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# Genome editing reveals reproductive and developmental dependencies on specific types of 2 vitellogenin in zebrafish (*Danio rerio*)

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1 **Title: Genome editing reveals reproductive and developmental dependencies on specific types of**  
2 **vitellogenin in zebrafish (*Danio rerio*)**

3  
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11

12 **ABSTRACT**

13 Oviparous vertebrates produce multiple forms of vitellogenin (Vtg), the major source of yolk  
14 nutrients, but little is known about their individual contributions to reproduction and development. This  
15 study employed a CRISPR/Cas9 genome editing to assess essentiality and functionality of zebrafish  
16 (*Danio rerio*) type-I and -III Vtgs. The multiple CRISPR approach employed to knock out (KO) all genes  
17 encoding type-I *vtgs* (*vtg1*, 4, 5, 6, and 7) simultaneously (*vtg1*-KO), and the type-III *vtg* (*vtg3*)  
18 individually (*vtg3*-KO). Results of PCR genotyping and sequencing, qPCR, LC-MS/MS and Western  
19 blotting showed that only *vtg6* and *vtg7* escaped Cas9 editing. In fish whose remaining type-I *vtgs* were  
20 incapacitated (*vtg1*-KO), and in *vtg3*-KO fish, significant increases in Vtg7 transcript and protein levels  
21 occurred in liver and eggs, a heretofore-unknown mechanism of genetic compensation to regulate Vtg  
22 homeostasis. Fecundity was more than doubled in *vtg1*-KO females, and fertility was ~halved in *vtg3*-KO  
23 females. Substantial mortality was evident in *vtg3*-KO eggs/embryos after only 8 h of incubation and in  
24 *vtg1*-KO embryos after 5 d. Hatching rate and timing were markedly impaired in *vtg* mutant embryos and  
25 pericardial and yolk sac/abdominal edema and spinal lordosis were evident in the larvae, with feeding and  
26 motor activities also being absent in *vtg1*-KO larvae. By late larval stages, *vtg* mutations were either

27 completely lethal (*vtg1*-KO) or nearly so (*vtg3*-KO). These novel findings offer the first experimental  
28 evidence that different types of vertebrate Vtg are essential and have disparate requisite functions at  
29 different times during both reproduction and development.

30

31 Keywords; *CRISPR/Cas9*, *knock out*, *vitellogenins*, *zebrafish*

32

### 33 1. INTRODUCTION

34 In oviparous animals, maternally supplied vitellogenins (Vtgs) are the major source of yolk  
35 nutrients supporting early development. Vertebrate Vtgs are specialized members of a superfamily of  
36 large lipid transfer proteins that are preferentially produced by the liver and transported via the  
37 bloodstream to the ovary (Babin et al., 2007). The Vtgs are taken up into growing oocytes via receptor-  
38 mediated endocytosis (Opresko and Wiley, 1987), where they are processed by the lysosomal  
39 endopeptidase, cathepsin D, into product yolk proteins that are stored in the ooplasm (Carnevali et al.,  
40 1999a,b, 2006). Jawed vertebrates produce three major forms of Vtg arising from a *vtg* gene cluster that  
41 was present in the ancestor of tetrapods and ray-finned fish (Babin, 2008; Finn et al., 2009). During  
42 vertebrate evolution these ancestral *vtg* genes were subject to whole genome duplications, loss of paralogs  
43 and lineage-specific tandem duplications, giving rise to substantial variation in the repertoire and number  
44 of *vtg* genes present in an individual species, especially among teleost fish (Andersen et al., 2017). The  
45 linear yolk protein domain structure of complete teleost Vtgs is: NH<sub>2</sub>-lipovitellin heavy chain (LvH)-  
46 phosphitin (Pv)-lipovitellin light chain (LvL)-beta component ( $\beta'$ c)-C-terminal component (Ct)-COOH  
47 (Patiño and Sullivan, 2002; Hiramatsu et al., 2005). Most teleosts possess from two to several forms of A-  
48 type Vtg (VtgA), which may be complete or incomplete, as well an incomplete C-type Vtg (VtgC)  
49 lacking both Pv and the two small C-terminal yolk protein domains ( $\beta'$ c and Ct). For example, the  
50 complex zebrafish (*Danio rerio*) Vtg repertoire includes five type-I Vtgs (Vtg 1, 4, 5, 6 and 7) that are  
51 incomplete, lacking  $\beta'$ -c and Ct domains (=ostariophysan VtgAo1), two type-II Vtgs (Vtg2 and Vtg8) that  
52 are complete (=VtgAo2), and one type-III Vtg (Vtg3), which is a typical VtgC (Yilmaz et al., 2018).

53           The multiplicity of teleost Vtgs and the roles that different types of Vtg play in oocyte growth and  
54 maturation and in embryonic and larval development has been target of attention for decades (Hiramatsu  
55 et al., 2005; Reading and Sullivan, 2011; Sullivan and Yilmaz, 2018). The most diverse group of fishes,  
56 the spiny-rayed teleosts (Acanthomorpha) generally possess two paralogous complete forms of VtgA  
57 (VtgAa, and VtgAb) in addition to VtgC, and these are orthologs of the zebrafish type-I, type-II and type-  
58 III Vtgs, respectively (Finn et al., 2009). In some marine species spawning pelagic eggs, the VtgAa has  
59 become neofunctionalized so that its product yolk proteins are highly susceptible to proteolytic  
60 degradation by cathepsins during oocyte maturation, yielding a pool of free amino acids (FAA) that  
61 osmotically assist oocyte hydration and acquisition of proper egg buoyancy (Matsubara et al., 1999; Finn  
62 and Kristoffersen, 2007) and that also serve as critical nutrients during early embryogenesis (Thorsen and  
63 Fyhn, 1996; Finn and Fyhn, 2010). The major yolk protein derived from the corresponding VtgAb  
64 (LvHAb) is less susceptible to maturational proteolysis. Based on its limited degradation during oocyte  
65 growth and maturation, and its utilization late in larval life in some species, it has been proposed that the  
66 VtgC may be specialized to deliver large lipoprotein nutrients to late stage larvae without affecting the  
67 osmotically active FAA pool (Reading et al., 2009; Reading and Sullivan, 2011). Aside from these few  
68 examples, very little is known about specific contributions of the different types of Vtg to developmental  
69 processes in acanthomorphs, virtually nothing is known about specialized functions of individual types of  
70 Vtg in other vertebrates, and no individual form of Vtg has been proven to be required for the  
71 developmental competence of eggs or offspring.

72           The zebrafish has become an established biomedical model for research on reproduction and  
73 developmental biology because they are small, easily bred in the laboratory with short generation time,  
74 and lay clutches of numerous large eggs every few days, with external fertilization of the transparent eggs  
75 in which embryonic development is easily observed (Ribas and Piferrer, 2013). A reference genome  
76 sequence is available, providing the needed databases and bioinformatics tools to conduct genomic and  
77 proteomic research on Vtgs in this species. Details on the genomic and protein domain structure of each  
78 individual zebrafish Vtg and on their transcript expression and protein abundance profiles were recently

79 made available by Yilmaz et al. (2018). Coupled with these advantages, the presence of multiple genes  
80 encoding the three classical major types of Vtg in zebrafish offers a unique opportunity to investigate  
81 their essentiality and functionality via application of CRISPR/Cas9 (clustered regularly interspaced short  
82 palindromic repeats (CRISPR)/CRISPR-associated protein 9) technology (Doudna and Charpentier,  
83 2014), a powerful gene-editing tool that provides a reliable process for making precise, targeted changes  
84 to the genome of living cells.

85 The extensive multiplicity of genes encoding type-I Vtgs, the major contributors to yolk proteins  
86 in zebrafish eggs, is a matter of interest considering their lack of  $\beta'$ - and Ct- domains, which contain 14  
87 highly conserved cysteine residues that are known to be involved in disulfide linkages required for  
88 complex folding of the Vtg polypeptide and possibly for the dimerization of native Vtg thought to be  
89 required for binding to its oocyte receptor (Reading et al., 2009; Reading and Sullivan 2011).  
90 Additionally, the type-III Vtg (VtgC), lacking all but Lv domains and usually being the least abundant  
91 form of Vtg, but one universally present in teleosts, begs investigation regarding its contributions to early  
92 development. Therefore, the main objectives of this study were to discover whether type-I Vtgs and type-  
93 III Vtg (VtgC) are required for zebrafish reproduction, and to identify specific developmental periods and  
94 processes to which they significantly contribute, by investigating the effects of knock out (KO) of their  
95 respective genes using the CRISPR/Cas9 gene-editing tool.

96

## 97 2. RESULTS

98 Large deletion mutations of 1821 bp and 1182 bp of gDNA were introduced in zebrafish type-I  
99 *vtgs* (*vtg1*-KO) and in *vtg3* (*vtg3*-KO), respectively, via CRISPR/Cas9 genome editing. The introduced  
100 deletions involved 703 bp and 714 bp of the respective transcripts, encoding 234 aa and 239 aa of their  
101 respective polypeptide sequences, and they resulted in double strand breaks in the ORF in both cases (**Fig**  
102 **1, S1 Fig**). For both *vtg1*-KO and *vtg3*-KO, the introduced mutation altered the structure of the deduced  
103 LvH chain of the Vtg polypeptide and, in the case of *vtg3*-KO, it extended into the Vtg receptor-binding  
104 domain (**Fig 2, S1 Fig**). Introduced mutations were detected by genotyping via PCR screening of gDNA

105 at each generation using combinations of primers flanking each altered target site (**Fig 1**). F0 generation  
106 individuals exhibiting a heterozygous mutant double banding pattern were retained as founders for  
107 production of stable mutant lines (**Fig 3**).

108 Microinjection efficiency was acceptable and high, resulting in 20% and 80% mutation positive  
109 embryos at 24h screening, for *vtg1*-KO and *vtg3*-KO, respectively. This efficiency was confirmed by  
110 finclip genotyping when these embryos reached adulthood. However, mutation transmission to F1  
111 offspring was as low as 0.010% for *vtg1*-KO and 0.025% for *vtg3*-KO, and only 2 heterozygous (Ht:  
112 *vtg1*+/+ and *vtg3*+/+) adult males were available to continue reproductive crosses with non-related wild  
113 type (Wt: *vtg1*+/+ and *vtg3*+/+) females for production of F2 generations. The rate of mutation  
114 transmission to the F2 generation produced from F1 Ht males and Wt females was 55% and 70% for  
115 *vtg1*-KO and *vtg3*-KO, respectively. Reproductive crosses of Ht males and Ht females revealed a  
116 Mendelian inheritance pattern with 25% wild type (wt: sibling wild type; *vtg1*+/+ and *vtg3*+/+), 52%  
117 heterozygous and 22% homozygous (Hm: *vtg1*-/- and *vtg3*-/-) individuals at the F3 generation. Hm F3  
118 females and males were crossed to produce the F4 generation yielding 100% homozygous offspring  
119 carrying only the mutated allele (**Fig 3**). As these Hm individuals are generally inviable (*see below*),  
120 production of subsequent generations of mutants requires crossbreeding of heterozygotes.

121 For both *vtg* KO lines, the relative level of expression of each individual *vtg* transcript in livers of  
122 Hm, Ht, and wt F3 generation females were compared to those obtained for Wt female liver. KO of type-I  
123 *vtgs* resulted in the absence of *vtg1*, *vtg4*, and *vtg5* transcripts in F3 Hm *vtg1*-KO female liver,  
124 representing a significant decrease in levels of these transcripts compared to Ht, wt and Wt females  
125 ( $p < 0.05$ ). Levels of *vtg6* and *vtg7* transcripts were still detectable, with *vtg7* transcript levels being  
126 significantly higher (~3-fold) in Hm *vtg1*-KO female liver as compared to Wt female liver ( $p < 0.05$ ). The  
127 *vtg1*-KO had no significant effect on *vtg2* and *vtg3* expression (**Fig 4, Panel A**). No *vtg3* transcripts were  
128 detected in F3 Hm *vtg3*-KO female livers, representing a significant decrease in *vtg3* transcript levels  
129 compared to Ht, wt and Wt females ( $p < 0.05$ ). The F3 Hm *vtg3*-KO females showed a statistically

130 significant ~3-fold increase in hepatic *vtg7* transcript levels relative to Wt fish ( $p < 0.05$ ). No significant  
131 effect of *vtg3*-KO on expression of other *vtg* genes was observed (**Fig 4, Panel B**).

132 The relative abundances of individual Vtgs or of their product yolk proteins in liver and eggs,  
133 respectively, of F3 Hm *vtg1*-KO females were evaluated as normalized spectral counts (N-SC) from LC-  
134 MS/MS and revealed no detectable amount of Vtg1, 4 and 5 ( $p < 0.05$ ) (**Fig 5A**). Similar to gene  
135 expression levels in these same samples, Vtg6 and 7 protein levels were still detectable and the Vtg7  
136 levels were significantly higher in Hm *vtg1*-KO female liver and eggs than in corresponding samples  
137 from Wt females. ( $p < 0.05$ ). The relative abundance of Vtg7 protein was ~4-fold and ~3-fold higher in  
138 Hm *vtg1*-KO liver and eggs, respectively, than in Wt females. Additionally, even though they were  
139 uniformly low, Vtg3 protein levels were also significantly higher (~2-fold) in Hm *vtg1*-KO eggs than in  
140 Wt eggs ( $p < 0.05$ ) (**Fig 5A**).

141 The *vtg3*-KO resulted in the absence of detectable Vtg3 protein in both liver and eggs of F3 Hm  
142 *vtg3*-KO females ( $p < 0.05$ ) but it did not seem to influence the relative abundances of Vtg1, 2, 4, 5, and 6  
143 or their yolk protein products in these samples (**Fig 5B**). However, Vtg7 protein levels were significantly  
144 higher (~1.5-fold) in *vtg3*-KO eggs than in Wt eggs ( $p < 0.05$ ), but *vtg3*-KO did not significantly alter the  
145 relative abundance of Vtg7 protein in the liver of the egg donors, although average levels were higher for  
146 the *vtg3*-KO fish (**Fig 5 Panel B**). For *vtg1*-KO, *vtg3*-KO and Wt females, relative protein abundances of  
147 all detected Vtgs were generally lower in liver in comparison to eggs. Among the various forms of Vtg  
148 protein, their relative abundance in eggs from Wt females ranged from 15 to 31 times higher than in livers  
149 of the same fish.

150 Domain-specific, affinity purified polyclonal antibodies were developed in rabbits against  
151 zebrafish (zf) Vtg Type-specific epitopes (Type-I Vtg: NEDPKANHIIVTKS on LvH1; Type-III Vtg:  
152 AQKDDIEMIVSEVG on LvL3. See **Fig 2**). The antibodies were used to detect these proteins by Western  
153 blotting in the respective Hm *vtg*-KO, Ht and Wt female livers, ovaries and eggs. The rabbit anti-zfLvH1  
154 antibody revealed the presence of high molecular weight bands corresponding in mass to LvH1 in all  
155 tested individuals and tissues (*data not shown*), consistent with the reported escape of the *vtg6* and *vtg7*

156 from Cas9 editing and the presence of Vtg6 and Vtg7 protein in liver, ovary and eggs from all groups of  
157 fish in the *vtg1*-KO experiment (Hm, Ht and Wt). In Western blots performed using anti-zfLvL3 in the  
158 *vtg3*-KO experiment, the antibody detected mainly a bold ~24 kDa band in samples of both ovary and  
159 eggs from Ht and Wt fish, but not from Hm fish, very close to the deduced mass of the LvL3 polypeptide  
160 (21.3 kDa) (Yilmaz et al., 2018) (**Fig 6**). The distinct absence of this ~24 kDa band only in samples of  
161 Hm ovary and eggs is considered to be evidence of successful *vtg3*-KO in this experiment. The very bold  
162 ~68 kDa band also present in samples of ovary and eggs from Ht and Wt females, but absent in samples  
163 from Hm *vtg3*-KO fish, which have a faint band in this position, may represent a degradation product of  
164 intact, covalently linked LvH-LvL conjugate (Vtg3) persisting after maturational proteolysis, as has been  
165 described for several species (Reading et al., 2009). Faint high molecular weight bands mainly  $\geq 68$  kDa  
166 were also evident for samples of liver, ovary and eggs from all groups of fish in the *vtg3*-KO experiment  
167 (Hm, Ht and Wt). For Hm *vtg3*-KO fish these bands are taken to indicate slight cross-reactivity of the  
168 antibody with yolk proteins other than LvL3 under the experimental conditions employed. For the  
169 corresponding Ht and Wt fish, some of these bands may represent high molecular weight Vtg3 products  
170 bearing intact or partially degraded LvL3, as noted above. No bands specific to Ht and Wt fish were  
171 detected in Western blots of liver performed using this antibody, consistent with absence of significant  
172 quantities of Vtg3 protein detectable in Wt liver by LC-MS/MS (**Fig 5**), a commonly observed  
173 phenomenon (see Yilmaz et al. (2016)) suggesting that Vtg3 is rapidly released into the bloodstream after  
174 synthesis.

175 Phenotypic parameters including fecundity (number of eggs per spawn), egg fertilization,  
176 hatching and survival rates, and egg diameter (embryo and chorion diameter) as well as larval size at 8  
177 days post spawning (dps), were measured to detect potential effects of *vtg* KO on zebrafish reproductive  
178 performance and development. There were no significant differences between Hm *vtg1*-KO and Wt eggs  
179 or offspring in fertilization rate, embryo size or larval size, respectively (**Fig 7**). However, F3 Hm *vtg1*-  
180 KO females produced significantly more eggs per spawn ( $593 \pm 40.06$ , mean  $\pm$  SEM) than did Wt  
181 females ( $280 \pm 28.97$ ) ( $p < 0.05$ ), although the final hatching rate of these eggs at 10 dps ( $64.9 \pm 6.45$  %)



182 was significantly lower than for eggs from Wt females ( $99.6 \pm 0.24$  %) ( $p < 0.05$ ). Eggs from F3 Hm *vtg1*-  
183 KO females also were strikingly delayed in hatching, completing hatching at 9 dps versus 5 dps for  
184 control fish (**Fig 7**). It was noted that the Hm *vtg1*-KO embryos appeared to have weaker heartbeats and  
185 body movements during incubation antecedent to hatching as compared to *vtg3*-KO embryos, which, even  
186 with malformations, exhibited apparently normal heartbeat rhythms and body movements comparable to  
187 those seen in Wt embryos. Embryo and larval survival rates of Hm *vtg1*-KO offspring were also  
188 significantly lower than for Wt offspring, beginning from 5 dps when their mean survival rate was  $57.14$   
189  $\pm 7.34$  % compared to  $79.40 \pm 5.75$  % for Wt fish. The survival rate of Wt offspring changed little  
190 thereafter, whereas the survival rate of Hm *vtg1*-KO offspring continued to decline, with *vtg1*-KO being  
191 completely lethal to the larvae by 16 dps (**Fig 8**).

192 There were no significant differences between Hm *vtg3*-KO fish and Wt fish in fecundity,  
193 embryo size or larval size (**Fig 7**). However, the fertility, hatching rate and overall survival of Hm *vtg3*-  
194 KO eggs and offspring, respectively, were significantly less than seen in Wt fish ( $p < 0.05$ ) (**Fig 7**). The  
195 fertilization rate of eggs from F3 Hm *vtg3*-KO females ( $35.5 \pm 7.7$  %) was substantially lower than for Wt  
196 eggs ( $81.6 \pm 7.0$  %), although hatching of eggs from these females was only slightly delayed, and to a  
197 much lesser extent than was observed for eggs from the F3 Hm *vtg1*-KO females (*see below*). The final  
198 hatching rate for eggs obtained from F3 Hm *vtg3*-KO females was  $74.3 \pm 7.7$  % at 10 dps compared to  
199  $99.6 \pm 0.24$  % for Wt eggs (**Fig 7**). Embryo and larval survival rates of Hm *vtg3*-KO offspring were  
200 significantly less than for Wt offspring ( $p < 0.05$ ), beginning from 8 hours post spawning (hps), with the  
201 difference from Wt fish increasing throughout the 22 d experiment (**Fig 8**). As previously reported  
202 (Yilmaz et al., 2017), at 2–4 hps eggs from low fertility spawns have a high incidence of abnormal  
203 embryos with asymmetric cell cleavage and/or developmental arrest at early cleavage stages. Such  
204 embryos may survive to 8 hps but not to 24 hps. The larval survival rate for Hm *vtg3*-KO offspring was  
205 only  $6.25 \pm 1.6$  % at 22 dps compared to  $69.2 \pm 3.8$  % for Wt offspring (**Fig 8**).

206 Separate panels in **Fig 9** illustrate morphological disorders observed during development of F4  
207 Hm *vtg1*-KO and Hm *vtg3*-KO fish in comparison to offspring from Wt females at 4 and 8 dps. In Hm

208 *vtg*-KO fish, these phenotypic disorders mainly involved pericardial and yolk sac/abdominal edema  
209 accompanied by spinal lordosis evidenced as curved or bent back deformities. The severity of these  
210 malformations, mainly the pericardial and yolk sac edema, appeared to be relatively lower in Hm *vtg1*-  
211 KO fish than in Hm *vtg3*-KO fish. However, the prevalence of deformity was much greater for Hm *vtg1*-  
212 KO fish, with nearly all larvae exhibiting some deformity versus approximately 30 % of Hm *vtg1*-KO  
213 larvae. Finally, the Hm *vtg1*-KO larvae exhibited no feeding activity or motor activities comparable to  
214 those seen in Hm *vtg3*-KO and Wt fish at the same times.

215

216

### 217 3. DISCUSSION

218 Vitellogenins are the ‘mother proteins’ that supply most yolk nutrients supporting early vertebrate  
219 development, and most species have evolved multiple forms of Vtg. However, little is known about  
220 specific functions of these different forms of Vtg and it is uncertain which forms are essential for  
221 successful development or at what stage(s) of development they are required. The present research was  
222 undertaken to address these questions using a zebrafish CRISPR/Cas9 *vtg* gene KO model. Three out of  
223 five type-I zebrafish *vtg* genes (*vtg1*, 4 and 5) were knocked out simultaneously (*vtg1*-KO experiment),  
224 and the type-III *vtg* gene (*vtg3*) was knocked out individually (*vtg3*-KO experiment), and the effects on  
225 maternal reproductive physiology and offspring development and survival were evaluated.

226 The efficacy of CRISPR/Cas9, which is reported to be the most practical and efficient tool  
227 available for genome editing, was lower in the *vtg1*-KO experiment (20 %), where five genes were  
228 targeted concomitantly, than in the *vtg3*-KO experiment (80 %) where only a single gene was targeted.  
229 Since the site-specific cleavage efficiency is mostly dependent on the concentrations of single guide (sg)  
230 RNAs and Cas9 endonuclease, Liu et al. (2018) related the low efficiency of simultaneous knockout of  
231 multiple homologous genes to the fact that more sgRNAs and gene target sites share the same Cas9  
232 enzyme. In addition to low efficiencies in the *vtg1*-KO experiment, the escape of the type-I *vtg6* and *vtg7*  
233 from Cas9 editing might be simply an outcome of an insufficient amount of administered Cas9 RNA.

