Cytosolic PrP can participate in prion-mediated toxicity

Alana M. Thackray, Chang Zhang, Tina Arndt, and Raymond Bujdoso#

University of Cambridge, Department of Veterinary Medicine,
Madingley Road, Cambridge, CB3 OES, UK

#Corresponding author

(Tel: 44-1223-337655; Fax 44-1223-337610; email: rb202@cam.ac.uk)

Running title: Cytosolic PrP transgenic *Drosophila*

Word count: Abstract; 231, Main text; 5,693

32 **Abstract**

33

34 Prion diseases are characterized by a conformational change in the normal host protein
35 PrPC. While the majority of mature PrPC is tethered to the plasma membrane by a
36 glycosylphosphatidyl-inositol anchor, topological variants of this protein can arise during its
37 biosynthesis. Here we have generated Drosophila transgenic for cytosolic ovine PrP in order
38 to investigate its toxic potential in the fly in the absence and presence of exogenous ovine
39 prions. While cytosolic ovine PrP expressed in Drosophila was predominantly detergent
40 insoluble and showed resistance to low concentrations of Proteinase K, it was not overtly
41 detrimental to the fly. However, Drosophila transgenic for cytosolic PrP expression exposed
42 to classical or atypical scrapie prion inocula showed an accelerated decrease in locomotor
43 activity compared to similar flies exposed to scrapie-free material. The susceptibility to
44 classical scrapie inocula could be assessed in Drosophila transgenic for pan neuronal
45 expression of cytosolic PrP whereas susceptibility to atypical scrapie required ubiquitous PrP
46 expression. Significantly, the toxic phenotype induced by ovine scrapie in cytosolic PrP
47 transgenic Drosophila was transmissible to recipient PrP transgenic flies. These data show
48 that while cytosolic PrP expression does not adversely affect Drosophila, this topological PrP
49 variant can participate in the generation of transmissible scrapie-induced toxicity. These
50 observations also show that PrP transgenic Drosophila are susceptible to classical and
51 atypical scrapie prion strains and highlight the utility of this invertebrate host to model
52 mammalian prion disease.

53

54

54 **Importance**
55
56 During prion diseases, the host protein PrPC converts into an abnormal conformer PrPSc, a
57 process coupled to the generation of transmissible prions and neurotoxicity. While PrPC is
58 principally a glycosylphosphatidyl-inositol-anchored membrane protein, the role of topological
59 variants, such as cytosolic PrP, in prion-mediated toxicity and prion formation is undefined.
60 Here we have generated *Drosophila* transgenic for cytosolic PrP expression in order to
61 investigate its toxic potential in the absence and presence of exogenous prions. Cytosolic
62 ovine PrP expressed in *Drosophila* was not overtly detrimental to the fly. However, cytosolic
63 PrP transgenic *Drosophila* exposed to ovine scrapie showed a toxic phenotype absent in
64 similar flies exposed to scrapie-free material. Significantly, the scrapie-induced toxic
65 phenotype in cytosolic transgenic *Drosophila* was transmissible to recipient PrP transgenic
66 flies. These data show that cytosolic PrP can participate in the generation of transmissible
67 prion-induced toxicity and highlight the utility of *Drosophila* to model mammalian prion
68 disease.
69
70
71
72
73

73 **Introduction**

74

75 Prion diseases, or transmissible spongiform encephalopathies (TSEs) are fatal
76 neurodegenerative disorders of humans and various other mammalian species (1). These
77 conditions include scrapie of sheep, Bovine Spongiform Encephalopathy (BSE) of cattle,
78 Creutzfeldt–Jakob disease (CJD) of humans and Chronic Wasting Disease (CWD) of cervids.
79 Susceptibility to prion disease requires expression of the host-encoded protein PrPC (2-5).
80 Furthermore, prion diseases are associated with conversion of PrPC, the normal form of the
81 prion protein, into an abnormal conformer PrPSc in a template-directed manner (6, 7).
82 Misfolding of PrP is associated with an increase in β-sheet content of the protein, which
83 accumulates principally in the central nervous system of affected individuals. There is now
84 considerable evidence to suggest that the transmissible prion agent comprises PrPSc (8-14).
85 However, despite intensive investigation, the molecular mechanisms of PrPC to PrPSc
86 conversion and of prion-mediated neurodegeneration remain unknown.

87

88 Although PrPC is highly conserved amongst different mammalian species its physiological
89 functions remain elusive. PrPC is a glycosylphosphatidyl-inositol (GPI)-anchored
90 sialoglycoprotein, principally located in lipid rafts on the outer leaf of the cell membrane (15,
91 16). Nascent PrPC is synthesized as a pre-protein of approximately 250 amino acid residues
92 in length. The N-terminal leader peptide is cleaved as PrP enters the endoplasmic reticulum
93 (ER) and the C-terminal signal sequence is cleaved upon attachment of the GPI anchor that
94 holds the protein to the membrane (15, 17, 18). Inside the ER lumen PrPC undergoes
95 additional post translational modification with the addition of carbohydrate structures at two
96 asparagine residues (19). In addition, a di-sulfide bond forms within the C-terminal globular
97 domain (20). During its biosynthesis, PrP may undergo aberrant translocation since leader
98 peptide inefficiency prevents all of the nascent protein entering into the lumen of the ER. As a
99 consequence, subpopulations of PrP are either retained fully in the cytosol (PrPcyt) or
100 produced as a membrane-bound protein with either N- or C-terminal residues exposed to the
101 cytosol (21-24). ER misfolded and aberrantly translocated proteins are targeted for
102 degradation by the ubiquitin proteasome system (UPS), the major cellular proteolytic
103 pathway, or via the autophagic / lysosomal system. While normal levels of cytosolic and
104 aberrantly translocated PrP are usually metabolized by the cell, these forms of PrP have
105 been reported to be neurotoxic when present in elevated amounts (25-28). Increased cellular
106 levels of cytosolic PrP may arise as a consequence of PrPSc-mediated inhibition of the

107 catalytic activity of the proteasome in cells (29-31). The role of cytosolic PrP in the generation
108 of infectious prions has yet to be determined.
109
110 Ovine scrapie is an important model of prion disease, not only for the natural host but for
111 mammalian species in general (32, 33). Polymorphisms within ovine PrPC correlate with
112 susceptibility to different types of scrapie in sheep. Four major polymorphisms in the ovine
113 prion protein, located at amino acid residues 136, 141, 154 and 171 are associated, in some
114 cases relatively (34, 35), with susceptibility to two classifications of scrapie disease (36-38).
115 Sheep that express $A^{136}L^{141}R^{154}Q^{171}$ (termed ARQ, where A, L, R and Q stand for alanine,
116 leucine, arginine and glutamine, respectively) or $V^{136}L^{141}R^{154}Q^{171}$ (termed VRQ, where V
117 stands for valine) ovine PrP are susceptible to classical scrapie, a transmissible prion
118 disease within the natural host (39). In contrast, a different ovine prion disease, referred to as
119 atypical or Nor98 scrapie, has been reported in classical scrapie-resistant PrP genotypes
120 including $A^{136}L^{141}R^{154}R^{171}$ (termed ARR), $A^{136}F^{141}R^{154}Q^{171}$ (termed AFRQ, where F stands for
121 phenylalanine) and $A^{136}L^{141}H^{154}Q^{171}$ (termed AHQ, where H stands for histidine) (38). It is
122 considered that atypical scrapie is a spontaneous disorder of PrP folding and/or metabolism
123 (38, 40), although transmission by the oral route cannot yet be excluded (41-44). We have
124 begun to model sheep scrapie in *Drosophila* in order to develop a more tractable model of
125 mammalian prion disease. In doing so, we have previously generated *Drosophila* transgenic
126 for polymorphic variants of ovine PrP expressed with a GPI anchor sequence [PrP(GPI)]
127 (45). Furthermore, we have shown that *Drosophila* transgenic for AHQ(GPI) ovine PrP show
128 a significant reduction in median survival time compared to flies transgenic for VRQ(GPI). It
129 has yet to be established whether the toxic potential of AHQ prion protein is mediated by a
130 cytosolic variant of this particular genotype of ovine PrP and whether cytosolic PrP *per se*
131 can participate in prion-mediated toxicity.
132
133 Here we have generated *Drosophila* transgenic for polymorphic variants of cytosolic ovine
134 PrP in order to investigate for the first time its toxic potential in the fly in the absence and
135 presence of exogenous ovine prions. While cytosolic ovine PrP expressed pan neuronally in
136 *Drosophila* was predominantly detergent insoluble and showed protease resistance to low
137 concentrations of Proteinase K (PK), it was not overtly detrimental to the fly. In contrast,
138 *Drosophila* transgenic for cytosolic PrP expression exposed to classical or atypical scrapie
139 prion inocula showed an accelerated decrease in locomotor activity compared to similar flies
140 exposed to scrapie-free material. The susceptibility to classical ovine scrapie was evident in
141 *Drosophila* transgenic for pan neuronal cytosolic PrP whereas susceptibility to atypical ovine

142 scrapie required ubiquitous expression. Significantly, the toxic phenotype induced by ovine
143 scrapie in cytosolic transgenic *Drosophila* was transmissible to PrP transgenic recipient flies.
144 These data show that while cytosolic ovine PrP is not inherently neurotoxic in *Drosophila*,
145 this topological variant can participate in the generation of a transmissible toxicity induced by
146 scrapie prion inocula. These novel observations highlight the utility of *Drosophila* to model
147 mammalian prion disease.
148
149

