

1 **Lipid droplets regulate actin remodeling via prostaglandin signaling during *Drosophila***
2 **oogenesis**

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21 **Abstract**

22

23 To ensure fertility, it is paramount to understand the factors controlling oocyte quality. One
24 incompletely characterized factor contributing to oocyte quality is lipids. In somatic cells, a key
25 regulator of lipid metabolism is lipid droplets (LDs), the sites of intracellular fat storage. Yet the
26 role of LDs in fertility is poorly understood. Here we use *Drosophila* oogenesis as a model for
27 uncovering if and how LDs promote egg development. LD accumulation in nurse cells coincides
28 with dynamic actin remodeling necessary for late-stage follicle morphogenesis and fertility. Loss
29 of major LD proteins, including PLIN2, Jabba, and ATGL, disrupts both actin bundle formation
30 and cortical actin integrity; this unusual phenotype is also seen when Pxt, the enzyme
31 responsible for prostaglandin (PG) synthesis, is missing. Further, both pharmacologic and
32 genetic loss of PG synthesis or loss of PLIN2 or Jabba impairs intracellular LD dispersal. These
33 similar phenotypes suggest that PGs and LD proteins act in the same pathway. Dominant
34 genetic interaction studies indicate that there are three actin regulatory pathways: PLIN2
35 regulates actin remodeling independent of PG signaling, whereas Jabba and ATGL act in two
36 separate PG-dependent pathways to regulate actin remodeling. We find that neither Jabba nor
37 ATGL modulate the levels of Pxt or its localization to the endoplasmic reticulum. As ATGL is a
38 triglyceride lipase, we hypothesize that it may release arachidonic acid (AA), the substrate for
39 PG production, from triglycerides stored in LDs. Indeed, lipidomic analysis reveals the presence
40 of AA-containing triglycerides in ovaries. In addition, exogenous AA is toxic and reduction of
41 ATGL ameliorates toxicity; these observations suggest that ATGL indeed generates free AA.
42 Our studies provide the first evidence that LDs and their associated proteins regulate PG
43 signaling to control actin remodeling. In particular, we propose that ATGL releases AA from LDs
44 to drive PG synthesis necessary for follicle development. We speculate that the same pathways
45 are conserved across organisms to regulate oocyte development and promote fertility.

46 **Introduction**

47 Rates of infertility have increased globally between 1990 and 2017 [1], and combatting this
48 increase is considered a public health priority [2]. Fertility requires the production of high-quality
49 oocytes. It is becoming increasingly clear that among the key factors ensuring oocyte quality are
50 the amount and types of lipids present during oogenesis [3, 4]. In mammalian follicles, for
51 example, fatty acids (FAs) likely contribute a critical source of energy since inhibitors of FA
52 oxidation impair oocyte maturation [5, 6]. In addition, lipid signaling molecules – from steroid
53 hormones to eicosanoids – control diverse aspects of oocyte development and fertility [7].
54 Indeed, prostaglandins (PGs), a class of eicosanoids, regulate oocyte development, ovulation,
55 fertilization, implantation, maintenance of pregnancy, and childbirth [8, 9]. Thus, control of lipid
56 metabolism is likely central for oocyte development and fertility, yet only few of the underlying
57 mechanisms have been elucidated.

58 Key regulatory steps in lipid metabolism are mediated by lipid droplets (LDs), the cellular
59 organelles dedicated to the storage of neutral lipids [10]. For example, LDs store excess
60 amounts of cholesterol and related sterols as sterol esters, and safely sequester toxic FAs in the
61 form of triglycerides. Once such FAs are released by LD-bound lipases, they can then be
62 shuttled to mitochondria for oxidative breakdown, used to generate various membrane
63 precursors, or turned into signaling molecules [11]. Since both triglycerides and LDs are
64 abundant in oocytes, LDs might perform similar regulatory roles during follicle development.
65 Indeed, LD accumulation, composition, and localization are dynamic during oocyte maturation
66 across organisms [3, 4, 12], and, in the context of obesity, oocytes display changes in LDs that
67 are associated with infertility [13-15]. However, the functions of LDs in oogenesis remain largely
68 undefined.

69 *Drosophila* oogenesis is a promising model for uncovering the roles of LDs in follicle
70 development. Adult female flies have two ovaries, each comprised of ~15 ovarioles or chains of
71 sequentially maturing follicles, also called egg chambers. Each follicle is made up of ~1000

72 epithelial cells termed follicle cells as well as 16 germline cells (15 nurse cells and one oocyte).
73 Follicles develop over the course of ~10 days through 14 stages. Over just a few hours in mid-
74 oogenesis, tens of thousands of LDs are newly formed and disperse throughout the nurse cells.
75 Strikingly, failure to form LDs, due to loss of diacylglycerol O-acyl transferase 1 (DGAT1,
76 *Drosophila* Midway), results in developmentally arrested follicles and an aberrant actin
77 cytoskeleton [16]. These observations suggest that LDs may have a role in follicle development
78 and directly or indirectly regulate actin.

79 Both LDs and the actin cytoskeleton undergo dramatic changes in mid-oogenesis (see
80 Fig 1A-1I) [17]. Prior to Stage 8, only a few scattered LDs are found throughout the follicle, and
81 the only obvious actin cytoskeletal structure in the nurse cells is F-actin (filamentous actin) at
82 the cell cortex. In Stage 9, LD biogenesis is massively upregulated in the nurse cells, so that by
83 Stage 10B, the nurse cell cytoplasm is full of uniformly sized LDs (~0.5 μm in diameter) [16, 18].
84 During Stage 10B, the actin cytoskeleton is also massively remodeled, parallel actin bundles
85 extend from the plasma membrane to the nuclei to form a cage, and the cortical actin
86 substantially thickens. Both of these actin structures are required during Stage 11 for the
87 process of nurse cell dumping, during which the nurse cells squeeze their cytoplasmic contents
88 into the oocyte via ring canals, intercellular bridges between the germline cells [19-21]; cortical
89 actin provides the contractile force, and actin bundles prevent the nuclei from being pushed into
90 and plugging the ring canals. Dumping also transfers the LDs from nurse cells to the oocyte.
91 These LDs then provide the future embryo with stores of energy and specific proteins needed
92 for embryo development; indeed, embryos with reduced numbers of LDs have reduced hatching
93 probability [18, 22]. From Stage 12 onwards, oocyte LD content remains stable [23], and nurse
94 cells undergo cell death [24].

95 The actin remodeling events during mid-oogenesis are regulated by lipid signaling via
96 PGs. PGs are derived from arachidonic acid (AA) [8], a poly-unsaturated FA, which is converted
97 first into PGH_2 and then into several types of bioactive PGs. These enzymatic steps are

98 mediated by cyclooxygenase (COX) enzymes and specific PG synthases, respectively.
99 *Drosophila* has a single COX-like enzyme called Pxt, and its absence during oogenesis results
100 in severe disruption in actin bundle formation, breakdown of cortical actin, and failure of nurse
101 cell contraction, ultimately leading to severe defects in follicle development and female sterility
102 [25, 26]. How PG production is temporally and spatially regulated during *Drosophila* oogenesis
103 is unknown, but in many cells the release of AA from cellular lipids is the rate limiting step [8,
104 27]. Those precursor lipids are generally thought to be phospholipids in cellular membranes, but
105 in mammalian immune cells neutral lipids are an essential source of AA for PG synthesis [28,
106 29].

107 Given the significance of lipid signals in actin remodeling during *Drosophila* oogenesis
108 and the central role of LDs in lipid metabolism in other cells, we sought to determine if LDs
109 contribute to *Drosophila* follicle development. We find that loss of each of three lipid droplet
110 proteins – LSD2/PLIN2 (subsequently referred to as PLIN2), Jabba, or ATGL (*Drosophila*
111 Brummer) – results in *pxt*-like actin remodeling defects during Stage 10B. Conversely, loss of
112 Pxt or pharmacological inhibition of PG synthesis results in aberrant LD clustering during Stage
113 10B; LD clustering also occurs in *PLIN2* and *Jabba* mutants. The similar phenotypes led us to
114 ask if LD proteins and Pxt function in the same pathway to regulate actin remodeling during
115 *Drosophila* oogenesis. Dominant genetic interaction studies support that there are three actin
116 regulatory pathways: PLIN2 regulates actin remodeling independent of PG signaling, whereas
117 Jabba and ATGL act in two separate PG-dependent pathways. The connection between LD
118 proteins and PG signaling is not due to Pxt localizing to LDs. Instead, our data support the
119 model that ATGL is required to release AA to provide the substrate for PG production.
120 Ultimately, these PGs control actin remodeling and thereby follicle development. Our studies
121 thus have uncovered a role for LDs in regulating a critical developmental transition during
122 oogenesis.

123

124 **Results**

125 *DGAT1 functions suggest a link between LDs and actin organization during oogenesis*

126 During *Drosophila* oogenesis, both actin and LDs undergo dramatic changes over the course of
127 just a few hours (Fig. 1A-I). F-actin present at the cortex of the nurse cells throughout early
128 stages (Fig. 1 F, G) is dramatically strengthened during Stage 10B (Fig 1H); in addition, actin
129 bundles form that hold the nurse cell nuclei in place (Fig. 1H-1J). LDs start to accumulate in
130 Stage 9, fill the nurse cells by Stage 10B, and are transferred to the oocyte by Stage 12 (Fig.
131 1B-D).

Fig 1. LD accumulation and actin cytoskeletal remodeling occur during mid-oogenesis. (A-D) Single confocal slices of wild-type follicles of the indicated stages (E) stained for LDs (Nile red) in white and DNA (Hoechst) in cyan. **(E)** Schematic depicting the progression of LD accumulation and actin remodeling during oogenesis. **(F-I)** Single confocal slices of wild-type follicles of the indicated stages (E) stained for F-Actin (phalloidin) in white and DNA (Hoechst) in cyan. Note that all images and diagram in A-I are on top of a black box. **(J)** Single confocal slice of a Stage 10B nurse cell stained for F-Actin (phalloidin) in white and DNA (Hoechst) in cyan; actin bundles are indicated by the green arrowhead and cortical actin is indicated by the magenta arrowhead. Scale bars=50 μ m (A-I) or 10 μ m (J). **(K)** Thin layer chromatograph of whole-lipid extracts from late-stage follicles. **(L)** Quantitation of (K), in which the ratio of TAG to cholesterol intensity is plotted; error bars, SD. Few LDs are present in the nurse cells during early oogenesis, but begin to accumulate by Stage 9 (B). By Stage 10B, LDs are present in large numbers and evenly distributed throughout the nurse cell cytoplasm (C). The LDs are dumped into the oocyte during Stage 11 and are highly abundant in the ooplasm by Stage 12 (D). This temporal accumulation is also reflected by the increase in triglycerides and sterol esters (K, L), neutral lipids stored in LDs. Massive remodeling of the nurse cell actin cytoskeleton begins when LDs are highly abundant. During Stage 10B (H), cortical actin thickens (magenta arrowhead, J) and parallel actin bundles extend from the nurse cell membrane to the nucleus (green arrowhead, J). During Stage 11, the actin bundles hold the nurse cell nuclei in place while the cortical actin contracts, squeezing the nurse cell cytoplasm into the oocyte in a process termed nurse cell dumping. This process ultimately results in the nurse cells shrinking and the oocyte expanding (I). Cortical actin surrounds each nurse cell throughout oogenesis(F-I); during Stage 10B, actin bundles form (J) and support nurse cell dumping.

132

133 The main neutral lipids stored in LDs are triglycerides and/or sterol esters [10]. Using
134 thin-layer chromatography on tissue extracts, we find that developing follicles accumulate both
135 classes of neutral lipids, with a large predominance of triglycerides (Fig 1K-1L). The final step of
136 triglyceride synthesis is mediated by DGATs, which catalyze the attachment of a third FA to

137 diacylglycerol. Like most animals, *Drosophila* encodes two such enzymes, DGAT1/Midway, and
138 DGAT2 [30]. Genome-wide expression data indicate that DGAT1 predominates in most tissues,
139 including in ovaries [31]. In *DGAT1* loss-of-function mutants, LDs fail to accumulate and follicles
140 die by Stage 8/9 of oogenesis [16]. We confirmed that LDs in nurse cells and oocytes (though
141 not follicle cells) are essentially abolished in follicles from *DGAT1* mutant animals and that
142 mutant ovaries have massively reduced amounts of neutral lipids (Fig 2A-2D).

Fig 2. DGAT1 is required for LD formation and actin cytoskeletal remodeling. (A-B) Single confocal slices of Stage 9 follicles stained for LDs (Nile red) in white and DNA (Hoechst) in cyan. **(A)** WT, wild-type (Oregon R). **(B)** *DGAT1*^{-/-} (*DGAT1*^{QX25}/*DGAT1*^{RF48}). **(C)** Thin layer chromatograph of whole-lipid extracts from whole ovary lysates of the indicated genotypes. **(D)** Graph of neutral lipid levels in the ovaries of the indicated genotypes normalized to cholesterol; TAG=triacylglycerol and SE=sterol esters. Error bars, SD. ***p*=0.0028, **p*=0.0234, Sidák's multiple comparisons test. **(E-G)** Single confocal slices of *DGAT1*^{-/-} follicles of the indicated stages stained for F-Actin (phalloidin) in white and DNA (Hoechst) in cyan. **(H-I)** Single confocal slices of germline RNAi knockdown of *DGAT1* (*mat-alpha4-Gal4-VP16>DGAT1* RNAi (TRiP.HMC06242)); same follicle, different focal plane, stained for F-Actin (H, phalloidin) or LDs (I, Nile red) in white and DNA (Hoechst) in cyan. Magenta arrowheads indicate cortical actin defects, and green arrowheads indicate actin bundle defects. Scale bars=50µm. Loss of DGAT1 results in decreased lipid accumulation in the ovaries (C-D) and a loss of LDs in nurse cells and oocyte (B compared to A). Loss of DGAT1 also results in defects within the actin cytoskeleton. In early and mid-oogenesis, cortical actin breaks down (magenta arrowheads) and actin bundles form prematurely (green arrowheads) (E-F). Ultimately, *DGAT1*^{-/-} follicles die at mid-oogenesis (G). Germline RNAi knockdown of *DGAT1* exhibits both actin remodeling defects (H) and decreased LD accumulation (I).

143
144 It had previously been reported that DGAT1 mutant follicles form premature actin
145 bundles [16]. We also observe such bundles in the *DGAT1* mutant, e.g., in Stage 9 (Fig 2F,
146 green arrowhead); in the wild type, they only appear by Stage 10B (Fig 1J). In addition, we also
147 detect defects in cortical actin, such as absence from the nurse cell periphery or aggregates
148 around ring canals between neighboring nuclei (Fig 2E-2F, magenta arrowhead). DGAT1 is
149 expressed throughout the adult fly, particularly in the fat body, the fly's adipose tissue [31]. Yet
150 knocking down DGAT1 expression specifically in the germline via RNAi was sufficient to
151 severely restrict LD accumulation and result in abnormal bundle formation and fractured cortical

152 actin (Fig 2H-2I). These tissue-autonomous actin defects suggest that DGAT1 and
153 consequently LDs in nurse cells are linked to actin remodeling.

154 Because lack of DGAT1 results in follicle death, it is conceivable that the actin defects
155 are a secondary consequence of the dramatic breakdown of cellular structures. This possibility
156 seems unlikely since germline specific knockdown of DGAT1 allowed follicles to progress
157 further, yet still resulted in fewer LDs and in actin defects (Fig 2H-2I). Nevertheless, to
158 circumvent this problem, we next sought to disrupt specific LD functions without preventing LD
159 accumulation or causing follicle death.

160

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

162 In early *Drosophila* embryos, the two most abundant LD-specific proteins are PLIN2 and Jabba
163 [32, 33]. PLIN2 is one of the two members of the perilipin family of LD proteins in flies and
164 regulates lipid metabolism by protecting LDs from the lipolytic machinery [34-36]. Jabba anchors
165 proteins from other cellular compartments to LDs [33, 37] and has been proposed to contribute
166 to lipid metabolism during oogenesis [38].

167 Similar to germline knockdown of DGAT1 (Fig 3B), females lacking either Jabba or
168 PLIN2 display two types of actin defects during oogenesis (Fig 3C-3D compared to 3A). First,
169 actin bundles are aberrant. Specifically, some nurse cell membranes lack bundles, and the
170 bundles that form are not uniformly distributed, shorter, and/or fail to project toward the nurse
171 cell nuclei (Fig 3C-3D, green arrowheads). Second, cortical actin is broken down, leading to the
172 appearance of multinucleate nurse cells (Fig 3C-3D, magenta arrowheads). Since in these
173 mutants LDs are abundant [18, 33] (see also Fig 4) and because the frequency of dying follicles
174 is similar to wild type (data not shown), we conclude that disruption of LD function indeed affects
175 actin remodeling.

Fig 3. LD proteins regulate actin bundling and cortical actin integrity in Stage 10B follicles. (A-F) Maximum projections of three confocal slices of S10B follicles stained for F-Actin (phalloidin) in white, and DNA (DAPI) in cyan. Arrowheads indicate examples of defective actin bundling (green) and disrupted cortical actin (magenta). Images were brightened by 30% to increase clarity. Black boxes were placed behind channel labels to improve visualization. Scale bars=50 μ m. (A) WT, *wild-type* (*yw*). (B) *DGAT1* RNAi (*osk3-GAL4>TRiP.HMC06242/+*). (C) *Jabba*^{-/-} (*Jabba^{z101}/Jabba^{z101}*). (D) *PLIN2*^{-/-} (*Lsd-2^{KG00149}/Lsd-2^{KG00149}*). (E) *ATGL*^{-/-} (*bmm¹/bmm¹*). (F) *pxt*^{-/-} (*pxt^{f01000}/pxt^{f01000}*). In wild-type late Stage 10B follicles, actin bundles extend from the nurse cell periphery to the nucleus, and the cortical actin is thickened relative to earlier stages (A). Actin bundles fail to form or form improperly, and cortical actin is disrupted upon RNAi knockdown of *DGAT1* (B), or loss of *Jabba* (C), *PLIN2* (D), or *ATGL* (E). These phenotypes resemble loss of the COX-like enzyme *Pxt* (F).

176

177 Most actin regulators impact either actin bundle formation or cortical actin, but not both
178 [17, 21]. However, the same combination of phenotypes is observed when PG signaling is lost:
179 lack of the COX-like enzyme *Pxt* causes delayed bundle development, collapsed, stunted or
180 absent actin bundles, and cortical actin breakdown with a failure of nurse cell contraction (Fig
181 3F compared to 3A) [25, 26]. In most cases of actin bundling defects, nurse cell nuclei are
182 pushed into the ring canals when the cortical actin contracts. However, this does not occur in
183 *Pxt* [26, 39], *Jabba*, or *PLIN2* mutants (data not shown), suggesting that cortical actin
184 contraction is also disrupted, presumably as result of the simultaneous breakdown of cortical
185 actin. The fact that loss of all three proteins results in this unusual combination of phenotypes
186 suggests a functional relationship between PGs and LD proteins in actin remodeling.

187

188 Both LD proteins and PG signaling control the intracellular distribution of LDs

189 The similarities between lack of PG signaling and lack of LD proteins extends to phenotypes
190 beyond F-actin: *Jabba*, *PLIN2*, and *pxt* mutants all display altered spatial distribution of LDs. In
191 wild-type Stage 10B follicles, LDs are dispersed throughout the entire nurse cell cytoplasm, with
192 only minor variations in LD density (Fig 4A-4A'). In contrast, in all three mutants, some regions
193 of the cytoplasm are depleted of LDs, while others have dense LD accumulation/clustering (Fig

194 4B-4D'). Clustering varies somewhat across the same follicle and between follicles. But when
195 Stage 10B follicles were scored as having either normal, mild, or severe LD clustering (see
196 Material and Methods for details), half or more of all follicles exhibited severe clustering in all
197 three mutants (Fig 4F).