234 Attempts at optimization of sgRNA/Cas9 concentrations may be useful in future studies. Taking into  
235 account the syntenic organization and close proximity of type-I *vtg* genes in zebrafish (Yilmaz et al.,  
236 2018), and the identity (100 %) of the common target sites for these genes, it is difficult to postulate  
237 criteria upon which any preference of Cas9 activity might be directed. No matter which gene-editing tool  
238 is used, low efficiency of germline mutant transmission has been a commonly faced problem among  
239 researchers, usually leading to labor intensive and time consuming screening work to acquire high-  
240 throughputs (Xie et al., 2016). The low ratios of mutation transmission to next generations in the present  
241 study (0.01 % - 0.025 %) emphasize the need for further research to improve germline transmission  
242 efficiencies in genome editing. Production of stable mutant lines was delayed an extra generation in both  
243 the *vtg1*-KO and *vtg3*-KO experiments since no mutation-positive female founders were obtained at the  
244 F1 generation for performing subsequent reproductive crosses.

245         The incapacitation of *vtg1*, *vtg4*, and *vtg5* in the *vtg1*-KO experiment, and of *vtg3* in the *vtg3*-KO  
246 experiment, was confirmed by conventional PCR, agarose gel electrophoresis and sequencing of gDNA  
247 and also by relative quantification of corresponding *vtg* transcript and Vtg protein abundances via qPCR  
248 and LC-MS/MS, respectively, with the absence of Vtg3 in Hm *vtg3*-KO ovary and eggs being  
249 additionally confirmed by Western blotting. Collectively, these procedures provided strong evidence of  
250 the success of genome editing and *vtg* gene KO. The introduced mutations were all large deletions (1182-  
251 1281 bp) that were achieved by administration of multiple sgRNAs. By disturbing the structure of the  
252 LvH chain in both the *vtg1*-KO and *vtg3*-KO experiments via the creation of large gaps in the respective  
253 LvH polypeptides, it was expected that the mutant proteins would not fold properly, or be able to bind to  
254 their receptor in the case of Vtg3, even if they were produced and partly expressed by the liver. There  
255 were no signs of hepatic synthesis of Vtg1, 4 or 5 in Hm *vtg1*-KO individuals or of Vtg3 in Hm *vtg3*-KO  
256 liver. While detection of the *vtg6* and *vtg7* transcripts and their product proteins was expected in the *vtg1*-  
257 KO experiment, since these two type-I *vtg* genes escaped Cas9 editing, the strikingly high abundance of  
258 Vtg7 (but not Vtg6) at both transcript and protein levels in both Hm *vtg1*-KO and Hm *vtg3*-KO  
259 individuals (**Figs 4 and 5**) was unexpected. These observations suggest an attempt of the organism to

260 compensate for the loss of other types of Vtgs by augmentation of Vtg7 levels, and they imply the  
261 existence of heretofore-unknown mechanisms for regulating Vtg homeostasis.

262         The lack of a mutant phenotype in Hm mutant individuals due to compensatory gene expression  
263 triggered upstream of protein function is known as ‘genetic compensation’ and this phenomenon has been  
264 encountered in gene editing studies of a wide range of model organisms. As examples, Marschang et al.  
265 (2004) related the normal development and lack of mutant phenotypes in LDL receptor-related protein 1b  
266 (*LRP1b*)-deficient mutant mice to functional compensation by *LRP1*, and Sztal et al. (2018) found that a  
267 genetic *actin1b* (*actc1b*) zebrafish mutant exhibits only mild muscle defects and is unaffected by injection  
268 of an *actc1b*-targeting morpholino due to compensatory transcriptional upregulation of an *actin* paralog in  
269 the same fish. In the present study, compensatory increases in relative levels of total Vtg protein  
270 attributable to upregulation of Vtg7 protein in F4 Hm *vtg1*-KO eggs offset only about half of the decrease  
271 in total Vtg protein attributable to KO of *vtg1*, 4 and 5 (**Fig 5A**). Therefore, these eggs/offspring were still  
272 deficient of type-I Vtg protein and they uniformly exhibited mutant and ultimately lethal phenotypes,  
273 perhaps due to the insufficient compensation. In contrast, the compensatory increase in total Vtg protein  
274 attributable to upregulation of Vtg7 in Hm *vtg3*-KO eggs was several-fold greater than the loss of Vtg  
275 protein attributable to *vtg3* KO (**Fig 5B**), yet many of these eggs/offspring still exhibited mutant  
276 phenotypes, with egg fertility being very low (*see below*) and most offspring not surviving for 22 d of  
277 development. Nonetheless, the incidence of mutant phenotypes in Hm *vtg3*-KO larvae (30 %) was far less  
278 than in Hm *vtg1*-KO larvae, all of which were malformed, and a low percentage (6.25 %) of Hm *vtg3*-KO  
279 larvae did survive for 22 d post fertilization, whereas no Hm *vtg1*-KO larvae did. These observations  
280 indicate that, while it is possible that upregulation of Vtg7 may have mitigated to some extent the effects  
281 *vtg3* KO owing to decreased total Vtg protein, Vtg7 cannot fully substitute for Vtg3 or eliminate the  
282 adverse effects of *vtg3* KO on egg fertility and offspring development. Therefore, Vtg3 must have  
283 functional properties distinct from Vtg7 and perhaps other type-I Vtgs.

284         Transcription of *vtg* genes is initiated when estrogen (E2)/estrogen receptor (Esr) complexes bind  
285 to estrogen response elements (ERE) located in the gene promoter regions (Babin, 2008; Nelson and

286 Habibi, 2013). E2-Esr complexes can also be tethered to transcription factor complexes targeting binding  
287 sites distinct from EREs, and several transcription factors other than Esrs have binding sites located in  
288 promoter regions of zebrafish *vtg* genes (reviewed by Lubzens et al., 2017). There is evidence that the  
289 multiple *vtg* genes in zebrafish exhibit differential sensitivities to estrogen induction as well as disparate  
290 patterns of ERE and other transcription factor binding sites in their promoter regions (Levi et al., 2009,  
291 2012). Bioinformatics analyses indicated that the promoter region of *vtg7* is comparatively rich in binding  
292 sites for transcription factors involved in retinoic acid signaling such as retinoic acid response elements  
293 (RAREs), and peroxisome proliferator-activated receptors (PPARs)/ retinoid X receptor (RXR), while  
294 having only a single ERE (most other *vtgs* having 2-3) (see Levi et al., 2012 Table 3). These types of  
295 differences between *vtg* promoters could underpin selective upregulation of Vtg7 in response to ablation  
296 of other forms of Vtg (other type-I Vtgs, Vtg3) via gene KO. Conspecific Vtg (type not specified) has  
297 been shown to downregulate plasma levels of E2 *in vivo* when injected into vitellogenic rainbow trout  
298 (*Oncorhynchus mykiss*) (Reis-Henriques et al., 1997) and to inhibit steroidogenesis leading to E2  
299 production *in vitro* by ovarian follicles of rainbow trout (Reis-Henriques et al., 1997, 2000) and greenback  
300 flounder, *Rhombosolea tapirina* (Sun and Pankhurst, 2006). Partial release from such inhibition in *vtg*-  
301 KO fish would increase vitellogenic signaling to the liver, activating estrogen responsive genes including  
302 those encoding Vtgs, Esr (Esrs) and PPARs.

303 Whether Vtg7 itself has vitellogenic properties remains to be determined. Certain conspecific  
304 Vtgs have been shown to upregulate vitellogenesis in Indian walking catfish (*Clarias batrachus*) (Jain et  
305 al., 2017; Bhattacharya et al., 2018) and comparisons of the available deduced catfish Vtg polypeptide  
306 sequences (85 and 152 residues; Jain et al., 2017, Fig. 3) to Vtgs from zebrafish and other teleosts using  
307 CLUSTAL W and BLASTP (*data not shown*) indicate that they are forms of VtgAol showing a high  
308 identity to type-I zebrafish Vtgs (up to 80% with Vtg7). The specific mechanism(s) by which Vtg7 is  
309 preferentially upregulated in *vtg*-KO zebrafish, and special properties of Vtg7 for regulation of Vtg  
310 homeostasis, are meaningful subjects for future research. Levels of Vtg3 protein were also upregulated in  
311 eggs from F3 Hm *vtg1*-KO females (**Fig 5A**) but the significance of this increase is difficult to interpret as

312 it was too slight to have much impact on total Vtg levels, and because hepatic levels of *vtg3* transcripts  
313 and of Vtg3 protein were not elevated in these fish (**Figs 4A and 5A**). Transcripts of *vtg3* are reported to  
314 be the most intensely upregulated transcripts in vitellogenic female and estrogenized male zebrafish (Levi  
315 et al., 2009) and there may not have been scope for further increases in the *vtg1*-KO fish. In this case post-  
316 transcriptional mechanisms for upregulating Vtg3 could have been at play (Flouriot et al., 1996; Ren et  
317 al., 1996). As noted above, Vtg3 may be released into the bloodstream immediately after synthesis, which  
318 would explain the lack of significant quantities of this protein in livers of Hm *vtg1*-KO and Wt fish (**Fig**  
319 **5**).

320         Neither *vtg1*-KO nor *vtg3*-KO influenced egg, embryo or larval size in spawns producing F4  
321 offspring of the stable mutant lines (**Fig 7**), and there were no apparent differences in ovary structure  
322 among the different groups of maternal F3 females (Hm, Ht, wt and Wt) sampled after spawning (*data*  
323 *not shown*). However, F3 Hm *vtg1*-KO females exhibited a 2-fold increase in fecundity (egg production)  
324 relative to Wt females, with normal egg fertility equivalent to that of Wt females (**Fig 7**). This response to  
325 elimination of three type-I Vtgs (including the most abundant one, Vtg1) implies that one or more of  
326 these Vtgs are normally involved in restriction of fecundity, perhaps via the aforementioned inhibition of  
327 follicular estrogenesis. It is also possible that Vtg7, which was highly elevated in Hm *vtg1*-KO females,  
328 might somehow positively modulate fecundity. The referenced VtgAo1 of walking catfish, when pelleted  
329 and implanted into pre-vitellogenic females, has been shown to stimulate vitellogenesis and complete  
330 oocyte growth all the way through the transition to final maturation (Bhattacharya et al., 2018). In the  
331 final analysis, any ‘compensation’ by Vtg7 for loss of other type-I Vtgs must be deemed ineffectual, as  
332 the resulting embryos unconditionally exhibited serious and lethal developmental abnormalities (*see*  
333 *below*).

334         The *vtg*-KO zebrafish larvae exhibited major phenotypic disorders, mainly pericardial and yolk  
335 sac/abdominal edemas and spinal lordosis associated with curved or arched back deformities. These  
336 abnormalities were observed to be much less prevalent, albeit usually more severe, in *vtg3*-KO larvae, but  
337 present to some extent in all *vtg1*-KO larvae along with the noted behavioral differences. Skeletal axis

338 malformations and pericardial and yolk sac/abdominal edema are among the most common deformities  
339 observed in cultured teleosts and they form an interrelated cluster of abnormalities that tend to be  
340 observed together (Alix et al., 2017). For example, in zebrafish pericardial edema tends to precede  
341 development of yolk sac edema, which when severe leads to notochord deformation (see Hanke et al.,  
342 2013, Fig. 1). These abnormalities have been associated with a broad variety of conditions including, as  
343 examples, rearing systems for Eurasian perch, *Perca fluviatilis* (Alix et al., 2017), larval rearing  
344 temperatures for Atlantic halibut, *Hippoglossus hippoglossus* L. (Ottesen and Bolla, 1998), embryo  
345 cryopreservation practices for streaked prochilod, *Prochilodus lineatus* (Costa et al., 2017), and, in  
346 zebrafish, phenanthroline toxicity (Ellis and Crawford, 2016), influenza A virus infection (Gabor et al.,  
347 2014), knockdown or KO of genes related to kidney function or development, respectively (Hanke et al.,  
348 2013; Zhang et al., 2018), knockdown of the *wwox* tumor suppressor gene (Tsuruwaka et al., 2015),  
349 deletion of a gene (*pr130*) encoding a protein essential for myocardium formation and cardiac contractile  
350 function (Yang et al., 2016), and mutagenesis of genes involved in thyroid morphogenesis and function  
351 (Trubiroha et al., 2018), among others. The edemas may ultimately result from many different proximal  
352 causes such as cardiac, kidney, liver or osmoregulatory failure, and researchers are just beginning to  
353 develop screens to differentiate between them (Hanke et al., 2013). Although they can occur under many  
354 different conditions and arise via several possible mechanisms, these major mutant phenotypes observed  
355 in the present study were not encountered in control Wt offspring and, therefore, they are clearly related  
356 to deficiencies of type-I Vtgs (Vtg1, 4 and 5) and of Vtg3.

357 Embryo and larval survival rates were severely diminished by *vtg* gene KO, but the magnitude,  
358 type and timing of losses differed between *vtg1*-KO and *vtg3*-KO fish (**Fig 8**). The fertility of Hm *vtg3*-  
359 KO eggs was only half that observed in Wt eggs (**Fig 7**), indicating that Vtg3 is an important contributor  
360 to fertility in zebrafish. Among the Vtgs examined, this dependency was specific to Vtg3, since fertility  
361 was not 'rescued' by the increase in Vtg7 levels in Hm *vtg3*-KO eggs, which was far greater than normal  
362 Vtg3 levels in Wt fish (**Fig 5B**), and this adverse effect on fertility was not seen in Hm *vtg1*-KO eggs.  
363 The substantial losses of Hm *vtg3*-KO eggs began early, at only 8 hps, and less than 30% survived to 24

364 hps (**Fig 8**). Both *vtg1*-KO and Wt eggs showed significant but much fewer losses ( $p < 0.05$ ) during this  
365 same interval. In this study, fertility was estimated conservatively, based on numbers of viable embryos  
366 showing normal cell division and subsequently developing to ~24 hps. It is uncertain whether the high  
367 mortality of Hm *vtg3*-KO eggs between 8 and 24 hps (**Fig 8**) resulted from a failure to be fertilized or  
368 from defects in early development involving zygotes that fail to initiate cell division or that briefly  
369 undergo abnormal cell divisions and then die. In future studies, some Hm mutant and Wt females should  
370 be bred with males bearing a unique germline marker gene, such as *vasa::egfp* (Krøvel and Olsen, 2002),  
371 that can be genotyped in resulting eggs and embryos to resolve this question.

372 The mechanism(s) whereby Vtg3 deficiency impairs fertility and/or early development of  
373 zebrafish are unknown. A recent study examining the proteomics of egg/embryo developmental  
374 competence in zebrafish identified disruption of normal oocyte maturation, including maturational  
375 proteolysis of Vtgs, as a likely cause of poor egg quality (Yilmaz et al., 2017). The proteolysis of Vtgs by  
376 cathepsins during oocyte maturation, a phenomenon that has been observed in zebrafish eggs undergoing  
377 maturation *in vitro* (Carnevali et al., 2006), releases FAA that steepen the osmotic gradient driving water  
378 influx through aquaporins on the cell surface, leading to oocyte hydration (Cerdà et al., 2007, 2013).  
379 These FAA are also major substrates for aerobic energy metabolism during early embryogenesis (Thorsen  
380 and Fyhn, 1996; Finn and Fyhn, 2010). In some species, Vtg3 (VtgC) is subjected to maturational  
381 proteolysis (see Yilmaz et al., 2016) and it is possible that zebrafish Vtg3 contributes to these critical  
382 processes ongoing during oocyte maturation, which are required for production of viable eggs. However,  
383 mass balance considerations seem to exclude the possibility that the early mortality of Hm *vtg3*-KO  
384 embryos results substantially from nutritional deficiencies. In this and prior studies of zebrafish, Vtg3 has  
385 been shown to be a very minor form of Vtg making only a miniscule contribution to stores of Vtg-derived  
386 yolk proteins in eggs (**Fig 5**; see also Yilmaz et al., 2018). Nonetheless, Vtg3 is clearly an important, if  
387 not essential, contributor to fertility and/or early development in zebrafish. The continuous mortality of  
388 Hm *vtg3*-KO embryos after 24 hps, leading to only ~6% survival at 22 dps, suggests that Vtg3 also  
389 contributes to late embryonic and larval development, as suggested in several prior studies (see below).



390 Survival of embryos emanating from Hm *vtg1*-KO females remained relatively high at 24 hps  
391 (~70%) but decreased continuously thereafter, becoming significantly less than survival of Wt embryos  
392 by 5 dps, and then decreasing to zero by 16 dps (**Fig 8**). The collective absence of Vtg1, 4 and 5 in  
393 zebrafish is lethal to offspring, and this effect could not be rescued via genetic compensation by Vtg7 or  
394 offset by the remaining intact Vtgs. This finding is not surprising as, collectively, these 3 type-I Vtgs  
395 account for the vast majority of Vtg-derived protein in Wt zebrafish eggs (**Fig 5**; see also Yilmaz et al.,  
396 2018). Since most mortality of *vtg1*-KO offspring occurred relatively late in development in larvae, with  
397 mortality rate increasing after 10 dps when yolk sac absorption was being completed (**Fig 8**), the  
398 collective contributions of Vtg1, 4 and 5 to survival could be largely nutritional, although this remains to  
399 be verified.

400 It is evermore apparent that the different types of vertebrate Vtg can have dissimilar effects on  
401 reproductive processes. As noted above (see also Introduction), in marine acanthomorphs spawning  
402 pelagic eggs in seawater the different types of Vtg can play disparate roles in oocyte hydration,  
403 acquisition of egg buoyancy, and early versus late embryonic and larval nutrition (Matsubara and Koya,  
404 1997; Matsubara et al., 1999, 2003; Reith et al., 2001; Sawaguchi et al., 2005, 2006a, 2006b; Finn, 2007).  
405 The type-specific ratios of circulating Vtgs (e.g. VtgAa:VtgAb:VtgC) may vary considerably during  
406 oocyte growth, but ratios of their derived yolk protein products present in eggs tend to be fixed and  
407 characteristic of species (Hiramatsu et al., 2015; Reading et al., 2017). This is also the case in zebrafish  
408 as evidenced by the similarity of Vtg profiles by type (and subtype) in Wt fish in the *vtg1*-KO and *vtg3*-  
409 KO experiments, and also in comparison to Wt fish in an earlier study (Yilmaz et al., 2018). It is thought  
410 that Vtg type-specific ratios of yolk proteins in eggs are maintained via activity of selective receptors for  
411 each type of Vtg, which target their specific ligand(s) into different compartments where their yolk  
412 protein products undergo disparate degrees of proteolysis during oocyte maturation. The initial abundance  
413 and degree of proteolysis of the yolk proteins determines their relative contribution(s) to oocyte  
414 hydration, egg buoyancy, FAA nutrition of early embryos and lipoprotein nutrition for late stage larvae  
415 (Hiramatsu et al., 2015; Reading et al., 2017). The collective findings of the present study introduce a new

416 point of view on the roles that multiple vitellogenins can play in vertebrate reproduction. Distinctively  
417 from what has been reported previously, the present study presents a mixed model of Vtg functionality  
418 covering both maternal reproductive physiology and early development of offspring, where type-I Vtgs  
419 regulate fecundity and make essential contributions to embryonic morphogenesis, hatching and larval  
420 kinesics and survival (Vtg1, 4 and 5), and also provide some homeostatic regulation of total Vtg levels  
421 (Vtg7), while Vtg3 (a typical VtgC) is critically important to fertility and early embryogenesis and also  
422 influences later development.

423 In summary, the present study, for the first time, targeted multiple forms of Vtgs for KO at  
424 family level using CRISPR/Cas9 technology in the zebrafish, a well-established biomedical model. The  
425 collective knock out of *vtg1*, *4*, and *5* and the individual knock out of *vtg3* were achieved successfully. A  
426 compensatory increase in *vtg7* at both transcript and protein levels was observed in both types of *vtg* KO  
427 mutants. However, this compensation was not effective in rescuing the serious developmental  
428 impairments and high mortalities resulting from ablation of three other type-I Vtgs or of Vtg3. By far the  
429 most abundant forms of Vtg in zebrafish, the type-I Vtgs appear to have essential developmental and  
430 nutritional functions in both embryos and larvae. In spite of being a very minor form of Vtg in zebrafish  
431 and most other species, and also the most divergent form, Vtg3 contributes importantly to the  
432 developmental potential of zygotes and/or early embryos. Finally, Vtgs appear to have previously  
433 unreported regulatory effects on the physiology of maternal females, including limitation of fecundity  
434 (type-I Vtgs) and maintenance of fertility (Vtg3). These novel findings represent the first steps toward  
435 discovery of the specific functions of multiple vertebrate Vtgs via genome editing. Further physiological  
436 studies are necessary to pinpoint the exact molecular mechanisms disturbed in the *vtg* mutants.

437

438

## 439 **4. MATERIAL AND METHODS**

440

### 441 **4.1. Animal care, spawning and phenotypic observations**

442 Zebrafish of the Tübingen strain originally emanating from the Nüsslein-Volhard Laboratory  
443 (Germany) were obtained from our zebrafish facility (INRA UR1037 LPGP, Rennes, France). The fish  
444 were ~15 months of age and of average length ~5.0 cm and average weight ~1.4 g. The zebrafish were  
445 housed under standard conditions of photoperiod (14 hours light and 10 hours dark) and temperature (28  
446 °C) in 10 L aquaria, and were fed three times a day *ad libitum* with a commercial diet (GEMMA,  
447 Skretting, Wincham, Northwich, UK). Females were bred at weekly intervals to obtain egg batches for  
448 CRISPR sgRNA microinjection (MI). The night before spawning, paired males and females bred from  
449 different parents were separated by an opaque divider in individual aquaria equipped with marbles at the  
450 bottom as the spawning substrate. The divider was removed in the morning, with the fish left undisturbed  
451 to spawn. Egg batches in majority containing intact, clean looking, well defined, activated eggs at the 1-  
452 cell stage were immediately transferred to microinjection facilities.

453 For phenotyping observations five couples formed from F3 Hm males and females and five Wt  
454 couples were spawned from 3 to 8 times and embryonic development, survival rate, hatching rate, and  
455 larval development were subsequently observed until 22 dps. Survival, fecundity and fertilization rate  
456 data was collected from 21, 24 and 5 spawns from *vtg1*-KO, *vtg3*-KO and Wt couples, respectively.  
457 Hatching rate was calculated based on the number of surviving embryos at 24h and only spawns with > 5  
458 % survival rates were considered, therefore, hatching rate data was collected from 21, 16, and 5 spawns  
459 from *vtg1*-KO, *vtg3*-KO and Wt couples, respectively, in this study. Fecundity (number of eggs per  
460 spawn) was recorded immediately after spawning and collected eggs were incubated in 100 mm Petri  
461 dishes filled with embryo medium (17.1 mM NaCl, 0.4 mM KCl, 0.65 mM MgSO<sub>4</sub>, 0.27 mM CaCl<sub>2</sub>,  
462 0.01 mg/L methylene blue) to assess embryonic development and phenotyping parameters. Incubated  
463 eggs/embryos were periodically observed at the early blastula (~256 cell) stage (~2-3 h post spawning  
464 hps), at mid-blastula transition stage (~4 hps), at the shield to 75% epiboly stages (~8 hps), at the early  
465 pharyngula stage (~24 hps), and during the hatching period at 48 and 72 hps (long-pec to protruding-  
466 mouth stages) following standard developmental staging (Kimmel et al., 1995). Fertilization rate was  
467 calculated based on viable embryos showing normal cell division and subsequent development to ~24 hps

468 since zygotes failing to initiate cell division, and embryos showing asymmetrical cell cleavage or early  
469 developmental arrest were dead by then. As noted above (see Discussion) it is uncertain whether these  
470 aberrant eggs/embryos result from infertility or developmental defects. The number of surviving  
471 eggs/embryos was recorded, those not surviving were removed and the number of abnormal embryos was  
472 recorded at each observation point. Hatched embryos were transferred into larger volume containers (1 L)  
473 filled with standard 28°C culture water and were fed *ad libitum* with artemia and GEMMA weaning diet  
474 mix after yolk sac absorption (at around 10 dps). At the time of feeding, larvae were also observed for  
475 motor and feeding activities. Observations were made daily up to 22 dps. Subsamples of 10-12 embryos  
476 and larvae from each clutch were taken for measurements of embryo and chorion diameter, and larval size  
477 at 2-3 hps and 8 dps, respectively. Measurements were made using an ocular micrometer under a Zeiss  
478 Stemi 2000-C stereomicroscope connected to a TouPCam 3,1 M pixels camera employing the TouPCview  
479 software.

