Title

Poly(A) binding protein is required for mRNP remodeling to form P-bodies in mammalian cells

Running title

Poly(A) binding protein and P-body formation

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Keywords

PABPC1, P-bodies, stress granules, mRNP remodeling, GW182

Summary statement

A novel role of poly(A) binding protein is reported in P-body formation

Abstract

Compartmentalization of mRNA through formation of RNA granules is involved in many cellular processes, yet it is not well understood. mRNP complexes undergo dramatic changes in protein compositions, reflected by markers of P-bodies and stress granules. Here, we show that PABPC1, albeit absent in P-bodies, plays important role in P-body formation. Depletion of PABPC1 decreases P-body population in unstressed cells. Upon stress in PABPC1 depleted cells, individual P-bodies fail to form and instead P-body proteins assemble on PABPC1-containing stress granules. We hypothesize that mRNP recruit proteins via PABPC1 to assemble P-bodies, before PABPC1 is displaced from mRNP. Further, we demonstrate that GW182 can mediate P-body assembly. These findings help us understand the early stages of mRNP remodeling and P-body formation.

Introduction

Cytoplasmic messenger ribonucleoproteins (mRNPs) are suggested to cycle among polysomes, stress granules (SGs) and P-bodies (PBs). In this cycle, mRNAs exist in different functional states from translating, non-translating to degradation. While polysomes consist of translating mRNAs and SGs paused mRNAs with translation initiation components, PBs contain mRNA decay machineries.

PBs are present in unstressed cells, and further induced upon inhibition of translation initiation (Teixeira et al., 2005, Kedersha et al., 2005). PBs are closely related to control of translation and mRNA degradation. Proteins at PBs are involved in mRNA decay and translation repression, including decapping enzyme complex Dcp1/Dcp2; decapping activator hedls/GE-1; translation repressor and decapping activator DDX6/RCK, and the CCR4/NOT deadenylase complex (Anderson and Kedersha, 2006, Eulalio et al., 2007a).

SGs can be juxtaposed with PBs in animal cells(Kedersha et al., 2005, Stoecklin and Kedersha, 2013), which suggests that they are close in origin. SGs share some common components with PBs, but distinctively contain translation initiation factors like PABPC1, eIF4G, eIF4A, eIF3 and eIF2 etc. (Decker and Parker, 2012).

Assembly of PBs and SGs may start from non-translating mRNPs, which aggregate into microscopic granules through certain protein-protein interactions. In yeast, a self-interacting domain of Edc3 protein and prion-like glutamine/asparagine (Q/N) rich region of Lsm4 can facilitate the aggregation (Reijns et al., 2008, Decker et al., 2007). However, in metazoans, Edc3 is not required for PB assembly (Eulalio et al., 2007b). Instead, depletion of GW182 or hedls/GE-1, two proteins containing low-complexity and Q/N rich regions, leads to decreased PBs in unstressed animal cells (Eulalio et al., 2007b, Liu et al., 2005a, Yu et al., 2005, Kato et al., 2012).

One interesting issue is the absence of most translation initiation factors, including poly(A) binding protein cytoplasmic 1 (PABPC1), in P-bodies of mammalian cells. Components of RNA granules are in dynamic exchange with cytoplasmic proteins (Kedersha et al., 2005,

63 Andrei et al., 2005). The association and dissociation of proteins, such as PABPC1, to and
64 from the cap and tail of mRNA may be an important step in the transitions of mRNAs from
65 translating to non-translating or decay states.
66
67 mRNAs have a poly(A) tail of about 50-100 nucleotides in mammalian cells (Chang et al.,
68 2014, Subtelny et al., 2014). PABPC1 occupies around 27-residues on the poly(A) tail and
69 forms repeating structures (Baer and Kornberg, 1983). PABPC1 is the major isoform out
70 of four known in mammals. PABPC1 consists of four RNA binding domains (RRM 1-4)
71 followed by a poorly conserved linker region and a protein-protein interaction (MLLE)
72 domain at the C-terminus. The RRM domains are pivotal for circularization of mRNA
73 through the binding of the poly(A) tail and eIF4G (Kahvejian et al., 2005, Deo et al., 1999,
74 Imataka et al., 1998, Safaee et al., 2012). The MLLE domain recognizes a conserved PAM2
75 peptide motif, found in a number of proteins including PB components GW182, Pan3 and
76 Tob1/2 (Xie et al., 2014).
77
78 PABPC1 plays important roles in decay besides translation of mRNA. Yeast poly(A)
79 binding protein (Pab1) can inhibit Ccr4/Pop2/Not deadenylase complex (Tucker et al.,
80 2002), possibly due to increased association and protection of Pab1 to the poly(A) tail. It
81 was recently shown that PABPC1 helps recruiting microRNA-induced silencing complex
82 (miRISC) through GW182, a major component of miRISC (Moretti et al., 2012). GW182,
83 in turn, facilitates PABPC1 dissociation from silenced mRNA without deadenylation
84 (Zekri et al., 2013).
85
86 Although PABPC1 displacement is a critical step in mRNA decay and can act as a scaffold
87 protein on mRNA to recruit PB proteins GW182 and Pan3 etc. through PAM2 motifs, it is
88 not known what role PABPC1 plays in assembling PBs. Here, we report that depletion of
89 PABPC1 affects PB numbers in unstressed cells and causes fusion of PB and SG
90 components upon cell stress. Further, we found that the availability of PABPC1, but not
91 the presence of SG structures, is critical in PB genesis. By engineering GW182 to
92 strengthen its binding to PABPC1, we simulated the fusion of SGs and PBs. Overall, we
93 conclude that PABPC1 serves as a platform for early mRNP remodeling to form PBs.

94

95 **Results**

96 **PABPC1 protein depletion decreases PBs in unstressed cells**

97 We were able to knock-down PABPC1 using siRNAs as previously shown (Yoshida et al.,
98 2006). The number of PBs decreased significantly upon PABPC1 knock-down in
99 unstressed HeLa and MEF cells (Fig. 1A). PABPC1 depletion affects bulk translation only
100 mildly and doesn't perturb the levels of the major translation factors (Yoshida et al., 2006).
101 However, Paip2, a PABPC1-interacting protein, decreases in cells following PABPC1
102 depletion (Yoshida et al., 2006). To exclude a role of Paip2 in PB formation, we knocked
103 down both isoforms, Paip2a and Paip2b, by siRNA and found little effect on the number
104 of PBs (Fig. 1B). It was reported that depletion of hedls or GW182 decreased PBs in cells
105 (Eulalio et al., 2007b, Liu et al., 2005b, Yu et al., 2005). However, PABPC1 depletion
106 didn't lower hedls or GW182 protein levels (Fig. 1C), which suggests that PABPC1 is
107 affecting PBs independently of changes in hedls or GW182.

108

109 **PBs cannot be re-induced in PABPC1 protein depleted cells**

110 PB population may be reduced by siRNAs unrelated to their silencing activities and PBs
111 can often be re-induced by stress (Serman et al., 2007). Therefore, we checked whether
112 PBs could be re-induced upon cell stress. In mammalian cells, PBs and SGs can be clearly
113 distinguished. PBs are relatively compact and dense, while SGs are bigger, loose and more
114 irregular (Stoecklin and Kedersha, 2013, Souquere et al., 2009, Kedersha et al., 2005).
115 When we treated PABPC1-depleted cells with arsenite to induce PBs and SGs, we were
116 surprised to find that compact dense PBs failed to form. Instead, the PB component hedls
117 was relocalized to SGs (Fig. 2). The colocalization was found across the cytoplasm by 3D
118 confocal microscopy. This suggests that PB assembly is affected by depletion of PABPC1.
119 To our knowledge, only DDX6, a RNA helicase also known as RCK/p54, can trigger
120 PB/SG fusions and prevent PB formation when depleted (Mollet et al., 2008, Serman et
121 al., 2007, Ayache et al., 2015). However, DDX6 protein levels remained unchanged after
122 PABPC1 depletion (Fig. 1C). Thus, PABPC1 affects PB formation independently of
123 DDX6.

