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1 ATP:Mg^{2+} shapes material properties of protein-RNA condensates
2 and its partitioning of clients

3

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11

12 ABSTRACT

13 Many cellular condensates are heterotypic mixtures of proteins and RNA formed in complex environments.
14 Magnesium ions (Mg^{2+}) and ATP can impact RNA folding, and local intracellular levels of these factors can vary
15 significantly. However, the effect of Mg^{2+}:ATP on the material properties of protein-RNA condensates is largely
16 unknown. Here, we use an *in vitro* condensate model of nucleoli, made from nucleophosmin 1 (NPM1) proteins
17 and ribosomal RNA (rRNA), to study the effect of Mg^{2+}:ATP. While NPM1 dynamics remains unchanged at
18 increasing Mg^{2+} concentrations, the internal RNA dynamics dramatically slowed until a critical point, where gel-
19 like states appeared, suggesting the RNA component alone forms a viscoelastic network that undergoes
20 maturation driven by weak multivalent interactions. ATP reverses this arrest and liquefies the gel-like structures.
21 Mg^{2+}:ATP also influenced the NPM1-rRNA composition of condensates, and enhanced the partitioning of two
22 clients: an arginine-rich peptide and a snoRNA. By contrast, larger ribosomes partitioning shows dependence on
23 Mg^{2+}:ATP, and can become reversibly trapped around NPM1-rRNA condensates. Lastly, we show that dissipative
24 enzymatic reactions that deplete ATP can be used to control the shape, composition and function of condensates.
25 Our results illustrate how intracellular environments may regulate the state and client partitioning of RNA-
26 containing condensates.

27

28

29 SIGNIFICANCE STATEMENT

30 • Heterotypic condensates made from protein and RNA, such as the nucleolus, can exhibit puzzling gel-
31 like morphologies that are at odds with fast protein dynamics that suggest liquid-like properties.
32 • Here, we highlight the importance of the RNA component.
33 • We show that the protein-RNA condensates are dynamic, adapting to local ATP concentrations through
34 Mg^{2+}-induced compaction of the RNA via enhanced RNA-RNA interactions, and reversible RNA
35 relaxation when ATP binds Mg^{2+} again.
36 • With this, and our observation that the RNA component exhibits temperature-dependent aging, suggests
37 the RNA component imparts viscoelastic properties onto protein-RNA condensates, driven by
38 multivalent RNA-RNA interactions.
39 • In contrast, the protein component is free to diffuse throughout the RNA network.
40 • Other condensates containing RNA probably respond in similar ways to Mg^{2+} and ATP.
41

INTRODUCTION

Biomolecular condensates facilitate the spatiotemporal organization of cellular processes. Most biomolecular condensates are heterotypic, and many arise from mixtures of proteins and nucleic acids, such as RNA (1). Forming and existing in complex intracellular environments, the membraneless nature of condensates exposes them to local environmental fluctuations. These fluctuating factors – ions, small molecules and temperature – can all regulate the molecular interactions within condensates, which in turn, shapes their biophysical properties to influence morphology and function (2,3). However, the intracellular parameter space of organisms is complex, variable and not fully mapped. Therefore, *in vitro* studies can establish valuable insights into the influence of such factors on condensate form and function.

It is well established that RNA structure is strongly influenced by magnesium ion (Mg^{2+}) concentrations (1,4,5), which can range from 0.5-1 mM in eukaryotic cells and 1-5 mM in bacterial cell, as well as having an astounding *in vivo* metabolite-bound concentration range of 20-100 mM (4,6). Indeed, the free Mg^{2+} concentrations can vary throughout the cell cycle due to changes in levels of adenosine triphosphate (ATP), a nucleotide that strongly complexes Mg^{2+} (5,7,8). Despite this, there has so far been no systematic analysis of the effect of Mg^{2+} and ATP on shaping protein-RNA condensates.

With this, we are inspired by the nucleolus, a protein-RNA condensate that emerges during eukaryotic interphase within a complex cellular environment. As the site of ribosome biogenesis, ribosomal RNA (rRNA) and various RNA-binding proteins are predominantly found in this dynamic, but highly viscous, condensate. *In vivo* nucleoli have slow fusion time scales of ~30 mins and non-spherical shapes (9-11), that are not well represented by *in vitro* models, which are often liquid-like. Other heterotypic biomolecular condensates show similar divergent dynamics between protein and RNA components (12). We hypothesize that RNA structuring and network formation, governed by Mg^{2+} and other environmental factors, can partly explain these differences, and shape the viscoelastic material properties of *in vitro* models of protein-RNA condensates.

In this work, we approximate part of the nucleolus by making model condensates from nucleophosmin 1 (NPM1) protein and rRNA. By altering Mg^{2+} in the environment of model protein-RNA condensates, we shed light on how this common divalent cation can dramatically slow RNA diffusion dynamics, until it arrests and non-spherical condensate morphologies were observed. Additionally, NPM1-rRNA condensates underwent temperature-dependent aging, particularly the rRNA component, which further supports the hypothesis that condensate maturation is governed by multivalent RNA-RNA interactions, suggesting RNA's role in imparting viscoelastic material properties to the condensate as a whole. The Mg^{2+}-stabilised arrested rRNA gel-like condensates were liquefied using either temperature or ATP, with morphologies becoming increasingly spherical, and these changes reflected in more fluid rRNA dynamics. We probed the functional consequence of different Mg^{2+}:ATP on NPM1-rRNA condensates, and showed that these small molecules can impact client molecule partitioning. In particular, we explored the intriguing formation of a 70S ribosome halo that transiently associated with the NPM1-rRNA condensates. To allude to the intracellular enzymatic control of ATP levels, we used a dissipative enzymatic reaction that depletes ATP, to regulate the biophysical properties of *in vitro* condensates and partitioning of ribosomes. Together, our results not only provide a useful perspective for interpreting *in vivo* observations, but also show that protein-RNA condensates are highly responsive to their environment, with the environment affecting condensate properties, form and function.

MATERIAL AND METHODS

Reagents
Unless otherwise stated, all materials were obtained from Sigma-Aldrich.

Cloning of NPM1-wt into pET28a plasmid
The human nucleophosmin 1 gene was extracted from the pET28a(+)-NPM1-eGFP construct (gifted from R.K.) and cloned into a pET28a(+) vector using *NdeI* and *XhoI* restriction sites. The correct insertion was confirmed by sequencing (BaseClear, Leiden), and this construct called pET28a(+)-NPM1-wt.