480

#### 481 **4.2. Single guide RNA (sgRNA) design, synthesis and microinjection**

482 Genomic DNA sequences from all five type-I zebrafish *vtgs* were aligned and three target sites  
483 common to all five genes were designed using CRISPR MultiTargeter (Prykhozhij et al., 2015) available  
484 online at <http://www.multicrispr.net>. Of proposed candidates, three target regions located on exons 4, 14  
485 and 17, corresponding to the LvH yolk protein domain were chosen for the *vtg1*-KO experiment. The  
486 *vtg3* genomic region was separately submitted to online available target designer tool at  
487 <http://zifit.partners.org/ZiFiT/ChoiceMenu.aspx> (Sander et al., 2007, 2010) and of proposed candidates,  
488 three gene specific target regions located on exons 4, 6 and 11, corresponding to the LvH yolk protein  
489 domain, were chosen. A schematic representation of the general strategy followed for CRISPR target  
490 design is presented in **Fig 1**. Forward and reverse oligonucleotides matching the chosen target sequences  
491 (given in **S1 Table**) were annealed and ligated to the pDR274 expression vector (Addgene). The vector  
492 was subsequently linearized by the *Dra*I restriction digestion enzyme (Promega) and *in vitro* transcribed  
493 using mMessage mMachine T7 Transcription Kit (Ambion) according instructions from the manufacturer.

494 The pCS2-nCas9n plasmid (Addgene Plasmid 47929) was digested with NotI restriction digestion  
495 enzyme (Promega) and transcribed using mMessage mMachine SP6 Transcription Kit (Ambion)  
496 according instructions from the manufacturer. The sgRNA concentration was measured on a Nanodrop  
497 1000 Spectrophotometer (Thermo Scientific, USA) and integrity was tested before use using an Agilent  
498 RNA 6000 Nano Kit (Agilent) on an Agilent 2100 Bioanalyzer.

499 Approximately 100 eggs per batch were injected with sgRNA mix containing sgRNAs for three  
500 target sites (at ~30 ng/ul (=30 mM) in 20ul of the final mix each) and nCas9n RNA (at ~200 ng/ul (=200  
501 mM) in the final mix) at the one-cell stage in both the *vtg1*-KO and *vtg3*-KO experiments. A total of 120  
502 pg sgRNA mix and ~800 pg Cas9 RNA was injected per embryo. Injected embryos were kept in 100 mm  
503 petri dishes filled with embryo medium (17.1 mM NaCl, 0.4 mM KCl, 0.65 mM MgSO<sub>4</sub>, 0.27 mM  
504 CaCl<sub>2</sub>, 0.01 mg/L methylene blue) to assess microinjection efficiency, embryo survival and development  
505 post injection.

506

### 507 **4.3. Genotyping by conventional PCR**

508 As representatives of their generation, ten embryos were sampled randomly and gDNA was  
509 extracted individually and used as a template in targeted conventional PCR reactions to screen for  
510 introduced mutations in the targeted *vtg* genes. For this purpose, embryos surviving for 24 h post-  
511 injection were incubated in 100 µl of 5 % Chelex® 100 Molecular Biology Grade Resin (BioRad) and 50  
512 µl of Proteinase K Solution (20 mg/ml, Ambion) initially for 2h at 55 °C and subsequently for 10 min 99  
513 °C with constant agitation at 12000 rpm. Extracts were then centrifuged at 5000 xg for 10 minutes and  
514 supernatant containing gDNA was transferred into new tubes and stored at -20 °C until use.

515 To evaluate generational transfer of introduced mutations, genotyping of ~2 month old offspring  
516 was conducted after extraction of gDNA from fin-clips. For this purpose, fish were anaesthetized in 2-  
517 phenoxyethanol (0.5 ml/L) and part of their caudal fin was excised with a sterile scalpel. Genomic DNA  
518 from fin tissues were then extracted using Chelex 5 % as described above.

519 One  $\mu$ l (~ 100 ng) of extracted gDNA was used in 20  $\mu$ l PCR reactions using AccuPrime™ Taq  
520 DNA Polymerase, High Fidelity (Invitrogen) and 10x AccuPrime™ PCR Buffer II in combination with  
521 gene specific primers (at 10  $\mu$ M each) anchoring target sites on the genomic sequence of targeted genes  
522 (**Fig 1**). PCR cycling conditions were as follows; 1 cycle of initial denaturation at 94 °C for 2 min, 35  
523 cycles of denaturation at 94 °C for 15–30 sec, annealing at 52–64 °C for 15–30 sec and extension at 68 °C  
524 for 1 min per kb plus 1 cycle of final extension at 68 °C for 5 min. Non-purified PCR products or gel  
525 purified DNA were sequenced using gene specific primers indicated in **S1 Table** by the Eurofins  
526 Genomics sequencing service (<https://www.eurofinsgenomics.eu/>). Obtained sequences were aligned to  
527 corresponding zebrafish genomic sequence using Clustal Omega (Sievers et al., 2011) for characterization  
528 and localization of introduced mutations, and then were blasted against all sequences available online  
529 using NCBI nucleotide Blast (Blastn) (Altschul et al., 1990) for confirmation of the consistency, accuracy  
530 and type of the mutations created at the target sites.

531

#### 532 **4.4. Generation of pure zebrafish lines carrying the introduced mutations**

533 In both the *vtg1*-KO and *vtg3*-KO experiments, embryos carrying introduced mutations were  
534 raised to adulthood, fin clipped and re-genotyped to confirm mutation of their type-I or III *vtgs*, and then  
535 heterozygous (Ht; *vtg1*<sup>+/-</sup> and *vtg3*<sup>+/-</sup>) males with the mutation on a single allele were outcrossed with  
536 non-related wild type (Wt; *vtg1*<sup>+/+</sup> and *vtg3*<sup>+/+</sup>) females with no genomic disturbance to produce the F1  
537 generation. Embryos from F1 generation were genotyped as stated above and remaining embryos were  
538 raised to adulthood. F1 offspring were screened again at ~2 months of age and, since mutation  
539 transmission occurred in two males only per group, these Ht males were crossed with Wt females to  
540 produce the F2 generation. Following the same genotyping strategy, F2 Ht males were crossed with Ht  
541 females to produce the F3 generation. Finally, F3 homozygous (Hm; *vtg1*<sup>-/-</sup> and *vtg3*<sup>-/-</sup>) males and Hm  
542 females with both alleles carrying the desired mutation were crossed to produce the F4 *vtg* mutants.

543

#### 544 **4.5. Tissue sampling and analyses**

545 Liver and ovary samples from *vlg1*-KO and *vlg3*-KO F3 Hm, Ht, wt and Wt female zebrafish  
546 were excised within 2-3 h after egg collection at the end of phenotyping experiment and after the fish  
547 were euthanized with a lethal dose of 2-phenoxyethanol (0.5 ml/L). Ovary samples were aliquoted into  
548 four pieces and stored according to subsequent analytical procedures; snap frozen for RNA and protein  
549 extraction or placed in Bouin's solution for histological analyses. Liver samples were aliquoted in two  
550 pieces and snap frozen until being used for LC-MS/MS or Western blotting.

551

#### 552 **4.6. Quantitative real time PCR**

553 Total RNA was extracted from frozen liver using TriReagent (SIGMA) and cDNA was  
554 synthesized using SuperScript III reverse transcriptase (Invitrogen, USA) from 1 µg of total RNA  
555 according to the manufacturer's instructions. Relative expression levels for all zebrafish *vlg*s (*vlg1*, 2, 3,  
556 4, 5, 6 and 7) in *vlg1*-KO female liver were measured using TaqMan real-time quantitative PCR (RT-  
557 qPCR) using gene specific primers and dual-labeled probes (FAM, 6-carboxyfluorescein and a BHQ-1,  
558 Black Hole Quencher 1 on 5' and 3' terminus, respectively). Sequences of these primers and probes used  
559 in this experiment are given in **S1 Table**. Each qPCR was performed in 10 µl reactions containing cDNA  
560 (diluted at 1:25), 600 nM of each primer, 400 nM of hydrolysis probe and 1× TaqMan Fast Advanced  
561 Master Mix (Applied Biosystems) according the manufacturer's instructions on a StepOnePlus real time  
562 PCR instrument (Applied Biosystems). PCR cycling conditions were as follows: 95°C for 20 seconds, 40  
563 cycles at 95°C for 1 second followed by an annealing-extension at 60°C for 20 seconds. The relative  
564 abundance of the target cDNA within a sample set was calculated from a serial dilution curve made from  
565 the cDNA pool, using StepOne software (Applied Biosystems). The  $2^{-\Delta\Delta CT}$  mean relative quantification of  
566 gene expression method with zebrafish *18S* as a reference gene was employed in this study. Relative  
567 expression levels of all zebrafish *vlg*s in *vlg3*-KO female liver were measured using SYBR GREEN  
568 qPCR Master Mix (SYBR Green Master Mix kit; Applied Biosystems) as indicated by the manufacturer  
569 in a total volume of 10 µl, containing RT products diluted at 1:1000 and 400 nM of each primer in order  
570 to obtain PCR efficiency between 95 and 100 %. Sequences of primers used in this experiment are given

571 in **S1 Table**. The RT-qPCR cycling protocol included 3 min initial denaturation at 95 °C followed by 40  
572 cycles of 95 °C for 3 sec and 60 °C for 30 sec on a StepOnePlus thermocycler (Applied Biosystem). The  
573 relative abundance of target cDNA within a sample set was calculated from a serially diluted cDNA pool  
574 (standard curve) using Applied Biosystem StepOne V.2.0 software. Similarly, the  $2^{-\Delta\Delta CT}$  mean relative  
575 quantification of gene expression method with the mean expression value of zebrafish elongation factor  
576 1a (*eif1a*), ribosomal protein 13a (*rpl13a*) and *18S* as reference were employed in this study. Primer  
577 sequences and properties for these genes are also given in **S1 Table**. Obtained data was subjected to  
578 independent samples Kruskal-Wallis nonparametric test ( $p < 0.05$ ) followed by Benjamini Hochberg  
579 correction for multiple tests ( $p < 0.1$ ) (IBM SPSS Statistics Version 19.0.0, Armonk, NY).

580

#### 581 **4.7. Western Blotting**

582 Samples of zebrafish liver, ovary and eggs were homogenized in 100µl of protein binding buffer  
583 containing 1mM AEBSF, 10mM Leupeptin, 1mM EDTA and 0.5 mM DTT as indicated by Hiramatsu et  
584 al. (2002) using a procellys tissue homogenizer (Bertin Instruments, France). Protein extracts were  
585 separated from homogenates with centrifugation at 13 000 rpm +4 °C for 30 minutes to generate  
586 supernatant samples for SDS-PAGE. Protein concentrations of the samples were estimated by Bradford  
587 Assay (Bradford, 1976) (Bio-Rad, Marnes-la-Coquette, France) and they were diluted to 4 µg protein µl<sup>-1</sup>  
588 in ultrapure water, mixed 1:1 v/v with Laemmli sample buffer (Laemmli, 1970) containing 2-  
589 mercaptoethanol, and boiled for 5 min before electrophoresis. A total of 10 µg of sample protein was  
590 loaded onto a precast 4–15 % acrylamide gradient Tris–HCl Ready Gel® (BioRad, Hercules, CA) with 4  
591 % acrylamide stacking gel and electrophoresed at 150 V for 45 min using a Tris–glycine buffer system  
592 (Laemmli, 1970). Biotinylated protein molecular weight markers (Vector Laboratories, USA) were used  
593 to estimate the mass of separated proteins.

594 Proteins in the gels were transferred to PVDF membranes using a Trans-Blot® Turbo™ Transfer  
595 Starter System (BioRad) at 25 mA for 15 min. Blots were blocked for 2 h with Casein solution in tris  
596 buffered saline (10 mM Tris HCl containing 15 mM NaCl) and 0.05% Tween 20 (TBST) to reduce non-



597 specific reactions. Affinity purified polyclonal primary antibody raised against a specific peptide epitope  
598 on lipovitellin light chain of zebrafish Vtg3 (anti-zfLvL3, GeneScript Custom Antibody production  
599 Service, USA) was employed to detect Vtg3 or its product yolk proteins in liver, ovary and eggs from F3  
600 *vtg3*-KO zebrafish. For this purpose, blots were incubated for 2 h at room temperature with the anti-  
601 zfLvL3 at a 1:000 dilution in phosphate buffered saline (10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.5, 150 mM NaCl).  
602 Membranes were washed three times for 5 minutes in TBST solution and incubated in biotinylated goat  
603 anti-rabbit IgG affinity purified secondary antibody diluted 1:8000 in casein solution for 30 minutes at  
604 room temperature. Membranes were washed in TBST solution three times for 5 min each and incubated  
605 in VECTASTAIN® ABC-AmP™ reagent (VECTASTAIN ABC-AmP Kit, for Rabbit IgG,  
606 Chemiluminescent Western Blot Detection, Vector Laboratories) for 10 minutes at room temperature.  
607 Following three washes of 5 minutes in TBST, membranes were equilibrated in 0.1 M Tris buffer, pH 9.5  
608 before development in DuoLuX™ Substrate (Vector Laboratories) and exposure to chemiluminescent  
609 signal detection on FUSION-FX7 advanced chemiluminescence/fluorescence system (Vilber Lourmat,  
610 Germany).

611

#### 612 **4.8. Liquid Chromatography Tandem Mass Spectrometry**

613 Protein extraction of liver and egg samples from *vtg1*-KO, *vtg3*-KO and Wt female zebrafish  
614 were done as described by Yilmaz et al. (2017). Briefly, samples were subjected to sonication in 20 mM,  
615 pH 7.4, HEPES buffer on ice, soluble protein extracts were recovered following centrifugation (15 000 x  
616 g) at +4 °C for 30 min and the remaining pellet was re-sonicated in 30 mM Tris / 8 M Urea / 4 % CHAPS  
617 buffer on ice. Ultracentrifugation (105,000 xg) of the pooled protein extracts for 1 h at 4 °C was followed  
618 by supernatant recovery and determination of the protein concentration by Bradford Assay (Bradford,  
619 1976) (Bio-Rad, Marnes-la-Coquette, France). Samples of extracts were mixed with sample buffer and  
620 DTT and denatured at 70 °C for 10 min before being subjected to SDS-PAGE (60 µg protein/sample  
621 lane). When protein samples had completely penetrated the stacking gel (~2 minutes at 200 V-400 mA  
622 (~23 W)), electrophoresis was stopped and gels were briefly rinsed in MilliQ ultrapure water (Millipore

623 S.A.S., Alsace, France) and then incubated in fixation solution containing 30 % EtOH / 10 % acetic acid /  
624 60 % MilliQ water for 15 min in order to fix proteins on the gel. Gels were then washed in MilliQ water  
625 three times for 5 min each and incubated in EZBlue™ Gel Staining Reagent (Sigma-Aldrich, Saint-  
626 Quentin Fallavier, France) at room temperature with slight agitation for 2 h, and de-stained in MilliQ  
627 water at room temperature overnight. Subsequently, protein bands were excised from the gel and the  
628 excised gel pieces were processed for tryptic digestion and peptide extraction as indicated by Yilmaz et  
629 al. (2017). Once peptide extraction was completed, pellets containing digested peptides were resolubilized  
630 in 30 µl of 95 % H<sub>2</sub>O : 5 % formic acid by vortex mixing for 10 min and diluted 10 times before being  
631 subjected to LC-MS/MS.

632 Peptide mixtures were analyzed using a nanoflow high-performance liquid chromatography  
633 (HPLC) system (LC Packings Ultimate 3000, Thermo Fisher Scientific, Courtaboeuf, France) connected  
634 to a hybrid LTQ-OrbiTrap XL spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray  
635 ion source (New Objective), as previously described (Lavigne et al., 2012; Yilmaz et al., 2017). The  
636 spectra search, protein identification, quantification by spectral counts, and spectral count normalization  
637 were conducted as described by Yilmaz et al. (2017). To detect significant differences between group  
638 mean N-SC values (*vtg1*-KO vs Wt or *vtg3*-KO vs Wt) for different zebrafish Vtgs from liver and eggs,  
639 an independent samples Kruskal-Wallis nonparametric test ( $p < 0.05$ ) followed by Benjamini Hochberg  
640 correction for multiple tests ( $p < 0.1$ ) was used (IBM SPSS Statistics Version 19.0.0, Armonk, NY).

641

#### 642 **4.9. Ethical Statement**

643 All experiments complied with French & European regulations ensuring 'animal welfare' and that  
644 'Animals will be held in the INRA UR1037 LPGP fish facility (DDCSPP approval # B35-238-6).'

645 Experimental protocols involving animals were approved by the Comité Rennais d'éthique pour  
646 l'expérimentation animale (CREEA).

647

648

649 **5. COMPETING INTERESTS**

650 Authors declare no competing interests.

651

652

653 **6. FUNDING**

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657

658

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663

664

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## 865 9. FIGURE LEGENDS

866 **Fig 1. Schematic representation of the general strategy for CRISPR target design in the zebrafish**  
867 ***vtg* knock out (KO) study.** **A)** Type-I *vtg* knock out (*vtg1*-KO). *vtg1* is depicted as representative of the  
868 five targeted type-I zebrafish *vtg* genes. **B)** Type-III *vtg* knock out (*vtg3*-KO). Target sites are shown by  
869 brown colored arrows labeled as sg followed by 1, 2 or 3 indicating the targeted zebrafish *vtg* type and the  
870 number of the target site (i.e. sg11, sg12, and sg13: single guide RNAs (sgRNAs) for target sites 1, 2, and  
871 3 for *vtg1*, respectively. sg31, sg32, and sg33: sgRNAs for target sites 1, 2, and 3 for *vtg3*, respectively).  
872 Arrows are oriented to indicate the sense/antisense orientation of each target. Numbers above each target  
873 site specify its exact location by nucleotide in the genomic sequence of the zebrafish *vtgs*. Primers used in  
874 screening for introduced mutations by PCR are shown as yellow arrowheads outlined in green, which are  
875 oriented to indicate the sense/antisense orientation of the primer. Numbers below each primer site indicate  
876 its exact position by nucleotide in the genomic sequence of the targeted gene (see also **S1 Fig**). Horizontal

877 brackets below indicate areas screened for mutations by PCR using selected primer combinations; bold  
878 green text below the brackets indicates the primer pair followed by the size of the band (bp) expected for  
879 wild type gDNA in agarose gel electrophoresis (see **Fig 3**). 11Fw, *vtg1* target1 forward primer; 12Rv,  
880 *vtg1* target2 reverse primer; 13Rv, *vtg1* target3 reverse primer; 12Fw, *vtg1* target2 forward primer; 31Fw,  
881 *vtg3* target1 forward primer; 32Rv, *vtg3* target2 reverse primer; 33Rv, *vtg3* target3 reverse primer; 32Fw,  
882 *vtg3* target2 forward primer. Primer sequences are given in **S1 Table**.

883

884 **Fig 2. Location of mutations introduced by CRISPR/Cas9 in the predicted polypeptide sequence of**  
885 **targeted zebrafish *vtgs*.** The yolk protein domain structures of Vtg1 (representative of zebrafish type-I  
886 Vtgs) and Vtg3 are pictured in 5' > 3' orientation above each panel. Light gray horizontal bars represent  
887 the lipovitellin heavy and light chain (LvH, LvL) and phosvitin (Pv) domains of the respective Vtg (Vtg3  
888 lacks a Pv domain) and are labeled above in large bold type. Sequences within these bars indicate the N-  
889 terminus of each yolk protein domain, the starting points of which are also indicated by vertical bars in  
890 the polypeptide sequence shown below. The 85-residue Vtg receptor-binding domain (***RbD***) and the  
891 critical 8-residue Vtg receptor-binding motif (***RbM***) located within this domain, which were identified by  
892 Li et al. (2003) in the LvH domain of blue tilapia (*Oreochromis aureus*) VtgAb, are shown in the  
893 polypeptide sequences in boldface italic type, with the ***RbM*** sequence being additionally underlined and  
894 also shown in the yolk protein domain map above. Residues encoded by nucleotide sequences targeted by  
895 sgRNAs for Cas9 editing are framed in magenta-shaded boxes. Cas9 created mutations (large deletions)  
896 are indicated with dashes replacing amino acid (aa) residues and the size of deletions in aa (234 aa and  
897 239 aa for *vtg1*-KO and *vtg3*-KO, respectively) in these regions are labeled by gray shaded text. Short  
898 sequences that were employed as epitopes to develop Vtg domain-specific antibodies against Vtg1-LvH  
899 (anti-LvH1) and Vtg3-LvL (anti-LvL3) are indicated by framed text on the LvH and LvL domains of  
900 Vtg1 and Vtg3, respectively, with their location also highlighted by black arrows labeled with the epitope  
901 names given by vertically-oriented text in the panel margins.

902

903 **Fig 3. Detection of CRISPR/Cas9-introduced mutations by embryo genotyping and production of**

904 **F4 generation *vtg*-KO mutants.** Left and middle panels illustrate genotyping of embryos at 24 h post-

905 fertilization (hpf) by PCR for *vtg1*-KO (representative of zebrafish type-I *vtgs*) and *vtg3*-KO lines,

906 respectively, from the F0 to F4 generation. F0 indicates the generation reared from microinjected embryos

907 and F1-4 represent offspring raised from each subsequent generation. The agarose gel electrophoresis

908 results shown here represent screening of 10 randomly sampled embryos as representatives of their

909 generations and two additional wild type embryos as controls. Bands comprised of wild type intact gDNA

910 (3642 bp and 1733 bp for *vtg1* and *vtg3*, respectively) and mutated gDNA (2361 bp and 551 bp for *vtg1*-

911 KO and *vtg3*-KO, respectively) are shown and highlighted by black arrowheads on the right side of each

912 panel. Open circles; non-related wild type fish (Wt) carrying intact (*vtg1*<sup>+/+</sup> or *vtg3*<sup>+/+</sup>) genomic DNA.

