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1 **Translation elongation factor 1A2 (eEF1A2) is encoded by one of four closely**
2 **related eef1a genes and is dispensable for survival in zebrafish**

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15 **Running title**: zebrafish eef1a2 is non-essential

16 **Key Words**: translation elongation, epilepsy model, zebrafish eef1a, eef1a2

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24 **Abstract**

25 Zebrafish are valuable model organisms for the study of human single-gene disorders:
26 they are genetically manipulable, their development is well understood, and mutant
27 lines with measurable, disease-appropriate phenotypic abnormalities can be used for
28 high throughput drug screening approaches. However, gene duplication events in
29 zebrafish can result in redundancy of gene function, masking loss of function
30 phenotypes and thus confounding this approach to disease modelling. Furthermore,
31 recent studies have yielded contrasting results depending on whether specific genes
32 are targeted using genome editing to make mutant lines, or whether morpholinos are
33 used (morphants). *De novo* missense mutations in the human gene *EEF1A*2,
34 encoding a tissue-specific translation elongation factor, cause severe
35 neurodevelopmental disorders; there is a real need for a model system in which to
36 study these disorders and we wanted to explore the possibility of a zebrafish model.
37 We identified four *eef1a* genes and examined their developmental and tissue-specific
38 expression patterns: *eef1a1l1* is first to be expressed whilst *eef1a2* is only detected
39 later during development. We then determined the effects of introducing null mutations
40 into eEF1A2 in zebrafish using CRISPR/Cas9 gene editing, in order to compare the
41 results with previously described morphants, and with the severe neurodegenerative
42 lethal phenotype of eEF1A2-null mice. In contrast with both earlier analysis in
43 zebrafish using morpholinos and with the mouse eEF1A2-null mice, disruption of the
44 *eef1a2* gene in zebrafish is compatible with normal lifespan. The resulting lines,
45 however, may provide a valuable platform for studying the effects of expression of
46 mutant human eEF1A2 mRNA.

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47 ## Introduction

48 Zebrafish represent a valuable model system for a range of human single gene
49 disorders because they are genetically manipulable and their development is well
50 understood. Importantly, if mutant zebrafish larvae have detectable phenotypes they
51 can be used for high throughput small molecule library screening for the discovery of
52 new therapeutically active molecules. However, redundancy of gene function as a
53 result of gene duplication can confound this approach, and recent studies have yielded
54 contrasting results that depend on whether specific genes are targeted using genome
55 editing approaches like CRISPR/Cas9, or whether morpholinos are used (Law and
56 Sargent, 2014; Kok *et al.*, 2015; Rossi *et al.*, 2015). One recently discovered human
57 single gene disorder is *EEF1A2* related epilepsy, for which model systems are badly
58 needed. In this study we sought to catalogue zebrafish *eef1a* genes, analyse their
59 expression, and determine the effects of ablating expression of translation elongation
60 factor eEF1A2 in zebrafish.

61

62 Translation elongation factor eEF1A, in its active GTP-bound form, is responsible for
63 the delivery of aminoacylated-tRNAs to the acceptor site of the ribosome during the
64 elongation step of protein synthesis. The elongation factor eEF1A is a member of the
65 G protein family and is typically encoded by more than one gene, often located on
66 distinct chromosomes in different eukaryotic species. Two sequence-redundant
67 eEF1A genes TEF1 and TEF2 are present in the yeast *Saccharomyces cerevisiae*
68 (Nagata et al., 1984; Nagashima, Nagata and Kaziro, 1986). In *Drosophila*
69 *melanogaster*, two genes, F1 and F2, have been described (Hovemann et al., 1988)
70 while four and five eEF1A genes has been reported in *Xenopus laevis* and *Solea*
71 *senegalensis* respectively (Djé et al., 1990; Infante *et al.*, 2008; Newbery et al., 2011).

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72 In mammals, although numerous pseudogenes exist, only two active genes, *EEF1A1*
73 and *EEF1A2*, encoding distinct but highly similar proteins (eEF1A1 and eEF1A2) have
74 been reported (Ann *et al.*, 1992; Knudsen *et al.*, 1993; Chambers, Peters and Abbott,
75 1998; Kahns *et al.*, 1998; Svobodová *et al.*, 2015). These genes exhibit a
76 developmental and tissue-specific pattern of expression: eEF1A1 is widely expressed
77 during development but is then down-regulated in neurons, skeletal and cardiac
78 muscle postnatally and replaced in these tissues with eEF1A2, which is concomitantly
79 upregulated (Knudsen et al., 1993; Lee, Wolfraim and Wang, 1993; Chambers, Peters
80 and Abbott, 1998; Svobodová et al., 2015).

81 In non-mammalian vertebrates the picture is less clear, but differential gene
82 expression among the eEF1A genes has been noted for *Solea senegalensis* during
83 larval development (Infante *et al.*, 2008). Expression of eEF1A genes in *Xenopus* is
84 regulated post-transcriptionally. Newbery *et al.*, 2011 showed overlapping expression
85 of eEF1A1 and eEF1A2 transcripts in the brain, heart and muscle tissues. However,
86 at the protein level they observed a down-regulation of eEF1A1 in the brain and spinal
87 cord and complete absence in *Xenopus* muscle. The eEF1A2 orthologue in *Xenopus*
88 showed the same expression pattern as that of mammals, with expression restricted
89 to the central nervous system and muscle tissues. While the importance of this isoform
90 switching remains to be elucidated, it has been suggested that the isoforms may have
91 additional distinct 'moonlighting' or non-canonical roles (reviewed in Ejiri, 2002;
92 Mateyak and Kinzy, 2010) that are required for the different cell types (Abbott *et al.*,
93 2009).

94 There are several lines of evidence implicating translation elongation factor 1A2
95 (eEF1A2) in neurological disorders. A spontaneous deletion spanning 15.8 kilobases
96 involving the promoter and first exon of *Eef1a2* is responsible for the wasted (*wst*)

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97 phenotype in mice (Chambers, Peters and Abbott, 1998; Newbery *et al.*, 2007). Mice
98 homozygous for this mutation initially develop normally but then develop muscle
99 wasting and neuronal degeneration from 21 days of age, the stage at which *Eef1a1* is
100 down-regulated to undetectable levels in these tissues (Chambers, Peters and Abbott,
101 1998; Khalyfa *et al.*, 2001). The severity of the wasted phenotype progresses rapidly,
102 leading to paralysis and death of the mouse by 28 days postnatal. On the other hand,
103 heterozygous mice are healthy and do not show any muscular or neuronal
104 abnormalities (Griffiths *et al.*, 2012).

105 More recently many heterozygous *de novo* missense mutations have been identified
106 in individuals with neurodevelopmental disorders encompassing epilepsy, intellectual
107 disability and autism (de Ligt *et al.*, 2012; Nakajima *et al.*, 2014; Veeramah *et al.*, 2014;
108 Inui *et al.*, 2016; Lam *et al.*, 2016; Lopes *et al.*, 2016). Subsequently, Cao *et al.*, 2017
109 reported a homozygous missense *EEF1A2* mutation (P333L) in siblings that resulted
110 in intractable seizures and death before the age of five from dilated cardiomyopathy .
111 The severity of these disorders makes it important that model systems are developed
112 for testing therapeutic strategies.

113 Zebrafish (*Danio rerio*) could provide a valuable model system for neurological
114 disorders resulting from mutations in eEF1A2, but relatively little is known about
115 eEF1A in zebrafish. In fact, it was first reported that only one eEF1A gene, which
116 appeared to be developmentally regulated, was present in the zebrafish genome (Gao
117 *et al.*, 1997). In addition, a gene identified as *eef1a* has been shown to be an essential
118 gene required for early embryonic development in zebrafish (Amsterdam et al, 2004).
119 More recently, Cao *et al*. 2017 reported that knockdown of *eef1a2* with morpholinos
120 resulted in small head, cardiac failure and skeletal muscle weakness at 2 days post

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121 fertilisation (dpf). Together these results would suggest that mutation of any *eef1a*
122 gene in zebrafish is lethal.

123 The complete sequence of the zebrafish genome is now available. Using this resource,
124 we have identified and characterised the expression pattern of four *eef1a* genes;
125 *eef1a1l1*, *eef1a1a*, *eef1a1b* and *eef1a2*, during development and in different adult
126 tissues. We show *eef1a1l1* to be the embryonic form being the first to be expressed
127 while *eef1a2* is the 'adult' form, detected later on during development. We went on to
128 generate *eef1a2* null zebrafish using CRISPR-Cas9 gene editing and show in contrast
129 with earlier analysis based on morpholinos, that disruption of the *eef1a2* gene is
130 compatible with normal function in zebrafish.

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132 **Materials and Methods**

133 ***Zebrafish husbandry and embryos and adult tissues collection***

134 Zebrafish of the AB strain were used for all experiments. They were maintained in the
135 MRC Human Genetics Unit (HGU) zebrafish facility at the University of Edinburgh
136 according to standard procedures (Westerfield, 2000). Embryos were raised at 28.5^{o}C
137 in Petri dishes containing E3 embryo medium and staged according to Kimmel *et al*
138 1995. For RNA isolation, embryos and larvae were collected, rinsed with water, snap-
139 frozen in dry ice and stored at -70°C until needed. Adult zebrafish were killed by
140 immersing in excess Tricaine. Tissues were quickly dissected, placed in RNAlater®
141 solution (Invitrogen) and stored at -70°C until RNA preparation. All procedures were
142 performed in accordance with the Home Office regulations and the University of
143 Edinburgh.

