

1 **Adipose triglyceride lipase promotes prostaglandin-dependent actin remodeling by**
2 **regulating substrate release from lipid droplets**

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18 Short Title: ATGL regulates actin remodeling via prostaglandin signaling

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20 Key words: actin cytoskeleton, lipid droplets, prostaglandin signaling, Drosophila, oogenesis

21 **Abstract**

22

23 A key factor controlling oocyte quality and fertility is lipids. Even though lipid droplets (LDs) are
24 crucial regulators of lipid metabolism, their roles in fertility are poorly understood. During
25 *Drosophila* oogenesis, LD accumulation in nurse cells coincides with dynamic actin remodeling
26 necessary for late-stage follicle morphogenesis and fertility. Loss of the LD-associated Adipose
27 Triglyceride Lipase (ATGL) disrupts both actin bundle formation and cortical actin integrity, an
28 unusual phenotype also seen when Pxt, the enzyme responsible for prostaglandin (PG)
29 synthesis, is missing. Dominant genetic interactions and PG treatment of follicles *in vitro* reveal
30 that ATGL and Pxt act in the same pathway to regulate actin remodeling, with ATGL upstream
31 of Pxt. Further, lipidomic analysis detects arachidonic acid (AA) containing triglycerides in
32 ovaries. Because AA is the substrate for Pxt, we propose that ATGL releases AA from LDs to
33 drive PG synthesis necessary for follicle development. We also find that exogenous AA is toxic
34 to follicles *in vitro*, and LDs modulate this toxicity. This leads to the model that LDs both
35 sequester AA to limit toxicity, and release AA via ATGL to drive PG production. We speculate
36 that the same pathways are conserved across organisms to regulate oocyte development and
37 promote fertility.

38 Introduction

39 Rates of infertility have increased globally between 1990 and 2017 (Sun *et al.*, 2019),
40 and combatting this increase is considered a public health priority (2014). Fertility requires the
41 production of high-quality oocytes. It is becoming increasingly clear that among the key factors
42 ensuring oocyte quality are the amount and types of lipids present during oogenesis (Dunning *et*
43 *al.*, 2014; Brusentsev *et al.*, 2019). In mammalian follicles, for example, fatty acids (FAs) likely
44 contribute a critical source of energy since inhibitors of FA oxidation impair oocyte maturation
45 (Dunning *et al.*, 2010; Dunning *et al.*, 2011). In addition, lipid signaling molecules – from steroid
46 hormones to eicosanoids – control diverse aspects of oocyte development and fertility (Prates *et*
47 *al.*, 2014). Indeed, prostaglandins (PGs), a class of eicosanoids, regulate oocyte development,
48 ovulation, fertilization, implantation, maintenance of pregnancy, and childbirth (Tootle, 2013;
49 Sugimoto *et al.*, 2015). Thus, control of lipid metabolism is likely central for oocyte development
50 and fertility, yet few of the underlying mechanisms have been elucidated.

51 Key regulatory steps in lipid metabolism are mediated by lipid droplets (LDs), the cellular
52 organelles dedicated to the storage of neutral lipids (Walther and Farese, 2012). For example,
53 LDs store excess amounts of cholesterol and related sterols as sterol esters, and safely
54 sequester toxic FAs in the form of triglycerides. Once such FAs are released by LD-bound
55 lipases, they can then be shuttled to mitochondria for oxidative breakdown, used to generate
56 various membrane precursors, or turned into signaling molecules (Welte, 2015). Since both
57 triglycerides and LDs are abundant in oocytes, LDs might perform similar regulatory roles during
58 follicle development. Indeed, LD accumulation, composition, and localization are dynamic during
59 oocyte maturation across organisms (Ami *et al.*, 2011; Dunning *et al.*, 2014; Brusentsev *et al.*,
60 2019), and, in the context of obesity, oocytes display changes in LDs that are associated with
61 infertility (Jungheim *et al.*, 2010; Cardozo *et al.*, 2011; Marei *et al.*, 2020). However, the
62 functions of LDs in oogenesis remain largely undefined.

63 *Drosophila* oogenesis is a promising model for uncovering the roles of LDs in follicle
64 development. Adult female flies have two ovaries, each comprised of ~15 ovarioles or chains of
65 sequentially maturing follicles, also called egg chambers. Each follicle is made up of ~1000
66 epithelial cells termed follicle cells as well as 16 germline cells (15 nurse cells and one oocyte).
67 Follicles develop over the course of ~10 days through 14 stages. LDs undergo dramatic
68 changes in mid-oogenesis (see Figure 1A-D) (Buszczak *et al.*, 2002). Prior to Stage 8 (S8), only
69 a few scattered LDs are found throughout the follicle. In S9, LD biogenesis is massively
70 upregulated in the nurse cells, so that by S10B, the nurse cell cytoplasm is full of uniformly
71 sized LDs (~0.5 μm in diameter) (Buszczak *et al.*, 2002; Teixeira *et al.*, 2003). Thus, in just a
72 few hours, tens of thousands of LDs are formed in the nurse cells. During S11, the LDs are
73 transferred into the oocyte in a process termed nurse cell dumping, in which cytoplasmic
74 contents of the nurse cells are squeezed into the oocyte through intercellular bridges called ring
75 canals. These LDs provide the future embryo with stores of energy and specific proteins needed
76 for embryo development (Li *et al.*, 2012); indeed, embryos with reduced numbers of LDs have
77 reduced hatching probability (Teixeira *et al.*, 2003; Parra-Peralbo and Culi, 2011). Yet whether
78 these LDs only provision the embryo or if they already play roles in follicle development remains
79 unclear.

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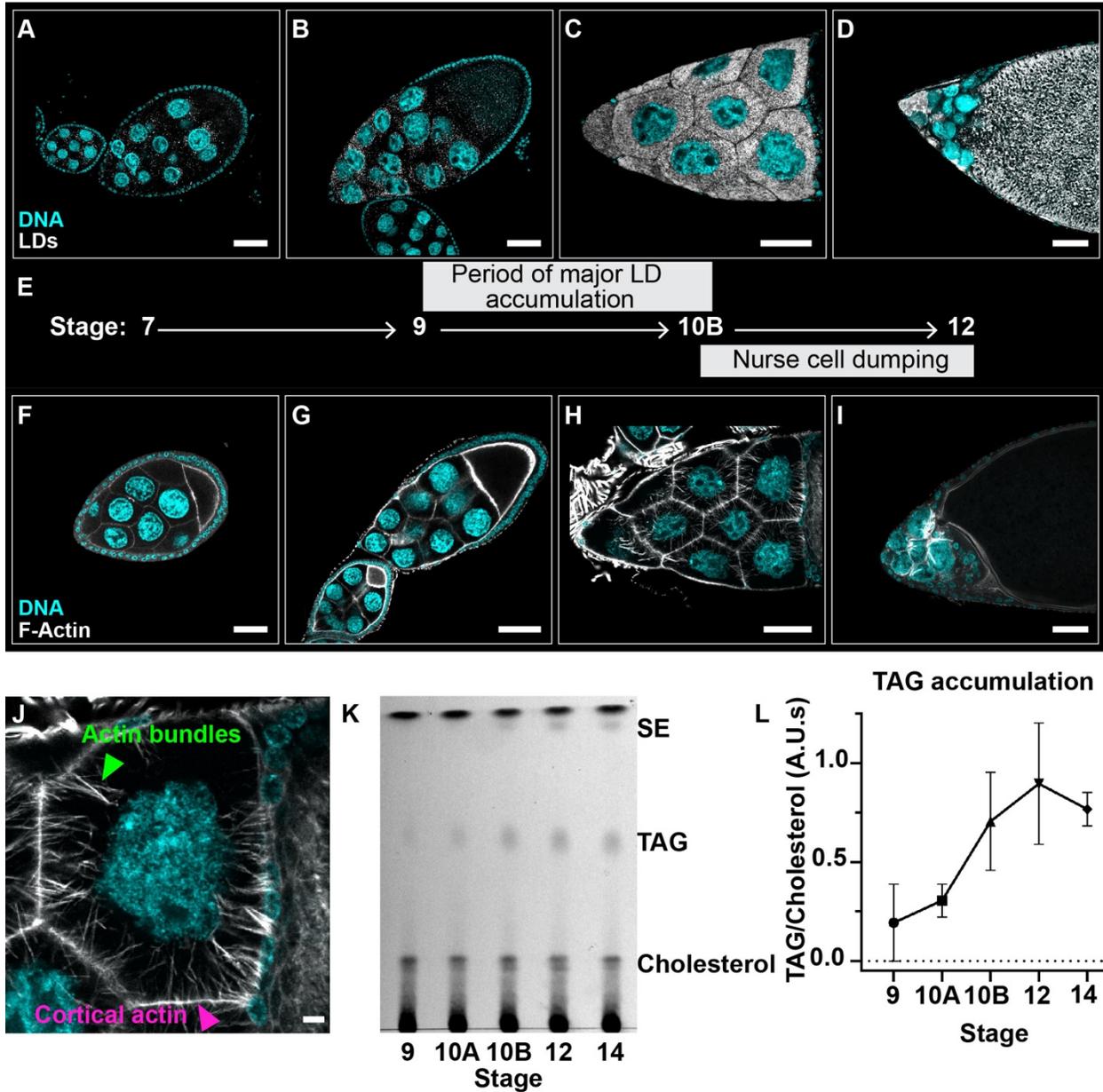
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90 **Figure 1: LD accumulation and actin cytoskeletal remodeling occur during mid-**

91 **oogenesis.**

92 **(A-D)** Single confocal slices of wild-type (Oregon R) follicles of the indicated stages (**E**) stained

93 for LDs (Nile red) in white and DNA (Hoechst) in cyan. **(E)** Schematic depicting the progression

94 of LD accumulation and actin remodeling during oogenesis. **(F-I)** Single confocal slices of wild-

95 type follicles (Oregon R) of the indicated stages (**E**) stained for F-Actin (phalloidin) in white and

96 DNA (Hoechst) in cyan. Note that all images and diagram in A-I are on top of a black box. **(J)**

97 Single confocal slice of a S10B nurse cell stained for F-Actin (phalloidin) in white and DNA
98 (Hoechst) in cyan; actin bundles are indicated by the green arrowhead and cortical actin is
99 indicated by the magenta arrowhead. Scale bars=50 μ m (**A-I**) or 10 μ m (**J**). (**K**) Thin layer
100 chromatograph of whole-lipid extracts from late-stage follicles. (**L**) Quantitation of (**K**), in which
101 the ratio of TAG to cholesterol intensity is plotted; error bars, SD. Few LDs are present in the
102 nurse cells during early oogenesis, but begin to accumulate by S9 (**B**). By S10B, LDs are
103 present in large numbers and evenly distributed throughout the nurse cell cytoplasm (**C**). The
104 LDs are dumped into the oocyte during S11 and are highly abundant in the ooplasm by S12 (**D**).
105 This temporal accumulation is also reflected by the increase in triglycerides and sterol esters (**K**,
106 **L**), neutral lipids stored in LDs. Massive remodeling of the nurse cell actin cytoskeleton begins
107 when LDs are highly abundant (**F-H**). Cortical actin surrounds each nurse cell throughout
108 oogenesis (**F-I**). During S10B (**H**), cortical actin thickens (magenta arrowhead, **J**) and parallel
109 actin bundles extend from the nurse cell membrane to the nucleus (green arrowhead, **J**). During
110 S11, the actin bundles hold the nurse cell nuclei in place while the cortical actin contracts,
111 squeezing the nurse cell cytoplasm into the oocyte in a process termed nurse cell dumping. This
112 process ultimately results in the nurse cells shrinking and the oocyte expanding (**I**).
113

114 One potential role of LDs during oogenesis may be to regulate lipid signaling. Indeed,
115 one class of lipid signaling molecules, prostaglandins (PGs), plays critical roles during mid-
116 oogenesis. PGs are derived from arachidonic acid (AA), a poly-unsaturated FA, which is
117 converted first into PGH₂ and then into several types of bioactive PGs (Tootle, 2013). These
118 enzymatic steps are mediated by cyclooxygenase (COX) enzymes and specific PG synthases,
119 respectively. *Drosophila* has a single COX-like enzyme called Pxt, and its absence during
120 oogenesis results in severe defects in actin cytoskeletal remodeling during S10B necessary for
121 late-stage follicle morphogenesis; this ultimately causes female sterility (Tootle and Spradling,
122 2008; Spracklen and Tootle, 2015). During S10B, the actin cytoskeleton is massively

123 remodeled: parallel actin bundles extend from the plasma membrane to the nuclei to form a
124 cage, and the cortical actin substantially thickens (Figure 1F-J). Both of these actin structures
125 are required during S11 for nurse cell dumping; the cortical actin provides the contractile force,
126 and the actin bundles prevent the nuclei from being pushed into the ring canals and thus
127 plugging them (Wheatley *et al.*, 1995; Guild *et al.*, 1997; Huelsmann *et al.*, 2013). Loss of Pxt
128 and the subsequent loss of all PG signaling results in severe disruption in actin bundle
129 formation, breakdown of cortical actin, and failure of nurse cell contraction (Tootle and
130 Spradling, 2008; Spracklen and Tootle, 2015). Genetic studies reveal PG signaling acts
131 upstream of critical actin regulators, including Fascin and Enabled, to drive actin remodeling
132 necessary for follicle morphogenesis (Groen *et al.*, 2012; Spracklen *et al.*, 2014; Spracklen *et al.*
133 *et al.*, 2019). However, how PG production is temporally and spatially regulated during *Drosophila*
134 oogenesis remains unknown, but in many cells the release of AA from cellular lipids is the rate
135 limiting step (Funk, 2001; Tootle, 2013). Those precursor lipids are generally thought to be
136 phospholipids in cellular membranes, but in mammalian immune cells neutral lipids are an
137 essential source of AA for PG synthesis (Dichlberger *et al.*, 2014; Schlager *et al.*, 2015). This
138 raises the question of whether LD accumulation during mid-oogenesis contributes to PG-
139 dependent actin remodeling necessary for late-stage follicle morphogenesis.

140 Given the significance of lipid signals in actin remodeling during *Drosophila* oogenesis
141 and the central role of LDs in lipid metabolism in other cells, we sought to determine if LDs
142 contribute to *Drosophila* follicle development. We find that loss of each of two lipid droplet
143 proteins – LSD-2/PLIN2 (subsequently referred to as PLIN2) or Brummer/ATGL (subsequently
144 referred to as ATGL) – results in *pxt*-like actin remodeling defects during S10B. The similar
145 phenotypes led us to ask if these LD proteins and Pxt function in the same pathway to regulate
146 actin remodeling during *Drosophila* oogenesis. Dominant genetic interaction studies support that
147 there are two actin regulatory pathways: PLIN2 regulates actin remodeling independent of PG
148 signaling, whereas ATGL acts in a PG-dependent pathway. We find that ATGL acts upstream of

149 PG synthesis, loss of ATGL increases ovarian levels of AA containing triglycerides, exogenous
150 AA is toxic to follicles, and impairing lipid droplet formation enhances, whereas reducing ATGL
151 suppresses the lipotoxicity of free AA. These data support the model that ATGL is required to
152 release AA from LDs to provide the substrate for PG production. Ultimately, these PGs control
153 actin remodeling and thereby follicle development. Our studies thus have uncovered new roles
154 for LDs in regulating oogenesis, to safely sequester a developmentally important, but potentially
155 toxic molecule, AA, and to control its release to drive specific processes. As LDs play a similar
156 role in regulating cytotoxic histones during embryogenesis (Li *et al.*, 2012; Stephenson *et al.*,
157 2021), this work suggests LDs may have a general role in tightly regulating the levels of critical
158 molecules to support proper development.

159

160 **Results**

161 Major lipid droplet proteins are required for actin remodeling in nurse cells

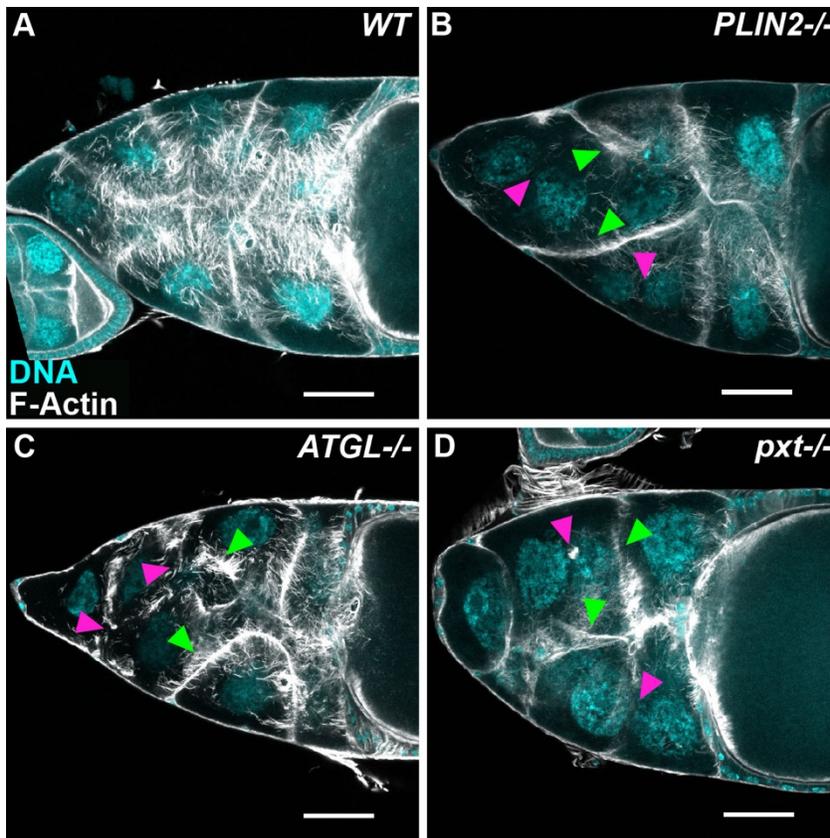
162 During *Drosophila* oogenesis, LDs undergo dramatic changes over the course of just a
163 few hours (Figure 1A-D). LDs start to accumulate in S9, fill the nurse cells by S10B, and are
164 transferred to the oocyte by S12 (Figure 1B-D). In most tissues, the main neutral lipids stored in
165 LDs are triglycerides and/or sterol esters (Walther and Farese, 2012). Using thin-layer
166 chromatography on tissue extracts, we find that developing follicles accumulate both classes of
167 neutral lipids, with a large predominance of triglycerides, and that neutral lipid accumulation
168 measured biochemically mirrors LD accumulation detected by microscopy (Figure 1K-L).

