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1 *Tudor domain containing protein 5-like* (*Tdrd5l*) identifies a novel germline granule that
2 regulates maternal RNAs
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19 Short title: Tdrd5l regulates maternally deposited RNAs
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21 Key words: Tudor-domain, RNA Granule, germ cell, maternal RNA, Drosophila, Tdrd5l, Tdrd5
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24 **Abstract**

25 Tudor domain-containing proteins are conserved across the animal kingdom for their important
26 role in germline development and fertility. Previously, we demonstrated that *Tudor domain-*
27 *containing protein 5-like (Tdrd5l)* plays an important role in the germline where it promotes
28 male identity. However, Tdrd5l is also expressed in both the ovary and testis during later stages
29 of germline development, suggesting that it may play a role in germline differentiation in both
30 sexes. Through immunohistochemistry and genetic interaction assays we found that Tdrd5l
31 localizes to a potentially novel germline RNA granule and plays a role in post-transcriptional
32 gene regulation. RNA sequencing of *Tdrd5l* mutant ovaries compared to wild-type showed an
33 enrichment for maternally deposited RNAs in differentially expressed genes. Additionally,
34 embryos laid by *Tdrd5l*-mutant females exhibited reduced viability and displayed dorsal
35 appendage defects suggesting a failure of proper dorsal-ventral patterning. We also observed
36 defects in posterior localization of the *oskar* mRNA and protein, as well as Kinesin-LacZ, which
37 indicate defects on anterior-posterior polarization of the oocyte. As both A/P and D/V
38 patterning are dependent on *gurken*, we examined Grk expression during oogenesis. We
39 observed premature accumulation of Gurken (Grk) protein, as well as a translational regulator
40 of Grk, Oo18 RNA-Binding Protein (Orb or CPEB), in nurse cells, indicating that translation of
41 these proteins is no longer properly repressed during mRNA transport to the oocyte. We also
42 found that decreased *orb* function suppressed the *Tdrd5l*-mutant phenotype, and so defects in
43 Orb are likely a primary cause of the defects in *Tdrd5l* mutants. Our data indicate that *Tdrd5l* is
44 important for translational repression of maternal mRNAs such as *orb*, and possibly others,

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45 following their synthesis in the nurse cells and during their transport and subsequent activation
46 in the oocyte.
47

48 **Author Summary**

49 Across the animal kingdom, Tudor-domain containing proteins are important for germline
50 development, gametogenesis and fertility. One important aspect of oogenesis is the post-
51 transcriptional regulation of maternal mRNAs that must be translationally repressed prior to
52 their activation at the correct time and place in the oocyte or early embryo. In this study we
53 show that *Drosophila Tudor-domain containing protein 5-like (Tdrd5l)* is important for post-
54 transcriptional regulation of maternal mRNAs. Tdrd5l localizes to an apparently new type of
55 RNA granule in the germline and interacts with post-transcriptional regulation pathways.
56 Embryos from *Tdrd5l*-mutant mothers exhibit patterning defects and lethality. We observe
57 premature accumulation of the important embryonic patterning factor Gurken (Grk/EGF) in
58 nurse cells during oogenesis. Grk is known to be regulated by a cytoplasmic polyadenylation
59 element binding protein (CPEB) called Orb (Oo18 RNA-Binding Protein), and we also observe
60 premature accumulation of Orb in nurse cells in *Tdrd5l* mutants. Decreased *orb* function
61 suppresses the *Tdrd5l*-mutant phenotype, indicating that this is a primary defect in these
62 mutants. Our data indicate that the "Tdrd5l Granule" is important for post-transcriptional
63 regulation of maternal mRNAs such as *orb*, leading to their repression until they are required in
64 the oocyte or early embryo.
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67 **Introduction**

68 Tudor domain-containing proteins are conserved across the animal kingdom, and one
69 important function of these proteins is germline specification and maintenance (1). Mutations
70 in the genes for Tudor proteins have been shown to cause infertility in flies and mammal alike
71 (2,3). While the molecular function of a tudor domain is to promote protein-protein interaction
72 by binding to dimethylated arginine and lysine residues (4,5), many tudor domain-containing
73 proteins also have additional domains such as RNA recognition motifs (6). Together this allows
74 for tudor domains to scaffold larger protein and ribonucleoprotein complexes (RNPs) such as in
75 RNA granules.

76 RNA granules allow for multiple modes of RNA regulation in locally concentrated and
77 protected membrane-less organelles (7). One germline specific granule that is enriched for
78 tudor domain-containing proteins is the nuage (8–10), which produces piRNAs that silence
79 transposons (11). In addition to the nuage, germ cells have a number of other types of granules
80 that can also be found in other tissues, the most common of which are the processing bodies
81 (P-bodies) (12). These granules can take on different functions all centered around post-
82 transcriptional regulation of mRNAs such as inducing mRNA degradation via removal of the 5'
83 cap and translational repression via regulation of the poly(A) tail. mRNAs are often targeted to
84 P-bodies through binding of miRNAs which leads to the formation of a protein complex around
85 the RNA (13,14). RNA granules are particularly important in the germline where RNAs are often
86 transcribed at a different time or place from their translation (15). mRNAs can be sent to these
87 granules for repression until they are needed for translation or to be repressed during transport

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88 to another location (16). In these granules germ cell RNAs can also associate with proteins to
89 form an RNP that targets RNA localization.
90 One special class of germline RNAs are the maternal RNAs generated during oogenesis
91 and deposited into the oocyte that are essential for early embryonic development, in particular
92 before activation of the zygotic genome (17). During Drosophila oogenesis, maternal RNAs are
93 transcribed in nurse cells and then transported into the oocyte where they are later translated.
94 While in the nurse cells, maternally deposited RNAs stay translationally silenced through
95 mechanisms that are not totally understood (18). Some of these RNAs become localized to
96 specific regions of the oocyte and act to establish the body axes of the embryo. *gurken (grk)* is
97 translated first at the posterior of the oocyte to specify posterior fate before localizing to the
98 dorsal-anterior corner of the oocyte to specify dorsal fate (19–21). Other important RNAs
99 include *oskar (osk),* which localizes to the posterior to help specify posterior identity and the
100 germline (22) (23) and *bicoid (bcd),* which specifies the anterior (24).
101 Previous work in our lab identified a novel tudor domain-containing protein Tdrd5l
102 (Tudor domain-containing protein 5-like*)* during a screen for genes involved in germline sex
103 determination (25). Tdrd5l is homologous to mouse Tdrd5 as well as to another Drosophila
104 protein, Tejas, both of which are required for fertility (10,26). All of these proteins have a highly
105 related tudor domain, while Tejas and mouse Tdrd5 also have LOTUS domains that interacts
106 with the RNA helicase Vasa (27). Drosophila Tejas and mouse Tdrd5 both localize with Vasa to
107 the nuage where they act in piRNA synthesis and retrotransposon repression (10,26).
108 Unlike its closest homologs, Tdrd5l does not localize to the nuage. Further, while Tdrd5l
109 is male-specific in the early germline, it is expressed in differentiating germ cells in both males

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110 and females, suggesting that it plays a role in post-transcriptional gene regulation in both
111 spermatogenesis and oogenesis. In this study we demonstrate that Tdrd5l localizes to a novel
112 germline RNA granule that associates with, but is distinct from, the nuage. Further, we
113 demonstrate that, in females, *Tdrd5l* is important for post-transcriptional regulation of
114 maternal RNAs. In *Tdrd5l* mutants, embryos exhibit defects in dorsal-ventral patterning and Grk
115 and Orb (Oo-18 RNA-Binding Protein) prematurely accumulate in nurse cells instead of being
116 primarily expressed in the oocyte. Thus, one important role for *Tdrd5l* is in regulation of
117 maternal RNAs as they are being transported from the nurse cells to the oocyte.
118

119 **Results**

120 **Tdrd5l localizes to cytoplasmic granules in the *Drosophila* germline**

121 To visualize the endogenous Tdrd5l protein, we used CRISPR/Cas9 to generate epitope-
122 tagged alleles of the endogenous *Tdrd5l* locus. Interestingly, both N- and C-terminally tagged
123 alleles behaved as loss of function alleles. Since we previously characterized Tdrd5l expression
124 using an N-terminally tagged BAC construct, it was particularly important to evaluate Tdrd5l
125 expression under normal conditions. Therefore, we generated internal hemagglutinin (HA) and
126 FLAG epitope-tagged alleles (Fig S1A), and these behaved as wt alleles of *Tdrd5l*. We also
127 generated a peptide guinea pig polyclonal antibody using amino acids 65-81 of Tdrd5l. This
128 antiserum yielded a similar staining pattern to the internally tagged *Tdrd5l* alleles and
129 immunostaining was eliminated in *Tdrd5l* mutants (Figure S2).
130 Using the internally FLAG-tagged allele, we observed anti-FLAG immunoreactivity in a
131 mixed population of small and larger cytoplasmic punctae or “granules” in the pre-meiotic male

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132 germline (Fig 1A), in agreement with our previous work using the exogenous HA-tagged BAC
133 construct (25). Interestingly, in the larger granules Tdrd5l immunostaining was only observed at
134 the periphery of the granule (Fig 1B white arrow, Fig 1E). In addition, while there were both
135 small and large granules present in the early germline, there was a progression from a mixed
136 population in the GSCs (Fig 1A, white arrow) to only large granules in later cysts (Fig 1A, yellow
137 arrow).

