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Supplemental information

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Supplemental information

Proteomics Reveal Cap-dependent Translation Inhibitors Remodel the Translation Machinery and Translatome

J.J. David Ho[1,2,3,*,#], Tyler A. Cunningham[1,4,5,*], Paola Manara[1,6], Caroline A. Coughlin[1,4,6], Artavazd Arumov[1,6], Evan R. Roberts[1,7], Ashanti Osteen[1,7], Preet Kumar[1,2], Daniel Bilbao[1,7], Jonathan R. Krieger[8], Stephen Lee[1,9], & Jonathan H. Schatz[1,2,#,†]

Inventory of Supplemental Information

1.
Supplemental Figures

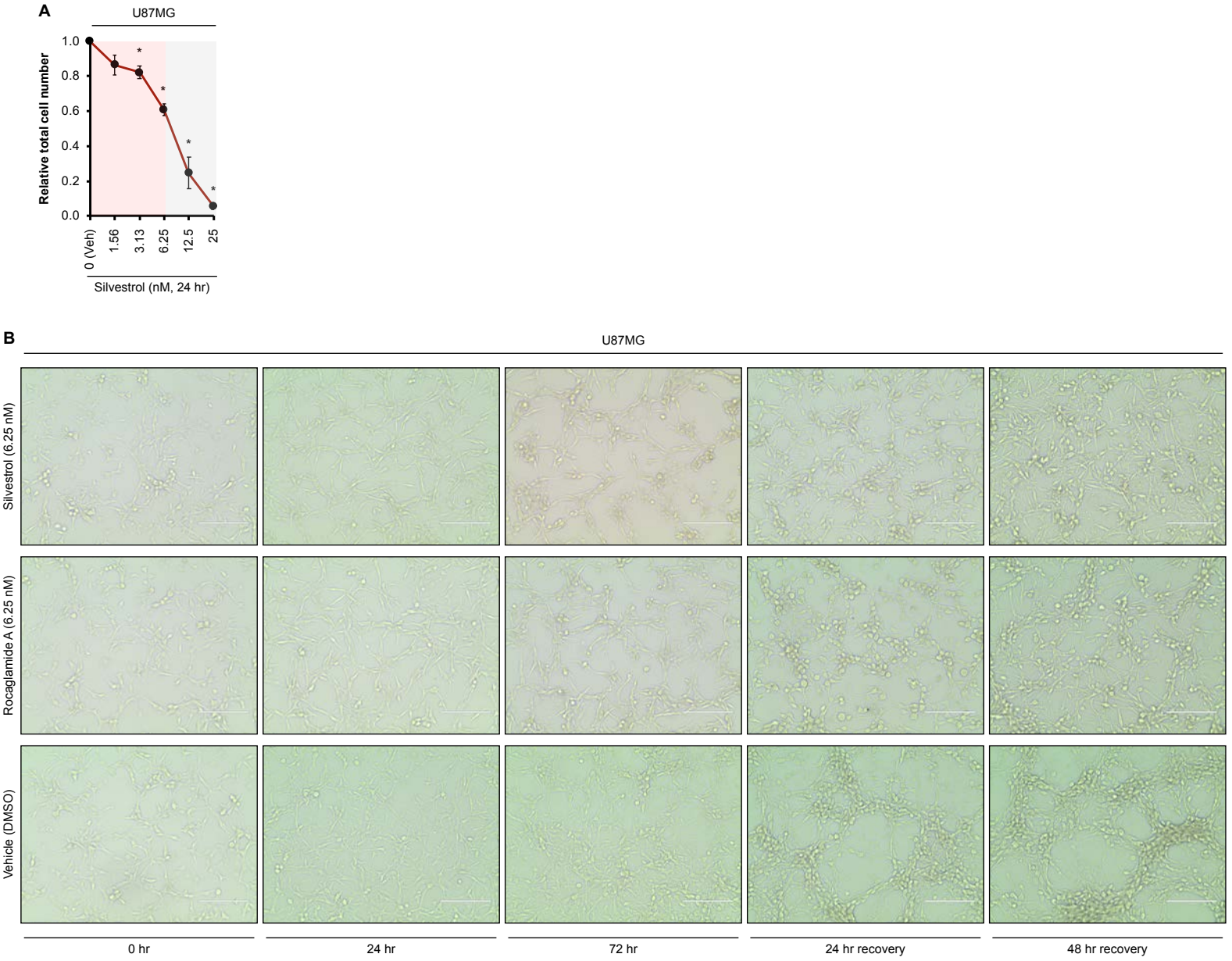
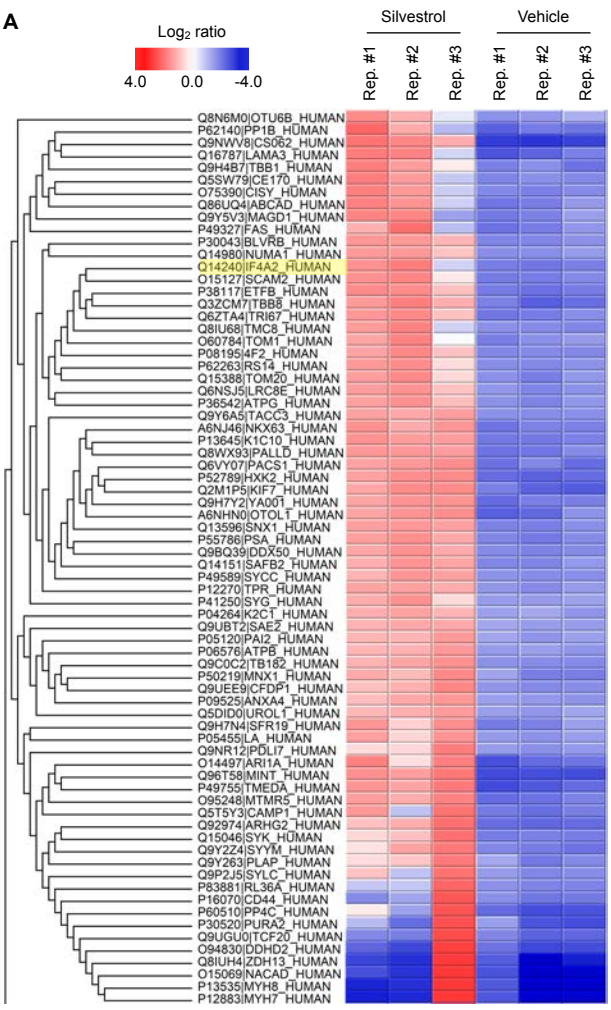
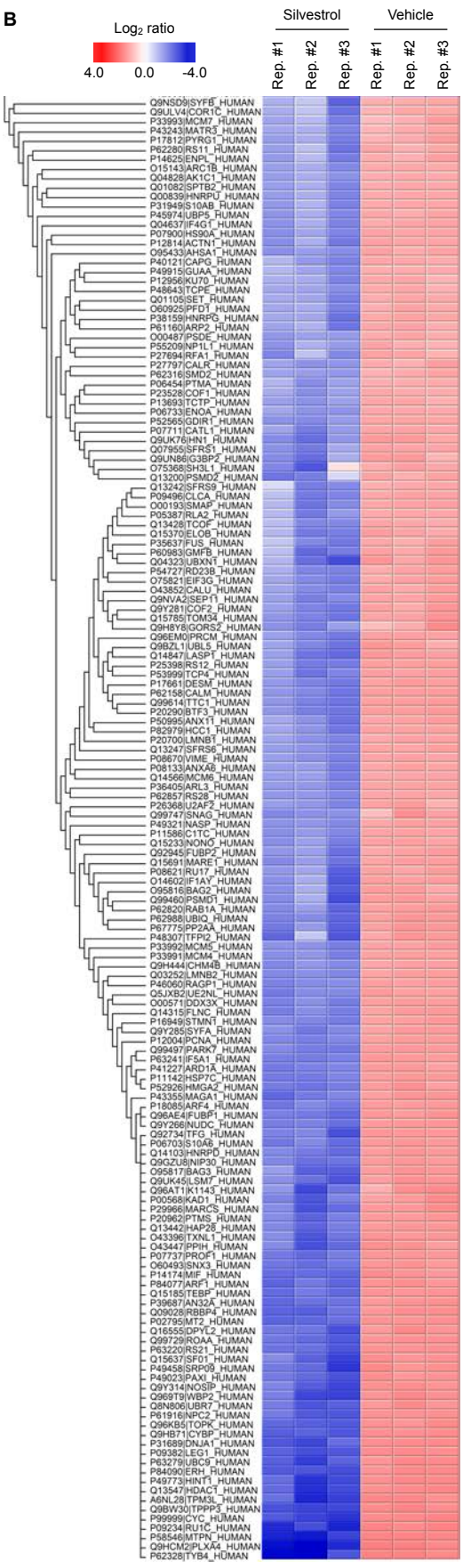