169 To begin to uncover the role of LDs during oogenesis, we focus on two LD-specific
170 proteins: ATGL and PLIN2. These proteins have critical roles in LD metabolism and – according
171 to RNA-seq data – are highly expressed in ovaries (Brown *et al.*, 2014). Both PLIN2 protein and
172 ATGL mRNA have previously been detected in nurse cells in mid-oogenesis (Teixeira *et al.*,
173 2003; Jambor *et al.*, 2015). ATGL catalyzes the conversion of triacylglycerol to diacylglycerol
174 and free FA (Gronke *et al.*, 2005), while PLIN2 is one of the two members of the perilipin family

175 of LD proteins in flies and regulates lipid metabolism by protecting the stored triglycerides from
176 the lipolytic machinery, including ATGL (Gronke *et al.*, 2003; Beller *et al.*, 2010; Bi *et al.*, 2012;
177 Zhao *et al.*, 2022).

178 Given that LD accumulation coincides with PG-dependent dynamic actin remodeling
179 (Figure 1E), we asked whether these LD regulators have a role in these events. F-actin present
180 at the cortex of the nurse cells throughout early stages of oogenesis (Figure 1F and G)
181 dramatically thickens during S10B (Figure 1H); in addition, actin bundles form that hold the
182 nurse cell nuclei in place during nurse cell dumping (Figure 1H-J). We find that females lacking
183 either PLIN2 or ATGL display two types of actin defects (Figure 2A-C). First, actin bundles are
184 aberrant. Specifically, some nurse cell membranes lack bundles, and the bundles that do form
185 are shorter, not uniformly distributed, and/or fail to project toward the nurse cell nuclei (Figure
186 2B and C, green arrowheads). Second, cortical actin is broken down, leading to the appearance
187 of multinucleate nurse cells (Figure 2B and C, magenta arrowheads).

188 The combination of actin defects due to loss of PLIN2 or ATGL are rarely seen in other
189 mutants, as most actin regulators impact either actin bundle formation or cortical actin, but not
190 both (Wheatley *et al.*, 1995; Buszczak and Cooley, 2000). However, the same combination of
191 phenotypes is observed when PG signaling is lost: lack of the COX-like enzyme Pxt causes
192 collapsed, stunted or absent actin bundles, and cortical actin breakdown with a failure of nurse
193 cell contraction (Figure 2D) (Tootle and Spradling, 2008; Spracklen and Tootle, 2015). In cases
194 of actin bundling defects, such as loss of Fascin, nurse cell nuclei are pushed into the ring
195 canals when the cortical actin contracts (Cant *et al.*, 1994). However, this does not occur in *pxt*
196 (Tootle and Spradling, 2008; Groen *et al.*, 2012), and is rarely observed in *ATGL* or *PLIN2*
197 mutants (data not shown), suggesting that cortical actin contraction is also disrupted,
198 presumably as result of the simultaneous breakdown of cortical actin. The fact that loss of all
199 three proteins results in this unusual combination of phenotypes suggests a functional
200 relationship between PGs and LD proteins in actin remodeling.



201

202 **Figure 2: Lipid droplet proteins regulate actin bundling and cortical actin integrity in**
203 **S10B follicles.**

204 (A-D) Maximum projections of three confocal slices of S10B follicles stained for F-actin
205 (phalloidin) in white, and DNA (DAPI) in cyan. Arrowheads indicate examples of defective actin

206 bundling (green) and disrupted cortical actin (magenta). Images were brightened by 30% to
207 increase clarity. Black box was placed behind channel label to improve visualization. Scale

208 bars=50 μ m. (A) WT, *wild-type* (*yw*). (B) *PLIN2*^{-/-} (*Lsd-2*^{KG00149}/*Lsd-2*^{KG00149}). (C) *ATGL*^{-/-} (*bmm*¹/
209 *bmm*¹). (D) *pxt*^{-/-} (*pxt*^{f01000}/*pxt*^{f01000}).

210 In wild-type late S10B follicles, actin bundles extend from
211 the nurse cell periphery to the nucleus, and the cortical actin is thickened relative to earlier
212 stages (A). Actin bundles fail to form or form improperly, and cortical actin is disrupted upon loss
213 of PLIN2 (B) or ATGL (C). These phenotypes resemble loss of the COX-like enzyme Pxt (D).

213

214 *Pxt functions in a linear pathway with ATGL, but not with PLIN2, to regulate actin remodeling*

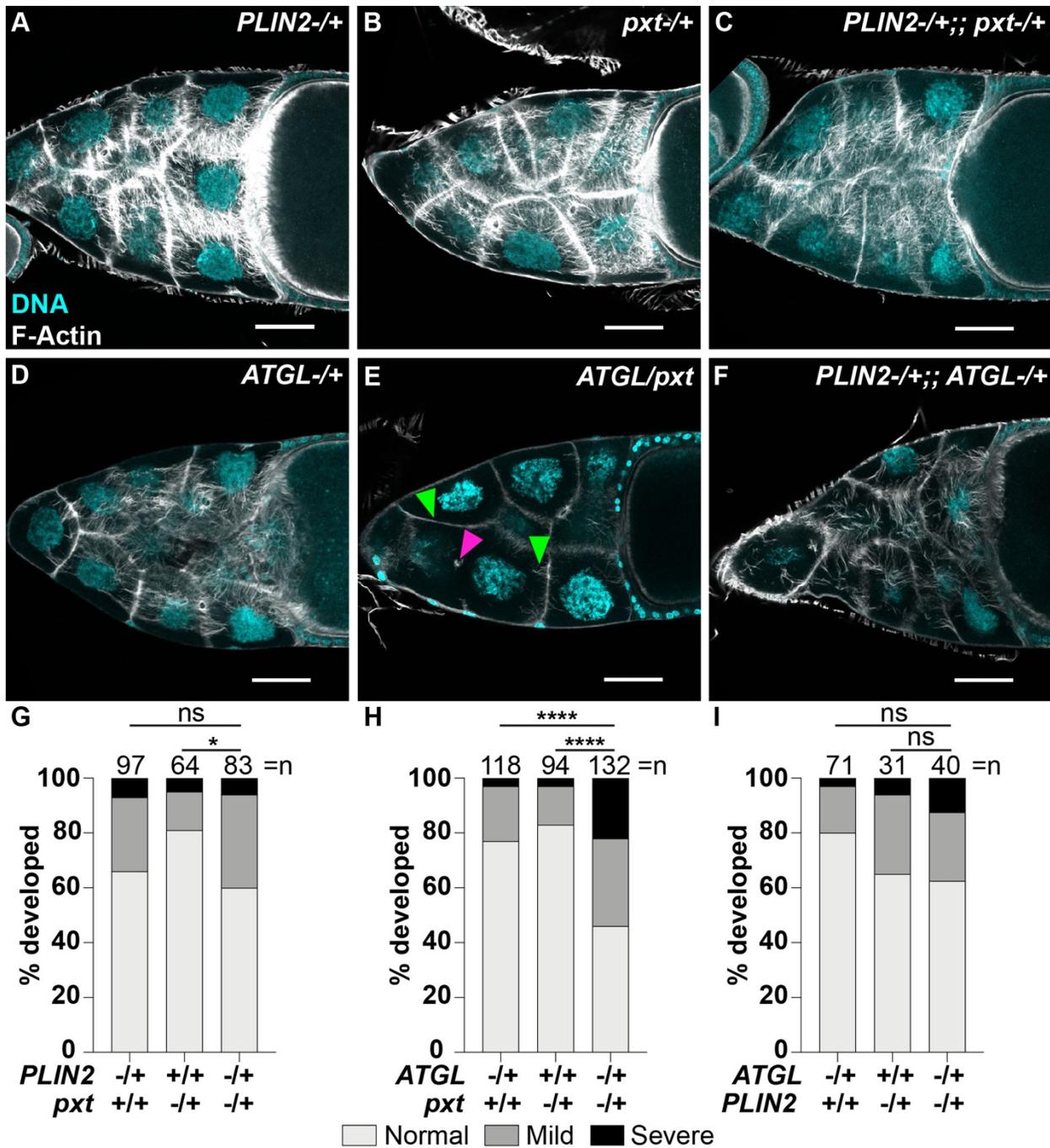
215 The similar actin phenotypes in *pxt*, *PLIN2*, and *ATGL* mutants suggest these proteins
216 may act in the same pathway to regulate actin remodeling. To test this hypothesis, we used a
217 dominant genetic interaction assay to assess actin remodeling defects. We developed a method
218 to quantify both actin bundle and cortical actin defects from confocal stacks of phalloidin-stained
219 S10B follicles. The degree of penetrance of actin bundle and cortical actin defects are scored
220 separately and summed to give an Actin Defect Index (ADI) that is used to classify each follicle
221 into three categories of defects: normal, mild, or severe (see the Material and Methods and
222 Figure 3- supplemental figure 1 for details).

223 Specifically, we asked whether reducing the level of *PLIN2* or *ATGL* causes actin
224 remodeling defects in the sensitized background of heterozygosity for *Pxt* (*pxt*+/+) (Figure 3-
225 supplemental figure 2). Heterozygosity for the individual mutations has limited effects on actin
226 remodeling, with the majority of follicles appearing normal: *PLIN2*+/+ (63% normal), *ATGL*+/+
227 (84% normal), and *pxt*+/+ (80-87% normal) (Figure 3A, B, D, G, H, and I). If either *PLIN2* and *Pxt*
228 or *ATGL* and *Pxt* function in the same pathway, then double heterozygotes for *PLIN2* and *pxt* or
229 *ATGL* and *pxt* will exhibit severe actin defects. Conversely, if they function in separate
230 pathways, then actin defects in the double heterozygotes will remain low or be additive of what
231 is observed in the single heterozygotes (Figure 3- supplemental figure 2).

232 In this assay, *PLIN2* and *ATGL* behaved very differently. Co-reduction of *PLIN2* and *Pxt*
233 (*PLIN2*+/+; *pxt*+/+) does not significantly increase the frequency of actin defects (Figure 3C and
234 G; 60% normal). This failure to exhibit a genetic interaction is not likely due to insufficient protein
235 reduction as *PLIN2* heterozygotes show *PLIN2* protein level of ~60% of wild type (Figure 3-
236 supplemental figure 3). Rather, it suggests that *Pxt* and *PLIN2* regulate actin remodeling via
237 separate pathways. In contrast, only 46% of the double heterozygotes of *pxt* and *ATGL*
238 (*pxt*/*ATGL*) exhibit normal actin remodeling, and the frequency of severe cases increases from
239 3% in the single heterozygotes to 22% in the double heterozygotes (Figure 3E and H). This

240 synergistic increase in actin defects indicates that ATGL and Pxt regulate actin remodeling via a
241 shared pathway.

242 Taken together, these data reveal an intricate relationship between Pxt and LD proteins
243 in which activity of both is necessary for proper actin remodeling. Specifically, our data reveals
244 two independent pathways control actin remodeling, one involving ATGL and Pxt, the other
245 involving PLIN2. Indeed, when we assessed dominant genetic interactions between PLIN2 and
246 ATGL, the frequency of actin remodeling defects observed in the double heterozygotes
247 (*PLIN2/+; ATGL/+*) is similar to that seen in the single heterozygotes (Figure 3F and I),
248 reinforcing that PLIN2 and ATGL/Pxt represent distinct pathways (see Figure 8).



249

250 **Figure 3: ATGL acts in a linear pathway with Pxt to regulate actin remodeling, while**

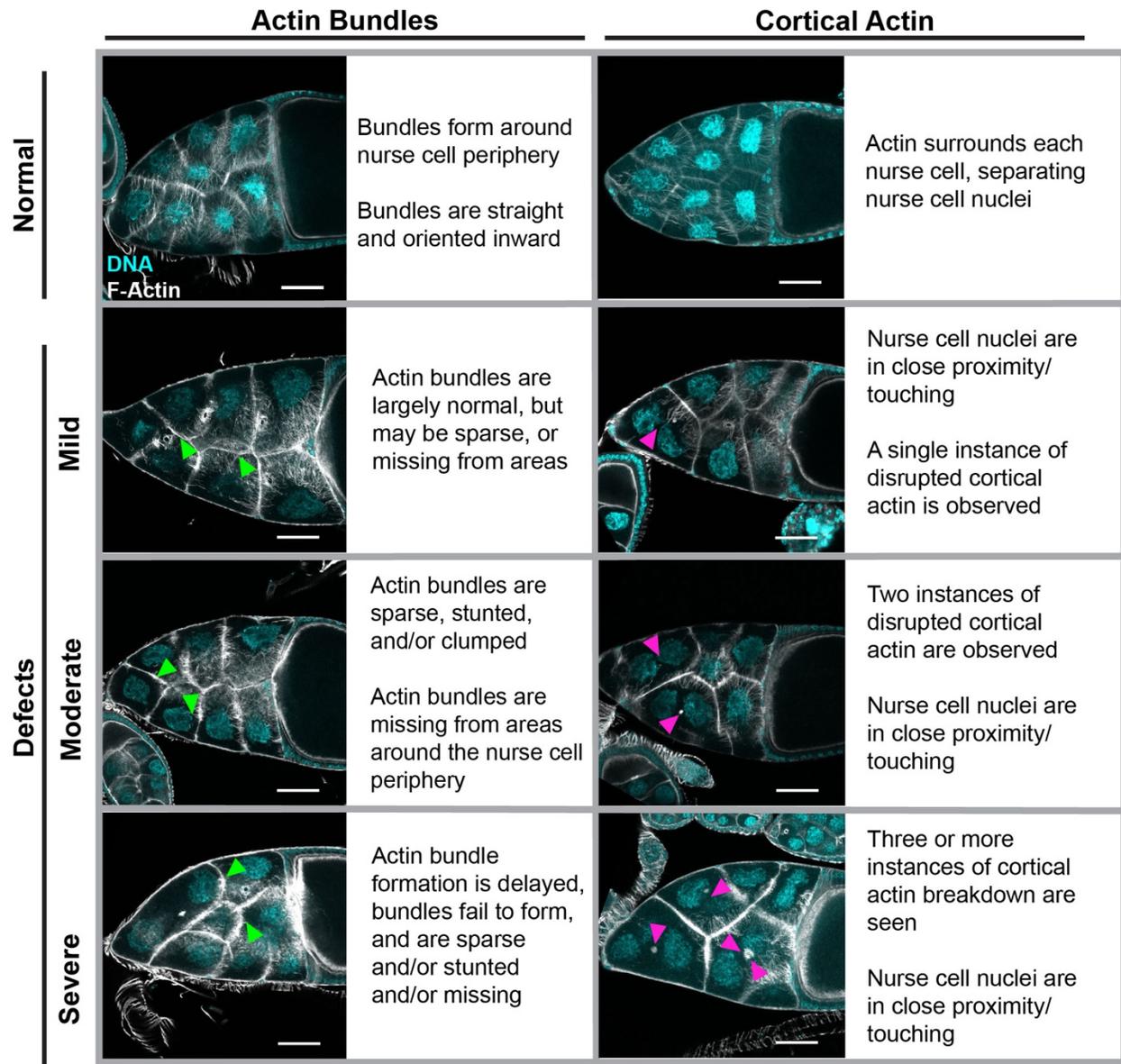
251 **PLIN2 controls it independently of ATGL and Pxt.**

252 **(A-F)** Maximum projections of three confocal slices of S10B follicles stained for F-Actin

253 **(Phalloidin)** in white, and DNA (DAPI) in cyan. Arrowheads indicate examples of defective actin

254 bundling (green) and disrupted cortical actin (magenta). Images were brightened by 30% to
255 increase clarity. Scale bars=50 μ m. **(A)** *PLIN2*^{-/+} (*Lsd-2*^{KG00149/+}). **(B)** *pxt*^{-/+} (*pxt*^{f01000/+}). **(C)**
256 *PLIN2*^{-/+;; pxt}^{-/+} (*Lsd-2*^{KG00149/+;; pxt}^{f01000/+}). **(D)** *ATGL*^{-/+} (*bmm*^{1/+}). **(E)** *ATGL/pxt*
257 (*bmm*^{1/pxt}^{f01000}). **(F)** *PLIN2*^{-/+;; ATGL}^{-/+} (*Lsd-2*^{KG00149/+;; bmm}^{1/+}). **(G-I)** Graphs of quantification
258 of actin phenotypes. Data were from the following genotypes: *PLIN2*^{-/+} = *Lsd-2*^{KG00149/+}; *pxt*^{-/+} =
259 *pxt*^{f01000/+} and *pxt*^{EY03052/+}; *PLIN2*^{-/+;; pxt}^{-/+} = *Lsd-2*^{KG00149/+;; pxt}^{f01000/+} and *Lsd-2*^{KG00149/+;;}
260 *pxt*^{EY03052/+}; *ATGL*^{-/+} = *bmm*^{1/+}; *ATGL/pxt* = *bmm*^{1/pxt}^{f01000} and *bmm*^{1/pxt}^{EY03052}; and *PLIN2*⁻
261 */+;;ATGL*^{-/+} = *Lsd-2*^{KG00149/+;;bmm}^{1/+}. Actin defects were quantified by scoring the penetrance
262 of actin bundle and cortical actin defects. Scores were summed and the total binned into one of
263 three categories: normal, mild defects, and severe defects. For a detailed description of the
264 quantification see the Materials and Methods and Figure 3 - supplemental figure 1. *p<0.01,
265 ****p<0.0001, ns>0.05, Pearson's chi-squared test. Error bars, SD. Both actin bundles and
266 cortical actin are largely normal in *PLIN2*^{-/+} (**A, G, I**), *PLIN2*^{-/+;; pxt}^{-/+} (**C, G**), and *PLIN2*^{-/+;;}
267 *ATGL*^{-/+} (**F, I**) S10B follicles. In contrast, in *ATGL/pxt* follicles (**E, H**), actin bundles are absent,
268 sparse, and stunted, and there are instances of cortical actin breakdown. The double
269 heterozygotes thus exhibit significantly more mild and severe actin defects (**H**) than either *pxt*^{-/+}
270 (**B**) or *ATGL*^{-/+} (**D**) follicles.

