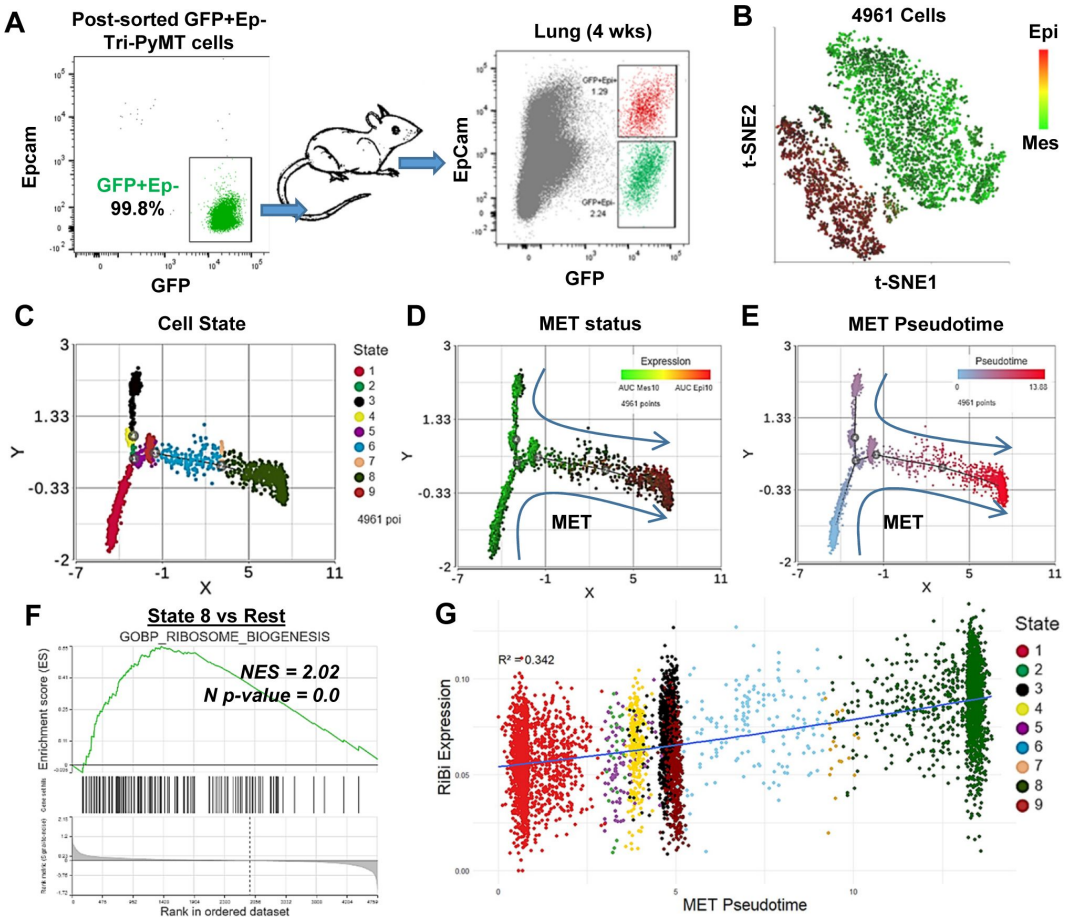

Figure 2.

Activation of RiBi pathway in MET during lung metastasis outgrowth.

A, Schematic of MET induction. GFP+ Tri-PyMT cells were sorted by flow cytometry (left) and injected into mice through the tail vein. Metastasis-bearing lungs were harvested after 4 weeks and analyzed for the gain of epithelial marker (EpCam) in tumor cells (right). Both Epcam+ and Epcam- cells were sorted and submitted for scRNA-seq analysis.

B, The *t*-SNE plot of scRNA-seq analysis of GFP+ Tri-PyMT cells sorted from metastatic lungs of 3 individual animals. Two major cell clusters with distinct EMT statuses were identified, as indicated by the AUC values of 10 epithelial genes (in red) and 10 mesenchymal genes (in green).

C, D, E, Monocle 2 model-based cell trajectory analysis identifies nine cell states (C). The MET process is emphasized by AUC values of mesenchymal and epithelial markers (D). MET Pseudotime is calculated with State 1 (the most mesenchymal state) as the root (E).

F, GSEA plot exhibits the enrichment of the GOBP_Ribosome Biogenesis (RiBi) pathway in State 8 cells (the most epithelial phenotype) compared to the other states.

G, Scatter plot illustrates the correlation between RiBi activity and MET pseudotime. A polynomial regression line (order = 3) emphasizes the elevated RiBi pathway during the MET process, $R^2 = 0.342$, $P < 0.01$.

Activation of ERK and mTOR signaling pathways are linked to the upregulation of ribosome biogenesis

Ribosome biogenesis was recognized as a crutial factor in cancer pathogenesis a century ago (15). To further investigate the signaling pathways responsible for RiBi upregulation in the EMT/MET process, we sorted RFP$^+$, Doub$^+$ and GFP$^+$ Tri-PyMT cells and probed the signaling activations in these cells. In response to serum stimulation, Doub$^+$ cells exhibited significantly higher levels of phosphorylated extracellular signal-regulated kinase (p-ERK) and phosphorylated mammalian target of rapamycin complex 1 (p-mTORC1) compared to either RFP$^+$ or GFP$^+$ cells (**Fig. 3A**). The phosphorylation of Rps6, an essential ribosome protein of the 40S subunit, is regulated by synergistic crosstalk between mTORC1 and ERK signaling(16,17). We thus explored the status of p-Rps6 and observed a significantly higher level of p-Rps6 in Doub$^+$ cells (**Fig. 3A**). These results imply a connection between differential signaling transductions and ribosome activities in EMT transitioning phase cells.

The primary function of ribosome is to synthesize proteins to support essential biological functions(18). Indeed, enhanced ribosome activity in Doub+ cells translated into distinct phenotypes in cell growth and nascent protein synthesis. Flow cytometry analysis showed that Doub+ cells possessed enlarged cell sizes compared to RFP$^+$ or GFP$^+$ cells (**Fig. 3B**). Using the O-propargyl-puromycin (OPP) incorporation assay, we found that Doub$^+$ cells exhibited an increased rate in nascent protein synthesis (**Fig. 3C**). Another hallmark of ribosome activity is rRNA transcription that occurs in nucleoli. By immunostaining of Fibrillarin, a nucleolar marker(19), we found that the Doub$^+$ cells had significantly more nucleoli compared to cells in epithelial (RFP$^+$) and mesenchymal (GFP$^+$) states (**Fig. 3D**). Consistently, using EU incorporation assay, we found significantly higher activity of transcription in Doub+ cells compared to RFP+ and GFP+ cells (**Supplementary Figure S7**).

Collectively, these results suggest that the elevation of RiBi pathway in cells at EMT transitioning phase is associated with aberrant activation of ERK and mTOR signalings, which may, in turn, confer nascent protein synthesis capability to tumor cells for completing phenotypic changes.

Suppression of ribosome biogenesis reduced the EMT/MET capability of tumor cells

The transcription of ribosomal RNA (rRNA) is mediated by RNA polymerase I (Pol I) in eukaryotic cells (20). Small molecules such as BMH21 and CX5461 are specific Pol I inhibitors, which inhibit rRNA transcription and disrupt ribosome assembly(21,22), providing a specific RiBi targeting strategy. We then evaluated whether these Pol I inhibitors would affact EMT/MET process.

Fluorescence switch from RFP+ to GFP+ of TriPyMT cells were employed to investigate the impact of Pol I inhibitors on EMT. RFP+/Epcam+ cells were sorted via flowcytometry and served as cells in Epithelial state. In contrast to the vehicle-treated RFP$^+$/Epcam+ cells, which transitioned to GFP$^+$ upon serum stimulation, most cells treated with either BMH21 or CX5461 stayed in the RFP$^+$ state (**Fig. 4A**, **Supplementary Fig. S8A**). Of note, the treatment of BMH21 or CX5461 indeed inhibited the transcription activity in cells as detected by EU incorporation assay (**Supplementary Fig 8B**). To confirm that the impaired fluorescence switch of RFP$^+$ Tri-PyMT cells is associated with the retention of epithelial phenotypes, we performed immunoblotting of EMT markers. Indeed, these Pol I inhibitors significantly blocked the expression of mesenchymal markers, including Vimentin and Snail, while preserving the expression of the epithelial marker (Ecadherin) (**Fig. 4B**).

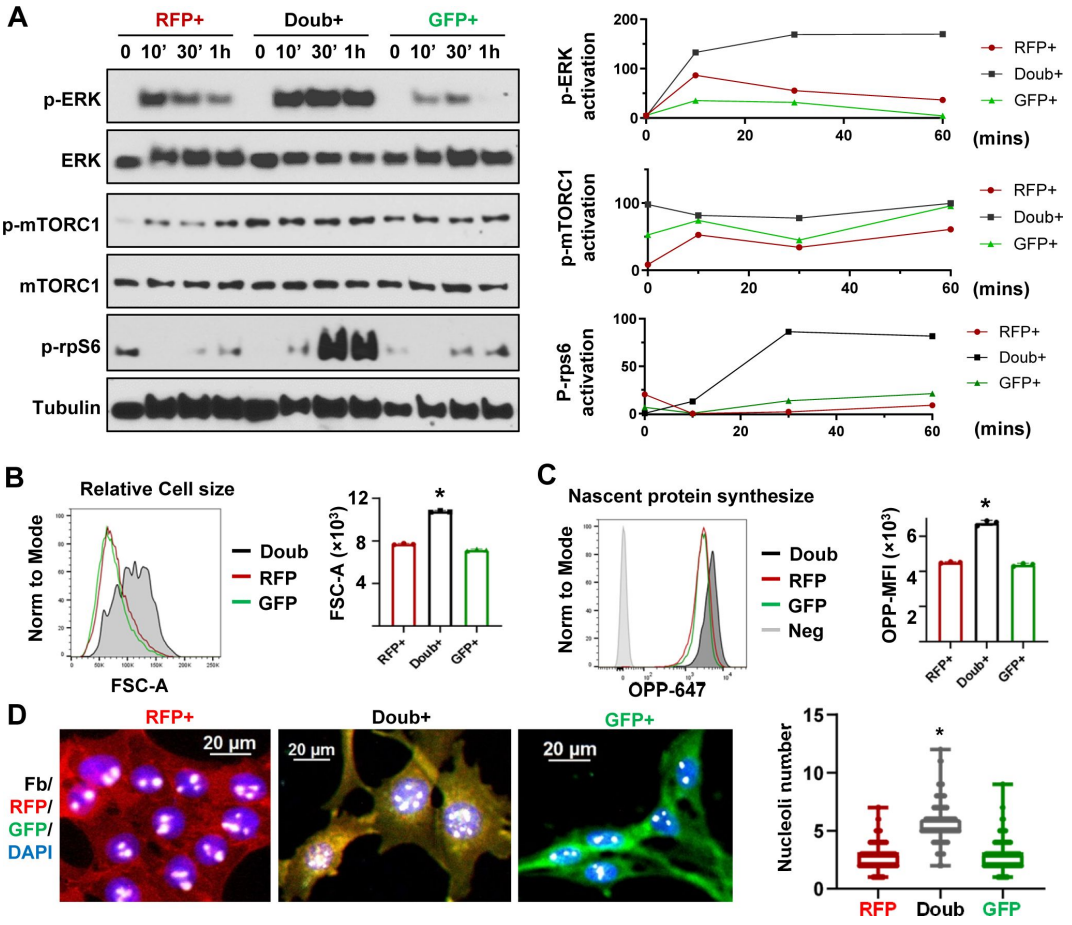

Figure 3.

MAPK and mTOR pathways mediated ribosome biogenesis activation in EMT transitioning phase cells.

A, Western blots show phospho-ERK, total ERK, phospho-mTORC1, total mTORC1 and p-rps6 in sorted RFP+, Doub+, and GFP+ Tri-PyMT cells following stimulation with 10% FBS at 0, 10, 30 and 60 mins. Activation of p-ERK, p-mTORC1 and p-Rps6 are quantified by the band intensity after normalizing to loading controls (right).

B, Cell size analysis. Tri-PyMT cells (p5) were analyzed by flow cytometry. The relative cell size was indicated by FSC-A, for individual RFP+, GFP+, and Doub+ cells. Data from three biological replicates.

C, Nascent protein synthesis assay. Histogram of OPP incorporation, as measured by flow cytometry, demonstrates enhanced nascent protein synthesis in Doub+ cells. The rate of new protein synthesis is assessed by adding fluorescently labeled O-propargyl-puromycin (OPP). Three biological replicates, One-way ANOVA, *P < 0.0001, Doub+ vs RFP+, and Doub+ vs GFP+.

D, Fluorescent images reveal increased RiBi activity in Doub+ Tri-PyMT cells, as evidenced by Fibrillarin staining. RFP+, Doub+, and GFP+ Tri-PyMT cells were sorted and stained for nucleoli using an anti-Fibrillarin antibody. The quantification of nucleoli per cell is shown on the right. n = 522 (RFP+), 141 (Doub+), 289 (GFP+). One-way ANOVA, *P < 0.0001.

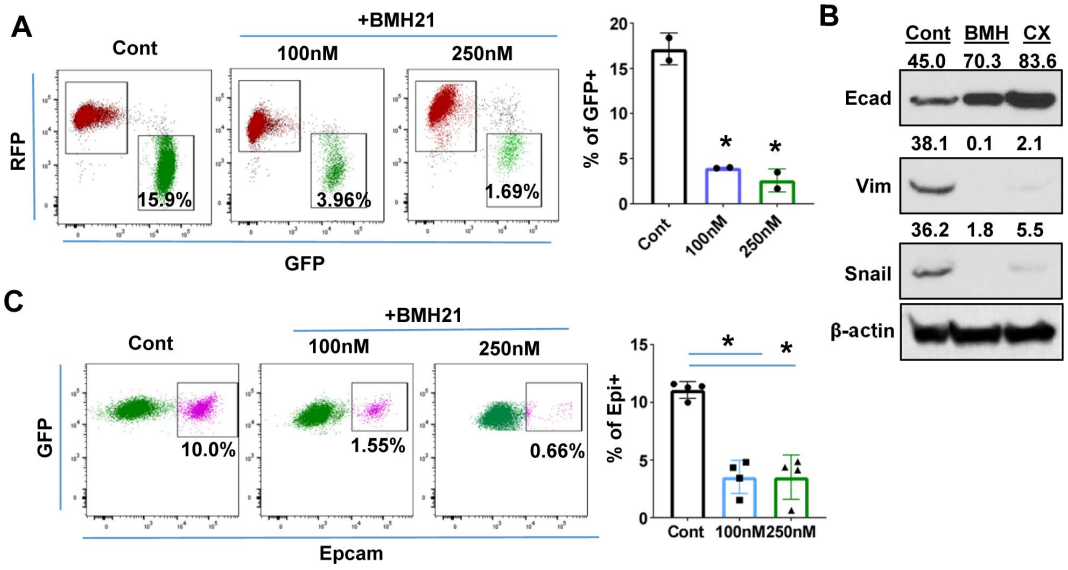

Figure 4.

RiBi inhibition by Pol I inhibitor reduces the EMT/MET capability of tumor cells.

A, Flow cytometry plots display the percentage of GFP+ cells following BMH21 treatment. RFP+/EpCam+ Tri-PyMT cells were sorted and cultured in growth medium with or without BMH21 for 5 days. Percentage of GFP+ cells was analyzed by flow cytometry. Biological repeat, n=2, One-way ANOVA, *P=0.0039, 100nM vs Cont; *P=0.0029, 200nM vs Cont.
B, Western blots reveal the expression of the epithelial marker (E-cad) and mesenchymal markers (Vim and Snail) in Tri-PyMT cells following a 5-day treatment with BMH21 (100nM) and CX5461 (20nM). The numbers indicate normalized intensity of the band according to the β-actin band of the sample.
C, Flow cytometry plots show the percentage of Epcam+/GFP+ Tri-PyMT cells treated with BMH21. GFP+/EpCam-Tri-PyMT cells were sorted and culture in 3D to induce MET for 14 days. The gain of Epcam expression was analyzed by flow cytometry. n=4, One way ANOVA, *P<0.0001, 100nM vs Cont; *P<0.0001, 200nM vs Cont.