149 **Materials and Methods**

150

151 **Fly stocks and generation of cytosolic ovine PrP transgenic *Drosophila***

152 The UAS-PrP(GPI) fly lines w; M{AHQ-PrP(GPI), 3xP3-RFP.attP}ZH-51D and w; M{ARQ-
153 PrP(GPI), 3xP3-RFP.attP}ZH-51D, that are transgenic for ovine $A^{136}H^{154}Q^{171}$ or $A^{136}R^{154}Q^{171}$
154 PrP, respectively, expressed with an N-terminal leader peptide and C-terminal
155 glycosylphosphatidyl-inositol (GPI) signal sequence [AHQ(GPI) and ARQ(GPI), respectively]
156 were generated by PhiC31 site-specific transformation as previously described (45). The
157 UAS-PrP(cyt) fly lines generated here were w; M{AHQ-PrP, 3xP3-RFP.attP}ZH-51D, w;
158 M{ARQ-PrP, 3xP3-RFP.attP}ZH-51D, and w; M{VRQ-PrP, 3xP3-RFP.attP}ZH-51D. The
159 ovine PrP(cyt) transgenes for insertion into the *Drosophila* genome were prepared by PCR
160 that generated a DNA fragment encoding ovine PrP amino acid residues 25-232. PCR was
161 carried out in the presence of Pfu DNA polymerase (Promega) using substrate plasmid DNA
162 that contained an insert encoding AHQ, ARQ or VRQ ovine PrP amino acid residues 25-252
163 (45) and oligonucleotide primers P2 (forward primer): 5' GATGA GAA TTC AAC ATG AAG
164 AAG CGA CCA AAA CCT GGC 3'; and P4 (reverse primer): 5' ACGATGAA CTC GAG CTA
165 CCC CCT TTG GTA ATA AG 3'. The PCR primers P2 and P4 contained *EcoR1* and *Xho1*
166 restriction sites, respectively, that allowed directional cloning of the 658bp PCR product into
167 the *Drosophila* transgenesis vector pUASTattB. A Kozak translation site (46) was
168 incorporated into the forward primer and a stop codon was incorporated into the reverse
169 primer ahead of the *Xho1* restriction site. The PCR reaction conditions comprised an initial
170 denaturation at 95 °C for 2 minutes followed by 30 cycles of denaturation at 95 °C for 30
171 seconds, primer annealing at 55 °C for 30 seconds and primer extension at 75 °C for 1
172 minute, and a final extension of the PCR product at 75 °C for 10 minutes. PCR products that
173 contained DNA encoding PrP(cyt) DNA were subsequently ligated into pUASTattB and
174 rescued by transformation in DH5α bacteria. Plasmid DNA was isolated from transformed
175 bacteria by an alkaline lysis method using the Qiagen maxiprep kit and the PrP construct
176 insert verified by DNA sequence analysis. Site-specific transformation of the pUASTattB-PrP
177 constructs into the 51D fly line (y[1] M{vas-int.Dm}ZH-2A w[*]; M{3xP3-RFP.attP}ZH-51D)
178 was performed by Bestgene Inc (California, USA). F1 flies were balanced and viable lines
179 were maintained as balanced stocks by conventional fly crosses. DNA sequence analysis
180 was performed on genomic DNA from each balanced fly line to confirm the presence of the
181 correct PrP transgene at the 51D site. *Cre*-mediated removal of RFP from the fly genome of
182 VRQ(cyt) PrP was performed by conventional fly crosses. *Elav-GAL4*
183 (P{w[+mW.hs]=GawB}elav[C155]) and *GMR-GAL4* (w; wg[Sp-1]/CyO; GMR-GAL4,

184 w+/TM6B) driver lines, and the control 51D (w; M{3xP3-RFP.attP}ZH-51D) fly line were
185 obtained from the Department of Genetics, University of Cambridge, UK. All fly lines were
186 raised on standard cornmeal media (47) at 25 °C and maintained at low to medium density.
187 Flies were used in the assays described below or harvested at various time points and then
188 frozen at -80 ˚C until required.
189

190 **Prion inoculation of *Drosophila***

191 *Primary passage of sheep scrapie (sheep-to-fly)*: *Drosophila* at the larval stage of
192 development were exposed to brain homogenates from confirmed scrapie-positive or known
193 scrapie-negative sheep. The classical scrapie-infected isolates were prepared from terminal
194 scrapie-affected sheep identified by routine statutory surveillance (VRQ/VRQ isolate
195 SE1848/0005; ARQ/ARQ isolate SE1848/0008) (50). The atypical scrapie-infected isolates
196 (n=2) were prepared from terminal AHQ/AHQ sheep challenged intracerebrally with atypical
197 scrapie and that were confirmed positive for the disease (43). New Zealand-derived
198 VRQ/VRQ (n=1), ARQ/ARQ (n=1) or AHQ/AHQ (n=2) scrapie-free brain tissue was used as
199 control material. Two hundred and fifty microlitres of a 1 % brain homogenate prepared in
200 PBS pH 7.4 were added to the top of the cornmeal that contained third instar *Drosophila*
201 larvae in 3” plastic vials. Flies were transferred to fresh, non-treated vials following eclosion.
202 *Secondary passage of sheep scrapie (fly-to-fly): Drosophila* brain homogenates were
203 prepared from 30 day old flies that had been exposed at the larval stage to scrapie-positive
204 or scrapie-negative sheep brain material. Two hundred and fifty microlitres of a 10 % dilution
205 (v/v) of the original fly brain homogenate were added to the top of the cornmeal that
206 contained third instar *Drosophila* larvae in 3” plastic vials. Flies were transferred to fresh,
207 non-treated vials following eclosion.
208

209 **Preparation of fly head homogenates**

210 Whole flies in an eppendorf tube were frozen in liquid nitrogen for 10 minutes followed by 2
211 minutes of vortexing. Individual fly heads were then isolated and placed in clean eppendorf
212 tubes using a paint brush. Homogenates were prepared by manual grinding of fly heads in
213 eppendorf tubes with sterilized plastic pestles. Homogenates for ELISA or western blot
214 without Proteinase K (PK) digestion of PrP were prepared by processing 20 fly heads in 20 μl
215 of lysis buffer [50 mM Tris pH 7.5, 100 mM NaCl, 0.5 % (v/v) Nonidet P-40 and 1 mM 4-(2-
216 Aminoethyl) benzenesulfonyl fluoride (AEBSF)]. In the case of PK digestion of PrP, AEBSF
217 was not added to the lysis buffer. Homogenates for conformational-dependent immunoassay
218 (CDI) were prepared by processing 40 fly heads in 8 μl of 8M GdnHCl, incubated at 18 ˚C for

219 15 minutes followed by a 1:50 dilution in assay buffer and assessed as described previously
220 (48). Homogenates for fly-to-fly transmission (secondary passage samples) were prepared
221 by processing 150 male and 150 female fly heads per group previously harvested at 30 days
222 of age. Each group of 300 fly heads was added to 300 μl of PBS (pH 7.4) prior to
223 homogenization.
224

225 **Preparation of soluble and insoluble prion protein fractions**

226 PrP fractions were prepared from fly head homogenates using a method adapted from
227 Fernandez-Funez et al. (49). A volume of fly head homogenate that was equivalent to 20 fly
228 heads was mixed with 20 μl of 10 % (w/v) Sarkosyl pH 7.4. The sample was shaken at 225
229 rpm for 10 minutes at 37 °C, 5 units of Benzonase were added and the sample was shaken
230 at 225 rpm for a further 10 minutes at 37 °C. Sodium phosphotungstic acid (diluted in PBS
231 pH 7.4) was added to the reaction mix to give 0.3 % w/v final concentration and the tubes
232 were shaken at 225 rpm for 30 minutes at 37 °C prior to centrifugation at 16,000 X g for 30
233 minutes at 4 °C. To obtain the soluble and insoluble PrP fractions, the supernatant (soluble
234 fraction, 40 μl) was transferred to a fresh tube and the pellet (insoluble fraction) was
235 resuspended in 40 μl of 0.1 % w/v Sarkosyl in PBS pH 7.4.
236

237 **Proteinase K digestion of fly head homogenate**

238 Fly head homogenates were prepared in 1.5 ml eppendorf tubes by processing 10 fly heads
239 in 9 μl of lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 0.5 % Nonidet P-40) using plastic
240 pestles. A 1 μl volume of PK at x10 the required concentration was added to the homogenate
241 and the mixture incubated at 37 ºC for 15 minutes. Proteolysis was stopped by the addition of
242 1.1 μl of 10 mM AEBSF and the samples analyzed by SDS-PAGE and western blot to detect
243 PrP.
244

245 **SDS-PAGE and western blot**

246 Fly head homogenate was mixed with an equal volume of x2-strength Laemmli loading
247 buffer, boiled for 10 minutes, cooled on ice and then centrifuged at 13,000 X g for 5 minutes
248 at 18 °C to remove debris. Fly head homogenate was subjected to SDS/PAGE run under
249 reducing conditions and western blot as described in detail previously (50) except that the
250 nitrocellulose membranes were probed with a 1:2000 dilution of anti-PrP monoclonal
251 antibody Sha31 (51).
252

253 **Capture-detector ELISA**

254 Duplicate 40 μl aliquots of fly head homogenate were diluted to 100 μl with PBS pH 7.4. PrP
255 was quantified by capture-detector ELISA carried out as described previously (52) except
256 that the capture reagent was anti-PrP monoclonal antibody 245 (53) and the detector
257 antibody was biotinylated SAF32 (51). The equivalent of 10 fly heads were assayed per well
258 in duplicate.
259

260 **Conformational-dependent immunoassay (CDI)**

261 Head homogenate was prepared as described above and PrP was quantified by CDI as
262 described previously (48) except that the capture reagent was anti-PrP monoclonal antibody
263 245 (53) and the detector antibody was biotinylated SAF32 (51). The equivalent of 20 fly
264 heads were assayed per well in duplicate.
265

266 **Survival assay**

267 Newly eclosed flies were allowed to mature and mate for 24 hours before the females were
268 separated and collected for survival assays. One hundred flies of each genotype were
269 housed in groups of 10 and the flies were flipped every 2 to 3 days onto fresh food. The
270 number of dead flies was recorded three times a week (45). Survival curves were calculated
271 using Kaplan-Meier plots and differences between them were analyzed by the log-rank
272 method using Prism (GraphPad Software Inc, San Diego, USA).
273

274 **Locomotor assay**

275 The locomotor ability of flies was assessed in a negative geotaxis climbing assay as
276 described previously (54). Briefly, age-matched, pre-mated female flies were placed in
277 adapted plastic 25 ml pipettes that were used as vertical climbing columns. The flies were
278 allowed to acclimatize for 30 minutes prior to assessment of their locomotor ability. Flies
279 were tapped to the bottom of the pipette (using the same number and intensity of taps) and
280 then allowed to climb for 45 seconds. At the end of the climbing period the number of flies
281 above the 25 ml mark, the number below the 2 ml mark and the number in between the 2 ml
282 and 25 ml mark were recorded. This procedure was performed three times at each time
283 point. The mean performance index (PI) ± SD for each group of flies was calculated as
284 described (54).
285

286 **Statistical analysis**

287 Statistical analysis of the data was performed by one-way analysis of variance, together with
288 Tukey highly significant difference (HSD) for *post hoc* analysis or the unpaired samples *t* test
289 using Prism (GraphPad Software Inc, San Diego, USA).
290
291
292

292 **Results**

293

294 **Cytosolic ovine PrP expression in *Drosophila***

295 Here we have generated *Drosophila* transgenic for polymorphic variants of cytosolic ovine
296 PrP in order to investigate the toxic potential of intracellular PrP expression in the absence or
297 presence of exogenous prions. The data in Figure 1 show the western blot detection of
298 cytosolic PrP [PrP(cyt)] expression in *Drosophila*. The analysis in Figure 1a shows that
299 ARQ(cyt), AHQ(cyt) and VRQ(cyt) were all efficiently expressed at a similar level pan
300 neuronally in the fly. The molecular mass of all three genotypes of PrP(cyt) was
301 approximately 27kDa, the same as that of non-glycosylated ovine recombinant PrP. The
302 analysis in Figure 1b shows that AHQ(cyt) and VRQ(cyt) transgenic *Drosophila* expressed
303 significantly higher levels of PrP compared to flies that expressed AHQ or VRQ PrP with a
304 GPI anchor sequence [PrP(GPI)]. The opposite trend was seen with *Drosophila* that
305 expressed the ARQ PrP genotype.