NPM1 protein expression and purification
E. coli BL21 (DE3) were transformed with pET28a(+)-NPM1-wt. Overnight cultures were used to inoculate large flasks of LB, then cells were grown at 37 ℃ to an OD_{600} = 0.5-0.7, before protein expression was induced with 1

94 mM IPTG. Protein expression was carried out at 20 ℃ for at least 16 hours, after which the cells were harvested
95 by centrifugation. The pellet was resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM β-
96 mercaptoethanol, 25 mM imidazole) containing 500 U Bezonase® Nuclease and Bovine Pancreas RNAse A
97 (VWR). The resuspended cells were lysed using a homogenizer. Lysed samples were left at 4 ℃ for 1 hour to allow
98 the enzymes to degrade the nucleic acids. The lysate was spun at 35000 *xg*, 30 minutes at 4 ℃ in a Beckman
99 JA25.50 rotor. The clarified supernatant was loaded onto a 5 mL HisTrapFF (Cytiva). After loading, the column
100 was washed with 50 mL lysis buffer, and the His-tagged NPM1 proteins were eluted using elution buffer (20 mM
101 Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM β-mercaptoethanol, 250 mM imidazole). Eluted proteins were concentrated
102 to <5 mL and loaded onto a Superdex 200 16/600 (GE Healthcare) size exclusion column connected to an AKTA
103 Basic FPLC (GE Healthcare), and pre-equilibrated in storage buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl).
104 Fractionation of proteins was carried out at 1 mL/min, and monitored at 280 nm and 260 nm. The fractions of the
105 main peak were pooled, and the protein concentration was determined using the NanoDrop One[C] (Thermo
106 Scientific). If the resulting UV spectrum had a 260/280 ratio of >0.6, the protein sample was further purified using
107 anion exchange chromatography to remove contaminating nucleic acids. All protein isolates were dialyzed into
108 storage buffer, aliquoted and snap frozen in liquid nitrogen and stored at -80 ℃.
109

Protein labelling

111 The purified NPM1 proteins were labelled using AlexaFluor488 C_5 maleimide (Thermofisher Scientific) based on
112 a previously published protocol (13). Excess dye was removed using anion exchange, and the protein dialyzed
113 into storage buffer. The final protein concentration was determined using the NanoDrop One[C]. A labelled NPM1
114 stock was mixed with unlabelled protein at a 1:9 ratio of labelled:unlabelled protein, and this mixture was called
115 NPM1-A488.
116   Sucrose gradient purified *E. coli* BL21 70S ribosomes were labelled using either ATTO488 NHS-ester
117 (ATTO-TEC GmbH), or Dylight650 NHS-ester (Thermofischer Scientific) dyes, as previously reported (14). The
118 purified and labelled ribosomes were stored in aliquots at -80°C until use. The final concentration of labelled 70S
119 ribosome was estimated to be 5.1 µM (A488 for ATTO488-labelled ribosomes) and 6.3 µM (D650 for Dylight650-
120 labelled ribosomes) determined using a Nanodrop 1000 (Isogen).
121

E. coli **rRNA purification**

123 *E. coli* BL21 cells were harvested from a 1 L culture in LB media (A_{600} ~1.5), and the cells were washed twice in
124 buffer A (50 mM Tris, pH 7.7, 60 mM potassium glutamate, 14 mM magnesium glutamate, 2 mM DTT). Washed
125 cells were lysed using the homogenizer, and insoluble debris was spun down at 20000 *xg* for 25 minutes. The
126 supernatant containing the ribosomes was removed and the ribosomes were pelleted by centrifugation for 3 hours
127 at 50000 rpm at 4 ℃ in a Beckman Ti70.1 rotor. The pellet containing the ribosomes was resuspended in buffer A,
128 and the rRNA was purified using standard phenol-chloroform extraction protocols. The final rRNA concentration
129 was determined using the Nanodrop One[C], where 1 OD_{260} = 40 µg/mL RNA.
130

RNA labelling using periodate oxidation

132 The 3'-hydroxyl of RNA was labelled with AlexaFluor™647-hydrazide (Thermofisher Scientific) using periodate
133 oxidation based on manufacturer's protocol. The RNAs labelled using this procedure included the purified rRNA,
134 polyadenylic acid potassium salt (P9403 Sigma) or polyuridylic acid potassium salt (P9528 Sigma). The nucleic
135 acids were purified using a standard isopropanol precipitation followed by 70% ethanol precipitation, or using the
136 Amicon®-Ultra spin concentrators (Millipore) (as per method described (15)). An agarose gel was used to double
137 check complete free dye removal, before the sample concentrations were calculated using the Nanodrop One[C],
138 where 1 OD_{260} = 40 µg/mL RNA, and the dye fluorescence concentration was calculated using the extinction
139 coefficient (at 649 nm) of 250,000 $cm^{-1}M^{-1}$ (as described by the manufacturer). The AlexaFluor647-labelled RNAs
140 were denoted with A647.
141

Making NPM1-RNA condensates

143 All experiments were performed using a standard base buffer (20 mM Tris-HCl, pH 7.2, 250 mM potassium
144 glutamate) with different concentrations of magnesium glutamate (often denoted as Mg^{2+}), as specified per
145 experiment. The buffers were made as a 4X concentrated stock and were diluted to a final 1X working
146 concentration, along with the other components, using Milli-Q water. The final concentrations of NPM1 protein and
147 RNA used in every experiment were 20 µM and 100 ng/µL, respectively. Under all buffer conditions, NPM1 was
148 required to nucleate NPM1-RNA condensate formation. In a typical experiment, the 4X buffer is first mixed with
149 Milli-Q water, followed by RNA, then the NPM1, after which samples were mixed and pipetted onto a functionalized

3

150 microscopy slide for imaging. Unless otherwise stated, samples were left to incubate on the glass slides at room
151 temperature for at least 45 minutes before imaging.
152