124

125 **SG assembly is not affected by PABPC1 depletion and is independent of PB**
126 **assembly**
127 Previously, it was observed that PABPC1 depletion induced SG in a low percentage of
128 unstressed cells (Mokas et al., 2009). We checked SG assembly in stressed cells, and found
129 SGs marked by G3BP, HuR (Fig. 3A & B) or Ago2 were not significantly affected by
130 PABPC1 depletion. This implies that SGs can form in the presence of low levels of
131 PABPC1 protein.
132 The assembly of PBs and SGs are independent processes (Kedersha et al., 2005). PBs exist
133 in unstressed cells in the absence of SGs. Meanwhile, PBs can be increased by arsenite
134 stress in MEF cells expressing non-phosphorylated eIF2α mutant, which prevents SG
135 formation (Kedersha et al., 2005). We treated HeLa cell with PP242, an mTOR inhibitor
136 inhibiting SG assembly (Fournier et al., 2013, Feldman et al., 2009), and found PBs were
137 increased by arsenite without SG formation (Fig. 3C). This implies that PBs do not require
138 SGs to form in agreement with previous studies (Kedersha et al., 2005).
139
140 **Endogenous PABPC1 can be substituted by over-expressed PABPC1-GFP**
141 We have shown that PB components can be found at PABPC1-rich SG (Fig. 2), and that
142 only a fraction of the SGs contain PABPC1 in knocked-down cells (Fig. 3). Thus PBs
143 require PABPC1, but not other SG components, for assembly.
144 It is known that overexpression of PABPC1 down-regulates the endogenous PABPC1
145 translation and protein abundance through self-regulation (Hornstein et al., 1999b, de Melo
146 Neto et al., 1995, Wu and Bag, 1998, Hornstein et al., 1999a). This provides an opportunity
147 to create cells where endogenous PABPC1 is replaced by exogenous PABPC1-GFP. We
148 took advantage of an antibody (Abcam ab21060) that recognized the tail region of PABPC1
149 but not the chimeric PABPC1-GFP protein. Overexpression of PABPC1-GFP reduced
150 endogenous PABPC1 to a very low level in cells (Fig. s1). However, the PBs assembled
151 as normal, which implies that the over-expressed PABPC1-GFP can substitute endogenous
152 PABPC1 in maintaining the PB population in unstressed cells.
153
154 **Separate PBs can be induced after SG inhibition**

155 PBs assembly is closely related to PABPC1-containing SGs at low PABPC1 levels. We
156 then asked whether structures of SG were required or not in such process. With PP242
157 inhibiting SG assembly, we stressed PABPC1-depleted cells with arsenite and individual
158 PBs began to form (Fig. 3D). This shows that the low level of PABPC1 dissipated in
159 cytoplasm can support PB assembly under stresses. It also confirms that SGs are not
160 required for PB assembly. On the contrary, when the low level of PABPC1 was packed
161 into SGs, it results in fusion of PBs and SGs. It is likely the tightly packed PABPC1 in SGs
162 will not easily dissociate for mRNP remodeling, and thus lead to fusion of PBs and SGs.
163

164 **Overexpressed Dcp1a, TNRC6A and DDX6 can induce PB formation in PABPC1-**
165 **depleted cells, and are found associated with the fusions of PBs and SGs**

166 Overexpression of PB proteins can generate PBs that are not functional (Cougot et al.,
167 2004). In PABPC1-depleted cells, overexpression of Dcp1a or DDX6 could induce PBs
168 (Fig. 4A). When cells were stressed with arsenite, sorbitol, or heat shock, we found Dcp1a-
169 GFP or HA-TNRC6A were present in PB/SG fusions (Fig. 4B & C). This confirms that
170 multiple PB components were assembling at the fusion structures. Meanwhile,
171 overexpression of Dcp1a or DDX6 could induce PB-like structures in stressed cells, as
172 observed by other groups. Therefore, depletion of PABPC1 did not prevent the aggregation
173 of overexpressed PB proteins into granules.
174

175 **Creating PB/SG fusions without PABPC1 depletion**

176 We next asked how PABPC1, a protein absent from PBs, could affect their formation. One
177 hypothesis is that PB assembly may initiate on PABPC1-containing mRNPs. When the
178 residual PABPC1 after depletion is packed into SGs, PB components are recruited there
179 and thus a PB/SG fusion emerges. We speculated that certain PB proteins interacting with
180 PABPC1 might mediate the processes. We used a protein engineering approach to test our
181 hypothesis.
182 GW182 proteins (TNRC6A/B/C in human) interact with PABPC1 through the MLLE
183 domain and are required for PB assembly (Eulalio et al., 2007b; Liu et al., 2005a). The
184 native affinity of the GW182 PAM2 motif for MLLE of PABPC1 is about 6 μM (Kozlov
185 et al., 2010, Jinek et al., 2010). We engineered the PAM2 motif in GW182 to increase its

186 affinity by including features from Paip2 PAM2 motif. The engineered GW182 protein
187 contained a super-PAM2, binding PABPC1 about 300 times tighter (Fig. 5A, construct
188 information in Materials and Methods). The super-PAM2 motif could efficiently localize
189 fused GFP protein to stress granules (Fig. 5B). Introduction of super-PAM2 greatly
190 increased association of GW182 and endogenous PABPC1 (Fig. 5C). When we stressed
191 cells overexpressing super-GW182-GFP, the super-GW182 along with other PB
192 components located to SGs to make PB/SG fusions (Fig. 6). This fusion is the combined
193 result of the increased affinity PAM2/MLLE interaction and tightly packed PABPC1 at
194 SG. It indicates that PABPC1 interacting proteins, like GW182, could play an important
195 role in PB assembly through interaction with PABPC1.
196
197 **Ago-binding region of super-GW182 is involved in recruiting other components**
198 Both the N-terminal Ago-binding and mid Q/N region of GW182 were required for PB
199 localization (Lazzaretti et al., 2009, Behm-Ansmant et al., 2006). We generated deletions
200 of super-GW182 (TNRC6A) and checked their capability to bring PB/SG components
201 together. Under arsenite stress, super-GW182ΔQ/N could induce fusion of PB/SG (Fig.
202 7B), while super-GW182ΔAgo was less efficient in fusing PB/SG (Fig. 7A). This suggests
203 that the Ago-binding region is critical in subsequent PB components recruiting and
204 assembly. This agrees with previous studies that showed that deletion of Q/N region in
205 TNRC6A affects its PB localization much less than deletion of Ago-binding region
206 (Lazzaretti et al., 2009). This indicates that the redirection of PB components to SGs by
207 super-GW182 uses a similar mechanism to TNRC6A assembly into PBs.
208
209
210 **GW182ΔPAM2 can aggregate in PABPC1 depleted cells and goes to SGs when**
211 **stressed**
212 We have shown that super-GW182 mimicked fusion of PB/SGs without depletion of
213 PABPC1. This led us to ask what if the PAM2/MLLE interaction was missing? We
214 transfected GW182ΔPAM2 (ΔT952–Q971) plasmid together with PABPC1 siRNA. HA-
215 GW182ΔPAM2 formed PB-like granules in unstressed cells (Fig. s2A) and could be found

216 in PB/SG fusions when stressed (Fig. s2B). The tendency of overexpressed PB proteins to
217 aggregate into granules makes it hard to dissect the role of PAM2/MLLE interaction here.
218

