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1 **Cell cycle-dependent mRNA localization in P-bodies**

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23 **Summary:**

24 Understanding the dynamics of RNA targeting to membraneless organelles is essential to
25 disentangle their functions. Here, we investigate how P-bodies (PBs) evolve during cell cycle
26 progression. PB purification across the cell cycle uncovers widespread changes in their RNA
27 content, which are partly uncoupled from cell cycle-dependent changes in RNA expression.
28 Single molecule FISH shows various mRNA localization patterns in PBs peaking in G1, S, or
29 G2, with examples illustrating the timely capture of mRNAs in PBs when their encoded protein

30 becomes dispensable. Yet, rather than directly reflecting absence of translation, cyclic mRNA
31 localization in PBs can be controlled by RBPs, such as HuR in G2, and by RNA features.
32 Indeed, while PB mRNAs are AU-rich at all cell cycle phases, they are specifically longer in G1,
33 possibly related to post-mitotic PB reassembly. Altogether, our study supports a model where
34 PBs are more than a default location for excess untranslated mRNAs.

35 Introduction

36 Proper compartmentalization of biological molecules is a fundamental aspect of cellular
37 organization. Recently, membraneless organelles received increasing attention for their
38 contribution in organizing subcellular space¹⁻⁵.

39 P-bodies (PBs) are ribonucleoprotein granules (RNPs) widespread throughout
40 eukaryotes and constitutively present in mammalian cells⁶. They contain a variety of RNA
41 binding proteins involved in translation regulation and RNA decay. Purification of PBs from
42 asynchronous human cells previously allowed the characterization of their RNA content⁷. PBs
43 accumulate one third of the coding transcriptome. These mRNAs are generally abundant,
44 inefficiently translated and strikingly AU-rich⁸. This nucleotide bias in the coding sequence
45 (CDS) results in a low-usage codon bias and poor protein yield. In the 3' untranslated regions
46 (3'UTRs) it favors accumulation in RNP granules, likely due to the binding of condensation-
47 prone RNA-binding proteins (RBP). PB-enriched mRNAs tend to encode regulatory proteins
48 whereas the mRNAs encoding house-keeping proteins tend to be excluded from PBs⁷. While
49 these studies provided a first picture of the PB transcriptome, further studies are required to
50 understand to which extent PBs adapt their content to cellular needs.

51 Various approaches have been used to investigate RNA localization in PBs. In situ
52 hybridization first showed that mRNA repressed by miRNA localize to PBs⁹. Then, single
53 molecule tracking of microinjected RNAs allowed examining their trafficking at a high temporal
54 resolution over minutes¹⁰. This revealed that miRNAs, repressed mRNAs, and lncRNAs can
55 associate transiently or stably with PBs. Other studies addressed RNA localization in PBs in
56 response to translational stress. For instance, relief of miR122-mediated silencing in response
57 to amino acid starvation or oxidative stress causes the release of its CAT-1 mRNA target from
58 PBs¹¹. Amino acid starvation leading to 60% translation reduction, also increased exogenous
59 RNA targeting to PBs¹². While such studies suggested a regulatory potential of mRNA targeting
60 to PBs under conditions of extreme translational reprogramming, the compositional dynamics of
61 PBs in unstressed conditions was not investigated.

62 The cell cycle is a program of physiological and molecular changes a cell undergoes to
63 produce two daughter cells. From an RNA metabolism perspective, cell cycle progression
64 involves specific waves of transcription¹³⁻¹⁸ and degradation¹⁹⁻²³. In terms of translation,
65 proliferative cells have a distinct tRNA signature compared to differentiating or arrested cells,
66 which may favor the translation of mRNAs with AU-rich codon usage²⁴. Regarding PBs, it was

67 shown that they dissolve at every mitosis, reform in G1 phase, and enlarge during S phase
68 progression²⁵. Interestingly, we previously found that, among the various regulatory proteins
69 encoded by PB mRNAs, cell cycle regulators were particularly enriched^{6,7}. We thus chose to
70 investigate the dynamics of RNA localization in PBs across the cell cycle.

71 Analyzing the composition of PBs purified at various cell cycle stages revealed
72 widespread changes in their RNA content during cell cycle progression. Some changes were
73 uncoupled from those occurring in the cytoplasm (due to variations in mRNA transcription or
74 stability), demonstrating a regulatory potential of PBs. Single molecule FISH (smFISH)
75 confirmed the diversity of PB localization patterns with some G2-induced mRNAs trafficking to
76 PBs specifically in early G1, when their encoded protein is no longer needed. Puromycin
77 experiments showed that the cyclic pattern of PB localization is not directly related to the
78 amount of untranslated transcripts in the cytoplasm. Rather, preventing HuR-mRNA interactions
79 abolished cyclic mRNA localization in PBs, particularly in G2. While we found no evidence of
80 phase-specific nucleotide or codon bias, we observed a marked bias in mRNA length in G1.
81 Altogether, our results demonstrate the existence of controlled differential RNA localization in
82 PBs across the cell cycle.

83 **Results**

84 **PBs enlarge during cell cycle progression**

85 Before analyzing the content of the PBs during the cell cycle, we first refined the description of
86 their global morphology and number across the cell cycle. To identify cell cycle stages, we relied
87 on the PIP-FUCCI system (see Methods, Figure S1A, B). PB labeling by immunofluorescence
88 (IF) against the classical PB marker DDX6 (Figure 1A) showed that PB size increased from G1
89 to G2 (Figure S1C) and DDX6 intensity in PBs increased in G2 (Figure S1D) regardless of PB
90 size (Figure 1B). However, the number of PBs per cell increased only modestly (Figure S1E).
91 As these changes could result from increased expression of PB proteins required for (DDX6,
92 LSM14A and 4E-T) or contributing to (PAT1B) human PB assembly²⁶, we separated HEK293-
93 FUCCI cells in G1, S and G2/M phases using fluorescence activated cell sorting (FACS).
94 However, none of the tested proteins was induced across the cell cycle (Figure S1F). In
95 summary, after dissolving in mitosis, PBs form in G1, progressively enlarge during interphase,
96 particularly in G2, while their number remains similar.

97 **PB purification reveals the same major PB proteins across the cell cycle**

98 To characterize changes in PB composition during cell cycle progression, we purified them from
99 different phases of the cell cycle using our previously developed Fluorescence Activated
100 Particle Sorting (FAPS) method⁷ (Figure 1C) and HEK293-GFP-LSM14A cells. In these cells,
101 GFP-LSM14A was expressed at levels similar to endogenous LSM14A and co-localized with the
102 PB marker DDX6 but not the stress granule marker TIA1 (Figure S1G-J). Cells were
103 synchronized in mid G1, G1S transition, mid S and G2M transition using a double thymidine
104 block or the selective CDK1 inhibitor²⁷ RO-3306 (see Methods, Figure 1D). Forty 15 cm plates
105 were synchronized per phase to obtain sufficient material for FAPS purification. Cytoplasmic
106 lysates were then prepared, with an aliquot kept aside for further analysis, termed the pre-
107 sorting fraction (PSF), while the remaining was used to sort PBs (see Methods, Figures 1E and
108 S2A). After sorting, a fraction of each purified sample was re-analyzed by flow cytometry and
109 visualized under microscope to verify that PBs were conserved while contaminants were
110 considerably reduced (Figures 1E and S2B).

111 After a total of >140 hr sorting, sufficient material was accumulated for proteomic
112 analysis of PBs from each cell cycle phase, using liquid chromatography-tandem mass
113 spectrometry (LC-MS-MS, Table S1). In all phases, the vast majority of known PB proteins (28-
114 43 proteins detected) were enriched in purified PBs compared to PSFs (Figures 1F, S2C-E).

115 These included translational repressors and RNA decay factors (DDX6, LSM14A, LSM14B, 4E-
116 T, EDC4 and DCP1A; a tentative list combining established PB proteins and FAPS-suggested
117 candidates is proposed in Table S1). In contrast, proteins such as mitochondrial proteins,
118 translation initiation factors, proteasome subunits, splicing factors and histones were mostly
119 depleted (Figures 1F, S2C-E, Table S1). The presence of FXR1 and FXR2 in PBs was
120 confirmed by IF, which revealed a particular crown-like localization around PBs (Figure 1G).
121 Given the inherently limited amounts of proteins obtained from purified PBs, we could not carry
122 out the replicates needed for quantitative MS. Nevertheless, a pairwise comparison of PBs from
123 successive cell cycle phases did not show drastic changes in the levels of the well-detected PB
124 proteins (Figure 1H). In summary, the proteomic analysis of purified PBs does not reveal major
125 changes of the main PB proteins across the cell cycle.

126 **PB RNA content is dynamic during cell cycle progression**

127 We then analyzed the RNA content of the purified PBs and their corresponding PSFs using
128 RNA sequencing. First, we assessed if the PSFs recapitulated known cell cycle-dependent RNA
129 regulation. Most RNAs previously reported as more expressed in G1S or in G2M in HeLa cells²⁸
130 were also more abundant in our G1S or G2M PSFs, respectively, despite the different cell line
131 and protocols (Figure S3A). Moreover, gene ontology (GO) analysis showed that RNAs more
132 abundant in our G2M sample encode proteins involved in chromatin condensation, chromosome
133 segregation, and cell division, while those more abundant in G1S encode proteins involved in
134 nucleotide metabolism, DNA replication and its regulation (Figure S3B,C). Altogether, these
135 analyses confirmed the reliability of our datasets. We then compared RNA levels before and
136 after sorting within each cell cycle phase (Figures 2A and S3D). Between 3,838 and 5,106
137 mRNAs were enriched in PBs ($p\text{-adj} < 0.05$, DEseq2 model²⁹) depending on the phase (Table
138 S2). Among them, 2043 were enriched in only a subset or a unique phase (Figure 2B).

139 Next, we compared the RNA content of purified PBs across the cell cycle (Table S3 and
140 S4, Figure S4A). Remarkably, this revealed widespread changes in RNA levels in PBs (Figures
141 2C, D and S4B). For example, from mid G1 to G1S, 2943 mRNAs had increased levels in PBs,
142 while 2599 decreased. To determine if the mRNAs with cyclic accumulation in PBs encode
143 proteins enriched for specific functions, we systematically performed GO analyses (Figure 2E).
144 We found that mRNAs accumulating in PBs between mid G1 and mid S were enriched (181 out
145 of 1889) for transcripts encoding proteins functioning in mitotic cell cycle phase transition,
146 chromosome condensation, and cytokinesis. Inversely, the mRNAs that decreased in PBs
147 between mid G1 and mid S were enriched (154 out of 1813) for transcripts encoding proteins

148 involved in transcription, transcriptional regulation, and RNA binding. This indicated that the
149 mRNA content of PBs is highly dynamic throughout the cell cycle.

150 **RNA accumulation in PBs is partly uncoupled from their cytoplasmic expression level**

151 Cell cycle variations in mRNA levels are controlled at the levels of transcription and/or
152 stability^{13,17–20,22,23,28}. The question here was whether there is an additional regulation at the level
153 of mRNA localization in PBs. If not, mRNA levels in PBs should follow their level in the
154 cytoplasm across phases. We therefore investigated the relationship between the RNA content
155 of PBs and the surrounding cytoplasm, using PSFs as a proxy. Comparing RNA changes in PBs
156 and PSFs from mid G1 to G1S showed an overall distribution along the diagonal (Figure 2F,
157 S4B-D), suggesting a broad coupling between the PB and cytoplasm contents. Yet mRNAs
158 decreasing in PBs tended to decrease twice less in the cytoplasm and some cases fell far from
159 the general distribution, as exemplified by FBXO5 and CLK1 mRNAs (studied later), indicating
160 some degree of uncoupling between the PB and cytoplasm contents. The pattern was similar
161 from G1S to mid S. In contrast, from mid S to G2M and G2M to mid G1, the PB content was
162 weakly coupled to the prominent cytoplasmic up and down regulations. For example, it did not
163 mirror the cytoplasmic increase in TOP2A, BUB1B, and CENPF levels (Figure 2F, studied later).
164 These data therefore suggested that the dynamics of the PB transcriptome can be uncoupled
165 from cytoplasmic variations, particularly during the second half of the cell cycle.

166 ***In-situ* confirmation of various patterns of cyclic RNA localization in PBs**

167 To support our transcriptomic analysis, we next analyzed several mRNAs (chosen based on
168 PB-enrichment pattern, p-adj values, and expression levels for reliable detection) using
169 smFISH, which allows *in-situ* localization and counting of individual RNA molecules^{30,31}.

170 We started with the mRNA of FBXO5, a major regulator of the anaphase promoting
171 complex (APC)^{32–37}. In our RNA-seq dataset, FBXO5 expression was highest in G1S and mid S
172 phases in the PSF, in accordance with its reported transcriptional induction at G1S³⁸. In purified
173 PBs, it also peaked at G1S and mid S, but with a much higher differential than mid G1 or G2
174 (Figure 3A). For smFISH, we synchronized HEK293-GFP-LSM14A cells as performed for PB
175 purification. In agreement with the RNA-seq data, we observed more FBXO5 mRNA molecules
176 in the cytoplasm, and considerably more in PBs, in G1S and mid S than in G2M and mid G1
177 (Figure 3B, C).

178 Similarly, we examined the mRNAs encoding the cell cycle-regulated splicing factor
179 CLK1³⁹ and cyclin E2 (CCNE2). In contrast to FBXO5, CLK1 transcripts gradually increased in
180 both PBs and the cytoplasm throughout the cell cycle, peaking at G2M (Figures 3A, D, E).
181 CCNE2 transcripts had a similar profile as FBXO5, with both sequencing data and smFISH
182 experiments showing maximal cytoplasmic and PB levels in G1S (Figure 3A, F, G). Thus,
183 CCNE2, FBXO5 and CLK1 mRNAs exhibited various PB localization patterns, peaking at G1S,
184 mid S, and G2M, respectively. These experiments provided single-molecule evidence of cyclic
185 mRNA accumulation in PBs.

186 **PBs capture a subset of cyclic mRNAs after mitotic exit**

187 We next focused on mRNAs harboring prominent cytoplasmic induction in G2 with limited
188 consequences on PB accumulation (Figure 2F). We first analyzed the mRNA encoding the G2
189 protein topoisomerase 2A (TOP2A), which regulates chromosome compaction⁴⁰ and relieves
190 topological DNA stress^{41,42}. In the sequencing dataset, expression of TOP2A peaked in G2M, as
191 previously described⁴³⁻⁴⁵. This increase however was not mirrored in PBs (Figure 4A). SmFISH
192 experiments on asynchronous cells revealed three TOP2A localization patterns (Figure 4B): (i)
193 few TOP2A mRNA molecules in both the cytoplasm and PBs; (ii) high TOP2A mRNA
194 expression in the cytoplasm but modest localization in PBs; and unexpectedly (iii) in a subset of
195 cells, heavy TOP2A mRNA accumulation in PBs with few TOP2A mRNA molecules free in the
196 cytoplasm. Quantitative image analysis further assessed this striking heterogeneity (Figure 4C).
197 TOP2A biology⁴⁶ and our RNA-seq data suggested that cells with high RNA expression and low
198 PB localization (pattern ii) corresponded to G2 cells, where the protein is needed and most
199 highly expressed. We speculated that after mitosis, in early G1 (a time point not included in the
200 PB purification pipeline, but accessible with smFISH), leftover TOP2A mRNA could be sent to
201 PBs (pattern iii). To test this hypothesis, we transiently expressed Halo-tagged LSM14A in
202 HEK293-FUCCI cells to allow for the simultaneous imaging of cytoplasmic TOP2A mRNAs, PBs
203 and nuclear PIP-FUCCI markers (Figure 4D). As expected, S and G2 cells showed high levels
204 of TOP2A mRNA with modest PB localization. In contrast, G1 cells showed two patterns, with
205 either minute or moderate TOP2A expression but striking accumulation in PBs. Since TOP2A
206 remains abundant during mitosis⁴⁵ (Figure 4D) and is transcriptionally induced only in G1S or
207 early S^{47,48}, we conclude that the latter cells correspond to early G1 where residual TOP2A
208 mRNAs are localized in PBs. To summarize, the burst of TOP2A mRNA expression in G2 cells
209 does not coincide with PB accumulation. Rather, localization in PBs occurs when they reform in
210 early G1, at a time when TOP2A mRNA is down-regulated and the protein no longer required.

