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2 **Active role of free RNA during the early phase of proteostasis stress**

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20 **ABSTRACT**

21 Transient sequestration of proteins and RNA is an essential principle of cellular reaction to stress.
22 Compared to polypeptides, less is known about the role of RNA released from polysomes during
23 acute proteostasis stress. Using quantitative mass spectrometry, we identified a set of proteins
24 assembled by free RNA in the heat-shocked mammalian cytosol. RNA-associated proteins displayed
25 higher disorder and larger size, which supports the role of multivalent interactions during the initial
26 phase of the RNA granule formation. Structural features of the free RNA interactors defined them as
27 a subset of RNA-binding proteins. The interactome contained preferentially the active form of eIF2 α .
28 The interaction between assembled proteins *in vivo* required RNA. The reconstitution of the
29 association process *in vitro* indicated to the multimolecular basis for the increased binding to RNA
30 upon heat shock in the cytosol. Our results reveal how free RNA can participate in reorganization of
31 cellular functions during proteostasis stress.

32

33 INTRODUCTION

34 Protein unfolding and aggregation during proteostasis stress can lead to the irreparable damage of the
35 cellular proteome and cell death. One of the strategies eukaryotic cells use to survive under stress is
36 the sequestration of proteins and RNA in transient membrane-less assemblies. Cytosolic stress
37 granules (SGs) is the best known example of those structures. Low complexity (LC) of the amino
38 acid sequence is characteristic of a fraction of proteins assembling into membrane-less structures ¹.
39 Another feature of the sequestering proteins is the multivalency of their interactions ². Compared to
40 proteins, less molecular details are known regarding the role of RNA during stress granulation. A
41 recent study uncovered the RNA-seeded formation of nuclear amyloid bodies upon heat shock and
42 during acidosis ³. It was reported that the secondary structure of mRNA affects the composition of
43 membrane-less granules ⁴. Importantly, RNA binding can modulate phase separation and toxicity of
44 solid-like assemblies ⁵.

45 The polypeptide-coding mRNAs are coated by specialized proteins at any stage of their lifecycle.
46 More than 1000 proteins can associate with mRNAs to participate in processing, transport, translation
47 and turnover of transcripts ⁶. Since many mRNA-binding proteins (RBPs) have LC domains ⁷, mRNA
48 can effectively increase the local concentration of RBPs, thus facilitating the associated phase
49 transition. Accordingly, a significant fraction of RBPs are found in mRNA-containing granules
50 formed *in vivo* and *in vitro* ^{8,9}. Although mRNAs lose the associated proteins during ribosomal
51 translation, a dense packing and particular arrangement of ribosomes seem to reduce the exposure of
52 stripped RNA to the cytosolic content ¹⁰. One of few circumstances when free mRNA appears in the
53 cytosol is the disassembly of polysomes during stress-induced shutdown of protein synthesis. It has
54 been proposed that the massive increase of free mRNA creates a relative insufficiency of RNA-
55 stabilizing proteins such as YB-1 ¹¹. Under these conditions, mRNAs would be able to associate with
56 granulation-prone proteins, an initial step of SG formation. Here, we aimed at a molecular

57 understanding of this early phase of the stress response. Quantitative mass spectrometry was used to
58 identify proteins in heat shocked HeLa lysates which interact with free RNA baits. Usually, UV
59 crosslinking is applied to stabilize direct protein-RNA interactions. We decided to avoid crosslinking
60 and use softer washing procedures in order to reveal the complexity of the assemblies. Secondly, we
61 used two defined RNA sequences, not the mix of all possible polyA-containing RNAs as it is done
62 during standard interactomics studies. An impressive interactivity potential of a given RNA sequence
63 was revealed. On the other hand, the significant overlap of the two interactomes argues for the
64 common molecular mechanism of free RNA processing during stress.

65

66 **EXPERIMENTAL SECTION**

67

68 **Reagents, Plasmids, Antibodies**

69 All chemicals were from Sigma-Aldrich (Saint Louis, MO) unless otherwise indicated.

70 Flag-tagged TRMT6 and TRMT61A mammalian expression vectors were purchased from Genscript
71 (Piscataway, NJ). Two additional Flag tags were inserted into the vector coding TRMT61A. Human
72 HSP70 under T7 promoter was cloned into pCH16 vector (a kind gift of H.-C. Chang) for *in vitro*
73 transcription. Human B-Raf under T7 promoter was a gift from Dustin Maly (Addgene #40775) and
74 was further modified by the insertion of two additional FLAG tags and of an RBS sequence.

75 The following antibodies were used: anti-TRMT6 (A303-008A-M) from Bethyl (Montgomery, TX);
76 anti-TRMT61A (sc-107105) and anti-S6 (sc-74459) from Santa Cruz Biotechnology (Dallas, TX);
77 anti-eIF2 α (9722), anti-eIF2 α (phosphor-Ser51) (3398), anti-eIF4E (9742), HRP-conjugated anti-
78 rabbit-IgG (7074) and Alexa Fluor 647-conjugated anti-rabbit IgG (4414) from Cell Signaling
79 (Danvers, MA); anti-Flag (F1804) and HRP-conjugated anti-mouse IgG (A9044) from Sigma-
80 Aldrich.

81

82 **Cell culture**

83 The human HeLa cell line was cultured in DMEM supplemented with 10% (vol/vol) FBS, 2 mM L-
84 glutamine, 100 IU/mL penicillin G, 100 μ g/mL streptomycin sulfate, and nonessential amino acids.

85

86 **RNA Electroporation and Imaging**

87 4×10^6 HeLa cells were transfected with 4 μ g Cy5-RNA or the molar equivalent of Cy5-55mer oligo
88 by electroporation in 400 μ L intracellular buffer (135 mM KCl, 0,2 mM CaCl₂, 2 mM MgCl₂, 5 mM
89 EGTA, 10 mM Hepes, pH 7.5) freshly supplemented with 2 mM ATP. Electroporation was performed
90 at 310 V and 950 μ F using a Gene Pulser Xcell system from Bio-Rad Laboratories (Hercules, CA).

91 After electroporation, the cells were washed and plated in a 12-well plate with polylysine-coated
92 cover slides at 1 million cells/well. They were left for recovery at 37°C for 6 h. Then cells were
93 washed in PBS and fixed with 4% paraformaldehyde/PBS at RT for 10 min. One slide was then
94 treated with 250 units Benzonase in PBS supplemented with 2 mM MgCl₂ at 37°C for 20 min. All
95 the slides were washed with PBS, stained with DAPI, mounted in PBS and imaged using a Zeiss
96 LSM-780 inverted confocal microscope with a 63x oil immersion objective. The quantitative
97 comparison was performed using CellProfiler¹² as follows. Nuclei were detected and cells were
98 identified by propagation from the nuclei. Intensity of Cy5 fluorescence was measured for each cell
99 and Cy5-positive cells were identified. The experiment was performed in triplicates and at least 300
100 cells were analyzed per condition and per repetition to determine the percentage of Cy5-positive cells.

101

102 **Analysis of Protein Translation Activity**

103 DMEM medium without methionine and cysteine was supplemented with 25 mM HEPES NaOH pH
104 7.5, 100 µg/mL streptomycin sulphate, 2 mM L-glutamine, 0.2 mM cystine and 0.02 mM methionine
105 and used to prepare a suspension of HeLa cells at 1x10⁷ cells/mL. Cells were pre-incubated for
106 indicated time at 37°C or 45°C before 50 µCi/mL of [35S]-methionine (Hartmann Analytics,
107 Germany) was added for 3 min labelling of nascent proteins. Samples were mixed immediately with
108 hot SDS sample buffer, nucleic acids were sheared using a 26 G needle with at least ten strokes and
109 run in 10% SDS-PAGE. Afterwards, the gel was dried at 70°C for 1 h and exposed on a
110 phosphorimager plate overnight. Typhoon 9400 imaging system (GE Healthcare) was used to
111 visualize and quantify the radioactive signal.

112

113 **Pulldowns, Immunoprecipitation, Western Blotting**

114 6x10⁷ HeLa cells were resuspended in 2 mL medium and heat-shocked at 45°C for 1 h. After two
115 PBS washes, the cells were resuspended in 1,5 volume of Hypotonic lysis buffer (HLB: 10 mM Hepes

116 KOH pH 7.6, 10 mM K acetate, 1.5 mM Mg acetate, 2 mM DTT) and kept on ice for 10 min. The
117 lysate was passed through a 20 G needle 20 times and centrifuged at 640 g for 5 min at 4°C. The
118 supernatant was collected, supplemented with 1/10 volume of 1 M potassium acetate and centrifuged
119 at 10,400 g for 20 min at 4°C. Protein concentration was normalized to 5 µg/µL. For each sample, 20
120 µL of Dynabeads M-280 Streptavidin (Thermo Fisher Scientific, Waltham, MA) were washed once
121 with BW (5 mM Tris HCl pH 7.5, 0.5 mM EDTA, 1 M NaCl), twice with Solution A (0.1 M NaOH,
122 0.05 M NaCl), once with Solution B (0.1 M NaCl) and once with BW. The beads were loaded with
123 2 µg biotinylated HSP70 mRNA (or DEPC-treated H₂O) in BW in a volume of 40 µL for 30 min at
124 RT, washed three times with BW and once with HLB. 100 µL of lysate was added to the beads and
125 incubated at 4°C for 2 h with gentle shaking. The beads were washed six times with 50 µL Washing
126 Buffer (WB: 25 mM Tris HCl pH 7.5, 10 mM MgCl₂, 2 mM DTT) and three times with 50 mM Tris
127 HCl pH 7.4, 150 mM NaCl. The beads were frozen at -80°C until further processing for mass
128 spectrometry. For biochemical verification, 100 µL of lysate were added to the beads and incubated
129 at 4°C for 3 h with gentle shaking, then washed six times with 50 µL WB. Elution was performed
130 with 250 units of Benzonase in 10 µL WB at 37°C for 15 min.

