

1 ***Nascent mutant Huntingtin exon 1 chains do not stall on ribosomes during translation but***
2 ***aggregates do recruit machinery involved in ribosome quality control.***

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8 ***Abstract***

9 Mutations that cause Huntington's Disease involve a polyglutamine (polyQ) sequence expansion beyond
10 35 repeats in exon 1 of Huntingtin. Intracellular inclusion bodies of mutant Huntingtin protein are a key
11 feature of Huntington's disease brain pathology. We previously showed that in cell culture the
12 formation of inclusions involved the assembly of disordered structures of mHtt exon 1 fragments
13 (Httex1) and they were enriched with translational machinery when first formed. We hypothesized that
14 nascent mutant Httex1 chains co-aggregate during translation by phase separation into liquid-like
15 disordered aggregates and then convert to more rigid, amyloid structures. Here we further examined
16 the mechanisms of inclusion assembly in a human epithelial kidney (AD293) cell culture model and
17 examined whether ribosome quality control machinery previously implicated in stalled ribosomes were
18 involved. We found mHttex1 did not appear to stall translation of its own nascent chain and there was
19 no recruitment of RNA into inclusions. However, proteins involved in translation or ribosome quality
20 control were co-recruited into the inclusions (Ltn1 and Rack1) compared to a protein not anticipated to
21 be involved (NACAD). Furthermore, we observed co-aggregation with other proteins previously
22 identified in inclusions, including Upf-1 and chaperone-like proteins Sgta and Hspb1, which also
23 suppressed aggregation at high co-expression levels. The newly formed inclusions contained immobile

24 mHttex1 molecules which points to the disordered aggregates being mechanically rigid prior to amyloid
25 formation.

26 **Keywords**

27 Huntington's Disease, flow cytometry, ribosome quality control, translation.

28

29 **INTRODUCTION**

30 Huntington Disease (HD) is an incurable and fatal neurodegenerative condition caused by dominant
31 trinucleotide expansion mutations in exon 1 of the *Huntingtin* gene [1]. These mutations expand a
32 polyglutamine (polyQ) sequence in the Huntington (Htt) protein to beyond a disease threshold of 35Q,
33 which makes the protein become aggregation prone [2]. N-terminal mutant Htt fragments accumulate in
34 intracellular inclusion bodies (inclusions) during disease progression, which represent a major hallmark
35 of disease pathology [2-4].

36 The transgenic expression of the Htt exon 1 fragment (Httex1) in polyQ-expanded form is sufficient to
37 produce a HD-like pathology in rodent and primate models, which is suggestive of these fragments
38 mediating proteotoxicity [5-7]. The mechanism of toxicity remains to be unequivocally determined but it
39 is thought to involve two distinct components; soluble and inclusion states of Httex1 [8]. Soluble states,
40 which may include monomeric or small nanometer-sized oligomers of mutant Httex1 cause oxidative
41 and mitochondrial stress and increase the risk of apoptosis in cell culture models of disease [9-12]. We
42 previously suggested that the toxicity of the soluble forms of mutant Httex1 may involve a quality
43 control feedback mechanism during translation involving stalled Httex1 nascent chains, which when
44 unresolved triggers apoptosis [8]. Once inclusions form survival times are improved in cell culture
45 models of disease, leading to a hypothesis that inclusion formation alleviates toxicity by sequestering
46 the soluble toxic forms away from harm (reviewed in [13]). However, rather than returning the cell to a

47 normal state of homeostasis, cells in culture with inclusions are metabolically quiescent and die at a
48 delayed rate by a non-apoptotic necrotic mechanism [8]. This finding suggests a second level of toxicity
49 from the inclusions distinct to that from the soluble states.

50 Here we sought to further investigate the molecular processes governing inclusion assembly in the
51 context of the hypothesis that newly synthesized mutant Httex1 stalls at the ribosome, attracts
52 ribosome quality control machinery to resolve the stress and when unresolved triggers the nucleation of
53 initially disordered aggregates into liquid-like droplets that then over time convert to amyloid. Since our
54 initial prediction that mutant Httex1 aggregates may arise through phase separation into liquid-like
55 structures, two studies have since reported evidence supporting this mechanisms of action [14, 15]. Our
56 findings suggest that nascent Httex1 does not stall on ribosomes during translation but there is an
57 enrichment of machinery involved in ribosome associated quality control into the inclusions. In our
58 hands and model, however, we found the early formed inclusions comprised immobile mutant Httex1
59 molecules with no evidence of liquid-like properties.

60 **METHODS**

61 **DNA vectors and constructs.** Human Httex1 and TC9-tagged Httex1 as fusions to fluorescent proteins
62 were expressed in pT-Rex vectors with CMV-promoters as described previously [8]. The pFN21A-HaloTag
63 constructs were purchased from Promega. The P2A stall construct was prepared as described previously
64 including the Httex1 constructs [16]. The tandem P2A T2A constructs were made using T2A sequences
65 from [17]. In essence the T2A sequence 5'
66 GGCGAGGGCAGGGAAAGTCTTCTAACATGCGGGACGTGGAGGAAAATCCGGCCA was inserted after the
67 P2A sequence of the existing Httex1(25Q) sequence in the pTriEx4 vector (GeneArt). The sequence of
68 the derived vector is shown in **Table 1**. From this, the Httex1(97Q) and 20K variants were made by
69 excising the gene fragments from the original stall reporter via NotI and BamHI restriction sites. The
70 control linker was made by PCR amplification using forward (5'

71 GCGGCCGCTATGCCTGGACCTACACCTAGCG) and reverse (5' GGATCCGCCGGTTTCAGGCCAGGGC)
72 primers and ligation into the P2A T2A Htt25Q Stall Reporter via the NotI and BamHI restriction sites.

73

74 **Table 1. Sequence of the base stall construct ***

*Sequences are highlighted as follows. GFP; P2A; T2A; Httex1(25Q); mCherry

CCATGGTGAGCAAGGGCGAGGAGCTGTTACCGGGGTGGTGCCCATCCTGGTCAGCTGGACGGCGACGTAAACGGCA
CAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCCTGAAGTTCATCTGCACCACCGCA
AGCTGCCCGTGCCCTGGCCCACCCCTCGTACCGACCCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCGACCACATGAA
GCAGCACGACTTCTCAAGTCCGCCATGCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTCAAGGACGACGGCAACTA
CAAGACCCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAG
GACGGCAACATCCTGGGGCACAAGCTGGAGTACAACCTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAAGAA
CGGCATCAAGGTGAACCTCAAGATCCGCCAACACATCGAGGACGGCAGCGTGCAGCTGCCGACCACTACCAGCAGAACAA
CCCCCATGGCGACGGCCCCGTGCTGCCGACAACCAACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACG
AGAACCGCGATCACATGGCCTGCTGGAGTTGCTGACCGCCGCCGGATCACTCTGGCATGGACGAGCTGTACAAGGCT
AGCGGAAGCGGAGCTACTAACCTCAGCCTGCTGAAGCAGGCTGGAGACGTGGAGGGAGAACCCCTGGACCTCTGCAGGGCT
CCGGCGAGGGCAGGGGAAGTCTTCTAACATGCGGGGACGTGGAGGAAAATCCGGCCCGCAGGGGGCGCCGCCCTT
CACAAatggcgaccctggaaaagctgtatggcgttccggcccttcacccctccacccctcaacttcctcaacccctccacaggcacgc
aacagcaacaacagcagcaacacaaccgcaccacccctccacccctcaacttcctcaacccctccacaggcacgc
agccacaacccctccacccctccacccctccacccctccacccctcaacttcctcaacccctccacaggcacgc
GATCCGGTACCGGAAGCGGAGCT
ACTAACTCAGCCTGCTGAAGCAGGCTGGAGACGTGGAGGGAGAACCCCTGGACCTACCGGTGGCTCCGGCAGGG
GAAGTCTTCTAACATGCGGGGACGTGGAGGAAAATCCGGCCCGAATTCTGAGCAAGGGCGAGGAGGATAACATGGC
CATCATCAAGGAGTTCATGCGCTCAAGGTGACATGGAGGGCTCCGTGAACGCCACGAGTTGAGATCGAGGGCGAGG
GCGAGGGCCGCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTGCCCTGGAC
ATCCTGTCCCCCTCAGTTCATGTACGGCTCAAGGCCTACGTGAAGCACCCGCCGACATCCCCGACTACTTGAAGCTGCTCCT

TCCCCGAGGGCTCAAGTGGAGCGCGTGATGAACCTCGAGGACGGCGGCGTGGTACCGTGACCCAGGACTCCTCCCTA
CAGGACGGCGAGTCATCTACAAGGTGAAGCTGCGCGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAC
TATGGGCTGGGAGGCCTCCGAGCGGATGTACCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAG
CTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGACCAAGGCAAGAACGCCCCGTGCAGCTGCCGGCGCTA
CAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCC
GCCACTCCACCGCGGCATGGACGAGCTGTACAAGTAACTCGAG

75

76 **Cell culture.** All experiments were performed with the HEK293 cell line derivative AD293, which was
77 maintained in DMEM supplemented with 10 % (w/v) fetal calf serum (FCS) and 1 mM glutamine in a
78 37°C humidified incubator with 5% v/v atmospheric CO₂. For microscopy experiments cells were plated
79 at 3 × 10⁴ cells per well in an 8-well μ-slide (Ibidi). For flow cytometry experiments cells were plated at
80 0.5 × 10⁵ cells in a 24-well plate. Cells were transiently transfected with the vectors using Lipofectamine
81 3000 reagent as per manufacturer's instructions (Life Technologies), and media was changed 6 hours
82 post transfection. For the HaloTag experiments, the transfection was done in a way so as to decouple
83 the correlated expression of the two plasmids. Specifically, this was achieved by mixing the plasmids
84 separately with Lipofectamine, before combining the lipofectamine:DNA complexes together to add to
85 the cells.