211 Looking for mRNAs with similar expression patterns, we identified the mRNAs encoding
212 the centromere protein F (CENPF) and, to a lesser extent, the mitotic checkpoint
213 serine/threonine kinase B (BUB1B) (Figure 4A). Like TOP2A, these mRNAs are most highly
214 expressed in G2 and encode proteins functioning mainly in G2 and mitosis⁴⁹⁻⁵². Using 2-color
215 smFISH, we found that CENPF and BUB1B mRNAs had similar dynamics as TOP2A: they co-
216 localized with it in PBs in early G1 cells and were highly expressed yet weakly present in PBs in
217 S or G2 cells (Figure 4E). As a benchmark, we used PUM2 mRNA, which displayed a stable
218 expression and PB localization throughout the cell cycle (Figure 4A) and in the 2-color smFISH
219 (Figure 4E). In summary, a group of transcripts encoding G2-M proteins traffic into PBs after cell
220 division, when their protein is no longer needed, further highlighting the uncoupling between PB
221 and cytoplasmic mRNA contents.

222 **A subset of mRNAs decaying at the mitosis-to-G1 transition accumulate in PBs in G1**

223 Independently of the FAPS approach, we also explored RNA localization in PBs across the cell
224 cycle using high-throughput smFISH (HT-smFISH, Figures 5A and S5). We selected 94 mRNAs
225 with or without known cyclic expression and carried out their analysis in non-synchronized
226 HEK293-GFP-LSM14A cells. Cells were classified in G1 or G2 phase based on their DAPI
227 signal (see Methods, Figure 5A, Table S7). While most transcripts displayed a similar fraction in
228 PBs in G1 and G2 cells, a subgroup of mRNAs showed higher accumulation in PBs in G1 than
229 in G2 (Figure 5B). Their fraction in PBs was particularly heterogeneous in G1 cells, readily
230 reaching values above 50% of the molecules (Figure 5C). Interestingly, expression of many of
231 these transcripts was reported to peak in G2 and decrease in G1, while their encoded proteins
232 are implicated in G2-to-mitosis progression (ECT2⁵³, TTK⁵⁴, and DLGAP5⁵⁵).

233 We hypothesized that mRNA accumulation in PBs may be concomitant with their
234 elimination after mitosis. In agreement, most mRNAs showing higher accumulation in PBs in G1
235 than in G2 belonged to a group of mRNAs previously reported to undergo decay at the mitosis-
236 to-G1 (M-G1) transition²² (Figure 5B-D and G). Conversely, mRNAs encoding G2 proteins but
237 not undergoing decay at the M-G1 transition accumulated similarly in PBs in G2 and G1 cells.
238 To extend these data, we considered the 161 mRNAs described as decaying at the M-G1
239 transition²² and well-detected in our FAPS dataset. 62% of them were significantly enriched in
240 PBs purified from G1 cells, which is higher than expected by chance (44%, Figure 5E). They
241 also tended to reach higher levels of enrichment (Figure 5F). Thus, HT-smFISH independently
242 provided evidence of cell cycle-dependent RNA localization in PBs, with mRNAs undergoing M-
243 G1 transition decay particularly accumulating in PBs in G1.

245 **Cyclic PB localization does not result from fluctuating amounts of non-polysomal**
246 **mRNAs**

247 To explain cyclic mRNA localization in PBs, we first envisioned a mechanism related to the
248 condensate nature of PBs. Indeed, it was previously proposed that condensates could attenuate
249 cell-to-cell expression differences⁵⁶. While the examples studied above clearly showed that
250 mRNA localization in PBs does not limit expression changes in the cytoplasm throughout the
251 cell cycle, we further challenged this hypothesis using the cell cycle-resolved TOP2A, FBXO5,
252 and CLK1 smFISH data. Even within the same cell cycle phase, the fraction of mRNAs in PBs
253 showed weak or no correlation with cytoplasmic expression (Figure S6A). This was true whether
254 RNA fractions in PBs were high or low (e.g. TOP2A mRNAs in early G1 vs S or G2,
255 respectively), and argued against a general role of PBs in recruiting excess cytoplasmic
256 mRNAs.

257 Alternatively, PBs could simply recruit free mRNAs in the cytoplasm. In this scenario, the
258 distinctive TOP2A, BUB1B and CENPF mRNA accumulation in PBs in early G1 could result
259 from their specific translational downregulation after mitosis, and disrupting polysomes should
260 be sufficient for their untimely recruitment in PBs in G2. However, treating HEK293-FUCCI cells
261 transiently expressing LSM14A-Halo with puromycin, which disrupts polysomes within a few
262 minutes⁵⁷, did not cause TOP2A mRNAs to massively relocalize into PBs in G2 cells (Figure
263 S6B-C). A 2-color smFISH of BUB1B or CENPF mRNAs with TOP2A mRNAs in HEK293-GFP-
264 LSM14A cells showed a similar behavior (Figure 6A-D). In summary, disrupting polysomes was
265 not sufficient to prematurely drive TOP2A, BUB1B, and CENPF mRNAs into PBs in G2.

266 **HuR-ARE interactions prevent mRNA localization in PBs in G2**

267 We then searched for RBPs contributing to cyclic mRNA localization and noticed a
268 particular overlap between 4E-T (a PB protein required for PB formation) and HuR (one of the
269 ARE-binding proteins) targets. Analyzing the top 1000 PB-enriched mRNAs, we observed that
270 targets of both 4E-T and HuR were less enriched in PBs in G2 than in the other phases, while
271 targets of 4E-T but not HuR were more enriched in PBs in G2 (Figure S6D). A similar analysis
272 of ARE-containing mRNAs showed this pattern was specific to HuR (Figure S6D). HuR shuttles
273 between the nucleus and cytoplasm where it stabilizes cell cycle-related mRNAs^{58,59}.
274 Furthermore, its cytoplasmic fraction increases in S and G2 phases⁵⁹, suggesting altogether that
275 it could prevent mRNA localization in PBs in G2.

276 To test this, we relied on a chemical inhibitor (CMLD-2) which specifically disrupts HuR-
277 ARE interactions⁶⁰ and we imaged several G2 mRNAs reported as HuR targets and
278 accumulating in PBs in G1 more than G2. Interestingly, a 24h treatment with CMLD-2 strikingly
279 increased the fraction of mRNA localized in PBs in G2, abolishing (CENPE and CENPF) or
280 reducing (TOP2A) the G2-G1 differential localization (Figure 6E, F). In contrast, control mRNAs,
281 either not HuR targets or HuR targets without cyclic PB localization, were not affected by the
282 drug (Figure 6E, F). In summary, we identified HuR as an RBP contributing to cyclic mRNA
283 localization by preventing their recruitment in PBs during G2.

284 **Length favors accumulation of AU-rich mRNAs in PBs in early G1**

285 We then searched for mRNA features that could participate in cyclic PB localization. We
286 previously reported that in asynchronous cells, the GC content of CDS and 3'UTR is the best
287 predictor of mRNA enrichment in PBs⁸. This held true at all cell cycle phases: PB-enriched
288 mRNAs were particularly AU-rich, with a striking correlation between PB enrichment and GC
289 content (r_s up to -0.79, Figure S7A, B). However, this compositional bias displayed only minor
290 changes across the cell cycle, even when focusing on the top 1000 PB-enriched mRNAs
291 (Figure S7C). Given the nucleotide bias in the CDS, NNA/U triplets were strongly
292 overrepresented in PB-enriched mRNAs, as observed previously⁸ (Figure S7D). While similar to
293 the codon usage bias reported for cell cycle-regulated genes⁶¹ (Figure S7E), it appeared
294 unrelated to cell cycle-regulated PB localization (Figure S7D).

295 We also reported that PB-enriched mRNAs tend to be longer than average⁸. While this
296 held true at all cell cycle phases (Figure S7F), the correlation between PB enrichment and
297 mRNA length was higher in G1 than in the other phases (Figure S7G). The analysis of the top
298 1000 PB-enriched mRNAs further revealed that mRNAs were particularly long in mid G1 (Figure
299 7A). The size differences seemed to mainly originate from the CDS compared to 3'UTRs (Figure
300 7A). Similarly, mRNAs revealed by HT-smFISH to accumulate more in PBs in G1 than in G2,
301 had particularly long CDS (Figure 7B). Strikingly, for the top 500 AU-rich mRNAs, mRNA length
302 appeared as a key feature associated with PB localization in G1 ($r_s=0.55$) but not in G2 ($r_s=-$
303 0.12, Figure 7C).

304 We therefore transiently transfected Renilla Luciferase reporters of different length in
305 HEK293-GFP-LSM14A cells and analyzed their localization using smFISH against Renilla along
306 with TOP2A mRNAs to distinguish early G1 from S and G2 cells (Figure 7D, E). Importantly, all
307 transcripts were similarly AU-rich (36-37% GC), making them amenable to PB localization⁸.

308 Lengthening the RLuc CDS from 0.9 to 1.7-1.8 kb using either GFP (Figure 7D-F) or RLuc itself
309 (Figure S7H, I), led to significantly more accumulation in PBs in early G1 cells, while reporters
310 minimally accumulated in PBs in S and G2 cells. The results were similar after introducing a
311 stop codon between RLuc and GFP, which reversed the CDS length to 0.9 kb without affecting
312 total length (Figure S7H, I). Therefore, the reporter assay pointed to the importance of total
313 rather than CDS length for specific accumulation in PBs in early G1. This apparent discrepancy
314 could be explained by the intrinsic features of G2-M mRNAs, among which many will
315 accumulate in PBs in G1. First, they are particularly AU-rich (Figure 7G). Second, they are
316 longer than average²⁸, and we found it is due to their CDS more than their 3'UTR (Figure 7G).

317 Similarly, we analyzed the features of M-G1 decay mRNAs, which also accumulate in
318 PBs in G1 (Figure 5). The M-G1 decay mRNAs that are part of the top1000 PB-enriched
319 mRNAs in G1 tend to have long CDS and to be particularly AU-rich, like other mRNAs of this
320 group (Figure 7H). In contrast, the M-G1 decay mRNAs not belonging to the top 1000-PB
321 enriched mRNAs have average CDS length and nucleotide composition. Thus, long CDS and
322 AU-rich composition are typical features of PB-enriched mRNAs in G1 rather than of M-G1
323 decay mRNAs. Taken together, these data show that mRNA length, in the context of an AU-rich
324 nucleotide composition, is a feature contributing to RNA accumulation in PBs in G1 cells.

325 **Discussion**

326 **The mRNA content of PBs is dynamic and partly uncoupled from the cytoplasmic content**

327 In this study, we set out to investigate the dynamics of mRNA localization in PBs at the scale of
328 hours in standard growth conditions. As a biological context, we chose the cell cycle since it
329 entails changes in cytoplasmic RNA abundance that have been well documented at various
330 RNA metabolism levels^{13–15,18,19,21,22}. During every cell cycle, PBs form in G1, then increase in
331 size more than in number, culminating in G2, before dissolving in mitosis. Cycling cells thus give
332 access to the cell-autonomous dynamics of the PB content in the absence of any stress.

333 The genome-wide landscape of PB RNAs during cell cycle progression allowed
334 answering two primary questions. Does the RNA content of PBs change during cell cycle
335 progression? Do these changes simply mirror changes of cytoplasmic mRNA abundance, or do
336 they behave distinctly? In fact, PBs undergo widespread changes in their mRNA content, and
337 part of these changes did not reflect those of the cytoplasm: (i) mRNAs could change in PBs
338 more strikingly than in the cytoplasm (particularly from mid G1 till mid S), (ii) and inversely, they
339 could markedly change in the cytoplasm without a similar change in PBs (particularly from mid S
340 to the next G1). Single molecule RNA imaging highlighted the remarkable variety in RNA
341 localization patterns: for example, TOP2A, BUB1B and CENPF strikingly accumulated in PBs in
342 early G1; many mRNAs peaking in G2 before undergoing decay accumulated in PBs in G1;
343 CCNE2 at the G1S transition; FBXO5 in mid S; and CLK1 at the G2M transition. Thus,
344 differential localization in PBs is not restricted to specific stages of the cell cycle. In particular, it
345 does not follow the evolution of PBs considered as a whole (increasing size and number) during
346 cell cycle progression.

347 **Biological relevance of differential mRNA accumulation in PBs**

348 For most mRNAs, a minority of molecules accumulates in PBs, arguing against a causative role
349 of PBs in regulating cyclic gene expression. Yet, some transcripts including TOP2A strongly
350 accumulated in PBs in early G1. These transcripts are transcriptionally and translationally
351 upregulated from late S to mitosis, periods when their encoded protein is needed^{46,47,50,52}, and
352 then subject to mitotic decay pathways²². TOP2A mRNA for instance is degraded by a CCR4-
353 NOT-dependent mechanism²². However, there is no mechanism for ensuring translation
354 inhibition of residual transcripts in G1^{22,62}. We hypothesize that PB localization could contribute
355 to the silencing of such transcripts in early G1. These examples were further corroborated by
356 HT-smFISH analysis, which revealed a group of mRNAs that specifically accumulate in PBs in
357 G1 and that are also subject to mitotic decay²². Yet, decay in G1 was not systematically

358 associated with accumulation in PBs in G1. Regardless of how and where their decay takes
359 place (not accessible through smFISH); our data suggest that PBs could compartmentalize
360 mRNAs away from the translation machinery at specific cell cycle phases.

361 **PBs do not buffer excess non-polysomal mRNAs**

362 To explain cyclic mRNA localization in PBs, we first envisioned a mechanism inspired by the
363 physical nature of membraneless organelles. Since liquid-liquid phase separation (LLPS)
364 implies the condensation of molecules that are over their saturation concentration,
365 membraneless organelles have been proposed as a buffering mechanism to limit cell-to-cell
366 variability of intracellular concentration⁵⁶. While this was observed for minimal condensates
367 (nanoclusters) forming upon massive mRNA storage in *C. elegans* arrested oocytes⁶³, our
368 smFISH data did not support such a hypothesis for PBs. Our results are rather consistent with
369 the conclusion that multicomponent cellular organelles like PBs and stress granules are not
370 governed by a fixed saturation concentration⁶⁴.

371 Then, since polysomal mRNAs are excluded from PBs⁷, we considered a scenario
372 where mRNA accumulation in PBs is governed by the amount of non-translating mRNAs. From
373 a transcriptome-wide perspective, it has been previously proposed that non-optimal codon
374 usage could generate cell cycle-dependent translation efficiency⁶¹. In all cell cycle phases, we
375 found that PB-enriched mRNAs were particularly AU-rich, leading to a non-optimal codon usage
376 associated with inefficient translation, as described previously⁸. However, neither their GC
377 content, nor their codon usage differed between phases, indicating that these features are not
378 involved in the cyclic pattern of mRNA accumulation in PBs. We also addressed the question
379 experimentally. Releasing TOP2A, BUB1B and CENPF mRNAs from polysomes in G2 cells was
380 not sufficient to relocate them in PBs, despite them having appropriate intrinsic features to
381 accumulate in PBs in early G1. Altogether, we did not find evidence that mRNA accumulation in
382 PBs represents the inverted mirror of mRNA translation.

383 **Intrinsic and extrinsic factors regulate cyclic localization in PBs**

384 This prompted us to look for other determinants of cyclic mRNA localization in PBs. In terms of
385 RBPs, we found that inhibiting HuR-ARE interaction causes the premature localization of HuR
386 target mRNAs to PBs in S or G2. This highlights a role for HuR in preventing mRNA recruitment
387 in PBs in later stages of the cell cycle. In terms of mRNA features, we previously reported that
388 mRNA accumulation in PBs in asynchronous cells moderately correlates with their length⁸. To
389 our surprise, this feature was cell cycle-dependent, with PB mRNAs longer in G1 than in G2.

390 Moreover, for the most AU-rich mRNAs of the transcriptome, PB enrichment correlated with
391 length in G1 but not G2. Such a phase specific impact of mRNA length was confirmed using
392 mRNA reporters. Overall, two mRNA features appear associated with PB localization: the
393 strongest is their GC content, in all phases; the second is their length, specifically in G1.

394 Interestingly, *in-silico* modeling showed that longer transcripts localize at the core of
395 RNA-protein condensates, where they augment the density of molecular interactions. This
396 promotes and stabilizes condensates, preventing them to collapse due to excessive RNA
397 recruitment^{65,66}. It is particularly tempting to speculate that long RNAs favor the seeding and
398 stabilize nascent PBs in early G1. These PBs could then progressively grow into "mature" PBs
399 without collapsing. Long RNAs may at a certain point lose their advantage, because the
400 interaction network becomes saturated, or also because the short RNAs at the surface⁶⁵ provide
401 a barrier to long RNA recruitment⁶⁷.