Given that the elevated RiBi activity occurs during MET process as well, we further assessed the impact of Pol I inhibitor on the retrieval of epithelial markers by mesenchymal tumor cells. The regain of EpCam expression by the sorted GFP+/EpCam-Tri-PyMT cells in a 3D culture was measured via flow cytometry. BMH21 significantly prevented the Epcam retrieval by GFP+/Epcam-Tri-PyMT cells, suggesting the impaired MET capability upon treatment (**Fig. 4C**). These results laid a foundation for pharmacological inhibition of RiBi pathway to block EMT/MET of tumor cells.

The requirement of RiBi activity during EMT/MET transitioning was also demonstrated by genetically modulating ribosome proteins. As the organelle for protein synthesis, ribosome comprises 4 ribosomal RNAs and approximately 80 structural ribosomal proteins(15). Depleting one r-protein usually causes decreases of other r-proteins in the same subunit, and ultimately compromises the overall ribosome assembly(23). We, therefore, employed Lenti-shRNAs targeting Rps24 and Rps28, two essential genes of 40S subunit, to genetically modulate the RiBi pathway in Tri-PyMT cells (**Supplementary Fig. S9A**). Effective knocking-down of Rps24 or Rps28 significantly reduced the number of nucleoli (**Supplementary Fig. S9B**). Accompanying with the downregulated RiBi activities were the impeded EMT (RFP-to-GFP switch) and the similarly reduced MET (regain of Epcam) (**Supplementary Fig. S9C, D**). These results suggested that the elevated RiBi activities are critical for tumor cells to maintain their abilities to shift between epithelial and mesenchymal states.

RiBi inhibition synergizes with chemotherapy drugs

To assess the overall impact of Pol I inhibitor on both epithelial and mesenchymal tumor cells, we treated unsorted Tri-PyMT cells (containing both RFP+ and GFP+ populations) with BMH21 for 7 days. Less accumulation of GFP+ cells was observed with BMH21 treatment compared with untreated controls (**Fig. 5A**). In contrast, treatment with the chemotherapy drug, cyclophosphamide (CTX), resulted in more GFP+ cells (**Fig. 5A**), consistent with our previous findings that chemoresistant features against CTX were acquired through EMT (6).

Based on these observations, we hypothesized that the blockade of EMT by Pol I inhibitor will reduce the EMT-mediated chemoresistance. The combination therapies of Pol I inhibitor and chemo drugs were therefore tested. We treated Tri-PyMT cells, which contain approximately 15% GFP+ cells, with a series of BMH21 concentrations with or without CTX. Cytotoxic assay after 3 days treatment revealed an enhanced sensitivity of tumor cells to the combination therapies (**Fig. 5B**). Interestingly, BMH21 and CTX exhibited optimal synergy (**Fig. 5C**). Particularly at concentrations of 100-200 nM, BMH21 showed significant high synergy scores with CTX (δ^{high} = 8.45). Such low concentrations of BMH21 were approximately 10-20% of the IC_{max} in Tri-PyMT cells and have also been shown to effectively block the EMT/MET process, suggesting the synergy was likely induced by EMT blockade, rather than cytotoxicity of BMH21. Importantly, the synergic effect between BMH21 and chemotherapy drugs is not limited to CTX. We performed the combination treatment of BMH21 with most commonly used chemodrugs of breast cancer therapy, including 5FU, Cisplatin, Doxorubicin, Gemcitabine and Paclitaxol. Trends toward synergies were also found with most of them, especially with lower concentrations of BMH21(100 – 200 nM) (**Supplementary Fig. S10**). These results suggest that RiBi blockade by the Pol I inhibitor may represent an effective approach to overcome EMT-related chemoresistance.

RiBi inhibition diminished chemoresistant metastasis of breast tumor cells in the lung

Both the reduced EMT-mediated chemoresistance and the diminished MET during metastatic outgrowth upon BMH21 treatment encouraged us to evaluate the efficacy of combination therapy for treating animals bearing metastatic breast tumors.

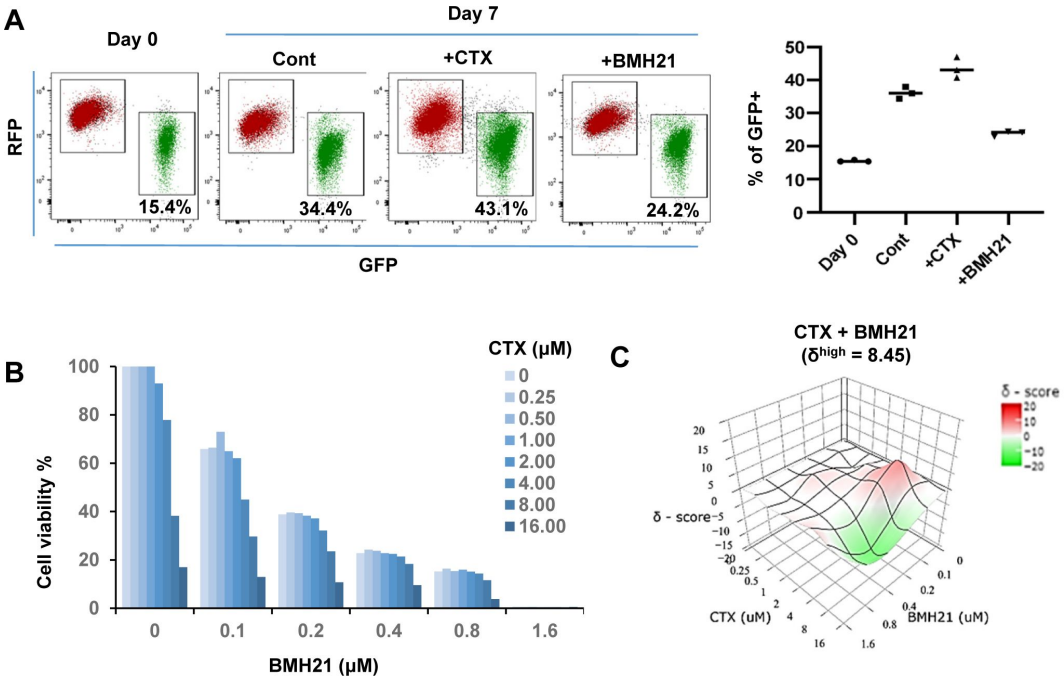

Figure 5.

RNA Pol I inhibitor synergizes with chemo drug *in vitro*.

A, Flow cytometry plots display the fluorescence switch of Tri-PyMT cells. Tri-PyMT cells (p5) were treat with cyclophosphamide (CTX, 2μM), Pol I inhibitor (BMH21, 0.1μM) or vehicle control for 7 days. Flow cytometry was performed to analyze the percentage of GFP+ cells. n=3 wells/treatment, One way ANOVA, *P=0.0050, Cont vs. CTX; *P=0.0002, Cont vs. BMH21.

B, Cytotoxic assay of BMH21 and CTX treatments. Tri-PyMT cells were treated with serial concentrations of BMH21 (0, 0.1, 0.2, 0.4, 0.8, and 1.6 μM) in combination with serial concentrations of CTX (0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 μM). Bars represent the mean value of cell viabilities from duplicated treatments, n=2 wells/treatment.

C, Synergy plots of BMH21 and Cyclophosphamide (CTX). Synergic scores (δ) for each combination were calculated using the ZIP reference model in SynergyFinder 3.0. Deviations between observed and expected responses indicate synergy (red) for positive values and antagonism (green) for negative values.

We established a competitive metastasis assay by injecting an equivalent number of GFP^+ and RFP^+ Tri-PyMT cells (GFP:RFP, 1:1, representing epithelial and mesenchymal tumor cells, respectively) into *Scid* mice via the tail vein. Tumor-bearing mice received the vehicle, single, or combination therapy of BMH21 (25mg/kg, 5 times/week for 3 weeks, *ip.*) and CTX (100mg/kg, once a week for 3 weeks, *i.p.*). Bioluminescent imaging (BLI) revealed that the combination treatment exhibited the highest restraint on the chemoresistant outgrowth of metastatic tumors compared to the vehicle, BMH21, or CTX mono-treatment groups (**Fig. 6A**). To analyze the differential impacts on epithelial and mesenchymal tumor cells by the therapies, we quantified the residual RFP+ and GFP+ cells by flow cytometry analysis. Significant decrease of both RFP+ and GFP+ cells were observed in mice receiving the combination therapy of BMH21 and CTX (**Supplementary Fig. S11**). Microscopic analyses also revealed that both RFP^+ and GFP^+ cells grew into macrometastases in the lung of vehicle-treated animals (**Fig. 6B**). Notably, many GFP^+ cells displayed epithelial markers, such as EpCam (**Fig. 6C**), indicating that a MET process was involved during the outgrowth of lung metastasis. CTX treatment eliminated the majority of RFP^+/epithelial metastases, while the GFP^+/mesenchymal cells showed survival advantages under chemotherapy, resulting in a higher ratio of GFP:RFP. BMH21 treatment inhibited metastatic tumor growth. Interestingly, most tumor cells under BMH21 treatment kept their EMT phenotypes as RFP^+/$Epcam^+$ or GFP^+/$Epcam^-$ (**Fig. 6C**), indicating the impaired EMT/MET transitioning by RiBi inhibition. Importantly, the combination of BMH21 and CTX eliminated most tumor cells including both RFP+ and GFP+ cells, and significantly inhibited the outgrowth of metastatic nodules under chemotherapy (**Fig. 6B-6C, and Supplementary Fig. S11**).

We further established an experimental lung metastasis model with human breast cancer cells (MDA-MB231-LM2). The LM2 cells predominantly exhibited a mesenchymal phenotype *in vitro* and gained Ecad expression during the outgrowth of lung nodules *in vivo* (**Supplementary Fig. S12**), indicating the involvement of the MET process. Consistent with the Tri-PyMT model, BMH21 synergized with CTX, leading to a significantly lower metastatic LM2 tumor burden than the mock or mono-treatment groups (**Fig. 6D**).

To further investigate the potential association of RiBi activity with EMT status of tumor cells in human breast cancer, we analyzed scRNA-seq data of primary tumor cells from two breast cancer patients (GSE 198745). A trend of relatively higher RiBi activity was detected in tumor cells with lower EMT pseudotime (**Supplementary Fig 13**). Interestingly, a pattern showing the highest RiBi activity in EMT/MET transitioning phase was detected in the sample of patient B (**Supplementary Fig 13F**), indicating the similar role of RiBi upregulation in EMT status of breast cancer patients. To examine whether RiBi activity associated with clinical outcomes of breast cancer patients, we analyze the RNAseq data in cBioPortal databases (*www.cbioportal.org*), including the TCGA PanCancer Atlas (1084 samples) and METABRIC (2500 samples). RiBi activities of tumors were quantified by the average z-scores of genes in RiBi pathway (303 genes). Patient samples with a score >1 were denoted as $RiBi^{High}$, while patients with a score <0.5 were denoted as $RiBi^{Low}$. The analyses of the survival data showed a significantly worse prognosis in the $RiBi^{High}$ group compared to the $RiBi^{Low}$ group (**Supplementary Fig 14**).

In summary, these results suggest that inhibition of RiBi activities diminished EMT/MET transitioning capability of tumor cells. In combination with chemotherapy drug, RiBi inhibition significantly reduced the outgrowth of chemoresistant metastasis. These results suggested that targeting RiBi-mediated EMT/MET process may provide a more effective therapeutic strategy for advanced breast cancer.

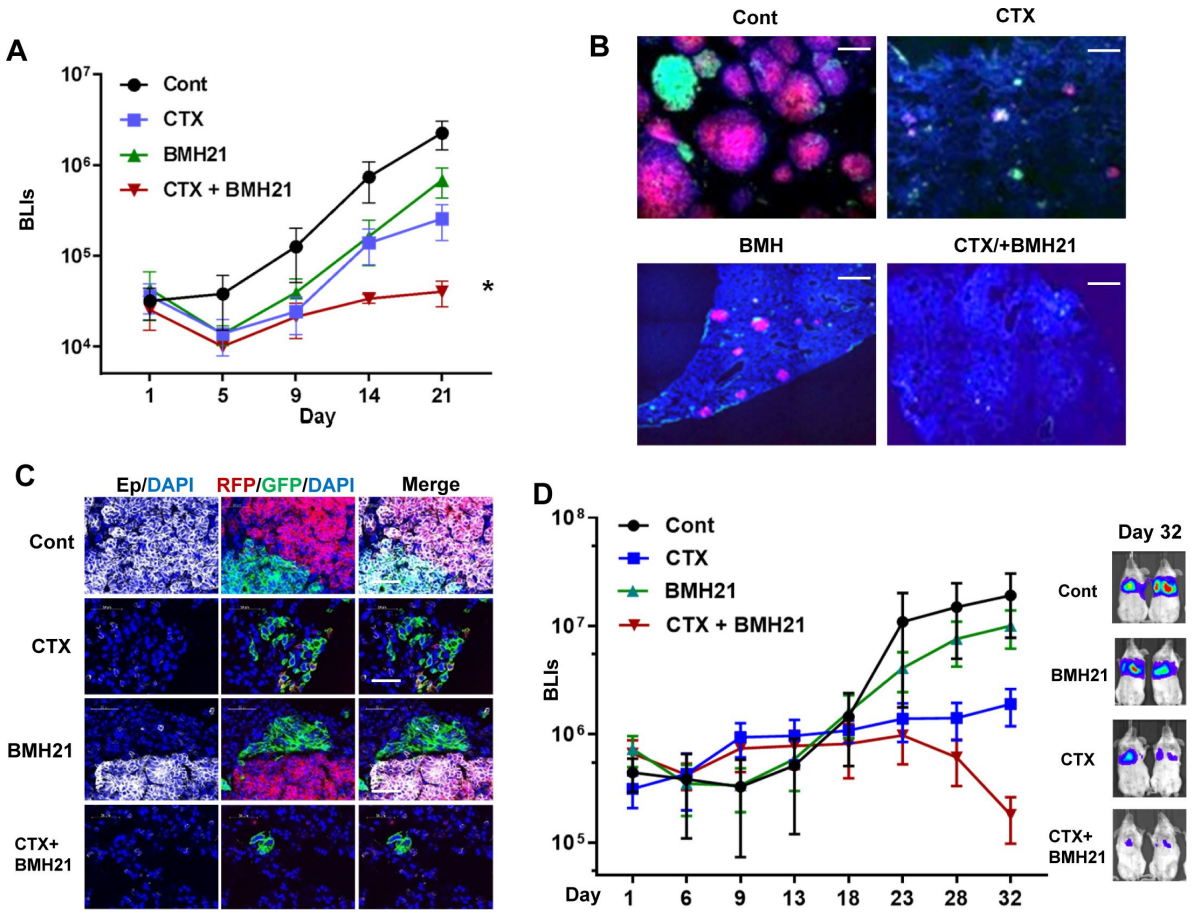

Figure 6.

RNA Pol I inhibition synergizes with chemo drug in treating lung metastatic tumors.

A-C, The same number of RFP+ and GFP+ Tri-PyMT cells were injected in animals via tail vein. Animals were treated CTX (100mg/kg, once a week, i.p.), BMH21 (20mg/kg, 5 times a week, i.p.) or both in combination.

A, Lung metastasis growth curves as measured by bioluminescent imaging (BLI). n=5, two-way ANOVA with Tukey's test, **P*=0.0154, Cont *vs*. CTX+BMH; **P*=0.0151, BMH21 *vs.* CTX+BMH21; **P*=0.0372, CTX *vs.* CTX+BMH21 at Day 21 post-inoculation.