131 For co-immunoprecipitation, 2x10⁶ HeLa cells were transfected with 5 µg 3xFlag-TRMT61A and 5
132 µg Flag-TRMT6 or 10 µg pcDNA3.1-Hygro. The cells were collected 24 h later, resuspended in
133 HEPES/DMEM and incubated at 37°C or 45°C for 1 h. Lysates without or with Benzonase treatment
134 were prepared as described above, centrifuged at 10,400 g for 20 min at 4°C. Protein concentration
135 in supernatants was normalized to 1,5 µg/µL. 10 µL anti-Flag M2 affinity gel were blocked with 1%
136 BSA in HLB for 1 h on ice, incubated with 200 µL lysate at 4°C for 3 h, washed four times with WB
137 and eluted with 50 µL 3xFLAG peptide solution at 100 µg/mL in TBS for 5 min at RT. Reducing
138 SDS sample buffer was added to the eluates, samples were resolved using 10% SDS-PAGE and
139 transferred onto nitrocellulose membranes. Membranes were blocked with 5% skim milk or 5% BSA
140 in TBST (TBS/0.1% Tween 20), probed with the indicated antibodies and developed using the

141 SuperSignal West Pico PLUS. Chemiluminescence images were acquired with the Chemidoc MP
142 imaging system and bands quantified using the Image Lab 5.0 software.

143
144 **PolyT Pulldowns**
145 5×10^6 HeLa cells were resuspended in 1 mL DMEM supplemented with 25 mM Hepes NaOH pH
146 7.5 and incubated at 45°C for 60 min, while control cells were kept at 37°C. The cells were washed
147 and lysed in 300 μ L lysis buffer (LB: 10 mM Tris HCl pH 7.4, 100 mM KCl, 5 mM MgCl₂, 0.5 %
148 sodium deoxycholate, 1% Triton X-100) supplemented with 2 mM DTT, Phosphatase Inhibitor
149 Cocktail 2 and RNAsin for 5 min at RT. Lysates were passed 5 times through a 20 G needle, protein
150 concentration was measured and normalized. 400 μ L of lysate at the protein concentration of 1,5
151 μ g/ μ L were added to 100 μ L of washed Oligo d(T)25 Magnetic Beads (NEB, Ipswich, MA) and
152 incubated at RT for 30 min. 2/3 of the beads were washed 3x with LB and resuspended in 10 μ L LB
153 supplemented with 250 units of Benzonase nuclease for protein elution. After 15 min at 37°C, the
154 supernatant was collected and analyzed by SDS-PAGE and western blotting using anti-TRMT6
155 antibody. The rest of the beads were processed for RNA elution. For this, the beads were washed 2x
156 with WB-I (20 mM Tris HCl pH7.5, 500 mM LiCl, 0.1% LiDS, 1 mM EDTA, 5 mM DTT), 2x with
157 WB-II (20 mM Tris-HCl pH 7.5, 500 mM LiCl, 1 mM EDTA) and 1x with Low Salt Buffer (20 mM
158 Tris HCl pH 7.5, 200 mM LiCl, 1 mM EDTA). The beads were eluted with 30 μ L of 20 mM Tris
159 HCl pH 7.5, 1 mM EDTA at 50°C for 2 min. RNA amounts were determined with RiboGreen and
160 used for normalization.

161
162 **Fluorescence Microscopy**
163 HeLa cells were seeded in a 12-well plate at 10^5 cells/well on a polylysine-coated cover slides. Next
164 day, medium was refreshed and supplemented with 25 mM Hepes. Cells were heat-shocked at 45°C
165 for 1 h or treated with 1 mM arsenite for 30 min at 37°C. Cells were washed with PBS, fixed with

166 3.7% paraformaldehyde, permeabilized with acetone at -20°C for 5 min, blocked with 1% BSA/PBS
167 for 1 h at RT and incubated with anti-eIF4E for 1 h. Incubation with anti-Rabbit-IgG Alexa Fluor647
168 conjugate for 1 h, three washes with PBS and staining with DAPI followed. Slides were imaged using
169 a Zeiss LSM-780 inverted confocal microscope with a 63x oil immersion objective. Quantification
170 was performed using CellProfiler. First, nuclei were detected, then cells were identified by
171 propagation from the nuclei. Stress granules were identified and counted. The experiment was
172 performed in triplicates and at least 100 cells were analyzed per condition and per repetition.

173

174 **Polysome and 80S Monosome Analyses**

175 HeLa cells were seeded at 5×10^6 per 10 cm-dish. Next day, DMEM was refreshed and supplemented
176 with 25 mM Hepes. The dishes were incubated at 45°C for 1 h or treated with 1 mM arsenite for 30
177 min at 37°C. The cells were washed twice with ice-cold PBS supplemented with 100 µg/mL
178 cycloheximide and resuspended in 600 µL lysis buffer (10 mM Tris HCl pH 7.4, 100 mM KCl, 5 mM
179 MgCl₂, 0,5% sodium deoxycholate, 1% Triton X-100) supplemented with Phosphatase inhibitor
180 cocktail 2 (1:100), RNasin (1:1000) and 100 µg/mL cycloheximide. After a 15 min incubation on ice,
181 the lysates were centrifuged at 10.000 g for 5 min at 4°C. 550 µL of supernatant was loaded on a cold
182 10-50% Sucrose gradient in an Open-top Polyclar 16,8 mL tube (Seton, Egelsbach, Germany) and
183 centrifuged in an Optima XPN-80 ultracentrifuge from Beckman Coulter (Brea, CA) using a SW28.1
184 rotor, at 100.000 g for 4 h at 4°C. Gradient absorbance at 254 nm was measured on a Gradient station
185 (Biocomp) at a speed of 0.2 mm/second.

186 For monosome analysis, the 80S fraction was collected. Samples were normalized according to the
187 absorbance at 260 nm and analyzed by western blotting using anti-S6 antibody.

188

189 **RNA Synthesis and Biotinylation *In Vitro***

190 For *in vitro* transcription, linearized plasmids (HSP70 using Xho I, BRaf using Xba I) were purified
191 by phenol/chloroform/isoamylalcohol extraction and ethanol precipitation. Ampliscribe T7 high
192 Yield kit (Epicenter, Madison, WI) was used to transcribe 1 µg of template for 4 h at 37°C. The
193 reaction mix was digested with DNaseI and RNA was purified. If the RNA had to be further
194 biotinylated, it was purified by phenol/chloroform/isoamylalcohol followed by ethanol precipitation
195 and resuspended in a small volume of DEPC-treated water. Otherwise, RNA was purified using
196 MegaClear transcription clean-up kit (Thermo Fisher Scientific). Quality of the mRNA was assessed
197 on a formaldehyde agarose gel. 50 pmol of *in vitro* transcribed RNA was biotinylated using RNA 3'
198 End biotinylation Kit (Thermo Fisher Scientific) overnight at 16°C. Biotinylated RNA was purified
199 using MegaClear kit.

200

201 **5'-labelling of RNA**

202 For Cy5-labeling of RNA, GMP was incorporated during *in vitro* transcription by adding it to the
203 transcription reaction in excess at 15 mM. The reaction was incubated at 37°C for 4 h, digested with
204 DNaseI and purified using Qiagen RNeasy Mini kit. Quality of the mRNA was assessed by running
205 an aliquote on a Formaldehyde agarose gel. EDC coupling: The RNA was dissolved in 75 µL 1x
206 Reaction Buffer (150 mM NaCl, 10 mM EDTA, 10 mM Potassium phosphate, pH 7) and added to a
207 tube containing 12.5 mg EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid*hydrochloride). 50
208 µL of 0.25 M ethylene diamine, 0.1 M imidazole in water were immediately added to the reaction.
209 The tube was vortexed and briefly centrifuged, then 200 µL of 0.1 M imidazole (pH 6) solution were
210 added. The reaction incubated at 37°C for 2.5-3 h. The RNA was ethanol precipitated twice in 0.3 M
211 NaOAc (pH 5.5). Dye coupling: One Cy5 Mono-Reactive Dye Pack was used for up to three labeling
212 reactions. The dye was dissolved in 60 µL RNase-free DMSO and split in 20 µL aliquots. The RNA
213 was dissolved in 20 µL of a freshly prepared 0.1 M NaHCO₃ solution. One 20 µL aliquot was added

214 to the RNA and the mixture was incubated for 90 min at RT in the dark. The RNA was ethanol
215 precipitated twice in 0.3 M NaOAc (pH 5.5), resuspended in water and stored at -20 °C.