Fluorescence confocal microscopy setups
154 Two fluorescence confocal microscopy setups were used for the experiments in this paper: (1) Olympus IX81
155 spinning disk confocal microscope, equipped with an Andor FRAPPA photobleach module and Yokogawa CSU-
156 X1 spinning disk. The Andor 400 series solid state lasers were used to bleach and image the samples. All the
157 images were recorded with a 100× oil immersion objective (NA 1.5) and an Andor iXon3 EM CCD camera. (2)
158 Leica SP8 Liachroic-beam splitting confocal laser scanning microscope, equipped with a PMT detector, 2 x HyD
159 SP GaAsP detectors and Leica DRC7000 GT monochrome camera. All the images recorded using this confocal
160 used a HC PL APO 63x/1.40 (oil) CS2 (0.14 mm) objective.
161   Two types of microscopy slides were used: the 18-well Ibidi chambers for quick imaging, as well as PDMS
162 chambers made in-house. The PDMS chambers were attached onto plasma-primed cover glass slides (No. 1.5H).
163 These PDMS chambers were used for experiments that exceeded 1 hour, as Vaseline-sealed coverslips were
164 applied on top to avoid evaporation. For both setups, the glass surfaces were cleaned using a plasma cleaner,
165 then incubated for 1 hour with 0.1 mg/mL PLL(2)-g[3.5]-PEG(2) (SuSoS AG, Switzerland) dissolved 10 mM
166 HEPES, pH 8.0, before the surface was thoroughly washed with Milli-Q water and dried with nitrogen gas.
167

Fluorescence recovery after photobleaching (FRAP)
169 Fluorescence recovery after photobleaching (FRAP) experiments were conducted on the Olympus IX81 spinning
170 disk confocal microscope set-up. FRAP measurements were carried out by selecting a small ROI in the middle of
171 a coacervate or aggregate of interest, and bleaching with the appropriate wavelength of the laser, depending on
172 the sample. The 488 nm laser line was set at 100% laser power using 75 pulses of 150 µs, and the 647 nm laser
173 line was set to at 100% laser power using 75 pulses of 100 µs. When both wavelengths were used, the 647 nm
174 laser bleaching preceded that of 488 nm laser. The recovery was imaged at reduced laser intensity (at least 5–
175 fold lower) and a regular time intervals, depending on the sample.
176   Using a MATLAB script, the experimental recovery was first normalized before being fitted to a simple
177 exponential, as a first-order approximation of 2D diffusion with a fixed boundary (i.e. droplet edge) (16). Briefly,
178 the exponential decay equation: $y = A(1 - e^{-bt}) + C$
179   From this equation, the recovery half-life (τ) was calculated by $\tau = \ln(2) / b$ and percentage recoveries
180 were extracted by multiplying A by 100. We note that the theoretical maximum recovery is limited by the size of
181 the bleached spot, which was 13.1 ± 4.4% of the droplet area in our experiments, corresponding to a maximum
182 theoretical recovery of ~87%.
183

Aging experiments
185 The CSU temperature stage (Tokai-HIT) was used to set the temperature of the samples within a range of 8 –
186 39℃. Sample temperatures were always confirmed using a thermometer at the end of each experiment. The
187 condensate components and buffers were pre-incubated at the temperature of interest prior to mixing, and being
188 immediately added to functionalized PMDS slides that were sealed with Vaseline. An Arrhenius plot of the τ
189 data revealed two straight lines with a transition around 21-22℃ (**Figure S2A**) and a transistivity plot (**Figure S2B**) was
190 derived from the Arrhenius plot to show this drastic transition.
191

Circularity (Figures 2C and D)
193 The NPM1-rRNA condensates were mixed and deposited onto the Vaseline-sealed PDMS slides incubating at
194 8℃ (sample temperature). Note the CSU temperature (Tokai-HIT) stage was set to 4℃, but sample temperature
195 was recorded throughout the experiment using a buffer blank in an adjacent well, and it is the sample temperature
196 that is reported in our figures. Samples were incubated for at least 45 mins before imaging began. Sample
197 temperature was adjusted incrementally with a 10-minute pause before imaging. At least three different images
198 were captured and the circularities calculated here were averaged across the population of condensates.
199   To monitor changes to the condensate circularity upon ATP addition, the NPM1-rRNA condensates were
200 made in 14 mM Mg^{2+} buffer and left to incubate in Ibidi slides for 50 minutes before 11 mM ATP was added to a
201 corner of the slide. Using the Olympus IX81 confocal microscope, the sample with focus on one large condensate
202 was imaged and the circularities calculated over time. We expect the circularity for the single condensate after
203 ATP addition to be less than 1 because the final free Mg^{2+} concentration was 3 mM. Indeed, the circularity of the
204 sample population was to 0.85 ± 0.13 after ATP addition.
205   Raw fluorescence confocal microscopy images and videos were processed and analysed with MATLAB
206 2021 Image Processing Toolbox. Objects smaller than 200 pixels were excluded from the analysis (to avoid
207 detecting smaller spherical droplets that tend to initially form as they settle onto the glass) and the area and mean

4

208 intensity were extracted for every object in each frame. For circularity calculations, the perimeter of each object
209 was calculated by taking the sum of all pixels that were directly adjacent to the dilute phase plus half the sum of
210 all pixels that were only diagonally adjacent to the dilute phase. The circularity (ϑ) of the object cross section was
211 then calculated as $\vartheta = 4\pi A/P^2$, where A is the area of the object and P the perimeter (17). Subsequently, a single
212 ϑ for each frame was calculated by taking the mean ϑ ± standard deviation.
213

ATP, ADP and AMP experiments (Figure 2 E-G)
215 Samples were prepared in a sequence as outlined above, and nucleotides were mixed last before everything was
216 deposited onto a glass slide for imaging. The samples were mixed in base buffer containing 14 mM Mg^{2+}, and
217 each nucleotide was at 10 mM final concentration. The FRAP experiments were performed as outlined above.
218 Errors are standard deviations from at least duplicate experiments.
219

SYBR Gold intensity calculations (Figure S6)
221 SYBR Gold was used to indirectly probe rRNA compaction within the condensates. According to Kolbeck *et al*, the
222 SYBR Gold 10000X stock is 12.4 ± 1.2 mM. Therefore, at 40 nM, the final SYBR Gold concentration used per 30
223 μL experiment is 62.5X below that of the reported 2.5 μM concentration where quenching effects were observed.
224 SYBR Gold fluorescence is dependent on not just concentration, but also local environment (18). To test this, the
225 condensate samples – using unlabelled components – were pre-mixed as outlined above and the SYBR Gold
226 was added last. In some cases, the Mg^{2+} and ATP were added sequentially after condensate formation. There
227 were no noticeable differences in fluorescence when comparing with pre-mixed and sequential samples. In fact,
228 our reported results are a combination of sequential and pre-mixed intensities.
229 Since we were comparing SYBR Gold fluorescence between samples with different Mg^{2+} and/or ATP
230 concentrations, the confocal images were taken using the Leica SP8 set-up. For all the images, the same laser
231 settings were used. The fluorescence intensity of SYBR Gold from confocal microscopy images and videos were
232 processed and analysed with MATLAB 2021 Image Processing Toolbox. The images were binarized with an
233 automatic intensity threshold computed with Otsu's method (19). Objects smaller than 50 pixels were excluded
234 from the analysis and the area and mean intensity were extracted for every object in each frame.
235

