Molecular Cell, Volume *31*

Supplemental Data

Codependent Functions of RSK2

and the Apoptosis-Promoting Factor, TIA-1,

in Stress Granule Assembly and Cell Survival

T.S. Karin Eisinger-Mathason, Josefa Andrade, Angela L. Groehler, David E. Clark, Tara L. Muratore-Schroeder, Lejla Pasic, Jeffrey A. Smith, Jeffrey Shabanowitz, Donald F. Hunt, Ian G. Macara, and Deborah A. Lannigan

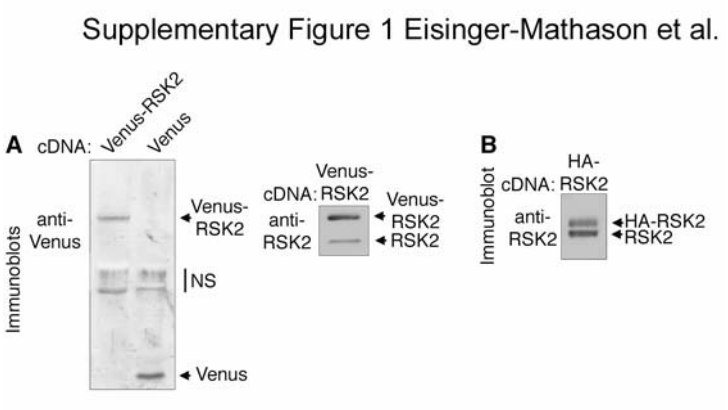

Figure S1. Characterization of MCF-7 clones. (A) Stably expressing Venus-RSK2 or Venus MCF-7 clones. (B) Stably expressing HA-RSK2 MCF-7 clone.