219 **Discussion**

220 Here, we have shown that PABPC1 protein level is critical to PB assembly. PABPC1 is a
221 translation initiation factor and plays important role in mRNA metabolism. Depletion of
222 PABPC1 decreases the number of PBs in unstressed cells. When the PABPC1-depleted
223 cells are stressed, PB components are recruited to PABPC1-enriched SG. However, if SG
224 assembly is inhibited, individual PBs can form in stressed cells.
225 PB assembly may initiate on PABPC1-containing mRNPs (Fig. 8). PABPC1 might be
226 required in mRNP remodeling into P-bodies, thus PABPC1 depletion decreases PB
227 numbers in unstressed conditions. The requirement of PABPC1 for PB formation drives
228 PB components to certain SGs, where the residual PABPC1 is enriched after depletion.
229 The displacement of PABPC1 at SG is difficult so individual PBs fail to form. When SG
230 is inhibited by PP242, the same residual amount of PABPC1 in cytoplasm can support
231 normal PB formation. It implies that the ability of PABPC1 to disassociate from mRNPs
232 is required for formation of individual PBs. However, the mechanisms regarding how and
233 when PABPC1 disassociates from mRNP are still to be investigated.
234 The displacement of PABPC1 from microscope-visible PBs is a critical step in PB
235 assembly as was proposed by other colleagues (Chen and Shyu, 2013). PABPC1 plays a
236 double role regarding poly(A) tail of mRNA. mRNA is stabilized and protected by
237 PABPC1 from deadenylation by Ccr4/Not deadenylases (Tucker et al., 2002). Meanwhile,
238 PABPC1 is required for deadenylation by Pan2/Pan3 deadenylases (Lowell et al., 1992,
239 Zheng et al., 2008). The absence of PABPC1 in PBs is likely due to shortening of poly(A)
240 in remodeling, as suggested by the absence of a signal for poly(A) RNA in PBs (Cougot et
241 al., 2004).
242 Tendency of PB proteins to aggregate when overexpressed adds to the difficulty of PB
243 assembly studies. Using super-PAM2 to relocate proteins to SGs is a novel way to evaluate
244 their roles in PB assembly and dissect specific interactions. In all, we show that PABPC1
245 functions in mRNP remodeling and PB formation. Many mechanistic details need further
246 studying. We look forward to future studies on roles of PABPC1 in mRNA metabolism.

247

248

249 **Materials and methods**

250 Plasmids and siRNA

251 pT7-EGFP-C1-HsTNRC6A, pT7-EGFP-C1-HsTNRC6B, pT7-EGFP-C1-HsRCK
252 (DDX6), pT7-EGFP-C1-HsDcp1a, and pT7-EGFP-C1-HsDCP2 were gifts from Elisa
253 Izaurralde (Addgene plasmid # 25030, 25031, 25033, 25034, 25035)(Tritschler et al.,
254 2009). Sequence (agcaatctgaatccaaatgca) encoding amino acids SNLNPNA was inserted
255 between nucleotides 4827A and 4828C of TNRC6A, or 4431T and 4432C of TNRC6B to
256 create super-GW182. Reverse-PCR was used for constructing TNRC6AΔAgo (Δ7-300)
257 and TNRC6AΔQ/N (Δ360-402). Plasmids expressing HA-MBP, HA-TNTC6A and HA-
258 TNRC6AΔPAM2 were generous gifts from Drs. Eric Huntzinger and Elisa Izaurralde
259 (Huntzinger et al., 2010). PABPC1 was inserted between BamH I and Not1 of pCDNA3-
260 EGFP. siRNAs were synthesized at Dharmacon. The siRNA sequences used were
261 siPABPC1 (5'-AAGGUGGUUUGUGAUGAAAAU-3', 5'-
262 AAUCGCUCCUGAACCAGAAUC-3'), siPaip2 (5'-
263 GAGUACAUGUGGAUGGAAAUU-3', 5'- UGGAAGAUCUUGUGGUCAAUU-3').
264 Control siRNA was purchased from Qiagen (SI03650318).

265

266 Cell culture and transfections

267 HeLa S3 or MEF cells were cultured in DMEM supplemented with 10% fetal bovine
268 serum. 10^5 cells were plated per well in 24-well plate the day before transfection. 60-90
269 pmol siRNA or 0.8 μg DNA plasmid was mixed with 2 μl Lipofectamine 2000 in Opti-
270 MEM and then added to cells. After 48 hours, cells were trypsin digested and split onto
271 cover slides. Cells were treated and fixed after 24 hours. For western analysis, cells were
272 changed into fresh medium for another day and harvested in SDS loading buffer.

273

274 Immunofluorescence and confocal microscopy

275 Cells were fixed with cold methanol (-20°C) for 10 minutes and then treated with 0.1%
276 Triton X-100 in 1x PBS. Cells were blocked with 5% normal goat serum (Millipore S26)
277 in PBS. Cells were incubated in 1x PBS, supplemented with anti-PABPC1 (Abcam

278 ab21060; Santa Cruz sc32318)(1:200), anti-hedls (Santa Cruz sc8418)(1:1000), anti-Paip2
279 (Sigma-Aldrich P0087)(1:500), anti-eIF4G1 (Sigma-Aldrich AB-1232)(1:200), anti-
280 Ataxin-2 (BD Biosciences 611378)(1:200), anti-HA (Covance MMS-101P)(1:200), anti-
281 Dcp1a (Abcam ab47811)(1:200), anti-DDX6 (Abcam ab40684)(1:200), anti-EDC4
282 (hedls)(Abcam ab72408)(1:200), anti-Ago1 (Santa Cruz sc53521)(1:200), anti-G3BP (gift
283 of Dr. Imed Gallouzi)(1:500) or anti-HuR (gift of Dr. Imed Gallouzi)(1:200). Cells were
284 washed in PBS 3 times, before incubated with corresponding second antibodies conjugated
285 with Alexa488 or Alexa647 (Sigma-Aldrich A31620, A31628, A31571) or Dylight550
286 (Bethyl A120-101D3) or Rhodamine (Millipore 12-509, 12-510) at 1:200 – 1:500
287 dilutions. DAPI (Roche) was added to washing buffer at 0.5μg/ml to treat cells for 10
288 minutes. Cover slides were finally mounted in ProLong Gold antifade reagent (Life
289 technology P36930). Images were collected on Zeiss LSM 310 confocal microscope in the
290 McGill University Life Sciences Complex Advanced BioImaging Facility (ABIF). PBs
291 were counted manually in a sample size of 50 cells for each group. Sample deviation was
292 calculated within group.
293
294 Western blotting
295 Protein samples were heated at 95°C and separated in SDS-PAGE. Proteins were then
296 transferred to PVDF membrane in Tris/glycine buffer, with 20% methanol in cold room.
297 PVDF membrane was blocked in 1x TBS (pH 7.5), containing 0.05% Tween-20 and 5%
298 skim milk powder or bovine serum albumin. Besides antibodies above, anti-GW182
299 (Novus NBP1-88232)(1:200) and anti-tubulin (Sigma-Aldrich T9028) were also used for
300 detection of related proteins.
301
302 Immunoprecipitation
303 Cells were lysed in 20 mM Hepes (pH 7.4), 150 mM NaCl, 0.5% NP-40, 2 mM DTT, 2
304 mM $MgCl_2$, 1 mM $CaCl_2$ and protease inhibitor tablet (Roche), and cleared by
305 centrifugation. Optimized amount of antibodies were added to cleared lysate for 2 hours.
306 Dynabeads protein A or protein G were washed and added to lysate for 0.5 hour.
307 Dynabeads were then washed with 1x PBS and boiled in SDS loading buffer for further
308 analysis.

