Atg1 and Marf collaborate to maintain germline stem cells in *Drosophila*

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Abstract

Mitochondrial dynamics (fusion and fission) has been demonstrated to necessary for stem cell maintenance and differentiation, however, the relation between mitophagy, mitochondrial dynamics and stem cell exhaustion is not clearly understood. Here we report the multifaceted role of Atg1 in mitophagy, mitochondrial dynamics and stem cell maintenance in female germline stem cells (GSCs) in *Drosophila*. We found that depletion of *Atg1* in GSCs leads to impaired autophagy (mitophagy) as measured by reduced formation of autophagosomes, increased accumulation of p62/Ref(2)P and accumulation of damaged mitochondria. We observed increased generation of mitochondrial reactive oxygen species (mROS) in GSCs with depleted *Atg1* and mitochondrial clustering/fusion in cysts with *Atg1* knockdown (Atg1KD). The mitochondrial clustering/fusion observed in Atg1KD cysts was due to increase in Marf levels and the mitochondrial phenotype could be rescued by overexpression of *Drp1* or by depleting *Marf* via RNAi in Atg1KD cyst cells. Interestingly, double knockdown of both *Atg1:Drp1* and *Atg1:Marf* affected ovariole size, number of GCs and the number of vitellogenic oocytes. Strikingly, *Atg1:Marf* double knockdown leads to loss of GSCs, differentiated GCs and a total loss of vitellogenic stages suggesting block in oogenesis. Overall, our results demonstrate that Drp1, Marf and Atg1 function together to influence female germline stem cell maintenance and differentiation of cystoblasts into cysts.

Keywords: mitochondria, mitophagy, homeostasis, Drp1, Mfn2, Drp1, oogenesis

Introduction

GSCs have ability to self-renew and differentiate into either the sperm or the egg. Their ability to self-renew or differentiate depends on the interaction with the niche-cells. GSC maintenance affects the reproductive fitness of the organism (Fuller and Spradling, 2007). Autophagy is a crucial homeostasis mechanism operational within cells including GSCs. Autophagy maintains proteostasis by removing toxic protein aggregates and redox homeostasis by removing damaged mitochondria via mitophagy (Aman et al., 2021; Boya et al., 2013; Mizushima, 2007). However, the molecular role of autophagy in maintenance and differentiation of GSCs is not clearly understood (Ishibashi et al., 2020). Autophagy is a highly regulated and a complex process that is initiated byt Ulk1/Atg1, a serine threonine kinase, that phosphorylates the downstream proteins that catalyze the formation of the autophagosome (Mulakkal et al., 2014; Scott et al., 2004; Scott et al., 2007). The autophagosome fuses with the lysosome to form autolysosome wherein the hydrolytic enzymes degrade the cargo and the contents released in the cytoplasm for recycling.

In metazoans mitochondria are inherited maternally and are important for generating energy in form of ATP. Mitophagy has been demonstrated to be crucial for in GSCs that generate sperms and eggs in *Drosophila* (Lieber et al., 2019; Onishi et al., 2021). Mitophagy is crucial for maintaining mitochondrial homeostasis by sensing and removal of dysfunctional mitochondria (Youle and Narendra, 2011). The PTEN- induced putative kinase 1 (PINK1) / Parkin pathway has a crucial role in mitophagy (Narendra and Youle, 2011; Von Stockum et al., 2016). Parkin, an E3 ubiquitin ligase resides in the cytosol in healthy cells while PINK1 is constantly imported into the mitochondrial intermembrane space, where it is cleaved by PARL, a rhomboid family protease (Meissner et al., 2011; Yamano and Youle, 2013). The cleavage of PINK1 prevents phosphorylation of downstream targets that facilitate mitophagy. Upon increase in oxidative stress or depolarization of mitochondria PINK1 can no longer be cleaved and so it accumulates on the outer mitochondrial membrane (Narendra et al., 2010). Stabilized PINK1 phosphorylates Parkin leading to activation of its ubiquitin E3 ligase activity subsequently ubiquitinating mitochondrial proteins and promoting mitophagy (Lazarou et al., 2015; Youle and Narendra, 2011).

Mitophagy is influenced by mitochondrial biogenesis, dynamics (fission and fusion), translocation (distribution) and degradation (Onishi et al., 2021; Pickles et al., 2018; Youle and

Narendra, 2011). Perturbation in any of these processes can lead to imbalance in redox homeostasis and lead to mitochondrial dysfunction. If mitochondrial dysfunction is beyond repair, the fusion machinery is inactivated, and mitochondrial fragmentation is promoted to facilitate mitophagy (van der Bliek et al., 2013; Youle and Narendra, 2011). Mitochondrial dynamics is controlled by activities of Dynamin-related protein 1 (Drp1), Mitochondrial assembly regulation factor (Marf)/ Mitofusin 2 (Mfn2) and Optic atrophy 1 (Opa1).These GTPases hydrolyse GTP and use the energy to execute changes in the structure of mitochondria (Grandemange et al., 2009; Seo et al., 2018). Mfn2 is required for the fusion of outer mitochondrial membranes of mitochondria. Opa1 is important for the fusion of the inner mitochondrial membrane (Dorn et al., 2011). Fission requires the cytosolic GTPase Dynamin related protein 1 (Drp1) which forms oligomers surrounding the mitochondria and cleaves the mitochondria (Taguchi et al., 2007). In *Drosophila* loss of Marf and Drp1 can lead to loss in the female GSCs (Amartuvshin et al., 2020; Garcez et al., 2021). Loss of Marf results in reduction of the pMad signaling which is a self-renewal factor for female GSCs. Mitophagy has also been demonstrated to be crucial for the development of the ovariole and the growing oocyte. Mitochondria exhibit maternal inheritance and it is vital to the egg to inherit healthy mitochondria for proper embryogenesis to occur. Mitochondria with mutated mtDNA are eliminated during oogenesis and this process requires the mitophagy proteins Atg1 and BNIP3 (Lieber et al., 2019). Despite these interesting observations the relation between mitophagy, mitochondrial dynamics and stem cell exhaustion is not clearly understood.

We used *Drosophila* female GSCs to address the question of how mitophagy, mitochondrial dynamics and stem cell exhaustion are related. Female *Drosophila* harbor a pair of ovaries with 16-20 individual units called ovarioles within each ovary. The GSCs are located within the anterior most structure called the germarium in each ovariole. It is surrounded by the niche cells that secrete factors required for maintenance of the 'stemness' while the daughter cells that exit the niche differentiate into cystoblast (CB). A single CB undergoes four asymmetric cell divisions to generate interconnected 16-celled cysts (Bratu and Gerard, 2015; McLaughlin and Bratu, 2015; Spradling, 1993). One of the 16-celled cysts is chosen as the future oocyte, which is then sustained by remaining 15 nurse cells. The *Drosophila* ovary presents a simple and genetically tractable model to study the mitophagy, mitochondrial dynamics and their role in stem cell maintenance (Cox and Spradling, 2003; Fuller and Spradling, 2007).

Here we demonstrate the importance of Atg1 in maintenance of basal autophagy and its role in mitophagy using RNAi and loss of function clones of *Atg1*. *Atg1* knockdown cyst cells also show a strikingly different mitochondrial morphology which implies a relation between the mitophagy machinery and the mitochondrial fission and fusion proteins. Using a combination of genetics and cell biology we demonstrate that Drp1, Marf and Atg1 collaborate to regulate stem cell maintenance in *Drosophila.*

Materials and Methods

Fly maintenance

All the transgenic fly stocks were maintained at 25°C on standard cornmeal sucrose malt agar. The following flies were used; *w; +; nosGal4VP16* (BL4937, RRID:BDSC_4937), *y sc v; +; Atg1-RNAi* (BL 35177, RRID:BDSC_35177), *y sc v; +; Atg17-RNAi, ywhsFLP12; NGT; Dr/TM6B, w; +;Drp1 RNAi, w:+; Marf RNAi, w;+; FIAsH-Flag-Drp1-HA, w;+; Marf-GFP; w;+; UAS-Drp1, w;+; UAS-Marf, Atg1Δ3D (Scott et. al., 2004), ywhsFLP12; +; FRT80BhismRFP, FIAsH-Flag-Drp1-HA and Marf-GFP* were combined with *ywhsFLP12; NGT; Dr/TM6B, nosP mCherry-Atg8a nos 3'UTR, nosP-Ref(2)P-GFP* (Nilangekar et al., 2019) and *mito-roGFP2-Grx1*(Nilangekar and Shravage, 2019).