306

307 We subsequently used capture-detector ELISA with C-terminal-specific anti-PrP monoclonal
308 antibodies in order to quantify the level of each genotype of cytosolic ovine PrP expressed in
309 *Drosophila*. The data in Figure 2a show that significantly lower levels of pan neuronally
310 expressed ovine PrP(cyt) were recognized by the anti-PrP-specific ELISA compared to ovine
311 PrP(GPI). This observation suggested that cytosolic ovine PrP may adopt a distinct
312 conformation compared to other forms of ovine PrP expressed in *Drosophila* that are
313 recognized by this ELISA (45). In order to test this, we used a conformational-dependent
314 immunoassay (CDI) whereby PrP(cyt) was denatured by guanidine prior to its recognition by
315 capture-detector immunoassay (48). The data in Figure 2b show that all of the genotypes of
316 cytosolic ovine PrP expressed pan neuronally in *Drosophila* were recognized by the
317 denaturant-based CDI.

318

319 **Cytosolic PrP is predominantly detergent insoluble and displays protease resistance**

320 We next investigated whether the immunobiochemical properties of PrP(cyt) expressed in
321 *Drosophila* correlated with distinct conformers of the ovine prion protein. Figure 3 shows a
322 comparison of ARQ(cyt) and ARQ(GPI) with respect to detergent solubility and relative
323 resistance to proteolytic digest. In order to determine the detergent solubility of cytosolic
324 ovine PrP we extracted fly head homogenates with Sarkosyl to prepare soluble and insoluble
325 fractions for subsequent analysis by western blot with anti-PrP monoclonal antibody Sha31.
326 The data in Figure 3a show that while *Elav*-driven ARQ(cyt) and ARQ(GPI) flies both

327 displayed a major band of approximately 27kDa in detergent soluble and insoluble head
328 homogenate fractions, the proportion of PrP present in these fractions varied. The level of
329 insoluble prion protein was greater than the level of soluble material in *Elav*-ARQ(cyt) flies
330 compared to that of the *Elav*-ARQ(GPI) fly line. The data in Figure 3b show the western blot
331 analysis of PK-digested fly head homogenate from pan neuronal ARQ(cyt) and ARQ(GPI)
332 flies. Pan neuronally expressed ARQ(GPI) PrP was readily cleaved by PK when treated with
333 3 - 9 μg/ml of proteolytic enzyme. In contrast, ARQ(cyt) was resistant to digestion with PK
334 when treated with the proteolytic enzyme used in the same concentration range and was only
335 susceptible to complete digest when PK was used in excess of 27 μg/ml. All three
336 polymorphic variants of cytosolic ovine PrP showed these trends (data not shown).
337
338 **Survival of cytosolic ovine PrP transgenic *Drosophila***
339 Cytosolic PrP accumulation is toxic to some neurons (26, 55) and may be part of the
340 neurotoxic mechanism associated with prion diseases (25). It was important therefore to
341 determine the effect of cytosolic PrP expression on the general well being of *Drosophila* prior
342 to prion infectivity studies in these fly lines.
343
344 The data in Figure 4 show the survival curves for PrP transgenic fly lines that pan neuronally
345 expressed cytosolic ovine prion protein in comparison with the survival curve for the control
346 *Elav*-51D fly line. Cytosolic PrP expression did not appear to be overtly detrimental to
347 *Drosophila* since the survival curve for each of the ovine PrP transgenic fly lines showed a
348 similar profile to that of the non-transgenic 51D control flies. However, log-rank test analysis
349 showed that the survival curves of all three genotypes of prion protein transgenic fly lines
350 were significantly different to that of the 51D control flies ($p \leq 0.002$) and this was reflected in
351 differences in median survival times, which were: 51D 86 days; AHQ(cyt) 81 days; ARQ(cyt)
352 79 days; and VRQ(cyt) 76 days. The general lack of toxicity in *Drosophila* as a consequence
353 of pan neuronal cytosolic PrP expression was also evident when PrP(cyt) was expressed
354 ubiquitously. For example, the percent survival for *β-actin*-driven and *elav*-driven VRQ(cyt)
355 PrP transgenic flies was similar at approximately 90 % and 95 %, respectively, when
356 assessed at 50 days of age.
357
358 **Cytosolic PrP transgenic *Drosophila* are susceptible to ovine prion inocula**
359 In order to assess whether cytosolic ovine PrP transgenic *Drosophila* were susceptible to the
360 toxic effect of exogenous ovine prion inocula, flies at the larval stage of development were
361 exposed to scrapie-infected sheep brain material and the locomotor activity of prion-exposed

362 flies assessed after eclosion (i.e. hatching). The prion inoculum used here was sheep brain
363 homogenate derived from natural cases of VRQ/VRQ and ARQ/ARQ classical (50) or
364 AHQ/AHQ experimental atypical (43) sheep scrapie. Genotype matched scrapie-free brain
365 homogenates were used as control material and 51D *Drosophila* were used as the control fly
366 line, which were similarly exposed to scrapie-infected and scrapie-free sheep brain
367 homogenate.
368
369 In order to assess the response to classical scrapie prion inocula, *β-actin*- or *Elav*-driven
370 VRQ(cyt) *Drosophila* were exposed at the larval stage to VRQ/VRQ scrapie-infected sheep
371 brain homogenate. Figure 5 shows the climbing ability expressed as a performance index of
372 prion exposed and control flies post-eclosion. The data in Figure 5a show that prion-exposed
373 *β-actin*-driven VRQ(cyt) *Drosophila* displayed a significantly accelerated decline in locomotor
374 activity compared to similar flies exposed to genotype-matched control brain homogenate
375 (p<0.001 over the whole assay). In contrast, *β-actin*-driven 51D flies showed a similar decline
376 in locomotor activity following exposure to scrapie-infected or genotype matched control
377 sheep brain homogenate. The data in Figure 5b show that prion-exposed *Elav*-driven
378 VRQ(cyt) *Drosophila* also showed a significantly accelerated decline in locomotor activity
379 compared to similar flies exposed to genotype-matched control brain homogenate (p<0.05
380 between days 2 and 51 of the assay), which was somewhat reduced compared to that seen
381 by *β-actin*-driven VRQ(cyt) *Drosophila*. In contrast, the performance index of *elav*-driven
382 VRQ(cyt) PrP transgenic *Drosophila* exposed to ARQ/ARQ scrapie-infected sheep brain
383 homogenate was not significantly different to that of similar flies exposed to scrapie-free
384 ARQ/ARQ sheep brain homogenate (data not shown). *Elav*-driven 51D flies showed no
385 difference in the decline of locomotor activity following exposure to scrapie-infected or
386 genotype matched control sheep brain homogenate.
387
388 We subjected head homogenates from prion-exposed flies to proteolytic digest followed by
389 western blot with anti-PrP monoclonal antibody in order to attempt to detect PK-resistant
390 PrPSc. The data in Figure 6 show that the majority of pan neuronally expressed VRQ(cyt)
391 from prion-exposed and control treated flies was digested with PK at 10 - 30 μg/ml and with
392 similar resultant molecular profiles. At 20 days of age a greater fraction of the VRQ(cyt) was
393 resistant to PK digestion at these concentrations of the proteolytic enzyme.
394
395 We subsequently investigated whether the toxic phenotype displayed by prion-exposed
396 VRQ(cyt) *Drosophila* was transmissible. In order to do so, we prepared homogenates from

397 the heads of 30 day-old *Drosophila* that had been exposed at the larval stage to either
398 VRQ/VRQ prion-infected or genotype-matched scrapie-free sheep brain homogenate. Fly
399 head homogenates were subsequently used to inoculate fresh batches of recipient VRQ(cyt)
400 *Drosophila* larvae. After hatching, the locomotor activity of fly head homogenate-exposed
401 *Drosophila* was assessed by a negative geotaxis climbing assay. The data in Figure 7 show
402 that head homogenate from prion-exposed VRQ(cyt) *Drosophila* induced a significantly
403 accelerated decline in locomotor activity compared to control fly head homogenates in
404 VRQ(cyt) recipient flies (p<0.01 over the whole assay) (Figure 7a). In contrast, no significant
405 differences were seen in the locomotor response of recipient VRQ(cyt) *Drosophila* to head
406 homogenate prepared from non-transgenic 51D flies previously exposed to either scrapie-
407 infected or scrapie-free sheep brain material (Figure 7b).
408
409 In order to assess the response to atypical scrapie prion inocula, *β-Actin*- or *Elav*-driven
410 cytosolic AHQ(cyt) ovine PrP transgenic *Drosophila* were exposed at the larval stage to
411 AHQ/AHQ prion-infected or genotype-matched scrapie-free sheep brain homogenate. *β-*
412 *Actin*- and *Elav*-driven AHQ(GPI) and *Elav*-driven ARQ(GPI) *Drosophila*, that both express
413 PrP with a GPI anchor sequence, were included for comparison. The data in Figure 8 show
414 the climbing ability of *Drosophila* with ubiquitous AHQ expression after exposure to atypical
415 scrapie-infected sheep brain homogenate. *β-Actin*-driven AHQ(cyt) flies showed an
416 accelerated decline in locomotor activity following exposure to atypical scrapie-infected
417 sheep brain homogenate compared to control brain homogenate (p=0.0226 between day 8
418 and day 39) (Figure 8a). Similarly, atypical prion-exposed *β-Actin*-driven AHQ(GPI) flies
419 showed a significantly enhanced decline in locomotor activity compared to similar flies
420 exposed to control inocula (P=0.0278 over the whole assay) (Figure 8b). *β-Actin*-driven
421 ARQ(GPI) flies also showed a significantly enhanced decline in locomotor activity following
422 exposure to AHQ/AHQ scrapie-infected brain homogenate compared to control inocula
423 (p=0.0351 between day 8 and day 39) (Figure 8c). In contrast, *β-Actin* -driven 51D flies
424 showed the same decline in locomotor activity following exposure to AHQ/AHQ scrapie-
425 infected or genotype-matched scrapie-free sheep brain homogenate (Figure 8d). Similar
426 trends were seen with both atypical scrapie inocula (data not shown).
427
428 *Elav*-driven AHQ(cyt), AHQ(GPI) or 51D flies showed no difference in decline of locomotor
429 activity following exposure to AHQ/AHQ scrapie-infected brain homogenate compared to
430 control brain homogenate. The data in Figure 9 show the climbing ability of *Drosophila* after
431 exposure to atypical scrapie-infected and control sheep brain homogenate. *Elav*-driven

432 AHQ(cyt) flies showed a similar decline in locomotor activity following exposure to AHQ/AHQ
433 scrapie-infected or genotype-matched scrapie-free sheep brain homogenate, or PBS (Figure
434 9a). In a similar manner, *Elav*-driven AHQ(GPI) flies showed no difference in decline of
435 locomotor activity following exposure to AHQ/AHQ scrapie-infected brain homogenate
436 compared to control inocula (Figure 9b) although a response was seen at day 33 with one
437 atypical scrapie inoculum (data not shown). In contrast to these data, *Elav*-driven ARQ(GPI)
438 flies showed a significantly enhanced decline in locomotor activity following exposure to
439 AHQ/AHQ scrapie-infected brain homogenate compared to control inocula ($p<0.05$ between
440 day 7 and day 40) **(**Figure 9c**)**. *Elav*-driven 51D flies showed the same decline in locomotor
441 activity following exposure to AHQ/AHQ scrapie-infected sheep brain homogenate or control
442 inocula (Figure 9d). Similar trends were seen with both atypical scrapie inocula (data not
443 shown).
444
445

445 **Discussion**

446

447 The pathogenesis that occurs during prion diseases is associated with the conformational
448 change of PrPC into PrPSc and concomitant neurodegeneration (1). However, the
449 mechanism of PrP conversion and its role in neurotoxicity are unknown. While PrPC is
450 primarily attached by a GPI anchor to the external side of the cell membrane, topological
451 variants of the protein can arise during its biogenesis and metabolism (25). Here we have
452 shown that one such variant, namely cytosolic PrP, can participate in the generation of a
453 transmissible toxicity induced by ovine prion inocula.