86 **Western Blot.** AD293 cells were transfected with P2A or P2A T2A stall constructs and harvested 24
87 hours post transfection. Cells were pelleted (200 g, 6 mins) and resuspended in RIPA lysis buffer
88 (150mM NaCl, 50 mM Tris pH 8.0, 1% v/v IGEPAL, 0.5% v/v sodium deoxycholate, 0.1% v/v SDS, 250 U
89 Benzonase and supplemented with cOmplete, EDTA-free Protease Inhibitor Cocktail pills (Roche)) and
90 incubated on ice for 30 minutes. Lysate was matched for total protein by BCA kit (Thermofisher
91 scientific, cat# 23225). 10 μg of total protein lysate was loaded on to an TGX Stain Free FastCast
92 Acrylamide gel (BioRad, cat# 1610185) and transferred using an iBlot2 gel transfer device (Thermofisher

93 scientific, cat# IB21001) and a PVDF iBlot2 transfer stack (Thermofisher scientific, cat# IB24001). The
94 membrane was blocked with 5% w/v skim milk powder in phosphate buffered saline (PBS) for 1 hour at
95 room temperature. Anti-GFP (Invitrogen, cat#A6455) and anti-Cherry (Abcam, cat#167453) were diluted
96 to 1:10,000 and 1:2500 respectively in PBS containing 0.1% v/v Tween 20 and incubated for 1 hour at
97 room temperature. The secondary antibody, goat anti-rabbit HRP antibody (Invitrogen, cat#656120),
98 was diluted 1:10,000 in PBS containing 0.1% v/v Tween 20 and incubated for 1 hour at room
99 temperature. HRP was detected by enhanced chemilumiscence.

100 **FlAsH staining.** Cells were stained with FlAsH as described previously to demarcate HBRI from PBRI cells
101 [8]. In essence, the ratio of Cerulean:FlAsH or mCherry:FlAsH fluorescence was determined and all cells
102 with a ratio greater than one standard error from the mean were classified as HBRI whereas all cells with
103 a ratio smaller than one standard error from the mean were classified as PBRI.

104 **RNA staining.** Cells were stained for RNA using the Click-iT Plus Alexa Fluor647 Picolyl Azide Toolkit (Life
105 technologies, cat#C10643) according to manufacture's instructions. In short, 6 hours post-transfection
106 5-ethynyl uridine (Click-Chemistry tools, cat# 1261-10) was added to cells to a final concentration of 0.4
107 mM. 24 hours post-transfection cells were stained with FlAsH as described above. Following staining,
108 cells were fixed with 4% w/v paraformaldehyde and permeabilised with 0.5% w/v Triton X-100 in PBS.
109 Cells were then washed with 3% w/v bovine serum albumin in PBS followed by Click-It staining according
110 to manufacturer's instructions using a 1:4 ratio of CuSO₄: Copper protectant. Nuclei were counter
111 stained with Hoechst (1:1000) and imaged by confocal microscopy.

112 **HaloTag staining.** After 6 hours post-transfection with the Halo-tagged constructs, TMRDirect ligand
113 (Promega) was diluted 1:1000 in complete media and added to cells. For imaging, cells were fixed at 24
114 hours post-transfection with 4% w/v paraformaldehyde. Imaging positions were marked using the "mark

115 and find" feature and then images captured on a Lecia TCS SP5. Cells were then stained with FlAsh as
116 described above and the positions were re-imaged.

117 **Flow Cytometry.** Cells were analyzed at high flow rate in an LSРFortessa flow cytometer, equipped with
118 488- and 561-nm lasers (BD Biosciences). 50,000–100,000 events were collected, using a forward scatter
119 threshold of 5,000. Data were collected in pulse height, area, and width parameters for each channel.
120 For Cerulean fluorescence, data were collected with the 405-nm laser and BV421 filter (450/50 nm).
121 TMR fluorescence was collected with the 561-nm laser and using the PE-Texas Red filter (610/20 nm). All
122 flow cytometry data were preprocessed with FlowJo (Tree Star Inc.) to assign live and inclusion-
123 containing cells. Cells with inclusions were assessed by Pulse Shape Analysis [18]. The fluorescence
124 intensity for individual cells in each channel was exported and analysed using custom python scripts
125 (available at <https://doi.org/10.5281/zenodo.3789864>). Briefly, cerulean fluorescence was assigned to
126 20 logarithmic bins spanning the range of recorded intensities. Four logarithmic bins for HaloTag
127 fluorescence were then assigned (none, low, medium, high) independently for each construct according
128 to the maximum intensity for that construct. Finally, the proportion of cells containing inclusions within
129 each combined cerulean-HaloTag bin was calculated.

130 **Immunofluorescence.** Cells were fixed at 24 hours post-transfection with 4% w/v paraformaldehyde for
131 15 min at room temperature. Cells were then permeabilized with 0.2% v/v Triton X-100 in PBS for 20
132 mins at room temperature. Samples were blocked in 5% w/v bovine serum albumin in PBS for 1 hour at
133 room temperature. Cells were stained with anti-GFP (1:300 dilution) (Invitrogen cat# A6455) diluted in
134 PBS containing 1 % w/v bovine serum albumin and 0.05% v/v Tween 20 for 1 hr at room temperature.
135 Samples were then incubated in goat anti-rabbit Alexa Fluro 647 (1:500) (Life technologies cat#A21244)
136 diluted in PBS containing 1 % w/v bovine serum albumin and 0.05% v/v Tween 20 for 30 mins at room
137 temperature.

138 **Fluorescence recovery after photobleaching.** Cells were imaged at 37°C and 5% atmospheric CO₂ on an
139 Olympus FV3000 confocal laser scanning microscope through a 60X 1.2NA water immersion objective.
140 mCherry fluorescence was excited with a 561 nm solid state laser diode. The resulting fluorescent
141 emission was directed through a 405/488/561 dichroic mirror to remove laser light and detected by an
142 internal GaAsP photomultiplier set to collect between 550–650 nm. 24 hours post transfection cells
143 were FIAsH stained as described above and then imaged. For FRAP, a pre-bleached image was taken
144 before half the inclusion was bleached for 5 seconds (10% laser power, 200 µs pixel dwell time).
145 Recovery was then monitored by imaging the inclusions every minute for 21 minutes.

146 **Cell imaging and analysis.** Images of live or fixed cells were acquired with a Leica TCS SP5 confocal
147 microscope. Anti-GFP images were taken on the Zeiss LSM800 Airyscan. Images were extracted from
148 proprietary formats using FIJI (v 2.0.0-rc-69/1.52p) equipped with the Bioformats plugin (v6.3.1). The
149 resultant tiff images were then processed using custom scripts written for FIJI and python (source code
150 available at <https://doi.org/10.5281/zenodo.3789864>). Briefly, in the case of RNA- and HaloTag- stained
151 images, regions of interest (ROI) were manually assigned and the mean fluorescence intensity within
152 each region exported. In the case of fluorescence recovery after photobleaching, bleached and whole-
153 cell ROI were assigned via automatic thresholding of the FIAsH and mCherry channels respectively, while
154 a circular background ROI with a nominal fixed radius (25 pixels in the images) was manually assigned
155 for each image. Pixel fluorescence intensities for each ROI were exported for all timepoints post-
156 recovery, and the bleached ROI determined by subtracting the unbleached pixel coordinates from the
157 whole-cell ROI. The relative recovery was then calculated by dividing the background-corrected mean
158 intensity in the bleached and non-bleached ROIs. Finally, in the case of anti-GFP antibody penetration,
159 inclusions were identified by automatic thresholding of the cerulean intensity and the resultant ROI was
160 scaled by 110% to yield the external inclusion boundary. The internal boundary was determined via
161 automatic thresholding of the inverted anti-GFP fluorescence. The Euclidean distance between the

162 centroid of the outer ROI and either the internal or external boundary pixels was then calculated, and
163 the penetration measured as the difference in the mean distance of the internal and external
164 boundaries.

165 **Statistics.** The statistical tests are described in the figure legends and *P* values shown directly on the
166 figures or coded as *, *P* < 0.05, **, *P* < 0.01, ***, *P* < 0.001; ****, *P* < 0.0001. Statistical tests were
167 performed in Graphpad Prism v6.

168 **RESULTS**

169 We previously postulated the presence of a translation-related quality control mechanism that clears
170 aggregating or misfolding proteins emerging from the ribosome in cells lacking inclusions. Prior data has
171 shown that polyglutamine (polyQ)-expanded Httex1 is more efficiently degraded than the wild-type
172 counterpart, which is consistent with an elevated clearance mechanism at this step [19]. If polyQ-
173 expanded Httex1 is engaging with quality control during synthesis we might anticipate this to lead to an
174 interruption of translation rates. Furthermore we previously found, by proteomics, that Upf1 (Rent1),
175 which plays a central role in non-sense mediated decay [20] was enriched in the inclusions [8]. This
176 finding raises the possibility that the stalled constructs, should they arise, proceed to nucleate the
177 aggregation process.