Partitioning experiments (Figure 3 A-C)
237 For partitioning of client molecules we prepared stock solutions at 33 μM 5,6-FAM-RP3 (CASLO) and 50 μM 5,6-
238 FAM-SNORD52 (IDT) in the standard base buffer containing 5 mM Mg^{2+}. The ribosome clients, 70S ribosomes-
239 A488 and 70S ribosomes-D650, were thawed and centrifuged for 10 minutes at 20000 *xg* to remove any
240 aggregates, and the supernatant was used for partitioning experiments. The ribosome concentration in the
241 supernatants did not diverge much from the stock concentrations described above.
242 For each partitioning experiment, 1 μL client was mixed with either the forming coacervates, or with the
243 RNA component prior to NPM1 addition. Both orders of addition yielded similar results. The mixtures were
244 incubated for 45 minutes on the glass slides at room temperature, before imaging.
245 For imaging, we used the Leica confocal setup with laser excitations of 488 nm (FAM, ATTO488) and 647
246 nm (Dylight650). For each laser setting, a blank was imaged made from NPM1-rRNA with one of the components
247 labelled, accordingly. The partitioning coefficient was then calculated using: K_p = ($I_{coacervate}$ - $I_{background}$)/(I_{dilute} -
248 $I_{background}$), where $I_{coacervate}$ is the average intensity as determined by a pixel gray value cut off above 40, and I_{dilute}
249 is the average intensity as determined by a pixel gray value cut-off below 10. The errors are standard deviations
250 of at least three sets of K_p values derived from three different images.
251

Ribosome halo formation
253 The ribosome halo formation was monitored over time using both confocal set-ups as highlighted in the video
254 captions. The pixel intensity measurement (**Figure 3F**) was from a time-lapse experiment of ATTO488-labelled
255 70S ribosomes where the video was taken shortly after sample mixing. Using ImageJ, a line of fixed pixel length
256 (equivalent to 9.23 μm) was drawn and the pixel intensity for each frame of the droplet was measured.
257

Apyrase enzyme experiments (Figure 6)
259 Apyrase Grade VII from Potato enzyme was dissolved in standard base buffer containing 5 mM Mg^{2+} (20 mM Tris-
260 HCl, pH 7.2, 250 mM potassium glutamate, 5 mM magnesium glutamate). For the FRAP experiment, the samples
261 were made using 5 mM Mg^{2+} base buffer with additives: ATP, AMP and ATP with apyrase. 5 U apyrase solution
262 was added to the forming coacervate mix and all the samples were left to equilibrate on the Ibidi microscope slides
263 for 30 minutes before imaging and FRAP experiments were performed. Error bars are standard deviations from at
264 least two different photobleaching experiments.

265   The morphology changes to the NPM1-rRNA condensates were monitored using the Leica SP8 confocal
266 microscope. Again, the samples were prepared as above, with 5U apyrase added to the forming coacervates. The
267 sample was left to incubate on functionalized Ibidi slides for 30 minutes prior to imaging using both the 488 nm
268 (NPM1-A488) and 647 nm (rRNA-A647).
269   Lastly, for the ribosome halo and apyrase experiments, 5 U apyrase solution was added to the formed
270 coacervates on one side of the well of the microscope slide, and the disappearance of the ribosome halo was
271 monitored every 1 second.

272   **RESULTS**

273

274   **Mg^{2+}-induced RNA compaction slows dynamics and leads to gelation**

275   In order to probe the influence of environmental factors on heterotypic condensates that contain both RNA and
276   protein components, we chose NPM1-rRNA as a model system (**Figure 1A**). NPM1 proteins are localized to the
277   outer layer of the nucleoli, in the so-called granular component, and was shown to readily form condensates with
278   rRNA *in vitro* (10). Similar to previous work (10), by mixing NPM1 proteins with rRNA in buffer containing no Mg^{2+},
279   spherical liquid-like droplets appeared. Simultaneous fluorescence after photobleaching (FRAP) analyses for both
280   protein and RNA components demonstrated a noticeable difference in FRAP recovery even at 0 mM Mg^{2+}
281   concentration, with a faster recovery half-life (τ) for NPM1 at 18.3 ± 2.4 s and slower recovery for rRNA at 30.7 ±
282   6.1 s (**Figure 1B and C**). The τ for NPM1 is comparable to *in vivo* values of around 20 s (10,20). The difference
283   between protein and RNA recoveries is reminiscent of other intracellular protein-RNA condensates, where the
284   constituent RNAs recover slower than protein components; this was attributed to multivalent RNA-RNA
285   interactions (12,21).

286   In order to promote such RNA-RNA interactions in the NPM1-rRNA condensates, we increased the Mg^{2+}
287   concentrations and checked the partitioning of each of the components within the condensates (**Figure 1D**). At
288   higher Mg^{2+}, rRNA partitions more strongly within condensates. We also observed the NPM1 protein and RNA
289   dynamics diverge, with rRNA recovering slower and to a lesser extent (**Figure 1E and F**), while NPM1 recovery
290   remained unchanged. At Mg^{2+} concentrations higher than 7 mM, rRNA is fully arrested with τ >1500 s and
291   percentage recoveries decreased to <10% (**Figure 1G**). These findings suggest a gradual transition from liquid to
292   an arrested, gel state of the RNA component of the nucleolus, which is supported by the scaling behavior of the
293   relaxation times near the critical point (**Figure 1E**) (22,23). The NPM1 component remained astonishingly mobile
294   within the rRNA gel network as the Mg^{2+} concentration increased, with low τ (**Figure 1E**), which is consistent with
295   other reported FRAP recoveries for proteins in tangled RNA networks (10,20). The percentage recovery of NPM1
296   proteins appeared to not significantly decrease until 20 mM Mg^{2+} was reached (**Figure 1F**). NPM1 thus shuttles
297   between different regions of the gelled RNA network. Finally, we note that when rRNA dynamics is fully arrested,
298   the condensates adopt an irregular-shaped morphology (**Figure 1G**) with gel-like states that can still slowly fuse
299   together within the time scale of several minutes (**Video S1**), reminiscent of nucleoli fusing *in vivo* (9). These
300   results suggest that RNA-RNA interactions, and by extension, Mg^{2+} concentrations, play an important role in
301   shaping condensate dynamics.