144 ***Bioinformatics***

145 Nucleotide and protein sequences of the eEF1A genes for zebrafish and other species
146 were obtained from the Ensembl genome browser
147 (https://www.ensembl.org/index.html). Multiple alignments of protein sequences were
148 carried out using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and
149 BoxShade v3.21 (https://embnet.vital-it.ch/software/BOX_form.html). A phylogenetic
150 tree was constructed with the MEGA version 6 software (Tamura et al., 2013) and
151 analysed using the maximum likelihood method based on the Poisson correction
152 model (Zuckerkandl and Pauling, 1965). The tree with the highest log likelihood (-
153 2809.8575) is shown. The percentage of trees in which the associated taxa clustered
154 together is shown next to the branches. Initial tree(s) for the heuristic search were
155 obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix

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156 of pairwise distances estimated using a JTT model, and then selecting the topology
157 with superior log likelihood value. The tree is drawn to scale, with branch lengths
158 measured in the number of substitutions per site. The reliability of each branch was
159 assessed using 1,000 bootstrap replicates and reliable assignment values indicated.
160 The analysis involved 24 amino acid sequences. All positions with less than 95% site
161 coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and
162 ambiguous amino acids were allowed at any position. There were a total of 462
163 positions in the final dataset.

164 ***RNA extraction and RT-PCR analysis***

165 Total RNA from adult fish tissues and approximately 50 embryos/larvae per
166 developmental stage was isolated using TRIzol® (Invitrogen) and cleaned up with the
167 RNeasy Mini Kit (Qiagen) with on-column DNase treatment using RNase-free DNase
168 (Qiagen) according to the manufacturer's instructions. RNA concentration and integrity
169 were analysed using the Agilent 2100 Bioanalyzer. Full-length cDNA was then
170 synthesised with a mix of random and oligo (dT) using the AffinityScript Multiple
171 Temperature cDNA Synthesis Kit (Agilent Genomics) according to the manufacturer's
172 protocol. RT-PCR was carried out using primers for the zebrafish eEF1A genes with
173 the Phusion High-Fidelity PCR master mix. Zebrafish *actb2* was amplified as an
174 internal control. Primer sequences are shown in Table 1. Primers for *actb2* were also
175 used to assess cDNA for genomic DNA contamination, with an additional amplicon of
176 684 bp seen if genomic DNA was present. Products were run on a 2% agarose gel.

177

178 ***Quantitative real time PCR (qRT-PCR)***

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179 RNA and cDNA preparation from brain, muscle and liver tissues of adult fish was
180 performed as described above. All qRT-PCR experiments were performed on diluted
181 cDNA (1:5 in nuclease-free water) using the Brilliant II SYBR Green qPCR Master Mix
182 (Agilent Technologies) and the 7900HT Real-Time PCR system (Applied Biosystems).
183 4 µl of each cDNA sample was added to 6 µl of qRT-PCR reaction mix following the
184 manufacturer's protocol. Reactions were performed under the following conditions: 95
185 °C for 10 minutes, 50 cycles of 95 °C for 30 seconds and 60 °C for 1 minute. For
186 normalisation of gene expression, three reference genes were used: ATPsynth, NADH
187 and 16S (selected using the geNorm kit from PrimerDesign Ltd UK). Prevalidated
188 primers (PrimerDesign Ltd UK) were used for these experiments unless otherwise
189 stated. Primer sequences shown in appendix table 1 are copyrighted by PrimerDesign
190 Ltd UK. Sequences for the reference genes are however not disclosed by the
191 company. To assess efficiencies, a standard curve was generated from seven 4-fold
192 serial dilutions of pooled cDNA from whole adult fish (1:4, 1:16, 1:64, 1:256, 1:1024,
193 1:4096 and 1:16384) for each primer pair (Appendix table 2). Gene expression was
194 quantified using the standard curve method. To compare the amount of each zebrafish
195 *eef1a* transcript, the Pfaffl method (Pfaffl, 2001) was used to calculate the gene
196 expression ratio of each target mRNA relative to the geometric average of the
197 reference genes for each tissue. Results are based on the analysis of three biological
198 replicates, each with triplicate technical replicates (a no-template control was included
199 for each gene). Significance testing was performed using the Mann Whitney test or
200 One-way ANOVA with Tukey multiple comparison tests where appropriate.

201 ***Expression vector construction and HEK293T cell line transfection***

202 Total RNA and full-length cDNA were prepared from whole adult fish as described
203 above. The zebrafish *eef1a* cDNAs were cloned into the destination vector,

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204 pcDNA6.2C-EmGFP for expression in mammalian cell lines using Gateway Cloning
205 technology (Invitrogen) following the manufacturer's instructions. The zebrafish *eef1a*
206 genes were expressed together with a GFP tag in order to be able to discriminate
207 between the exogenously expressed eEF1As and endogenous eEF1A1 of HEK293T
208 cells. Each of the constructs were transfected into HEK293T cells using the TurboFect
209 Transfection reagent (Thermo Fisher Scientific) according to the manufacturer's
210 protocol, with an empty vector control included. 24 hours before transfection, 2.4×10^4
211 cells were seeded per well of a 6-well cell culture plate containing 4ml of DMEM
212 (Gibco) with 10% Fetal Bovine Serum (FBS) growth medium. For each transfection
213 reaction, 4 µg of plasmid DNA was diluted in 400 µl of serum-free DMEM (Gibco). The
214 transfection reagent was briefly vortexed, then 6 µl was added to the diluted DNA. The
215 reaction was mixed gently and incubated for 15 minutes at room temperature. The
216 transfection mix (400 µl) was added to each well and mixed by rocking the plate gently.
217 Cells were incubated at 37 °C in a CO_2 incubator for 24 hours, after which they were
218 analysed for transgene expression.

219 ***Protein analysis***

220 Protein lysates from adult zebrafish tissues and transfected HEK293T cells were
221 prepared using RIPA lysing buffer with EDTA-free protease inhibitor (Roche).
222 Concentration of protein lysates was determined using either the Pierce BCA protein
223 assay kit (Pierce) or the DC Protein Assay (Bio-Rad) following the manufacturers'
224 instructions. Western blotting was carried out using near-infrared detection method by
225 LICOR as previously described in Davies *et al*, 2017. Protein detection was also
226 performed using the chemiluminescence method. In this case, after quantification of
227 total protein using Sypro Ruby Blot Stain (Invitrogen) was analysed, blots were
228 blocked at room temperature for 1 hour in 5% dried skimmed milk in TBS-0.1% Tween

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229 20 (TBST). Membranes were incubated with primary antibody in blocking buffer
230 overnight at 4°C, washed three times in TBST for 5 minutes and then incubated with
231 the appropriate horseradish peroxidase-conjugated secondary antibody diluted in
232 blocking buffer for 1 hour at room temperature. Finally, membranes were washed three
233 times for five minutes each in TBST and protein detected using Clarity western ECL
234 substrate (Bio-Rad) according to the manufacturer's instructions.

235 ***CRISPR/Cas9 experiment***

236 Single guide RNA (sgRNA) targeting the zebrafish *eef1a2* gene was designed using
237 the online tool CHOPCHOP (http://chopchop.cbu.uib.no/) and the oligonucleotides
238 TAGGATAAGTTGAAGGCTGAGA and AAACTCTCAGCCTTCAACTTAT purchased
239 from Integrated DNA Technologies (IDT) with a 5' phosphate modification to increase
240 ligation efficiency. The sgRNA construct was made by inserting annealed pairs of
241 oligonucleotides into BsaI (New England Biolabs) digested pDR274 (Addgene #42250,
242 Hwang *et al*, 2013) backbone. The single guide RNA plasmid was used as a template
243 to amplify gRNA sequences, which were then transcribed using the Ambion
244 MAXIscript T7 kit (Thermo Fisher Scientific). Cas9 mRNA was synthesised by
245 transcribing NotI-digested pCS2-nCas9n (Addgene #47929, Jao *et al*, 2013) using the
246 SP6 mMESSAGE mMACHINE kit (Thermo Fisher Scientific) to generate capped
247 mRNA. Purification of synthesised mRNA was performed using SigmaSpin
248 sequencing reaction clean-up kit (Sigma Aldrich) according to the manufacturer's
249 instructions.

250 *Microinjection*: Injection mixture containing 300 ng/µl Cas9 mRNA and 92 ng/µ sgRNA
251 was prepared and injected into the cell of one-cell-stage zebrafish embryos. At 2 days
252 post fertilisation (dpf), genomic DNA was extracted from a pool of 5-10 microinjected

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253 healthy embryos and the target region was amplified using the primer set 5'
254 CACCTTTATTTTTGCGTGAACA and 5' TCAAAAACATGATCACTGGGAC. In order
255 to assess the mutagenic efficiency of the gRNA, PCR products were TOPO cloned
256 using the TOPO-TA cloning kit (Invitrogen) and individual clones were sequenced.
257 Founder fish (3 months) were screened by amplifying target region using genomic
258 DNA extracted from tail fin clippings and analysing the amplicons on the Agilent 2100
259 Bioanalyser.