A
U87MG
Relative total cell number
1.0
0.8
0.6
0.4
0.2
0.0
0 (Veh)
1.56
3.13
6.25
12.5
25
Silvestrol (nM, 24 hr)
B
U87MG
Silvestrol (6.25 nM)
Rocaglamide A (6.25 nM)
Vehicle (DMSO)
0 hr
24 hr
72 hr
24 hr recovery
48 hr recovery

Figure S1.

Figure S1. TMT-pSILAC reveals rocaglate-dependent translatome remodeling, related to Figure 1. (**A**) Relative cell number of U87MG treated with silvestrol (indicated concentrations) or vehicle (DMSO) for 24 hr. Data represent mean ± SEM (error bars) of three independent experiments. Asterisk denotes statistical significance calculated using two-sided Student's t-tests compared to vehicle (DMSO). Pink-shaded area highlights concentrations at which cell viability was largely maintained (> 80%). (**B**) Representative bright field images of U87MG treated with silvestrol (6.25 nM), rocaglamide A (6.25 nM), or DMSO, for 72 hr followed by 48 hr of treatment recovery. Scale bars represent 200 μm.





C PANTHER Overrepresentation Test

Gene Ontology term	Fold enrichment	P value	FDR corrected p
Polymeric cytoskeletal fiber (GO:0099513)	4.07x e.g. **GEF-H1**	4.51e-06	3.02e-03
Extracellular space (GO:0005615)	2.01x e.g. **CD98hc**	8.86e-05	1.27e-02
Aminoacyl-tRNA ligase activity (GO:0004812)	16.2x **CARS, GARS, KARS, LARS, YARS2**	6.66e-05	3.90e-02

Figure S2.

Figure S2. TMT-pSILAC reveals rocaglate-dependent translatome remodeling, related to Figure 1. (**A**) Unsupervised hierarchical clustering of silvestrol-inducible proteins based on TMT-pSILAC analysis. Cell color represents the $\log_2$ ratio to the average abundance across different samples. The following filters were applied: target detected across all 6 samples, fold change ≥2, spectrum quality ≥10. eIF4A2 (IF4A2) was confirmed as a positive control (highlighted). 72 proteins identified. (**B**) Unsupervised hierarchical clustering of silvestrol-repressed proteins based on TMT-pSILAC. PTGES3 (TEBP) was confirmed as a positive control (highlighted). 165 proteins identified. (**C**) Gene Ontology (GO) pathway enrichment analysis performed on the 101 silvestrol-induced proteins using the PANTHER classification algorithm. P values were calculated using the Fisher's Exact test, and corrected for multiple comparisons using the False Discovery Rate (FDR) method.