454

455 To do so, we have generated *Drosophila* that express cytosolic AHQ, ARQ or VRQ ovine
456 PrP. All three cytosolic PrP variants were expressed at a similar level in the fly and
457 comprised predominantly detergent insoluble material that showed resistance to proteolytic
458 digest with relatively low concentrations of Proteinase K enzyme. In addition, epitopes
459 normally exposed in ovine PrPC were either hidden or buried in PrP(cyt) since the latter
460 required denaturation prior to its immunodetection by capture-detector immunoassay. The
461 molecular profile and conformational properties of the PrP(cyt) variants expressed in
462 *Drosophila* are distinct from those of the same polymorphic variants expressed with a GPI
463 anchor in this host (45). This is likely to be due to the lack of post translational modifications
464 experienced by PrP(cyt) as a consequence of its failure to enter the ER during biosynthesis.
465 The modifications that PrPC normally experiences during its biogenesis include glycosylation
466 and the introduction of a disulfide bond to the polypeptide chain, both of which influence
467 protein folding and thermodynamic stability. Despite the acquisition of properties of misfolded
468 prion protein, the pan neuronal expression of PrP(cyt) in *Drosophila* was not overtly
469 detrimental to the fly. We have previously shown that *Drosophila* transgenic for AHQ
470 expressed with a GPI anchor sequence displayed a median life-span that was significantly
471 reduced compared to control 51D flies (45). Ovine AHQ PrP is associated with susceptibility
472 to atypical scrapie in sheep, which is considered to be a spontaneous disorder of PrP folding
473 and/or metabolism (38, 40) rather than an acquired condition (41-43). Our observation here
474 that cytosolic AHQ does not induce a comparable toxicity to AHQ(GPI) suggests that the
475 toxicity associated with AHQ targeted to the cell membrane is a consequence of this protein
476 trafficking through the secretory pathway of the cell. The expression of AHQ variants of ovine
477 PrP in *Drosophila* provides a novel model system to investigate the potential spontaneous
478 misfolding of this genotype of ovine prion protein.

479

480 We assessed the response of PrP(cyt) transgenic *Drosophila* to exogenous ovine prions in a
481 negative geotaxis climbing assay, a versatile and robust method used to assess locomotor
482 defects in fly models of mammalian neurodegenerative conditions (54). *Drosophila*
483 transgenic for VRQ(cyt) or AHQ(cyt) expression showed a decreased climbing ability after
484 exposure at the larval stage to classical or atypical scrapie-infected sheep brain
485 homogenate, respectively. The toxic effect of classical and atypical scrapie in PrP(cyt)
486 transgenic *Drosophila* is suggestive of a prion-mediated effect as it was not induced by
487 exposure to normal sheep brain homogenate and it was PrP dependent, since scrapie-
488 infected sheep brain homogenate was not toxic to non transgenic 51D flies. Importantly, we
489 have shown that the toxic phenotype of prion-exposed PrP(cyt) flies was transmissible. Fly
490 head homogenate from prion-exposed VRQ(cyt) PrP transgenic *Drosophila* efficiently
491 induced a toxic phenotype in recipient flies of the same genotype. This was not due to carry
492 over of scrapie-infected sheep brain inocula in fly head homogenate since no effect was
493 induced in recipient PrP(cyt) flies by prion-exposed non-transgenic 51D fly head inocula.
494 These observations are suggestive of the generation of an infectious moiety, analogous to
495 prion replication, during primary passage of scrapie in VRQ(cyt) flies (i.e. sheep-to-fly
496 transmission) that was subsequently transmitted at secondary passage (i.e. fly-to-fly
497 transmission). However, we were unable to demonstrate an increase in PK-resistant
498 VRQ(cyt) PrP in prion exposed flies of this genotype. In other studies we have shown that
499 protein misfolding cyclic amplification can be used to detect PK-resistant PrPSc in prion
500 exposed VRQ(GPI) transgenic *Drosophila* but not similarly treated VRQ(cyt) flies (Thackray
501 et al. *submitted*).
502
503 A feature of the response by AHQ(cyt) transgenic *Drosophila* to atypical scrapie toxicity was
504 the requirement for ubiquitous PrP expression in the fly. The lack of susceptibility of pan
505 neuronal AHQ(cyt) but not VRQ(cyt) transgenic *Drosophila* to scrapie prions would not
506 appear to be due to the level of ovine PrP expressed in these flies since cytosolic PrP was
507 expressed at a similar level in both *Elav*-driven fly lines. Furthermore, resistance to atypical
508 scrapie toxicity by *Drosophila* transgenic for pan neuronal expression of AHQ(cyt) did not
509 appear to be due to the topological expression of PrP in this fly line since *Drosophila*
510 transgenic for pan neuronal expression of AHQ(GPI) were also refractive to the same
511 inocula. The need for ubiquitous PrP expression in AHQ PrP transgenic *Drosophila* for
512 susceptibility to atypical scrapie toxicity may reflect a low infectious titre in these particular
513 prion-infected isolates compared to classical scrapie material. Alternatively, atypical scrapie
514 infectivity may be more unstable than its classical scrapie counterpart. It is known that the

515 PrPSc associated with atypical scrapie is less PK resistant compared to that associated with
516 classical scrapie (56, 57). Whatever the case, ubiquitous expression of PrP in *Drosophila*
517 may provide an environment for enhanced uptake and neuroinvasion of scrapie-infected
518 material and generation of the toxic agent compared to pan neuronal expression, which may
519 be more important for the response to atypical scrapie prion inocula. In mammalian species
520 PrPC is ubiquitously expressed, a feature that plays an essential role in the transmission of
521 prion infectivity in naturally acquired cases of prion disease (58), which may include atypical
522 scrapie (41, 42). However, not all of the ovine PrP transgenic fly lines used here required
523 ubiquitous expression of PrP in order to succumb to atypical scrapie prion inocula.
524 *Drosophila* with pan neuronal expression of ovine ARQ(GPI) showed susceptibility to
525 AHQ/AHQ atypical scrapie-infected sheep brain homogenate, as they do to ARQ/ARQ and
526 VRQ/VRQ classical scrapie prion inocula (59). The promiscuous susceptibility of ARQ(GPI)
527 PrP flies to atypical and classical scrapie-induced toxicity correlates with the high level of
528 ovine prion protein expressed by this fly line (45). It is known that the transmission barrier
529 effect (1) can be circumvented by elevated levels of PrP expression. For example, tg338
530 mice that express high levels of ovine VRQ PrP are susceptible to atypical scrapie isolates
531 whereas VRQ/VRQ sheep are resistant (34, 42). Collectively, these observations suggest
532 that *Drosophila* engineered for elevated levels of ubiquitous cell surface or cytosolic PrP
533 expression will be susceptible to a greater diversity of scrapie prion isolates and potentially
534 lower quantities of associated toxicity. This suggests that PrP transgenic *Drosophila* could
535 provide the basis of a new animal model to bioassay low levels of infectious toxicity in
536 peripheral tissues and blood of prion-affected animals.
537
538 Our studies with cytosolic PrP transgenic *Drosophila* presented here begin to contribute to an
539 understanding of the potential role of topological variants in prion-induced neurotoxicity.
540 While the mechanism of prion toxicity remains to be defined, it is established that PrP
541 expression is required for susceptibility to the neurotoxic agent. The essential requirement for
542 PrP expression in prion-induced neurotoxicity may suggest an intermediate in the conversion
543 of PrPC to PrPSc is the neurotoxic agent (60, 61). An alternative possibility is that
544 neurotoxicity results from PrPSc interference with the normal biosynthesis and metabolism of
545 PrPC (25). PrP can accumulate in the cytosol in a misfolded form when proteasomal activity
546 is compromised (28, 31) and cytosolic PrP has been reported to be neurotoxic in some
547 neurons (26, 55). However, the neurotoxicity of cytosolic PrP *per se* has been debated (62,
548 63). Our observations here have shown that while cytosolic PrP can adopt a conformation
549 distinct from PrP targeted to the cell membrane, expression of PrP(cyt) in *Drosophila* does

550 not result in the accumulation of a transmissible toxic moiety without prior exposure of these
551 flies to exogenous prion inocula. Collectively, our data presented here suggest that cytosolic
552 PrP is not overtly toxic to neurons *per se* but may participate in a toxicity mediated by scrapie
553 prion inocula, possibly by acting as a substrate for the generation of PrP-dependent
554 transmissible moiety that initiates or maintains repression of neuronal proteostasis (29, 30,
555 64-66). The tractable nature of *Drosophila* as a genetically and biochemically well-defined
556 experimental model will allow us to test the validity of this hypothesis.
557
558
559
560
561
562 **Acknowledgements**
563
564 We thank John Roote for useful discussion regarding fly genetics. We thank Drs. Marion
565 Simmons and John Spiropoulos for the supply of sheep atypical scrapie samples. This work
566 was supported by funds from the China Scholarship Council and Cambridge Overseas Trust
567 who provided PhD studentship support for C.Z. We acknowledge funding support from the
568 NC3Rs.
569
570
571
572
573
574
575
576

576 **Legends**

577

578 **Figure 1. Western blot detection of cytosolic ovine PrP expression in *Drosophila***
579 Head homogenates were prepared from 5 day old ovine PrP transgenic *Drosophila* or 51D
580 control flies crossed with the *Elav-GAL4* driver fly line. Samples were analyzed by SDS-
581 PAGE and western blot with anti-PrP monoclonal antibody Sha31. **(a)** Molecular profile of
582 ARQ(cyt); AHQ(cyt); VRQ(cyt) all at the equivalent of 10 fly heads per track. Mature length
583 ovine VRQ recombinant PrP (rPrP) was used at 10 ng. Molecular mass marker values (kDa)
584 are shown on the left hand side. **(b)** Comparison of ovine PrP(cyt) and PrP(GPI) expression
585 in *Drosophila*. Tracks 1 and 2: PrP(cyt); tracks 3 and 4: PrP(GPI); tracks 1 and 3; male flies;
586 tracks 2 and 4; female flies. The equivalent of 5 fly heads were run per track. Molecular mass
587 marker values (kDa) are shown on the left hand side. The ovine PrP genotype is indicated
588 on the right hand side.