260 *Establishing stable mutant lines*: Putative founders were outcrossed with wild-type AB
261 fish. To determine whether the Cas9-induced mutations were heritable, genomic DNA
262 from 10 individual embryos were assessed for indels, while the others were raised to
263 adulthood. Mutant alleles were identified in F1 fish by Sanger sequencing and fish with
264 identical mutations were mated. Confirmation of the sequence of the alleles was
265 achieved with the homozygous F2 fish using Phusion High-Fidelity DNA Polymerase
266 (NEB) and Sanger sequencing.

267

268 ***Histology***

269 Adult zebrafish from both *eef1a2* mutant lines and age-matched wild-type controls
270 were fixed in 10% neutral buffered formalin (Sigma-Aldrich). Spinal cord sections were
271 cut at a thickness of 3 μM. Paraffin-embedded spinal cord sections were dewaxed with
272 xylene and rehydrated through a decreasing series of ethanol. Antigen retrieval was
273 carried out using Proteinase K for 10 minutes at room temperature and slides were
274 then treated with 3% hydrogen peroxide to block endogenous peroxidase. Sections
275 were blocked with goat serum (1:5 in PBS) for 10 minutes. They were incubated
276 overnight with anti-GFAP rabbit antibody (Dako) diluted at 1:500 in PBS, washed twice

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277 with PBS for 5 minutes and then incubated with anti-rabbit biotinylated antibody (Dako)
278 at a concentration of 1:500 in PBS for 30 minutes at room temperature. Sections were
279 then treated with Strept ABC reagent (Vector Laboratories) for 30 minutes and with
280 Diaminobenzidine (DAB; Abcam) for 10 minutes. Sections were counterstained in
281 haematoxylin solution (Shandon), dehydrated and finally mounted in DPX (VWR).

282

283 **Table 1 Sequences of primers**

Expression analysis of *eef1a* during development and adult tissues Grey highlight- indicate primer sequences are copyrighted by Primerdesign Ltd.	
***eef1a1l1* RT-PCR F**	ACCTACCCTCCTCTTGGTCG
***eef1a1l1* RT-PCR R**	GGAACGGTGTGATTGAGGGA
***eef1a1a* RT-PCR F**	TCCTCCTCTGGGTCGTTTTG
***eef1a1a* RT-PCR R**	GTAACCTTTCCGCTTGTCGC
***eef1a1b* RT-PCR F**	TCCTCTTGGTCGTTTTGCAGT
***eef1a1b* RT-PCR R**	TGTGGCTGACCCAAGTGTTT
[a]***eef1a2* RT-PCR F**	TACTGTTCTCTCTTGCCGCC
[a]***eef1a2* RT-PCR R**	TTTTCCCATCTCAGCTGCCT
actb2 F	GATCAAGATCATTGCCCCACC
actb2 R	GAGTCGGCGTGAAGTGGTAA
Expression analyses in Del and Ins4 *eef1a2* mutant lines[a]	
eef1a2 F (Primerdesign)	AGGCGGATTGTGCTGTCTT
eef1a2 R (Primerdesign)	GGCGTGTTCCCTTGTTTGG
eef1a1l1 F (Primerdesign)	GAGGAAATCACCAAGGAAGTCA
eef1a1l1 R (Primerdesign)	GTTGTCACCGTGCCATCC

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eef1a1a F (Primerdesign)	GATTGTGCTGTGCTGATTGTG
eef1a1a R (Primerdesign)	GTAAGCCAGAAGAGCGTGTT
eef1a1b F (Primerdesign)	CTTGCTGGCGTACACTCTC
eef1a1b R (Primerdesign)	GACTTCCTTCACAATCTCCTCAT
3' eef1a2 RT-PCR F	AGTATCCTCCACTGGGACGC
3' eef1a2 RT-PCR R	AGCTGATTTGGTCACTCTCCC

284

285 **Table 2: Slope, intercept and correlation coefficient (R^2) output from SDS**
286 **software to estimate efficiency of primers used for qPCR analyses.**

Primer	**Slope**	**Y-Intercept**	**Correl. Coeff. (R^2**
eef1a1l1	-3.296	11.766	0.997
eef1a1a	-3.223	19.571	0.997
eef1a1b	-3.283	20.197	0.996
eef1a2P	-3.294	21.767	0.994
eef1a2S	-3.495	20.357	0.981
3' eef1a2	-3.208	22.445	0.992
ATPsynth	-3.237	14.832	0.999
NADH	-3.227	16.500	0.996
16S	-3.018	10.194	0.991

287

288

289 ***Data availability***

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290 The authors state that all data necessary for confirming the conclusions presented in
291 the article are represented fully within the article. All fish lines and plasmids are
292 available upon request.
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297 **Results**

298 ***Characterisation of the zebrafish eef1a genes***

299 *Bioinformatics analysis of eef1a genes in the zebrafish*

300 To determine the full complement of *eef1a* genes in zebrafish, we identified four *eef1a*
301 genes using Ensembl, namely, *eef1a1l1*, *eef1a1a*, *eef1a1b* and *eef1a2* located on
302 chromosome 19, 13, 1 and 23 respectively. Each contained eight exons, seven introns
303 and an open reading frame encoding distinct proteins of 462 (Eef1a1l1, Eef1a1a and
304 Eef1a1b) or 463 (Eef1a2) amino acids. They also had similar exon-intron organisation
305 with the 5'UTRs extending into exon 2 and the 3'UTR starting in exon 8. Whilst coding
306 exons were of a consistent size, some introns of *eef1a1a*, *eef1a1b* and *eef1a2* are
307 greatly expanded compared to the compact introns of *eef1a1l1* (Fig.1a). The zebrafish
308 *eef1a* genes shared high sequence similarity at the nucleotide level within the coding
309 region and also at the amino acid level, with *eef1a1a* and *eef1a1b* being particularly
310 closely related (Fig. 1b)

311 The phylogenetic relationships of the four zebrafish Eef1a protein sequences and
312 those from other vertebrate species were analysed using the maximum likelihood
313 method (Fig. 1c). The phylogenetic tree obtained from this analysis showed both
314 Eef1a1a and Eef1a1b to fall into the eEF1A1 clade while the zebrafish Eef1a2
315 segregated with eEF1A2 from the other vertebrates. In contrast, Eef1a1l1 did not
316 cluster with any of the well-supported clades but appears to possess sequence
317 features similar to both eEF1A1 and eEF1A2.

318 Alignment of the protein sequences of eEF1A protein sequences from zebrafish,
319 mouse and human using Clustal omega (Fig. 2) also showed Eef1a1a and Eef1a1b to
320 have higher sequence identity of ~95% with mouse and human eEF1A1 than those

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321 obtained for Eef1a1l1 and Eef1a2. The zebrafish Eef1a2 showed higher sequence
322 identity of ~94% with mouse and human eEF1A2 than those obtained for Eef1a1l1,
323 Eef1a1a and Eef1a1b. On the other hand, Eef1a1l1 had similar sequence identity to
324 the eEF1A orthologues in mouse and human, with ~92% for eEF1A1 and ~90% for
325 eEF1A2 in both species.

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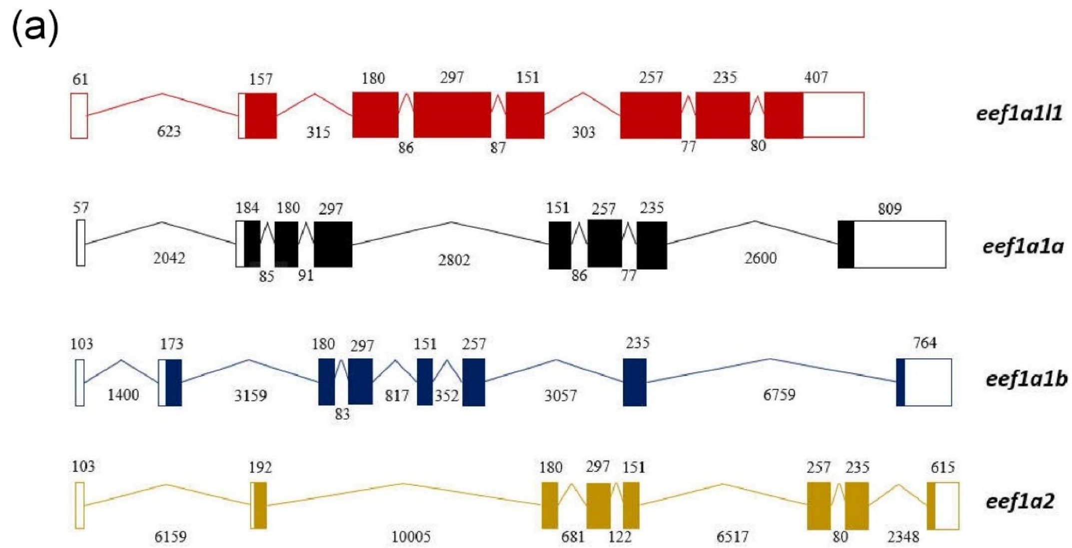
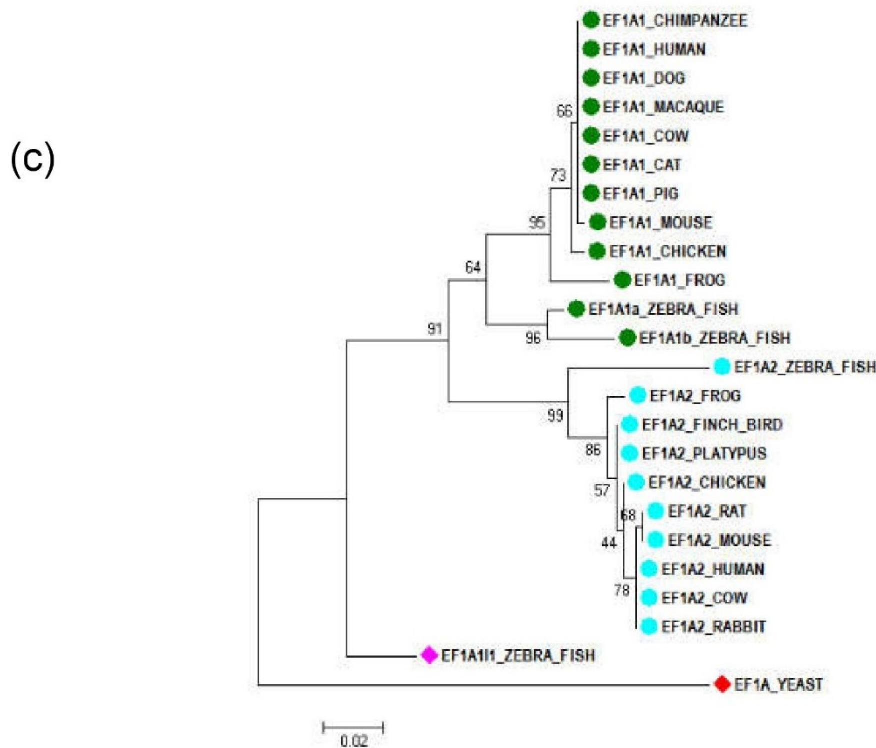


