Supporting Information

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SI Materials and Methods

Cell Proliferation Assays. Cell proliferation was monitored using the BrdU incorporation assay (Cell Proliferation ELISA BrdU kit; Roche). Cells were seeded in 96-well plates (1,000 cells per well) and treated with drug concentrations as indicated in the text or a vehicle for 48 h. Absorbance at 370 nm (reference wavelength 492 nm) was measured using a Varioskan microplate reader (Thermo Electron Corporation). Concentration of the drug, which inhibits proliferation by ∼50% of the control (aIC50), was determined by dose–response curve fitting using Graph prism software.

Western Blot Analysis. Cells were cultured in 15-cm dishes and treated with indicated concentration of drugs or a vehicle (DMSO) for 12 h. One-tenth of the cells was used for Western blot analysis and nine-tenths were used for polysome analysis. Cell lysates were prepared using RIPA buffer supplemented with phosphatase and protease inhibitors. Western blotting was carried out as previously described (1). Antibodies against eukaryotic translation initiation factor 4E-binding protein (4E-BP)1, 4E-BP2, phospho-4E-BP1 (Thr37/46 and Ser65), S6 kinase 1 and 2, phospho-S6 kinase 1 (Thr389), rpS6, phospho-rpS6 (Ser240/244), and cyclin D3 were from Cell Signaling Technology. Other antibodies used in this study were anti-ODC (BIOMOL), anti-cyclin E1 and anti-cyclin E2 (Santa Cruz Biotechnology), and anti–β-actin (AC-15; Sigma). Secondary HRP-conjugated anti-rabbit IgG and anti-mouse IgG were from Amersham Biosciences and the signals were revealed using Western Lightning ECL Enhanced Chemiluminescence Substrate (PerkinElmer). Densitometry was performed using ImageJ.

Polysome Preparations. Polysome profile analysis was carried out as previously described (1). Briefly, cells were cultured in 15-cm dishes and treated with indicated concentration of drugs or a vehicle (DMSO) for 12 h, washed with cold PBS containing 100 μg/mL cycloheximide, collected, and lysed in a hypotonic lysis buffer [5 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 1.5 mM KCl, 100 μg/mL cycloheximide, 2 mM DTT, 0.5% Triton X-100, and 0.5% sodium deoxycholate]. A sample was collected from the lysate and cytoplamsic RNA was isolated using TRIzol (Invitrogen). Lysates were loaded onto 10–50% (wt/vol) sucrose density gradients [20 mM Hepes-KOH (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$] and centrifuged at 36,000 rpm [SW 40 Ti rotor (Beckman Coulter, Inc.)] for 2 h at 4 °C. Gradients were fractionated and the optical density at 254 nm was continuously recorded using an ISCO fractionator (Teledyne ISCO). RNA from each fraction was isolated using TRIzol (Invitrogen). Fractions with mRNA associated with >3 ribosomes were pooled (polysome-associated mRNA).

Data Analysis. For analysis of microarray data, we extracted and normalized data using robust mutiarray averaging implemented in the R package "affy" (www.r-project.org). We used updated probe set definitions because these provide improved precision and accuracy. We used the random variance model (RVM) (2) ANOVA to assess differential expression between conditions using data from cytoplasmic or polysome-associated mRNA (Fig. 2*A* and Fig. S1*A*) and the RVM *t* test to identify differential expression between each treatment and control (Fig. 2 *B–D* and Fig. S1 *B–D*). To identify differential mRNA translation we used analysis of partial variance (APV) (3) and applied RVM using both an omnibus test (to assess differential translation between the conditions) (Fig. 2*A* and Fig. S1*A*) and treatment contrasts (to identify the effect on mRNA translation from each drug) (Fig. 2 *B–D* and Fig. S1 *B–D*) as implemented in the anota R package (4). In anota we applied the following settings for gene selection: slopeP = 0.01; ΔPT = $\log_2$ (1) (as defined in the anotaPlotSigGenes function in anota). For identification of differential translation of each drug treatment to control we used a cut off of false-discovery rate (FDR) < 0.15 for differential translation and an effect < $-\log_2(1.5)$ (Table S2). To group genes based on drug sensitivity patterns we collected all genes that showed differential mRNA translation under at least one drug treatment compared with control, extracted their translational activity under all conditions (i.e., the intercepts from APV), performed a per gene centering, and used *k*-means clustering (in R) to identify 14 clusters which were manually annotated to drug sensitivity patterns (Fig. 3*A* and Fig. S2). We used GO::Termfinder (5) to identify enriched cellular functions as defined by the gene ontology (GO) consortium (6) within subsets of differentially regulated mRNAs corresponding to different drug-sensitivity patterns. Only categories from the "cellular process" system that defined >5 mRNAs in the drug sensitivity group and show a >twofold enrichment compared with the background with an FDR < 0.05 were collected (Fig. S3).