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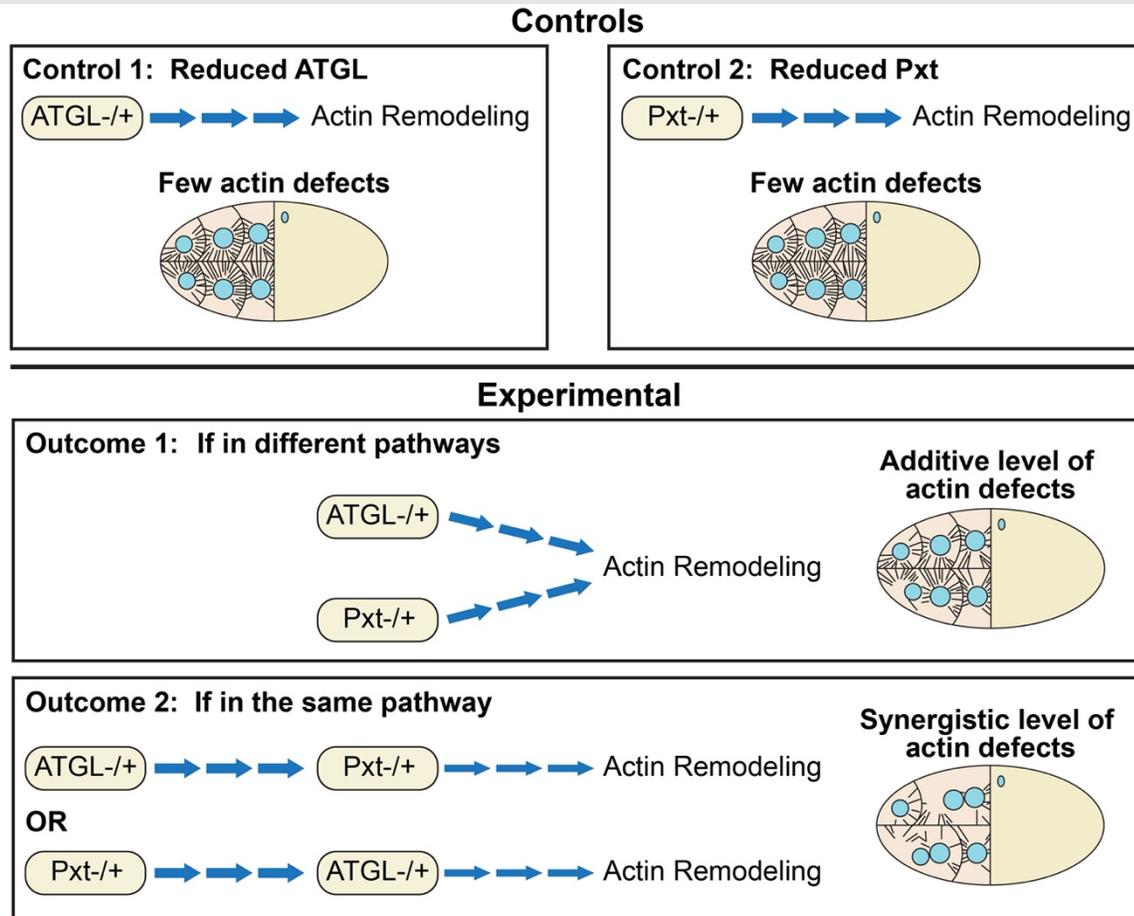


272

273 **Figure 3 – supplemental figure 1: Examples and criteria used for scoring actin**
 274 **remodeling phenotypes.**

275 Maximum projection of three confocal slices of representative S10B follicles stained for F-Actin
 276 (phalloidin) in white, and DNA (DAPI) in cyan, and written definitions of the indicated actin
 277 bundle and cortical actin phenotypes. Actin bundle and cortical actin phenotypes were
 278 independently binned into four categories: Normal, Mild Defects, Moderate Defects, or Severe
 279 Defects. Images were brightened by 30% to increase clarity. Green arrowheads indicate

280 examples of actin bundle defects, and magenta arrowheads indicate examples of cortical actin
281 breakdown. Scale bar=50 μ m.



282

283 **Figure 3 – supplemental figure 2: Schematic of dominant genetic interactions.**

284 Schematic of the premise of how dominant genetic interactions can be used to determine if two

285 factors act in the same pathway. Specifically shown are the controls and potential experimental

286 outcomes for assessing if ATGL and Pxt function separately or together to control actin

287 remodeling during S10B. The top depicts the Controls, single heterozygous follicles that have

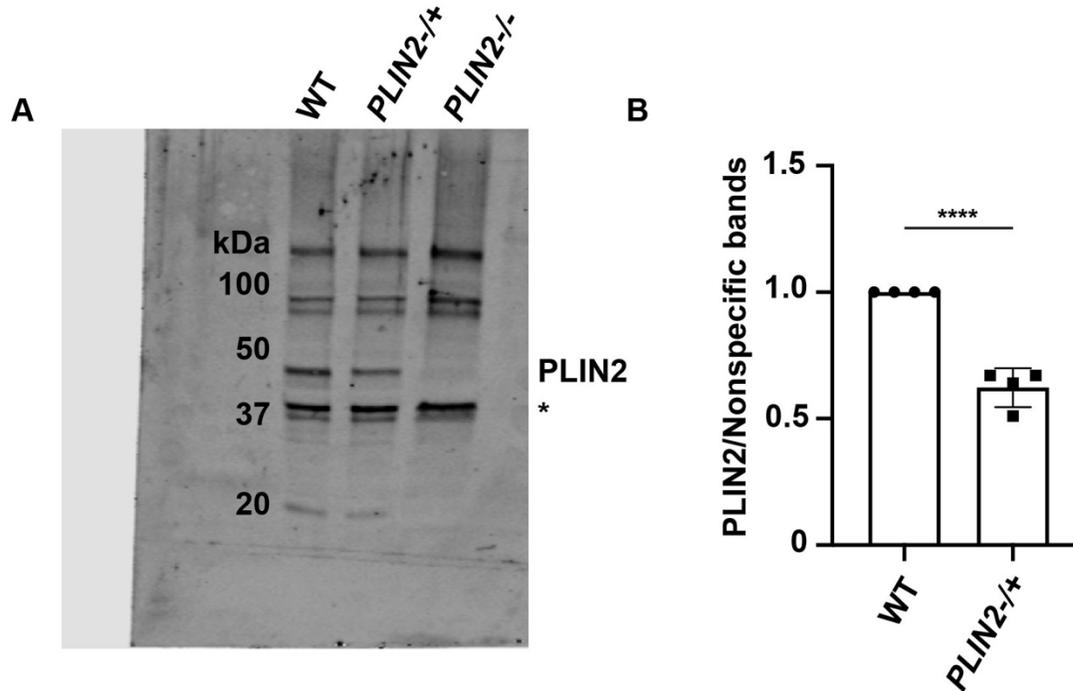
288 either reduced ATGL (**Control 1**) or reduced Pxt (**Control 2**); these control follicles are

289 expected to have few actin defects. The bottom depicts the two possible experimental

290 outcomes. **Outcome 1** depicts that ATGL and Pxt function in two separate pathways to regulate

291 actin remodeling. In this case, the double heterozygous follicles will have actin defects that are

292 not significantly greater than those seen in the control conditions. **Outcome 2** depicts that ATGL
293 and Pxt function in the same pathway, with either ATGL acting upstream of Pxt or vice versa, to
294 control actin remodeling. In this case, the double heterozygous follicles will exhibit a significant
295 increase in actin defects compared to the control conditions. The latter outcome is what is
296 observed in Figure 3H.



297

298 **Figure 3 – supplemental figure 3: *PLIN2*^{-/+} heterozygotes have reduced PLIN2 protein.**
299 (A) Immunoblot comparing PLIN2 protein levels in wild type (WT, Oregon R), *PLIN2*^{-/+} (*Lsd-*
300 *2*^{KG00149/+}), and *PLIN2*^{-/-} (*Lsd-2*^{KG00149/Lsd-2}^{KG00149}) S10B follicles. (B) Quantification of (A), in
301 which PLIN2 band intensity was normalized to the nonspecific bands (asterisk). $p < 0.0001$,
302 unpaired t-test, two-tailed. PLIN2 protein levels are reduced by approximately half in *PLIN2*^{-/+}
303 S10B follicles.

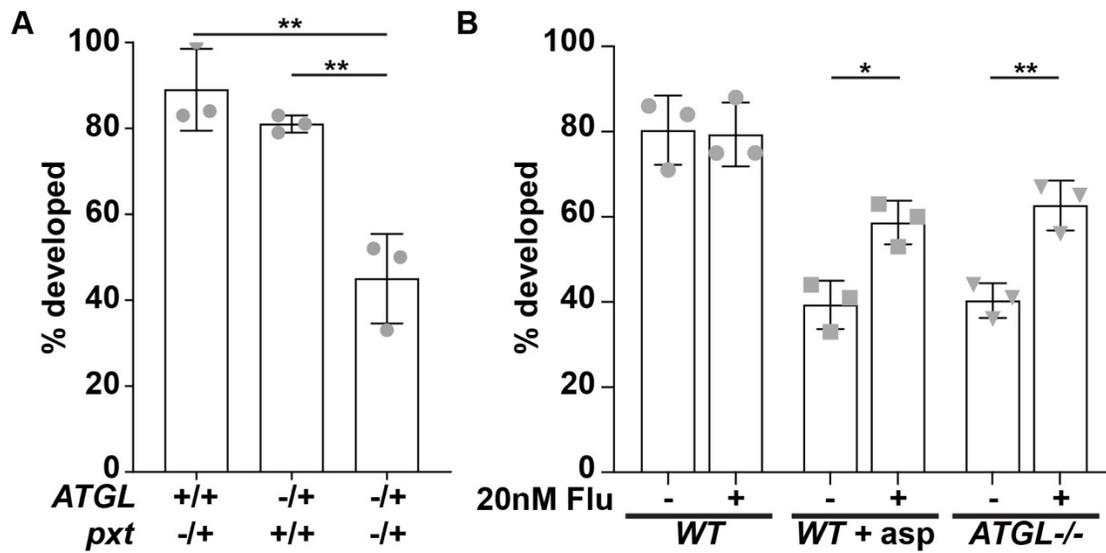
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306 *ATGL acts upstream of Pxt*

307 Our genetic analysis indicates that ATGL and Pxt act in the same pathway to regulate
308 actin remodeling during S10B, but does not address whether ATGL acts upstream of Pxt or vice
309 versa. For example, PGs produced by Pxt might control the ability of ATGL to sequester actin
310 regulators to LDs and thus effectively inactivate them, altering actin dynamics. Alternatively,
311 ATGL might control Pxt expression or the availability of the substrate Pxt acts on. To determine
312 the order in which ATGL and Pxt act, we took advantage of the fact that application of
313 exogenous $\text{PGF}_{2\alpha}$, the PG responsible for S10B actin remodeling, can suppress the actin
314 defects due to loss of Pxt (Tootle and Spradling, 2008); a stabilized $\text{PGF}_{2\alpha}$ analog, Fluprostenol
315 (Flu), is used in these studies. If ATGL acts upstream of Pxt, $\text{PGF}_{2\alpha}$ should similarly suppress
316 defects due to loss of ATGL. If, in contrast, Pxt acts upstream of ATGL, $\text{PGF}_{2\alpha}$ should not be
317 able to modulate the *ATGL* mutant phenotype.

318 We utilized our *in vitro* egg maturation (IVEM) assay, in which isolated S10B follicles
319 mature *in vitro* in a simple culture medium (Spracklen and Tootle, 2013). This assay does detect
320 the genetic interaction between Pxt and ATGL, as the majority of S10B follicles from single
321 heterozygotes of *pxt* and *ATGL* develop *in vitro*, while 55% of the follicles from double
322 heterozygotes fail to develop (Figure 4A). These data recapitulate what we observed when we
323 quantitatively assessed the actin defects in the double heterozygotes (Figure 3H). Using the
324 same assay, we then tested the role of $\text{PGF}_{2\alpha}$ downstream of ATGL. Treatment of S10B follicles
325 with 1.5mM aspirin (an inhibitor of COX enzymes, including Pxt) inhibits ~50% of follicle
326 development, and this is suppressed by addition of $\text{PGF}_{2\alpha}$ (Figure 4B). Of the *ATGL* mutant
327 follicles, only ~38% develop, but addition of $\text{PGF}_{2\alpha}$ results in significant improvements, with
328 ~58% developing (Figure 4B). These data indicate that Pxt and $\text{PGF}_{2\alpha}$ act downstream of ATGL.



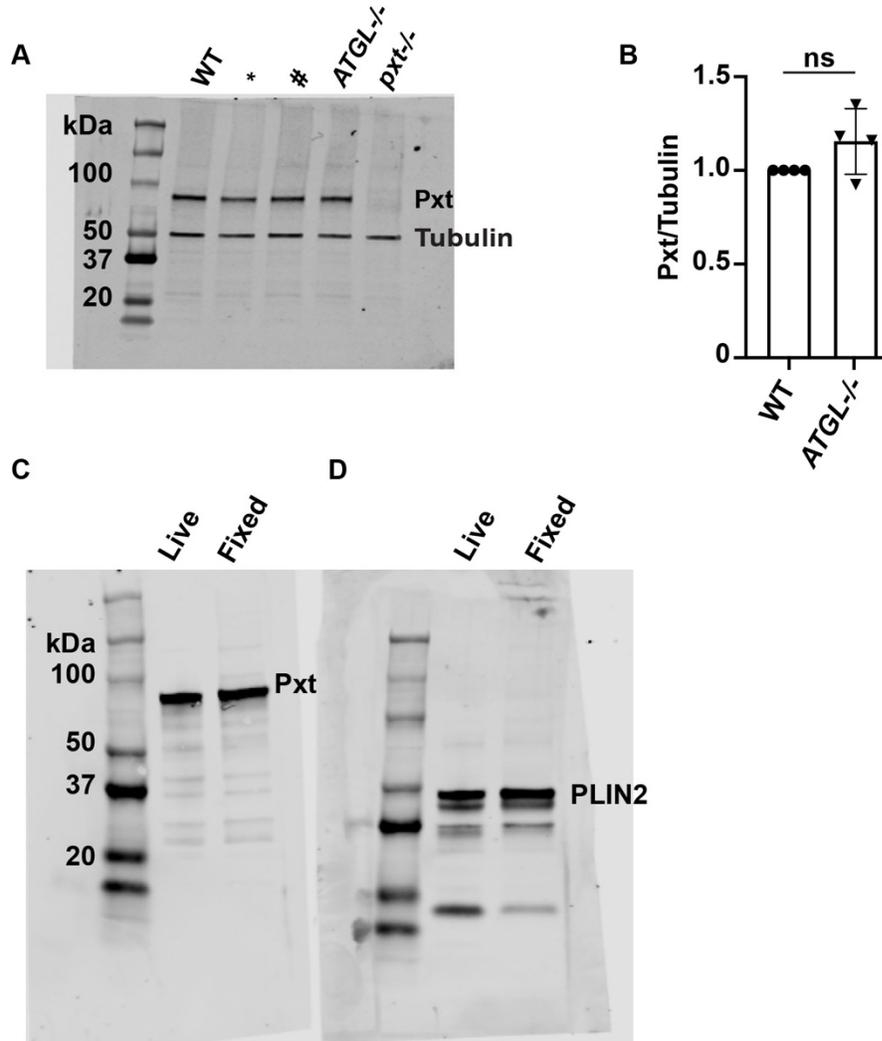
329

330 **Figure 4: ATGL functions upstream of PGF_{2α}.**

331 **(A)** Graph of the percentage of S10B follicles developing in the IVEM assay, normalized to wild-
 332 type (*yw*) development, for the following genotypes: *ATGL*^{-/+} (*bmm*^{1/+}), *pxt*^{-/+} (*pxt*^{f01000/+}), and
 333 *ATGL/pxt* (*bmm*^{1/pxt}^{f01000}). ***p*<0.005, ns>0.05, unpaired t-test, two-tailed. Error bars, SD. **(B)**

334 Graph of the percentage of S10B follicles developing in the IVEM assay with either control
 335 (ethanol, EtOH) or 20nM PGF_{2α} analog (Fluprostenol, Flu) treatment of wild-type (*yw*) and
 336 *ATGL*^{-/-} (*bmm*^{1/bmm}¹). **p*<0.05, ***p*<0.005, ns>0.05, unpaired t-test, two-tailed. Error bars, SD.