913 Open diamonds; sibling wild type individuals, which do not carry the desired mutation in either allele

914 (*vtg1*<sup>+/+</sup> or *vtg3*<sup>+/+</sup>) of their gDNA. Open triangles; heterozygous (Ht) individuals carrying the

915 introduced mutation on only a single allele (*vtg1*<sup>-/+</sup> or *vtg3*<sup>-/+</sup>) in their genomic DNA. Asterisks;

916 homozygous embryos (Hm) carrying the introduced mutation in both alleles (*vtg1*<sup>-/-</sup> or *vtg3*<sup>-/-</sup>) of their

917 genomic DNA. The panel on the far right illustrates the general strategy followed to establish pure

918 zebrafish lines bearing the desired Cas9 introduced mutation. This process involved stepwise reproductive

919 crosses (indicated by X) between males (♂) and females (♀) indicated here with zebrafish icons. F0-4

920 represents the zebrafish generations produced in the process. Images of sub-adult fish are shown for

921 simplicity at generation F4; all or most of these fish were actually inviable and did not survive past early

922 developmental stages (see text for details).

923

924 **Fig 4. Relative quantification of *vtg* gene expression in *vtg*-KO zebrafish female liver. A)**

925 Comparison of gene expression levels for all *vtgs* in F3 *vtg1*-KO female liver (Hm, homozygous; Ht,

926 heterozygous; wt, sibling wild type) versus non-related wild type female liver (Wt). TaqMan qPCR-2<sup>-ΔΔCT</sup>

927 mean relative quantification of gene expression was employed using zebrafish 18S ribosomal RNA (*18S*)

928 as the reference gene. Data were statistically analyzed using a Kruskal Wallis nonparametric test  $p < 0.05$   
929 followed by Benjamini Hochberg corrections for multiple tests  $p < 0.1$ . **B)** Comparison of gene expression  
930 levels for all *vtgs* in F3 *vtg3*-KO female liver (Hm, Ht and wt) versus Wt female liver. SYBR Green  
931 qPCR- $2^{-\Delta\Delta CT}$  mean\_relative quantification of gene expression normalized to the geometric mean  
932 expression of zebrafish elongation factor 1a (*ef1a*), ribosomal protein L13a (*rpl13a*) and *18S* was  
933 employed. Data were statistically analyzed using a Kruskal Wallis nonparametric test  $p < 0.05$  followed by  
934 Benjamini Hochberg corrections for multiple tests  $p < 0.1$ . In the box plots, the centerlines indicate the  
935 median for each data set, upper boxes indicate the difference of the 3<sup>rd</sup> quartile from the median, lower  
936 boxes indicate the difference of the 1<sup>st</sup> quartile from the median. Top whiskers indicate difference of the  
937 maximum value from the 3<sup>rd</sup> quartile and the bottom whiskers indicate the difference of the minimum  
938 values from the 1<sup>st</sup> quartile in each data set. In both panels, numbers below x-axis labels indicate sample  
939 size and lowercase letters above the error bars represent significant differences between means ( $p < 0.05$ ).  
940 For box plots sharing a common letter superscript, the means are not significantly different.

941  
942 **Fig 5. Relative quantification of multiple vitellogenins by LC-MS/MS in *vtg*-KO zebrafish female**  
943 **liver and eggs.** **A)** Comparisons of mean normalized spectral counts (N-SC) for Vtg protein levels in Wt  
944 versus Hm F3 *vtg1*-KO female zebrafish livers and in eggs obtained from these females, indicated by dark  
945 and light gray vertical bars, respectively. Vertical brackets indicate SEM. **B)** Corresponding comparison  
946 of N-SC for Vtg protein levels in Wt versus F3 Hm *vtg3*-KO female zebrafish livers and in eggs obtained  
947 from these females. Asterisks indicate statistically significant differences between group means detected  
948 by an independent samples Kruskal Wallis non-parametric test ( $p < 0.05$ ) followed by Benjamini Hochberg  
949 correction for multiple tests ( $p < 0.1$ )

950  
951 **Fig 6. Detection of Vtg3 in *vtg3*-KO versus wild type female liver, ovary and eggs by Western**  
952 **blotting.** An affinity-purified, polyclonal anti-zfLvL3 antibody was employed to detect LvL3 in this  
953 experiment. Numbers on the left of each panel indicate the mass of molecular weight marker proteins

954 (kDa). M, marker protein ladder; Hm, homozygous; Ht, heterozygous; Wt, non-related wild type. Bands  
955 that were detected in Ht and Wt zebrafish whose mass corresponds to that of zebrafish Vtg3 LvL (LvL3,  
956 ~24 kDa) are indicated with brackets and labels immediately underneath (LvL3).

957 **Fig 7. Phenotypic measurements of F3 *vtg*-KO females and their F4 progeny.** Bar graphs indicate  
958 mean values ( $\pm$ SEM) for measurements of each parameter and labels below the x-axes indicate the groups  
959 that were compared. In the panel at the bottom right, mean hatching percentages for Hm *vtg1*-KO, Hm  
960 *vtg3*-KO, and Wt eggs are shown as circles, triangles and diamonds, respectively. Numbers on the x-axis  
961 accompanied by dashed- and solid-lined arrows represent sampling times in hours or days post spawning,  
962 respectively. In all graphs, asterisks and black stars indicate mean values that are statistically significantly  
963 different from corresponding Wt mean values based upon results of an independent samples t-test  
964 ( $p < 0.01$ ) followed by Benjamini Hochberg corrections for multiple tests in the case of hatching  
965 percentage ( $p < 0.05$ ).

966  
967 **Fig 8. Comparisons of survival percentages for homozygous F4 *vtg1*-KO and *vtg3*-KO zebrafish**  
968 **embryos and larvae versus wild type offspring.** Line plots represent mean survival percentages and  
969 numbers on the x-axis accompanied by dashed- and solid-lined arrows represent sampling times in hours  
970 or days post spawning during the observation period. Mean survival percentages for Hm *vtg1*-KO, Hm  
971 *vtg3*-KO and unrelated Wt embryos and larvae at each time point are indicated by circles, triangles and  
972 diamonds, respectively, and vertical lines indicate SEM. Asterisks and black stars indicate mean values  
973 that are statistically significantly different from corresponding mean wild type (Wt) values based upon  
974 results of an independent samples t-test ( $p < 0.01$ ) followed by Benjamini Hochberg corrections for  
975 multiple tests ( $p < 0.05$ ).

976  
977 **Fig 9. Observed phenotypes of F4 *vtg*-KO offspring compared to wild type offspring.** **A) Hm *vtg1*-**  
978 **KO unhatched embryos and hatched larvae at 4 dps. B) Wt larva at 4 dps. C) Hm *vtg3*-KO larvae at 4 dps.**  
979 **D) Hm *vtg1*-KO larvae at 8 dps. E) Wt larva at 8 dps. F) Hm *vtg3*-KO larvae at 8 dps.** Special features of

980 observed phenotypes are indicated by pointed arrows (see text for details). In all images, horizontal bars  
981 indicate 1000  $\mu\text{m}$ .

982

983

## 984 **10. SUPPORTING INFORMATION LEGENDS**

### 985 **S1 Fig. Location and character of mutations introduced by CRISPR/Cas9 in zebrafish *vtgs* A-B)**

986 Location on genomic DNA. Schematic representations of the intron/exon structure of zebrafish *vtg1*  
987 (representative of type-I *vtgs*) and *vtg3* are given at the top of panels **A** and **B**, respectively. Horizontal  
988 line segments indicate introns and filled gold boxes indicate exons. Exons bearing CRISPR/Cas9 target  
989 sequences are indicated by magenta-colored arrows pointing upwards to the target name (sg11, sg12, and  
990 sg13 for *vtg1*; sg31, sg32, and sg33 for *vtg3*). Horizontal dashed lines bearing dual arrowheads indicate  
991 regions where mutations were introduced, with the size of deletions in bp given below the arrows (1281  
992 bp and 1182 bp for *vtg1*-KO and *vtg3*-KO, respectively). The lower sections of panels **A** and **B** show  
993 Clustal Omega alignments for partial genomic sequences of the *vtg1* and *vtg3* genes, respectively,  
994 covering regions where Cas9 introduced targeted mutations. Sequences of undisturbed wild type alleles  
995 are labeled *vtg1*<sup>+/+</sup> and *vtg3*<sup>+/+</sup>, and sequences of homozygous mutated alleles are labeled *vtg1*<sup>-/-</sup> and  
996 *vtg3*<sup>-/-</sup>, respectively. Dashes were introduced to illustrate regions where deletions occurred in the *vtg1*<sup>-/-</sup>  
997 and *vtg3*<sup>-/-</sup>-sequences. Nucleotide positions are indicated by numbers on the right and asterisks indicate  
998 nucleotide identity. Target sequences are enclosed in magenta-colored boxes emphasized by magenta-  
999 colored arrows on the right. Intron sequences are given in dark gray font enclosed in light gray filled  
1000 frames and are labeled by Intron on the right with the same formatting. Exons are shown in regular black  
1001 font and labeled on the right with exon numbers (e.g. Exon 6, 7, 8...). Exons bearing the target sites are  
1002 also labeled with the target name below in parenthesis (e.g. Exon 14/(sg12)). **C-D)** Location on predicted  
1003 cDNA. Nucleotide sequences targeted by sgRNAs for Cas9 editing and present in the predicted transcript  
1004 are framed in magenta-shaded boxes. The deleted region of the transcript is indicated by dashes replacing  
1005 nucleotide residues and the size of the deletion in bp is given by gray highlighted text in this region (703



1006 bp deletion for *vtg1* and 714 bp deletion for *vtg3*). The sequence encoding the receptor-binding domain  
1007 (***RbD***) on the LvH of the respective Vtg is shown in italic bold typeface with the sequence encoding the  
1008 critical, short receptor-binding motif (***RbM***) being additionally underlined. **E-F**) Location on predicted  
1009 polypeptide sequences. Schematic representations of the yolk protein domain structures of Vtg1  
1010 (representative of zebrafish type-I Vtgs) and Vtg3 are given in 5' > 3' orientation above each panel. Light  
1011 gray horizontal bars represent the lipovitellin heavy and light chain (LvH, LvL) and the phosvitin (Pv)  
1012 yolk protein domains of the respective Vtg (Vtg3 lacks a Pv domain) and are labeled above in large bold  
1013 type. Sequences within these bars indicate the N-terminus of each yolk protein domain, the start of which  
1014 is also indicated by vertical bars in the polypeptide sequence shown below. The ***RbM*** is shown in bold  
1015 italic underlined font on the gray horizontal bars in the LvH1 and LvH3 domains. The ***RbD*** and ***RbM*** are  
1016 also indicated in the polypeptide sequences shown below by bold italic font with the ***RbM*** being  
1017 additionally underlined. Residues encoded by nucleotide sequences targeted by sgRNAs for Cas9 editing  
1018 are framed in magenta-shaded boxes. Cas9 created mutations (large deletions) are indicated with dashes  
1019 replacing amino acid (aa) residues and the size of deletions in aa (234 aa and 239 aa for *vtg1*-KO and  
1020 *vtg3*-KO, respectively) in these regions are labeled by gray shaded text. Short sequences that were  
1021 employed as epitopes to develop Vtg domain-specific antibodies against Vtg1-LvH (anti-zfLvH1) and  
1022 Vtg3-LvL (anti-zfLvL3) are indicated by framed text on the LvH and LvL domains of Vtg1 and Vtg3,  
1023 respectively, with their location also highlighted by black arrows labeled with the epitope names given by  
1024 vertically-oriented text in the panel margins.

1025

1026 **S1 Table. Targets, primers and probes utilized in *vtg1*-KO and *vtg3*-KO studies.** Target oligo an  
1027 screening primer names are given according Figure 1. CRISPR recognition NGG motifs are highlighted by bold  
1028 typeface on sequences. Position of primers, target sites and probes on vitellogenin (Vtg) yolk protein (YP)  
1029 domains are given on the far right columns.

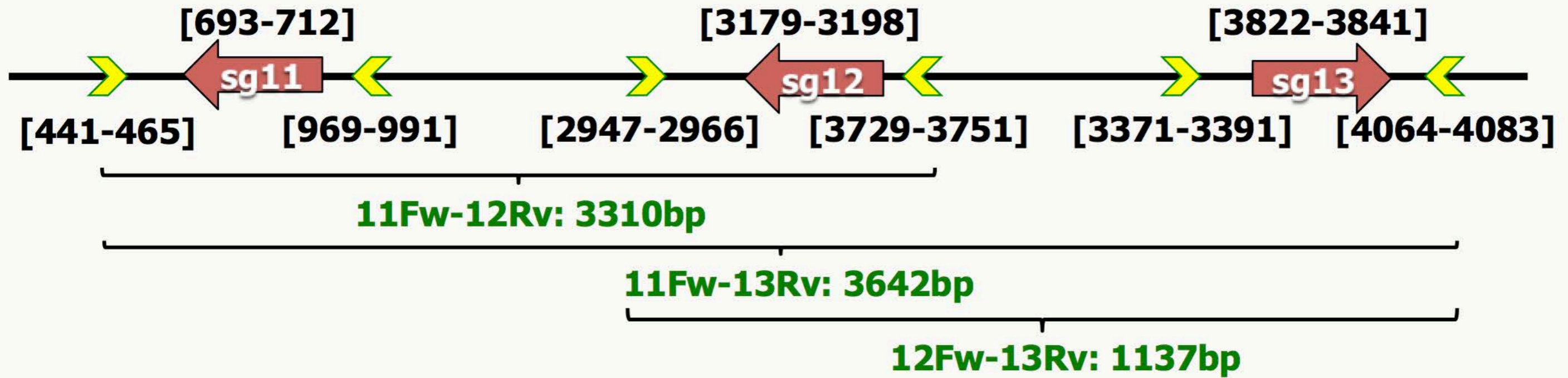
1030

1031

# A) *vtg1*-KO

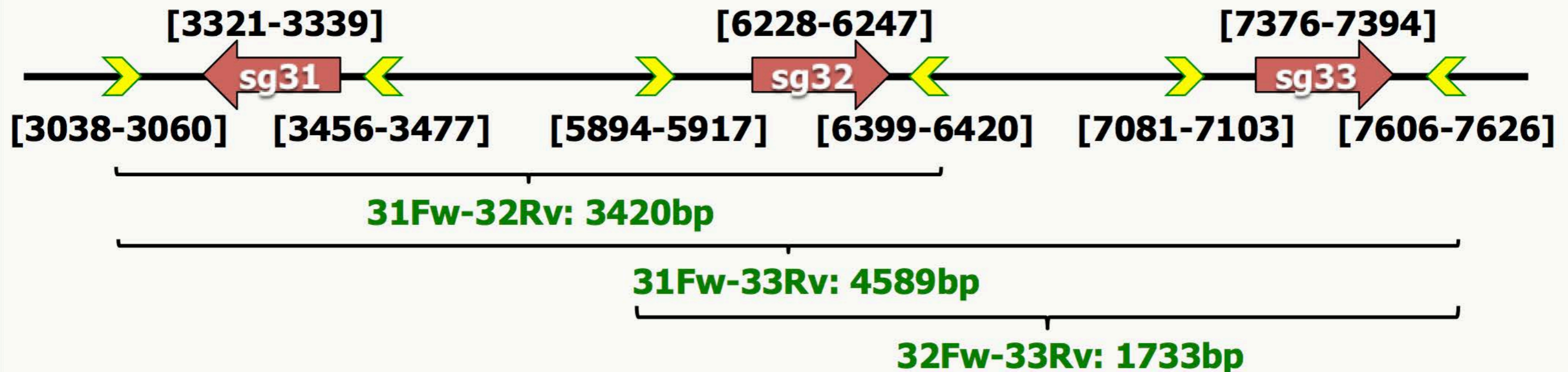
*vtg1, 4, 5, 6 & 7* = 6671-7493 bp gDNA

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# B) *vtg3*-KO

*vtg3* = 20947 bp gDNA



**LvH1**

**Pv1 LvL1**

5' QQFNLVPEFAHDKTYVYKYEALLLGGLPQEGGLARAGIKVSSKVL REILDTRMSKTAT-- 3'

anti-zfL<sub>v</sub>H1 epitope

>ENSDART0000050238 peptide: ENSDARP0000050237 pep:KNOWN\_protein\_coding  
MRAVVLALTVALVAC | QQFNLVPEFAHDKTYVYKYEALLLGGLPQEGGLARAGIKVSSKVL  
ISATTENTYLMKLM DPLL EYAGTWP KDPFVPATKLT SALAAQLQIP IKFEYANGVVGKV  
FAPAGVSP TVMNLHRGI **LNILQL** NLKKTQNI YEMQEAGA QGVC RTHYVI **NEDPKANHI IV**  
**TKSKDL** SHCQERIMKDVGLAYTERCAECTERVKSLIETATYNYIMKPADNGALIAEATVE  
EVYQFS PFNE IHGAAMMEAKQTLAFVEIEKTPVVP IKADYMPRGS LQYEFATEILQTP IQ  
LMKISDAPAQ IVEVLKHLVSNKDMVHDDAPFKFVQLVQLLRVASLEKIEAIWSQFKDKP  
VYRRWLLDALPAVGTPV I IKFIKEKFLAGEFTTPEF IQTLVIALQMV TADPETIKMTASL  
ATHEKFATIPALREV VMLGYGSLIAKYCVAVPTCPAELLRP IHEIATEAISKNDIPEITL  
ALKVMGNAGHPSS LKPIMKLLPGLRTAANALP IRVQVD AI  
-----  
----- 234 aa deletion -----  
-----  
-----MATGPKPRALL **KEALKAI** QEGVAFQYA  
KPLLAAEVRRILPTAVGVPM EFSWYTA AVAAASVNVQATITPALPEKLESMTYEQLKKT D  
VQFQAEARPSVALQTFAVMGVNTAF IQAAVMARGKIRTIAPGKVAARADILKGN YKVEAL  
PVELPEHIASASFETYAVVRNIEDHSAERSVPLVPELSLQNSQASYAGDLSSEMSSVASV  
RAPAPFDRTLCYAVPYIEIKGCVEVHSHNAAFIRNSTLFYIIGHHSVRAAVARAEGPAVE  
RLEFEVQVGPRAAERLVKQINI IDDDTPEGQAFLLKL | REILDTEAKNAPVSSSESSSRN  
SRSSSSRSTSTSTSSSSSSSSSSSSSSSSSSSSSSSSMSSS | RMSKTATIIEPFRKFHKDRYLAHHS  
ATKDTSSGSAASFEQMOKQNRFLGNDIPPVFAI IARAVRADQKLLGYQLAAYFDKPTAR  
VQLIVSSIAENDNMKICADGALLSKHKVTGKFSWGAECKQYAVFAKAEAGVLGEFP AARL  
EVEWERLP IIVTTYAKKLGKHILTAAYDTGFRFERATNSEKEIELTAALPSQRSLNI IAR  
IPEITMSKRDIYLPVAVPINPDGTF SIETYEDFLAWIQKYIKEE

RbM / RbD

**LvH3**

**LvL3**

5' MANYEPF-- **IVTRIVDIT**-----TQDL D----- 3'

>ENSDART0000014979 peptide: ENSDARP0000023158 pep:KNOWN\_protein\_coding  
RLCLCLLVALAASE | MANYEPFLNSKKT YEYKYEGLVQV GRELPHLVESALKLRCTFKI I  
GESPHTFVLQVSNVDFEDFNGIP **GKSVFS** PSKNITKHL SAEISQPI IFEYSKGQITDIRT  
APGVSNTV VNIVRGILGFLQVTVKT TQS FYELIELGIHGLCQSSYTV **DEDSNAKELIVTR**  
**IVDITNCQQPASLYRGMALAPEDKLSKQRGESVSVSTVKHTYTVKSTADGGQITKAFAQER**  
**QYFSPFNVKGGNF**-----  
----- 239 aa deletion -----  
-----  
-----KTL LKFLPGYSNGAEKLSTRVQGA AVQAFRL LASRASHSVQDIVLNL FV  
QKHLPAEIRMLACIVLLETKPSTALISV VSEVLL EADLQVASFSYSLLKGF AKSRTPDN  
QHLSIACNIAMKILTRKLG HLSYRYSKNLHFDWFHDDFLFGTSADV YMLQNESPIPTKLM  
LKGKFHF IGRILQFLEFGIRADGLKDLFAGKIP ELTKDLGISDLASILKILSNWQSLPKD  
KPLL TAYARVFGQEAFLMDVSRDSVQSI IKSFS PSAGKESKVWERIQDVQKGT SWHWTKP  
HLVYEARFIQPTCLGLPVEISKYYSV VNAV TMKAKAEINPPPKEHLGELLSSDISM QTDG  
FIGVTKDHFLFHGIN TDLFQCGTE LKSKVSMGLPWAFDLKINPKEQTYEMNLTPSKSVTE  
LFSVSSNVYAVLRNIEDPTSSKITPMP PETGESWQGVPLRMLPPLRDEQTKKSGMKFRQC  
AEAKIYG TALCIEAEAKRAHYLHEYPLYLLGD THFSYSLEPAKDAKPIEKIQI QVSASR  
QHPSVMSGMVNLNQRVFKETRDENTSCEERKTS SSSLPV | TQDL DVTPDPVVTVKALSLS P  
QAKPLGYEGVAFYLPT **AQKDDIEMIVSEVGE**EANWKM CANAHFDKTH TSAKAHLRWGAEC  
QTYDVSMRVSAACQPE SKPSISTKINWGTLP SVFTTVGQIVQEYVPGVSYIMGFYQKKEE  
NPERQASVIVVASSPETFDLKV KIPERTIYKKKIPSP IELLGIEAANLTMST