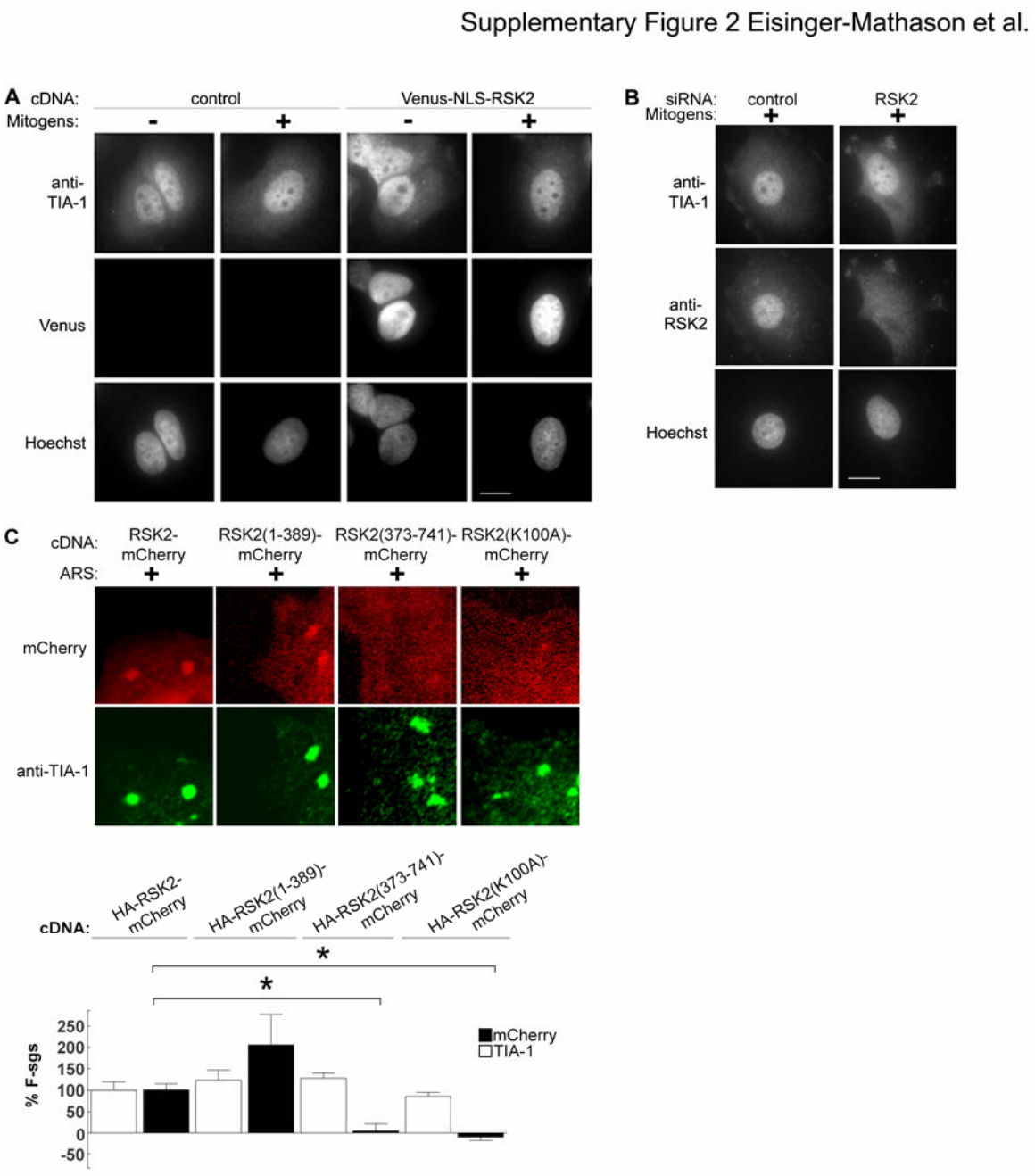

Figure S2. TIA-1 accumulates in the nucleus independently of RSK2. (A) MCF-7 cells transfected with Venus-NLS-RSK2 or control vector. (B) MCF-7 cells transfected with RSK2-specific or control siRNA. (C) MCF-7 cells transfected with various C-terminal mcherry-tagged RSK2 constructs. Individual image panels represent 10 μm. Columns, mean (n=10); SEM, $p < 0.001$.