337 In the IVEM assay, ATGL and Pxt genetically interact, as *ATGL/pxt* follicle development is
 338 reduced by ~50% compared to the *ATGL*^{-/+} and *pxt*^{-/+} controls **(A)**. Complete loss of ATGL
 339 strikingly reduces the percentage of follicles developing in control media; this impairment of
 340 development is significantly suppressed by supplying a PGF_{2α} analog exogenously **(B)**. This
 341 finding indicates that PGF_{2α} functions downstream of ATGL.



342

343 **Figure 4 – supplemental figure 1: ATGL does not affect Pxt expression.**

344 (A) Immunoblot for Pxt and α -Tubulin of the indicated genotypes: WT, wild-type (Oregon R),

345 ATGL^{-/-} (*bmm¹/bmm¹*) and *pxt*^{-/-} (*pxt^{f01000}/pxt^{f01000}*). The * and # lanes correspond to unrelated

346 genotypes. (B) Quantification of A, in which band intensity was normalized as indicated.

347 ns= $p > 0.05$, unpaired t-test, two-tailed. (C-D) Simultaneously scanned immunoblots comparing

348 the blotting patterns of live versus formaldehyde fixed wild-type whole ovary samples, 5 ovaries

349 per lane, for Pxt (C) and PLIN2 (D). Loss of ATGL has no effect on Pxt levels (A-B). The live vs.

350 fixed banding pattern is comparable for both antibodies tested (C-D). Fixed samples were used

351 in panel A and Figure 3 – supplemental figure 3.

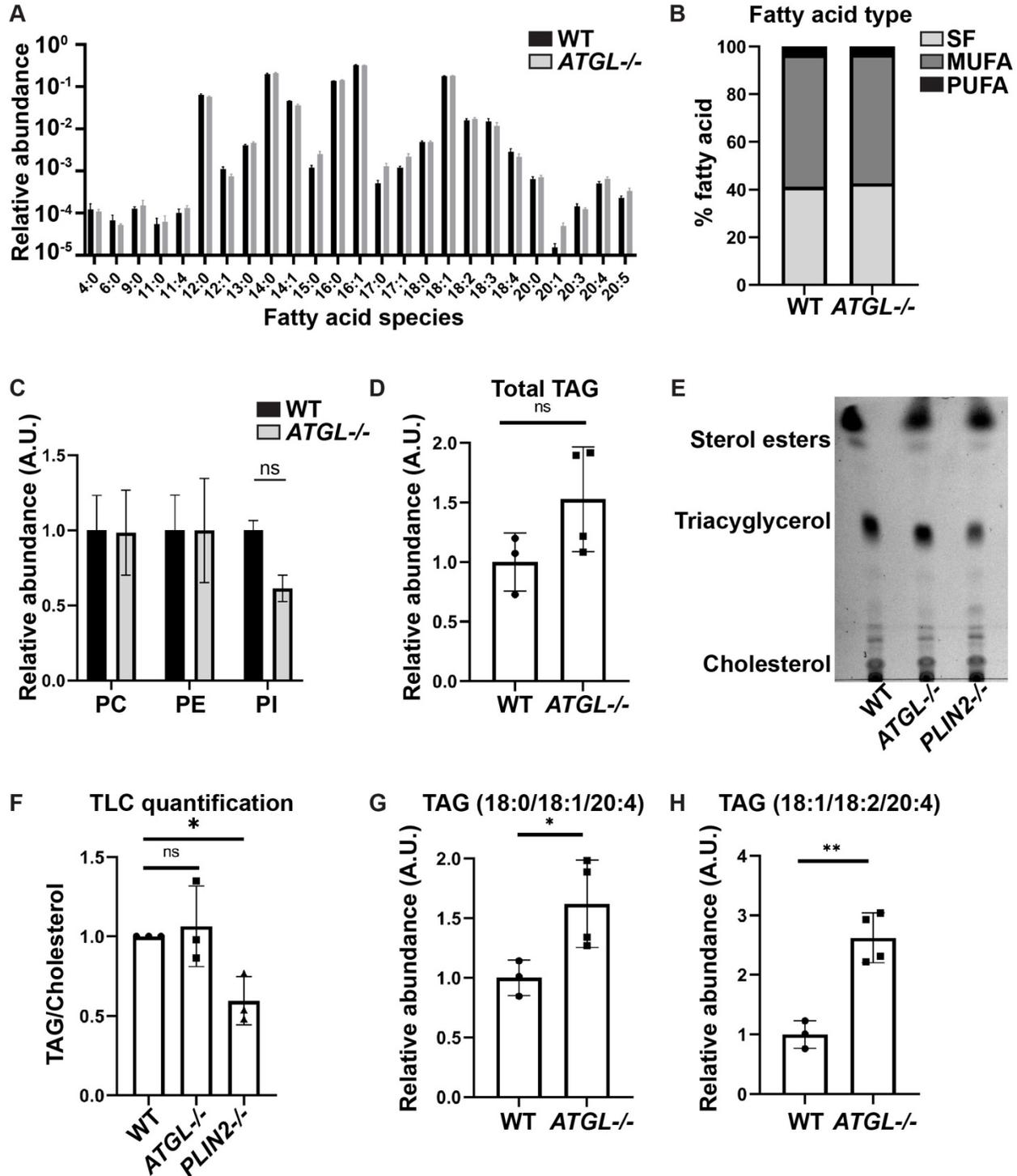
352 AA is present in ovary triglycerides

353 We next sought to determine how ATGL functions upstream of PGs. One means by
354 which ATGL could act upstream of PG production is by regulating the expression of Pxt, the
355 enzyme responsible for PG synthesis. This is not the case, as loss of ATGL does not alter Pxt
356 levels (Figure 4 – supplemental figure 1). Studies in mammalian cells suggest an alternative
357 possibility. There, ATGL releases AA, the substrate for COX enzymes, from triglycerides stored
358 in LDs (Dichlberger *et al.*, 2014). If ATGL similarly generates the substrate for Pxt, *Drosophila*
359 follicles should have AA-containing triglycerides. We therefore extracted lipids from wild-type
360 and *ATGL* mutant ovaries and analyzed them by LC-MS/MS mass spectrometry. Among the 98
361 different triglyceride species identified (Figure 5- supplemental table 1), two contain a twenty-
362 carbon FA with four double bonds (20:4), presumably AA. AA was relatively rare, making up on
363 the order of 0.06-0.07% of all FAs detected in triglycerides (Figure 5A), with four other FA
364 species accounting for the bulk (~85%) of FAs: the mono-unsaturated palmitoleic (16:1) and
365 oleic (18:1) acids, and the saturated myristic (14:0) and palmitic (16:0) acids. We did not detect
366 AA in the phospholipids identified in our analysis (data not shown).

367 Our lipidomic analysis did not uncover a significant difference in the content of
368 phospholipids (Figure 5C) or of total triglycerides (Figure 5D) between wild-type and *ATGL*
369 mutant ovaries. The overall FA profile in triglycerides was also similar between the two
370 genotypes (Figure 5A and B). In addition, there was no significant difference in total triglycerides
371 in S14 oocytes, as detected by thin layer chromatography (Figure 5E and F), even though we
372 could confirm the previously described reduced triglyceride loading in *PLIN2* mutants (Teixeira
373 *et al.*, 2003). Thus, ATGL mediated lipolysis during oogenesis does not appear to lead to bulk
374 turnover of LDs and may be restricted to supporting lipid signaling.

375 Supporting this possibility, wild-type and *ATGL* mutant ovaries exhibited differences in
376 the AA-containing triglycerides, both of which were elevated in the absence of ATGL (Figure 5G
377 and H). This trend persisted even when measurements were normalized to total triglycerides

378 (not shown) or all lipids in the sample (Figure 5- supplemental figure 1). These observations are
 379 consistent with the possibility that in the *ATGL* mutants less AA is released from triglycerides,
 380 resulting in a reduced pool of free AA available for signaling.



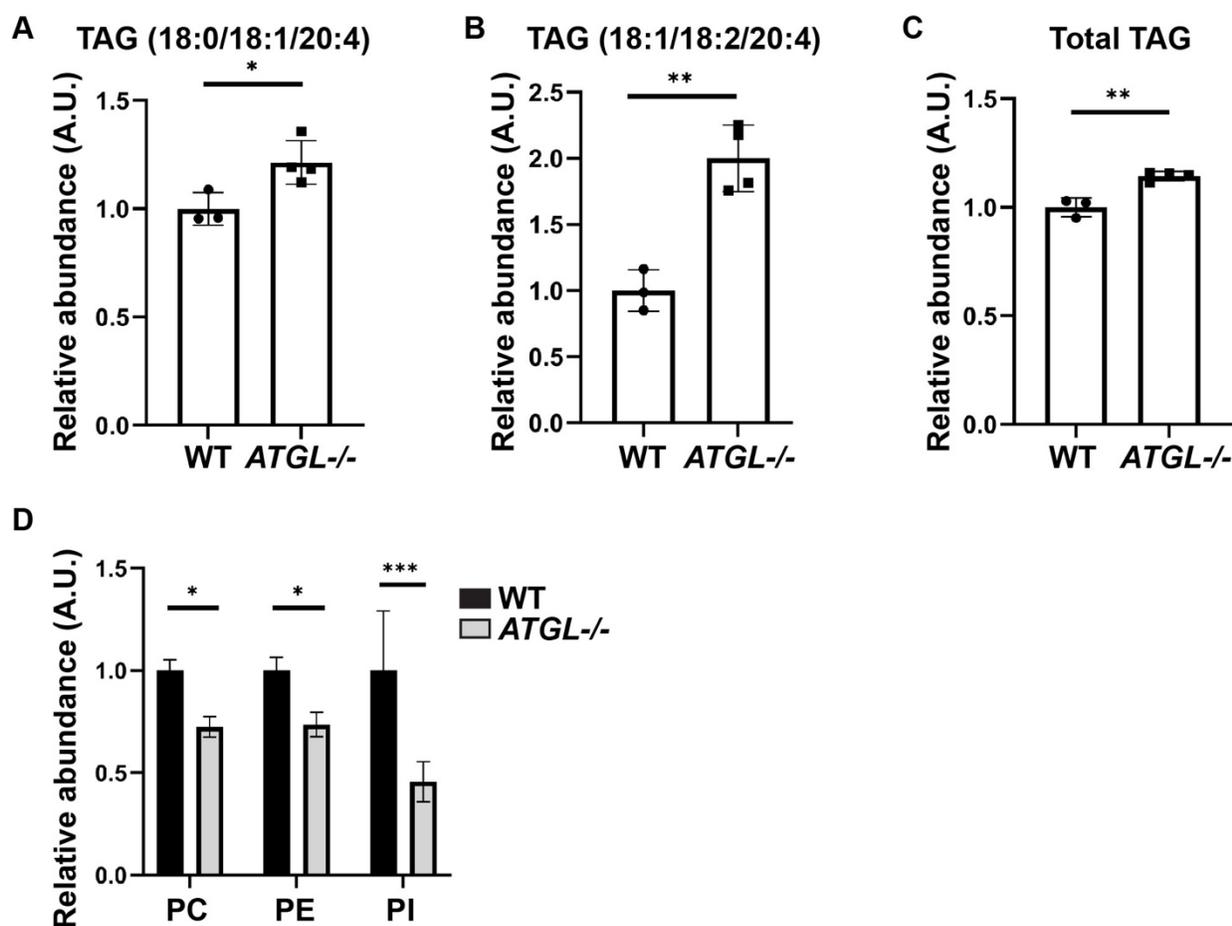
381

382 **Figure 5. Ovary triglycerides contain arachidonic acid.**
383 **(A-F)** Lipids were extracted from wild-type (Oregon R) and *ATGL*^{-/-} (*bmm*¹/*bmm*¹) ovaries and
384 analyzed by mass spectrometry. Error bars, SD. **(A)** Abundance of individual fatty acids in
385 triglycerides expressed as fraction of all such fatty acids; significance evaluated using Sidak's
386 multiple comparisons test. **(B)** Total amount of saturated (SF), monounsaturated (MUFA) or
387 polyunsaturated (PUFA) fatty acids relative to all fatty acids in triglycerides; significance
388 evaluated using Tukey's multiple comparisons test. **(C)** Levels of the three phospholipid classes
389 phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI);
390 significance evaluated using Sidak's multiple comparisons test. **(D)** Overall triglyceride levels. ns
391 = $p = 0.1239$, unpaired t-test, two-tailed. **(E)** Thin layer chromatograph of whole-lipid extracts
392 from S14 follicles of the indicated genotypes. **(F)** Quantitation of the triacylglycerol (TAG) to
393 cholesterol ratio in **(D)**. * $p=0.0477$, Dunnett's multiple comparisons test. **(G, H)** Quantification of
394 two triglyceride species containing arachidonic acid (AA). * $p=0.0419$, ** $p=0.0019$, unpaired t-
395 tests, two-tailed. Wild-type and *ATGL* mutant ovaries exhibit a similar abundance of fatty acids
396 in triglycerides **(A, B)**, phospholipids **(C)**, and total triglycerides **(D)** by lipidomic analysis.
397 Similarly, thin layer chromatographic analysis of Stage 14 follicles reveals no significant
398 differences in triglycerides **(E-F)**. However, two AA-containing triglyceride species are present in
399 wild-type ovaries, and their levels are elevated in the absence of *ATGL* **(G, H)**.

400

401 **Figure 5 – supplemental table 1: Quantitation of triglyceride species in ovaries**

402 Column A lists the fatty acid content of various triglyceride species detected in ovaries by
403 lipidomics. Columns B through H show the relative amount of those species in three wild-type
404 (Oregon R) and four *ATGL*^{-/-} (*bmm*¹/*bmm*¹) samples. Reads are background corrected. A fourth
405 wild-type sample was discarded as an outlier because the detected lipid amounts were an order
406 of magnitude lower than in the other samples.



407

408 **Figure 5 – supplemental figure 1: AA-containing TAG and total TAG normalized to total**
 409 **lipids in sample.**

410 (A-D) Lipids were extracted from wild-type (Oregon R) and ATGL^{-/-} (*bmm¹/bmm¹*) ovaries and
 411 analyzed by mass spectrometry. Data from Figure 5 re-plotted as relative abundance
 412 normalized to total lipids in the sample. Error bars, SD. (A, B) Two triglyceride species
 413 containing arachidonic acid (AA). Error bars, SD, **p*=0.0279, ***p*=0.0019, unpaired t-tests, two-
 414 tailed. (C) Overall triglyceride levels are slightly increased in ATGL^{-/-} ovary lipids. Error bars,
 415 SD, ***p*=0.002, unpaired t-test, two-tailed. (D) Relative amounts of phosphatidylcholine (PC),
 416 phosphatidylethanolamine (PE), and phosphatidylinositol (PI) in wild-type versus ATGL^{-/-} ovary
 417 lipids. Error bars, SD, **p*=0.0329, **p*=0.0416, ****p*=0.0001, Sidak's multiple comparisons test.
 418 While overall triglyceride levels are similar between the two genotypes (C), the AA-containing

419 triglycerides are increased (**A**, **B**), and three classes of phospholipids are decreased (**D**) in the
420 absence of ATGL. The reason for the decrease in phospholipids is not clear. One possibility is
421 that ATGL breaks down triglycerides to generate precursors for phospholipid production;
422 alternatively, in the absence of ATGL, ovaries may contain a different mix of follicle stages (and
423 thus different levels of triglyceride accumulation) due to altered developmental progression.

