

1        **Inclusion bodies formed by polyglutamine and poly(glycine-alanine) are enriched with**  
2        **distinct proteomes but converge in proteins that are risk factors for disease and involved in**  
3        **protein degradation**

4        Mona Radwan<sup>1</sup>, Jordan D. Lilley<sup>1</sup>, Ching-Seng Ang<sup>2</sup>, Gavin E. Reid<sup>1,3</sup>, Danny M. Hatters<sup>1\*</sup>

5        *<sup>1</sup>Department of Biochemistry and Molecular Biology; and Bio21 Molecular Science and*  
6        *Biotechnology Institute, The University of Melbourne, VIC 3010. Australia*

7        *<sup>2</sup>Bio21 Mass Spectrometry and Proteomics Facility, The University of Melbourne, Parkville,*  
8        *Victoria, Australia*

9        *<sup>3</sup>School of Chemistry, The University of Melbourne, VIC 3010. Australia*

10        *\*Correspondence: dhatters@unimelb.edu (D.M. Hatters). Twitter: @DannyHatters*

11        **ABSTRACT**

12        **Poly(glycine-alanine) (polyGA) is one of the dipolypeptides expressed in Motor Neuron**  
13        **Disease caused by C9ORF72 mutations and accumulates as inclusion bodies in the brain of**  
14        **patients. Superficially these inclusions are similar to those formed by polyglutamine (polyQ)**  
15        **in Huntington's disease and both have been reported to form an amyloid-like structure**  
16        **suggesting they might aggregate via similar mechanisms to confer cellular dysfunction**  
17        **similarly. Here we investigated which endogenous proteins were enriched in these inclusions**  
18        **and whether aggregation-prone lengths of polyQ (Q<sub>97</sub>), in context of Huntingtin exon 1,**  
19        **shared similar patterns to aggregation-prone lengths of polyGA (101<sub>GA</sub>). When co-expressed**  
20        **in the same cell, polyGA<sub>101</sub> and HttQ<sub>97</sub> inclusions adopted distinct phases with no overlap**  
21        **suggesting different endogenous proteins would be enriched. Proteomic analyses indeed**  
22        **yielded distinct sets of endogenous proteins recruited into the inclusion types. The**  
23        **proteosome, microtubules, TriC chaperones, and translational machinery were enriched in**  
24        **polyGA aggregates, whereas Dnaj chaperones, nuclear envelope and RNA splicing proteins**  
25        **were enriched in polyQ aggregates. Both structures revealed a synergy of degradation**

26 **machinery including proteins in the polyQ aggregates that are risk factors for other**  
27 **neurodegenerative diseases involving protein aggregation when mutated, which suggests a**  
28 **convergence point in the pathomechanisms of these diseases.**

## 29 INTRODUCTION

30 The formation of protein inclusions is a hallmark of many neurodegenerative diseases.  
31 In Huntington's disease, amino-terminal fragments of mutant Huntingtin (Htt) protein  
32 aggregate into intraneuronal inclusions [1]. The aggregation of mutant Htt is triggered by  
33 an abnormally expanded polyglutamine (polyQ) sequence encoded in exon 1 that arises  
34 by CAG trinucleotide repeat expansions [2, 3]. Long polyglutamine sequences form  
35 cytoplasmic or nuclear inclusions in animal and mouse models and is associated with a  
36 pathological cascade of events (reviewed in [4]).

37 In motor neuron disease caused by *C9ORF72* GGGGCC hexanucleotide repeat  
38 expansion mutations, protein inclusions arise from the aggregation of dipolydipeptides  
39 (DPRs) expressed abnormally from the expanded GGGGCC hexanucleotide repeat  
40 sequence. 5 different DPRs are expressed, namely dipeptide polymers of glycine-alanine  
41 (polyGA), proline-arginine (polyPR), glycine-arginine (polyGR), proline-alanine (polyPA),  
42 and proline-glycine (PolyPG). Of these polyPR and polyGR are profoundly toxic when  
43 expressed in cell culture and animal models targeting mechanisms in ribosome  
44 biogenesis, translation, actin cytoskeleton among others [5-12]. PolyGA appears less toxic  
45 than the others although has been reported in some models to confer toxicity [13-21].  
46 We previously reported polyGA to be mildly toxic to cultured Neuro2a cells and to  
47 induce a distinct network of proteome changes that occur compared to the arg-rich DPRs  
48 [12]. We also noted a distinction to the other DPRs in forming large inclusions that  
49 morphologically are similar to the inclusions formed by polyQ. Furthermore, it has been  
50 reported that polyGA inclusions are, like polyQ, SDS-insoluble and amyloid-like [22, 23].

51 PolyGA is also more widespread in MND-patient brain tissue compared to the other DPRs  
52 [24].

53 Here we investigated the proteinaceous composition of polyGA inclusions and  
54 compared the profile quantitatively to inclusions of polyQ using a Huntingtin exon 1  
55 model (Httex1Q<sub>97</sub>) in a mouse neuroblastoma cell culture model using a novel  
56 proteomics-based approach to enrich the inclusions from cells under mild lysis  
57 conditions. We find distinct recruitment patterns to each inclusion type and also some  
58 similarities in biological mechanisms pertaining to degradation and a convergent  
59 pathomechanism for neurodegenerative diseases involving inappropriate protein  
60 aggregation.

61

## 62 **METHODS**

63 **Plasmids.** A pEGFP-based construct expressing polyGA dipeptide repeat length of  
64 101 dipeptides (polyGA<sub>101</sub>) was generated as described previously [12]. This construct  
65 expresses a GFP fusion tag at N-terminus of the polyGA. pT-REx vector expressing exon  
66 1 of Htt (Httex1) with polyQ sequence length of 97 and C-terminal mCherry or GFP  
67 fluorescent tags and pT-REx-mCherry were prepared as previously described [25, 26].

68 **Cell lines.** Neuro-2a cells, obtained originally from the American Type Culture  
69 Collection (ATCC), were maintained in Opti-MEM (Life Technologies). The medium was  
70 supplemented with 10% v/v fetal calf serum, 1 mM glutamine, and 100 Unit mL<sup>-1</sup> penicillin  
71 and 100 µg mL<sup>-1</sup> streptomycin, and cells were kept in a humidified incubator with 5% v/v  
72 atmospheric CO<sub>2</sub> at 37 °C.

73 **Transfections.** Neuro2a cells were transiently transfected with the vectors using  
74 Lipofectamine 2000 reagent (Life Technologies). Specific transfection conditions for the  
75 different culture vessel types at densities of  $9 \times 10^4$  (Ibidi 8-well µ-chamber) or  $6 \times 10^6$  (T75

76 flasks). The following day cells (confluency of 80 – 90 %) were transiently transfected with  
77 1.25 or 60  $\mu$ L Lipofectamine 2000 and 0.5 or 24  $\mu$ g vector DNA, respectively, as per the  
78 manufacturer's instructions (Life Technologies). The next day, the medium was changed  
79 to Opti-MEM, and for the time course the medium was refreshed daily.

80 ***Confocal imaging.*** Cells co-transfected with EGFP2-GA<sub>101</sub> and Httex1Q<sub>97</sub>-mCherry  
81 were fixed 24 h after transfection in 4% w/v paraformaldehyde for 15 min at room  
82 temperature. Nuclei were counterstained with Hoechst 33342 at 1:200 dilution (Thermo  
83 Fisher Scientific, San Jose, CA) for 30 min then washed twice in phosphate buffered saline  
84 (PBS). Fixed cells were imaged on a Leica SP5 confocal microscope using HCX PL APO CS  
85 40 $\times$  or 63 $\times$  oil-immersion objective lens (NA 1.4) at room temperature. Laser used: 405 nm  
86 excitation, 445–500 nm emission– Hoechst 33342; 488 nm excitation, 520–570 nm  
87 emission–GFP; 561 nm excitation, 590 nm emission– mCherry. Single colour controls  
88 were used to establish and adjust to remove bleed through of the emission filter  
89 bandwidths. FIJI version of ImageJ [27] and Inkscape software were used for image  
90 processing.

91 ***Longitudinal live-cell imaging.*** Neuro2a cells were co-transfected with pT-REx-  
92 mCherry and either EGFP2-GA<sub>101</sub> or pT-REx-Httex1Q<sub>97</sub>-GFP. Medium was refreshed 24 hr  
93 post transfection and cells were imaged longitudinally with a JuLI-stage fluorescence  
94 microscope (NanoEnTek) at 15 min intervals for 96 hours. Channels used: GFP for EGFP  
95 (466/40 nm excitation, 525/50 nm emission), RFP for mCherry (525/50 nm excitation, 580 nm  
96 emission). Measurement of time of inclusion formation were extracted from files  
97 generated with automated imaging using the FIJI version of ImageJ [28]. Image  
98 processing was performed with the FIJI version of ImageJ [27] and visual inspection.  
99 Differences in inclusion formation rates were assessed by survival curve analysis in  
100 GraphPad Prism 7.05 (Graphpad Software Inc., San Diego, CA).

101           ***Purification of PolyGA and polyQ Aggregates.*** Neuro-2a cells expressing either GFP-  
102 tagged 101xGA or Httex1Q<sub>97</sub> in 3 replicates were harvested by pelleting (200 *g*; 5 min; 24  
103 °C) 24 h post transfection. Cell pellets were resuspended in lysis buffer (20 mM Tris, pH  
104 8.0; 2 mM MgCl<sub>2</sub>; 150 mM NaCl; 1% (w/v) Triton X-100; 20 Units/mL Benzonase, Novagen;  
105 1× complete mini-protease cocktail; Roche) and then incubated for 30 min on ice. Lysates  
106 were diluted 2 times with PBS supplemented with protease inhibitor and aggregates were  
107 pelleted at 1000 *g* for 6 minutes. The aggregates were washed twice with 1 mL PBS, then  
108 resuspended in 1 ml PBS and subjected to fluorescence-activated cell sorting (FACS) on a  
109 BD FACS Aria III instrument with an outlet nozzle of 100 μm in diameter. The flow rate  
110 was adjusted to ~500 events/min, and EGFP fluorescence was monitored for sorting.  
111 Sorted aggregates were pelleted (12,000 *g*; 5 min; 4 °C), resuspended in PBS and washed 3  
112 times by pelleting as above and resuspension in PBS. The final pellets were harvested by  
113 pelleting (21,000 *g*, 6 min, 4 °C) and dissolved in 10 μL neat formic acid for 30 min at 37 °C,  
114 vortexed for 20 seconds and sonicated for 1 min three times then incubated in a shaking  
115 microfuge tube incubator (30 min, 37 °C). Samples were neutralized to pH 7.0 by titration  
116 with unbuffered 3 M Tris. The protein concentration in the sample was determined by a  
117 Bradford assay using bovine serum albumin as mass standard. A total protein of 200 μg  
118 was further processed for mass spectrometry analysis.