178 First to test for stalling, we implemented a translational stall assay in AD293 cells which are sensitive to
179 the proteotoxicity of polyQ-expanded Httex1 [8]. The assay involves a reporter cassette containing two
180 fluorescent reporters on each side of the peptide sequence to be tested for stalling (GFP at the N-
181 terminus and mCherry at the C-terminus) [21] (**Fig 1A**). Each construct is encoded in frame without stop
182 codons however the test sequence is flanked by viral P2A sequences, which causes the ribosome to skip
183 the formation of a peptide bond but otherwise continue translation elongation uninterrupted [22].
184 Complete translation of the cassette from one ribosome will generate three independent proteins (GFP,

185 test protein, and mCherry). However, should the ribosome stall during synthesis (such as through the
186 previously established poly-lysine (20K) sequence used here as a control [21]), mCherry is produced at
187 lower stoichiometries than the GFP. In our hands, we noted that there was a small fraction of protein
188 synthesized reading through the P2A sequences (**Fig 1B**). Of particular note was the appearance of SDS-
189 insoluble material in the stacking gel for the mutant polyQ-length form of Httex1 (97Q), which is
190 indicative of SDS-insoluble mHtt products that arises from aggregation [3] (**Fig 1B**). Because
191 fluorescence resonance energy transfer (FRET) from GFP to mCherry is anticipated to inflate the
192 mCherry/GFP fluorescence ratio, and be particularly high in the aggregates, we sought to remove this
193 confounding factor by redesigning the stall construct to have a more efficient skip sequence [17]. This
194 involved tandem P2A and T2A sequences, which appeared to reduce the read through effect below
195 detection by Western Blot (**Fig 1C**). The data shows Httex1 in wild-type (25Q) or mutant (97Q) form to
196 have an insignificant difference in mCherry/GFP ratios compared to the control construct, although
197 there was a very small significant difference between the 25Q and 97Q constructs. Collectively these
198 data suggest that long polyQ sequences do not lead to ribosome stalling, or very small amounts, and
199 therefore point to the proteotoxicity of soluble Httex1 most likely arising from other factors.

200

201 **Figure 1. Translation of Httex1 is not stalled during synthesis at short or long polyQ lengths in AD293**
202 **cells. A.** Schematic of the design of the stall reporter assays. GFP and mCherry sequences flank a test
203 sequence and are expressed in frame. The 20K sequence is a positive stall control containing repeat
204 AAA sequences as the test sequence. The Control linker is a non-stalling sequence for the test
205 sequence. In the original assay, the P2A sequence causes the ribosome to skip the formation of a
206 peptide bond leading to independent protein expression of the three components. If the ribosome
207 stalls during translation of the test sequence, then the yield of mCherry decreases relative to GFP. In the
208 modified assay, an additional alternative skip sequence (T2A) was added in tandem to the P2A to reduce

209 the rate of readthrough **B.** Western blots of cells expressing the stall reporters probed with GFP or
210 mCherry antibodies. Marker molecular weight masses are shown on the left. Products labeled on the
211 right. **C.** mCherry:GFP fluorescence ratios measured by flow cytometry.

212

213 Next, we assessed whether the inclusions were enriched with RNA on the possibility that translational
214 machinery, mRNA and ribosomes included, are coaggregated into the newly formed inclusions. For this
215 we used a biosensor form of Httex1 (TC9-Httex1) we previously developed that enables us to demarcate
216 early-formed inclusions from older inclusions [8, 18]. This biosensor involves a tetracysteine tag
217 embedded near the start of the polyglutamine sequence that can bind to the biarsenical dye FlAsH when
218 Httex1 is monomeric or forms a disordered aggregate in the early-formed inclusions. In contrast, when
219 Httex1 is in an amyloid conformation and in the more mature inclusions, it is unable to bind [8]. Using a
220 flow cytometry method called Pulse Shape analysis, cells containing Httex1 diffusely distributed (ni) or
221 within inclusions (i) can be separated and then further divided into those Highly Biarsenical-Reactive
222 (HBR) or Poorly Biarsenical-Reactive (PBR) [18, 23]. The result is the detection of cells with recently
223 formed inclusions which are reactive to FlAsH (HBri), and cells with mature inclusions which are PBri
224 [8]. HBri and PBri status can also be determined by microscopy [8].

225 To test for incorporation of RNA we used a Click-It RNA imaging approach, which involves adding 5-
226 ethynyl uridine (EU) nucleoside to the media that then gets incorporated into newly synthesized RNA
227 molecules and can be labelled with a fluorophore by click chemistry [24]. EU was rapidly incorporated
228 into nuclear RNA pools and to a lesser extent the cytoplasm (**Fig 2A-B**). The levels of EU inside the
229 inclusions was lower than the surrounding cytosol but was higher than background levels of
230 fluorescence determined by control cells labelled with Alexa 647 in the absence of EU (**Fig 2C**). This
231 suggests that at best there was a small incorporation of mRNA into the inclusions, however there was no

232 difference between early-formed and later-formed inclusions in the amount of RNA. Hence, if nascent
233 chain Httex1-ribosome complexes are major drivers of the first stages of aggregation, it is likely that this
234 occurs after the 40S and 60S ribosome components have split, which would liberate the mRNA (and 40S
235 subunit) from the stalled 60S-nascent chain complex [25, 26].

236

237 **Figure 2. mRNA is not enriched inside Httex1 inclusions. A.** Confocal images of AD293 cells transfected
238 with TC9-Httex1(97Q)-Cerulean and stained with FlAsH 16 h after growing in nucleoside analogue 5-
239 ethynyl uridine. Cells were stained with Hoecsht 33342 (nuclei stain), and Alexafluor 647 (RNA). Scale
240 bar: 10 μ m. **B.** FlAsH:Cerulean fluorescence ratios with ranges shown that was used to classify the HBRi
241 and PBRi cells. Bar shows mean and SEM of individual inclusions. **C.** Quantitation of individual images of
242 cells for intensity of Alexafluor 647 in one focal plane (measured by confocal microscopy). Data points
243 indicate individual cells. Bars show means and SD. Differences were assessed by ordinary 2 way ANOVA.

244

245 We then examined whether components of the 60S ribosome and other candidate machinery involved
246 in ribosome quality control (RQC) are enriched in the inclusions. The RQC system is responsible for
247 monitoring partially synthesised proteins and labelling nascent chains that stall before reaching the stop
248 codon for destruction [25, 26]. The RQC forms a stable complex with the 60S ribosome which triggers
249 degradation of the nascent chain via ubiquitinylolation [26]. The sequence of events include (1) splitting
250 of the stalled ribosome; (2) assembly of the RQC and ubiquitinylolation of the nascent chain; (3) extraction
251 of the nascent chain and then degradation [25].

252 For these experiments, we co-expressed Halo-tagged candidate proteins previously suggested to be
253 involved in ribosome quality control with TC9-Httex1(97Q), allowed inclusions to form and then stained
254 for location of the candidate proteins via the HaloTags. This included Rack1 (Gnb2l1), which is a

255 component of the 40S ribosome and is involved in translational repression [27, 28]. Rack1 has previously
256 been reported to bind to Httex1, which in mutant form was suggested to interfere with protein
257 translation [29], and is involved in initiating RQC by promoting ubiquitinylation of 40S ribosome subunits
258 to resolve poly(A)-induced ribosome stalling [30]. We examined listerin (Ltn1) which is an E3 ubiquitin
259 ligase that is recruited to 60S ribosome subunits close to the nascent chain and ubiquitinylates nascent
260 chains that become stalled during synthesis [26]. We also investigated another protein not observed as
261 enriched in inclusions [8] or predicted to be involved in this biology as a negative control, the α domain-
262 containing protein 1 (Nacad). Nacad did not appear to colocalize with the inclusions based on HaloTag
263 staining, yet both Ltn1 and Rack1 were enriched in the outer layer of the inclusions suggesting a specific
264 enrichment of the RQC machinery to the aggregates (**Fig 3A and B**). The over-expression of these
265 proteins did not appear to influence the formation of inclusions of Httex1(97Q) (**Fig 3C**).

266

267 **Figure 3. RQC proteins are recruited and enrich in the outer layer of inclusions whilst having no affect**
268 **on inclusion formation.** AD293 cells were co-transfected with TC9-Httex1(97Q)-Cerulean and Halo-
269 tagged RQC proteins Rack1 (**A**), Ltn1 (**B**) and Nacad (**C**). HaloTag proteins were visualized by staining with
270 TMRDirect ligand 24 hour after transfection (scale bar; 10 μ m). Images (*upper panels*) were then
271 quantified for enrichment of Halo-tagged proteins in inclusions (*middle panels*). The outer ring denotes
272 the outer edge of the inclusion which was defined as the outer most area of cerulean fluorescence in the
273 inclusions. Data points indicate individual cells, with lines connecting matched data. Means are shown
274 as red dashes. Differences were assessed by repeated measures one way ANOVA with Tukey multiple
275 comparisons test. Impact of HaloTag protein expression on Httex1(97Q)-Cerulean inclusion formation
276 was determined by flow cytometry analysis (*lower panels*). Data indicates fraction of cells with
277 inclusions, as measured by pulse shape analysis for cells categorized into different expression level bins
278 (Htt levels, Cerulean fluorescence and Halo-tagged protein level, TMR fluorescence). The TMR was

279 categorized into four evenly-spaced expression-level bins for each protein. Points and lines show means,
280 shaded regions indicate SD.