For NanoString data analysis, the obtained counts were $\log_2$-transformed. Per sample normalization was performed using geometric means from three housekeeping genes (*GAPDH*, *MRPS24*, and *TBCD*). Based on the linearity of the positive control signals we identified signals with ≤64 counts as outside the dynamic range. All genes that did not have at least four signals with >64 counts were therefore removed (34 genes passed filtering). We then applied anota to identify mRNAs whose translation was suppressed under each drug treatment compared with control (FDR < 0.15). The translational activities from all conditions (i.e., the intercepts from APV) were obtained for those mRNAs that are translationally suppressed, and normalized to the vehicle control to obtain relative effects (shown in Fig. 3*B*).

1. Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N (2007) Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. *Cancer Res* 67:10804–10812.
2. Wright GW, Simon RM (2003) A random variance model for detection of differential gene expression in small microarray experiments. *Bioinformatics* 19:2448–2455.
3. Larsson O, Sonenberg N, Nadon R (2010) Identification of differential translation in genome wide studies. *Proc Natl Acad Sci USA* 107:21487–21492.
4. Larsson O, Sonenberg N, Nadon R (2011) anota: Analysis of differential translation in genome-wide studies. *Bioinformatics* 27:1440–1441.
5. Boyle EI, et al. (2004) GO:TermFinder—Open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. *Bioinformatics* 20:3710–3715.
6. Anonymous; Gene Ontology Consortium (2008) The Gene Ontology project in 2008. *Nucleic Acids Res* 36(Database issue):D440–D444.