B, Fluorescent images display RFP+ and GFP+ metastatic nodules in the lungs treated with CTX and BMH21. Cell nuclei were stained with DAPI. Scale bar: 200μm.

C, Fluorescent images exhibit EMT statuses of RFP+ and GFP+ metastases. Sections were stained with anti-EpCam antibody; cell nuclei were stained with DAPI. Scale bar: 50μm.

D, Lung metastasis growth curves for LM2 model. Lung metastatic breast tumor cells (LM2 cells) were injected into animals via tail vein. Animals were treated CTX (100mg/kg, once a week, i.p.), BMH21 (20mg/kg, 5 times a week, i.p.) or both in combination. n=5, two-way ANOVA with Tukey's test, **P*=0.0404, Cont *vs.* CTX+BMH21; **P*=0.0161, BMH21 *vs.* CTX+BMH21; **P*=0.0187, CTX *vs.* CTX+BMH at Day 32 post-inoculation. Representative BLI images of Day 32 were presented on the right.

Discussion

Targeting EMT for cancer therapy has been challenging due to the complexity of the EMT process and controversies of promoting MET which also favors tumor progression. Using the EMT-lineage-tracing model, we found an upregulation of RiBi pathway at the transitioning phase of both EMT and MET (**Supplementary Fig 15**). The transcient activation of RiBi pathway during the EMT process has been reported previously. Prakash et al. found that elevated rRNA synthesis/RiBi pathway was concomitant with cell cycle arrest induced by TGFβ, fueling the EMT program in breast tumor cells(24). Using the Tri-PyMT model, we found that the EMT transitioning (Doub+) cells had higher percentages of cells in the S and G2/M phases compared to the RFP+ and GFP+ cells. This is inconsistent with the observation that RiBi activity was higher in G1 arrested cells treated with TGFβ (24). This discrepancy may be due to the different EMT stimuli used in the experiment systems. Additionally, further investigations are needed to determine whether a cell could complete the EMT process within a single cell cycle or requires multiple cell divisions.

A more recent study identified a subpopulation of circulating tumor cells (CTCs) in which high RiBi activities persisted to maintain their high metastatic potentials(25). Interestingly, the RiBi activity was associated with epithelial phenotypes rather than mesenchymal ones in CTCs(25). Indeed, EMT induction by TGFβ primarily suppressed ribosome gene expression and global translational activity (25). By employing our unique EMT-lineage-tracing model, we discovered that the RiBi pathway was transiently elevated during the transitioning phases of EMT/MET program. The enhanced activation of RiBi pathway diminished as tumor cells accomplished phenotype changes. In general, a lower RiBi activity was observed in the mesenchymal tumor cells as compared to that in the epithelial ones. Importantly, the involvement of unwonted RiBi activities during both EMT and MET processes makes RiBi a new and better target for overcoming EMT-related chemoresistance and chemoresistant metastasis.

Targeting RiBi pathway by RNase Pol I inhibitor impaired the EMT/MET transitioning capability of tumor cells and significantly synergized with common chemotherapeutics. These observations also suggest that malignant cells might require certain ease to “ping pong” between epithelial and mesenchymal states, so that they could adapt themselves to the challenging microenvironment. Of note, some commonly used chemotherapeutics (Cisplatin, 5FU, Doxorubicin, etc), although primarily targeting DNA duplications, may also affect RiBi pathway by inhibiting rRNA processing(26). Therefore, the synergic effects with Pol I inhibitor varied among different combinations. Moreover, RiBi is a process dysregulated in most, if not all, cancers. Its involvement in EMT/MET process make it a feasible targeted pathway for treating patients with advanced breast cancer.

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Data access statement

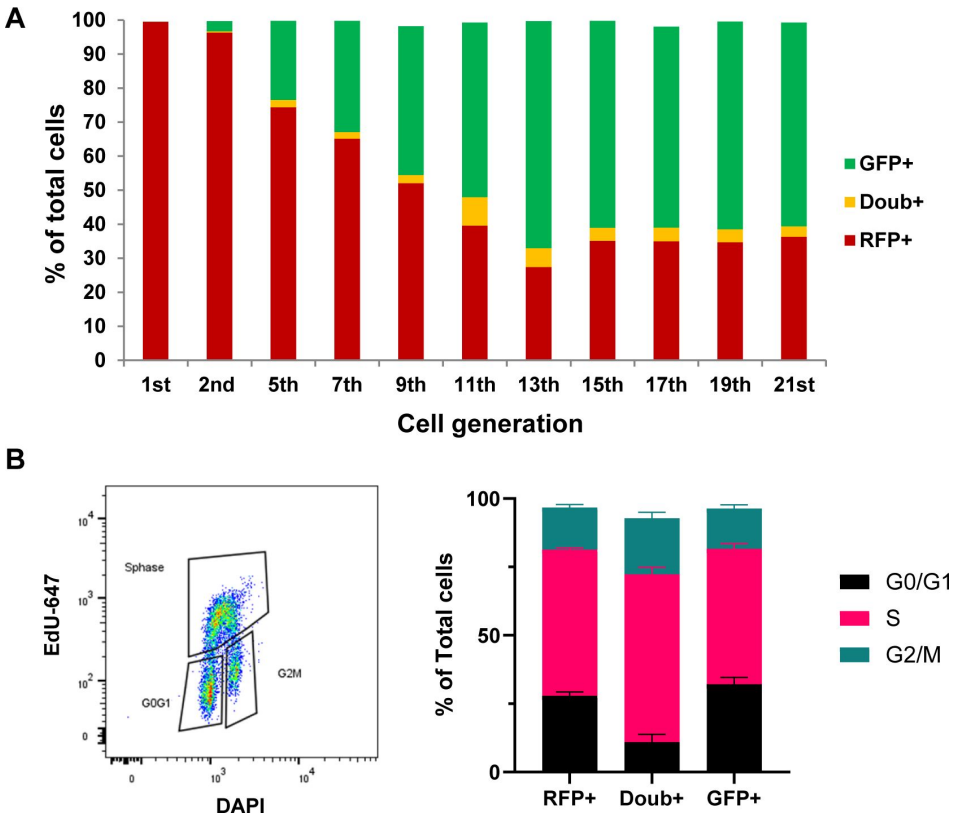
Data generated in the study is either publicly available or by request to the corresponding authors.

Author contributions

Y.B. and D.G. designed the experiments. Y.B., and Y.Z., performed the experiments. Y.B., D.G., J.S., Y.C., and S.W. performed bulk and single-cell RNA sequencing analyses. S.L and R.B. performed some animal works for experiments. D.G. supervised this study. Y.B. and D.G. wrote the manuscript. All authors discussed the results and conclusions drawn from the studies.

Declaration of interests

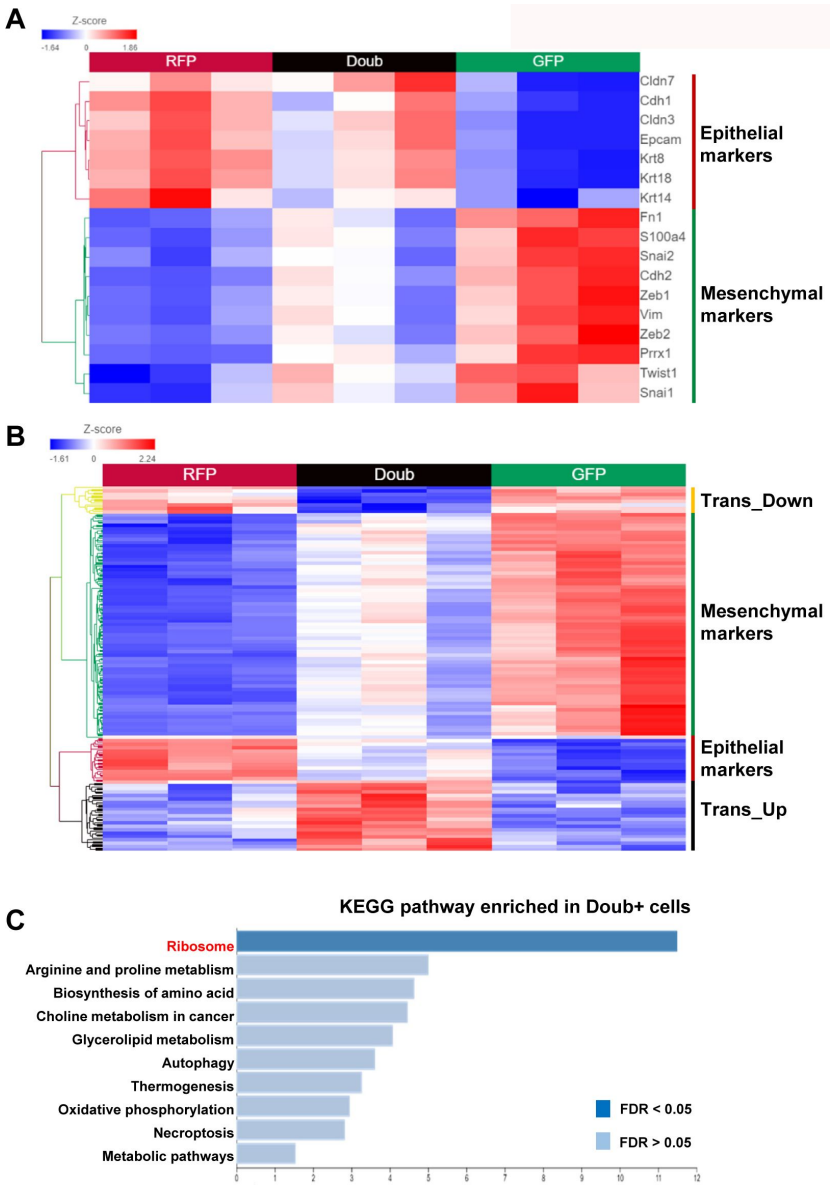
The authors declare no competing financial interests.



Supplementary Fig S1.

Characterization of fluorescent marker switch in Tri-PyMT cells.

A, RFP+ Tri-PyMT cells were sorted via flow cytometry and cultured in DMEM with 10% FBS. Plot shows the percentage of RFP+, GFP+ and Doub+ cells at different generations of cell culture. The experiment was repeated twice with similar results. **B,** Cell cycle analysis of Tri-PyMT cells. Tri-PyMT cells (p7) composed by both RFP+ and GFP+ cells were cultured in growth medium. Cell cycle analysis was performed by using EdU incorporation and Click-chemistry. Cells in G0/G1, S and G2/M phases were quantified by flow cytometry. n =4, two-way Anova, P=0.0242, for S phase RFP+ vs GFP+; P < 0.0001, for S phases Doub+ vs RFP+ and Doub+ vs GFP+ cells.



Supplementary Fig S2.

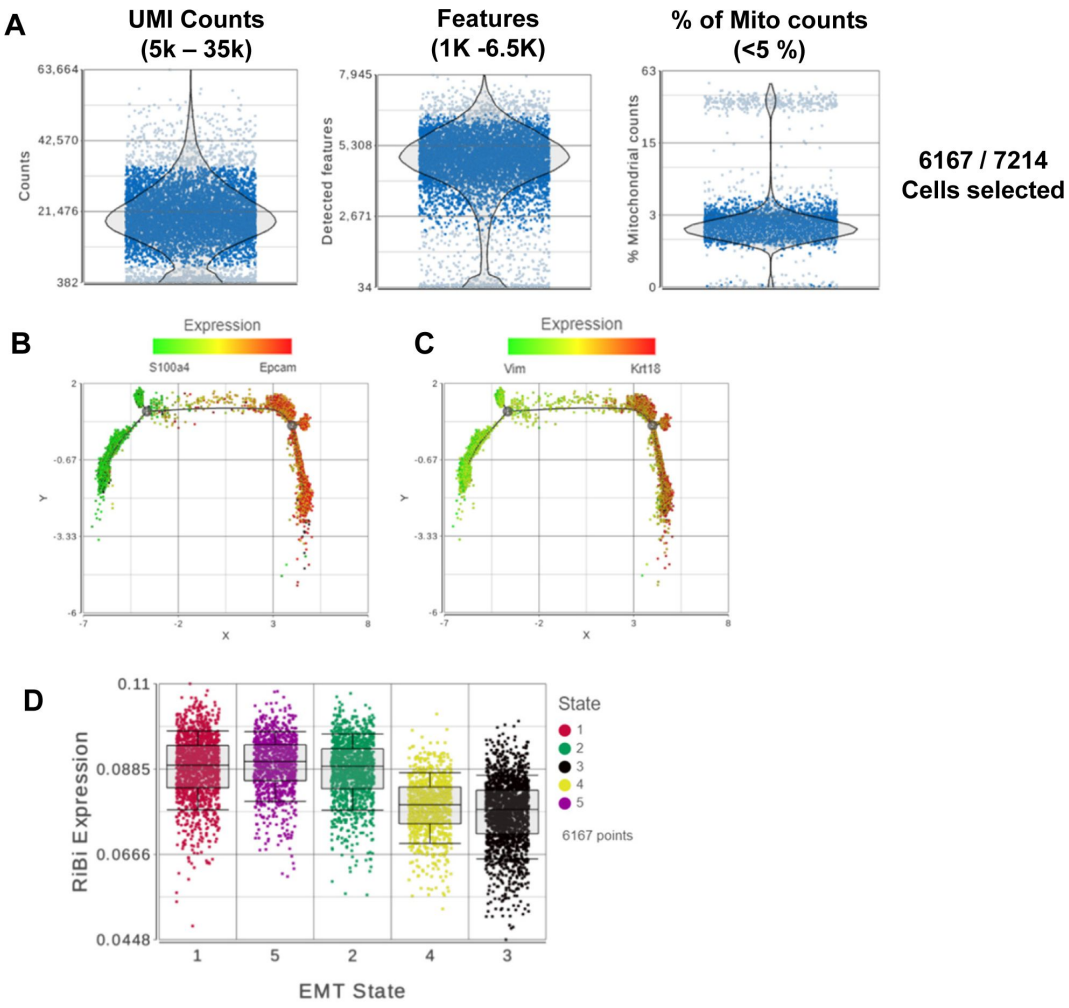
RNA-sequencing analysis of Tri-PyMT cells.

RFP+ Doub+ and GFP+ Tri-PyMT cells were purified using flow cytometry sorting, total RNA was extracted and submitted for RNA-sequencing analysis.

A, Heatmap of selected EMT marker genes in RFP+, Doub+ and GFP+ cells. Of note, the intermediate expression of both epithelial and mesenchymal markers in Doub+ cells.

B, Heatmap of differentially expressed genes in Doub+ cells compared to RFP+ and GFP+ cells. In total, 213 genes are presented in 4 clusters, P<0.01, Doub+ vs RFP+ and GFP+.

C, Gene set over-representative assay with upregulated genes in Doub+ cells showing the significant enrichment of Ribosome pathway (326 KEGG pathways analyzed in total).



Supplementary Fig S3.

The scRNA-sequencing analysis of Tri-PyMT cells.

RFP+, Doub+, and GFP+ Tri-PyMT cells were sorted via flow cytometry and submitted for sc-RNA sequencing analysis.
A. Quality controls of the scRNA-seq. Plots show the single-cell QA/QC analyses including total counts of UMI, detected gene features, and the percentage of mitochondria genes. Totally, 6167 cells out of 7214 cells were filtered according to the criteria for downstream analyses.
B. C. EMT trajectory analysis plots. Cell trajectory analysis was performed with filtered EMT-related genes using Monocle 2 model. Pairs of epithelial and mesenchymal marker genes, S100a4 vs Epcam (B) and Vim vs Krt18 (C), are used to highlight the EMT status of individual cells.
D. Scatter plot shows the expression of Ribosome biogenesis genes (AUC values of RiBi pathway genes) according to EMT cell states. A trend of highest RiBi activation was detected in State 5 cells ($p=0.072$ for 5 vs 1, and $p<0.01$ for 5 vs 2, 3, 4, One-way ANOVA).