119           ***Collection of cells by Pulse Shape Analysis.*** To assess the impact of polyGA  
120 aggregation on whole proteome, Neuro2a cells expressing GFP-tagged polyGA in 3  
121 replicates were harvested 48 h post transfection by resuspension in PBS with a cell  
122 scraper. Cells were pelleted (120 *g*; 6 min) and resuspended in 2 mL PBS supplemented  
123 with 10 units/mL DNase I and filtered through 100 μm nylon mesh before analysis by flow  
124 cytometry. DAPI or Sytox (Thermo Fisher Scientific) was spiked into cell suspensions just  
125 before sorting to stain dead cells. Cells were analyzed by a FACS ARIA III cell sorter (BD  
126 Biosciences) equipped with 405-nm, 488-nm, 561-nm and 640-nm lasers. Live cells were

127 gated using side and forward scatter as described previously [29]. Cells were further  
128 gated into cells with polyGA<sub>101</sub> in the soluble form (ni) and those with polyGA<sub>101</sub>  
129 inclusions (i) by pulse shape analysis (PulSA) as previously described [29]. Each gate  
130 recovered between  $0.8-1 \times 10^6$  cells which were sorted directly into PBS and then snap  
131 frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until used.

132 ***Sample preparation for whole proteome analysis.*** Sorted cell populations were  
133 thawed and resuspended in 100  $\mu\text{l}$  RIPA lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM  
134 NaCl, 1% v/v NP-40, 0.1% w/v SDS, 1% w/v sodium deoxycholate, 1 $\times$  complete mini-  
135 protease mixture; Roche), and incubated on ice for 30 min. The concentration of proteins  
136 was measured by the Pierce microBCA Protein Assay according to the manufacturer's  
137 instruction (Thermo Fisher Scientific). Equal amounts of protein for each sample were  
138 precipitated with six volumes of pre-chilled ( $-20^\circ\text{C}$ ) acetone, and incubation overnight at  
139  $-20^\circ\text{C}$ . Samples were then pelleted (21,000  $g$ , 10 min,  $4^\circ\text{C}$ ). Acetone was decanted without  
140 disturbing the protein pellet. The pellets were washed once with pre-chilled acetone  
141 then allowed to dry for 10 min. The protein precipitates were resuspended in 100  $\mu\text{l}$  0.1 M  
142 triethylammonium bicarbonate (TEAB) and were vortexed and then sonicated 3 times for  
143 30 s. The samples were further processed for mass spectrometry analysis.

144 ***Protein sample preparation for mass spectrometry.*** Proteins were subjected to  
145 reduction with 10 mM tris(2-carboxyethyl)phosphine (TCEP), pH 8.0, and alkylation with  
146 55 mM iodoacetamide for 45 min, followed by trypsin digestion (0.25  $\mu\text{g}$ ,  $37^\circ\text{C}$ ,  
147 overnight). The resultant peptides were adjusted to contain 1% v/v formic acid then  
148 desalted by solid-phase extraction with an SPE cartridge (Oasis HLB 1 cc Vac Cartridge,  
149 Waters Corp., Milford, MA) pre-washed with 1 ml of 80% v/v acetonitrile (ACN)  
150 containing 0.1% v/v trifluoroacetic acid (TFA) and equilibrated with 1.2 ml of 0.1% v/v TFA  
151 three times. Samples were then loaded on the cartridge and washed with 1.5 ml of 0.1%  
152 v/v TFA before being eluted with 0.8 ml of 80% v/v ACN containing 0.1% v/v TFA and

153 collected in 1.5 ml microcentrifuge tubes. Peptides were then lyophilized by freeze  
154 drying (Virtis, SP Scientific, Warminster, PA). The peptides were resuspended in 100  $\mu$ l  
155 distilled water and quantified using microBCA assay with bovine serum albumin as the  
156 mass standard. Then, 10  $\mu$ g of each sample (in a volume of 50  $\mu$ l containing 100 mM TEAB)  
157 were differentially labelled by reductive dimethyl labelling using equal volumes (2  $\mu$ l) of  
158 4% light formaldehyde ( $\text{CH}_2\text{O}$ ) or 4% medium formaldehyde ( $\text{CD}_2\text{O}$ , 98% D) and 0.6 M  
159 Sodium cyanoborohydride ( $\text{NaCNBH}_3$ ). The peptide solutions were incubated on an  
160 Eppendorf Thermomixer (Eppendorf South Pacific Pty. Ltd., Macquarie Park, NSW,  
161 Australia) at room temperature for 1 h. After quenching with 8  $\mu$ l of 1% v/v ammonium  
162 hydroxide followed by further quenching with 8  $\mu$ l of neat formic acid, dimethyl-labelled  
163 peptides were combined in equal amounts prior to liquid chromatography-nano  
164 electrospray ionization-tandem mass spectrometry (LC-nESI-MS/MS) analysis.

165 ***NanoESI-LC-MS/MS analysis.*** Peptides were analyzed by LC-nESI-MS/MS using an  
166 Orbitrap Lumos mass spectrometer (Thermo Fisher Scientific) fitted with nanoflow  
167 reversed-phase-HPLC (Ultimate 3000 RSLC, Dionex, Thermo Fisher Scientific). The nano-  
168 LC system was equipped with an Acclaim Pepmap nano-trap column (Dionex - C18, 100  $\text{\AA}$ ,  
169 75  $\mu\text{m} \times 2$  cm) and an Acclaim Pepmap RSLC analytical column (Dionex - C18, 100  $\text{\AA}$ , 75  $\mu\text{m}$   
170  $\times 50$  cm, Thermo Fisher Scientific). For each LC-MS/MS experiment, 1  $\mu\text{g}$  (whole  
171 proteome) or 0.135  $\mu\text{g}$  (aggregate proteome) of the peptide mix was loaded onto the  
172 enrichment (trap) column at a flow of 5  $\mu\text{l}/\text{min}$  in 3%  $\text{CH}_3\text{CN}$  containing 0.1% v/v formic  
173 acid for 6 min before the enrichment column was switched in-line with the analytical  
174 column. The eluents used for the LC were 5% DMSO/0.1% v/v formic acid (solvent A) and  
175 100%  $\text{CH}_3\text{CN}/5\%$  DMSO/0.1% formic acid v/v. The gradient used was 3% v/v B to 20% B for  
176 95 min, 20% B to 40% B in 10 min, 40% B to 80% B in 5 min and maintained at 80% B for  
177 the final 5 min before equilibration for 10 min at 3% B prior to the next analysis.



178 The mass spectrometer was operated in positive-ionization mode with spray  
179 voltage set at 1.9 kV and source temperature at 275 °C. Lockmass of 401.92272 from  
180 DMSO was used. The mass spectrometer was operated in the data-dependent acquisition  
181 mode, with MS spectra acquired by scanning from m/z 400–1500 at 120,000 resolution with  
182 an AGC target of 5e5. For MS/MS, the “top speed” acquisition method mode (3 s cycle  
183 time) on the most intense precursor was used whereby peptide ions with charge states  $\geq 2$   
184 were isolated with an isolation window of 1.6 m/z and fragmented with high energy  
185 collision (HCD) mode, with a stepped collision energy of  $30 \pm 5\%$ . Product ion spectra  
186 were acquired in the Orbitrap at 15,000 resolution. Dynamic exclusion was activated for  
187 30s.

188 ***Proteomic data analysis.*** Raw data were analyzed using Proteome Discoverer  
189 (version 2.3; Thermo Scientific) with the Mascot search engine (Matrix Science version  
190 2.4.1). Database searches were conducted against the Swissprot *Mus musculus* database  
191 (version 2016\_07; 16794 proteins) combined with common contaminant proteins. GFP  
192 sequence (UniProt ID: P42212) was also concatenated to the Httex1Q<sub>97</sub> and PolyGA<sub>101</sub>  
193 sequences. Search was conducted with 20 ppm MS tolerance, 0.2 Da MS/MS tolerance  
194 and 2 missed cleavages allowed. Variable modifications were used for all experiments:  
195 oxidation (M), acetylation (Protein N-term), dimethylation (K), dimethylation (N-Term),  
196 2H(4) dimethylation: (K) and 2H(4) dimethylation (N-term). A fixed modification used for  
197 all experiments was carbamidomethyl (C). The false discovery rate (FDR) was calculated  
198 by the Percolator node in Proteome Discoverer v 2.3.0.81 and was set to 0.5 % at the  
199 peptide identification level and 1 % at the protein identification level. Proteins were  
200 filtered for those containing at least two unique peptides in 3 biological replicates.  
201 Peptide quantitation was performed in Proteome Discoverer v.2.3 using the precursor ion  
202 quantifier node. Dimethyl labelled peptide pairs were established with a 2 ppm mass  
203 precision and a signal to noise threshold of 3. The retention time tolerance of isotope  
204 pattern multiplex was set to 0.6 min. Two single peak or missing channels were allowed



205 for peptide identification. The protein abundance in each replicate was calculated by  
206 summation of the unique peptide abundances that were used for quantitation (light or  
207 medium derivatives). Missing quantitation values were replaced with a constant (zero-  
208 filling). The peptide group abundance and protein abundance values were normalized to  
209 account for sample loading. In brief, the total peptide abundances for each sample was  
210 calculated and the maximum sum for all files was determined. The normalization factor  
211 was the factor of the sum of the sample and the maximum sum in all files. After  
212 calculating the normalization factors, the Peptide and Protein Quantifier node  
213 normalized peptide group abundances and protein abundances by dividing abundances  
214 with the normalization factor over all samples. The normalized protein abundances were  
215 imported into Perseus software (v 1.6.5.0). Protein abundances were transformed to log<sub>2</sub>  
216 scale. The samples were then grouped according to the replicates. For pairwise  
217 comparison of proteomes and determination of significant differences in protein  
218 abundances, paired Student's t test based on permutation-based FDR statistics was then  
219 applied (250 permutations; FDR = 0.05; S<sub>0</sub> = 0.1). This was justified on the basis the  
220 proteomics abundance data is normally distributed.