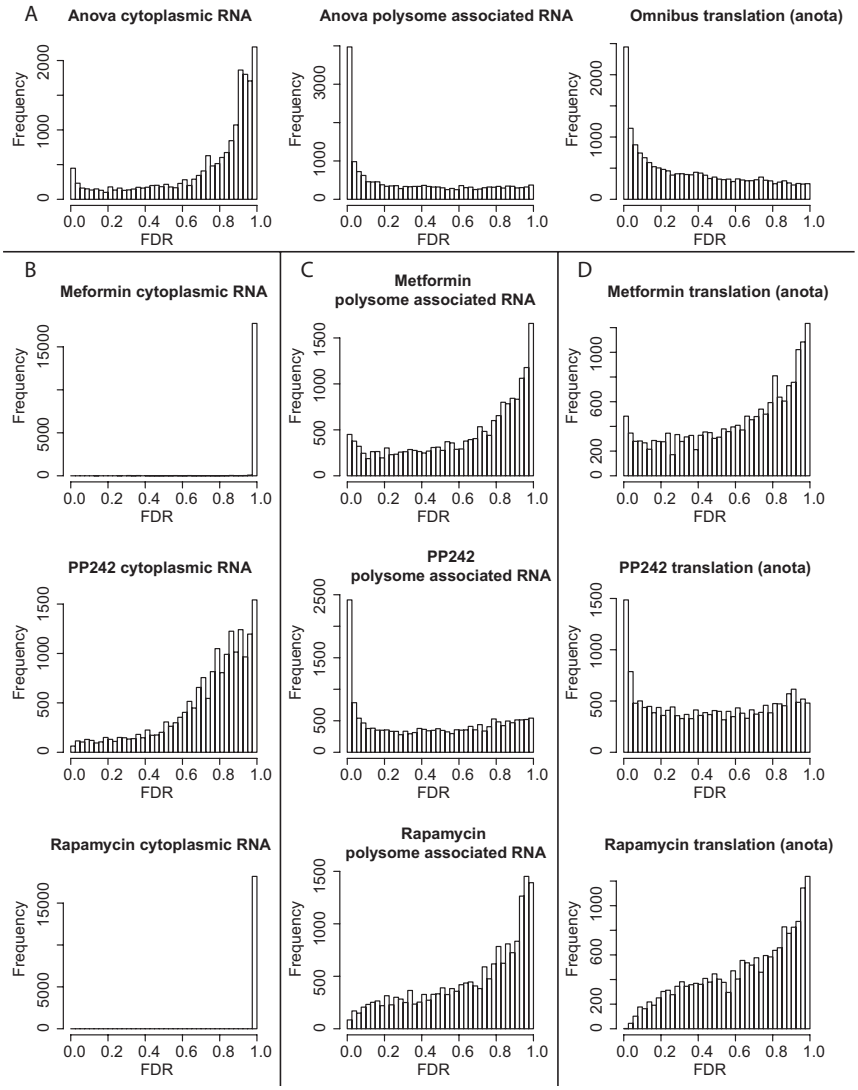
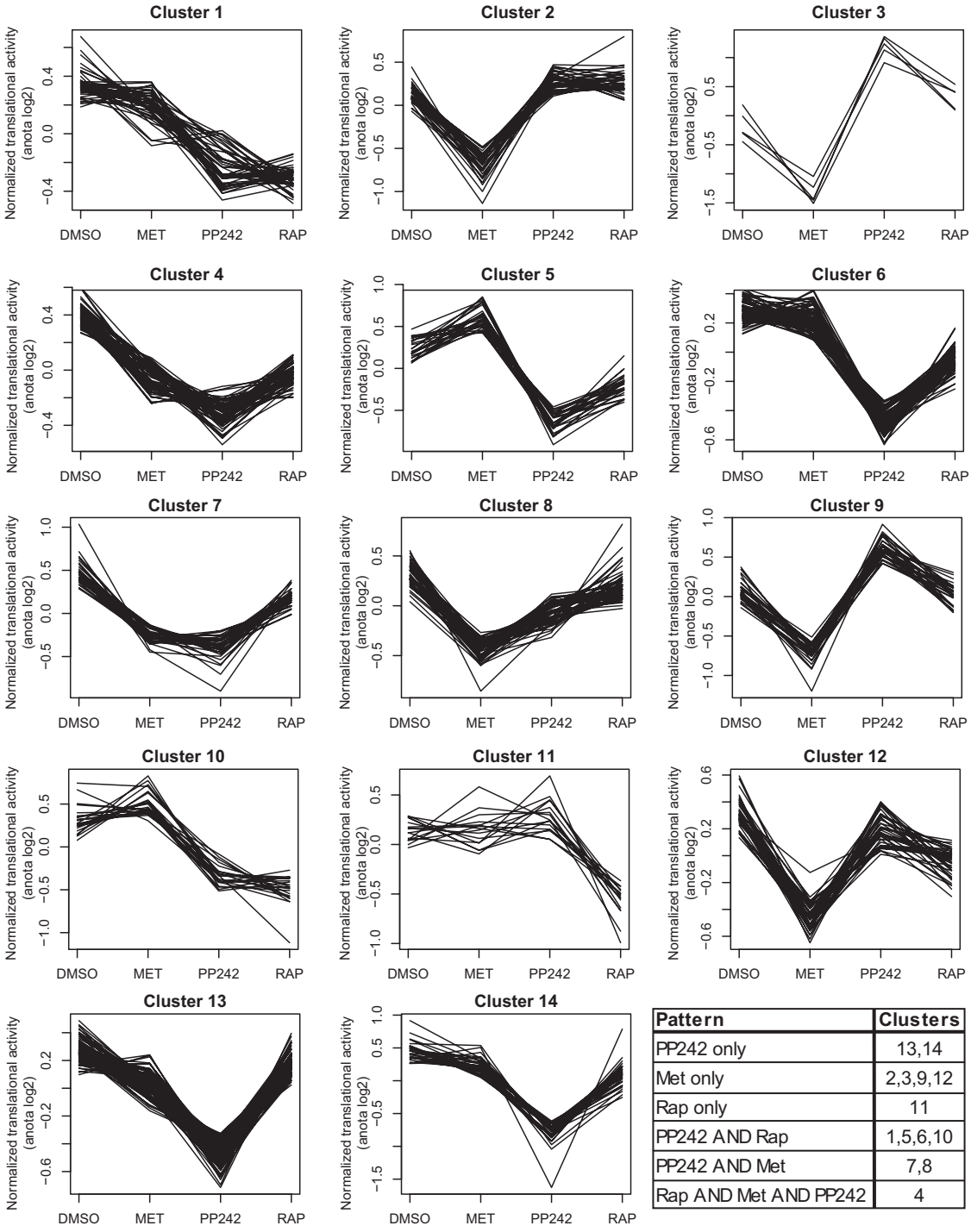


Fig. S1. Mammalian target of rapamycin (mTOR) inhibitors and metformin suppress gene expression at the level of mRNA translation. (*A*) Histograms of adjusted *P* values (FDRs) from ANOVAs comparing all conditions using data obtained from cytoplasmic or polysome-associated mRNA and from analysis of translational activity using anota. Histograms of adjusted *P* values (FDRs) from treatment (metformin, PP242, or rapamycin) compared with vehicle using data obtained from cytoplasmic (*B*), polysome-associated mRNA (*C*), or translation as analyzed using anota (*D*).



