

1 **Eukaryotic translation initiation factor eIF4E-5 is required for spermiogenesis in**
2 *Drosophila melanogaster*

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65 **Introduction**

66 Translation of mRNA into protein is frequently targeted by genetic regulatory
67 mechanisms (Kugler and Lakso, 2009; Lin and Holt, 2007; Mingle et al., 2005; Moor et al.,
68 2017). Protein synthesis can be divided into three steps (initiation, elongation, termination), yet
69 much of the regulation occurs at the first step (Hershey et al., 2018; Pelletier and Sonenberg,
70 2019; Sonenberg and Hinnebusch, 2009). During initiation, the eukaryotic initiation factor 4F
71 (eIF4F) cap-binding complex is recruited to the 7-methylguanylate cap located at the 5' end of
72 mRNAs. eIF4F is composed of a cap-binding protein (eIF4E) and an RNA helicase (eIF4A) held
73 together by a scaffolding protein (eIF4G). eIF4G binds poly(A)-binding protein (PABP) and
74 eIF3 to recruit the 40S ribosomal subunit. Translational repression can occur when eIF4E-
75 binding proteins (4E-BPs) bind eIF4E to block its association with eIF4G or when eIF4E-
76 homologous proteins (4E-HPs) bind the 5' cap to block the recruitment of eIF4E to the mRNA.
77 Therefore, eIF4E plays an essential role in eukaryotic cap-mediated translation initiation.

78 Most eukaryotic genomes encode several paralogs of eIF4E, but their roles and regulation
79 are not well understood. Mammals have three paralogs (eIF4E1 to eIF4E3; Joshi et al., 2004):
80 mouse eIF4E1 and eIF4E2/4EHP mutants exhibit behavioral defects (Aguilar-Valles et al., 2018;
81 Wiebe et al., 2020). *C. elegans* has five paralogs (IFE-1 to IFE-5): IFE-3, which is most similar
82 to mammalian eIF4E1 (*i.e.*, canonical), is essential for viability; IFE-1 is required for
83 spermatogenesis and oocyte maturation; IFE-2 is needed for meiotic recombination; and IFE-4 is
84 required to translate mRNAs involved in egg laying (Amiri et al., 2001; Dinkova et al., 2005;
85 Henderson et al., 2009; Kawasaki et al., 2011; Keiper et al., 2000; Song et al., 2010). *Drosophila*
86 has eight paralogs (eIF4E-1 to eIF4E-8/4E-HP) that bind to mRNA 5' caps with varying
87 affinities *in vitro* (Zuberek et al., 2016). Canonical eIF4E-1 and 4E-HP are essential for viability,
88 and eIF4E-3 is needed for meiotic chromosome segregation and cytokinesis during
89 spermatogenesis (Brown et al., 2014; Chintapalli et al., 2007; Ghosh and Lasko, 2015;
90 Hernández et al., 2012). However, the roles of the remaining paralogs are uncharacterized.
91 Whereas *eIF4E-1* and *4E-HP* mRNAs are ubiquitously expressed, *eIF4E-3*, *eIF4E-4*, *eIF4E-5*
92 and *eIF4E-7* mRNAs are highly and specifically enriched in the testis (Graveley et al., 2011).
93 This suggests that these paralogs may have distinct cellular or developmental roles during sperm
94 development.

95 The stages of *Drosophila* male germ cell development are organized in a spatiotemporal
96 manner (Fig. S1). The stem cell niche is located at the apical tip of the testis and germ cell
97 development progresses toward the basal end of the testis where mature sperm will eventually
98 exit (reviewed in Fabian and Brill 2012; Fuller 1993; Lindsley and Tokuyasu, 1980; Renkawitz-
99 Pohl et al., 2005). Male germline stem cells divide asymmetrically to form new stem cells and
100 differentiating daughter cells called gonialblasts. Each gonialblast undergoes four rounds of
101 mitosis with incomplete cytokinesis to generate a cyst of 16 primary spermatocytes. After an
102 extended period of growth and gene expression, the 16 spermatocytes undergo meiosis with
103 incomplete cytokinesis to form a cyst of 64 interconnected haploid spermatids. A series of
104 dramatic morphological changes convert these round spermatids into 1.85mm long mature sperm
105 through a process called spermiogenesis. These changes include polarization of spermatid cysts
106 relative to the long axis of the testis; assembly of flagellar axonemes that make up the sperm
107 tails; elongation of spermatid cysts by membrane addition at the distal, growing ends; and
108 individualization, which separates fully elongated, interconnected spermatids into individual
109 sperm. During individualization, unneeded organelles and other cellular materials are stripped
110 from elongated spermatid cysts by an actin-based individualization complex that moves down
111 the length of the spermatids, forming a cystic bulge whose contents eventually pinch off into a
112 structure called the waste bag.

113 Post-transcriptional regulation, possibly at the level of translation, is a crucial aspect of
114 *Drosophila* spermatogenesis. Many genes needed post-meiotically are transcribed in primary
115 spermatocytes and translationally repressed until later stages (Jayaramaiah-Raja and Renkawitz-
116 Pohl, 2005; Renkawitz-Pohl et al., 2005; Santel et al., 1997; Schäfer et al., 1993; White-Cooper,
117 2010; White-Cooper and Caporilli, 2013; Zhao et al., 2010). In addition, a small subset of “cup”
118 and “comet” genes, named based on their mRNA distribution, are transcribed post-meiotically,
119 and the corresponding mRNAs localize at the growing ends of elongating spermatid cysts
120 (Barreau et al., 2008a,b). One of these genes, *soti*, encodes a protein translated late in sperm
121 development that regulates non-apoptotic caspase activity during individualization (Kaplan et al.,
122 2010). Although little is known about how translation of cup and comet mRNAs is regulated, the
123 cytoplasmic polyadenylation element binding protein (CPEB) Orb2 binds the 3' UTR of *soti* and
124 *f-cup*, suggesting it might regulate their translation (Xu et al., 2012). Orb2, together with atypical

125 protein kinase C (aPKC), ensures polarization of spermatid cysts relative to the long axis of the
126 testis (Xu et al., 2014).

127 Here, we show that the testis-enriched translation initiation factor eIF4E-5 is essential for
128 male fertility, localizes to the distal ends of elongated spermatid cysts, is required for normal
129 accumulation of Soti at the distal ends of elongated spermatids, and interacts genetically with
130 *orb2* and *apk* during spermatid cyst polarization. Thus, eIF4E-5 is novel player in post-
131 transcriptional regulation during spermiogenesis.

132

133 **Results**

134 ***eIF4E-5* is required for male fertility**

135 *eIF4E-5* encodes a single predicted polypeptide of 232 amino acids that contains
136 conserved residues needed to bind the mRNA cap (Asp108, Trp120, Glu121; Hernández et al.
137 2005). To examine the role of eIF4E-5 in *Drosophila* spermatogenesis, CRISPR/Cas9 mediated
138 mutagenesis was used to produce *eIF4E-5* alleles with deletions in the coding sequence of the
139 gene (Fig. 1A). The deletions in *eIF4E-5^{B8a}* (12 bp), *eIF4E-5^{B8b}* (3 bp) and *eIF4E-5^{D19a}* (1 bp)
140 are predicted to produce a 4-amino acid in-frame deletion, a 1-amino acid in-frame deletion, and
141 a frame-shift resulting in a 77-amino acid truncated protein lacking the eIF4E domain, which
142 contains the cap-binding site (Fig. 1B). All three mutations affect the conserved motif required
143 for binding to the eIF4E-binding motif found in eIF4Gs and other eIF4E-binding proteins
144 (His55, Pro56, Leu57; Grüner et al., 2016): *eIF4E-5^{B8a}* removes Pro56 and Leu57; *eIF4E-5^{B8b}*
145 removes His55; and *eIF4E-5^{D19a}* has a frameshift after His55 that removes all subsequent amino
146 acids, replacing them with 22 amino acids from an alternate reading frame. To investigate
147 whether levels of eIF4E-5 were reduced in these mutants, polyclonal antibodies were raised
148 against the full-length sequence of eIF4E-5. The antibodies strongly recognized a protein at the
149 predicted molecular weight of approximately 26.9 kDa on immunoblots of testis extracts from
150 wild type. The level of this protein was substantially reduced in testis extracts from *eIF4E-5^{B8a}*
151 or *eIF4E-5^{B8b}*, and not detectable in *eIF4E-5^{D19a}* homozygous mutants (Figs 1C and S2). Thus,
152 all three *eIF4E-5* mutations are predicted to interfere with eIF4G binding and eIF4F complex
153 formation and result in reduced levels of eIF4E-5 protein.

154 Males homozygous mutant for *eIF4E-5* were viable and sterile, as were male *eIF4E-5^{B8a}*,
155 *eIF4E-5^{B8b}* and *eIF4E-5^{D19a}* mutants *in trans* with two different deficiencies (*Df(3L)BSC631* and

156 *Df(3L)Exel6279*) that uncover the *eIF4E-5* locus (Fig. 1D). A 1610 bp genomic rescue construct
157 containing an N-terminal 3xFLAG in frame with the *eIF4E-5* coding region restored male
158 fertility in *eIF4E-5^{B8b}* and *eIF4E-5^{D19a}* homozygous mutants. These results confirm that the male
159 sterility of *eIF4E-5* mutants is due to loss of *eIF4E-5* function rather than a second-site mutation
160 generated by CRISPR/Cas9 mutagenesis. Closer inspection revealed that sperm failed to
161 accumulate in the seminal vesicles of *eIF4E-5* mutants (Fig. 1E-H) and that expression of the
162 transgene resulted in the presence of mature, motile sperm (Fig. S3A-G). Hence, *eIF4E-5* is
163 required for male fertility.

164

165 **eIF4E-5 localizes to the distal ends of elongated spermatid cysts**

166 To begin to decipher the requirement for *eIF4E-5* in male fertility, we examined *eIF4E-5*
167 protein distribution during sperm development. Immunostaining revealed that *eIF4E-5* (yellow
168 arrowheads) localized near the membrane skeletal protein Adducin (red arrowheads) at the distal
169 ends of elongated spermatid cysts (Fig. 2A-A''). In contrast, elongated spermatid cysts from
170 *eIF4E-5^{B8a}*, *eIF4E-5^{B8b}* and *eIF4E-5^{D19a}* homozygotes retained Adducin localization (red
171 arrowheads) but lacked detectable *eIF4E-5* at the distal ends (Fig. 2B-D''). To confirm the
172 localization of *eIF4E-5*, we examined the distribution of 3xFLAG-*eIF4E-5* expressed from the
173 rescuing transgene. Immunostaining of testes from *3xFLAG-eIF4E-5;eIF4E-5^{D19a}* males with
174 anti-FLAG and anti-*eIF4E-5* antibodies revealed that these antibodies stained the same region at
175 the distal ends of elongated spermatid cysts (Fig. 2E-E''). Moreover, testes from *eIF4E-5^{D19a}*
176 homozygotes lacked similar staining (Fig. 2F-F''), confirming that the signals were specific to
177 *eIF4E-5*. In addition, immunostaining of testes from *3xFLAG-eIF4E-5;eIF4E-5^{D19a}* males
178 revealed that 3xFLAG-*eIF4E-5* was present in the cytoplasm of spermatocytes and elongating
179 spermatids (Fig. 2G-G''). In contrast, testes from *eIF4E-5^{D19a}* mutants revealed non-specific
180 staining with anti-FLAG antibodies at the tip of the testis and along a subset of elongated
181 spermatid cysts, as well as non-specific staining with anti-*eIF4E-5* antibodies in the nuclei of
182 spermatogonia, spermatocytes and spermatids (Fig. 2F-F'',H-H''). Together, these results reveal
183 that *eIF4E-5* is expressed in early spermatocytes and persists through spermiogenesis, with
184 enrichment at the distal ends of elongated spermatid cysts.

185

186 **eIF4E-5 is required for spermatid individualization**

187 To determine the cause of male sterility in *eIF4E-5* mutants, we examined testes by
188 phase-contrast microscopy. Although overall testis morphology appeared normal, waste bags
189 (Fig. 3A', red arrowheads) were absent, suggesting that loss of *eIF4E-5* caused defects in
190 spermatid individualization (Fig. 3A-D'). Waste bag formation was rescued by the 3xFLAG-
191 *eIF4E-5* transgene (Fig. S3G,G'). Whole testes stained for activated (cleaved) caspase-3 revealed
192 normal cystic bulges in wild-type testes (Fig. 3E,E', yellow arrowheads), whereas these appeared
193 flattened in *eIF4E-5* mutants (Fig. 3F-H', yellow arrowheads). The mutants also displayed an
194 elevated level of active effector caspases starting at the cystic bulges and extending further
195 towards the distal ends of the tails (Fig. 3F'-H'), as compared to wild type (Fig. 3E').
196 Furthermore, unlike the synchronous movement of the 64 actin-based investment cones within
197 caspase-positive cystic bulges in wild type (Fig. 3E''-E'''), the actin cones in *eIF4E-5* mutants
198 appeared scattered and disorganized within the cystic bulges (Fig. 3F''-H'''). As
199 individualization is an essential step in the formation of mature sperm, this fully penetrant
200 phenotype is likely the cause of male infertility in *eIF4E-5* mutants.