Generation of Atg1 clones

FRT80BAtg1Δ3D mutants crossed to FRT80BmRFP or FRT80BGFP were collected and fed on yeast for 2 days. The clones were generated using the FLP-FRT technique described previously (Xu and Rubin 1994). For induction of clones a 2-hour heat-shock at 37°C was given to the adults twice a day in a waterbath. These heat-shocks were separated by an interval of at least 4 hours. The protocol was continued for 6 consecutive days.

Immunostaining

Ovaries were dissected and fixed in 4% PFA for 15 minutes, washed three times with 0.1% PBTx for 5 minutes each and blocked with 1% PBTx containing 0.5% BSA for 1 hour at RT (RT). The ovaries were incubated with primary antibody in 0.3% PBTx containing 0.5% BSA overnight at 4°C. The next day 1° antibody was removed and the sample was washed with 0.1% PBTx for 15 minutes then blocked with 10% NGS in 0.1% PBTx for 2 hours at RT. Ovaries were then stained with 2° antibody in 0.1% PBTx containing 10% NGS for 2 hours at RT protected from light. Excess antibody was removed by three washes with 0.1% PBTX for 15 minutes each. 1µg/ml DAPI solution in 0.1% PBTx was added to the samples and incubated at RT for 10 minutes followed by washing with 0.1% PBTx solution 3 times for 5 minutes each. Ovarioles were mounted in Prolong Gold. Antibodies and dilutions used; anti-CathepsinL 1:400 (Abcam Cat# ab58991, RRID:AB_940826); anti-ATP5α 1:250 (ThermoFisher Scientific Cat# 43-9800, RRID:AB_2533548), anti-GFP 1:10 (DSHB Cat#12A6, RRID:AB_2617417) anti-α-Spectrin 1:20 (DSHB Cat#3A9 (323 or M10-2), RRID:AB_528473), anti-HA (CST Cat# 3724, RRID:AB_1549585), anti-Vasa (DSHB Cat#

anti-vasa, RRID:AB_760351), anti-Hts (DSHB Cat# 1B1, RRID:AB_528070), anti-Lamin C (DSHB Cat# lc28.26, RRID:AB_528339). 2° antibodies used: Goat anti-rabbit Alexafluor555 1:250 (Molecular Probes Cat# A-21429, RRID:AB_141761) and Goat anti-rabbit Alexafluor488 1:250 (Molecular Probes Cat# A-11070, RRID:AB_142134), Goat anti-mouse (Molecular Probes Cat# A-11001, RRID:AB_142134).

Redox chemical treatment

Ovaries of *mito-roGFP2-Orp1* and *mito-roGFP2-Grx1* flies were dissected in Grace's medium and washed in 1xPBS for 2 minutes. The redox treatment procedure was followed according to Nilangekar et al., 2019.

Imaging and Analysis

All imaging was performed on Leica SP8 Confocal microscope using 63x oil objective. Images acquired were 8 bit, 1024x1024 pixel resolution at 100Hz scanning. Frame accumulation was performed with 6 frames for mCherry-Atg8a and GFP-Ref(2)P. Images were analysed using ImageJ. For mCherry-Atg8a puncta measurements in *Atg1Δ3D (Atg1-/-)* clones, an ROI was drawn around the GSCs and *Atg1-/-* clones. GSCs were identified by their close association with the niche cells and size of their nuclei while clones were recognized by absence of GFP. mCherry-Atg8a and GFP-Ref(2)P punctae were counted manually in *RNAi* mediated knockdown studies and the area of germarium were measured using ImageJ. Analysis of mitochondrial size was done by the MitoAnalyzer plugin. For colocalization analysis, we - analyzed overlap of mCherry-Atg8a and CathepsinL, and GFP-Ref(2)P with CathepsinL puncta. Mitophagy flux was measured as colocalization between mitochondria (ATP5α) and lysosomes (Cathepsin L). Microsoft Excel was used for statistical analysis. Student's T-Test of two samples assuming unequal variance was performed for all comparisons. Graphs were plotted in GraphPad Prism7.

Imaging and image analysis of mito-roGFP2-Grx1

Mito-roGFP2-Grx1 imaging and image analysis was performed as described in (Nilangekar and Shravage, 2019).

DHE staining protocol

DHE staining and live imaging was performed according to Amartuvshin et al., 2020. Five pair of ovaries were dissected in Grace's medium (GM) followed by washing with 300µl GM for 2 min at RT. 30µM DHE in 300µl GM was added (protected from light) and incubated for 20 min at RT. The dye was washed off with one wash of 300µl GM for 5 min. The sample was incubated in 1g/ml DAPI solution in 300µl 0.1 % PBTx for 5 minutes. Final washes of 0.1 % PBTx for five minutes each were performed at RT.

RNA isolation

20 pair of ovaries were used for RNA extraction using Trizol method (Invitrogen, USA). RNA extraction was carried out as per manufacturer's instructions. The aqueous phase obtained after phase separation was then transferred to a fresh microcentrifuge tube followed by addition of equal volumes of 95% ethanol and mixed thoroughly to precipitate the RNA. Further steps were performed using Monarch Total RNA Miniprep Kit (NEB #T2010). RNA was eluted in 20µl DEPC treated water.

cDNA synthesis

1µg of tissue specific (ovary) RNA was used for First strand cDNA synthesis using PrimeScriptTM 1st strand cDNA Synthesis Kit (TakaraBio, India, Cat# 6110A). The synthesized cDNA was diluted (1:7.5) and validated with the help of PCR using reference genes at different cycles (25,30,32).

Real-time PCR:

qPCR was performed on EcoMax (Bibby Scientific, UK). Reactions were run in triplicates in a 48-well plate with 10µl reaction volume. Following is the program used: 95°C for 10 minutes, 95°C for 15 seconds, 60° for 1 minute, 70°C for 5 seconds, 70°C for 5 seconds, 90°C for 2 minutes. The mean of housekeeping gene *Tubulin* was used as an internal control for normalization. The expression data was analysed using 2- ΔΔCT described by (Livak and Schmittgen, 2001).

Results

Atg1 is necessary for basal autophagy during oogenesis

Atg1 has been shown to be necessary and sufficient to induce autophagy in several tissues in *Drosophila* (Barth et al., 2011; Chang and Neufeld, 2009; Scott et al., 2004; Scott et al., 2007). However, if Atg1 is required for autophagy in females GSCs is unclear. To address this, we examined localization of autophagosome marker mCherry-Atg8a in GSC specific knockdown of *Atg1* by expression of a double-stranded inverse-repeat (IR) construct designed to target and knockdown Atg1 (UAS-*Atg1RNAi*) during oogenesis using nanos-Gal4VP16 (Brand and Perrimon, 1993; Rørth, 1998; Yan et al., 2014). Atg8a, the *Drosophila* ortholog of mammalian LC3 (microtubule-associated protein 1 light chain 3), displays diffuse cytoplasmic localization in the germarium and developing egg chambers, but becomes incorporated into autophagosome membranes upon upregulation of autophagy and is visualized as punctate spots in the cytoplasm (Bali and Shravage, 2017; Klionsky et al., 2021; Nilangekar et al., 2019; Scott et al., 2004; Scott et al., 2007). GSCs and germ cells (GCs, also known as cyst cells) expressing *Atg1RNAi* had significantly fewer number of mCherry-Atg8a puncta as compared to the control (**Figure 1A-D and Supplmentary Figure 1C**). Significant reduction of autophagy flux in *Atg1RNAi* germarium as well as GSCs was observed as seen by reduced colocalization between mCherry-Atg8a and CathepsinL (autolysosomes) puncta (**Figure1C-D, Supplementary Figure 1D**). Further, we utilized FLP-FRT sites to generate *Atg1Δ30* mitotic mutant cell clones in the germarium, resulting in tissue composed of control (either wild type or heterozygous *Atg1Δ30* /wild type) and homozygous *Atg1Δ30/Atg1Δ30* (termed *Atg1-/-* henceforth) mutant cells (Theodosiou and Xu, 1998). Control cells, which were marked by the presence of green fluorescent protein (GFP) contained several mCherry-Atg8a puncta, whereas homozygous *Atg1-/-* mutant cells (lacking GFP) displayed diffuse localization of mCherry-Atg8a (**Supplementary Figure 1E-E", F**). These data suggest that Atg1 is necessary for autophagy in germline cells including the GSCs.