216

217 **tRNA Synthesis *In Vitro***

218 Human initiation tRNA (iMet) was synthesized *in vitro* using Ampliscribe T7 High Yield
219 transcription kit. Two oligonucleotides were used at 1 µM for this purpose. One oligonucleotide (3'-
220 T-TCG-AAT-TAT-GCT-GAG-TGA-TAT-CCG-TCT-CAC-CGC-GTC-GCC-TTC-GCA-CGA-
221 CCC-GGG-TAT-TGG-GTC-TCC-AGC-TAC-CTA-GCT-TTG-GTA-GGA-GAC-GGT-GGT-5')
222 was used as a template. The resulting tRNA starts with G instead of A. The complementary base in
223 the acceptor stem was accordingly exchanged into C. Both changes in the template are underlined.
224 The second oligonucleotide (5'-A-AGC-TTA-ATA-CGA-CTC-ACT-ATA- 3') was used to prime
225 the T7 RNA polymerase. The reaction was carried at 37°C for 4.5 h. The final product was purified
226 by Phenol/Chloroform/Isoamylalcohol extraction and Ethanol precipitation.

227

228 **Protein Purification**

229 Human his-tagged TRMT6/61A dimer was purified from Escherichia coli BL21 cells using 1 mL
230 HisTrap HP affinity chromatography columns (GE Healthcare). Eluted fractions containing the
231 recombinant proteins were pooled and incubated overnight with PreScission protease at 4°C. Protein
232 solution was then subjected to size-exclusion chromatography using a HiLoad Superdex 200 column
233 (GE Healthcare) in 50 mM HEPES NaOH pH 7.5, 150 mM NaCl and 1 mM DTT.

234

235 **Protein Melting Analysis**

236 A fluorescent dye-based thermal melting assay was carried out using a CFX96 Real-Time PCR
237 detection system. 10 µM of purified recombinant heterodimer TRMT6/61A were mixed with or
238 without 10 µM tRNA and with SYPRO Orange dye (1:5000) in 100 mM Tris HCl pH 7.6, 100 mM

239 NH₄Cl, 10 mM MgCl₂, 0.1 mM EDTA, and 1 mM DTT (added fresh). Fluorescence change was
240 recorded while gradually increasing the temperature from 20°C to 80°C in 0.5°C/15 s steps
241 (excitation at 450-490 nm, detection at 510-530 nm). Background fluorescence from the buffer as
242 well as from the tRNA were subtracted before further analysis. The apparent melting temperature
243 values were obtained by calculating the first derivative of the fluorescence change.

244

245 **CD Spectroscopy**

246 CD spectra of proteins were recorded using a Jasco J-810 spectropolarimeter at 37°C and 45°C in 50
247 mM HEPES NaOH pH 7.5 and 150 mM NaCl. Proteins at 5 µM were placed in a 1-mm path cell
248 (Hellma Analytics, Müllheim, Germany) and samples were scanned three times from 260 nm to 200
249 nm in 0.5 nm steps at 50 nm/min. The values from the buffer were subtracted.

250

251 **Protease Sensitivity Analysis**

252 Recombinant proteins were diluted to 1.5 µM in 25 mM Hepes KOH pH 7.5, 150 mM NaCl, 5 mM
253 CaCl₂ and 2 mM DTT and incubated for 20 min at the indicated temperature. Proteinase K was added
254 at 0,1 ng/µL and samples were collected after 3, 6 and 9 min. Hydrolysis was stopped by addition of
255 PMSF followed by hot SDS sample buffer. The samples were analyzed by SDS-PAGE and
256 Coomassie BB G-250 (CBB) staining. Images were acquired with the Chemidoc MP imaging system
257 and bands quantified using the Image Lab 5.0 software (both from Bio-Rad).

258

259 **TRMT6/61A Binding to RNA *In Vitro***

260 *In vitro* transcribed tRNA was heated at 85°C for 3 min and then refolded in 100 mM Tris HCl pH
261 7.6, 100 mM NH₄Cl, 10 mM MgCl₂, 0.1 mM EDTA and 1 mM DTT at 30°C for 15 min. Recombinant
262 TRMT6/61A was diluted to 10 nM in hypotonic lysis buffer supplemented with RNasin and
263 phosphatase inhibitors and incubated at indicated temperature for 20 min. tRNA was added at a final

264 concentration of 1 μ M and the mixture was further incubated at room temperature for 20 min with
265 20 μ L M-280 Streptavidin beads coated with biotinylated RNA. The beads were washed and eluted
266 as described for RNA pulldowns.

267
268 **TRMT6/61A Binding to tRNA *In Vitro***
269 M200 Pro Microplate Reader (Tecan, Männedorf, Switzerland) was used to record the change in
270 tRNA UV light absorbance upon binding to the TRMT6/61A heterodimer. tRNA (1 μ M) and protein
271 solution was prepared in 100 mM Tris HCl pH 7.6, 100 mM NH₄Cl, 10 mM MgCl₂, 0.1 mM EDTA,
272 and 1 mM DTT (added fresh). The mix was pre-incubated for 10 min at 25°C in a UV-transparent
273 96-well plates (Corning). Then temperature was raised to 37°C or 42°C for another 10 min before
274 measuring the absorbance between 230 nm and 350 nm in 2-nm steps. The formula ($\lambda_{\text{max}} -$
275 λ_{260})/ λ_{max} was used to determine the binding of the tRNA to the protein, where λ_{max} is the 260 nm
276 absorbance of the pure tRNA and λ_{260} is the 260 nm absorbance from the tRNA protein mix. The
277 background absorbance from the buffer as well as from the protein only solution was subtracted
278 before calculations.

279
280 **Quantitative Mass Spectrometry**
281 Sample preparation. Pulled down proteins were processed on-beads for LC-MS/MS analysis as
282 following. Beads were re-suspended in 50 μ L 8M urea/50 mM Tris HCl pH 8.5, reduced with 10 mM
283 DTT for 30 min and alkylated with 40 mM chloroacetamide for 20 min at 22°C. Urea was diluted to
284 a final concentration of 2 M with 25 mM Tris HCl pH 8.5, 10% acetonitrile and proteins were digested
285 with trypsin/lys-C mix overnight at 22°C. Acidified peptides (0.1% trifluoroacetic acid) were
286 desalted and fractionated on combined C18/SCX stage tips (3 fractions). Peptides were dried and
287 resolved in 1% acetonitrile, 0.1% formic acid.

288 LC-MS/MS. LC-MS/MS was performed on a Q Exactive Plus equipped with an ultra-high pressure
289 liquid chromatography unit (Easy-nLC1000) and a Nanospray Flex Ion-Source (all three from
290 Thermo Fisher Scientific). Peptides were separated on an in-house packed column (100 μ m inner
291 diameter, 30 cm length, 2.4 μ m Repronil-Pur C18 resin) using a gradient from mobile phase A (4%
292 acetonitrile, 0.1% formic acid) to 30% mobile phase B (80% acetonitrile, 0.1% formic acid) for 60
293 min followed by a second step to 60% B for 30 min, with a flow rate of 300 nl/min. MS data were
294 recorded in data-dependent mode selecting the 10 most abundant precursor ions for HCD with a
295 normalized collision energy of 30. The full MS scan range was set from 300 to 2000 m/z with a
296 resolution of 70000. Ions with a charge ≥ 2 were selected for MS/MS scan with a resolution of 17500
297 and an isolation window of 2 m/z. The maximum ion injection time for the survey scan and the
298 MS/MS scans was 120 ms, and the ion target values were set to 3×10^6 and 10^5 , respectively. Dynamic
299 exclusion of selected ions was set to 30 s. Data were acquired using Xcalibur software.

300 Data analysis with MaxQuant. MS raw files from five (Hsp70 coding region interactome) or three
301 biological replicates (B-Raf coding region interactome) of pulldown and background samples were
302 analyzed with Max Quant (version 1.5.3.30) (Cox and Mann, 2008) using default parameters.
303 Enzyme specificity was set to trypsin and lysC and a maximum of 2 missed cleavages were allowed.
304 A minimal peptide length of 7 amino acids was required. Carbamidomethylcysteine was set as a fixed
305 modification, while N-terminal acetylation and methionine oxidation were set as variable
306 modifications. The spectra were searched against the UniProtKB human FASTA database
307 (downloaded in November 2015, 70075 entries) for protein identification with a false discovery rate
308 of 1%. Unidentified features were matched between runs in a time window of 2 min. In the case of
309 identified peptides that were shared between two or more proteins, these were combined and reported
310 in protein group. Hits in three categories (false positives, only identified by site, and known
311 contaminants) were excluded from further analysis. For label-free quantification (LFQ), the
312 minimum ratio count was set to 1.

313 Data analysis with Perseus. Bioinformatic data analysis was performed using Perseus (version
314 1.5.2.6) (Tyanova et al., 2016). Proteins identified in the pulldown experiments were further included
315 in the analysis if they were quantified in at least 4 out of 5 biological replicates in at least one group
316 (pulldown/background) for the Hsp70 coding region RNA and in at least 3 out of 3 biological
317 replicates in at least one group (pulldown/background) for the B-Raf coding region RNA control.
318 Missing LFQ values were imputed on the basis of normal distribution with a width of 0.3 and a
319 downshift of 1.8. Proteins enriched in the pulldown (RNA over background binding) were identified
320 by two-sample t-test at a permutation-based FDR cutoff of 0.001 and $s_0 = 0.1$ for the Hsp70 coding
321 region interactome and at a p-value cutoff of 0.01 for the B-Raf coding region interactome.
322 Categorical annotations were added in Perseus and a Fisher's exact test with a p-value threshold of
323 0.001 was run for GO term enrichment analysis.