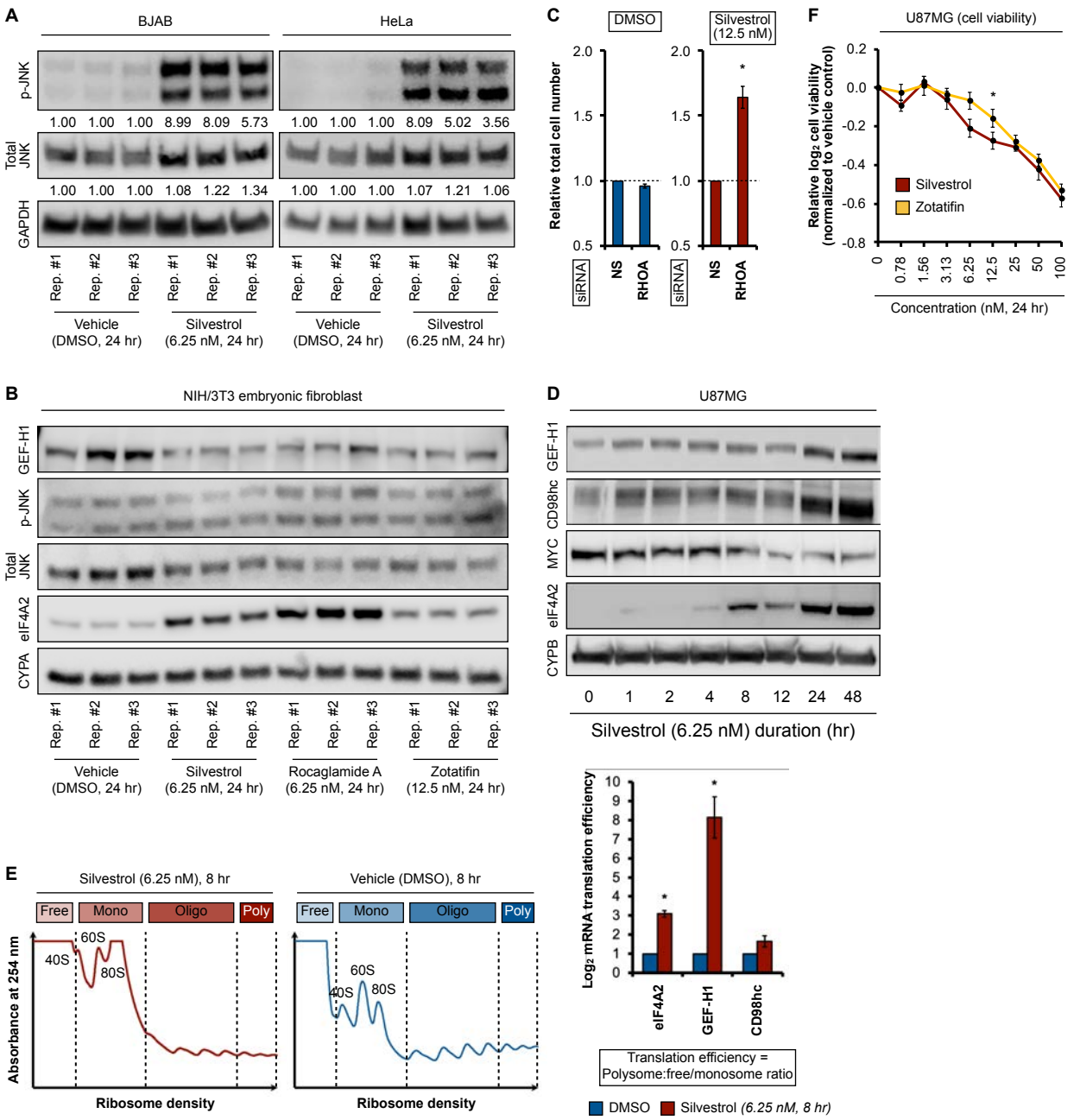

A
BJAB
HeLa
p-JNK
Total JNK
GAPDH
1.00 1.00 1.00 8.99 8.09 5.73
1.00 1.00 1.00 8.09 5.02 3.56
1.00 1.00 1.00 1.08 1.22 1.34
1.00 1.00 1.00 1.07 1.21 1.06
Rep. #1
Rep. #2
Rep. #3
Vehicle (DMSO, 24 hr)
Silvestrol (6.25 nM, 24 hr)
B
NIH/3T3 embryonic fibroblast
GEF-H1
p-JNK
Total JNK
eIF4A2
CYPA
Vehicle (DMSO, 24 hr)
Silvestrol (6.25 nM, 24 hr)
Rocaglamide A (6.25 nM, 24 hr)
Zotatifin (12.5 nM, 24 hr)
C
DMSO
Silvestrol (12.5 nM)
Relative total cell number
siRNA
NS
RHOA
D
U87MG
GEF-H1
CD98hc
MYC
eIF4A2
CYPB
0 1 2 4 8 12 24 48
Silvestrol (6.25 nM) duration (hr)
Log2 mRNA translation efficiency
eIF4A2
GEF-H1
CD98hc
Translation efficiency = Polysome:free/monosome ratio
DMSO
Silvestrol (6.25 nM, 8 hr)
E
Silvestrol (6.25 nM), 8 hr
Vehicle (DMSO), 8 hr
Free
Mono
Oligo
Poly
Absorbance at 254 nm
40S
60S
80S
Ribosome density
F
U87MG (cell viability)
Relative log2 cell viability (normalized to vehicle control)
Silvestrol
Zotatifin
0 0.78 1.56 3.13 6.25 12.5 25 50 100
Concentration (nM, 24 hr)

Figure S3.

Figure S3. Rocaglate-inducible GEF-H1 regulates JNK signaling via RHOA in malignant cells, related to Figure 2. (**A**) Representative immunoblots of BJAB and HeLa treated with silvestrol (6.25 nM) or vehicle (DMSO) for 24 hr. Densitometry values are normalized to loading control. (**B**) Representative immunoblots of NIH/3T3 mouse embryonic fibroblasts treated with silvestrol (6.25 nM), rocaglamide A (6.25 nM), zotatifin (12.5 nM) or vehicle (DMSO) for 24 hr. (**C**) Relative total cell number of U87MG treated with indicated siRNA pools (100 nM final concentration) for 72 hr, followed by treatment with silvestrol (12.5 nM) or vehicle (DMSO) for 24 hr. Data represent mean ± SEM (error bars) of three independent experiments. * indicates $p < 0.05$ compared to NS (non-silencing) siRNA control. (**D**) Representative immunoblots of U87MG treated with silvestrol (indicated concentrations) for indicated durations. (**E**) Ribosome density profiles (left panel) and mRNA translation efficiency (abundance ratio of polysome-associated mRNA to ribosome-free and monosome-associated mRNA) (right panel) of U87MG treated with silvestrol (6.25 nM) or vehicle (DMSO) for 8 hr. Data represent mean ± SEM (error bars) of five independent experiments. * denotes $p < 0.05$ compared to vehicle (DMSO) treatment. of U87MG treated with silvestrol (6.25 nM) or vehicle (DMSO) for 8 hr. (**F**) Relative cell viability based on ATP levels in U87MG treated with indicated concentrations of silvestrol or zotatifin for 24 hr (left panel) or 48 hr (right panel). Data represent mean ± SEM (error bars) of four independent cell populations. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively compared to vehicle-treated condition.