281

282 To further probe the role of other proteins previously shown to be enriched in Httex1(97Q) inclusions
283 [8] we overexpressed HaloTagged versions of proteins most enriched proteins in PBRi inclusions (Hspb1,
284 Sgta, Upf1) and HBRi inclusions (Snu13, Rpl18). Hspb1 (Hsp27), which is a small heat shock protein
285 involved in chaperone activity, appeared to enrich in the outer layer of Httex1(97Q) inclusions (**Fig 4A**).
286 Furthermore, at high expression levels, Hspb1 lowered the potential of Httex1 to form inclusions which
287 suggests it plays a role in mitigating (or reversing) aggregation (**Fig 4A**). Similarly, Sgta, which is a co-
288 chaperone involved in the Bag6 system and ERAD also enriched to the inner and outer layer of Httex1
289 and had a more potent effect on suppressing aggregation of Httex1(97Q) at high co-expression levels
290 (**Fig 4B**). Snu13, which is involved in pre-mRNA splicing, also was enriched in the inner and outer layers
291 of the Httex1(97Q) inclusions and could suppress the aggregation of Httex1(97Q) (**Fig 4C**). Upf1 also
292 enriched in the inner and outer edges of the inclusions but did not appear to affect the aggregation
293 process of Httex1 (**Fig 4D**). Previously we found RPL18 as the most enriched protein in Httex1 inclusions
294 by proteomics [8]. Halo-tagged Rpl18 was mostly present in the nucleus as punctate structures but
295 small levels were seen in the cytosol (**Fig 4E**). Endogenous Rpl18 is anticipated to mostly reside in the
296 nucleolus, ER and cytoplasm based on the Human Protein Atlas database [31]. We did not observe any
297 evidence of enrichment of Halo-tagged Rpl18 with Httex1 inclusions (HBRi or PBRi). Furthermore, there
298 was no evidence that the expression level of Halo-tagged Rpl18 affected the aggregation of Httex1 into
299 inclusions (**Fig 4E**). However, the strong nuclear localization of the Halo-tagged Rpl18 suggests it might
300 not be properly forming complexes with the ribosomes under these conditions.

301

302 **Figure 4. Recruitment patterns of proteins previously shown to be enriched in Httex1(97Q) inclusions**
303 **and impact on inclusion assembly** AD293 cells were co-transfected with TC9-Httex1(97Q)-Cerulean and
304 either chaperones Hspb1 (**A**) or Sgta (**B**), splicing proteins Snu13 (**C**) or Upf1 (**D**), or ribosome protein
305 Rpl18 (**E**). Data is presented in the same manner as Fig 3. Scale bar; 10 μ m. For proteins where cytosolic
306 TMR staining could not be seen when other structures were not saturated a second ‘high PMT’ image
307 was taken where the PMT was increased to a point when the cytoplasm could then be seen.

308
309 Our last set of experiments examined the diffusibility of mHttex1 in HBRi versus PBRi to determine
310 whether the newly formed inclusions comprised of liquid-like mutant Httex1 aggregates as previously
311 postulated [8, 14, 15]. For this experiment we performed fluorescence recovery after photobleaching
312 on live AD293 cells expressing the TC9- Httex1(97Q)-mCherry and stained with FlAsH. After bleaching,
313 both HBRi and PBRi inclusions did not recover over a period of 20 mins, which is consistent with a non-
314 liquid aggregation state (**Fig 5A–B**). As a further probe for porosity we immunostained cells with a GFP
315 antibody to test whether the antibody was able to penetrate the inclusions formed by TC9-Httex1(97Q)
316 fused to GFP derivative Cerulean. The antibody formed a tight ring around the inclusions of both PBRi
317 and HBRi, which suggested both inclusion states formed impenetrable aggregates (**Fig 5C**) and there was
318 no statistical difference in penetration distance of the antibody staining between the HBRi and PBRi
319 suggesting that inclusions form a dense, and immobile core structure quickly after formation (**Fig 5D**).

320
321 **Figure 5. Httex1 molecules are immobile within early and later-formed inclusions.** FRAP experiments
322 tracking the recovery of mCherry fluorescence in regions of bleached HBRi (**A**) and PBRi (**B**) inclusions.
323 Cells were expressing TC9-tagged Httex1 as a fusion to mCherry. Shown are representative images of
324 inclusions before (pre-bleached), immediately after bleaching (t0) and at the endpoint (t21) following

325 recovery. Plots show fluorescence recovery in the bleached region for individual inclusions. **C**.
326 Inclusions in cells expressing Httex1 fusions to Cerulean are impervious to anti-GFP antibodies. Shown
327 are representative images of anti-GFP stained inclusions for HBRi and PBRi (scale bar: 10 μ m) **D**.
328 Quantitation of antibody penetration distance into inclusions (pixels) from confocal images of
329 immunostained inclusions *in situ* in cells. Individual inclusions are shown as well as means \pm SD. There
330 was no statistical difference as assessed by a two tailed t-test ($P=0.1641$).

331

332 **DISCUSSION**

333 Our data here suggests that expression of Httex1, at wild-type or mutant polyQ lengths, does not stall
334 on the ribosome during translation. As we wrote this manuscript a study was published that suggested
335 Htt has a physiological function of binding to ribosomes and slowing the speed of ribosome
336 translocation of many target mRNAs [32]. Intriguingly, mutant Htt further slowed protein synthesis rates
337 [32]. These data suggest that Httex1 may be slowing translation in trans rather than in cis (through
338 nascent Httex1-driven stalling). This data therefore raises the possibility that mutant Httex1 does not
339 itself stall during synthesis but upon aggregation can sequester proteins similarly involved in regulating
340 ribosome translocation, and potentially those involved in ribosome quality control, into mutant Httex1
341 inclusions. This mechanism likely would involve the co-aggregation of Httex1 with the endogenous full
342 length Htt that exerts the physiological activity. It is important to note that our constructs contained
343 mixed CAG and CAA codons of glutamine, which is different to the mostly homogenous CAG repeats
344 seen in the human disease [1]. Other studies of polylysine encoded by repeating AAA codons stall much
345 more effectively than polylysine encoded by mixed AAG and AAA lysine codons, or just AAG codons[33,
346 34]. The mechanism of stalling was explained in this case by contributions from both RNA and protein
347 [34].

348 Previously it was shown that inclusions formed in yeast constitutively expressing mHtt(72Q)-GFP had a
349 diffusible core suggestive of a liquid-like state [15]. Also it has been shown that Httex1 can form
350 droplets in vitro and liquid-like states in cells [14]. We did not observe any evidence of liquid-like states
351 in early or late-formed inclusions. It remains possible that the early-formed inclusions detected by our
352 dyes have already solidified into a gel state but remain disordered by the time we assessed them. It also
353 remains possible that the structures observed in other studies are distinct to what we observed.

354 Of the proteins that were co-aggregated Sgta, Snu13 and Upf1 were mildly enriched inside the inclusion
355 whereas Rack1, Ltn1 and Hspb1 were only or more extensively enriched on the outside edge of the
356 inclusion. Previously it was shown that Hspb1 can form molecular condensates, which raises the
357 possibility of a mixed phase separation process with polyQ that may explain some of the co-aggregation
358 mechanism [35, 36]. Nonetheless, the overexpression of the proteins involved in ribosome quality
359 control did not alter the aggregation propensity. This result is more consistent with them playing non
360 rate-limiting roles if they are involved in aggregation or clearance, or indeed acting as bystanders that
361 do not play a critical role in mediating inclusion formation but are co-aggregated.

362 In conclusion, our data suggests that nascent chains of mutant Httex1 emergent from the ribosome are
363 unlikely to stall and therefore unlikely to drive inclusion formation as stalled entities. However, given
364 that we did see some ribosome-associated proteins co-aggregating as well as other proteins we
365 previously identified as enriched in inclusions by proteomics, it remains possible that newly synthesized
366 nascent Httex1 contributes to the aggregation process substoichiometrically by nucleating further
367 association of post translated pools of Httex1. Alternatively, it is possible that aggregation of mHttex1
368 can co-aggregate with endogenous Htt that is engaging in a physiological function of regulating
369 ribosome translocation rates, and thereby drawing translation machinery into the inclusions in trans.
370 Both contexts are consistent with other reports of pre-existing pools of Httex1 monomer and small
371 oligomers being quickly absconded into the inclusion once they form [37].