Fig 4. PG signaling and LD proteins promote LD dispersal. (A-E') Single confocal slices of follicles of Stage 10B follicles stained for LDs (Nile red) in white and DNA (Hoechst) in cyan; A'-E' are zoomed in images of A-E. A black box was added under the channel labels in A' to aid in visualization. **(A)** WT, wild-type (Oregon R). **(B)** *pxt*^{-/-} (*pxt*^{f01000}/*pxt*^{f01000}). **(C)** *Jabba*^{-/-} (*Jabba*^{DL}/*Jabba*^{DL}). **(D)** *PLIN2*^{-/-} (*LSD-2*^{KG}/*LSD-2*^{KG}). **(E)** *ATGL*^{-/-} (*bmm*¹/*bmm*¹). **(F)** Quantification of LD clustering in Stage 10B follicles for the indicated genotypes. Follicles were classified as normal or as exhibiting moderate or severe clustering, and the percentage of each class was calculated. **(G)** Quantification of actin cytoskeletal and LD clustering defects in control versus 3mM aspirin-treated Stage 10B follicles. Dots represent the average of separate experiments; control n=49 and aspirin n=63. Error bars, SD. ****p*=0.0001, ****=*p*<0.0001, Sidák's multiple comparisons test. **(H-I')** Single confocal slices of Stage 10B follicles treated with vehicle (control, EtOH or DMSO) or 3mM aspirin for 1 hour, and then stained for F-Actin (H, I, phalloidin) or LDs (H', I', Nile red) in white; H'' and I'' are zoomed in images of H' and (I'). Note that images in panels H-H' are on black box, and a black box was added under the I'' label to aid in visualization. Scale bars=50µm (A-E, H-H', I-I') or 10µm (A'-E', H'', I''). Loss of Pxt or two LD proteins (Jabba and PLIN2) result in similar LD clustering/reorganization phenotypes. Similarly, treatment of Stage 10B follicles with 3mM aspirin, a COX inhibitor, for 1 hour results in both LD clustering (H'-H'' compared to I'-I'', and G) and actin cytoskeletal defects (H compared to I, and G).

198

199 To test whether clustering is caused by defective PG signaling within the follicle or some
200 as-yet uncharacterized function of Pxt, we pharmacologically inhibited Pxt to abolish PG
201 production [26]. Wild-type Stage 10B follicles were incubated *in vitro* for 1-1.5 hours in medium
202 containing either the solvent control or 3mM aspirin, an inhibitor of COX enzymes. Follicles were
203 then stained for both LDs and F-actin. Under control conditions, follicles maintain uniform LD
204 distribution and intact actin bundles and cortical actin, very similar to follicles without any
205 treatment (Fig 4G-4H''). In the aspirin treated samples, the actin cytoskeleton is disrupted, as
206 expected from previous studies (Fig 4G-4I) [26]. This treatment causes LD clustering (Fig 4G,
207 4I'-4I''). Such a rapid response supports the idea that PG signaling within the follicle not only
208 promotes actin remodeling but also maintains LD dispersal. Together, our observations indicate

209 that LDs and PG signaling may reciprocally influence each other. As the molecular mechanisms
210 underlying LD dispersal are not well understood, we focus the rest of the study on the actin
211 remodeling effects.

212

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

214 The similar actin phenotypes in *pxt*, *PLIN2*, and *Jabba* mutants suggest these proteins act in the
215 same pathway to regulate actin remodeling. To test this hypothesis, we used a dominant
216 genetic interaction assay to assess actin remodeling defects. As the severity of actin disruption
217 can exhibit considerable variability within a given genotype, we developed a method to quantify
218 both actin bundle and cortical actin defects from confocal stacks of phalloidin-stained Stage 10B
219 follicles. Specifically, the degree of penetrance of actin bundle and cortical actin defects are
220 scored separately and summed to give an Actin Defect Index (ADI) that is used to classify each
221 follicle into three categories of defects: normal, mild, or severe (see the Material and Methods
222 and S1 Fig for details).

S1 Fig. Examples and criteria used for scoring actin remodeling phenotypes.

Maximum projection of three confocal slices of representative Stage 10B follicles stained for F-Actin (phalloidin) in white, and DNA (DAPI) in cyan, and written definitions of the indicated actin bundle and cortical actin phenotypes. Actin bundle and cortical actin phenotypes were independently binned into four categories: Normal, Mild Defects, Moderate Defects, or Severe Defects. Images were brightened by 30% to increase clarity. Green arrowheads indicate examples of actin bundle defects, and magenta arrowheads indicate examples of cortical actin breakdown. Scale bar=50 μ m.

223

224 We expect heterozygosity for mutations in *pxt*, *PLIN2*, or *Jabba* alone to have little to no
225 effect on actin dynamics. If either *PLIN2* and *Pxt* or *Jabba* and *Pxt* function in the same
226 pathway, then double heterozygotes for *PLIN2* and *pxt* or *Jabba* and *pxt* will exhibit severe actin
227 defects. Conversely, if they function in separate pathways, then actin defects in the double
228 heterozygotes will remain low or be additive of what is observed in the single heterozygotes.

229 As expected, there are few actin defects in single heterozygotes of PLIN2 (63% normal)
230 and *pxt* (80% normal) (Fig 5A-5B, 5G). Co-reduction of both PLIN2 and Pxt (*PLIN2*+/+;*pxt*+/+)
231 does not significantly increase the frequency of actin defects (Fig 5C, 5G; 60% normal). This
232 failure to exhibit a genetic interaction is not likely due to insufficient protein reduction as PLIN2
233 heterozygotes show a 40% reduction in PLIN2 protein level (S2 Fig), but instead suggests that
234 Pxt and PLIN2 regulate actin remodeling via separate pathways.

Fig 5. PLIN2 acts independently of Pxt, while Jabba acts in a linear pathway with Pxt to regulate actin remodeling. (A-F) Maximum projections of three confocal slices of S10B follicles stained for F-Actin (phalloidin) in white, and DNA (DAPI) in cyan. Arrowheads indicate examples of defective actin bundling (green) and disrupted cortical actin (magenta). Images were brightened by 30% to increase clarity. Scale bars=50µm. (A) *PLIN2*+/+ (*Lsd-2*^{KG00149}/+). (B) *pxt*+/+ (*pxt*^{f01000}/+). (C) *PLIN2*+/+; *pxt*+/+ (*Lsd-2*^{KG00149}/+;; *pxt*^{f01000}/+). (D) *Jabba*+/+ (*Jabba*^{z101}/+). (E) *Jabba*+/+; *pxt*+/+ (*Jabba*^{z101}/+; *pxt*^{f01000}/+). (F) *PLIN2*+/+; *Jabba*+/+ (*Lsd-2*^{KG00149}/+; *Jabba*^{z101}/+). (G-I) Graphs of quantification of actin phenotypes. Data were from the following genotypes: *pxt*+/+ = *pxt*^{f01000}/+ and *pxt*^{EY03052}/+; *Jabba*+/+ = *Jabba*^{z101}/+ and *Jabba*^{DL}/+; *Jabba*+/+; *pxt*+/+ = *Jabba*^{z101}/+; *pxt*^{f01000}/+, *Jabba*^{z101}/+; *pxt*^{EY03052}/+, *Jabba*^{DL}/+; *pxt*^{f01000}/+, and *Jabba*^{DL}/+; *pxt*^{EY03052}/+; *PLIN2*+/+; *pxt*+/+ = *Lsd-2*^{KG00149}/+;; *pxt*^{f01000}/+ and *Lsd-2*^{KG00149}/+;; *pxt*^{EY03052}/+; and *PLIN2*+/+; *Jabba*+/+ = *Lsd-2*^{KG00149}/+; *Jabba*^{z101}/+. Actin defects were quantified by scoring the penetrance of actin bundle and cortical actin defects. Scores were summed and the total binned into one of three categories: normal, mildly defective, and severely defective. For a detailed description of the quantification see the Materials and Methods and Supplemental Figure 1. *****p*<0.0001, Pearson's chi-squared test. Error bars, SD. Both actin bundles and cortical actin are largely normal in *PLIN2*+/+ (A, G, I), *PLIN2*+/+; *pxt*+/+ (C, G), or *PLIN2*+/+; *Jabba*+/+ (F, I) Stage 10B follicles. In contrast, in *Jabba*+/+; *pxt*+/+ follicles (E), actin bundles are absent, sparse, and stunted and there are instances of cortical actin breakdown. The double heterozygotes thus exhibit significantly more mild and severe actin defects than either *pxt*+/+ (B, H) or *Jabba*+/+ (D, H) follicles (H).

S2 Fig. *PLIN2*+/+ heterozygotes have reduced PLIN2 protein. (A) Immunoblot comparing PLIN2 protein levels in wild type (WT, Oregon R), *PLIN2*+/+ (*Lsd-2*^{KG00149}/+), and *PLIN2*-/- (*Lsd-2*^{KG00149}/*Lsd-2*^{KG00149}) Stage 10B follicles. (B) Quantification of (A), in which PLIN2 band intensity was normalized to the nonspecific bands (asterisk). *p*<0.0001, unpaired t-test, two-tailed. PLIN2 protein levels are reduced by approximately half in *PLIN2*+/+ Stage 10B follicles.

235
236 We next examined the relationship between Jabba and Pxt. Like before, the single
237 heterozygotes have only minor actin defects, with the vast majority of all follicles exhibiting
238 normal actin remodeling (Fig 5B, 5D, 5H). In contrast, only 11% of follicles from double

239 heterozygotes (*Jabba*^{+/+}; *pxt*^{+/+}) were normal, and the frequency of the severe class increased
240 almost tenfold (Fig 5E, 5H). This synergistic increase in actin defects suggests Pxt and Jabba
241 act in the same pathway to regulate actin remodeling.

242 For the relationship between Jabba and PLIN2, we considered two scenarios. In the first,
243 Jabba acts in two pathways, one with PLIN2 and one with Pxt, to regulate actin remodeling. In
244 the second, PLIN2 regulates actin remodeling independently of both Pxt and Jabba. The actin
245 phenotypes of *PLIN2*^{+/+}; *Jabba*^{+/+} double heterozygotes are mild, with the frequency of each
246 category similar to those seen in the single heterozygotes (Fig 5F, 5I). We conclude that actin
247 remodeling is regulated by two separate pathways involving LD proteins, with Jabba and Pxt
248 acting in the same pathway (see Fig 10).

249

250 *Pxt is not enriched on LDs*

251 The observed genetic interaction between Jabba and Pxt could be explained if the two proteins
252 directly or indirectly regulate each other's expression. However, Western analysis on extracts
253 from Stage 10B follicles revealed that Jabba protein levels in wild-type and *pxt* mutant follicles
254 were indistinguishable, as were Pxt levels in wild-type and *Jabba* mutant follicles (S3A-S3D
255 Fig). Thus, we find no evidence that Jabba and Pxt interact by regulating each other's
256 expression. We also find no effect of PLIN2 on the levels of Jabba and Pxt, and vice versa
257 (S3A, S3E-S3F Fig).

S3 Fig. Pxt and LD proteins do not affect each other's expression. (A, C, E)

Immunoblots for Pxt and alpha-Tubulin (A), Jabba (C), and PLIN2 (E) of the indicated genotypes: WT, wild-type (Oregon R), *Jabba*^{-/-} (*Jabba*^{z101}/*Jabba*^{z101}), *PLIN2*^{-/-} (*Lsd-2*^{KG00149}/*Lsd-2*^{KG00149}), *ATGL*^{-/-} (*bmm*¹/*bmm*¹) and *pxt*^{-/-} (*pxt*^{f01000}/*pxt*^{f01000}). Asterisk indicates non-specific band in C and E. (B, D, F) Quantification of A, C, and E, respectively, in which band intensity was normalized as indicated. ns=p>0.05, unpaired t-test, two-tailed. Loss of Jabba, PLIN2, or ATGL have no effect on Pxt levels (A-B), and loss of Pxt has no effect on Jabba (C-D) or PLIN2 (E-F) levels.

258

259 Jabba and Pxt might also regulate each other's activity by being physically present in the
260 same cellular compartment. Jabba localizes to LDs in embryos [33] and in nurse cells [23], and
261 in other systems, components of the PG synthesis machinery are sometimes enriched on LDs
262 [40]. Using immunostaining, LD purification, and *in vivo* centrifugation, we assessed Pxt's
263 relationship to LDs in wild-type nurse cells. All three methods show that Pxt is not enriched on
264 LDs. By immunostaining, Pxt localizes to the Golgi compartments prior to Stage 9 and to the
265 Endoplasmic reticulum (ER) by Stage 10B, but not obviously to LDs (Fig 6A-6C"). We also
266 prepared an LD enriched biochemical fraction from ovary lysates, as previously described for
267 embryos [33], and analyzed it by Western blotting for Pxt, PLIN2 (as a marker for LDs), and
268 Calnexin (as marker for the ER). Pxt behaves like an ER protein, not like an LD protein (Fig 6D-
269 6E). Finally, we separated LDs from other cellular components by *in vivo* centrifugation: when
270 living *Drosophila* follicles are centrifuged, their contents separate by density within each nurse
271 cell/oocyte, with LDs accumulating on the side that faced up [32, 33]. Staining of fixed
272 centrifuged follicles reveals no enrichment of Pxt (magenta) in the LD layer (green, marked by
273 yellow arrowhead) (Fig 6F). We conclude that the majority of Pxt is not present on LDs.

Fig 6. Pxt is not enriched on LDs. (A-C") Single confocal slices of nurse cells of the indicated stages, stained for Pxt (A-C), organelle marker (A'-C'), and DNA (D"-G", Hoechst). Merged image (A""-C""): Pxt, magenta; organelle marker, green; and DNA, cyan. Organelle marker: LDs (A', A""; Nile red); Golgi-eYFP (B', B""); and ER-eYFP (C', C""). Scale bars=10µm. **(D)** Western blots of purified LDs and a dilution series of ovary lysate (OL) for Calnexin (magenta) and Pxt (green) in the top half of the blot and α-Tubulin (magenta) and PLIN2 (green) in the bottom half of the blot; dashed line indicates where the membrane was cut. **(E)** Quantification the abundance of proteins in the LD fraction relative to undiluted ovary lysate (OL). The ratio of signal for the LD and OL lane in Western blots like in (F) were computed for the indicated proteins and normalized to the average value for PLIN2. Error bars, SD. ** $p < 0.0028$, Tukey's multiple comparisons test. **(F-F")** Single confocal slices of centrifuged wild-type Stage 10B follicle stained for Pxt (F), LDs (F', Nile red), and DNA (F", Hoechst). Merged image (A""-C""): Pxt, magenta; LDs, green; and DNA, cyan. Scale bars=50µm. Pxt does not colocalize with LDs (A-A""), is not enriched on purified LDs (D-E), and does not co-accumulate with LDs in centrifuged follicles (F-F"). Instead, Pxt localizes to the Golgi during Stage 8 (B-B") and the ER during Stage 10B (C-C").

274

275 Alternatively, Pxt and Jabba might genetically interact because they influence each
276 other's localization. We find that this is not the case, as in Stage 10B follicles, Pxt still robustly
277 colocalizes with the ER in *Jabba* mutants (S4B-S4B''' compared to S4A-S4A''' Fig), and Jabba
278 still localizes to LDs in *pxt* mutants (S4F-S4F''' compared to S4E-S4E'''). There are some
279 changes in the overall spatial distribution of LDs and ER in these mutants: LDs are clustered in
280 *pxt* mutant (consistent with Fig 4B-4B') and, in *Jabba* mutants, the ER has a more diffuse
281 appearance and is excluded from areas occupied by LD clusters (S4B-S4B''' compared to S4A-
282 S4A''' Fig). However, in either mutant condition, Jabba and Pxt remain enriched on LDs and ER,
283 respectively, as in the wild type. Similarly, loss of PLIN2 has no effect on Pxt localization (S4C-
284 S4C''' Fig).

S4 Fig. LD proteins do not affect Pxt's localization to the ER. (A-D''''') Single confocal slices of Stage 10B nurse cells, stained for Pxt (A-D), Calnexin (A'-D', ER marker), and DNA (A''-D'', Hoechst). Merged image (A''', A''''-D''', D'''''): Pxt, magenta; Calnexin (ER marker), green; and DNA (Hoechst), cyan. **(E-F''''')** Single confocal slices of Stage 10B nurse cells, stained for Jabba (E-F), LDs (E'-F', Nile red) and DNA (E''-F'', Hoechst). Merged image (E''', E''''', F''', F'''''): Jabba, magenta; LDs (Nile red), green; and DNA (Hoechst), cyan. Genotypes: WT, wild-type (Oregon R); *Jabba* *-/-* (*Jabba*^{z101}/*Jabba*^{z101}); *PLIN2* *-/-* (*LSD-2*^{KG00149}/*LSD-2*^{KG00149}); *ATGL* *-/-* (*bmm*¹/*bmm*¹), and *pxt* *-/-* (*pxt*^{f01000}/*pxt*^{f01000}). Scale bars=10µm. Note that black boxes were added under some panel and/or channel labels to aid in visualization (A, A', A''', D, E, F' and F'''). In Stage 10B follicles, Pxt's localization to the ER is similar to wild-type in *Jabba* (B-B'''), *PLIN2* (C-C'''), and *ATGL* mutants (D-D'''). As in the wild type (E-E'''), Jabba localizes to LDs in *pxt* mutants (F-F'''), even though the spatial distribution of LDs is altered.

285
286 *The triglyceride lipase ATGL/Brummer and Pxt act in the same pathway to regulate actin*
287 If there is no clear physical relationship between LDs and Pxt, how else might LDs modulate PG
288 production? In some mammalian cells, the substrate for COX enzymes, arachidonic acid (AA),
289 can be released from triglycerides stored in LDs by the triglyceride lipase ATGL [28]. These FAs
290 can either be directly used as COX substrate or be first incorporated into membrane
291 phospholipids and subsequently released by phospholipases [41]. In the latter case, diffusing
292 phospholipids may deliver AA to ER regions spatially distant from LDs. Thus, AA availability for
293 PG production may ultimately depend on the breakdown of LD triglycerides.

294 The *Drosophila* homolog of ATGL is called Brummer (Bmm; referred thereafter as ATGL
295 for clarity); it catalyzes the conversion of triacylglycerol to diacylglycerol and free FA [42]. Based
296 on RNA-seq analyses, ATGL is highly expressed in ovaries [43], and in-situ analysis reveals
297 that ATGL mRNA is expressed in the nurse cells and oocytes in early and mid-oogenesis [44].
298 Intriguingly, we find that loss of ATGL causes actin remodeling defects in Stage 10B follicles
299 that resemble those seen in *pxt* and *Jabba* mutants, *i.e.*, defects in both cortical actin and actin
300 bundles (Fig 3C, 3E, 3F compared to 3A); conversely, loss of ATGL does not alter LD dispersal
301 (Fig. 4E-4F). Additionally, ATGL does not regulate the level or localization of Pxt (S3A and S4D-
302 S4D''' Fig), similar to our findings related to *Jabba* and PLIN2.

303 To test if ATGL acts in the same pathway as Pxt to regulate actin remodeling, we used
304 our quantitative dominant genetic interaction assay to assess actin remodeling during Stage
305 10B. As expected, actin remodeling is largely normal in single heterozygotes of *pxt* (87%) and
306 *ATGL* (84%) (Fig 7A, 7D). In contrast, only 46% of the double heterozygotes of *pxt* and *ATGL*
307 (*pxt/ATGL*) exhibit normal actin remodeling, and the frequency of severe cases is increased
308 almost tenfold (Fig 7B, 7D). This synergistic increase in actin defects indicates that ATGL and
309 Pxt regulate actin remodeling via a shared pathway.

Fig 7. ATGL acts in a linear pathway with Pxt but not with Jabba to regulate actin remodeling. (A-C) Maximum projections of three confocal slices of Stage 10B follicles stained for F-Actin (phalloidin) in white, and DNA (DAPI) in cyan. Arrowheads indicate examples of defective actin bundling (green) and disrupted cortical actin (magenta). Images were brightened by 30% to increase clarity. Scale bars=50 μ m. (A) *ATGL*^{-/+} (*bmm*^{1/+}). (B) *ATGL/pxt* (*bmm*^{1/pxt}^{f01000}). (C) *Jabba*^{-/+}; *ATGL*^{-/+} (*Jabba*^{z101/+}; *bmm*^{1/+}). (D-E) Graphs of quantification of actin phenotypes of the indicated genotypes. Data were from the following genotypes: *pxt*^{-/+} = *pxt*^{f01000}^{-/+} and *pxt*^{EY03052}^{-/+}, *ATGL*^{-/+} = *bmm*^{1/+}, *ATGL/pxt* = *bmm*^{1/pxt}^{f01000} and *bmm*^{1/pxt}^{EY03052}; *Jabba*^{-/+} = *Jabba*^{z101/+}; and *Jabba*^{-/+}; *ATGL*^{-/+} = *Jabba*^{z101/+}; *bmm*^{1/+}. Actin defects were quantified by scoring the penetrance of actin bundle and cortical actin defects. Scores were summed and the total binned into one of three categories: normal, mildly defective, and severely defective. For a detailed description of the quantification see the Materials and Methods and Supplemental Figure 1. ****p*<0.0001, Pearson's chi-squared test. Error bars, SD. Actin bundles and cortical actin are largely normal in the *ATGL*^{-/+} and *pxt*^{-/+} Stage 10B follicles (A-B, D-E). In contrast, *ATGL/pxt* Stage 10B follicles have stunted and incomplete bundles (green arrowheads) and breakdown of cortical actin (magenta arrowheads, B, D). Actin remodeling is normal in *Jabba*^{-/+}; *ATGL*^{-/+} Stage 10B follicles (C, E).