324
325 **Bioinformatics Analysis**
326 pI and molecular weight values were calculated using the pI/MW tool on the ExPASy website.
327 Hydrophobicity and disorder propensity were analyzed with the boxplotter function of cleverSuite
328 (Klus et al., 2014) using Kyte and Doolittle (Kyte and Doolittle, 1982) and TOP-IDB (Campen et al.,
329 2008) scales, respectively. To analyze aggregation propensity the Zyggregator algorithm was used
330 (Tartaglia and Vendruscolo, 2010). To analyze granulation propensity the catGRANULE algorithm
331 was used (Bolognesi et al., 2016). Hsp70 mRNA interactors were compared to the human proteome
332 as provided by Perseus (20592 proteins) and to the human RBPs (Gerstberger et al., 2014). Statistical
333 significance of observed differences was assessed by Mann-Whitney test.

334
335 **Statistical Analysis**

336 All repetitions in this study were independent biological repetitions performed at least three times if
337 not specified differently. To identify significantly increased proteins in pulldowns (RNA over
338 background binding) in mass spectrometry analyses, a two-sample t-test analysis of grouped
339 biological replicates was performed using a FDR cutoff of 0.001 with $s_0 = 0.1$ and a p-value cutoff
340 of 0.01 for the Hsp70 and B-Raf coding region interactome, respectively. Means and standard
341 deviations were calculated from at least three independent experiments.

342

343 **RESULTS**

344

345 **Free RNA Forms Granules *In Vivo***

346 The majority of mRNA-specific regulatory associations are anchored on the untranslated regions
347 (UTRs) of transcripts. Specifically, all known regulation of the HSP70 mRNA operates through these
348 regions¹³. To reduce the mRNA-specific bias, UTRs were deleted from the mRNAs used in this
349 study (Figure 1A). Electroporation of *in vitro* transcribed and 5'-labelled RNA led to the efficient
350 granule formation in up to 95% of transfected human HeLa cells (Figure 1B). The granulation
351 reassured us in our strategy to reduce sequence specificity of the RNA, since native HSP70 mRNA
352 is known to escape granulation¹⁴. The formed structures contained RNA because nuclease treatment
353 before imaging significantly reduced the percentage of cells containing them (Figure S1A). Cy5-
354 labeled oligonucleotide was used to exclude that the fluorophore freed from RNA by intracellular
355 hydrolysis accumulates to fluorescent foci in the cytosol. (Figure S1A).

356 We then used the *in vitro* transcribed and biotin-labelled mRNA as a bait to identify RNA interactors
357 in the heat-shocked HeLa cytosol, under conditions of polysome disassembly, free RNA release and
358 stress granule (SG) formation. Cell treatment with 1 mM arsenite was used as a reference. The
359 polysome profile indicated an early stage of cellular reorganization (Figure 1C), because it was
360 reduced, yet not fully absent as in the case of the arsenite treatment. The monosome peak (rRNAs
361 plus associated mRNAs) was strongly increased (Figure 1C) but had higher fraction of ribosomes
362 (higher rRNA fraction means smaller fraction of mRNA per absorbance unit) as measured by anti-
363 S6 western blot (Figure 1D). This observation confirmed that mRNAs from disassembled polysomes
364 did not remain associated with ribosomes but were freed into the cytosol. Microscopically, imminent
365 formation of stress granules (SG) was observed confirming the early phase of stress response (Figure
366 S1B).

367

368 **Free RNA Assembles a Set of Proteins in the Heat-Shocked Cytosol**

369 The bait RNA was incubated with the lysate of heat shocked cells and associated proteins were
370 identified by means of mass spectrometry (Figure 2A and Table S1). We did not perform experiments
371 with non-shocked lysates because they are not supposed to have free RNAs. Instead, we used binding
372 to uncoated beads as background control. Five independent biological repetitions yielded highly
373 reproducible label-free quantification of RNA interacting proteins (Figure S2A). When averaged and
374 corrected for background binding to beads without RNA, a distinct set of 79 interacting proteins
375 unveiled as visualized by the volcano plot (Figure 2B). More than half of the interactors (46) had
376 been shown or are predicted to be RNA-binders¹⁵ (Table S2). At the same time, proteostasis and
377 signaling networks were represented by a number of proteins, such as the cochaperone DNAJC21,
378 ubiquitin ligase RBB6, APC protein or casein kinase I.

379 To reassure that the interactome was not dominated by the sequence identity, we determined the
380 interactome of another UTR-deleted RNA of similar length (2295 nucleotides of the human BRAf
381 coding region plus 184 nucleotides from the vector). This independent RNA is further called ‘control
382 RNA’ (Table S3). With three repetitions the analysis was less deep, nevertheless, we identified 58
383 free RNA-interacting proteins in the heat-shocked cells (Figure S2B and Table S4). Importantly, ca.
384 half of the interactome of the control RNA overlapped with the interactome of the first bait RNA
385 with the high significance of $p < 4.7 \times 10^{-63}$ based on hypergeometric distribution (Figure S2C).

386

387 **Interactors with Free RNA Display Distinct Structural Features**

388 A possible concern while analyzing nucleic acid interactors are the trivial electrostatic interactions
389 between negatively charged RNA and positively charged proteins. The analysis of the isoelectric
390 point (pI) distribution showed only a slight increase of the mean pI value of the interactors above the
391 proteome average (Figure 3A). This finding suggests that the electrostatics did not critically steer the
392 associations. In contrast, the molecular size distribution was strongly shifted when compared to the

393 distribution of the whole human proteome (Figure 3B). An average interactor is 74 kDa big while the
394 human proteins have a molecular weight of 38 kDa on average (Figure 3C). The fractional difference
395 was especially obvious for proteins larger than 100 kDa. The large size is indicative of a multidomain
396 structure of the respective proteins and thus suggests the importance of multivalent interactions in
397 the identified assembly.

398 The mean hydrophobicity calculated according to the Kyte-Doolittle scale was significantly lower
399 for the RNA interactors (Figure 3D). A low hydrophobicity and lack of a hydrophobic core usually
400 correlate with the lack of a defined structure. Consequently, a significant increase of structural
401 disorder was predicted for the RNA interactors (Figure 3E). These properties might partially explain
402 the reduced aggregation propensity of the interactors (Figure 3F), because, the presence of a
403 hydrophobic core is a key requirement for aggregation. Finally, we used the recently developed
404 algorithm *cat*GRANULE which estimates the granule-forming propensity of proteins ¹⁶. This
405 parameter also turned out to be strongly discriminatory between the human proteome and the RNA
406 interactors (Figure 3G).

407 Gene ontology (GO) analysis revealed an expected highly significant enrichment of nucleic
408 acid/RNA binding categories among the 79 interactors (Figure S3A). Two further categories,
409 “nucleolus” and “non-membrane-bound organelle”, pointed to the involvement of the interactome in
410 the membrane-less protein sequestration, presumably in its early phase since the lysates were
411 prepared from cells with few nascent SGs (Figure S1B). The comparison of the human RNA binding
412 proteins ¹⁵ with the identified free-RNA interactome of heat shocked cells revealed differences
413 between these groups at several structural parameters, including the granulation propensity (Figures
414 S3B-S3G).

415

416 **Heat Shock Promotes RNA-Protein Association**

417 Attenuation of the cap-dependent protein translation is an obligatory reaction to different stress
418 stimuli, including the conformational stress that causes protein misfolding. eIF2 α phosphorylation
419 on serine 51 (S51) is the key to the shutdown of translation and the assembly of SG¹⁷. We were
420 intrigued to discover all three subunits of the eIF2 among the RNA interactors in the heat shocked
421 lysate (Figure 4A). The enrichment of the individual proteins over the background was not high, yet
422 significant, and came along with extensive sequence coverage by mass spectrometry (Figure S4A).
423 The interaction could be verified biochemically (Figure 4B) and was found to increase significantly
424 during heat shock. The association cannot be considered trivial, because the current translation
425 initiation model states that eIF2 binds mRNAs after a complex with the 40S ribosome and eIF3 is
426 formed. Neither 40S nor eIF3 were found in the interactome.
427 Notably, only the S51-non phosphorylated eIF2 α could be detected in the pulldowns. The phospho-
428 S51 eIF2 α , through a tight interaction with eIF2B, leads to slower GDP-to-GTP exchange which
429 results in an impaired translation initiation. Control experiments showed that the phosphorylated
430 eIF2 α accumulated in the lysate as expected (Figure S4B) along with an efficient shutdown of the
431 bulk protein translation (Figure S4C). Thus, association of the active fraction of eIF2 on free RNA
432 in the heat shocked lysates suggested a functional relevance of the interaction.
433 Next, we analyzed the TRMT6/61A complex, one of the top enrichments among the RNA interactors
434 (Table S2, Figure 4C and Figure S4D). TRMT6/61A has been known as a tRNA methyltransferase
435 able to methylate adenine 58 at N1 in tRNAs¹⁸. Recently, m¹A was identified also in eukaryotic
436 mRNAs which implicated the modification in posttranscriptional regulation of gene expression^{19,20}.
437 Two studies established TRMT6/61A as an m¹A writer on mRNAs^{21,22}. Similarly to eIF2, heat shock
438 enhanced the docking of TRMT6/61A on free mRNA (Figure 4D). TRMT6 increasingly associated
439 with endogenous mRNA during heat shock (Figure 4E).