An additional approach was used to determine the influence of Atg1 function on autophagy in the GCs. The *Drosophila* ortholog of p62 (SQSTM1) is termed as Ref(2)P and has been demonstrated to bind ubiquitylated substrates and aid in their recruitment into autophagosomes (Klionsky et al., 2021; Nezis et al., 2008; Nezis et al., 2010; Nilangekar et al., 2019). GCs expressing *Atg1RNAi* accumulated GFP-Ref(2)P as compared to control germaria (**Figure 1E-F; Supplementary Figure 1B)**.We also observed reduced colocalization between

Ref(2)P and CathepsinL suggesting a significant decrease in degradation of protein aggregates (**Figure 1G,H**) This indicates that Atg1 is necessary for clearance of Ref(2)P during oogenesis via autophagy.

To determine whether other autophagy genes in the Atg1 complex are required for autophagy during oogenesis, we specifically knocked down Atg genes belonging to the Atg1 complex. The Atg1 complex in *Drosophila* consists of Atg1 kinase, Atg13, Atg17 and Atg101(Nagy et al., 2014). Knockdown of *Atg17* mediated by *Atg17IR* within the GSCs and GCs had significantly fewer numbers of mCherry-Atg8a puncta as compared to the controls (**Supplementary Fig.1**). These data suggest that both Atg1 and Atg17 which are components of Atg1 complex are necessary for basal autophagy in the germline cells including GSCs during oogenesis.

Atg1 is necessary to maintain redox homeostasis within the GSCs and GCs during oogenesis.

Metabolic reactions within cells produce ROS like hydroxyl ion ($OH^{-)}$, Superoxide (0_2^-) and hydrogen peroxide (H_2O_2) as byproducts which irreversibly oxidize macromolecules including nucleic acids, proteins lipids etc., and as a result render them non-functional (Nilangekar and Shravage, 2019; Nilangekar et al., 2019; Scherz-Shouval and Elazar, 2011). Deficiency in autophagy leads to increase in ROS levels within cells (Scherz-Shouval and Elazar, 2011; Shadel and Horvath, 2015). To test if ROS levels are perturbed during oogenesis, we stained *Atg1RNAi* expressing ovarioles with dihydroethidium (DHE). DHE exhibits blue fluorescence in the cytosol and when oxidized by superoxide molecules intercalates cell's DNA and stains the nucleus leading to emission of bright red fluorescence which peaks at 610 nm when excited at 535 nm (Bucana et al., 1986). GCs including GSCs within the germaria and developing egg chambers expressing *Atg1RNAi* had significantly higher levels of ROS evident by an increase in the red fluorescence as compared to the control (**Figure 2A-D**). This increase in red fluorescence was also detected in nurse cells (GCs) of older *Atg1RNAi* egg chambers indicating an increase in the production of superoxide molecules (**Figure 2A,B and Supplementary Figure 2B**).

In addition, we used a mitochondrially targeted redox sensitive green fluorescent protein (roGFP2) fused to Glutaredoxin 1 (Grx1). Grx1 is oxidized nonenzymatically during conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). The redox state of Grx1 leads to reduction or oxidation of the roGFP2 cysteine residues. This changed redox state of roGFP2 is responsible for the switch in excitation of fluorescence green to blue (488–405

nm) which is a reliable measure of H_2O_2 production (Nilangekar et al., 2019) GCs expressing *Atg1RNAi* had significantly higher levels of roGFP2-Grx1 oxidation compared to the control GCs. The green to blue fluorescence shift was more pronounced in the GSCs with depleted *Atg1* (**Figure 2E-H**). In summary, knockdown of *Atg1* leads to disruption of redox homeostasis in both the cytoplasm and the mitochondria during oogenesis.

Nrf2 and Keap1 are two transcription factors that are involved in H_2O_2 sensing within cells and are upregulated at both transcriptional and translational level in response to H_2O_2 (Bellezza et al., 2018; Yamamoto et al., 2018). Further, SOD2 and Catalase are two enzymes that are involved in detoxifying H_2O_2 and their transcription is also increased in response to elevated levels of H_2O_2 (Dan Dunn et al., 2015; Marinho et al., 2014). qPCR data revealed that the transcript levels of these genes were elevated and specifically Nrf2 was found to be significantly upregulated (**SupplementaryFigure 2A**). Taken together our data suggest that Atg1 is necessary to maintain redox homeostasis during oogenesis.

Atg1 is necessary for mitophagy and influences mitochondrial morphology during oogenesis

Mitochondria are crucial for generating ATP within cells and their dysfunction is detrimental to cells. Mitochondrial ROS increases in cases wherein defective mitochondria accumulate within the cytoplasm and are not cleared by the process of mitophagy (Dan Dunn et al., 2015; Lemasters, 2005; Sena and Chandel, 2012; Shadel and Horvath, 2015). Several Atgs have been shown to be required for mitophagy in *Drosophila* (Chang et al., 2013; Lieber et al., 2019). We tested if mitophagy is reduced in the germline cells during oogenesis in absence of Atg1. To measure mitophagy flux we used an antibody against a lysosomal resident protease CathepsinL, in combination with ATP5alpha (Klionsky et al., 2021). Mitophagy flux was measured using colocalization of ATP5 alpha (red staining) and CathepsinL (green staining) staining and subsequent analysis using colocalization analyses and Pearson's coefficient correlation (Klionsky et al., 2021). Mitophagy flux was found to be significantly reduced in *Atg1RNAi* expressing GSCs and GCs as compared to the controls evident by reduced Pearson's coefficient correlation (**Figure 3A-D**). p62/Ref(2)P is recruited to damaged mitochondria and has been shown to be necessary for mitophagy in mammals and *Drosophila* by facilitating the recruitment of damaged mitochondria to autophagosomes (Amartuvshin et al., 2020; Aparicio et al., 2019; Rana et al., 2017). Further, p62/Ref(2)P has been shown to be essential for PINK1-Parkin mediated mitophagy (de Castro et al., 2013). We examined p62/Ref(2)P co-localization with mitochondria in germaria expressing *Atg1RNAi*. Recent reports demonstrate that decline

of mitophagy is associated with increased co-localization with p62/Ref(2)P (Rana et al., 2017). Strikingly, a significant number of punctate p62/Ref(2)P structures were found to colocalize with mitochondria in *Atg1RNAi* cells as compared to controls consistent with disruption of mitophagy (**Figure 3E-H**). Taken together, these data suggest that mitophagy is disrupted in GSCs and their progeny upon Atg1 knockdown (*Atg1KD*).

Mitochondrial morphology and dynamics during oogenesis in the GCs have been studied previously. Mitochondria within GSCs are elongated and become fragmented as cystoblasts differentiate into cysts cells (Amartuvshin et al., 2020; Cox and Spradling, 2003). In cyst cells, the mitochondria are predominantly fragmented and appear spherical. During our analysis of mitophagy flux, we frequently observed that mitochondria were clustered/aggregated/fused together in germline cells expressing *Atg1RNAi* (**See Figure 3A,B,E,F**). Loss of *Atg1* had been reported to disrupt mitophagy, however, we observed that mitochondria were predominantly clustered/aggregated/fused particularly the cyst cells which was surprising. We tested if the mitochondrial clustering/fusion phenotype was also observed in *Atg1-/-* mitotic clones generated using FLP-FRT as described earlier. We generated *Atg1-/-* mitotic clones stained the germaria with ATP5alpha. We observed clustering/fusion of mitochondria *Atg1-/-* mitotic mutant cell clones in the germarium and the progeny within the germarium similar to *Atg1RNAi* (**Supplementary Figure 3A**). We also tested if other components of Atg1 complex have similar mitochondrial phenotypes. *Atg17RNAi* exhibited a clustered/fused mitochondrial phenotype similar to the *Atg1RNAi* mitochondrial phenotype (**Supplementary Figure 3B**). Thus, these data suggest that disruption of Atg1 complex in GCs leads to mitochondrial clustering during oogenesis indicating that it controls mitochondrial dynamics in addition to its role in mitophagy.