138 In the female germline, Tdrd5l immunostaining was greatly reduced compared to the
139 male germline when using identical confocal settings (Fig S3A-B), but still localized to
140 cytoplasmic granules (Fig 1C). Unlike in the male GSCs (Fig 1B brackets), Tdrd5l granules were
141 absent from the germline stem cells (GSCs) in females (Fig 1D, brackets). Since Sxl represses
142 Tdrd5l expression (25), this likely accounts for the lack of Tdrd5l expressing in female GSCs
143 where Sxl is at highest levels (28). At later stages in the female germline, we observed Tdrd5l
144 localizing to granules in the early differentiating germline of the germarium (Fig 1C), as well as
145 in the nurse cells (Fig 1E and 1F, white arrow) and oocyte (Fig 1E, yellow arrow) until mid-
146 oogenesis.

147

148 **Tdrd5l localizes to a novel RNA granule**

149 We next analyzed the "Tdrd5l granule" to determine whether it corresponds to any
150 previously identified RNA granules. To test whether these granules were dependent on RNA,
151 we treated adult testes with RNase A (29). Testes treated with RNase A had a severe reduction
152 in Tdrd5l staining which suggests that Tdrd5l granules depend on RNA for their stability (Fig S4C
153 and D).

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154 One prominent germline RNA granule is the perinuclear nuage, which acts as the site of
155 piRNA production for transposon repression (30–32) and contains other tudor-domain
156 containing proteins (8,9,33). One hallmark of the nuage is the presence of Vasa protein (34).
157 We observed that the Tdrd5l-containing granules often associate with or are imbedded in
158 regions of Vasa immunoreactivity, but that Vasa staining is not detected within the Tdrd5l
159 granules themselves (Fig 2A, arrows). Analysis of the distribution of Tdrd5l granules revealed
160 that there was an even distribution between granules that exhibit cytoplasmic and perinuclear
161 localization, and granules in both locations were associated with Vasa immunoreactivity (Fig
162 S4A-B). These data indicate that there may be a relationship between the Tdrd5l granules and
163 the nuage, but that the Tdrd5l granules themselves are not part of the nuage as defined by
164 Vasa staining.

165 Two additional RNA granules that are commonly found in many cell types are the P
166 bodies, sites of post-transcriptional RNA regulation, and U bodies, which are involved in snRNP
167 maturation (35). A common P body component is Decapping protein 1 (Dcp1) (36) and we
168 visualized Dcp1 immunofluorescence in combination with Tdrd5l (Fig 2B). We did not observe
169 co-localization between Dcp1-containing punctae and Tdrd5l, and the Dcp1-containing bodies
170 were also of much smaller size than Tdrd5l granules. In 63% of Tdrd5l granules, we did observe
171 Dcp1 labeled P-bodies bodies associated with the periphery of Tdrd5l granules (e.g. Fig 2B
172 arrow). This suggests that there may be a relationship between P bodies and Tdrd5l bodies or
173 exchange of materials between these structures, but these data indicate that the two types of
174 bodies are distinct. We visualized U bodies using an endogenously tagged allele of Survival
175 Motor Neuron (SMN) (35) and we failed to observe co-localization between SMN-containing

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176 bodies and Tdrd5l (Fig 2C). Taken together, these data indicate that the Tdrd5l granules are
177 distinct structures from the nuage, P bodies and U bodies.
178

179 **Tdrd5l genetically interacts with post-transcriptional regulatory factors.**

180 Since cytoplasmic RNA granules are often important for post-transcriptional gene
181 regulation, we determined whether *Tdrd5l* exhibited genetic interaction with known post-
182 transcriptional regulation pathways. To ask whether Tdrd5l functions in regulating translation
183 and RNA stability by influencing poly(A) tail length, we tested for a genetic interaction between
184 *Tdrd5l* and *twin*, which encodes the CCR4 homolog and major deadenylase in *Drosophila* (37).
185 Expression of a weak RNAi trigger for *twin* in the germline of wild-type (wt) females (*nos-Gal4,*
186 UAS-*twin* RNAi) caused no morphological or fertility defects (Fig 3A). *Tdrd5l* heterozygous
187 animals also exhibit no morphological or fertility defects on their own. However, when the *twin*
188 RNAi trigger was expressed in the germline of females heterozygous for a mutation in *Tdrd5l*
189 (*nos-Gal4,* UAS-*twin* RNAi, *Tdrd5l/+*), we observed severely disorganized ovaries that lacked
190 recognizable egg chambers (Fig 3B) and these animals were completely sterile. Additionally, to
191 test whether Tdrd5l functions in regulating mRNA stability through removal of the mRNA 5’ cap,
192 we tested for a genetic interaction with *Dcp1,* which encodes one of the major mRNA
193 decapping enzymes in Drosophila (13). As in the case of the *twin* knockdown, there were no
194 morphological or fertility defects when our *Dcp1* RNAi line was expressed in the germline of wt
195 females (*nos-Gal4, UAS-Dcp1 RNAi,* Fig 3C). However, when *Dcp1* was knocked down in the
196 germline of females heterozygous for a mutation in *Tdrd5l,* the ovaries exhibited a severe
197 morphological defect and lack of germline (Fig 3D), and these animals were also completely

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198 sterile. These dramatic, dose-sensitive genetic interactions between *Tdrd5l* and *twin* or *Dcp1*
199 indicate that Tdrd5l normally functions in post-transcriptional repression of mRNAs through
200 both the deadenylation and decapping pathways.
201

202 **Tdrd5l is important for proper egg development**

203 One place where post-transcriptional gene regulation is particularly important is in the
204 developing egg chambers of the female germline. Females trans-heterozygous for predicted
205 null mutant alleles of *Tdrd5l* exhibit a relatively normal ovary morphology but lay slightly
206 reduced numbers of eggs (Fig S5A). To determine whether the levels of any mRNAs are
207 regulated by *Tdrd5l* we conducted RNAseq in wildtype vs *Tdrd5l*-mutant ovaries. Interestingly,
208 the mRNAs altered in *Tdrd5l* mutants were enriched for genes that are maternally deposited in
209 the embryo, as defined by genes present in 0-2 hr old embryos (38). While maternal genes
210 represent 23.3% of all Drosophila genes, they represent 30% of genes altered in *Tdrd5l* mutants
211 (Fig S6A-B). Further, when the analysis is restricted to genes with a 2-fold or greater change in
212 mRNA level in *Tdrd5l* mutants, the percentage of maternally-expressed genes rises to 94% (Fig
213 S6C). Additionally, we compared our RNAseq data to previously published data identifying RNAs
214 associated with the BicaucalD (BicD)/Egalitarian (Egl) complex involved in transport of RNAs
215 from nurse cells to the oocyte (39). While mRNAs identified in our RNA-seq analysis represent
216 10.4% of all genes in the Drosophila genome, they represent 30% of the top 100 mRNAs
217 associated with the BicD/Egl complex, further suggesting a role for Tdrd5l in regulating mRNAs
218 that are deposited maternally in the oocyte during oogenesis (Fig S6D).