(b)

	eef1a1l1	***eef1a1a***	***eef1a1b***	***eef1a2***
eef1a1l1	100 (**100**)	80 (**92**)	79 (**91**)	75 (**89**)
eef1a1a	80 (**92**)	100 (**100**)	83 (**97**)	77 (**91**)
eef1a1b	79 (**91**)	83 (**97**)	100 (**100**)	77 (**90**)
eef1a2	75 (**89**)	77 (**91**)	77 (**90**)	100(**100**)



326

327 **Fig. 1** Four *eef1a* genes identified in the zebrafish genome. **a** Schematic representation of the exon-
328 intron organisation of *eef1a1l1* (red), *eef1a1a* (black), *eef1a1b* (blue) and *eef1a2* (yellow) structures
329 obtained from the Ensembl database. Length (in base pairs) of exons and introns, which are not drawn

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330 to scale, are indicated above and below respectively. **b** Percentage identity matrix for zebrafish eEF1As
331 at the nucleotide and amino acid sequence level (in brackets) calculated using Clustal Omega. **c**
332 Phylogenetic relationship among the zebrafish eEF1As and other vertebrate eEF1As using the
333 Maximum Likelihood method.

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```
eEf1al1     1 MGKEKTHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAEMGKGSFKYAWVL
eEf1a1a     1 MGKEKLHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAEMGKGSFKYAWVL
eEf1a1b     1 MGKEKLHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAEMGKGSFKYAWVL
heEF1A1     1 MGKEKTHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAEMGKGSFKYAWVL
meEF1A1     1 MGKEKTHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAEMGKGSFKYAWVL
eEf1a2      1 MGKEKIHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAEMGKGSFKYAWVL
heEF1A2     1 MGKEKTHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAEMGKGSFKYAWVL
meEF1A2     1 MGKEKTHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAAEMGKGSFKYAWVL

eEf1al1    61 DKLKAERERGITIDIALWKFETSKYYVTIIDAPGHRDFIKNMITGTSQADCAVLIVAGGV
eEf1a1a    61 DKLKAERERGITIDISLWKFETSKYYVTIIDAPGHRDFIKNMITGTSQADCAVLIVAAGV
eEf1a1b    61 DKLKAERERGITIDISLWKFETSKYYVTIIDAPGHRDFIKNMITGTSQADCAVLIVAAGV
heEF1A1    61 DKLKAERERGITIDISLWKFETSKYYVTIIDAPGHRDFIKNMITGTSQADCAVLIVAAGV
meEF1A1    61 DKLKAERERGITIDISLWKFETSKYYVTIIDAPGHRDFIKNMITGTSQADCAVLIVAAGV
eEf1a2     61 DKLKAERERGITIDISLWKFETTKYYITIIDAPGHRDFIKNMITGTSQADCAVLIVAAGV
heEF1A2    61 DKLKAERERGITIDISLWKFETTKYYITIIDAPGHRDFIKNMITGTSQADCAVLIVAAGV
meEF1A2    61 DKLKAERERGITIDISLWKFETTKYYITIIDAPGHRDFIKNMITGTSQADCAVLIVAAGV

eEf1al1   121 GEFEAGISKNGQTREHALLAFTLGVKQLIVGVNKMDSTEPPYSQARFEEITKEVSAYIKK
eEf1a1a   121 GEFEAGISKNGQTREHALLAYTLGVKQLIVGVNKMDSTEPSYSQKRYEEIVKEVSTYIKK
eEf1a1b   121 GEFEAGISKNGQTREHALLAYTLGVKQLIVGVNKMDSTEPNYSQKRYEEIVKEVSTYIKK
heEF1A1   121 GEFEAGISKNGQTREHALLAYTLGVKQLIVGVNKMDSTEPPYSQKRYEEIVKEVSTYIKK
meEF1A1   121 GEFEAGISKNGQTREHALLAYTLGVKQLIVGVNKMDSTEPPYSQKRYEEIVKEVSTYIKK
eEf1a2    121 GEFEAGISKNGQTREHALLAYTLGVKQLIVAVNKMDSTEPSYSEKRYDEIVKEVSAYIKK
heEF1A2   121 GEFEAGISKNGQTREHALLAYTLGVKQLIVGVNKMDSTEPAYSEKRYDEIVKEVSAYIKK
meEF1A2   121 GEFEAGISKNGQTREHALLAYTLGVKQLIVGVNKMDSTEPAYSEKRYDEIVKEVSAYIKK

eEf1al1   181 IGYNPASVAFVPISGWHGDNMLEASSNMGWFKGWKIERKEGNASGTTLLDALDAILPPSR
eEf1a1a   181 IGYNPDTVAFVPISGWNGDNMLEASPNMSWFKGWKITRKEGNAAGTTLLEALDAIQPPTR
eEf1a1b   181 IGYNPDTVAFVPISGWNGDNMLEASPNMTWFKGWKITRKDGSSSGTTLLEALDAIQPPTR
heEF1A1   181 IGYNPDTVAFVPISGWNGDNMLEPSANMPWFKGWKVTRKDGNASGTTLLEALDCILPPTR
meEF1A1   181 IGYNPDTVAFVPISGWNGDNMLEPSANMPWFKGWKVTRKDGSASGTTLLEALDCILPPTR
eEf1a2    181 IGYSPASVPFVPISGWHGDNMLEPSSNMPWFKGWKLDRKEHHAGGVTLLEALDTIMPPTR
heEF1A2   181 IGYNPATVPFVPISGWHGDNMLEPSPNMPWFKGWKVERKEGNASGVSLLEALDTILPPTR
meEF1A2   181 IGYNPATVPFVPISGWHGDNMLEPSPNMPWFKGWKVERKEGNASGVSLLEALDTILPPTR

eEf1al1   241 PTDKPLRLPLQDVYKIGGIGTVPVGRVETGVLKPGMVVTFAPANVTTEVKSVEMHHESLT
eEf1a1a   241 PTDKPLRLPLQDVYKIGGIGTVPVGRVETGLLKPGMVVTFAPVNVTTEVKSVEMHHEALS
eEf1a1b   241 PTDKPLRLPLQDVYKIGGIGTVPVGRVETGILKPGLVVTFAPVNVTTEVKSVEMHHEALS
heEF1A1   241 PTDKPLRLPLQDVYKIGGIGTVPVGRVETGVLKPGMVVTFAPVNVTTEVKSVEMHHEALS
meEF1A1   241 PTDKPLRLPLQDVYKIGGIGTVPVGRVETGVLKPGMVVTFAPVNVTTEVKSVEMHHEALS
eEf1a2    241 PTDKPLRLPLQDVYKIGGIGTVPVGRVETGVLRPSMVVTFAPVNITTEVKSVEMHHESLS
heEF1A2   241 PTDKPLRLPLQDVYKIGGIGTVPVGRVETGILRPGMVVTFAPVNITTEVKSVEMHHEALS
meEF1A2   241 PTDKPLRLPLQDVYKIGGIGTVPVGRVETGILRPGMVVTFAPVNITTEVKSVEMHHEALS