402 Taken together, mRNA accumulation in PBs appears to be a multifaceted process
403 involving the cumulative effect of extrinsic (HuR) and intrinsic (GC content, length) factors. If
404 PBs are dynamic in a cell-autonomous process like cell cycle progression, they could be even
405 more so across a whole organism, or during development and differentiation.

406 **Limitations of the study**

407 For feasibility reasons, PBs were purified following cell cycle synchronization procedures, which
408 could entail changes in gene expression. However, the majority of *in situ* RNA imaging
409 experiments was performed on non-synchronized cells. Second, it wasn't possible to perform
410 deep and quantitative proteomic analysis from purified PBs, due to insufficient material. While
411 our gross mass spectrometry analysis did not reveal drastic loss of the main known PB proteins
412 at particular cell cycle phases, the observed variations of their mRNA content strongly suggest
413 associated changes in terms of RBP content. Third, puromycin being a global inhibitor of
414 translation elongation, may have a different impact on PB recruitment compared to endogenous
415 transcript-specific translation initiation repression. Finally, further studies will be required to
416 identify other factors that add to HuR binding, mRNA nucleotide composition and length, to
417 control cell cycle-dependent localization in PBs.

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434

435 **Figure legends:**

436 **Figure 1: PBs enlarge during cell cycle progression while maintaining a similar**
437 **proteome.** (A) HEK-FUCCI cells with DDX6-labeled PBs. Anti-DDX6 IF reveals cytoplasmic
438 PBs while nuclear Cdt1-mVenus signal indicates cells in G1 or G2 phase (both in green in the
439 merge). Nuclear mCherry-Gem signal indicates cells in S or G2 phase (red in the merge). (B)
440 Scatter plot of PB size and DDX6 intensity across the cell cycle (2 independent experiments).
441 (C) Main steps of PB purification. (D) Cell cycle analysis from synchronized samples, with cells
442 in G1, S and G2M colored in green, red and yellow, respectively. (E) FAPS profiles of pre-
443 sorting lysate and sorted PBs from cells synchronized at the G1S transition. (F) Enrichment or
444 depletion of several protein groups after sorting (ratio of MS area after/before sorting) in cells at
445 the G1S transition. The number of detected proteins (MS area >1) is indicated in brackets. (G)
446 IF of FXR1 and FXR2 in HEK293-GFP-LSM14A cells. The IF and PB signals are green and red,
447 respectively. (H) Pairwise comparison of the abundance (MS area) of known PB proteins (list in
448 Table S1) in the PB fraction between successive cell cycle stages. Scale bars: 10 μ m in panels,
449 1 μ m in insets.

450 **Figure 2: Analysis of purified PBs uncovers their dynamic transcriptome.** (A) RNA levels
451 (normalized DEseq2 counts) before and after PB sorting from mid S cells. All expressed mRNAs
452 are shown. The mRNAs significantly enriched and depleted in PBs ($p\text{-adj}<0.05$) are in red and
453 green, respectively. (B) Venn diagram of PB-enriched mRNAs in the different cell cycle phases.
454 (C) Volcano plot showing the changes in PB mRNA content from mid G1 to G1S. All expressed
455 mRNAs are shown with significant changes ($p\text{-adj}<0.05$) in red. (D) Summary of significant
456 changes in PBs ($p\text{-adj}<0.05$) across the cell cycle. (E) GO analyses of PB mRNAs with
457 significant positive (left panel) and negative (right panel) changes in mid S compared to mid G1.
458 The 10 most enriched GO categories are shown. (F) Quadrant plots comparing mRNA fold-
459 changes before and after sorting (normalized counts >50) between successive cell cycle stages.
460 mRNAs further studied by smFISH are indicated. Associated volcano plots are in Figure S4B-D.

461 **Figure 3: mRNA localization in PBs can peak at any cell cycle phase.** (A) Evolution of
462 FBXO5, CLK1 and CCNE2 mRNA levels (in normalized counts) in purified PBs and pre-sorting
463 fractions across the cell cycle. Each dot corresponds to one replicate, with the line connecting
464 their mean. (B) Synchronized HEK293-GFP-LSM14A cells after smFISH of FBXO5 mRNA. For
465 better visualization, the Cy3 smFISH signal is in green and GFP-LSM14A in red. Nuclei were
466 stained with DAPI (blue). Individual mRNA molecules appear as small dots (indicated by the
467 arrow). Insets present enlargements of representative PBs, illustrating the heterogeneity of RNA
468 amounts in PBs. Scale bars: 10 μm in panels, 1 μm in insets. (C) Number of FBXO5 mRNA
469 molecules in the cytoplasm or in PBs, and the fraction of mRNA in PBs across the cell cycle.
470 Each dot corresponds to one cell (2 independent experiments). Horizontal lines, median; error
471 bars, 95% CI. Two-tailed Mann-Whitney statistical tests: ****, $p<0.0001$; *, $p<0.05$; ns, non-
472 significant ($p>0.05$). (D, E) Same as in B and C, for CLK1 mRNA. (F, G) Same as in B and C,
473 for CCNE2 mRNA.

474 **Figure 4: TOP2A, CENPF, BUB1B mRNAs concentrate in PBs after cell division.** (A)
475 Evolution of TOP2A, CENPF, BUB1B and PUM2 mRNA levels (in normalized counts) in purified
476 PBs and pre-sorting fractions across the cell cycle. (B) Asynchronous HEK293-GFP-LSM14A
477 cells after smFISH of TOP2A mRNA. The Cy3 smFISH signal is in green and GFP-LSM14A in
478 red. Nuclei were stained with DAPI (blue). Insets present enlargements of representative PBs,
479 illustrating the intercellular heterogeneity of RNA concentration in PBs. (C) Fraction of TOP2A
480 mRNAs localized in PBs as a function of their number in the cytoplasm. Each dot corresponds
481 to one cell. (D) Left panel: HEK-FUCCI cells transiently expressing LSM14A-Halo to label PBs
482 (in far-red, shown in blue), after smFISH of TOP2A mRNA (Cy3, in red). The green, red, yellow,

483 and white arrows point to cells in G1, S, G2, and M, respectively, identified based on the FUCCI
484 system. These cells are enlarged in the middle panels. Insets on the right present enlargements
485 of the representative PBs framed in the middle panels. (E) 2-color smFISH of TOP2A mRNAs
486 and BUB1B, CENPF or PUM2 mRNAs in asynchronous HEK293-GFP-LSM14A cells. In the
487 merge, TOP2A mRNAs (in Cy5, upper row) are in red, co-detected mRNAs (in Cy3, middle row)
488 are in green, PBs are in blue, and DAPI-staining of the nuclei in white. Blue arrows point to early
489 G1 cells and yellow ones to S or G2 cells, as revealed by the TOP2A mRNA labeling pattern.
490 Green circles surround mRNA clustered in PBs. The pink arrows point to nuclear transcription
491 sites. Scale bars: 10 μ m in panels, 1 μ m in insets.

492 **Figure 5: A high-throughput smFISH screen reveals PB localization of an mRNA subset**
493 **after mitosis.** (A) HT-smFISH workflow and typical cell cycle profiling based on DAPI staining.
494 (B) Scatter plot comparing the fraction of mRNA molecules in PBs in G1 and G2 cells for the 94
495 screened mRNAs. The mRNAs decayed or not during M-G1 transition were taken from
496 Krenning et al., 2022²². The indicated mRNAs are those shown in (G). (C) Fraction of mRNA
497 molecules in PBs for indicated transcripts, in G1 and G2 cells. The number of analyzed cells is
498 in Figure S5C. Horizontal lines, mean; error bars, 95% CI. Two-tailed Mann-Whitney test: ****,
499 $p < 0.0001$; *, $p < 0.05$; ns, non-significant ($p > 0.05$). (D) Boxplot showing the fold-change of the
500 mRNA fraction in PBs in G1 versus G2 cells for mRNAs decaying at the M-G1 transition and
501 other screened mRNAs. Two-tailed Mann-Whitney test: ****, $p < 0.0001$. (E) Stacked bar graph
502 showing the proportion of PB-enriched (red), PB-depleted (green), or neither (grey) mRNAs in
503 G1 in the whole coding transcriptome and in M-G1 transition decay mRNAs (normalized counts
504 > 100). Chi-squared test: ****, $p < 0.0001$. (F) Box plot showing PB enrichment in G1 for M-G1
505 transition decay mRNAs and for the whole coding transcriptome (normalized counts > 100).
506 Two-tailed Mann-Whitney test: ****, $p < 0.0001$. (G) HEK293-GFP-LSM14A cells hybridized using
507 HT-smFISH. Examples of mRNAs undergoing M-G1 transition decay or not. mRNA is shown in
508 green, PBs in red, and nuclei in blue. DAPI-based cell cycle classification is indicated within
509 cells. Scale bars: 10 μ m in panels, 1 μ m in insets.

510 **Figure 6: Determinants of cyclic localization of mRNAs in PBs.** (A) 2-color smFISH of
511 BUB1B and TOP2A mRNAs in asynchronous HEK293 cells expressing LSM14A-GFP, treated
512 or not with puromycin for 1 hr. In the merge, BUB1B mRNAs (Cy3) are in red, TOP2A mRNAs
513 (Cy5) in green, LSM14A-GFP-labelled PBs in blue and DAPI-staining of the nuclei in white.
514 Cells were classified in early G1, or in S or G2 based on TOP2A mRNA labeling pattern. Scale
515 bars: 10 μ m in panels, 1 μ m in insets. (B) Fraction of BUB1B and TOP2A mRNAs localized in

516 PBs in early G1 and in S or G2, in control and puromycin-treated cells. Each dot corresponds to
517 one cell (2 independent experiments). (C and D) Same as A and B for CENPF and TOP2A
518 mRNAs. (E) Localization of TOP2A or CENPF mRNAs (in green) in HEK293-GFP-LSM14A
519 cells after a 24h DMSO or CMLD-2 treatment. PBs are in red and nuclei in blue. (F) Fraction of
520 test and control mRNAs localized in PBs in DMSO or CMLD-2-treated cells (2-3 independent
521 experiments). TOP2A and CENPF were detected by smiFISH and the others by HT-smFISH.
522 Horizontal lines, mean; error bars, 95% CI. Two-tailed Mann-Whitney tests: ****, $p < 0.0001$; ***,
523 $p < 0.0005$; **, $p < 0.005$; *, $p < 0.05$; ns, non-significant ($p > 0.05$). Scale bars: 10 μm in panels, 1
524 μm in insets.

525 **Figure 7: Length increases RNA accumulation in PBs in G1.** (A) Lengths of the full mRNA,
526 CDS, and 3'UTR for all mRNAs (normalized counts > 100) or for the top 1000 PB-enriched
527 mRNAs ($FC > 0$, $p\text{-adj} < 0.05$, PSF normalized counts > 100) in the various cell cycle phases.
528 Whiskers represent the 10 to 90% percentile. (B) CDS or 3'UTR length and GC content of the
529 mRNAs imaged by HT-smFISH. mRNAs accumulating in PBs more in G1 than G2 are
530 highlighted in red. Dotted lines indicate the median length and GC content of the coding
531 transcriptome (PSF normalized counts > 100). (C) Scatter plot of total length and PB enrichment
532 of the top 500 AU-rich mRNAs, in mid G1 and G2M, with Spearman correlation (r_s). (D)
533 Schematic of the length reporter experiment, with smFISH probes in red. (E) 2-color smFISH of
534 the reporter mRNA and TOP2A mRNA in asynchronous HEK293-GFP-LSM14A cells, after
535 transfection of the long or short form of the Renilla Luciferase reporter. Renilla Luciferase
536 mRNAs (Cy3) are in red, TOP2A mRNAs (Cy5) in green, GFP-LSM14A-labelled PBs in blue,
537 and DAPI in white. Cells in early G1 and in S or G2 were classified based on TOP2A mRNA
538 labeling pattern. Scale bars: 10 μm in panels, 1 μm in insets. (F) Fraction of long and short
539 Renilla Luciferase mRNAs in PBs, with TOP2A mRNAs as an internal control (2 independent
540 experiments). Horizontal lines, median; error bars, 95% CI. Two-tailed Mann-Whitney tests: ****,
541 $p < 0.0001$; **, $p < 0.005$; *, $p < 0.05$; ns, non-significant ($p > 0.05$). (G and H) Features of G2M and
542 M-G1 decay mRNAs compared to all mRNAs or the top 1000 PB-enriched mRNAs. Two-tailed
543 Mann-Whitney tests: ****, $p < 0.0001$; **, $p < 0.005$; ns, non-significant ($p > 0.05$).

544

545 **STAR Methods**

546

547 **Resource availability**

548 **Lead contact**

549 Further information and requests for resources and reagents should be directed to and will be
550 fulfilled by the lead contact, Dominique Weil (dominique.weil@upmc.fr)

551 **Materials availability**

552 All unique reagents generated by this study are available from the lead contact either without
553 restriction or with a completed Materials Transfer Agreement.

554 **Data and code availability**

- 555 • Raw RNA-seq data is available at ArrayExpress under the accession number E-MTAB-
556 12923
- 557 • All original code has been deposited at Zenodo and is publicly available as of the date of
558 publication. DOIs are listed in the key resources table. The code used to analyze RNA
559 PB localization is available at DOI: 10.5281/zenodo.12742387 or
560 https://github.com/15bonte/p_bodies_cycle_2023 for standard smFISH experiments, and
561 at DOI: 10.5281/zenodo.12666004 or [https://github.com/Flo3333/Cell-cycle-and-HT-](https://github.com/Flo3333/Cell-cycle-and-HT-smFISH-analysis-of-RNA-localization-in-PBs)
562 [smFISH-analysis-of-RNA-localization-in-PBs](https://github.com/Flo3333/Cell-cycle-and-HT-smFISH-analysis-of-RNA-localization-in-PBs) for HT-smFISH analysis.
- 563 • Raw microscopy images and blots for all figures were deposited on Mendeley Data at
564 DOI:10.17632/67s7c3dyc9.1
- 565 • Any additional information required to reanalyze the data reported in this paper is
566 available from the lead contact upon request.

567 **Experimental model and subject details**

568 **Cell culture**

569 HEK293 and HeLa cells were grown in Dulbecco's modified Eagle's Medium (DMEM, Gibco)
570 supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), and 100 U/mL
571 penicillin/streptomycin (Sigma-Aldrich). HEK293-GFP-LSM14A cells were obtained by
572 transfecting HEK293 cells with a pCMV-GFP-LSM1A plasmid and selecting stable clone under
573 500 µg/mL G418 (Gibco). HEK293-FUCCI cells were obtained via CRISPR-Cas9-mediated
574 insertion of the PIP-FUCCI reporter in the AAVS1 genomic locus and selection under 1 µg/mL
575 puromycin (Sigma-Aldrich). All cells were grown at 37 °C with 5% CO₂.

576

577 **Method details**

578 **Cell cycle determination, drug treatments, and transfections**

579 PIP-FUCCI allows accurate determination of cell cycle phases by expressing decay-
580 sensitive fragments of two known cyclic proteins fused to fluorescent markers: (i) Cdt₁₁₋₁₇-
581 mVenus expressed in G1, G2 and mitosis (G2/M); and (ii) mCherry-Gem₁₋₁₁₀ expressed in S and
582 G2/M phases^{68,69}. Both markers are fused with nuclear localization elements to make them
583 compatible with cytoplasmic co-labeling. The accurate cell cycle-dependent expression of PIP-
584 FUCCI was verified by flow cytometry analysis of live cells after DNA labeling with Hoechst. The
585 mVenus positive, mCherry positive, and double positive cells showed the expected quantity of
586 DNA (1N to 2N) for cells in G1, S, and G2/M phases, respectively (Figure S1A, B).

587 To inhibit translation, cells were treated with 100 µg/mL puromycin for 1 hr or 15 min, as
588 indicated. The efficiency of the puromycin batch was controlled using HeLa cells expressing
589 endogenous ASPM-MS2 mRNA that localizes to centrosomes in a translation-dependent
590 manner^{70,71}. As expected, centrosomal localization of this mRNA was abolished after a 15 min
591 puromycin treatment (Figure S6C). To inhibit HuR-ARE interactions, cells were treated with
592 20 µM CMLD-2 (MedChemExpress) for 24 h. Control cells were treated with DMSO (Sigma-
593 Aldrich) for the same time.