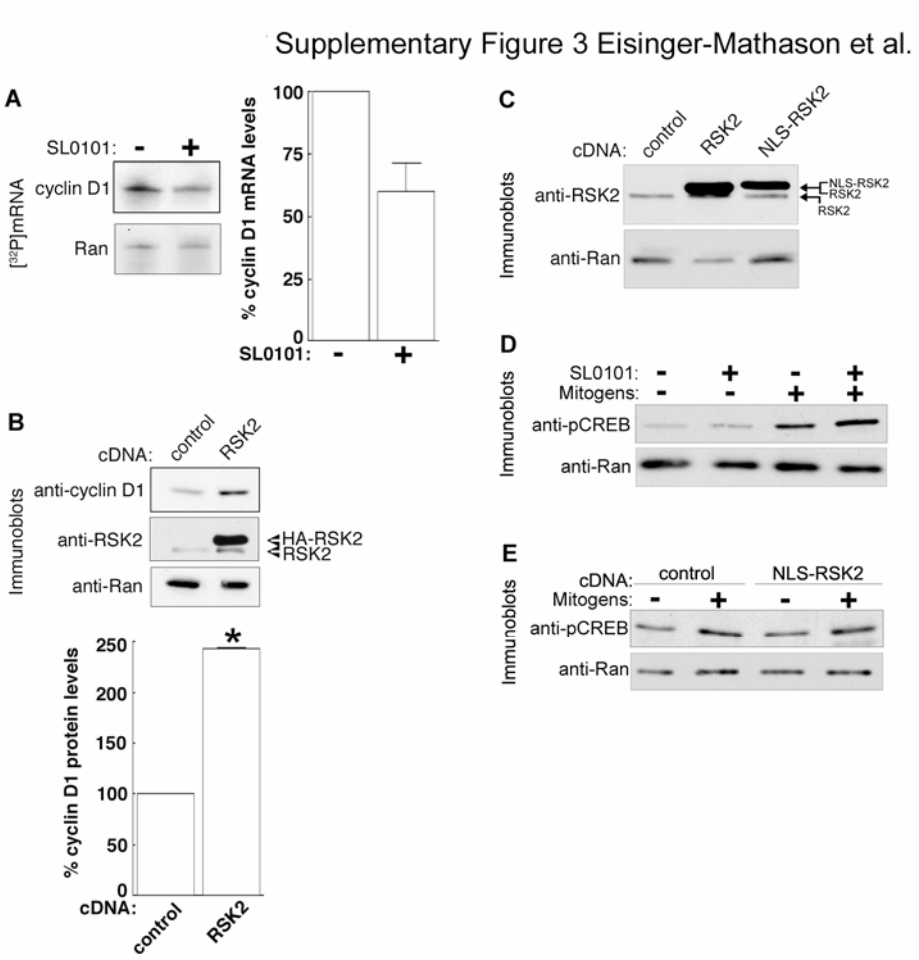

Figure S3. RSK2 controls cyclin D1 levels independently of CREB phosphorylation. (A) Relative levels of cyclin D1 mRNA determined by RNase protection assay from MCF-7 cells pre-treated with vehicle or SL0101 (4 h). Columns, mean (n=3); bars, SEM, $p < 0.05$. (B) MCF-7 cells transfected with control or vector encoding RSK2. Columns, mean (n=3); bars, SD, $p < 0.005$. (C) Serum-starved MCF-7 cells transfected with vector control or HA-RSK2 or NLS-HA-RSK2. (D) MCF-7 cells treated with or without SL0101 (4 h) and mitogen. (E) MCF-7 cells transfected with NLS-RSK2 or vector control.

Supplementary Table 1 Eisinger-Mathason et al.

Protein Class	SG Protein	Reference
Ribosomes	Ribosomal Protein S3	Kedersha et al., 2002
	Ribosomal Protein S3a	Kedersha et al., 2002
	Ribosomal Protein S19	Kedersha et al., 2002
Translation	eIF3	Kedersha et al., 2002
	PABP	Kedersha et al., 1999
	TIA-1/R	Kedersha et al., 1999

Table S1. Stress granule proteins identified by mass spectrometry that associate with RSK2 in serum starved MCF-7 cells.

Supplemental Experimental Procedures

Cell culture and treatment

MCF-7, T47D, MCF-10A, and MDA-MB231 cells were cultured as directed by American Type Culture Collection (ATCC) (Manassas, VA). Venus-HA-RSK2 and HA-RSK2 MCF-7 stables were cultured under G418 selection. Primary human mammary epithelial cells (HME) were cultured in DMEM/F12 (Invitrogen, Carlsbad, CA) containing 1X insulin-transferrin-selenium (Invitrogen, Carlsbad, CA), 100 μM ethanolamine and 1 μg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO), 10 ng/mL epidermal growth factor (EGF) (EMD Biosciences, San Diego, CA), and 5 ng/mL fibroblast growth factor 2 (R&D Systems, Inc., Minneapolis, MN). HME cells were grown in 3D culture using growth factor reduced matrigel (BD Biosciences, San Jose, CA) at 37^{o}C and 5% CO_2. Mitogenic stimulation consisted of base media containing 3 μg/mL insulin, 150 ng/mL epidermal growth factor and 5% FBS. For survival assays cells were harvested as previously described (Smith et al., 2005). In experiments with SL0101 (100 μM) cells were pre-treated for 4 h and with sodium arsenite (Sigma-Aldrich, St.Louis, MO) (500 μM; 1h).

RNase protection assay (RPA)

Cells were treated with 100 μM SL0101 or DMSO for 4 h prior to RNA extraction. The RPA was performed using RPAIII (Ambion, Austin, TX). Briefly, PCR was used to generate a 208bp riboprobe from a portion of the full-length CCND1 cDNA, corresponding to nucleotides 210-417, and a flanking T7 promoter was added. Results were normalized in parallel to the housekeeping gene, Ran. A 308bp Ran riboprobe corresponding to nucleotides 118-449, was generated. Antisense RNA was transcribed in the presence of [$\tilde{\alpha}^{32}$P] UTP using a MAXIscript kit (Ambion, Austin, TX). Total RNA (5 μg) was hybridized with excess riboprobe corresponding to 8 x 10^4 cpm. Precipitated RNA was electrophoresed on a 5% polyacrylamide, 8 M urea denaturing gel. The signals were quantitated using the PhosphorImager 445 SI system (Molecular Dynamics, Piscataway, NJ).