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219 In *Drosophila,* maternally deposited RNAs are transcribed in the nurse cells where they
220 are silenced until they get to their proper location in the oocyte and are then translationally
221 activated at specific times (40). To investigate whether Tdrd5l plays a role in this process we
222 tested whether the eggs laid by *Tdrd5l*-mutant females exhibited defects in development when
223 crossed to wildtype males. Indeed, we observed that these eggs were greatly reduced in their
224 ability to hatch and give rise to viable larvae (Fig 4A). To determine if the decrease in hatch rate
225 was due to patterning defects, we examined the dorsal appendages (DA) on the offspring, an
226 indicator of Dorsal/Ventral patterning (41). Offspring from trans-heterozygous *Tdrd5l*-mutant
227 females exhibited a greatly increased frequency of DA defects (Fig 4B), which ranged from
228 alterations of DA length (Fig 4D) to a fusion of DA material into a ring (Fig 4E) or a single fused
229 appendage (Fig 4F). If the defects in dorsal-ventral patterning are the major reason behind the
230 embryonic defects, then we would expect that the majority of unhatched embryos would
231 exhibit DA defects. However, when we examined the DA defects specifically of those embryos
232 that failed to hatch, we did not observe a greater percentage of DA defects as we observed
233 when examining all embryos. This indicates that, while DA defects and possibly D/V defects are
234 the probable cause of death for many of the embryos, other embryos are likely to die for other
235 reasons. When we attempted to examine the cuticle pattern of unhatched embryos we
236 observed no discernable cuticle pattern, indicating that these embryos die prior to cuticle
237 deposition.
238
239 ***Tdrd5l* is required for repression of Grk expression in nurse cells**

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240 The D/V and anterior/posterior (A/P) axes in the developing oocytes are set up by
241 localized translation of maternally deposited RNAs. These RNAs are transcribed in the nurse
242 cells and post-transcriptionally silenced during transport to the oocyte (40). Three of the classic
243 maternally deposited RNAs are *gurken (grk)*, which regulates D/V patterning and DA formation
244 (20,21), *bicoid (bcd)* which determines anterior fate (24), and *oskar (osk)* which specifies
245 posterior identity and the germ plasm (22). To determine if Tdrd5l regulates maternally
246 deposited RNAs we immunostained for the protein products of these three mRNAs in wildtype
247 and *Tdrd5l*-mutant ovaries. Both Grk and Osk protein staining were altered in *Tdrd5l*-mutant
248 ovaries (Fig 5A-H), whereas no Bcd staining was observed in the mutant ovaries, similar to wt
249 (data not shown). In wt ovaries, Grk immunoreactivity is normally observed in the dorsal-
250 anterior corner of the oocyte (Fig 5A, arrowhead) and is absent from the nurse cells of
251 developing egg chambers (Fig 5C). However, in *Tdrd5l* mutant ovaries we observed Grk staining
252 in the nurse cells of mid-stage egg chambers of 80% of ovaries tested (Fig 5D, outlined and
253 inset), suggesting the *grk* mRNA is no longer translationally repressed in the nurse cells. In
254 addition, we still observed Grk immunoreactivity at the dorsal-anterior corner of developing
255 oocytes (Fig 5B) in *Tdrd5l* mutants.

256 In wt ovaries, Osk immunostaining is normally restricted to the posterior pole of
257 developing oocytes beginning at stage 10 (Fig 5E, arrowhead) (22). In stage 10 *Tdrd5l*-mutant
258 egg chambers we still observed Osk protein correctly localized to the posterior pole (Fig 5F).
259 However, in 77% of stage 9 *Tdrd5l*-mutant egg chambers we observed premature staining for
260 Osk, and in these cases Osk protein was localized to the middle of the oocyte, rather than the

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261 posterior end (Fig 5H). However unlike with Grk, we did not observe Osk protein expressed in
262 the nurse cells of *Tdrd5l* mutants (Fig 5H).
263 To determine if the ectopic expression of Grk and Osk proteins was due to defects in
264 RNA transport from the nurse cells or RNA localization in the oocyte, we conducted fluorescent
265 *in situ* hybridization (FISH) to visualize the *grk* and *osk* mRNAs (Fig 5I-L). We observed no
266 changes in *grk* mRNA localization between wildtype and *Tdrd5*-mutant ovaries (Fig 5I-J).
267 Contrary to what we observed with the *grk* RNA, we observed altered *osk* mRNA localization in
268 *Tdrd5l*-mutant oocytes (Fig 5L). In *Tdrd5l* mutant oocytes, *osk* mRNA was not tightly localized to
269 the posterior and was instead observed throughout the oocyte in a 31% of ovaries (Fig 5L).
270 Grk is first required at the posterior of the oocyte to specify the posterior follicle cells,
271 which are then required for proper microtubule orientation and localization of *osk* RNA and
272 protein to the posterior pole (42). Thus, it is possible that the mis-localization of *osk* we
273 observed could be secondary to defects in *grk* function. To test whether the microtubule
274 network is properly oriented in *Tdrd5l* mutants, we used Kinesin-LacZ (Kin-LacZ) to mark the
275 plus ends of the microtubules (21). In wt ovaries the microtubule network in the oocyte is
276 polarized with plus ends at the posterior, therefore Kin-LacZ should be localized tightly to the
277 posterior by stages 9 and 10 of egg chamber development (Fig S7a white arrow, and Fig S7B
278 yellow arrow). In *Tdrd5l*-mutant ovaries we detected a range of Kin-LacZ localization. Some
279 oocytes had tight posterior localization, while others had some posterior localization of Kin-LacZ
280 with plumes of staining extending away from the posterior pole. In the most extreme cases we
281 observed diffuse Kin-LacZ localization in the center of the oocyte (Fig S7C white arrow and Fig

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282 S7D yellow arrow). These data indicate that the defects in *osk* posterior localization may be
283 caused by loss of *grk* function rather than defects in the direct regulation of *osk* itself.
284

285 ***Tdrd5l* represses Orb protein expression in nurse cells**

286 Work by other labs has shown that Grk translation in the oocyte is activated by Oo18
287 RNA binding protein (Orb) (43–45). Orb is a cytoplasmic polyadenylation element binding
288 protein (CPEB) that is highly expressed in the oocyte where it recruits Wispy to lengthen the *grk*
289 poly(A) tail to activate its translation (44). It has also been shown that ectopic expression of Orb
290 in the nurse cells is sufficient to activate Grk translation in the nurse cells (45). To test whether
291 ectopic expression of Orb in the nurse cells could also be the cause of Grk translation in *Tdrd5l*-
292 mutant nurse cells, we stained for Orb protein in *Tdrd5l*-mutant ovaries. As previously
293 described, in wt ovaries we observed Orb immunofluorescence primarily in the oocyte and it
294 was largely absent from the nurse cells of later egg chambers, except the nurse cell closest to
295 the oocyte (Fig 6A, squared region). In *Tdrd5l*-mutant ovaries we saw an expansion of Orb
296 protein expression into almost all the nurse cells suggesting Orb translation had been de-
297 repressed in *Tdrd5l* mutants (Fig 6B, squared region).

298 To test whether the phenotype observed in *Tdrd5l* mutants is due to increased Orb
299 expression, we determined whether reduction of *orb* function could suppress the *Tdrd5l*-
300 mutant phenotype by examining egg laying and hatch rates. *Tdrd5l* mutants that were also
301 heterozygous for an *orb* loss of function allele (*Tdrd5l-/Tdrd5l-; orb*^{mel}*/+* females) laid more eggs
302 compared to their *Tdrd5l-/Tdrd5l-* siblings (Fig 6C), and also exhibited and increase in egg hatch

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303 rate (Fig 6D). This suggests that the defects observed in *Tdrd5l* mutants are partially due to the
304 increased Orb expression observed in the nurse cells.
305
306 **Discussion**
307 Work from a number of labs has demonstrated the importance of RNA granules for
308 proper germ cell identity and function (15,22,46,47). Here we have extended our study of
309 Tdrd5l and show that it localizes to the periphery of what is likely a cytoplasmic RNA granule.
310 The "Tdrd5l" granule does not exhibit the characteristics of previously identified RNA granules
311 in the Drosophila germline, meaning that it is likely a novel structure. While we previously
312 demonstrated that Tdrd5l expression is highly male-biased in the undifferentiated germline, we
313 show here that Tdrd5l granules are also present during germline differentiation in females.
314 Rather than regulating piRNA production and transposon expression like other Tdrd5 proteins,
315 Tdrd5l regulates the expression of maternal RNAs that need to be silenced in nurse cells as they
316 are transported to the oocyte.
317
318 **The "Tdrd5l granule" represents a new type of germline RNA granule in Drosophila**
319 We used both an epitope-tag placed into the endogenous *Tdrd5l* locus, as well as a
320 peptide polyclonal antibody generated against Tdrd5l, to verify and characterize further the
321 localization of Tdrd5l. We were able to verify that Tdrd5l is more highly expressed in the male
322 germline than the female germline, particularly in the germline stem cells where Tdrd5l is
323 specific to male GSCs. This is consistent with *Sex lethal* being a repressor of *Tdrd5l* expression as
324 we previously found (25). However, we also describe the expression of Tdrd5l in the female

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325 germline, where it is found in punctae in the germarium, in germ cells that have begun to
326 differentiate (Fig 1C-D), and in nurse cells and oocytes in the developing egg chambers (Fig 1E-
327 F). Interestingly, the punctae of Tdrd5l expression appear “hollow” (Fig 2) indicating that Tdrd5l
328 resides on the surface of the structures it identifies. The fact that Tdrd5l immunostaining is
329 sensitive to RNase treatment (Fig 4C-D) further suggests that these are ribonuclear protein
330 granules that form independent domains within the germ cell cytoplasm.