201

202 ***eIF4E-5 binds translational regulators 4E-BP, eIF4G-2, 4E-T and Cup***

203 To begin to examine the biochemical properties of *eIF4E-5*, we tested whether *eIF4E-5*
204 binds known *eIF4E* binding proteins, including *eIF4G* and *eIF4G-2*, which have roles in sperm
205 development and male fertility (Baker and Fuller 2007; Franklin-Dumont et al., 2007; Ghosh and
206 Lasko 2015). We performed yeast two-hybrid assays using *eIF4E-5* as “bait” and constructs
207 containing 4E-BP (Miron et al. 2001), *eIF4G* (Hernández et al. 1998), *eIF4G-2* (Baker and Fuller
2007; Franklin-Dumont et al., 2007; Ghosh and Lasko 2015), 4E-T (Kamenska et al. 2014), Cup
209 (Nelson et al, 2004; Zappavigna, et al., 2004) and GIGYF (Russica et al., 2019) as “prey” (Fig.
210 S4). Positive interactions were detected between *eIF4E-5* and 4E-BP, *eIF4G-2*, 4E-T, and Cup,
211 but not *eIF4G*. The lack of interaction between *eIF4E-5* and *eIF4G* was unexpected, as we
212 previously detected a weak interaction of these proteins in a more sensitive yeast two-hybrid
213 assay (Hernández et al., 2005) and in fluorescent binding assays with a short *eIF4G* peptide
214 containing the *eIF4E*-binding motif (Zuberek et al., 2016). In addition, unlike binding of *eIF4E-5*
215 to 4E-BP, 4E-T and Cup, binding to *eIF4G-2* was sensitive to more stringent selection
216 conditions, indicating that *eIF4E-5* may preferentially bind inhibitors of translation rather than

217 translational activators. Overall, these results suggest eIF4E-5 may bind known translational
218 regulators in various complexes during sperm development.

219

220 eIF4E-5 is required for localized accumulation of Soti protein

221 As eIF4E-5 is essential for spermatid individualization and localizes at the distal ends of
222 elongated spermatid cysts, we sought potential eIF4E-5 translational targets that show similar
223 localization during spermiogenesis. Among the cup and comet genes, *soti* encodes a testis-
224 specific E3 ubiquitin ligase inhibitor that controls caspase activation and is concentrated at the
225 distal ends of elongated spermatid cysts where its mRNA is found (Barreau et al., 2008a; Kaplan
226 et al., 2010). Because of Soti's role in individualization and similar localization of the Soti and
227 eIF4E-5 proteins, we investigated whether Soti protein distribution was affected in *eIF4E-5*
228 mutants. In wild type, Soti was concentrated at the distal ends of elongated spermatid cysts (Fig.
229 4A, yellow arrowheads), where it colocalized with the membrane skeletal protein Adducin (Fig.
230 4D, red arrowheads; Hime et al., 1996). Soti protein levels were reduced in *eIF4E-5*^{D19a} mutants
231 (Fig. 4B,E), and normal levels of Soti protein were rescued by expression of the 3xFLAG-eIF4E-
232 5 genomic transgene (Fig. 4C, yellow arrowheads). Single molecule RNA fluorescent *in situ*
233 hybridization (smFISH) revealed that loss of *eIF4E-5* did not alter *soti* mRNA expression or
234 localization (Fig. S5A-F). These results demonstrate that eIF4E-5 is required for localized
235 accumulation of Soti protein at the distal ends of elongated spermatid cysts.

236 Because of their similar distribution in elongated spermatid cysts, we examined whether
237 eIF4E-5 colocalizes with *soti* mRNA or Soti protein. smFISH of *soti* mRNA combined with
238 immunofluorescence of 3xFLAG-eIF4E-5 protein (Fig. 5A-A'') or endogenous eIF4E-5 (Fig.
239 S5G-G'') revealed that *soti* mRNA (cysts with dashed outlines) and eIF4E-5 protein (red
240 arrowheads) did not colocalize and were concentrated at the distal ends of different elongated
241 spermatid cysts. In addition, smFISH showed that *eIF4E-5* mRNA was diffusely cytoplasmic
242 starting in primary spermatocytes and did not colocalize with *soti* mRNA or become
243 concentrated at the distal ends of spermatid cysts (Fig. S5H-J). In contrast, immunofluorescence
244 revealed colocalization of 3xFLAG-eIF4E-5 and Soti protein (Fig. 5B-B''), with some cysts
245 exhibiting higher (Fig. 5Bi), similar (Fig. 5Bii) or lower (Fig. 5Biii) levels of eIF4E-5 relative to
246 Soti at the distal ends the elongated spermatid cysts. These results suggest either that eIF4E-5

247 promotes Soti translation concomitant with *soti* mRNA degradation or that eIF4E-5 affects
248 accumulation of Soti at the distal ends of elongated spermatid cysts by an alternate mechanism.

249

250 **eIF4E-5 is dispensable for translation of axonemal dyneins**

251 Because flagellar axoneme assembly occurs at the distal ends of elongating spermatids
252 and proper axoneme assembly is required for individualization (Fabian and Brill, 2012; Gottardo
253 et al., 2013; Riparbelli et al., 2012; Tokuyasu, 1975), we examined whether eIF4E-5 is needed
254 for the translation of axonemal proteins. Transcripts encoding the testis-specific axonemal
255 dynein heavy chain Kl-3 localize at the distal ends of spermatid cysts (Fingerhut and Yamashita,
256 2020). We used smFISH to test whether *soti* and *kl-3* mRNAs colocalize in the same cysts and
257 found that although both transcripts were present at the distal ends of early elongating spermatid
258 cysts, they did not colocalize (Fig. 6A). In addition, *soti* mRNA was present at the distal ends of
259 late elongating spermatid cysts at stages when *kl-3* mRNA was no longer enriched (Fig. 6B,C).
260 Similar to *soti* mRNA, *kl-3* mRNA (cysts with dashed outlines) did not colocalize with eIF4E-5
261 protein (red arrowheads) at the distal ends of elongating cysts (Fig. 6D-F). Immunoblotting of
262 endogenously tagged Kl-3 3xFLAG revealed that Kl-3 protein levels were unaffected in *eIF4E-5*
263 mutants (Figs 6G and S6). Hence, *soti* and *kl-3* transcript localization appears to be distinct, and
264 the translational regulation involved in axoneme assembly is likely independent of eIF4E-5.

265

266 **eIF4E-5 affects polarization of spermatid cysts**

267 In addition to defects in individualization, *eIF4E-5* mutants exhibited spermatid cysts that
268 were mispolarized relative to the long axis of the testis (Fig. 7A-D). In wild-type testes,
269 spermatid cysts with elongated nuclei (~50 cysts/testis; Zhou et al., 2011) were oriented such that
270 all 64 nuclei within a cyst point towards the basal end of the testis and the tails point towards the
271 tip (Fig. 7A,C). In contrast, in *eIF4E-5* mutants, a small subset of cysts (at most three) was
272 mispolarized relative to the long axis of the testis, such that clusters of elongated nuclei faced the
273 tip (Fig. 7B, inset, yellow arrowheads and Fig. 7D, insets; see also Fig. 2C,C'', cyan
274 arrowheads). Whereas mispolarized cysts were rarely seen in wild-type testes (5%), 30-50% of
275 *eIF4E-5* mutant testes exhibited this phenotype ($p < 0.05$), which appeared to be more common in
276 *eIF4E-5^{B8b}* mutants than *eIF4E-5^{B8a}* mutants (Fig. 7E). The cyst mispolarization phenotype was
277 also observed but not quantified in *eIF4E-5^{D19a}* mutants (Fig. 4B'', white arrowhead). However,

278 because nearly all spermatid cysts are oriented properly in testes from *eIF4E-5* mutants, this
279 defect is likely not the cause of the observed fertility defects.

280

281 **eIF4E-5 acts with Orb2 and aPKC to promote spermatid cyst polarization**

282 The cyst polarization defect in *eIF4E-5* mutants was reminiscent of phenotypes observed
283 in mutants for the cell polarity regulator atypical protein kinase C (aPKC) and the cytoplasmic
284 polyadenylation-element binding protein (CPEB) Orb2 (Xu et al., 2014). Heterozygosity for a
285 null allele of either *aPKC* (*aPKC*^{k06403}) or *orb2* (*orb2*³⁶) leads to mispolarized spermatid cysts.
286 To determine whether *eIF4E-5* acts in the same manner as aPKC and Orb2, we examined testes
287 from males transheterozygous for *eIF4E-5^{B8a}* or *eIF4E-5^{B8b}* and *aPKC*^{k06403} or *orb2*³⁶ (Fig. 7F).
288 In comparison to the single heterozygous mutants, which exhibited low levels of cyst
289 mispolarization (7-17% of testes), this phenotype was enhanced in the transheterozygotes (29-
290 37% testes) ($p < 0.05$). These genetic results suggest that *eIF4E-5* has at least a partially
291 overlapping role with Orb2 and aPKC in controlling spermatid cyst polarization.

292

293 **Discussion**

294 ***eIF4E-5* is required for spermiogenesis and male fertility**

295 Our data indicate that *eIF4E-5* is essential for *Drosophila* male fertility and needed for
296 faithful polarization of spermatid cysts and individualization of spermatids to form mature
297 sperm. These post-meiotic defects in *eIF4E-5* mutants are distinct from the earlier defects
298 observed in *eIF4E-3* mutants (Hernández et al., 2012), demonstrating that two of the four testis-
299 enriched eIF4Es have distinct spatiotemporal roles during *Drosophila* spermatogenesis. *eIF4E-3*
300 is most highly enriched in primary spermatocytes, where it is needed for meiotic chromosome
301 segregation and cytokinesis (Hernández et al., 2012). In contrast, *eIF4E-5* concentrates at the
302 distal end of elongated spermatid cysts, a site important for regulating individualization.
303 Although both *eIF4E-3* and *eIF4E-5* are transcribed in primary spermatocytes, enrichment of
304 these eIF4E proteins coincides with the stages they regulate, supporting the idea that the
305 evolutionary expansion of these non-canonical testis-enriched eIF4Es allows for distinct roles
306 and regulation during spermatogenesis. Indeed, complementation experiments revealed that
307 *eIF4E-1* and *eIF4E-3*, but not *eIF4E-5*, were able to rescue growth of *Saccharomyces cerevisiae*
308 lacking its only eIF4E paralog, *Cdc33*, suggesting that *eIF4E-1/3* and *eIF4E-5* likely have

309 different activities or require distinct binding partners *in vivo*. Sorting out the corresponding
310 mechanisms will provide a better understanding of post-transcriptional regulation in *Drosophila*
311 sperm development.

312

313 **eIF4E-5 is needed for spermatid individualization and localized Soti protein accumulation**

314 Non-apoptotic caspase activity is needed for progression of actin cones and degradation
315 of unneeded organelles during individualization (Arama *et al.*, 2003; Huh *et al.*, 2004; Arama *et*
316 *al.*, 2007; Muro *et al.*, 2006). In elongated spermatid cysts, caspases are initially activated at the
317 nuclear end, where actin cones form, and repressed along the spermatid tails. As the actin cones
318 move away from the nuclei, the peak of caspase activity remains associated with the cystic
319 bulge. This localized caspase activity allows for controlled degradation of organelles, and
320 disruption of this activity leads to failure of individualization, characterized by scattered actin
321 cones. In this study, we show that localized caspase activation is disrupted and actin cones are
322 scattered in individualizing spermatids of *eIF4E-5* mutants. These results suggest that *eIF4E-5*
323 post-transcriptionally regulates non-apoptotic caspase activity during spermiogenesis.

324 Caspase activity is regulated during individualization by the inhibitor of apoptosis (IAP)-
325 like protein dBruce. dBruce is ubiquitinated by the cullin E3 ubiquitin ligase complex CRL3 and
326 degraded at the nuclear end, allowing caspase activation (Arama *et al.*, 2007). At the distal end,
327 Soti binds and prevents CRL3 from binding dBruce and promoting its degradation (Kaplan *et al.*,
328 2010). In *soti* mutants, CRL3 activity leads to dBruce destruction and caspase activation along
329 the entire length of the spermatid cysts, inhibiting proper individualization. Here, we show that
330 localized accumulation of Soti protein is reduced at the distal end of elongated spermatids in
331 *eIF4E-5* mutants, which exhibit defects in individualization that resemble *soti* mutants.

332 Although Soti protein and *eIF4E-5* colocalize at the distal ends of elongated spermatid
333 cysts, *soti* mRNA and *eIF4E-5* do not. One possibility is that Soti translation depends on *eIF4E-5*
334 and that *soti* mRNA is degraded in a co-translational manner, as seen for many transcripts in
335 yeast and mammals (Pelechano *et al.*, 2015; Tuck *et al.*, 2020). However, this seems unlikely, as
336 some Soti protein remains present in *eIF4E-5* mutants. Another possibility is that one of the
337 other testis *eIF4Es* might be able to translate *soti*, but with less efficiency than *eIF4E-5*, resulting
338 in production of a smaller amount of Soti protein. Yet another explanation could be that *eIF4E-5*
339 regulates Soti translation indirectly or that it regulates other mRNA targets that are needed for

340 individualization. In addition to *soti*, many transcripts have been identified that are post-
341 meiotically transcribed and accumulate at the distal ends of elongating spermatid cysts in cup or
342 comet patterns (Rathke et al., 2007; Barreau et al., 2008a,b; Vibranovski et al., 2009, 2010). It is
343 possible that one or more of these transcripts is needed for individualization and requires eIF4E-
344 5 for its localization or localized translation. Alternatively, eIF4E-5 might promote Soti
345 accumulation independent of any role it may have in mRNA regulation. Thus, although our
346 results reveal a novel requirement for eIF4E-5 in promoting regulation of non-apoptotic caspase
347 activity during *Drosophila* spermatogenesis, its mechanism of action remains obscure.
348 Distinguishing among these possibilities will be the subject of future studies.