309

310 Surface plasmon resonance

311 GST-MLLE protein was applied to Series S sensor chip CM5 bound with anti-GST
312 antibodies (GE Healthcare BR100223), in Biacore T100. After wash, various
313 concentrations of super-PAM2 (SNLNPNAPEFHPGVPWKGLQ) or super-PAM2-
314 Phe10Ala (SNLNPNAPEAHPGVPWKGLQ) were added to bind GST-MLLE captured on
315 chip. The corresponding steady states measured in relative units (RU) were plotted versus
316 concentrations of peptides in the flow system to estimate dissociate constants.

317

318 **Acknowledgements**

319 We acknowledge Drs. Elisa Izaurralde, Witold Filipowicz and Imed Gallouzi for
320 generously sharing plasmids or antibodies. We are grateful for suggestions and help from
321 colleagues during the project.

322

323 **Competing interests**

324 No competing interests declared.

325

326 **Author contributions**

327 J.X. designed the experiments. Y.C. and X.W. assisted J.X. in carrying out the experiments.
328 G.K. designed the super-PAM2 sequence. J.X. and K.G. prepared the manuscript.

329

330 **Funding**

331 This study was supported by Canadian Institutes of Health Research grant MOP-14219.

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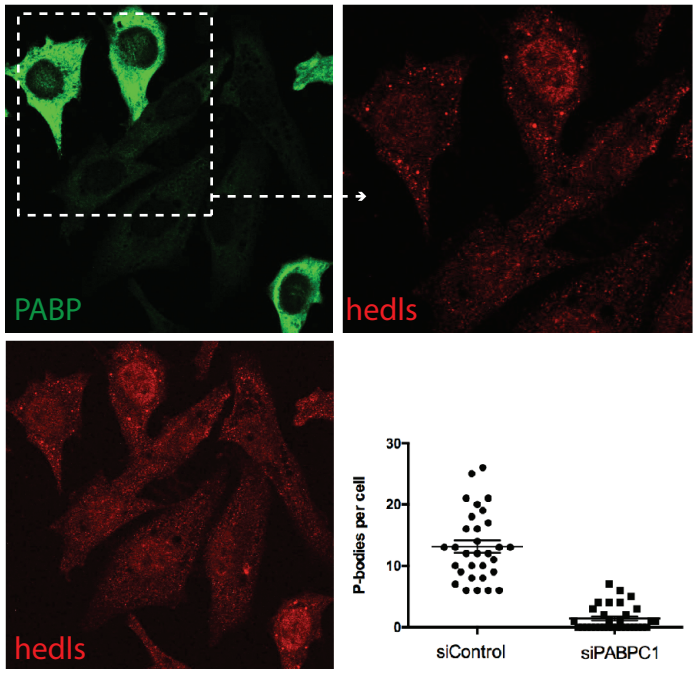
478
479
480
481
482 **Figure legends**
483
484 Figure 1. PB number and protein levels after PABPC1 or Paip2 depletion. (A-B) HeLa
485 cells transfected with scramble siRNA were mixed with siPaip2 or siPABPC1 treated cells,
486 to highlight knockdown effects and compare cells in the same environment. Staining of
487 PABPC1 or Paip2 (*green*) indicated knockdown effects in cells. PB number was monitored
488 by hedls (*red*). Visible P-bodies of 50 cells were counted manually and sample deviation
489 was calculated. The PB number in siPABP cells was significantly lower at 0.01
490 significance level using two-tailed t-test for two samples with unequal variance. (C) Levels
491 of PABPC1, Paip2, hedls, DDX6, Dcp1a, G3BP, GW182 and tubulin 72 hours after siRNA
492 knockdown. The Paip2 antibody recognized both Paip2a and Paip2b.
493
494 Figure 2. Relocalization of hedls or Dcp1a to stress granules in PABPC1-depleted cells.
495 (A-B) HeLa cells were treated with 0.5 mM sodium arsenite for 0.5 hour before fixation.
496 The PB components hedls (*red*) formed loose and irregular granules, colocalized to stress
497 granules (*green*).
498
499 Figure 3. (A-B) Stress granule assembly is not affected by PABPC1 depletion. PABPC1
500 depleted cells (Red) displayed similar stress granule pattern to control cells, stained by anti-
501 G3BP or anti-HuR (*green*). (C) P-bodies form independent of stress granules. HeLa cells
502 treated by PP242 overnight failed to form stress granules (*red*), but had normal PBs present.
503 (D) Separate PBs can be induced after SG inhibition in depletion of PABPC1. HeLa cells
504 were treated with 10μM PP242 for overnight before stressed in 0.5 mM sodium arsenite
505 for 0.5 hour. Individual PBs (*green*) formed in PABPC1 depleted cells (*red*).
506

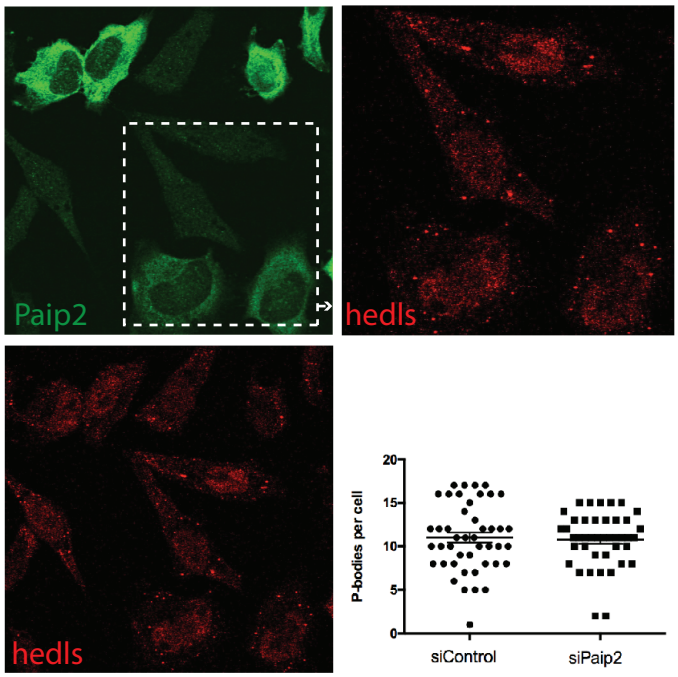
507 Figure 4. P-bodies can be induced in PABPC1 depleted cells. (A) Dcp1a or DDX6
508 overexpression (*green*) could induce P-body like granules in cells with low PABPC1 (*red*).
509 (B-C) Dcp1a or TNRC6A (*green*) were partly localized to stress granules in arsenite
510 stressed PABPC1 depleted cells. HeLa cells were transfected with siRNA and related
511 plasmids simultaneously.
512
513 Figure 5. Engineering of super-GW182 to interact with PABPC1. (A) Super-PAM2
514 integrated sequences of Paip2-PAM2 and GW182-PAM2 to bind an extended surface on
515 MLLE domain (PDB: 3KUS and 3KTP). Super-PAM2 bound MLLE of PABPC1 at around
516 20 nM measured by surface plasmon resonance using Biacore T100. The Phe10Ala mutant
517 was not binding MLLE and used as control. (B) Overexpressed super-PAM2-GFP (*green*)
518 localized to PABP or G3BP (*red*) marked stress granules upon sodium arsenite treatment.
519 (C) HA-tagged MBP, GW182 (TNRC6A), GW182ΔPAM2 or super-GW182 were
520 transfected in HeLa cells. Immunoprecipitation with anti-HA antibodies showed stronger
521 interaction of super-GW182 with endogenous PABPC1. The weak association of natural
522 GW182 and PABPC1 was not shown due to harsh washings.
523
524 Figure 6. Simulation of P-body/Stress granule fusion using super-GW182. HeLa cells
525 overexpressing super-GW182-GFP (*green*) were stressed by sodium arsenite and stained
526 with anti-hedls (*cyan*) for P-bodies and anti-PABPC1 (*red*) for stress granules.
527
528 Figure 7. Ago-binding region of GW182 (TNRC6A) is important for recruitment of PB
529 components. (A-B) Super-GW182ΔAgo or super-GW182ΔQ/N (*green*) was co-
530 transfected with siPABPC1. PBs were labeled by anti-hedls (*cyan*) and stress granules were
531 by anti-PABPC1 (*red*). Super-GW182ΔAgo was less efficient in redirecting PB
532 components to SGs (A) compared with super-GW182ΔQ/N (B).
533
534 Figure 8. P-body formation on mRNPs or stress granules. PB assembly may initiate on
535 PABPC1-containing mRNPs. The requirement of PABPC1 in PB formation drives PB
536 components to stress granules, where the residual PABPC1 is enriched after depletion.