eEf1al1   301 EATPGDNVGFNVKNVSVKDIRRGNVAGDSKNDPPMEAANFNAQVIILNHPGQISQGYAPV
eEf1a1a   301 EALPGDNVGFNVKNVSVKDIRRGNVAGDSKNDPPQEAANFTAQVIILNHPGQISAGYAPV
eEf1a1b   301 EALPGDNVGFNVKNVSVKDIRRGNVAGDSKNDPPQEAASFTAQVIILNHPGQISAGYAPV
heEF1A1   301 EALPGDNVGFNVKNVSVKDVRRGNVAGDSKNDPPMEAAGFTAQVIILNHPGQISAGYAPV
meEF1A1   301 EALPGDNVGFNVKNVSVKDVRRGNVAGDSKNDPPMEAAGFTAQVIILNHPGQISAGYAPV
eEf1a2    301 EALPGDNVGFNVKNVSVKDIRRGNVCGDSKSDPPQEASGFTAQVIILNHPGQISSGYSPV
heEF1A2   301 EALPGDNVGFNVKNVSVKDIRRGNVCGDSKSDPPQEAAQFTSQVIILNHPGQISAGYSPV
meEF1A2   301 EALPGDNVGFNVKNVSVKDIRRGNVCGDSKADPPQEAAQFTSQVIILNHPGQISAGYSPV

eEf1al1   361 LDCHTAHIACKFAELKEKIDRRSGKKLEDNPKALKSGDAAIVEMVPGKPMCVESFSTYPP
eEf1a1a   361 LDCHTAHIACKFAELKEKIDRRSGKKLEDNPKSLKSGDAAIVEMIPGKPMCVESFSEYPP
eEf1a1b   361 LDCHTAHIACKFAELKEKIDRRSGKKLEDNPKSLKSGDAAIVDMIPGKPMCVESFSEYPP
heEF1A1   361 LDCHTAHIACKFAELKEKIDRRSGKKLEDGPKFLKSGDAAIVDMVPGKPMCVESFSDYPP
meEF1A1   361 LDCHTAHIACKFAELKEKIDRRSGKKLEDGPKFLKSGDAAIVDMVPGKPMCVESFSDYPP
eEf1a2    361 IDCHTAHIACKFAELKEKIDRRSGKKLEDNPKSLKSGDAAIVDMIPGKPMCVESFSQYPP
heEF1A2   361 IDCHTAHIACKFAELKEKIDRRSGKKLEDNPKSLKSGDAAIVEMVPGKPMCVESFSQYPP
meEF1A2   361 IDCHTAHIACKFAELKEKIDRRSGKKLEDNPKSLKSGDAAIVEMVPGKPMCVESFSQYPP