349

350 **eIF4E-5 acts with aPKC and Orb2 to regulate spermatid cyst polarization**

351 For successful transfer of mature sperm to the seminal vesicle, spermatid cysts must
352 polarize such that nuclei face the basal end of the testis, and the tails point towards the tip. aPKC
353 and Orb2 are involved in polarization of the cysts; heterozygous *aPKC* or *orb2* mutants exhibit
354 bundles of 64 spermatids whose polarity is reversed relative to the long axis of the tissue (Xu et
355 al., 2014). Orb2 ensures localization and localized translation of *aPKC* mRNA to establish
356 spermatid cyst polarity, and transheterozygotes of *aPKC* and *orb2* have a more severe defect
357 than heterozygotes alone (Xu et al., 2014). Here, we show that *eIF4E-5* mutants exhibit the same
358 polarization defect as *aPKC* and *orb2* mutants, with a subset of spermatid cysts pointing towards
359 the wrong end of the testis. Transheterozygous mutants of *eIF4E-5* and *aPKC* or *orb2* have a
360 more severe polarity defect than the heterozygous mutants alone, suggesting that eIF4E-5, aPKC
361 and Orb2 might act in the same pathway to establish spermatid cyst orientation. aPKC protein
362 localizes at the growing ends of elongating spermatids where its mRNA is also found (Xu et al.,
363 2014), raising the possibility that *aPKC* mRNA or protein accumulation might be regulated by
364 eIF4E-5.

365 In addition to localized translation of *aPKC* in spermatids, there is evidence that polarity
366 proteins are post-transcriptionally regulated in different cell contexts (Barr et al., 2016). For
367 example, *Par-3* mRNA is locally translated for axonal outgrowth in embryonic rat neurons
368 (Hengst et al., 2009; Macara et al., 2009). This raises the possibility that polarity proteins other
369 than aPKC are similarly regulated in spermatids. Xu et al. (2014) showed that Bazooka, Dlg and
370 Par-1 do not localize at the growing ends of spermatid cysts, suggesting they are unlikely targets

371 of post-transcriptional regulation. However, the authors describe a subset of cysts as
372 mispolarized in *par-6* heterozygotes. Thus, Par-6 might also act with eIF4E-5, aPKC and Orb2 to
373 control spermatid cyst polarization, and that its transcript could be a potential target of eIF4E-5
374 regulation. Our results add to the existing literature that post-transcriptional regulation plays an
375 important role in cyst polarization during *Drosophila* spermatogenesis and indicate that eIF4E-5
376 participates in this process.

377

378 **Regulation of eIF4E-5 during spermiogenesis**

379 Although the relationship of eIF4E-5 to aPKC translation is unclear, our data show that
380 *Soti* translation does not rely solely on eIF4E-5. In addition, eIF4E-5 is dispensable for
381 translation of at least one other transcript found at the distal ends of spermatid cysts, *kl-3*. Indeed,
382 it appears that eIF4E-5 accumulates in elongated spermatids at a later stage than *soti* and *kl-3*
383 mRNAs. Because *Kl-3* is required for construction of the flagellar axoneme during elongation,
384 and *eIF4E-5* is required for individualization at a later stage, eIF4E-5 localization to the distal
385 end likely initiates around the time spermatids become fully elongated. Because eIF4E-5 protein
386 is present in the cytoplasm of male germ cells in primary spermatocytes and spermatids, and its
387 transcript also shows a diffuse localization at these stages, it appears unlikely that eIF4E-5 itself
388 is post-transcriptionally regulated. Thus, it remains unclear how eIF4E-5 protein becomes
389 concentrated at the distal end. Perhaps the mRNA localization mechanism involved in
390 transporting the cup and comet transcripts from nuclei also regulates localization of eIF4E-5
391 protein. Alternatively, it is possible that local translation of eIF4E-5 depends on translational
392 machinery that is present at the distal end. It will be of interest to identify the factors needed for
393 mRNA transport and translation at the distal ends of elongated spermatid cysts.

394 Our results indicate that eIF4E-5 directly binds several known eIF4E binding partners
395 (4E-BP, eIF4G-2, 4E-T, Cup) and might act in the same pathway as Orb2. As all of these
396 proteins regulate mRNA translation (Miron et al. 2001; Kamenska et al. 2014; Nelson et al,
397 2004; Zappavigna, et al., 2004; Baker and Fuller 2007; Franklin-Dumont et al., 2007),
398 interactions with these proteins may help facilitate or repress translational activity of eIF4E-5
399 and its target mRNAs at different stages of spermiogenesis. Although canonical eIF4E-1 and
400 testis-specific eIF4E-3 both associate with canonical eIF4G and testis-specific eIF4G-2 (Ghosh

401 and Lasko, 2015), eIF4E-5 shows a stronger interaction with eIF4G-2 than eIF4G. Thus, an
402 eIF4F complex formed by eIF4E-5 and eIF4G-2 might target transcripts for translation *in vivo*.
403

404 Conclusion

405 Here, we show that the testis-specific *Drosophila* eIF4E paralog eIF4E-5 is essential for
406 male fertility. Loss of eIF4E-5 disrupts localized accumulation of the caspase inhibitor Soti
407 during individualization and hence regulated activation of Caspase-3. In addition, eIF4E-5 acts
408 with Orb2 and aPKC to promote spermatid cyst polarization. Our study provides evidence of
409 localized post-transcriptional regulation by eIF4E-5 during two developmental stages of
410 *Drosophila* spermatogenesis. Future experiments will reveal the mechanism by which eIF4E-5
411 acts to promote male fertility.

412 Although there are apparent differences between *Drosophila* and human
413 spermatogenesis, activation of pro-apoptotic proteins without causing the death of the entire cell
414 is also used to eliminate cytoplasmic components during terminal differentiation of mammalian
415 spermatids (Shaha et al., 2010). Because one known cause of human male infertility is
416 incomplete extrusion of cytoplasm (Rengan et al., 2012), it would be of interest to know if there
417 is a similar role for post-transcriptional regulation in promoting this aspect of male fertility in
418 humans.

419

420 Materials and Methods

421 Fly strains and husbandry

422 Flies were raised on standard cornmeal molasses agar at 25°C (Ashburner, 1990). *w¹¹¹⁸*
423 was used as the experimental control. *w¹¹¹⁸; PBac{vas-Cas9}* (Bloomington *Drosophila* Stock
424 Center (BDSC) #56552, Bloomington, USA) was used to generate *eIF4E-5* CRISPR/Cas9
425 mutants. *y^l M{3xP3-RFP-3xP3-GFP-vas-int.DM}ZH-2A w^{*}; P{CaryP}attP40* (BestGene Inc.)
426 contains a second chromosome attP site (25C6) and was used to generate 3xFLAG-eIF4E-5
427 transgenic lines. Double-balancer stock *w¹¹¹⁸; Sco/CyO; MKRS/TM6B* was used for balancing
428 mutants. *eIF4E-5* alleles were examined *in trans* to chromosomal deletions *Df(3L)BSC631*
429 (BDSC #25722) and *Df(3L)Exel6279* (BDSC #7745) lacking the entire *eIF4E-5* coding region.
430 *KL-3^{3xFLAG}* carries a 3xFLAG tag at the endogenous C-terminus of the *kl-3* coding region,
431 generated by CRISPR/Cas9 mediated knock-in (Fingerhut et al., 2019). *UAS-kl-3^{TriP.HMC03546}*

432 (BDSC #53317) expresses dsRNA for RNAi directed against *kl-3* under UAS control (Perkins et
433 al., 2015). *aPKC^{k06403}* (BDSC #10622) carries a *P{lacW}* insertion between two promoters in the
434 third intron, resulting in a loss of function allele (Xu et al., 2014). *Orb2³⁶* (BDSC #58479) carries
435 a deletion of the Orb2 coding region generated through FRT-mediated recombination between
436 two flanking progenitor insertions of *PBac{WH}CG43783^{f04965}* and *PBac{WH}orb2^{f01556}* (Xu et
437 al., 2012).

438 Fertility tests were performed by mating individual males of each genotype with five
439 virgin *w¹¹¹⁸* females at 25°C. After 5 days, crosses were observed for the presence of progeny.
440

441 **Generation of *eIF4E-5* mutant strains**

442 gRNAs targeting two different regions in *eIF4E-5* with no predicted off-targets were
443 selected using the CRISPR Optimal Target Finder (<http://targetfinder.flycrispr.neuro.brown.edu>;
444 Gratz et al., 2014): 5'-GAATTTGTCGCGATTCGAG-3' (gRNA1) and 5'-
445 GAGTCGAGTACAAGCATCCTT-3' (gRNA2). The two selected gRNAs were cloned into
446 pCFD4d under two promoters, U6-1 and U6-3 (Addgene plasmid #83954, Watertown, USA; Ge
447 et al., 2016). pCFD4d was digested with *Bbs*I (New England Biolabs, R3539L, Waltham, USA)
448 and gel purified. Inserts were generated by PCR using the following primers and pCFD4d as a
449 template: 5'-

450 TATATAGGAAAGATATCCGGGTGAACCTCGGAATTTGTCGCGATTCGAGGTTTAG
451 AGCTAGAAATAGCAAG-3' and 5'-
452 ATTTAACTTGCTATTCTAGCTCTAAAACAAGGATGCTGTACTCGACTCGACGTTA
453 AATTGAAAATAGGTC-3'. The backbone and insert were combined by Gibson Assembly
454 Master Mix (New England Biolabs, E2611L). The gRNA plasmid was confirmed by sequencing
455 and injected into transgenic embryos expressing Vasa-Cas9 and allowed to develop to adulthood
456 (BestGene Inc., Chino Hills, USA). Each adult fly was individually crossed with a balancer stock
457 to generate stocks of putative mutants. Genomic DNA was extracted from homozygous putative
458 mutants of each stock for PCR amplification of *eIF4E-5* and sequenced using primers: 5'-
459 GGTGATGACACTACTGACGC-3' and 5'-AACGCCAACAACTGAAAC-3' (The Centre
460 for Applied Genomics, The Hospital for Sick Children, Toronto, Canada). This experiment was
461 repeated twice; the initial round identified two different mutant alleles from the same founder

462 parent (*eIF4E-5^{B8a}* and *eIF4E-5^{B8b}*) and the second round identified a frame-shift allele (*eIF4E-*
463 *5^{D19a}*).

464

465 **Generation of genomic 3xFLAG-eIF4E-5 rescue construct and transgenic flies**

466 The rescue construct consisted of the genomic DNA starting 359 bp upstream of the
467 ATG, 69 bp 3xFLAG tag (5'-
468 GACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGA
469 CGATGACAAG-3'), 12 bp linker (5'- GGCAGCGAATTC-3'), all 791 bp of eIF4E-5 protein
470 coding sequence including introns, and 362 bp downstream of the stop codon including the
471 putative poly(A) site. The first three regions (5' region, 3xFLAG, linker) was synthesized
472 (BioBasic Inc., Markham, Canada) and subcloned into the pattB plasmid (Drosophila Genomics
473 Research Center (DGRC), 1420, Bloomington, USA) using *Bam*HI (New England Biolabs,
474 R3136S) and *Eco*RI (New England Biolabs, R3101S). The last two regions were PCR-amplified
475 from genomic DNA with *Eco*RI and *Not*I (New England Biolabs, R3189S) added to the primers
476 (5'-ATGACAAGGGCAGCGAATTCATGGCCAGTGCACAAGTG -3' and 5'-
477 GTACCCTCGAGCCGCGGCCGCGCTTGAGTAGGCAATTACGAC-3'), and subcloned into
478 pattB downstream and in-frame with the 5' genomic region, 3xFLAG and linker. The pattB-
479 3xFLAG-eIF4E-5 plasmid was confirmed by sequencing and integrated into the attP40 site on
480 the second chromosome via PhiC31 integrase-mediated transgenesis by injection into *y^l*
481 *M{3xP3-RFP-3xP3-GFP-vas-int.DM}ZH-2A w^{*}*; *P{CaryP}attP40* embryos (BestGene Inc.).
482

483 **Generation of anti-eIF4E-5 antibodies**

484 The full-length coding region of eIF4E-5 was PCR-amplified using the plasmid 4E5-
485 pCR2.1 as a template (Hernández et al., 2005) and subcloned into pRSET (Invitrogen, V35120)
486 to create an expression construct. The plasmid was transformed into *E. coli* BL21 (DE3)
487 (Novagen, 71012) to produce a His6X fusion recombinant protein according to the
488 manufacturer's instructions. The fusion protein was purified using Ni-NTA beads under
489 denaturing conditions (Thermo Fisher Scientific, R90101, Waltham, USA). Polyclonal anti-
490 eIF4E-5 antibodies were raised in rabbit against this recombinant protein (Comparative Medicine
491 and Animal Resources Centre, McGill University, Montreal, Canada). The sera were separated
492 and NaN₃ was added at a concentration of 0.02%.

493

494 **Squashed preparations of *Drosophila* testes**

495 Testes were dissected from 0 to 2-day old males (unless otherwise stated) in cold testis
496 isolation buffer (TIB) (Casal et al., 1990). Whole testes were mounted on polylysine coated
497 slides in TIB and squashed with a coverslip (Polysine, P4681-001, Thermo Fisher Scientific).
498 Live images for Fig. 3 were acquired on an upright Zeiss Axioplan 2E epifluorescence
499 microscope equipped with a 20x phase-contrast objective and an Axiocam black and white CCD
500 camera using Axiovision software (Carl Zeiss, Oberkochen, Germany). Live images for Fig. S3
501 were acquired on an inverted Leica DMi8 epifluorescence microscope equipped with a 20x
502 phase-contrast objective and a Leica K5 camera using Thunder Imaging System. All images
503 were uniformly processed for brightness and contrast using Adobe Photoshop (San Jose, USA).