310

311 We also assessed dominant genetic interactions between ATGL and Jabba, and ATGL
312 and PLIN2. We find that frequency of actin remodeling defects observed in the double
313 heterozygotes (*Jabba*^{+/+}; *ATGL*^{+/+}) is similar to that seen in the single heterozygotes (Fig 7C,
314 7E), indicating that Jabba and ATGL act in different pathways to regulate actin remodeling.
315 Similarly, we find that PLIN2 and ATGL do not interact genetically (S5 Fig).

S5 Fig. ATGL and PLIN2 regulate actin remodeling via separate pathways. (A-C)

Maximum projections of three confocal slices of S10B follicles stained for F-Actin (phalloidin) in white, and DNA (DAPI) in cyan. Images were brightened by 30% to increase clarity. Scale bars=50µm. (A) *PLIN2*^{-/+} (*Lsd-2*^{KG00149}^{-/+}). (B) *ATGL*^{-/+} (*bmm*¹^{-/+}). (C) *PLIN2*^{-/+}; *ATGL*^{-/+} (*Lsd-2*^{KG00149}^{-/+}; *bmm*¹^{-/+}). (D) Graph of quantification of actin phenotypes of the indicated genotypes. Actin defects were quantified by scoring the penetrance of actin bundle and cortical actin defects. Scores were summed and the total binned into one of three categories: normal, mildly defective, and severely defective. For a detailed description of the quantification see the Materials and Methods and Supplemental Figure 1. ns=p>0.05, Pearson's chi-squared test. Error bars, SD. The level of actin defects in *PLIN2*^{-/+}; *ATGL*^{-/+} (C-D) is not significantly different from that in *PLIN2*^{-/+} (A, D) or *ATGL*^{-/+} (B, D).

316

317 Taken together, these data reveal an intricate relationship between Pxt and LD proteins
318 in which activity of both is necessary for proper actin remodeling. Specifically, our data supports
319 that three pathways control actin remodeling. PLIN2 regulates actin dynamics independently of
320 Jabba, ATGL, and Pxt. Conversely, Pxt acts in two separate pathways with Jabba and ATGL to
321 regulate actin remodeling (see Fig 10). While there are no obvious mechanisms for how Pxt and
322 Jabba work together to regulate actin remodeling, ATGL may regulate the availability of the
323 substrate, AA, necessary for PG production.

324

325 *AA is present in ovary triglycerides*

326 If ATGL is indeed responsible for generating the substrate for Pxt, our model implies that AA-
327 containing triglycerides are present in *Drosophila* follicles. We therefore extracted lipids from
328 wild-type and *ATGL* mutant ovaries and analyzed them by LC-MS/MS mass spectrometry.

329 Among the 98 different triglyceride species identified (STable 1), two contain a twenty-carbon
330 FA with four double bonds (20:4), presumably AA. AA was relatively rare, making up on the
331 order of 0.06-0.07% of all FAs detected in triglycerides (Fig 8A), with four FA species
332 accounting for the bulk (~85%) of FAs: the mono-unsaturated palmitoleic (16:1) and oleic (18:1)
333 acids, and the saturated myristic (14:0) and palmitic (16:0) acids. We did not detect AA in the
334 phospholipids recovered in our analysis (data not shown).

STable 1. Quantitation of triglyceride species in ovaries

Column A lists the fatty acid content of various triglyceride species detected in ovaries by lipidomics.

Columns B through H show the relative amount of those species in three wild-type and four ATGL^{-/-} samples. Reads are background corrected. A fourth wild-type sample was discarded as an outlier because the detected lipid amounts were an order of magnitude lower than in the other samples.

Fig 8. Ovary triglycerides contain arachidonic acid. (A-F) Lipids were extracted from wild-type and ATGL^{-/-} (*bmm¹/bmm¹*) ovaries and analyzed by mass spectrometry. Error bars, SD. **(A)** Abundance of individual fatty acids in triglycerides expressed as fraction of all such fatty acids; significance evaluated using Sidak's multiple comparisons test. **(B)** Total amount of saturated (SF), monounsaturated (MUFA) or polyunsaturated (PUFA) fatty acids relative to all fatty acids in triglycerides; significance evaluated using Tukey's multiple comparisons test. **(C)** Levels of the three phospholipid classes phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI); significance evaluated using Sidak's multiple comparisons test. **(D)** Overall triglyceride levels. ns = $p = 0.1239$, unpaired t-test, two-tailed. **(E)** Thin layer chromatograph of whole-lipid extracts from Stage 14 follicles of the indicated genotypes. **(F)** Quantitation of the triacylglycerol (TAG) to cholesterol ratio in (D). $*p=0.0477$, Dunnett's multiple comparisons test. **(G, H)** Quantification of two triglyceride species containing arachidonic acid (AA). $*p=0.0419$, $**p=0.0019$, unpaired t-tests, two-tailed. Wild-type and ATGL mutant ovaries exhibit a similar abundance of fatty acids in triglycerides (A, B), phospholipids (C), and total triglycerides (D) by lipidomic analysis. Similarly, thin layer chromatographic analysis of Stage 14 follicles reveals no significant differences in triglycerides (E-F). However, two AA-containing triglyceride species are present in wild-type ovaries, and their levels are elevated in the absence of ATGL (G, H).

335

336 Our lipidomic analysis did not uncover a significant difference in the content of
337 phospholipids (Fig 8C) or of total triglycerides (Fig 8D) between wild-type and ATGL mutant
338 ovaries. The overall FA profile in triglycerides was also similar between the two genotypes (Fig
339 8A-B). In addition, there was no significant difference in total triglycerides in Stage 14 oocytes,

340 as detected by thin layer chromatography (Fig 8E-8F), even though we could confirm the
341 previously described reduced triglyceride loading in PLIN2 mutants [18]. Thus, ATGL mediated
342 lipolysis during oogenesis does not appear to lead to bulk turnover of LDs and may be restricted
343 to supporting lipid signaling.

344 However, wild-type and *ATGL* mutant ovaries exhibited differences in the AA-containing
345 triglycerides, both of which were elevated in the absence of ATGL (Fig 8G-8H). This trend
346 persisted even when measurements were normalized to total triglycerides (not shown) or all
347 lipids in the sample (S6A-S6B Fig). These observations are consistent with the possibility that in
348 the *ATGL* mutants less AA is released from triglycerides, resulting in a reduced pool of free AA
349 available for signaling or phospholipid production.

S6 Fig. AA-containing TAG and total TAG normalized to total lipids in sample. Lipids were extracted from wild-type and *ATGL*^{-/-} (*bmm*¹/*bmm*¹) ovaries and analyzed by mass spectrometry. Data from main Figure 8 has been re-plotted as relative abundance normalized to total lipids in the sample. Error bars, SD. **(A, B)** Two triglyceride species containing arachidonic acid (AA). Error bars, SD, **p*=0.0279, ***p*=0.0019, unpaired t-tests, two-tailed. **(C)** Overall triglyceride levels are slightly increased in *ATGL*^{-/-} ovary lipids. Error bars, SD, ***p*=0.002, unpaired t-test, two-tailed. **(D)** Relative amounts of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) in wild-type versus *ATGL*^{-/-} ovary lipids. Error bars, SD, **p*=0.0329, **p*=0.0416, ****p*=0.0001, Sidak's multiple comparisons test. While overall triglyceride levels are similar between the two genotypes (C), the AA-containing triglycerides are increased (A, B), and three classes of phospholipids are decreased (D) in the absence of ATGL. The reason for the decrease in phospholipids is not clear. One possibility is that ATGL breaks down triglycerides to generate precursors for phospholipid production; alternatively, in the absence of ATGL, ovaries may contain a different mix of follicle stages (and thus different levels of triglyceride accumulation) due to altered developmental progression.

350

351 *ATGL limits arachidonic acid levels to control PG-dependent actin remodeling*

352 We hypothesize that ATGL acts on LD triglycerides to release AA, which then serves as the
353 substrate for Pxt-dependent PG production; PG, in turn, controls actin remodeling necessary for
354 follicle development. Thus, the actin defects due to reduction or loss of ATGL, or co-reduction in
355 ATGL and Pxt should be suppressed by exogenous free AA.

356 We utilized our *in vitro* egg maturation (IVEM) assay, in which isolated Stage 10B
357 follicles mature *in vitro* in a simple culture medium [45]. One of its key components is fetal
358 bovine serum (FBS), which supplies numerous nutrients and growth factors. Using this assay,
359 we find that the majority of Stage 10B follicles from single heterozygotes of *pxt* and *ATGL*
360 develop *in vitro*, while 55% of the follicles from double heterozygotes fail to develop (S7 Fig).
361 These data recapitulate what we observed when we quantitatively assessed the actin defects in
362 the double heterozygotes (Fig 7B, 7D). To test if *ATGL* supplies AA for PG production, we
363 hoped to use the IVEM assay to determine if exogenous AA rescues follicle development in the
364 double heterozygotes (*pxt/ATGL*). Unfortunately, this experiment yielded highly variable results
365 (data not shown), likely because FBS contains unknown amounts of FAs, including AA.

S7 Fig. *ATGL* and *Pxt* genetically interact in the *in vitro* egg maturation assay. Graph of the percentage of Stage 10B follicles developing *in vitro*, normalized to wild-type (*yw*) development, for the following genotypes: *ATGL*^{-/+} (*bmm*¹^{-/+}), *pxt*^{-/+} (*pxt*^{f01000}^{-/+}), and *ATGL/pxt* (*bmm*¹/*pxt*^{f01000}). *ATGL/pxt* follicle development is reduced by ~50% compared to the *ATGL*^{-/+} and *pxt*^{-/+} controls.

366
367 As an alternative approach, we reduced the amount of FBS in the medium and assessed
368 follicle response to exogenous free AA (Fig 9A). At ~60μM, a slightly higher percentage of
369 follicles developed than in control medium, but that fraction dropped steadily as AA
370 concentrations were raised further. This finding indicates that high levels of AA are toxic to
371 follicle development; AA is known to be cytotoxic for cultured cells, at least in part due to
372 pleotropic effects unrelated to PG production [46].

373

Fig 9. ATGL controls free arachidonic acid. (A) Graph of the percentage of wild-type (*yw*) Stage 10B follicles that develop *in vitro* in control (EtOH, ethanol) or arachidonic acid (AA) media. Error bars, SD. (B-C') Maximum projection of three confocal slices of wild-type (*yw*) S10B follicles treated with either IVEM medium containing EtOH or 500 μ M AA for 1 hour and stained for LDs (Nile red) in white; B' and C' are zoomed in images of the boxed regions in B and C. All images were brightened by 70% to improve visibility. B and C, scale bars=50 μ m. B' and C', scale bars=10 μ m. (D) Graph of quantification of LD clustering in nurse cells of wild-type (*yw*) S10B follicles exposed to either EtOH or 500 μ M AA for one hour. Pearson's chi-square test, $p>0.05$. (E) Schematic depicting the rationale of the experimental approach used in F (created with Biorender.com). (F) Graph of relative percentage of follicles from the indicated genotypes developing in 500 μ M AA media versus control media (EtOH). Genotypes tested are wild-type (*yw*), *pxt*^{-/+} (*pxt*^{f01000}/+), *ATGL*^{-/+} (*bmm*¹/+), and *ATGL/pxt* (*bmm*¹/*pxt*^{f01000}). Error bars=SD. * $p<0.05$, ** $p<.001$ by paired t-test. Wild-type follicle development, *in vitro*, is inhibited with increasing AA concentration (A). Exposure to high doses of AA causes LD clustering (C-D), suggesting AA is accumulating in the LDs. If the release of AA from LDs is mediated by ATGL, and this free AA contributed to impairing *in vitro* follicle development when exogenous AA is provided, then reducing ATGL will improve the percentage of follicles developing (E). While reducing Pxt does not alter the relative percentage of follicles developing in 500 μ M AA media compared to wild-type follicles, reducing ATGL alone or in combination with reduced Pxt results in increased follicle development compared to wild-type follicles (F).

374

375 In other systems, exogenous AA is initially stored in LDs [40, 47, 48]. Similarly, we find
376 that high-doses of AA (500 μ M AA) result in LD clustering in 60% of the follicles, and perhaps an
377 increase in LD size (Fig 9B-9D). These observations are consistent with the notion that some
378 exogenous free AA can be sequestered by LDs, but at higher levels AA inhibits follicle
379 development.

380 The dose-dependent AA inhibition of follicle development provided a new approach to
381 determine the contribution of ATGL in this system. We reasoned that if ATGL is required to
382 release AA from internal stores and thus generate free AA, then reducing ATGL levels will
383 partially suppress the inhibition of follicle development by high levels of exogenous AA (Fig 9E)
384 as total free AA levels will be lower. We find that development of *pxt* heterozygous follicles is
385 impaired to the same level as wild-type follicles in the presence of 500 μ M AA (Fig 9F). In
386 contrast, two-fold more Stage10B follicles from *ATGL* heterozygotes or double heterozygotes of

387 *ATGL* and *pxt* develop in the presence of 500 μ M AA than wild-type follicles (Fig 9F). These data
388 are consistent with the model that *ATGL* releases AA, which is required for PG production (Fig
389 10). These PGs then regulate actin remodeling necessary for follicle development.
390

Fig 10. LD proteins regulate actin remodeling via three pathways. Schematic (created with Biorender.com) summarizing the findings presented. Synthesis of TAG (triglyceride) by DGAT1 (magenta) leads to accumulation of TAG between the inner and outer leaflets of the ER membrane (dashed inset=zoomed in schematic) and eventually LD budding from the ER. Arachidonic acid (AA, red lines) is stored either in the form of TAG or is incorporated into the phospholipid membrane. We identified three pathways by which LD proteins regulate actin remodeling. In the first, PLIN2 (blue) regulates actin remodeling by a PG-independent pathway. In the second, Jabba (green) and Pxt act together to regulate actin remodeling, but the precise nature of this relationship is unknown (dashed arrow). In the third pathway, *ATGL* (red) can liberate AA from TAG stored in LDs, providing the substrate, either directly or indirectly, for Pxt and PG production (orange) to regulate actin remodeling. The AA freed from TAG by *ATGL* (red) could be supplied directly to Pxt or be first incorporated into phospholipids (surrounding the neutral lipid core of LDs or present in the ER or other cellular membranes). In a second step, cPLA2 can then release AA from these phospholipids to provide the substrate for PG production.

391 Discussion

392 Our data reveal that multiple LD proteins have critical roles in regulating actin cytoskeletal
393 dynamics during *Drosophila* follicle development. Loss of either ATGL, PLIN2, or Jabba results
394 in cortical actin breakdown, cortical contraction failure, and defective actin bundle formation
395 during Stage 10B of oogenesis (Fig 3). This phenotype is uncommon, but is observed in flies
396 lacking Pxt, the *Drosophila* COX-like enzyme [26]. Dominant genetic interactions reveal that
397 LDs participate in three distinct pathways to regulate actin remodeling (Fig 10): PLIN2 acts
398 independent of PGs, and Jabba and ATGL act in two separate PG-dependent pathways (Figs 5
399 and 7). For the ATGL-dependent pathway, we provide evidence that ATGL controls actin
400 remodeling by regulating the availability of AA for PG synthesis (Fig 9). While PG signaling is
401 known to have a conserved role in regulating actin remodeling across organisms and cell-types
402 [49-54], this study provides the first evidence linking LDs to PG-dependent actin remodeling.

403

404 LDs provide the substrate for PG production

405 The rate limiting step for PG production is the enzymatic release of AA from cellular lipids [8,
406 27]. To explain the genetic interaction between Pxt and the triglyceride lipase ATGL, we
407 propose that ATGL regulates the release of AA from triglyceride stores, and that this AA serves
408 – directly or indirectly – as substrate for Pxt-dependent PG production. Consistent with our
409 model, we detect triglycerides containing AA in the ovary (Fig 8) and find that the toxic effect of
410 exogenous AA on development is modulated by ATGL levels (Fig 9). While 500 μ M AA severely
411 impairs *in vitro* wild-type follicle development, reducing ATGL dosage significantly increases the
412 number of developing follicles (Fig 9F). These finding suggest that release of endogenous AA is
413 impaired in ATGL-/+ follicles and therefore total free AA levels do not as readily reach toxic
414 levels (Fig 9E); free AA is likely toxic due to its many diverse roles in cellular signaling [55, 56].

415 Our studies also provide the first evidence that AA has a functional role during
416 *Drosophila* oogenesis: AA is present in ovary triglycerides; exogenous AA has both beneficial

417 and detrimental effects on follicle development, depending on concentration; and ATGL
418 heterozygosity shifts the sensitivity of follicles to exogenous AA (Figs. 8 and 9). These findings
419 are particularly important as it has been debated whether AA is present in flies. Some studies
420 report that AA is not detectable in various life stages and body parts [57-59], while others find it
421 [60-62]. These differences may be due to varying detection sensitivities and the fact that lipid
422 profiles vary dramatically between tissues and/or on different diets [63].

423 The free AA that serves as Pxt substrate might originate from two different sources:
424 phospholipids and triglycerides. In mammalian systems, both sources make contributions to PG
425 synthesis [47]. Cytoplasmic phospholipase A2 (cPLA2) releases AA from phospholipids, and in
426 many cell types, inhibiting cPLA2 severely impairs PG production [64-66]. Conversely,
427 knockdown/inhibition of ATGL decreases PG production in mast cells [28] and leukocytes [29].
428 The AA released by ATGL might either directly serve as substrate for PG synthesis, or it might
429 first be incorporated into phospholipids, to be released by cPLA2 in a subsequent step (Fig 10).

430 The site of PG synthesis in *Drosophila* Stage 10B nurse cells remains to be determined.
431 In a variety of mammalian cell types, the PG synthesis machinery localizes to LDs [40]. For
432 example, cPLA2, and its activators, localize to LDs and provide AA for PG production [67, 68],
433 and COX2 and PGE₂ synthases localize to and produce PGE₂ on LDs in cultured colon
434 adenocarcinoma cells [69]. Similarly, PGE₂ production is observed on LDs in normal rat
435 intestinal epithelial cells [67] and in macrophages during parasitic infection [70]. In fly ovaries,
436 PG may also be produced directly on LDs, or LDs may regulate the concentration of AA-
437 containing phospholipids and free AA and thus indirectly control PG synthesis on the ER. As the
438 cytoplasm of Stage 10B follicles is full of both ER and LDs (S4A-S4A''' and S4E-S4E''' Fig), the
439 two organelles are in very close proximity; in addition, in many cells, LDs remain physically
440 connected to the ER via lipidic bridges, which could convey AA or AA-containing phospholipids
441 to the ER [71]. Further, although we do not observe enrichment of Pxt on LDs by
442 immunofluorescence or biochemical purification (Fig 6), these observations do not rule out that

443 a fraction of Pxt is intimately associated with LDs. To resolve exactly where in nurse cells PG
444 synthesis occurs, it will be important to determine if cPLA2 and PG-type specific synthases are
445 enriched on LDs or elsewhere and to directly visualize the site of PG production.

446

447 Why LDs?

448 As Pxt resides in the ER and AA can be released from ER phospholipids, why would the
449 organism use LDs to control AA availability? LDs are increasingly recognized as a critical way
450 station during FA trafficking in cells [11] and often transiently sequester excess FAs from
451 external and internal sources to prevent cellular damage, such as ER stress and mitochondrial
452 dysfunction [72, 73]. Once safely stored in LDs, FAs can then be released in a regulated
453 manner and directed to specific intracellular fates, like energy production or signaling [74, 75].
454 Thus, LDs serve as FA trafficking hubs to both channel FAs to the correct intracellular
455 destinations and buffer the FA supply.

456 Proper channeling is particularly important for AA because it is cytotoxic, even at
457 concentrations that overlap physiological ones [46]. For example, in cultured hippocampal
458 neurons, low concentrations of AA support survival, but higher concentrations are quite toxic,
459 much more so than other FAs [76]. This pattern also holds true for oogenesis: a recent study on
460 bovine granulosa cells found that while low doses increase survival, high levels (200 μ M) induce
461 apoptosis [77]. We observe the same trend when we incubate *Drosophila* follicles, *in vitro*, in
462 AA. Low doses support a small increase in follicle development, while higher doses largely
463 inhibit development (Fig 9A). This sensitivity to AA suggests that cells need to carefully buffer
464 AA availability. Indeed, microscopic autoradiographic experiments reveal that in both immune
465 and epithelial cells exogenous AA is initially almost exclusively incorporated into LDs, in both
466 the phospholipid and neutral lipid pools [40, 47, 48].