221 **Bioinformatics.** Protein interaction networks were generated using Cytoscape  
222 3.7.1[30] built-in STRING (v11.0) [31] using active interaction sources parameters on for  
223 Experiments, Databases, Co-expression neighborhood, Gene Fusion and Cooccurrence.  
224 The minimum required interaction score setting was 0.9 (highest confidence). The  
225 corresponding enriched GO annotation terms were determined by calculating their  
226 enrichment *P*-value, which we compute using a Hypergeometric test, as explained in [32].  
227 The *P*-values are corrected for multiple testing using the method of Benjamini and  
228 Hochberg [33]. Selected GO terms were used to manually re-arrange nodes and were  
229 added to protein interaction network using Inkscape.  
230 IUPred [34] were applied to predict the intrinsically unstructured/disordered regions of  
231 proteins significantly enriched in polyGA or Httex1Q<sub>97</sub> aggregates. Glutamine content

232 was analyzed with the web-server COPid [35] (<http://www.imtech.res.in/raghava/copid/>). A  
233 control set of 100 random proteins (Table S1) was generated from a list of the mouse  
234 proteome obtained from the Uni-ProtKB database  
235 (<http://www.uniprot.org/uniprot/?query=reviewed:yes+AND+organism:10090&random=yes>  
236 [s](#)). The Mann-Whitney- Wilcoxon test was employed to determine significant differences.

237 ***Statistical Analysis.*** The details of the tests were reported in the figure legends. All  
238 statistical analyses were performed with GraphPad Prism v 7.05 (Graphpad Software Inc.,  
239 San Diego, CA). Significant results were defined on the figures for  $P < 0.05$ .

240 ***Data availability.*** The MS proteomic data have been deposited to the  
241 ProteomeXchange Consortium via the PRIDE [36] partner repository with the dataset  
242 identifiers PXD018505 for aggregate proteome data and PXD018824 for whole proteome  
243 data.

## 244 **RESULTS & DISCUSSION**

245 Previously we found that polyGA<sub>101</sub> as a fusion to GFP formed cytosolic inclusions in  
246 Neuro2a cells when transiently transfected. To further investigate the rate that this  
247 occurs, we used live cell imaging to track cells from 24 h after transfection onwards. Of  
248 cells with detectable levels of expression by 24 h almost all of them had formed  
249 inclusions by 60 h (Fig 1A). This was faster than comparable experiments with Httex1Q<sub>97</sub>  
250 as a fusion to mCherry, which is well known to extensively aggregate in cell culture [37,  
251 38] (Fig 1A). We noted that some lower-expressing cells showed detectable expression of  
252 polyGA only after 24 h (which we did not track) and that these were likely to form  
253 aggregates more slowly. When we co-expressed Httex1Q<sub>97</sub> as a fusion to mCherry, we  
254 found the polyGA<sub>101</sub> and Httex1Q<sub>97</sub> formed discrete inclusions in the same cell with no  
255 apparent colocalization (Fig 1B). This suggested that any concomitant co-aggregation  
256 patterns that arise with endogenous proteins may involve distinct proteins.

257

258 **Figure 1. Httex1Q<sub>97</sub> and polyGA<sub>101</sub> rapidly form distinct inclusions in neuro2a cells. A.**  
259 Kaplan-Meier curves of polyGA- or Httex1Q<sub>97</sub> expressing cells that form visible inclusions  
260 as assessed by longitudinal imaging. Cells were tracked from 24 h post-transfection. *P*-  
261 values correspond to log-rank (Mantel-Cox) test. **B.** Confocal micrographs of neuro2a  
262 cells co-expressing GFP-tagged GA<sub>101</sub> and mCherry-tagged Httex1Q<sub>97</sub>, fixed 24 hr post-  
263 transfection and stained with Hoechst33258 (cyan) to visualize nuclei. Scale bar  
264 represents 5  $\mu$ m.

265  
266 To investigate these potential differences, pellets recovered from lysates of neuro2a cells  
267 expressing GFP-tagged Httex1Q<sub>97</sub> or GFP-tagged polyGA<sub>101</sub> were sorted to purify the  
268 aggregates using flow cytometry via monitoring the GFP fluorescence. Quantitative  
269 proteomics, by way of dimethyl isotope labelling, was used to define the proteins  
270 enriched in each aggregate class after normalization to total mass of protein. We  
271 observed 737 proteins. Of these 63 were significantly enriched in polyGA inclusions (3  
272 replicates, a permutation-based FDR cut-off of 5% and *S*<sub>0</sub> of 0.1) and 48 were enriched in  
273 Httex1Q<sub>97</sub> (Table 1, Table S2 and Fig 2A).

274  
275 **Figure 2. Proteome recruitment patterns to polyGA<sub>101</sub> and Httex1Q<sub>97</sub> inclusions. A.**  
276 Volcano plot of proteins identified in the inclusions. *P*-values were calculated by a two-  
277 sided one samples t-test with null hypothesis that abundances were unchanged and the  
278 Log<sub>2</sub> ratio was equal to 0. Proteins meeting stringency thresholds (hyperbolic curves,  
279 FDR $\leq$ 0.05, *S*<sub>0</sub>=0.1) are shown as colored circles. **B.** STRING interaction maps (v.11)  
280 determined in Cytoscape (v3.7) for proteins significantly enriched in the inclusions (the  
281 full list of proteins are in Table S2). The analysis was done at the highest confidence  
282 setting. Each protein was represented by a colored circle sized proportionally to  $-\log_{10}$  (*P*-  
283 value). The color scale represents logarithm of fold change. Selected significantly

284 enriched GO terms (GOCC, GOPB, and UniProt keywords) are displayed (Full terms are  
285 shown in Table S3). C. Analysis of enriched proteomes for low-complexity regions  
286 (IUPred-L) and high glutamine content. Significance of difference was assessed against a  
287 control dataset of random mouse proteins (Table S1) with the Mann–Whitney–Wilcoxon  
288 test. Whiskers extend from 10 to 90%.

289

290 We observed notable features in this dataset consistent with known pathological markers  
291 of polyGA inclusions. Namely in C9ORF72 mediated MND, a subset of inclusions is non-  
292 reactive to TDP-43 [39]. In most other forms of MND, TDP-43-reactive inclusions are a key  
293 pathological signature of neurons in disease [40]. These TDP-43 negative inclusions were  
294 previously found to be immunoreactive for polyGA, suggesting they form by polyGA  
295 aggregation [41, 42]. We observed a lack of TDP43 in the polyGA inclusions by virtue of  
296 an enrichment in the Httex1Q<sub>97</sub> inclusions (Table 1). In addition, the TDP43-negative  
297 inclusions seen in vivo are immunoreactive to p62 [43] and lack immunoreactivity to FUS,  
298 optineurin, alpha-internexin and neurofilament [44, 45]. In our data p62 (also called  
299 sequestosome 1) is one of the most enriched proteins in polyGA inclusions, whereas Fus  
300 appeared excluded by virtue of its enrichment in Httex1Q<sub>97</sub> inclusions, which has been  
301 observed previously in cell models of polyQ aggregation and human pathology [25, 46-  
302 48]. Hence these data point to the cell model of polyGA inclusions mimicking the  
303 process of aggregation and recruitment seen in vivo and also providing specificity of co-  
304 recruitment relative to Httex1Q<sub>97</sub>.

305 Analysis of the differences is shown visually in Fig 2B by a (STRING) protein-protein  
306 interaction map and annotation to functional networks. Overall both inclusions yielded  
307 an enrichment for gene ontology and KEGG networks of microtubule cytoskeleton,  
308 proteasome complex, chaperones, RNA splicing and nuclear envelope (Fig 2C; Table S3).  
309 These findings are in accordance with prior findings that protein aggregation impacts

310 these biological processes and in particular an involvement in machinery for their  
311 clearance and degradation [49-52].

312 In addition, the data points more directly to proteins and genes implicated in MND  
313 phenotype and mechanisms. Phlda1 was one of the proteins enriched in polyGA  
314 inclusions. Previously it was found that Phlda1 was upregulated in Fus-mutant motor  
315 neurons and that this was an adaptive response to protect against apoptosis [53]. Phlda1  
316 was also observed upregulated in sporadic MND fibroblasts treated to stress compared  
317 to controls [54]. Nudt5 was also found enriched in inclusions, and expression of this  
318 gene was significantly increased in motor neurons derived from induced pluripotent  
319 cells from MND patients over controls [55]. Another protein of note enriched in the  
320 polyGA aggregates was Dpysl3. A missense mutation that has been linked to MND risk in  
321 French population and in culture expression of the mutation leads to shortened neuronal  
322 survival [56]. Hence it remains plausible that co-aggregation of these proteins into  
323 polyGA inclusions sequesters their activity and renders cells less resilient to stress  
324 triggers.

325 The Httex1Q<sub>97</sub>-enriched proteome also yielded noteworthy findings. Previously it was  
326 found that polyQ can preferentially co-recruit proteins containing intrinsically  
327 disordered domains and proteins enriched in glutamine (IDRs) [25, 47]. These patterns  
328 were also observed in our data (Fig 2C). However, polyGA did not show these  
329 enrichment patterns, indicative of specificity for polyQ in recruiting IDRs and Q-rich  
330 proteins.

331 To assess whether the changes in polyGA inclusion formation had other effects on  
332 proteome abundance, we expressed polyGA<sub>101</sub> and at 48 h after transfection sorted live  
333 cells into those with visible aggregates from those without by flow cytometry sorting  
334 method and pulse shape analysis [57] (Fig 3A). We found cells with inclusions were more  
335 reactive to Sytox (Fig 3A inset), which is indicative of dying and dead cells, than cells

336 without inclusions so we excluded these cells from analysis. 35% of the remaining live  
337 cells expressing polyGA had inclusions (Fig 3A). This was a lower yield of aggregates than  
338 we measured by live cell imaging at 48h in Fig 1A (about 90%), which we attribute to this  
339 experiment being more inclusive of the lower-expressing cells and other differences in  
340 the experimental conditions that affect aggregation rates such as phototoxicity of live cell  
341 imaging. Nonetheless, the yield obtained made the experiment amenable for comparing  
342 cells with versus without inclusions. Out of 2420 proteins identified, we observed 56  
343 proteins that significantly changed abundance in these sorted cell populations (Fig 3B;  
344 Table S4). There was no overlap in the proteins seen enriched in polyGA inclusions with  
345 proteins that changed expression due to polyGA aggregation. This provides firmer  
346 confidence that the enrichment seen in the polyGA aggregates arises from co-  
347 aggregation rather than changes in gene expression. Of the genes that changed  
348 expression, protein interaction networks yielded significant enrichment in networks  
349 including nuclear speck (GO: 0016607), ribosome biogenesis (GO: 0042254), chromosome  
350 (GO:0005694), mitochondrion (GO:0005739) and Golgi-to-ER-traffic (MMU-6811442) (Table  
351 S5). These pathways would be anticipated to be activated by stress responses incurred by  
352 protein aggregation, however, we did not note any striking changes that pertained to  
353 novel mechanisms other than that from this data.