504

505 **Immunohistochemistry on squashed testis preparations**

506 Testes were prepared as for live microscopy (above) and were processed for
507 immunofluorescence as previously described (Hime et al., 1996). In brief, after mounting on
508 polylysine coated slides (Polysine, Thermo Fisher Scientific, P4681-001, Waltham, USA),
509 samples were squashed with a coverslip and frozen in liquid nitrogen. Coverslips were removed
510 with a razor blade, and samples were immediately chilled in 95% ethanol for at least 10 minutes.
511 Samples were fixed in PBS with 4% paraformaldehyde (Electron Microscope Sciences, 15710,
512 Hadfield, USA) for 7-10 minutes, permeabilized in PBS with 0.37% Triton X-100 and 0.3%
513 sodium deoxycholate for 30 minutes, and blocked in PBS with 0.1% Triton X-100 and 5%
514 bovine serum albumin (PBTB; Sigma-Aldrich, A3912-100G, St. Louis, USA). Samples were
515 incubated at 4°C overnight with primary antibodies, then washed with PBTB three times for 5
516 minutes and once for 15 minutes and incubated for 1 hour at room temperature with secondary
517 antibodies diluted in PBTB. Samples were then washed with PBTB once for 15 minutes, stained
518 (when applicable) with rhodamine-phalloidin in PBTB (1:200; Invitrogen, R415) for 30 minutes,
519 washed with PBS with 0.1% Triton X-100 (PBT) for 15 minutes, stained with 4',6-diamidino-2-
520 phenylindole (DAPI) in PBT (1:1000; VWR, 89139-054, Radnor, USA) for 10 minutes and
521 washed with PBT twice for 15 minutes. Samples were mounted in ProLong Diamond Antifade
522 Mountant (Molecular Probes, P36961, Eugene, USA), sealed with nail polish, and examined
523 within 1-2 days. Fluorescence micrographs were acquired on a Nikon A1R inverted laser

524 scanning confocal equipped with 10x, 20x, 40x, and 60x objectives, photomultiplier tube (PMT)
525 detectors for DAPI channel, and gallium arsenide phosphide (GaAsP) PMT detectors for green
526 and red channels using NIS Elements software (SickKids Imaging Facility, The Hospital for Sick
527 Children, Toronto, Canada). All images were uniformly processed for brightness and contrast
528 using Adobe Photoshop (San Jose, USA). Apart from Fig. 3H, all images from the same
529 experiment were adjusted for brightness and contrast in an identical manner.

530 Primary antibodies used for immunofluorescence were rabbit anti-eIF4E-5 (1:500; #4524,
531 this work), rabbit anti-caspase-3 (1:400; Asp175, Cell Signaling Technology, Danvers, USA),
532 guinea pig anti-Soti (1:100; Kaplan et al., 2010; a kind gift of Eli Arama, Weitzmann Institute,
533 Rohovot, Israel), mouse anti-Adducin 1B1 (1:20; Zaccai and Lipshitz, 1996; a kind gift of
534 Howard Lipshitz, University of Toronto, Toronto, Canada) and mouse anti-FLAG (1:200; M2,
535 Sigma-Aldrich, F1804). Secondary antibodies used for immunofluorescence were Alexa Fluor
536 488-conjugated anti-rabbit (1:1000; Invitrogen, A-11008), Alexa Fluor 488-conjugated anti-
537 guinea pig (1:1000; Invitrogen, A-11073), or Alexa Fluor 568-conjugated anti-mouse IgG
538 (1:1000; Invitrogen, A-31043).

539

540 **Quantifying spermatid cyst polarity defects**

541 Testes were dissected and stained following the immunohistochemistry protocol
542 described above. The percentage of testes with spermatid nuclei near the tip was recorded (Xu et
543 al., 2014). For Fig. 7E-F, statistical analysis and graphing were performed using GraphPad Prism
544 versions 8 for Macintosh, respectively (GraphPad Software). Differences observed between
545 genotypes were analyzed with unpaired two-tailed Student *t*-test. Results were considered
546 statistically significant at *p*<0.05.

547

548 **Immunofluorescence with single molecule RNA FISH**

549 All solutions used were RNase free. Testes were dissected in 1xPBS (Invitrogen,
550 AM9624) and fixed in 4% formaldehyde (Polysciences, Inc., 18814-10) in 1xPBS for 30
551 minutes, washed briefly in PBS, and permeabilized in 70% ethanol overnight at 4°C. Samples
552 were then washed with 1xPBS and blocked for 30 minutes at 37°C in blocking buffer (1xPBS,
553 0.05% BSA [Invitrogen, Am2616], 50µg/mL yeast tRNA [Sigma-Aldrich, R8759], 10mM
554 Vanadyl Ribonucleoside complex [New England Biolabs, S1402S], 0.2% Tween-20 [Sigma-

555 Aldrich, P7949]). Samples were incubated with primary antibodies diluted in blocking buffer
556 overnight at 4°C, washed with 1xPBS containing 0.2% Tween-20, re-blocked for 5 minutes at
557 37°C in blocking buffer, and incubated 4°C overnight in blocking buffer containing secondary
558 antibodies. Testes were then washed with 1xPBS containing 0.2% Tween-20, and re-fixed for 10
559 minutes before being briefly rinsed with wash buffer (2x saline-sodium citrate [SSC, Invitrogen,
560 AM9770], 10% formamide [Fisher Scientific, BP227]), and then hybridized overnight at 37°C in
561 hybridization buffer (2xSSC, 10% dextran sulfate [Sigma-Aldrich, D8906], 1mg/mL yeast
562 tRNA, 2mM Vanadyl Ribonucleoside complex, 0.5% BSA, 10% formamide). Following
563 hybridization, samples were washed three times in wash buffer for 20 minutes each at 37°C and
564 mounted in Vectashield with DAPI (Vector Laboratory, H-1200, Burlingame, USA). Images
565 were acquired using an upright Leica Stellaris 8 confocal microscope with a 63X oil immersion
566 objective lens (NA = 1.4) and processed using ImageJ software (National Institutes of Health,
567 Bethesda, USA).

568 Primary antibodies were rabbit anti-eIF4E-5 (1:500; #4524, this work) or rabbit anti-
569 FLAG (1:500; Invitrogen, PA1-984B), and secondary antibodies were Alexa Fluor 488-
570 conjugated anti-rabbit (1:200; Life Technologies, Carlsbad, USA). Fluorescently labeled probes
571 were added to the hybridization buffer to a final concentration of 100nM. Probes against *kl-3*,
572 *soti*, and *eIF4E-5* mRNA were designed using the Stellaris® RNA FISH Probe Designer
573 (Biosearch Technologies, Inc., Novato, USA) available online at
574 www.biosearchtech.com/stellarisdesigner. Probe sets are listed in Table S1.

575 For smFISH alone, hybridization immediately followed the overnight incubation in 70%
576 ethanol and short wash with wash buffer.

577

578 Immunoblotting

579 Three methods were used for immunoblotting. For Fig. 1, approximately 20 pairs of
580 testes per genotype were dissected in TIB with protease inhibitor cocktail (1:100; Halt, Thermo
581 Fisher Scientific, 87786), then lysed in radioimmunoprecipitation assay (RIPA) buffer with
582 protease inhibitors (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% Nonidet P-40, 0.5% sodium
583 deoxycholate, 0.1% SDS). NuPAGE LDS Sample Buffer was added, and samples were boiled
584 for 10 minutes at 98°C (Invitrogen, NP0007). Proteins were run on gradient pre-cast SDS
585 polyacrylamide gels (8-16%, ExpressPlu, GenScript, M81610, Piscataway, USA) before being

586 transferred to nitrocellulose membranes (0.45 μ m, Amersham Protran, GE Healthcare Life
587 Sciences, 10600020 Chicago, USA) with NuPAGE Transfer Buffer (Invitrogen, NP0006).
588 Membranes were rinsed in TBST (Tris-buffered saline with 0.05% Tween-20), blocked in TBST
589 containing 5% nonfat milk, and incubated overnight at 4°C with primary antibodies diluted in
590 TBST containing 5% nonfat milk. Membranes were washed with TBST and incubated with
591 secondary antibodies diluted in TBST containing 1% nonfat milk. Membranes were washed with
592 TBST, and detection was performed using Novex ECL Chemiluminescent Substrate Reagents
593 Kit (Invitrogen, WP20005). Primary antibodies used were rabbit anti-eIF4E-5 (1:5000; #4524,
594 this work) and mouse anti- α -tubulin (5 μ g/mL; AA4.3, Developmental Studies Hybridoma Bank,
595 Iowa City, USA). Secondary antibodies were HRP-conjugated anti-rabbit (1:10,000; Jackson
596 ImmunoResearch Laboratories, 111-035-003, West Grove, USA) or anti-mouse IgG (1:10,000;
597 Jackson ImmunoResearch Laboratories, 715-035-150).

598 For Fig. 6, testes (40 pairs/sample) were dissected in Schneider's medium (Gibco, 21720-
599 024) at room temperature within 30 minutes, the medium was removed, and samples were frozen
600 at -80°C until use. Tissues were then lysed in equal volumes of 2xLaemmli Sample Buffer (Bio-
601 Rad Laboratories, 1610737, Hercules, USA) + β ME (Bio-Rad Laboratories, 1610710, Hercules,
602 USA) and equal volumes were run on a NuPAGE Tris-Acetate gel (3-8%, 1.5mm, Invitrogen,
603 EA0378BOX) with Tris-Acetate SDS Running Buffer (Invitrogen, LA0041) before being
604 transferred onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, 1620177,
605 Hercules, USA) using NuPAGE transfer buffer (Invitrogen, NP0006) without added methanol.
606 Membranes were blocked in 1xTBST (0.1% Tween-20) containing 5% nonfat milk (Bio-Rad,
607 1706404, Hercules, USA), followed by incubation with primary antibodies diluted in 1X TBST
608 containing 5% nonfat milk. Membranes were then washed with 1xTBST, followed by incubation
609 with secondary antibodies diluted in 1xTBST containing 5% nonfat milk. After washing with
610 1xTBST, detection was performed using the Pierce® ECL Western Blotting Substrate enhanced
611 chemiluminescence system (Thermo Fisher Scientific, 32106). Primary antibodies used were
612 mouse anti- α -tubulin (1:2,000; clone DM1a, Sigma-Aldrich) and mouse anti-FLAG (1:2,500;
613 M2, Sigma-Aldrich). Secondary antibody was HRP-conjugated anti-mouse IgG (1:10,000;
614 Abcam, ab6789, Cambridge, UK).

615 For Fig. S6, immunoblotting was carried out as described for Fig. 6 except that samples
616 were run on a Novex Tris-Glycine gel (14%, 1mm, Invitrogen, XP00140BOX) with running

617 buffer (25mM Tris base, 192mM glycine, 0.1% SDS) and transferred to the PVDF membrane
618 using transfer buffer (25mM Tris base, 192mM glycine, 20% methanol). Primary antibodies used
619 were mouse anti- α -tubulin (1:2,000; clone DM1a, Sigma-Aldrich) and rabbit anti-eIF4E-5
620 (1:5000; #4524, this work). Secondary antibodies were HRP-conjugated anti-mouse IgG
621 (1:10,000; Abcam, ab6789, Cambridge, UK) and HRP-conjugated anti-rabbit IgG (1:10,000;
622 Abcam, ab6721, Cambridge, UK).

623

624 Yeast two-hybrid system

625 A cDNA encoding *Drosophila* eIF4E-5 (CG8277) was PCR-amplified using the plasmid
626 4E5-pCR2.1 as a template (Hernández et al., 2005) and subcloned into the vector pOAD (“prey”)
627 vector; Cagney et al., 2000) in-frame with the activator domain sequence of GAL4 to generate
628 the construct eIF4E-5-AD. *Drosophila* GRB10-interacting GYF (glycine-tyrosine-phenylalanine
629 domain) protein (GIGYF, CG11148; Russica et al., 2019; a kind gift of Catia Igreja, Max Planck
630 for Developmental Biology, Tübingen, Germany), CUP (CG11181; Nelson et al., 2004;
631 Zappavigna, et al., 2004; a kind gift of Nancy Standart, Cambridge University, Cambridge, UK),
632 eIF4E transporter (4E-T, CG32016), eIF4G (CG10811; Hernández et al. 1998), eIF4G-2
633 (CG10192; Baker and Fuller 2007), and 4E-BP (CG8846; Miron et al., 2001) cDNAs were
634 subcloned into the pOBD2 vector (“bait” vector; Cagney et al., 2000) in-frame with the DNA-
635 binding domain sequence of GAL4 to create the respective plasmids pGIGYF-BD, pCUP-BD,
636 p4E-T-BD, p ϵ IF4G-2 (313-1164)-BD, p ϵ IF4G-BD and p4E-BP-BD.