In *Drosophila*, mitochondrial dynamics is regulated primarily by specific GTPases. Marf (Mitochondrial assembly regulatory factor) or Mitofusin 2, which regulates mitochondrial outer-membrane fusion while *Opa1* mediates fusion of the inner membrane of mitochondria (Amartuvshin et al., 2020; Garcez et al., 2021; Sandoval et al., 2014; Seo et al., 2018; von Stockum et al., 2016). Mitochondrial fission is catalyzed by *Drp1 (Dynamin-related protein 1)* which associates itself with the mitochondrial outer membrane and constricts mitochondria (Ma et al., 2018; Verstreken et al., 2005). Our data show that mitochondria are clustered/fused in *Atg1RNAi* expressing GCs, suggesting that Atg1 regulates mitochondrial dynamics during oogenesis. Mitochondrial fusion events can occur when Drp1 levels are reduced or when Marf and Opa1 levels increase (Amartuvshin et al., 2020; Garcez et al., 2021; Seo et al., 2018). It is possible that GCs expressing *Atg1RNAi* have reduced activity of Drp1

or increased Marf activity. To test these possibilities, we decided to monitor the levels of Drp1 and Marf in *Atg1RNAi* cells. We utilized two separate protein fusion reporters Drp1-HA and Marf-GFP (Mfn2-GFP) that are expressed from their respective endogenous promoters (Sandoval et al., 2014; Verstreken et al., 2005). These reporters have been characterized and validated previously in *Drosophila* muscle tissue. No detectable difference in Drp1-HA levels was observed as quantified from anti-HA staining (green) in *Atg1RNAi* expressing GSCs (and GCs) as compared to the controls (**Figure 4A, B, D, E**). However, the levels of Drp1 are higher in region 1 as compared to region 2 of the germarium in *Atg1RNAi*/+ controls. This difference in Drp1 expression in region 1 Vs region 2 of germanium is disrupted in *Atg1RNAi* (**Supplementary Figure 4A)**. Thus, Drp1 expression is reduced in region 2 vs region 1 in *Atg1RNAi* GCs suggesting that Atg1 influences Drp1 expression.

We next tested if disrupting Drp1 function through expression of *Drp1RNAi* could phenocopy *Atg1RNAi* mitochondrial clustering/fusion phenotype. Previous studies have demonstrated that depleting *Drp1* in *Drosophila* muscle cells and in the GCs leads to mitochondrial fusion (Amartuvshin et al., 2020; Garcez et al., 2021; Ma et al., 2018). Compared to the control, GCs expressing *Drp1RNAi* showed mitochondrial clustering in GSCs as well as in region 2b of the germarium (**Figure 4C, F and Supplementary Figure 4B, C and F**). Strikingly, the mitochondrial phenotype observed in *Atg1RNAi* is similar to that of *Drp1RNAi*. Thus, the clustering of mitochondria in *Atg1RNAi* is consistent with the decrease in mitochondrial fission events caused by depletion of fission regulator Drp1. Interestingly, our data also suggest that depleting *Drp1* leads to reduction in *Atg1* transcription indicating a genetic interaction between Drp1 and Atg1 (**Supplementary Figure 4D)**.

We also tested if Marf levels were altered in *Atg1RNAi* expressing GSCs and GCs. Marf levels in *Atg1RNAi* GCs were at least 2-fold higher as compared to the controls. These data are consistent with the clustered/fused mitochondrial phenotype observed in *Atg1RNAi* (**Figure 4G, H, J and K and Supplementary Figure 4D**). Further, we could detect significant increase in Marf levels in region 1 and region 2 of *Atg1RNAi* expressing germaria as compared to the controls (**Supplementary Figure 4E**). Taken together, our results indicate that Atg1 is necessary to maintain fissed/fragmented mitochondria in the GCs located within the germarium by regulating Marf levels in these cells. Further, we interfered with Marf function using RNAi-mediated knockdown and tested whether this affects mitochondrial morphology (Amartuvshin et al., 2020; Garcez et al., 2021). RNAi of Marf caused fragmentation of mitochondria within germarium including GSCs which is consistent with the reduction in fusion events upon depletion of fusion regulators (**Figure 4I, L and Supplementary Figure 4G**). Taken together

our data show that *Atg1RNAi* disrupts mitochondrial dynamics leading to fused/clustered mitochondrial morphology during early oogenesis.

Atg1 genetically interacts with mitochondrial fusion machinery components

We next sought to test whether enhancing fission can rescue the *Atg1RNAi* mitochondrial fusion phenotype given that Marf levels are elevated in *Atg1RNAi* expressing germline cells. Fission could be enhanced by either elevating levels of Drp1 protein or by reducing Marf levels (Amartuvshin et al., 2020; Garcez et al., 2021; Rana et al., 2017). We hypothesized that forcing mitochondrial fragmentation by expressing Drp1 in *Atg1RNAi* expressing cells should rescue the mitochondrial fusion/clustering phenotype. Similarly, interfering with mitochondrial fusion by reducing Marf levels through *MarfRNAi* in combination with *Atg1RNAi* expressing cells should lead to the rescue of the fused mitochondrial phenotype. We recombined *UAS-Drp1(called Drp1^{OE} hereafter*) with *Atg1RNAi* to generate *UAS-Drp1:Atg1RNAi* recombinants and crossed them to *nanosGal4VP16* to co-express *Atg1RNAi* and *Drp1*, assessed the mitochondrial clustering/fusion phenotype. *Drp1^{OE}* in *Atg1RNAi* expressing GCs led to suppression of the clustering/fusion mitochondrial phenotype as observed by dispersed and punctate ATP5A1 staining (**Figure 5A-C, E and Supplementary Figure 5A, B**). The mitochondrial area was significantly reduced as compared to GCs expressing *Atg1RNAi* suggesting that *Atg1* and *Drp1* genetically interact with each other to control mitochondrial dynamics. However, it is important to note that as compared to wt controls the complete rescue of the mitochondrial phenotype was not observed.

We further tested if mitochondrial fusion phenotype can be rescued by reducing Marf levels in *Atg1RNAi*. We co-expressed *Atg1RNAi* and *MarfRNAi* together in all GCs including GSCs using the *nanos-Gal4VP16* driver line. Previous studies have shown that reduction in Marf levels within the GSCs/GCs leads to the fragmentation of mitochondria (Amartuvshin et al 2020 and Garcez et al 2021). GSCs/GCs co-expressing *Atg1RNAi* and *MarfRNAi* exhibited fragmented mitochondrial phenotype suggesting a suppression of fused mitochondrial phenotype in GCs (**Figure 5A, B, D and E and Supplementary Figure5A, C)**. The total mitochondrial area was significantly reduced in combined knockdown to *Atg1* and *Marf*. Together these observations suggest that Atg1, Drp1 and Marf interact genetically to control mitochondrial dynamics during oogenesis in *Drosophila*.