eEf1al1   421 LGRFAVRDMRQTVAVGVIKSVEKKIGGAGKVTKSAQKAAKTK-
eEf1a1a   421 LGRFAVRDMRQTVAVGVIKGVEKKTATSGKVTKSAQKAQKAK-
eEf1a1b   421 LGRFAVRDMRQTVAVGVIKGVEKKTSTSGKVTKSAQKAQKNK-
heEF1A1   421 LGRFAVRDMRQTVAVGVIKAVDKKAAGAGKVTKSAQKAQKAK-
meEF1A1   421 LGRFAVRDMRQTVAVGVIKAVDKKAAGAGKVTKSAQKAQKAK-
eEf1a2    421 LGRFAVRDMRQTVAVGVIKNVEKKIGGSGRVTKSAQKAQKSSK
heEF1A2   421 LGRFAVRDMRQTVAVGVIKNVEKKSGGAGKVTKSAQKAQKAGK
meEF1A2   421 LGRFAVRDMRQTVAVGVIKNVEKKSGGAGKVTKSAQKAQKAGK
```

334

335

336 **Fig. 2** Multiple amino acid sequence alignment of eEF1A orthologues from zebrafish, human and

337 mouse. The human eEF1A: heEF1A1 and heEF1A2, mouse eEF1A: meEF1A1 and meEF1A2. Identical

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338 and similar amino acid residues are indicated by black and grey backgrounds. Red asterisks (*) indicate
339 some of the clinically important human eEF1A2 mutations, each of which involves residues that are
340 completely conserved in the four zebrafish eEF1A isoforms (de Ligt *et al.*, 2012; Nakajima *et al.*, 2014;
341 Veeramah *et al.*, 2014; Inui *et al.*, 2016; Lam *et al.*, 2016; Lopes *et al.*, 2016).

342

343 *Expression of eef1a genes during development and adult tissue*

344 The expression of each zebrafish *eef1a* gene was analysed at each of twelve different
345 developmental stages; 1-cell, 2-cell, 4-cell, 8-cell, 16-cell, 256-cell, high, 50%-epiboly,
346 90%-epiboly, 24 hours post- fertilisation (hpf), 48 hpf and 72 hpf stages (Fig. 3a). Only
347 *eef1a1l1* transcripts were detected at all the stages examined. Expression of *eef1a1a*
348 and *eef1a1b* transcripts were detected at the 24 hpf, 48 hpf and 72 hpf developmental
349 stages. The zebrafish *eef1a2* gene was the last to be expressed, being detected only
350 at 48 and 72 hpf (Fig. 3b).

351 We used RT-PCR to examine the expression of the zebrafish *eef1a* genes in adult
352 tissues using total RNA extracted from brain, muscle, spleen, testis, intestine, liver and
353 ovary (Fig. 3c). Three of the zebrafish *eef1a* genes, *eef1a1l1*, *eef1a1a* and *eef1a1b*
354 were readily detected in all the tissues examined. Expression of zebrafish *eef1a2* was
355 detected in brain, muscle, spleen, testis and ovary tissues but was only just detectable
356 in the intestine. No *eef1a2* expression was seen in the liver. Using qPCR, we then
357 analysed the level of expression for each *eef1a* gene in the brain, muscle and liver
358 (Fig. 3d). The expression level of *eef1a1l1* was significantly higher in liver than in brain
359 ($p<0.01$) and muscle ($p < 0.05$). Similar expression were seen for *eef1a1a*, *eef1a1b*
360 and *eef1a2* with brain showing the highest level, followed by muscle and liver. The
361 relative amount of the four *eef1a* transcripts in these tissues were calculated (Fig. 3e).
362 In general, the most abundant transcript was that of *eef1a1l1* with approximately

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363 7,980, 7,830 and 240-fold higher overall expression ratios than *eef1a1a, eef1a1b* and
364 *eef1a2* respectively. While the relative amount of all the *eef1a* transcripts was similar
365 in brain, *eef1a1l1* showed the highest value in muscle (1,040, 1,280 and 490-fold
366 higher than *eef1a1a*, *eef1a1b* and *eef1a2* respectively) and liver (22,900 and 22,200-
367 fold higher in *eef1a1a* and *eef1a1b* respectively) tissues. The relative amount of
368 *eef1a2* transcripts in muscle was approximately two and three-fold higher than that of
369 *eef1a1a* and *eef1a1b* respectively, both of which showed similar amounts in all three
370 tissues examined.

371 *Validation of commercially available eEF1A2 antibody*

372 To investigate whether the *eef1a* mRNAs detected are translated into stable proteins
373 we needed to identify a suitable antibody. Three commercially available antibodies
374 against Eef1a2 were tested on protein lysates from adult zebrafish brain, liver and
375 muscle tissues. The Genetex and Proteintech anti-eEF1A2 antibodies detected a band
376 in liver (data not shown) in spite of the absence of Eef1a2 at the mRNA level (Fig. 3 c
377 and d). However, an antibody from Abcam detected a band only in lysates from adult
378 zebrafish brain and not in liver, consistent with our qRT-PCR data for Eef1a2 (Fig. 3f).
379 This antibody was then used to test a range of other adult tissues: muscle, spinal cord,
380 intestine, ovary and heart. Interestingly, expression was only detected in spinal cord
381 and not muscle, in contrast to our RT-PCR results (data not shown).

382 Since the interpretation of results using this antibody could be complicated by the
383 presence of other Eef1a paralogues, we carried out an antibody validation test. GFP-
384 tagged constructs were made for each of the four zebrafish *eef1a* genes, transfected
385 into HEK293T cells and lysates analysed with the eEF1A2-Abcam antibody. A band
386 of the expected size was observed in lanes containing lysates isolated from cells

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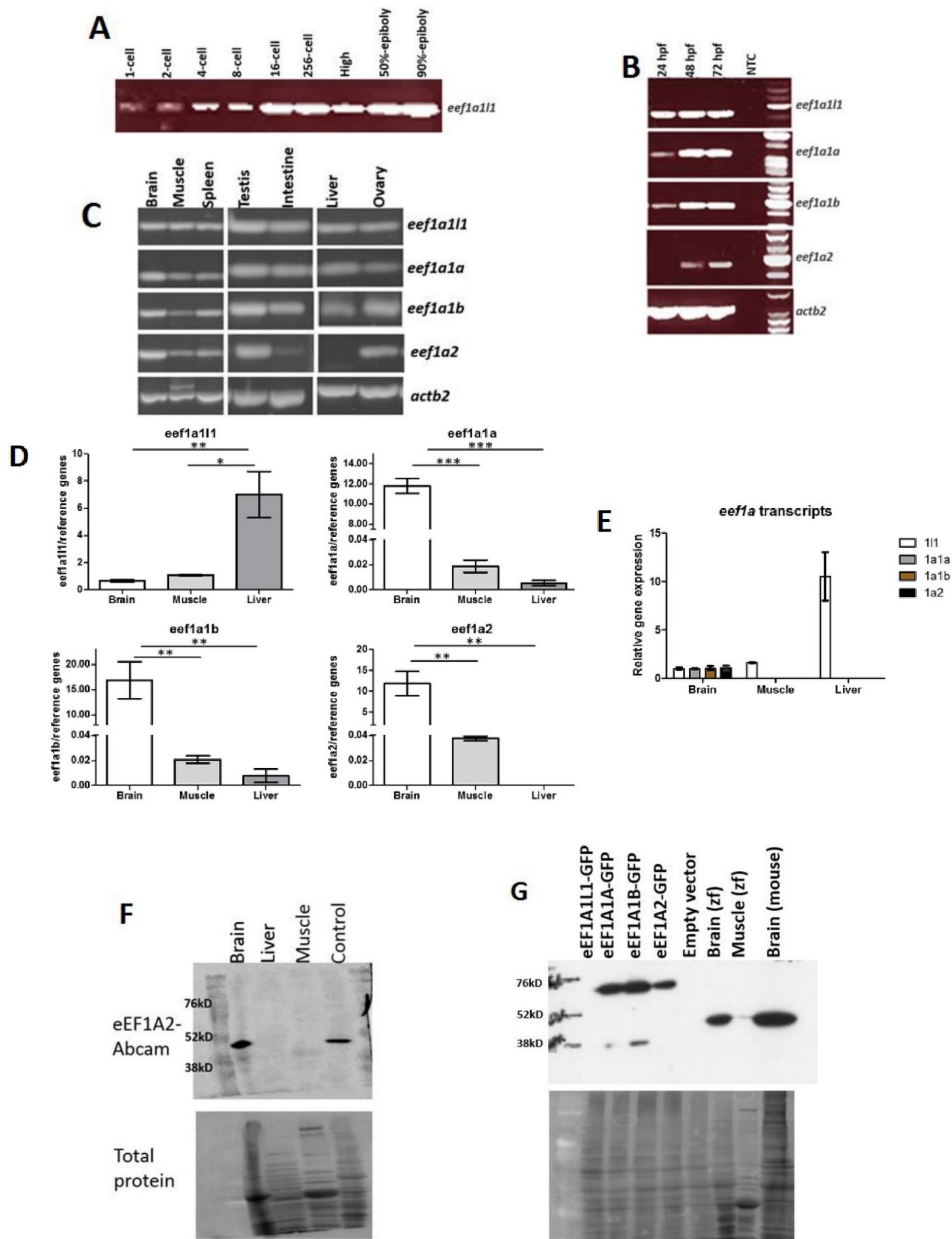
387 transfected with GFP-tagged Eef1a1a, Eef1a1b and Eef1a2 (Fig. 3g), suggesting that

388 this antibody cross-reacts with other Eef1a paralogues.



389

390 **Fig. 3** Expression analysis of the zebrafish *eef1a* genes. **a** Expression of *eef1a1l1* in different early

391 embryonic stages detected by RT-PCR. The other eef1a genes were undetected at these stages (data

392 not shown). **b** Expression of *eef1a* genes in 24 hpf, 48 hpf and 72 hpf developmental stages using RT-

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393 PCR. NTC – no template control. **c** Expression of *eef1a* genes in different tissues of adult zebrafish
394 detected by RT-PCR. **d** Expression levels of *eef1a* genes in brain, muscle and liver tissues **e** and
395 comparison of the relative levels of their transcripts in these tissues. Expression values were normalised
396 to those of *ATPsynth*, *NADH* and *16S*. Results are means $\pm$ S.E.M, n=3. *p < 0.05; **p < 0.01; ***p <
397 0.0001. For comparison, data were presented as the gene expression ratio of the target mRNA to the
398 geometric mean of reference genes for each tissue. **f** Western blot showing Eef1a2 expression in brain,
399 liver and muscle zebrafish tissues using eEF1A2-Abcam antibody (1:1000). Control is muscle tissue
400 from mouse. **g** Validation of eEF1A2-Abcam antibody specificity using lysates isolated from individually
401 transfected HEK293T cells with GFP-tagged Eef1a constructs. Zf- zebrafish.

402

403 ***CRISPR/Cas9 generated Eef1a2-null zebrafish survive to adulthood***

404 We next sought to investigate the effect of loss of Eef1a2 in zebrafish using the
405 CRISPR/Cas9 system. We wanted to establish whether zebrafish, like mice, undergo
406 fatal neurodegeneration in response to the loss of eEF1A2 after developmental down-
407 regulation of eEF1A1; consistent with the lethal phenotype arising from the use of
408 morpholinos against eEF1A2 as shown by Cao *et al.*, 2017 or whether the presence
409 of three paralogues in zebrafish gives rise to redundancy. A single gRNA targeting the
410 zebrafish *eef1a2* gene was designed and microinjected, together with Cas9 mRNA,
411 into one-cell embryos. The gRNA showed a mutagenic activity rate of ~77% and a
412 survival rate of 93% was observed within the CRISPR-injected embryos. At 2 months,
413 adult (F0) injected zebrafish were genotyped using genomic DNA from tail fin clipping
414 in order to identify potential founders. PCR amplicons containing the target region
415 were analysed on the Agilent 2100 Bioanalyser which showed several distinct
416 mutations at the target site were present in these fish (supplementary figure 1). In
417 order to establish stable null mutant lines, F1 embryos were obtained from one of the
418 mosaic putative founders outcrossed with wild-type fish. These embryos were then

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419 raised to adulthood and three mutant alleles, a 12 base pair deletion, a 4 base pair
420 insertion and a 2 base pair deletion, were recovered (Fig. 4a). The 4 base pair insertion
421 (hereafter referred to as Ins4) and 2 base pair deletion (hereafter referred to as Del2)
422 were chosen for further analysis since they were each predicted to give rise to