302   Mg^{2+} stabilization of RNA-RNA interactions and corresponding compaction of RNA chains is well reported (1,4,5).
303   The observed rRNA arrest at high Mg^{2+} is likely due to the compaction of RNA, promoted by enhanced RNA-RNA
304   base-paring and stacking interactions, leading to slowed diffusion of these entangled polymers in the dense phase.
305   To further explore this process, NPM1 condensates were made with other homopolymeric RNAs with different
306   propensities for RNA-RNA interactions. Poly-adenosine (pA) RNA is known to form base-stacking interactions with
307   Mg^{2+}, whereas poly-uridine (pU) remains largely unstructured (24). At 20 mM Mg^{2+}, condensates made from rRNA
308   or pA both formed gels, whereas condensates made from pU remained spherical, liquid droplets (**Figure 1H**) that
309   still readily fused with one another within seconds (**Video S2**). The FRAP parameters also reflected this, with τ
310   increasing for pA at increasing Mg^{2+}, but pU τ's were unaffected by Mg^{2+} (**Figure 1I**). The gelation induced by RNA
311   compaction can also be seen in decreasing percentage recoveries for pA at increasing Mg^{2+} concentrations, while
312   pU percentages remained relatively unchanged (**Figure 1J)**, in agreement with previous studies on Mg^{2+}-induced
313   folding of single 16S rRNA and pU mRNA molecules (25). Together, these results suggest that RNA compaction
314   is facilitated by Mg^{2+} induced RNA-RNA interactions, which occur in RNAs with strong base pairing and base
315   stacking interactions, such as rRNA and pA, but not in RNAs with weak base stacking interactions, such as pU.
316   Together, our results suggest that, in systems where RNA-RNA interactions can be stabilized with Mg^{2+}, the RNA
317   component imbues protein-RNA condensates with viscoelastic material properties.

318

319   **Tuning rRNA compaction with temperature and ATP**

320   We hypothesize that RNA forms a viscoelastic network in heterotypic protein-RNA condensates, such as for this
321   NPM1-rRNA system, based indirectly on the partial FRAP recoveries and slowing dynamics with increasing Mg^{2+}.
322   Additional evidence for the viscoelastic nature of the rRNA component is its gradual aging to a more structured
323   state, with slower relaxation observed as an increasing τ over time that reaches a plateau (**Figure 2A**). In contrast,

324   the τ of NPM1 remains relatively constant over time (**Figures 2B and S1**). This relaxation varies non-linearly with
325   the inverse temperature ($1/T$), which further supports the viscoelastic nature of rRNA in the condensates. In a pure
326   viscous liquid, where FRAP recovery is commonly attributed to simple Stokes-Einstein diffusion, the diffusion
327   coefficient should increase as $1/T$, and the recovery time should be linearly proportional to temperature. However,
328   this is clearly not the case for NPM1/rRNA condensates, as there is a strong non-linear dependence of τ on T
329   (**Figure 2B**). A closer inspection reveals that the temperature dependence shows signs of an activated process
330   that leads to structuring and relaxation. Our data can be fitted using an Arrhenius equation with a fixed activation
331   energy for temperatures above the gel point (**Figure S2A**). Below that point, the activation energy increases to a
332   higher level, as shown by the transitivity plot (**Figure S2B**), which is characteristic of gels and glasses (26).
333   Therefore, even at 0 mM Mg^{2+}, the rRNA network appears to be a viscoelastic material, held together by multivalent
334   intra- and intermolecular RNA-RNA interactions. In contrast, the NPM1 protein can diffuse freely under all
335   conditions, consistent with observations that nucleoplasmic NPM1 concentrations increase with higher overall
336   NPM1 expression levels (27).

337   Since Mg^{2+} stabilizes RNA-RNA interactions, it was anticipated that this could affect the τ at maturation of
338   condensates formed in Mg^{2+} buffer. Indeed, condensates made in 5 mM Mg^{2+} had slower observed τ at maturation,
339   which decreased at higher temperature (**Figure S3**). This result made us curious about the effect of temperature
340   on Mg^{2+}-stabilized RNA-RNA interactions, and how this could impact condensate morphology. NPM1-rRNA
341   condensates formed at 8 ℃, in either 5 mM or 10 mM Mg^{2+} buffer after 60 minutes of incubation, had a striking
342   non-spherical morphology (**Figures 2C and S4**). The 10 mM Mg^{2+} condensates appeared smaller and had lower
343   average circularity than the 5 mM Mg^{2+} condensates, but both circularities were lower than at higher temperatures,
344   where values increased closer to 1, which is a characteristic of spherical liquid droplets(17). These results suggest
345   that RNA is fully arrested at lower temperatures in 10 mM Mg^{2+}, with the small gel-like condensates unable to
346   completely fuse together. Instead, the small gel droplets partially fuse, at the time of formation, to form irregular-
347   shaped condensates similar to other gel-like RNA condensates (12). As temperature was increased, the gel-like
348   condensates relaxed and coalesced, eventually, into spherical droplets, which resulted in increased average
349   circularity of the condensate population (**Figure 2C**). In fact, the condensates appeared spherical at 35 ℃, with
350   circularities close to 1, for both Mg^{2+} concentrations. This temperature-dependent relaxation of the NPM1-rRNA
351   condensates manifesting as a morphological change provides further evidence for gelled states being caused by
352   RNA compaction that results in incomplete fusion of gel condensates. Since circularity changes are a one-way
353   process, we demonstrated thermo-reversibility of the rRNA component in condensates using FRAP (**Figure S5**).
354   The responsive morphology changes of NPM1-condensates within the temperature range of living systems, hints
355   that this could be an important parameter to consider for nucleoli dynamics.

356   In homeostatic eukaryotic cells, Mg^{2+} concentrations and temperature are often well regulated (28,29), but local
357   ATP levels are known to fluctuate (7,8). ATP strongly chelates Mg^{2+}, changing the effective free Mg^{2+}
358   concentrations. When we tested the effect of ATP addition to the NPM1-rRNA condensates that were incubated
359   with Mg^{2+}, we observed that ATP caused the gel-like morphologies to liquefy, and the circularity increased over
360   20 minutes (**Figure 2D**). ATP relaxes the rRNA network, with FRAP recoveries corresponding to the remaining
361   free Mg^{2+} concentrations (**Figure S6**). In order to probe the influence of ATP on RNA compaction, we used SYBR
362   Gold fluorescence to probe the RNA-RNA interactions within the condensates. SYBR Gold is a fluorescent dye
363   that intercalates double stranded nucleic acids (18), and we hypothesize that Mg^{2+} will stabilize such interactions
364   between the rRNAs. This was indeed the case, as the average SYBR Gold fluorescence detected inside the
365   NPM1-rRNA condensates increased by ~41% on Mg^{2+} addition (**Figure S7**). Subsequently, the SYBR Gold
366   fluorescence decreased when ATP was added, providing indirect evidence for decreased RNA compaction due
367   to ATP chelating Mg^{2+} (**Figure S7**). This liquefying effect is less pronounced for other adenosine nucleotides (ADP
368   and AMP) that do not chelate Mg^{2+} as effectively (**Figure 2E-G**). These results demonstrate how ATP can be a
369   key regulator of free Mg^{2+} concentrations that can impact NPM1-rRNA condensate dynamics.