637 Interactions between proteins expressed as “prey” or “bait” fusions were detected
638 following a yeast interaction-mating method using the strains PJ69-4a and PJ69-4 α (Cagney et
639 al., 2000). Diploid cells containing both bait and pray plasmids were grown in –Trp, –Leu
640 selective media (Clontech, 630417, Mountain View, USA) and shown as controls for growth.
641 Protein interactions were detected by replica-plating diploid cells onto –Trp, –Leu, –Ade (20
642 ug/mL L-His HCl monohydrate (A-9126, Sigma) added to –Trp, –Leu, –Ade, –His (630428,
643 Clontech) or –Trp, –Leu, –His (630419, Clontech) selective media +3 mM, 10 mM or 30 mM 3-
644 amino-1,2,4-triazole (3AT, Sigma-Aldrich). Growth was scored after 4 days of growth at 30°C.
645

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653

654 **Conflict of interest**

655 The authors declare no competing interests.

656

657 **Author contributions**

658 Conceptualization, L.S., J.A.B.; Methodology, L.S., J.M.F., B.L.F., H.H., G.M., Y.Q., V.L.,
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661 J.A.B.; Resources, H.H., P.L.; Writing – original draft, L.S., J.A.B.; Writing – review and
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874

875 **Figure Legends**

876

877 **Fig. 1. *eIF4E-5* mutants are male sterile.** (A) CRISPR/Cas9 mutagenesis was used to generate
878 mutants of *eIF4E-5*. Diagram showing nucleotide position of *eIF4E-5* on chromosome 3L (top).
879 Gene structure: 5' and 3' UTRs (grey boxes), coding exons (black boxes), introns (lines).
880 Locations of gRNAs in 5'UTR (gRNA1) and first exon (gRNA2) are indicated. *eIF4E-5^{B8a}*,
881 *eIF4E-5^{B8b}* and *eIF4E-5^{D19a}* mutants contain deletions in the first exon, overlapping gRNA2.
882 Genomic sequence: protospacer adjacent motif (blue), target region of the gRNA (red), deletions
883 (dashed lines). (B) Diagram showing structure of predicted wild-type (top) and mutant *eIF4E-5*
884 proteins. Wild-type *eIF4E-5* has a non-conserved N-terminus (amino acids 1-54; white box) and
885 conserved C-terminus (amino acids 54-232; dark grey box). In-frame deletions encoded by
886 *eIF4E-5^{B8a}* and *eIF4E-5^{B8b}* and frameshift (fs) mutation encoded by *eIF4E-5^{D19a}* (out-of-frame
887 amino acids 56-77; light grey box) are indicated. (C) Immunoblot of total testis extracts probed
888 with the indicated antibodies reveal reduced (*eIF4E-5^{B8a}* and *eIF4E-5^{B8b}*) or undetectable
889 (*eIF4E-5^{D19a}*) levels of *eIF4E-5* protein. Note that these levels correlate with the severity of the
890 phenotypes shown in Figs 2, 3 and 7. (D) Fertility tests reveal that all tested combinations of
891 *eIF4E-5* alleles and deficiencies are male sterile and that a genomic transgene expressing
892 3xFLAG-*eIF4E-5* restores fertility to *eIF4E-5^{B8b}* and *eIF4E-5^{D19a}* mutant males (*eIF4E-5^{B8a}* not
893 tested). (E-H) Laser-scanning confocal fluorescence micrographs demonstrate the presence of
894 needle-shaped sperm nuclei in wild-type (E) but not *eIF4E-5^{B8a}* (F), *eIF4E-5^{B8b}* (G) or *eIF4E-*
895 *5^{D19a}* (H) seminal vesicles stained with DAPI. Scale bars: 20 μ m.

896

897 **Fig. 2. *eIF4E-5* localizes to the distal ends of elongated spermatid cysts.** Laser-scanning
898 confocal fluorescence micrographs (A-D) Wild-type (A), *eIF4E-5^{B8a}* (B), *eIF4E-5^{B8b}* (C) and
899 *eIF4E-5^{D19a}* (D) whole adult testes stained for Adducin (magenta, red arrowheads) and *eIF4E-5*
900 (green, yellow arrowheads) reveals that *eIF4E-5* localizes just distal to Adducin at the ends of
901 elongated spermatid cysts in wild-type but not *eIF4E-5* mutant spermatid cysts. Note that anti-
902 *eIF4E-5* antibodies non-specifically stain individualization complexes, as shown for the
903 mispolarized cyst in *eIF4E-5^{B8b}* (C-C'', cyan arrowheads). Scale bars: 20 μ m. (E-H) 3x-FLAG-
904 *eIF4E-5;eIF4E-5^{D19a}* (E, G) and *eIF4E-5^{D19a}* (F, H) whole adult testes stained for 3xFLAG-
905 *eIF4E-5* (magenta) and endogenous *eIF4E-5* (green). 3xFLAG-*eIF4E-5* and *eIF4E-5* colocalize

906 at the distal end of elongated spermatid cysts (E; orange arrowheads). Note non-specific staining
907 of spermatogonia and spermatid tails with anti-FLAG antibodies and non-specific staining of
908 nuclei in spermatogonia, spermatocytes and round spermatids with anti-eIF4E-5 antibodies (E-
909 H'') in *eIF4E-5^{D19a}* mutants. Scale bars: 20 μ m (E-F), 100 μ m (G-H).

910

911 **Fig. 3. *eIF4E-5* mutants exhibit defects in individualization. (A-D)** Phase-contrast images of
912 4-day old wild-type (A), *eIF4E-5^{B8a}* (B), *eIF4E-5^{B8b}* (C) or *eIF4E-5^{D19a}* (D) testes reveal an
913 absence of waste bags (red arrowheads) near the testis tip in *eIF4E-5* mutants. Scale bars: 100
914 μ m. **(E-H)** Laser-scanning confocal fluorescence micrographs of wild type (E), *eIF4E-5^{B8a}* (F),
915 *eIF4E-5^{B8b}* (G) or *eIF4E-5^{D19a}* (H) whole adult testes stained for DNA (magenta), activated
916 caspase-3 (green) and F-actin (shown only in insets). Activated caspase (yellow arrowheads) is
917 restricted to cystic bulges in wild type (E) but not *eIF4E-5* mutants (F-H). Boxed areas are
918 magnified 10-fold in insets. Groups of actin cones in individualization complexes move
919 synchronously down the length of cysts in wild type (E'') but become scattered prior to
920 reaching the distal end of elongated spermatid cysts in *eIF4E-5* mutants (F''-H''). Brightness
921 and contrast for Fig. 3H'' were adjusted separately from the rest of the images for visualizing
922 individualization complexes. Scale bars: 200 μ m (whole tissue), 20 μ m (insets).

923

924 **Fig. 4. *eIF4E-5* is required for localized accumulation of Soti, a caspase inhibitor.** Laser-
925 scanning confocal fluorescence micrographs. **(A-C)** Wild-type (A), *eIF4E-5^{D19a}* (B) and
926 *3xFLAG-eIF4E-5;eIF4E-5^{D19a}* rescue (C) whole adult testes stained for DNA (magenta) and Soti
927 (green). Boxed areas are magnified 4 to 5-fold in insets. Soti is enriched at distal ends of
928 elongated spermatid cysts in wild type and rescue but not *eIF4E-5^{D19a}* (yellow arrowheads). Note
929 presence of nuclei from mispolarized spermatid cyst in *eIF4E-5^{D19a}* (B'', white arrowhead).
930 Scale bars: 100 μ m. **(D-E)** Tip regions of testes stained for Soti (green, yellow arrowheads) and
931 Adducin (magenta, red arrowheads). Soti localizes near Adducin at the distal ends of elongated
932 spermatid cysts in wild-type (C-C'') but is reduced in *eIF4E-5^{D19a}* (D-D'') testes. Scale bar: 100
933 μ m.

934

935 **Fig. 5. *eIF4E-5* colocalizes with Soti protein but not *soti* mRNA at the distal ends of**
936 **elongated spermatid cysts.** Laser-scanning confocal fluorescence micrographs. **(A)** *3xFLAG-*

937 *eIF4E-5;eIF4E-5^{D19a}* adult testis probed for *soti* mRNA (magenta; in cysts outline by dotted
938 yellow line) and stained for 3xFLAG-eIF4E-5 protein (green; red arrowheads). Scale bar: 50 μ m.
939 **(B)** *3xFLAG-eIF4E-5;eIF4E-5^{D19a}* adult testis stained for Soti protein (magenta) and 3xFLAG-
940 eIF4E-5 protein (green) reveals varying extents of eIF4E-5 and Soti colocalization at the distal
941 ends of elongated spermatid cysts (i-iii). Boxed areas are magnified 3-fold in insets. Scale bar:
942 10 μ m.

943

944 **Fig. 6. Accumulation of axonemal dynein Kl-3 is independent of eIF4E-5. (A-F)** Laser-
945 scanning confocal fluorescence micrographs. **(A-C)** Wild-type whole adult testes probed for *kl-3*
946 mRNA (magenta) and *soti* mRNA (green) and stained for DNA (white). *kl-3* mRNA (cysts
947 outlined by dotted yellow lines) does not colocalize with *soti* mRNA at the distal end of
948 elongating spermatid cysts. Scale bars: 50 μ m. **(D-F)** Wild-type whole adult testes probed for *kl-3*
949 mRNA (magenta) and stained for 3xFLAG-eIF4E-5 protein (green) and DNA (white). *kl-3*
950 mRNA (cysts outlined by dotted yellow lines) does not colocalize with eIF4E-5 protein (red
951 arrowheads) at the distal ends of early or late elongating spermatid cysts. Scale bars: 50 μ m. **(G)**
952 Immunoblots of whole testis extracts revealing Kl-3 3xFLAG levels in the indicated genotypes
953 expressing endogenously tagged Kl-3 3xFLAG. Kl-3 3xFLAG protein levels are unaffected in
954 *eIF4E-5* mutants.

955

956 **Fig. 7. eIF4E-5 acts with Orb2 and aPKC to regulate spermatid cyst polarity. (A-D)** Laser-
957 scanning confocal fluorescence micrographs of wild-type (A, C) or *eIF4E-5^{B8b}* (B, D) whole
958 adult testes stained for DNA (magenta) and F-actin (green). Nuclei in elongated spermatid cysts
959 orient towards the basal end in wild-type testes, whereas occasional clusters of nuclei orient
960 towards the testis tip in *eIF4E-5^{B8b}* mutants (B, D). Scale bars: 100 μ m. **(E-F)** Percentage of
961 testes that have at least one cluster of spermatid nuclei found at the tip instead of the basal end of
962 the testes. Error bars show standard deviation based on three sets of experiments. Student *t*-tests
963 were unpaired. **(E)** The percentage of testes with misoriented spermatid cysts was significantly
964 higher in homozygous *eIF4E-5* mutants as compared to wild type: *eIF4E-5^{B8a}* ($p<0.05$) and
965 *eIF4E-5^{B8b}* ($p<0.01$). Number of testes scored for each genotype, from left to right: wild type =
966 17; *eIF4E-5^{B8a}* = 32; *eIF4E-5^{B8b}* = 25. **(F)** The percentage of testes with misoriented spermatid
967 cysts was significantly higher in each of the four transheterozygotes relative to their respective

968 heterozygous controls: *aPKC⁰⁶⁴⁰³/+*; *eIF4E-5^{B8a}/+* and *aPKC⁰⁶⁴⁰³/+* ($p<0.05$) and *eIF4E-5^{B8a}/+*
969 ($p<0.05$), *aPKC⁰⁶⁴⁰³/+*; *eIF4E-5^{B8b}/+* and *aPKC⁰⁶⁴⁰³/+* ($p<0.01$) and *eIF4E-5^{B8b}/+* ($p<0.01$),
970 *orb2³⁶/eIF4E-5^{B8a}* and *orb2³⁶/+* ($p<0.05$) and *eIF4E-5^{B8a}/+* ($p<0.05$), *orb2³⁶/eIF4E-5^{B8b}* and
971 *orb2³⁶/+* ($p<0.01$) and *eIF4E-5^{B8b}/+* ($p=0.01$). Number of testes scored for each genotype, from
972 left to right: *aPKC⁰⁶⁴⁰³/+* = 30, *orb2³⁶/+* = 28, *eIF4E-5^{B8a}/+* = 31, *eIF4E-5^{B8b}/+* = 72,
973 *aPKC⁰⁶⁴⁰³/+*; *eIF4E-5^{B8a}/+* = 145, *aPKC⁰⁶⁴⁰³/+*; *eIF4E-5^{B8b}/+* = 108, *orb2³⁶/eIF4E-5^{B8a}* = 117,
974 *orb2³⁶/eIF4E-5^{B8b}* = 125.

975

976 **Fig. S1. Stages of spermatogenesis are organized in a spatiotemporal manner within the**
977 ***Drosophila* testis.** Developing germline cells are enclosed by somatic cyst cells throughout
978 spermatogenesis. Cysts undergoing elongation and individualization have 64 spermatids but only
979 four are shown in this schematic for simplicity.

980

981 **Fig. S2. eIF4E-5 is reduced in eIF4E-5 mutants.** Whole immunoblots of total testis extracts
982 probed with anti-eIF4E-5 (A) or anti- α -tubulin (B) reveal reduced (*eIF4E-5^{B8a}* and *eIF4E-5^{B8b}*)
983 or undetectable (*eIF4E-5^{D19}*) levels of eIF4E-5 protein while levels of tubulin loading control
984 remained the same.

985

986 **Fig. S3. 3xFLAG-eIF4E-5 transgene restores formation of mature sperm and waste bags in**
987 ***eIF4E-5* mutants.** Phase-contrast micrographs. (A-F) 7-day old wild type (A), *eIF4E-5^{B8a}* (B),
988 *eIF4E-5^{B8b}* (C), *eIF4E-5^{D19a}* (D), 3xFLAG-eIF4E-5; *eIF4E-5^{B8b}* (E), or 3xFLAG-eIF4E-5; *eIF4E-*
989 *5^{D19a}* (F) revealing accumulation of mature sperm in seminal vesicles in mutants carrying
990 3xFLAG-eIF4E-5 transgene. Scale bar: 50 μ m. (G) 3-day old 3xFLAG-eIF4E-5; *eIF4E-5^{D19a}*
991 testis revealing presence of waste bag (yellow arrowheads). Scale bars: 50 μ m.

992

993 **Fig. S4. eIF4E-5 interacts with multiple translational regulators in yeast two-hybrid assays.**
994 eIF4E-5 interacts with Cup, 4E-T, eIF4G-2, and 4EBP in yeast two-hybrid assays, as revealed by
995 growth on selective medium. Empty vectors (pOAD and pOBD2) were used as negative
996 controls. L, leucine; W, tryptophan; A, adenine; H, histidine; 3AT, 3-amino-1,2,4-triazole.