589

590 **Figure 2. Capture-detector immunoassay analysis of cytosolic ovine PrP expression**
591 **in *Drosophila***
592 Head homogenates were prepared from 5 day old ovine PrP transgenic *Drosophila* or 51D
593 control flies crossed with the *Elav-GAL4* driver fly line. Samples were analyzed by: **(a)** ELISA
594 using anti-PrP monoclonal antibody 245 as capture and biotinylated anti-PrP monoclonal
595 antibody SAF32 as detector. The equivalent of 10 fly heads were measured per well and the
596 results shown are OD_{415nm} means ± SD for duplicate wells; **(b)** Conformational-dependent
597 immunoassay (CDI). Fly head homogenates were treated with 8M GdnHCl prior to
598 immunoassay using anti-PrP monoclonal antibody 245 as capture and biotinylated anti-PrP
599 monoclonal antibody SAF32 as detector. The equivalent of 20 fly heads were measured per
600 well. Mature length ovine ARQ recombinant PrP (rPrP) was used at 122 ng/well. The results
601 are shown as time resolved fluorescence (TRF) counts per second (cps) ± SD for duplicate
602 wells.

603

604 **Figure 3. Cytosolic ovine PrP is characterized by reduced solubility and increased PK**
605 **resistance**
606 Head homogenates were prepared from 5 day old ovine ARQ(cyt) or ARQ(GPI) PrP
607 transgenic *Drosophila* crossed with the *Elav-GAL4* driver fly line. After various treatments fly
608 head homogenate samples were analyzed by SDS-PAGE and western blot with anti-PrP
609 monoclonal antibody Sha31. The equivalent of 10 fly heads were loaded per track. Molecular
610 mass marker values (kDa) are shown on the left hand side of each gel. **(a)** Total (T), soluble

611 (S) and insoluble (I) fractions of PrP were prepared from fly heads as described in the
612 Materials and Methods. **(b)** Reaction products of fly head homogenates incubated with
613 various concentrations of PK at 37 °C for 30 minutes.
614
615 **Figure 4. Survival curves for cytosolic ovine PrP transgenic *Drosophila***
616 Groups of 100 age-matched *Elav*-PrP or control *Elav*-51D flies were selected for survival
617 assays. The number of surviving flies was recorded three times a week as described in the
618 Materials and Methods. Survival curves were calculated using Kaplan-Meier plots and
619 differences between them were analyzed by the log-rank method using Prism (GraphPad
620 Software Inc, San Diego, USA).
621
622 **Figure 5. Primary transmission of classical ovine scrapie in cytosolic VRQ PrP**
623 **transgenic *Drosophila***
624 VRQ(cyt) PrP transgenic (squares) or 51D control flies (circles) crossed with either the *β-*
625 *actin-GAL4* or *Elav-GAL4* driver line were assessed for locomotor activity by a negative
626 geotaxis climbing assay following exposure at the larval stage to VRQ/VRQ scrapie-infected
627 (filled symbols) or scrapie-free (open symbols and dashed lines) sheep brain homogenate. *β-*
628 *actin-GAL4*-VRQ(cyt) PrP flies were non RFP. The mean performance index ± SD is shown
629 for three groups of n=15 flies of each genotype per time point (45 flies in total for each
630 group). Statistical analysis of the linear regression plots was performed using one-way
631 analysis of variance (ANOVA) and *post hoc* Tukey honestly significant difference.
632
633 **Figure 6. PK digestion of prion-exposed cytosolic VRQ fly head homogenate**
634 Fly head homogenates were prepared from ovine VRQ(cyt) PrP transgenic *Drosophila*
635 crossed with the *Elav-GAL4* driver fly line following exposure at the larval stage to VRQ/VRQ
636 scrapie-free (tracks 1 - 3) or scrapie-infected (tracks 4 - 6) sheep brain homogenate.
637 Samples were incubated with 0, 10 or 30 μg/ml PK at 37 ˚C for 15 minutes and the reaction
638 products analyzed by SDS-PAGE and western blot with anti-PrP monoclonal antibody
639 Sha31. The equivalent of 10 fly heads were loaded per track. Molecular mass marker values
640 (kDa) are shown on the left hand side of each gel. Age of flies (in days) is shown on the right.
641
642 **Figure 7. Fly-to-fly transmission of prion-induced toxic phenotype**
643 VRQ(cyt) PrP (non RFP) transgenic flies crossed with the *β-actin-GAL4* driver line were
644 assessed for locomotor activity by a negative geotaxis climbing assay following exposure at
645 the larval stage to a 10 % (v/v) dilution of head homogenate derived from 30 day old

646 *Drosophila* exposed at the larval stage to either scrapie-infected (filled squares) or scrapie-
647 free (open squares and dashed line) sheep brain homogenate. The mean performance index
648 ± SD is shown for three groups of n=15 flies of each genotype per time point (45 flies in total
649 for each group). Statistical analysis of the linear regression plots was performed using one-
650 way analysis of variance (ANOVA) and *post hoc* Tukey honestly significant difference.
651
652 **Figure 8. Primary transmission of atypical scrapie in *β-actin*-driven PrP transgenic**
653 ***Drosophila***
654 PrP transgenic or 51D control flies crossed with the *β-actin-GAL4* driver line were assessed
655 for locomotor activity by a negative geotaxis climbing assay following exposure at the larval
656 stage to AHQ/AHQ scrapie-infected (filled circles) or scrapie-free (open circles and dashed
657 lines) sheep brain homogenate. The mean performance index ± SD is shown for three
658 groups of n=15 flies of each genotype per time point (45 flies in total for each group).
659 Statistical analysis of the scrapie-infected and scrapie-free linear regression plots for each fly
660 line was compared by the unpaired samples *t* test.
661
662 **Figure 9. Lack of response by *Elav*-driven AHQ PrP transgenic *Drosophila to* atypical**
663 **scrapie**
664 PrP transgenic or 51D control flies crossed with the *Elav-GAL4* driver line were assessed for
665 locomotor activity by a negative geotaxis climbing assay following exposure at the larval
666 stage to AHQ/AHQ scrapie-infected (closed squares and continuous line) or scrapie-free
667 (closed circles and dashed line) sheep brain homogenate or PBS (closed triangles and
668 dotted line). The mean performance index ± SD is shown for three groups of n=15 flies of
669 each genotype per time point (45 flies in total for each group). Statistical analysis of the linear
670 regression plots was performed using one-way analysis of variance (ANOVA) and *post hoc*
671 Tukey honestly significant difference.
672
673
674
675

675 **References**

676

677 1. Prusiner SB. 2004. Prion biology and diseases (second edition). Cold Spring Harbor
678 Laboratory Press, New York.
679 2. Brandner S, Isenmann S, Raeber A, Fischer M, Sailer A, Kobayashi Y, Marino S,
680 Weissmann C, Aguzzi A. 1996. Normal host prion protein necessary for scrapie-
681 induced neurotoxicity. Nature 379:339-343.
682 3. Bueler H, Aguzzi A, Sailer A, Greiner RA, Autenried P, Aguet M, Weissmann C. 1993.
683 Mice devoid of PrP are resistant to scrapie. Cell 73:1339-1347.
684 4. Mallucci G, Dickinson A, Linehan J, Klohn PC, Brandner S, Collinge J. 2003.
685 Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis.
686 Science 302:871-874.
687 5. Ciccotosto GD, Cappai R, White AR. 2008. Neurotoxicity of prion peptides on cultured
688 cerebellar neurons. Methods Mol Biol 459:83-96.
689 6. Collinge J. 2001. Prion diseases of humans and animals: their causes and molecular
690 basis. Annu Rev Neurosci 24:519-550.
691 7. Aguzzi A, Baumann F, Bremer J. 2008. The prion's elusive reason for being. Annu
692 Rev Neurosci 31:439-477.
693 8. Jackson WS, Borkowski AW, Faas H, Steele AD, King OD, Watson N, Jasanoff A,
694 Lindquist S. 2009. Spontaneous generation of prion infectivity in fatal familial
695 insomnia knockin mice. Neuron 63:438-450.
696 9. Sigurdson CJ, Nilsson KP, Hornemann S, Heikenwalder M, Manco G, Schwarz P, Ott
697 D, Rulicke T, Liberski PP, Julius C, Falsig J, Stitz L, Wuthrich K, Aguzzi A. 2009. De
698 novo generation of a transmissible spongiform encephalopathy by mouse
699 transgenesis. Proc Natl Acad Sci USA 106:304-309.
700 10. Watts JC, Giles K, Stohr J, Oehler A, Bhardwaj S, Grillo SK, Patel S, DeArmond SJ,
701 Prusiner SB. 2012. Spontaneous generation of rapidly transmissible prions in
702 transgenic mice expressing wild-type bank vole prion protein. Proc Natl Acad Sci USA
703 109:3498-3503.
704 11. Saborio GP, Permanne B, Soto C. 2001. Sensitive detection of pathological prion
705 protein by cyclic amplification of protein misfolding. Nature 411:810-813.
706 12. Castilla J, Saa P, Hetz C, Soto C. 2005. In vitro generation of infectious scrapie
707 prions. Cell 121:195-206.
708 13. Deleault NR, Harris BT, Rees JR, Supattapone S. 2007. Formation of native prions
709 from minimal components in vitro. Proc Natl Acad Sci USA 104:9741-9746.