354

355 **Figure 3. Cellular protein abundance changes arising from polyGA<sub>101</sub> aggregation. A.**

356 Schematic of flow cytometry method of pulse shape analysis (PuSA) to sort cells  
357 enriched with inclusions (i) from those without inclusions (ni). Cells with inclusions  
358 display shorter width (W) fluorescence values versus cells with soluble protein, and  
359 typically higher height values (H) arising from the condense foci of fluorescence inside  
360 the cells. Cells were sorted to exclude dead cells by Sytox reactivity. Inset shows  
361 percentage of transfected cells reactive to Sytox by time after transfection.  $n=4$ , means  $\pm$   
362 SD shown. B. Volcano plots of proteins that changed their abundance upon polyGA

363 aggregation. The data for all proteins are plotted as log<sub>2</sub>-fold change versus the  $-\log_{10}$  of  
364 the  $P$ -value. The dotted line indicates significance cut-off (hyperbolic curves,  $FDR \leq 0.05$ ,  
365  $S_0=0.1$ ) and proteins meeting stringency thresholds are shown as colored circles. **C.**  
366 Protein-protein interaction network (STRING v11) of proteins significantly changed in  
367 abundance upon polyGA aggregation (the full list of proteins are in Table S4). The  
368 analysis was done at the highest confidence setting. Each protein was represented by a  
369 colored circle sized proportionally to  $-\log_{10}$  ( $P$ -value). The color scale represents  
370 logarithm of fold change. Selected significantly enriched GO terms (GOCC, GOPB, and  
371 UniProt keywords) are displayed (Table S5).

372

373 Lastly we investigated the overlap of proteins enriched in Httex1Q<sub>97</sub> inclusions with our  
374 previously reported changes in solubility of whole cell proteome before versus after  
375 inclusions had formed [58]. In that dataset we observed 17 proteins that significantly  
376 decreased in solubility as cells expressing Httex1Q<sub>97</sub> shifted from a dispersed  
377 unaggregated state to forming inclusions [58]. Of these, 5 proteins were found in our list  
378 of 48 proteins significantly enriched in Httex1Q<sub>97</sub> inclusions (Picalm, Hgs, Clint1, Ubqln2  
379 and Dnajb1). Four of these proteins (all except Dnajb1) form a robust protein-protein  
380 interaction network with a significant gene ontology enrichment for clathrin coat  
381 assembly (GO:0048268; FDR of 0.0031) suggestive that this mechanism is involved in  
382 polyQ aggregation. Clint1 and Ubqln2 were previously shown to colocalize to polyQ  
383 inclusions, supporting this conclusion [47, 59]. An interesting note with respect to  
384 mechanism is that UBQLN2 targets ubiquitinated substrates for degradation in ERAD and  
385 autophagy [60]. Furthermore mutations in UBQLN2 cause MND, and appear to operate  
386 by impairing protein degradation of ubiquitinated proteins [61]. Further supporting an  
387 important role linking protein aggregation, degradation more broadly to these  
388 neurodegenerative diseases is the enrichment of Picalm in the polyQ inclusions. Picalm  
389 is an phosphatidylinositol-binding clathrin assembly protein and has been shown via



390 GWAS as a top ten risk for Alzheimer's disease [62, 63]. It has been reported to modulate  
391 intracellular APP processing and plaque pathogenesis [64], modulate autophagy and alter  
392 tau clearance [65].

393 Collectively the data here reports proteins that co-aggregate into two very different  
394 neurodegenerative disease proteinaceous deposits. The findings provide specificity of  
395 proteins to the aggregation type that provide useful perspective to that reported by  
396 others. Moreover, the mechanisms of protein clearance mechanism appear relevant to  
397 both aggregation types and notably of a number of proteins in the Httex1Q<sub>97</sub> aggregates  
398 that when mutated are modifiers of MND risk. Therefore, the findings identify a synergy  
399 of biological mechanisms involved in protein degradation that appear central to at least  
400 two different neurodegenerative diseases, and possibly more applicable to the other  
401 neurodegenerative diseases involving inappropriate protein aggregation.

402

#### 403 **ACKNOWLEDGMENTS**

404 We thank the Bio21 Melbourne Mass Spectrometry and Proteomics facility. This work  
405 was funded by grants to DMH (National Health and Medical Research Council  
406 APP1161803 and Motor Neuron Disease Research Institute, Australia small grant) and to  
407 DMH and GER (Australian Research Council DP170103093). MR acknowledges support  
408 from an Australian Government Research Training Program (RTP) Scholarship and an  
409 Egyptian Ministry of Higher Education and Scientific Research PhD scholarship.

410

#### 411 **REFERENCES**

- 412 1. DiFiglia M, Sapp E, Chase KO, Davies SW, Bates GP, Vonsattel JP, et al. Aggregation  
413 of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain.  
414 Science. 1997;277(5334):1990-3. PubMed PMID: 9302293.

- 415 2. MacDonald ME, Ambrose CM, Duyao MP, Myers RH, Lin C, Srinidhi L, et al. A  
416 Novel Gene Containing a Trinucleotide Repeat that is Expanded and Unstable on  
417 Huntington's Disease Chromosomes. *Cell*. 1993;72(6):971-83.
- 418 3. Scherzinger E, Sittler A, Schweiger K, Heiser V, Lurz R, Hasenbank R, et al. Self-  
419 assembly of polyglutamine-containing huntingtin fragments into amyloid-like  
420 fibrils: implications for Huntington's disease pathology. *Proc Natl Acad Sci U S A*.  
421 1999;96(8):4604-9. Epub 1999/04/14. doi: 10.1073/pnas.96.8.4604. PubMed PMID:  
422 10200309; PubMed Central PMCID: PMC16379.
- 423 4. Cox D, Raeburn C, Sui X, Hatters DM. Protein aggregation in cell biology: An  
424 aggregomics perspective of health and disease. *Semin Cell Dev Biol*. 2018. Epub  
425 2018/05/14. doi: 10.1016/j.semcdb.2018.05.003. PubMed PMID: 29753879.
- 426 5. Kwon I, Xiang S, Kato M, Wu L, Theodoropoulos P, Wang T, et al. Poly-dipeptides  
427 encoded by the C9orf72 repeats bind nucleoli, impede RNA biogenesis, and kill  
428 cells. *Science*. 2014;345(6201):1139-45. Epub 2014/08/02. doi: 10.1126/science.1254917.  
429 PubMed PMID: 25081482; PubMed Central PMCID: PMC4459787.
- 430 6. Tao Z, Wang H, Xia Q, Li K, Li K, Jiang X, et al. Nucleolar stress and impaired stress  
431 granule formation contribute to C9orf72 RAN translation-induced cytotoxicity.  
432 *Hum Mol Genet*. 2015;24(9):2426-41. Epub 2015/01/13. doi: 10.1093/hmg/ddv005.  
433 PubMed PMID: 25575510.
- 434 7. Zhang K, Donnelly CJ, Haeusler AR, Grima JC, Machamer JB, Steinwald P, et al. The  
435 C9orf72 repeat expansion disrupts nucleocytoplasmic transport. *Nature*.  
436 2015;525(7567):56-61. Epub 2015/08/27. doi: 10.1038/nature14973. PubMed PMID:  
437 26308891; PubMed Central PMCID: PMC4800742.
- 438 8. Kanekura K, Yagi T, Cammack AJ, Mahadevan J, Kuroda M, Harms MB, et al. Poly-  
439 dipeptides encoded by the C9ORF72 repeats block global protein translation.  
440 *Hum Mol Genet*. 2016;25(9):1803-13. Epub 2016/03/05. doi: 10.1093/hmg/ddw052.  
441 PubMed PMID: 26931465; PubMed Central PMCID: PMC4986334.

- 442 9. Lee KH, Zhang P, Kim HJ, Mitrea DM, Sarkar M, Freibaum BD, et al. C9orf72  
443 Dipeptide Repeats Impair the Assembly, Dynamics, and Function of Membrane-  
444 Less Organelles. *Cell*. 2016;167(3):774-88 e17. Epub 2016/10/22. doi:  
445 10.1016/j.cell.2016.10.002. PubMed PMID: 27768896; PubMed Central PMCID:  
446 PMCPMC5079111.
- 447 10. Freibaum BD, Taylor JP. The Role of Dipeptide Repeats in C9ORF72-Related ALS-  
448 FTD. *Front Mol Neurosci*. 2017;10(35):35. Epub 2017/03/01. doi:  
449 10.3389/fnmol.2017.00035. PubMed PMID: 28243191; PubMed Central PMCID:  
450 PMCPMC5303742.
- 451 11. Zhang YJ, Gendron TF, Ebbert MTW, O'Raw AD, Yue M, Jansen-West K, et al.  
452 Poly(GR) impairs protein translation and stress granule dynamics in C9orf72-  
453 associated frontotemporal dementia and amyotrophic lateral sclerosis. *Nat Med*.  
454 2018;24(8):1136-42. Epub 2018/06/27. doi: 10.1038/s41591-018-0071-1. PubMed PMID:  
455 29942091; PubMed Central PMCID: PMCPMC6520050.
- 456 12. Radwan M, Ang CS, Ormsby AR, Cox D, Daly JC, Reid GE, et al. Arginine in  
457 C9ORF72 dipolypeptides mediates promiscuous proteome binding and multiple  
458 modes of toxicity. *Mol Cell Proteomics*. 2020. Epub 2020/02/23. doi:  
459 10.1074/mcp.RA119.001888. PubMed PMID: 32086375.
- 460 13. Al-Sarraj S, King A, Troakes C, Smith B, Maekawa S, Bodi I, et al. p62 positive, TDP-  
461 43 negative, neuronal cytoplasmic and intranuclear inclusions in the cerebellum  
462 and hippocampus define the pathology of C9orf72-linked FTLD and MND/ALS.  
463 *Acta Neuropathol*. 2011;122(6):691-702. Epub 2011/11/22. doi: 10.1007/s00401-011-  
464 0911-2. PubMed PMID: 22101323.
- 465 14. Bigio EH, Weintraub S, Rademakers R, Baker M, Ahmadian SS, Rademaker A, et al.  
466 Frontotemporal lobar degeneration with TDP-43 proteinopathy and chromosome  
467 9p repeat expansion in C9ORF72: clinicopathologic correlation. *Neuropathology*.