467 We hypothesize that *Drosophila* follicles face a similar challenge. During Stages 9 and
468 10, nurse cells generate tens of thousands of LDs (Fig 1A-C) from lipids taken up from the

469 hemolymph [22]. This massive influx of FAs presumably includes AA, and AA, like other FAs, is
470 likely initially sequestered into LDs upon arrival. Exposing follicles to AA promoted LD clustering
471 and possibly LD enlargement (Fig 9B-D), consistent with the idea that AA is taken up by the
472 follicles and incorporated into LDs. Currently, we cannot tell if all the AA present in Stage 10B
473 follicles passed through LDs at some point or whether some is incorporated into phospholipids
474 directly. The penetrance and severity of actin defects in Stage 10B follicles of *ATGL* mutants is
475 somewhat variable, but can approach those of *pxt* mutants (Fig 3). These data are consistent
476 with the model that ATGL-dependent release of AA from LDs is a substantial, and possibly the
477 primary, source of AA for PG production during late-stage *Drosophila* oogenesis.

478

479 *ATGL function in Drosophila*

480 Given this critical role of ATGL in controlling PG synthesis during specific periods of *Drosophila*
481 follicle development, it will be interesting to determine whether it contributes to PG signaling in
482 other tissues. ATGL is best known for its role in fat storage [42] and is highly expressed in the
483 larval and adult fat body, the adipose tissue of the fly. Loss of ATGL causes triglyceride
484 overaccumulation in this tissue [42]. Notably, PG may be involved in regulating fat body
485 development: normally, cells of the larval fat body persist through pupal stages and are cleared
486 in early adulthood [78]; however, loss of Pxt results in their accumulation in adult abdomens
487 (TLT, unpublished observation). In males, ATGL function in neurons and testes regulates
488 whole-body energy storage by an as-yet uncharacterized mechanism thought to involve
489 systemic signaling. And ectopic ATGL expression has beneficial roles in two *Drosophila* disease
490 models, neurodegeneration due to mitochondrial dysfunction [79] and impairment of
491 nephrocytes, components of the renal system, due to high fat diet [80]. Here, tissue-specific
492 overexpression of ATGL in glia or nephrocytes, respectively, reverts disease-induced LD
493 accumulation as well as disease phenotypes. Intriguingly, glia cells in other organisms activate
494 PG signaling during brain inflammation/neurodegeneration [81, 82]. In the nephrocyte example,

495 ectopic ATGL affects transcription via lipid signaling, but exact mechanisms remain unknown.
496 Our work identifies a specific signaling pathway, PG signaling, that is regulated by ATGL during
497 oogenesis and provides evidence that AA is the relevant lipid released by ATGL. It will be
498 important to determine how widely used this new signaling pathway is, given the strong potential
499 for PGs to be playing roles in tissues with known ATGL functions.

500

501 *LDs regulate actin remodeling via multiple mechanisms*

502 ATGL is not the only LD protein promoting actin remodeling in nurse cells. The actin
503 cytoskeleton is similarly disturbed in mutants that lack two other LD proteins, Jabba and PLIN2
504 (Fig 3). Our genetic interaction tests suggest that PLIN2 affects actin remodeling via a
505 mechanism that does not involve PGs (Fig 5) and that Jabba and ATGL act in distinct PG-
506 dependent pathways (Fig 7). Thus, neither PLIN2 nor Jabba are likely to regulate ATGL activity,
507 suggesting the existence of additional mechanisms by which LDs control actin remodeling in
508 nurse cells.

509 One such mechanism might be an alternate means of generating AA. This possibility
510 seems unlikely for PLIN2 since there is no genetic interaction with Pxt. Jabba, however, might
511 regulate a distinct lipase pathway that acts in parallel to ATGL to release AA. Indeed, Jabba has
512 been proposed to regulate lipid metabolism in the ovary [38]. Jabba recruits numerous proteins
513 to LDs [32, 33], which might include lipases or their regulators, such as the ATGL paralog Dob
514 [42] or a cPLA2 ortholog. Another intriguing candidate is Hsl, an enzyme involved in the
515 breakdown of sterol esters in *Drosophila* [83]. Hsl is among the most abundant proteins on
516 maternally inherited LDs of early embryos [32], and its substrates, sterol esters, are present in
517 ovary LDs (Fig 1K and 8E-8F).

518 Jabba might alternatively regulate the localization of PG synthesis machinery. The direct
519 product of COX-like Pxt is the intermediate PGH₂ which is then converted by various synthases
520 into PGs with distinct functions. Previous work suggests that Stage 9 follicles produce an

521 unknown PG that inhibits actin remodeling, while during Stage 10B $\text{PGF}_{2\alpha}$ promotes remodeling
522 [84]. Because Pxt relocalizes from the Golgi to the ER during this time (Fig 6), we hypothesize
523 that these two compartments recruit different PG synthases, leading to the production of distinct
524 PGs. If this hypothesis is correct, a role of Jabba might be to directly or indirectly control the
525 targeting of PG synthases between these compartments, e.g., Jabba might recruit certain
526 synthases to ER regions adjacent to LDs. Intriguingly, work in mammalian systems has
527 suggested that spatial segregation of PG synthesis enzymes between Golgi and ER is
528 functionally important [85].

529 A final possibility is that PLIN2 or Jabba regulate actin dynamics by recruiting actin and
530 actin binding proteins to LDs. Actin and actin-regulators have been found in purified LD
531 preparations in numerous biochemical and proteomic studies [e.g., 86, 87-89]. LDs might
532 therefore regulate the concentration of these molecules free in the cytosol and thus their
533 availability for actin assembly. If Jabba's ability to recruit these proteins is regulated by PG
534 signaling, it would explain how Pxt and Jabba act in the same pathway. Further, since LDs are
535 highly motile [88], including in nurse cells [1], they may deliver actin and its binding partners to
536 sites of actin remodeling within the cell. Intriguingly, PLIN2 regulates LD transport during early
537 *Drosophila* embryogenesis [90], and the extensive LD clustering in *Jabba* mutants may interfere
538 with their motility (Fig 4C-4C", 4F).

539 While many questions remain, our findings have uncovered a critical role of LD proteins
540 in regulating actin cytoskeletal remodeling. Given the conservation of ATGL and PLIN2 protein
541 and metabolic functions, it is likely this new actin regulatory function is also conserved across
542 organisms.

543

544 LD dispersal

545 The intracellular distribution of LDs is highly regulated and can change with the cell cycle,
546 developmental stage, and nutritional state [88, 91]. We find that PG signaling promotes LD

547 dispersal as loss of Pxt causes severe LD clustering in Stage 10B (Fig 4B-4B', 4F). Because LD
548 clustering also occurs after just ~1hr treatment with the COX inhibitor aspirin (Fig 4G, 4H-H"),
549 this phenotype is indeed due to loss of PG synthesis and signaling within the follicle, and is not
550 an artifact of poor health of the follicles. These findings are exciting as there is little evidence yet
551 in the literature that PGs regulate LDs. One study implicated PGE₂ signaling in inhibiting
552 triglyceride storage in LDs in white adipose tissue [92]. Conversely, in granulosa cells of
553 periovulatory follicles, PGE₂ upregulates PLIN2 [93]. And in human trophoblastic cells, COX2
554 inhibition reduces LD production induced by the intracellular parasite *Toxoplasma gondii* [94].
555 These limited studies suggest that PGs may have cell-type specific roles in regulating LD
556 dynamics and lipid storage. Our results now reveal a dramatic effect of PGs on the spatial
557 distribution of LDs in a genetically tractable model, setting the stage for a mechanistic dissection
558 in the future.

559 PGs may regulate LD distribution by controlling actin dynamics. Indeed, actomyosin is
560 known to regulate LD transport, distribution, and shape from plants to mammals [95-97]. In
561 osteosarcoma cells, non-muscle myosin-dependent actin remodeling is required to drive
562 clustered LDs apart [87]. Whether actin cytoskeletal dynamics regulate LDs during *Drosophila*
563 oogenesis is unknown, but it is tempting to speculate that the LD clustering observed in *pxt*
564 mutants is due to defective actin remodeling. Indeed, evidence suggests that PGs promote
565 myosin activity during Stage 10B [98]. Alternatively, PGs may control ER integrity and/or LD
566 biogenesis or lipolysis and thereby regulate LD distribution.

567 We also find that LD proteins regulate LD distribution. Specifically, loss of PLIN2 or
568 Jabba causes dramatically clustered LDs (Fig 4). In embryos, LDs are normally dispersed in
569 *PLIN2* mutants [90] and less dramatically clustered in *Jabba* mutants [33], suggesting that these
570 proteins promote oogenesis-specific dispersal. A key question is whether the LD clustering is
571 related to the disrupted actin remodeling observed in the LD protein mutants. We deem this
572 unlikely, as the severity of LD clustering does not correlate with the level of actin defects when

573 the LD proteins are lost. For example, *Jabba* mutants have mild actin defects, but severe LD
574 clustering, whereas *ATGL* mutants have limited LD clustering and severe actin defects (Figs 3,
575 4, 5, and 7). Thus, we favor the model that the LD proteins regulate LD clustering at least in part
576 independently of PG signaling controlled actin remodeling.

577 Live imaging of *ex vivo* follicles and inhibition of various cytoskeletal components will
578 likely be critical to determine how LD clustering arises. For example, clustering may arise during
579 LD biogenesis, if LDs originate from a limited region of the ER and do not disperse. *PLIN2*
580 mutants have been proposed to exhibit LD biogenesis defects in nurse cells [18]. If like in
581 oocytes and embryos, nurse cell LDs move along microtubules, defects in the arrangement of
582 microtubules or in kinesin-1 and cytoplasmic dynein activity could also induce clustering [99-
583 101]. *PLIN2* regulates the relative activity of these motors on embryonic LDs [90]. Alternatively,
584 if like in osteosarcoma cells, LDs nucleate actin filaments to segregate from each other [87],
585 *Jabba* and *PLIN2* may recruit actin and actin-regulators, and PG signaling may regulate their
586 actin remodeling activity.

587

588 *LDs as regulators of development and female fertility*

589 Animal development requires careful regulation of metabolism, specific to tissue type and
590 developmental stage [102]. Yet despite the fact that LDs have a central role in lipid metabolism
591 and energy homeostasis in normal physiology and diseases [10, 11, 103], they have so far been
592 implicated as regulators of development in only a handful of cases [23, 33, 37, 104]. Our
593 analysis now reveals that LDs not only play a critical role at a specific transition during follicle
594 development, but identifies PG signaling as one of the mechanisms by which they exert their
595 regulatory role.

596 We speculate that this pathway is conserved across organisms. Indeed, LD
597 accumulation, composition, and localization are dynamic during oocyte maturation [4, 12], LDs
598 are hubs for FA trafficking, and FA levels (including that of AA) are critical for oocyte

599 development in many species [7, 13, 105]. Further, in both mouse models and human patients,
600 metabolic syndrome causes LD clustering in oocytes, failures in oocyte maturation, and
601 decreased fertility [13-15, 106, 107]. But does this relate to PG signaling? It is already well-
602 established that PG signaling plays critical roles in oocyte development across organisms [108-
603 114]. Recent evidence implicates LDs as a foci of PG production during reproduction. For
604 example, COX enzymes localize to LDs in the rat corpus luteum [115], and in fetal membranes
605 during advanced gestation and at induction of labor, times when PG synthesis and signaling are
606 upregulated [116, 117]. In mammals, it is not yet known whether the PG effects on oogenesis
607 and reproduction are due to changes in the actin cytoskeleton. However, cytoplasmic actin
608 density and cortical actin thickness increase during oocyte maturation, contribute to meiotic
609 resumption, and play roles in fertilization and early embryonic divisions [118, 119]. Given these
610 data, and our findings connecting LDs, PG signaling, and actin remodeling during *Drosophila*
611 oogenesis, we speculate the same pathways that regulate oocyte development in the fly are
612 conserved across organisms to regulate oocyte development, and contribute to infertility issues
613 due to limited or excess nutrition.

614

615 **Materials and Methods**

616

617 Fly stocks

618 Flies used in actin quantification and *in vitro* egg maturation experiments were maintained on
619 cornmeal/agar/yeast food at room temperature except as noted below. Flies used in all other
620 experiments were maintained on molasses/agar/yeast/malt extract/corn flour/soy flour food at
621 room temperature except as noted below. Stocks used were *y¹w¹* (BDSC1495), Oregon R
622 (BDSC 5), *pxt^{f01000}* (Harvard Exelixis Collection, [120]), *pxt^{EY03052}* (BDSC 15620), *Jabba^{DL}* [33],
623 *Jabba^{z101}* [33], *bmm¹* [34], *Lsd-2^{KG00149}* (BDSC13382), *osk3-GAL4* (BDSC 44242), *DGAT1 RNAi*
624 (TRiP.HMC06242, BDSC 65963), *mdy^{QX25}* (BDSC5095), *mdy^{RF48}* [16], *mat-alpha4 GAL4-VP16*

625 (BDSC 7063), *Lsd-2* RNAi (TRiP.HMS00629, BDSC 32846), *Lsd-2* RNAi (TRiP.HMS01292,
626 BDSC 34617), *sqh-EYFP-ER* (BDSC7195, [121]), and *sqh-EYFP-Golgi* (BDSC7193, [121]). For
627 the *DGAT1* RNAi germline knockdown, either *mat-alpha4 GAL4-VP16>DGAT1* RNAi or *osk3-*
628 *GAL4>DGAT1* RNAi crosses were performed at room temperature. For *mat-alpha4-GAL4*
629 knockdown, adult progeny were shifted to 29°C for 5 days and were fed on wet yeast paste
630 (paste replaced once on day 2 or 3). For *osk3-GAL4* knockdown, progeny were fed wet yeast
631 paste at room temp for 48 hours, then shifted to 29°C for 24 hours. Ovaries were subsequently
632 dissected and treated as described.

633

634 Immunofluorescence and fluorescent reagent staining

635 For Figures 1, 2, 4, 6, and Supplemental Figure 4 the following method, referred to as Staining
636 Method 1, was used. Adult female and male flies (to allow for mating) younger than two weeks
637 old were fed dry yeast for 48 hours at room temperature before ovary dissection in PBS-T
638 (phosphate buffered saline, 0.1% Triton X-100), except for RNAi knockdown as noted above.
639 Ovaries were fixed with 3.6% formaldehyde in PBS for 12 minutes at room temperature. Ovaries
640 were washed with PBS-T, and follicles of desired stages were isolated using forceps and pin
641 vises. Follicles were blocked in ovary block (10% BSA, PBS, 0.1% Triton X-100, 0.02% sodium
642 azide) overnight at 4°C. Primary antibodies were diluted in ovary block and incubated overnight
643 at 4°C. The following primary antibodies were obtained from the Developmental Studies
644 Hybridoma Bank (DSHB) developed under the auspices of the National Institute of Child Health
645 and Human Development and maintained by the Department of Biology, University of Iowa
646 (Iowa City, Iowa): mouse anti-Calnexin99A at 1:1000 (Cnx99A 6-2-1, Munro, S.) and mouse
647 anti-Golgin84 at 1:1000 (Golgin84 12-1, Munro, S.) [122]. Additionally, the following primary
648 antibodies were used: rabbit anti-Jabba preabsorbed at 1:10 against *Jabba* *-/-* embryos in ovary
649 block and used at 1:1000 [37] and rabbit anti-Pxt preabsorbed at 1:10 on *pxt* *-/-* ovaries in ovary
650 block and used at 1:1000 [84]. Samples were washed 3x with PBS-T, and then protected from

651 light for the remainder of the experiment. Samples were incubated in the following secondary
652 antibodies diluted to 1:1000 in ovary block overnight at 4°C: goat anti-rabbit IgG Alexa Fluor 633
653 (Invitrogen), goat anti-mouse IgG Alexa Fluor 488 (Invitrogen), and goat anti-rabbit IgG Alexa
654 Fluor 488 (Invitrogen). Samples were washed 3x with PBS-T and then stained with the following
655 reagents diluted in ovary block: Phalloidin Alexa Fluor 633 (Invitrogen) 1:150, 1 hour at room
656 temperature; Nile Red (1 mg/ml, Sigma-Aldrich) 1:50, 1 hour at room temperature; and Hoechst
657 33342 (1 mg/ml, Thermo Fisher Scientific) 1:1000, 20 minutes room temperature. Samples were
658 washed 3x with PBS-T, and then mounted on coverslips in Aqua Polymount (PolySciences).

659 For Figures 3, 5, 7, 9, and Supplemental Figures 1 and 5 the following protocol, referred
660 to as Staining Method 2, was used: Adult female and male flies (to allow for mating) within 24hrs
661 of eclosion were fed wet yeast paste for 72 hours at room temperature before dissection in
662 room-temperature Grace's medium (Lonza). Ovaries were fixed in 4% paraformaldehyde in
663 Grace's medium for 10 minutes at room temperature. Ovaries were washed 6x 10 minutes in
664 antibody wash (1X PBS-T + 0.1% Bovine Serum Albumin (BSA)). Samples were stained
665 overnight at 4°C with Alexa Fluor-488, -568, or -647 Phalloidin (Invitrogen) at 1:250. Samples
666 were then washed 5-6 times in 1X PBS for 10 minutes each and stained with 4'6'-diamidino-2-
667 phenylindole (DAPI, 5 mg/mL) at 1:5000 in 1X PBS for 10 minutes. Samples were rinsed in 1X
668 PBS and mounted in 1 mg/mL phenylenediamine in 50% glycerol, pH 9 [123].

669

670 Image acquisition and processing

671 Microscope images of fixed and stained *Drosophila* follicles were obtained using the following
672 microscopes: Leica SPE confocal microscope with an ACS APO x20/0.60 IMM CORR-/D or
673 ACS APO x40/1.5 oil CS objective (Leica Microsystems), Zeiss 700 confocal microscope or
674 Zeiss 880 confocal microscope (Carl Zeiss Microscopy) using a Plan-Apochromat x20/0.8
675 working distance (WD)=0.55 M27 objective, or Leica SP5 confocal microscope (Leica
676 Microsystems) using an HCX PL APO CS 63.0x1.40 oil UV objective and Leica HyD detectors.

677 Maximum projections of images, image cropping, and image rotation were performed in
678 Fiji/ImageJ software [124], scale bars were added in either Adobe Illustrator or Adobe
679 Photoshop (Adobe, San Jose, CA) and figures were assembled using Adobe Illustrator. Images
680 in Figures 3, 5, 7, and Supplemental Figures 1 and 5 were brightened by 30% and images in
681 Figure 9 were brightened 70% in Adobe Photoshop to improve visualization.

682

683 Quantification of actin defects

684 Confocal images of phalloidin stained Stage 10B follicles were collected as described. Actin
685 bundle and cortical actin defects were scored by scanning through confocal z-stacks of Stage
686 10B follicles in ImageJ in a genotypically blinded manner. Representative images of follicles and
687 scoring criteria are provided in Supplemental Figure 1. Briefly, actin bundles were scored as
688 normal (score = 0) if they were straight, forming from the nurse cell plasma membrane and
689 oriented inwards toward the nurse cell nuclei. Mild defects in bundling (score = 1) were defined
690 as sparse bundle formation or slight delay in bundle growth relative to follicle development.
691 Moderate defects (score = 2) were defined as collapsed, thick, and/or missing bundles from
692 regions of the nurse cell plasma membrane. Severe defects (score=3) included those previously
693 described, as well as a complete failure of bundles to form. Cortical actin was scored based on
694 whether the cortical actin was intact or disrupted. Defects in cortical actin are evident by an
695 absence or incomplete phalloidin staining between nurse cells and/or by nurse cell nuclei in
696 close proximity or contacting each other. Normal cortical actin (score = 0) is defined as being
697 completely intact and fully surrounding each nurse cell. Degree of severity of cortical actin
698 defect was determined by the relative number of disruptions in cortical actin observed with mild
699 defects (score=1) having a single instance, moderate defects (score=2) having two instances,
700 and severe defects (score = 3) having three or more instances of disrupted cortical actin. The
701 actin defect index (ADI) was then calculated by adding the bundle and cortical actin defect
702 scores and binning them into normal (total score = 0-1), mildly defective (total score = 2-3), or

703 severely defective (score = 4-6). Pearson's chi-squared analysis was performed using R
704 (www.r-project.org).