594 For all transient transfections, 5 µl Lipofectamine 2000 (Thermo Fisher Scientific) was
595 used with the following DNA quantities per well of a 6-well plate: 1 µg LSM14A-Halo tag, and
596 100 ng of each Renilla luciferase construct with 900 ng of a non-transcribed plasmid. All
597 transfection were done for 5 hr after which the reagent was washed off and cells allowed to
598 grow for 24-48 hr before fixation. Cell fixation was done with 4% paraformaldehyde PFA
599 (Electron Microscopy Sciences) for 20 min at room temperature (25°C). The Renilla Luciferase
600 plasmids were expressed under a CMV promoter.

601

602 **Synchronizations**

603 All synchronizations were performed in HEK293-GFP-LSM14A cells. For PB purification,
604 synchronizations were done in 15 cm dishes. For double thymidine blocks, 4 million cells were
605 seeded. The next day, 7.5 mM thymidine was added to the culture medium for 18 hr. Cells were
606 then washed gently with warm PBS three times and further incubated in fresh complete
607 medium. Cells were released for 9 hr after which 7.5 mM thymidine was added again for
608 another 18 hr. This results in cells blocked at the G1S transition. To obtain cells in mid S phase,
609 cells were further washed three times in warm PBS after which fresh complete medium was
610 added. Cells were released for 5 hr to obtain a population at mid S phase. For CDK1 inhibition,

611 8 million cells were seeded. The next day the CDK1 inhibitor RO-3306 was added at 9 μ M. After
612 22 hr, cells blocked at the G2M transition were harvested. To obtain cells at mid G1, we found
613 that half the drug's concentration (with the same blockage time) provides a better release from
614 G2M into mid G1 (with three PBS washes and an 8 hr release in fresh complete medium). Forty
615 to fifty 15 cm dishes for each cell cycle phase were synchronized. After each synchronization, a
616 small fraction of each 15 cm dish was tested in flow cytometry for the quality of the
617 synchronization and, if synchronization reached 55% for mid G1, 80% for G1S or mid S, and
618 70% for G2M, the rest was used for PB purification. For microscopy experiments, identical
619 synchronizations were done for cells grown on 22x22 cm coverslips in 6-well plates, with the
620 addition of poly-L-lysine for better cell adhesion.

621 For RNA-seq, 6 independent synchronization experiments were performed to obtain a
622 sufficient number of cells in mid G1, G1S, G2M, and 7 for mid S. Each synchronization
623 experiment was performed on 6-8 15 cm cell plates. For each FAPS, at least 2 synchronization
624 experiments were pooled together. For each cell cycle stage, 2-3 days FAPS were required to
625 purify enough material for three independent RNA extractions.

626

627 **Cell cycle related flow cytometry analysis and FACS purification**

628 To fix cells for flow cytometry analysis, ~100,000 cells were washed twice in PBS, trypsinized,
629 and suspended in PBS. Cells were pelleted at 500 g for 5 min at 4°C and the pellet
630 resuspended in 300 μ l ice-cold PBS to which 900 μ l cold ethanol (100% vol/vol) was slowly
631 added. Cells were stored at -20°C overnight to allow fixation. The next day, cells were pelleted
632 at 500 g for 5 min at 4°C. To label DNA, cells were resuspended in a 500 μ l solution of 20
633 μ g/mL RNaseA and 50 μ g/mL propidium iodide in PBS and incubated at 37°C for 30 min. Flow
634 cytometry was performed on a MACSQuant® Analyzer 10 Flow Cytometer (Miltenyi Biotec)
635 using the 561 nm laser and acquisitions analyzed using FlowJo (v10.6.2). For flow cytometry
636 analysis of living HEK293-FUCCI cells: cells grown to 80% confluency in 6-well plates were
637 washed three times in PBS, trypsinized, and suspended in PBS. Cells were then pelleted for 5
638 min at 500 g at room temperature (25°C). The pellet was then resuspended in complete DMEM
639 medium containing 15 μ g/mL Hoechst 33342 (Life technologies) and incubated for 30 min at
640 37°C before flow cytometry analysis. Cytometry and analysis were performed using the same
641 hardware (with a 405 nm laser) and software.

642 To sort HEK293-FUCCI cells by FACS: cell were grown to 80% confluency in 10 cm
643 dishes and processed identically, minus the Hoechst 33342 staining. Around 1.5-2 million cells
644 were sorted for each cell cycle phase (G1, S, or G2/M) using a MoFlo Astrios EQ (Beckman-
645 Coulter, 488 and 561 nm lasers) and collected cells were pelleted for 5 min at 500 g. Cell pellets
646 were flash frozen every 30 min to minimize the amount of cells transitioning into the next cell
647 cycle phase and accumulated for western blotting.

648

649 **PB purification by FAPS**

650 PB purification by FAPS is based on particle size and PB fluorescence. It was performed as
651 described previously⁷, with a few modifications. HEK293-GFP-LSM14A cells (synchronized or
652 not) were allowed to grow to ~70-80% confluency in 15 cm dishes. After two PBS washes, cells
653 were scrapped off, collected in 2 ml Eppendorf tubes and pelleted at 500 g for 5 min. Pellets
654 were flash frozen in liquid nitrogen and stored at -80°C until the day before sorting. Each cell
655 pellet was then resuspended in 1.5 mL ice-cold lysis buffer containing 50 mM Tris pH 8, 1 mM
656 EDTA, 150 mM NaCl, and 0.2% Triton X-100 supplemented with 65 U/mL RNase out (Life
657 Technologies) and 2x protease inhibitor cocktail (Roche), pipetted a few times and kept on ice
658 for 20 min. Then, nuclei were pelleted by centrifugation for 5 min at 500 g, and the supernatant
659 containing cytoplasmic organelles, including PBs, was transferred to a new tube. After
660 supplementation with 10 mM MgSO₄, 1 mM CaCl₂, and 4 U/mL RQ1 DNase (Promega),
661 samples were incubated at room temperature (25°C) for 30 min while avoiding direct sources of
662 light to avoid GFP fluorescence bleaching. Next, the samples were centrifuged at 10,000 g for 7
663 min at 4°C. The supernatant was gently aspirated and the pellet was resuspended in 30 µl lysis
664 buffer supplemented with 40 U RNase out. This was called the pre-sorting fraction. A small
665 aliquot (1-3 µL) was stained with ethidium bromide and visualized using widefield fluorescence
666 microscopy for a reference image before sorting. For each cell cycle phase, 3 independent pre-
667 sorting fractions were kept aside for RNA-seq and mass spectrometry analyses to compare with
668 their sorted counterparts.

669 Fluorescence activated particle sorting (FAPS) was carried out on a MoFlo Astrios EQ
670 (Beckman-Coulter) using the 488 nm excitation laser. The PB sorting gate was determined
671 using control samples of lysis buffer alone, or an identical cell preparation but made with cells
672 expressing a truncated GFP-LSM14A protein that only displays diffuse fluorescence (GFP-
673 LSM14A-Δ, described in ⁷) (Figure S1J). The pre-sorting sample was diluted 30 times in lysis

674 buffer at the time of sorting (10 μ L in 300 μ L lysis buffer). The differential pressure during sorting
675 was maintained around 0.8 and the sorting speed was around 8,000 events per second on
676 average. The sorting purity was >95%. After sorting, a small aliquot was stained with ethidium
677 bromide and visualized under widefield fluorescence microscopy. Purified PBs were pelleted at
678 10,000 g for 7 min in 2 mL low binding Eppendorf tubes, while the remaining was stored at -
679 80°C. ~6 hr of active sorting were done per day and 2-3 days of sorting output were combined
680 together to constitute a single replicate for RNA-seq for each cell cycle phase. A total of >140 hr
681 of FAPS was needed to accumulate sufficient material for RNA-seq in triplicates and 1 mass
682 spectrometry analysis for each of the 4 cell cycle conditions. Protein and RNA were extracted
683 using TRIzol (Thermo Fisher Scientific). A total of ~400 ng protein per condition and 5-10 ng
684 RNA per replicate per condition were obtained.

685

686 **Mass spectrometry**

687 Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was performed at the
688 proteomics platform at Institut Jacques Monod, Paris France. Proteins from the purified PB or
689 pre-sorting fractions were dissolved in a 8 M urea solution (Sigma-Aldrich) before trypsin
690 digestion. Samples were analyzed using an Orbitrap Fusion Lumos Tribrid Mass Spectrometer
691 (Thermo Fisher Scientific) with the following settings: Ion Source: ESI (nano-spray),
692 Fragmentation Mode: high energy CID, MS Scan Mode: FT-ICR/Orbitrap. Peptide and protein
693 signals were processed using PEAKS Studio (v10.6 build 20201015) with the following
694 parameters: Max Missed Cleavages: 2, Database: Homo Sapiens SwissProt Release_2020_06,
695 Parent Mass Error Tolerance: 15.0 ppm, and Fragment Mass Error Tolerance: 0.5 Da. Protein
696 and peptide signals were selected using a <1% false discovery rate filter. To classify proteins as
697 PB-enriched or –depleted proteins, the Fisher’s exact test was performed in R. The significance
698 cutoff was set to <0.025. The functional annotation used in Figures 1 and S2 was performed
699 manually and is provided in Table S1.

700

701 **cDNA library generation and RNA sequencing**

702 Library preparation and sequencing were performed at Ecole normale supérieure Génomique
703 ENS core facility (Paris, France). cDNAs were synthesized using a combination of random
704 primers and oligodT to amplify RNAs independently of their polyadenylation status. 2 ng of total

705 RNA were amplified and converted to cDNA using Ovation RNA-Seq system v2 (TECAN).
706 Following amplification, libraries were generated using the Nextera XT DNA Library Preparation
707 Kit from Illumina. Libraries were multiplexed, and after single-end 75 bp sequencing (NextSeq
708 500, Illumina), 40 to 60 million reads per sample passed the Illumina filters. Three replicates per
709 fraction and per cell cycle phase were made, for a total of 24 libraries.

710

711 **RNA-seq analysis**

712 RNA-seq analysis was performed on a local Galaxy server. RNA STAR^{72,73} (version 2.7.8a) was
713 used to align reads to the GRCh38p.13 assembly of the human genome with haplotypic
714 duplications removed. The following parameters were used: 2 maximum mismatches per read
715 alignment, a maximum ratio of mismatches to mapped length of 0.3, a minimum alignment
716 score, normalized to read length of 0.66. All other parameters were set to default. More than
717 95% of reads mapped to the human genome, with the rest too short to be mapped.
718 FeatureCounts⁷⁴ (version 2.0.1) was used to count read using the Gencode v38 gene
719 annotation. All parameters were set to default. Differential expression analysis was done using
720 DEseq2 with the default settings. Normalized counts showed high reproducibility between
721 replicates in both the PB and pre-sorting fractions across the cell cycle (Figure S4A), after
722 exclusion of one PB replicate in G1S and one pre-sorting replicate in mid G1, due to lower
723 reproducibility ($R < 0.9$). Depending on the analysis, two normalizations were performed. (i) For
724 PB enrichment calculation, PB replicates were normalized to pre-sorting replicates within each
725 cell cycle phase, as performed previously⁷; (ii) to follow the evolution of RNA content in the PB
726 and pre-sorting fractions across the cell cycle, all PB replicates were normalized together on the
727 one hand, and all pre-sorting replicates together on the other hand. It can be noted that PB
728 enlargement throughout interphase may lead to some underestimation of RNA content in PBs,
729 since large PBs likely accumulate a larger pool of RNAs, while RNA-seq only measures relative
730 RNA abundance.

731

732 **Gene ontology analysis**

733 GO term analyses were performed using clusterProfiler^{75,76} in R. For GO analysis of PB mRNAs
734 (Figure 2E), the reference list was a compilation of all mRNAs detected (normalized counts > 0)
735 in the PB fraction of at least one cell cycle phase, and the test lists contained mRNAs displaying

736 differential abundance in the PB fraction ($p\text{-adj} < 0.05$, DEseq2) between 2 cell cycle phases.
737 Similarly, for GO analysis of the pre-sorted fraction mRNAs (Figure S3B, C), the reference list
738 was a compilation of all mRNAs detected (normalized counts > 0) in this fraction and the test lists
739 contained the mRNAs differentially expressed ($p\text{-adj} < 0.05$, DEseq2). The Benjamini &
740 Hochberg p -value adjustment method was applied and the significance cutoff was set to < 0.05 .
741 To limit GO term redundancy, simplified GO terms were used with a cutoff of 0.6 using the
742 adjusted p -value, in Figure 2E. Dot plots were used to represent the top 10 GO categories with
743 associated mRNAs count and $p\text{-adj}$ values. Full detailed GO term lists are provided in Table S5.

744

745 **Immunofluorescence**

746 Cells grown on 22x22 mm glass coverslips were fixed for 20 min at room temperature (25°C)
747 with 4% PFA (Electron Microscopy Sciences) diluted in PBS and stored at 4°C. Before IF
748 labeling, cells were permeabilized with 0.1% Triton X-100 in PBS (Sigma-Aldrich) for 15 min at
749 room temperature (25°C). All primary and secondary antibodies were diluted in PBS 0.1% BSA.
750 To label DDX6, we used a rabbit polyclonal anti-DDX6 antibody recognizing its C-ter extremity
751 (BIOTECHNE, NB200-192, dilution 1/1000) and a secondary F(ab)2 goat anti-rabbit antibody
752 labeled with AF488 (Life technologies, A11070, dilution 1/1000). To label the stress granule
753 marker TIA1, we used goat polyclonal anti-TIA1 antibody (Santa Cruz, SC1751, dilution 1/200)
754 and a secondary donkey anti-goat antibody labeled with Cy3 (Jackson ImmunoResearch, 705-
755 166-147, dilution 1/800). To label FXR1, we used rabbit polyclonal anti-FXR1 antibody (Sigma-
756 Aldrich, HPA018246, dilution 1/500) and a secondary goat anti rabbit antibody labeled with
757 AF488 (Life technologies, A11070, dilution 1/1000). To label FXR2, we used mouse monoclonal
758 anti-FXR2 antibody (Life Technologies, MA1-16767, dilution 1/200) and a secondary goat anti
759 mouse antibody labeled with AF488 (Life technologies, A11029, dilution 1/1000). Primary and
760 secondary antibodies were incubated for 1 hr at room temperature after which coverslips were
761 washed 3 times in PBS. Coverslips were then mounted using DAPI-containing Vectashield
762 (Vector Laboratories).

763

764 **Single molecule FISH**

765 Cells grown on 22x22 mm glass coverslips were fixed for 20 min at room temperature with 4%
766 PFA (Electron Microscopy Sciences) diluted in PBS and permeabilized with 70% ethanol

767 overnight at 4°C. We used the single molecule inexpensive variant of smFISH (smiFISH)³¹,
768 which uses 24 primary probes for each RNA target each made of a gene-specific sequence that
769 hybridizes to the RNA, and a common overhang that serve as platforms for recruiting
770 fluorescently labeled oligos (either Cy3 or Cy5). To this end, 40 pmoles of primary probes were
771 first pre-hybridized to 50 pmoles of fluorescently labeled oligos in 10 µL of 100 mM NaCl, 50
772 mM Tris-HCl, 10 mM MgCl₂, pH 7.9. This was performed on a thermocycler with the following
773 program: 85 °C for 3 min, 65 °C for 3 min, and 25 °C for 5 min. The resulting fluorescent probe
774 duplexes were then used in a hybridization mixture consisting of: 1x SSC, 0.34 mg/mL tRNA,
775 15% formamide (Sigma-Aldrich), 2 mM vanadyl ribonucleoside complexes (Sigma-Aldrich), 0.2
776 mg/mL RNase-free bovine serum albumin (Roche Diagnostics), 10% Dextran sulfate (Eurobio),
777 and 2 µL fluorescent duplexes per 100 µl hybridization volume. Hybridization was performed
778 overnight at 37°C. The next day, coverslips were washed in a 15% formamide 1x SSC solution
779 for 40 min twice. Coverslips were then mounted using DAPI-containing Vectashield (Vector
780 Laboratories, Inc.). All probe sequence are available in Table S6.