440 The heat shock increased the association of both complexes either directly or via common binding
441 partners (Figure 5A). Hydrolysis of RNA in the lysate abolished this association (Figure 5B) which
442 suggested that free RNA provides a scaffold to assemble regulatory components of the translation
443 machinery during the early phase of the stress response.

444 Importantly, subunits of the eIF2 and TRMT6/61A were identified as significant interactors of the
445 control RNA as well (Table S4).

446

447 **Structural Changes of TRMT6/61A at Higher Temperature**

448 To understand the reasons for the heat-activated association of TRMT6/61A with the free RNA, the
449 recombinant methyltransferase was purified (Figure S5A) and analyzed *in vitro*. The stability analysis
450 of the proteins TRMT6/61A revealed two melting points (Figure 6A). The major unfolding happened
451 around 51°C (T_m). However, at temperature around 43°C, an additional partial transition could be
452 detected (T_m'). The presence of a TRMT6/61A substrate, the unmethylated tRNA, slightly increased
453 T_m but decreased T_m' (Figure 6A, right panel). The early transition did not involve a substantial loss
454 of secondary structure (Figure S5B). The lack of an appreciable difference in the circular dichroism
455 (CD) spectrum suggested two possibilities. From one side, the T_m' at ca. 43°C might reflect the
456 complex dissociation. TRMT6/61A exists as a tetramer formed by two heterodimers²³. tRNA binds
457 across the dimer interface and involves TRMT6 and TRMT61A from the opposing heterodimers.
458 tRNA was able to shift the major T_m (Figure 6A) which indicates intactness of the tRNA binding site
459 up to at least 51°C and argues against the tetramer dissociation into dimers at lower temperature. The
460 interface and predicted free energy between the monomers in the heterodimer are almost two times
461 larger than those between the heterodimers in the tetramer²³. This makes the dissociation of
462 heterodimers into monomers at 43°C even less probable. Alternatively, a subtle local unfolding might
463 take place at this temperature. Analysis of the protease sensitivity of the complex supported the latter
464 interpretation (Figure 6B). Interestingly, only the subunit TRMT6 showed an increased sensitivity.

465 **TRMT6/61A Interacts Directly with mRNA *in Vitro***

466 The binding of tRNA to the TRMT6/61A complex was investigated in a direct assay *in vitro* (Figure
467 S6A). The assay revealed an unchanged capacity of the endogenous tRNA to associate with
468 TRMT6/61A also at higher temperature (Figure 7A). We considered the possibility that the
469 endogenous tRNA was not a faithful substrate for the methyltransferase because it is already N1-
470 methylated at A58. To exclude this, we next analyzed the binding of the *in vitro* synthesized, i.e.,
471 unmethylated, tRNA and again did not observe significant changes (Figure 7B).
472 Finally, we succeeded to reconstitute the association of TRMT6/61A with mRNA *in vitro*. According
473 to our estimations, the methyltransferase is found in the cytosol of HeLa cells at 1-10 nM (Figure
474 S6B) and the tRNA concentration is known to be in low micromolar range. At these concentrations
475 *in vitro*, very efficient direct interaction of TRMT6/61A and free RNA was detected (Figure 7C).
476 This interaction did not depend on the presence of tRNA, regardless whether methylated or not.
477 Contrary to the result in the lysate, increased temperature not only failed to enhance the interaction,
478 but actually abolished it (Figure 7C).

479 **DISCUSSION**

480

481 Inhibition of the cap-dependent translation initiation and disassembly of polysomes are two well-

482 documented components of cellular reaction to heat shock, which are involved in stress granule (SG)

483 formation. Our study uncovered the early step of this response in mammalian cells, namely, the

484 capacity of mRNA - when freed from polysomes - to recruit a set of proteins involved in RNA

485 metabolism and proteostasis. We sought to reduce sequence-specific effects by deleting 5'-UTR and

486 3'-UTR from both bait RNAs. Indeed, known regulation of the HSP70 mRNA operates through these

487 regions, for example, the increased stability of the transcript²⁴ and its enhanced translation²⁵ during

488 stress are due to the ARE in the 3'-UTR, cap-independent translation is due to IRES in the 5'-UTR

489^{26,27}, ribosomal shunting during heat shock operates via the 18S rRNA complementarity sequence in

490 the 5'-UTR²⁸, transcription and translation are coupled by eEF1A1 via the 3'-UTR²⁹. It is known

491 that HSP70 mRNA escapes TIA-positive SG¹⁴. Our data indicate that the escape involves the UTRs

492 as well because the UTR-free HSP70 RNA accumulated in granules when introduced into cells

493 (Figure 1B).

494

495 It is not trivial to setup RNA association analyses devoid of evolutionary established sequence-

496 specific interactions. Yet, the significant overlap of the interactomes of two different RNA sequences

497 is reassuring that we uncovered, at least partially, the common processing machinery of free RNA

498 released from polysomes during stress. Among interactors, there was G3BP1 known to be required

499 for SG assembly³⁰. G3BP1 promotes phase separation in association with Caprin1³¹ which was also

500 among the hits. Another indication that the interactome we report does represent an early snapshot of

501 free RNA processing in the cytosol is the abundance of RNA helicases which would be capable of

502 supporting the dynamic packing of long polynucleotides into SGs. At the same time, one should avoid

503 overgeneralization of results from this kind of analysis because of the heterogeneity of SGs formed

504 by different stressors or in different cells³². Secondly, it cannot be excluded that initial stages of
505 granulation are mechanistically similar between different types of RNA granules, for example,
506 between SGs and P-bodies. For long, P-bodies were believed to be main sites of RNA decay, but also
507 for storage³³. In this regard, the presence of the endonuclease Dicer in the free RNA interactome is
508 especially intriguing.

509
510 Recent studies suggested that a network of proteins exists pre-formed under normal conditions and
511 is recruited to build up granules during stress^{32,34}. If so, only a few additional components might be
512 needed to induce granule formation. Our *in vitro* experiments using recombinant TRMT6/61A
513 support this scenario. TRMT6/61A, one of the highest enrichments in the interactome, has been
514 known as N1-adenine methyltransferase of A58 in tRNAs and was recently shown to methylate
515 mRNAs as well. Why does proteostasis stress increase the association of TRMT6/61A with mRNAs?
516 We observed a local unfolding of the TRMT6 subunit around the temperature of the heat shock. Yet,
517 the unfolding did not weaken its affinity towards tRNA and thus cannot explain the increase of
518 methyltransferase binding to mRNA due to reduced competition with tRNA. We could also exclude
519 that the local unfolding increases the affinity to mRNA since TRMT6/61 completely lost its
520 interaction with mRNA in an *in vitro* reconstitution under higher temperature (Figure 7C). One
521 possible explanation is that a collaborative association of several proteins with free RNA is taking
522 place at increased temperature. A pre-existing or acutely formed network of proteins could then
523 facilitate complex association of further proteins, such as TRMT6/61A. Thus, additional components
524 would be needed to reconstitute the enhanced binding of some interactors to RNA upon heat shock.
525 The nuclease sensitivity of the co-precipitation of TRMT6/61A and eIF2 α supports this possibility
526 (Figures 5A and 5B).

527

528 A number of neurodegenerative disorders are associated with repeat expansions in the disease-linked
529 genes ³⁵. As a result, faulty polypeptide products of the mutant genes misfold and aggregate
530 sequestering a number of regulatory proteins, which interferes with diverse cellular functions ^{36,37}.
531 RNA-binding proteins constitute a significant part of coaggregating species. On the other side, repeat-
532 containing RNAs can form disease-associated granules as well ³⁸. Moreover, even bulk RNA as a
533 biopolymer seems to modulate phase separation of prion-like proteins ⁵. Our data supports the active
534 role of non-mutant RNA in granulation processes during proteostasis stress.
535

536 **CONCLUSIONS**

537 The present study directly links to the recent advances in the field of RNA-protein granulation,
538 namely, an increasing appreciation of the active role of RNAs in this process. Composition of several
539 types of RNA-protein granules could be determined recently. The challenge now is to understand the
540 mechanisms of their formation. Our study provides a molecular snapshot of the RNA-driven protein
541 association early during heat shock and thus sets the ground towards this understanding. The
542 significant overlap of the interactomes of two different RNAs argues for the general validity of the
543 identified protein set. We hope that the data will prompt in-depth mechanistic and functional studies
544 of individual interactors in the future.

545

546 **ASSOCIATED CONTENT**

547 **Supporting Information**

548 Supporting Information includes six figures (PDF) and four tables (XLSX).

549 **Table S1.** MaxLFQ Quantitative Data and Identifiers of mRNA (HSP70 Coding Region) Interactors.