423 frameshifts resulting in premature stop codons (Fig. 4a). Heterozygous F1 zebrafish
424 carrying the same mutations were then intercrossed and the embryos raised to
425 adulthood. Interestingly, homozygous Ins4 and Del2 fish survived to adulthood with no
426 obvious phenotypic differences from their wild type siblings, and were fertile (Fig. 4b).

427

428 *Expression analysis shows reduced eef1a2 transcripts in Ins4 and Del2 lines*

429 We then went on to investigate the effect of the mutant alleles in each of the Ins4 and
430 Del2 lines. The expression level of *eef1a2* mRNA was assessed using two different
431 set of primers, eef1a2P and 3'eefla2, for both Ins4 and Del2 lines and a third primer
432 set, eef1a2S, for Ins4 only (supplementary figure 2). The position of the primers in
433 relation to the target site is shown in figure 4c.The primer 3'eefla2 was designed such
434 that it is located towards the extreme 3' end of the *eef1a2* mRNA such that any
435 transcripts downstream of the target site that could lead to translation of a protein
436 would be detected. A marked decrease of *eef1a2* mRNA was seen in both Ins4 and
437 Del2 lines compared to their wild type siblings using each of the different sets of
438 primers (Fig. 4c). Approximately 81% and 92% reduction in the levels of *eef1a2*
439 expression was seen in Del2 homozygous adult brains when compared to their wild-
440 type siblings and a reduction of approximately 86% and 95% in brain tissues of
441 homozygous Ins4 adult fish was seen compared to wild-type. These results suggest
442 that each of the mutant alleles, Ins4 and Del2, lead to decreased messenger RNA

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443 levels possibly through nonsense-mediated decay (NMD). Homozygous mutant fish
444 for both lines are thus effectively *eef1a2*-null.

445

446 *Immunohistochemical assessment of Ins4 and Del2 mutants*

447 Complete loss of eEF1A2 in mice has been well characterised and causes motor
448 neuron degeneration of the anterior horn of the spinal cord and muscle wasting, which
449 is of neurogenic origin (Doig *et al.*, 2013). We examined spinal cord sections from
450 homozygous Ins4 and Del2 adult fish to evaluate them for the presence of gliosis (a
451 reactive response to injuries such as neurodegeneration in the central nervous
452 system) using immunohistochemistry for glial fibrillary acidic protein (GFAP). High
453 levels of GFAP staining is seen in human cases of motor neuron degeneration and in
454 animal models, including in the anterior horn of the spinal cord of eEF1A2-null mice
455 (Newbery *et al.*, 2005). However, no increased staining of GFAP was seen in the spinal
456 cord sections of homozygous Del2 and Ins4 mutants when compared to wild-type (Fig.
457 4d). This result, together with apparently normal histology and normal survival,
458 demonstrates that there is no evidence of neurodegeneration in the spinal cord of
459 either *eef1a2*-mutant zebrafish lines.

460

461 *Other eef1a*s *mRNA remained unchanged in Ins4 and Del2 lines*

462 We next quantified the mRNA level of the other zebrafish *eef1a* genes in order to
463 address both whether the lack of phenotype was a result of a compensatory
464 mechanism from the other genes and also whether any off-target effect involving
465 homologous *eef1a* genes had occurred. The results obtained showed no significant
466 change in the mRNA level of any of the other *eef1a* genes in the brain of adult

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467 homozygous Ins4 and Del2 fish (Fig. 4e), suggesting that they were unaffected by the
468 CRISPR/Cas9 targeting of *eef1a2*, and also that no compensatory mechanism had
469 occurred, at least at the mRNA level.

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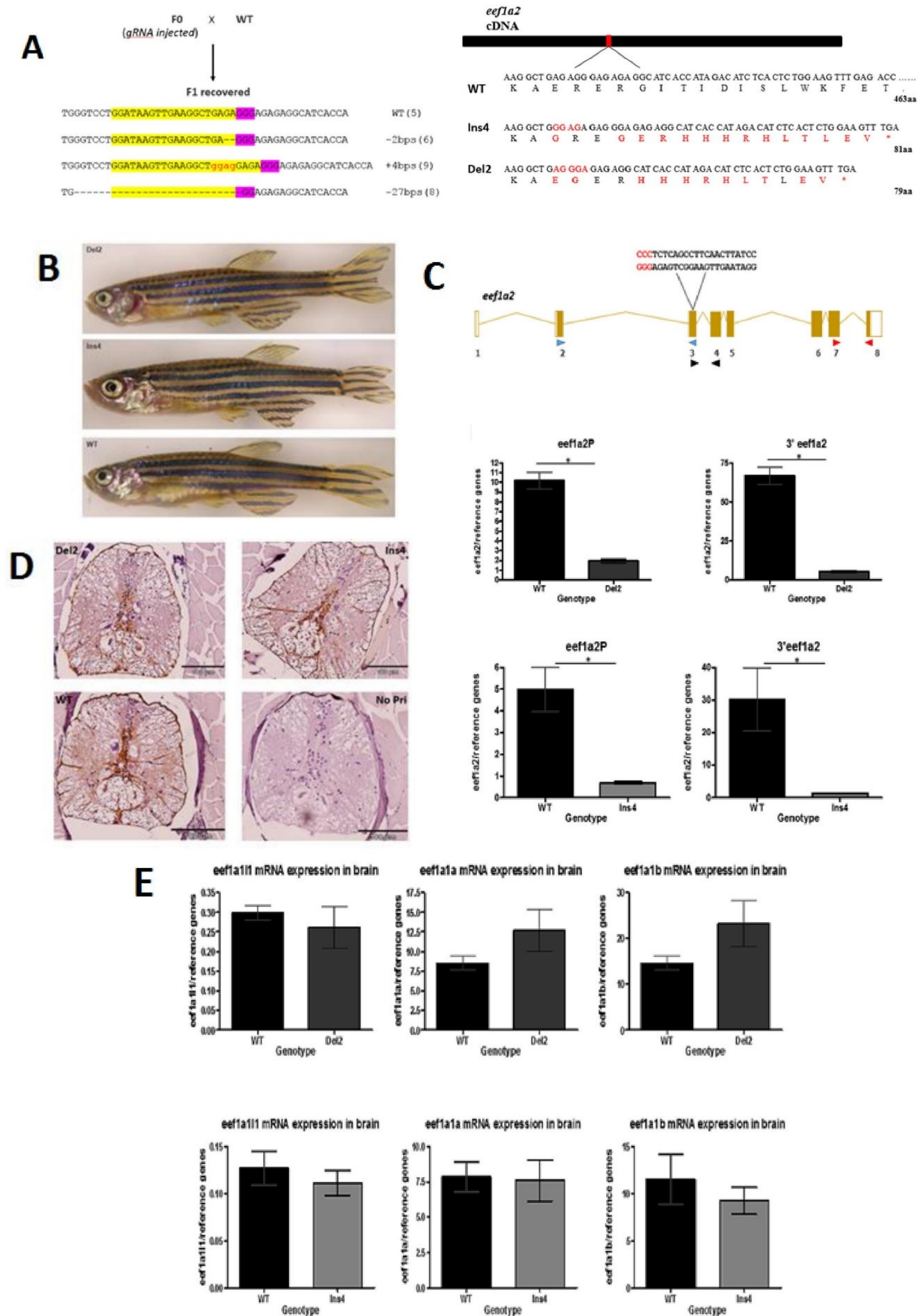

470

471 **Fig. 4 a** Schematic showing outcross mating of founder (F0) fish and wild-type fish showing recovered

472 F1 sequences (number of F1 fish for each allele are indicated in brackets, target sequences (yellow

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473 highlight) and PAM site (purple) with red showing inserted bases) and the predicted effect of Ins4 and
474 Del2 mutant allele with aberrant residues shown in red (right). **b** No overt difference in homozygous
475 Del2 (6 months) and homozygous Ins4 (8 months) adult fish from wild-type (6 months) adult fish. **c** The
476 position of the three different primer sets; eef1a2S (Blue triangle), eef1a2P (black triangle) and 3'eef1a2
477 (red triangle) is illustrated in relation to the gRNA target site (PAM site sequence in red). Expression
478 levels of *eef1a2* in homozygote Del2 (top panel) and homozygote Ins4 (bottom panel) fish using
479 eef1a2P and 3'eef1a2 primer sets. Results were normalised to *ATPsynth*, *NADH* and *16S*. (means $\pm$
480 S.E.M; N=3) *$p < 0.05$. **d** Anti-GFAP antibody stained transverse sections of spinal cords of
481 homozygous Del2 and Ins4 adult fish showed no sign of neurodegeneration. Negative control of a no
482 primary (No Pri) was included which showed no staining. Scale bar = 100μm **e** expression levels of
483 *eef1a1l1* (left), *eef1a1a* (middle) and *eef1a1b* (right) mRNA in homozygous Del2 (top panel) and Ins4
484 (bottom panel) adult brain. Date are normalised to *ATPsynth*, *NADH* and *16S* and were presented as
485 means $\pm$ S.E.M.; n=3.

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488 **Discussion**

489 Four different eEF1A genes, namely *eef1a1l1*, *eef1a1a*, *eef1a1b* and *eef1a2* are
490 present in the zebrafish genome and share high sequence identity both at the
491 nucleotide (75 - 83 %) and amino acid (89 – 97 %) levels. The nucleotide and amino
492 acid sequences of *eef1a1a* and *eef1a1b* are more similar to each other than the other
493 paralogous genes, suggesting that they arose from the additional teleost-specific
494 genome duplication event which took place at the base of the teleost fish evolutionary
495 lineage (Christoffels et al., 2004). It is clear from our phylogenetic and sequence
496 alignment analyses that the zebrafish Eef1a1a and Eef1a1b are co-orthologues of
497 mammalian eEF1A1. Whilst Eef1a1l1 appears to lack a mammalian orthologue, the
498 data also give strong support to zebrafish Eef1a2 being the sole orthologue of
499 mammalian genes encoding eEF1A2.

500 We have also demonstrated that all four zebrafish *eef1a* genes are actively transcribed
501 and are expressed in a developmental-specific pattern, consistent with the pattern
502 seen in other vertebrates. During embryogenesis, *eef1a1l1* is the only gene shown to
503 have maternal contribution in addition to zygotic expression as it was detected at all
504 embryonic stages analysed. Interestingly, *eef1a1l1* (formerly referred to as *eef1a*) has
505 been shown to be an essential gene required for early embryonic development in
506 zebrafish (Amsterdam et al, 2004). In this study, mutation in *eef1a1l1* was shown to
507 result in abnormal phenotypes such as small head and eyes from 2 dpf and eventually
508 death at 5 dpf from failure of the swim bladder to inflate. We found that the next *eef1a*
509 genes to be detected were *eef1a1a* and *eef1a1b*, while *eef1a2* was the last to be
510 expressed, at 48 hpf. The detection of *eef1a2* at a later developmental stage is
511 consistent with that of mammals, where its expression is observed much later in
512 development than eEF1A1, gradually replacing it in skeletal muscle and neurons

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513 (Knudsen *et al.*, 1993; Lee, Wolfraim and Wang, 1993; Chambers, Peters and Abbott,
514 1998; Svobodová e*t al.*, 2015).

515 In all adult tissues analysed, we detected mRNA derived from the *eef1a1l1*, *eef1a1a*
516 and *eef1a1b* genes. On the other hand, *eef1a2* showed a tissue-specific expression
517 pattern as its mRNA was not present in the liver and was only just detected in the
518 intestine, again similar to the expression seen in mammals. The difference between
519 zebrafish *eef1a2* and the mammalian and *Xenopus* eEF1A2 orthologues however, is
520 the presence of zebrafish *eef1a2* mRNA in spleen and ovary tissue samples. Whilst
521 the expression pattern of *eef1a1a* and *eef1a1b* is in contrast to that of their mammalian