781

782 **High-throughput smFISH**

783 HT-smFISH was performed as described previously⁷⁷. Probesets against mRNAs of interest
784 were generated starting from a pool of DNA oligonucleotides (GenScript). The design of DNA
785 oligonucleotides was based on the Oligostan script³¹. Briefly, oligos belonging to the same
786 probeset (hybridizing to the same mRNA target) share a common barcode which allows their
787 specific amplification through 2 rounds of PCR. Then, *in-vitro* transcription was used to
788 generate transcript-specific primary RNA probesets. Each primary RNA probe contains a
789 hybridization sequence recognizing the target of interest, flanked by 2 readout sequences.
790 These readout sequences serve as platforms to recruit fluorescent TYE-563-labeled locked
791 nuclei acids (similar to the smiFISH technique³¹). For each mRNA target, 25 ng of each of the
792 fluorescent locked nuclei acids were prehybridized with 50 ng of primary RNA probeset in 100
793 µL of a solution containing 7.5 M urea (Sigma-Aldrich), 0.34 mg/mL tRNA, and 10% Dextran
794 sulfate. Pre-hybridization was done using thermocycler with the following program: 90°C for 3
795 min, 53°C for 15 min, giving rise to fluorescent duplexes. Cells were grown in 96-well plates with
796 glass bottoms, fixed with 4% PFA for 20 min, and permeabilized with 70% ethanol overnight.
797 Cells were then washed with PBS and hybridization buffer (1x SSC, 7.5 M urea), and the 100
798 µL solution containing fluorescent duplexes was added. Cells were incubated at 48 °C
799 overnight. The next morning, plates were washed 8 times for 20 min each in 1x SSC, and 7.5 M

800 urea at 48 °C. Finally, cells were washed with PBs, labeled with DAPI at 5 µg/mL, and mounted
801 in 90% glycerol (VWR), 1 mg/mL p-Phenylenediamine (Sigma-Aldrich) and PBS pH 8.

802

803 **Imaging**

804 smFISH and IF imaging were performed using an inverted Zeiss Z1 widefield fluorescence
805 microscope equipped with a motorized stage. A 63x oil objective with a numerical aperture of
806 1.4 and an Axiocam 506 mono camera (Zeiss) with a pixel size of 72 nm were used. The
807 microscope was controlled by the Zeiss ZEN blue software (version 3.5.093.00003). Z stacks
808 were acquired with a 0.3 µm spacing between each plane. This spacing provided adequate
809 single molecule detection without oversampling. Maximum intensity projection (MIP) was used
810 to obtain 2D images for visualization, mounting, and analysis. The following exposure duration
811 and laser powers were used: 300-500 ms exposure at 100% laser power to image RNA in the
812 Cy3 or Cy5 channels and PBs labeled with anti-DDX6 in the GFP channel; 50 ms at 50% laser
813 power to image GFP-LSM14A-labeled PBs in the GFP channel and DNA in the DAPI channel.
814 For the HT-smFISH screen, 96-well plates were imaged on an Opera Phenix High-Content
815 Screening System (PerkinElmer), with a 63x water-immersion objective (NA 1.15). Three-
816 dimensional images were acquired, with a spacing of 0.3 µm. Figures were mounted using Fiji⁷⁸,
817 Adobe illustrator, and the OMERO Figure tool⁷⁹.

818

819 **Image analysis**

820 To analyze PB characteristics in HEK293-FUCCI cells, nuclei and cell segmentation was done
821 using the Cellpose⁸⁰ model with watershed. Nuclei were segmented using the DAPI channel,
822 and the cytoplasm using the DDX6 IF channel using an intensity threshold of 350-450. To
823 determine the cell cycle phase based on the nuclear FUCCI signal, we used the Otsu method to
824 classify nuclei as positive or negative in the green and red channels. All images were inspected
825 to verify the reliability of the classification. PBs were detected based on DDX6 IF using the
826 BigFISH⁸¹ Segmentation subpackage applied to the cytoplasm, and PB GFP masks were
827 generated based on a fluorescence intensity threshold of 1200-1500. PBs were then assigned
828 to individual cells and the mean PB fluorescence intensity was computed. The fluorescence
829 background (250), estimated from areas of the image without cells, was subtracted from PB
830 intensity values. PB size was computed as the maximal rectangular perimeter occupied by a

831 PB, multiplied by the pixel size (72 nm) and divided by 2. PBs with size appearing smaller than
832 200 nm were excluded as they approach the diffraction limit of the microscope in the green
833 channel. Images from 2 independent experiments were normalized to have the same PB
834 intensity distribution means. The few PBs overlapping the nucleus in the MIPs were not taken
835 into consideration.

836 smFISH images were analysed using BigFISH⁸¹, a python implementation of FISH-
837 quant⁸² available at <https://github.com/fish-quant/big-fish>. The specific code used to analyze
838 RNA localization in PBs is available at https://github.com/15bonte/p_bodies_cycle_2023. Nuclei
839 and cell segmentation was done using the Cellpose⁸⁰ model with watershed. Nuclei were
840 segmented using the DAPI channel. The cytoplasm was segmented using background from the
841 smFISH channel using an intensity threshold of 400-500. Spot detection was done using the
842 Detection subpackage of BigFISH, which uses a Laplacian of Gaussian filter to enhance the
843 spot signal. A local maximal algorithm then localizes every peak in the image, and a threshold
844 was applied to discriminate the actual spots from the nonspecific background signal. One
845 advantage BigFISH provides is the ability to automatically set an optimal RNA spot detection
846 threshold regardless of the signal-to-background ratio of the smFISH. This parameter-free
847 detection was used for all RNAs imaged in this study and manually adjusted when needed to
848 obtain a unimodal distribution of single molecule fluorescence intensities. Next, we decomposed
849 dense areas of RNA signal, firstly by removing background noise with a Gaussian background
850 subtraction and secondly using the cluster decomposition tool. This tool computes the median
851 detected spot intensity distribution, fits it with a Gaussian distribution signal, and uses it to
852 compute the number of spots that best fit within each cluster. Next we detected PBs as masks
853 using the GFP channel and the BigFISH Segmentation subpackage with a fluorescence
854 intensity threshold of 2200 for cells synchronized in G1S, mid S and G2M, and 1600 for cells
855 synchronized in mid G1, since PBs were less bright. We then computed two RNA populations:
856 (i) RNA molecules in the cytoplasm (i.e. outside the nucleus), (ii) and RNA molecules in PBs
857 (i.e. within the GFP mask). Since BigFISH was applied to MIPs, some PBs overlapped with the
858 nucleus in a minority of cells and were excluded from the analysis. Cells without labeled PBs,
859 with improper segmentation, or incomplete cell fragments were also excluded. In experiments
860 involving transient transfection (Figure 7, S7, Renilla Luciferase reporters), cells with fewer than
861 15 mRNA molecules or with too high expression for proper BigFISH detection were excluded
862 and the total mRNA was used to calculate the fraction of mRNA in PBs in cells with PBs
863 appearing in the nucleus.

864 For HT-smFISH analysis (Figure 5, 6), a similar BigFISH pipeline was used to segment
865 cells, nuclei and detect RNA spots. The specific code used to analyze HT-smFISH images is
866 available here: [https://github.com/Flo3333/Cell-cycle-and-HT-smFISH-analysis-of-RNA-](https://github.com/Flo3333/Cell-cycle-and-HT-smFISH-analysis-of-RNA-localization-in-PBs)
867 [localization-in-PBs](https://github.com/Flo3333/Cell-cycle-and-HT-smFISH-analysis-of-RNA-localization-in-PBs). PBs were detected as 2D mask using a log filter ($\sigma = 2.25$) to detect PB
868 edges from the average projection of the image stack. A threshold was then applied to segment
869 PBs edges by detecting positive gradients of intensity on the filtered image. Finally, PBs masks
870 were filled and artifacts smaller than 10 pixels are removed. Cells without labeled PBs or RNA
871 spot, with improper segmentation, and incomplete cells were excluded from the image analysis.
872 To classify cells in G1 or G2, the DAPI signal was used to construct a cell cycle profile, as
873 follows. We first calculated the integrated DAPI signal in each nucleus (nucleus area multiplied
874 by the mean DAPI signal). The obtained profile was then fitted, using a ranking-based
875 classification, to a cell cycle profile of HEK293-GFP-LSM14A cells obtained with flow cytometry
876 and analyzed using the Dean-Jett model (~50% cells in G1 and ~16.5% of cells in G2, see
877 example in Figure 5A).

878

879 **Western blots**

880 Cytoplasmic proteins were extracted as described previously⁸³, separated on a NuPage 4%–
881 12% gel (Invitrogen, Life Technologies) and transferred to Optitran BA-S83 nitrocellulose
882 membrane (Fisher Scientific). After blocking in PBS containing 5% (wt/vol) nonfat dry milk for 1
883 hr at room temperature, membranes were incubated with the primary antibody overnight at 4°C,
884 washed in PBS, and incubated with horseradish peroxidase–conjugated anti-rabbit secondary
885 antibody (Interchim, Cat#111-036-003) diluted 1/10,000 for 1 hr at room temperature. Primary
886 antibodies were: rabbit anti-DDX6 (BIOTECHNE, Cat# NB200-192, diluted 1/15,000), rabbit
887 anti-4E-T (Abcam, Cat#ab95030, 1/2,500), rabbit anti-LSM14A (Bethyl, Cat#A305-102A,
888 1/5,000), rabbit anti-PAT1b (Cell Signaling, Cat#14288S, 1/1,000), and rabbit anti- rProt S6
889 (5G10, Cell signaling, Cat#2217, 1/5,000). After washing in PBS, proteins were detected using
890 the Western lightning plus ECL kit (Perkin Elmer) and visualized by exposure to CL-XPosure
891 film (Thermo Scientific).

892

893 **Statistical analysis**

894 Graphical representations and statistical tests were performed using the GraphPad Prism
895 software (v8, GraphPad software, Inc), the R suite (v 4.2.0, <https://www.R-project.org>, R Core
896 Team 2018, R: A language and environment for statistical computing, R Foundation for
897 Statistical Computing, Vienna, Austria), R studio (v 2022.12.0), and Excel 2016 and the Excel
898 Analysis ToolPak (Microsoft). Venn diagrams were generated using a tool available at
899 <https://bioinformatics.psb.ugent.be/webtools/Venn>. For imaging experiments, cell population
900 distributions were compared using a Two-tailed Mann-Whitney test.

901 **Supplemental tables:**

902 **Table S1: Mass spectrometry analysis before and after sorting, related to Figure 1.** The
903 proteomic data for each cell cycle phase are shown in independent tabs. The two other tabs
904 provide the tentative list of PB proteins and the functional protein annotation used to build
905 Figures 1 and S2.

906 **Table S2: DEseq2 results comparing RNA-seq before and after sorting, related to Figure**
907 **2.** The normalized RNA expression levels (in normalized counts) for each replicate per cell cycle
908 phase before and after sorting are shown, as well as their mean value.

909 **Table S3: DEseq2 results comparing RNA-seq before and after sorting throughout the**
910 **cell cycle, related to Figure 2.** Every tab compares two successive cell cycle phases in the PB
911 or the presorting fractions. The periodic phase of cyclic RNAs obtained from Dominguez et al.,
912 2016 is also included²⁸.

913 **Table S4: Normalized RNA expression levels (in normalized counts) before and after**
914 **sorting across the cell cycle, related to Figure 2.** These correspond to the normalization
915 used in Table S3. Values are shown for each replicate in both fractions throughout the cell
916 cycle.

917 **Table S5: Gene ontology analysis results, related to Figure 2.** P-values, gene IDs, and gene
918 counts are provided for each GO category shown in Figures 2E and S7B, C.

919 **Table S6: Sequences of smFISH probes used throughout the study, related to Figures 3-7**
920 **and STAR Methods.**

921 **Table S7: Data related to the HT-smFISH experiments, related to Figure 5.** RNA names, cell
922 numbers, total mRNA counts, mRNA counts in PBs, mean fraction of mRNA in PBs, fraction of
923 mRNA in PBs per cell, and decay status in G1 are indicated.