550 **Table S2.** Free RNA (HSP70 Coding Region) Interactors in the Heat-Shocked Mammalian Cytosol.

551 **Table S3.** MaxLFQ Quantitative Data and Identifiers of Control mRNA (BRaf Coding Region)
552 Interactors.

553 **Table S4.** Free Control RNA (BRaf Coding Region) Interactors in the Heat-Shocked Mammalian
554 Cytosol.

555

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559 **Notes**

560 The authors declare no competing financial interest.

561

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568 **ABBREVIATIONS**

569 MS, mass spectrometry; RBPs, RNA-binding proteins; UTRs, untranslated regions; SGs, stress
570 granules; GO, gene ontology; CD, circular dichroism.

571 **REFERENCES**

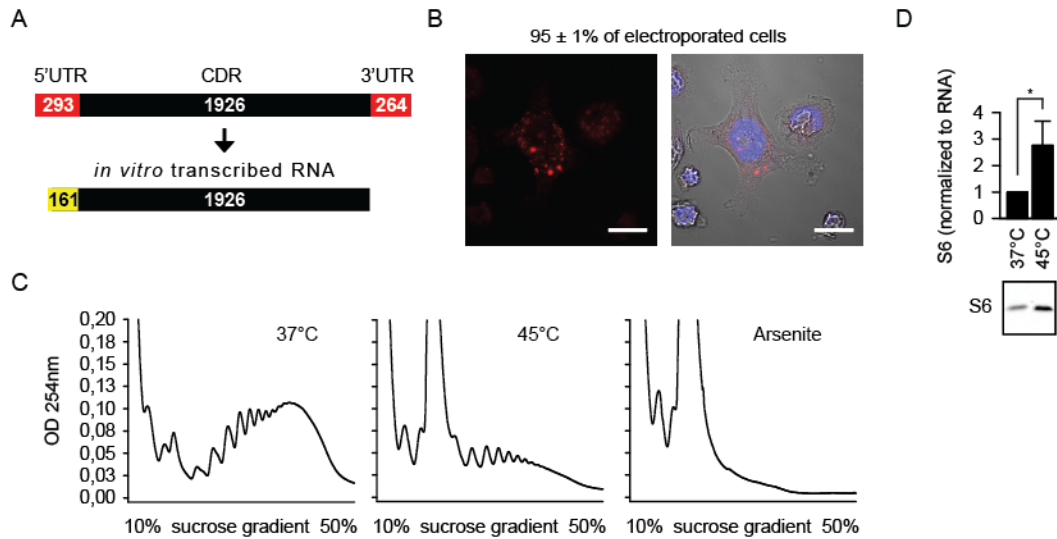
- 572 (1) Brangwynne, C. P.; Tompa, P.; Pappu, R. V. Polymer Physics of Intracellular Phase
573 Transitions. *Nature Physics* **2015**, *11* (11), 899.
- 574 (2) Li, P.; Banjade, S.; Cheng, H.-C.; Kim, S.; Chen, B.; Guo, L.; Llaguno, M.; Hollingsworth, J.
575 V.; King, D. S.; Banani, S. F.; et al. Phase Transitions in the Assembly of Multivalent
576 Signalling Proteins. *Nature* **2012**, *483* (7389), 336–340.
- 577 (3) Audas, T. E.; Audas, D. E.; Jacob, M. D.; Ho, J. J. D.; Khacho, M.; Wang, M.; Perera, J. K.;
578 Gardiner, C.; Bennett, C. A.; Head, T.; et al. Adaptation to Stressors by Systemic Protein
579 Amyloidogenesis. *Dev. Cell* **2016**, *39* (2), 155–168.
- 580 (4) Langdon, E. M.; Qiu, Y.; Ghanbari Niaki, A.; McLaughlin, G. A.; Weidmann, C.; Gerbich, T.
581 M.; Smith, J. A.; Crutchley, J. M.; Termini, C. M.; Weeks, K. M.; et al. MRNA Structure
582 Determines Specificity of a PolyQ-Driven Phase Separation. *Science* **2018**, *360* (6391), 922-
583 927.
- 584 (5) Maharana, S.; Wang, J.; Papadopoulos, D. K.; Richter, D.; Pozniakovsky, A.; Poser, I.; Bickle,
585 M.; Rizk, S.; Guillén-Boixet, J.; Franzmann, T.; et al. RNA Buffers the Phase Separation
586 Behavior of Prion-like RNA Binding Proteins. *Science* **2018**, *360* (6391), 918-921.
- 587 (6) Singh, G.; Pratt, G.; Yeo, G. W.; Moore, M. J. The Clothes Make the MRNA: Past and Present
588 Trends in MRNP Fashion. *Annu. Rev. Biochem.* **2015**, *84*, 325–354.
- 589 (7) King, O. D.; Gitler, A. D.; Shorter, J. The Tip of the Iceberg: RNA-Binding Proteins with
590 Prion-like Domains in Neurodegenerative Disease. *Brain Res.* **2012**, *1462*, 61–80.
- 591 (8) Kato, M.; Han, T. W.; Xie, S.; Shi, K.; Du, X.; Wu, L. C.; Mirzaei, H.; Goldsmith, E. J.;
592 Longgood, J.; Pei, J.; et al. Cell-Free Formation of RNA Granules: Low Complexity Sequence
593 Domains Form Dynamic Fibers within Hydrogels. *Cell* **2012**, *149* (4), 753–767.

- 594 (9) Jain, S.; Wheeler, J. R.; Walters, R. W.; Agrawal, A.; Barsic, A.; Parker, R. ATPase-Modulated
595 Stress Granules Contain a Diverse Proteome and Substructure. *Cell* **2016**, *164* (3), 487–498.
- 596 (10) Brandt, F.; Carlson, L.-A.; Hartl, F. U.; Baumeister, W.; Grünwald, K. The Three-
597 Dimensional Organization of Polyribosomes in Intact Human Cells. *Mol. Cell* **2010**, *39* (4),
598 560–569.
- 599 (11) Boundedjah, O.; Desforges, B.; Wu, T.-D.; Pioche-Durieu, C.; Marco, S.; Hamon, L.; Curmi,
600 P. A.; Guerquin-Kern, J.-L.; Piétrement, O.; Pastré, D. Free mRNA in Excess upon Polysome
601 Dissociation Is a Scaffold for Protein Multimerization to Form Stress Granules. *Nucleic Acids*
602 *Res.* **2014**, *42* (13), 8678–8691.
- 603 (12) McQuin, C.; Goodman, A.; Chernyshev, V.; Kametsky, L.; Cimini, B. A.; Karhohs, K. W.;
604 Doan, M.; Ding, L.; Rafelski, S. M.; Thirstrup, D.; et al. CellProfiler 3.0: Next-Generation
605 Image Processing for Biology. *PLoS Biol.* **2018**, *16* (7), e2005970.
- 606 (13) Silver, J. T.; Noble, E. G. Regulation of Survival Gene Hsp70. *Cell Stress Chaperones* **2012**,
607 *17* (1), 1–9.
- 608 (14) Kedersha, N.; Anderson, P. Stress Granules: Sites of mRNA Triage That Regulate mRNA
609 Stability and Translatability. *Biochem. Soc. Trans.* **2002**, *30* (Pt 6), 963–969.
- 610 (15) Gerstberger, S.; Hafner, M.; Tuschl, T. A Census of Human RNA-Binding Proteins. *Nat. Rev.*
611 *Genet.* **2014**, *15* (12), 829–845.
- 612 (16) Bolognesi, B.; Lorenzo Gotor, N.; Dhar, R.; Cirillo, D.; Baldrighi, M.; Tartaglia, G. G.; Lehner,
613 B. A Concentration-Dependent Liquid Phase Separation Can Cause Toxicity upon Increased
614 Protein Expression. *Cell Rep* **2016**, *16* (1), 222–231.
- 615 (17) Holcik, M.; Sonenberg, N. Translational Control in Stress and Apoptosis. *Nat. Rev. Mol. Cell*
616 *Biol.* **2005**, *6* (4), 318–327.
- 617 (18) Oerum, S.; Dégut, C.; Barraud, P.; Tisné, C. M1A Post-Transcriptional Modification in
618 TRNAs. *Biomolecules* **2017**, *7* (1), pii: E20.