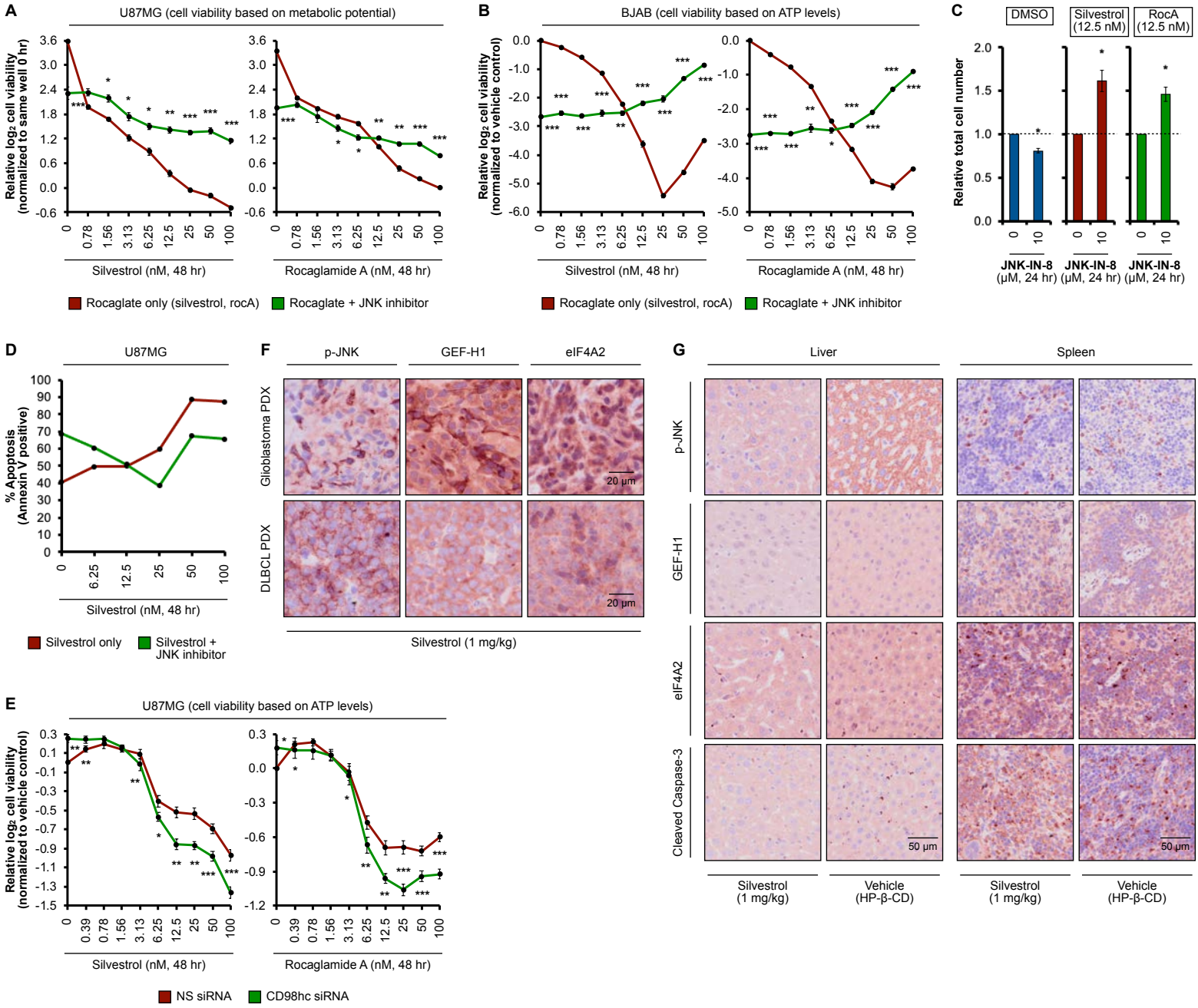

A
U87MG (cell viability based on metabolic potential)
Relative log2 cell viability (normalized to same well 0 hr)
Silvestrol (nM, 48 hr)
Rocaglamide A (nM, 48 hr)
Rocaglate only (silvestrol, rocA)
Rocaglate + JNK inhibitor
B
BJAB (cell viability based on ATP levels)
Relative log2 cell viability (normalized to vehicle control)
Silvestrol (nM, 48 hr)
Rocaglamide A (nM, 48 hr)
Rocaglate only (silvestrol, rocA)
Rocaglate + JNK inhibitor
C
DMSO
Silvestrol (12.5 nM)
RocA (12.5 nM)
Relative total cell number
JNK-IN-8 (μM, 24 hr)
D
U87MG
% Apoptosis (Annexin V positive)
Silvestrol (nM, 48 hr)
Silvestrol only
Silvestrol + JNK inhibitor
E
U87MG (cell viability based on ATP levels)
Relative log2 cell viability (normalized to vehicle control)
Silvestrol (nM, 48 hr)
Rocaglamide A (nM, 48 hr)
NS siRNA
CD98hc siRNA
F
p-JNK
GEF-H1
eIF4A2
Glioblastoma PDX
DLBCL PDX
20 μm
Silvestrol (1 mg/kg)
G
Liver
Spleen
p-JNK
GEF-H1
eIF4A2
Cleaved Caspase-3
50 μm
Silvestrol (1 mg/kg)
Vehicle (HP-β-CD)

Figure S4.

Figure S4. Rocaglate-inducible JNK phosphorylation mediates cellular toxicity signaling, related to Figure 3. (**A**) Relative cell viability based on cellular metabolic potential in U87MG treated with indicated concentrations of silvestrol (left panel) or rocaglamide A (right panel) with and without JNK inhibitor JNK-IN-8 (10 μM) for 48 hr. Data represent mean ± SEM (error bars) of four independent cell populations. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to corresponding rocaglate only condition. (**B**) Relative cell viability based on ATP levels in BJAB treated with indicated concentrations of silvestrol and rocaglamide A, with and without JNK inhibitor JNK-IN-8 (10 μM) for 48 hr. Data represent mean ± SEM (error bars) of four independent cell populations. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to corresponding rocaglate only condition. (**C**) Relative total cell number of U87MG treated with silvestrol (12.5 nM), rocaglamide A (rocA, 12.5 nM), or vehicle (DMSO), with and without JNK inhibitor JNK-IN-8 (10 μM) for 24 hr. Data represent mean ± SEM (error bars) of three independent experiments. * denotes statistical significance compared to vehicle (DMSO). (**D**) Percent apoptosis in U87MG treated with indicated concentrations of silvestrol or vehicle (DMSO) with and without JNK inhibitor JNK-IN-8 (10 μM) for 48 hr. Representative experiment shown. Three independent experiments were performed. (**E**) Relative cell viability of U87MG treated with indicated siRNA pools (50 nM final concentration) for 48 hr, followed by indicated concentrations of silvestrol (left panel) or rocaglamide A (right panel) for 48 hr. Data represent mean ± SEM (error bars) of four independent cell populations. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to corresponding NS siRNA control. Representative immunohistochemistry images of (**F**) glioblastoma and DLBCL PDX tumors (scale bar represents 20 μm) , (**G**) liver (left panel) and spleen (right panel) of mice treated with silvestrol (1 mg/kg) or vehicle (2-hydroxypropyl-β-cyclodextrin) (daily intraperitoneal injection for three days) (scale bar represents 50 μm).