370

371   **Consequences of ATP:Mg^{2+} on condensate function: partitioning of clients**

372   Alongside chelating Mg^{2+}, ATP can also bind to the nucleic acid interacting domain of NPM1 (30,31), which could
373   lead to an altered chemical microenvironment inside the condensates. Inspired by the profound changes to
374   condensate dynamics observed with Mg^{2+} and ATP, we were curious whether these small molecules would
375   influence the partitioning of component proteins, RNAs and client molecules inside the condensates. Indeed, we
376   observed that, at higher Mg^{2+} when there is no ATP, the partitioning coefficient (K_p) of rRNA increased on
377   compaction, and the NPM1 protein K_p decreased, as more RNA-RNA interactions could exclude protein-RNA

8

378 interactions (**Figure 3A and B**). ATP increased the K_p of both RNA and protein components. We speculate that
379 ATP, when bound to the nucleic acid interacting domain (30,31), can also stabilize the interaction between NPM1-
380 rRNA, because the nucleic acid interacting domain is also where NPM1 binds rRNA (32). As a result, a synergistic
381 increase in partitioning of both components inside the condensates occurred only when Mg^{2+} and ATP were
382 present, and not when ADP or AMP were present (**Figure S8**).

383 We then tested whether the altered microenvironment has a functional consequence in the partitioning of two
384 model clients, the $(RRASL)_3$ peptide (RP3) and the SNORD52 RNA. RP3 resembles arginine-rich peptides that
385 can electrostatically interact with both the NPM1 protein and rRNA (33), whereas SNORD52 is a small nucleolar
386 RNA that binds specifically to NPM1 (34) (**Figure 3C**). The K_p of both clients followed trends that are expected
387 based on their interaction strengths with the condensate components (**Figure 3C**). The SNORD52 RNA K_p
388 increased at higher ATP concentrations as more NPM1 partitioned into the condensates. Conversely, the RP3 K_p
389 decreased as less binding surface was expected due to the synergistic partitioning and interaction of NPM1 and
390 rRNA at higher ATP concentrations. These results indicate that Mg^{2+}:ATP can be used to tune client partitioning
391 within NPM1-rRNA condensates.

392 As nucleoli are the sites of ribosome biogenesis, we next investigated the effect of Mg^{2+} and ATP on the partitioning
393 of ribosomes into NPM1-rRNA condensates. Here, we used labelled mature 70S ribosomes that should remain
394 intact at Mg^{2+} concentrations above 5 mM (6). Therefore, it is unsurprising that at increasing Mg^{2+}, the K_p
395 decreased as the ribosomes remained intact in solution (**Figure S9**). In contrast, ATP addition resulted in a 30%
396 increase in K_p, likely due to chelation of Mg^{2+} that was previously bound to the ribosomes, thereby destabilizing
397 the ribosome structure and causing it to partition inside the NPM1-rRNA condensate (**Figure S9**). This
398 destabilization of ribosome structure was previously reported when free Mg^{2+} concentrations were below 5 mM
399 (35). Interestingly, under conditions where Mg^{2+} concentrations exceeded ATP concentrations, the ribosomes
400 formed a striking halo around the NPM1-rRNA condensates (**Figures 3D and E**). The ribosome halo excluded the
401 rRNA, and resulted in a deformed rRNA condensate shape (**Figure 3D**). In contrast, the NPM1 fluorescence is
402 homogeneously distributed throughout the condensates localizing to both the ribosome and rRNA (**Figure 3E**),
403 which is expected from a protein that can bind to all rRNAs present. Indeed, these heterotypic multicomponent
404 interactions of NPM1 with rRNA and the pre-ribosomal subunits, have been suggested to drive ribosome assembly
405 (27), and here we show that NPM1 bridges the interactions between the rRNA component within the condensates
406 and the 70S ribosomes in the halo.

407 In order to examine our hypothesis that the halo appeared due to partly destabilized 70S ribosomes, we monitored
408 the condensate growth over time. The ribosome halo appeared after ~25 minutes, with an increased fluorescence
409 intensity observed around the edge of the condensates (**Figure 3F**). Here, the ribosome-NPM1-rRNA condensates
410 exhibited decelerated fusion between larger droplets, in the order of tens of minutes (**Video S3**). A possible reason
411 for slow droplet fusion could be the viscoelastic nature of the ribosome halo and its rearrangement that would be
412 necessary for rRNA-NPM1 condensates to fuse. Indeed, we found that the ribosome halo had slow FRAP
413 dynamics with an average τ of 490 ± 10 s, and percentage recoveries of 45 ± 11%. As the droplets fused, the
414 ribosome halo seemed to become incorporated into the condensate and appeared inside as bright spots, with
415 some droplets stalled mid-fusion (**Video S3**). As a control, we made NPM1-rRNA condensates in 10 mM Mg^{2+} and
416 left these to mature for 2 hours, before 5 mM ATP was added, to verify that the observed halo was not a transient
417 phenomenon linked to nucleoli maturation, but rather a result of destabilized ribosomes that originate from the
418 dilute phase. Here, the ribosome halo also appeared after approximately ~10 minutes of ATP addition, alongside
419 separate droplets composed of NPM1 and ribosomes (**Video S4, Figure S10**), which is reminiscent of *in vivo*
420 experiments showing NPM1 interacting with 60S pre-ribosomes (36). Our results suggest that the ATP addition
421 destabilizes the ribosomes in solution, which in turn could liberate ribosome-associated rRNA to interact with
422 NPM1, forming the halo. The exclusion of partially destabilized ribosomes from the NPM1-rRNA condensates is
423 an exciting result as this corroborates the *in vivo* behavior of nucleoli (27), but to our knowledge this is the first
424 time this phenomenon has been observed *in vitro*. Therefore this work not only provides a useful platform for
425 further studying thermodynamic exclusion from condensates, but also suggests that ATP:Mg^{2+} could be one of the
426 ways cells can alter the condensate environment to drive ribosome formation.