- 619 (19) Dominissini, D.; Nachtergaele, S.; Moshitch-Moshkovitz, S.; Peer, E.; Kol, N.; Ben-Haim, M.
620 S.; Dai, Q.; Di Segni, A.; Salmon-Divon, M.; Clark, W. C.; et al. The Dynamic N(1)-
621 Methyladenosine Methylome in Eukaryotic Messenger RNA. *Nature* **2016**, *530* (7591), 441–
622 446.
- 623 (20) Li, X.; Xiong, X.; Wang, K.; Wang, L.; Shu, X.; Ma, S.; Yi, C. Transcriptome-Wide Mapping
624 Reveals Reversible and Dynamic N(1)-Methyladenosine Methylome. *Nat. Chem. Biol.* **2016**,
625 *12* (5), 311–316.
- 626 (21) Safra, M.; Sas-Chen, A.; Nir, R.; Winkler, R.; Nachshon, A.; Bar-Yaacov, D.; Erlacher, M.;
627 Rossmannith, W.; Stern-Ginossar, N.; Schwartz, S. The M1A Landscape on Cytosolic and
628 Mitochondrial mRNA at Single-Base Resolution. *Nature* **2017**, *551* (7679), 251–255.
- 629 (22) Li, X.; Xiong, X.; Zhang, M.; Wang, K.; Chen, Y.; Zhou, J.; Mao, Y.; Lv, J.; Yi, D.; Chen, X.-
630 W.; et al. Base-Resolution Mapping Reveals Distinct M1A Methylome in Nuclear- and
631 Mitochondrial-Encoded Transcripts. *Mol. Cell* **2017**, *68* (5), 993-1005.e9.
- 632 (23) Finer-Moore, J.; Czudnochowski, N.; O’Connell, J. D.; Wang, A. L.; Stroud, R. M. Crystal
633 Structure of the Human TRNA m(1)A58 Methyltransferase-TRNA(3)(Lys) Complex:
634 Refolding of Substrate TRNA Allows Access to the Methylation Target. *J. Mol. Biol.* **2015**,
635 *427* (24), 3862–3876.
- 636 (24) Zhao, M.; Tang, D.; Lechpammer, S.; Hoffman, A.; Asea, A.; Stevenson, M. A.; Calderwood,
637 S. K. Double-Stranded RNA-Dependent Protein Kinase (Pkr) Is Essential for
638 Thermotolerance, Accumulation of HSP70, and Stabilization of ARE-Containing HSP70
639 mRNA during Stress. *J. Biol. Chem.* **2002**, *277* (46), 44539–44547.
- 640 (25) Moseley, P. L.; Wallen, E. S.; McCafferty, J. D.; Flanagan, S.; Kern, J. A. Heat Stress
641 Regulates the Human 70-KDa Heat-Shock Gene through the 3’-Untranslated Region. *Am. J.*
642 *Physiol.* **1993**, *264* (6 Pt 1), L533-537.

- 643 (26) Rubtsova, M. P.; Sizova, D. V.; Dmitriev, S. E.; Ivanov, D. S.; Prassolov, V. S.; Shatsky, I. N.
644 Distinctive Properties of the 5'-Untranslated Region of Human Hsp70 mRNA. *J. Biol. Chem.*
645 **2003**, *278* (25), 22350–22356.
- 646 (27) Hernández, G.; Vázquez-Pianzola, P.; Sierra, J. M.; Rivera-Pomar, R. Internal Ribosome Entry
647 Site Drives Cap-Independent Translation of Reaper and Heat Shock Protein 70 MRNAs in
648 *Drosophila* Embryos. *RNA* **2004**, *10* (11), 1783–1797.
- 649 (28) Yueh, A.; Schneider, R. J. Translation by Ribosome Shunting on Adenovirus and Hsp70
650 MRNAs Facilitated by Complementarity to 18S RRNA. *Genes Dev.* **2000**, *14* (4), 414–421.
- 651 (29) Vera, M.; Pani, B.; Griffiths, L. A.; Muchardt, C.; Abbott, C. M.; Singer, R. H.; Nudler, E. The
652 Translation Elongation Factor EEF1A1 Couples Transcription to Translation during Heat
653 Shock Response. *Elife* **2014**, *3*, e03164.
- 654 (30) Matsuki, H.; Takahashi, M.; Higuchi, M.; Makokha, G. N.; Oie, M.; Fujii, M. Both G3BP1
655 and G3BP2 Contribute to Stress Granule Formation. *Genes Cells* **2013**, *18* (2), 135–146.
- 656 (31) Kedersha, N.; Panas, M. D.; Achorn, C. A.; Lyons, S.; Tisdale, S.; Hickman, T.; Thomas, M.;
657 Lieberman, J.; McInerney, G. M.; Ivanov, P.; et al. G3BP-Caprin1-USP10 Complexes Mediate
658 Stress Granule Condensation and Associate with 40S Subunits. *J. Cell Biol.* **2016**, *212* (7),
659 845–860.
- 660 (32) Markmiller, S.; Soltanieh, S.; Server, K. L.; Mak, R.; Jin, W.; Fang, M. Y.; Luo, E.-C.; Krach,
661 F.; Yang, D.; Sen, A.; et al. Context-Dependent and Disease-Specific Diversity in Protein
662 Interactions within Stress Granules. *Cell* **2018**, *172* (3), 590-604.e13.
- 663 (33) Hubstenberger, A.; Courel, M.; Bénard, M.; Souquere, S.; Ernoult-Lange, M.; Chouaib, R.;
664 Yi, Z.; Morlot, J.-B.; Munier, A.; Fradet, M.; et al. P-Body Purification Reveals the
665 Condensation of Repressed mRNA Regulons. *Mol. Cell* **2017**, *68* (1), 144-157.e5.
- 666 (34) Youn, J.-Y.; Dunham, W. H.; Hong, S. J.; Knight, J. D. R.; Bashkurov, M.; Chen, G. I.; Bagci,
667 H.; Rathod, B.; MacLeod, G.; Eng, S. W. M.; et al. High-Density Proximity Mapping Reveals

- 668 the Subcellular Organization of mRNA-Associated Granules and Bodies. *Mol. Cell* **2018**, 69
669 (3), 517-532.e11.
- 670 (35) La Spada, A. R.; Taylor, J. P. Repeat Expansion Disease: Progress and Puzzles in Disease
671 Pathogenesis. *Nat. Rev. Genet.* **2010**, 11 (4), 247–258.
- 672 (36) Schaffar, G.; Breuer, P.; Boteva, R.; Behrends, C.; Tzvetkov, N.; Strippel, N.; Sakahira, H.;
673 Siegers, K.; Hayer-Hartl, M.; Hartl, F. U. Cellular Toxicity of Polyglutamine Expansion
674 Proteins: Mechanism of Transcription Factor Deactivation. *Mol. Cell* **2004**, 15 (1), 95–105.
- 675 (37) Kim, Y. E.; Hosp, F.; Frottin, F.; Ge, H.; Mann, M.; Hayer-Hartl, M.; Hartl, F. U. Soluble
676 Oligomers of PolyQ-Expanded Huntingtin Target a Multiplicity of Key Cellular Factors. *Mol.*
677 *Cell* **2016**, 63 (6), 951–964.
- 678 (38) Jain, A.; Vale, R. D. RNA Phase Transitions in Repeat Expansion Disorders. *Nature* **2017**, 546
679 (7657), 243–247.
- 680



681

682

683 **Figure 1. Free RNA Forms Granules *In Vivo*.**

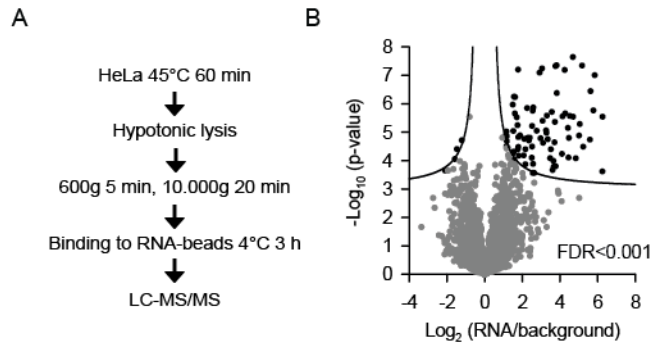
684 (A) Schematic depiction of the RNA (gene *HSPA1A*) used in the study. The nucleotide length of
685 different elements is indicated. UTR, untranslated region; CDR, coding region. Yellow, an additional
686 fragment from the expression vector.

687 (B) HeLa cells 6 h after electroporation with Cy5 (red) 5'-labelled RNA. DAPI staining (blue), nuclei.
688 Scale bar 20 μ m. Fraction of transfected cells with granules is indicated (N=3 independent
689 experiments). Right, merged image.

690 (C) Polysome profile under the indicated conditions. One representative out of three independent
691 experiments is shown.

692 (D) Ribosome amount in the monosome fraction as measured by anti-S6 western blot. *p<0.05, two-
693 tailed t-test; N=3 independent experiments (mean + SD).

694



695

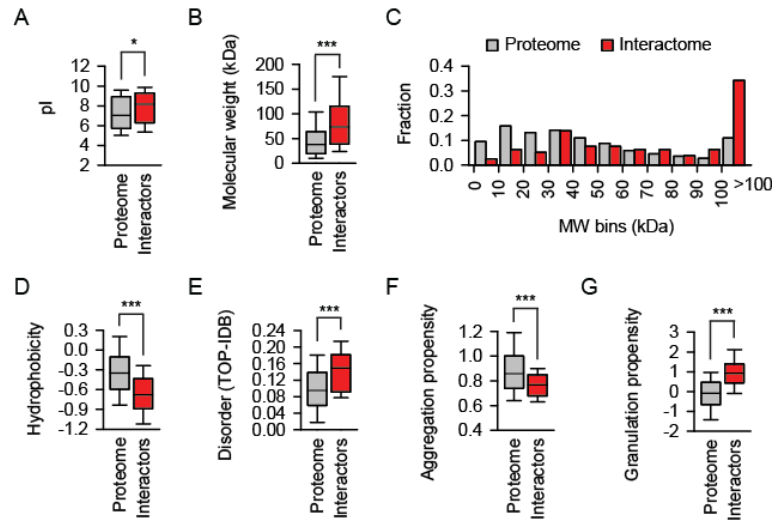
696

697 **Figure 2. Free RNA Assembles a Set of Proteins in the Heat-Shocked Cytosol.**

698 (A) Experimental steps to identify the free RNA-assembled machinery in the heat shocked
699 mammalian cytosol.