424

425 LDs modulate the toxicity of exogenous AA

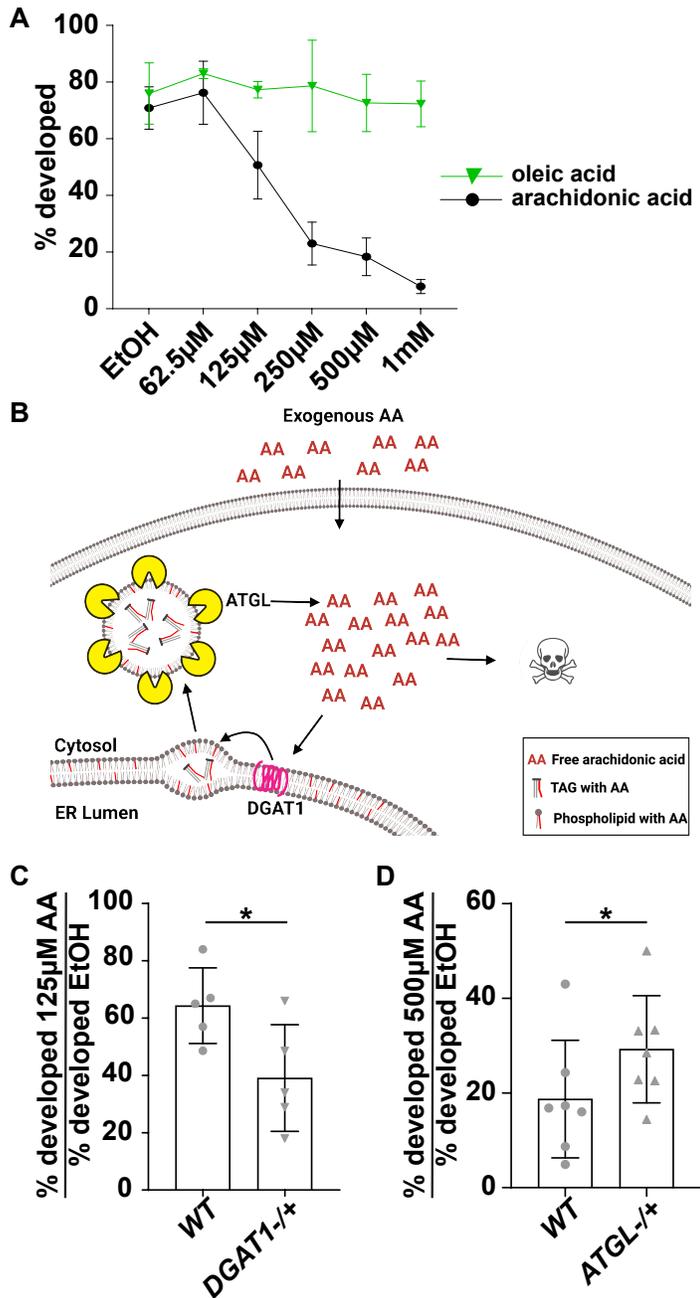
426 Our lipidomics data supports the model that AA is incorporated into triglycerides that are
427 stored in LDs. This storage may be critical because for many cells free AA is toxic at near
428 physiological levels (Pompeia *et al.*, 2003). Indeed, exogenously applied AA is typically first
429 routed to LDs (Weller and Dvorak, 1994; Bozza *et al.*, 2009; Bozza *et al.*, 2011), likely a
430 mechanism to prevent free AA from reaching toxic levels. To test if free AA is also toxic to
431 *Drosophila* follicles, we assessed the dose-dependent effects of AA on wild-type S10B follicle
432 development using a modified IVEM assay. One of the IVEM medium's key components is fetal
433 bovine serum (FBS), which supplies growth factors and numerous nutrients, including unknown
434 amounts of FAs such as AA. We therefore reduced the amount of FBS from 10% to 2.5%, which
435 still allows the majority of follicles to mature, and then assessed the effects of AA. At ~60 μ M AA,
436 a slightly higher percentage of follicles developed than in control medium, but that fraction
437 dropped steadily as AA concentrations were raised further (Figure 6A). Notably, oleic acid (OA)
438 does not impair follicle development at any concentration (Figure 6A). Thus, it is specifically free
439 AA that is dangerous to the follicles, which provides a rationale for why free AA is rapidly
440 sequestered into LDs.

441 Free FAs must be incorporated into triglycerides to be stored in LDs. The final step of
442 triglyceride synthesis is mediated by diacylglycerol O-acyl transferases (DGATs), which catalyze
443 the attachment of a third FA to diacylglycerol. Like most animals, *Drosophila* encodes two such
444 enzymes, DGAT1/Midway and DGAT2 (Yen *et al.*, 2008). Genome-wide expression data

445 indicate that DGAT1 predominates in most tissues, including in ovaries (Chintapalli *et al.*, 2007).
446 In *DGAT1* loss-of-function mutants, LDs fail to accumulate and follicles die by S8/9 of oogenesis
447 (Buszczak *et al.*, 2002). If exogenous AA, as we hypothesize, is incorporated into LDs to protect
448 follicles, then reducing DGAT1 levels should enhance AA toxicity (Figure 6B). We tested this
449 idea with our modified IVEM assay and treated follicles with 125 μ M AA. At this concentration,
450 64% of wild-type follicles develop whereas only 39% of *DGAT1*^{-/+} follicles develop (Figure 6C).
451 These data support the model that exogenous AA is sequestered into LDs.

452 We next asked whether ATGL is required to release AA from internal LDs stores and
453 thus generate free AA. If this is true, then reducing ATGL levels should partially suppress the
454 toxicity of high levels of exogenous AA (Figure 6B), as total free AA levels will be lower. Indeed,
455 two-fold more S10B follicles from *ATGL* heterozygotes develop in the presence of 500 μ M AA
456 than wild-type follicles (Figure 6C). These data are consistent with the model that ATGL
457 releases AA from LDs.

458 Together these findings support the model that AA is stored in LDs to prevent toxicity
459 and that ATGL is required to release AA from LDs. This AA can then be used for PG production
460 and thus promotes actin remodeling necessary for follicle development.



461

462

Figure 6: LDs buffer follicles against damage from free arachidonic acid.

463

(A) Dose curve of the percent of *wild-type* (*yw*) S10B follicles developing in the modified IVEM

464

assay with either increasing concentrations of arachidonic acid (black circles and line, AA) or

465

oleic acid (green triangles and line). Error bars, SD. (B) Schematic depicting the proposed

466

mechanisms whereby LDs buffer AA toxicity. Exogenous AA is rapidly sequestered into LDs by

467 DGAT1, and ATGL can release AA from LD triglycerides. Too much free AA results in toxicity.
468 **(C-D)** Graph of the percentage of S10B follicles developing in the modified IVEM assay for the
469 indicated genotypes and conditions. Error bars, SD, * $p < 0.05$, paired t-test, two-tailed. In **C**, *wild-*
470 *type* (*WT*, *yw*) and *DGAT1*^{-/+} (*mdy*^{QX25/+}) follicles were treated with 125 μ m AA; this is an
471 intermediate dose of AA. In **D**, *wild-type* (*WT*, *yw*) and *ATGL*^{-/+} (*bmm*^{1/+}) follicles were treated
472 with 500 μ m AA; this is a high dose of AA. High levels of AA suppress S10B follicle development
473 **(A)**. In contrast, similar levels of oleic acid have no effect on follicle development **(B)**. Reducing
474 the level of DGAT1, which is expected to decrease LD formation, significantly enhances the
475 ability of an intermediate dose of AA to block *in vitro* follicle development **(C)**. Conversely,
476 reducing ATGL significantly suppresses the ability of a high dose of AA to block *in vitro* follicle
477 development; this supports the model that ATGL is required to release AA from LDs **(D)**.

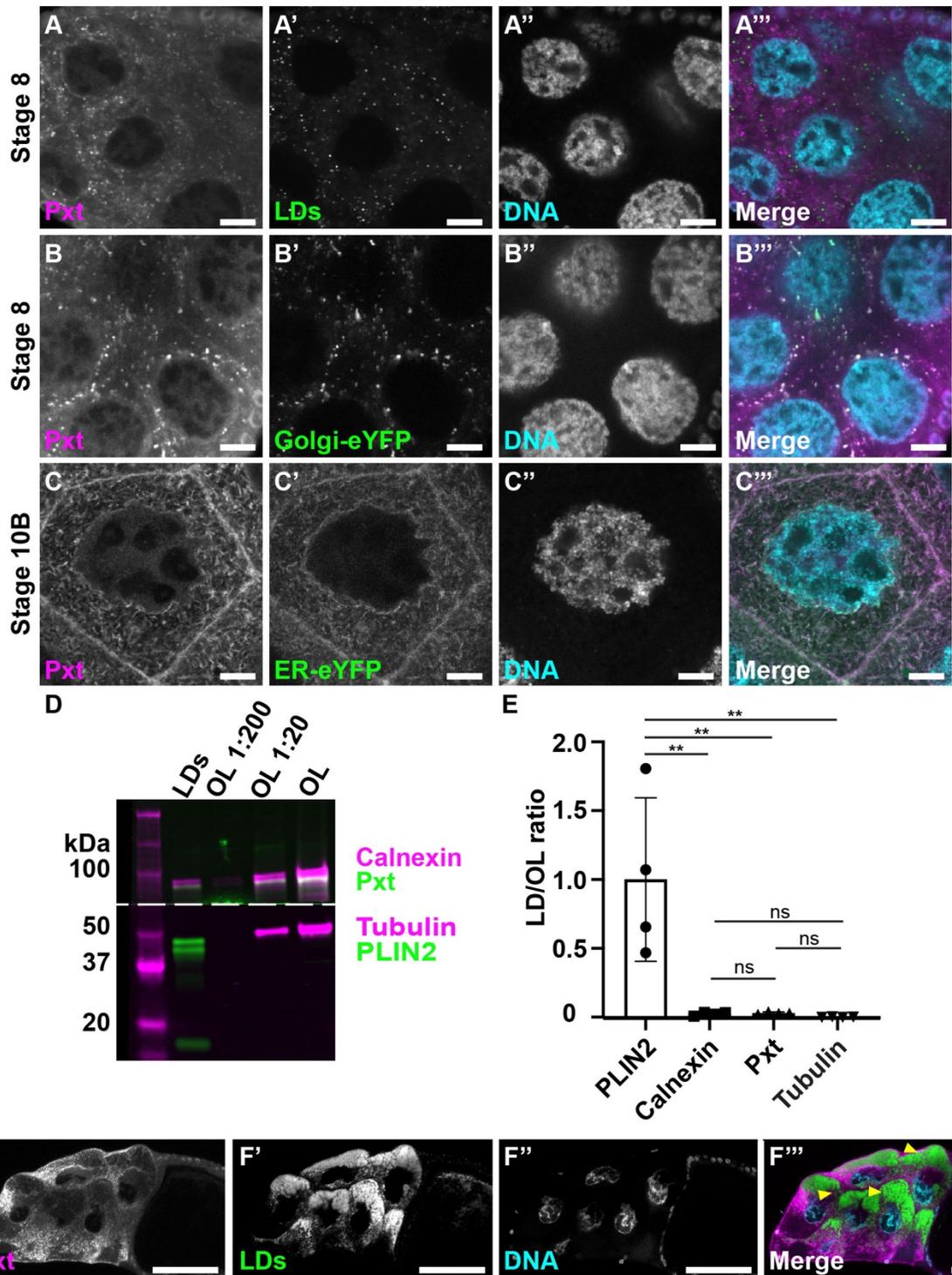
478

479 *Pxt is not enriched on LDs*

480 How does ATGL provide AA to Pxt for PG synthesis? PG production could occur on
481 LDs, as in other systems components of the PG synthesis machinery are sometimes enriched
482 on LDs (Bozza *et al.*, 2011). To determine whether this is true during *Drosophila* oogenesis, we
483 used immunostaining, LD purification, and *in vivo* centrifugation to assess Pxt's relationship to
484 LDs in wild-type nurse cells. All three methods show that Pxt is not enriched on LDs. By
485 immunostaining, Pxt localizes to the Golgi compartments prior to S9 and to the Endoplasmic
486 reticulum (ER) by S10B, but not obviously to LDs (Figure 7A-C'''). Further, Pxt localization is not
487 affected by loss of ATGL or PLIN2 (Figure 7- supplemental figure 1). We also prepared a LD
488 enriched biochemical fraction from ovary lysates, as previously described for embryos (Li *et al.*,
489 2012), and analyzed it by Western blotting for Pxt, PLIN2 (as a marker for LDs), and Calnexin
490 (as marker for the ER). Pxt behaves like an ER protein, not like an LD protein (Figure 7D-E).
491 Finally, we separated LDs from other cellular components by *in vivo* centrifugation: when living

492 *Drosophila* follicles are centrifuged, their contents separate by density within each nurse
493 cell/oocyte, with LDs accumulating on the side that faced up during centrifugation (Cermelli *et*
494 *al.*, 2006; Li *et al.*, 2012). Staining of fixed centrifuged follicles reveals no enrichment of Pxt
495 (magenta) in the LD layer (green, marked by yellow arrowhead) (Figure 7F-F’’’). We conclude
496 that the majority of Pxt is not present on LDs. This finding, in combination with our other data,
497 reveals that LDs and ATGL can regulate PG production even when the PG synthesis machinery
498 is localized at other cellular sites. Given that in most cells COX enzymes localize to the ER and
499 the contiguous nuclear envelope, the role of LDs in regulating PG signaling is likely much more
500 widespread than previously thought.

501 Together our data support the model that during Stage 10B, AA stored in LD
502 triglycerides is released by ATGL and serves as the substrate for PGF_{2α} production by ER
503 localized Pxt. PGF_{2α} signaling then drives actin remodeling necessary for late-stage follicle
504 development (Figure 8).



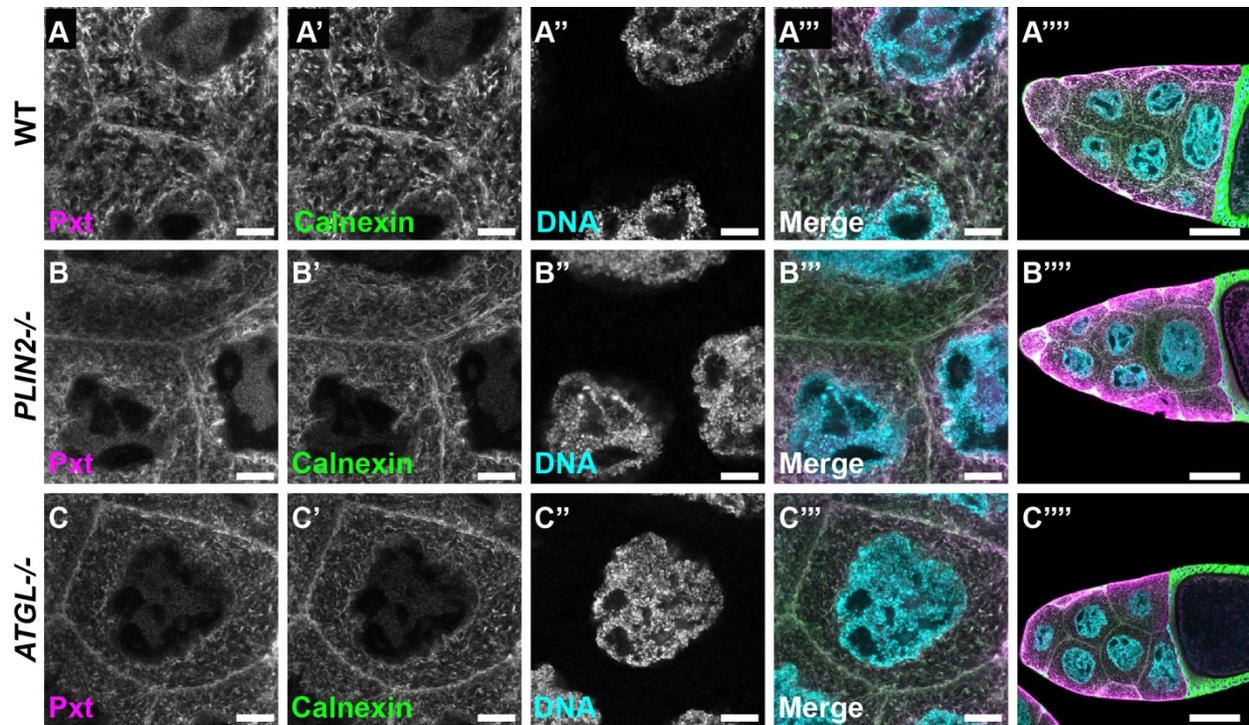
505

506 **Figure 7: Pxt is not enriched on LDs.**

507 (A-C''') Single confocal slices of wild-type (Oregon R) nurse cells of the indicated stages,

508 stained for Pxt (A-C), organelle marker (A', B', and C'), and DNA (A'', B'', and C'', Hoechst).