A. Epithelial and mesenchymal marker genes of Tri-PyMT cells*:

Epithelial marker genes:	Cdh1, Cldn3, Cldn7, Epcam, Rfp, Krt8, Krt7, Krt18, Serpinb5, Wfdc2
Mesenchymal marker genes:	Col6a1, Col6a2, Fn1, Gfp, Il33, Mgp, Pdgfrb, S100a4, Vim, Zeb1

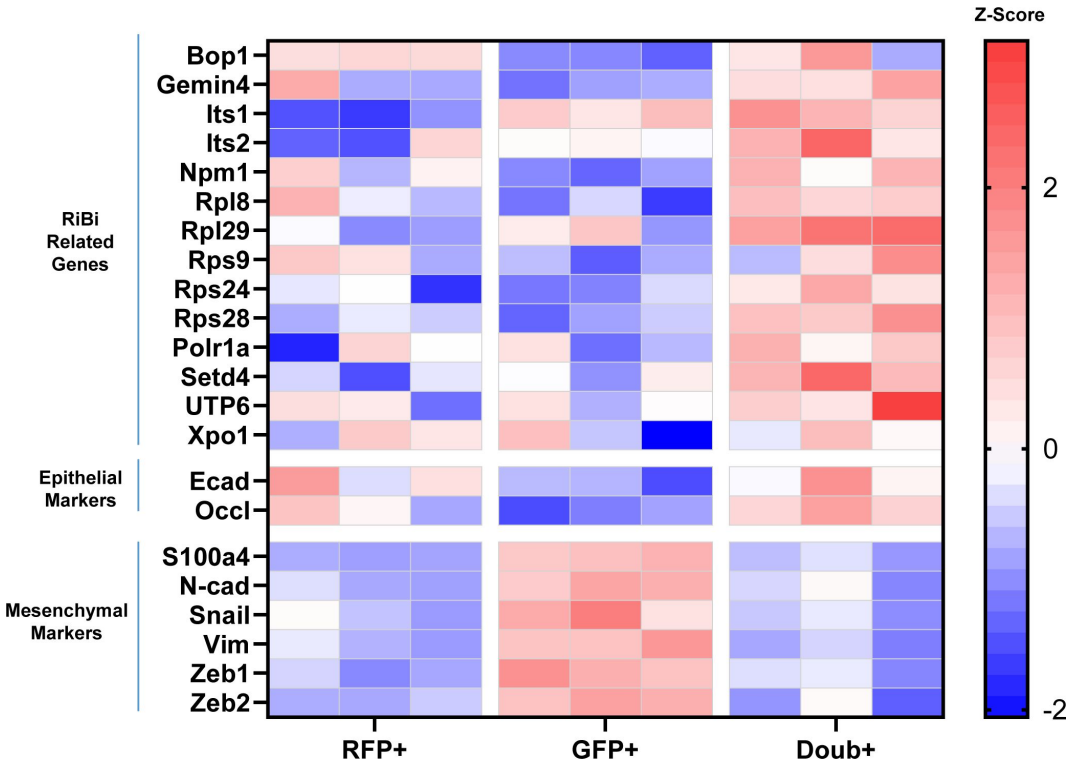
* Epithelial and mesenchymal marker genes that were used in calculation of AUCell value of EMT status.

B. Enriched pathways in GSEAs of EMT transitioning cells:**

		Trans vs Epi		Trans vs Mes	
PATHWAY NAME	**SIZE**	**NES**	**NOM p-value**	**NES**	**NOM p-value**
GOBP_CELL_CELL_JUNCTION_ORGANIZATION	123	1.570	0.008	2.450	0.000
GOBP_SKIN_DEVELOPMENT	170	1.760	0.000	2.270	0.000
GOBP_EPIDERMIS_DEVELOPMENT	194	1.690	0.000	2.240	0.000
GOBP_KERATINOCYTE_DIFFERENTIATION	70	1.730	0.000	2.210	0.000
GOBP_RIBOSOMAL_LARGE_SUBUNIT_BIOGENESIS	68	1.730	0.008	2.190	0.000
GOBP_RIBOSOME_BIOGENESIS	291	1.640	0.008	2.190	0.000
GOBP_RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS	392	1.630	0.010	2.180	0.000
GOBP_EPIDERMAL_CELL_DIFFERENTIATION	104	1.690	0.002	2.150	0.000
GOBP_RRNA_METABOLIC_PROCESS	233	1.750	0.004	2.150	0.000
GOBP_RNA_PHOSPHODIESTER_BOND_HYDROLYSIS_ENDONUCLEOLYTIC	26	1.830	0.000	2.040	0.002
GOBP_EPITHELIAL_CELL_DIFFERENTIATION	346	1.580	0.000	2.020	0.000
GOBP_ACTIN_FILAMENT_BASED_MOVEMENT	57	1.770	0.000	2.000	0.000
GOBP_CELL_JUNCTION_ASSEMBLY	232	1.850	0.000	1.980	0.000
GOBP_CELL_CELL_ADHESION	371	1.620	0.000	1.980	0.000
GOBP_ENDONUCLEOLYTIC_CLEAVAGE_INVOLVED_IN_RRNA_PROCESSING	16	1.890	0.000	1.920	0.006
GOBP_RNA_LOCALIZATION	143	1.690	0.006	1.900	0.000
GOBP_POSITIVE_REGULATION_OF_MRNA_PROCESSING	34	1.670	0.006	1.890	0.000
GOBP_POSITIVE_REGULATION_OF_MRNA_METABOLIC_PROCESS	112	1.730	0.002	1.880	0.000
GOBP_ESTABLISHMENT_OR_MAINTENANCE_OF_CELL_POLARITY	158	1.830	0.000	1.870	0.000
GOBP_ESTABLISHMENT_OF_RNA_LOCALIZATION	121	1.690	0.008	1.860	0.002
GOBP_NUCLEAR_TRANSPORT	281	1.700	0.000	1.850	0.000
GOBP_REGULATION_OF_MRNA_METABOLIC_PROCESS	236	1.590	0.010	1.830	0.000
GOBP_TISSUE_MORPHOGENESIS	401	1.600	0.000	1.820	0.000
GOBP_ACTIN_MEDIATED_CELL_CONTRACTION	39	1.770	0.002	1.820	0.002
GOBP_MUSCLE_CONTRACTION	118	1.610	0.008	1.820	0.002
GOBP_NUCLEAR_EXPORT	141	1.710	0.000	1.810	0.000
GOBP_GLAND_MORPHOGENESIS	95	1.560	0.000	1.810	0.000
GOBP_MRNA_TRANSPORT	95	1.710	0.006	1.810	0.002
GOBP_RIBOSOMAL_SUBUNIT_EXPORT_FROM_NUCLEUS	16	1.850	0.000	1.800	0.002
GOBP_CELL_JUNCTION_ORGANIZATION	428	1.700	0.000	1.800	0.000
GOBP_ESTABLISHMENT_OF_CELL_POLARITY	118	1.740	0.004	1.790	0.002
GOBP_IMPORT_INTO_NUCLEUS	140	1.590	0.008	1.790	0.000
GOBP_POSITIVE_REGULATION_OF_LOCOMOTION	366	1.660	0.000	1.760	0.000
GOBP_REGULATION_OF_ERBB_SIGNALING_PATHWAY	56	1.670	0.002	1.760	0.004
GOBP_REGULATION_OF_CELL_ADHESION	419	1.590	0.000	1.750	0.000
GOBP_CELL_MATRIX_ADHESION	127	1.820	0.002	1.750	0.002
GOBP_NUCLEOBASE_CONTAINING_COMPOUND_TRANSPORT	151	1.690	0.008	1.730	0.004
GOBP_ACTIN_FILAMENT_BASED_PROCESS	471	1.720	0.000	1.720	0.000
GOBP_REGULATION_OF_NUCLEOCYTOPLASMIC_TRANSPORT	104	1.750	0.000	1.710	0.008
GOBP_PROTEIN_LOCALIZATION_TO_NUCLEUS	253	1.640	0.004	1.680	0.000
GOBP_ACTOMYOSIN_STRUCTURE_ORGANIZATION	122	1.590	0.006	1.680	0.004
GOBP_ERBB_SIGNALING_PATHWAY	82	1.800	0.000	1.670	0.008
GOBP_RNA_DESTABILIZATION	78	1.690	0.006	1.670	0.004
GOBP_CELL_SUBSTRATE_ADHESION	200	1.710	0.002	1.660	0.002
GOBP_REGULATION_OF_PROTEIN_LOCALIZATION_TO_NUCLEUS	117	1.570	0.004	1.660	0.002
GOBP_RNA_MEDIATED_GENE_SILENCING	87	1.650	0.010	1.650	0.009
GOBP_NUCLEUS_ORGANIZATION	114	1.790	0.000	1.640	0.006
GOBP_HEART_PROCESS	108	1.690	0.000	1.600	0.004
GOBP_MUSCLE_SYSTEM_PROCESS	184	1.680	0.006	1.540	0.006

** GSEAs of scRNA-seq data were performed with BP subset of GO pathway (3185 gene sets with a filter of 15 – 500 genes/set). Totally, 49 gene sets were overlapped with the significantly enriched gene sets ($P < 0.01$) in comparisons of Trans vs Epi and Trans vs Mes. Gene set size (number of genes), normalized enrich scores (NES) and nominal P values (NOM p-val) are shown in the table. Pathways related to Ribosome or RRNA processing were highlighted in red.

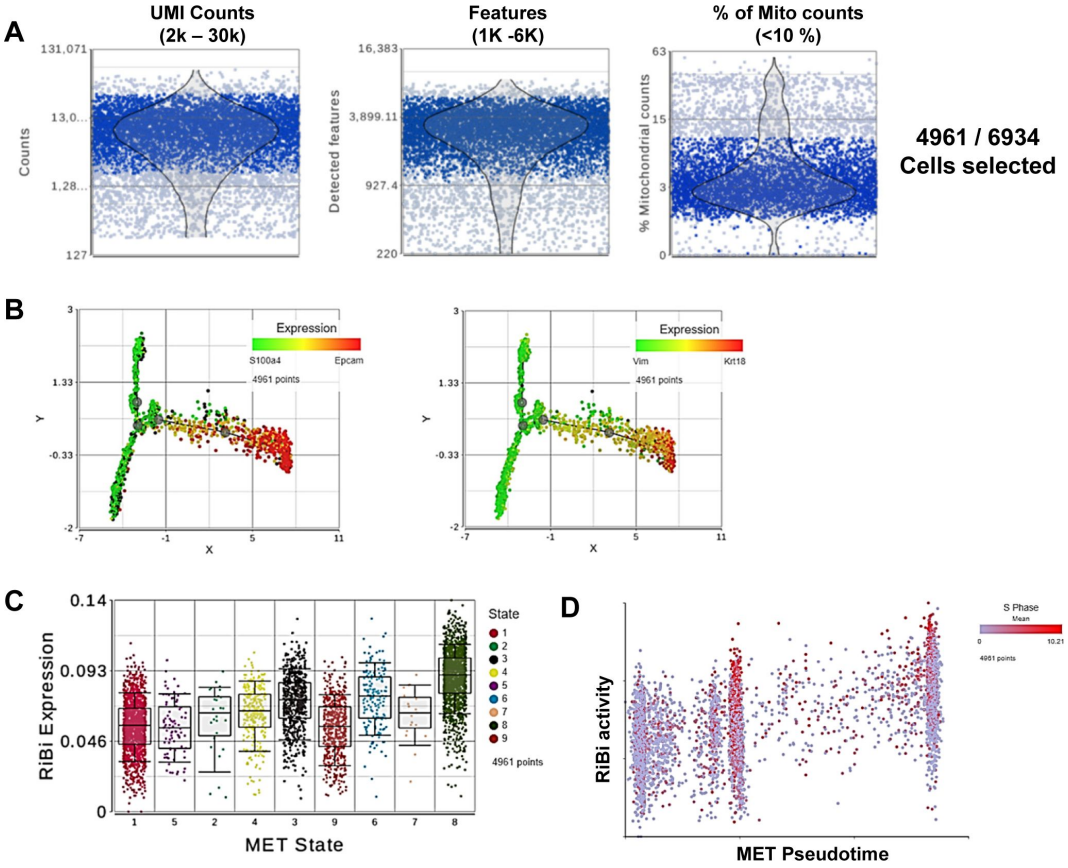
Supplementary Figure S4



Supplementary Fig S5.

RT-PCR analysis of RiBi genes in Tri-PyMT cells.

RFP+, Doub+ and GFP+ cells were purified from Tri-PyMT (p7) culture using flow cytometry sorting. Total RNA was extracted and analyzed by RT-PCR. The heatmap shows z-score of RiBi related genes, epithelial and mesenchymal marker genes expression with Gapdh as internal control, n=3.



Supplementary Fig S6.

The scRNA-sequencing analysis of GFP+ Tri-PyMT cells.

GFP+ Tri-PyMT cells, including Epcam+ and Epcam-cells were sorted from metastasis-bearing lungs and submitted for single cells RNA sequencing analysis.

A. Quality controls of the scRNA-seq. Plots show the single-cell QA/QC analyses of the total counts of UMI, detected gene features and the percentage of mitochondria genes. Totally, 4961 cells out of 6934 cells were included in the downstream analyses.

B. Plots of MET trajectory analysis. Cell trajectory analysis was performed with filtered EMTome genes using Monocle 2 model. Pairs of epithelial and mesenchymal marker genes, S100a4 vs Epcam (left) and Vim vs Krt18 (right) are used to highlight the MET status of individual cells.

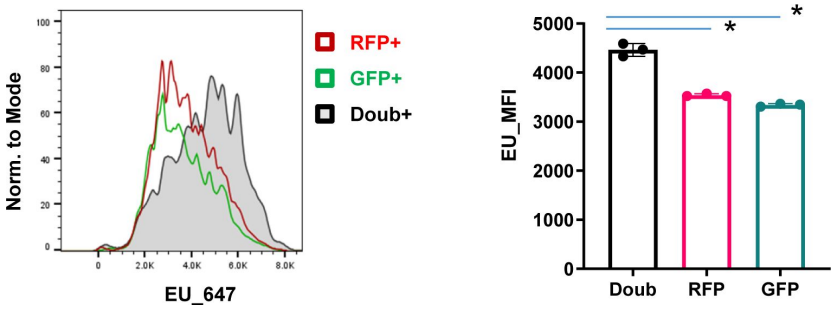
C. Elevated ribosome biogenesis pathway during MET. Cells were classified by their MET state. The activation of RiBi pathway was indicated by AUC value of genes in the ribosome biogenesis pathway. Of note, the highest RiBi expression in MET State 8 cells, which exhibit the most epithelial phenotypes.

D. Scatter plot illustrates the correlation between RiBi activity and MET pseudotime. Proliferating cells (in S phase) are highlighted in red color.

Supplementary Fig S7.

Elevated RNA transcription activity in the Doub+ Tri-PyMT cells.

Tri-PyMT cells (p7) including both RFP+ and GFP+ cells were cultured in growth medium. EU incorporation assay was performed and detected with click chemistry using Alexa A647-azide. Labeled cells were analyzed by flow cytometry (Left). The median fluorescence intensity (MFI) of EU staining of RFP+, GFP+ and Doub+ cells were quantified, n =3, One-way ANOVA, *P < 0.0001, Doub+ vs RFP+; *P < 0.0001, Doub+ vs GFP+.