372 **Competing interests**

373 No competing interests were disclosed.

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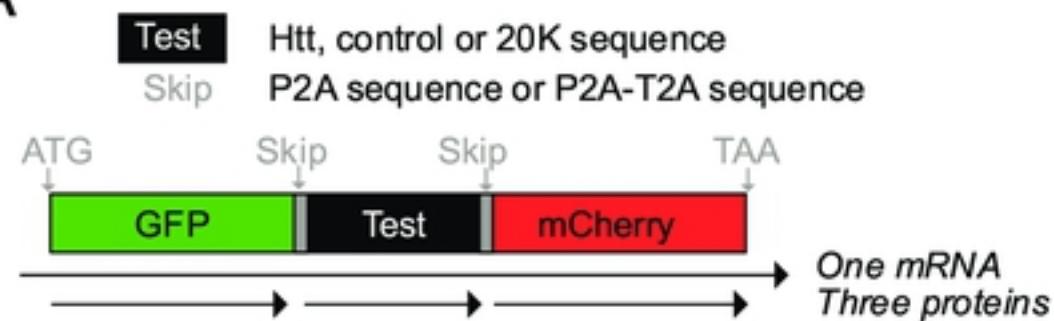
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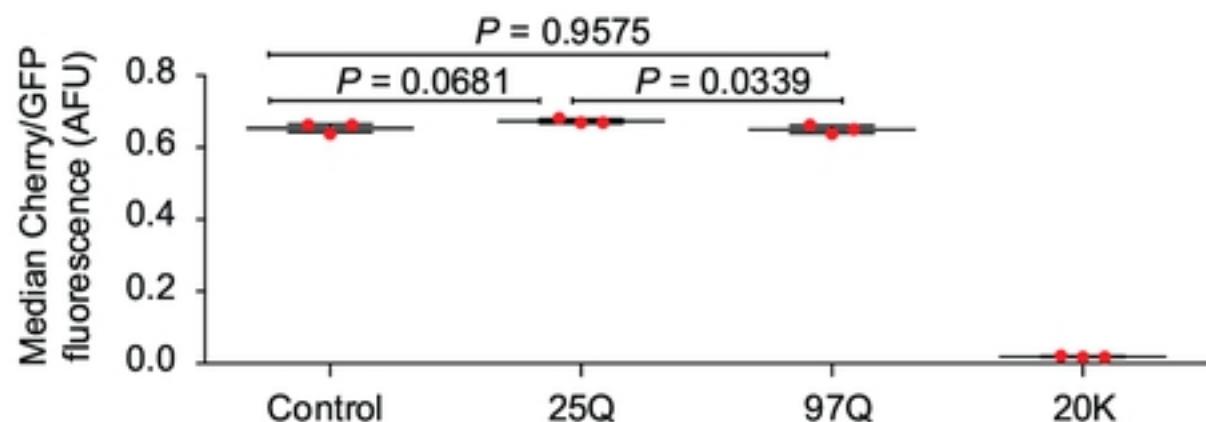
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A