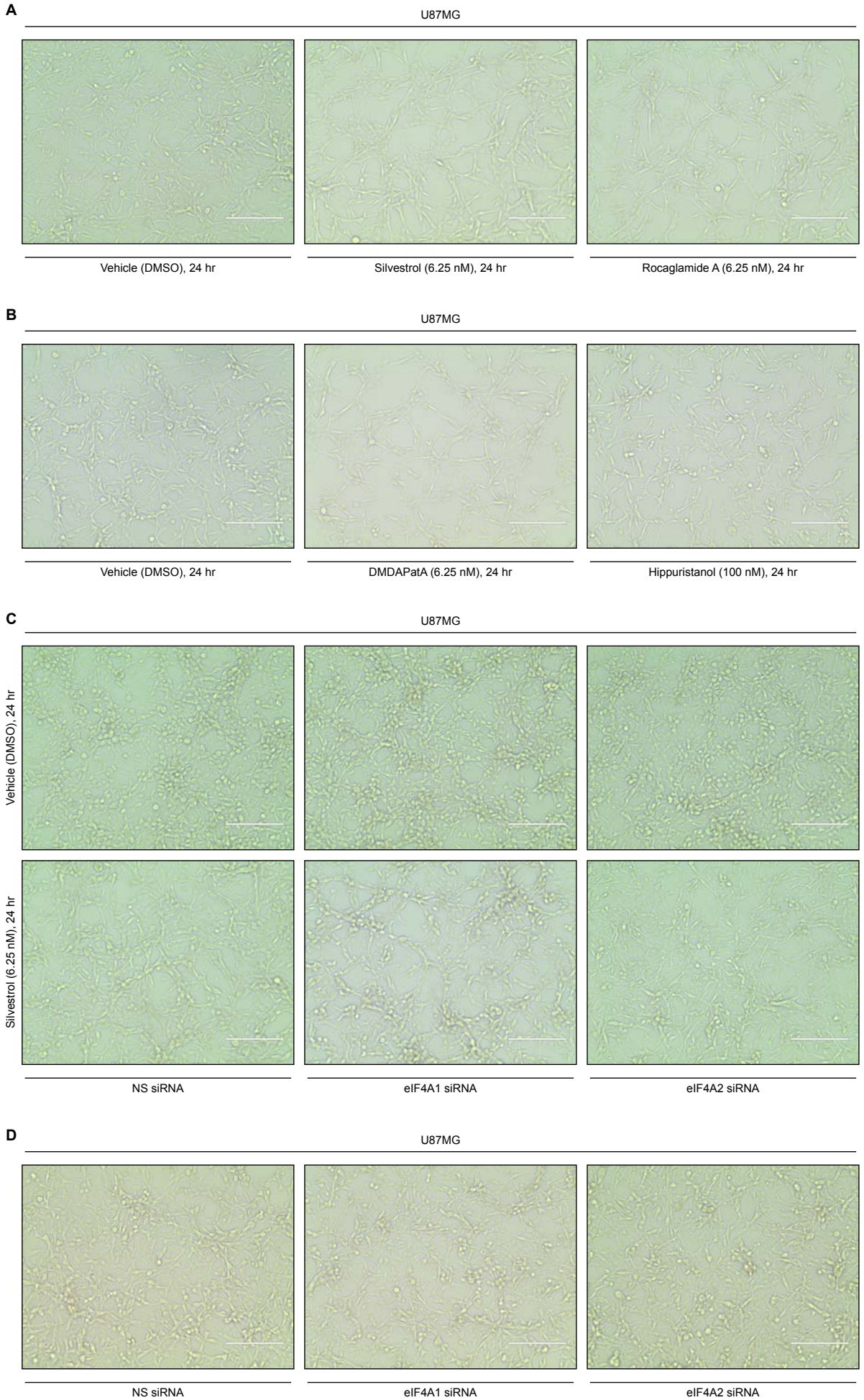

A
U87MG
Vehicle (DMSO), 24 hr
Silvestrol (6.25 nM), 24 hr
Rocaglamide A (6.25 nM), 24 hr
B
U87MG
Vehicle (DMSO), 24 hr
DMDAPatA (6.25 nM), 24 hr
Hippuristanol (100 nM), 24 hr
C
U87MG
Vehicle (DMSO), 24 hr
Silvestrol (6.25 nM), 24 hr
NS siRNA
eIF4A1 siRNA
eIF4A2 siRNA
D
U87MG
NS siRNA
eIF4A1 siRNA
eIF4A2 siRNA

Figure S5.

Figure S5. Mechanisms of rocaglate-dependent protein induction, related to Figure 4. (**A**) Representative bright field images of U87MG treated with silvestrol (6.25 nM), rocaglamide A (6.25 nM), or vehicle (DMSO) for 24 hr. (**B**) Representative bright field images of U87MG treated with DMDAPatA (6.25 nM), hippuristanol (100 nM), or vehicle (DMSO) for 24 hr. Scale bars in (a) to (d) represent 200 μm. (**C**) Representative bright field images of U87MG treated with indicated siRNA pools (50 nM final concentration) for 48 hr, followed by silvestrol treatment (6.25 nM) or vehicle (DMSO) for 24 hr. (**D**) Representative bright field images of U87MG treated with indicated siRNA pools (50 nM final concentration) for 48 hr. NS: non-silencing.