However, the displacement of PABPC1 is difficult in SG and then individual PBs fail to form.

A


PABP
hedls
hedls
P-bodies per cell
30
20
10
0
siControl
siPABPC1

B


Paip2
hedls
hedls
P-bodies per cell
20
15
10
5
0
siControl
siPaip2

C

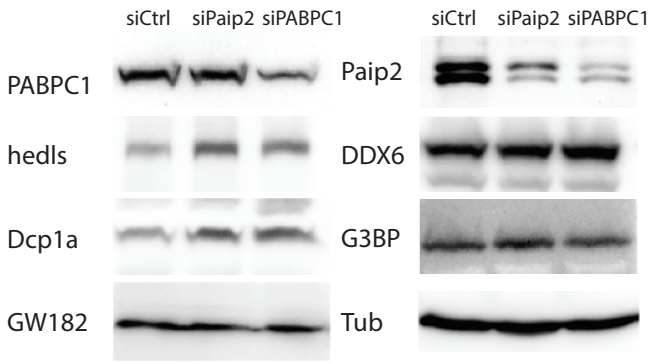
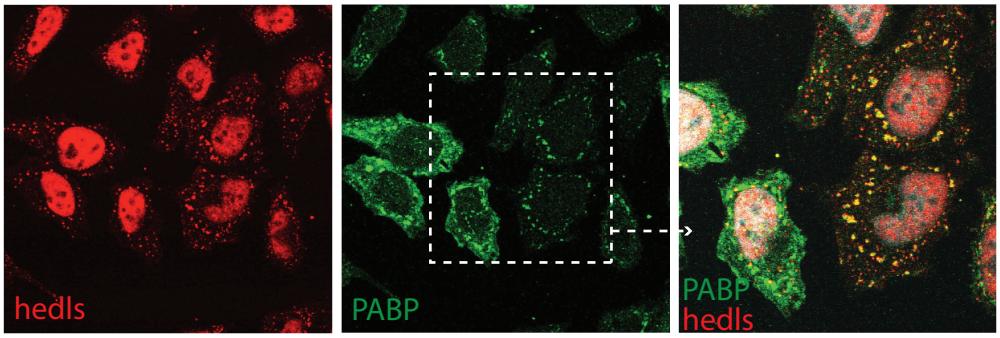

siCtrl
siPaip2
siPABPC1
siCtrl
siPaip2
siPABPC1
PABPC1
hedls
Dcp1a
GW182
Paip2
DDX6
G3BP
Tub

Figure 2

A



B

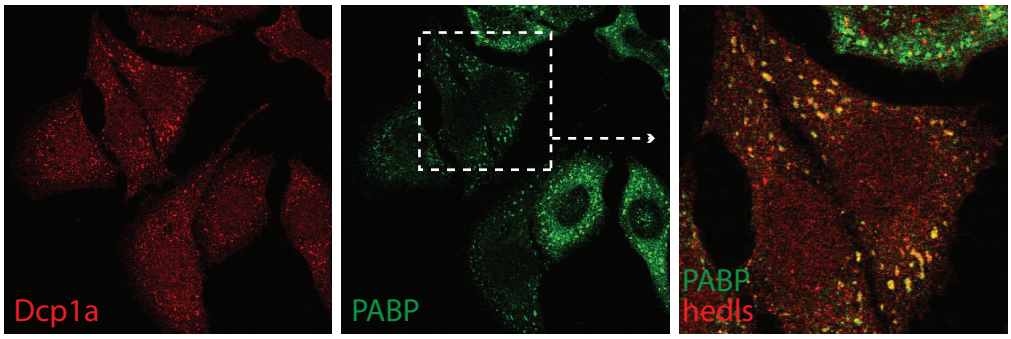
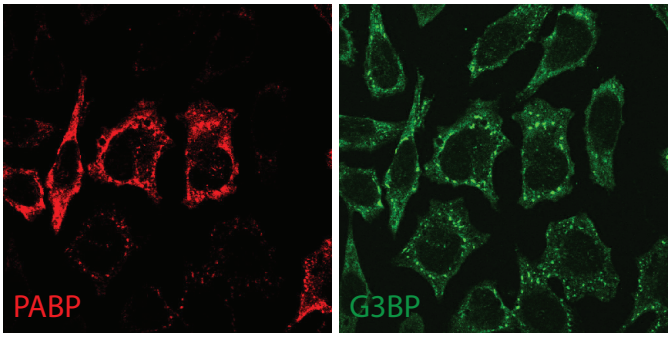
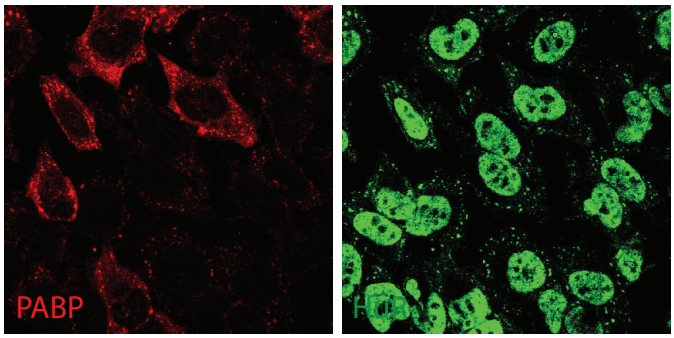