522 orthologues, it is consistent with that of *Xenopus* where eEF1A1 mRNA, in addition to
523 eEF1A2, was detected in adult muscle (Newbery *et al*, 2011). Despite the *eef1a* genes
524 being co-expressed in the tissues, quantification of their expression levels in the brain,
525 muscle and liver suggests that they are not present in equal amounts. As a whole,
526 *eef1a1l1* transcripts are the most abundant, in muscle and liver compared to the other
527 *eef1a* genes, while the levels of all the *eef1a* mRNA species were the same in the
528 brain. The *eef1a2* transcript was the second most abundant in the muscle. In line with
529 being co-paralogues, *eef1a1a* and *eef1a1b* exhibited the same expression pattern in
530 these tissues. The finding that the zebrafish *eef1a* genes display distinct expression
531 profiles suggest they may have evolved unique roles hence their being retained after
532 the duplication events.

533 We were unable to establish specific expression patterns for zebrafish eef1a genes at
534 the protein level as all commercially available eEF1A antibodies we tested failed to
535 distinguish between the products of the different zebrafish eef1a genes. We were,
536 however, able to see strong expression of *eef1a2* specifically in brain.

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537 We went on to generate two *eef1a2* mutant lines, Ins4 and Del2, using CRISPR/Cas9
538 genome editing. Our qPCR data showed a substantial decrease in *eef1a2* expression
539 in each of the two mutant lines with either of two sets of primers. This finding indicates
540 that the mutant transcripts are likely targets of nonsense-mediated decay, suggesting
541 that both the Ins4 and Del2 mutations are effectively null, since any remaining
542 transcript would not encode a functional protein. Homozygous loss of *eef1a2* was not
543 lethal in either of our zebrafish mutant lines, in contrast to the situation in mice, which
544 die before 4 weeks in the absence of eEF1A2. Adult homozygous Ins4 and Del2
545 mutants showed no obvious phenotypic abnormalities, were fertile and produced
546 viable embryos. There are three immediate possible explanations for this discrepancy.
547 Firstly the regenerative capacity of the zebrafish CNS could be masking any
548 neurodegeneration. Secondly, it remains possible the Ins4 and Del2 mutants retain
549 some degree of residual function of *eef1a2*. However, the consistency of the *eef1a2*
550 reduction observed with three different set of primers at different locations from the
551 target site makes this explanation unlikely, and any protein produced from the residual
552 transcripts would be so truncated that they would be highly unlikely to be functional.
553 The third explanation, which seems most likely, is that there is functional redundancy
554 as a result of the three additional *eef1a* genes which were found to be co-expressed
555 with *eef1a2* at the mRNA level. During the course of this work, Cao *et al*. 2017 reported
556 that knockdown of *eef1a2* with morpholinos resulted in abnormal phenotypes including
557 small head size, cardiac failure and skeletal muscle weakness in 2 dpf morphants.
558 Since the development of efficient genome editing techniques in zebrafish it has been
559 increasingly recognised that CRISPR-induced mutants can fail to replicate
560 morpholino-induced phenotypes (Kok et al, 2015). Cao *et al*, did not investigate
561 whether other *eef1a* gene(s) had been down-regulated by the morpholinos used, and

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562 it is thus likely that the phenotypes observed were not specific to *eef1a2* but rather the
563 combined effect of the knockdown of one or more of the other *eef1a* genes.
564 Interestingly, a small head, which was one of the phenotypes observed in the *eef1a2*
565 morphants, was also reported in 2 dpf *eef1a1l1* mutant recovered from a large
566 retroviral-mediated insertional mutagenesis screen (Amsterdam *et al*. 2004). Although
567 the phenotypes observed were consistent between different types of morpholinos,
568 translational and splice-site targeting, it is still possible that they may have been the
569 result of a common off-target toxic effect induced by both morpholinos. This type of
570 situation has been demonstrated in the study by Robu *et al.*, 2007, who observed that
571 these two different types of morpholinos induced off-target effects mediated through
572 p53 activation in the zebrafish embryo. Furthermore, Kok et al showed that many
573 morpholino-induced phenotypes in zebrafish, even those that could be rescued by co-
574 injecting with the wild-type mRNA, were likely due to off-target effects and that off-
575 target phenotypes induced by the use of morpholinos occurred much more frequently
576 than was previously thought (Kok et al 2015). The discrepancy between our findings
577 and those of Cao *et al*, 2017 might be due to the different approaches used which in
578 turn induced different responses to *eef1a2* inactivation in zebrafish. Rossi *et al.*, 2015
579 demonstrated that genetically induced severe mutations resulted in compensatory
580 upregulation of specific proteins which rescued the phenotypes observed. However,
581 our qPCR results show that no upregulation of any of the other *eef1a* genes occur at
582 the mRNA level. Again, this is consistent with the idea of functional redundancy of
583 *eef1a* genes as the most likely explanation for the lack of abnormalities after the loss
584 of *eef1a2* in Ins4 and Del2 mutant lines. In contrast to morpholinos, CRISPR/Cas9 has
585 been shown to have negligible off-target effects in zebrafish. Using next-generation
586 sequencing (NGS), Hruscha *et al.*, 2013 demonstrated that off-target effects were

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587 limited in founder fish. This study was small but was supported by another larger study
588 in which the target sites for five gRNAs targeted to different genes were analysed. One
589 3 base pair deletion was found, in only one of the 25 off-target loci tested (Varshney
590 *et al.*, 2015). The possibility of random off-target events occurring in the Ins4 and Del2
591 lines cannot be ruled out, but, the use of mutant fish starting from the F2 generation
592 and resulting from an outcross of the F0 fish with wild-type fish should minimise the
593 risks as off-target mutations should segregate away from the Del2 and Ins4 *eef1a2*
594 mutation (Schulte-Merker and Stainier, 2014). Furthermore, the comparison of two
595 independent mutations and the lack of any observable abnormalities suggest that off-
596 target effects are not a concern.

597 Overall, our results suggest that ablating the expression of eEF1A2 in zebrafish is
598 unlikely to provide a model system in which to study disease-causing loss of function
599 mutations. However, if the epilepsy-causing missense mutations seen in humans in
600 fact represent a toxic gain of function, our new Del2 and Ins4 lines could provide an
601 important resource in which to test the effects of expression of mutant eEF1A2 in the
602 form of human mRNA.

603 **Acknowledgements**

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607 support and helpful advice.

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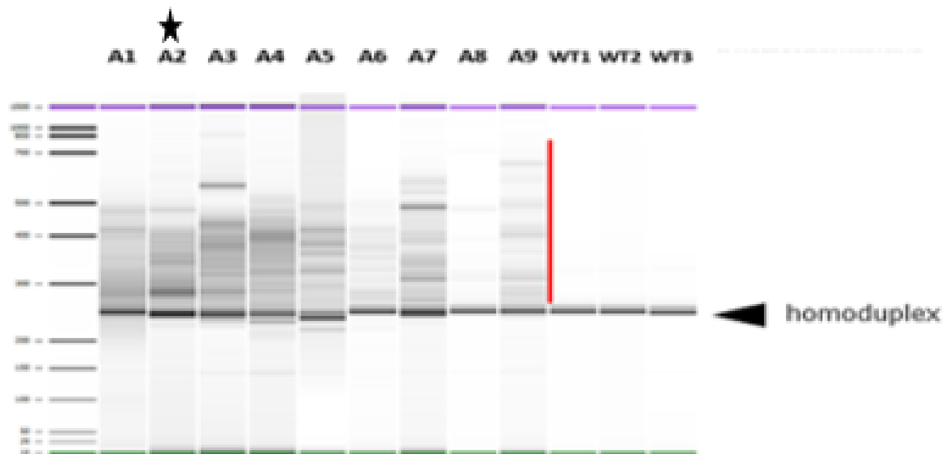
611 **Supplementary figures**

612 **Supplementary figure 1**

613



614

615 **Screening of potential F0 mutants injected with gRNA by running PCR**
616 **amplicons of target site on the Agilent 2100 Bioanalyser**. A mismatch between
617 wild type and mutant strands gives rise to heteroduplex ((shown by the red line)), which
618 indicates the presence of indels in these fish which are mosaic at this stage. Black star
619 indicates founder (F0) fish used to generate Ins4 and Del2 lines. WT1, WT2 and WT3
620 indicate PCR products obtained from three different uninjected wild-type fish fin-
621 clippings.

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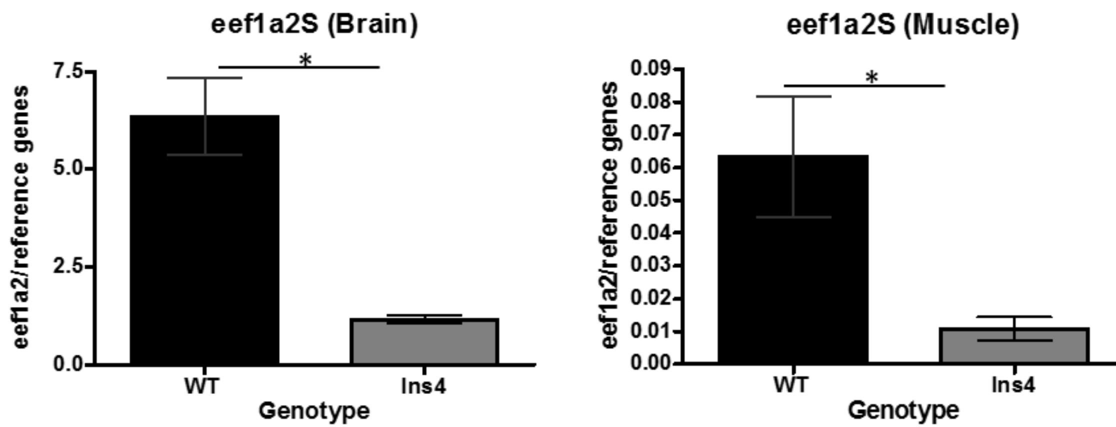
624 **Supplementary figure 2**



625

626

627 **Analysis of *eef1a2* transcripts in Ins4 mutants using eef1a2S**. Reduced *eef1a2*
628 transcript levels in F2 Ins4 homozygous (3 months) brain and muscle tissues was also
629 noted using this set of primers.

630