427

428 **Enzymatic depletion of ATP levels changes condensate properties and function**

429 Within cells, ATP levels fluctuate due to a variety of enzymes: from ATP-dependent chaperones to reactions that
430 consume ATP (5,7,8). Here we demonstrate that ATP removal using a dissipative enzymatic reaction can also

431 control the condensate properties of the nucleolus-like condensates made from NPM1 and rRNA. Using apyrase,
432 an enzyme that converts ATP to AMP (**Figure 4A**), we showed that the rRNA recovery of samples without and
433 with apyrase reflected those for the ATP and AMP nucleotides, respectively, similar to observations in Figure 2
434 (**Figure 4B-D**). Apyrase can effectively liberate Mg^{2+} from ATP, thereby causing rRNA arrest and morphology
435 changes for samples made in high Mg^{2+} concentrations (**Figure 4E-G**). This change in morphology from spherical
436 liquid-like to irregular-shaped gel-like states is remarkably similar to observations for purified and *in vivo* nucleoli
437 when ATP was depleted (9,11). For the purified and *in vivo* nucleoli, ATP-dependent chaperones were
438 hypothesized to facilitate liquid-like condensate dynamics. However, our results highlight the impact of liberated
439 Mg^{2+} due to ATP depletion, and demonstrate that changes to free Mg^{2+} levels may corroborate these *in vivo* and
440 our *in vitro* observations. Here, enzymes that consume ATP, and not necessarily active ATP-dependent
441 chaperones, are a possible route that cells use to regulate Mg^{2+} levels and impact nucleoli dynamics.

442 As nucleoli should exclude fully folded ribosomes (27), we were curious whether ATP removal would affect the
443 previously observed ribosome halo around the NPM1-rRNA condensates. We hypothesized that the ribosome
444 halo was formed from destabilized ribosomes interacting with NPM1, but to what extent the ribosomes were
445 destabilized and whether this was reversible was not yet clear. ATP was depleted using apyrase, which resulted
446 in increased available Mg^{2+} concentrations, and as a result, we observed the striking disappearance of the
447 ribosome halo over time (**Figure 4H**). Additionally, the ribosome-NPM1 puncta formed outside the NPM1-rRNA
448 condensates (**Video S5**) also vanished on apyrase addition, suggesting that fully folded ribosomes are excluded
449 from the NPM1-rRNA condensates. With the disappearance of the ribosome halo, the NPM1-rRNA condensates
450 that were previously stabilized mid-fusion were able to relax and fuse again, and the resulting condensates
451 appeared unexpectedly spherical in shape (**Video S6**), despite the higher available Mg^{2+} concentrations. The more
452 spherical final condensates are hypothesized to be a result of altered Mg^{2+} ions concentrations: although liberated
453 from ATP, the Mg^{2+} will likely bind to the 70S ribosomes making the ion less available to stabilize rRNA-rRNA
454 interactions. These results demonstrate that enzymatic changes to ATP concentrations can have a profound effect
455 on client localization around the NPM1-rRNA condensates, and could indeed be a way how cells change the flux
456 of client molecules interacting with nucleoli.

457

458 **DISCUSSION**

459 Many biomolecular condensates are portrayed as heterotypic protein-RNA droplets with viscoelastic material
460 properties, governed by primarily by interactions between proteins and RNA (2,3,37,38). However, here we show
461 that the RNA component itself has a determining role in the shape, dynamics and material properties of protein-
462 RNA condensates, especially in systems where the RNA network is formed via RNA-RNA interactions tuneable
463 by changes in Mg^{2+}:ATP, or temperature. The viscoelastic properties of the RNA network depend on the structure
464 of the RNA and the strengths of the intermolecular RNA-RNA interactions. NPM1-pU condensates, for example,
465 remained largely liquid-like even at high Mg^{2+}, suggesting pU RNAs do not form a strong network, while NPM1-
466 rRNA and NPM1-pA condensates showed dynamic arrest at high Mg^{2+}. Indeed, our results at high Mg^{2+} for rRNA
467 and pA condensates are reminiscent of the gel-like morphologies observed in reconstituted protein-RNA
468 condensates, where increased intermolecular interactions are engineered for one component, either protein (39)
469 or RNA (12,24), within the network. Here, we demonstrate that also the environment can influence the
470 intermolecular interactions in RNA, and this can be a way to control the dynamic material properties of protein-
471 RNA condensates, like NPM1-rRNA condensates.

472 Homotypic protein-based condensates were previously shown to mature over time as viscoelastic Maxwell fluids
473 (40), with slowed aging at increased temperatures. The aging of NPM1-rRNA condensates is similarly
474 temperature-dependent with faster т at maturation for RNA at higher temperatures. This plateau in т observed after
475 1h is also reminiscent to another *in vitro* system of protein-only condensates formed via Ni^{2+} interactions (41).
476 Additionally, an aging phenomenon was also documented for *in vivo* protein-RNA condensates where FRAP
477 recoveries of the protein component at 5 hours after formation was faster than at 16 hours (12). Although the
478 physiological relevance is still unknown, aging seems to be a common feature of viscoelastic condensates formed
479 via multivalent interactions, and here, we show for the first time, that the RNA component also undergoes aging.

480 Since nucleoli are rich in RNA (42), our work on NPM1-rRNA condensates, highlighting the considerable influence
481 of Mg^{2+}:ATP on the dynamics of the RNA network, can in turn help us better interpret the puzzling observations of
482 actual nucleoli. Indeed, *in vivo* nucleoli are gel-like, with slow fusion time scales of ~30 minutes and non-spherical
483 shapes (9-11), however the localised proteins had fast dynamics, in the order of seconds (20,43), which never

10

484   explained the material properties of nucleoli. By considering the RNA component, our work shows that NPM1-
485   rRNA condensates at 5 mM Mg^{2+} fused in the order of minutes (**Video S1**), and at Mg^{2+} concentrations >7 mM,
486   gel-like morphologies were observed. Our reconstituted system approximates nucleoli and, along with recent
487   findings of rRNA movement through the nucleolus (44), supports this hypothesis that the viscoelastic contribution
488   of RNA in nucleoli accounts for its irregular shapes and slow fusion dynamics.