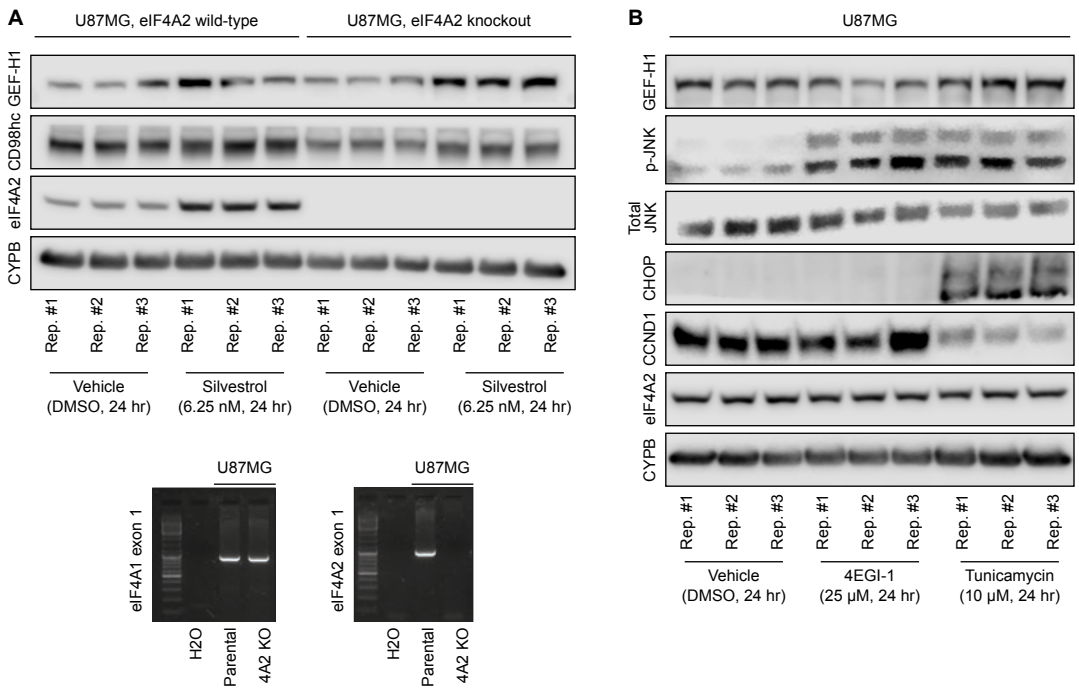

A
U87MG, eIF4A2 wild-type
U87MG, eIF4A2 knockout
GEF-H1
CD98hc
eIF4A2
CYPB
Rep. #1
Rep. #2
Rep. #3
Vehicle
(DMSO, 24 hr)
Silvestrol
(6.25 nM, 24 hr)
U87MG
eIF4A1 exon 1
eIF4A2 exon 1
H2O
Parental
4A2 KO
B
U87MG
GEF-H1
p-JNK
Total JNK
CHOP
CCND1
eIF4A2
CYPB
4EGI-1
(25 μM, 24 hr)
Tunicamycin
(10 μM, 24 hr)

Figure S6.

Figure S6. Mechanisms of rocaglate-dependent protein induction, related to Figure 4
(**A**) Representative immunoblots of U87MG eIF4A2 wild-type or eIF4A2 knockout cells treated with silvestrol (6.25 nM) or vehicle (DMSO) for 24 hr (top panel). Genomic DNA PCR analysis of exon 1 of eIF4A1 and eIF4A2 in U87MG parental and 4A2 KO cells (bottom panel). (**B**) Representative immunoblots of U87MG treated with 4EGI-1 (25 μM), tunicamycin (10 μM) or vehicle (DMSO) for 24 hr.