Figure 3

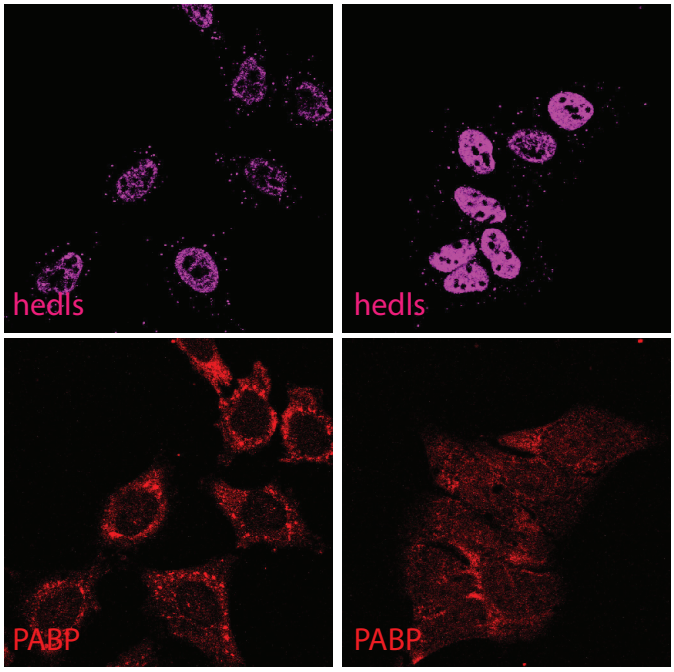
A



B



C



D

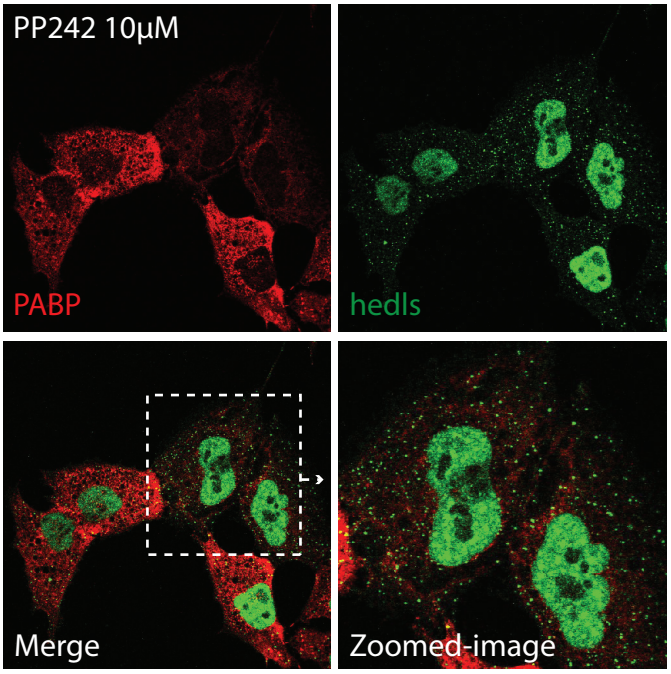


Figure 4

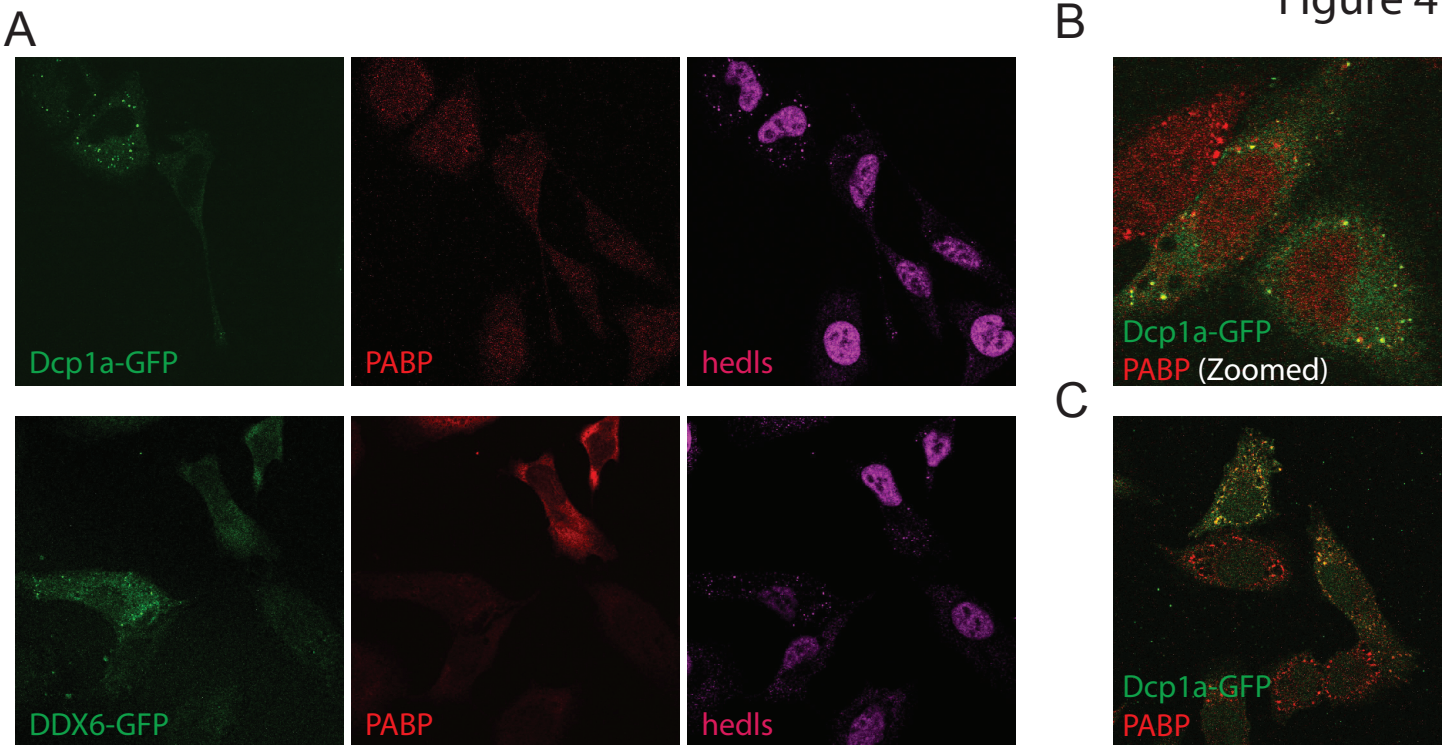
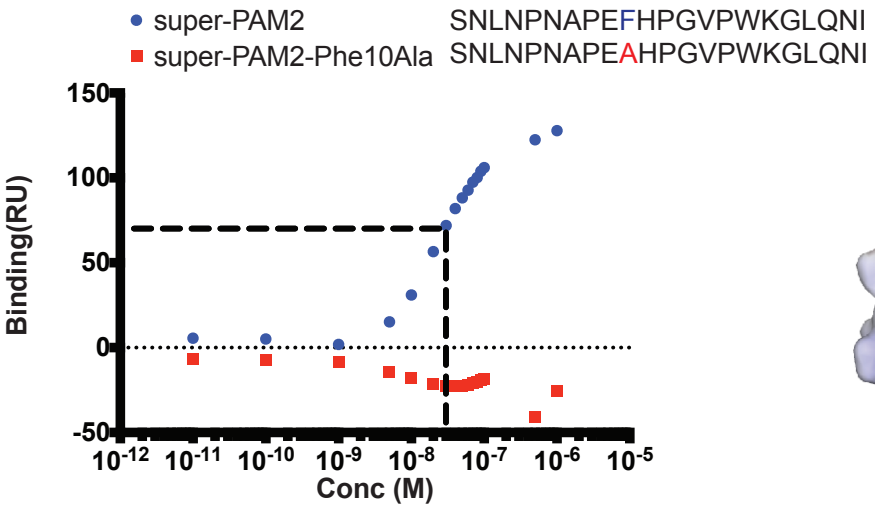
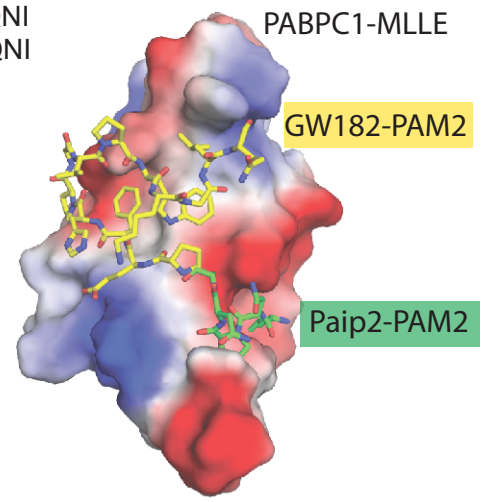

A
Dcp1a-GFP
PABP
hedls
DDX6-GFP
PABP
hedls
B
Dcp1a-GFP
PABP (Zoomed)
C
Dcp1a-GFP
PABP