705

706 *In vitro egg maturation (IVEM) assay*

707 Adult female and male flies (to allow for mating) within 24hrs of eclosion were fed wet yeast
708 paste daily for 3 days prior to dissection. Ovaries were dissected in room temperature Grace's
709 medium (Lonza) containing 1x penicillin/streptomycin (100x, Gibco) and either 10% (S7 Fig) or
710 2.5% (Fig 9) fetal bovine serum (FBS, Atlanta Biologicals); this is termed IVEM medium. Stage
711 10B follicles were isolated and distributed between wells of a 24-well plastic tissue culture plate
712 (Falcon) and 1ml of fresh media was added with no additions (S7 Fig), arachidonic acid (AA,
713 Cayman Chemicals) diluted in 100% ethanol (EtOH) at the final concentrations listed, or an
714 equivalent amount of EtOH. Follicles were incubated overnight at room temperature in the dark.
715 The next day the number of follicles at each developmental stage were counted, and the
716 percentage developing was calculated; follicles that reached Stage 12 and older were
717 considered developed. For Supplemental Figure 7, the percent developing was normalized for
718 each experiment to the percent of wild-type follicles developing. Statistical analysis was
719 performed using the paired t-test function in Prism 8 (GraphPad Software).

720

721 *Aspirin or arachidonic treatment, staining, and phenotypic quantification of follicles*

722 Adult female and male flies (to allow for mating) within 24hrs of eclosion were fed wet yeast
723 paste daily for 3 days prior to dissection. Ovaries were dissected in room temperature Grace's
724 medium containing 1x penicillin/streptomycin and either 10% (Fig 4) or 2.5% (Fig 9) FBS. Stage
725 10B follicles were isolated and transferred to wells of a 24 well plastic cell culture plate. Fresh
726 medium was added to each well, to which 3mM aspirin (Cayman Chemicals) dissolved in EtOH
727 or DMSO, 500µM AA (dissolved in EtOH), or the same volume of solvent was added. Follicles
728 were incubated at room temperature in the dark for 1-1.5 hours. For Figure 4, samples were

729 stained with Phalloidin and Nile Red as described above in Staining Method 1. For Figure 9, the
730 medium was removed and follicles were fixed in 4% paraformaldehyde in 1X PBS for 10
731 minutes. Follicles were washed 3 times in PBS-T and stained for 2 hours at room temperature
732 with Nile Red 1:5000 (1 mg/mL) and Alexa 647 Phalloidin 1:250 (Invitrogen). Follicles were then
733 stained with DAPI (5 mg/mL) at 1:5000 in 1X PBS for 10 minutes and then washed 2 times in
734 1X PBS. Samples were mounted in Aqua-Polymount (PolySciences, Inc.).

735 Confocal images of Stage 10B follicles were scored in a genotypically blinded manner
736 for LD clustering and actin defects. LD clustering was scored based on the penetrance of the
737 phenotype with normal defined as ≤ 2 nurse cells exhibiting clustering, mild being defined as 3 to
738 4 nurse cells with clustering, and severe defined as >4 nurse cells with clustering. Follicles were
739 scored as having actin defects if actin bundle defects and/or disrupted cortical actin were
740 observed. Pearson's chi squares analysis was performed using R.

741

742 Western blots

743 Follicles from well-fed females of the indicated genotypes and stage were dissected and fixed
744 as described in Staining Method 1. Note that proteins from such samples run at the same
745 molecular weight as those from lysates prepared from live ovary tissue (S8 Fig). 25-50 follicles
746 were collected per sample and boiled in sample buffer (Laemmli Sample Buffer, Bio-Rad + 2-
747 mercaptoethanol, Bio-Rad). Samples were run on 4-15% gradient gels (Bio-Rad) at 80-120V
748 and transferred onto PVDF membrane (Immobilon-FL, EMD Millipore) in either Towbin (10%
749 Tris-Glycine + 20% MeOH; 80V for 30 minutes; for anti-PLIN2, anti-Pxt, anti-Calnexin) or CAPS
750 (Sigma-Aldrich; 10mM CAPS + 20% MeOH; 50V for 40 minutes; for anti-Jabba) transfer buffer.
751 Membranes were blocked for 1 hour at room temperature in Li-COR Odyssey Block (LI-COR
752 Biosciences). Primary antibodies diluted in Odyssey Block were performed overnight at 4
753 degrees. The following primary antibodies were used rabbit anti-Jabba (1:5000) [37], rabbit anti-
754 Pxt (1:5000) [84], rabbit anti-PLIN2 (1:5000) [90], mouse anti-Calnexin99A (1:5000, DSHB),

755 Mouse anti- α -Tubulin (1:5000, Cell Signaling Technologies). Before use, anti-Jabba had been
756 preabsorbed against PVDF membranes with samples from *Jabba* *-/-* ovaries. Membranes were
757 washed 3 times in 1X PBS-0.1% Tween 20 (PBS-Tween). The following secondary antibody
758 incubations were performed for 1 hour at room temperature: IRDye 800CW Goat anti-Rabbit
759 IgG (1:5000, Li-COR), and IRDy3 680RD Goat anti-Mouse IgG (1:5000, Li-COR). After
760 secondary antibody incubation, membranes were washed two times in PBS-Tween and once in
761 1X PBS. Membranes were imaged on a Li-COR Odyssey CLx imager and blots were processed
762 and quantified in Image Studio Lite 5.2 (Li-COR). To quantify, fluorescence intensity of each
763 band was measured and normalized to respective experimental controls. Data were analyzed
764 and plotted using Prism (GraphPad).

765

S8 Fig. Immunoblots comparing live vs. fixed samples. (A-C) Three simultaneously scanned immunoblots comparing the blotting patterns of live versus formaldehyde fixed wild-type whole-ovary samples, 5 ovaries per lane. **(A)** anti-Jabba immunoblot. **(B)** anti-Pxt immunoblot. **(C)** anti-PLIN2 immunoblot. The live vs. fixed banding pattern is comparable for all three antibodies tested. Fixed samples were used in S1 and S2 Fig immunoblots.

766

767 Ovary centrifugation

768 Mated adult females of mixed ages fed dry yeast were anesthetized and beheaded before being
769 mounted in Eppendorf tubes filled with 2.5% apple juice agar as described in [125]. Tubes were
770 spun for 10 minutes at 10,000 rpm at room temperature using an Eppendorf Microcentrifuge
771 (Model 5415D). Flies were removed from agar with forceps and ovaries were isolated and
772 treated as described in Staining Method 1 above.

773

774 Lipid droplet purification

775 250-300 ovaries per sample were rapidly dissected from mated and dry yeast fed females and
776 kept on ice in TSS (68 mM Na Cl₂, 0.03% Triton X-100) in 2mL Eppendorf tubes. Ovaries were
777 then washed 3x with TKM (50 mM Tris, pH 7.4, 25 mM KCl, 5 mM MgCl₂) to remove detergent.

778 TKM was removed and the volume of ovaries was estimated visually. Ovaries were kept on ice,
779 to which were added: 2 times the estimated ovary volume of TKM + 1M sucrose, protease
780 inhibitor cocktail to final concentration of 1X protease inhibitor cocktail (Sigma-Aldrich), and
781 calyculin A serine/threonine phosphatase inhibitor (10 μ L per mL of volume; Cell Signaling).
782 Ovaries were homogenized on ice by grinding with an automated tissue grinder (KONTES pellet
783 pestle) for 1-2 minutes, and then 20 μ L ovary lysate samples were transferred from the total
784 lysate to 0.65mL Eppendorf tubes and stored at -80°C. The remaining ovary lysates were spun
785 at 13200rpm for 10 minutes in an Eppendorf Microcentrifuge (Model 5145D) at 4°C. The
786 following was then added to samples slowly to avoid disturbing the LD layer: 300 μ L TKM + 0.5M
787 sucrose, 300 μ L TKM + 0.25M sucrose, 400 μ L TKM (no sucrose). Tubes were spun for 20
788 minutes at 4°C, with the speed adjusted as follows: 1000rpm for 5 minutes, 5000rpm for 5
789 minutes, 13200rpm for 10 minutes. Purified LDs were then scooped off the top of the sucrose
790 gradient using a drawn Pasteur pipette loop (Fisher Scientific, loop drawn by holding over a
791 flame). LDs were washed off the pipette loop with 20 μ L TKM (no sucrose) and stored at -80°C
792 until analyzed.

793 In past Western analyses, we loaded equal amounts of protein from LD preparations and
794 original tissue lysate for easy comparison [33]. This approach was not feasible here because of
795 the relatively low amounts of LDs recovered from the large volume of ovaries used. Instead, we
796 compared the LD sample to dilutions of ovary lysate. Purified LDs and ovary lysates were
797 diluted as indicated in 2X Laemmli Sample Buffer before being boiled for 30 minutes and
798 subsequently run on 4-15% SDS-PAGE gradient gels (Western blot protocol as described
799 above). Before the blocking step, the membrane was cut with a razor blade between 50 and
800 75kDa and each half was treated separately. Quantification was performed as described above
801 in Western blots.

802

803 Lipid extractions

804 25 ovaries per sample were dissected from mated, mixed-age, and dry yeast fed females and
805 homogenized in a 2mL Eppendorf tube with a motorized pestle (KONTES pellet pestle) in 1X
806 PBS and kept on ice. Lysates were then incubated 1:1 in a 2:1 chloroform/methanol mixture
807 overnight at 4°C. Samples were spun in an Eppendorf Microcentrifuge (model 5415D) at max
808 speed at room temperature for 1 minute, and the bottom organic layer was transferred to an
809 0.65mL Eppendorf tube. Samples were then vacuum dried and either analyzed immediately or
810 stored at -80°C for thin layer chromatography (TLC) analysis, or stored at -80°C before being
811 shipped on dry ice for mass spectrometry analysis.

812

813 Thin layer chromatography

814 Evaporated lipids were resuspended in 10µL 2:1 chloroform/methanol and spotted on
815 dehydrated silica plates (EMD Millipore; dehydrated by baking for 30 minutes at 100°C). Plates
816 were placed in a chamber (Millipore Sigma/Sigma-Aldrich Z266000) pre-saturated for 30
817 minutes with Petroleum Ether/Diethyl Ether/acetic acid (32:8:0.8) and allowed to develop until
818 the solvent almost reached the top of the plates, and the solvent line was quickly marked with a
819 pencil after removing plates from the chamber. The plates were then air-dried and briefly
820 submerged in charring solution (50% ethanol, 3.2% H₂SO₄, 0.5% MgCl₂). Plates were air-dried
821 briefly and were charred for 30 minutes at 120°C. Plates were imaged using a Bio-Rad Gel Doc
822 and bands were quantified using Fiji. Lipids were identified according to Knittelfelder & Kohlwein
823 [126] and by comparing whole lipid extracts to lipid standards (Millipore Sigma, data not shown).
824 Colorimetric band intensities were quantified in Fiji and triglyceride or sterol ester bands were
825 normalized to cholesterol band intensity. Statistical tests were run in GraphPad Prism as
826 follows: Figure 2D, Sidak's multiple comparisons test and Figure 8F, Dunnett's multiple
827 comparisons test.

828

829 LC-MS/MS Mass Spectrometry Analysis

830 Extracted lipids were dissolved in corresponding volumes of 2:1 methanol:chloroform (v/v) and 5
831 μ l of each sample was injected for positive and negative acquisition modes, respectively. Mobile
832 phase A consisted of 3:2 (v/v) water/acetonitrile, including 10 mM ammonium formate and 0.1%
833 formic acid, and mobile phase B consisted of 9:1 (v/v) 2- propanol/acetonitrile, also including 10
834 mM ammonium formate and 0.1% formic acid. Lipids were separated using an UltiMate 3000
835 UHPLC (Thermo Fisher Scientific) under a 90 min gradient; during 0–7 minutes, elution starts
836 with 40% B and increases to 55%; from 7 to 8 min, increases to 65% B; from 8 to 12 min,
837 elution is maintained with 65% B; from 12 to 30 min, increase to 70% B; from 30 to 31 min,
838 increase to 88% B; from 31 to 51 min, increase to 95% B; from 51 to 53 min, increase to 100%
839 B; during 53 to 73 min, 100% B is maintained; from 73 to 73.1 min, solvent B was decreased to
840 40% and then maintained for another 16.9 min for column re-equilibration. The flow-rate was set
841 to 0.2 mL/min. The column oven temperature was set to 55° C, and the temperature of the
842 autosampler tray was set to 4° C. Eluted lipids were analyzed using Orbitrap Q Exactive
843 (Thermo Fisher Scientific) Orbitrap mass analyzer. The spray voltage was set to 4.2 kV, and the
844 heated capillary and the HESI were held at 320° C and 300° C, respectively. The S-lens RF
845 level was set to 50, and the sheath and auxiliary gas were set to 35 and 3 units, respectively.
846 These conditions were held constant for both positive and negative ionization mode
847 acquisitions. External mass calibration was performed using the standard calibration mixture
848 every 7 days. MS spectra of lipids were acquired in full-scan/data-dependent MS2 mode. For
849 the full-scan acquisition, the resolution was set to 70,000, the AGC target was 1e6, the
850 maximum integration time was 50 msec, and the scan range was $m/z = 133.4\text{--}2000$. For data-
851 dependent MS2, the top 10 ions in each full scan were isolated with a 1.0 Da window,
852 fragmented at a stepped normalized collision energy of 15, 25, and 35 units, and analyzed at a
853 resolution of 17,500 with an AGC target of 2e5 and a maximum integration time of 100 msec.
854 The underfill ratio was set to 0. The selection of the top 10 ions was subject to isotopic exclusion
855 with a dynamic exclusion window of 5.0 sec. Mass spectrometry data was analyzed using

856 LipidSearch version 4.1 SP (Thermo Fisher Scientific). Identified lipid species with grade A and
857 B were manually curated. A total of 98 different triglyceride species were identified (STable 1).

858 For the quantitative analysis in Figures 8A-AD, 8G-H and Supplemental Figure 6, four
859 ovaries each from wild-type and *ATGL* mutant females were analyzed and the quantity of the 98
860 triglyceride species determined, relative to background. One wild-type sample contained an
861 order of magnitude less lipid and was discarded as an outlier (STable 1). In total, 25 different
862 types of FAs were identified in the ovary triglycerides. The abundance of each FA was
863 estimated by summing the amount of each triglyceride species containing that FA, weighted by
864 how many times that FA is represented in that triglyceride. For Figures 8A-8B, this abundance is
865 expressed as fraction of the total amount of fatty acids such identified. For Figure 8C, the signal
866 for all species of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol,
867 respectively, was summed and normalized to the average of the wild type. For Figure 8D, the
868 signal for all triglyceride species was summed and normalized to the average of the wild type.
869 To express the abundance of various lipid species relative to total lipids recovered (S6 Fig),
870 values for various lipid species were divided by the sum of all lipids in the sample. For Figure
871 8G and 8H, the signal for two AA-containing triglyceride species was computed, and normalized
872 to the average of the wild-type signal. Amounts and fractions were calculated using Microsoft
873 Excel and graphed using Prism 8 (GraphPad Software). Statistical tests for Figure 8 were as
874 follows: 8A and 8C was Sidak's multiple comparisons test, 8B was Tukey's multiple
875 comparisons test, 8D was an unpaired t-test, two-tailed, and 8G-H were unpaired t-tests, two-
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877

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889

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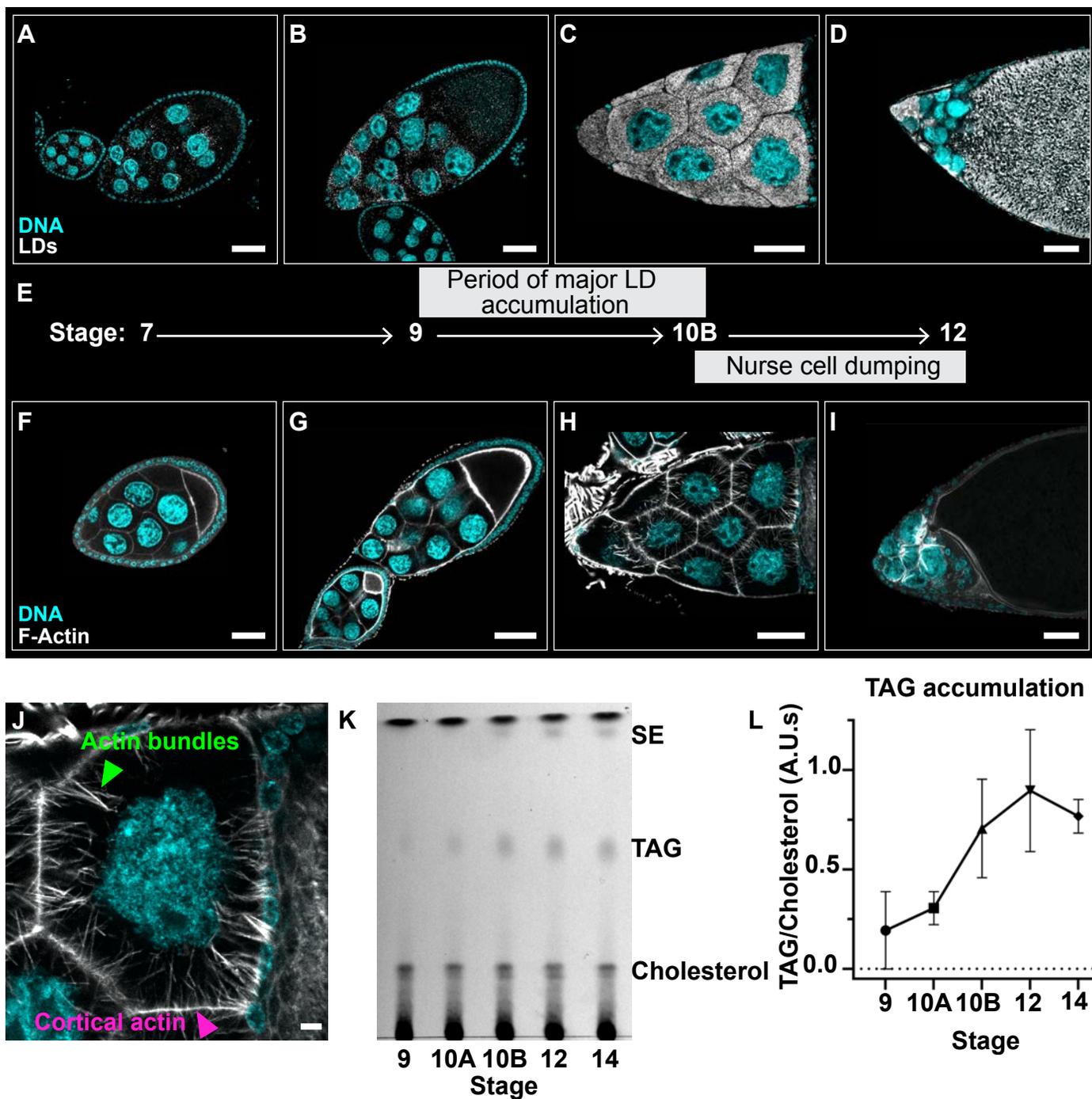
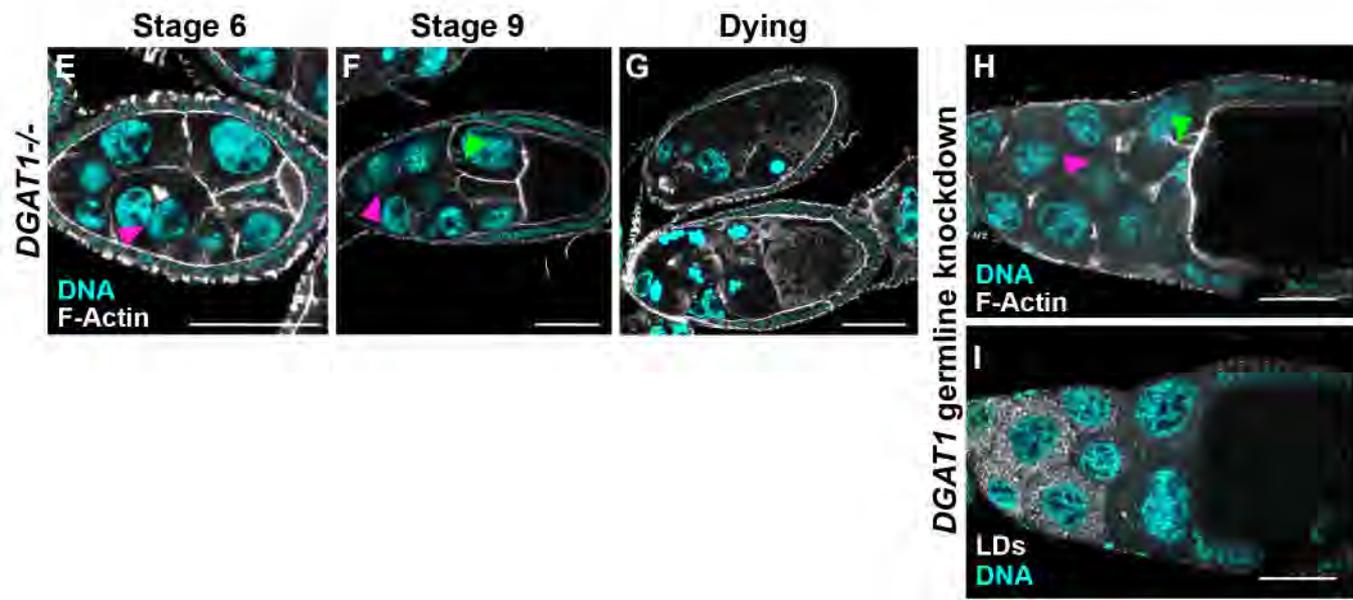
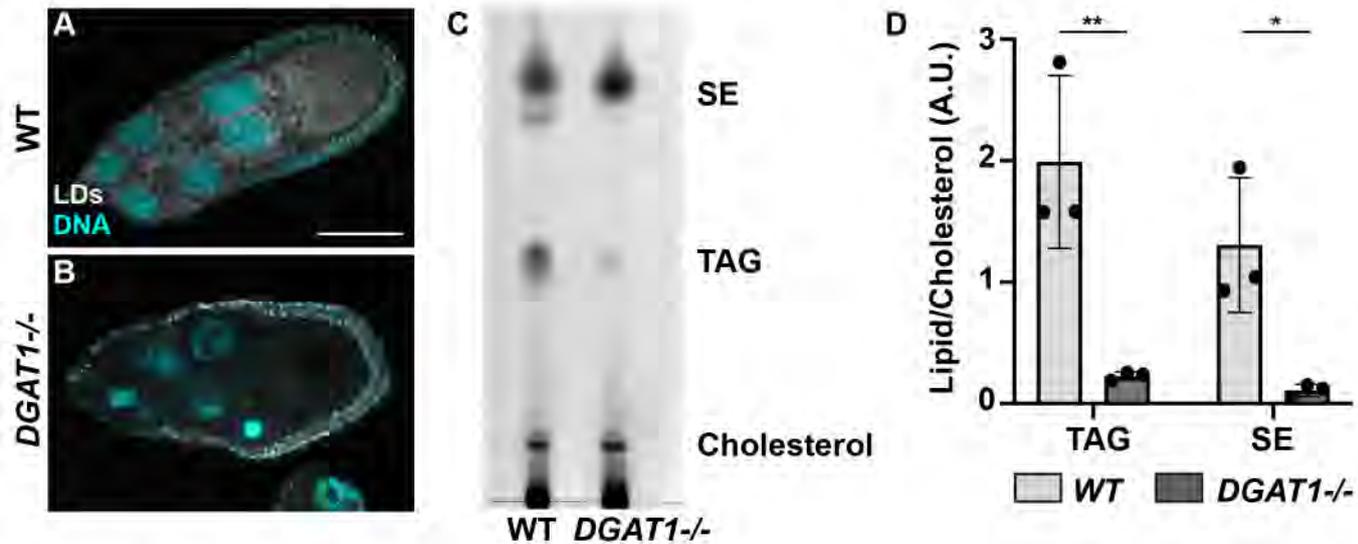