700 (B) Volcano plot of quantified proteins plotted according to their enrichment on RNA over
701 background with the statistical significance of the respective ratios plotted on the y-axis. False
702 discovery rate (FDR) used to define the 79 interactors (black symbols) is indicated. N=5 independent
703 experiments.

704



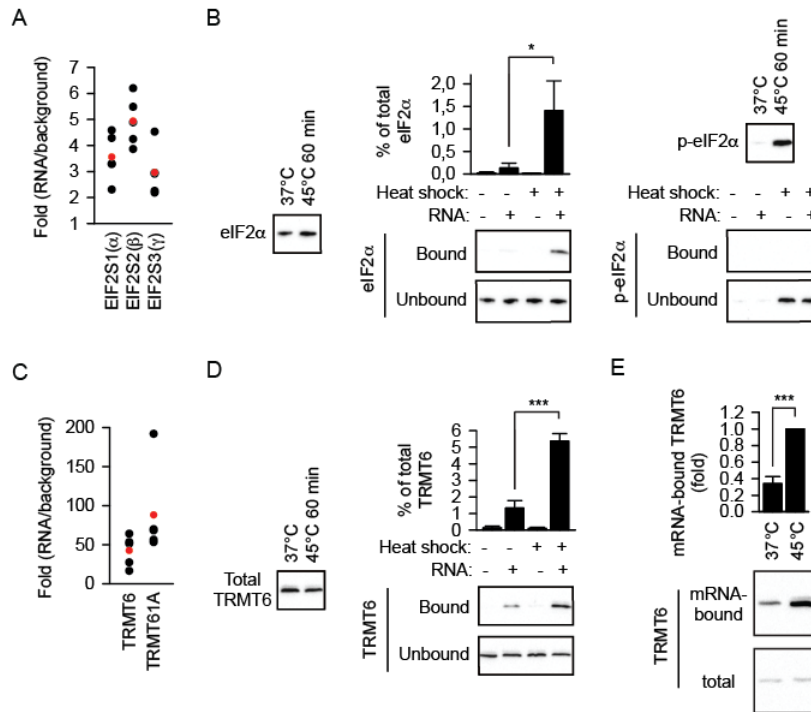
705

706

707 **Figure 3. Interactors with Free RNA Display Distinct Structural Features.**

708 Distribution of isoelectric points (A), molecular weights (B and C), Kyte-Doolittle hydrophobicity
709 scores (D), predicted disorder (E), aggregation propensity (F) and granulation propensity (G) scores
710 of the indicated sets of proteins. * $p < 0.05$, *** $p < 0.001$, Mann-Whitney test.

711



712

713

714 **Figure 4. Heat Shock Promotes RNA-Protein Association in HeLa cells.**

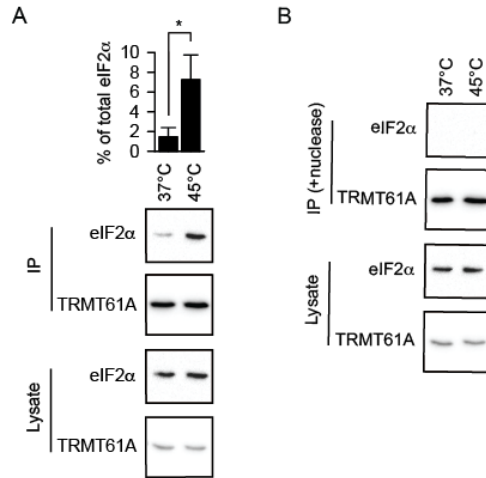
715 (A) Enrichment values of the subunits of eIF2 in individual experiments (black symbols) and the
 716 enrichment averages (red symbols).

717 (B) Left panels, amounts of total eIF2α and its serine 51-phosphorylated form (p-eIF2α) as determined
 718 by western blotting. Right panels, enrichment of non-phosphorylated eIF2α on RNA upon heat
 719 shock. *p<0.05, two-tailed t-test; N=3 independent experiments (mean + SD).

720 (C) Enrichment values of the subunits of the m¹A methyltransferase TRMT6/61A in individual
 721 experiments (black symbols) and the enrichment averages (red symbols).

722 (D) Upper panel, amounts of TRMT6 as determined by western blotting. Lower panel, enrichment
 723 of TRMT6 on RNA upon heat shock. ***p<0.001, two-tailed t-test; N=3 independent experiments
 724 (mean + SD).

725 (E) Increased association of TRMT6 with mRNAs during heat shock as determined by western blot
726 analysis of PolyT pulldowns. *** $p < 0.001$, two-tailed t-test, N=3 independent experiments (mean +
727 SD).
728



729

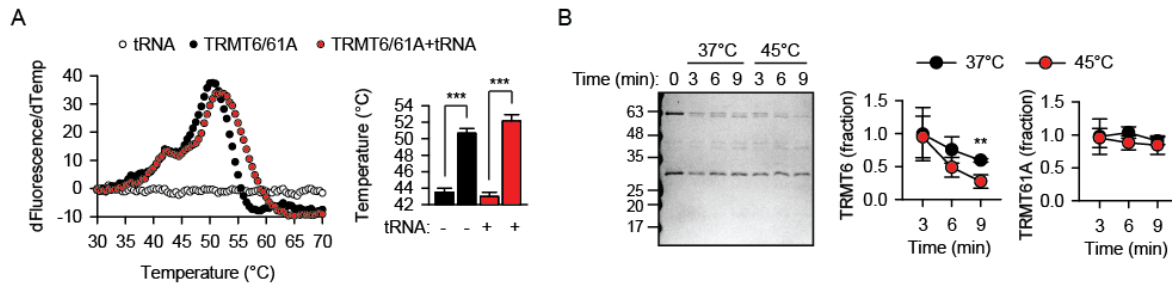
730

731 **Figure 5. RNA Scaffolds Protein Complexes in Heat-Shocked HeLa cells.**

732 (A) Increased association of eIF2 α with TRMT61A-containing complexes during heat shock as
733 determined by immunoprecipitation (IP) of Flag-tagged TRMT6/61A. * $p < 0.05$, two-tailed t-test;
734 $N = 3$ independent experiments (mean + SD).

735 (B) RNA hydrolysis (+nuclease) abolishes association of eIF2 α with the TRMT61A-containing
736 complexes. One representative out of three independent experiments is shown.

737



738

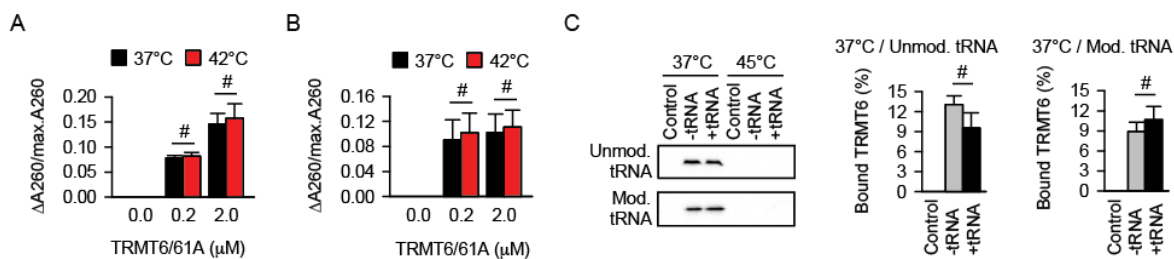
739

740 **Figure 6. TRMT6/61A Undergoes Minor Structural Changes at Higher Temperature.**

741 (A) Left panel, Melting analysis of TRMT6/61A without and with tRNA. First derivatives of the
742 fluorescence change are plotted and indicate two melting points (T_m' and T_m) upon increase of the
743 temperature. Right panel, T_m' is significantly lower than T_m . *** $p < 0.001$, two-tailed t-test; $N = 3$
744 independent experiments (mean + SD).

745 (B) Proteinase K sensitivity indicates partial unfolding of the RNA-binding TRMT6 subunit at 45°C.
746 Right panel, the amount of protein at timepoint 0 was set as 1. ** $p < 0.01$, two-tailed t-test; $N = 3$
747 independent experiments (mean + SD).

748



749

750

751 **Figure 7. TRMT6/61A Interacts Directly with mRNA.**

752 (A and B) Comparison of TRMT6/61A binding to 1 μM endogenous tRNA (A) or 1 μM *in vitro*
 753 transcribed tRNA (B) at physiological and febrile temperature. #, not significant difference; two-
 754 tailed t-test; N=3 independent experiments (mean + SD).

755 (C) Direct association of RNA and TRMT6/61A *in vitro*. The efficiency of TRMT6/61A pulldowns
 756 by RNA-coated beads (+/- 1 μM tRNA) was estimated by western blotting. *In vitro* transcribed tRNA
 757 (Unmod. tRNA) or endogenous tRNA (Mod. tRNA) were used. Control, beads without RNA. #, not
 758 significant difference; two-tailed t-test; N=3 (mean+SD).

759