- 468 2013;33(2):122-33. Epub 2012/06/19. doi: 10.1111/j.1440-1789.2012.01332.x. PubMed  
469 PMID: 22702520; PubMed Central PMCID: PMC3449045.
- 470 15. Ash PE, Bieniek KF, Gendron TF, Caulfield T, Lin WL, Dejesus-Hernandez M, et al.  
471 Unconventional translation of C9ORF72 GGGGCC expansion generates insoluble  
472 polypeptides specific to c9FTD/ALS. *Neuron*. 2013;77(4):639-46. Epub 2013/02/19.  
473 doi: 10.1016/j.neuron.2013.02.004. PubMed PMID: 23415312; PubMed Central  
474 PMCID: PMC3593233.
- 475 16. Gendron TF, Bieniek KF, Zhang YJ, Jansen-West K, Ash PE, Caulfield T, et al.  
476 Antisense transcripts of the expanded C9ORF72 hexanucleotide repeat form  
477 nuclear RNA foci and undergo repeat-associated non-ATG translation in  
478 c9FTD/ALS. *Acta Neuropathol*. 2013;126(6):829-44. Epub 2013/10/17. doi:  
479 10.1007/s00401-013-1192-8. PubMed PMID: 24129584; PubMed Central PMCID:  
480 PMC3830741.
- 481 17. Mann DM, Rollinson S, Robinson A, Bennion Callister J, Thompson JC, Snowden  
482 JS, et al. Dipeptide repeat proteins are present in the p62 positive inclusions in  
483 patients with frontotemporal lobar degeneration and motor neurone disease  
484 associated with expansions in C9ORF72. *Acta Neuropathol Commun*. 2013;1(1):68.  
485 Epub 2013/11/21. doi: 10.1186/2051-5960-1-68. PubMed PMID: 24252525; PubMed  
486 Central PMCID: PMC3893586.
- 487 18. Mori K, Arzberger T, Grasser FA, Gijssels I, May S, Rentzsch K, et al. Bidirectional  
488 transcripts of the expanded C9orf72 hexanucleotide repeat are translated into  
489 aggregating dipeptide repeat proteins. *Acta Neuropathol*. 2013;126(6):881-93. Epub  
490 2013/10/18. doi: 10.1007/s00401-013-1189-3. PubMed PMID: 24132570.
- 491 19. Zu T, Liu Y, Banez-Coronel M, Reid T, Pletnikova O, Lewis J, et al. RAN proteins  
492 and RNA foci from antisense transcripts in C9ORF72 ALS and frontotemporal  
493 dementia. *Proc Natl Acad Sci U S A*. 2013;110(51):E4968-77. Epub 2013/11/20. doi:

- 494 10.1073/pnas.1315438110. PubMed PMID: 24248382; PubMed Central PMCID:  
495 PMCPMC3870665.
- 496 20. May S, Hornburg D, Schludi MH, Arzberger T, Rentzsch K, Schwenk BM, et al.  
497 C9orf72 FTL/ALS-associated Gly-Ala dipeptide repeat proteins cause neuronal  
498 toxicity and Unc119 sequestration. *Acta Neuropathologica*. 2014;128(4):485-503.  
499 doi: 10.1007/s00401-014-1329-4. PubMed PMID: PMC4159571.
- 500 21. Mizielinska S, Isaacs AM. C9orf72 amyotrophic lateral sclerosis and  
501 frontotemporal dementia: gain or loss of function? *Curr Opin Neurol*.  
502 2014;27(5):515-23. doi: 10.1097/WCO.000000000000130. PubMed PMID: 25188012.
- 503 22. May S, Hornburg D, Schludi MH, Arzberger T, Rentzsch K, Schwenk BM, et al.  
504 C9orf72 FTL/ALS-associated Gly-Ala dipeptide repeat proteins cause neuronal  
505 toxicity and Unc119 sequestration. *Acta Neuropathol*. 2014;128(4):485-503. Epub  
506 2014/08/15. doi: 10.1007/s00401-014-1329-4. PubMed PMID: 25120191; PubMed  
507 Central PMCID: PMC4159571.
- 508 23. Chang YJ, Jeng US, Chiang YL, Hwang IS, Chen YR. The Glycine-Alanine Dipeptide  
509 Repeat from C9orf72 Hexanucleotide Expansions Forms Toxic Amyloids  
510 Possessing Cell-to-Cell Transmission Properties. *J Biol Chem*. 2016;291(10):4903-11.  
511 Epub 2016/01/16. doi: 10.1074/jbc.M115.694273. PubMed PMID: 26769963; PubMed  
512 Central PMCID: PMC4777828.
- 513 24. Mackenzie IR, Frick P, Grasser FA, Gendron TF, Petrucelli L, Cashman NR, et al.  
514 Quantitative analysis and clinico-pathological correlations of different dipeptide  
515 repeat protein pathologies in C9ORF72 mutation carriers. *Acta Neuropathol*.  
516 2015;130(6):845-61. Epub 2015/09/17. doi: 10.1007/s00401-015-1476-2. PubMed PMID:  
517 26374446.
- 518 25. Ramdzan YM, Trubetskov MM, Ormsby AR, Newcombe EA, Sui X, Tobin MJ, et al.  
519 Huntingtin Inclusions Trigger Cellular Quiescence, Deactivate Apoptosis, and

- 520           Lead to Delayed Necrosis. *Cell Rep.* 2017;19(5):919-27. Epub 2nd May 2017. doi:  
521           10.1016/j.celrep.2017.04.029. PubMed PMID: 28467905.
- 522   26.    Tsvetkov AS, Miller J, Arrasate M, Wong JS, Pleiss MA, Finkbeiner S. A small-  
523           molecule scaffold induces autophagy in primary neurons and protects against  
524           toxicity in a Huntington disease model. *Proc Natl Acad Sc USA.* 2010;107(39):16982-  
525           7. doi: 10.1073/pnas.1004498107.
- 526   27.    Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji:  
527           an open-source platform for biological-image analysis. *Nature Methods.*  
528           2012;9(7):676-82. doi: 10.1038/nmeth.2019.
- 529   28.    Abramoff MD, Magalhaes PJ, Ram SJ. Image Processing with ImageJ. *Biophotonics*  
530           International. 2004;11(7):36-42.
- 531   29.    Ramdzan YM, Polling S, Chia CPZ, Ng IHW, Ormsby AR, Croft NP, et al. Tracking  
532           protein aggregation and mislocalization in cells with flow cytometry. *Nature*  
533           Methods. 2012;9:467. doi: 10.1038/nmeth.1930
- 534   <https://www.nature.com/articles/nmeth.1930#supplementary-information>.
- 535   30.    Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A  
536           Software Environment for Integrated Models of Biomolecular Interaction  
537           Networks. *Genome Research.* 2003;13(11):2498-504.
- 538   31.    Szkarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, et al. The  
539           STRING database in 2017: quality-controlled protein–protein association networks,  
540           made broadly accessible. *Nucleic Acids Research.* 2016;45(D1):D362-D8. doi:  
541           10.1093/nar/gkw937.
- 542   32.    Rivals I, Personnaz L, Taing L, Potier M-C. Enrichment or depletion of a GO  
543           category within a class of genes: which test? *Bioinformatics.* 2006;23(4):401-7. doi:  
544           10.1093/bioinformatics/btl633.
- 545   33.    Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and  
546           Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society:*

- 547 Series B (Methodological). 1995;57(1):289-300. doi: 10.1111/j.2517-  
548 6161.1995.tb02031.x.
- 549 34. Dosztányi Z, Csizmok V, Tompa P, Simon I. IUPred: web server for the prediction  
550 of intrinsically unstructured regions of proteins based on estimated energy  
551 content. *Bioinformatics*. 2005;21(16):3433-4. doi: 10.1093/bioinformatics/bti541.
- 552 35. Kumar M, Thakur V, Raghava GPS. COPid: Composition Based Protein  
553 Identification. *In Silico Biology*. 2008;8(2):121-8.
- 554 36. Vizcaíno JA, Csordas A, del-Toro N, Dianas JA, Griss J, Lavidas I, et al. 2016 update  
555 of the PRIDE database and its related tools. *Nucleic Acids Research*.  
556 2015;44(D1):D447-D56. doi: 10.1093/nar/gkv1145.
- 557 37. Arrasate M, Mitra S, Schweitzer ES, Segal MR, Finkbeiner S. Inclusion body  
558 formation reduces levels of mutant huntingtin and the risk of neuronal death.  
559 *Nature*. 2004;431(7010):805-10. doi: 10.1038/nature02998. PubMed PMID: 15483602.
- 560 38. Ormsby AR, Ramdzan YM, Mok YF, Jovanoski KD, Hatters DM. A platform to view  
561 huntingtin exon 1 aggregation flux in the cell reveals divergent influences from  
562 chaperones hsp40 and hsp70. *J Biol Chem*. 2013;288(52):37192-203. doi:  
563 10.1074/jbc.M113.486944. PubMed PMID: 24196953; PubMed Central PMCID:  
564 PMC3873573.
- 565 39. Cruts M, Gijselinck I, Van Langenhove T, van der Zee J, Van Broeckhoven C.  
566 Current insights into the C9orf72 repeat expansion diseases of the FTLD/ALS  
567 spectrum. *Trends Neurosci*. 2013;36(8):450-9. Epub 2013/06/12. doi:  
568 10.1016/j.tins.2013.04.010. PubMed PMID: 23746459.
- 569 40. Neumann M, Sampathu DM, Kwong LK, Truax AC, Micsenyi MC, Chou TT, et al.  
570 Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic  
571 lateral sclerosis. *Science*. 2006;314(5796):130-3. Epub 2006/10/07. doi:  
572 10.1126/science.1134108. PubMed PMID: 17023659.