997

998 **Fig. S5. eIF4E-5 is dispensable for expression and localization of *soti* mRNA. (A-F)** Laser-
999 scanning confocal fluorescence micrographs of wild-type (A-C) and *eIF4E-5^{D19a}* (D-F) whole
1000 adult testes probed for *soti* mRNA (magenta) and DNA (white). Expression and localization of
1001 *soti* mRNA at the distal ends of elongated cysts (cysts outlined by dotted yellow lines) appear
1002 similar in wild type and *eIF4E-5* mutants. Scale bars: 25 μ m (A, D), 50 μ m (B-C, E-F). (G)
1003 Laser-scanning confocal fluorescence micrographs of a wild-type whole adult testis probed for
1004 *soti* mRNA (magenta) and endogenous eIF4E-5 (green). *soti* mRNA (cysts outlined by dotted
1005 yellow lines) does not colocalize with eIF4E-5 at the distal ends of elongated spermatid cysts
1006 (red arrowheads). Scale bar: 50 μ m. (H-J) Laser-scanning confocal fluorescence micrographs of
1007 wild-type whole adult testes probed for *soti* mRNA (magenta) *eIF4E-5* mRNA (green) and
1008 stained for DNA (white). Expression and localization of *eIF4E-5* mRNA is diffuse during early
1009 and late stages of spermatogenesis. Scale bar: 25 μ m.

1010

1011 **Fig. S6. eIF4E-5 levels remain reduced in *Kl-3 3xFLAG;eIF4E-5* mutants.** Immunoblots of
1012 whole testis extracts revealing eIF4E-5 levels in the indicated genotypes expressing
1013 endogenously tagged *Kl-3 3xFLAG*. eIF4E-5 protein levels are reduced in *eIF4E-5* mutants.

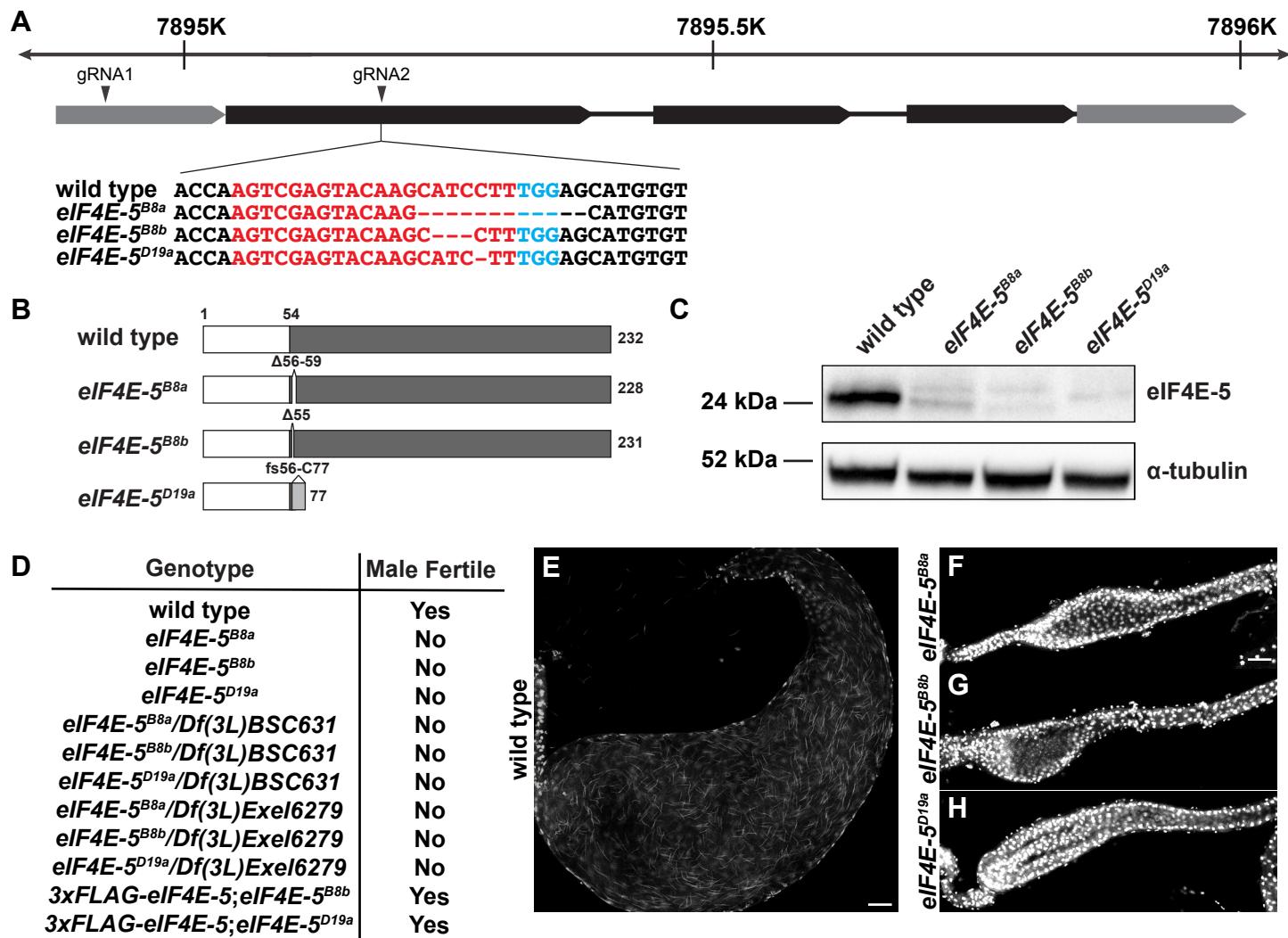
1014

1015 **Table S1. Probes for RNA FISH**

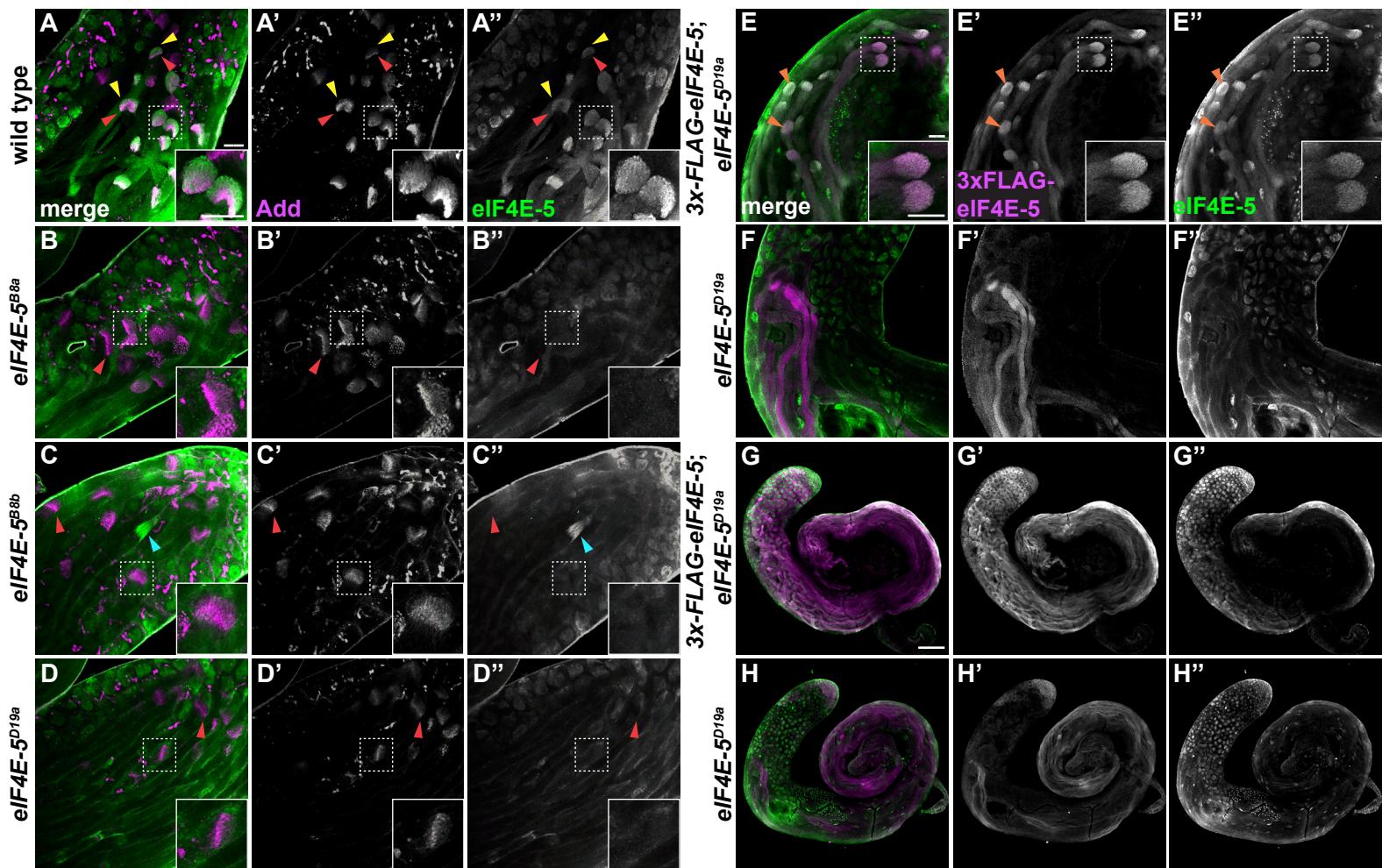
| Probe Target | Fluorophore | 5'-Sequence-3' |
|----------------------|-------------|--|
| <i>kl-3</i> , Exon 1 | Quasar® 670 | taacattccttctggatcc, cgcgaaacgccaaagagtt, gcagcacgcctaacatgtt, ttggtaacttacactaggtc, tatcgcttcttggtc, cctcatttcgaagtaact, caggcttgaatccgttgtt, aaacataccgttgttggg, atacccaacaatctgcaca, gttactattcctcaggatc, ttgcttcatccacaatacc, accatttacattctcaacat, gggacctttcctcaaata, cgttacttacattatggcc, cggaatcagtggatatcct, tttaagctttcctggtagc, agagcggtgaatttcagtgt, actagatccgaccaaaca, gtcgatatacacaacgtccac, accgaacggttatcaatcga, aaatattgccacacatcac, aagcaagagttcgtcttc, cggcttaaaacgtgatcca, caaattcggcacagttct, |

| | | |
|----------------|-------------|---|
| | | tggagattgcgaatagtcca, tgcataccagtccgatgaat, tcctgccttaaaactgtta, cgttgccatgtcatcaata |
| <i>soti</i> | Quasar® 570 | Tctcgacgaggtaatttg, tccgtgttagtacgtccat, gctcatcgtagcagatcgt, ccgactcgatcgattagc, atcttcattcaccgcgtc, tgtccaagtcatcgccag, tgctgtccatcctccat, tgacgattgactcccagg, gtccaggagtagtgcct, caacggggcttggg, gtcgggaa, ctcccttgcggaaaaaa, acgtgggtccatttgg, aacttcgttctccgc, ggagtgggttggcata, ctcctgacttggcatgg, ttaggaggcacatctcg, attgccctcgtagactg, atccctcgacgtgacg, caaagtactcgccctcg, gggtagttctgactggc, tggcagaccataccatt, agaactgaccccaatgct |
| <i>eIF4E-5</i> | Quasar® 670 | ttgcacttgcactggc, tgatcctgctgcttcata, ctattcagctgtatgttc, cttcgtgggtatgtga, ttgctccatgttgc, atcgatagggtctggct, gcttgactcgacttgg, gtccacacatgtccaaa, ggtgcgatcgatccaa, catgcctccatgtc, atctcgtaatctcg, aaggctccacactgtca, gtatggtacaagctccag, tccgctgggttgc, tcgcataatctc, tgcccttgcacacgg, cttcataatctccatgg, cgaccaccctgtatgttgc, ttactcaggtgaccagc, aattccgtttggcactc, gcagtagaaatgtccagcc, aattttggccaccatca, atctcgatggatattca, ttgatgacagatccgc, ttgttgcttgc, ttgcgttactccgttgc, cgatctccagaatggcc, gtatctcagatctggc, tttggacattgc, aaaccgatttgacgccccg |