924

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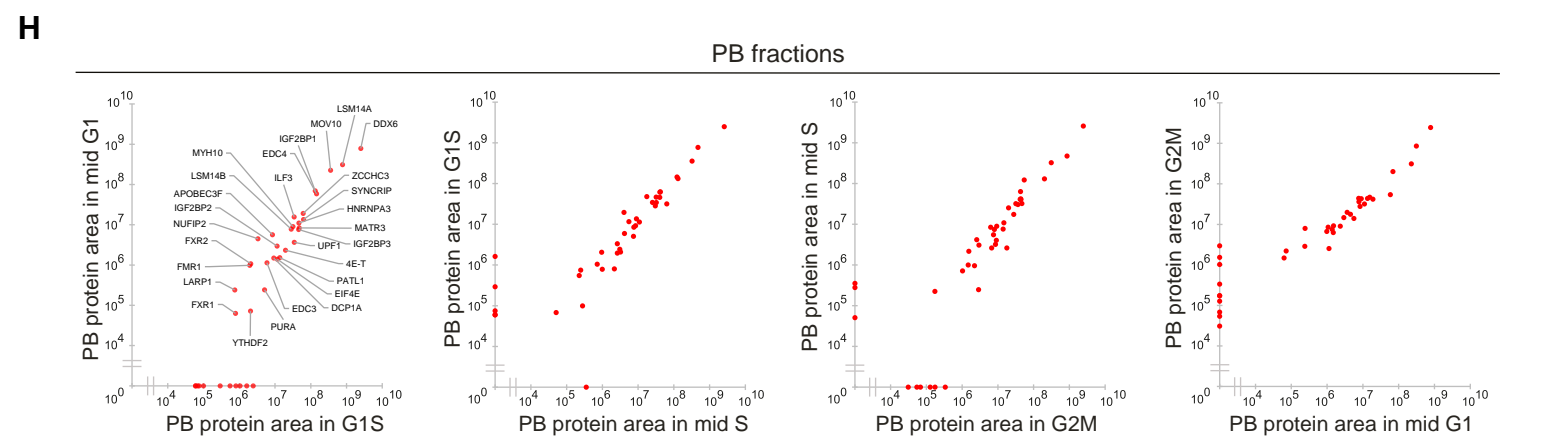
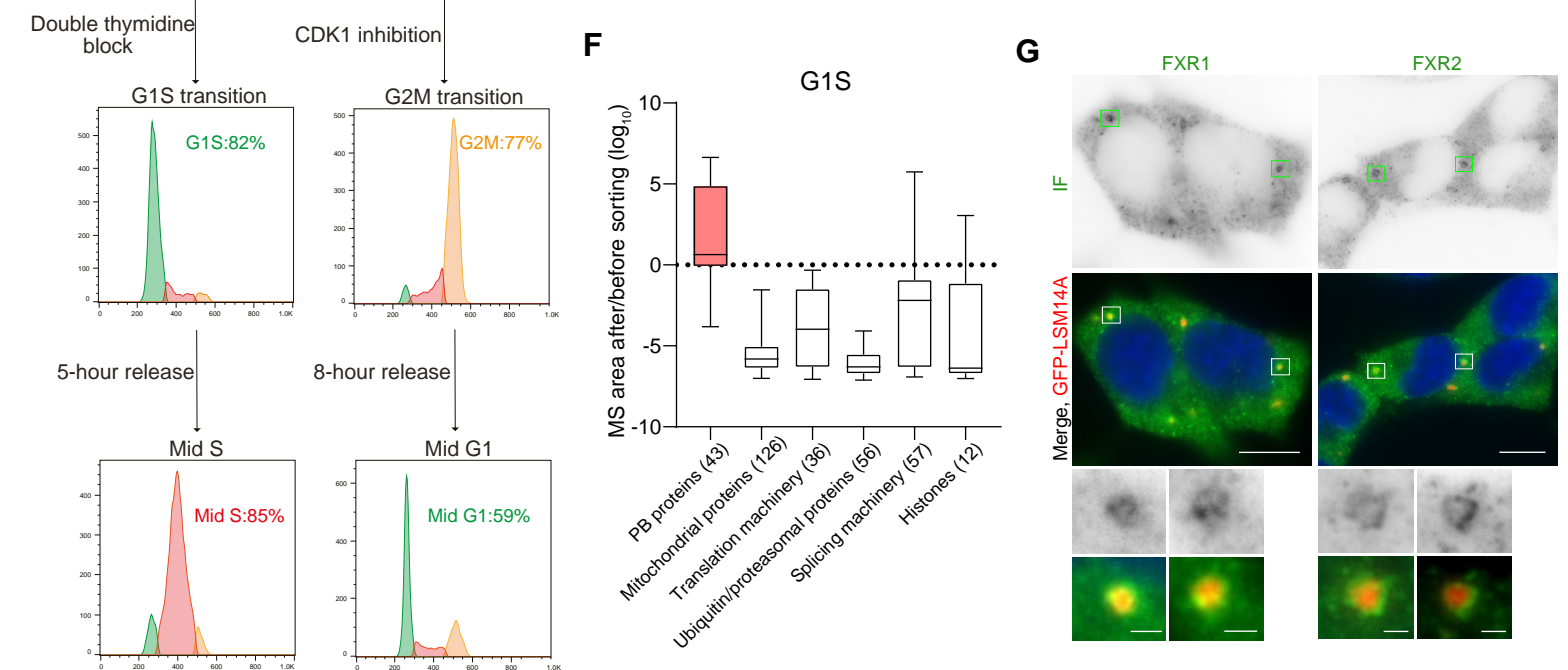
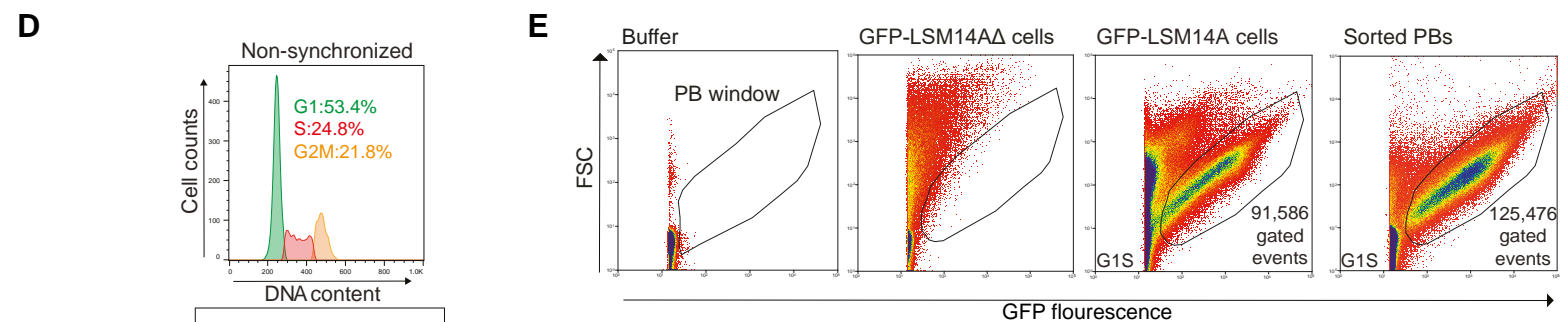
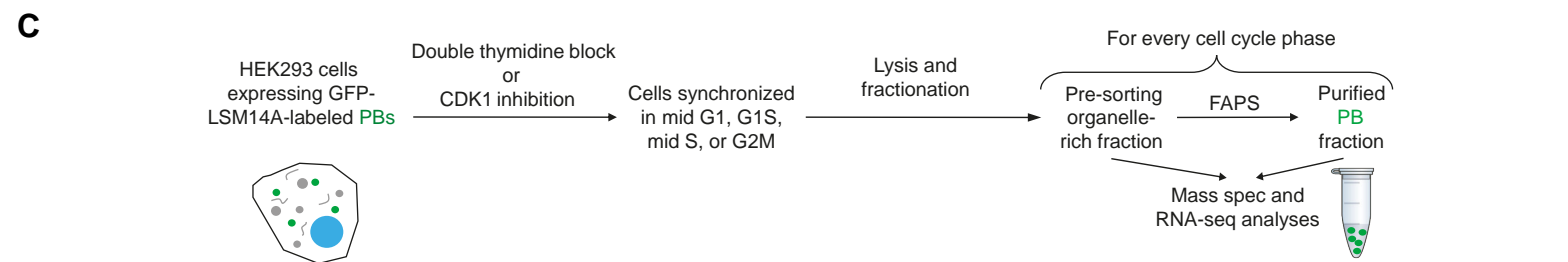
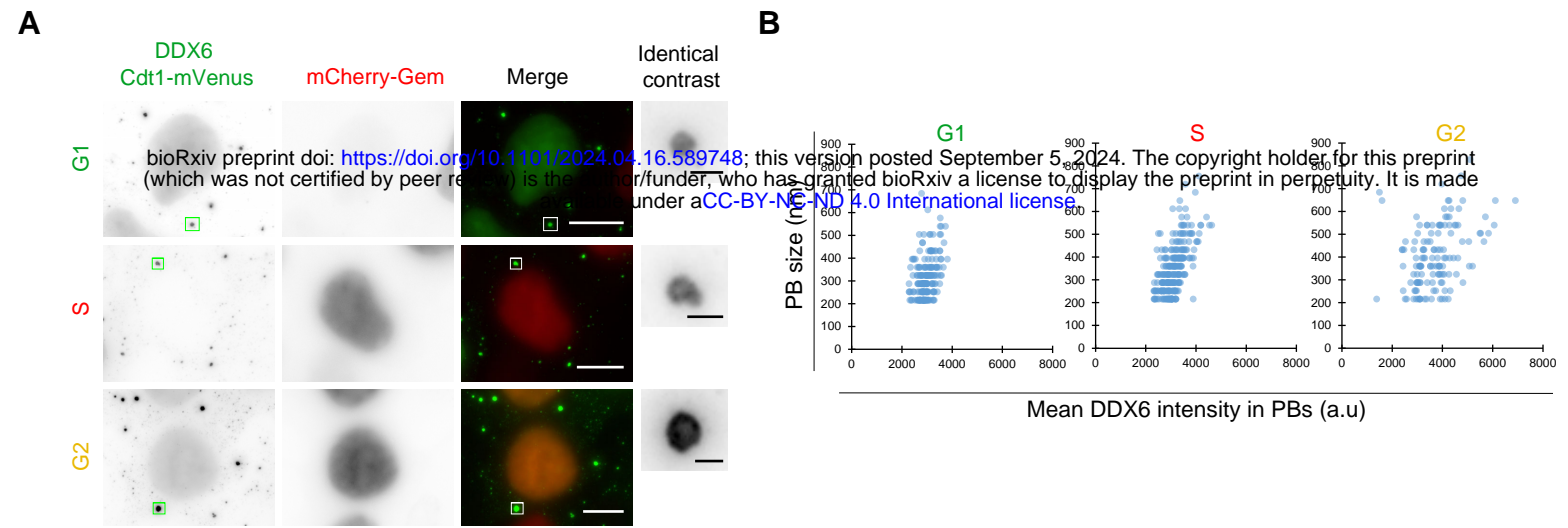
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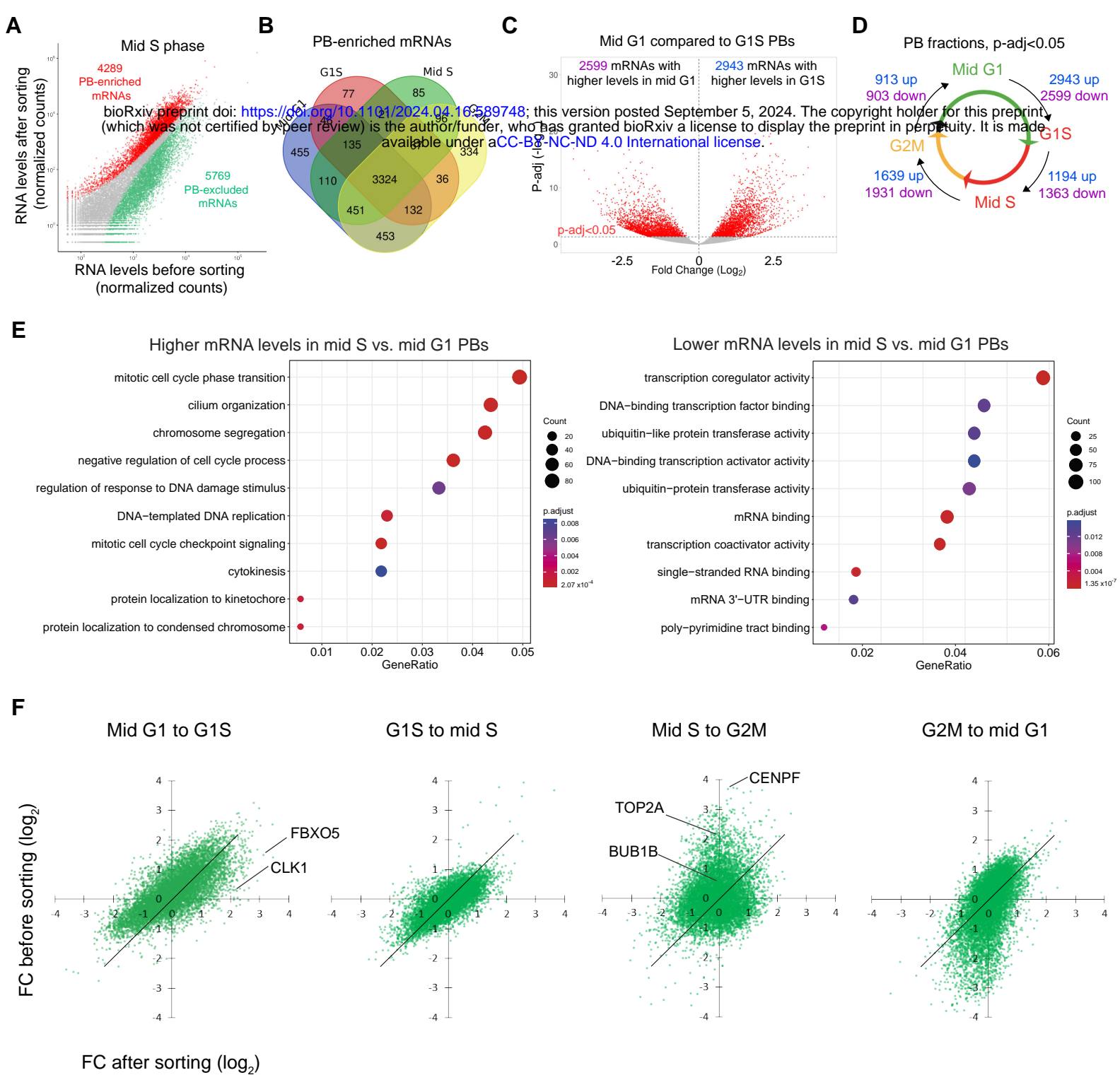
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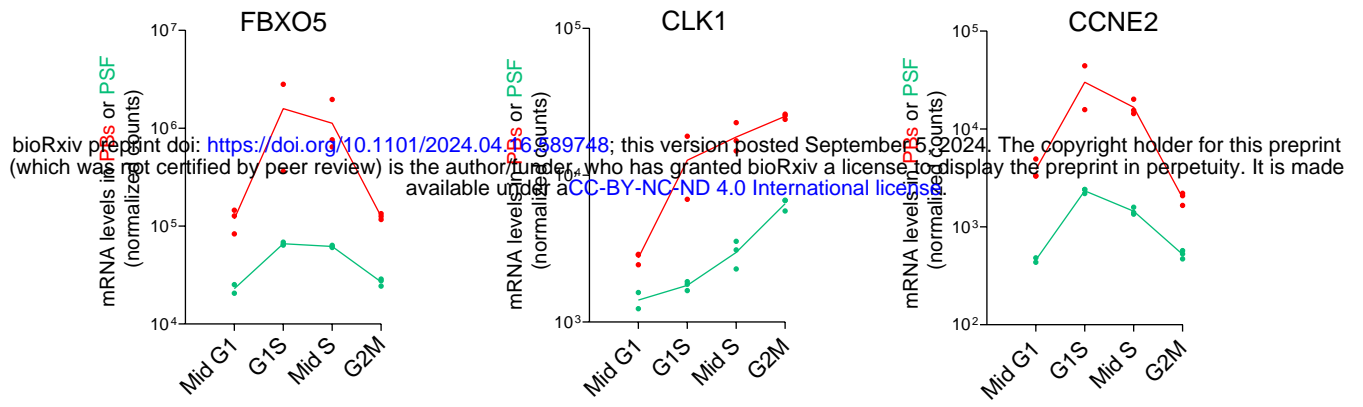
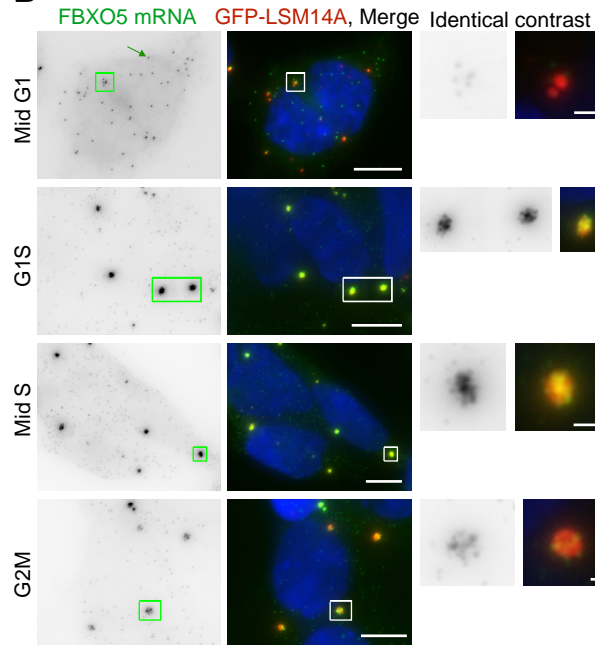
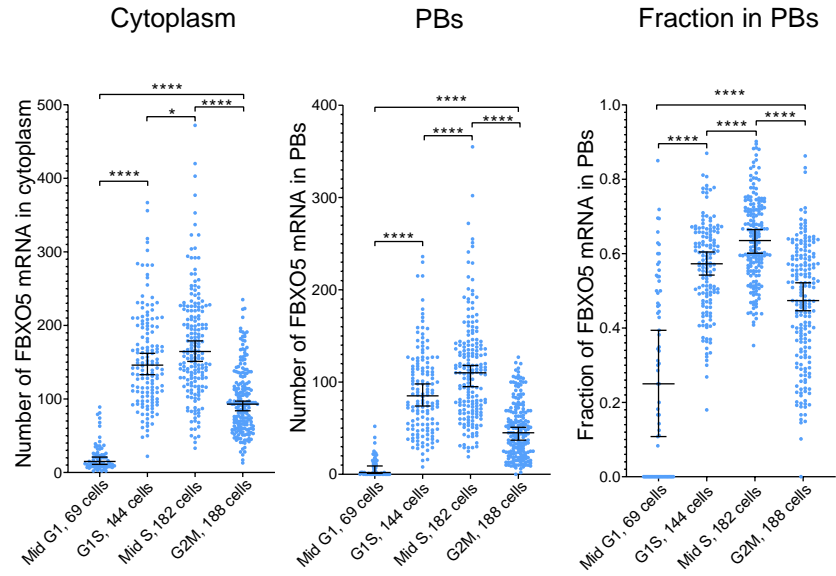
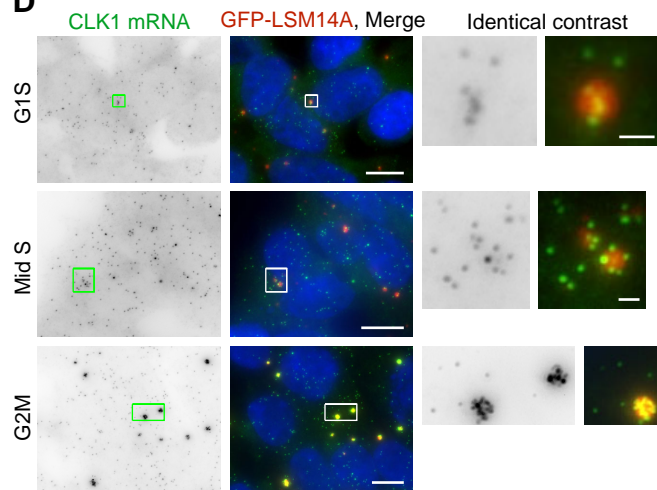
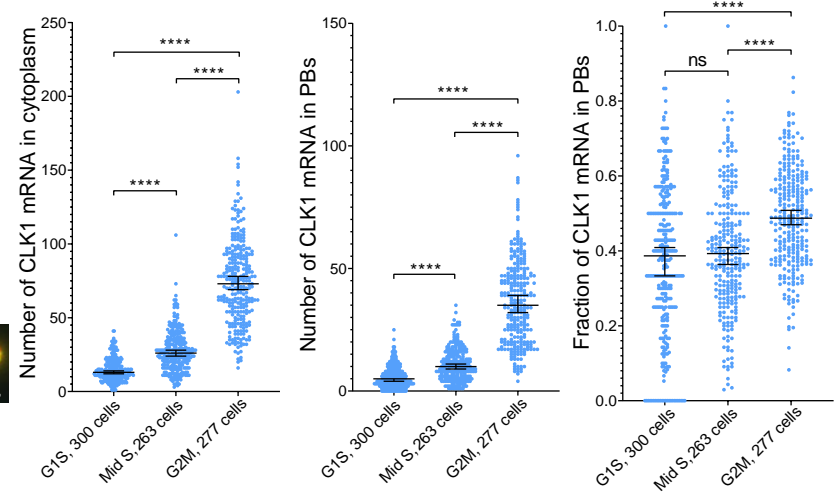