- 573 41. DeJesus-Hernandez M, Mackenzie IR, Boeve BF, Boxer AL, Baker M, Rutherford  
574 NJ, et al. Expanded GGGGCC hexanucleotide repeat in noncoding region of  
575 C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron*. 2011;72(2):245-56.  
576 Epub 2011/09/29. doi: 10.1016/j.neuron.2011.09.011. PubMed PMID: 21944778;  
577 PubMed Central PMCID: PMC3202986.
- 578 42. Renton AE, Majounie E, Waite A, Simon-Sanchez J, Rollinson S, Gibbs JR, et al. A  
579 hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-  
580 linked ALS-FTD. *Neuron*. 2011;72(2):257-68. Epub 2011/09/29. doi:  
581 10.1016/j.neuron.2011.09.010. PubMed PMID: 21944779; PubMed Central PMCID:  
582 PMC3200438.
- 583 43. Pikkarainen M, Hartikainen P, Alafuzoff I. Ubiquitinated p62-positive, TDP-43-  
584 negative inclusions in cerebellum in frontotemporal lobar degeneration with TAR  
585 DNA binding protein 43. *Neuropathology*. 2010;30(2):197-9. Epub 2009/07/23. doi:  
586 10.1111/j.1440-1789.2009.01043.x. PubMed PMID: 19622109.
- 587 44. King A, Al-Sarraj S, Shaw C. Frontotemporal lobar degeneration with ubiquitinated  
588 tau-negative inclusions and additional alpha-synuclein pathology but also unusual  
589 cerebellar ubiquitinated p62-positive, TDP-43-negative inclusions.  
590 *Neuropathology*. 2009;29(4):466-71. Epub 2008/08/22. doi: 10.1111/j.1440-  
591 1789.2008.00966.x. PubMed PMID: 18715271.
- 592 45. King A, Maekawa S, Bodi I, Troakes C, Al-Sarraj S. Ubiquitinated, p62  
593 immunopositive cerebellar cortical neuronal inclusions are evident across the  
594 spectrum of TDP-43 proteinopathies but are only rarely additionally  
595 immunopositive for phosphorylation-dependent TDP-43. *Neuropathology*.  
596 2011;31(3):239-49. Epub 2010/12/02. doi: 10.1111/j.1440-1789.2010.01171.x. PubMed  
597 PMID: 21118398.
- 598 46. Doi H, Okamura K, Bauer PO, Furukawa Y, Shimizu H, Kurosawa M, et al. RNA-  
599 binding protein TLS is a major nuclear aggregate-interacting protein in huntingtin

- 600 exon 1 with expanded polyglutamine-expressing cells. *J Biol Chem*.  
601 2008;283(10):6489-500. Epub 2008/01/03. doi: 10.1074/jbc.M705306200. PubMed PMID:  
602 18167354.
- 603 47. Wear MP, Kryndushkin D, O'Meally R, Sonnenberg JL, Cole RN, Shewmaker FP.  
604 Proteins with Intrinsically Disordered Domains Are Preferentially Recruited to  
605 Polyglutamine Aggregates. *PLoS One*. 2015;10(8):e0136362. Epub 2015/09/01. doi:  
606 10.1371/journal.pone.0136362. PubMed PMID: 26317359; PubMed Central PMCID:  
607 PMCPMC4552826.
- 608 48. Doi H, Koyano S, Suzuki Y, Nukina N, Kuroiwa Y. The RNA-binding protein  
609 FUS/TLS is a common aggregate-interacting protein in polyglutamine diseases.  
610 *Neurosci Res*. 2010;66(1):131-3. Epub 2009/10/17. doi: 10.1016/j.neures.2009.10.004.  
611 PubMed PMID: 19833157.
- 612 49. Ross CA, Poirier MA. What is the role of protein aggregation in  
613 neurodegeneration? *Nat Rev Mol Cell Biol*. 2005;6(11):891-8.
- 614 50. Woerner AC, Frottin F, Hornburg D, Feng LR, Meissner F, Patra M, et al.  
615 Cytoplasmic protein aggregates interfere with nucleocytoplasmic transport of  
616 protein and RNA. *Science*. 2016;351(6269):173-6. Epub 2015/12/05. doi:  
617 10.1126/science.aad2033. PubMed PMID: 26634439.
- 618 51. Boland B, Yu WH, Corti O, Mollereau B, Henriques A, Bezard E, et al. Promoting  
619 the clearance of neurotoxic proteins in neurodegenerative disorders of ageing.  
620 *Nat Rev Drug Discov*. 2018;17(9):660-88. Epub 2018/08/18. doi: 10.1038/nrd.2018.109.  
621 PubMed PMID: 30116051; PubMed Central PMCID: PMCPMC6456907.
- 622 52. Nussbacher JK, Tabet R, Yeo GW, Lagier-Tourenne C. Disruption of RNA  
623 Metabolism in Neurological Diseases and Emerging Therapeutic Interventions.  
624 *Neuron*. 2019;102(2):294-320. Epub 2019/04/19. doi: 10.1016/j.neuron.2019.03.014.  
625 PubMed PMID: 30998900; PubMed Central PMCID: PMCPMC6545120.

- 626 53. De Santis R, Santini L, Colantoni A, Peruzzi G, de Turris V, Alfano V, et al. FUS  
627 Mutant Human Motoneurons Display Altered Transcriptome and microRNA  
628 Pathways with Implications for ALS Pathogenesis. *Stem Cell Reports*.  
629 2017;9(5):1450-62. Epub 2017/10/11. doi: 10.1016/j.stemcr.2017.09.004. PubMed PMID:  
630 28988989; PubMed Central PMCID: PMC5830977.
- 631 54. Raman R, Allen SP, Goodall EF, Kramer S, Ponger LL, Heath PR, et al. Gene  
632 expression signatures in motor neurone disease fibroblasts reveal dysregulation  
633 of metabolism, hypoxia-response and RNA processing functions. *Neuropathol  
634 Appl Neurobiol*. 2015;41(2):201-26. Epub 2014/04/23. doi: 10.1111/nan.12147. PubMed  
635 PMID: 24750211; PubMed Central PMCID: PMC4329387.
- 636 55. Egawa N, Kitaoka S, Tsukita K, Naitoh M, Takahashi K, Yamamoto T, et al. Drug  
637 screening for ALS using patient-specific induced pluripotent stem cells. *Sci Transl  
638 Med*. 2012;4(145):145ra04. Epub 2012/08/03. doi: 10.1126/scitranslmed.3004052.  
639 PubMed PMID: 22855461.
- 640 56. Blasco H, Bernard-Marissal N, Vourc'h P, Guettard YO, Sunyach C, Augereau O, et  
641 al. A rare motor neuron deleterious missense mutation in the DPYSL3 (CRMP4)  
642 gene is associated with ALS. *Hum Mutat*. 2013;34(7):953-60. Epub 2013/04/10. doi:  
643 10.1002/humu.22329. PubMed PMID: 23568759.
- 644 57. Ramdzan YM, Polling S, Chia CP, Ng IH, Ormsby AR, Croft NP, et al. Tracking  
645 protein aggregation and mislocalization in cells with flow cytometry. *Nat Methods*.  
646 2012;9(5):467-70. Epub 2012/03/20. doi: 10.1038/nmeth.1930. PubMed PMID:  
647 22426490.
- 648 58. Sui X, Pires DEV, Ormsby AR, Cox D, Nie S, Vecchi G, et al. Widespread  
649 remodeling of proteome solubility in response to different protein homeostasis  
650 stresses. *Proc Natl Acad Sci U S A*. 2020. Epub 2020/01/23. doi:  
651 10.1073/pnas.1912897117. PubMed PMID: 31964829.

- 652 59. Yang H, Yue HW, He WT, Hong JY, Jiang LL, Hu HY. PolyQ-expanded huntingtin  
653 and ataxin-3 sequester ubiquitin adaptors hHR23B and UBQLN2 into aggregates  
654 via conjugated ubiquitin. *FASEB J.* 2018;32(6):2923-33. Epub 2018/02/07. doi:  
655 10.1096/fj.201700801RR. PubMed PMID: 29401586.
- 656 60. Renaud L, Picher-Martel V, Codron P, Julien JP. Key role of UBQLN2 in  
657 pathogenesis of amyotrophic lateral sclerosis and frontotemporal dementia. *Acta*  
658 *Neuropathol Commun.* 2019;7(1):103. Epub 2019/07/20. doi: 10.1186/s40478-019-0758-  
659 7. PubMed PMID: 31319884; PubMed Central PMCID: PMC6889556.
- 660 61. Deng HX, Chen W, Hong ST, Boycott KM, Gorrie GH, Siddique N, et al. Mutations  
661 in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and  
662 ALS/dementia. *Nature.* 2011;477(7363):211-5. Epub 2011/08/23. doi:  
663 10.1038/nature10353. PubMed PMID: 21857683; PubMed Central PMCID:  
664 PMCPMC3169705.
- 665 62. Harold D, Abraham R, Hollingworth P, Sims R, Gerrish A, Hamshere ML, et al.  
666 Genome-wide association study identifies variants at CLU and PICALM associated  
667 with Alzheimer's disease. *Nat Genet.* 2009;41(10):1088-93. Epub 2009/09/08. doi:  
668 10.1038/ng.440. PubMed PMID: 19734902; PubMed Central PMCID:  
669 PMCPMC2845877.
- 670 63. Elias-Sonnenschein LS, Bertram L, Visser PJ. Relationship between genetic risk  
671 factors and markers for Alzheimer's disease pathology. *Biomark Med.*  
672 2012;6(4):477-95. Epub 2012/08/25. doi: 10.2217/bmm.12.56. PubMed PMID: 22917148.
- 673 64. Xiao Q, Gil SC, Yan P, Wang Y, Han S, Gonzales E, et al. Role of  
674 phosphatidylinositol clathrin assembly lymphoid-myeloid leukemia (PICALM) in  
675 intracellular amyloid precursor protein (APP) processing and amyloid plaque  
676 pathogenesis. *J Biol Chem.* 2012;287(25):21279-89. Epub 2012/04/28. doi:  
677 10.1074/jbc.M111.338376. PubMed PMID: 22539346; PubMed Central PMCID:  
678 PMCPMC3375549.

- 679 65. Moreau K, Fleming A, Imarisio S, Lopez Ramirez A, Mercer JL, Jimenez-Sanchez M,  
680 et al. PICALM modulates autophagy activity and tau accumulation. Nat Commun.  
681 2014;5:4998. Epub 2014/09/23. doi: 10.1038/ncomms5998. PubMed PMID: 25241929;  
682 PubMed Central PMCID: PMC4199285.
- 683
- 684

685 **SUPPORTING INFORMATION CAPTIONS**

686 **Table S1. List of random proteins from mouse Uniprot database.**

687 **Table S2. Proteins enriched in inclusions of polyGA<sub>101</sub> and Httex1Q<sub>97</sub>. Relates to Table 1 &**  
688 **Fig 2.**

689 **Table S3. Gene Ontology terms enriched among proteins identified in polyGA<sub>101</sub> or**  
690 **Httex1Q<sub>97</sub> inclusions. Relates to Fig 2.**

691 **Table S4. Cellular abundances of proteins caused by polyGA<sub>101</sub> aggregation. Relates to Fig 3.**

692 **Table S5. Gene Ontology terms enriched among proteins that changed abundance upon**  
693 **polyGA<sub>101</sub> aggregation. Relates to Fig 3.**