331 Our analysis also indicates that the Tdrd5l granules are not representative of any type of
332 granule previously described in the Drosophila germline. The most prominent germline RNA
333 granule is the perinuclear nuage which contains the helicase Vasa. While the Tdrd5l granules
334 are often observed in a perinuclear location (Fig 2A), these granules notably lack Vasa (Fig 2A
335 and S4A-B). Thus, while Tdrd5l granules may interact with the nuage, they appear to be distinct
336 entities. A large granule, the piRNA nuage giant body, has been described that is associated
337 with the nuage (48). However, unlike the Tdrd5l body, this body also contains Vasa and appears
338 only in primary spermatocytes, and so is distinct from the Tdrd5l granule. The localization of the
339 Tdrd5l granule is also inconsistent with it being part of the germ plasm or sponge bodies found
340 in the oocyte (46,49–51). The Tdrd5l granule also appears to be distinct from other RNA
341 granules found more commonly in different cell types, such as P bodies, U bodies and stress
342 granules. P bodies characteristically contain Dcp1 and are much smaller in size than Tdrd5l
343 granules. Our previous work suggested some overlap in localization between Tdrd5l and Dcp1
344 (25) but the more detailed analysis described here reveals that smaller, Dcp1-positive bodies
345 can be seen associating with Tdrd5l granules, but that Dcp1 is not present in the Tdrd5l
346 granules themselves (Fig 2B). We also do not see overlap between Tdrd5l and the U body

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347 protein Smn (Fig 2C). Lastly, the Tdrd5l granules are observed constitutively in the germline and
348 are not affected by stresses such as starvation or changes in temperature (C.P. unpublished
349 data) indicating that they are not stress granules.

350 Germline granules have been most well-studied in C. elegans, and a number of distinct
351 regions of the nuage have been described in this species (52,53). At many stages of germline
352 development, P granules are associated with the nuclear periphery and, like Drosophila nuage,
353 contain Vasa-class helicases (the GLH's) as well as Ago proteins (54–56). However, associated
354 with the P granules are other perinuclear regions known as the Mutator foci, SIMR foci and the
355 Z granules, that have distinct protein components (53). One possibility is that the Tdrd5l
356 granule represents a similar sub-structure associated with the nuage in *Drosophila*. In addition
357 to studying the function of *Tdrd5l*, identifying other proteins and possibly RNAs that are present
358 in the Tdrd5l granule will be an important next step.
359

360 **Function of the Tdrd5 proteins**

361 There are many types of Tudor domain containing proteins and they can be classified
362 into sub-families based on the homology of their Tudor domains. In diverse animal species,
363 including mouse and humans, there is a single Tdrd5 protein. Interestingly, flies have two such
364 proteins, Tdrd5l and Tejas (10,25). Many Tdrd5 proteins, like mouse Tdrd5 and Drosophila
365 Tejas, also contain an N-terminal LOTUS domain which is known to bind Vasa-type helicases
366 (27), but Drosophila Tdrd5l lacks this domain. However, the N-terminus of Tdrd5l must be
367 important for its function since placing even a small epitope tag in this position compromises
368 *Tdrd5l* function. Consistent with its Vasa-binding LOTUS domain, Tejas associates with the

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369 nuage where it acts in the piRNA pathway to repress germline transposon expression (10). In
370 contrast, we did not observe Tdrd5l co-localizing with the Vasa-positive nuage (Fig 2A), and we
371 have observed no change in transposon expression in *Tdrd5l* mutants (25). Instead, we find that
372 *Tdrd5l* exhibits potent genetic interaction with pathways that repress mRNAs at the post-
373 transcriptional level, including the mRNA decapping and deadenylation pathways (Fig 3). This is
374 also consistent with a role for Tdrd5l in regulating mRNA repression in nurse cells (see below).
375 Thus, it may be that Tejas is more specific for transposon regulation while Tdrd5l is involved in
376 post-transcriptional regulation of germline mRNAs.

377 Mouse Tdrd5 associates with the Chromatoid Body, a nuage-related structure. In
378 addition, mice mutant for Tdrd5 exhibit defects in transposon regulation and spermatogenesis
379 (26), similar to Drosophila *tejas* (10). Interestingly, Tdrd5 mutant mice also exhibit defects in
380 regulation of pachytene piRNAs (57,58), which are proposed to regulate mRNAs important for
381 spermatogenesis rather than to repress transposons. One intriguing hypothesis is that the role
382 of the single Tdrd5 protein in mice in regulating both transposon repression and mRNA
383 expression has been divided between the two Tdrd5 proteins in Drosophila, Tejas and Tdrd5l. In
384 addition, both Drosophila proteins have functions in oogenesis in addition to spermatogenesis
385 (10,25, this work). Similarly, knock down of a Tdrd5 homolog in Locust also affects both
386 oogenesis and spermatogenesis (59). While a role for mouse Tdrd5 in the female germline has
387 not been described, consortium data indicate that Tdrd5 is expressed in the mouse ovary (60),
388 making this an interesting place to look for additional functions for Tdrd5 in the mouse.

389

390 **Regulation of Maternally deposited RNAs**

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391 Prior work from our lab demonstrated that Tdrd5l promotes male identity in the
392 germline. Consistent with this, Tdrd5l is expressed in male GSCs but is repressed in female GSCs
393 by the action of *Sxl* (25). However, Tdrd5l is also expressed in differentiating germ cells in both
394 males and females ((25), and Fig 1C-F). This suggests that Tdrd5l might promote male identity in
395 early germ cells and germline stem cells but also regulate aspects of germline differentiation in
396 both sexes. One process in the female germline that relies heavily on post-transcriptional
397 regulation of RNAs is the production and transport of maternal RNAs from nurse cells into the
398 oocyte.

399 Maternal contribution of RNAs, proteins, and organelles is conserved from flies through
400 vertebrates (61,62), and supports embryonic patterning and embryogenesis prior to activation
401 of the zygotic genome and beyond. In Drosophila, maternal RNAs are transcribed in the nurse
402 cells, and are post-transcriptionally silenced to prevent their translation during transport to the
403 oocyte and prior to the specific time they should be activated. The cytoplasmic polyadenylation
404 element binding protein (CPEB) Orb is known to activate translation of maternal RNAs such as
405 *osk* and *grk* in the oocyte (44,45,63). Like other maternal RNAs, *orb* is transcribed in nurse cells
406 and transported to the oocyte where it is translated. Interestingly, Orb/CPEB homologs are
407 critical for regulating translation of maternal RNAs in vertebrate systems as well (64).