Immunoprecipitation and mass spectrometry

Cells were lysed in lysis buffer (100 mM HEPES (pH 7.9), 15 mM $MgCl_2$, 100 mM KCl, 100 μM 1,4-dithiothreitol (DTT) (Roche Diagnostics, Indianaoplis, IN), and protease inhibitor cocktail (PIC) (Sigma-Aldrich, St.Louis MO)) and fractionated by centrifugation. The nuclei were isolated from the pellet with extraction buffer (20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 100 μM DTT, 25% glycerol and PIC). Samples were incubated with monoclonal anti-HA (12CA5) antibody (1 μg antibody/10 mg of protein) (Lymphocyte Culture Center, University of Virginia, Charlottesville, VA) overnight in a Thermomixer at 900 rpm and 4°C. Washed Dynabeads (80 μL) coated with sheep anti-mouse secondary antibody (Invitrogen, Carlsbad, CA) were incubated with lysate for 2 hours in a Thermomixer at 900 rpm and 4°C. Immunoprecipitate was washed 2x in hypotonic wash buffer (1 mM EDTA (pH 8.0), 10 mM HEPES pH7.4, 10mM KCl, 1 μM microcystin, and PIC), 5x in high salt buffer (1 mM EDTA (pH 8.0), 10 mM HEPES pH7.4, 10 mM KCl, 300 mM NaCl, 1 μM microcystin, and PIC), 3x in low salt buffer (1 mM EDTA pH 8.0, 10 mM HEPES pH7.4, 10 mM KCl), and 3x in NH_4HCO_3 buffer (100 mM NH_4HCO_3, 30 mM NaCl). 5% of the sample was boiled in SDS-PAGE loading buffer for 5 min, removed from the beads, electrophoresed, and immunoblotted with monoclonal anti-RSK2 (C-21**).**

Beads bound with HA-RSK2 were exchanged to 100 mM ammonium bicarbonate (pH 7.8) (Sigma-Aldrich, St. Louis, MO) and were reduced with 1 mM DTT at 25°C for 1 hr and alkylated with 2mM iodoacetamide in the dark at 25°C for 45 min, followed by digestion with trypsin (Promega Corp., Madison, WI) at an enzyme/substrate ratio of 1:20 (wt:wt) for 6 hrs at 25°C (under agitation). Beads were compacted using a magnet and the supernatant containing the peptides was transferred into a new tube and acidified with glacial acetic acid to pH 3.5. Peptide mixture was stored at -35°C until analysis.

Aliquots of peptide mixtures were pressure loaded onto a polyimide coated, fused silica microcapillary pre-column (360 μm OD x 75 μm ID, Polymicro Technologies, Phoenix, AZ) packed with 6 cm of irregular (5-20 μm) C18 packing material (YMC, Kyoto, Japan) and equipped with a LiChrosorb® Si 60 frit (EM Science). The pre-column was then connected with polytetrafluoroethylene tubing (0.06 in OD x 0.02 in ID, Zeus Industrial Products, Orangeburg, SC) to an analytical column (360 μm OD x 50 μm ID) packed with 4 cm of regular (5 μm) C18 packing material with an integrated, laser-pulled emitter tip (~3

μm) (Martin et al., 2000). Peptide mixtures were gradient eluted into the mass spectrometer via an Agilent 1100 series binary LC pump (Palo Alto, CA). A 90 min gradient, 0-60% B in 60 min, 60-100% B in 5 min, 100-0% B in 5 min and a 20 min rinse with 0% B, was used for each sample analysis. Solvent A = 100 mM acetic acid (Sigma-Aldrich, St. Louis, MO) in water, Solvent B = 100 mM acetic acid in 70% aqueous acetonitrile (Mallinckrodt, Paris, KY). All mass spectra were recorded on a linear quadrupole ion trap Fourier-transform ion cyclotron resonance mass spectrometer (Finnigan LTQ-FTMS, Thermo Electron Corp., San Jose, CA) equipped with a modified microelectrospray ionization source. The instrument method was employed in a data-dependent manner to select abundant precursor ions for fragmentation. DTA files were generated for all raw data files using Bioworks 3.2 and were searched against a human database using SEQUEST™ (Eng et al., 1994). Search parameters were as follows: precursor mass +/- 0.01 Da; differential modifications +80 atomic mass unit (amu) on Ser, Thr and Tyr residues, +16 amu on Met and a static modification of +57 amu on Cys; and trypsin digest (cleavage of Arg and Lys) allowing for two missed cleavages. All spectra of interest were validated manually.