Fig 1



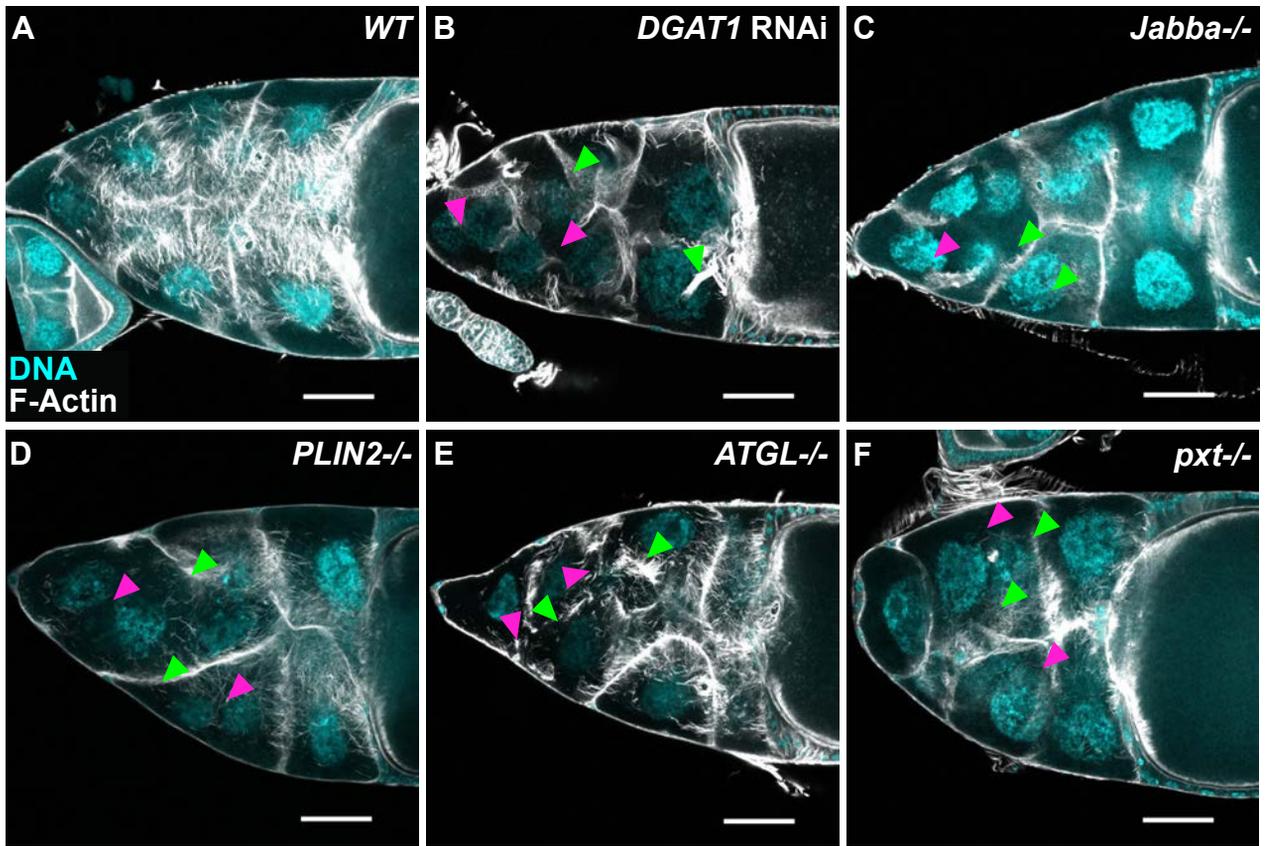


Fig 3

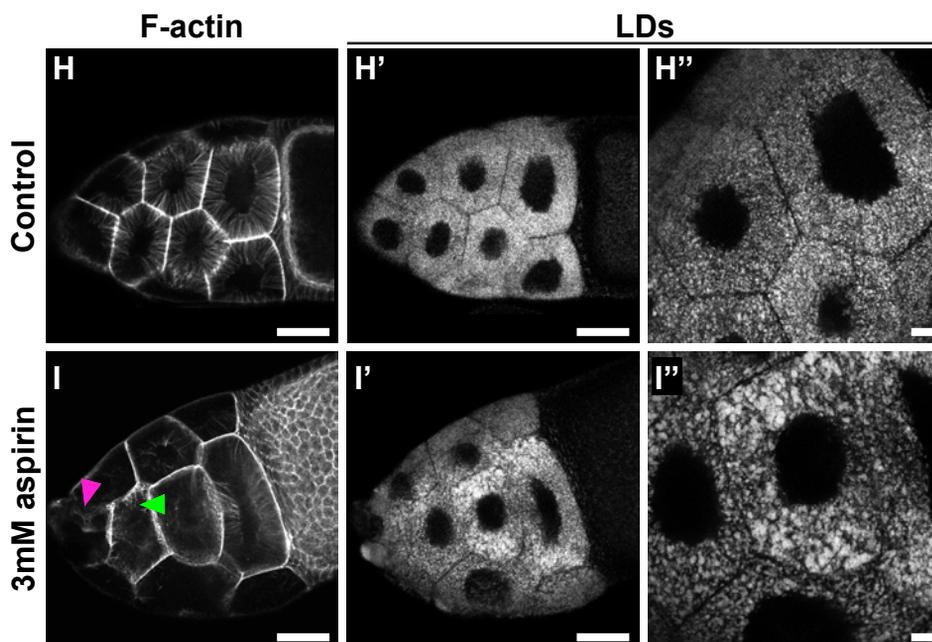
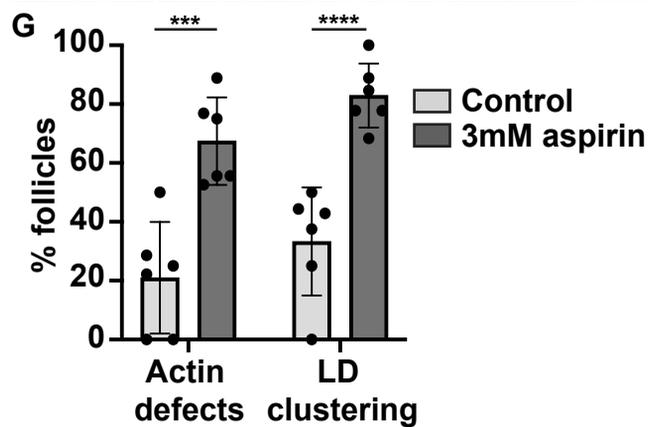
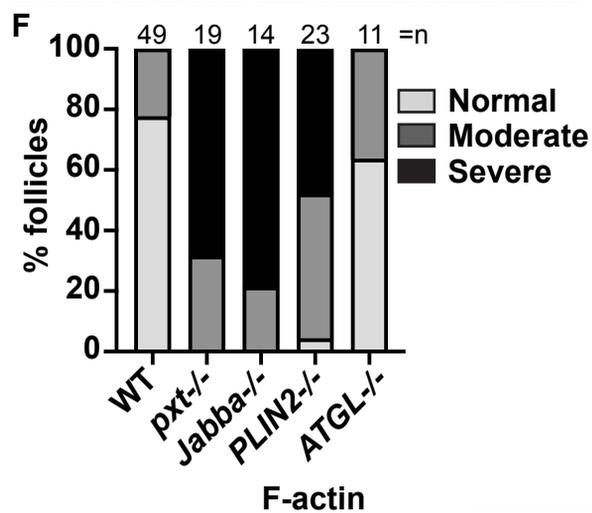
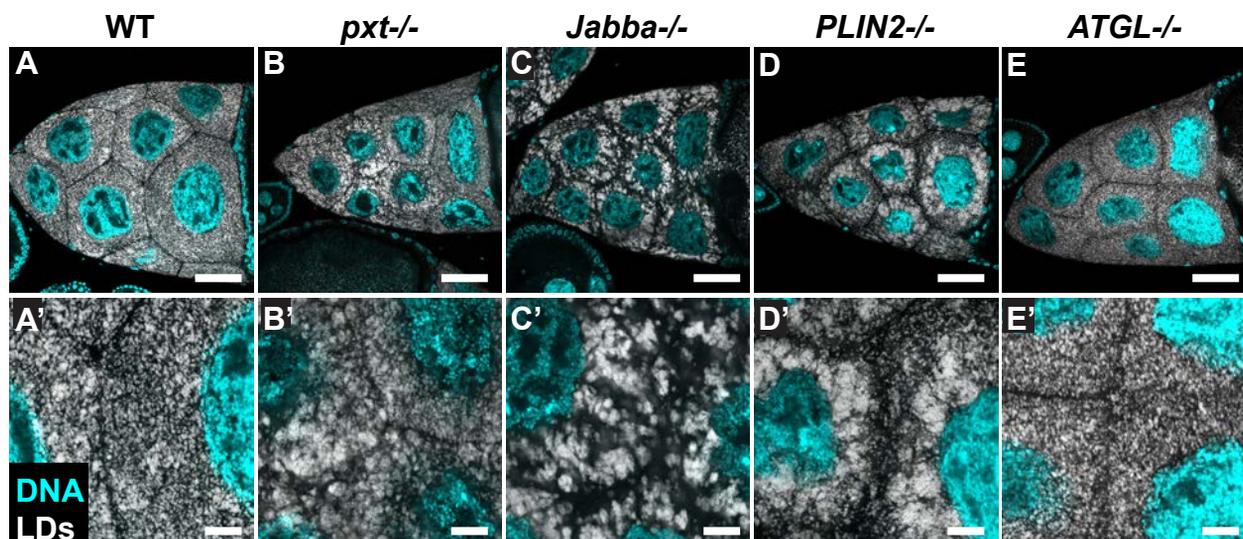


Fig 4

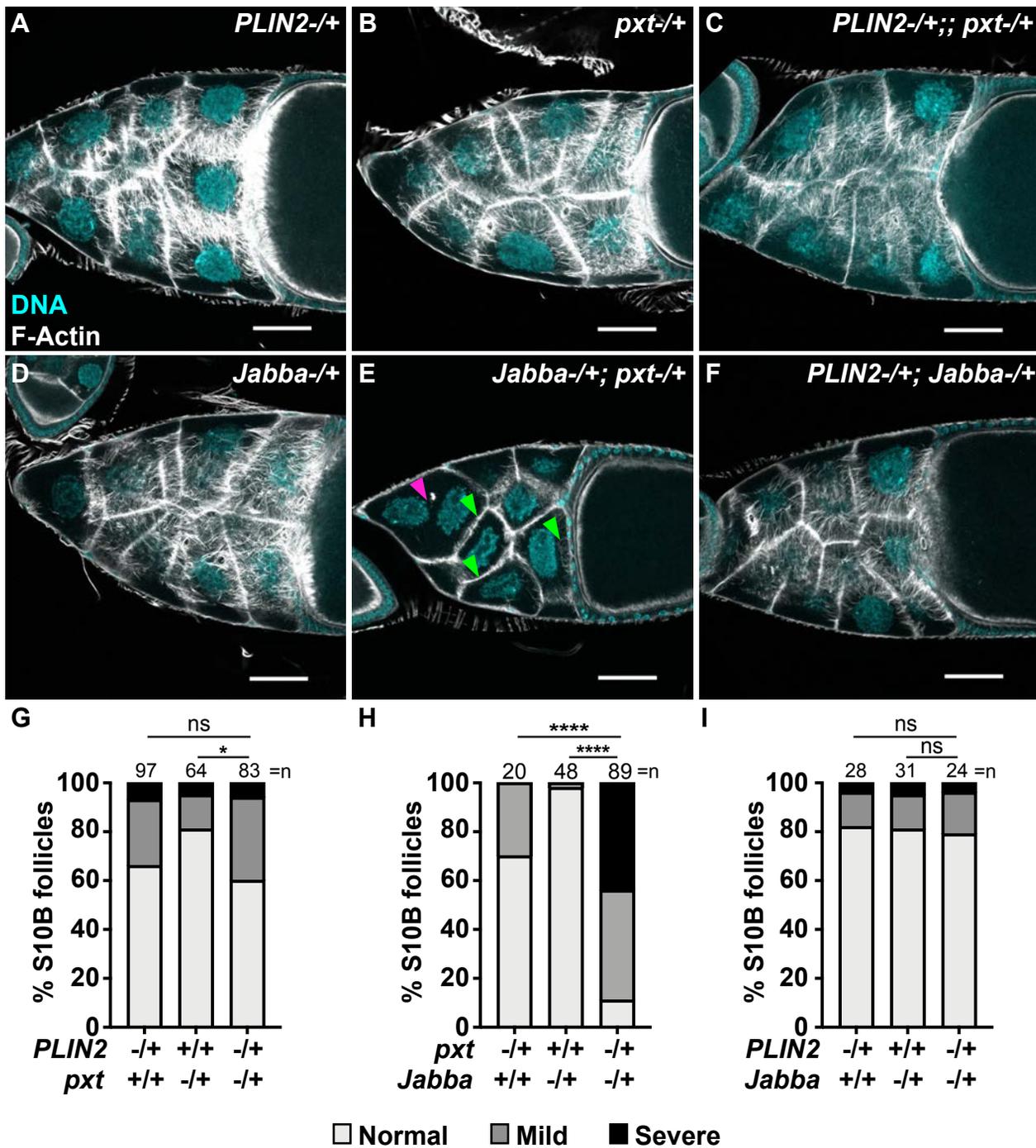


Fig 5

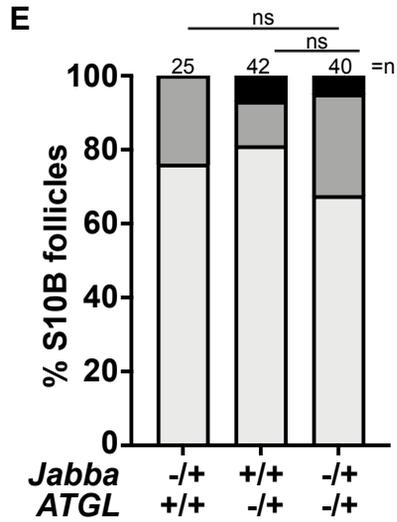
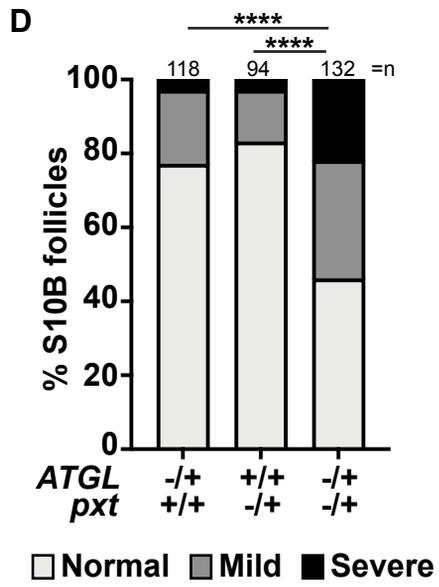
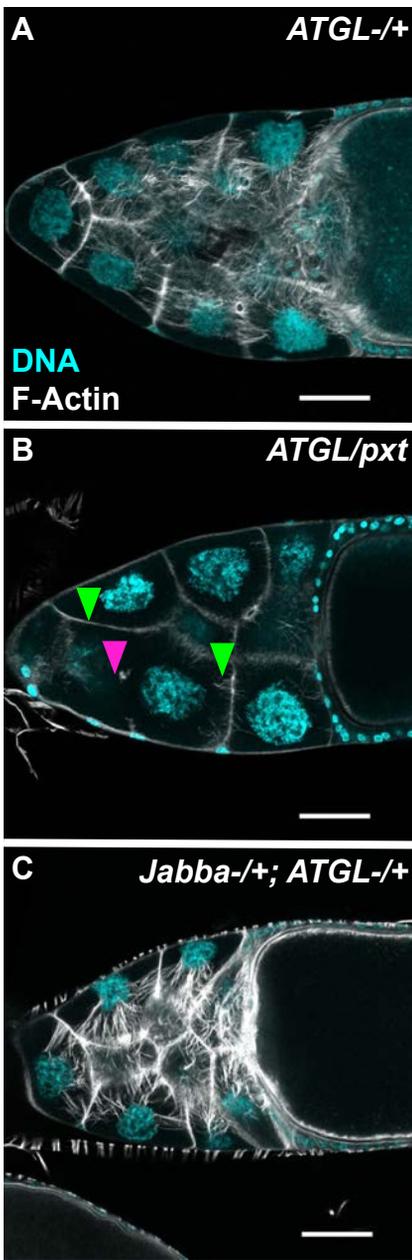