710 14. Wang F, Wang X, Yuan CG, Ma J. 2010. Generating a prion with bacterially
711 expressed recombinant prion protein. Science 327:1132-1135.
712 15. Stahl N, Borchelt DR, Hsiao K, Prusiner SB. 1987. Scrapie prion protein contains a
713 phosphatidylinositol glycolipid. Cell 51:229-240.
714 16. Taylor DR, Hooper NM. 2006. The prion protein and lipid rafts. Mol Membr Biol 23:89-
715 99.
716 17. Basler K, Oesch B, Scott M, Westaway D, Walchli M, Groth DF, McKinley MP,
717 Prusiner SB, Weissmann C. 1986. Scrapie and cellular PrP isoforms are encoded by
718 the same chromosomal gene. Cell 46:417-428.
719 18. Turk E, Teplow DB, Hood LE, Prusiner SB. 1988. Purification and properties of the
720 cellular and scrapie hamster prion proteins. Eur J Biochem 176:21-30.
721 19. Haraguchi T, Fisher S, Olofsson S, Endo T, Groth D, Tarentino A, Borchelt DR,
722 Teplow D, Hood L, Burlingame A, et al. 1989. Asparagine-linked glycosylation of the
723 scrapie and cellular prion proteins. Arch Biochem Biophys 274:1-13.
724 20. Riek R, Hornemann S, Wider G, Glockshuber R, Wuthrich K. 1997. NMR
725 characterization of the full-length recombinant murine prion protein, mPrP(23-231).
726 FEBS Lett 413:282-288.
727 21. Stewart RS, Harris DA. 2003. Mutational analysis of topological determinants in prion
728 protein (PrP) and measurement of transmembrane and cytosolic PrP during prion
729 infection. J Biol Chem 278:45960-45968.
730 22. Kim SJ, Hegde RS. 2002. Cotranslational partitioning of nascent prion protein into
731 multiple populations at the translocation channel. Mol Biol Cell 13:3775-3786.
732 23. Hay B, Barry RA, Lieberburg I, Prusiner SB, Lingappa VR. 1987. Biogenesis and
733 transmembrane orientation of the cellular isoform of the scrapie prion protein
734 [published erratum appears in Mol Cell Biol 1987 May;7(5):2035]. Mol Cell Biol 7:914-
735 920.
736 24. Hegde RS, Mastrianni JA, Scott MR, DeFea KA, Tremblay P, Torchia M, DeArmond
737 SJ, Prusiner SB, Lingappa VR. 1998. A transmembrane form of the prion protein in
738 neurodegenerative disease. Science 279:827-834.
739 25. Chakrabarti O, Ashok A, Hegde RS. 2009. Prion protein biosynthesis and its
740 emerging role in neurodegeneration. Trends Biochem Sci 34:287-295.
741 26. Ma J, Wollmann R, Lindquist S. 2002. Neurotoxicity and neurodegeneration when PrP
742 accumulates in the cytosol. Science 298:1781-1785.

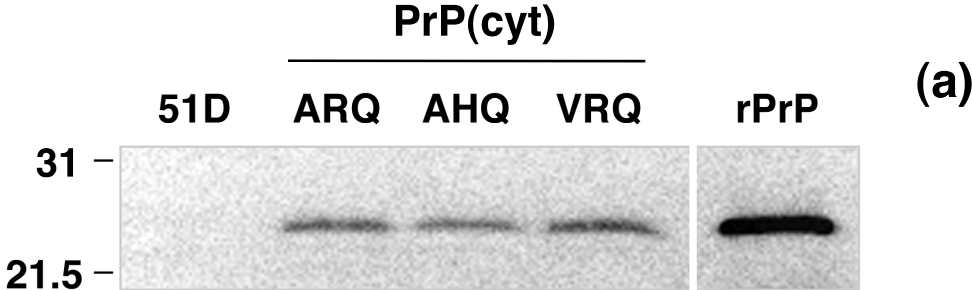
743 27. Grenier C, Bissonnette C, Volkov L, Roucou X. 2006. Molecular morphology and
744 toxicity of cytoplasmic prion protein aggregates in neuronal and non-neuronal cells. J
745 Neurochem 97:1456-1466.
746 28. Ma J, Lindquist S. 2001. Wild-type PrP and a mutant associated with prion disease
747 are subject to retrograde transport and proteasome degradation. Proc Natl Acad Sci
748 USA 98:14955-14960.
749 29. Kristiansen M, Deriziotis P, Dimcheff DE, Jackson GS, Ovaa H, Naumann H, Clarke
750 AR, van Leeuwen FW, Menendez-Benito V, Dantuma NP, Portis JL, Collinge J,
751 Tabrizi SJ. 2007. Disease-associated prion protein oligomers inhibit the 26S
752 proteasome. Mol Cell 26:175-188.
753 30. Deriziotis P, Andre R, Smith DM, Goold R, Kinghorn KJ, Kristiansen M, Nathan JA,
754 Rosenzweig R, Krutauz D, Glickman MH, Collinge J, Goldberg AL, Tabrizi SJ. 2011.
755 Misfolded PrP impairs the UPS by interaction with the 20S proteasome and inhibition
756 of substrate entry. EMBO J 30:3065-3077.
757 31. Ma J, Lindquist S. 2002. Conversion of PrP to a self-perpetuating PrPSc-like
758 conformation in the cytosol. Science 298:1785-1788.
759 32. Bujdoso R, Thackray AM. 2013. The emergence of a Drosophila model to measure
760 ovine prion infectivity. CAB Reviews 8 1-15.
761 33. Windl O, Dawson M. 2012. Animal prion diseases. Subcell Biochem 65:497-516.
762 34. Saunders GC, Cawthraw S, Mountjoy SJ, Hope J, Windl O. 2006. PrP genotypes of
763 atypical scrapie cases in Great Britain. J Gen Virol 87:3141-3149.
764 35. Moum T, Olsaker I, Hopp P, Moldal T, Valheim M, Benestad SL. 2005.
765 Polymorphisms at codons 141 and 154 in the ovine prion protein gene are associated
766 with scrapie Nor98 cases. J Gen Virol 86:231-235.
767 36. Clouscard C, Beaudry P, Elsen JM, Milan D, Dussaucy M, Bounneau C, Schelcher F,
768 Chatelain J, Launay JM, Laplanche JL. 1995. Different allelic effects of the codons
769 136 and 171 of the prion protein gene in sheep with natural scrapie. J Gen Virol
770 76:2097-2101.
771 37. Goldmann W, Hunter N, Smith G, Foster J, Hope J. 1994. PrP genotype and agent
772 effects in scrapie: change in allelic interaction with different isolates of agent in sheep,
773 a natural host of scrapie. J Gen Virol 75:989-995.
774 38. Benestad SL, Arsac JN, Goldmann W, Noremark M. 2008. Atypical/Nor98 scrapie:
775 properties of the agent, genetics, and epidemiology. Vet Res 39:19.
776 39. Hunter N. 1997. PrP genetics in sheep and the applications for scrapie and BSE.
777 Trends Microbiol 5:331-334.

778 40. Fediaevsky A, Maurella C, Noremark M, Ingravalle F, Thorgeirsdottir S, Orge L,
779 Poizat R, Hautaniemi M, Liam B, Calavas D, Ru G, Hopp P. 2010. The prevalence of
780 atypical scrapie in sheep from positive flocks is not higher than in the general sheep
781 population in 11 European countries. BMC Vet Res 6:9.
782 41. Simmons MM, Moore SJ, Konold T, Thurston L, Terry LA, Thorne L, Lockey R,
783 Vickery C, Hawkins SA, Chaplin MJ, Spiropoulos J. 2011. Experimental oral
784 transmission of atypical scrapie to sheep. Emerg Infect Dis 17:848-854.
785 42. Andreoletti O, Orge L, Benestad SL, Beringue V, Litaise C, Simon S, Le Dur A, Laude
786 H, Simmons H, Lugan S, Corbiere F, Costes P, Morel N, Schelcher F, Lacroux C.
787 2011. Atypical/Nor98 scrapie infectivity in sheep peripheral tissues. PLoS Pathog
788 7:e1001285.
789 43. Simmons MM, Konold T, Simmons HA, Spencer YI, Lockey R, Spiropoulos J, Everitt
790 S, Clifford D. 2007. Experimental transmission of atypical scrapie to sheep. BMC Vet
791 Res 3:20.
792 44. Le Dur A, Beringue V, Andreoletti O, Reine F, Lai TL, Baron T, Bratberg B, Vilotte JL,
793 Sarradin P, Benestad SL, Laude H. 2005. A newly identified type of scrapie agent can
794 naturally infect sheep with resistant PrP genotypes. Proc Natl Acad Sci USA
795 102:16031-16036.
796 45. Thackray AM, Muhammad F, Zhang C, Di Y, Jahn TR, Landgraf M, Crowther DC,
797 Evers JF, Bujdoso R. 2012. Ovine PrP transgenic Drosophila show reduced
798 locomotor activity and decreased survival. Biochem J 444:487-495.
799 46. Kozak M. 1986. Point mutations define a sequence flanking the AUG initiator codon
800 that modulates translation by eukaryotic ribosomes. Cell 44:283-292.
801 47. Lewis EB. 1960. A new standard food medium. Drosophila Information Service
802 34:117–118.
803 48. Thackray AM, Hopkins L, Bujdoso R. 2007. Proteinase K-sensitive disease-
804 associated ovine prion protein revealed by conformation-dependent immunoassay.
805 Biochem J 401:475-483.
806 49. Fernandez-Funez P, Zhang Y, Casas-Tinto S, Xiao X, Zou WQ, Rincon-Limas DE.
807 2010. Sequence-dependent prion protein misfolding and neurotoxicity. J Biol Chem
808 285:36897-36908.
809 50. Thackray AM, Hopkins L, Spiropoulos J, Bujdoso R. 2008. Molecular and
810 transmission characteristics of primary-passaged ovine scrapie isolates in
811 conventional and ovine PrP transgenic mice. J Virol 82:11197-11207.

812 51. Feraudet C, Morel N, Simon S, Volland H, Frobert Y, Creminon C, Vilette D, Lehmann
813 S, Grassi J. 2005. Screening of 145 anti-PrP monoclonal antibodies for their capacity
814 to inhibit PrPSc replication in infected cells. J Biol Chem 280:11247-11258.
815 52. Thackray AM, Fitzmaurice TJ, Hopkins L, Bujdoso R. 2006. Ovine plasma prion
816 protein levels show genotypic variation detected by C-terminal epitopes not exposed
817 in cell-surface PrPC. Biochem J 400:349-358.
818 53. Thackray AM, Madec JY, Wong E, Morgan-Warren R, Brown DR, Baron T, Bujdoso
819 R. 2003. Detection of bovine spongiform encephalopathy, ovine scrapie prion-related
820 protein (PrPSc) and normal PrPc by monoclonal antibodies raised to copper-refolded
821 prion protein. Biochem J 370:81-90.
822 54. White KE, Humphrey DM, Hirth F. 2010. The dopaminergic system in the aging brain
823 of Drosophila. Front Neurosci 4:205.
824 55. Wang X, Bowers SL, Wang F, Pu XA, Nelson RJ, Ma J. 2009. Cytoplasmic prion
825 protein induces forebrain neurotoxicity. Biochim Biophys Acta 1792:555-563.
826 56. Benestad SL, Sarradin P, Thu B, Schonheit J, Tranulis MA, Bratberg B. 2003. Cases
827 of scrapie with unusual features in Norway and designation of a new type, Nor98. Vet
828 Rec 153:202-208.
829 57. Buschmann A, Biacabe AG, Ziegler U, Bencsik A, Madec JY, Erhardt G, Luhken G,
830 Baron T, Groschup MH. 2004. Atypical scrapie cases in Germany and France are
831 identified by discrepant reaction patterns in BSE rapid tests. J Virol Methods 117:27-
832 36.
833 58. Gough KC, Maddison BC. 2010. Prion transmission: prion excretion and occurrence
834 in the environment. Prion 4:275-282.
835 59. Thackray AM, Muhammad F, Zhang C, Denyer M, Spiropoulos J, Crowther DC,
836 Bujdoso R. 2012. Prion-induced toxicity in PrP transgenic Drosophila. Exp Mol Pathol
837 92:194-201.
838 60. Sandberg MK, Al-Doujaily H, Sharps B, Clarke AR, Collinge J. 2011. Prion
839 propagation and toxicity in vivo occur in two distinct mechanistic phases. Nature
840 470:540-542.
841 61. Zhou M, Ottenberg G, Sferrazza GF, Lasmezas CI. 2012. Highly neurotoxic
842 monomeric alpha-helical prion protein. Proc Natl Acad Sci USA 109:3113-3118.
843 62. Fioriti L, Dossena S, Stewart LR, Stewart RS, Harris DA, Forloni G, Chiesa R. 2005.
844 Cytosolic prion protein (PrP) is not toxic in N2a cells and primary neurons expressing
845 pathogenic PrP mutations. J Biol Chem 280:11320-11328.