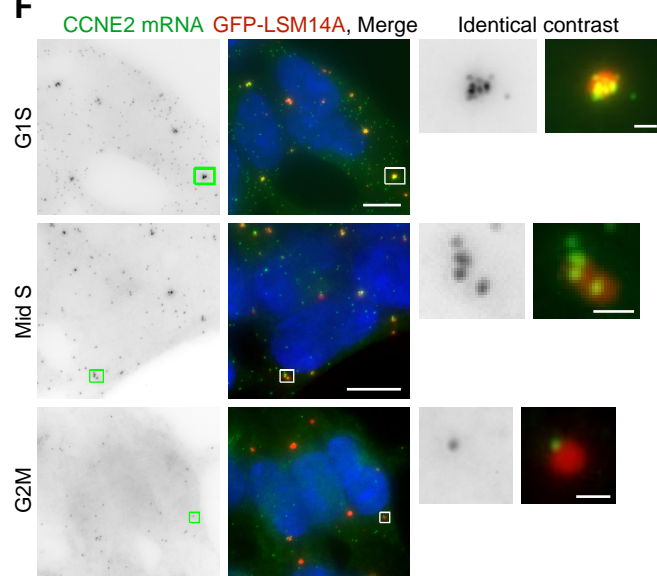
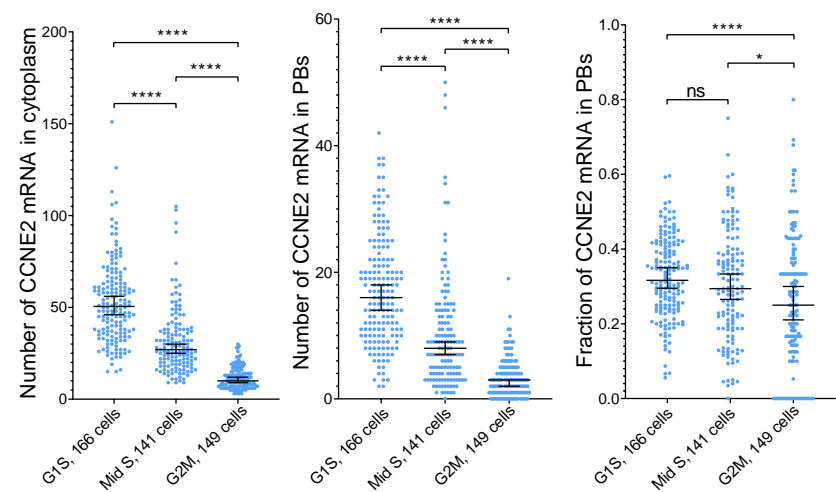
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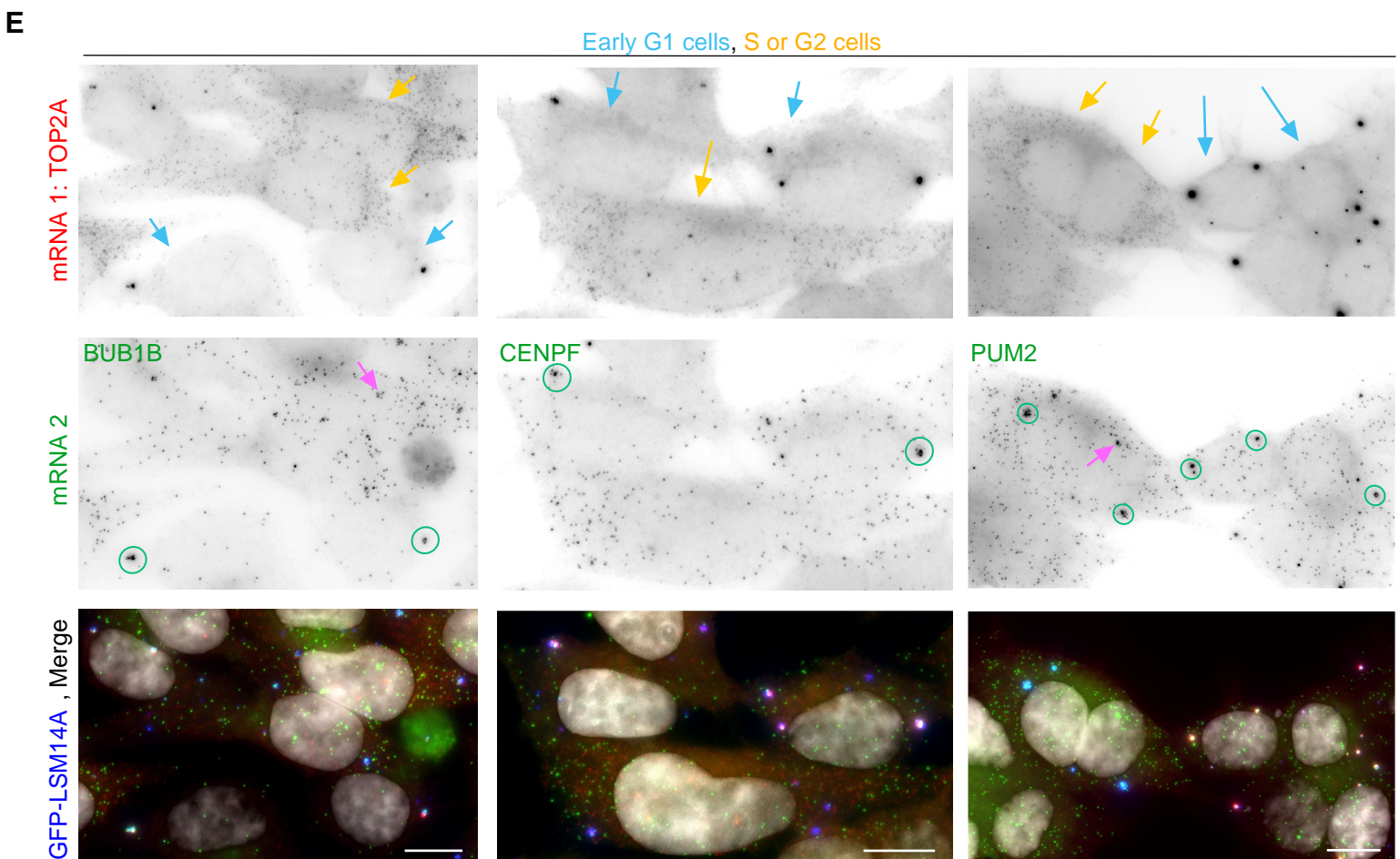
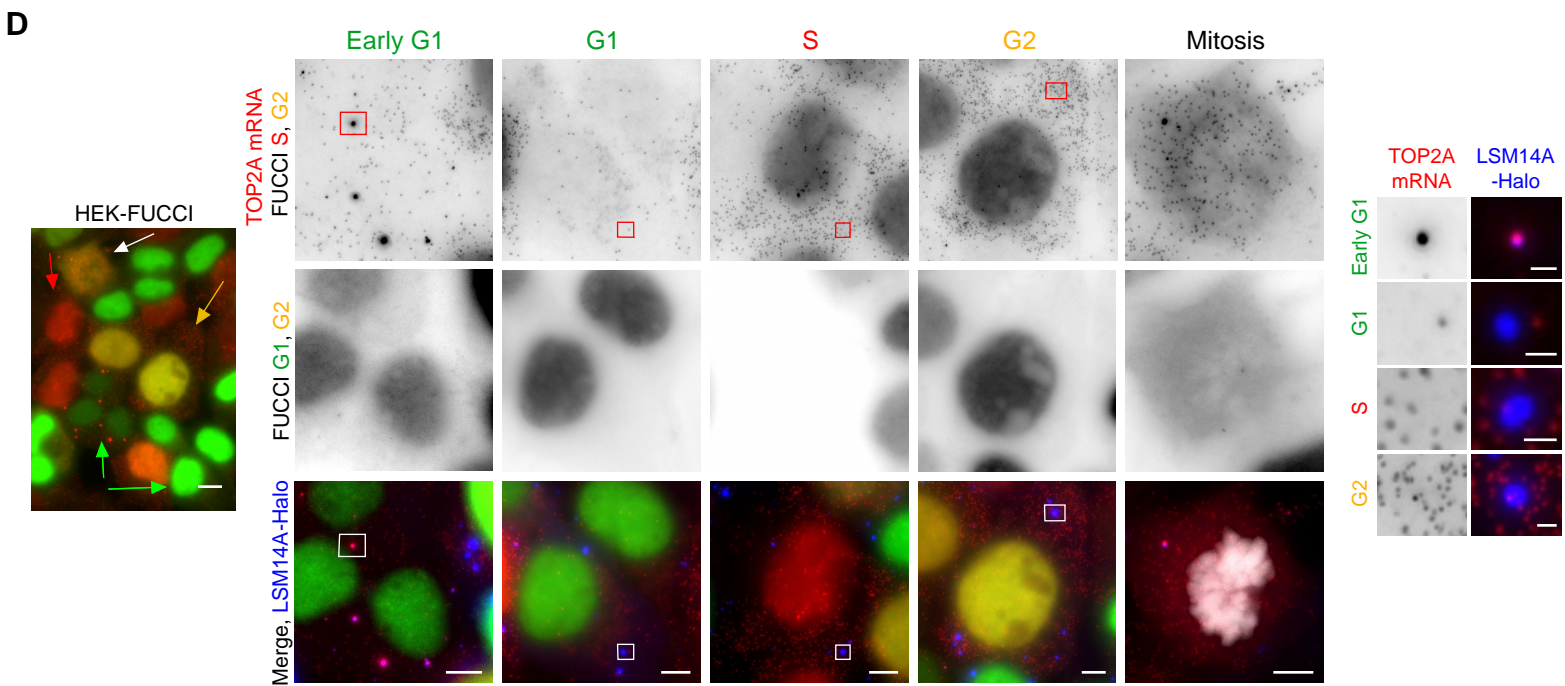
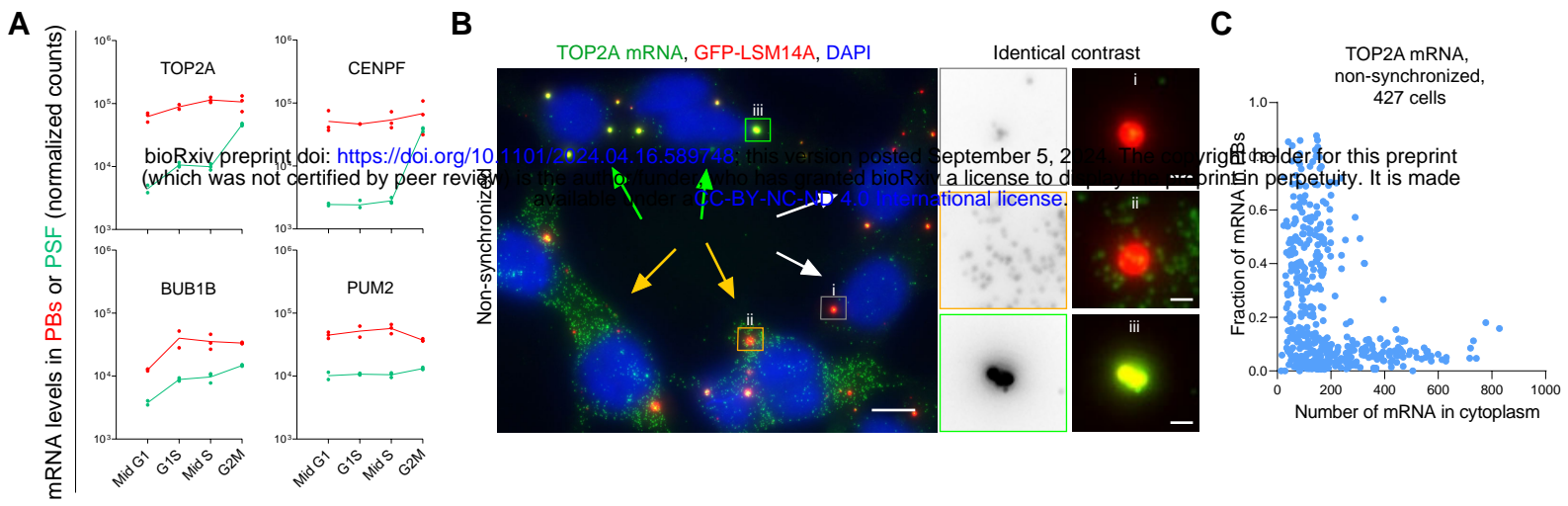
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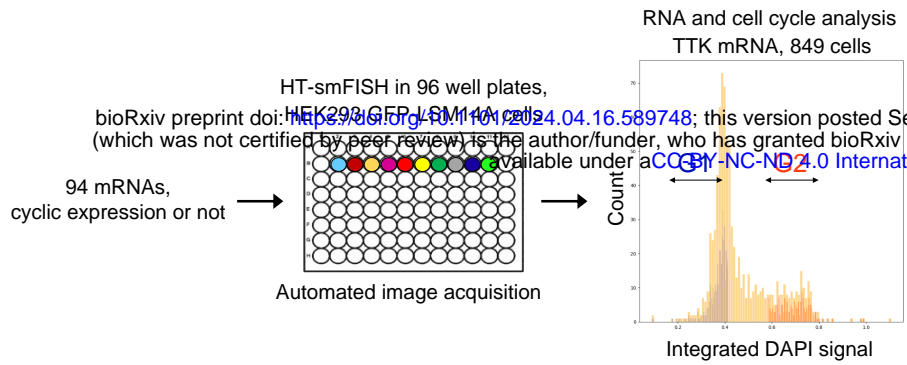
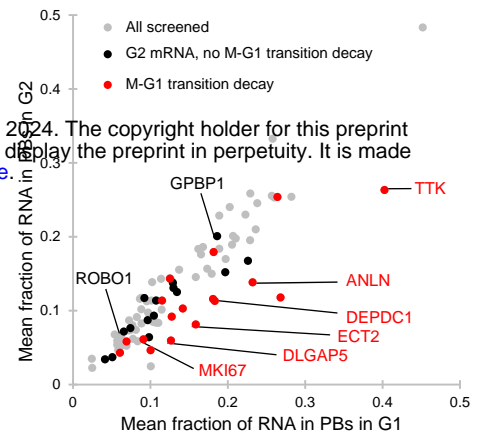
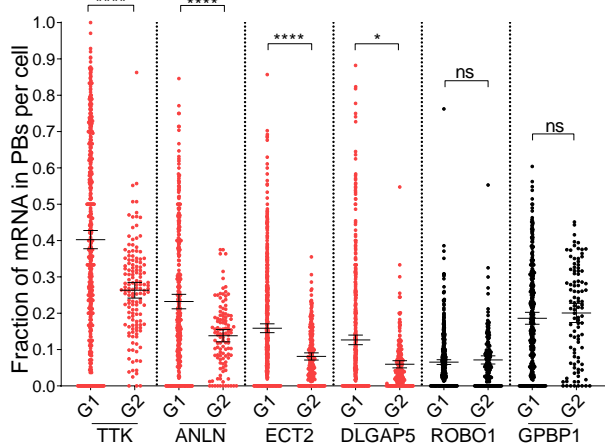
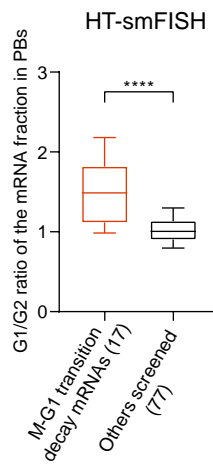
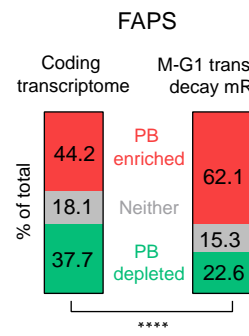
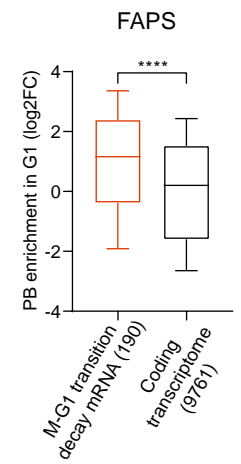
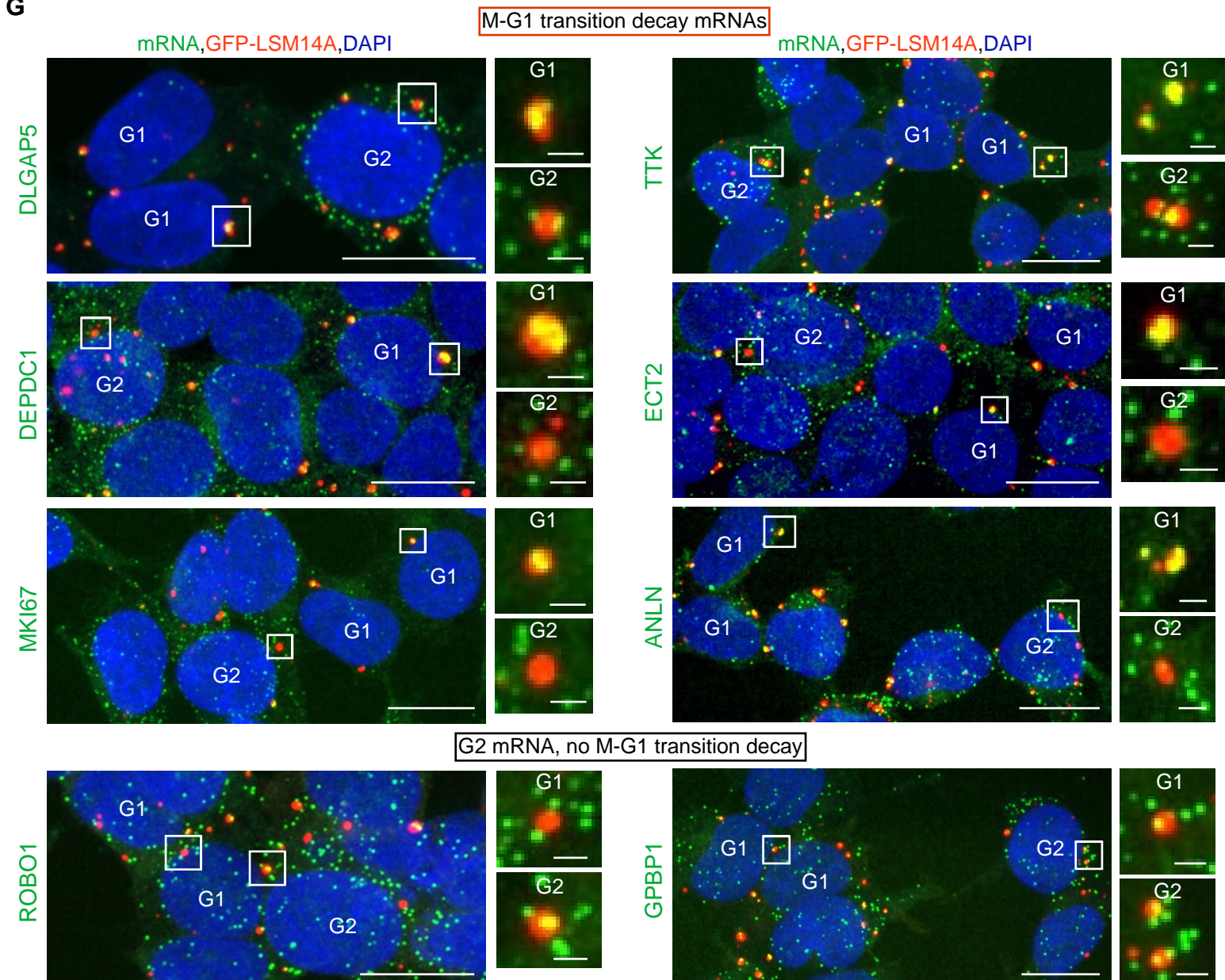