489   Additionally, our *in vitro* studies provides a valuable caveat for the interpretation of underlying causes observed in
490   ATP depletion studies. Numerous works on ATP-depleted nucleoli, purified and *in vivo*, resulted in irregularly
491   shaped structures reminiscent of incomplete fusion (9,11,45,46), and a similar observation of slowed recovery
492   dynamics was observed in ATP-depleted cells containing CAG-repeat RNA condensates (47). The slowed
493   condensate dynamics upon ATP depletion in all cases was attributed to an unknown ATP-dependent
494   process/enzyme required to maintain condensate fluidity. However, our ATP-depleted *in vitro* model, when
495   apyrase was added, challenges this perspective. Here, the depleted ATP liberates Mg^{2+}, resulting in the morphed
496   gel-like appearance of the protein-RNA condensates. Our results can help explain these *in vivo* observations,
497   without the need for ATP-dependent enzymatic activity to maintain condensate fluidity, by highlighting the
498   importance of considering free Mg^{2+} in the interpretation of ATP depletion results. Beyond enzymes, it is also
499   valuable to consider the changes in ATP levels during a cell cycle (8,48), and how this could impact the fluidity
500   and condensate properties of nucleoli and other protein-RNA condensates.

501   Together, our results show that protein-RNA condensates are responsive, responding to changes in the local
502   environment: from temperature to differences in Mg^{2+} and ATP concentrations. The small molecules influenced
503   partitioning of constituent condensate components, which in turn affected client partitioning. Transient changes to
504   ATP levels may be how cells regulate the amount of free Mg^{2+} and thereby control the dynamics of RNA-based
505   condensates, such as the nucleolus.

506
507   **SUPPLEMENTARY DATA**

508   Supplementary Data are available online.

509   **FUNDING**

512

513   **CONFLICT OF INTEREST**

514   The authors have no conflict of interest.

515

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520

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665 **TABLE AND FIGURE LEGENDS**

666

667 **Figure 1.** Mg^{2+}-induced RNA compaction leads to slow dynamics and gelation. (**A**) NPM1 protein and rRNA, both
668 labelled with different fluorophores, are mixed together to form condensates that show differences in FRAP
669 recovery (**B** & **C**), where NPM1 (green) recovers faster than rRNA (red). (**D**) The recovery of rRNA slows to a halt
670 at Mg^{2+} ion concentrations > 7 mM (shaded in yellow) and shows critical scaling behavior (purple fitted line)
671 indicating it forms a gel, whereas the NPM1 protein remains mobile. (**E**) The decrease in percentage recovery for
672 both NPM1 and rRNA reflects the gel environment at higher Mg^{2+} concentrations. (**F**) These droplets become gels
673 at higher Mg^{2+} where the rRNA is fully arrested, indicated by bleached regions not recovering (white arrows). In
674 order to test the RNA compaction hypothesis, NPM1-rRNA condensate morphology (**G**) and FRAP recovery
675 parameters (**H** & **I**) were compared with NPM1-pA and NPM1-pU condensates. The errors in this figure are
676 standard deviations from triplicate measurements. Scale bars are all 10 μm.

677

678 **Figure 2.** Temperature and ATP can reverse the effect of Mg^{2+}-induced RNA condensation. (**A**) NPM1-rRNA
679 condensates aging at 20℃ with individual τ for rRNA measured over time (dots) and a fitted exponential curve
680 (line). (**B**) The extracted plateau τ at maturation for rRNA (red) and NPM1 (green) was plotted for aged droplets at
681 different temperatures in 0 mM Mg^{2+} buffer. The errors here are derived from the exponential fits. (**C**) Forming
682 NPM1-rRNA condensates at 8℃ in buffer containing magnesium resulted in irregular gel-like morphologies that
683 changed to spherical droplets at increasing temperatures, with increased circularities. (**D**) The gels formed in 14
684 mM Mg^{2+} buffer also liquefied after 11 mM ATP addition, with increasing circularity reflected in the changed
685 morphology of the NPM1-rRNA condensates. (**E**) The FRAP recovery over times for samples that contain ATP
686 (yellow), ADP (orange) or AMP (blue) compared with just the 5 mM Mg^{2+} buffer (grey). The extracted τ (**F**) and
687 percentage recoveries (**G**) indicate that the better Mg^{2+}-chelating ability of ATP compared to other adenosine
688 nucleotides caused the condensates to liquefy. The errors in figures E-G are standard deviations from at least
689 duplicate measurements. Scale bars are all 10 μm.

690

691 **Figure 3:** The effect of Mg^{2+} and ATP on partitioning of components, clients and 70S ribosomes. The partitioning
692 coefficients of rRNA (**A**) and NPM1 (**B**) at different Mg^{2+} and ATP concentrations. (**C**) The partitioning coefficients
693 of 5,6-FAM-RP3 peptide (blue) and 5,6-FAM-SNORD52 RNA (orange) at different ATP concentrations in a 5 mM
694 Mg^{2+} base buffer. Confocal microscope images of NPM1-rRNA condensates with 70S ribosome as the client (**D** &
695 **E**). (**D**) The images show that rRNA-A647 is excluded from the ribosome fluorescence, whereas (**E**) shows NPM1-
696 A488 fluorescence as spherical droplets that occupy both locations where there is 70S ribosome and rRNA. (**F**)
697 The formation of the ribosome halo over time as indicated by an increase in fluorescence pixel intensity as peaks
698 at the edges of the droplet. The errors in this figure are standard deviations from triplicate measurements. Scale
699 bars are all 10 μm.

700

701 **Figure 4**: Enzymatic control of ATP concentrations influences condensate morphology and dynamics. (**A**) Apyrase
702 enzymes catalyze the removal of phosphate groups from ATP to form AMP, a nucleotide that poorly chelates Mg^{2+}.
703 (**B**) The effect of apyrase on the dynamics of rRNA after FRAP is clearly shown (**B**-**D**) where rRNA recovery in the
704 presence of ATP (yellow) drops when apyrase (purple) is added. In fact, the resulting FRAP parameters (**C** & **D**)
705 show that apyrase converts ATP to AMP which imbues the rRNA with gel-like dynamics. The errors in this figure
706 are standard deviations from triplicate measurements. The morphology of NPM1-rRNA condensates at 18℃ in 5
707 mM Mg^{2+} buffer (**E**) with 5 mM ATP added (**F**), and when apyrase is also added (**G**) shows the changes in
708 morphology that is expected from gel-like condensates in buffer containing Mg^{2+} that stabilizes RNA-RNA
709 interactions, and spherical morphology in conditions where ATP chelates the Mg^{2+} and liquefies these interactions.
710 (**H**) This series of confocal images over time of labelled 70S ribosomes show the disappearance of the ribosome
711 halo and puncta, as apyrase was added. ATP depletion results in higher Mg^{2+}, which stabilizes the ribosomes and
712 causes them to dissipate back into the dilute phase. Scale bars are all 10 μm.