C



B

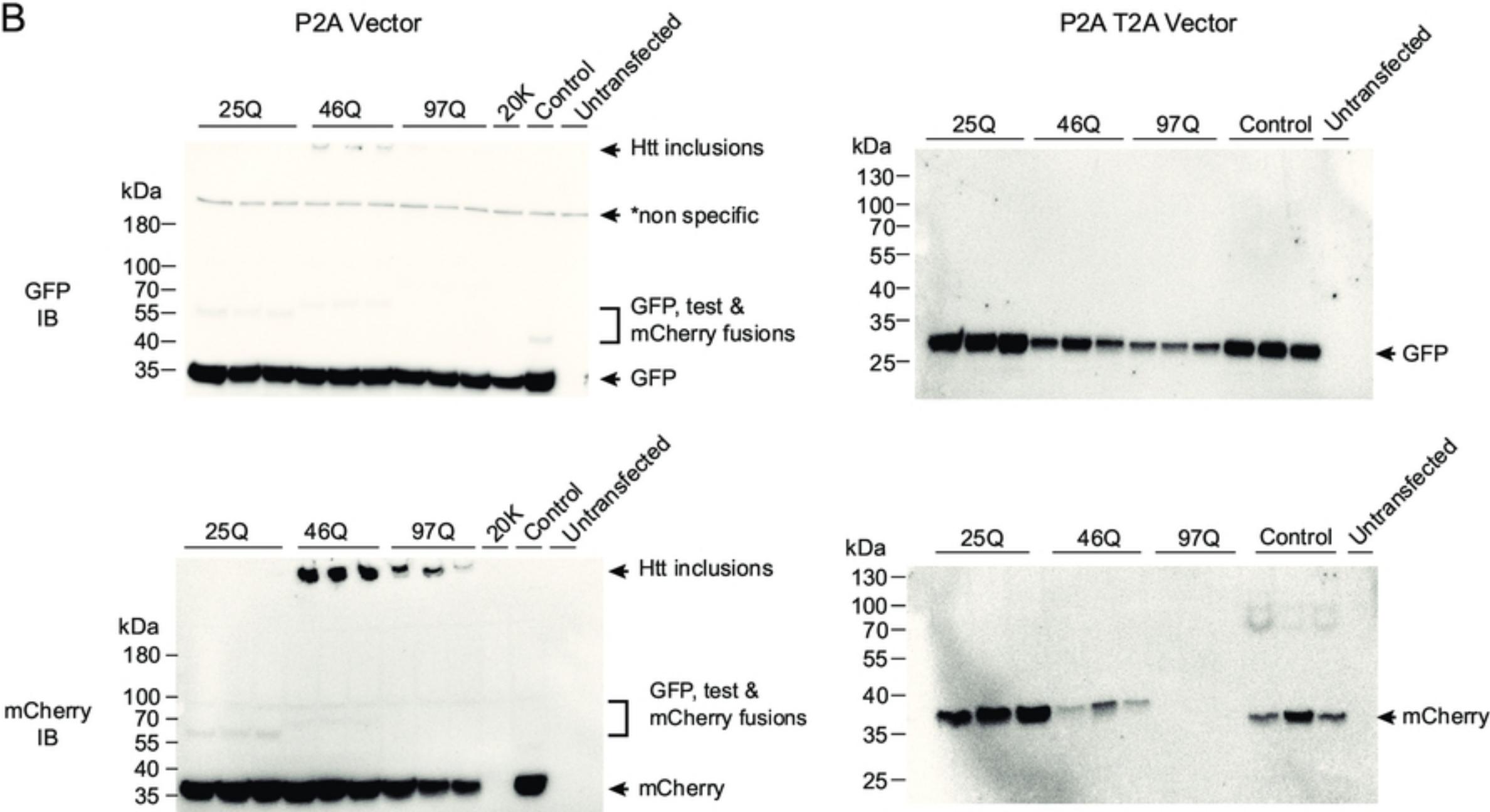
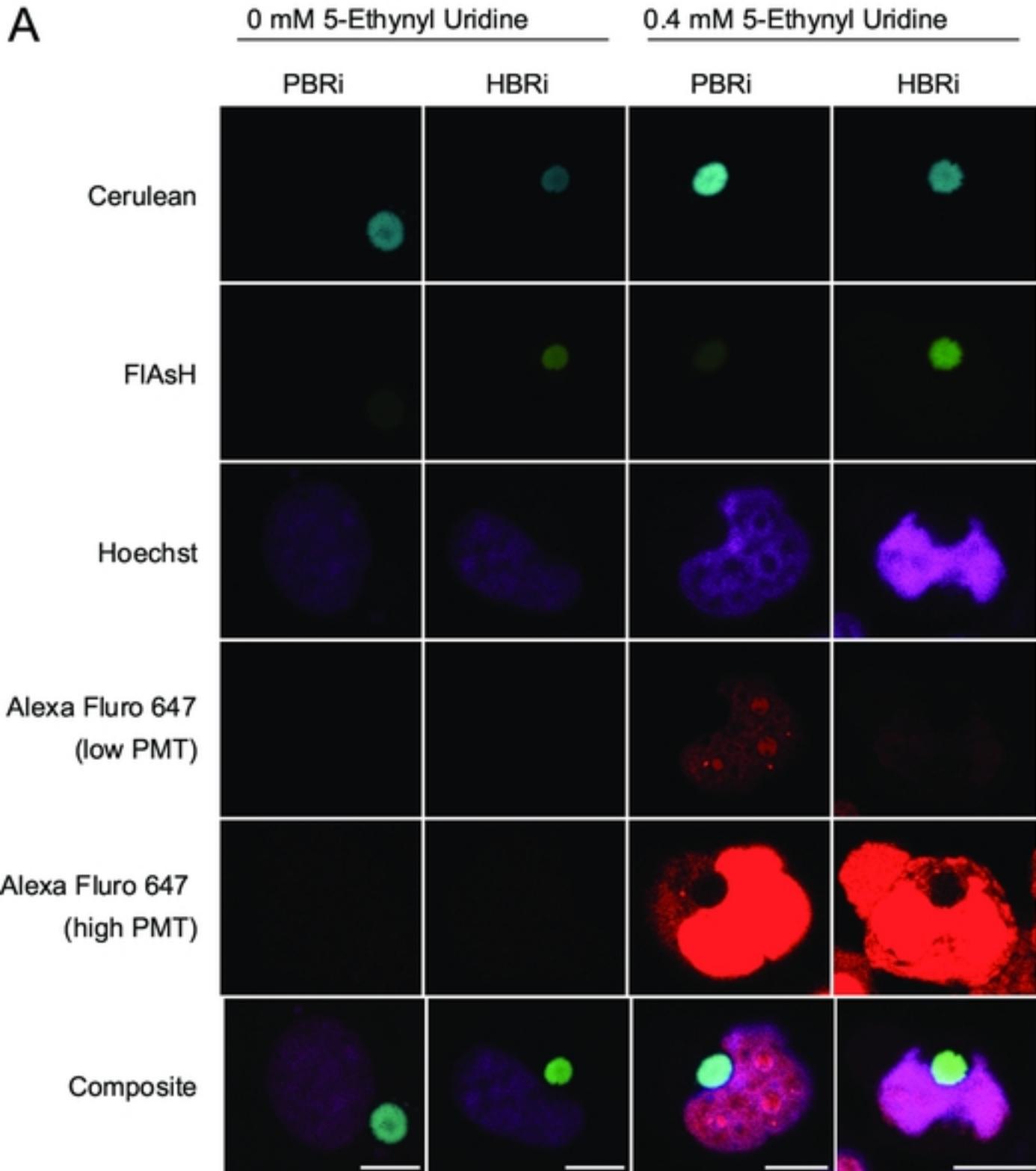
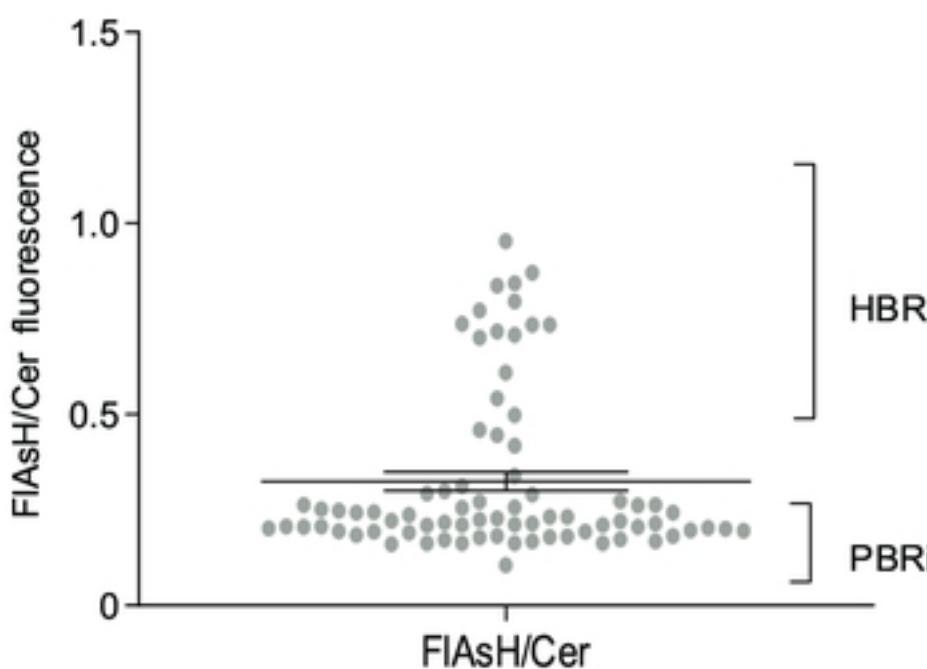
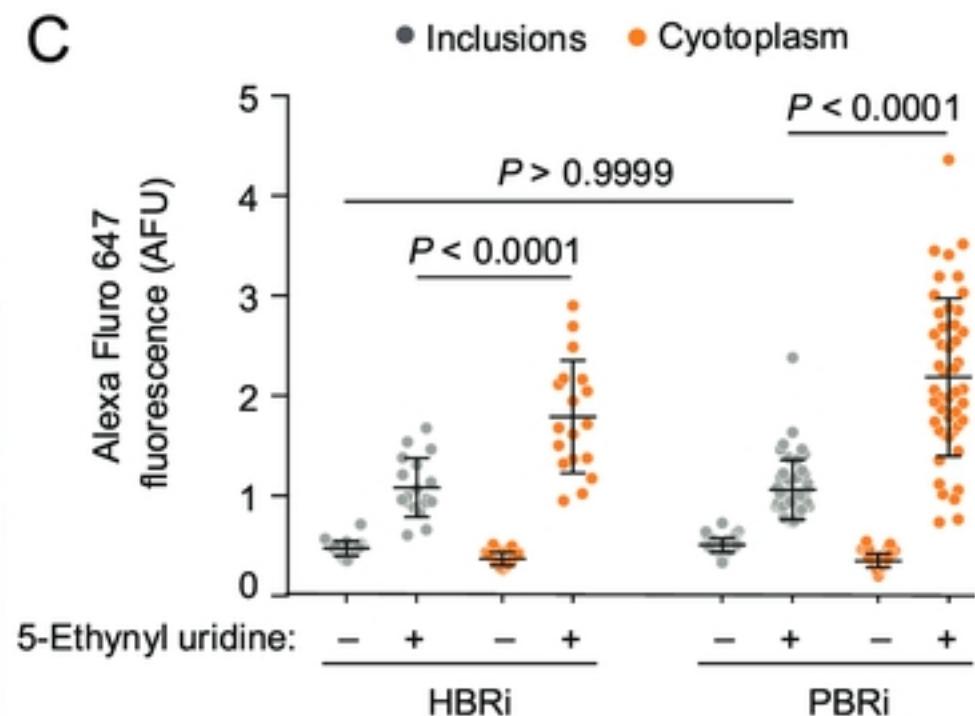
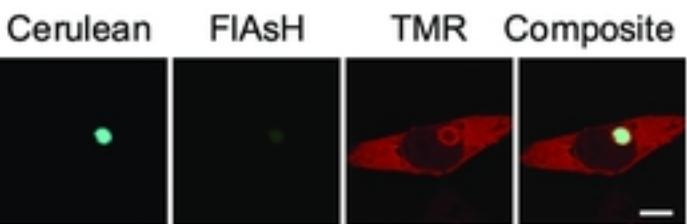
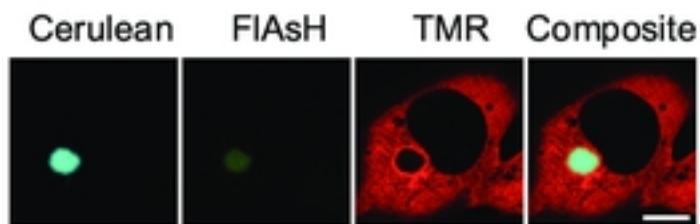
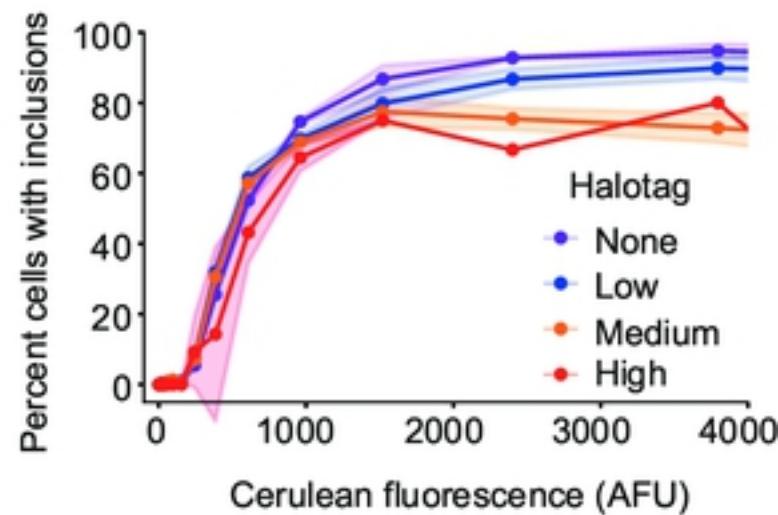
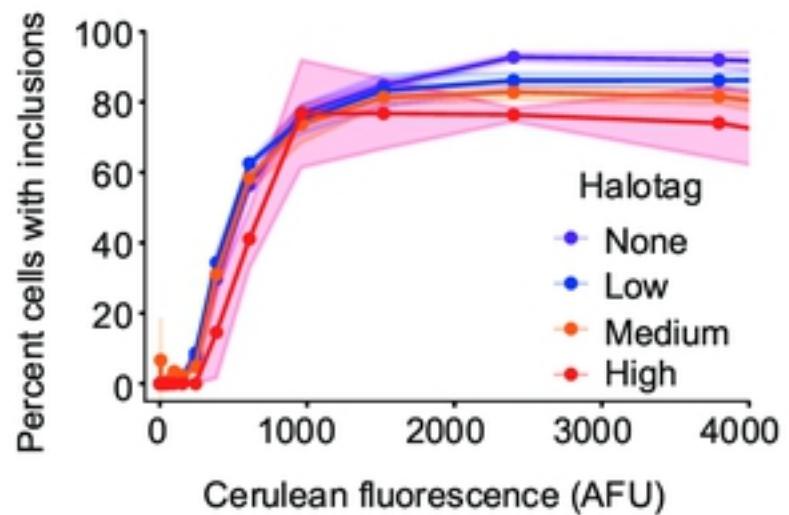
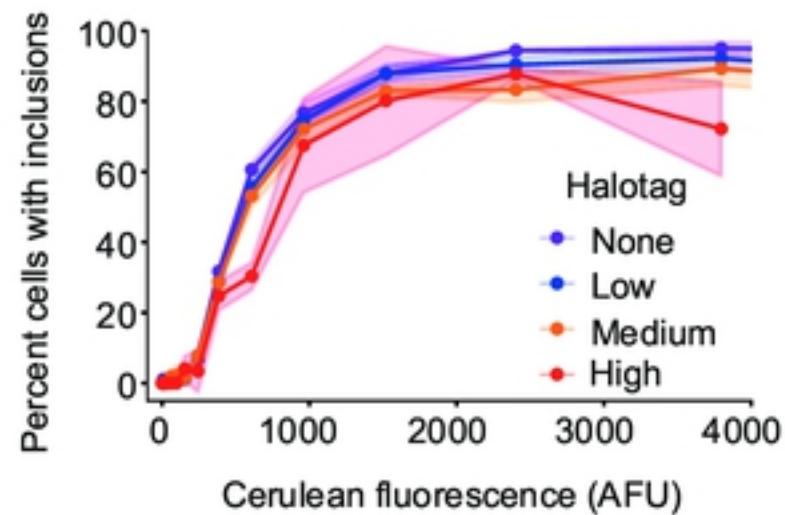
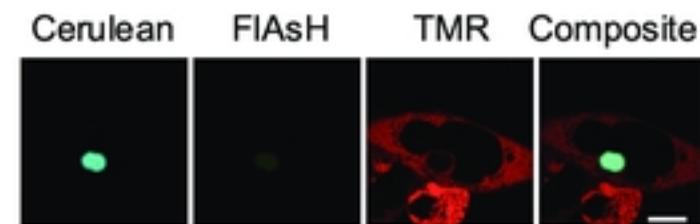