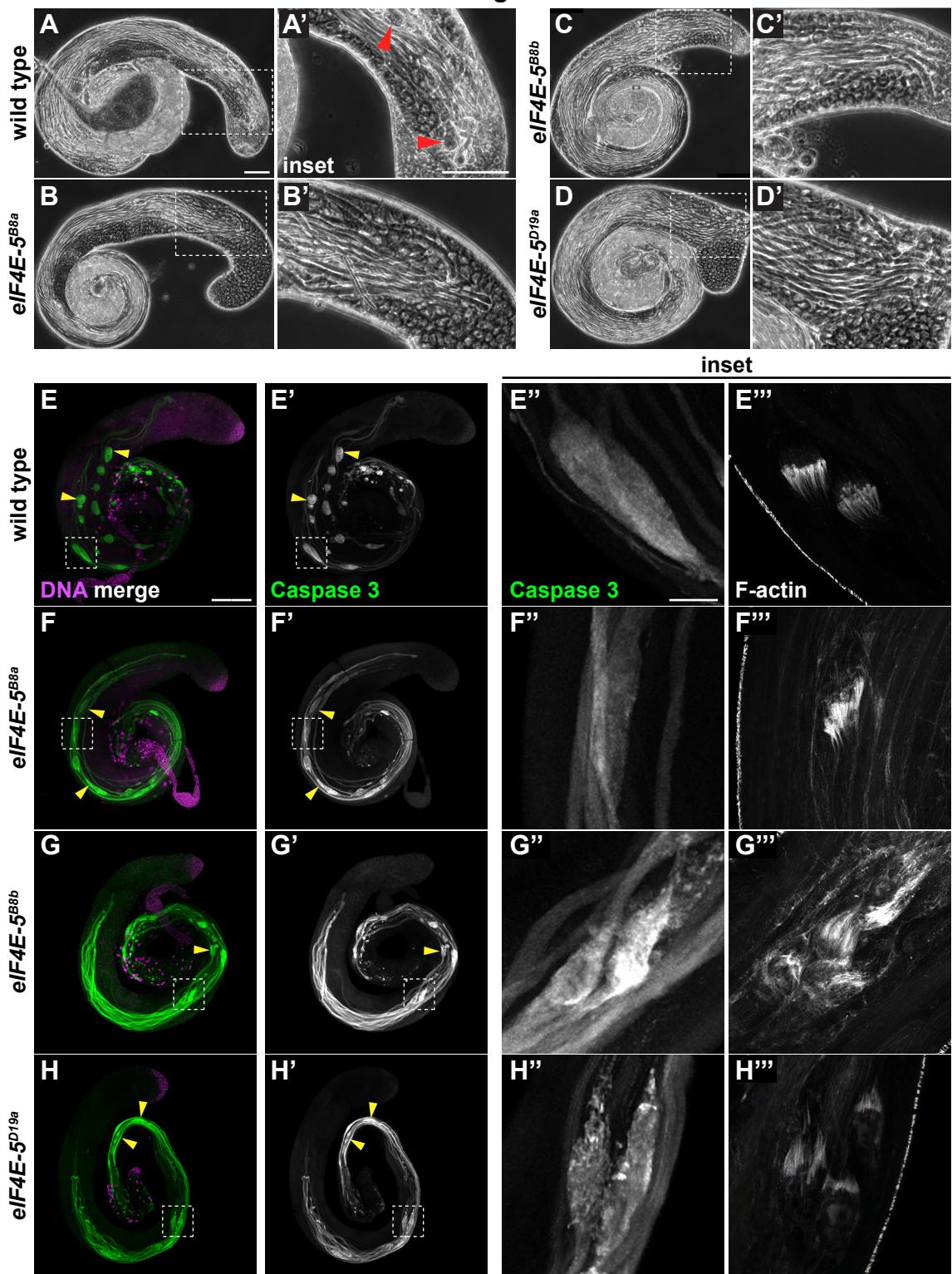
Shao Figure 1



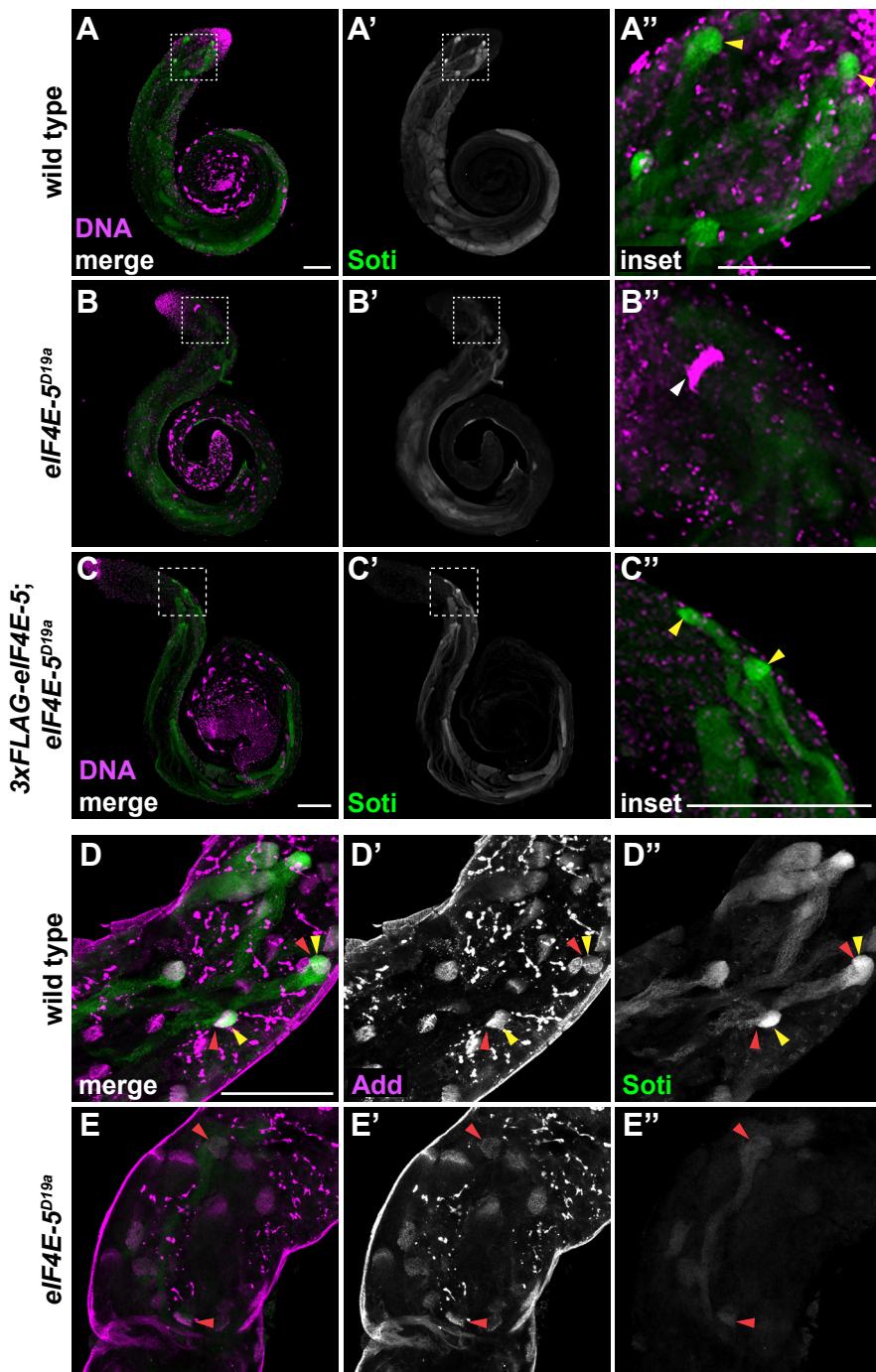
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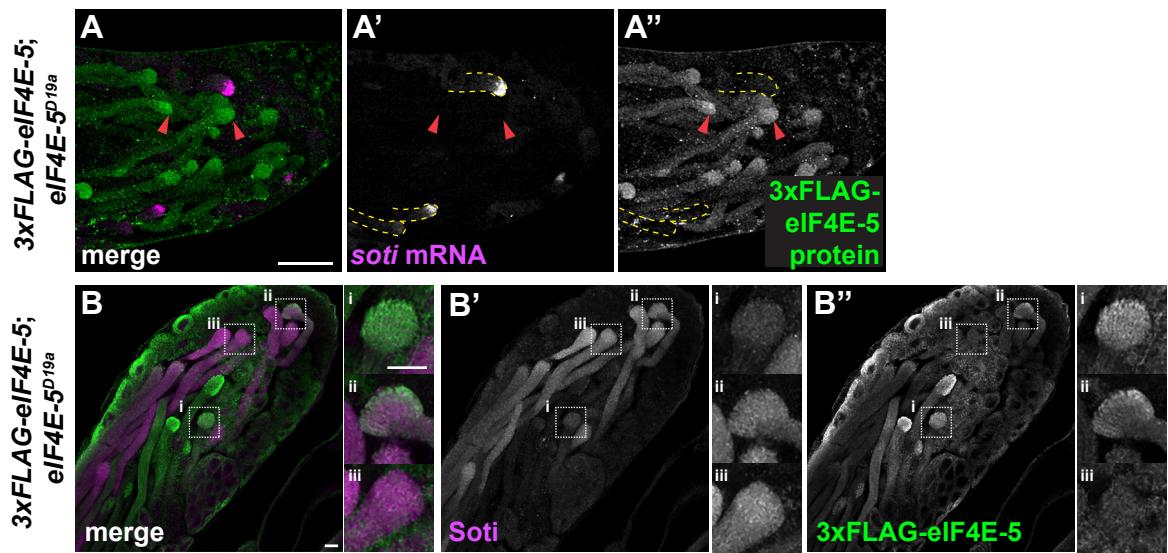
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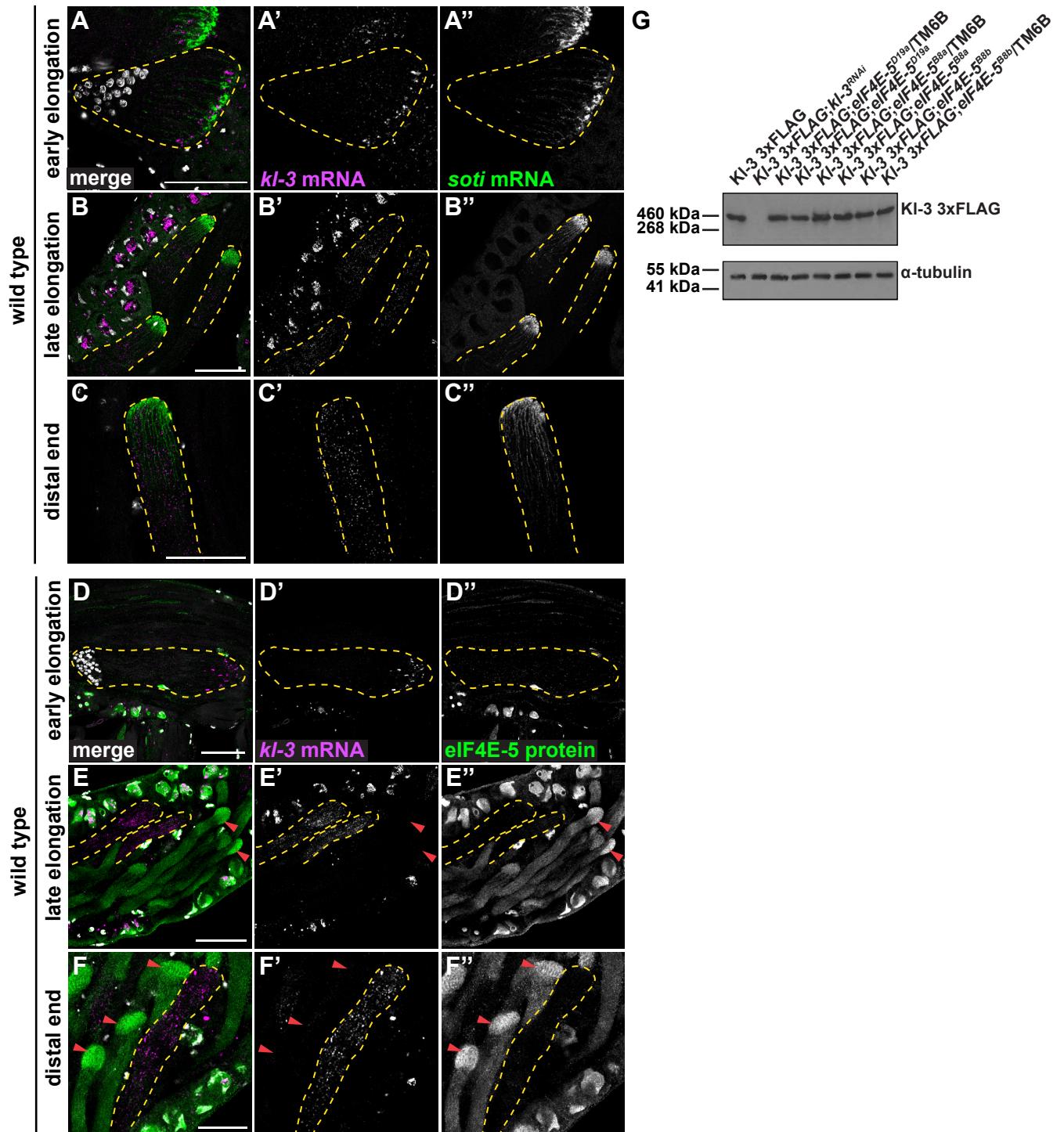
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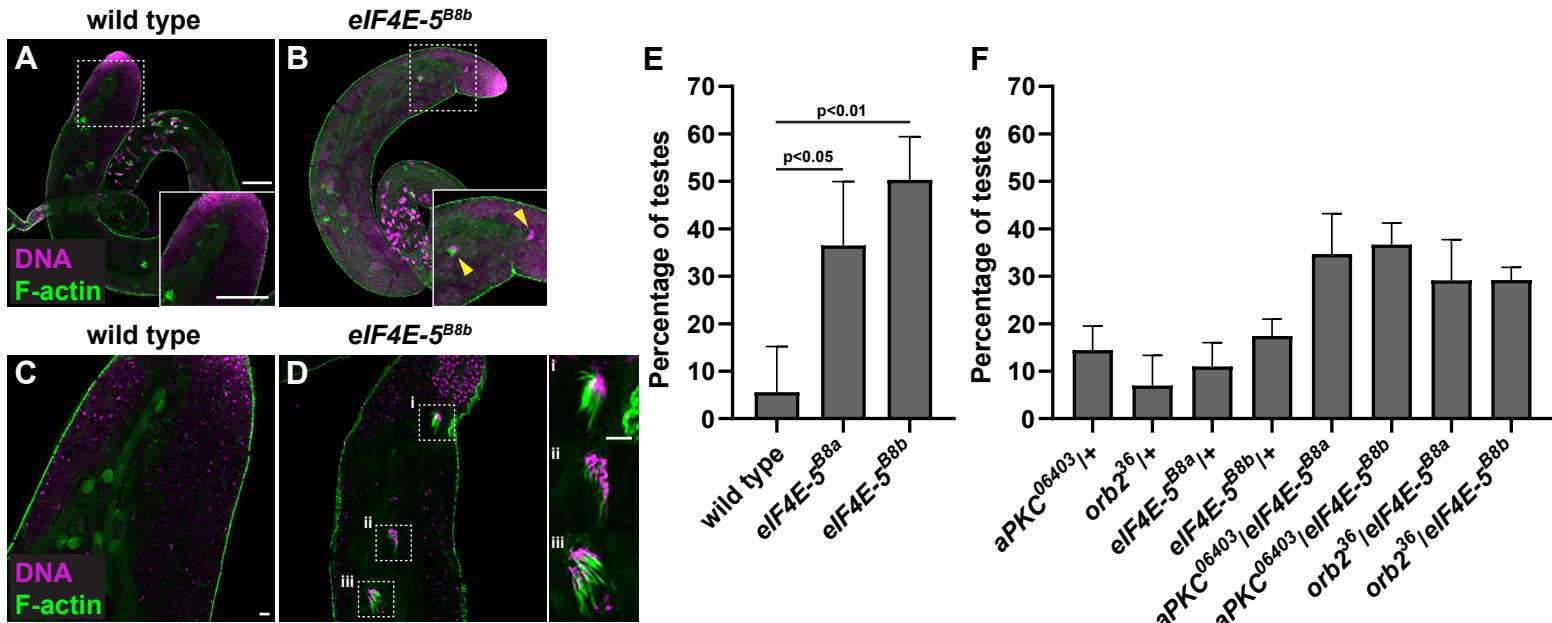
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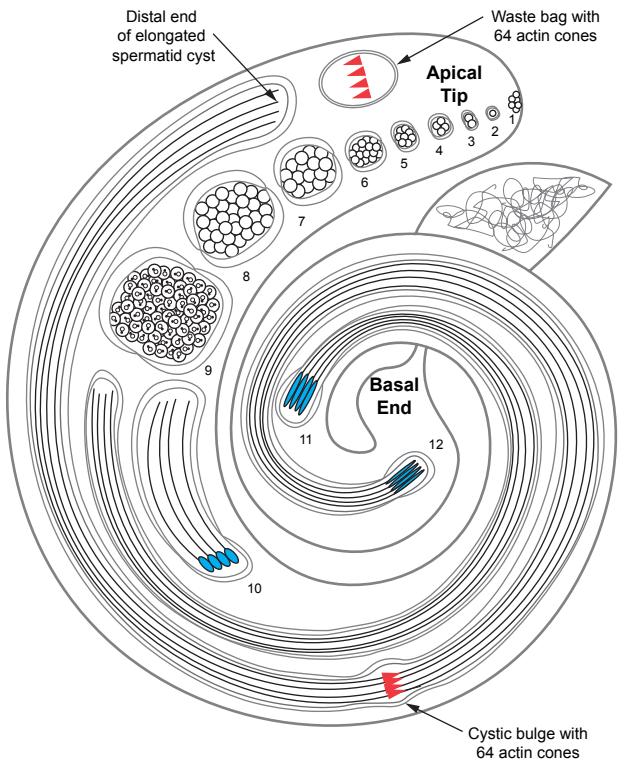
Shao Figure 6