408 We observed defects in expression of Orb, Grk and Osk proteins in *Tdrd5l* mutants (Fig 5
409 and Fig 6). We saw ectopic accumulation of both Orb and Grk in nurse cells (Fig 5D and Fig 6B),
410 while Osk protein was sometimes observed in the middle of the oocyte instead of its normal
411 location at the posterior pole (Fig 5H). We also observed maternal-effect lethality of embryos
412 from *Tdrd5l*-mutant mothers that included dorsal appendage defects consistent with Grk's role

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413 in dorsal-ventral patterning. One possible explanation for all of these phenotypes is a failure of
414 Orb to be properly repressed in nurse cells. Ectopic Orb translation in nurse cells can cause
415 improper activation of Grk translation in these cells (45), and this might also lead to insufficient
416 translation of Grk in the oocyte. Early Grk expression at the posterior pole of the oocyte is
417 necessary for proper posterior determination and defects in this process can lead to disruption
418 of *osk* mRNA and protein localization to the posterior pole (19,21) as observed in *Tdrd5l*
419 mutants (Fig 5L). Further, impaired Grk expression in the dorsal-anterior corner of the oocyte
420 can cause improper specification of dorsal follicle cells, and defects in dorsal appendage
421 formation and dorsal-ventral patterning of the embryo. Thus, the primary defect in *Tdrd5l*
422 mutants could be a failure to properly repress *orb* translation in the nurse cells.

423 Other factors have been implicated in repressing *orb* translation in nurse cells, including
424 FMR1, the Drosophila homolog of Fragile X mental retardation protein (65), and Cup, a
425 translational repressor (66). Mutations in both *FMR1* and *cup* cause Orb accumulation in nurse
426 cells similar to what we observe in *Tdrd5l* mutants. One intriguing hypothesis is that the Tdrd5l
427 granule in nurse cells is a site where mRNAs such as *orb* get marked for translational repression
428 by factors such as FMR and Cup. Since the nuage and nuage-related material in flies, worms and
429 mice all function in small RNA regulatory pathways, the marking of mRNAs like *orb* in the Tdrd5l
430 granule may also involve such regulatory RNAs.

431

432 Materials and Methods

433 Fly stocks and CRISPR tagging

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434 Fly stocks used in this paper were obtained from the Bloomington stock center unless
435 otherwise noted. *Nos*-gal4 (67), *twin* RNAi(BDSC# 32901), *Dcp1* RNAi(BDSC# 67874), *mcherry*
436 RNAi(BDSC# 35785), *orb* RNAi(BDSC# 43143), Vasa-Cas9(BDSC# 56552), and piggyBac (BDSC#
437 32070), Kin-LacZ (T. Schupbach) . *Tdrd5l* mutant alleles were generated previously by our lab
438 (25). *Tdrd5l*:GFP and *Tdrd5l*:FLAG alleles were generated using CRISPR as described by the fly
439 CRISPR group (68). The GFP and FLAG donor plasmids and pU6 gRNA plasmid were obtained
440 from the *drosophila* genetics resources center and were stock numbers 1365, 1367, 1363
441 respectively.
442

443 **Immunofluorescence and antibody generation**

444 All gonads were dissected, and fixed, and stained as previously published (69). All images were
445 taken on a Zeiss LSM 700 or LSM 800 with airyscan detector (when noted). Primary antibodies
446 used were Guinea pig anti-Tdrd5l 1:10,000 (this paper), rat anti-HA 1:100 (Roche 3F10), rabbit
447 anti- Vasa 1:10,000 (R, Lehman), Guinea pig anti-TJ 1:1000 (J. Jemc), mouse anti Armadillo
448 1:100 (DSHB), Mouse anti Orb 1:40 (DSHB), mouse anti- Gurken 1:20 (DSHB), rabbit anti-oskar
449 1:1000 (A. Ephrussi). Secondary antibodies were used at 1:500 (Alexa Flour). Samples were
450 stained in DAPI solution and mounted in DABCO.
451

452 **Quantification of nuage association**

453 To quantify the association of Tdrd5l granules with Vasa, we calculated the number of Tdrd5l
454 granules in the 8 and 16 cell cysts that localized either to the nuclear periphery (perinuclear) or

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455 to the cytoplasm. For each group we then calculated the number of Tdrd5l granules that are
456 directly adjacent to vasa granules, co-localize, or do not associate with vasa at all

457 **Egg lay assays**

458 Trans-heterozygous *Tdrd5l* mutant females and control females were aged 7days with *OregonR*
459 males. Individual females and males were placed in condos on grape juice plates with wet yeast
460 paste for 24hrs, and then taken off the plate. The number of eggs laid and those with dorsal
461 appendage defects were counted for each female. At 48hrs these counts were done again to
462 count how many had hatched.

463 **Genetic interaction assays**

464 UAS-RNAi lines were crossed to Nos-gal4 for controls or *Tdrd5l*^{Q5}*;;nos-gal4* for mutants. To
465 conduct this screen each gene was knocked down using a UASp-shRNAi driven in the germline
466 by nanos-gal4 in a wildtype background and in a *Tdrd5l-* background. *Tdrd5l* males analyzed
467 were hemizygous for a null allele of *Tdrd5l* (*Tdrd5l/Y*) while females were heterozygous (*Tdrd5l/*
468 *+*) Progeny were aged 5-10days then dissected immunostained as described above using anti-
469 vasa, anti-TJ, anti-Arm and DAPI.

470 **Fluorescence in situ hybridization**

471 FISH for *gurken* and *oskar* were conducted as published by the Berg lab (70). cDNA clones 2169
472 and 7305 for probes were obtained from the Drosophila genetics resource center.

473 **RNA sequencing**

474 For testis RNA sequencing, Testes were dissected from 5d old males, RNA was prepped using
475 RNA-Bee(Tel-test) 3 biological replicated were used for each genotype. Libraries were prepped

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476 using a protocol (71). 100bp paired end sequencing was conducted by the Johns Hopkins
477 Genetics Resources Core facility
478 For ovary RNA sequencing, ovaries were dissected from virgin females, and 3 biological
479 replicates were dissected per genotype. RNA was prepped using The Direct-zol RNA micro prep
480 kit (zymogen). Library construction and 100bp paired end sequencing was conducted by the
481 Johns Hopkins Genetics Resources Core facility.

482 **RNAseq Analysis**

483 Read quality for both the ovary and testis RNAseq data was determined using the fastQC kit.
484 Reads were mapped using Tophat and HTSeq to the *Drosophila* genome using Ensemble release
485 BDGP6. Differential gene expression analysis was conducted using DESeq.
486

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488

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494
495
496

497 **Figure legend**

498 **Figure 1: Tdrd5l localized to cytoplasmic granules in the *drosophila* germline**

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499 (A-E) immunofluorescence of Tdrd5l in the germline using FLAG tagged Tdrd5l. A) Tdrd5l
500 localizes to granules in the male germline. Small granules are marked by a white arrow head,
501 and a large granule is marked by a yellow arrowhead. B) Tdrd5l is expressed in male germline
502 stem cells (GSCs) marked by brackets. Granule with Tdrd5l localized to the periphery is marked
503 by the white arrow C) Tdrd5l localizes to granules in the germaria of female ovaries as outlined
504 by the dashed line. D) Tdrd5l is not expressed in female GSCs as marked by brackets. E) Tdrd5l
505 is expressed in nurse cells in the female germline. Oocyte staining is marked by a yellow
506 arrowhead and a nurse cell granule is marked by a white arrowhead.
507
508 **Figure 2: Tdrd5l localizes to a novel RNA granule**
509 A) Immunostaining of gonads for Vasa positive nuage and Tdrd5l granules showed that Tdrd5l
510 granules are closely adjacent to the Nuage but did not colocalize as marked by white arrows.
511 B) Tdrd5l granules and processing bodies marked by Dcp1:YFP as marked by yellow arrows. C)
512 Tdrd5l granules marked by the yellow granule do not colocalize with U-bodies marked by a
513 white arrowhead
514
515 **Figure 3: Tdrd5l genetically interacts with post-transcriptional gene regulatory factors**
516 (A-F) immunofluorescence of gonads from *Tdrd5l* genetic interaction assays with *twin* and *dcp1*.
517 A) *twin* RNAi in the germline of wildtype males produced normal gonads. B) *twin* RNAi in the
518 germline of *Tdrd5l* mutant males resulted in sterile gonads. C) *twin* RNAi in the germline of
519 wildtype females produced normal gonads. D) *twin* RNAi in the germline of *Tdrd5l-* females
520 results in tumorous and sterile ovaries. E) *dcp1* RNAi in the germline of wildtype females
521 produced normal gonads. F) *dcp1* RNAi in the germline of *Tdrd5l-* females resulted in germline
522 loss.
523
524 **Figure 4: Tdrd5l is important for proper egg development**
525 A) quantification of hatch rate of eggs laid during single female fecundity assays. Eggs laid by
526 *Tdrd5l* mutants are represented by orange dots and eggs laid by control flies are represented by
527 blue dots. B) Quantification of dorsal appendage defects found in eggs laid during single female
528 fecundity assays. (C-F) examples of dorsal appendages quantified in B. C) example of an egg
529 with normal dorsal appendages. (D-F) examples of eggs with dorsal appendage defects.
530
531 **Figure 5: Tdrd5l regulates maternally deposited RNAs**
532 (A-H) immunofluorescence of proteins produced by maternally deposited RNAs using
533 antibodies as indicated. A) Grk protein localizes to the dorsal anterior corner of wildtype
534 oocytes as marked by the white arrowhead. B) diminished levels of Grk protein localize to the
535 dorsal anterior corner of *Tdrd5l-* oocytes as marked by white arrows. C) Grk protein is absent
536 from wildtype nurse cells. Outlined egg chamber is enlarged in bottom left inset. D) Grk protein
537 was present in nurse cells of *Tdrd5l-* egg chambers. Outlined egg chamber is enlarged in bottom
538 left inset. E) Osk protein was localized to the posterior of wild type oocytes as marked by
539 yellow arrowhead. F) Osk protein is localized at increased levels to the posterior of *Tdrd5l-*
540 oocytes as marked by the yellow arrow head. G) Osk protein was absent from mis stage
541 wildtype nurse cells and oocytes. Oocyte is outlined with a yellow dashed line H) Osk protein