RbM / RbD

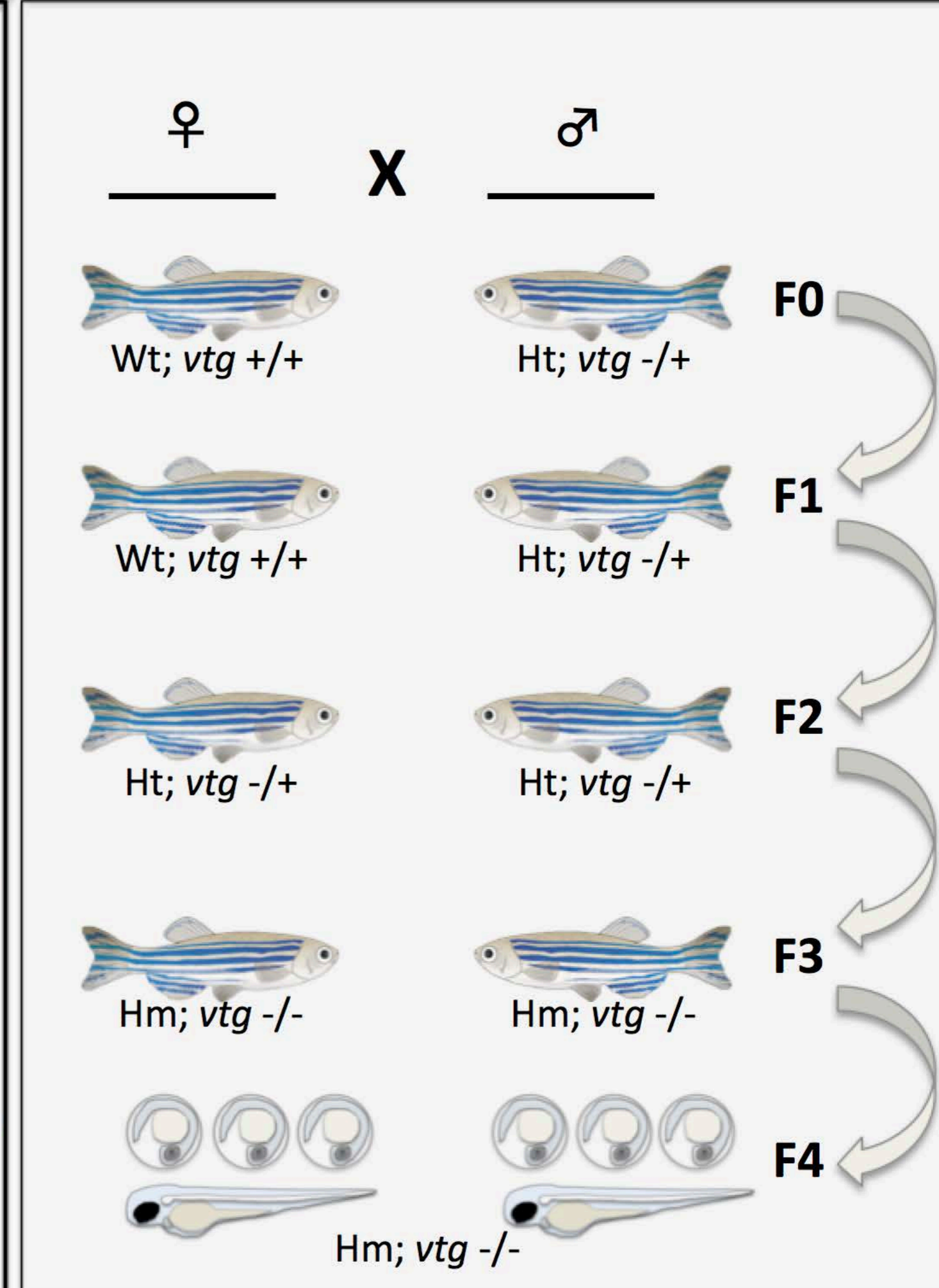
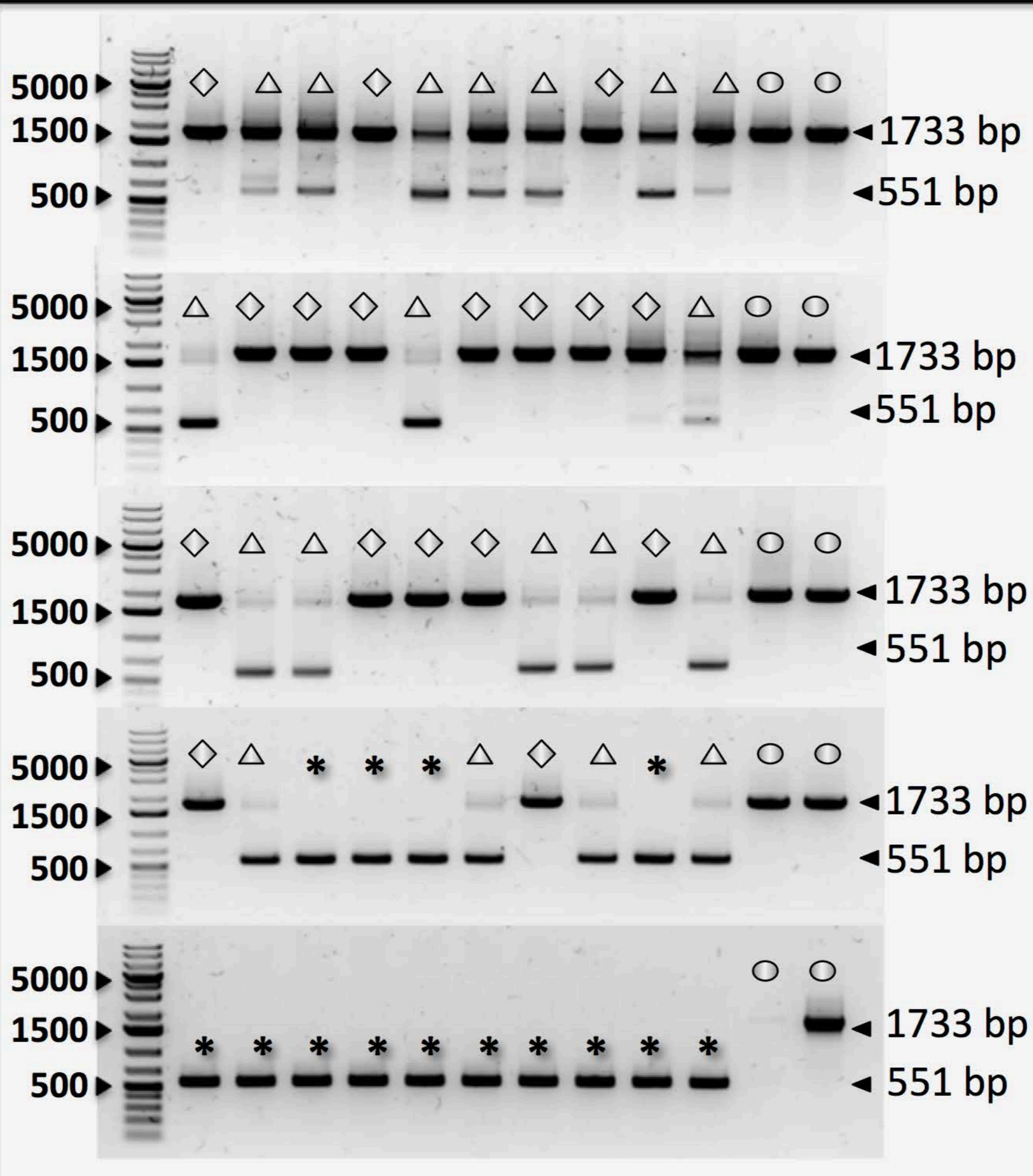
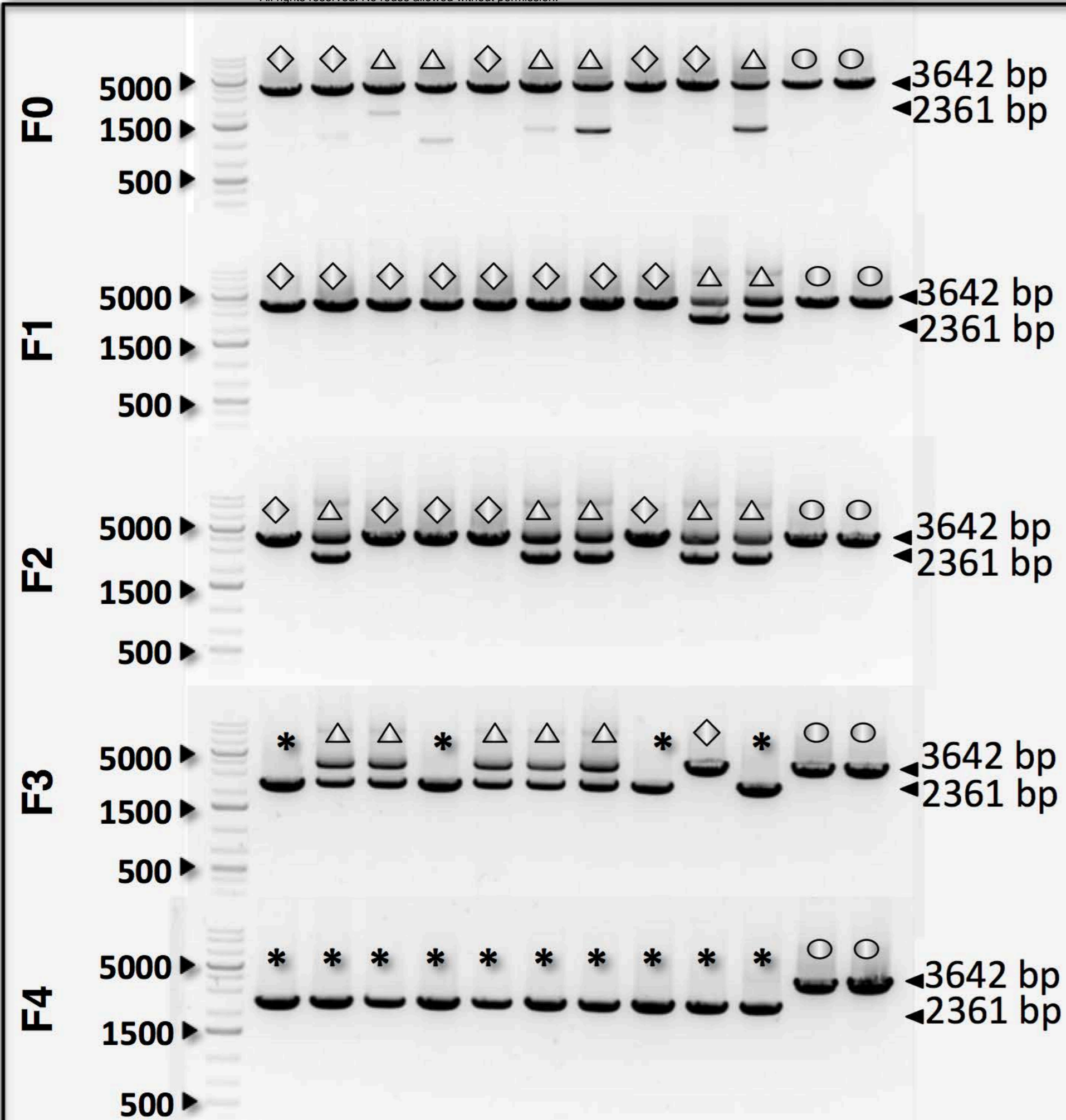
anti-zfL<sub>v</sub>L3 epitope

# *vtg1*-KO Embryo Genotyping

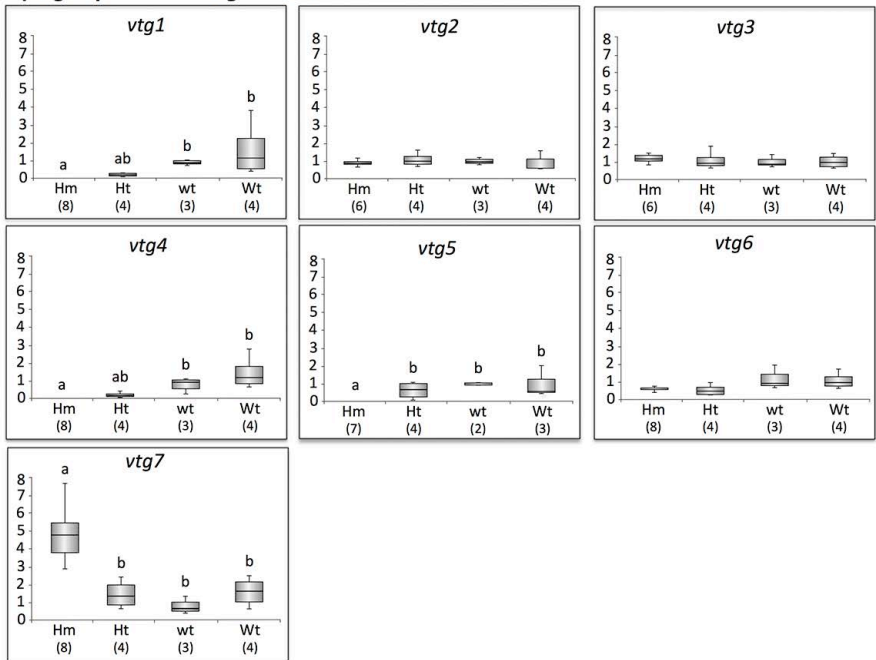
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# *vtg3*-KO Embryo Genotyping

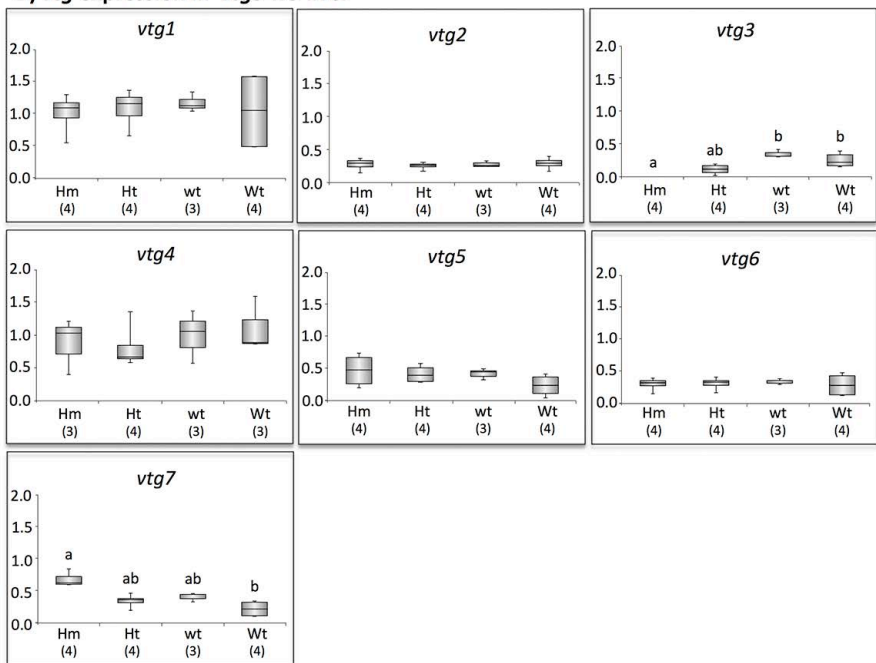
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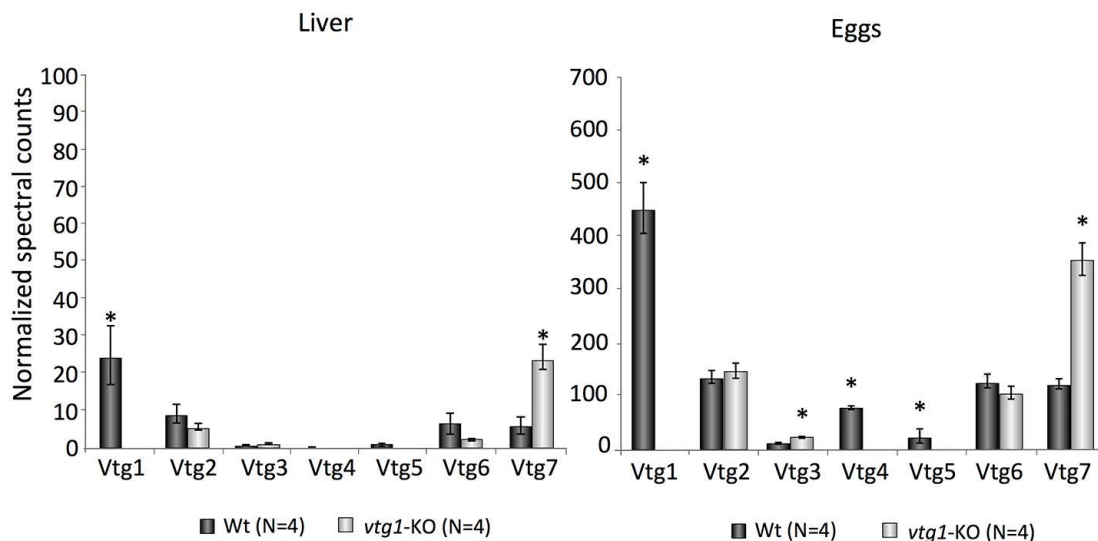
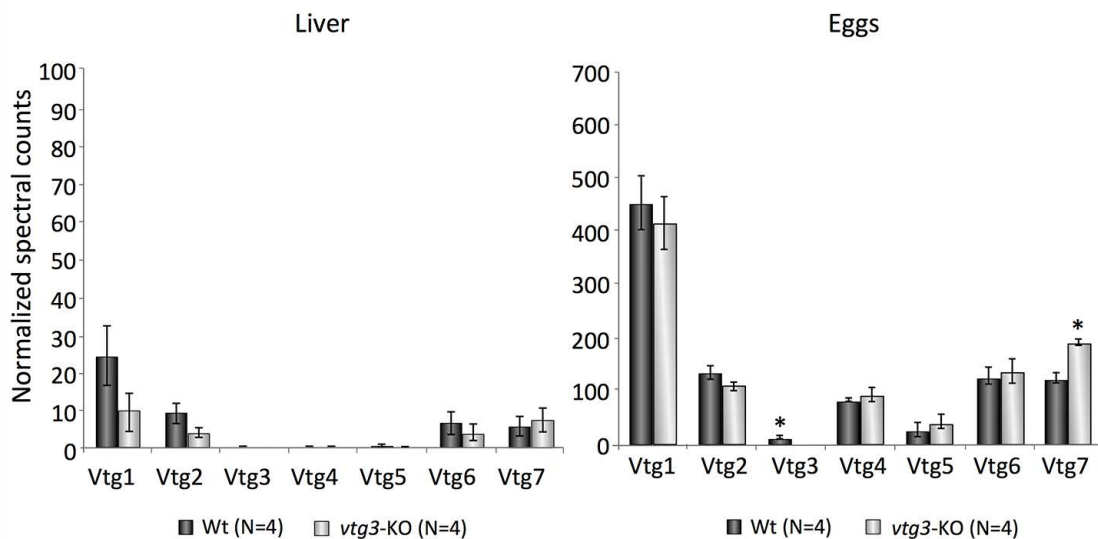