694

**Table 1: Proteins enriched in inclusions of polyGA<sub>101</sub> and Httex1Q<sub>97</sub>\***

Enriched in polyGA <sub>101</sub>			Enriched in Httex1Q <sub>97</sub>		
Description	Gene ID	<i>log2</i>	Description	Gene ID	<i>log2</i>
		<i>enrichment</i>			<i>enrichment</i>
		(mean SD)			(mean SD)
Pleckstrin homology-like domain family A member 1	Phlda1	3.91 ±1.18	Hsc70-interacting protein	St13	4.78 ±0.36
DNA replication licensing factor MCM3	Mcm3	3.73 ±10.23	Histone H3.1	Hist1h3 a	4.3 ±1.28
Eukaryotic translation initiation factor 2 subunit 1	Eif2s1	3.65 ±5.69	<i>Clathrin interactor 1</i>	<i>Clint1</i>	4.28 0.91
Proteasome subunit beta type-4	Psmb4	2.6 ±2.39	Coiled-coil-helix-coiled-coil-helix domain-containing protein 2	Chchd2	4.02 ±0.73
<b>Sequestosome-1 (p62)</b>	Sqstm1	2.47 ±0.11	<b>RNA-binding protein FUS</b>	<b>Fus</b>	<b>3.26 ±1.23</b>
Interferon-inducible double-stranded RNA-dependent protein kinase activator A	Prkra	2.4 ±2.63	Tight junction protein ZO-1	Tjp1	3.25 ±0.93
Sorting nexin-3	Snx3	2.36 ±1.05	Ubiquitin-associated protein 2	Ubp2	3.24 ±0.36



Electron transfer flavoprotein subunit alpha, mitochondrial	Etfa	2.24 ±2.41	Small glutamine-rich tetratricopeptide repeat-containing protein alpha	Sgta	3.22 ±0.8
Cytochrome c oxidase subunit 5B, mitochondrial	Cox5b	2.19 ±2.64	<i>Dnal homolog subfamily B member 1</i>	<i>Dnajb1</i>	3.11 1.11
Nuclear migration protein nudC	Nudc	2.17 ±1.67	Chromobox protein homolog 1	Cbx1	3.1 ±0.36
Receptor of activated protein C kinase 1	Rack1	2.17 ±0.91	<b><i>Ubiquilin-2</i></b>	<b><i>Ubqln2</i></b>	<b>3.06 ±0.77</b>
40S ribosomal protein S2	Rps2	2.12 ±1.85	<b><i>Phosphatidylinositol-binding clathrin assembly protein</i></b>	<b><i>Picalm</i></b>	<b>2.81 ±0.25</b>
Nuclear fragile X mental retardation-interacting protein 2	Nufip2	2.11 ±1.41	CUGBP Elav-like family member 1	Celf1	2.51 ±0.37
26S proteasome non-ATPase regulatory subunit 12	Psm12	2.09 ±2.28	Transgelin-2	Tagln2	2.17 ±0.49
Vigilin	Hdlbp	2.07 ±0.36	RNA-binding protein 25	Rbm25	2.16 ±0.51
Insulin-like growth factor 2 mRNA-binding protein 3	Igf2bp3	2.06 ±2.16	Nucleolysin TIAR	Tial1	2.06 ±0.19
GTP cyclohydrolase 1	Gch1	2.04 ±2.12	Caprin-1	Caprin1	2.04 ±0.43
60S ribosomal protein L10	Rpl10	1.99 ±1.03	Probable ATP-dependent RNA helicase DDX17	Ddx17	2.02 ±0.18
ATPase WRNIP1	Wrnip1	1.98 ±2.39	Protein PRRC2C	Prrc2c	2.01 ±0.48
Protein SOGA3	Soga3	1.96 ±0.27	RNA-binding motif, single-stranded-interacting protein	Rbms1	1.98 ±0.13

Ubiquitin fusion degradation protein 1 homolog	Ufd1l	1.95 ±1.62	1	Ankyrin repeat domain-containing protein 17	Ankrd17	1.95 ±0.26
Proteasome subunit alpha type-6	Psmα6	1.93 ±1.54		Pre-mRNA-processing factor 40 homolog A	Prpf40a	1.82 ±0.23
26S proteasome non-ATPase regulatory subunit 3	Psmδ3	1.91 ±2.13		Dnaj homolog subfamily C member 9	Dnajc9	1.78 ±0.32
40S ribosomal protein S27	Rps27	1.91 ±0.84		<i>Hepatocyte growth factor-regulated tyrosine kinase substrate</i>	<i>Hgs</i>	<i>1.73 0.25</i>
Adenine phosphoribosyltransferase	Aprt	1.84 ±1.62		Ubiquitin-associated protein 2-like	Ubap2l	1.65 ±0.2
COP9 signalosome complex subunit 7a	Cops7a	1.83 ±1.14		Nuclear pore complex protein Nup214	Nup214	1.65 ±0.17
Cytochrome c oxidase subunit NDUF4A	Ndufa4	1.82 ±0.68		Poly [ADP-ribose] polymerase 1	Parp1	1.6 ±0.47
Interferon-induced protein with tetratricopeptide repeats 1	Ifit1	1.76 ±1.24		Calponin-3	Cnn3	1.6 ±0.32
Proteasome subunit beta type-5	Psmβ5	1.74 ±0.84		Dnaj homolog subfamily A member 2	Dnaja2	1.58 ±0.63
60S ribosomal protein L23	Rpl23	1.73 ±0.47		Serine/arginine repetitive matrix protein 2	Srrm2	1.55 ±0.6
E3 ubiquitin-protein ligase TRIM32	Trim32	1.72 ±1		Muscleblind-like protein 2	Mbnl2	1.54 ±0.34
T-complex protein 1 subunit eta	Cct7	1.72 ±0.86		Protein phosphatase 1 regulatory subunit 12A	Ppp1r12a	1.5 ±0.44

ZW10 interactor	Zwint	1.68 ±0.74	Poly(rC)-binding protein 1	Pcbp1	1.41 ±0.33
Cyclin-dependent kinase 1	Cdk1	1.59 ±0.65	<b>TAR DNA-binding protein 43</b>	<b>Tardbp</b>	<b>1.41 ±0.15</b>
ATP-dependent 6-phosphofructokinase, platelet type	Pfkp	1.57 ±0.67	Poly(rC)-binding protein 3	Pcbp3	1.35 ±0.16
Nuclear protein localization protein 4 homolog	Nploc4	1.54 ±1.38	5'-3' exoribonuclease 2	Xrn2	1.32 ±0.36
Large proline-rich protein BAG6	Bag6	1.48 ±0.66	Heterogeneous nuclear ribonucleoprotein F	Hnrnpf	1.29 ±0.43
26S proteasome non-ATPase regulatory subunit 14	Psm14	1.48 ±0.56	Lamina-associated polypeptide 2, isoforms alpha/zeta	Tmpo	1.22 ±0.46
Malate dehydrogenase, cytoplasmic	Mdh1	1.48 ±0.43	Pumilio homolog 1	Pum1	1.22 ±0.07
ADP-sugar pyrophosphatase	Nudt5	1.47 ±0.21	Tropomodulin-3	Tmod3	1.21 ±0.37
26S protease regulatory subunit 6A	Psmc3	1.42 ±0.87	14-3-3 protein beta/alpha	Ywhab	1.15 ±0.43
Bifunctional glutamate/proline--tRNA ligase	Eprs	1.39 ±0.81	Plectin	Plec	0.97 ±0.2
Aminoacyl tRNA synthase complex-interacting multifunctional protein 1	Aimp1	1.3 ±0.91	Small ubiquitin-related modifier 1	Sumo1	0.83 ±0.22
Ribosome-binding protein 1	Rrbp1	1.26 ±0.71	Regulator of nonsense transcripts 1	Upf1	0.82 ±0.21
40S ribosomal protein S27-like	Rps27l	1.23 ±0.68	Vimentin	Vim	0.72 ±0.13

Ras GTPase-activating protein-binding protein 1	G3bp1	1.18 ±0.66	Nuclear pore complex protein Nup98-Nup96	Nup98	0.68 ±0.15
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	1.15 ±0.48	Importin subunit alpha-1	Kpna2	0.58 ±0.1
Dihydropyrimidinase-related protein 2	Dpysl2	1.09 ±0.45	Protein C10	Grcc10	0.56 ±0.01
Ataxin-10	Atxn10	1.05 ±0.29			
<b>Dihydropyrimidinase-related protein 3</b>	<b>Dpysl3</b>	<b>1.05 ±0.08</b>			
ATP-binding cassette sub-family E member 1	Abce1	1.04 ±0.34			
60S ribosomal protein L38	Rpl38	1.03 ±0.39			
Green fluorescent protein	GFP	1.01 ±0.4			
Multifunctional protein ADE2	Paics	0.98 ±0.35			
Polymerase delta-interacting protein 3	Poldip3	0.97 ±0.15			
Melanoma-associated antigen D1	Maged1	0.94 ±0.31			
Dihydropyrimidinase-related protein 1	Crmp1	0.92 ±0.19			
60S acidic ribosomal protein P0	Rplp0	0.89 ±0.35			
ADP/ATP translocase 2	Slc25a5	0.89 ±0.17			
T-complex protein 1 subunit beta	Cct2	0.88 ±0.22			
Eukaryotic translation initiation factor 3 subunit D	Eif3d	0.87 ±0.32			

T-complex protein 1 subunit delta	Cct4	0.84 ±0.27
Tubulin beta-5 chain	Tubb5	0.79 ±0.14
26S protease regulatory subunit 10B	Psmc6	0.76 ±0.15
Golgi-associated plant pathogenesis-related protein 1	Glipr2	0.75 ±0.22
IgE-binding protein	Iap	0.75 ±0.22
Cell division control protein 42 homolog	Cdc42	0.71 ±0.15
Non-POU domain-containing octamer-binding protein	Nono	0.67 ±0.1
Aspartate aminotransferase, cytoplasmic	Got1	0.45 ±0.05

\* Only proteins that meet significance cut-off (hyperbolic curves, permutation-based  $FDR \leq 0.05$ ,  $S_0 = 0.1$ ). Full table of proteins are shown in Table S2; **Bold** are genes with known causes or risk factors for MND (or other neurodegenerative diseases in the case of Picalm); *Italics* are cellular proteins previously seen to become more insoluble when Httex1Q<sub>97</sub> formed inclusions [58]