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542 was localized to the center of mid stage *Tdrd5l-* oocytes. Oocyte is outlined by dashed yellow
543 line.
544
545 **Figure 6: Tdrd5l represses Orb translation**
546 (A-B) Orb immunofluorescence in ovaries. A) Orb protein expression was detected at high levels
547 in wildtype oocytes. A') boxed area is enlarged in A'' B) Orb protein expression was detected in
548 oocytes and nurse cells of *Tdrd5l-* ovaries. B') boxed area is enlarged in B'' (C-D) genetic
549 interaction assay between *Tdrd5l* and *orb.* C) actin localized to the oocyte cortex in wildtype
550 ovaries. D) cortical actin pulls away from C) fewer eggs are laid by *Tdrd5l-* females than by
551 *Tdrd5l-* mutant females with an *orb*^{mel} allele. D) decreased hatch rate in eggs laid by *Tdrd5l-*
552 mutants is partially rescued by *orb*^{mel}.
553
554 **Supplemental figure 1: Tdrd5l localized to cytoplasmic granules in the *drosophila* germline**
555 A) Schematic of the *Tdrd5l* gene locus, Includes where tags were inserted and the antibody
556 epitope. (B-D) immunostaining of gonads with Tdrd5l N-terminally tagged by GFP. Antibodies
557 used as indicated. B) GFP staining localizes to granules in females with one untagged copy of
558 Tdrd5l. C) No GFP staining was detected in ovaries with only tagged Tdrd5l. D) No GFP staining
559 was detected in testes with tagged Tdrd5l.
560
561 **Supplemental figure 2: Validation of Tdrd5l antibody**
562 (A-B) immunofluorescence of the anti-Tdrd5l antibody. A) Tdrd5l staining was detected in
563 wildtype testes. B) Tdrd5l staining was not detected in *Tdrd5l-* testes.
564
565 **Supplemental figure 3: Tdrd5l expression is higher in males than females**
566 A) Tdrd5l-Flag staining in testes compared to B) Tdrd5l staining in ovaries.
567
568 **Supplemental figure 4: Tdrd5l granules are RNA dependent**
569 A) quantification of Tdrd5l granule association with Vasa stained nuage. (B-C) RNase assay. B)
570 Tdrd5l granules are present in testes treated with PBS. C) Tdrd5l granules were diminished in
571 testes treated with PBS and RNaseA
572
573 **Supplemental figure 5: Tdrd5l regulates maternally deposited RNAs**
574 (A-C) quantification of changes in maternal RNA expression in *Tdrd5l-* ovaries. A) 30% of
575 differentially expressed genes in *Tdrd5l* mutant ovaries are maternally deposited B) 23% of all
576 genes in the genome are maternally deposited. C) 90% of genes with a greater than 2 fold
577 change in gene expression in *Tdrd5l* mutant ovaries are maternally deposited. D) 25% of RNAs
578 pulled down in a previously published BicD/Egl RIP data set are upregulated in *Tdrd5l-* ovaries.
579 E) Comparison of male and female *Tdrd5l* mutant RNAseq data
580
581 **Supplemental figure 6: Tdrd5l is important for proper egg development**
582 (A-B) quantification of single female fecundity assays. A) fewer eggs are laid by *Tdrd5l-* females.
583 B) unhatched eggs from *Tdrd5l-* tend to have dorsal appendage defects
584
585 **Supplemental figure 7: Kinesin mis localizes in *Tdrd5l* mutant oocytes**

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586 (A) Kin-LacZ staining in wildtype stage 9 egg chambers and in (B) wild type stage 10 egg
587 chambers. (C) Kin lacZ staining in *Tdrd5l* mutant stage 9 egg chambers and in (D) *Tdrd5l* mutant
588 stage 10 egg chambers. (E) Quantification of A-D
589
590
591 **Supplemental table 1: Male RNAseq**
592
593 **Supplemental table 2: Female RNAseq**
594

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Figure 1

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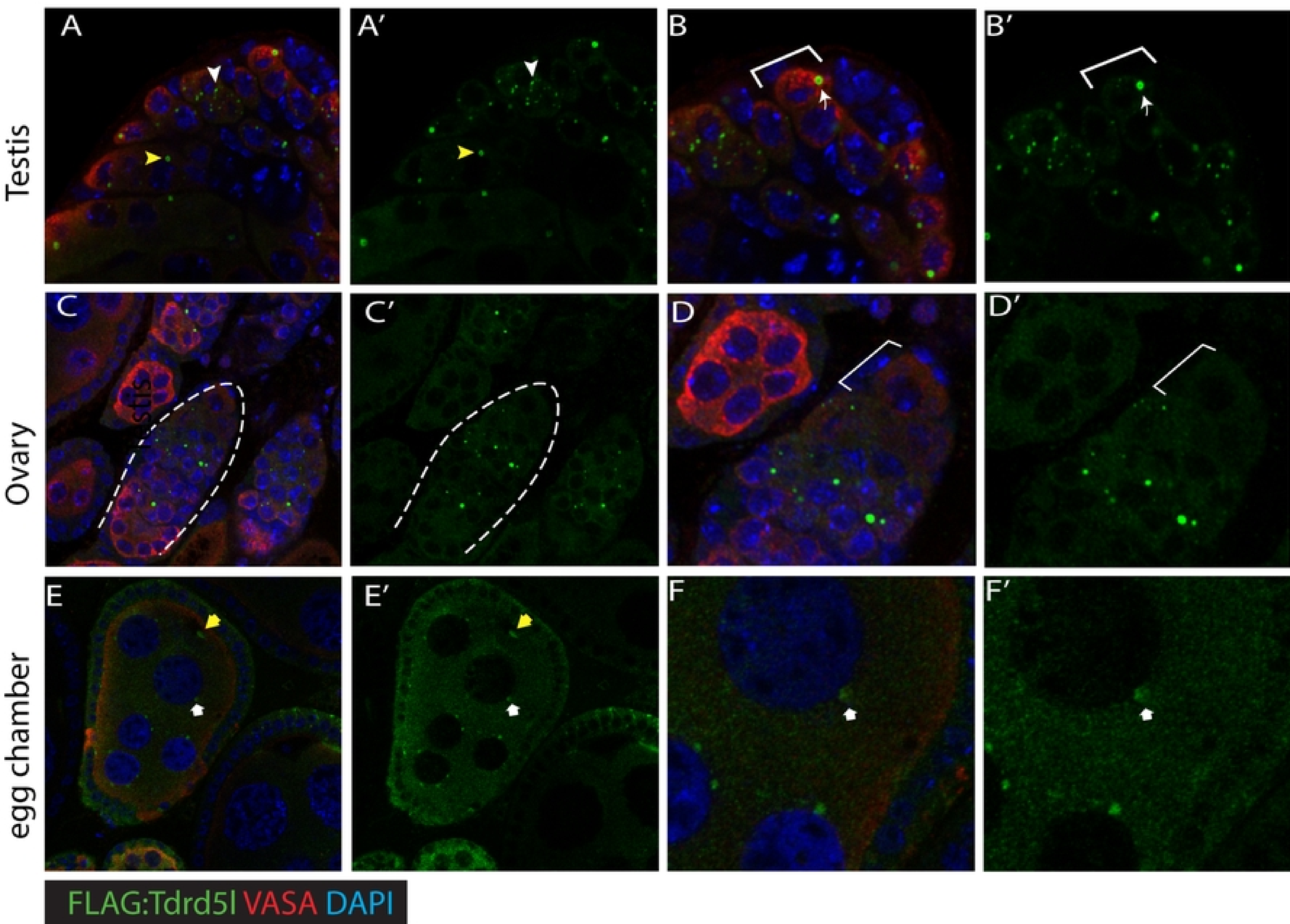