**A) *vtg* expression in *vtg1*-KO liver**

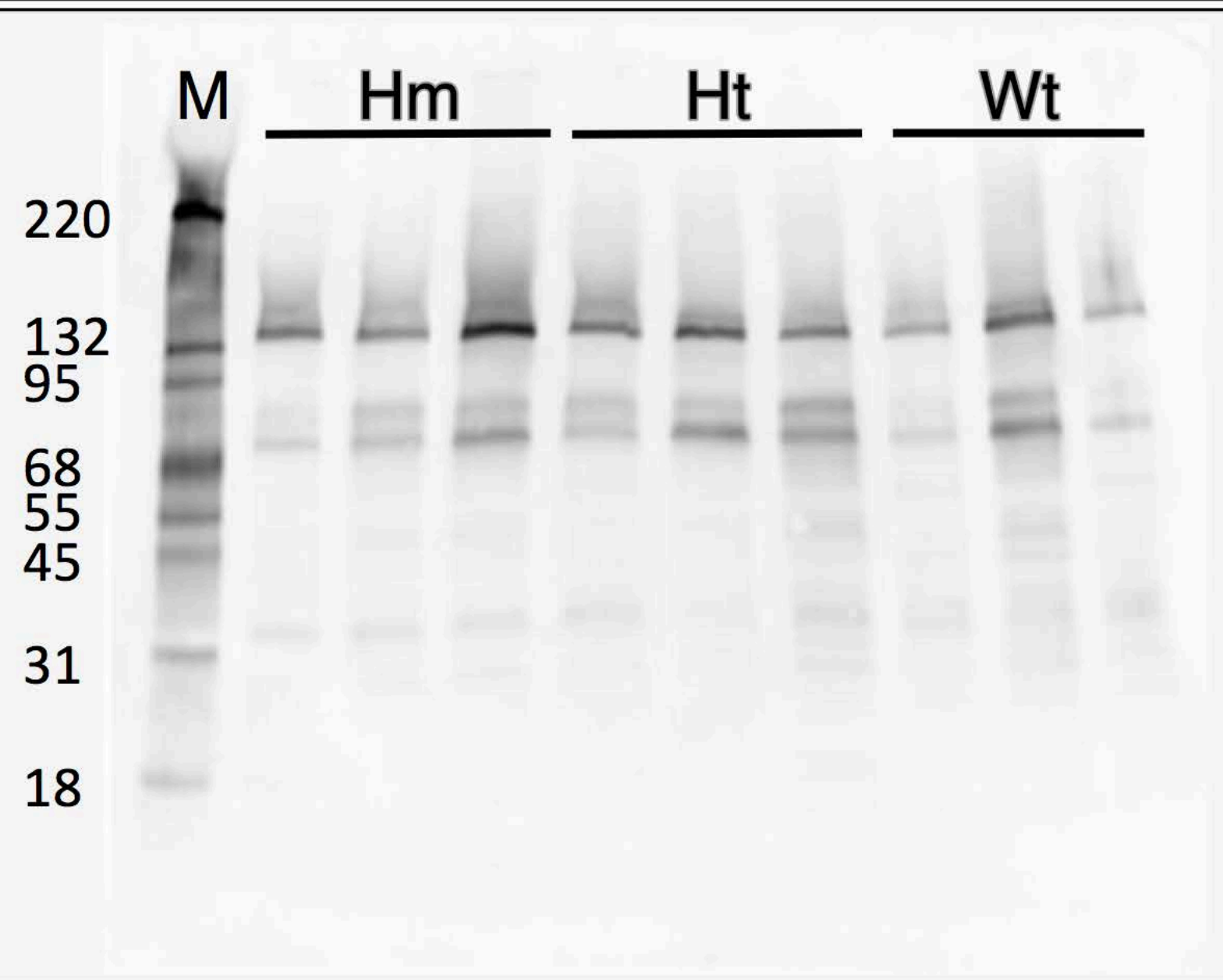


**B) *vtg* expression in *vtg3*-KO liver**

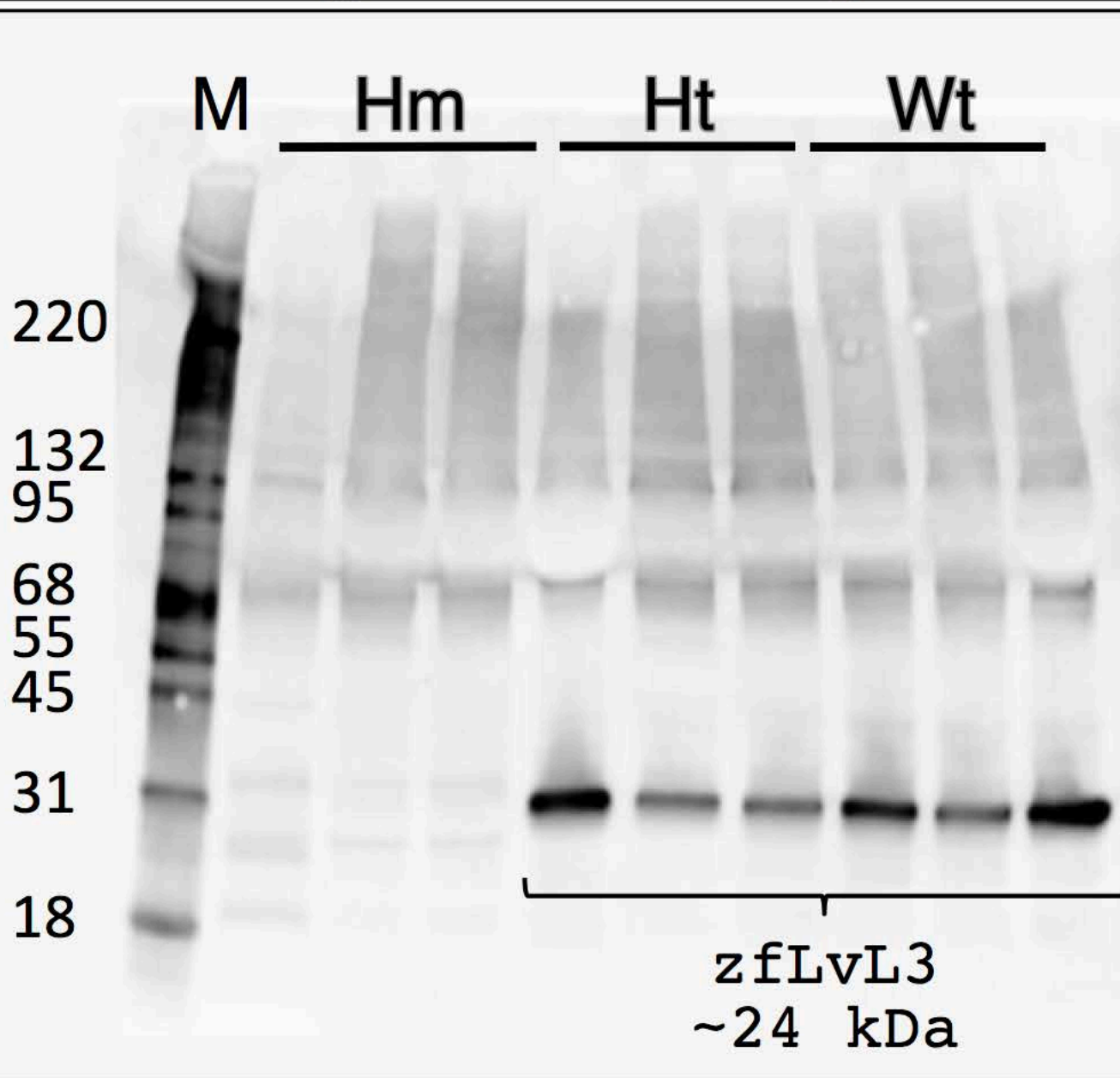


**A) *vtg1*-KO****B) *vtg3*-KO**

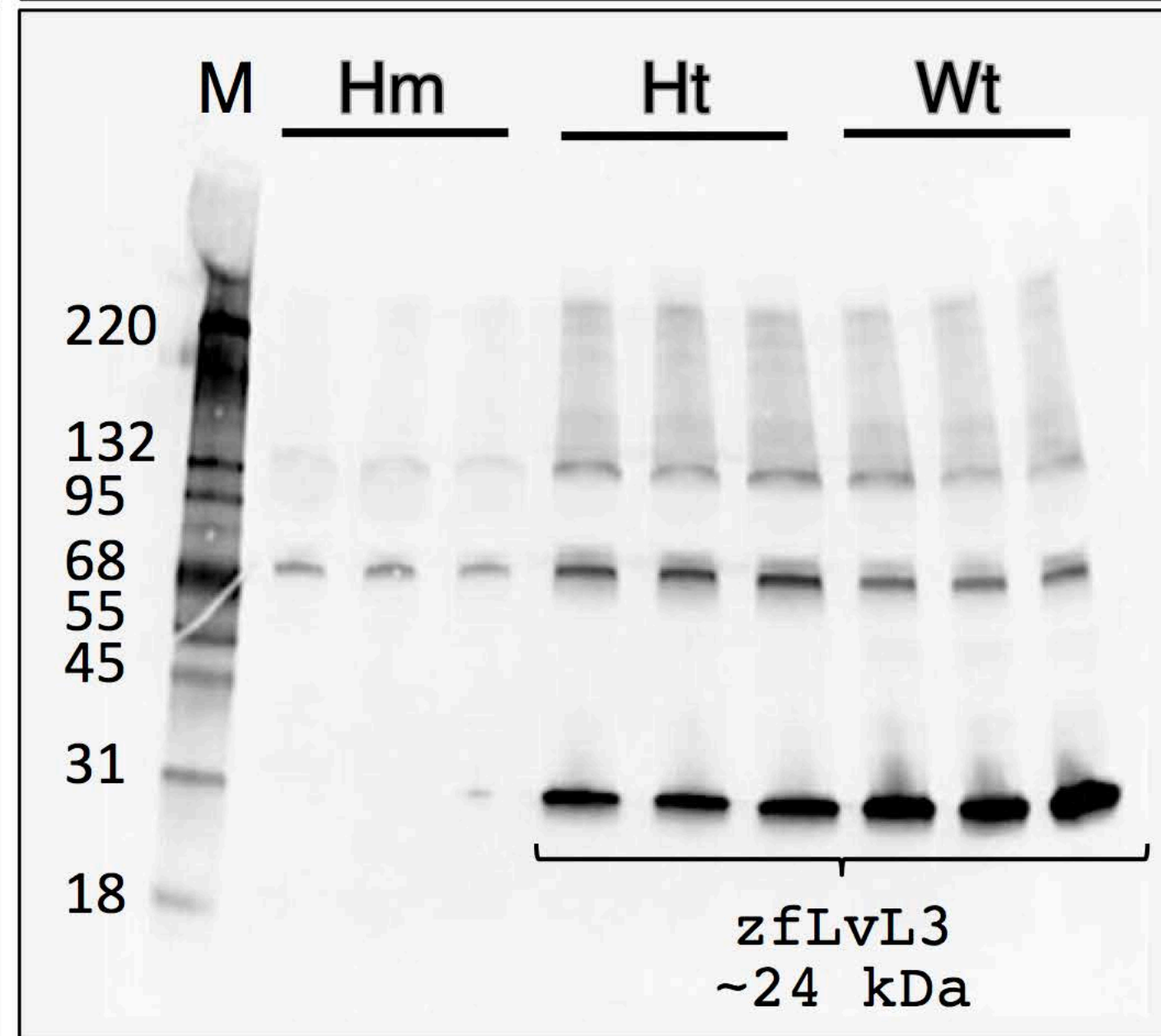
### *vtg3*-KO Liver

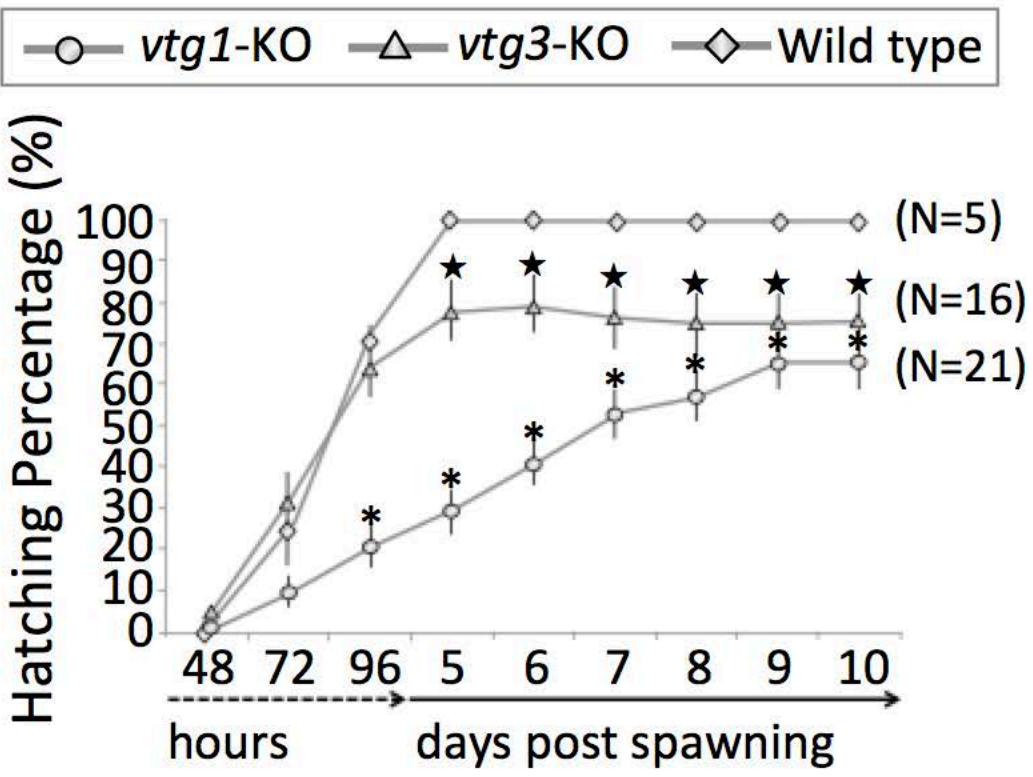
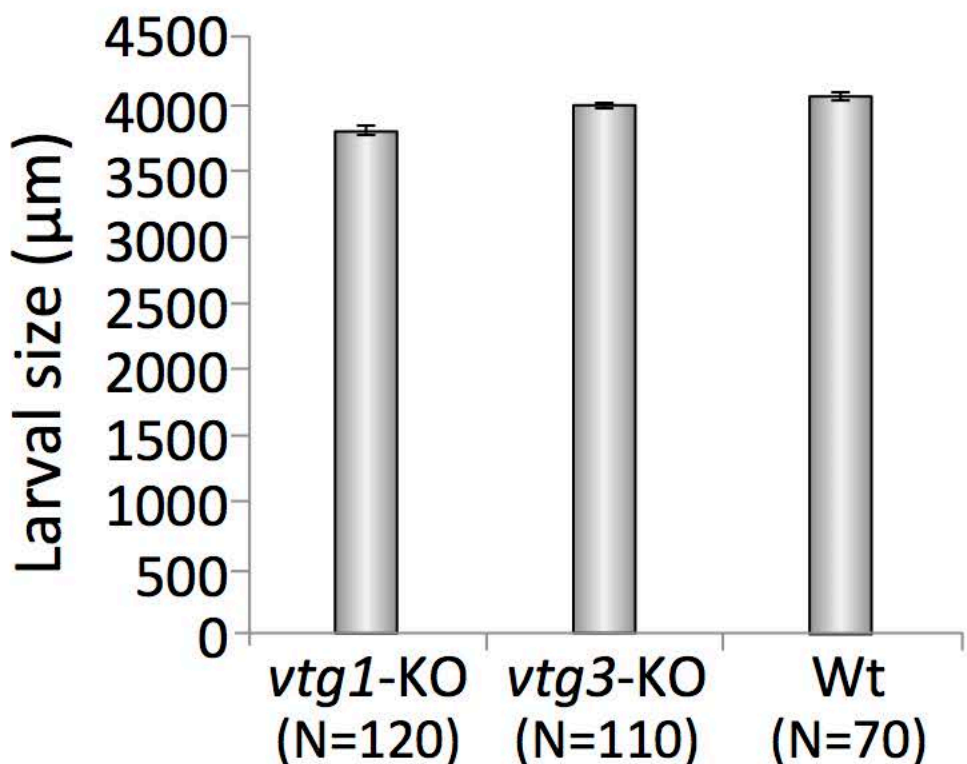
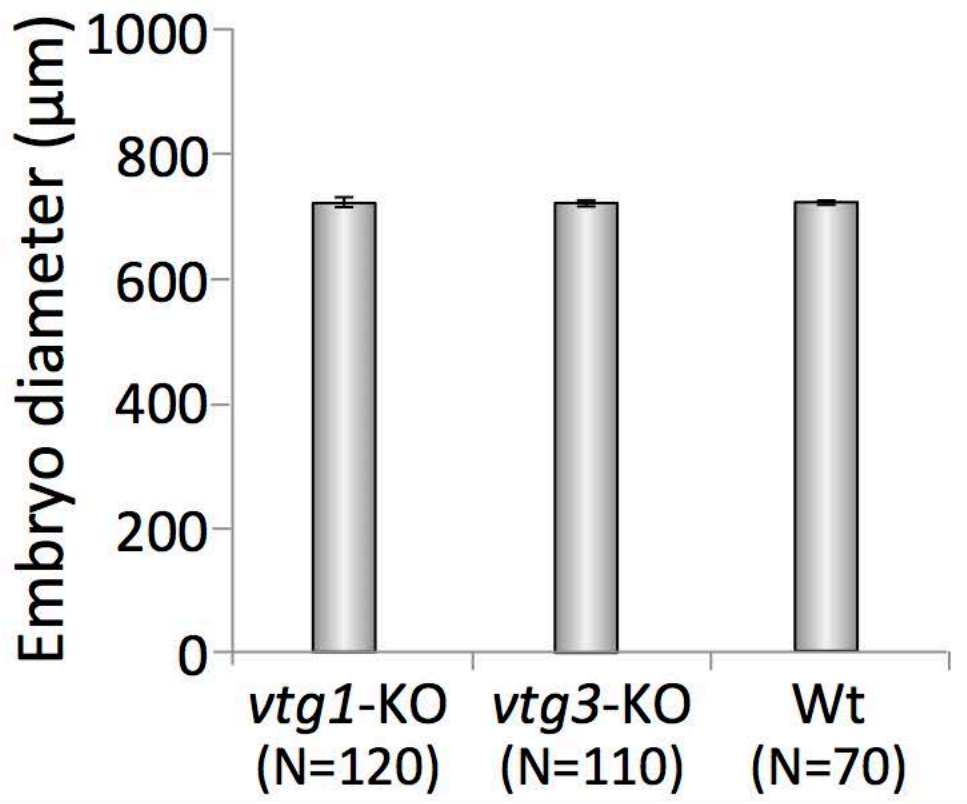
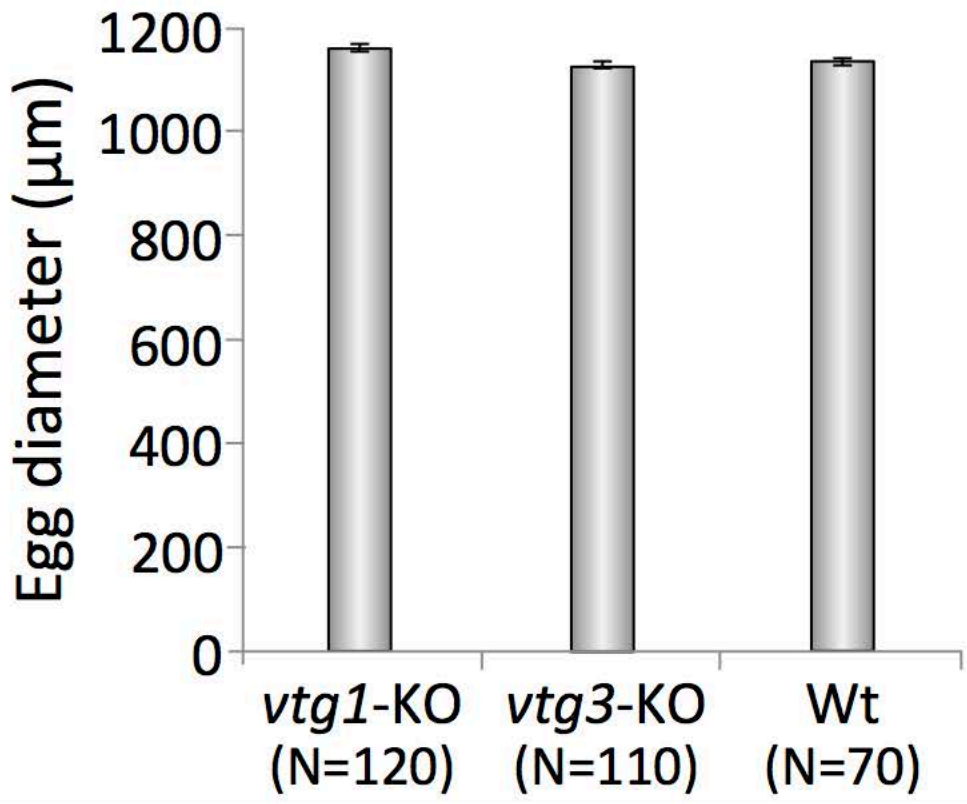
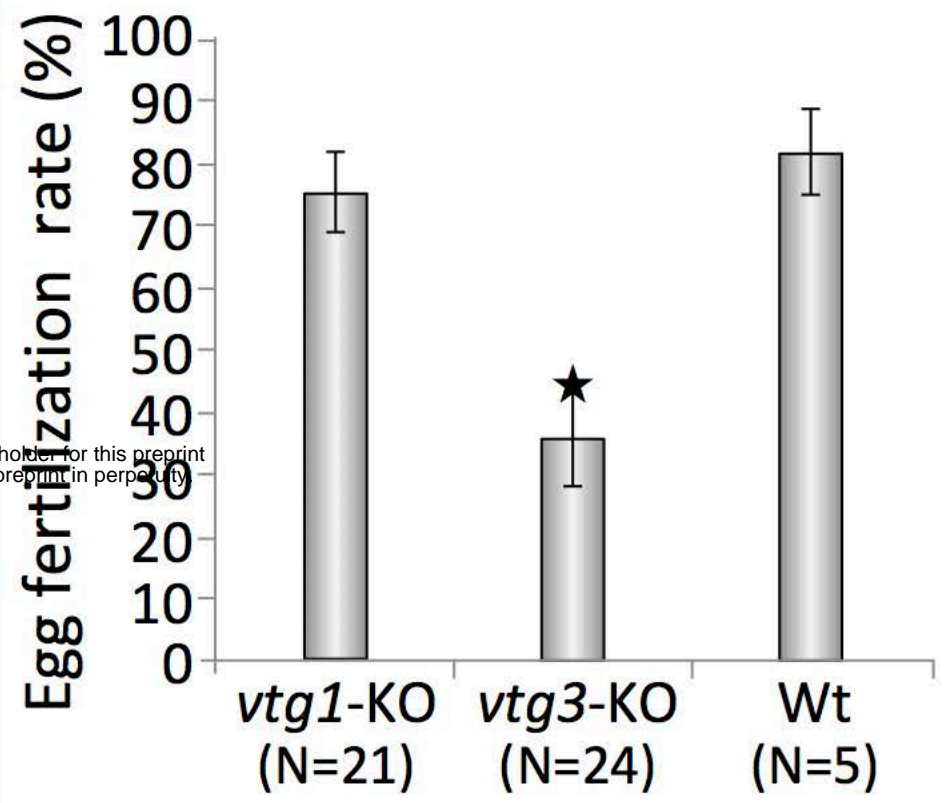
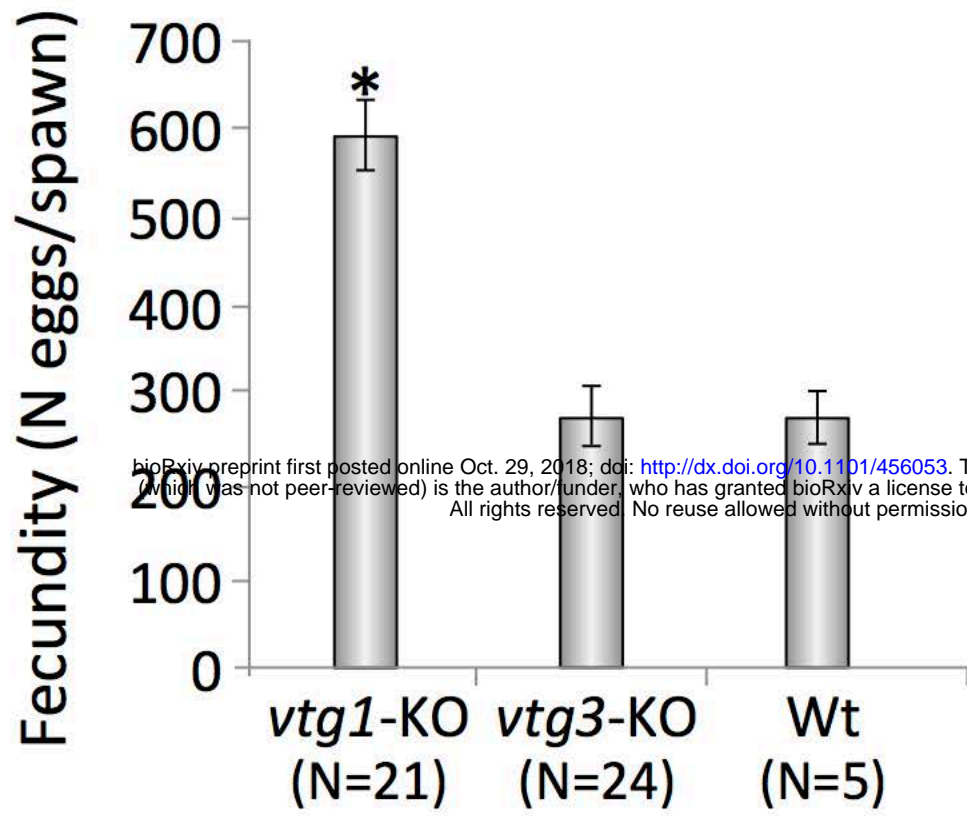