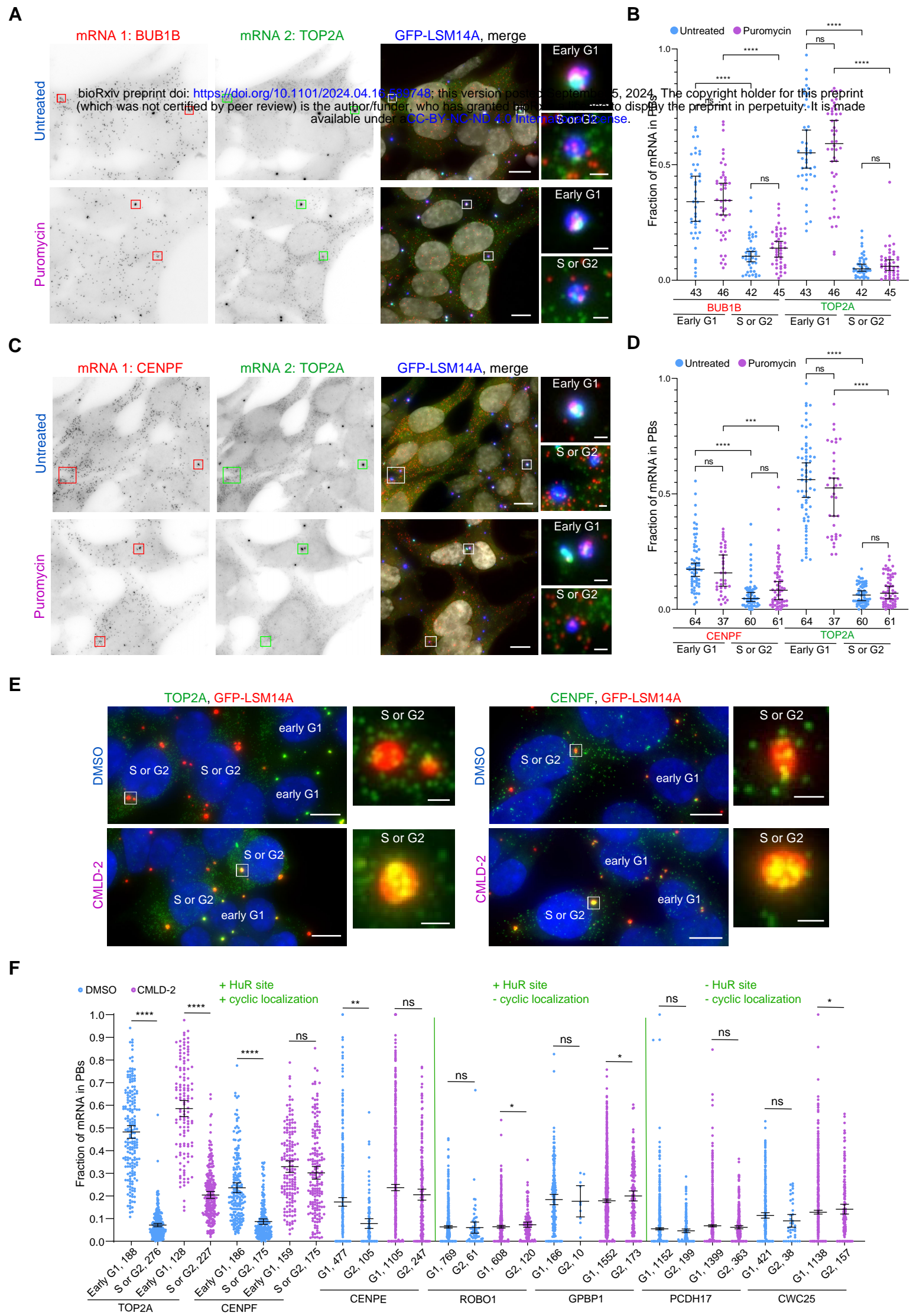


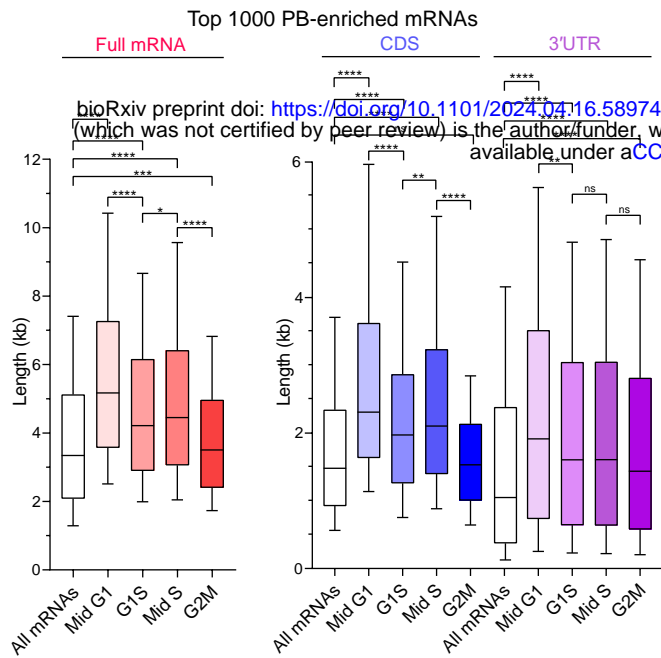
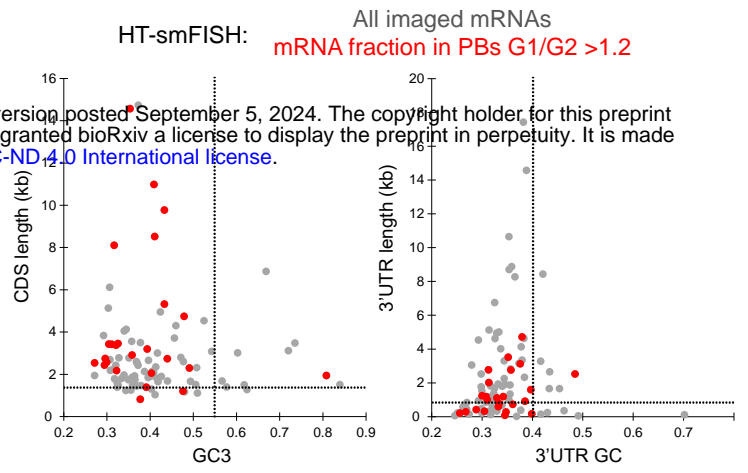
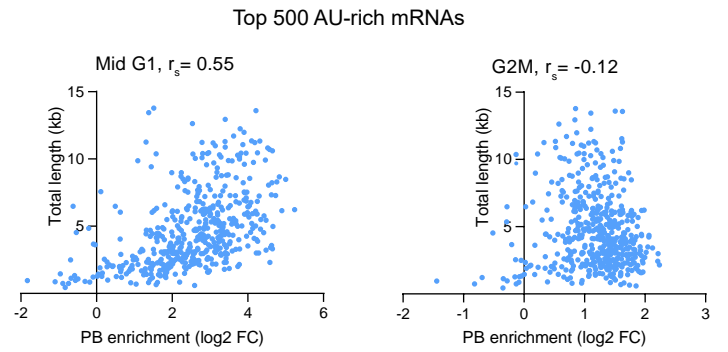
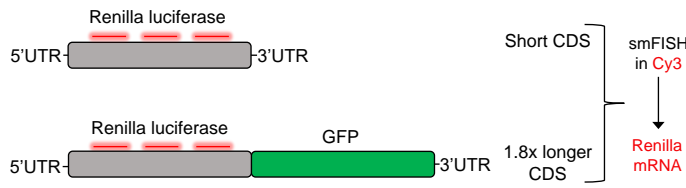
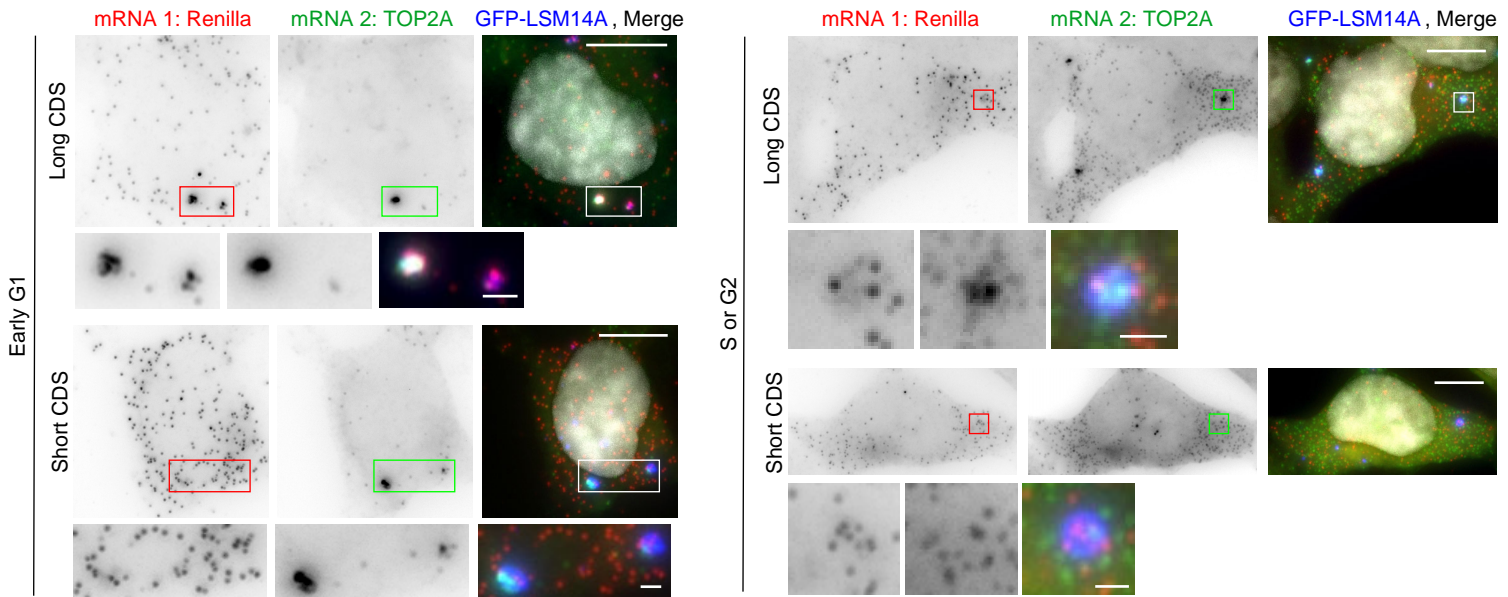
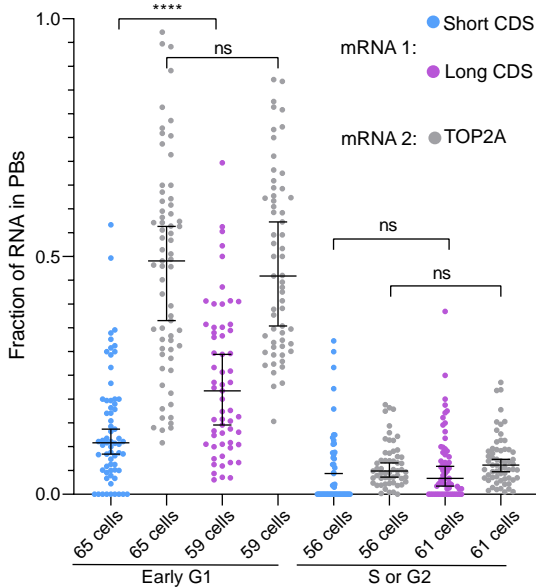
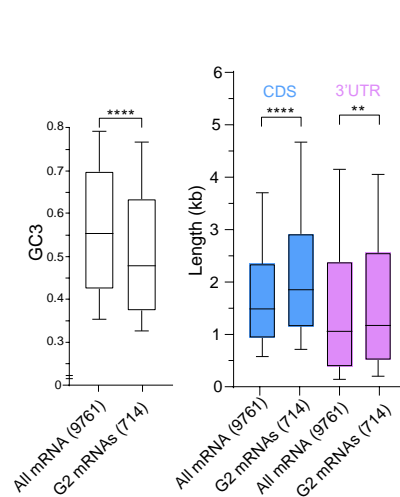
FC after sorting (log_2)

A**B****C****D****E****F****G**



A**B****C****D****E****F****G**



A**B****C****D****E****F****G****H**