Atg1, Drp1 and Marf collaborate to regulate oogenesis in *Drosophila*

Stem cells undergo differentiation and proceed through multiple stages of development in egg chambers where they undergo further development and are supplemented with necessary factors and are sustained for embryogenesis (Spradling,1993). Previous reports suggest that both Drp1 and Marf are crucial for oogenesis and their absence leads to detrimental effects on development of the ovary (Amartuvshin et al., 2020; Garcez et al., 2021). Atg1 has been demonstrated to be dispensable for oogenesis as knockdown or null mutants within the GCs do not disrupt egg development (Barth et al., 2011). Our data also suggests that overall egg development is not disrupted in *Atg1RNAi*. However, total area of the ovary is significantly reduced (**Figure 6A, B, G and Supplementary Figure 6A, F-I**). In addition, number of GCs and the percentage of ovarioles showing development of pre-vitellogenic and vitellogenic stages (stage 8 and after) also does not change in *Atg1RNAi* as compared to the controls (**Figure 6H, I**).

We measured various parameters of ovarian development like the ovary size, ovariole area, distribution and germ cell differentiation in individual and combined RNAis of *Atg1*, *Drp1 Marf,* and (Garcez et al., 2021; Spradling., 1993). We observed that individual knockdown as well as combined knockdown of *Atg1* and *Drp1* and, *Atg1* and *Marf* led to significant reduction in the size of the ovary, ovariole and decreased production of egg chambers, both pre-vitellogenic and vitellogenic stages (Garcez et al., 2021)(**Figure 6C-C'-F-F', G-J and Supplementary Figure 6A-E"and F-I**). Strikingly, double knockdown of *Atg1* and *Marf* show poorly developed ovaries an ovarioles that have almost no older egg chambers (Stage 3 and above) (**Figure 6B-B', D-D', F-F', G, H and Supplementary Figure A-C", E-E" and F-I**). We used GC specific marker Vasa, to identify GCs and to assess their distribution throughout the germarium in different *RNAi*. In all controls and *Atg1RNAi* the germarium showed near normal distribution of GSCs and GCs including 2, 4, 8 and 16-celled cysts (**Figure 6A-A'", B-B"' and Supplementary Figure A-E""**). Interestingly, loss of *Drp1*, *Marf* alone exhibited germaria with significant reduction in the number of GCs as seen by reduced number of Vasa positive cells in the germarium (**Figure 6A"-D"**). We observed further reduction in number of GCs in combined *RNAi*s of *Atg1*:*Drp1* and, *Atg1*:*Marf*. *Atg1*:*Marf* double RNAi exhibited the strongest GC loss phenotype and had detrimental effect on oogenesis. There was upto 90% reduction in GSCs and GCs in *Atg1:Marf* double RNAi as compared to controls (**Figure 6I, J and Supplmentary Figure D-E"', H and I**). Depletion of GSCs and their differentiated progeny GCs has been previously linked to failure of oogenesis (Khurana and Theurkauf, 2010; Peng et al., 2016; Xie and Spradling, 1998). Taken together, these data

suggest that Drp1, Marf and Atg1 function together to regulate both maintenance and differentiation of GSCs during *Drosophila* oogenesis.

Discussion

Our work demonstrates that *Autophagy related gene-1* (*Atg1*) in addition to its role in regulation of autophagy (and mitophagy) in *Drosophila* also regulates mitochondrial dynamics during oogenesis. Atg1 is a core autophagy protein in *Drosophila* that exerts its function through its kinase activity by phosphorylating substrates necessary for upregulation of autophagy in several tissues (Barth et al., 2011; Chang and Neufeld, 2009; Scott et al., 2004; Scott et al., 2007). Loss of *Atg1* has been previously reported to disrupt autophagosome formation and we have reported reduced formation of autophagosomes both in *Atg1RNAi* as well as *Atg1-/-* clones in the germarium. Impaired autophagy in *Atg1-/-* mutants (and *Atg1RNAi*) has been shown due to disruption in autophagosome formation. Additionally, we also observed accumulation of Ref(2)P in *Atg1RNAi* germaria which is associated with impaired autophagy clearance of protein aggregates as shown earlier in mutants of *Atg* genes in several *Drosophila* tissues (Barth et al., 2011; Mulakkal et al., 2014; Scott et al., 2004; Scott et al., 2007).

Our data demonstrates that Atg1 is necessary in GSCs and their progeny for mitochondrial clearance (Lieber et al., 2019; Ma et al., 2018). We demonstrate that mitophagy is dependent on Ref(2)P during oogenesis (Kawajiri et al., 2010; Lee et al., 2018). Ref(2)P has been proposed to facilitate recruitment of damaged mitochondria to autophagosomes. Recent work has shown that Ref(2)P, plays a critical role in the PINK1-Parkin mitophagy (Aparicio et al., 2019; Kawajiri et al., 2010; Rana et al., 2017). In these studies the authors observed colocalization of Ref(2)P with mitochondria in aged muscle cells of flies consistent with a decline in mitophagy. The authors further demonstrated that mitophagy is dependent on Atg1 function in muscle tissue similar to our observations of impaired mitophagy in the *Drosophila* ovary upon depletion of *Atg1*. Additionally, Atg1 in yeast and ULK1 the mammalian homologue of Atg1, are known to regulate mitophagy by phosphorylating BNIP3, FUNDC1 and Parkin (Hung et al., 2021; Poole et al., 2021; Wu et al., 2014). However, if Atg1 phosphorylates BNIP3, FUNDC1 and Parkin in *Drosophila* during oogenesis needs to be explored.

Dysfunctional mitochondria are a major source of ROS within the cell and autophagy is crucial for maintaining redox homeostasis. Excess production of ROS like 0_2^-, H_2O_2 and OH^- within the mitochondria can lead to damage to macromolecules within the cells (Scherz-Shouval and Elazar, 2011, Dan Dunn et al., 2015; Sena and Chandel, 2012). Depletion of *Atg1* leads to increased production of ROS within the cytoplasm as measured by DHE. Additionally, we observed increased oxidation of glutaredoxin-1 as measured using roGFP2-Grx1

suggesting that blocking autophagy leads to accumulation of damaged mitochondria. This is in agreement with various reports as several *Atg* gene mutants in *Drosophila* have been demonstrated to have increased ROS production due to failure in removal of superfluous mitochondria (Chang and Neufeld, 2009; Chang et al., 2013; Juhasz et al., 2007). In cells where redox homeostasis is maintained, Nrf2 is expressed at low levels, however, disruption of redox homeostasis leads to increase in Nrf2 expression which is facilitated by Keap1 inactivation (Bellezza et al., 2018; Pajares et al., 2016; Yamamoto et al., 2018). Disruption of redox homeostasis in *Atg1RNAi* germaria is corroborated by corresponding increase in Nrf2 expression (**Supplementary Figure** 2). High levels of ROS disrupt germline stem cell maintenance and differentiation.

We expected that knockdown of *Atg1* will affect mitophagy and this had been demonstrated in *Drosophila* by several researchers using tissue specific knockdown of *Atg1* or *Atg1-/-* mutants (Ma et al., 2018; Vincow et al., 2013). However, our data suggests that Atg1 also regulates mitochondrial dynamics during oogenesis. This phenotype was observed earlier in the nurse cells and in context of cell death mediated by Bcl2 family members during *Drosophila* oogenesis (Wang et al., 2016). In their study *Atg1-/-* nurse cell clones lead to clustering/fusion leading to large masses of mitochondria termed as "mitochondrial islands" similar to our findings of *Atg1RNAi* (Tanner et al., 2011). However, the researchers did not co-relate the mitochondrial phenotype with Drp1/Marf expression levels in *Atg1-/-* cells. In our study mitochondrial clustering/fusion ("mitochondrial island") phenotype in *Atg1RNAi* and, Drp1 and Marf expression levels revealed an interesting co-relation. Drp1 levels remained largely unchanged in *Atg1RNAi*, however, Marf levels exhibited a significant increase in the GCs where the mitochondrial clustering/fusion phenotype was most prominent. This suggested that the fusion/clustering of mitochondria to form "islands" is mediated by elevated Marf and not by reduction of Drp1 levels as detected by anti-HA staining. Interestingly, we observed that impaired Drp1 function can cause mitochondrial fusion/clustering leading to formation of mitochondrial islands similar to *Atg1RNAi* (**Figure 4**). Thus, it is possible that the mitochondrial clustering phenotype could be due to change in the ratio of expression Drp1 to Marf (ratio of levels of fission proteins to fusion proteins) rather than changes in the levels of proteins at the individual levels. These findings need to be further explored in the context of mitophagy and mitochondrial dynamics during *Drosophila* oogenesis.