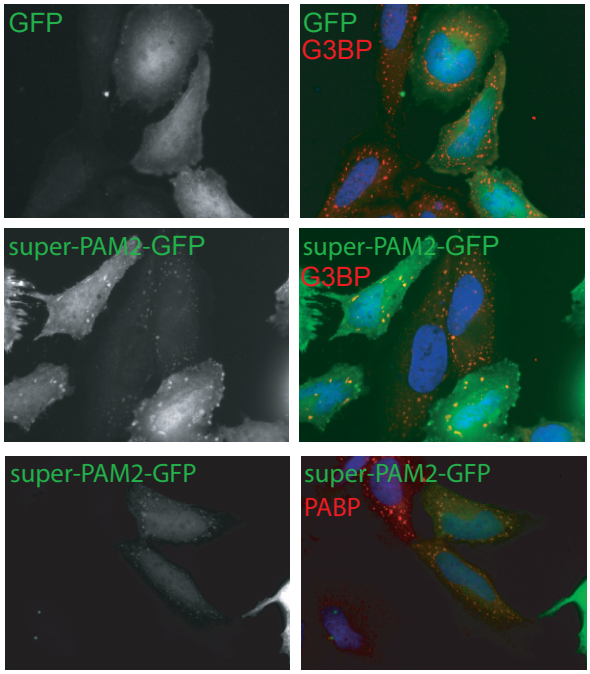
Figure 5

A


super-PAM2 SNLNPNAPEFHPGVPWKGLQNI
super-PAM2-Phe10Ala SNLNPNAPEAHPGVPWKGLQNI
Binding(RU)
150
100
50
0
-50
10-12 10-11 10-10 10-9 10-8 10-7 10-6 10-5
Conc (M)


PABPC1-MLLE
GW182-PAM2
Paip2-PAM2

B


GFP
GFP
G3BP
super-PAM2-GFP
super-PAM2-GFP
G3BP
super-PAM2-GFP
super-PAM2-GFP
PABP

C

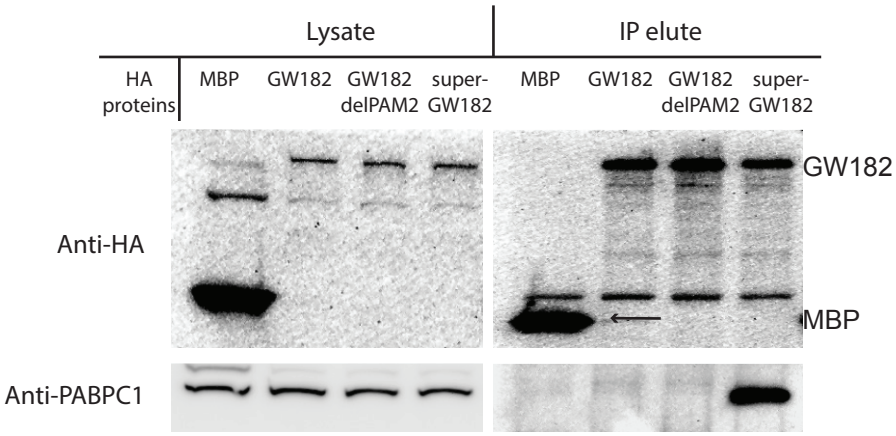

Lysate
IP elute
HA proteins
MBP
GW182
GW182 delPAM2
super-GW182
MBP
GW182
GW182 delPAM2
super-GW182
Anti-HA
GW182
MBP
Anti-PABPC1

Figure 6

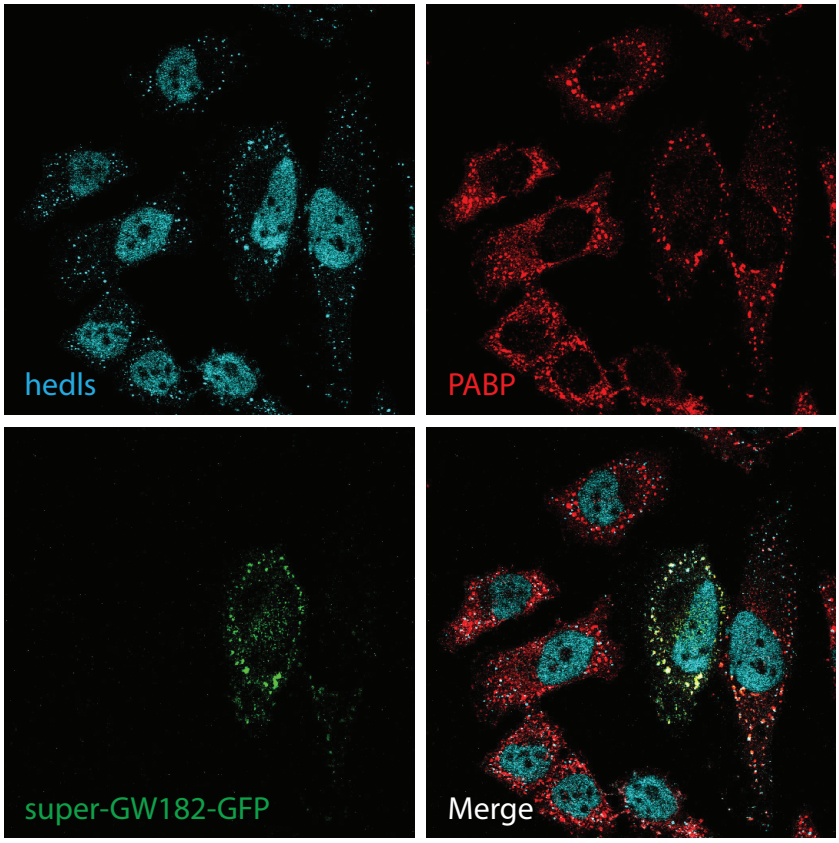
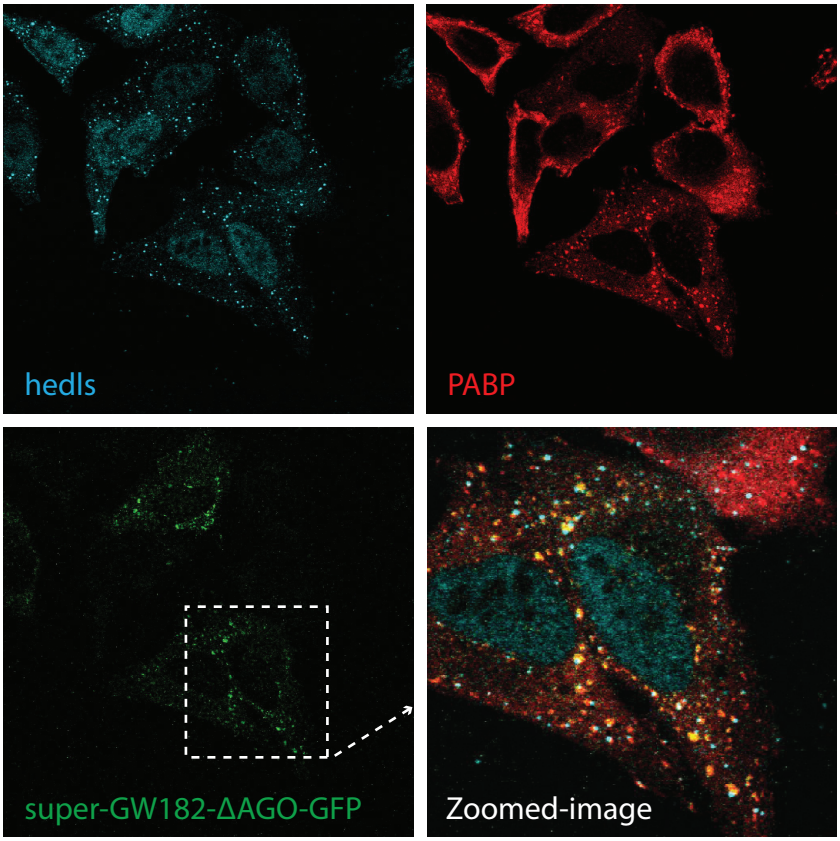