Pattern	Clusters
PP242 only	13,14
Met only	2,3,9,12
Rap only	11
PP242 AND Rap	1,5,6,10
PP242 AND Met	7,8
Rap AND Met AND PP242	4

Fig. S2. Expression patterns following drug treatments. *k*-Means clustering of genes that were translationally suppressed by at least one drug (anota FDR < 0.15 and fold-change >1.5). The table indicates the clusters that were assigned to the various drug sensitivity patterns.

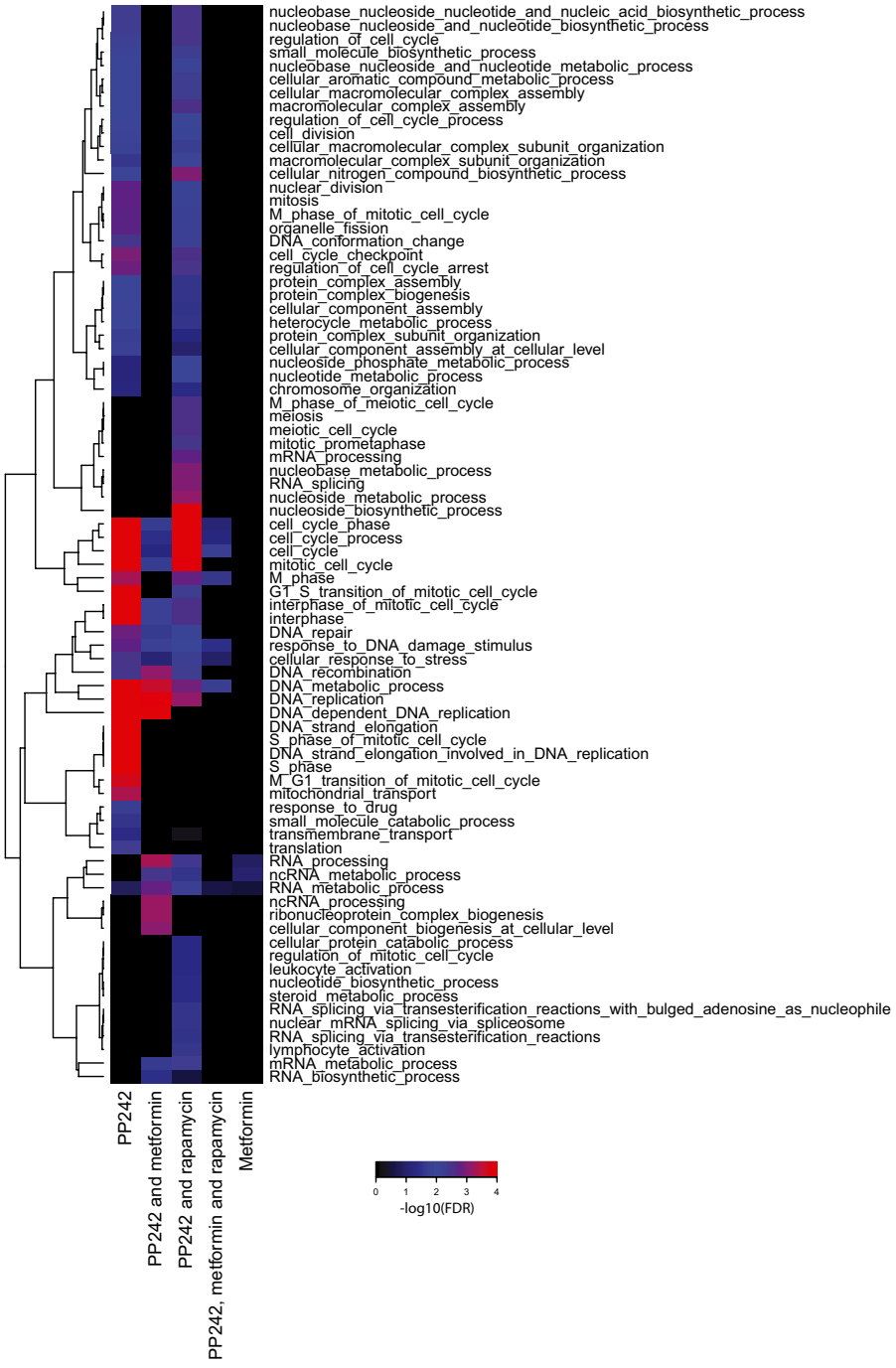


Fig. S3. mRNAs encoding proteins involved in cell cycle, metabolism, and RNA processing are enriched among mRNAs that are distinctly or commonly translationally suppressed following drug treatment. Shown are $-\log_{10}$ (FDRs) for enrichments of GO categories (rows) among encoded mRNAs that were grouped as distinctly or commonly translationally suppressed as indicated in Fig. 3*A*. Those categories that showed >twofold enrichment with an FDR < 0.05 and involved >5 members of the group (columns) were considered enriched.

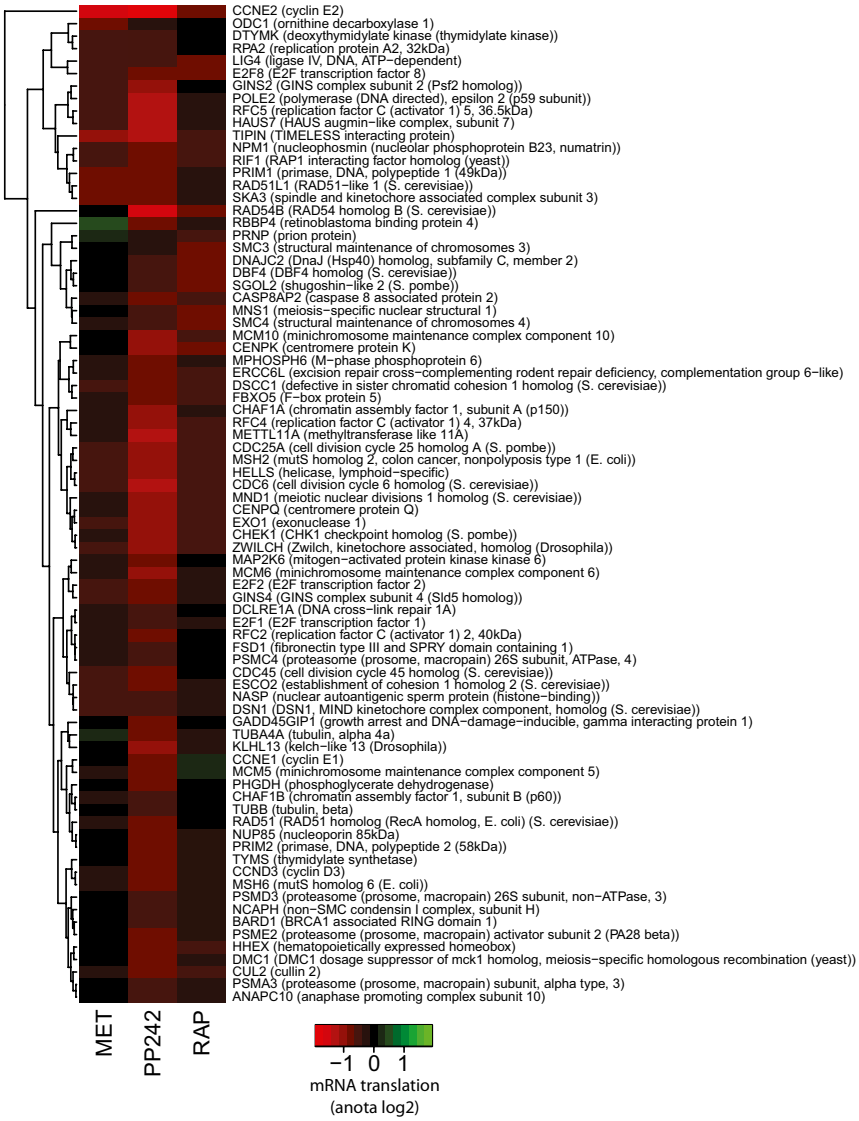


Fig. S4. Rapamycin, PP242 or metformin show overlapping and distinct translational suppression of mRNAs that encode for cell-cycle factors. Translational activity calculated with anota after treatment with metformin, PP242, or rapamycin compared with vehicle (log_2 scale) for mRNAs whose encoded proteins are annotated to cell cycle according to GO.

Other Supporting Information Files

Table S1 (DOC)
Table S2 (DOC)