Fig 7

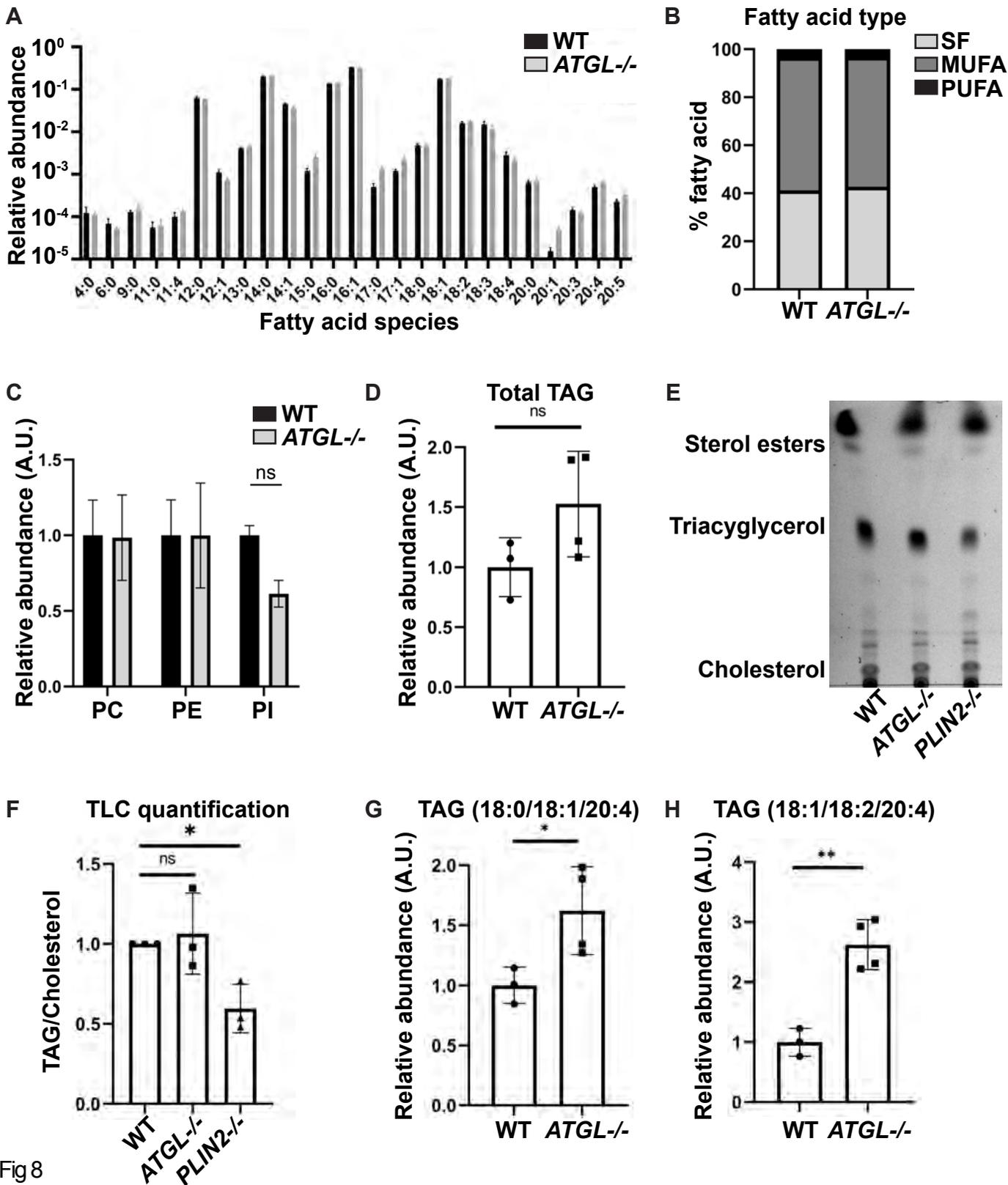


Fig 8

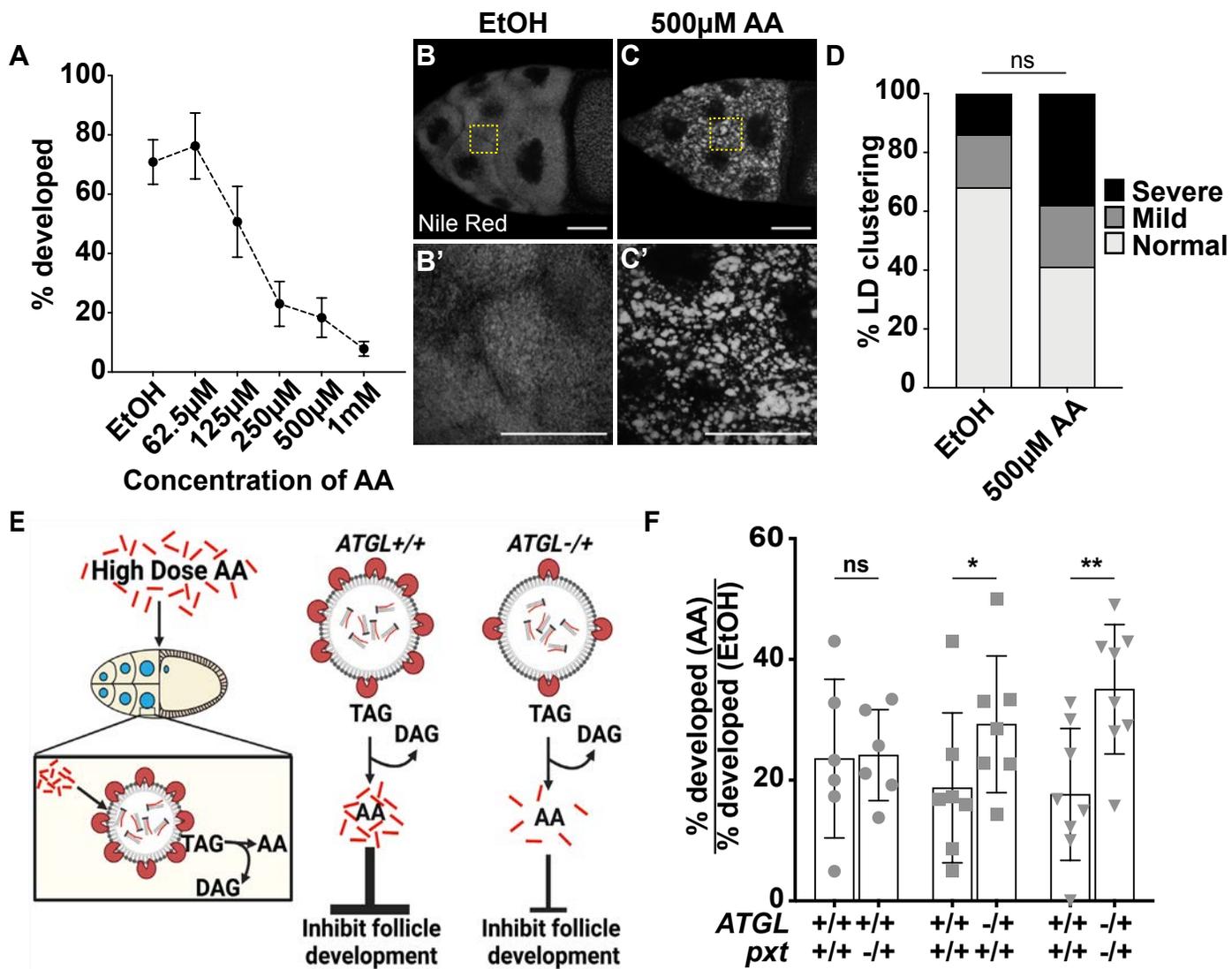


Fig 9

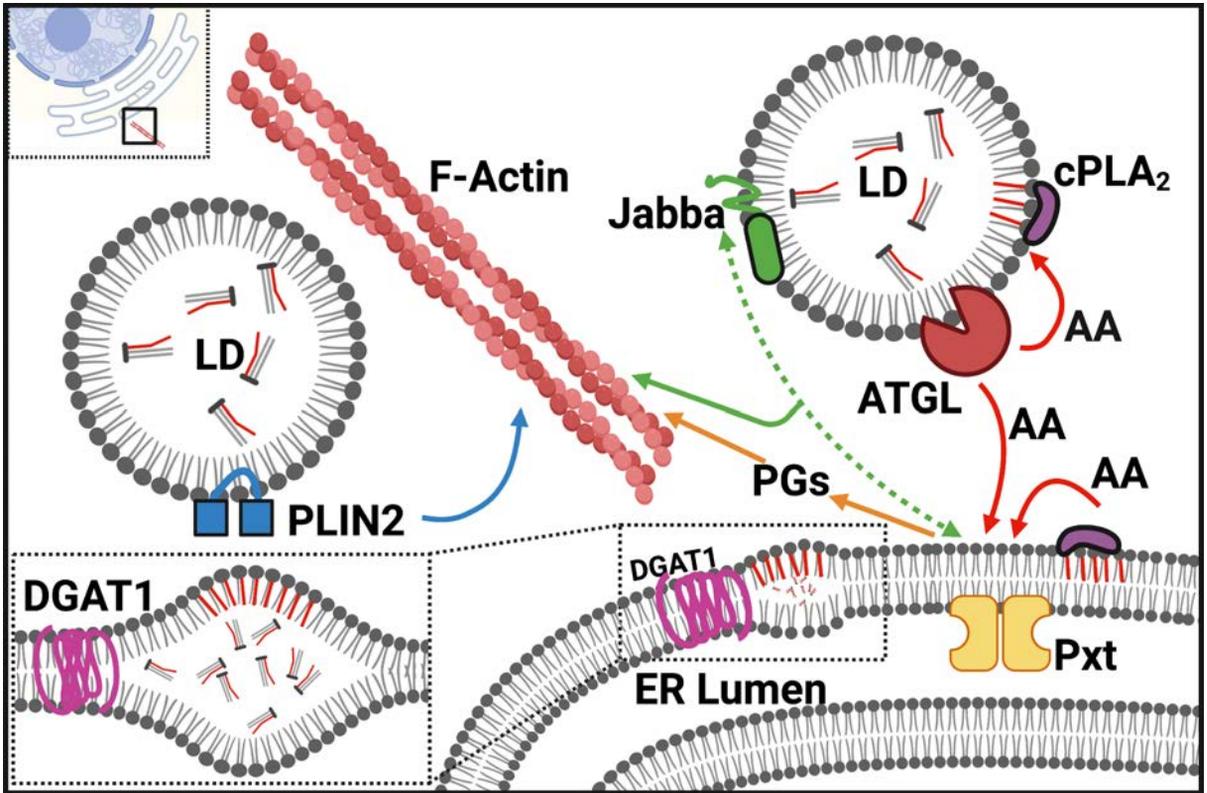
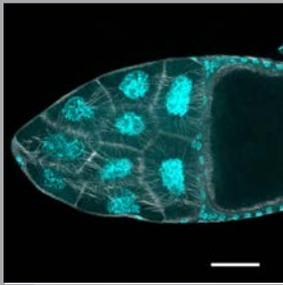
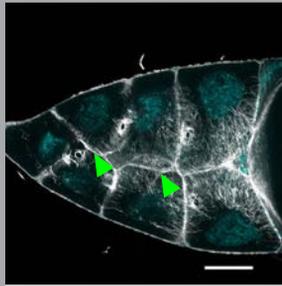
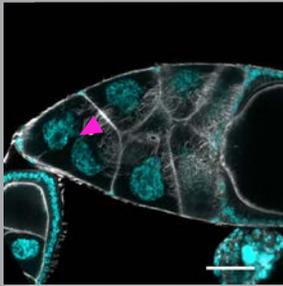
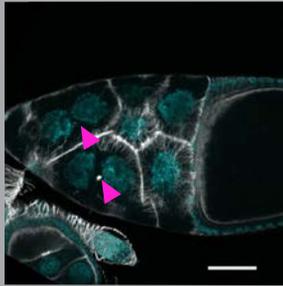
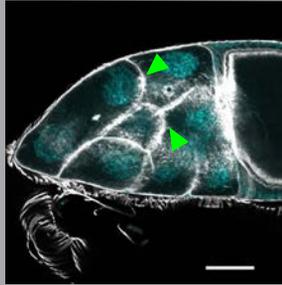
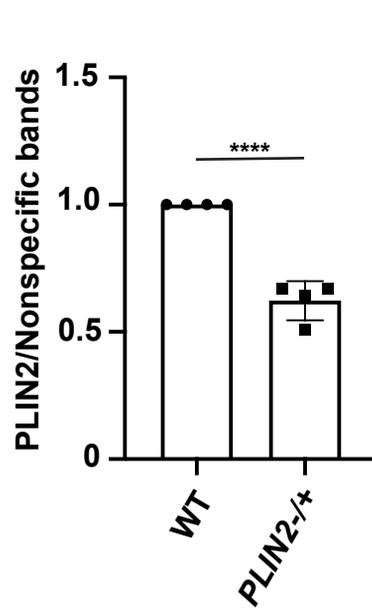
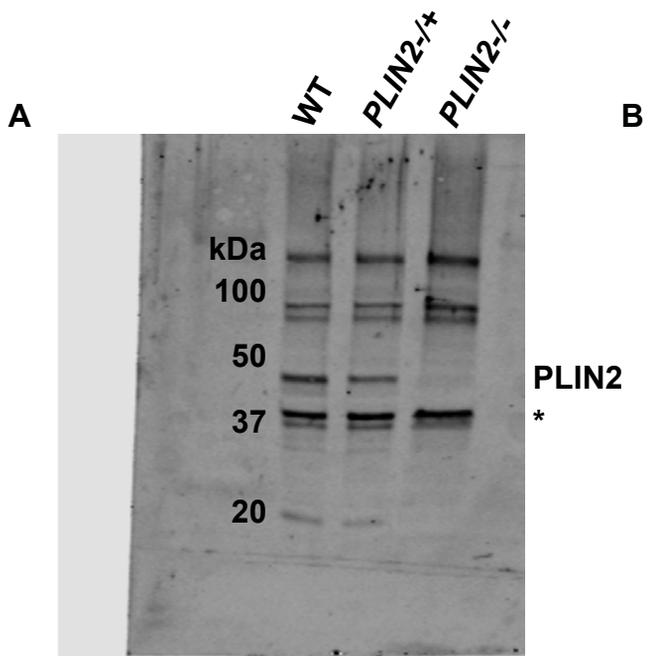


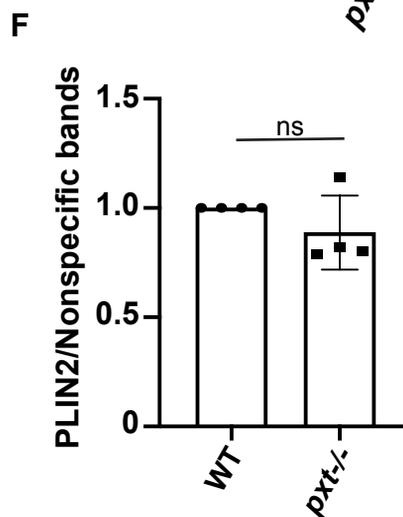
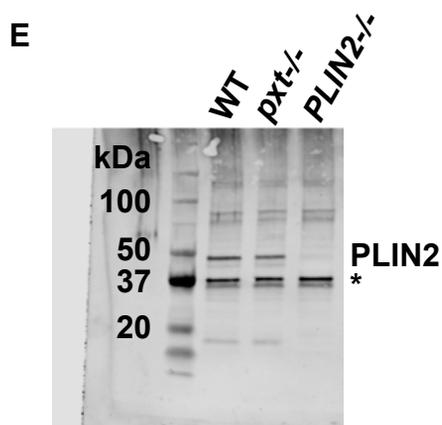
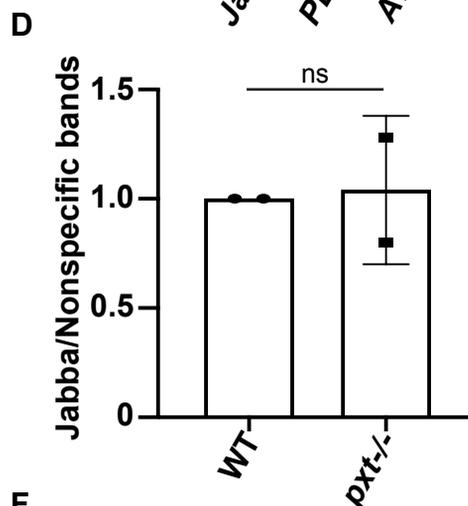
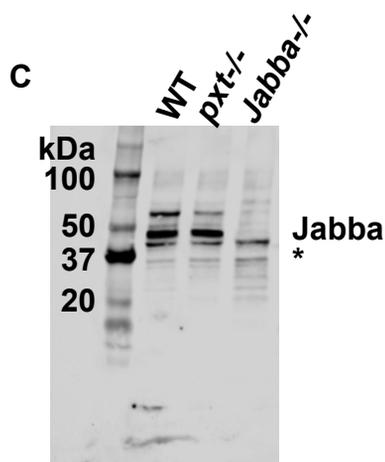
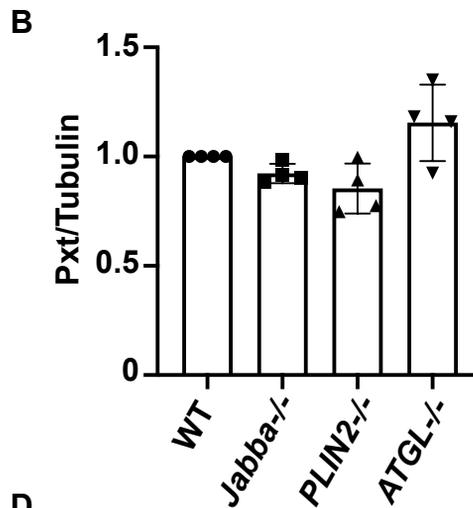
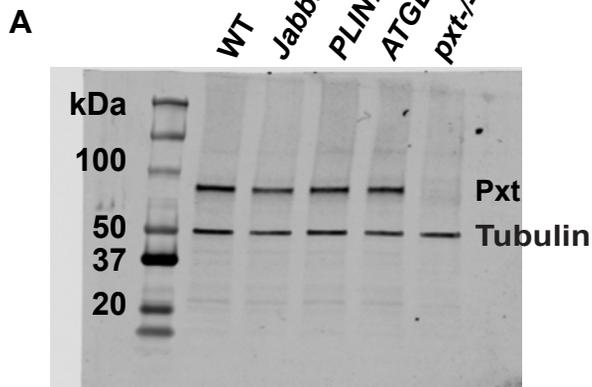
Fig 10

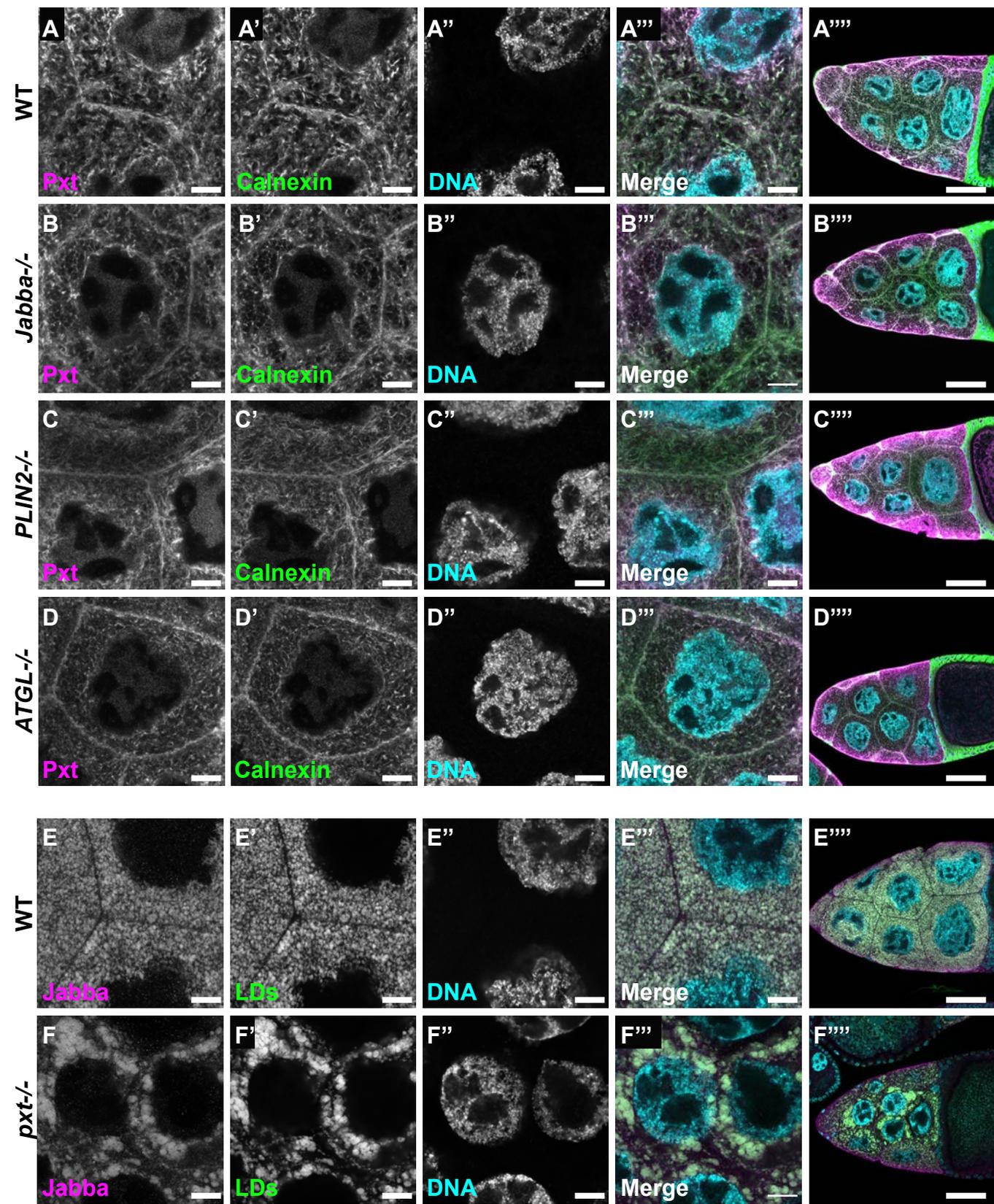
		Actin Bundles	Cortical Actin
Defects	Normal	 <p>Bundles form around nurse cell periphery</p> <p>Bundles are straight and oriented inward</p>	 <p>Actin surrounds each nurse cell, separating nurse cell nuclei</p>
	Mild	 <p>Actin bundles are largely normal, but may be sparse, or missing from areas</p>	 <p>Nurse cell nuclei are in close proximity/ touching</p> <p>A single instance of disrupted cortical actin is observed</p>
	Moderate	 <p>Actin bundles are sparse, stunted, and/or clumped</p> <p>Actin bundles are missing from areas around the nurse cell periphery</p>	 <p>Two instances of disrupted cortical actin are observed</p> <p>Nurse cell nuclei are in close proximity/ touching</p>
	Severe	 <p>Actin bundle formation is delayed, bundles fail to form, and are sparse and/or stunted and/or missing</p>	 <p>Three or more instances of cortical actin breakdown are seen</p> <p>Nurse cell nuclei are in close proximity/ touching</p>