### *vtg3*-KO Ovary

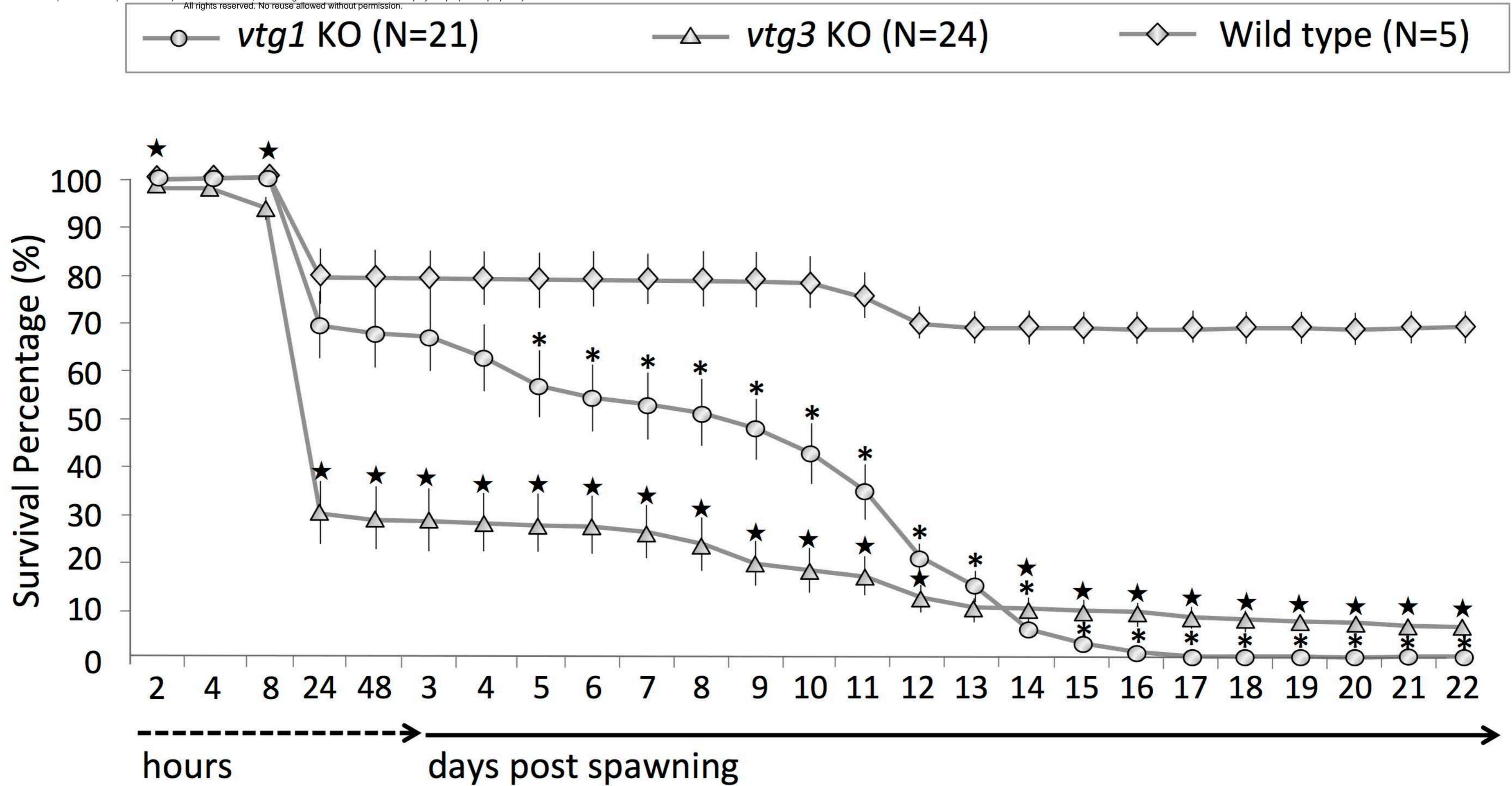


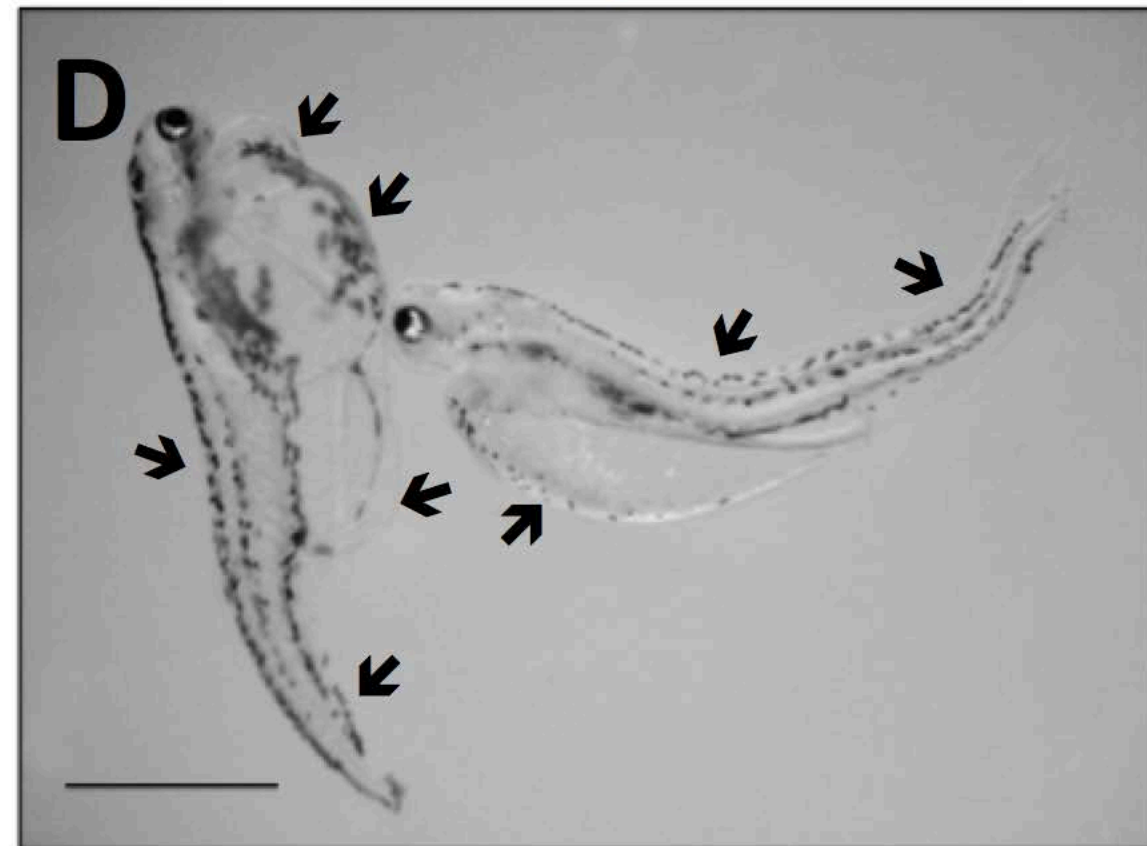
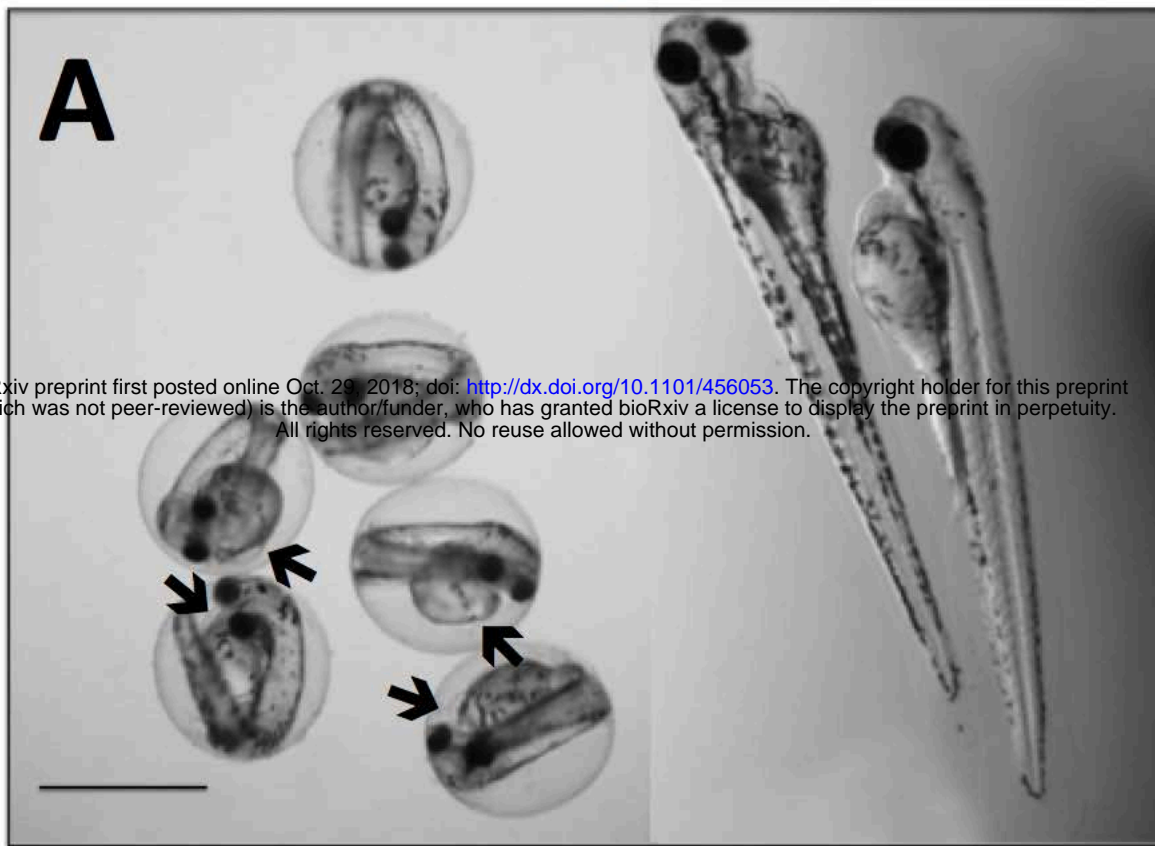
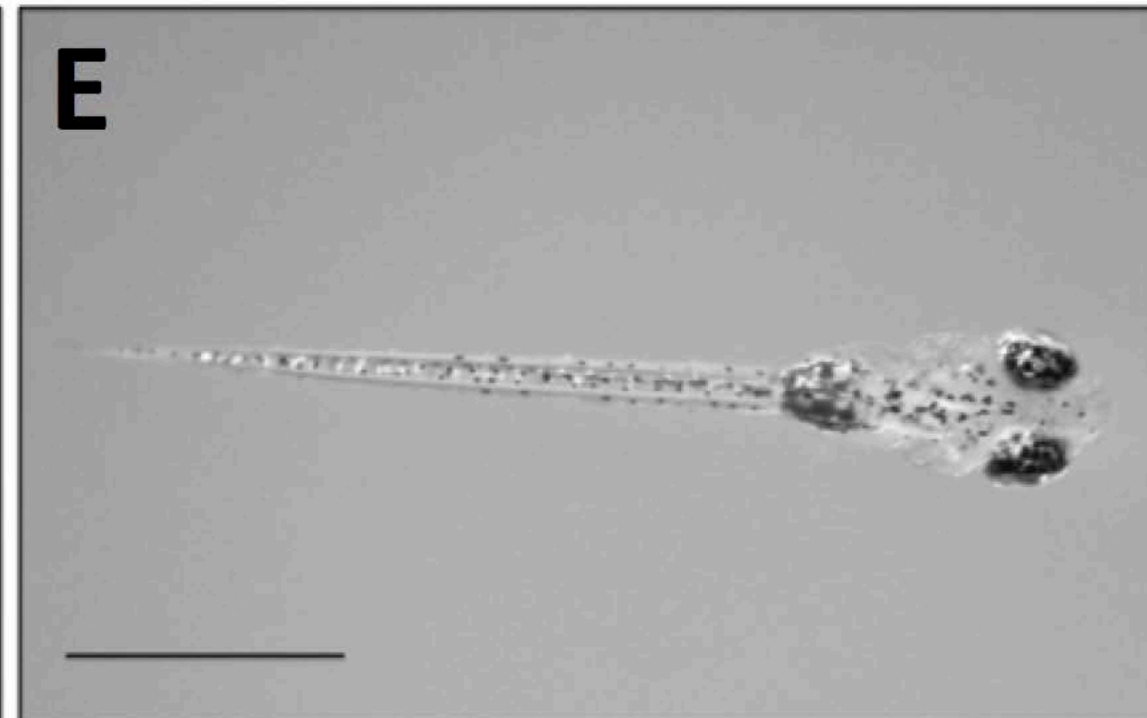
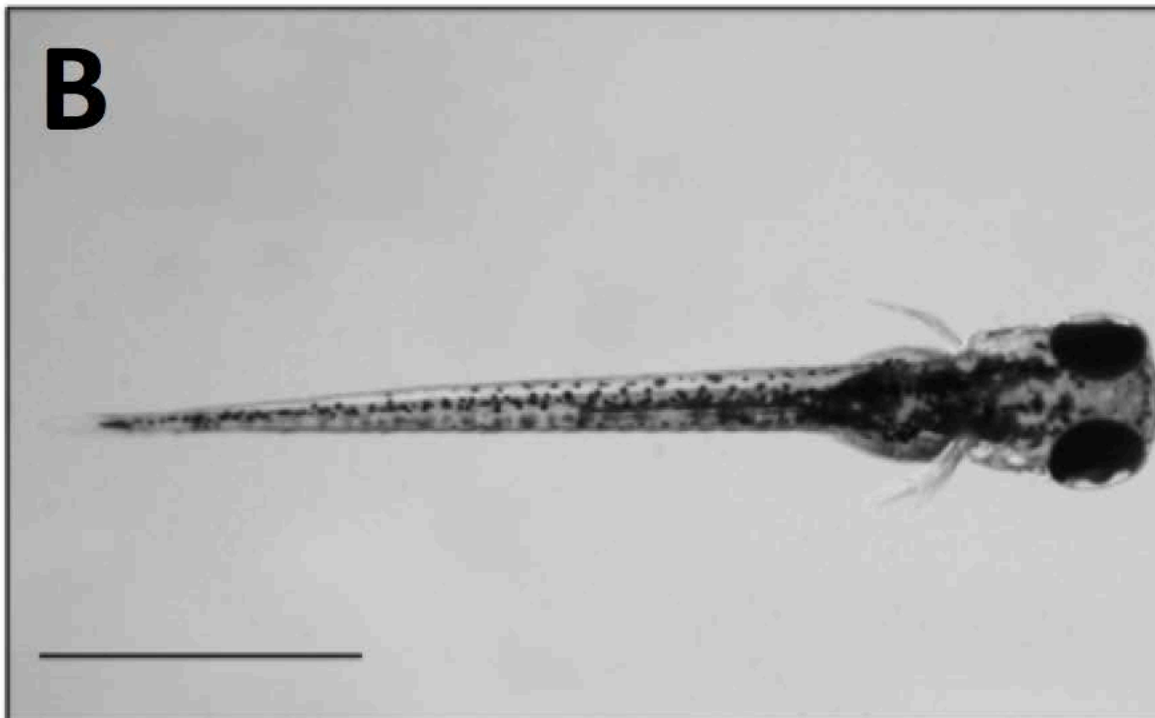
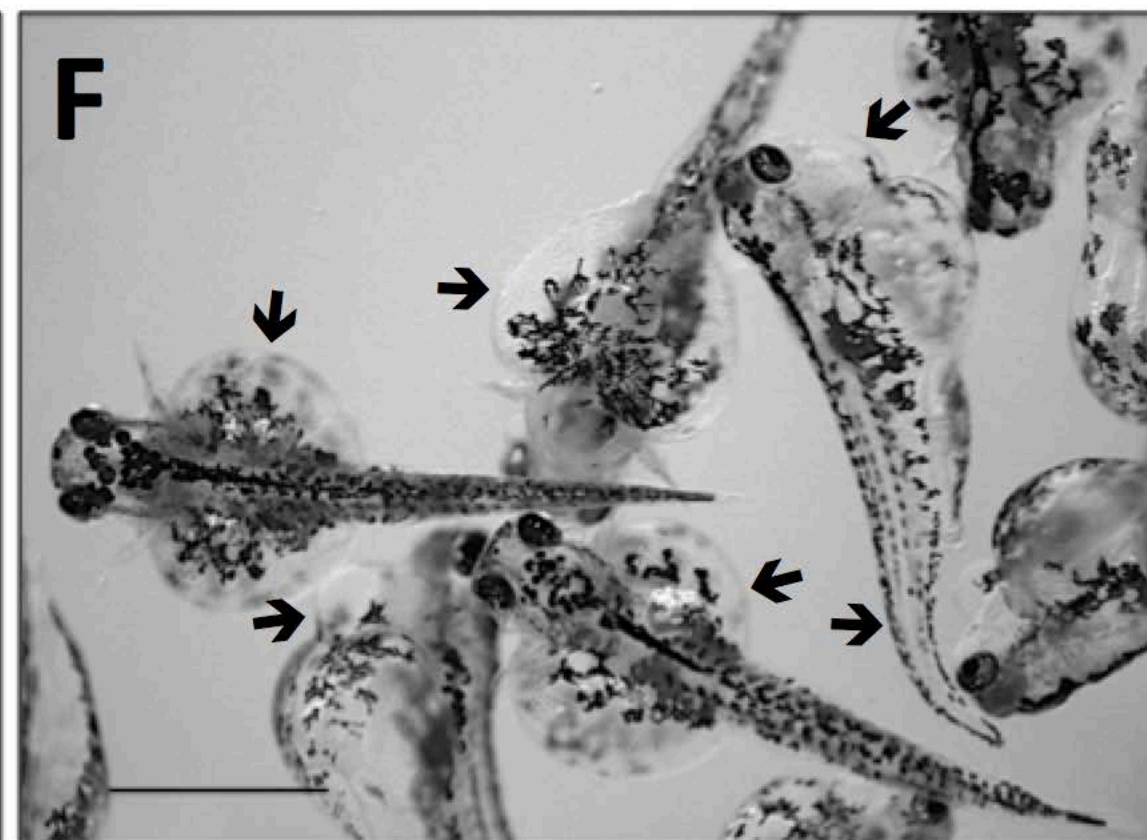
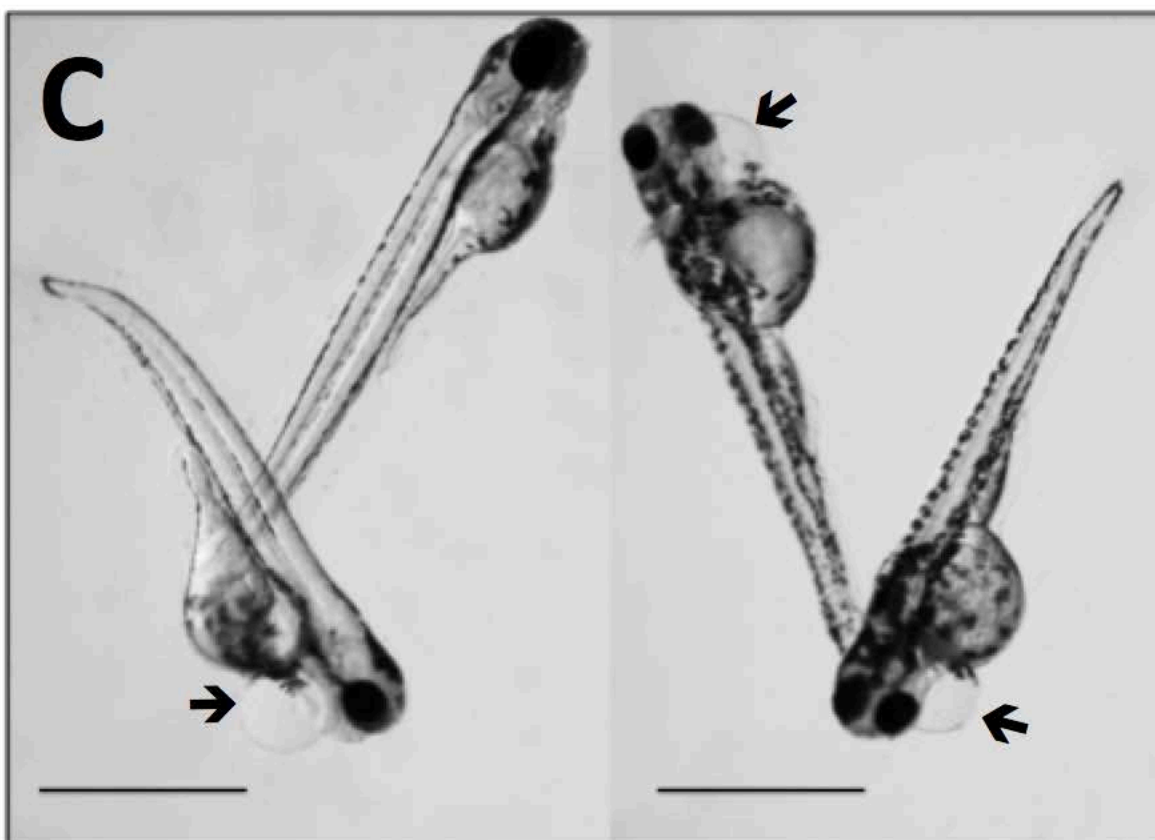
### *vtg3*-KO Eggs



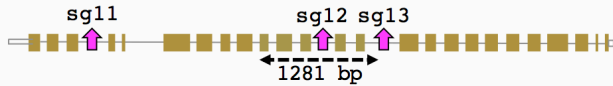






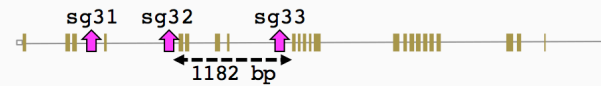
**4 dps****8 dps*****vtg1*-KO****Wild type*****vtg3*-KO**