Figure 7

A


hedls
PABP
super-GW182-ΔAGO-GFP
Zoomed-image

B

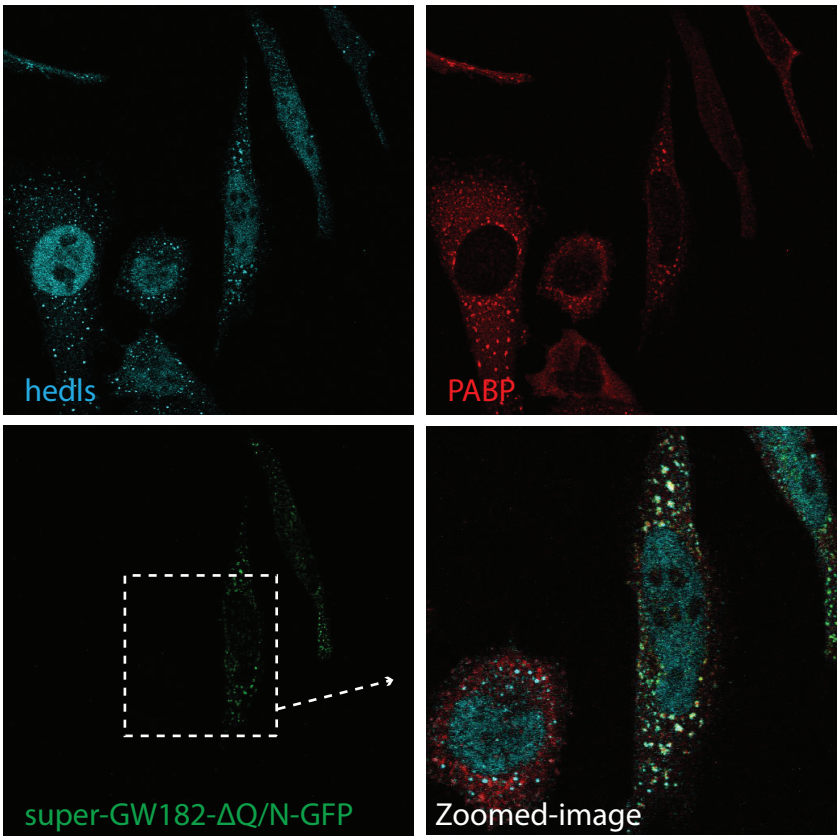
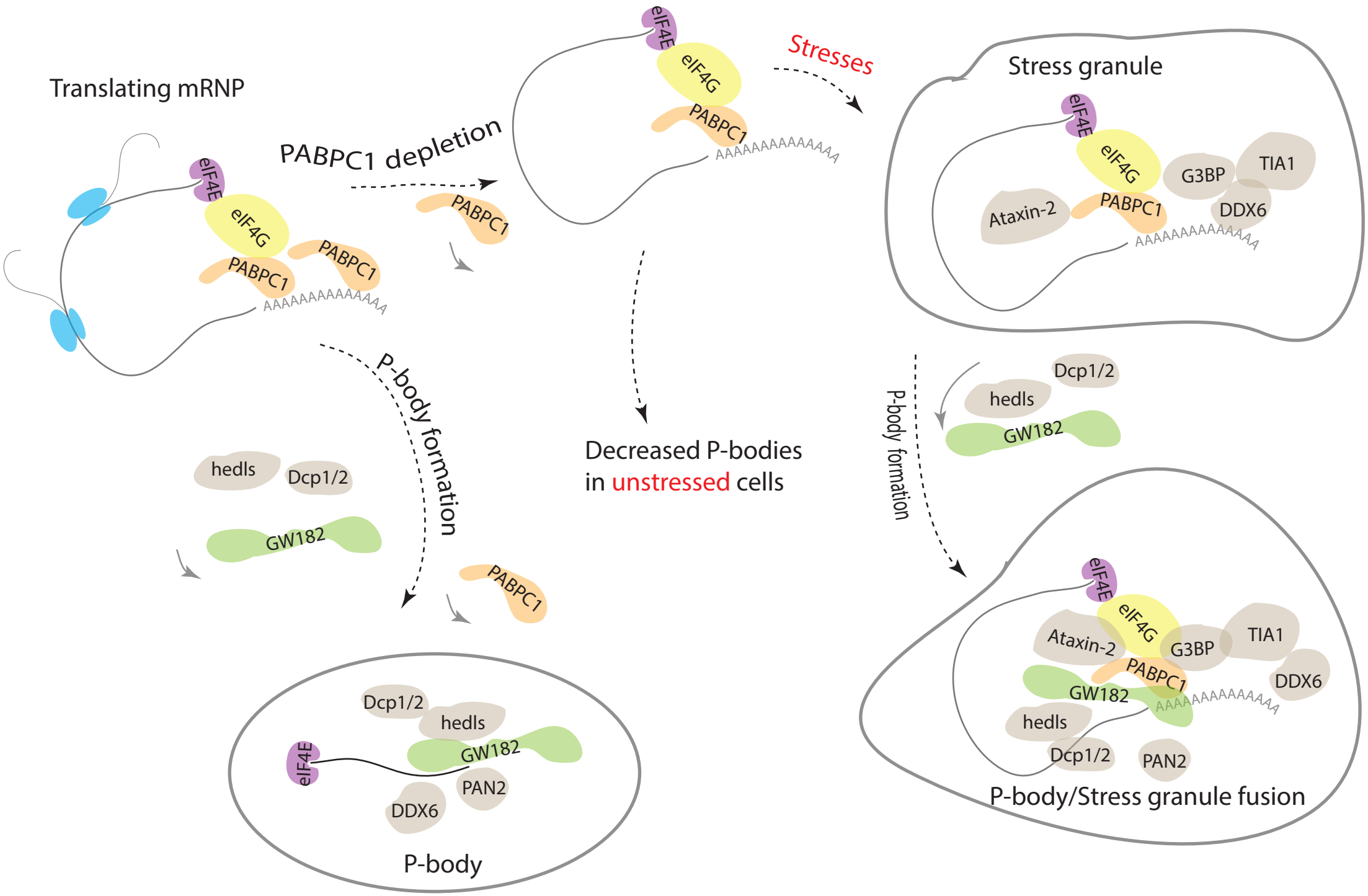

hedls
PABP
super-GW182-ΔQ/N-GFP
Zoomed-image

Figure 8


Translating mRNP
eIF4E
eIF4G
PABPC1
PABPC1
AAAAAAAAAAAAAA
PABPC1 depletion
PABPC1
eIF4E
eIF4G
PABPC1
AAAAAAAAAAAAAA
Stresses
Stress granule
eIF4E
eIF4G
G3BP
TIA1
Ataxin-2
PABPC1
DDX6
AAAAAAAAAAAAAA
P-body formation
hedls
Dcp1/2
GW182
PABPC1
Decreased P-bodies
in unstressed cells
P-body formation
Dcp1/2
hedls
GW182
Dcp1/2
hedls
eIF4E
GW182
PAN2
DDX6
P-body
eIF4E
eIF4G
Ataxin-2
G3BP
TIA1
PABPC1
DDX6
GW182
AAAAAAAAAAAAAA
hedls
Dcp1/2
PAN2
P-body/Stress granule fusion