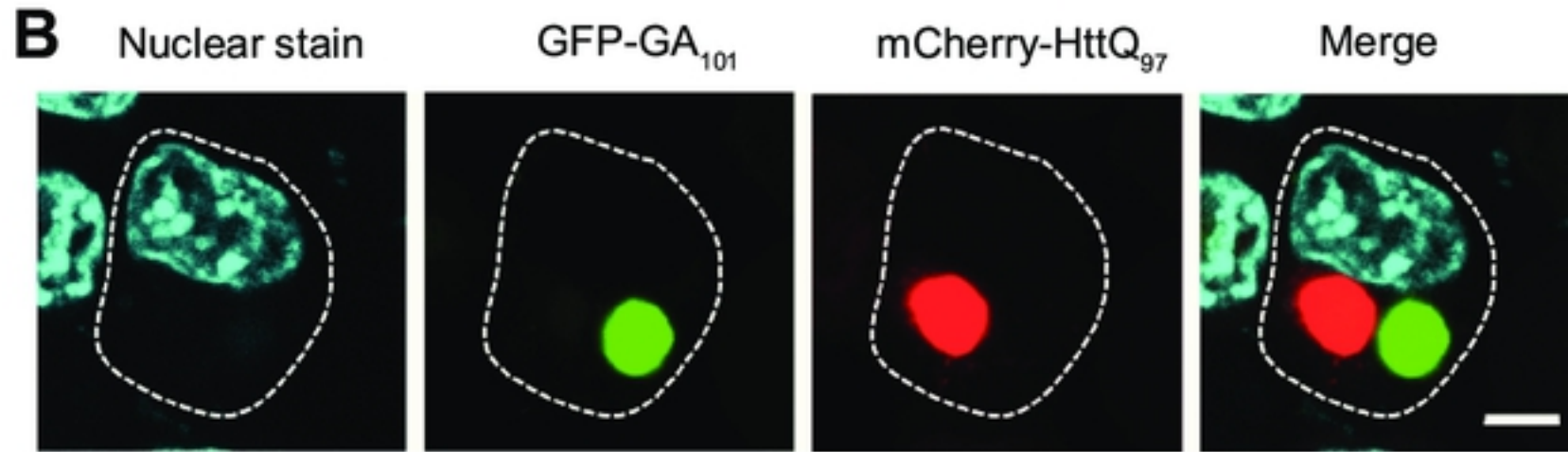
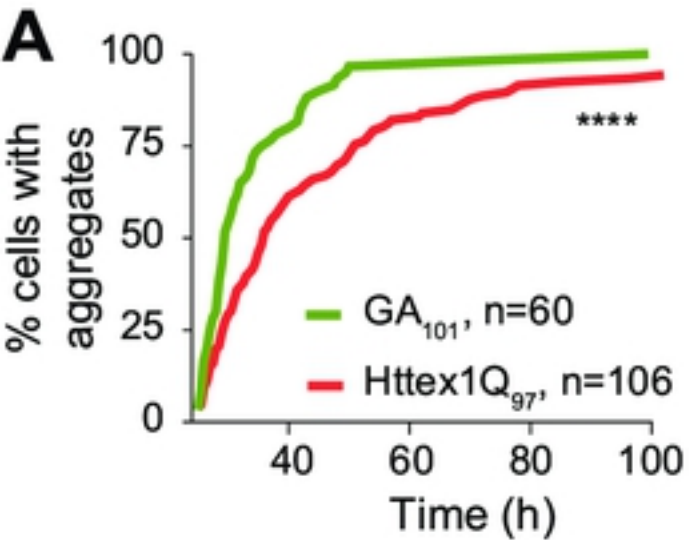


Figure 1



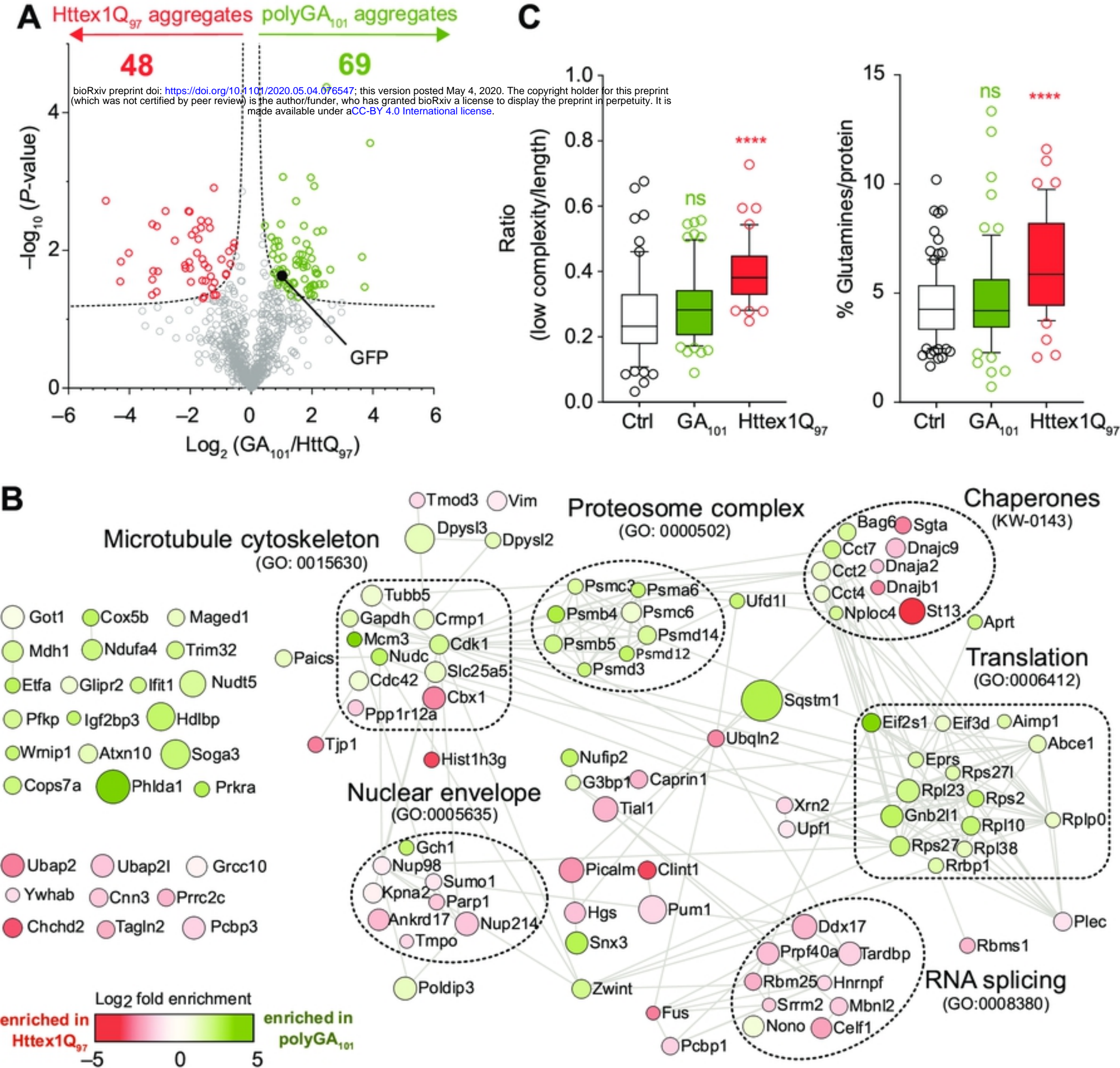


Figure 2



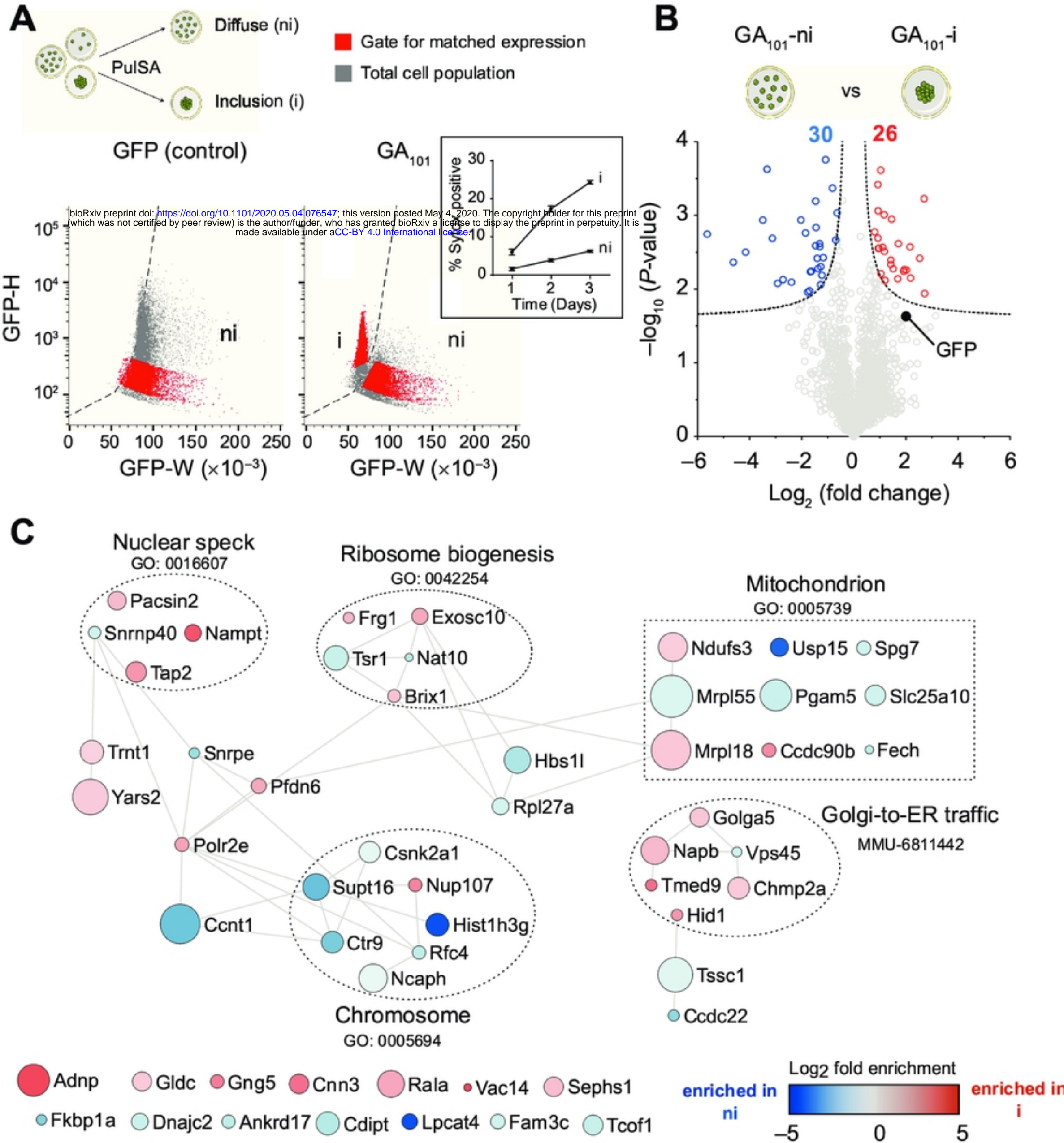


Figure 3