Plasmids

The SV40 nuclear localization signal (NLS) (PKKKRKV) was subcloned in-frame at the 5' end of RSK2 to generate the vector pKH3-NLS-RSK2. pKH3-RSK2(373-741) and pKH3-RSK2(1-389) were generated by amplifying, using PCR, either the C-terminus or the N-terminus of RSK2 and inserting in-frame at the 3' end of the HA tag of the pKH3 vector. pCMV-HA-RSK2 was generated by subcloning HA-RSK2 into pCMV (Stratagene, La Jolla, CA). pCMV-Venus-HA-RSK2 was generated by subcloning Venus at the 5' end of HA-RSK2 in pCMV. pKR7-Venus-NLS-RSK2 was generated by subcloning NLS-RSK2 into pKR7-Venus. The fluorescent tag mCherry was subcloned in-frame at the 3' end of RSK2 in which the stop codon had been removed by the polymerase chain reaction (PCR) to generate the RSK2-mCherry fusion protein. The mCherry protein is a derivative of RFP1.4 containing the following mutations: M163Q, N6aD, R17H, K194N, T195V, D196N. RSK2-mCherry was subcloned into pKR7. pKR7-RFP-TIA-1 was generated by subcloning TIA-1 in-frame into pKR7-RFP. GST-TIA-1 was generated by subcloning TIA-1 into the 3' end of GST in the pGEX2T vector (GE Healthcare, Piscataway, NJ). pKR7-RSK2 (373-741)-mCherry and pKR7-RSK2 (1-389)-mCherry were generated by amplifying, using PCR, either the C-terminus or the N-

terminus of RSK2 and inserting in-frame at the 5' end of the mCherry tag of pKR7-mCherry. RSK2(K100A) was also inserted-in-frame at the 5' end of the mCherry tag of pKR7-mCherry to generated pkR7-RSK2(K100A)-mCherry. HA-cyclin D1 was produced by amplification of the open reading frame and inserted in-frame with the HA-tag of the eukaryotic expression vector, pKH3. Constructs used in lentiviral production including psPAX2, pLVTHM, pMD2G were generously provided by Didier Trono M.D. (Swiss Federal Institute of Technology, Lausanne, Switzerland) (Zufferey et al., 1997). The short hairpin sequence, including the RSK2 targeting shRNA (**in bold**), is 5'-GATCCCC**GGAACGTGATATCTTGGTA**TTCAAGAGA**TACCAAGATATCACGTTCC**TTTTTGGAAA. All sequences were verified by the Biomolecular Research Core (University of Virginia, Charlottesville, VA).

Supplemental References

Eng, J., McCormack, A.L., and Yates, J.R.I. (1994). J. Am. Soc. Mass Spectrom *5*, 976-989.

Kedersha, N., Chen, S., Gilks, N., Li, W., Miller, I.J., Stahl, J., and Anderson, P. (2002). Evidence that ternary complex (eIF2-GTP-tRNA(i)(Met))-deficient preinitiation complexes are core constituents of mammalian stress granules. Mol Biol Cell *13*, 195-210.

Kedersha, N.L., Gupta, M., Li, W., Miller, I., and Anderson, P.(1999). RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules. J Cell Biol *147*, 1431-1442.

Martin, S.E., Shabanowitz, J., Hunt, D.F., and Marto, J.A. (2000). Subfemtomole MS and MS/MS peptide sequence analysis using nano-HPLC micro-ESI fourier transform ion cyclotron resonance mass spectrometry. Anal Chem *72*, 4266-4274.

Smith, J.A., Poteet-Smith, C.E., Xu, Y., Errington, T.M., Hecht, S.M., and Lannigan, D.A. (2005). Identification of the first specific inhibitor of p90 Ribosomal S6 Kinase (RSK) reveals an unexpected role for RSK in cancer cell proliferation. Cancer Res *65*, 1027-1034.