Fig 1

A**B****C****Fig 2**

A**Rack1****B****Ltn1****C****Nacad****Fig 3**

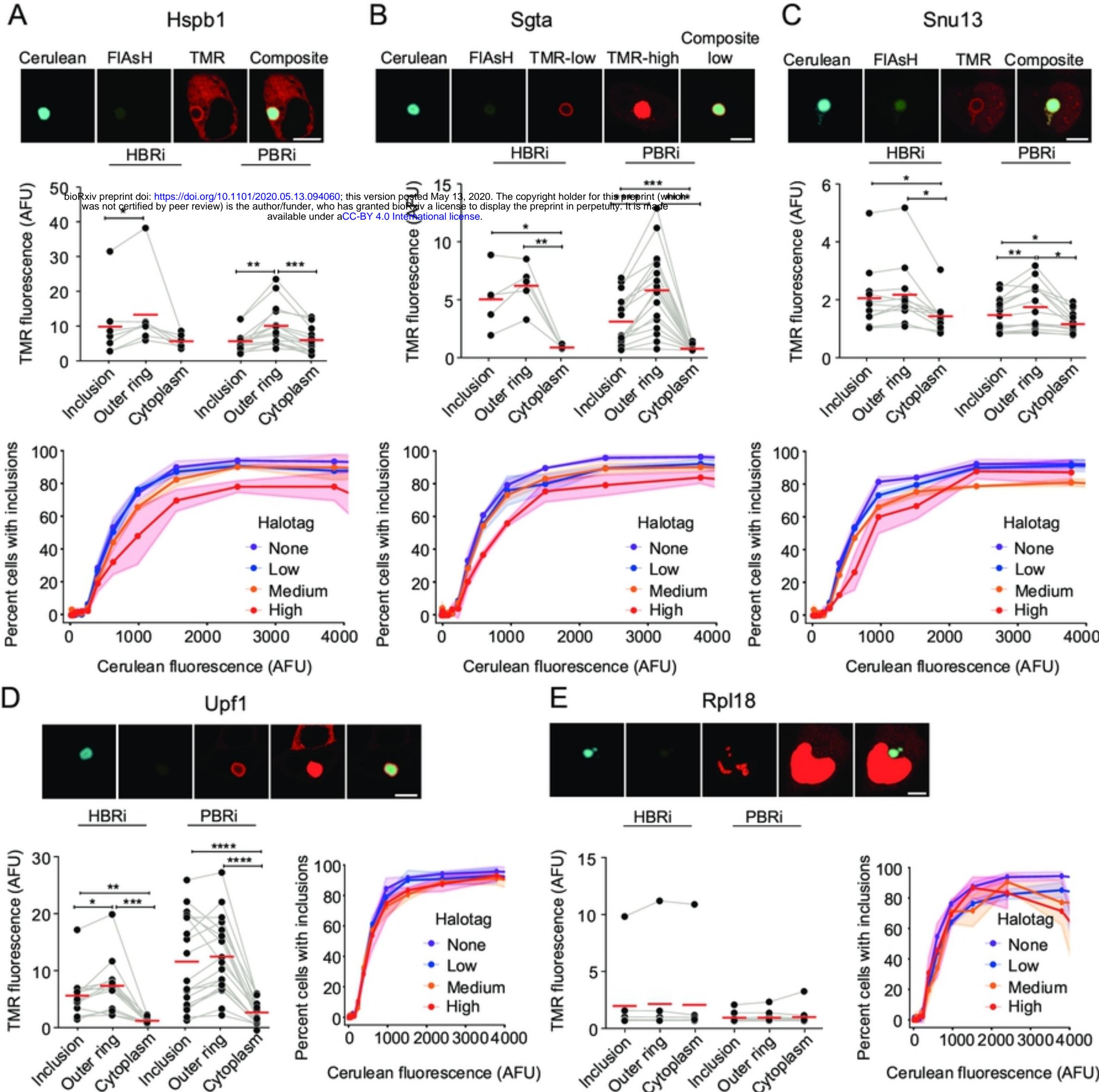
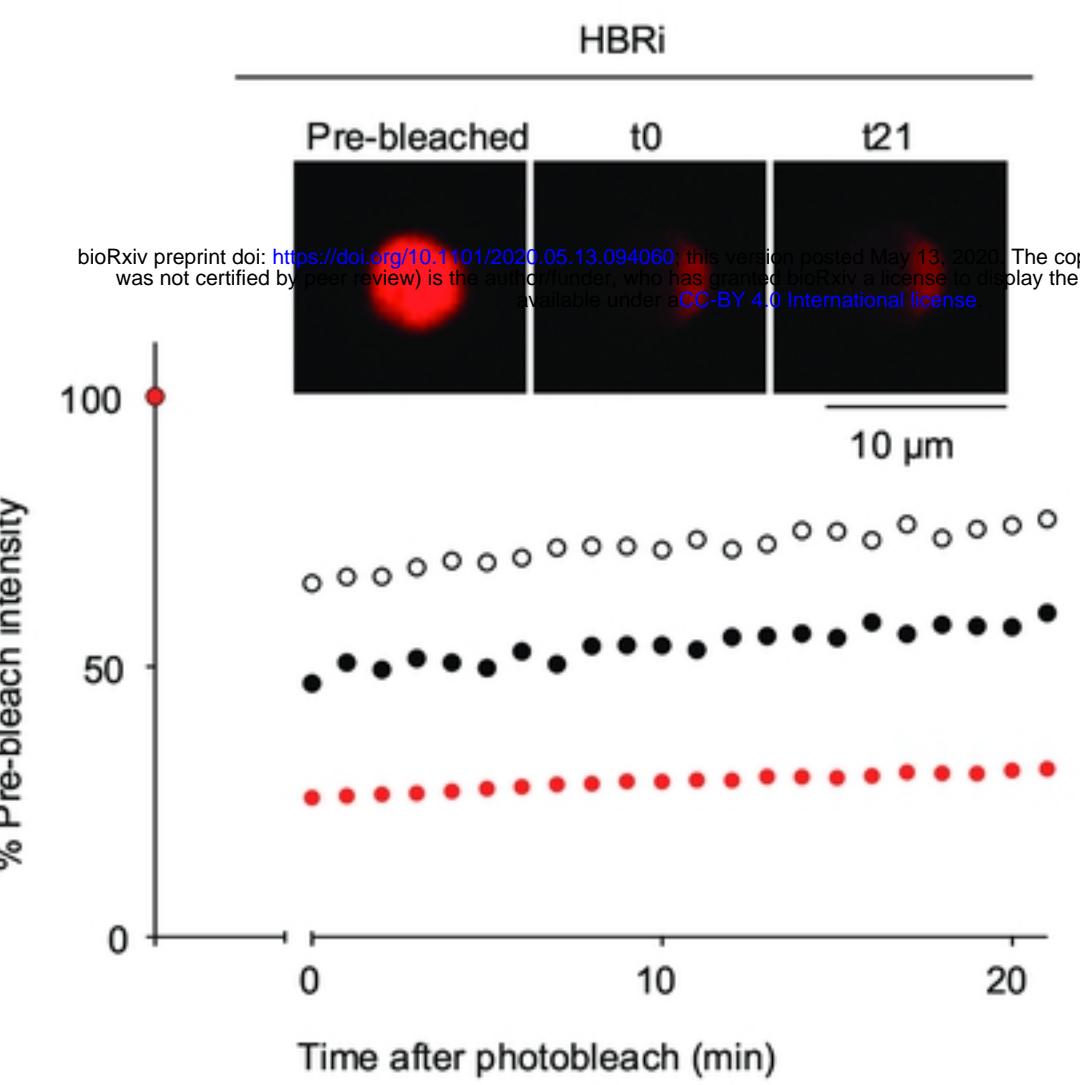
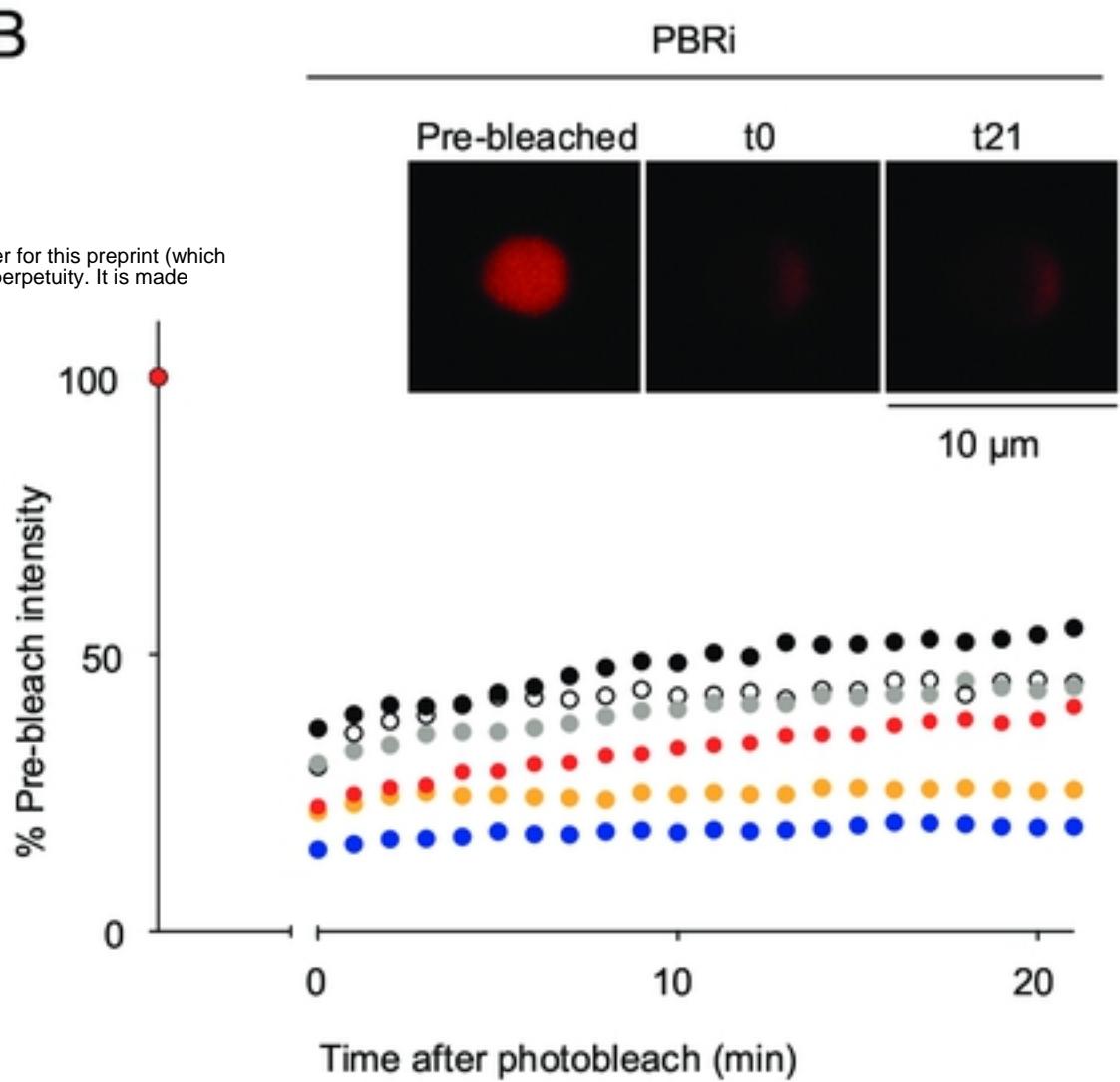