Mitochondrial dynamics could be force-shifted towards fission or fusion by overexpression or *RNAi of Drp1*, *Marf* or *Opa1*. For instance, mitochondrial fragmentation/fission could be achieved by either overexpressing *Drp1* or by reducing *Marf*

levels through *MarfRNAi* or mutants. The Atg1 mitochondrial clustering phenotype could be rescued by *Drp1*^{OE} suggesting that Drp1 and Atg1 interact genetically. This was also demonstrated in the context of Parkinson's disease (PD) where Atg1 has been shown to regulate mitochondrial dynamics. Mutations in Parkinson's disease related genes *Pink1/Parkin* cause mitochondrial dysfunction and this leads to formation of elongated mitochondrial clumps in the indirect flight muscle cells of adult *Drosophila*. Atg1 overexpression (*Atg1*^{OE}) rescues the *Pink1/Parkin* mutant associated mitochondrial phenotype by driving mitochondrial fission (Ma et al., 2018). Further the authors demonstrated that this rescue is mediated by Drp1 as *Drp1* knockdown abrogates the rescue of PD associated mitochondrial phenotype by *Atg1* overexpression. Interestingly, in another study the beneficial effects of *Drp1* overexpression in prolonging lifespan of *Drosophila* could be abrogated by *Atg1* knockdown suggesting that *Drp1* and *Atg1* interact genetically (Aparicio et al., 2019; Rana et al., 2017). As expected, our data show that *Marf* knockdown in *Atg1RNAi* rescues the mitochondrial fusion phenotype of *Atg1* knockdown indicating that Atg1 also interacts with *Marf* genetically. However, the average mitochondrial area significantly reduced in the double KD of *Atg1* and *Marf*. If Atg1 physically interacts with Drp1 and Marf in the GCs needs to be explored further. Both Drp1 and Marf are regulated by phosphorylation and given that Atg1 is a serine/threonine kinase it is possible that Atg1 phosphorylates Drp1 or Marf or both. However, it is also likely that Drp1 and Marf could be regulated indirectly by Atg1 through other kinases like AMPK, Pink1, MAPK.

Although Atg1 has a crucial role in induction of autophagy in the GCs of *Drosophila* ovary previous studies and our data suggest that it is not necessary for oogenesis. Atg1 is primarily required in follicle cells for the process of oogenesis egg production (Barth et al., 2011; Kuhn et al., 2015). Depletion of *Atg1* leads to increased levels of Marf in GSCs and this could be beneficial to the GSCs and may lead to their longer retention in the GSC-niche. However, disruption of Atg1 function in the GCs affects early embryogenesis and this has been shown to be partly due to inefficient utilization of lipids (Kuhn et al., 2015). It remains to be tested if the disruption of embryogenesis due to inefficient utilization of lipids is due to inheritance of damaged mitochondria within the developing egg (Kuhn et al., 2015; Lieber et al., 2019). Drp1 and Marf have been reported to regulate both germline stem cell maintenance, differentiation, ovariole development and fecundity (Amartuvshin et al., 2020; Garcez et al., 2021). Additionally, Drp1 and Marf disrupt mitochondrial function and this is important for ovariole development and for development of vitellogenic stages. *Atg1* and *Drp1* double *RNAi* did not enhance the *Drp1RNAi* oogenesis phenotypes (**Supplementary Figure 5D, E**). This

was expected as combined knockdown of *Atg1* and *Drp1* did not lead to a severe mitochondrial fusion phenotype (**Supplementary figure 5D-I"**). However, *Marf* and *Atg1* combined knockdown lead to a dramatic phenotype leading to an arrested growth of ovariole, a drastic reduction in both GSCs and GCs. These data indicate that Atg1, Drp1 and Marf collaborate to regulate germ cell specification and GSC maintenance.

Figure legends

Figure 1 Atg1 is required for induction of autophagy during oogenesis.

(A,B) Control (A-A") and *Atg1RNAi* (B-B") germarium showing mCherryAtg8a (A,B red) and CathepsinL (A', B" green) staining. Inset in A", B" shows enlarged region of colocalization (yellow puncta). (C, D) Interleaved scatter graph representing overall decrease in autolysosomes (Cathepsin and mCherryAtg8a) in control and *Atg1RNAi*germarium (C) and GSCs (D). (E,F) Control (E-E") and *Atg1RNAi* (F-F") germarium showing Ref(2)PGFP (E',F' green) and CathepsinL (E",F" red) staining. Arrowheads point to colocalized punctae. Inset shows enlarged region of complete colocalization. Arrowheads point to colocalized punctae and arrows indicate Ref(2)PGFP punctae (cargo). (G,H) Interleaved scatter graph representing overall decrease in percentage colocalization between puncta/structures positive for CathepsinL and Ref(2)PGFP in control and *Atg1RNAi*germarium (G) and GSCs (H).Nuclei is marked in blue (DAPI). Dotted ovals mark the GSCs and asterisk mark the cap cells. Scale bar- 10µm. Error bars represent SD in red and the mean is represented in blue. n=20, **$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

Figure 2

Depletion of Atg1 results in increased ROS during oogenesis.

(A,B) DHE (red) staining in control (A-A') and *Atg1RNAi* (B-B') ovariole.(C,D) Interleaved scatter graph showing mean intensity of DHE incontrol and *Atg1RNAi*GSCs (C) and germarium (D) per unit area.(E,F) mito-roGFP2-Grx1 monitors the redox state in the GSC and the germarium. Ratio of emission at 405 and 488 nm obtained as a response of the reporter in control (E-E") and *Atg1RNAi* (F-F"). (G,H) Interleaved scatter graph showing ratiometric shift of excitation in control and *Atg1RNAi*GSC (G) and germarium (H). DR stands for dynamic range.Nuclei are marked in blue. Dotted ovals mark the GSCs and asterisk mark the cap cells. Scale bar- 10µm. Error bars represent SD in red and the mean is represented in blue. n=20, **$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. Ovals and asterisks indicate GSCs and cap cells respectively.

Figure 3. Atg1 is required for mitophagy in GSCs and GCs.

(A,B). Control (A-A") and *Atg1RNAi* (B-B") flies immunostained with ATP5α (red) and CathepsinL (green).(C,D) Interleaved scatter graph showing colocalization of CathepsinL and ATP5α puncta in control and *Atg1RNAi*germarium(C) and GSCs (D). (E,F) Control (E-E") and *Atg1RNAi* (F-F") germarium showing Ref(2)PGFP (green) and ATP5α (red) staining.(G,H)

Interleaved scatter graph showing colocalization between Ref(2)PGFP and ATP5α puncta in germarium(G) and GSCs (H) of control and *Atg1RNAi* flies. Error bars represent SD in red and the mean is blue. Nuclei are marked in blue. Ovals and asterisks indicate GSCs and cap cells respectively. Error bars represent SD in red and the mean is blue. n=20, **p < 0.05, **p < 0.01, ****p < 0.0001 . Scale bar 10 µm

Figure 4.Atg1 regulates Drp1 and Marfand*Drp1RNAi* phenocopies *Atg1RNAi* mitochondrial phenotype.

(A,B) Control (A-A") and *Atg1RNAi* (B-B") germaria immunostained with ATP5α (red) and HA (green) antibody to visualise HA tagged Drp1 protein.(C-C") Germaria from Drp1RNAi stained with ATP5α (red) showing large clumped mitochondria (C), thresholded mitochondria (C') and merged image with DAPI (C"). (D,E) Interleaved scatter graph showing the mean intensity of HA per unit area in germarium(C) and GSCs (D) of control and *Atg1RNAi* flies.(F) Box and whisker plot representing mean area of mitochondria measured per unit area of the germarium in C-C".(G,H) Control (G-G") and *Atg1RNAi* (H-H") germarium immunostained for ATP5α (red) and GFP (green) and to visualise GFP tagged Marf protein.(J,K) Interleaved scatter graph showing the mean intensity of GFP per unit area in germarium (J) and GSCs (K) of control flies and *Atg1RNAi*.(I-I") Germaria from MarfRNAi stained with ATP5α (red) showing fragmented mitochondria(I), thresholded mitochondria (I') and merged image with DAPI (I"). (L) Box and whisker plot representing mean area of particles measured per unit area of the germarium in I-I". Nuclei are marked in blue. Ovals and asterisks indicate GSCs and Cap cells respectively. Error bars represent SD in red and the mean is blue. n=20, **p < 0.05, **p < 0.01, ****p < 0.0001 . Scale bar 10 µm.