Shao Figure 7



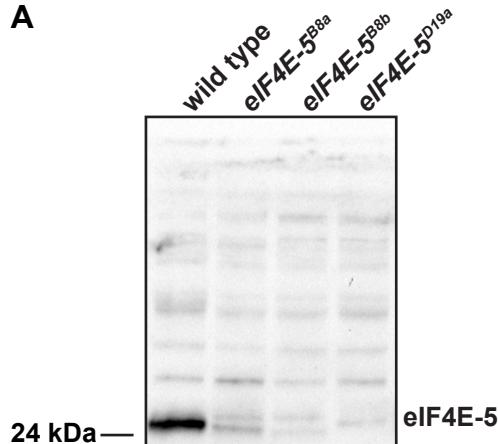
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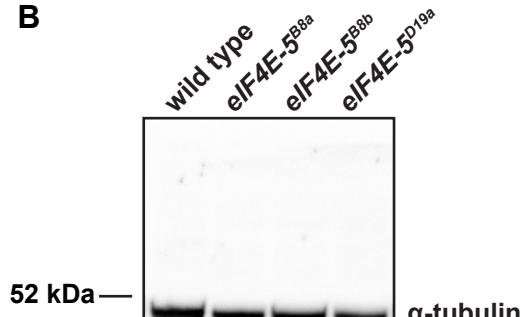
| Cell type | Stages of spermatogenesis |
|--------------------------------|---------------------------|
| Germline stem cells | 1 |
| 1 Gonialblast | 2 |
| 2 Spermatogonia | 3 |
| 4 Spermatogonia | 4 |
| 8 Spermatogonia | 5 |
| 16 Spermatogonia | 6 |
| 16 Primary spermatocytes | 7 |
| 32 Secondary spermatocytes | 8 |
| 64 Round spermatids | 9 |
| 64 Early elongating spermatids | 10 |
| 64 Late elongating spermatids | 11 |
| 64 Individualizing spermatids | 12 |

Shao Figure S2

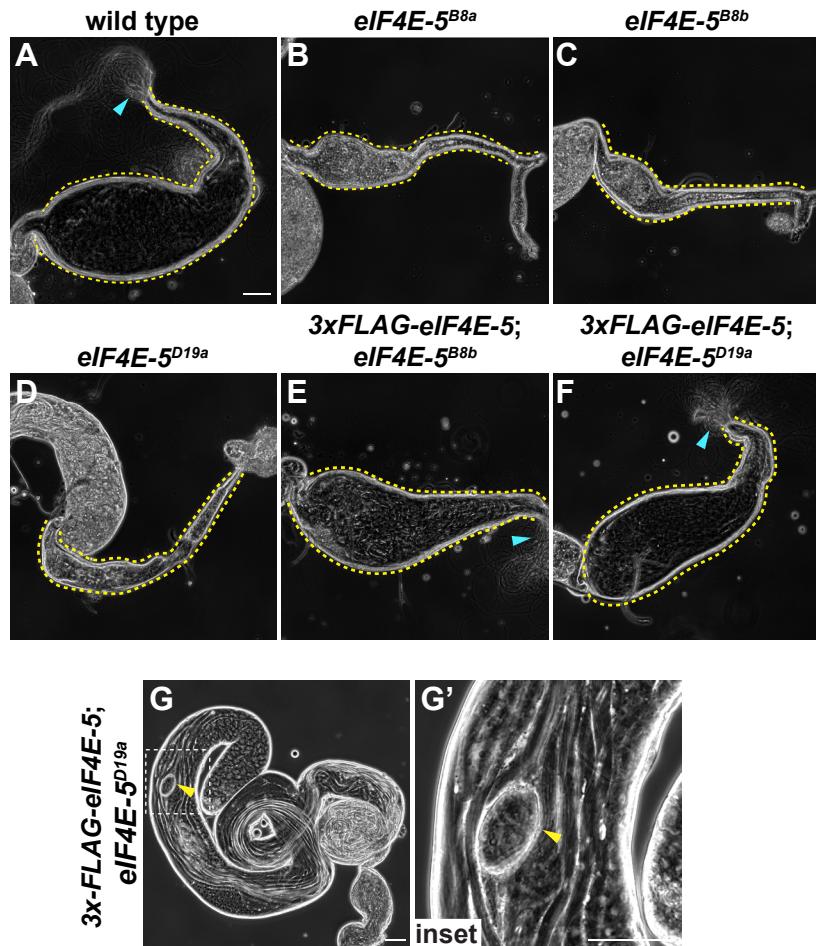
A



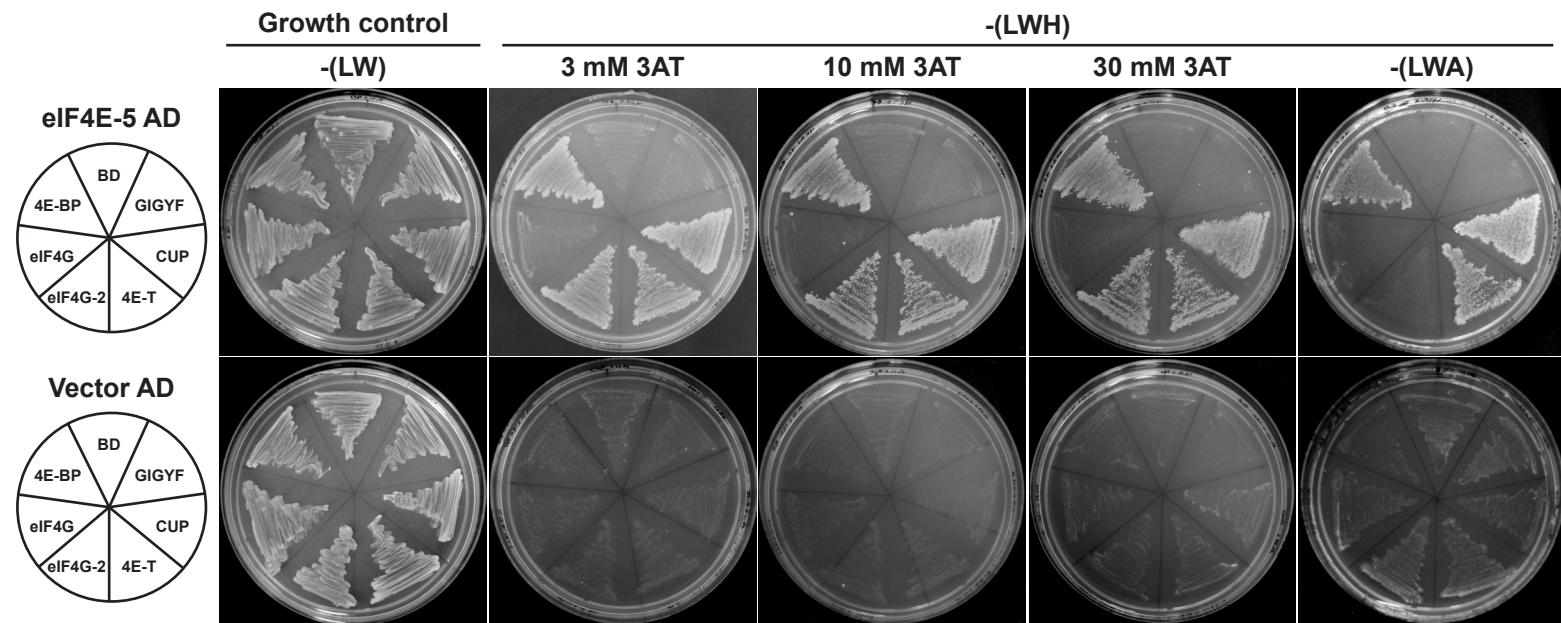
B



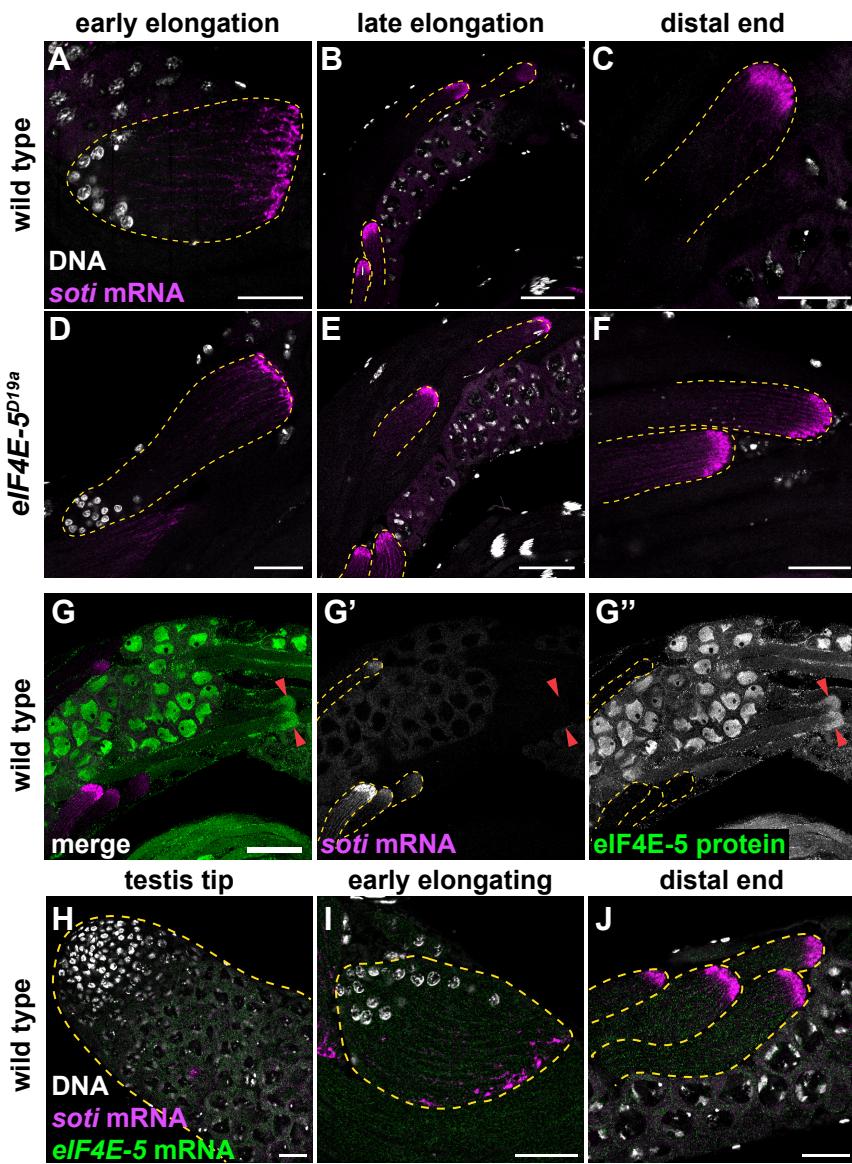
Shao Figure S3



Shao Figure S4



Shao Figure S5



Shao Figure S6