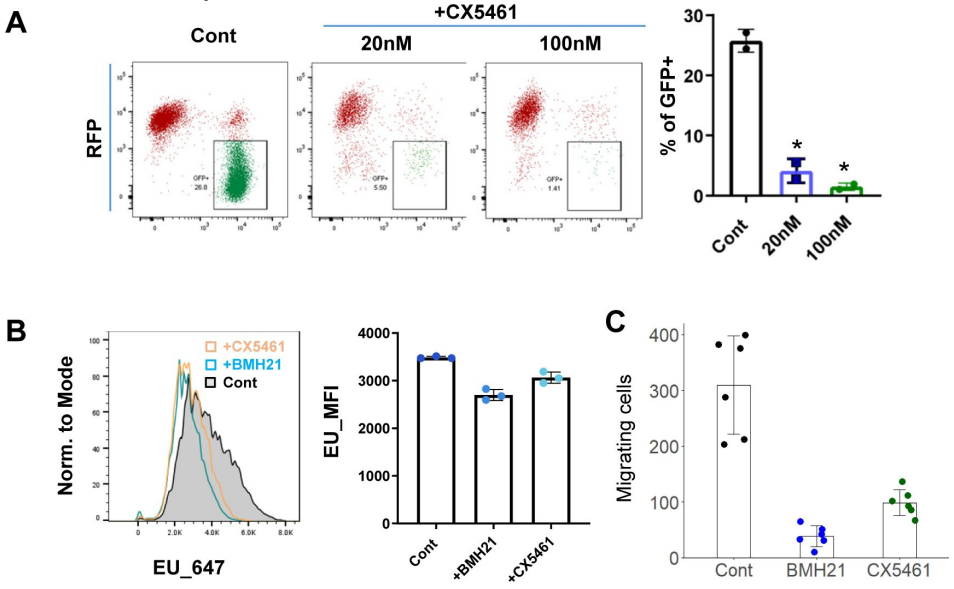
Supplementary Fig. S8.

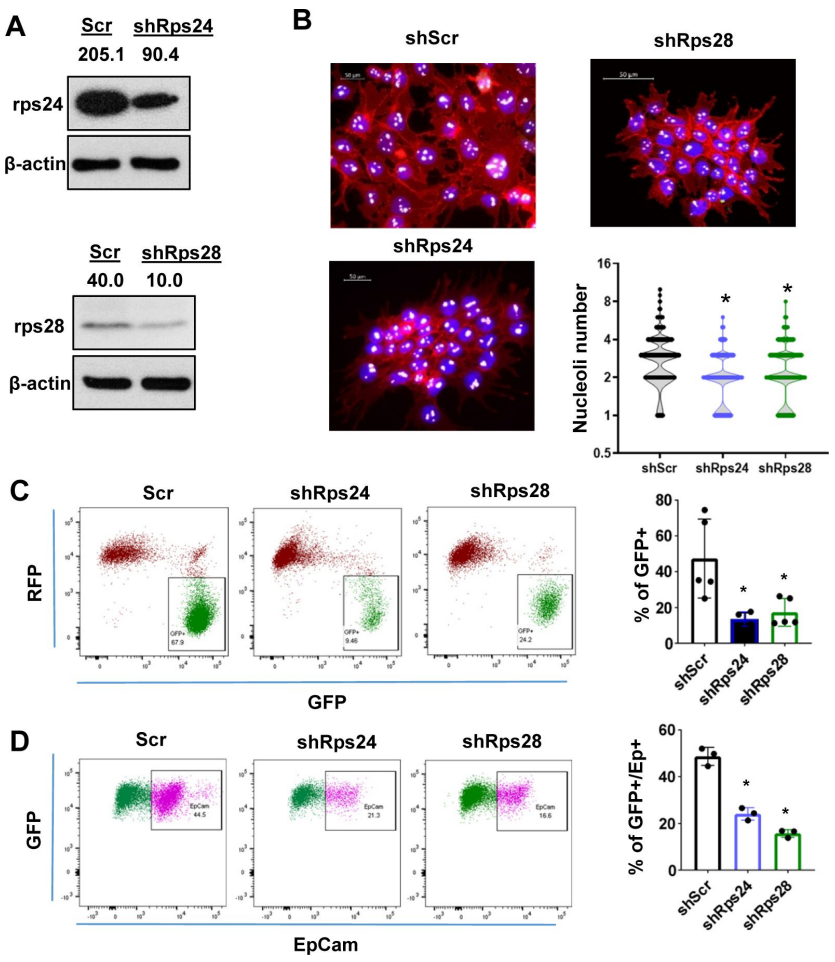
RiBi inhibition impairs RNA transcription activity and reduces EMT capability of tumor cells.

A, Fluorescence switch in Tri-PyMT cells with CX5461 treatment. Flow cytometry plots exhibit the percentage of GFP+ Tri-PyMT cells after treatment with the Pol I inhibitor CX5461. Biological repeat, n=2, One-way ANOVA, *P=0.0015, 20nM vs Cont; *P=0.0011, 100nM vs Cont.

B, EU incorporation assay. Tri-PyMT cells (p10) were treated with RiBi inhibitors, BMH21 at 100nM, or CX5461 at 20nM, for 2 days. EU incorporation assay was performed and detected with click chemistry using Alexa A647-azide. Labeled cells were analyzed by flow cytometry (Left). The median fluorescence intensity (MFI) of EU staining of cells were quantified, n =3, One-way ANOVA, P = 0.0001, +BMH21 vs Cont; P = 0.0032, +CX5461 vs Cont.

C, Cell migration assay. Tri-PyMT cells (p7) were seeded in the inserts of migration plate and treated BMH21 (100nM) or CX5461 (20nM) for 5 days. Cell migration was induced by FBS gradient for 24 hours. Migrating cells were counted from the bottom of the insert. n=6, One-way ANOVA, * P < 0.001 BMH21 vs Cont and CX5461 vs Cont.

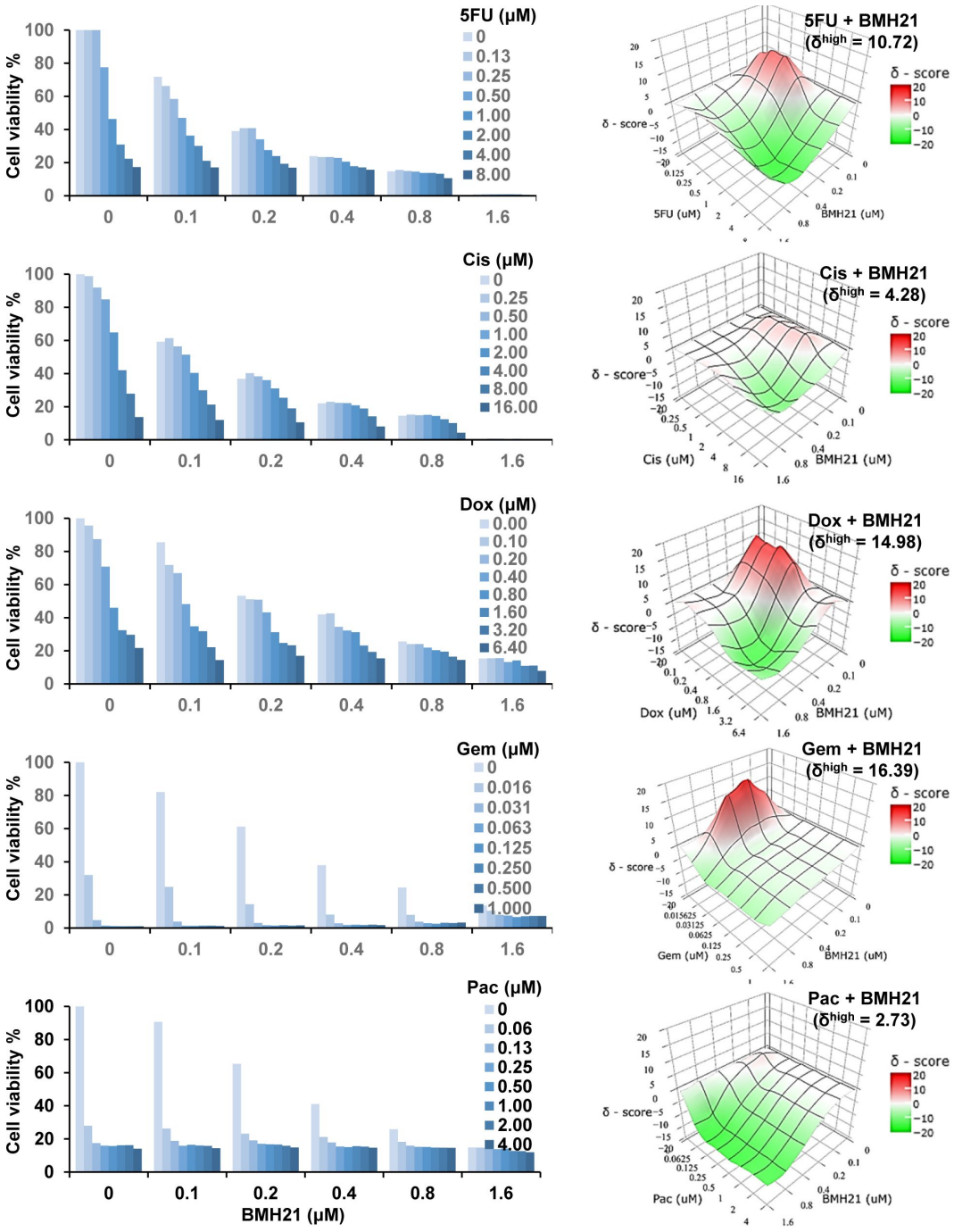




Supplementary Fig S9.

RiBi modulating by knocking down ribosome proteins reduces EMT/MET capability of tumor cells.

A, Western blots show the knockdown expression of Rps24 and Rps28 in targeted Tri-PyMT cells. The numbers indicate relative intensity of the band normalizing to the β-actin band of the sample.
B, Impact on nucleoli numbers by Rps knockdown in RFP+ Tri-PyMT cells. Fluorescent images show the reduced number of nucleoli in Rps24 and Rps28 knockdown cells. n = 623 cells (shScr), 490 cells (shRps24), 553 cells (shRps28). One-way ANOVA with Dunnett multiple comparisons, *P < 0.0001 for shScr vs shRps24 and shScr vs shRps28.
C, Flow cytometry plots show the percentage of GFP+ cells in Rps24 and Rps28 knockdown Tri-PyMT cells. 4 biological repeats, One-way ANOVA, *P=0.0085, shScr vs. shRps24, *P=0.0117, shScr vs. shRps28.
D, Flow cytometry plots show the Epcam+/GFP+ cells in Rps24 or Rps28 knockdown Tri-PyMT cells. 3 biological repeats, one-way ANOVA, *P=0.0055, shScr vs. shRps24, *P=0.0124, shScr vs. shRps28.



Supplementary Fig S10.

Combination therapy of RNA Pol I inhibitor and chemo drugs.

Left, Bar graphs display Tri-PyMT cell viability following combination treatment with BMH21 and chemotherapeutic drugs at various concentrations. Bars represent the mean value of duplicated treatments, n=2 wells/treatment. Right, Synergy plots of BMH21 and chemotherapeutic drugs. Synergistic scores (δ) for each combination were calculated using the ZIP model in SynergyFinder 3.0. The highest δ numbers among combinations is shown.

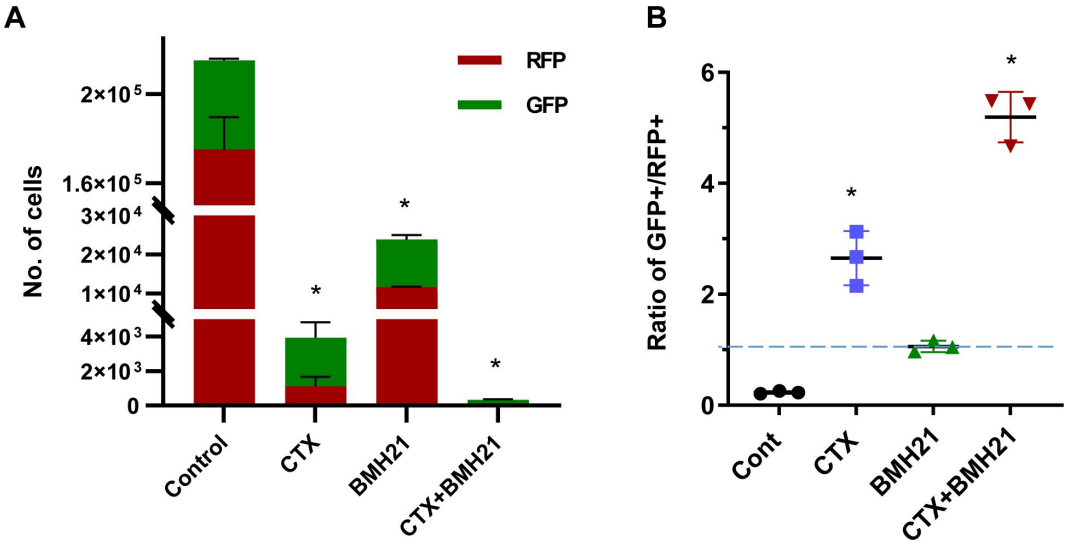
Supplementary Fig S11.

Differential impact of Pol I inhibitor and chemo drug on epithelial and mesenchymal Tri-PyMT cells *in vivo*.

The same number of RFP+ and GFP+ Tri-PyMT cells were injected in animals via tail vein. Animals were treated CTX (100mg/kg, once a week, i.p.), BMH21 (20mg/kg, 5 times a week, i.p.) or both in combination. Number of RFP+ and GFP+ cells were quantified by flow cytometry at day 21 after inoculation.

A, Plots show the number of RFP+ and GFP+ Tri-PyMT cells in the lung treated with CTX and/or BMH21. n=3 mice/treatment, One way ANOVA, *P<0.0001 for Cont vs CTX, Cont vs BMH21 and Cont vs CTX+BMH21.

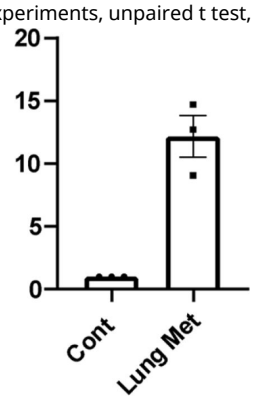
B, The ratio of GFP+ vs RFP+ cells in the lung treated with CTX and/or BMH21. n=3 mice/treatment, One way ANOVA, *P=0.0001 for Cont vs CTX; *P=0.0661 for Cont vs BMH21 and *P<0.0001 for Cont vs CTX+BMH21.



Supplementary Fig S12.

E-cadherin expression by LM2 cells in lung metastases.

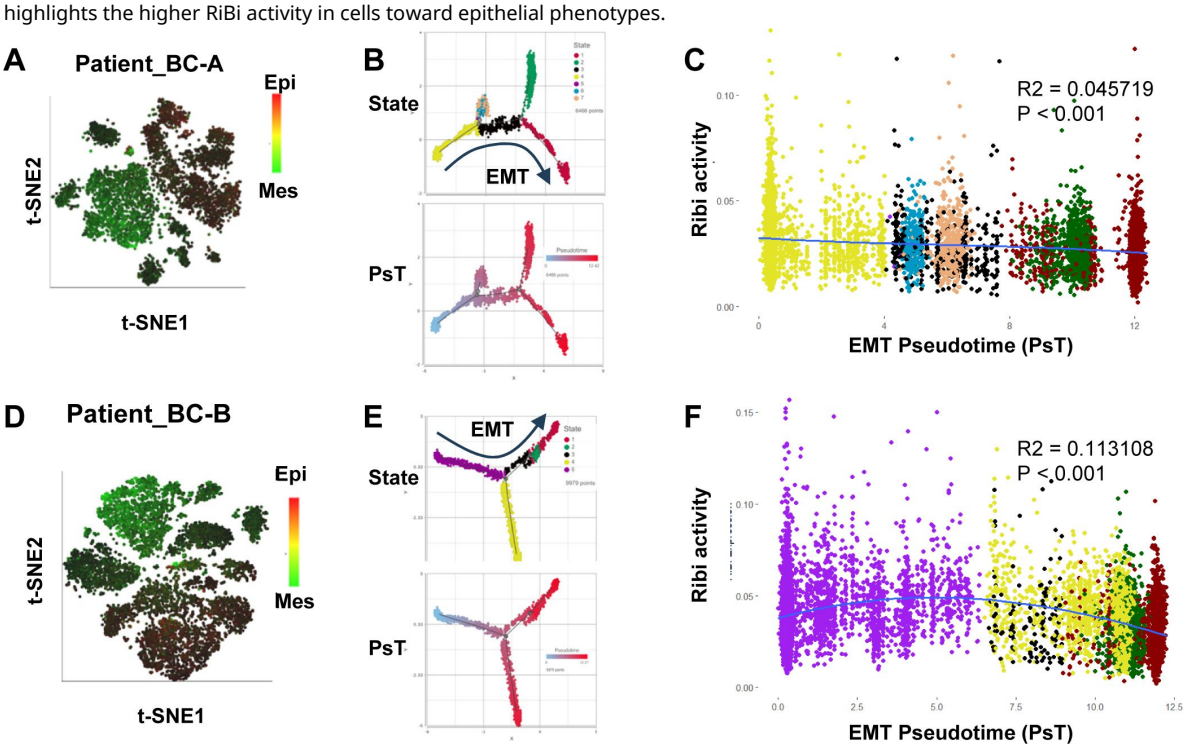
qRT-PCR analysis of Ecadherin expression in LM2 cells isolated from lung metastases. LM2 cells in culture served as control. GAPDH served as internal control for PCR. n=3 experiments, unpaired t test, * P=0.0025.