Figure 5. *Atg1*, *Drp1* and *Marf* interact genetically to regulate mitochondrial dynamics during oogenesis.

(A-D") Germaria from*wildtype*, *Atg1RNAi*, *Atg1RNAi:UASDrp1* and *Atg1RNAi:Marf* stained with ATP5α (A-D red), thresholded mitochondria (A'-D') and merged image with DAPI (A"-D").(E) Box and whisker plot representing mean area of mitochondria measured per unit area of the germarium.Dotted ovals mark the GSCs and asterisk mark the cap cells. Scale bar-10µm. Error bars represent SD in red and the mean is represented in blue. n=20, **p < 0.05, **p < 0.01, ****p < 0.0001.

Figure 6.Atg1, Drp1 and Marf collaborate to regulate several aspects of oogenesis

(A-F) Ovaries from *wildtype*, *Atg1RNAi*, *Drp1RNAi, Marf RNAi*, *Atg1RNAi:UASDrp1* and *Atg1RNAi:Marf*(A'-F')Ovaries from*wildtype*, *Atg1RNAi*, *Drp1RNAi, Marf RNAi*, *Atg1RNAi:UASDrp1* and *Atg1RNAi:Marf*.(A"-F")germarium immunostained with Vasa (green) to mark the GCs in *wildtype*, *Atg1RNAi*, *Drp1RNAi, Marf RNAi*, *Atg1RNAi:UASDrp1* and *Atg1RNAi:Marf*.(G) Interleaved scatter plot showing change in overall size of the ovary of *wildtype*, *Atg1RNAi*, *Drp1RNAi, Marf RNAi*, *Atg1RNAi:UASDrp1* and *Atg1RNAi:Marf* (n> 10). (H) Stacked bar graph representing the percentage of ovarioles with only pre-vitellogenic and vitellogenic stages in ovary of *wildtype*, *Atg1RNAi*, *Drp1RNAi, Marf RNAi*, *Atg1RNAi:UASDrp1* and *Atg1RNAi:Marf* (n>100).(I) Interleaved scatter plot showing the change in absolute counts of GCc (Vasa positive cells), quantified in images taken across the central plane of the germaria of ovary of *wildtype*, *Atg1RNAi*, *Drp1RNAi, Marf RNAi*, *Atg1RNAi:UASDrp1* and *Atg1RNAi:Marf* (n=20). (J) Stacked bar graph showing the decrease in GSC percentage in *Atg1:Marf*double knockdown as compared to *Atg1* knockdown n=10. Dotted ovals mark the GSCs. Scale bar- 10µm. Error bars represent SD in red and the mean is represented in blue., **$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

Author Contributions

MA and BVS designed the experiments. MA performed the experiments. BVS and MA wrote the manuscript. All authorscommented onand edited the manuscript.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data Availability Statement

Data available on request from the authors

References

1. **Aman, Y., Schmauck-Medina, T., Hansen, M., Morimoto, R. I., Simon, A. K., Bjedov, I., Palikaras, K., Simonsen, A., Johansen, T., Tavernarakis, N., et al.** (2021). Autophagy in healthy aging and disease. *Nat Aging* **1**, 634–650.
2. **Amartuvshin, O., Lin, C. H., Hsu, S. C., Kao, S. H., Chen, A., Tang, W. C., Chou, H. L., Chang, D. L., Hsu, Y. Y., Hsiao, B. S., et al.** (2020). Aging shifts mitochondrial dynamics toward fission to promote germline stem cell loss. *Aging Cell* **19**,.
3. **Aparicio, R., Rana, A. and Walker, D. W.** (2019). Upregulation of the Autophagy Adaptor p62/SQSTM1 Prolongs Health and Lifespan in Middle-Aged Drosophila. *Cell Rep* **28**,.
4. **Bali, A. and Shravage, B. V.** (2017). Characterization of the autophagy related gene-8a (Atg8a) promoter in drosophila melanogaster. *International Journal of Developmental Biology* **61**, 551–555.
5. **Barth, J. M. I. I., Szabad, J., Hafen, E. and Köhler, K.** (2011). Autophagy in Drosophila ovaries is induced by starvation and is required for oogenesis. *Cell Death Differ* **18**, 915–924.
6. **Bellezza, I., Giambanco, I., Minelli, A. and Donato, R.** (2018). Nrf2-Keap1 signaling in oxidative and reductive stress. *Biochim Biophys Acta Mol Cell Res* **1865**,.
7. **Boya, P., Reggiori, F. and Codogno, P.** (2013). Emerging regulation and functions of autophagy. *Nat Cell Biol* **15**, 713–20.
8. **Brand, A. H. and Perrimon, N.** (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*.
9. **Bratu, D. and Gerard, M. P.** (2015). *Drosophila Oogenesis*.
10. **Bucana, C., Saiki, I. and Nayar, R.** (1986). Uptake and accumulation of the vital dye hydroethidine in neoplastic cells. *Journal of Histochemistry and Cytochemistry* **34**,.
11. **Chang, Y. Y. and Neufeld, T. P.** (2009). An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. *Mol Biol Cell* **20**, 2004–2014.
12. **Chang, T.-K., Shravage, B. v., Hayes, S. D., Powers, C. M., Simin, R. T., Wade Harper, J. and Baehrecke, E. H.** (2013). Uba1 functions in Atg7- and Atg3-independent autophagy. *Nat Cell Biol* **15**, 1067–78.
13. **Cox, R. T. and Spradling, A. C.** (2003). A Balbiani body and the fusome mediate mitochondrial inheritance during Drosophila oogenesis. *Development* **130**,.
14. **Dan Dunn, J., Alvarez, L. A. J., Zhang, X. and Soldati, T.** (2015). Reactive oxygen species and mitochondria: A nexus of cellular homeostasis. *Redox Biol* **6**, 472–485.
15. **de Castro, I. P., Costa, A. C., Celardo, I., Tufi, R., Dinsdale, D., Loh, S. H. Y. and Martins, L. M.** (2013). Drosophila ref(2)P is required for the parkin-mediated suppression of mitochondrial dysfunction in pink1 mutants. *Cell Death Dis*.
16. **Denton, D., Shravage, B., Simin, R., Mills, K., Berry, D. L., Baehrecke, E. H. and Kumar, S.** (2009). Autophagy, Not Apoptosis, Is Essential for Midgut Cell Death in Drosophila. *Current Biology* **19**, 1741–1746.