509 Merged image (**A'''**, **B'''** and **C'''**): Pxt, magenta; organelle marker, green; and DNA, cyan.
510 Organelle marker: LDs (**A'**, **A'''**; Nile red); Golgi-eYFP (**B'**, **B'''**); and ER-eYFP (**C'**, **C'''**). Scale
511 bars=10 μ m. (**D**) Western blots of purified LDs and a dilution series of ovary lysate (OL) for
512 Calnexin (magenta) and Pxt (green) in the top half of the blot and α -Tubulin (magenta) and
513 PLIN2 (green) in the bottom half of the blot; dashed line indicates where the membrane was cut.
514 (**E**) Quantification the abundance of proteins in the LD fraction relative to undiluted ovary lysate
515 (OL). The ratio of signal for the LD and OL lane in Western blots like in (**D**) were computed for
516 the indicated proteins and normalized to the average value for PLIN2. Error bars, SD.
517 $**p < 0.0028$, Tukey's multiple comparisons test. (**F-F'''**) Single confocal slices of centrifuged
518 wild-type Stage 10B follicle stained for Pxt (**F**), LDs (**F'**, Nile red), and DNA (**F''**, Hoechst).
519 Merged image (**F'''**): Pxt, magenta; LDs, green; and DNA, cyan. Scale bars=50 μ m. Pxt does not
520 colocalize with LDs (**A-A'''**), is not enriched on purified LDs (**D-E**), and does not co-accumulate
521 with LDs in centrifuged follicles (**F-F'''**). Instead, Pxt localizes to the Golgi during S 8 (**B-B'''**)
522 and the ER during S10B (**C-C'''**).
523
524



525

526 **Figure 7 – supplemental figure 1: ATGL and PLIN2 do not affect Pxt's localization to the**

527 **ER.**

528 (A-C''''') Single confocal slices of S10B nurse cells of the indicated genotypes, stained for Pxt

529 (A-C), Calnexin (A', B' and C', ER marker), and DNA (A'', B'' and C'', Hoechst). Merged image

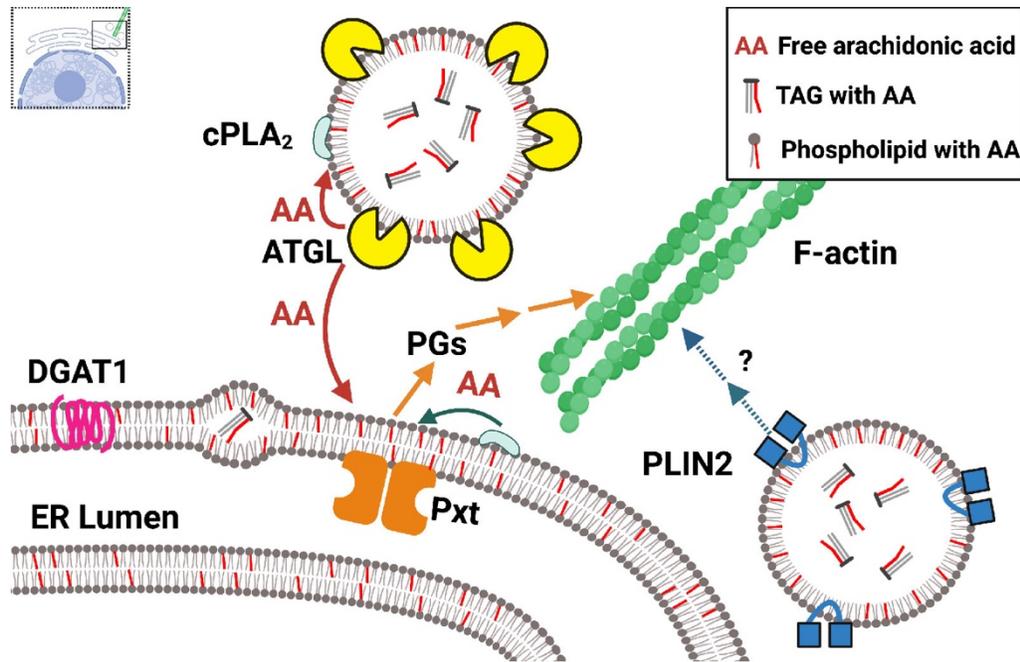
530 (A''', B''' and C'''''): Pxt, magenta; Calnexin, green; and DNA, cyan. Genotypes: WT, wild-type

531 (Oregon R); *PLIN2*^{-/-} (*LSD-2*^{KG00149}/*LSD-2*^{KG00149}); *ATGL*^{-/-} (*bmm*¹/*bmm*¹), and *pxt*^{-/-}

532 (*pxt*^{#01000}/*pxt*^{#01000}). Scale bars = 10µm. Black boxes were added under panel and/or channel

533 labels in A, A' and A''' to aid in visualization. In S10B follicles, Pxt's localization to the ER is

534 similar to wild-type (A-A''') in *PLIN2* (B-B''') and *ATGL* mutants (C-C''').



535

536 **Figure 8: LD proteins regulate actin remodeling via two pathways.**

537 Schematic (created with Biorender.com) summarizing the findings presented. Synthesis of TAG
538 (triglyceride) by DGAT1 (magenta) leads to accumulation of TAG between the inner and outer
539 leaflets of the ER membrane and eventually LD budding from the ER (not shown). Arachidonic
540 acid (AA, red lines) is stored either in the form of TAG or is incorporated into the phospholipid
541 membrane. We identified two pathways by which LD proteins regulate actin remodeling. In the
542 first pathway, ATGL (yellow) can liberate AA from TAG stored in LDs, providing the substrate,
543 either directly or indirectly, for Pxt and PG production (orange) to regulate actin remodeling. The
544 AA freed from TAG by ATGL (red) might be supplied directly to Pxt or be first incorporated into
545 phospholipids (surrounding the neutral lipid core of LDs or present in the ER or other cellular
546 membranes). In a subsequent step, cPLA₂ (light blue) would then release AA from these
547 phospholipids to provide the substrate for PG production. In the second pathway, PLIN2 (blue)
548 regulates actin remodeling by a PG- and ATGL-independent mechanism.

549

550

551 **Discussion**

552 Our data reveal that the LDs produced during *Drosophila* oogenesis are not just lipid and
553 protein stores for the future embryo, but already play a critical role during follicle development.
554 In particular, loss of the triglyceride lipase ATGL results in cortical actin breakdown, cortical
555 contraction failure, and defective actin bundle formation during S10B of oogenesis, an unusual
556 combination of phenotypes also observed in flies lacking Pxt, the *Drosophila* COX-like enzyme
557 (Tootle and Spradling, 2008). Dominant genetic interactions and PG treatment of follicles *in vitro*
558 reveal that ATGL and Pxt act in the same pathway to regulate actin remodeling, with ATGL
559 upstream of Pxt. We propose that LDs provide AA as substrate for Pxt, and that the PGs
560 subsequently produced regulate actin dynamics. While PG signaling is known to have a
561 conserved role in regulating actin remodeling across organisms and cell-types (Peppelenbosch
562 *et al.*, 1993; Pierce *et al.*, 1999; Dormond *et al.*, 2002; Tamma *et al.*, 2003; Bulin *et al.*, 2005;
563 Birukova *et al.*, 2007), this study provides the first evidence linking LDs to PG-dependent actin
564 remodeling.

565

566 *LDs provide the substrate for PG production*

567 The enzymatic release of AA from cellular lipids is the rate limiting step for PG
568 production (Funk, 2001; Tootle, 2013). The free AA that serves as Pxt substrate might originate
569 from two different sources: phospholipids and triglycerides. In mammalian systems, both
570 sources make contributions to PG synthesis (Weller and Dvorak, 1994). Cytoplasmic
571 phospholipase A2 (cPLA2) releases AA from phospholipids, and in many cell types, inhibiting
572 cPLA2 severely impairs PG production (Barbour and Dennis, 1993; Miyaura *et al.*, 2003; Ghosh
573 *et al.*, 2004). Conversely, knockdown/inhibition of ATGL decreases PG production in mast cells
574 (Dichlberger *et al.*, 2014) and leukocytes (Schlager *et al.*, 2015).

575 Our data support the model that in *Drosophila* follicles LD triglycerides are a major
576 source of AA for PG production and that ATGL is responsible for its release. This AA might

577 either directly serve as substrate for Pxt, or it might first be incorporated into phospholipids, to
578 be released by cPLA2 in a subsequent step (Figure 8). Such a model explains the genetic
579 interaction between Pxt and ATGL (Figure 3B, D, E, and H and Figure 4A), the increase in AA-
580 containing ovary triglycerides in *ATGL* mutants (Figure 5G and H), the fact that exogenous
581 $\text{PGF}_{2\alpha}$ can promote maturation of *ATGL* mutant follicles (Figure 4B), and that reducing ATGL
582 suppresses the lipotoxicity of exogenous AA (Figure 6D).

583 Our studies also provide the first direct evidence that AA has a functional role during
584 *Drosophila* oogenesis: AA is present in ovary triglycerides (Figure 5A, G and H); exogenous AA
585 has both beneficial and detrimental effects on follicle development (Figure 6A), depending on
586 concentration; and DGAT1 and ATGL heterozygosity shift the sensitivity of follicles to
587 exogenous AA (Figure 6B-D). These findings are particularly important as it has been debated
588 whether AA is present in flies. Some studies report that AA is not detectable in various life
589 stages and body parts (Yoshioka *et al.*, 1985; Shen *et al.*, 2010; Tan *et al.*, 2016), while others
590 find it (Steinhauer *et al.*, 2009; Parisi *et al.*, 2011; Sieber and Spradling, 2015). These
591 differences may be due to varying detection sensitivities and the fact that lipid profiles vary
592 dramatically between tissues and/or on different diets (Carvalho *et al.*, 2012).

593

594 Site of PG synthesis

595 In a variety of mammalian cell types, the PG synthesis machinery localizes to LDs and
596 produces PGs there (Bozza *et al.*, 2011). For example, cPLA2, and its activators, localize to
597 LDs and provide AA for PG production (Yu *et al.*, 1998; Moreira *et al.*, 2009), and COX2 and
598 PGE_2 synthases localize to and produce PGE_2 on LDs in cultured colon adenocarcinoma cells
599 (Accioly *et al.*, 2008). Similarly, PGE_2 production is observed on LDs in normal rat intestinal
600 epithelial cells (Moreira *et al.*, 2009) and in macrophages during parasitic infection (D'Avila *et*
601 *al.*, 2011).

602 In fly ovaries, in contrast, the site of PG synthesis remains unknown. By
603 immunofluorescence or biochemical purification, Pxt is not enriched on LDs, but is present
604 throughout the ER (Figure 7). Two different scenarios might account for these observations. On
605 one hand, AA released from LDs may reach only Pxt molecules that are in closely associated
606 ER regions, while Pxt further away never sees substrate. As both ER and LDs are highly
607 abundant in the cytoplasm of S10B follicles (Figure 1C and Figure 7C'), AA from LDs could
608 quickly reach Pxt in close proximity. In addition, in many cells, LDs remain physically connected
609 to the ER via lipidic bridges, which could convey AA to nearby ER (Hugenroth and Bohnert,
610 2020). On the other hand, AA released from LDs might get incorporated into phospholipids in
611 the ER, which can then diffuse throughout the entire ER; in a second step, AA is then released
612 by cPLA2. In this scenario, LD derived AA would indirectly provide substrate to Pxt anywhere in
613 the ER. To resolve exactly where in nurse cells PG synthesis occurs, it will be important to
614 determine if cPLA2 and PG-type specific synthases are enriched on LDs or elsewhere and to
615 directly visualize the site of PG production.

616 We find that LDs can still function as critical regulators of PG signaling even when the
617 relevant COX enzyme shows no particular LD association. This finding should prompt a careful
618 re-examination of the functional impact of LDs in the many PG-producing cell types in which
619 COX enzymes are distributed throughout the ER, nuclear envelope, and Golgi, similar to
620 *Drosophila* follicles. Given the emerging role of LDs as central hubs for cellular fatty acid
621 trafficking, they may regulate PG synthesis much more widely than previously recognized.

622

623 Why LDs?

624 If AA released from phospholipids can serve as substrate for Pxt, why is AA incorporated into
625 LDs at all? AA in triglycerides may act as a storage form of AA for the embryo, to be
626 incorporated there into new membranes and possibly to also sustain PG production. However,
627 our experiments in Figure 6 raise the additional possibility that LDs are needed to buffer the

628 toxicity of free AA. We find that high levels of exogenous AA inhibit follicle development (Figure
629 6A), similar to the case of bovine granulosa cells where AA induces apoptosis (Zhang *et al.*,
630 2019). In fact, AA is cytotoxic for many cells, e.g., cultured hippocampal neurons (Okuda *et al.*,
631 1994), even at concentrations that overlap physiological ones (Pompeia *et al.*, 2003). This
632 toxicity is likely due to AA's many diverse roles in cellular signaling (Brash, 2001; Tallima and El
633 Ridi, 2018). Because of such toxicity, levels of free AA are tightly regulated across organisms,
634 often by turning excess AA into inert triglycerides stored in LDs. Indeed, in mammalian cells,
635 exogenous AA is initially almost exclusively incorporated into LDs (Weller and Dvorak, 1994;
636 Bozza *et al.*, 2009; Bozza *et al.*, 2011). LDs likely have a similar buffering role in *Drosophila*
637 follicles, as reduced ability to incorporate AA into triglycerides (in DGAT1 heterozygotes)
638 increases AA toxicity, while reduced ability to release AA from triglycerides (in ATGL
639 heterozygotes) ameliorates toxicity to exogenous AA (Figure 6B-D).

640 It seems likely that LDs also buffer AA released from endogenous sources. Because
641 *Drosophila* lacks the enzymes to synthesize AA (Shen *et al.*, 2010; Suito *et al.*, 2020), AA
642 present in the ovary must be derived from food and be transported, like other FAs, through the
643 hemolymph via lipoprotein particles. These lipoproteins are captured by lipophorin receptors at
644 the surface of nurse cells which deliver FAs to the germ line (Parra-Peralbo and Culi, 2011).
645 Because in the wild nutrient availability fluctuates, AA flux into follicles is likely variable and
646 unpredictable, potentially putting follicles at risk. Thus, we propose that storing AA in
647 triglycerides in LDs avoids the dangers of high levels of free AA while allowing release of just
648 enough AA to support PG synthesis.

649 Such an AA buffering function of LDs aligns with the recent recognition of LDs as a
650 critical way station during FA trafficking in cells (Welte, 2015); they transiently sequester excess
651 FAs from external and internal sources to prevent cellular damage, such as ER stress and
652 mitochondrial dysfunction (Chitraju *et al.*, 2017; Nguyen *et al.*, 2017). Once safely stored in LDs,
653 FAs can then be released in a regulated manner and directed to specific intracellular fates, like

654 energy production or signaling (Haemmerle *et al.*, 2011; Rambold *et al.*, 2015). Thus, LDs serve
655 as FA trafficking hubs to both channel FAs to the correct intracellular destinations and buffer the
656 FA supply.

657 Our finding may reflect a broader function of LDs to handle essential, but potentially
658 harmful factors during development. Indeed, *Drosophila* oocytes accumulate large amounts of
659 histones needed to support embryonic development, but free histones are cytotoxic. For a
660 subset of histones this problem is solved by sequestering them to LDs (Li *et al.*, 2012;
661 Stephenson *et al.*, 2021). Thus, for both histones and AA, LDs limit the availability of potentially
662 toxic molecules by sequestering them and thus keeping them available for later use.

663

664 ATGL functions in development

665 Like in mammals, ATGL in flies is best known for its role in fat storage and energy
666 homeostasis in adipose tissue (Gronke *et al.*, 2005). ATGL is highly expressed in the larval and
667 adult fat body; its absence causes triglyceride overaccumulation in this tissue, while
668 overexpression depletes organismal fat stores (Gronke *et al.*, 2005). In this context, ATGL and
669 PLIN2 act antagonistically, with ATGL promoting triglyceride breakdown and PLIN2 preventing it
670 (Gronke *et al.*, 2003; Beller *et al.*, 2010; Bi *et al.*, 2012; Zhao *et al.*, 2022). While our
671 experiments do not address whether a similar antagonism operates in follicles, PLIN2 must
672 affect actin remodeling via a PG-independent mechanism since loss of PLIN2 causes actin
673 defects (Figure 2B) and there is no synergistic effect between PLIN2 and ATGL/Pxt in our
674 dominant genetic interaction tests (Figure 3C, G and I). One speculative possibility is that PLIN2
675 recruits actin and actin binding proteins to LDs and either controls their concentration in the
676 cytoplasm or delivers them to sites of actin remodeling. Indeed, actin and actin regulators are
677 candidate LD proteins in a number of cell types (e.g., Fong *et al.*, 2001; Pfisterer *et al.*, 2017;
678 Bersuker *et al.*, 2018; Kilwein and Welte, 2019; Tan *et al.*, 2021), and PLIN2 regulates LD
679 motility (Welte *et al.*, 2005).