Supplementary Fig 13.

Correlation of ribosome biogenesis pathway and EMT status in human breast cancer cells.

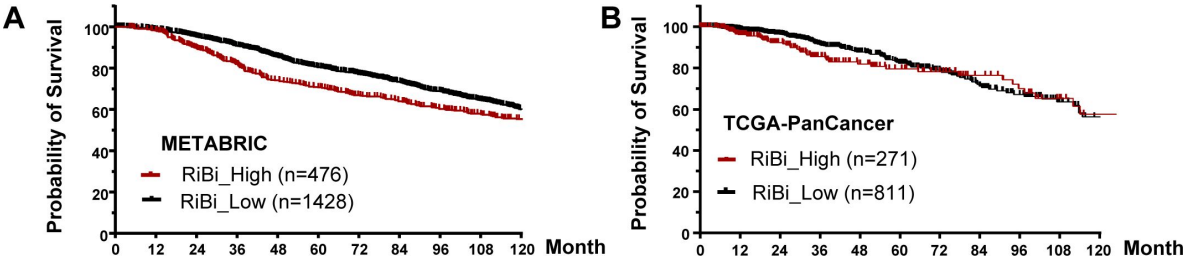
Single-nucleus cell RNA sequencing analysis of primary tumor cells from breast cancer patients (BC-A and BC-B, GEO database, GSE 198745).
A,D, The *t*-SNE plots show the heterogeneity in EMT statuses of tumor cells with AUC values of epithelial genes (in red) and mesenchymal genes (in green).
B,E, Cell trajectory analysis was performed with filtered EMT-related genes (EMTome genes) using Monocle 2 model. Cell states were identified with the most epithelial cells as root for calculation of EMT pseudotime (PsT).
C,F, The scatter plot displays the correlation of Ribi activity to EMT pseudotime. The polynomial regression line (order = 3) highlights the higher RiBi activity in cells toward epithelial phenotypes.

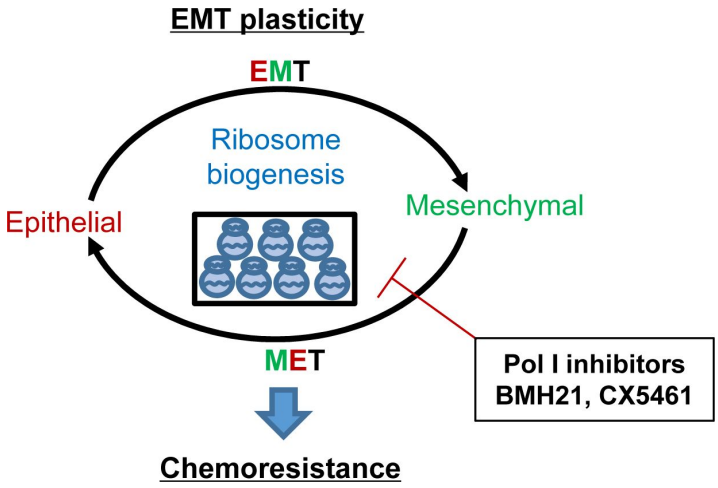


Supplementary Fig S14.

Survival curves of breast cancer patients with different RiBi activities.

Gene expression data of METABRIC and TCGA-PanCancer was obtained from CBioPortal. Patients were grouped according to the average z-score of RiBi gene expression, RiBi High (>1) and RiBi Low (RiBi score <0.5). Upper, METABRIC dataset, Gehan-Breslow-Wilcoxon test *P*-value = 0.0019. Lower, TCGA data, Gehan-Breslow-Wilcoxon test *P*-value = 0.0225.





Supplementary Fig S15.

Working model of ribosome biogenesis in EMP plasticity.

Tumor cells exhibit plasticity by undergoing EMT (epithelial-mesenchymal transition) and MET (mesenchymal-epithelial transition), contributing to the development of resistance to chemotherapies. Elevated ribosome biogenesis is essential for maintaining this EMT plasticity. Inhibition of ribosome biogenesis by RNA Pol I inhibitors synergizes with chemotherapeutic drugs to overcome resistance development.

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Editors

Reviewer #1 (Public Review):

The process of EMT is a major contributor of metastasis and chemoresistance in breast cancer. By using a modified PyMT model that allows identification of cells undergoing EMT and their decedents via S100A4-Cre mediated recombination of the mTmG allele, Ban et al. tackle a very important question of how tumor metastasis and therapy resistance by EMT can be blocked. They identified that pathways associated with ribosome biogenesis (RiBi) are activated during transition cell states. This finding represents a promising therapeutic target to block any transition from E to M (activated during cell dissemination and invasion) as well as from M to E (activated during metastatic colonization). Inhibition of RiBi-blocked EMT also reduced the establishment of chemoresistance that is associated with an EMT phenotype. Hence, RiBi blockage together with standard chemotherapy showed synergistic effects, resulting in impaired colonization/metastatic outgrowth in an animal model. The study is of great interest and of high clinical relevance as the authors show that blocking the transition from E to M or vice versa targets both aspects of metastasis, dissemination form the primary tumor and colonization in distant organs.

The study is done with high skill using state of the art technology and the conclusions are convincing and solid, but some aspects require some additional experimental support and clarification. It remains elusive whether blocking of EMT/MET is necessary for the synergistic effect of standard chemotherapy together with RiBi blockage or whether a general growth disadvantage of RiBi treated cells independent of blocking transition is responsible. How can specific effect on state transition by RiBI block be seperated from global effects attributed to overall reduced protein biosynthesis, proliferation etc.? Some other aspects are misleading or need extension:

In the revised version, the authors appropriately addressed all my comments. I'd like to congratulate the authors for this wonderful work!

https://doi.org/10.7554/eLife.89486.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Public Review):***
>
> *The process of EMT is a major contributor to metastasis and chemoresistance in breast cancer. By using a modified PyMT model that allows the identification of cells undergoing EMT and their decedents via S100A4-Cre mediated recombination of the mTmG allele, Ban et al. tackle a very important question of how tumor metastasis and therapy resistance by EMT can be blocked. They identified that pathways associated with ribosome biogenesis (RiBi) are activated during transition cell states. This finding represents a promising therapeutic target to block any transition from E to M (activated during cell dissemination and invasion) as well as from M to E (activated during metastatic colonization). Inhibition of RiBi-blocked EMT also reduced the establishment of chemoresistance that is associated with an EMT phenotype. Hence, RiBi blockage together with standard chemotherapy showed synergistic effects, resulting in impaired colonization/metastatic outgrowth in an animal model. The study is of great interest and of high clinical relevance as the authors show that blocking the transition from E to M or vice versa targets both aspects of metastasis, dissemination from the primary tumor, and colonization in distant organs.*

We appreciate the positive acknowledgment of our work.

> *The study is done with high skill using state-of-the-art technology and the conclusions are convincing and solid, but some aspects require some additional experimental support and clarification. It remains elusive whether blocking of EMT/MET is necessary for the synergistic effect of standard chemotherapy together with RiBi blockage or whether a general growth disadvantage of RiBi-treated cells independent of blocking transition is responsible.*

We appreciate the reviewer for raising the pertinent query regarding the interrelation between EMT/MET blocking by RiBi inhibition and its synergistic effect with chemotherapy drugs. Our experimental data suggests a potential consequence of these events. Specifically, when assessing the potency of RiBi inhibitors (BMH21 and CX5410), we observed a pronounced EMT/MET blocking effect at concentrations preceding the emergence of cytotoxic effects (refer to Fig. 4 and Supplementary Fig S8). Notably, the IC50 for BMH21 was approximately 200nM, which is a concentration surpassing those that manifested the EMT/MET blocking effects. Crucially, the enhanced synergy of RiBi inhibitors with chemotherapy drugs was predominantly seen at these lower concentrations (as illustrated in Supplementary Fig S10). Therefore, the EMT/MET blocking by RiBi inhibition, rather than the cytotoxic effect, is likely instrumental for the synergy with chemotherapy drugs. The result was highlighted in Page#16.

> *How can specific effects on state transition by RiBI block be separated from global effects attributed to overall reduced protein biosynthesis, proliferation etc.?*

We appreciate the reviewer's insightful query. We agree that RiBi activity and associated protein synthesis are fundamental processes for cell viability, making it challenging to clearly delineate the overall effects of RiBi blockage to the specific effects of EMT state transition. Our results showed an elevated RiBi activity during the EMT transitioning phases, concomitant with enhanced nascent protein synthesis, indicating a higher-than-normal requirement of new proteins for cells to switch their phenotype. This would provide us a chance to target the excessive activities of RiBi to block EMT/MET transition. Based on a similar consideration, we chose to apply shRNA instead of CRISPR technology to modulate RiBi gene expression. By comparing to scramble controls, the growth rates of the Rps knockdown cells (both RFP+ and GFP+ cells) were not significantly affected, while the EMT/MET transitioning was impaired (Supplementary Fig 9). These results may provide evidence of uncoupling the cell proliferation and EMT/MET status changes by inhibiting RiBi pathway.

> *Some other aspects are misleading or need extension.*
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *(1) The analysis of RiBi expression during EMT in Fig. 1K shows that transition states have high RiBi levels, whereas E and M states are low. Analyses of MET in Fig.2G indicate that M states have the lowest, transition states upregulate RiBi while E states have the highest levels of RiBi expression. This is puzzling and how can it be explained? It would be helpful to demonstrate how these two settings are related by combining results from Figs 1 and 2 in an E-Trans-M-Trans-E state graph (in a sequence of EMT/MET). Does it mean that the initial E state starts with lower RiBi and the final E state displays the highest RiBi expression? In other words, are the initial E state and the one after MET different?*

Thank the reviewer for raising the concern about which EMT/MET state exhibits the highest RiBi activity. Following the reviewer's suggestions, we merged the scRNA-seq data of EMT and MET cells and performed the trajectory analysis. Similar epithelial-mesenchymal spectrums were detected from these cells (For reviewers Fig 1). Notably, the highest RiBi activity was detected in the early EMT transitioning or the late MET transitioning cells (revised For

reviewers Fig 1D). Addressing the question of the reviewer, the initial E state (of EMT cells) did not show significant differences to the final E state (of MET cells) in comparisons of EMT pseudotime and RiBi activities. In addition, the analysis with merged cells also revealed:

(1) Both the EMT (In_Vitro_Mix) and MET (In_Vivo_GFP) cells were generally divided into two major clusters representing epithelial and mesenchymal phenotypes (For reviewers Fig 1A, 1B).

(2) The EMT and MET cells exhibited similar EMT spectrums (EMT/MET status, and pseudotime) in the trajectory analysis (For reviewers Fig 1C, 1D).

(3) Cells with high RiBi activity were mostly from the transitioning cell during EMT (In_Vitro_Mix) cells (For reviewers Fig 1D).

> *(2) It needs to be elaborated on how the experiment in Fig. 4A was exactly done. Are there cells isolated directly from the autochthonous TriPyMT tumor in contrast to steady-state cultures from Fig. 1? Does the control graph represent 0d in culture or have the cells been cultured for the same amount of time as the treated samples? How do these observed 15% GFP+ cells are related to the 15% GFP+ cells obtained at day 0 and 34% at d7 control condition in Fig. 5A?*

Following the reviewer's suggestion, we have amended the figure legend to clarify the experiment settings. In Fig. 4A, we initiated the experiment with sorted RFP+/Epcam+ cells. The control cells were cultured for the same period of time (5 days) as drug-treated cells did. We apologize for the unclear description. The percentage of GFP+ cells in this experiment is not related to the experiment in Fig 5A, where the initial cell population comprised an unsorted mix of RFP/GFP cells.

> *(3) Fig. 4B: Since the bulk population is loaded in the WB, does that suggest that the epithelial state is stabilized/enhanced or does it reflect only different cell ratios? So, it would be important to show the WB for RFP+ and GFP+ cells separately.*

Thank the reviewer for the query regarding Fig. 4B. We apologize for the unclear explanation. The experimental setup for Fig 4B was identical to that of Fig 4A, where the sorted RFP+ cells were utilized at the start. Indeed, the observed increase in epithelial markers and decrease in mesenchymal markers in cells treated with BMH and CX suggest a higher proportion of cells maintaining the RFP+ state.

Performing WB for RFP+ and GFP+ cells separately may not address the question we asked since the experiment was initialed with pure RFP+ cells. Also, the expression of the fluorescent markers is closely aligned with the EMT status of the cells with and without drug treatment.

> *(4) Figs. 4-6: The authors claim that there is less EMT under treatment. If the experiment was done over 5 days (as indicated in Fig.4b legend), it is necessary to rule out that shifts in E/M ratios are attributed to the effects of treatment on proliferation/survival affecting both populations differently. How do the same cells grow under treatment when injected orthotopically/subcutaneously?*

We apologized for the unclear descriptions. The effect of blocking the transitioning of EMT with RiBi inhibitors were performed with purified RFP+/EpCam+ cells. All GFP+ cells in this experiment setting were transformed from RFP+ cells. Given the fluorescence switch was well correlated with EMT status of cells, RFP and GFP were used as EMT reporters. Similarly, we used purified GFP+/EpCam- cells as the initial population to study the MET process of tumor cells.

To address the reviewer's concern regarding how RiBi inhibition may differentially affect the growth of RFP+ and GFP+ cells, we conducted a cell cycle assay using Tri-PyMT cells, which include both RFP+ and GFP+ populations. Our results demonstrated that both RFP+ and GFP+ cells exhibited a trend towards G2/M phase accumulation when treated with BMH21. It is important to note that the impact of BMH21 on the cell cycle was less pronounced than previously reported by Fu et al. (Oncol Rep, 2017). This is likely because the dose used for EMT inhibition in our study was approximately one-tenth of the dose known to inhibit cell growth (For Reviewers Fig 2). Also, no significantly differential impacts were detected between RFP+ and GFP+ cells.

We have previously characterized the proliferation rate of RFP+ and GFP+ populations (Lourenco et al 2020). RFP+ cells proliferate faster than GFP+ cells. Primary tumor cells derived from RFP+ cells also grew faster than GFP+ tumors (Lourenco et al 2020).

> *(5) Fig. 6B: this image is puzzling. Only in the lower two panels the outline of the lung is visualized by DAPI staining. The upper two panels look like there is no lung tissue in ctrl (no DAPI+GFP-RFP- cells) or show almost exclusively DAPI+GFP-RFP- cells that are present in a clustered assembly. Do the latter represent lymphoid cell clusters or normal lung tissue?*

To improve the clarity of fluorescent images in Fig 6B, we enlarged the merge images with higher contrast (Revised Fig. 6B). The DAPI+/RFP-/GFP- region represent normal lung tissue. Nodules with either RFP or GFP signals represent tumor lesions.