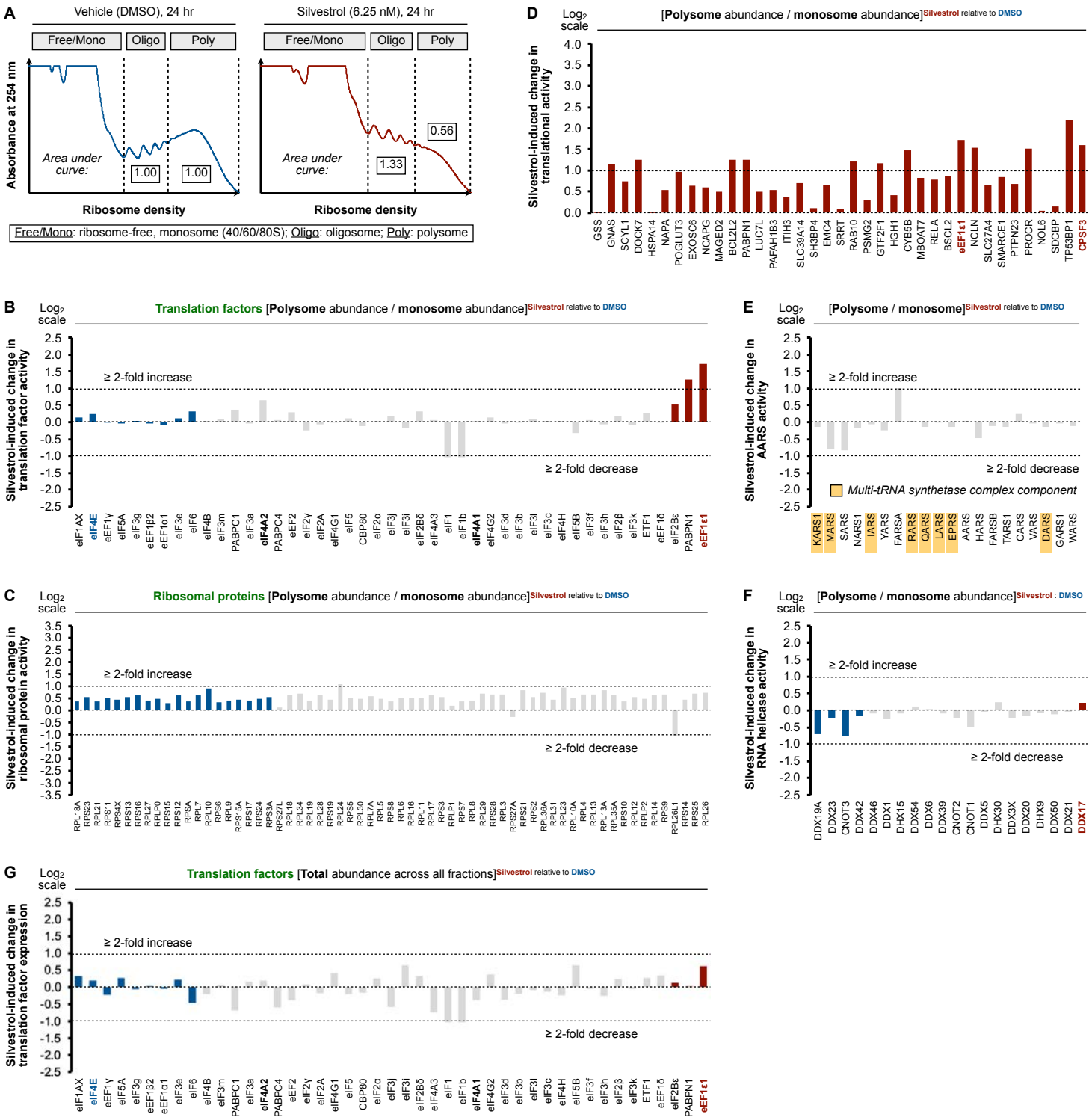

A
Vehicle (DMSO), 24 hr
Silvestrol (6.25 nM), 24 hr
Free/Mono
Oligo
Poly
Absorbance at 254 nm
Area under curve:
1.00
1.00
1.33
0.56
Ribosome density
Free/Mono: ribosome-free, monosome (40/60/80S); Oligo: oligosome; Poly: polysome
D
Log2 scale
[Polysome abundance / monosome abundance]Silvestrol relative to DMSO
Silvestrol-induced change in translational activity
GSS GNAS SCYL1 DOCK7 HSPA14 NAPA POGLUT3 EXOSC6 NCAPG MAGED2 BCL2L2 PABPN1 LUC7L PAFAH1B3 ITIH3 SLC39A14 SH3BP4 EMC4 SRRT RAB10 PSMG2 GTF2F1 HGH1 CYB5B MBOAT7 RELA BSCL2 eEF1ε1 NCLN SLC27A4 SMARCE1 PTPN23 PROCR NOL6 SDCBP TP53BP1 CPSF3
B
Translation factors [Polysome abundance / monosome abundance]Silvestrol relative to DMSO
Silvestrol-induced change in translation factor activity
≥ 2-fold increase
≥ 2-fold decrease
eIF1AX eIF4E eEF1γ eIF5A eIF3g eEF1β2 eEF1α1 eIF3e eIF6 eIF4B eIF3m PABPC1 eIF3a eIF4A2 PABPC4 eEF2 eIF2γ eIF2A eIF4G1 eIF5 CBP80 eIF2α eIF3j eIF3i eIF2Bδ eIF4A3 eIF1 eIF1b eIF4A1 eIF4G2 eIF3d eIF3b eIF3l eIF3c eIF4H eIF5B eIF3f eIF3h eIF2β eIF3k ETF1 eEF1δ eIF2Bε PABPN1 eEF1ε1
E
Log2 scale
[Polysome / monosome]Silvestrol relative to DMSO
Silvestrol-induced change in AARS activity
≥ 2-fold increase
≥ 2-fold decrease
Multi-tRNA synthetase complex component
KARS1 MARS SARS NARS1 IARS YARS FARSA RARS QARS LARS EPRS AARS HARS FARSB TARS1 CARS VARS DARS GARS1 WARS
C
Log2 scale
Ribosomal proteins [Polysome abundance / monosome abundance]Silvestrol relative to DMSO
Silvestrol-induced change in ribosomal protein activity
≥ 2-fold increase
≥ 2-fold decrease
RPL18A RPS23 RPL21 RPS11 RPS4X RPS13 RPS16 RPL27 RPLP0 RPS15 RPS12 RPSA RPL7 RPL10 RPS6 RPL9 RPS15A RPS17 RPS24 RPS3A RPS27L RPL18 RPL34 RPL19 RPL28 RPS19 RPL24 RPS5 RPL30 RPL7A RPL5 RPS8 RPL6 RPL16 RPL11 RPL17 RPS3 RPLP1 RPS7 RPL8 RPL29 RPS28 RPL13 RPS27A RPS21 RPS2 RPL36A RPL31 RPL23 RPL10A RPL4 RPL13 RPL13A RPL35A RPS10 RPL12 RPLP2 RPL14 RPS9 RPL26L1 RPS14 RPS25 RPL26
F
Log2 scale
[Polysome / monosome abundance]Silvestrol : DMSO
Silvestrol-induced change in RNA helicase activity
≥ 2-fold increase
≥ 2-fold decrease
DDX19A DDX23 CNOT3 DDX42 DDX46 DDX1 DHX15 DDX54 DDX6 DDX39 CNOT2 CNOT1 DDX5 DHX30 DDX3X DDX20 DHX9 DDX50 DDX21 DDX17
G
Log2 scale
Translation factors [Total abundance across all fractions]Silvestrol relative to DMSO
Silvestrol-induced change in translation factor expression
≥ 2-fold increase
≥ 2-fold decrease
eIF1AX eIF4E eEF1γ eIF5A eIF3g eEF1β2 eEF1α1 eIF3e eIF6 eIF4B eIF3m PABPC1 eIF3a eIF4A2 PABPC4 eEF2 eIF2γ eIF2A eIF4G1 eIF5 CBP80 eIF2α eIF3j eIF3i eIF2Bδ eIF4A3 eIF1 eIF1b eIF4A1 eIF4G2 eIF3d eIF3b eIF3l eIF3c eIF4H eIF5B eIF3f eIF3h eIF2β eIF3k ETF1 eEF1δ eIF2Bε PABPN1 eEF1ε1

Figure S7.

Figure S7. System-wide survey of rocaglate-dependent changes in the translation machinery, related to Figure 5. (**A**) Ribosome density profiles of U87MG treated with vehicle (DMSO) or silvestrol (6.25 nM) for 24 hr. Samples were loaded based on equal total RNA content. 2x more total RNA was loaded compared to Figure 4A. MATRIX analysis of silvestrol-induced changes in translational activity (i.e. ratio of protein abundance in polysome versus monosome (40/60/80S) fraction) for (**B**) canonical translation factors, (**C**) ribosomal proteins, (**D**) most highly induced proteins (≥ 3x activity enrichment), (**E**) aminoacyl-tRNA synthetases (AARSs), and (**F**) DEAD/DEAH RNA helicases. (**G**) Total sum of protein abundances across all MATRIX fractions in silvestrol- versus vehicle-treated samples. Proteins (x-axis) are arranged in the same order as panels B-F of Figure 5. Blue and red indicate silvestrol-repressed and silvestrol-activated translational assets (≥ 2x difference in activity), respectively, based on [polysome/free] protein abundance ratios as shown in Figure 5.