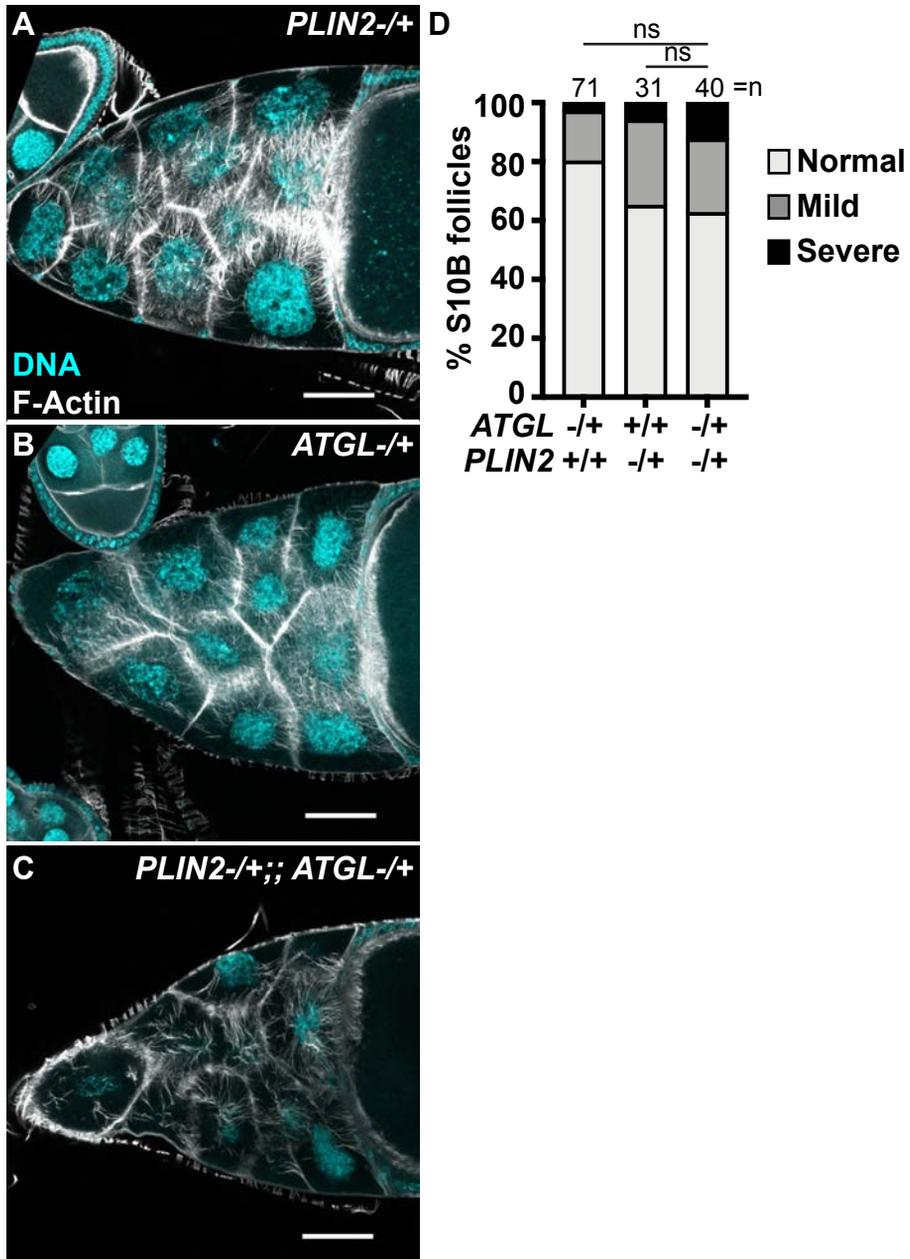
SFig 1



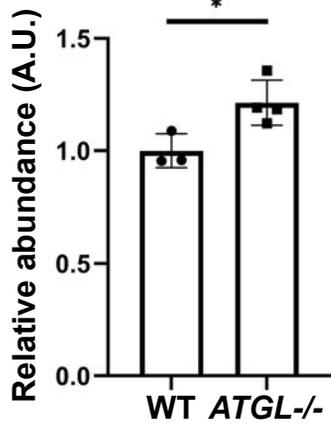
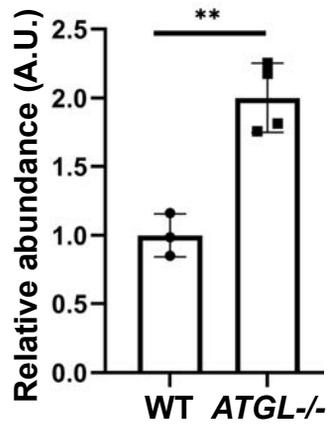
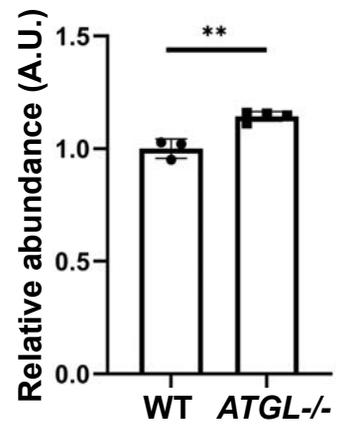
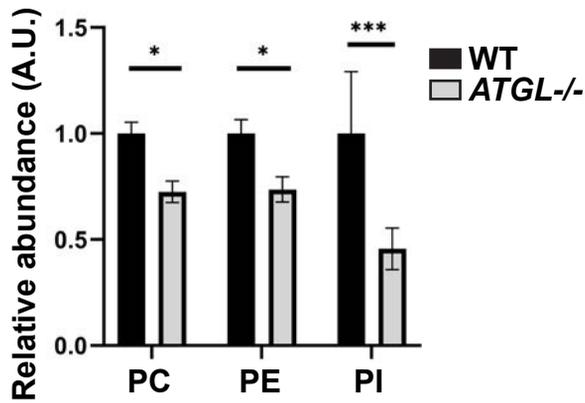
SFig 2

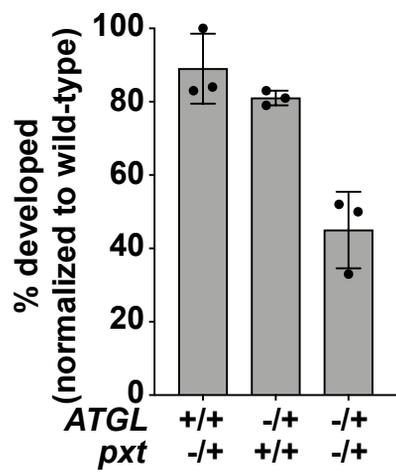




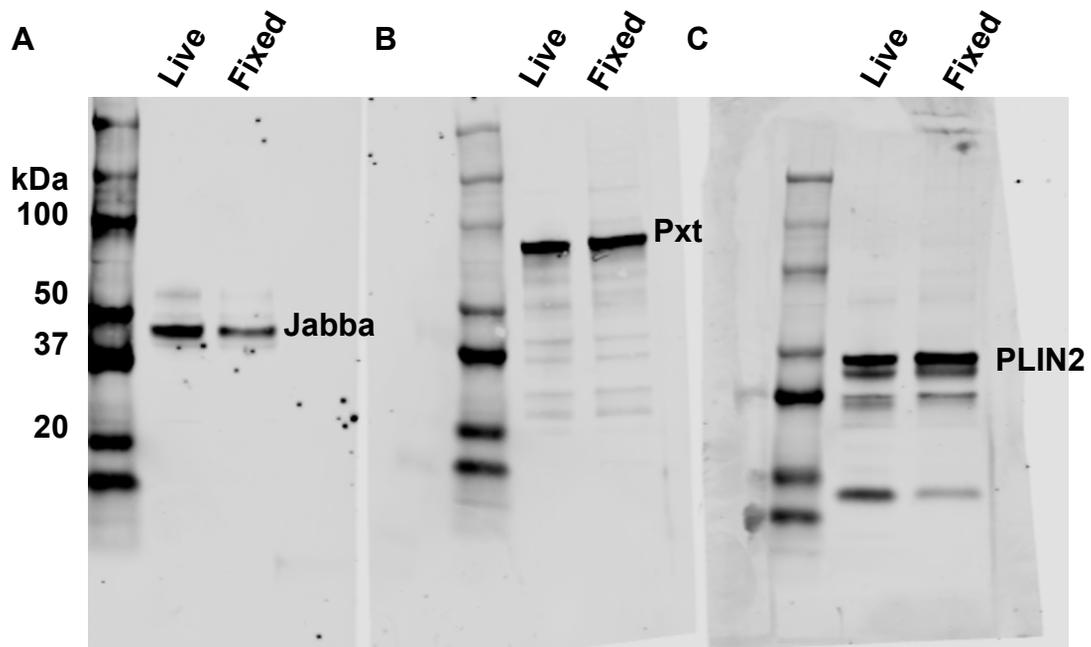


SFig 5

A TAG (18:0/18:1/20:4)**B** TAG (18:1/18:2/20:4)**C** Total TAG**D**



SFig 7



SFig 8

Triglyceride species	wild type 2	wild type 3	wild type 4	ATGL 1	ATGL 2	ATGL 3	ATGL 4
16:1/17:1/18:2	4028800	5894800	5037800	9625800	8910800	17357800	11937800
16:1/17:1/18:1	12114670	30224670	23104670	33724670	52254670	81364670	76874670
16:0/17:0/18:1	9373400	16235400	9343400	51165400	28065400	65165400	90555400
16:0/17:1/18:1	4137000	16844000	13074000	100434000	36974000	93664000	209534000
16:1/18:2/18:3	6618000	7563000	8387000	8656000	7124000	11580000	12230000
16:1/18:2/18:2	60132600	76802600	84792600	96572600	84822600	154002600	144702600
16:0/18:3/18:3	239213200	265713200	280313200	289213200	293313200	336713200	351213200
16:0/18:1/18:1	651417000	889417000	1044717000	1131717000	1049717000	1967717000	1790717000
16:1/18:1/18:1	350998000	620098000	728198000	575298000	611898000	1101198000	1008198000
18:0/16:0/18:0	-1351000	5946000	-274000	766000	404000	293000	5596000
18:0/16:0/18:1	204881000	251781000	294481000	255881000	261981000	460181000	484981000
18:1/17:1/18:1	3689500	5196500	5905500	11836500	11186500	19696500	21746500
17:0/18:1/18:1	3882870	10355870	5462870	14605870	14895870	26285870	30315870
18:0/17:0/18:1	1815370	2666370	2927370	6245370	6207370	13873370	14393370
16:0/18:2/18:3	358861000	410861000	423761000	401361000	383261000	456261000	453961000
16:1/18:1/18:2	197861810	362561810	252361810	516761810	494661810	520561810	554361810
16:0/18:1/18:3	180684000	230684000	236784000	242384000	220484000	312984000	284684000
18:1/17:1/18:2	2040932	2523932	2585932	4208932	4480932	9307932	8063932
18:1/18:1/18:2	41067000	59897000	82277000	146257000	151157000	270257000	256057000
18:1/18:1/18:1	57566000	82996000	97366000	176266000	148266000	264166000	236166000
18:1/18:2/18:2	23786000	24666000	23886000	37276000	37106000	194856000	62736000
18:1/18:2/18:3	4033000	4218000	4060000	8135000	8378000	8587000	11959000
18:0/18:0/18:0	874000	415000	863000	16000	412000	1362000	726000
20:0/16:0/18:1	32296500	38566500	51966500	40066500	42846500	73046500	70666500
18:0/18:1/18:1	81152000	101912000	122712000	128612000	121612000	227012000	235612000
18:0/18:1/20:0	4287500	2946500	5775500	5189500	6357500	10104500	8550500
20:0/18:1/18:1	7320500	7999500	10952500	16302500	19632500	30372500	29632500
20:0/18:1/18:2	1844160	2363160	2575160	6299160	7283160	11603160	11413160
18:0/18:1/20:3	10737520	11887520	15497520	12437520	13607520	20487520	20097520
20:1/18:1/18:2	902700	1274200	2044200	4523200	6021200	8181200	7727200
18:0/18:1/20:4	25554100	30534100	34514100	38384100	40504100	57014100	59884100
18:0/18:1/20:5	6982000	10821000	8221000	18731000	19941000	31471000	28641000

18:1/18:2/20:4	10681000	17171000	14131000	32331000	31021000	42521000	40971000
16:1/13:0/14:0	13329030	20259030	19019030	45249030	28799030	56029030	70829030
16:0/13:0/14:0	6196000	10599000	11059000	36299000	23969000	32019000	49869000
12:0/14:1/18:3	8790000	8677000	11500000	8853000	8726000	14370000	16240000
16:1/12:0/16:1	678033300	1046033300	1335033300	1390033300	955133300	2383033300	2367033300
16:1/14:1/14:1	149165090	289265090	378165090	218965090	132165090	373365090	368365090
16:0/14:0/14:0	150501000	206901000	227501000	317201000	269201000	517701000	517801000
16:1/14:0/14:0	865006000	1521306000	1564306000	1717306000	1310306000	2807306000	2376306000
16:0/9:0/20:5	9265960	11568960	13068960	23668960	15348960	22308960	17968960
16:1/13:0/16:1	40157500	59137500	68267500	62877500	63237500	92627500	88377500
16:1/12:1/17:1	15681520	11441520	19681520	19411520	16531520	24631520	20631520
16:0/13:0/16:1	16147540	27777540	25897540	65397540	39867540	116777540	105977540
15:0/14:0/16:0	2747700	5173700	5266700	10177700	11527700	16297700	19007700
16:1/12:0/18:3	30040190	39840190	68370190	51410190	52560190	81240190	81230190
16:1/14:1/16:1	567743500	854043500	1023643500	754243500	774043500	1347643500	1289643500
16:1/14:0/16:1	1200527000	2320527000	2475527000	2450527000	2309527000	3902527000	4107527000
16:0/14:0/16:1	1199890000	2039890000	2212890000	2472890000	2038890000	3763890000	3703890000
16:0/14:0/16:0	33530000	41780000	45330000	66210000	62020000	108340000	114840000
18:4/13:0/16:1	15020980	20390980	23860980	22120980	25580980	37290980	31310980
16:1/13:0/18:3	12905110	25995110	23495110	20325110	15845110	39395110	30755110
16:1/13:0/18:2	22309630	27299630	29549630	32819630	24079630	58839630	48109630
15:0/16:0/16:0	-3774000	-4006000	-3577000	588000	-2416000	4224000	1917000
15:0/16:0/16:1	21870000	41550000	31210000	79930000	76170000	134650000	156050000
15:0/16:1/16:1	12790500	19000500	21280500	58390500	37030500	72210500	87500500
18:4/14:0/16:0	181187000	205787000	210187000	210787000	195387000	229187000	222787000
16:1/14:1/18:2	77313700	104483700	107483700	80713700	84323700	139483700	128883700
16:1/14:1/18:3	18850000	24380000	19030000	24080000	18310000	28730000	18480000
16:0/16:0/16:0	10890000	11380000	16000000	19130000	17710000	24150000	37880000
16:0/14:0/18:1	1462227000	2093227000	2285227000	2493227000	2257227000	4009227000	4207227000
16:1/14:0/18:1	2413634000	3721634000	4113634000	3295634000	3620634000	6420634000	6523634000
16:1/16:1/16:1	1259614000	1910614000	1894614000	1870614000	1771614000	2733614000	2811614000
18:4/15:0/16:1	24866790	34216790	25226790	44996790	5740790	54936790	58736790
16:1/16:1/17:1	29614120	53894120	53674120	58864120	52714120	94444120	104034120

16:0/16:0/18:1	940998000	1337998000	1505998000	1632998000	1397998000	2759998000	2529998000
16:1/16:1/17:0	19234000	26384000	25904000	82114000	53734000	105094000	122294000
15:0/16:0/18:1	20373000	32913000	27363000	103293000	67123000	146293000	157693000
16:1/16:1/18:3	48716010	65426010	75796010	61616010	58326010	106266010	104066010
18:0/16:0/16:0	2430000	3115000	3538000	2519000	4656000	7666000	9286000
16:1/16:1/18:1	1445819000	1048819000	1532819000	2460819000	1112819000	1927819000	1663819000
16:0/16:1/18:1	2259521000	3282521000	3769521000	3557521000	3427521000	5693521000	6088521000
16:1/16:1/18:2	222845400	333245400	407745400	398745400	357645400	657445400	678045400
4:0/14:0/16:1	4557000	3758000	7911000	6126000	6512000	9974000	10230000
12:0/12:0/12:1	3052000	4825000	6376000	6618000	7058000	11680000	11850000
4:0/16:1/16:1	4847000	3396000	8107000	5018000	5778000	7704000	7573000
12:0/14:1/14:1	82885700	97715700	173695700	64675700	97045700	135895700	101295700
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12:0/13:0/14:0	7012600	9015600	7736600	25022600	19612600	33282600	28552600
14:1/12:1/14:1	5199510	12032510	18392510	5524510	10192510	18972510	7462510
11:0/14:1/14:1	2981000	7452000	4521000	6734000	8740000	11010000	5697000
12:0/13:0/14:1	13711210	21751210	24651210	44951210	49221210	29041210	31341210
12:0/12:0/14:0	295054000	487154000	597854000	534654000	443354000	959254000	877354000
12:0/12:0/14:1	109223650	168123650	211323650	122223650	150823650	209823650	185123650
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12:0/12:0/13:0	6437660	12098660	12798660	9628660	10388660	14908660	15368660
12:0/12:1/14:1	6960950	9858950	16655950	8248950	11065950	15255950	16115950
12:0/12:0/12:0	37670000	82170000	98680000	75030000	80380000	138180000	136280000
16:1/13:0/14:1	42614890	53754890	58024890	55934890	48574890	69304890	70244890
16:1/11:4/16:1	5941000	7661000	13750000	12660000	15270000	22180000	21430000
14:0/14:0/14:0	385915000	601715000	632015000	841015000	711715000	1287615000	1369615000
14:0/14:0/14:1	675182000	1052482000	1138482000	1231482000	976882000	1697482000	1848482000
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14:0/13:0/14:0	11909370	16269370	15669370	37449370	24689370	50449370	51789370
16:1/12:0/13:0	39732960	57002960	61132960	55512960	79152960	82472960	76482960
13:0/14:1/14:1	28310000	28410000	30680000	17060000	17810000	44780000	41760000
12:0/14:0/14:0	459324000	715924000	841324000	938724000	777924000	1409724000	1287724000

sum of all triglyceride values	2.1588E+10	3.1807E+10	3.5577E+10	3.6082E+10	3.2121E+10	5.6826E+10	5.6153E+10
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