846 63. Roucou X, Guo Q, Zhang Y, Goodyer CG, LeBlanc AC. 2003. Cytosolic prion protein
847 is not toxic and protects against Bax-mediated cell death in human primary neurons.
848 J Biol Chem 278:40877-40881.
849 64. Morimoto RI. 2008. Proteotoxic stress and inducible chaperone networks in
850 neurodegenerative disease and aging. Genes Dev 22:1427-1438.
851 65. Walter P, Ron D. 2011. The unfolded protein response: from stress pathway to
852 homeostatic regulation. Science 334:1081-1086.
853 66. Moreno JA, Radford H, Peretti D, Steinert JR, Verity N, Martin MG, Halliday M,
854 Morgan J, Dinsdale D, Ortori CA, Barrett DA, Tsaytler P, Bertolotti A, Willis AE,
855 Bushell M, Mallucci GR. 2012. Sustained translational repression by eIF2alpha-P
856 mediates prion neurodegeneration. Nature 485:507-511.
857
858

Figure 1. Western blot detection of cytosolic ovine PrP expression in *Drosophila*



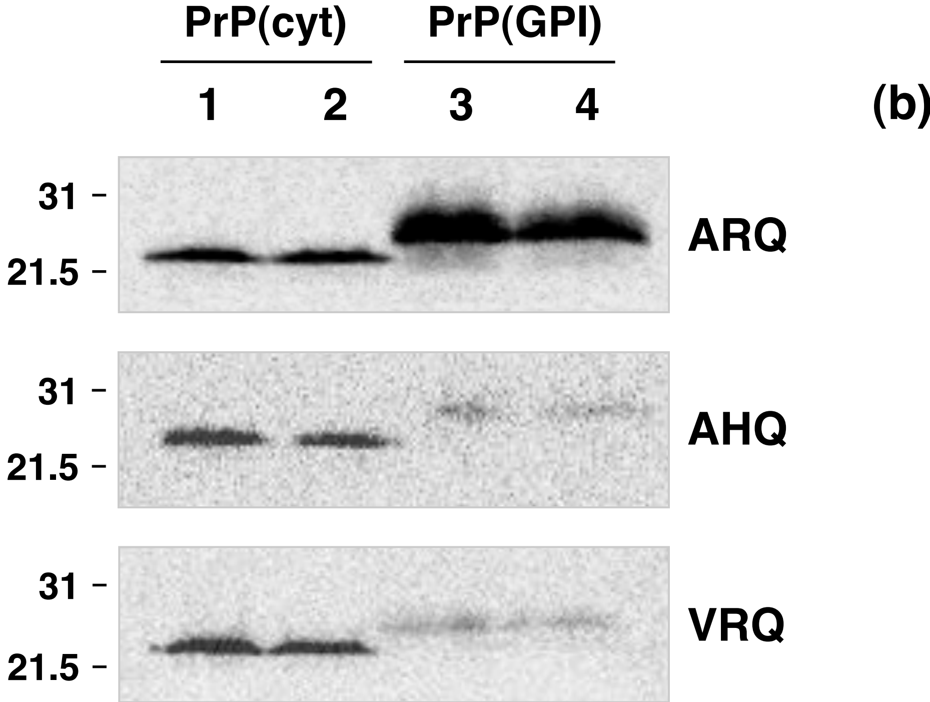


Figure 2. Capture-detector immunoassay analysis of cytosolic ovine PrP expression in *Drosophila*

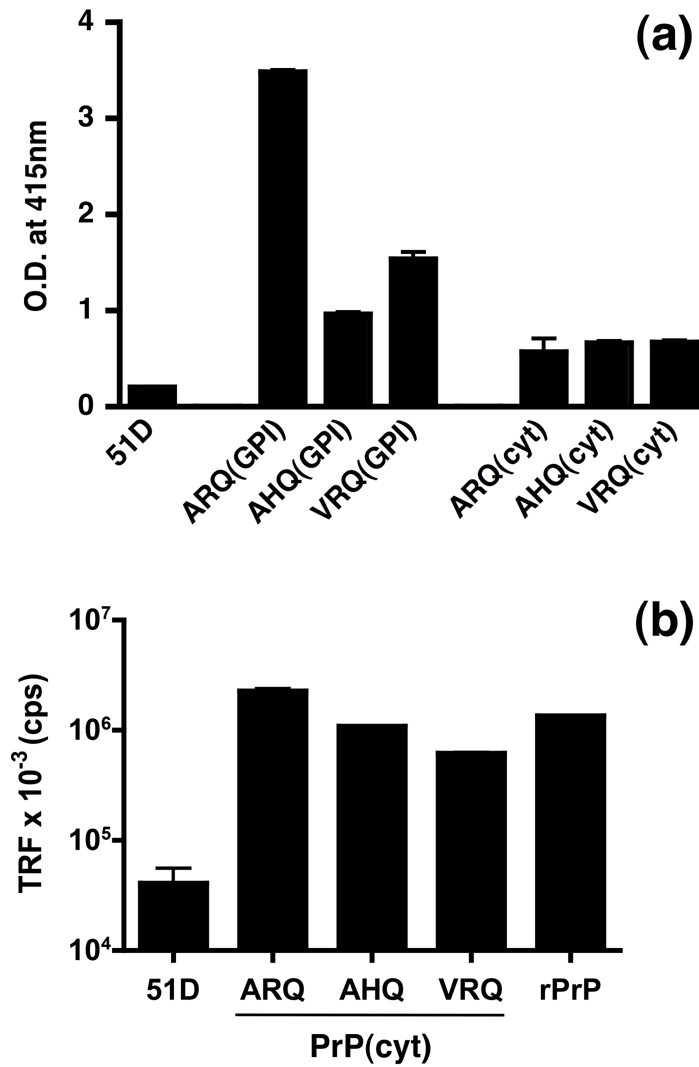


Figure 3. Cytosolic ovine PrP is characterized by reduced solubility and increased PK resistance

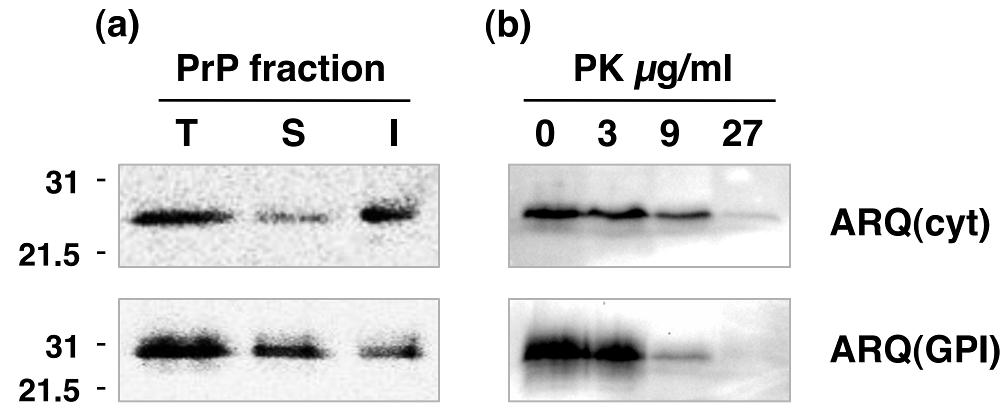


Figure 4. Survival curves for cytosolic ovine PrP transgenic *Drosophila*

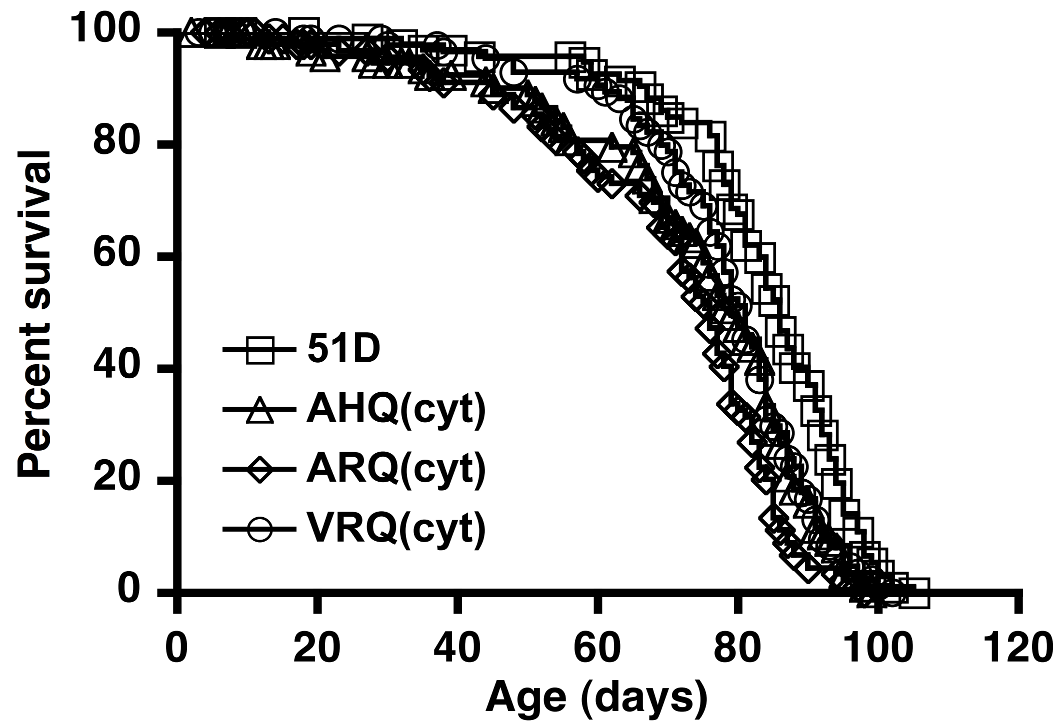
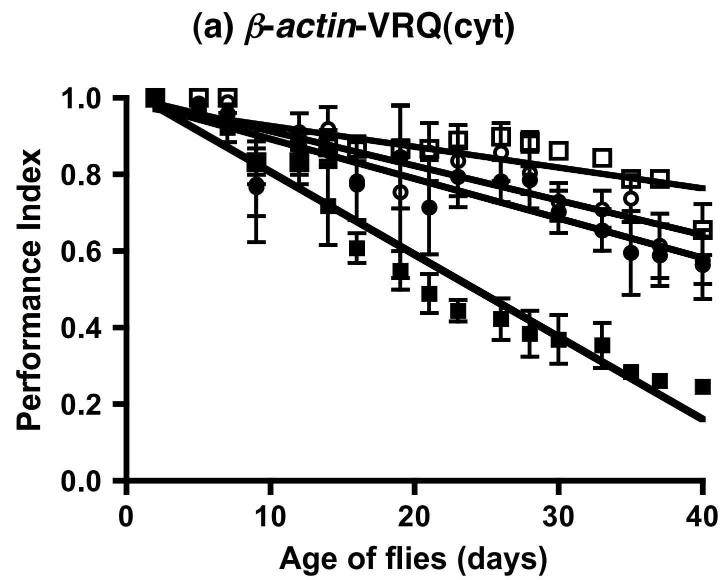