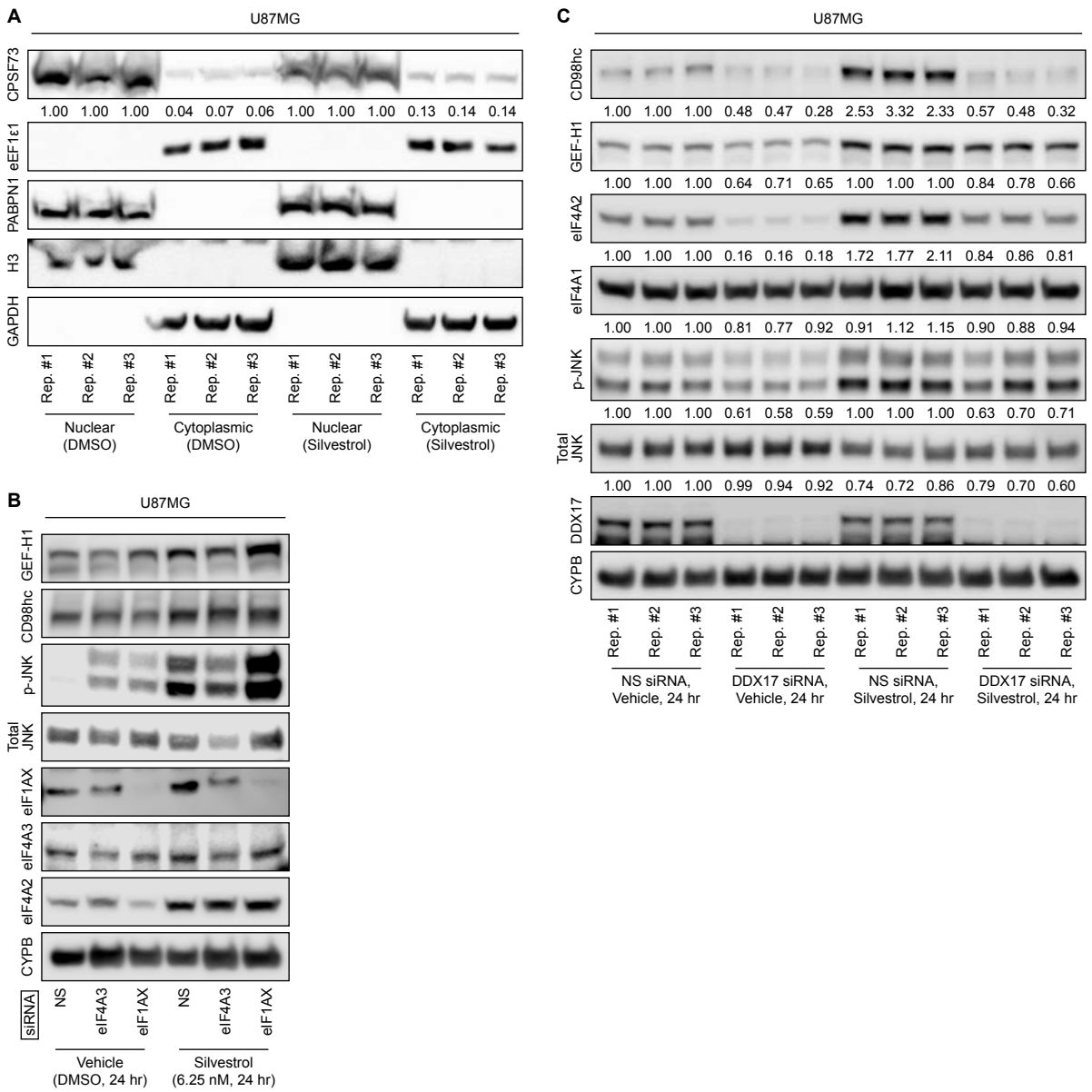

A
U87MG
CPSF73
1.00 1.00 1.00 0.04 0.07 0.06 1.00 1.00 1.00 0.13 0.14 0.14
eEF1ε1
PABPN1
H3
GAPDH
Rep. #1 Rep. #2 Rep. #3 Rep. #1 Rep. #2 Rep. #3 Rep. #1 Rep. #2 Rep. #3 Rep. #1 Rep. #2 Rep. #3
Nuclear (DMSO)
Cytoplasmic (DMSO)
Nuclear (Silvestrol)
Cytoplasmic (Silvestrol)
B
U87MG
GEF-H1
CD98hc
p-JNK
Total JNK
eIF1AX
eIF4A3
eIF4A2
CYPB
siRNA
NS eIF4A3 eIF1AX NS eIF4A3 eIF1AX
Vehicle (DMSO, 24 hr)
Silvestrol (6.25 nM, 24 hr)
C
U87MG
CD98hc
1.00 1.00 1.00 0.48 0.47 0.28 2.53 3.32 2.33 0.57 0.48 0.32
GEF-H1
1.00 1.00 1.00 0.64 0.71 0.65 1.00 1.00 1.00 0.84 0.78 0.66
eIF4A2
1.00 1.00 1.00 0.16 0.16 0.18 1.72 1.77 2.11 0.84 0.86 0.81
eIF4A1
1.00 1.00 1.00 0.81 0.77 0.92 0.91 1.12 1.15 0.90 0.88 0.94
p-JNK
1.00 1.00 1.00 0.61 0.58 0.59 1.00 1.00 1.00 0.63 0.70 0.71
Total JNK
1.00 1.00 1.00 0.99 0.94 0.92 0.74 0.72 0.86 0.79 0.70 0.60
DDX17
CYPB
Rep. #1 Rep. #2 Rep. #3 Rep. #1 Rep. #2 Rep. #3 Rep. #1 Rep. #2 Rep. #3 Rep. #1 Rep. #2 Rep. #3
NS siRNA, Vehicle, 24 hr
DDX17 siRNA, Vehicle, 24 hr
NS siRNA, Silvestrol, 24 hr
DDX17 siRNA, Silvestrol, 24 hr

Figure S8.

Figure S8. Increased eEF1ε1 activity enables rocaglate-driven protein synthesis, related to Figure 6. (**A**) Representative immunoblots of nuclear and cytoplasmic fractions from U87MG treated with silvestrol (6.25 nM) or vehicle (DMSO) for 24 hr. Histone H3 and GAPDH are shown as nuclear and cytoplasmic controls, respectively. (**B**) Representative immunoblots of U87MG treated with silvestrol (6.25 nM) or vehicle (DMSO) for 24 hr. (**C**) Representative immunoblots of U87MG treated with indicated siRNA pools for 48 hr, followed by treatment with silvestrol (6.25 nM) or vehicle (DMSO) for 24 hr. Densitometry values in are normalized to loading control.