Figure 1

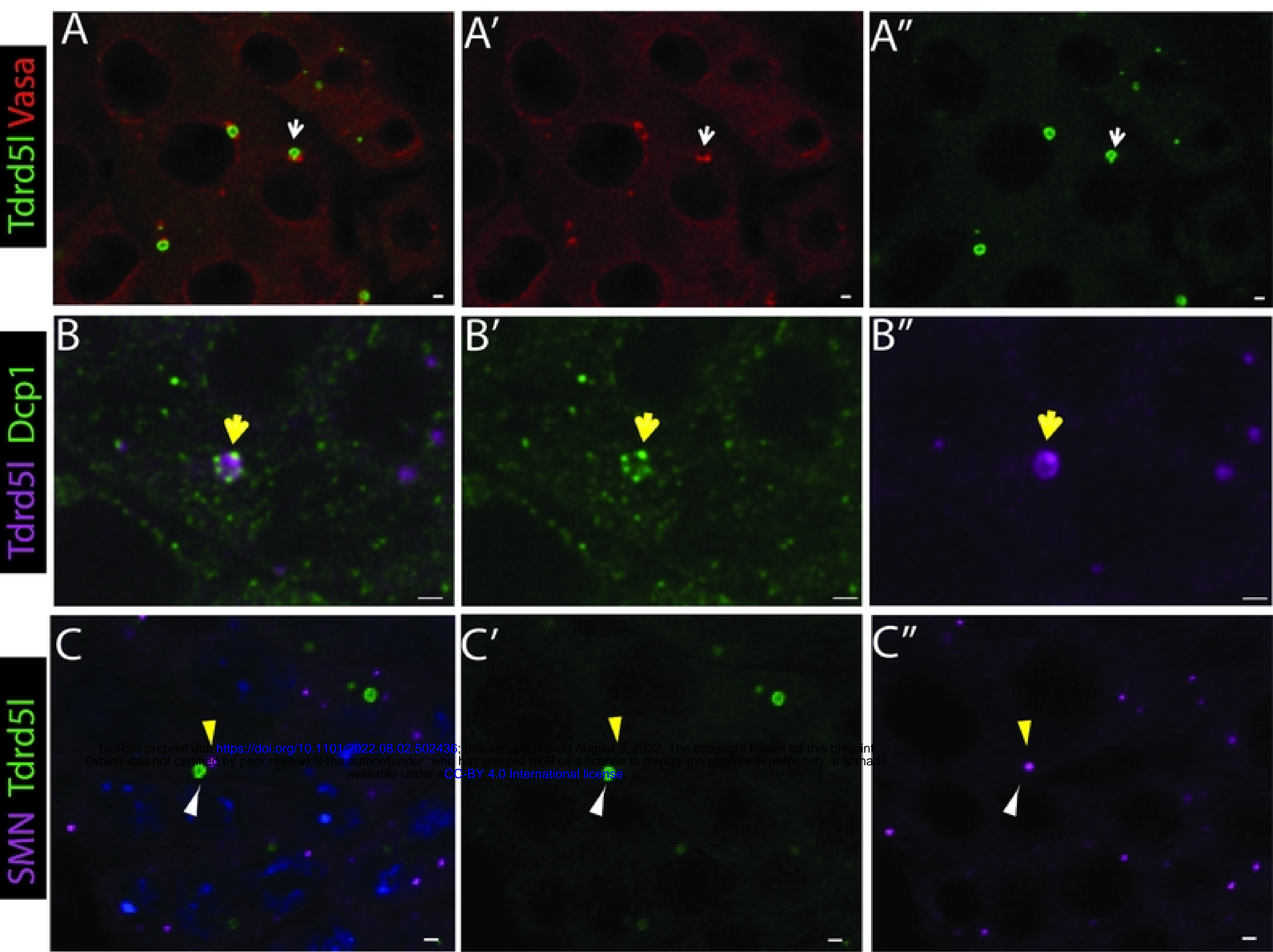

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Figure 2

Figure 3

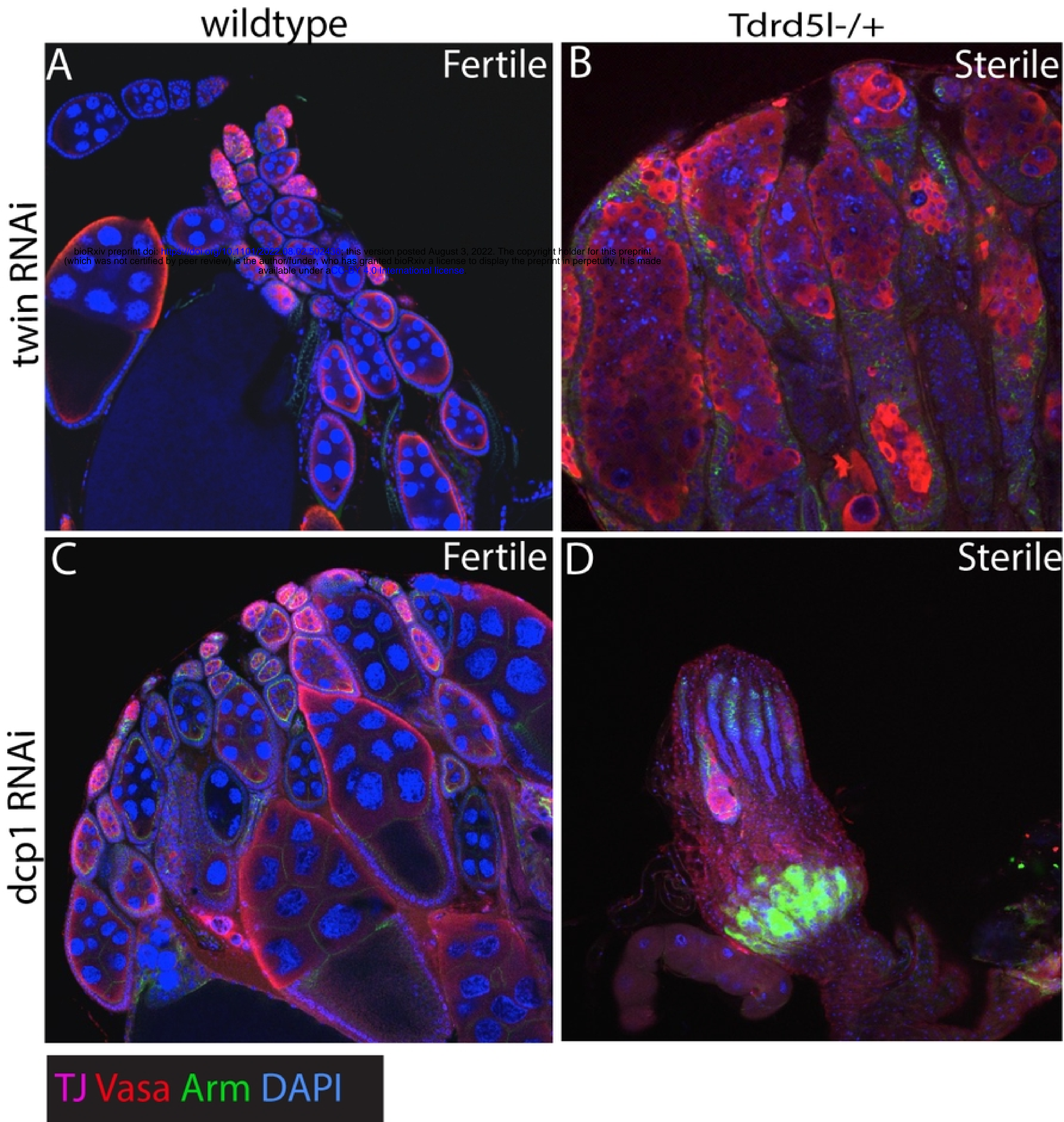

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Figure 3

Figure 4

A

Hatch rate

percent of eggs hatched

100

50

0

2.997e-007

Control

tdrd5l-

Genotype

B

Dorsal appendage defects