> *(6) Text: Several typos and sentences should be revised, including p. 3 "Le et al. discovered" which should read as "Li et al. discovered", p.8 "Vimten", p.10 "Cells were then classified cells into three main categories", GSEA should be spelled out as Gene Set Enrichment Analysis (not Assay), p. 13 "cells, suggesting the impaired MET capability with upon treatment".*

We apologize for the typos. All were corrected in the revised manuscript.

> *(7) Figures: Color gradient indicator in Fig. 1E does not reflect the colors of the cells, Fig. S5A+C are not referenced in the text, there is mislabeling of S5B,C,D in the legend, graph in Fig. 3D is placed two times and overlapping, Fig. 6C labeling needs adjustments, labeling of Fig. 6D should be similar to Fig. 6A: CTX blue and BMH21 green.*

We apologize for these errors and made corrections. Color in Fig.1E represents the EMT status of tumor cells as indicated in the revised figure, red for more epithelial, and green for more mesenchymal features. Fig S5 is now Fig S6, and referred in the revised manuscript. Legend for figures were corrected. Labels of Fig 6 were adjusted.

> ***Reviewer #2 (Public Review):***
>
> *(1) The current manuscript by Ban et al describes that cells undergoing EMT have increased rRNA synthesis, as analyzed by RNA seq-based gene expression analysis, and that the increased rRNA synthesis provides a therapeutic opportunity to target chemoresistance. The cells utilized in this manuscript were isolated from the authors' Tri-PyMT EMT lineage tracing model published a few years ago which demonstrated that cells undergoing EMT are not the cells that are contributing to metastasis but rather to tumor chemoresistance (Fischer, Nature 2015). This in vivo model has since then been criticized for not capturing all relevant EMT events which the authors also acknowledge in the introduction. The authors therefore reason that they use this lineage tracing model to better understand the role of EMT in chemoresistance.*

> *A major problem with the current manuscript is that the authors present many of their findings as a novel without the proper acknowledgment of previously published literature in particular, Prakash et al., Nature Communications, 2019 and Dermitt, Dev Cell, 2020. In the studies by Prakash, the authors demonstrate that maintaining ongoing rRNA biogenesis is essential for the execution of the EMT program, and thus the ability of cancer cells to become migratory and invasive. Further, Prakash et al showed that blocking rRNA biogenesis with a small molecule inhibitor, CX-5461 (which is also used in the study by Ban et al) specifically inhibits breast cancer growth, invasion, EMT, and metastasis in animal models without significant toxicity to normal tissues. As such a significant revision that is necessary at this time is a rewrite of the manuscript especially the introduction and the discussion to more accurately describe and cite previously published findings and then highlight the current work by Ban et al which nicely builds on the previously published literature as it highlights the contribution of EMT to chemoresistance rather than metastasis. The suggestion for the authors is that they therefore should focus on highlighting the chemotherapy resistance angle as their Tri-PyMT EMT lineage tracing was chosen to test this angle and as such focus on both primary tumor growth and metastasis.*

We appreciate the reviewer's insightful feedback. In response, we have revised a section in the discussion to better highlight how our study builds upon and extends the work of others. We acknowledge that the link between ribosome biogenesis (RiBi) and the epithelial-mesenchymal transition (EMT) pathway was noted by prior researches (Prakash et al. 2019; Ebright et al. 2020). In the revised manuscript, we have included extra discussion about the topic. Our findings, however, contribute to this knowledge by elucidating increased activities of RiBi during both EMT and mesenchymal-epithelial transition (MET) processes, thereby deepening our understanding of its role. Additionally, we have clarified our novel stance on EMT-targeting strategies. Rather than solely targeting the mesenchymal phenotype, we propose that inhibiting the phenotypic switching ability of tumor cells (a round trip encompassing both EMT and MET) could be more effective, as described in the introduction part.

> *Additional major revisions:*
>
> *(2) The authors use the FSP1-Cre Model which in the field has been questioned as to not capture all the relevant EMT events and therefore their findings should be corroborated by another EMT model system.*

We agree with the reviewer that the Fsp1-Cre model could not capture ALL the relevant EMT events. However, the fidelity and accuracy of Fsp1-Cre model in reporting EMT process of Tri-PyMT cells have also been demonstrated in our previous studies (Lourenco et al. 2020). Also, we have included additional results to further characterize this model: 1) Continuous fluorescence switching from RFP+ to GFP+ was observed in Tri-PyMT cells (Supplementary Fig S1); 2) Bulk RNA-seq data showed the differential expression of EMT marker genes with the RFP+ and GFP+ cells (Supplementary Fig S2A); 3) Single-cell RNA-seq data showed the EMT spectrum and EMT status distributions according to Fsp1(S100a4)/Epcam, and Vim/Krt18 expression (revised Supplementary Fig S3B, 3C). Hope these results clarify the reviewer's doubt about the Fsp1-Cre model in reporting EMT of tumor cells. Of note, the evaluation of EMT status with RiBi activity does not rely solely on the fluorescent marker switch but on the ETM-related transcriptome (EMTome) of the Tri-PyMT cells.

Again, we agree with the reviewer that the Tri-PyMT model does not report ALL relevant EMT events. In the manuscript, we have included experiments with MD-MB231-LM2 cells (Fig 6D) and analyzed the sequencing databases of breast cancer patients (revised Supplementary Fig S13, S14), to validate the findings of the association between EMT status and RiBi activity.

> *(3) In the current version of the manuscript, there are no measurements of rRNA synthesis, but the gene expression profiles are used as a proxy for rRNA synthesis. The authors therefore need to include measurements of rRNA synthesis corroborating the RNA sequencing data to support their scientific findings and claims. This can be accomplished by qPCR, Northern blot, or EU staining of the respective sorted cell population. Quantification of rRNA synthesis is also needed for the CX5461/BMH-21 and silencing studies.*

We agree that direct measure rRNA synthesis is important to validate the association of RiBi activity with the EMT/MET process. Following the reviewer's suggestion, we performed EU incorporation assay with RFP+, Double+, and GFP+ Tri-PyMT cells with and without RiBi inhibitors. Under the treatment-naïve condition, the double+ (EMT-transitioning) cells exhibited highest activity of rRNA synthesis compared to either RFP+ (E) and GFP+ (M) cells (revised Supplementary Fig S7). Also, as expected, the treatment of BMH21 or CX-5461 could significantly inhibit the rRNA synthesis (revised Supplementary Fig S8B).

> *(4) Currently, there is no mechanistic insight as to how rRNA synthesis is increased during EMT, which would also strengthen the manuscript. This could be done through targeted ChIP analysis.*

The experimental data in the current manuscript suggest that the activation of RiBi is upstream of the EMT process, as the impaired RiBi pathway hinders the EMT of tumor cells. We are uncertain about the suggestion regarding ChIP analysis. If the reviewer refers to ChIP analysis with EMT transcription factors (i.e., Snail, Twist, and Zeb1), it may not elucidate the mechanisms by which the EMT process is associated with rRNA synthesis. Using sorted GFP/RFP double-positive Tri-PyMT cells, we found enhanced activations in the ERK and mTOR pathways in the EMT-transitioning cells (Figure 3A). It is well-documented that the ERK and mTOR pathways are key coordinators of EMT (Xie et al., Neoplasia 2004; Shin et al., PNAS 2019; Lamouille et al., J. Cell Sci. 2012; Roshan et al., Biochimie 2019). Interestingly, we also observed significantly higher phosphorylation of rpS6, a downstream indicator of mTOR pathway activation, in the Doub+ cells. As an indispensable ribosome protein, rpS6 phosphorylation could impact ribosome functions of protein translation (Bohlen et al., Nucleic Acid Res. 2021; Mieulet et al., 2007).

> *(5) rRNA synthesis has canonically been linked to the cell cycle therefore it will be necessary for the authors to determine the cell cycle state of their respective cell populations throughout the manuscript.*

Following the reviewer's suggestion, we analyzed the cell cycles of RFP+, GFP+, and Doub+ Tri-PyMT cells. Our analysis revealed that the proportion of proliferating RFP+ cells (in the S phase) was higher than that of proliferating GFP+ cells. Interestingly, the Doub+ cells also exhibited a higher ratio of proliferation, which was significantly greater compared to both RFP+ and GFP+ cells (revised supplementary Figure S1B).

> *(6) Statistics and quantifications are currently missing in several figures and need to be better explained throughout the manuscript to strengthen the scientific rigor of the studies.*

We have improved the clarity of our manuscript. Proper statistics descriptions of experiments have been carefully reviewed and adequate information was edited in the revised manuscript.

> *(7) Only metastasis studies are shown in the current version of the manuscript. These studies should be complemented with primary tumor studies as the main focus of the paper is the contribution of EMT to chemoresistance.*

We appreciate the reviewer's suggestion regarding the primary tumor studies. We apologize for not stating clearly in our manuscript. In response, we have revised the manuscript to outline the rationale for establishing a competitive model by injecting a mixture of RFP+ and GFP+ cells in a 1:1 ratio via the tail vein. This model is designed to study of both EMT and MET processes under chemotherapy at a distal site, where tumor cells need phenotypic switches (both EMT and MET) to adapt to and overcome chemo/environmental challenges in this context. Indeed, we have studied the primary tumor growth with the pre-EMT (RFP+) and postEMT (GFP+) cells. Their differential contribution to tumor growth was published in another paper (Lourenco etal. Cancer Res 2020).

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Figure 1 and associated supplementary figure panels*
>
> *Fig. 1A. More details are needed about the Tri-PyMT model and the induction of EMT in vitro. The authors mention that when growing the isolated cells they spontaneously undergo EMT when grown in 10% FBS. What is the timeline for this transition and how reproducible is it? This information is not clear from Supp. 1. When were cells taken for analysis and also how long is plasticity maintained? According to Supp 1. cell generation 15-21 seems to have a stable cell population of green, red, and yellow cells. Are these cell populations changing if one stimulates the whole cell population with a pro-EMT stimulus? Since cell proliferation is linked to rRNA synthesis the authors also need to include markers of cell cycle for the individual cell population to identify which cell cycle state each sorted cell population is associated with.*

We thank the reviewer for recommending further analysis of the cell cycle among RFP+, GFP+, and Doub+ cells. As illustrated in the revised Supplementary Figure 1B, an increased proportion of RFP+ cells was observed in the S phases in comparison to GFP+ cells. Conversely, Doub+ cells demonstrated a proliferation rate even higher than to that of RFP+ cells.

Upon sorting, RFP+ cells were found to spontaneously undergo epithelial-mesenchymal transition (EMT) when cultured in 10% FBS media, thereby converting to GFP+. We quantified the GFP+ cell percentage within the total cell population, noting a consistent transition of a certain proportion of RFP+ cells to EMT, leading to an accumulation of GFP+ cells. This accumulation stabilizes as approximately 60-70% of the entire population become GFP+. Remarkably, re-sorting RFP+ cells from this balanced tumor cell population resulted in a similar fluorescent transition pattern as observed in the parental population. The mechanisms by which tumor cells regulate the EMT phenotypes across the entire population remain unclear. Nevertheless, the equilibrium between RFP+ and GFP+ cells may be attributed in part to the more rapid proliferation of RFP+ cells and the limited proportion of tumor cells undergoing EMT.

We conducted repeated long-term cultures (up to 20 passages) of the Tri-PyMT cells, yielding consistent results. The fluorescence transition pattern in Tri-PyMT cells proved highly reliable. Further details regarding the Tri-PyMT cells have been incorporated into the Methods section.

> *Fig. 1B. The loading control is not even and quantification is missing, in the text, it states Vimten instead of Vimentin.*

The less loading with Doub+ cells was due to the limited number of EMT transitioning cells we could purify by flow sorting. Even though, the expression of both epithelial and mesenchymal markers in the Doub+ cells were clear. In the revised manuscript, we have quantified the Western blot results. We also apologize for the type errors and have corrected the spelling of "Vimentin."

> *Fig. 1K. In this figure, the authors write: 'It is worth noting that with the 2-phase classifications (Epi or Mes), the elevated RiBi activity was associated with the transitioning cells still exhibiting overall epithelial phenotypes; RiBi activities diminished as cells completed their transition to the mesenchymal phase'. But in Fig. 1K, the Ribi activity is already at a peak during the epithelial state and starts declining already at the beginning of the transition, can the authors please explain this data a bit more? The finding that ribosome biogenesis diminishes once the cells have completed their transition was shown in Prakash et al, Fig. 1 J, I, and accordingly their scientific findings should be discussed in the context of published work.*

We acknowledge the reviewer's concerns regarding the comparison of the timeline for EMT in our model with that in Prakash's study. In our model, EMT-transitioning cells are identified by their EMT marker genes and fluorescence expression. We enriched the EMT transitioning cells by sorting the Doub+ cells. Due to the RFP protein's half-life, cells remain RFP+ for 2-3 days after the reporter cassette has switched to GFP expression. In Prakash's study, the EMT transitioning phase was defines by the duration of TGF-β stimulation.

In Figure 1K, cells are categorized based on their EMT pseudotime, calculated from their expression of EMT marker genes in the EMTome. Ribosome biogenesis (RiBi) activity is highest in cells transitioning between phase 1 (Red) and phase 2 (Green), with both phases displaying predominantly epithelial phenotypes (Figures 1C, 1D, and 1E). RiBi activity declines in cells in phases 4, 5, and 3, which exhibit a mesenchymal phenotype. We have expanded the discussion to include more details in comparison with Prakash's study in the revised manuscript.

> *Supp Fig S4. The authors should provide a rationale for how and why the specific marker genes were selected to calculate the AUC values.*

We have chosen the specific EMT marker genes based on their overall expression levels in Tri-PyMT cells, ensuring consistency with the reported associations of their expression patterns to epithelial or mesenchymal phenotypes in the literature. We provide a detailed rationale for the selection of these genes in the Method of revised manuscript (Page #7).

> *Figure 2 and associated supplementary figure panel. In this figure, rRNA synthesis needs to be evaluated in the cells isolated from the lungs to corroborate the RNA sequencing findings.*

Following the reviewer's suggestion, we performed an RT-PCR of Ribi related genes including Bop1, Gemin4, Its1, Its2, Npm1, Rpl8, Rpl29, Rps9, Rps24, Rps28, Polr1a, Setd4, Utp6, and Xpo1. Consistent with the bulk and single cell RNA sequencing, relatively higher expression of Ribi related genes were detected in Doub+ cells compared to that of RFP+ and GFP+ cells (revised Supplementary Fig S5).

> *Fig 2C, as per figure Supp Fig S4 please explain the rationale for how and why the specific marker genes were selected.*

The same marker genes used for the calculation of the EMT AUC value as in Fig. 1. These marker genes were selected because their overall expression levels are readily detectable in

Tri-PyMT cells, their expression patterns are consistent with their epithelial or mesenchymal phenotypes, and the associations between expression of marker genes and phenotypes are in line with the previous reports in literature. Description of AUCell value quantification was included in the revised manuscript (Page #7).

> *Fig. 2G. The high Ribi during the epithelial state is most likely due to the resumption of cell proliferation of these cells. The authors should check the cell cycle states of these different sets of cells.*

We agree with the reviewer that higher Ribi activity could be related to the resumption of cell proliferation of mesenchymal tumor cells. To clarify this, we revisited the scRNAseq data, and project the S phase score to the scatter plot of Ribi activity/MET pseudotime. Indeed, cells in the far mesenchymal state show low S phase score, while the proliferating cells were mostly detected in the MET transitioning phase and epithelial phase (revised Supplementary Figure S6D).

> *Suppl Fig. 5 Please correct the figure legends as there is no figure D.*

We apologize for the mislabeling. We have corrected the figure legend accordingly.