680 Here we show that ATGL has a critical role in follicle development, via PG signaling and
681 actin remodeling. ATGL has previously been implicated in other critical developmental
682 transitions, though the underlying mechanisms remain unclear. In male flies, ATGL in neurons
683 and testes regulates whole-body energy storage by an as-yet uncharacterized mechanism
684 thought to involve systemic signaling (Wat *et al.*, 2020). And in mammalian muscle, ATGL in
685 satellite cells is important for proper differentiation and efficient recovery from muscle injury
686 (Yue *et al.*, 2022). It will be interesting to determine whether in these instances ATGL also acts
687 via AA release to drive PG production. Finally, ectopic ATGL expression has beneficial roles in
688 two *Drosophila* disease models: neurodegeneration due to mitochondrial dysfunction (Liu *et al.*,
689 2015) and impairment of nephrocytes, components of the renal system, due to high fat diet
690 (Lubojevska *et al.*, 2021). Here, tissue-specific overexpression of ATGL in glia or nephrocytes,
691 respectively, reverts disease-induced LD accumulation as well as disease phenotypes.
692 Intriguingly, glia cells in other organisms activate PG signaling during brain
693 inflammation/neurodegeneration (Tzeng *et al.*, 2005; Lima *et al.*, 2012); and in the nephrocyte
694 example, ectopic ATGL affects transcription via lipid signaling, but exact mechanisms remain
695 unknown. Our work identifies a specific signaling pathway, PG signaling, regulated by ATGL
696 during oogenesis. It will be important to determine how widely used this new signaling pathway
697 is, given the strong potential for PGs to play roles in tissues with known ATGL functions.

698

699 *LDs as regulators of female fertility*

700 Animal development requires careful regulation of metabolism, specific to tissue type
701 and developmental stage (Sieber and Spradling, 2017). Yet despite the fact that LDs have a
702 central role in lipid metabolism and energy homeostasis in normal physiology and diseases
703 (Walther and Farese, 2012; Hashemi and Goodman, 2015; Welte, 2015), they have so far been
704 implicated as regulators of development in only a handful of cases (Chan *et al.*, 2007; Li *et al.*,
705 2012; Johnson *et al.*, 2018; Stephenson *et al.*, 2020; Yue *et al.*, 2022). Our analysis now reveals

706 that LDs not only play a critical role at a specific transition during follicle development, but
707 identifies PG signaling as one of the mechanisms by which LDs exert their regulatory role.

708 We speculate that this pathway is conserved across organisms. Indeed, LD
709 accumulation, composition, and localization are dynamic during oocyte maturation (Ami *et al.*,
710 2011; Dunning *et al.*, 2014), LDs are hubs for FA trafficking, and FA levels (including that of AA)
711 are critical for oocyte development in many species (Dunning *et al.*, 2014; Prates *et al.*, 2014;
712 Brusentsev *et al.*, 2019). Further, in both mouse models and human patients, metabolic
713 syndrome causes failures in oocyte maturation and decreased fertility (Wattanakumtornkul *et*
714 *al.*, 2003; Jungheim *et al.*, 2010; Cardozo *et al.*, 2011; Marei *et al.*, 2020). Specifically, obesity
715 seems to directly impair oocyte quality, as fertilized oocytes from healthy donors can be
716 successfully transplanted into obese recipients without affecting implantation rates or pregnancy
717 success (Styne-Gross *et al.*, 2005). In addition, it is well-established that PG signaling plays
718 critical roles in oocyte development across organisms (Akil *et al.*, 1996; Smith *et al.*, 1996; Lim
719 *et al.*, 1997; Pall *et al.*, 2001; Wang *et al.*, 2004; Takahashi *et al.*, 2006; Takahashi *et al.*, 2010).
720 Recent evidence also points to a link between LDs and PGs, and implicates LDs as a foci of PG
721 production during reproduction. For example, COX enzymes localize to LDs in the rat corpus
722 luteum (Arend *et al.*, 2004), and in fetal membranes during advanced gestation and at induction
723 of labor, times when PG synthesis and signaling are upregulated (Meadows *et al.*, 2003;
724 Meadows *et al.*, 2005). Whether the PG effects on oogenesis and reproduction in mammals are
725 due to changes in the actin cytoskeleton is not yet known. However, cytoplasmic actin density
726 and cortical actin thickness increase during oocyte maturation, contribute to meiotic resumption,
727 and play roles in fertilization and early embryonic divisions (Coticchio *et al.*, 2015; Namgoong
728 and Kim, 2016). Given these data, and our findings connecting LDs, PG signaling, and actin
729 remodeling during *Drosophila* oogenesis, we speculate the same pathways that regulate oocyte
730 development in the fly are conserved across organisms to regulate oocyte development, and
731 contribute to infertility issues due to limited or excess nutrition.

732 **Materials and Methods**

733 **Key Resources Table**

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
genetic reagent (<i>Drosophila melanogaster</i>)	<i>pxt</i> ^{EY03052}	Bloomington <i>Drosophila</i> Stock Center	FLYB: FBgn0261987; RRID:BDSC_15620	
genetic reagent (<i>Drosophila melanogaster</i>)	<i>pxt</i> ^{f01000}	Harvard Exelixis Collection; Thibault, 2004 PMID: 14981521		
genetic reagent (<i>Drosophila melanogaster</i>)	<i>Lsd-2</i> ^{KG00149}	Bloomington <i>Drosophila</i> Stock Center	FLYB: FBgn0030608; RRID:BDSC_13382	
genetic reagent (<i>Drosophila melanogaster</i>)	<i>bmm</i> ¹	Gronke et al., 2005 PMID: 16054079		
genetic reagent (<i>Drosophila melanogaster</i>)	<i>y</i> ¹ <i>w</i> ¹	Bloomington <i>Drosophila</i> Stock Center	RRID:BDSC_1495	
genetic reagent (<i>Drosophila melanogaster</i>)	<i>Oregon R</i>	Bloomington <i>Drosophila</i> Stock Center	RRID:BDSC_5	
genetic reagent (<i>Drosophila melanogaster</i>)	<i>mdy</i> ^{QX25}	Bloomington <i>Drosophila</i> Stock Center	RRID:BDSC_5095	
genetic reagent (<i>Drosophila melanogaster</i>)	<i>ER-eYFP</i>	Bloomington <i>Drosophila</i> Stock Center; LaJeunesse, 2004	RRID:BDSC_7195	
genetic reagent (<i>Drosophila melanogaster</i>)	<i>Golgi-eYFP</i>	Bloomington <i>Drosophila</i> Stock Center; LaJeunesse, 2004	RRID:BDSC_7193	
antibody	Alexa Fluor 488 Phalloidin	Thermo Fisher Scientific (Invitrogen)	Cat#A12379	lot 1262669 (1:250)

antibody	Alexa Fluor 647 Phalloidin	Thermo Fisher Scientific (Invitrogen)	Cat#A22287	lot 2151755 (1:250)
antibody	Alexa Fluor 568 Phalloidin	Thermo Fisher Scientific (Invitrogen)	Cat#A12380	lot 2170291 (1:250)
antibody	Nile Red	Sigma-Aldrich	Cat#72485 CAS Number 7385-67-3	(1:5000)
antibody	mouse anti-Calnexin99A	Developmental Studies Hybridoma Bank, Munro, S.	Cat#Cnx99A 6-2-1 RRID:AB272201 1	(1:1000)
antibody	mouse anti-Golgin84	Developmental Studies Hybridoma Bank, PMID:30910009	Cat#Golgin84 12-1 RRID:AB272211 3	(1:1000)
antibody	rabbit anti-pxt	Spracklen, 2014 PMID:24284900	RRID:AB_2569638	3873S (1:1000)
antibody	mouse anti-Tubulin	Cell Signaling Technologies, Welte, 2005 PMID:15647372	DM1A RRID:AB_1904178	(1:5000)
antibody	rabbit anti-PLIN2			(1:5000)
antibody	goat anti-mouse IgG Alexa Fluor 488	Thermo Fisher (Invitrogen)	RRID:AB_2536161	(1:1000)
antibody	goat anti-rabbit IgG Alexa Fluor 633	Thermo Fisher (Invitrogen)		(1:1000)
antibody	goat anti-rabbit IgG Alexa Fluor 488	Thermo Fisher (Invitrogen)		(1:1000)
chemical compound, drug	arachidonic acid	Cayman Chemical Company	Cat#90010 CAS Number 506-32-1	Batch No. 6570304-49
chemical compound, drug	aspirin	Cayman Chemical Company	Cat#70260 CAS Number 50-78-2	Batch No. 0479934-28

chemical compound, drug	oleic acid	Cayman Chemical Company	Cat#90260 CAS Number 112-80-1	
chemical compound, drug	Hoechst	Thermo Fisher	Cat#62249	(1:1000)
chemical compound, drug	DAPI (4'6-diamidino-2-phenylindole)	Thermo Fisher (Invitrogen)	D3571	lot 699931 (1:1000)
chemical compound, drug	Fluprostenol	Cayman Chemical Company	Cat#16768 CAS Number 54276-17-4	
chemical compound, drug	Calyculin A (10g) (Serine/Threonine Phosphatase Inhibitor)	Cell Signaling	Cat#9902S	
chemical compound, drug	Protease Inhibitor Cocktail	Sigma-Aldrich	Cat#P2714-1BTL	
software, algorithm	R Project for Statistical Computing	www.r-project.org	RRID:SCR_001905	
software, algorithm	FIJI-FIJI is Just ImageJ	PMID 22743772	RRID:SCR_002285	software, algorithm
software, algorithm	ZEN Digital Imaging for Light Microscopy	Carl Zeiss	RRID:SCR_013672	software, algorithm
software, algorithm	GraphPad Prism	GraphPad	RRID:SCR_002798	software, algorithm
software, algorithm	Image Studio Lite	Li-COR	RRID:SCR_013715	software, algorithm
software, algorithm	Leica Application Suite X	Leica Microsystems	RRID:SCR_013673	software, algorithm
other	Zeiss LSM 700 microscope	Carl Zeiss	RRID:SCR_017377	other
other	Zeiss LSM 880 with Airy Scan Cofocal microscope	Carl Zeiss	RRID:SCR_020925	other
other	Leica TCS SPE confocal microscope	Leica Microsystems	RRID:SCR_002140	other
other	Aqua Polymount	PolySciences	Cat#18606-100	other

735 Fly stocks

736 Flies used in actin quantification and IVEM experiments were maintained on
737 cornmeal/agar/yeast food at room temperature except as noted below. Flies used in all other
738 experiments were maintained on molasses/agar/yeast/malt extract/corn flour/soy flour food at
739 room temperature except as noted below. Stocks used were y^1w^1 (BDSC1495), Oregon R
740 (BDSC 5), pxt^{f01000} (Harvard Exelixis Collection, (Thibault *et al.*, 2004)), $pxt^{EY03052}$ (BDSC 15620),
741 bmm^1 (Gronke *et al.*, 2003), $Lsd-2^{KG00149}$ (BDSC 13382), mdy^{QX25} (BDSC 5095), $sqh-EYFP-ER$
742 (BDSC 7195, (LaJeunesse *et al.*, 2004)), and $sqh-EYFP-Golgi$ (BDSC 7193, (LaJeunesse *et al.*,
743 2004)).

744

745 Immunofluorescence and fluorescent reagent staining

746 For Figures 1, 7 and 7 – supplemental figure 1 the following method, referred to as Staining
747 Method 1, was used. Adult female and male flies (to allow for mating) younger than two weeks
748 old were fed dry yeast for 48 hours at room temperature before ovary dissection in PBS-T
749 (phosphate buffered saline, 0.1% Triton X-100). Ovaries were fixed with 3.6% formaldehyde in
750 PBS for 12 minutes at room temperature. Ovaries were washed with PBS-T, and follicles of
751 desired stages were isolated using forceps and pin vices. Follicles were blocked in ovary block
752 (10% BSA, PBS, 0.1% Triton X-100, 0.02% sodium azide) overnight at 4°C. Primary antibodies
753 were diluted in ovary block and incubated overnight at 4°C. The following primary antibodies
754 were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the
755 auspices of the National Institute of Child Health and Human Development and maintained by
756 the Department of Biology, University of Iowa (Iowa City, Iowa): mouse anti-Calnexin99A at
757 1:1000 (Cnx99A 6-2-1, Munro, S.) and mouse anti-Golgin84 at 1:1000 (Golgin84 12-1, Munro,
758 S.) (Riedel *et al.*, 2016). Additionally, the following primary antibodies were used: rabbit anti-Pxt
759 preabsorbed at 1:10 on $pxt^{-/-}$ ovaries in ovary block and used at 1:1000 (Spracklen *et al.*, 2014).
760 Samples were washed 3x with PBS-T, and then protected from light for the remainder of the

761 experiment. Samples were incubated in the following secondary antibodies diluted to 1:1000 in
762 ovary block overnight at 4°C: goat anti-rabbit IgG Alexa Fluor 633 (Invitrogen), goat anti-mouse
763 IgG Alexa Fluor 488 (Invitrogen), and goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen). Samples
764 were washed 3x with PBS-T and then stained with the following reagents diluted in ovary block:
765 Phalloidin Alexa Fluor 633 (Invitrogen) 1:150, 1 hour at room temperature; Nile Red (1 mg/ml,
766 Sigma-Aldrich) 1:50, 1 hour at room temperature; and Hoechst 33342 (1 mg/ml, Thermo Fisher
767 Scientific) 1:1000, 20 minutes at room temperature. Samples were washed 3x with PBS-T, and
768 then mounted on coverslips in Aqua Polymount (PolySciences).

769 For Figures 2, 3, and 3 - supplemental figure 1 the following protocol, referred to as
770 Staining Method 2, was used: Adult female and male flies (to allow for mating) within 24hrs of
771 eclosion were fed wet yeast paste for 72 hours at room temperature before dissection in room-
772 temperature Grace's medium (Lonza). Ovaries were fixed in 4% paraformaldehyde in Grace's
773 medium for 10 minutes at room temperature. Ovaries were washed 6x 10 minutes in antibody
774 wash (1X PBS-T + 0.1% Bovine Serum Albumin (BSA)). Samples were stained overnight at 4°C
775 with Alexa Fluor-488, -568, or -647 Phalloidin (Invitrogen) at 1:250. Samples were then washed
776 5-6 times in 1X PBS-T for 10 minutes each and stained with 4'6'-diamidino-2-phenylindole
777 (DAPI, 5 mg/mL) at 1:5000 in 1X PBS for 10 minutes. Samples were rinsed in 1X PBS and
778 mounted in 1 mg/mL phenylenediamine in 50% glycerol, pH 9 (Platt and Michael, 1983).

779

780 Image acquisition and processing

781 Microscope images of fixed and stained *Drosophila* follicles were obtained using the following
782 microscopes: Leica SPE confocal microscope with an ACS APO x20/0.60 IMM CORR-/D or
783 ACS APO x40/1.5 oil CS objective (Leica Microsystems), Zeiss 700 confocal microscope or
784 Zeiss 880 confocal microscope (Carl Zeiss Microscopy) using a Plan-Apochromat x20/0.8
785 working distance (WD)=0.55 M27 objective, Zeiss 980 confocal microscope (Carl Zeiss
786 Microscopy) using a PIn Apo x20/0.8 or PIn Apo 40x/1.3 oil objective, or Leica SP5 confocal

787 microscope (Leica Microsystems) using an HCX PL APO CS 63.0x1.40 oil UV objective and
788 Leica HyD detectors. Maximum projections of images, image cropping, and image rotation were
789 performed in Fiji/ImageJ software (Abramoff *et al.*, 2004), scale bars were added in either
790 Adobe Illustrator or Adobe Photoshop (Adobe, San Jose, CA) and Figures were assembled
791 using Adobe Illustrator. Images in Figures 2, 3, and 3 – supplemental figure 1 were brightened
792 by 30% in Adobe Photoshop to improve visualization.

793

794 Quantification of actin defects

795 Confocal images of phalloidin stained S10B follicles were collected as described. Actin bundle
796 and cortical actin defects were scored by scanning through confocal z-stacks of S10B follicles in
797 ImageJ in a genotypically blinded manner. Representative images of follicles and scoring
798 criteria are provided in Figure 3 – supplemental figure 1. Briefly, actin bundles were scored as
799 normal (score = 0) if they were straight, forming from the nurse cell plasma membrane and
800 oriented inwards toward the nurse cell nuclei. Mild defects in bundling (score = 1) were defined
801 as sparse bundle formation or slight delay in bundle growth relative to follicle development.
802 Moderate defects (score = 2) were defined as collapsed, thick, and/or missing bundles from
803 regions of the nurse cell plasma membrane. Severe defects (score=3) included those previously
804 described, as well as a complete failure of bundles to form. Cortical actin was scored based on
805 whether the cortical actin was intact or disrupted. Defects in cortical actin are evident by an
806 absence or incomplete phalloidin staining between nurse cells and/or by nurse cell nuclei in
807 close proximity or contacting each other. Normal cortical actin (score = 0) is defined as being
808 completely intact and fully surrounding each nurse cell. Degree of severity of cortical actin
809 defect was determined by the relative number of disruptions in cortical actin observed with mild
810 defects (score=1) having a single instance, moderate defects (score=2) having two instances,
811 and severe defects (score = 3) having three or more instances of disrupted cortical actin. The
812 actin defect index (ADI) was then calculated by adding the bundle and cortical actin defect

813 scores and binning them into normal (total score = 0-1), mild defects (total score = 2-3), or
814 severe defects (score = 4-6). Pearson's chi-squared analysis was performed using R ([www.r-](http://www.r-project.org)
815 [project.org](http://www.r-project.org)).