% of eggs with defects

100

50

0

1.257e-012

control

tdrd5l-

Genotype

C

D

E

F

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Figure 4

Figure 5

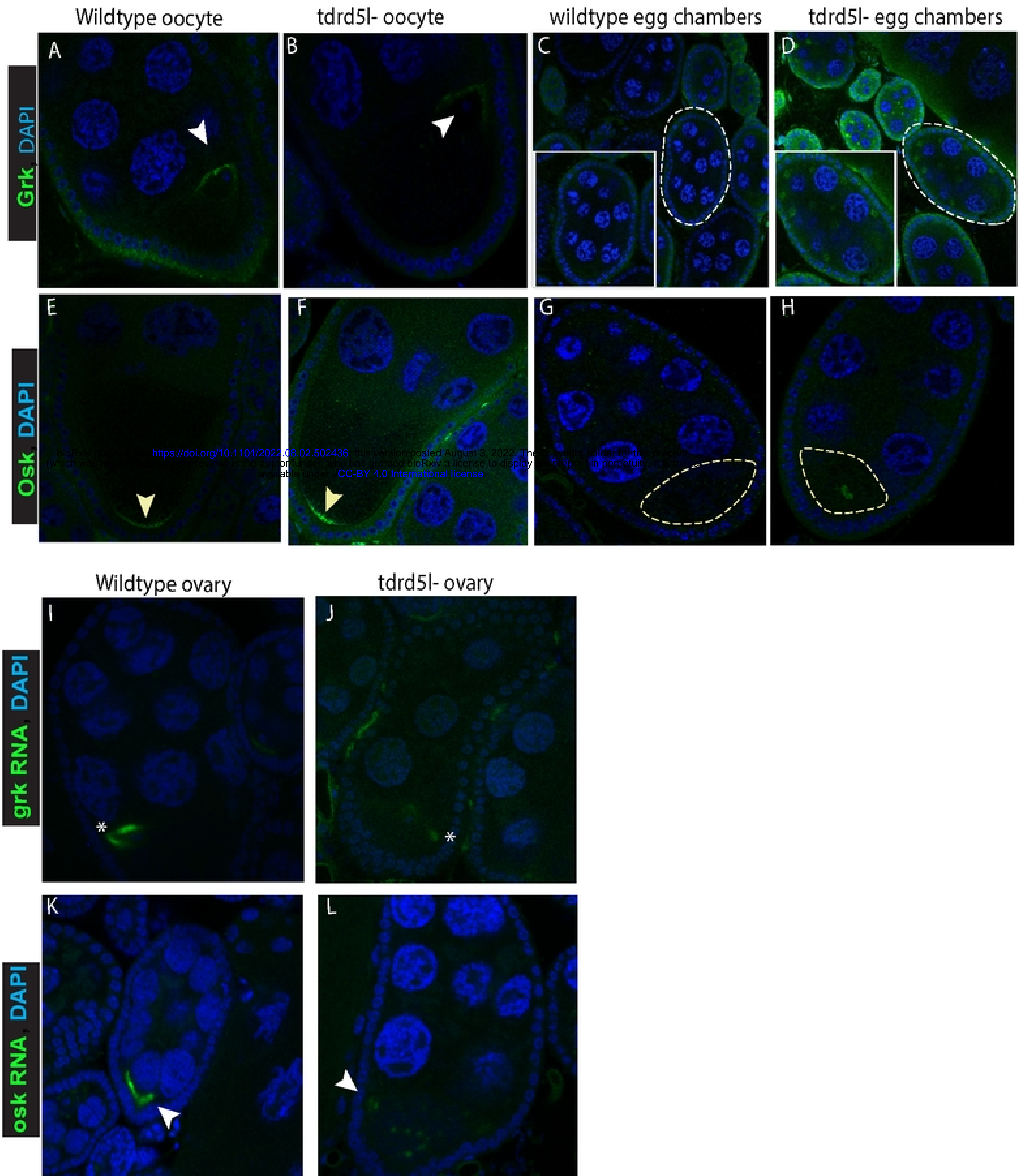

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Figure 5

Figure 6

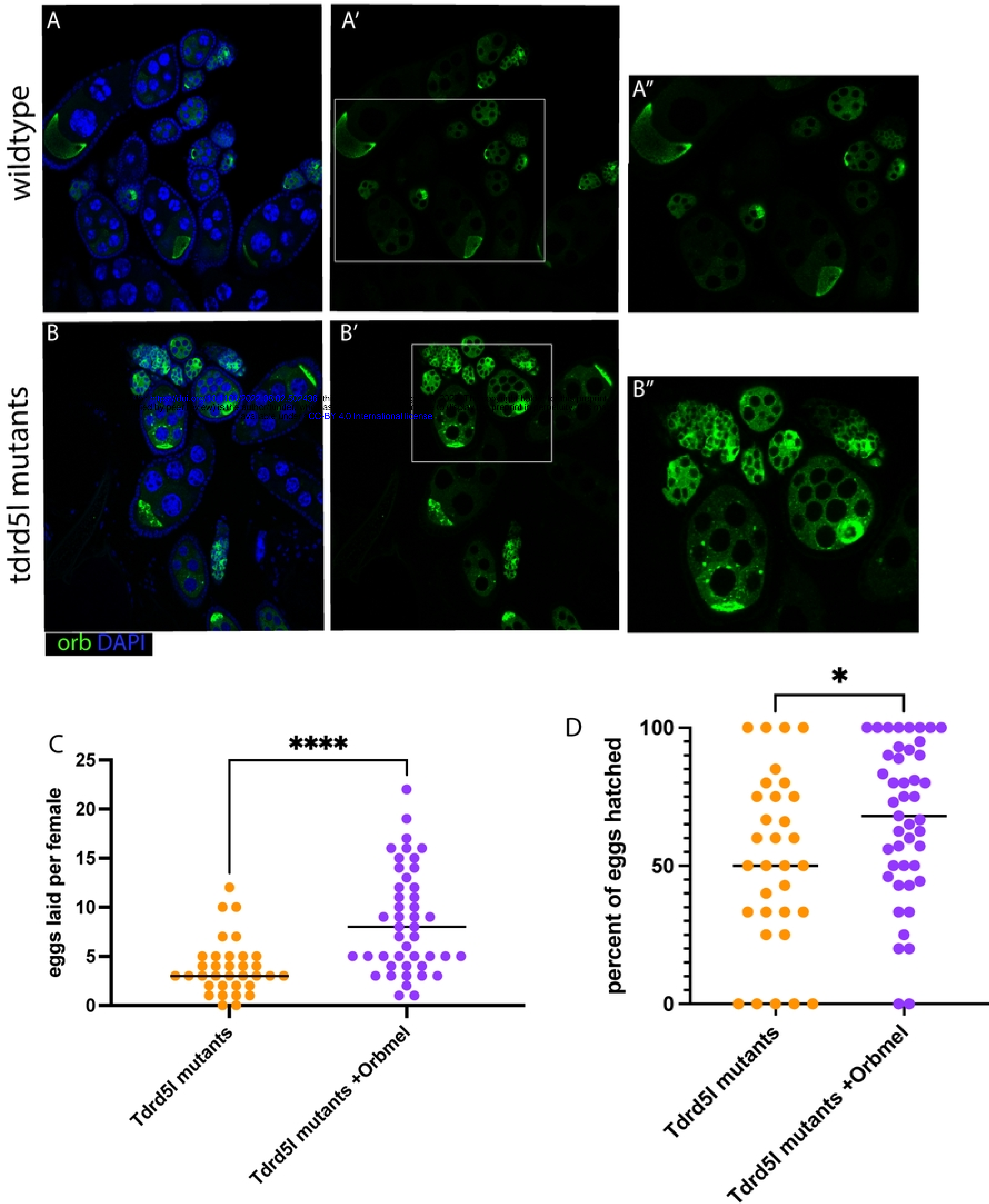

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Figure 6