> *Figure 3. Please explain the rationale for stimulating cells with FBS for the selected time points.*
>
> *Fig. 3A. The loading control is not even, and quantification is missing. In addition, the authors should explain why the different time points were chosen and why FBS was chosen as a stimulus. In addition, from which passage of cells were these cells?*

The RFP+ Tri-PyMT cells underwent EMT and switched their expression of fluorescent marker to GFP+ when cultured with FBS. To investigate the response of cells at varying EMT statuses to an FBS-enriched environment, we isolated RFP+, Doub+, and GFP+ cells from the 4th and 5th passages of Tri-PyMT cells and probed downstream signaling pathways after FBS stimuli. The timeline for stimulation was informed by the innate activation profile of these phosphorylation-dependent signals, spanning from 10 minutes to 1 hour. We noted that ERK signaling activation in RFP+ cells occurred within minutes of FBS exposure and diminished within approximately one hour. This ERK signal was more pronounced and persisted longer in Doub+ cells. In contrast, GFP+ cells exhibited a more transient and lower ERK activation (see revised Fig 3A). To address concerns regarding potential uneven loading in our previous assays, we have now included the quantification of Western blots in the revised Fig 3A.

> *How and why were ERK and mTORC1 pathways chosen for analysis downstream of increased rRNA synthesis? ERK and mTORC1 have mostly been investigated in the role of cell proliferation which is why the cell cycle status of these cell populations will be important to consider in the context of their findings.*

The regulation of ribosome biogenesis (RiBi) is mediated by multiple pathways, including the myelocytomatosis oncogene (Myc), mammalian targets of rapamycin (mTOR), and noncoding RNAs, as detailed by Jiao et al. in Signal Transduction and Targeted Therapy (2023). There was no significant difference in Myc expression between tumor cells with epithelial and mesenchymal phenotypes. We thus investigated the activation of the mTOR pathway in sorted RFP+, Doub+, and GFP+ cells. Additionally, given the recognized role of the ERK/MAPK signaling pathway in regulating protein synthesis and cell proliferation, we also analyzed the activation of ERK signals.

In alignment with the reviewer's observation regarding the potential correlation between cell proliferation rate and RiBi activation, we further characterized the cell cycle distributions of

RFP+, Doub+, and GFP+ cells. Notably, the Doub+ cells exhibited a higher ratio of cells in the proliferative state (including S and G2/M phases) compared to RFP+ and GFP+ cells. Also, higher percentage of S phase cells were detected in RFP+ cells than GFP+ cells (revised Supplementary Figure S1B).

> *Figure 3 B, C, D. Please provide more information about which cells are analyzed in this figure.*

We apologize for the previous ambiguity regarding the cells analyzed in these figures. To clarify, the figure legend has been revised to specify that Tri-PyMT cells from the 5th to 10th passages were the subjects of analysis for cell size and nascent protein synthesis, utilizing flow cytometry.

> *Figure 3D. The selected images show enlarged nucleoli/ fibrillarin which is an indicator of increased rRNA synthesis however, the authors need to show an increase in rRNA transcripts by q-PCR or Northern blot and also show EU staining in these different cell states to support their claim.*

We appreciate the reviewer's recommendation to further validate the enhanced ribosome biogenesis (RiBi) in Doub+ cells. In response, we conducted RT-PCR analysis of several RiBi-related genes (revised Supplementary Fig S5). Additionally, we carried out an EU incorporation assay to illustrate the rRNA transcription activity within these cells. The new results have been incorporated into the revised manuscript (Supplementary Fig S7).

> *Figure 4 and associated supplementary. In this figure, the authors show that using small molecule Pol I assembly inhibitors (BMH-21 and CX-5461) reduces the expression of mesenchymal proteins. As mentioned in previous comments these results should be put in the context of published work by Prakash et al which demonstrate that upon CX-5461 and genetic silencing of Pol I EMT is hampered as demonstrated by gene expression profiles as well as functional assays.*

We revised the description of our experiments with Pol I inhibitors in the revised manuscript by including the citation context (Prakash et al Nat Commun, 2019) as mentioned above.

> *Figure 4A. Please provide an explanation of how the doses of Pol I assembly inhibitors were determined and also the selected time points. The Pol I assembly inhibitors should have an effect within a few hours (Drygin, Cancer Research, 2011, Peltonen, Cancer Cell, 24). The authors also need to show that the BMH-21 and CX5461 at selected doses are indeed inhibiting rRNA synthesis in the selected cell populations. The data would also be strengthened by performing ChIP analysis demonstrating that indeed the Pol I complex is disassociated from the rDNA genes upon inhibition.*
>
> *In addition, why are there only 2 reports and how were the statistics done? Were the data normalized to the total number of cells? The graph visually shows a difference in cell numbers. Are cells dying at this concentration? More controls must be included including markers for cell stress, p53, autophagy, and apoptosis.*

The dose of Pol inhibitors was selected based on prior studies, as noted by the reviewer. Peltonen et al. demonstrated that BMH-21 inhibits growth across a wide spectrum of cancer cell lines, achieving a mean half-maximal inhibition of cell proliferation (GI50) at 160 nM (Peltonen K., et al. Cancer Cell. 2014). Consistently, in our experiments, the growth inhibitory effect of BMH-21 on Tri-PyMT cells fell within this range, at approximately 200 nM (Fig 5B, Supplementary Fig S10).

To address the reviewer's suggestion and verify that RiBi inhibitor effectively inhibits rRNA synthesis in our study, we conducted an EU incorporation assay. This assay revealed significant inhibition of rRNA synthesis by BMH-21 and CX5461 in Tri-PyMT cells (revised Supplementary Fig S8B). Furthermore, to enhance the robustness of our findings, we repeated the BMH-21 treatment on sorted RFP+ Tri-PyMT cells across three biological replicates, which yielded consistent results.

> *Figure 4B. How many replicates were done for this experiment and please provide quantification as per previous comments on WB experiments. The authors should provide a rationale for why Snail and Vimentin were chosen for these studies. Also, the authors should provide a functional assay and demonstrate that cells are less migratory post-treatment and not only markers.*

Western blots with sorted Tri-PyMT cells were performed twice. We have added the quantification of these blot in the revised manuscript. Snail and Vimentin were chosen as mesenchymal markers to indicate EMT phenotype switches as those were well-studied and commonly used mesenchymal markers of EMT. The association of fluorescent marker switch and

EMT phenotype such as cell migration was well established in our previous study (Fischer et al., 2015, Lourenco et al., 2020). The morphology and migration property of GFP+ were well distinguished from RFP+ counterparts. Also, following reviewer's suggestion, we performed migration assay with BMH21 treatment (revised Supplementary Fig 8C). Indeed, the treatment with BMH21 or CX5461 inhibited cell migration as expected.

> *Supplementary figure 7. The authors need to provide a rationale as to why the two Rps were chosen to inhibit ribosome biogenesis.*

The two Rps targets were chosen based on their differential expression in Doub+ cells compared with RFP+ and GFP+ cells. Also, we considered the overall expression level of these genes in Tri-PyMT cells. We have edited the according text in the revised manuscript.

> *Figure S7B. In the images shown there does not appear to be a significant change in the number of nucleoli however the cells seem to be smaller. This should be explained.*

We agree with the reviewer that the box plot does not clearly show the nucleoli differences between these cells. We present the data with a violin plot, which more clearly exhibit the result (revised Supplementary Fig S9B). It was also true that the sizes of the Rps knockdown cells were relatively smaller than control cells. This is consistent with the finding that the EMT transitioning cell size was bigger than the non-transitioning cells (Fig 3B)

> *Figure 5 and Supp 8. The authors should provide the background as to why the specific chemotherapeutic drugs were chosen.*

The chemotherapeutic agents employed in this study are widely used in the treatment of breast cancer. For instance, Cyclophosphamide (CTX) hampers both DNA replication and RNA transcription; Doxorubicin inhibits DNA replication by disrupting topoisomerase activity; Paclitaxel prevents cell division by stabilizing microtubules; and 5-Fluorouracil (5-FU), a pyrimidine analog, blocks thymidylate synthase, thereby disrupting DNA synthesis. Additionally, some of these agents, such as CTX and 5-FU, may directly or indirectly affect RNA polymerase, prompting us to investigate the synergistic effects of these drugs when used in combination with BMH21. We have included the information in revised manuscript.

> *Fig 5B/Supp 8. Can the authors please explain why only 2 replicates were done and provide a rationale for future statistics?*

Using serial concentrations of drugs tested—6 doses for BMH21 and 8 doses for CTX—it is logical to arrange the experiment in duplicates on 96-well plates. For the statistical analysis, we conducted dose-response analysis to ascertain the IC50 values for each drug alone and in combination. Additionally, we calculated the synergy score to assess the interactions between the drugs. The methodology section of the manuscript has been enhanced to provide a clearer description of these processes in the revised version.

> *Figure 6. The authors should provide a rationale of why tail veins were chosen as their in vivo model system as the EMT cells do not cause metastasis and if chemoresistance is the main focus of their studies both primary and secondary tumors should be considered. Why was not the MMTVPyMT mouse model chosen where the cells were originally isolated from to test the role of the dual treatment? How was the drug concentration decided and the interval of treatments?*

We acknowledge the reviewer's concerns regarding the choice of experimental setup for our metastasis model. Certainly, utilizing the original MMTV-PyMT mice for the combination therapy experiment would be the ideal scenario. However, there are potential drawbacks to using these transgenic mice: 1) The occurrence of multiple primary tumors that develop simultaneously but without synchronized timelines (in mice aged 6-9 weeks), and the unsynchronized development of lung metastasis (from 10-16 weeks of age). This leads to uncontrollable variations in the experimental setup, particularly when establishing multiple treatment groups; 2) Gathering a sufficient number of female transgenic mice of a similar age poses another challenge; 3) The absence of tumor cell labeling complicates the focus on assays for EMT/MET phenotype changes during tumor progression. Consequently, we have chosen to employ our Tri-PyMT model for this experiment. The drug treatment protocol was established after reviewing literature on the in vivo application of CTX and BMH21 treatment (Peltonen etal. Cancer Cell 2014; Jacobs etal. JBC 2022).

> *Figure 6B, C. The authors should provide quantification for these data, how many mice were analyzed, and how many sections were stained and analyzed.*

We have improved the quality of these fluorescent images and clarify the methodology, including the mouse/section numbers per group, for obtaining these fluorescent images in the legend. To quantify the differential impact of BMH21 on RFP+ and GFP+ tumor cells, we performed flow cytometry (revised Supplementary Fig S11). We have also changed the presentation of these flow data to improve the clarity of these results.

> *Fig 6D. How were the treatment timeline and dosing chosen? LM2 cells are derived from a metastatic site, so they are not transitioning cells they are stably mesenchymal why was this chosen as their in vivo model?*

LM2 cells were derived from the lung metastasis of MDA-MB-231 cell line. These cells exhibit predominantly mesenchymal phenotype in culture. While growing into metastasis in the lung, expressions of epithelial markers such as E-cad were upregulated (Supplementary Fig S12), suggesting a MET process may be involved the outgrowth of lung metastasis. Therefore, we choose the LM2 cells as our experimental model for assessing the effect of RiBi inhibitor on MET. The treatment timeline was determined based on previous studies of BMH21 and chemotherapy applications in vivo (Peltonen etal. Cancer Cell 2014; Jacobs etal. JBC 2022).

> ***Reviewer #3 (Public Review):***
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> *Summary:*
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> *Ban et al. investigated the role of ribosome biogenesis (RiBi) in epithelial-to-mesenchymal transition (EMT) and its contribution to chemoresistance in breast cancer. They used a Tri-PyMT EMT lineage-tracing model and scRNA-seq to analyze EMT status and found that RiBi was elevated during both EMT and mesenchymal-to-epithelial transition (MET) of cancer cells. They further revealed that nascent protein synthesis mediated by ERK and mTOR signaling pathways was essential for the completion of RiBi. Inhibiting excessive RiBi impaired EMT and MET capability. More importantly, combinatorial treatment with RiBi inhibitors and chemotherapy drugs reduced metastatic outgrowth of both epithelial and mesenchymal tumor cells. These results suggest that targeting the RiBi pathway may be an effective strategy for treating advanced breast cancer with EMT-related chemoresistance.*
>
> *Strengths:*
>
> *The conclusions of this study are generally supported by the data. However, some weaknesses still exist as mentioned below.*
>
> *Weaknesses:*
>
> *(1) The study predominantly focused on RiBi as a target for overcoming EMT-related chemoresistance. Thus, it will be necessary to provide some canonical outcomes after upregulating ribosome biogenesis, such as translation activity. I would suggest ribosome profiling or puromycin-incorporation assay, or other more suitable experiments.*

EU incorporation assay (revised Supplementary Fig S7) and puromycin incorporation assay (Fig 3C) were performed.

> *(2) The results were basically obtained from mice and in vitro experiments. While these results provide valuable insights, it will be valuable to validate part of the findings using some tissue samples from patients (e.g. RiBi activity) to determine the clinical relevance and potential therapeutic applications.*

We agree. We have added the analyses on the correlation between patients' survival and RiBi activation (revised Supplementary Fig S13, S14).

> *(3) The results revealed that mTORC1 and ERK mediated RiBi activation. How about mTORC2? It will be informative to evaluate mTORC2 signaling.*

We investigated the role of the mTORC1 pathway in regulating RiBi activation. It is pertinent to acknowledge that the mTORC1 complex is known to positively regulate protein synthesis through the phosphorylation of ribosomal protein S6 kinase, among other mechanisms. Additionally, Rps6 is recognized as an essential component of the 40S subunit in the ribosome. We agree with the reviewer that mTORC2 may also be involved in RiBi activity, as its activation is mediated through ribosome association (Zinzalla et al., Cell 2011; Prakash et al., Nat Comm 2019). However, this association is more likely to be downstream of RiBi activation, as the RiBi inhibitor CX5461 can block the translocation of Rictor into the nucleus (Prakash et al., Nat Comm 2019).

We also revisited our sequencing data of RFP+, GFP+, and Doub+ cells. While there was no significant change in the expression of either Rptor or Rictor among these cells, the LSMean (overall expression level) of Rptor was higher than that of Rictor; for example, 163.77 vs 29.95

in RFP+ cells. This suggests that mTORC1 may play a dominant role in regulating RiBi activity in our model.

Furthermore, we analyzed how Rapamycin (an mTORC1 inhibitor) affects the EMT process in TriPyMT cells. As expected, Rapamycin-treated cells exhibited higher expression of the epithelial marker E-cadherin (Ecad) and lower expression of the mesenchymal markers Snail and Vimentin (Vim) compared to the control (For Reviewers Figure 3).

> *(4) The results also demonstrated promising synergic effects of Pol I inhibitor (BMH21) and chemotherapy drug (CTX) on chemo-resistant metastasis. How about using the inhibitors of mTORC1 together with CTX?*

Several mTOR inhibitors (e.g., sirolimus, temsirolimus, ridaforolimus) have demonstrated antitumor activity. The combination of mTOR inhibitors with various targeted therapies or chemotherapies is being examined in numerous clinical trials, showing promising results. Although the combination therapy of mTORC inhibitors and CTX is beyond the scope of our study, we analyzed how mTOR inhibitors may affect the EMT process in our model, as mentioned above. Western blot analysis of EMT markers (E-cadherin, Snail, and Vimentin) showed that rapamycin treatment inhibited the EMT transition of Tri-PyMT cells. (For Reviewers Figure 3).

> *(5) While the results demonstrate the potential efficacy of RiBi inhibitors in reducing metastatic outgrowth, other factors and mechanisms contributing to chemoresistance may exist and need further investigation. I would suggest some discussion about this aspect.*

Following reviewer's suggestion, we have edited the discussion section with more future directions.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *(1) Please provide the quantified data for all western blots, rather than solely show some representative blots.*

We quantified the western blot images as shown in the revised figures. Thanks for reviewer's suggestion.

> *(2) Please add a graphic abstract or schematic to help the readers understand the whole story.*

We have summarized a schematic graph of our findings in the revised manuscript (Supplementary Fig S15).

> *(3) It is hard to read the numbers inside all plots of flow cytometry.*

High-resolution figures of flow plots are included in the revised manuscript.

> *(4) Please provide high-resolution figures for all the synergy plots.*

High-resolution figures of synergy plots are included in the revised manuscript.

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