**A) vtg1-KO**

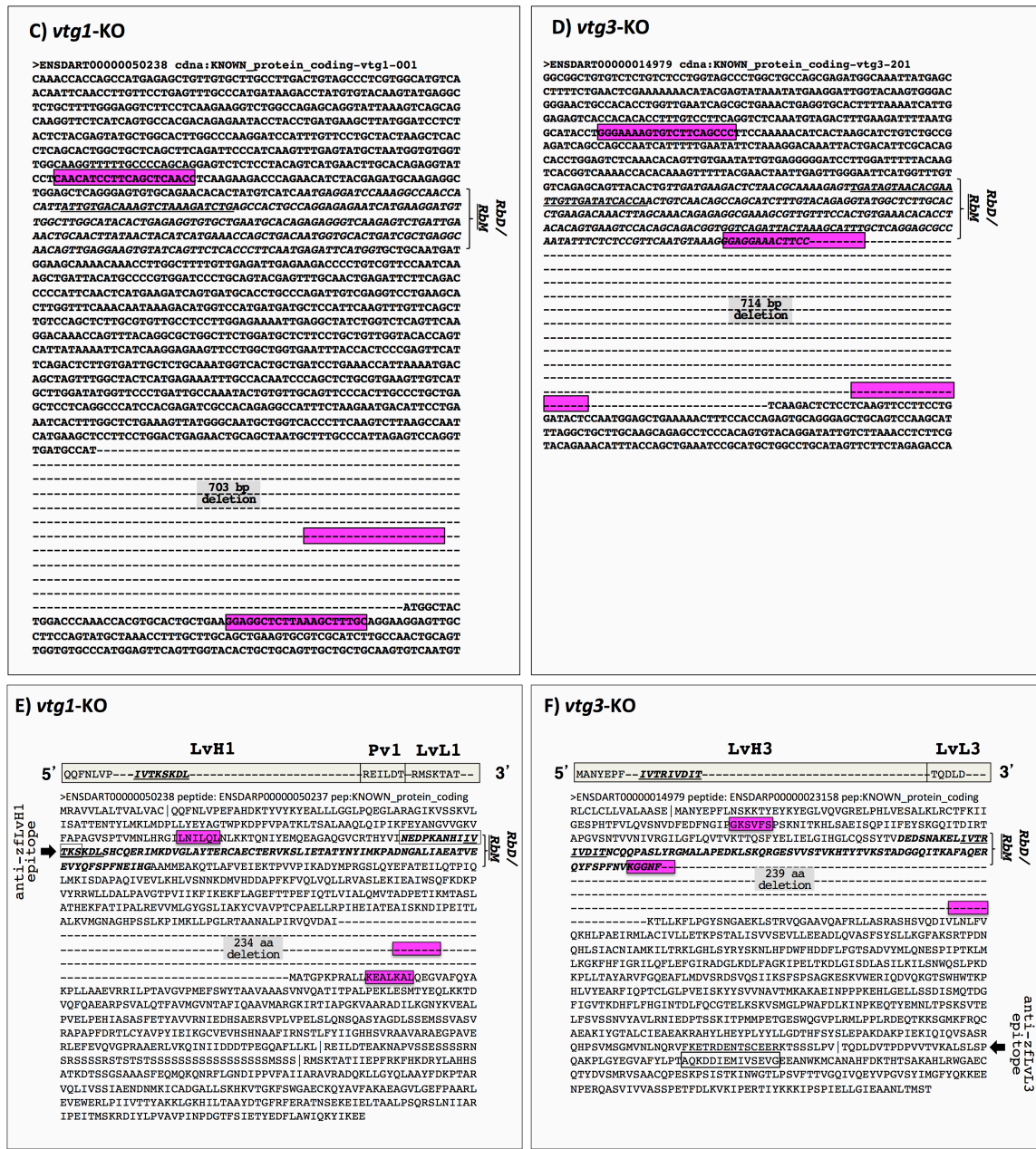


vtg1-/-	CTTCCTGGACTGGAACCTGCAGCTAATGCTTTGGCCATTAGAGTCCAGGTTGATGCCAT-	2458	
vtg1+/+	CTTCCTGGACTGGAACCTGCAGCTAATGCTTTGGCCATTAGAGTCCAGGTTGATGCCATC	2460	Exon 11
vtg1-/-	TTGGCCCTGAGAAACATTGCTAAGAAAGAGCCAAACTGTAAGATTTCAGGTTATTTAGA	2458	
vtg1+/+	TTGGCCCTGAGAAACATTGCTAAGAAAGAGCCAAACTGTAAGATTTCAGGTTATTTAGA	2520	
vtg1-/-	TAATGAACCTGCTGTAATGTTATGCAATGTTTCTTCCAATGAAAAAACTTTGTTCTCTGT	2458	Intron
vtg1+/+	TAATGAACCTGCTGTAATGTTATGCAATGTTTCTTCCAATGAAAAAACTTTGTTCTCTGT	2580	
vtg1-/-	ATCAGGTTTCAGCCTGTGCCCTGCAGCTTTGTTGGACAGAGCTCCACCCCGAGGTGC	2458	
vtg1+/+	ATCAGGTTTCAGCCTGTGCCCTGCAGCTTTGTTGGACAGAGCTCCACCCCGAGGTGC	2640	
vtg1-/-	GCATGGTTCCTTGTATTTGCTGTTTGGAGCTGAGCCCTCAGTGGCATTGCTCTAGTC	2458	Exon 12
vtg1+/+	GCATGGTTCCTTGTATTTGCTGTTTGGAGCTGAGCCCTCAGTGGCATTGCTCTAGTC	2700	
vtg1-/-	TTGCTGGAGCTTAAGGATTGAGCCAAACATGCATGTTGCAAGCTTTGCCTATCCCA	2458	
vtg1+/+	TTGCTGGAGCTTAAGGATTGAGCCAAACATGCATGTTGCAAGCTTTGCCTATCCCA	2760	
vtg1-/-	TCAGTCCCTGACAGAACTCACTGCTCTGATATGGCATCTGTAAAGAAAGCCAAAT	2458	
vtg1+/+	TCAGTCCCTGACAGAACTCACTGCTCTGATATGGCATCTGTAAAGAAAGCCAAAT	2820	Intron
vtg1-/-	ATTTTCAGTTTGTGTTTTTACATTAATTTCCATGTTTTTTCACACTTTACACATGAA	2458	
vtg1+/+	ATTTTCAGTTTGTGTTTTTACATTAATTTCCATGTTTTTTCACACTTTACACATGAA	2880	
vtg1-/-	GAGTACAAGCCTAGGTAATCATGGCTATTTATTTTCAGTGGTGCAGCTAATGTTCA	2458	Exon 13
vtg1+/+	GAGTACAAGCCTAGGTAATCATGGCTATTTATTTTCAGTGGTGCAGCTAATGTTCA	2940	
vtg1-/-	ATCAAGCTTATGAGCCGAAACTGGACAGACTTAACTACCGTTACAGCAGAGCTTTTC	2458	
vtg1+/+	ATCAAGCTTATGAGCCGAAACTGGACAGACTTAACTACCGTTACAGCAGAGCTTTTC	3000	
vtg1-/-	ATGGACTATTATATATAAAGAAATTTCAATTTCTTAAGAGCTAAGAAATTTATATCG	2458	
vtg1+/+	ATGGACTATTATATATAAAGAAATTTCAATTTCTTAAGAGCTAAGAAATTTATATCG	3060	Intron
vtg1-/-	GTTTCTTAAACTGCATTAATAATTTGCTGACATACAGATTGCAAAATTTATCTTTAA	2458	
vtg1+/+	GTTTCTTAAACTGCATTAATAATTTGCTGACATACAGATTGCAAAATTTATCTTTAA	3120	
vtg1-/-	GTATCTTTTAAATTTGCTCAATTAAGTCTCTTATGATGGAGCTGCTGGTAGTGCC	2458	Exon 14 (sg12)
vtg1+/+	GTATCTTTTAAATTTGCTCAATTAAGTCTCTTATGATGGAGCTGCTGGTAGTGCC	3180	
vtg1-/-	ATGATCAATGATGCTGCCACCATCCCTGCCAGAGCTGTTAGCTAAAGCTCGTCTTAC	2458	
vtg1+/+	ATGATCAATGATGCTGCCACCATCCCTGCCAGAGCTGTTAGCTAAAGCTCGTCTTAC	3240	
vtg1-/-	CTGGCTGGAGCTGCTGCTGATGTTATTGAGTGAACCACTTAAAATTTCTTACTAG	2458	
vtg1+/+	CTGGCTGGAGCTGCTGCTGATGTTATTGAGTGAACCACTTAAAATTTCTTACTAG	3300	Intron
vtg1-/-	TTTGACTTACTATTTAAATCAATTTTAAAATGCATCACTAAATTTGCTTCTAAA	2458	
vtg1+/+	TTTGACTTACTATTTAAATCAATTTTAAAATGCATCACTAAATTTGCTTCTAAA	3360	
vtg1-/-	TTGGTGTGAGAACTGGAGGAATCCATGAAGCTCTCTAAAATCTCTCTGACAGATG	2458	Exon 15
vtg1+/+	TTGGTGTGAGAACTGGAGGAATCCATGAAGCTCTCTAAAATCTCTCTGACAGATG	3420	
vtg1-/-	GTGCTGACCGTATCACAAGATTAAAGCTACACTGAGAGCAATAAGTTTTTTTTATG	2458	
vtg1+/+	GTGCTGACCGTATCACAAGATTAAAGCTACACTGAGAGCAATAAGTTTTTTTTATG	3480	Intron
vtg1-/-	ATAGTATCTTAAATTTTACACTTAAATATAAAAATGAAAAAGAAAAAAGTCAAAA	2458	
vtg1+/+	ATAGTATCTTAAATTTTACACTTAAATATAAAAATGAAAAAGAAAAAAGTCAAAA	3540	
vtg1-/-	TGATTTGTTTCTTCAAGCTCACAAAAGTGAAGGCTTTGCCAACCGATAAACCACT	2458	Exon 16
vtg1+/+	TGATTTGTTTCTTCAAGCTCACAAAAGTGAAGGCTTTGCCAACCGATAAACCACT	3600	
vtg1-/-	CAGCCTATCTCAAAGTATTTGGACAAGAGTGGCTTATGTCAACTTTGACACAG	2458	
vtg1+/+	CAGCCTATCTCAAAGTATTTGGACAAGAGTGGCTTATGTCAACTTTGACACAG	3660	
vtg1-/-	TTGAAGAAGCCATACCGTATTTGTTGTTGCTATTTATCTCTCTCCATCATATGCT	2458	
vtg1+/+	TTGAAGAAGCCATACCGTATTTGTTGTTGCTATTTATCTCTCTCCATCATATGCT	3720	Intron
vtg1-/-	CTAAGAACTTGAACCTGACTGACTCACAATTTGAATTAAGCTTCTAATAAACACT	2458	
vtg1+/+	CTAAGAACTTGAACCTGACTGACTCACAATTTGAATTAAGCTTCTAATAAACACT	3780	
vtg1-/-	CTTCAGATGGCTACTGGACCCAAACACAGTGCAGTCTGAAAGAGGCTCTTAAAGCT	2559	Exon 17 (sg13)
vtg1+/+	CTTCAGATGGCTACTGGACCCAAACACAGTGCAGTCTGAAAGAGGCTCTTAAAGCT	3840	

**B) vtg3-KO**



vtg3-/-	AAAGCATTGCTCAGGAGCGCCAAATATTTCTCCGTTCAATGTAAGGAGGAAACTTC	337	Exon 6 (sg32)
vtg3+/+	AAAGCATTGCTCAGGAGCGCCAAATATTTCTCCGTTCAATGTAAGGAGGAAACTTC	6240	
vtg3-/-	CGAATGTTGGCATTCTAAGTGCCTTAACTGTTATCAACTTTCAGTGGTGCACCTCTG	339	Intron
vtg3+/+	CGAATGTTGGCATTCTAAGTGCCTTAACTGTTATCAACTTTCAGTGGTGCACCTCTG	6300	
vtg3-/-	ATTAATCAATGATGTTGTGCTTCTTTTACCAGCGGGACATTGAACTCTTAAAGTTTC	339	
vtg3+/+	ATTAATCAATGATGTTGTGCTTCTTTTACCAGCGGGACATTGAACTCTTAAAGTTTC	6360	
vtg3-/-	AGACACAACCTGACAAAGTAGTACTGGACAGGTGCAGAGCAGAGGCAACCTGATGTATA	339	Exon 7
vtg3+/+	AGACACAACCTGACAAAGTAGTACTGGACAGGTGCAGAGCAGAGGCAACCTGATGTATA	6420	
vtg3-/-	GACAATAAGGACCTCAAACCAATACCTGTTGTGATGCTTAACTGAAACCCAGCTGC	339	
vtg3+/+	GACAATAAGGACCTCAAACCAATACCTGTTGTGATGCTTAACTGAAACCCAGCTGC	6480	
vtg3-/-	CAAGSCAACTTCAAAGATTATATAAACATTTAAGCATATACAGACATGAGAATTA	339	Intron
vtg3+/+	CAAGSCAACTTCAAAGATTATATAAACATTTAAGCATATACAGACATGAGAATTA	6540	
vtg3-/-	ATTAATCGTTTAAATCATACTTTACTCCAACAGATTTTAGATTTAATCAAGCCCTCG	339	
vtg3+/+	ATTAATCGTTTAAATCATACTTTACTCCAACAGATTTTAGATTTAATCAAGCCCTCG	6600	
vtg3-/-	CAGGCTAATATATATATCATGTGGACAGTGAACACGACAGAAATCTGGACCTAATTC	339	Exon 8
vtg3+/+	CAGGCTAATATATATATCATGTGGACAGTGAACACGACAGAAATCTGGACCTAATTC	6660	
vtg3-/-	TTGATCGGGTAACAACACTTGATAATCTAGACATTTATGGAAGCAGGCTTCAGGAA	339	
vtg3+/+	TTGATCGGGTAACAACACTTGATAATCTAGACATTTATGGAAGCAGGCTTCAGGAA	6720	
vtg3-/-	GATGAGCACAGTATGTTTCAACCTTCTCTAAATACTATACAATTAATCAAGATG	339	Intron
vtg3+/+	GATGAGCACAGTATGTTTCAACCTTCTCTAAATACTATACAATTAATCAAGATG	6780	
vtg3-/-	CAATTTCCCTGTTGACTTTCTTTTTTTTTTTTGGTCCGGCGTGGTCTTGGACTGG	339	
vtg3+/+	CAATTTCCCTGTTGACTTTCTTTTTTTTTTTTGGTCCGGCGTGGTCTTGGACTGG	6840	
vtg3-/-	TGGAGGTAACAGATGAAAGAATTTCAAATTCCTTGGCCAGATACAAGCAGGAGACA	339	Exon 9
vtg3+/+	TGGAGGTAACAGATGAAAGAATTTCAAATTCCTTGGCCAGATACAAGCAGGAGACA	6900	
vtg3-/-	TCACAGCAATGAGCCAGGACAAGCACTGGTAGTGGCATTAAACCTTGTCCGCTGAC	339	
vtg3+/+	TCACAGCAATGAGCCAGGACAAGCACTGGTAGTGGCATTAAACCTTGTCCGCTGAC	6960	
vtg3-/-	CTGTGTCGGTGGCATTAGCTCAGTGGACAGTGAACCAAGTTAAATGATGTAGAA	339	Intron
vtg3+/+	CTGTGTCGGTGGCATTAGCTCAGTGGACAGTGAACCAAGTTAAATGATGTAGAA	7020	
vtg3-/-	TTTTTTATCTGCGCTGTGATACGCTCTCCGCTCTCCAGGATTCCTGACCATCT	339	
vtg3+/+	TTTTTTATCTGCGCTGTGATACGCTCTCCGCTCTCCAGGATTCCTGACCATCT	7080	Exon 10
vtg3-/-	AGTAATCCATCTCTCCTGTGGAACACTGTAGTTTGGCATATGGATCTTGTACAC	339	
vtg3+/+	AGTAATCCATCTCTCCTGTGGAACACTGTAGTTTGGCATATGGATCTTGTACAC	7140	
vtg3-/-	AGATACTGTGTATACTGACCCCTGCCCTATCACTGGTACAGTATCTGAGATG	339	Intron
vtg3+/+	AGATACTGTGTATACTGACCCCTGCCCTATCACTGGTACAGTATCTGAGATG	7200	
vtg3-/-	TACACCTCTATGTTGATTGATTCATACACCTATCATAGTAAATTTTGTAGTTTATG	339	
vtg3+/+	TACACCTCTATGTTGATTGATTCATACACCTATCATAGTAAATTTTGTAGTTTATG	7260	
vtg3-/-	CAAACCTAGTCTTTGATATTTACAGTACATCTCACTGCTTTTTGTACATACTATG	339	
vtg3+/+	CAAACCTAGTCTTTGATATTTACAGTACATCTCACTGCTTTTTGTACATACTATG	7320	
vtg3-/-	AGCCATTGCTGAATATGGCTGCAAGTAGCTTAAGTAAAACCTGAGGATGAAATGG	339	
vtg3+/+	AGCCATTGCTGAATATGGCTGCAAGTAGCTTAAGTAAAACCTGAGGATGAAATGG	7380	
vtg3-/-	TTGCGTGAAGTCTTTGGAAACCGAGCTCATCTTCCAGCTCAAGATCTCTCAAGT	357	Exon 11 (sg33)
vtg3+/+	TTGCGTGAAGTCTTTGGAAACCGAGCTCATCTTCCAGCTCAAGATCTCTCAAGT	7440	
vtg3-/-	TCCTTCTGGATACTCCAATGGAGCTGAAAACTTCCACAGAGTGCAGGAGCTGCAG	417	
vtg3+/+	TCCTTCTGGATACTCCAATGGAGCTGAAAACTTCCACAGAGTGCAGGAGCTGCAG	7500	



**S1 Fig. Location and character of mutations introduced by CRISPR/Cas9 in zebrafish *vtg*s**

A-B) Location on genomic DNA. Schematic representations of the intron/exon structure of zebrafish *vtg1* (representative of type-I *vtg*s) and *vtg3* are given at the top of panels A and B, respectively. Horizontal line segments indicate introns and filled gold boxes indicate exons. Exons bearing CRISPR/Cas9 target sequences are indicated by magenta-colored arrows pointing upwards to the target name (sg11, sg12, and sg13 for *vtg1*; sg31, sg32, and sg33 for *vtg3*).

Horizontal dashed lines bearing dual arrowheads indicate regions where mutations were introduced, with the size of deletions in bp given below the arrows (1281 bp and 1182 bp for *vtg1*-KO and *vtg3*-KO, respectively). The lower sections of panels **A** and **B** show Clustal Omega alignments for partial genomic sequences of the *vtg1* and *vtg3* genes, respectively, covering regions where Cas9 introduced targeted mutations. Sequences of undisturbed wild type alleles are labeled *vtg1*<sup>+/+</sup> and *vtg3*<sup>+/+</sup>, and sequences of homozygous mutated alleles are labeled *vtg1*<sup>-/-</sup> and *vtg3*<sup>-/-</sup>, respectively. Dashes were introduced to illustrate regions where deletions occurred in the *vtg1*<sup>-/-</sup> and *vtg3*<sup>-/-</sup>-sequences. Nucleotide positions are indicated by numbers on the right and asterisks indicate nucleotide identity. Target sequences are enclosed in magenta-colored boxes emphasized by magenta-colored arrows on the right. Intron sequences are given in dark gray font enclosed in light gray filled frames and are labeled by Intron on the right with the same formatting. Exons are shown in regular black font and labeled on the right with exon numbers (e.g. Exon 6, 7, 8...). Exons bearing the target sites are also labeled with the target name below in parenthesis (e.g. Exon 14/(sg12)). **C-D**) Location on predicted cDNA. Nucleotide sequences targeted by sgRNAs for Cas9 editing and present in the predicted transcript are framed in magenta-shaded boxes. The deleted region of the transcript is indicated by dashes replacing nucleotide residues and the size of the deletion in bp is given by gray highlighted text in this region (703 bp deletion for *vtg1* and 714 bp deletion for *vtg3*). The sequence encoding the receptor-binding domain (***RbD***) on the LvH of the respective Vtg is shown in italic bold typeface with the sequence encoding the critical, short receptor-binding motif (***RbM***) being additionally underlined. **E-F**) Location on predicted polypeptide sequences. Schematic representations of the yolk protein domain structures of Vtg1 (representative of zebrafish type-I Vtgs) and Vtg3 are given in 5' > 3' orientation above each panel. Light gray horizontal bars represent the lipovitellin heavy and light chain (LvH, LvL) and the phosvitin (Pv) yolk protein domains of the respective Vtg (Vtg3 lacks a Pv domain) and are labeled above in large bold type. Sequences within these bars indicate the N-terminus of each yolk protein domain, the start of which is also indicated by

vertical bars in the polypeptide sequence shown below. The ***RbM*** is shown in bold italic underlined font on the gray horizontal bars in the LvH1 and LvH3 domains. The ***RbD*** and ***RbM*** are also indicated in the polypeptide sequences shown below by bold italic font with the ***RbM*** being additionally underlined. Residues encoded by nucleotide sequences targeted by sgRNAs for Cas9 editing are framed in magenta-shaded boxes. Cas9 created mutations (large deletions) are indicated with dashes replacing amino acid (aa) residues and the size of deletions in aa (234 aa and 239 aa for *vtg1*-KO and *vtg3*-KO, respectively) in these regions are labeled by gray shaded text. Short sequences that were employed as epitopes to develop Vtg domain-specific antibodies against Vtg1-LvH (anti-zfLvH1) and Vtg3-LvL (anti-zfLvL3) are indicated by framed text on the LvH and LvL domains of Vtg1 and Vtg3, respectively, with their location also highlighted by black arrows labeled with the epitope names given by vertically-oriented text in the panel margins.

**S1 Table. Targets, primers and probes utilized in *vtg1*-KO and *vtg3*-KO studies.** Target oligo and screening primer names are given according Figure 1. CRISPR recognition NGG motifs are highlighted by bold typeface on sequences. Position of primers, target sites and probes on vitellogenin (Vtg) yolk protein (YP) domains are given on the far right columns.

Target Oligos	Sequence	Vtg YP domain
sg11_Rv	<b>GGTTGAGCTGAAGGATGTTG</b>	LvH
sg12_Rv	<b>GGCAGCATCATTGATCATAT</b>	LvH
sg13_Fw	<b>GGAGGCTCTTAAAGCTTTC</b>	LvH
sg31_Rv	<b>GGGCTGAAGACACTTTTCCC</b>	LvH
sg32_Fw	<b>GGGAGGAAACTCCGAATGT</b>	LvH
sg33_Fw	<b>GGTCCTTGCGCTGAAGTCT</b>	LvH
Screening Primers	Sequence	Vtg YP domain
11_Fw	GAAGCAACACTTAATAAGCAATGG	LvH
11_Rv	CTTATTACCTCTGTGCATTACAGC	LvH
12_Fw	CTTATGAGCCGCAAACCTGGA	LvH
12_Rv	TGTGAGTATCAGTCACAGTTCAA	LvH
13_Fw	AACTGGAGGAATCCATGAAGC	LvH
13_Rv	AGGTGTAATGGTGGCCTGAA	LvH
31_Fw	TATGAAGGATTGGTACAAGTGGG	LvH
31_Rv	AGGATCCCCCTCACAAATATCA	LvH
32_Fw	TGATGAAGACTCTAACGCAAAAAGA	LvH
32_Rv	TTATACATCAGGTTCCTCTGC	LvH
33_Fw	AGTAAATCCCATCCTCTCTGTG	LvH
33_Rv	TTAGTGCGCAACCAGATGAA	LvH
qPCR Primers (SybrGreen)	Sequence	Vtg YP domain
vtg1_Fw	GATTAAGCGTACACTGAGACCA	LvH
vtg1_Rv	AGCCACTTCTGTCCAAATACT	
vtg2_Fw	TGCCGCATGAAACTTGAATCT	Ct
vtg2_Rv	GTTCTTACTGGTGCACAGCC	
vtg3_Fw	GGGAAAGGATTCAAGATGTTCAGA	LvH
vtg3_Rv	ATTTGCTGATTCAACTGGGAGAC	
vtg4_Fw	TCCAGACGGTACTTTCACCA	LvL
vtg4_Rv	CTGACAGTTCTGCATCAACACA	
vtg5_Fw	ATTGCCAAGAAAAGAGCCCAA	LvH
vtg5_Rv	TTCAGCCTCAAACAGCACAA	
vtg6_Fw	TTGGTGTGAGAACTGGAGG	LvH
vtg6_Rv	CCAGTTTGTGAGTGCCTTCAG	
vtg7_Fw	TTGGTGTGAGAACTGGAGGA	LvH
vtg7_Rv	TTGCAAGTGCCTTCAGTGTA	
rpl13a_Fw	TCTGGAGGACTGTAAGAGGTATGC	N/A
rpl13a_Rv	AGACGCACAATCTTGAGAGCAG	
eif1a_Fw	CTGGAGGCCAGCTCAAACAT	N/A
eif1a_Rv	ATCAAGAAGAGTAGTACCGCTAGCATTAC	
18S_Fw	TCGCTAGTTGGCATCGTTTATG	N/A
18S_Rv	CGGAGGTTCGAAGACGATCA	
qPCR Primers and probes (TaqMan)	Sequence	Vtg YP domain
vtg1_Fw	CCATGAAGCTCTCCTAAAATCTC	LvH
vtg1_probe	[FAM]CACTGAGAGCAGTCACAAACTGGAAGG[BHQ1]	
vtg1_Rv	TAGGCTGATGCTAGTGGTTTATC	
vtg2_Fw	GGCTGATGGTTTTGCACTTTATG	Ct
vtg2_probe	[FAM]TTGCCAATGGTACTGGAAGATCCAAG[BHQ1]	
vtg2_Rv	TGTCCTTTCATCCAGTCTGC	
vtg3_Fw	GCGTGTCTTACATTATGGGTTTC	LvL
vtg3_probe	[FAM]CGGCAGGCATCTGTCATCGTTGTAG[BHQ1]	
vtg3_Rv	TCACTTTCAGGTCAAAGGTCTC	
vtg4_Fw	TAGCTGGTGAATTTACAACCTCCC	LvH
vtg4_probe	[FAM]CTCTGCAAAGGGTTCTGCTGATCTTG[BHQ1]	
vtg4_Rv	GCATGGCAACTTCACGCAGA	
vtg5_Fw	AGGAACATTGCCAAGAAAAGAGC	LvH
vtg5_probe	[FAM]AGGCTGAACCTCAGTGGCTCTCAT[BHQ1]	
vtg5_Rv	GTCTCAATGTCCAGCAAG	
vtg6_Fw	GCCAAAAATTGTCACCCACTATG	LvL
vtg6_probe	[FAM]CTTAATGCAGCTTATGACACAGGATTCAGG[BHQ1]	
vtg6_Rv	TGACGGGGAGGTAAATATCCC	
vtg7_Fw	TATTCAGACTCTCGTGGTTGCTTT	LvH
vtg7_probe	[FAM]CAACGCATGAGAAGTTACCACAAATCC[BHQ1]	
vtg7_Rv	GGTAATCTCGTGGATGGGCT	