816

817 *In vitro egg maturation (IVEM) assays*

818 For both the standard IVEM and modified IVEM assays, adult female and male flies (to allow for
819 mating) within 24hrs of eclosion were fed wet yeast paste daily for 3 days prior to dissection.
820 Ovaries were dissected in room temperature IVEM or modified IVEM medium. IVEM medium:
821 Grace's medium (Lonza), 1x penicillin/streptomycin (100x, Gibco) and 10% fetal bovine serum
822 (FBS, Atlanta Biologicals). Modified IVEM medium: Grace's medium, 1x penicillin/streptomycin,
823 and 2.5% FBS. S10B follicles were isolated and distributed between wells of a 24-well plastic
824 tissue culture plate (Falcon) and 1ml of fresh medium was added with or without additions
825 (described below). Follicles were incubated overnight at room temperature in the dark. The next
826 day the number of follicles at each developmental stage were counted, and the percentage
827 developing was calculated; follicles that reached S12 and older were considered developed.
828 Statistical analysis was performed using the unpaired t-test function in Prism 8 or 9 (GraphPad
829 Software).

830 For the exogenous PGF_{2α} analog (Fluprostenol, Flu) experiments the standard IVEM
831 was performed and stock solutions of 10μM Flu (Cayman Chemical Company) and 0.5M aspirin
832 (Cayman Chemical Company) were prepared in 100% EtOH. For each genotype, there were
833 two wells of S10B follicles with one well treated with EtOH (control) and the other with Flu (final
834 concentration of 20nM). As an additional control, wild-type follicles were also treated with
835 1.5mM aspirin to verify that Flu rescues the loss of PG synthesis; any experiment where Flu
836 failed to suppress the effects of aspirin was excluded from the analysis. In all conditions the total
837 amount of EtOH was kept constant. Follicle development was assessed as described above.

838 For the exogenous fatty acid (AA or OA) experiments the modified IVEM was performed,
839 AA and OA stock solutions were made in 100% EtOH, and total EtOH volumes were kept
840 constant in each experiment. Follicle development was assessed as described above.

841

842 Western blots

843 Follicles from well-fed females of the indicated genotypes and stage were dissected and fixed
844 as described in Staining Method 1. Note that proteins from such samples run at the same
845 molecular weight as those from lysates prepared from live ovary tissue (Figure 4- supplemental
846 figure 1C-D). 25-50 follicles were collected per sample and boiled in sample buffer (Laemmli
847 Sample Buffer, Bio-Rad + 2-mercaptoethanol, Bio-Rad). Samples were run on 4-15% gradient
848 gels (Bio-Rad) at 80-120V and transferred onto PVDF membrane (Immobilon-FL, EMD
849 Millipore) in Towbin (10% Tris-Glycine + 20% MeOH; 80V for 30 minutes) transfer buffer.
850 Membranes were blocked for 1 hour at room temperature in Li-COR Odyssey Block (LI-COR
851 Biosciences). Primary antibodies diluted in Odyssey Block were performed overnight at 4
852 degrees. The following primary antibodies were used: rabbit anti-Pxt (1:5000) (Spracklen *et al.*,
853 2014), rabbit anti-PLIN2 (1:5000) (Welte *et al.*, 2005), mouse anti-Calnexin99A (1:5000, DSHB),
854 and mouse anti- α -Tubulin (1:5000, Cell Signaling Technologies). Membranes were washed 3
855 times in 1X PBS-0.1% Tween 20 (PBS-Tween). The following secondary antibody incubations
856 were performed for 1 hour at room temperature: IRDye 800CW Goat anti-Rabbit IgG (1:5000,
857 Li-COR), and IRDy3 680RD Goat anti-Mouse IgG (1:5000, Li-COR). After secondary antibody
858 incubation, membranes were washed two times in PBS-Tween and once in 1X PBS.
859 Membranes were imaged on a Li-COR Odyssey CLx imager and blots were processed and
860 quantified in Image Studio Lite 5.2 (Li-COR). To quantify, fluorescence intensity of each band
861 was measured and normalized to respective experimental controls. Data were analyzed and
862 plotted using Prism (GraphPad).

863

864 Lipid extractions

865 25 ovaries per sample were dissected from mated, mixed-age, and dry yeast fed females and
866 homogenized in a 2mL Eppendorf tube with a motorized pestle (KONTES pellet pestle) in 1X
867 PBS and kept on ice. Lysates were then incubated 1:1 in a 2:1 chloroform/methanol mixture
868 overnight at 4°C. Samples were spun in an Eppendorf Microcentrifuge (model 5415D) at max
869 speed at room temperature for 1 minute, and the bottom organic layer was transferred to a
870 0.65mL Eppendorf tube. Samples were then vacuum dried and either analyzed immediately or
871 stored at -80°C for thin layer chromatography (TLC) analysis or before being shipped on dry ice
872 for mass spectrometry analysis.

873

874 Thin layer chromatography

875 Evaporated lipids were resuspended in 10µL 2:1 chloroform/methanol and spotted on
876 dehydrated silica plates (EMD Millipore; dehydrated by baking for 30 minutes at 100°C). Plates
877 were placed in a chamber (Millipore Sigma/Sigma-Aldrich Z266000) pre-saturated for 30
878 minutes with Petroleum Ether/Diethyl Ether/acetic acid (32:8:0.8)) and allowed to develop until
879 the solvent almost reached the top of the plates, and the solvent line was quickly marked with a
880 pencil after removing plates from the chamber. The plates were then air-dried and briefly
881 submerged in charring solution (50% ethanol, 3.2% H₂SO₄, 0.5% MgCl₂). Plates were air-dried
882 briefly and were charred for 30 minutes at 120°C. Plates were imaged using a Bio-Rad Gel Doc.
883 Lipids were identified according to Knittelfelder & Kohlwein (Knittelfelder and Kohlwein, 2017)
884 and by comparing whole lipid extracts to lipid standards (Millipore Sigma, data not shown).
885 Colorimetric band intensities were quantified in Fiji and triglyceride or sterol ester bands were
886 normalized to cholesterol band intensity. Dunnett's multiple comparisons test was run in
887 GraphPad Prism for Figure 5F.

888

889 LC-MS/MS Mass Spectrometry Analysis

890 Extracted lipids were dissolved in corresponding volumes of 2:1 methanol:chloroform (v/v) and
891 5 μ l of each sample was injected for positive and negative acquisition modes, respectively.
892 Mobile phase A consisted of 3:2 (v/v) water/acetonitrile, including 10mM ammonium formate
893 and 0.1% formic acid, and mobile phase B consisted of 9:1 (v/v) 2- propanol/acetonitrile, also
894 including 10mM ammonium formate and 0.1% formic acid. Lipids were separated using an
895 UltiMate 3000 UHPLC (Thermo Fisher Scientific) under a 90 min gradient; during 0–7 minutes,
896 elution starts with 40% B and increases to 55%; from 7 to 8 min, increases to 65% B; from 8 to
897 12 min, elution is maintained with 65% B; from 12 to 30 min, increase to 70% B; from 30 to 31
898 min, increase to 88% B; from 31 to 51 min, increase to 95% B; from 51 to 53 min, increase to
899 100% B; during 53 to 73 min, 100% B is maintained; from 73 to 73.1 min, solvent B was
900 decreased to 40% and then maintained for another 16.9 min for column re-equilibration. The
901 flow-rate was set to 0.2mL/min. The column oven temperature was set to 55°C, and the
902 temperature of the autosampler tray was set to 4°C. Eluted lipids were analyzed using Orbitrap
903 Q Exactive (Thermo Fisher Scientific) Orbitrap mass analyzer. The spray voltage was set to 4.2
904 kV, and the heated capillary and the HESI were held at 320°C and 300°C, respectively. The S-
905 lens RF level was set to 50, and the sheath and auxiliary gas were set to 35 and 3 units,
906 respectively. These conditions were held constant for both positive and negative ionization
907 mode acquisitions. External mass calibration was performed using the standard calibration
908 mixture every 7 days. MS spectra of lipids were acquired in full-scan/data-dependent MS2
909 mode. For the full-scan acquisition, the resolution was set to 70,000, the AGC target was 1e6,
910 the maximum integration time was 50msec, and the scan range was m/z = 133.4–2000. For
911 data-dependent MS2, the top 10 ions in each full scan were isolated with a 1.0Da window,
912 fragmented at a stepped normalized collision energy of 15, 25, and 35 units, and analyzed at a
913 resolution of 17,500 with an AGC target of 2e5 and a maximum integration time of 100msec.
914 The underfill ratio was set to 0. The selection of the top 10 ions was subject to isotopic exclusion
915 with a dynamic exclusion window of 5.0sec. Mass spectrometry data was analyzed using

916 LipidSearch version 4.1 SP (Thermo Fisher Scientific). Identified lipid species with grade A and
917 B were manually curated. A total of 98 different triglyceride species were identified (Figure 5 –
918 supplemental table 1).

919 For the quantitative analysis in Figures 5A-D, 5G-H and Figure 5- supplemental figure 1,
920 four biological replicates containing 25 ovaries each from both wild-type and *ATGL* mutant
921 females were analyzed and the quantity of the 98 triglyceride species determined, relative to
922 background. One wild-type sample contained an order of magnitude less lipid and was
923 discarded as an outlier (Figure 5- supplemental table 1). In total, 25 different types of FAs were
924 identified in the ovary triglycerides. The abundance of each FA was estimated by summing the
925 amount of each triglyceride species containing that FA, weighted by how many times that FA is
926 represented in that triglyceride. For Figures 5A and B, this abundance is expressed as fraction
927 of the total amount of fatty acids such identified. For Figure 5C, the signal for all species of
928 phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, respectively, was
929 summed and normalized to the average of the wild type. For Figure 5D, the signal for all
930 triglyceride species was summed and normalized to the average of the wild type. To express
931 the abundance of various lipid species relative to total lipids recovered (Figure 5- supplemental
932 figure 1), values for various lipid species were divided by the sum of all lipids in the sample. For
933 Figure 5G and 5H, the signal for two AA-containing triglyceride species was computed, and
934 normalized to the average of the wild-type signal. Amounts and fractions were calculated using
935 Microsoft Excel and graphed using Prism 8 (GraphPad Software). Statistical tests for Figure 5
936 were as follows: 5A and 5C was Sidak's multiple comparisons test, 5B was Tukey's multiple
937 comparisons test, 5D was an unpaired t-test, two-tailed, and 5G-H were unpaired t-tests, two-
938 tailed.

939

940 Ovary centrifugation

941 Mated adult females of mixed ages fed dry yeast were anesthetized and beheaded before being
942 mounted in Eppendorf tubes filled with 2.5% apple juice agar, adapting a method previously
943 described for embryos (Tran and Welte, 2010). Tubes were spun for 10 minutes at 10,000 rpm
944 at room temperature using an Eppendorf Microcentrifuge (Model 5415D). Flies were removed
945 from agar with forceps and ovaries were isolated and treated as described in Staining Method 1
946 above.

947

948 Lipid droplet purification

949 250-300 ovaries per sample were rapidly dissected from mated and dry yeast fed females and
950 kept on ice in TSS (68 mM Na Cl₂, 0.03% Triton X-100) in 2mL Eppendorf tubes. Ovaries were
951 then washed 3x with TKM (50 mM Tris, pH 7.4, 25 mM KCl, 5 mM MgCl₂) to remove detergent.
952 TKM was removed and the volume of ovaries was estimated visually. Ovaries were kept on ice,
953 to which were added: 2 times the estimated ovary volume of TKM + 1M sucrose, protease
954 inhibitor cocktail to final concentration of 1X protease inhibitor cocktail (Sigma-Aldrich), and
955 calyculin A serine/threonine phosphatase inhibitor (10 μ L per mL of volume; Cell Signaling).
956 Ovaries were homogenized on ice by grinding with an automated tissue grinder (KONTES pellet
957 pestle) for 1-2 minutes, and then 20 μ L ovary lysate samples were transferred from the total
958 lysate to 0.65mL Eppendorf tubes and stored at -80°C. The remaining ovary lysates were spun
959 at 13200rpm for 10 minutes in an Eppendorf Microcentrifuge (Model 5145D) at 4°C. The
960 following was then added to samples slowly to avoid disturbing the LD layer: 300 μ L TKM + 0.5M
961 sucrose, 300 μ L TKM + 0.25M sucrose, and 400 μ L TKM (no sucrose). Tubes were spun for 20
962 minutes at 4°C, with the speed adjusted as follows: 1000rpm for 5 minutes, 5000rpm for 5
963 minutes, 13200rpm for 10 minutes. Purified LDs were then scooped off the top of the sucrose
964 gradient using a drawn Pasteur pipette loop (Fisher Scientific, loop drawn by holding over a
965 flame). LDs were washed off the pipette loop with 20 μ L TKM (no sucrose) and stored at -80°C
966 until analyzed.

967 In past Western analyses, we loaded equal amounts of protein from LD preparations and
 968 original tissue lysate for easy comparison (Li *et al.*, 2012). This approach was not feasible here
 969 because of the relatively low amounts of LDs recovered from the large volume of ovaries used.
 970 Instead, we compared the LD sample to dilutions of ovary lysate. Purified LDs and ovary lysates
 971 were diluted as indicated in 2X Laemmli Sample Buffer before being boiled for 30 minutes and
 972 subsequently run on 4-15% SDS-PAGE gradient gels (Western blot protocol as described
 973 above). Before the blocking step, the membrane was cut with a razor blade between 50 and
 974 75kDa and each half was treated separately. Quantification was performed as described above
 975 in Western blots.

976

977 **Table 1: Genotype by figures.** List of genotypes shown in the figures. * phenotypic example of
 978 genotype not related to the data presented in the manuscript.

Figure	Panel	Genotype
Figure 1	A	<i>Oregon R</i>
	B	<i>Oregon R</i>
	C	<i>Oregon R</i>
	D	<i>Oregon R</i>
	F	<i>Oregon R</i>
	G	<i>Oregon R</i>
	H	<i>Oregon R</i>
	I	<i>Oregon R</i>
	J	<i>Oregon R</i>
Figure 2	A	<i>y¹w¹</i>
	B	<i>Lsd-2^{KG00149}/Lsd-2^{KG00149}</i>
	C	<i>bmm¹/bmm¹</i>
	D	<i>pxt^{#01000}/pxt^{#01000}</i>
Figure 3	A	<i>Lsd-2^{KG00149}/+</i>
	B	<i>pxt^{#01000}/+</i>
	C	<i>Lsd-2^{KG00149}/+;; pxt^{#01000}/+</i>
	D	<i>bmm¹/+</i>
	E	<i>bmm¹/pxt^{#01000}</i>

	F	<i>Lsd-2</i> ^{KG00149/+;;} <i>bmm</i> ^{1/+}
	G	<i>Lsd-2</i> ^{KG00149/+}
		<i>pxt</i> ^{#01000/+}
		<i>Lsd-2</i> ^{KG00149/+;;} <i>pxt</i> ^{#01000/+}
	H	<i>bmm</i> ^{1/+}
		<i>bmm</i> ^{1/pxt} ^{#01000}
		<i>pxt</i> ^{#01000/+}
	I	<i>Lsd-2</i> ^{KG00149/+}
		<i>bmm</i> ^{1/+}
		<i>Lsd-2</i> ^{KG00149/+;;} <i>bmm</i> ^{1/+}
Figure 3- supplemental figure 1	Normal Actin Bundles	<i>y</i> ¹ <i>w</i> ¹
	Normal Cortical Actin	<i>y</i> ¹ <i>w</i> ¹
	Mild Actin Bundle Defects	*
	Mild Cortical Actin Defects	<i>bmm</i> ^{1/pxt} ^{#01000}
	Moderate Actin Bundle Defects	*
	Moderate Cortical Actin Defects	*
	Severe Actin Bundle Defects	*
	Severe Cortical Actin Defects	<i>bmm</i> ^{1/pxt} ^{#01000}
Figure 3- supplemental figure 2	A	Oregon R
		<i>Lsd-2</i> ^{KG00149/+}
		<i>Lsd-2</i> ^{KG00149/Lsd-2} ^{KG00149}
Figure 4	A	<i>bmm</i> ^{1/+}
		<i>pxt</i> ^{#01000/+}
		<i>bmm</i> ^{1/pxt} ^{#01000}
	B	<i>y</i> ¹ <i>w</i> ¹
		<i>bmm</i> ^{1/bmm} ¹
Figure 4- supplemental figure 1	A	Oregon R
		<i>bmm</i> ^{1/bmm} ¹
		<i>pxt</i> ^{#01000/pxt} ^{#01000}
	C-D	Oregon R
Figure 5	A-H	Oregon R
		<i>bmm</i> ^{1/bmm} ¹
Figure 5- supplemental Table 1		Oregon R
		<i>bmm</i> ^{1/bmm} ¹
Figure 5- supplement figure 1	A-D	Oregon R
		<i>bmm</i> ^{1/bmm} ¹

Figure 6	A	y^1w^1
	C	y^1w^1
		<i>mdyQX25/+</i>
	D	y^1w^1
<i>bmm¹/+</i>		
Figure 7	A-F'''	<i>Oregon R</i>
Figure 7- supplemental figure 1	A-A'''''	<i>Oregon R</i>
	B-B'''''	<i>Lsd-2^{KG00149}/Lsd-2^{KG00149}</i>
	C-C'''''	<i>bmm¹/bmm¹</i>

979

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