Fig 4

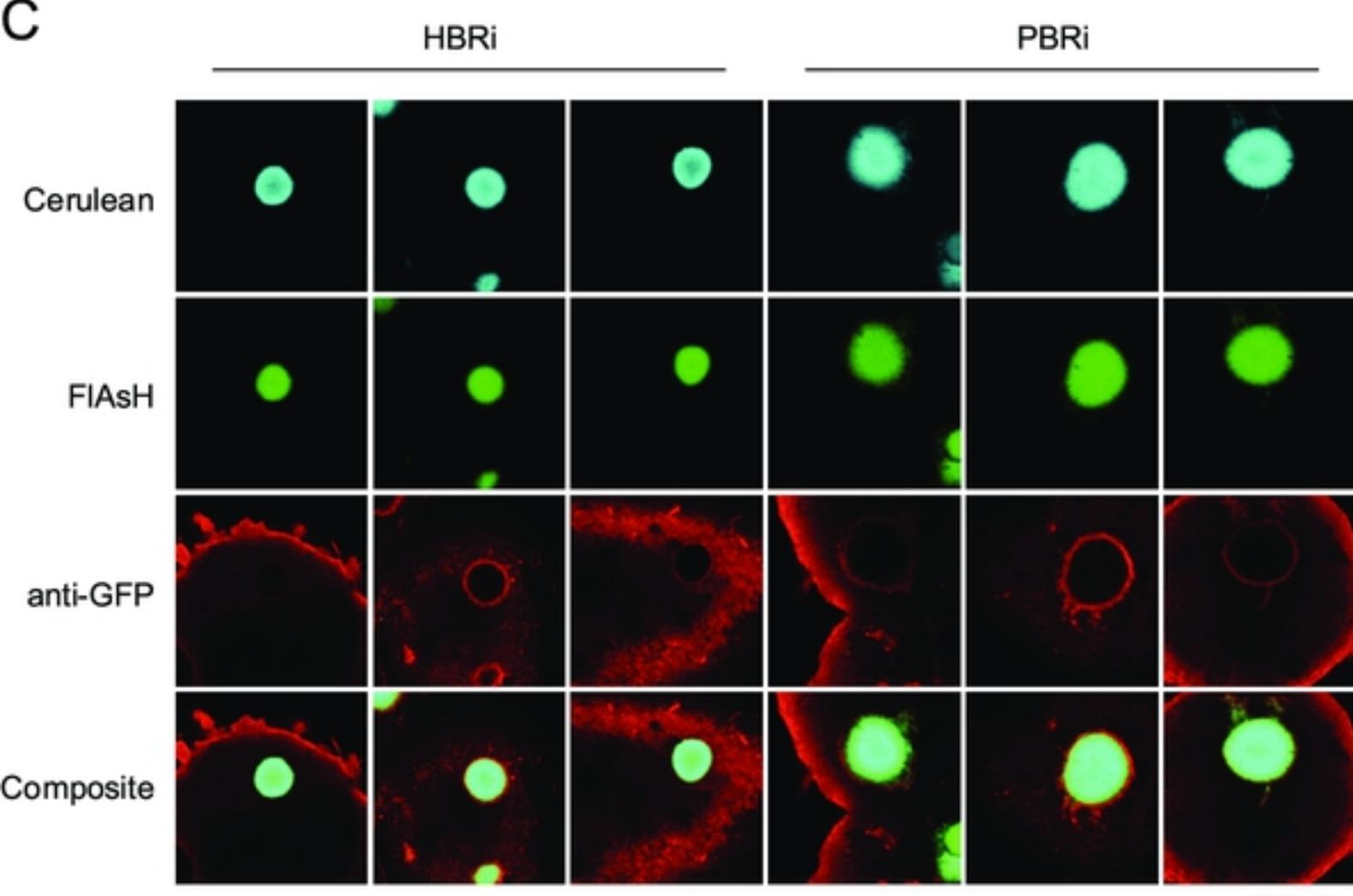
A



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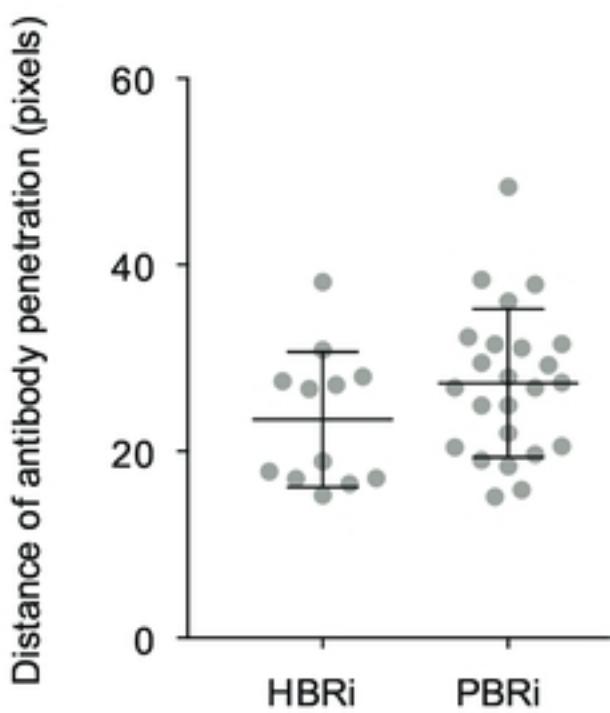


Fig 5