Figure 5. Primary transmission of classical ovine scrapie in cytosolic VRQ PrP transgenic *Drosophila*



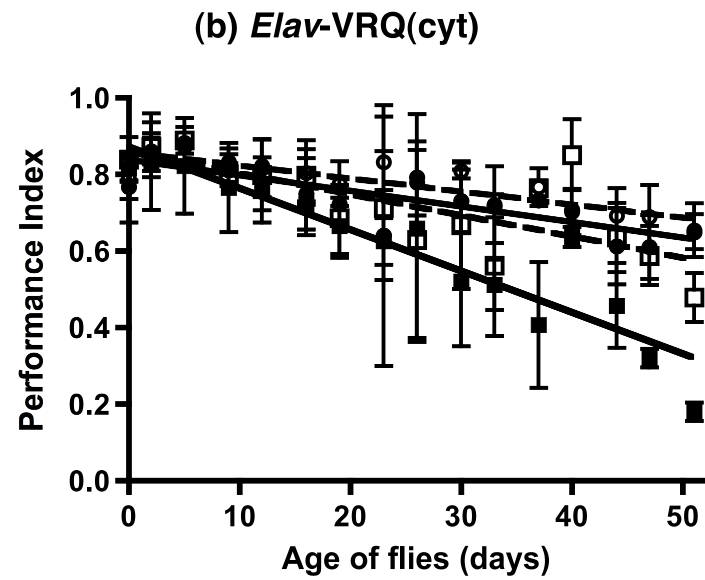


Figure 6. PK digestion of prion-exposed cytosolic VRQ fly head homogenate

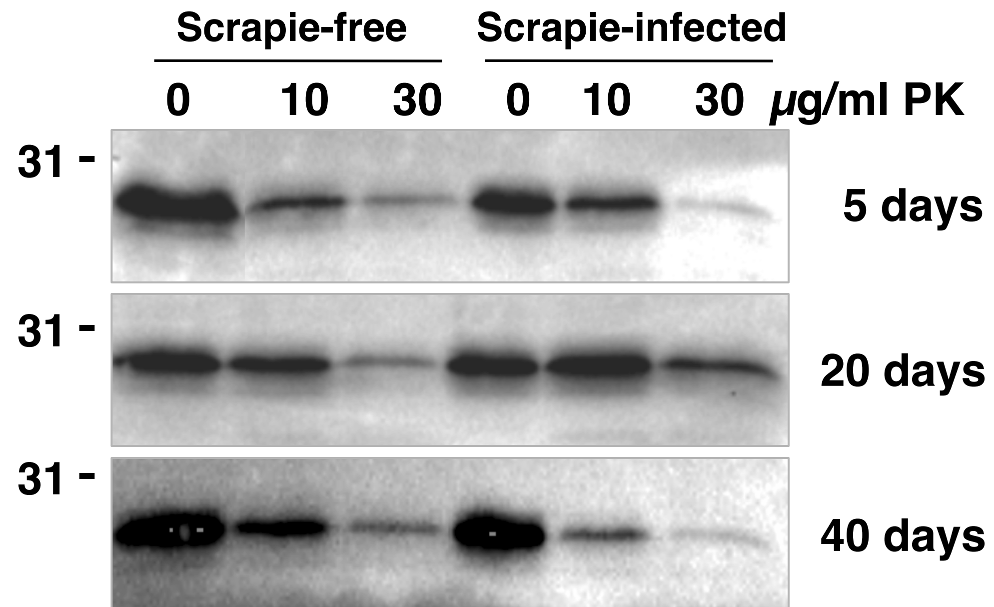


Figure 7. Fly-to-fly transmission of prion-induced toxic phenotype

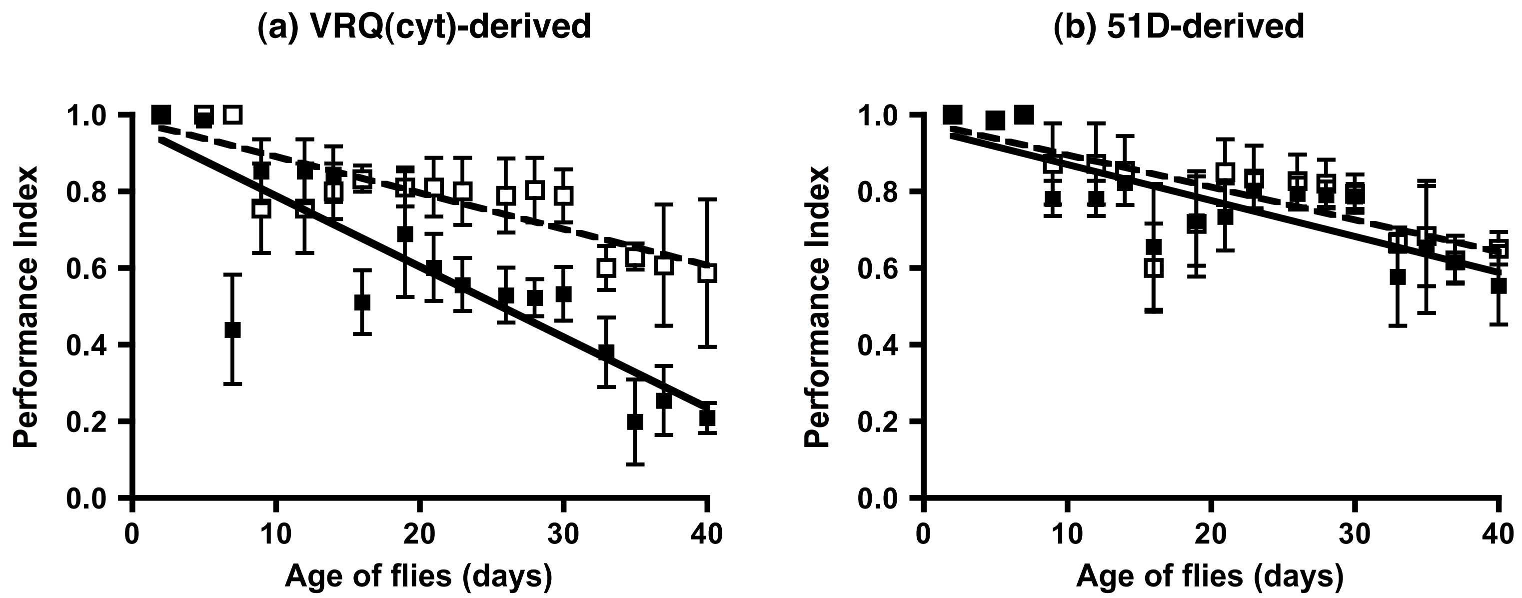
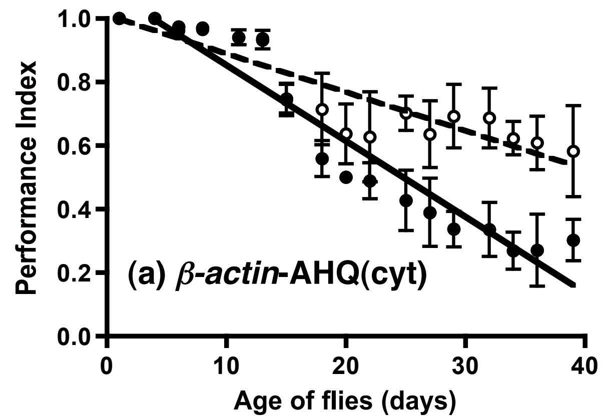
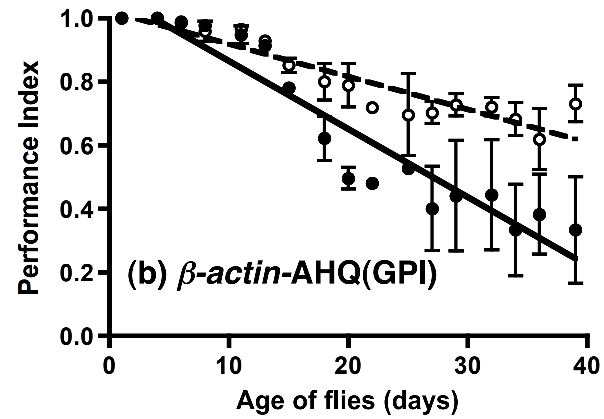
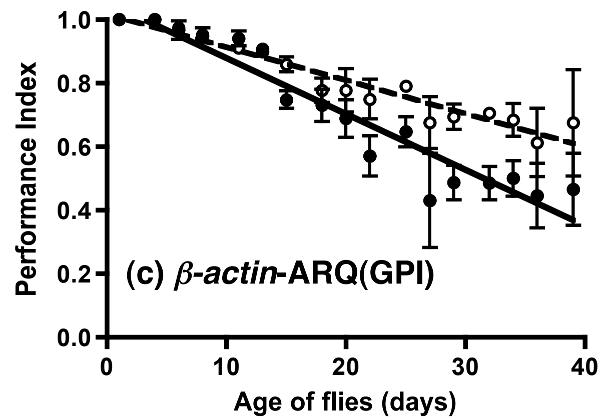


Figure 8. Primary transmission of atypical scrapie in *β-actin*-driven PrP transgenic *Drosophila*







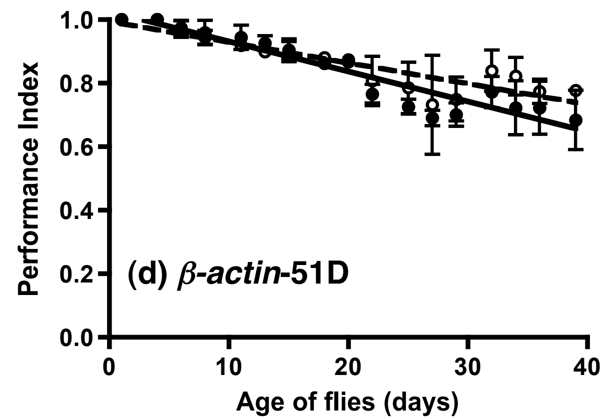


Figure 9: Lack of response by *Elav*-driven AHQ PrP transgenic *Drosophila to* atypical scrapie