17. **Dorn, G. W., Clark, C. F., Eschenbacher, W. H., Kang, M. Y., Engelhard, J. T., Warner, S. J., Matkovich, S. J. and Jowdy, C. C.** (2011). MARF and Opa1 control mitochondrial and cardiac function in Drosophila. *Circ Res*.
18. **Fuller, M. T. and Spradling, A. C.** (2007). Male and female Drosophila germline stem cells: Two versions of immortality. *Science (1979)*.
19. **Garcez, M., Branco-Santos, J., Gracio, P. C. and Homem, C. C. F.** (2021). Mitochondrial Dynamics in the Drosophila Ovary Regulates Germ Stem Cell Number, Cell Fate, and Female Fertility. *Front Cell Dev Biol* **8**,.
20. **Grandemange, S., Herzig, S. and Martinou, J. C.** (2009). Mitochondrial dynamics and cancer. *Semin Cancer Biol*.
21. **Hung, C. M., Lombardo, P. S., Malik, N., Brun, S. N., Hellberg, K., van Nostrand, J. L., Garcia, D., Baumgart, J., Diffenderfer, K., Asara, J. M., et al.** (2021). AMPK/ULK1-mediated phosphorylation of Parkin ACT domain mediates an early step in mitophagy. *Sci Adv* **7**,.
22. **Ishibashi, J. R., Taslim, T. H. and Ruohola-Baker, H.** (2020). Germline stem cell aging in the Drosophila ovary. *Curr Opin Insect Sci* **37**,.
23. **Juhasz, G., Erdi, B., Sass, M. and Neufeld, T. P.** (2007). Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila. *Genes Dev* **21**, 3061–3066.
24. **Kawajiri, S., Saiki, S., Sato, S., Sato, F., Hatano, T., Eguchi, H. and Hattori, N.** (2010). PINK1 is recruited to mitochondria with parkin and associates with LC3 in mitophagy. *FEBS Lett* **584**,.
25. **Khurana, J. S. and Theurkauf, W.** (2010). piRNAs, transposon silencing, and Drosophila germline development. *Journal of Cell Biology* **191**,.
26. **Klionsky, D. J., Abdel-Aziz, A. K., Abdelfatah, S., Abdellatif, M., Abdoli, A., Abel, S., Abeliovich, H., Abildgaard, M. H., Abudu, Y. P., Acevedo-Arozena, A., et al.** (2021). Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition)1. *Autophagy* **17**,.
27. **Kuhn, H., Sopko, R., Coughlin, M., Perrimon, N. and Mitchison, T.** (2015). The Atg1-Tor pathway regulates yolk catabolism in Drosophila embryos. *Development (Cambridge)* **142**,.
28. **Lazarou, M., Sliter, D. A., Kane, L. A., Sarraf, S. A., Wang, C., Burman, J. L., Sideris, D. P., Fogel, A. I. and Youle, R. J.** (2015). The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. *Nature* **524**,.
29. **Lee, J. J., Sanchez-Martinez, A., Zarate, A. M., Benincá, C., Mayor, U., Clague, M. J. and Whitworth, A. J.** (2018). Basal mitophagy is widespread in Drosophila but minimally affected by loss of Pink1 or parkin. *Journal of Cell Biology* **217**,.
30. **Lemasters, J. J.** (2005). Selective Mitochondrial Autophagy, or Mitophagy, as a Targeted Defense Against Oxidative Stress, Mitochondrial Dysfunction, and Aging. *Rejuvenation Res* **8**, 3–5.
31. **Lieber, T., Jeedigunta, S. P., Palozzi, J. M., Lehmann, R. and Hurd, T. R.** (2019). Mitochondrial fragmentation drives selective removal of deleterious mtDNA in the germline. *Nature*.
32. **Ma, P., Yun, J., Deng, H. and Guo, M.** (2018). Atg1-mediated autophagy suppresses tissue degeneration in pink1/parkin mutants by promoting mitochondrial fission in Drosophila. *Mol Biol Cell*.

33. **Marinho, H. S., Real, C., Cyrne, L., Soares, H. and Antunes, F.** (2014). Hydrogen peroxide sensing, signaling and regulation of transcription factors. *Redox Biol* **2**,.
34. **McLaughlin, J. M. and Bratu, D. P.** (2015). Drosophila melanogaster oogenesis: An overview. *Methods in Molecular Biology* **1328**,.
35. **Meissner, C., Lorenz, H., Weihofen, A., Selkoe, D. J. and Lemberg, M. K.** (2011). The mitochondrial intramembrane protease PARL cleaves human Pink1 to regulate Pink1 trafficking. *J Neurochem* **117**,.
36. **Mizushima, N.** (2007). Autophagy: Process and function. *Genes Dev* **21**, 2861–2873.
37. **Mulakkal, N. C., Nagy, P., Takats, S., Tusco, R., Juhász, G. and Nezis, I. P.** (2014). Review Article Autophagy in Drosophila : From Historical Studies to Current Knowledge. *Biomed Res Int* **2014**,.
38. **Nagy, P., Kárpáti, M., Varga, Á., Pircs, K., Venkei, Z., Takáts, S., Varga, K. and Érdi, B.** (2014). Atg17 / FIP200 localizes to perilysosomal Ref (2) P aggregates and promotes autophagy by activation of Atg1 in Drosophila. 453–467.
39. **Narendra, D. P., Jin, S. M., Tanaka, A., Suen, D. F., Gautier, C. A., Shen, J., Cookson, M. R. and Youle, R. J.** (2010). PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol* **8**,.
40. **Nezis, I. P., Simonsen, A., Sagona, A. P., Finley, K., Gaumer, S., Contamine, D., Rusten, T. E., Stenmark, H. and Brech, A.** (2008). Ref(2)P, the Drosophila melanogaster homologue of mammalian p62, is required for the formation of protein aggregates in adult brain. *J Cell Biol* **180**, 1065–1071.
41. **Nezis, I. P., Shravage, B. v., Sagona, A. P., Lamark, T., Bjørkøy, G., Johansen, T., Rusten, T. E., Brech, A., Baehrecke, E. H. and Stenmark, H.** (2010). Autophagic degradation of dBruce controls DNA fragmentation in nurse cells during late Drosophila melanogaster oogenesis. *J Cell Biol* **190**, 523–531.
42. **Nilangekar, K. S. and Shravage, B. V.** (2019). *Mitochondrial redox sensor for drosophila female germline stem cells*.
43. **Nilangekar, K., Murmu, N., Sahu, G. and Shravage, B. v.** (2019). Generation and characterization of germline-specific autophagy and mitochondrial reactive oxygen species reporters in Drosophila. *Front Cell Dev Biol* **7**,.
44. **Onishi, M., Yamano, K., Sato, M., Matsuda, N. and Okamoto, K.** (2021). Molecular mechanisms and physiological functions of mitophagy. *EMBO J* **40**,.
45. **Pajares, M., Jiménez-moreno, N., García-yagüe, Á. J., Ceballos, M. L. de, Leuven, F. van, Rábano, A., Rojo, A. I., Cuadrado, A., Pajares, M., Jiménez-moreno, N., et al.** (2016). Transcription factor NFE2L2 / NRF2 is a regulator of macroautophagy genes. **8627**,.
46. **Peng, J. C., Valouev, A., Liu, N. and Lin, H.** (2016). Piwi maintains germline stem cells and oogenesis in Drosophila through negative regulation of Polycomb group proteins. *Nat Genet* **48**,.
47. **Pickles, S., Vigié, P. and Youle, R. J.** (2018). Mitophagy and Quality Control Mechanisms in Mitochondrial Maintenance. *Current Biology* **28**, R170–R185.
48. **Poole, L. P., Bock-Hughes, A., Berardi, D. E. and Macleod, K. F.** (2021). ULK1 promotes mitophagy via phosphorylation and stabilization of BNIP3. *Sci Rep* **11**,.
49. **Rana, A., Oliveira, M. P., Khamoui, A. v., Aparicio, R., Rera, M., Rossiter, H. B. and Walker, D. W.** (2017). Promoting Drp1-mediated mitochondrial fission in midlife prolongs healthy lifespan of Drosophila melanogaster. *Nat Commun*.

50. **Rørth, P.** (1998). Gal4 in the Drosophila female germline. *Mech Dev* **78**, 113–118.
51. **Sandoval, H., Yao, C. K., Chen, K., Jaiswal, M., Donti, T., Lin, Y. Q., Bayat, V., Xiong, B., Zhang, K., David, G., et al.** (2014). Mitochondrial fusion but not fission regulates larval growth and synaptic development through steroid hormone production. *Elife*.
52. **Scherz-Shouval, R. and Elazar, Z.** (2011). Regulation of autophagy by ROS: Physiology and pathology. *Trends Biochem Sci*.
53. **Scott, R. C., Schuldiner, O. and Neufeld, T. P.** (2004). Role and regulation of starvation-induced autophagy in the Drosophila fat body. *Dev Cell* **7**, 167–178.
54. **Scott, R. C., Juhász, G. and Neufeld, T. P.** (2007). Direct Induction of Autophagy by Atg1 Inhibits Cell Growth and Induces Apoptotic Cell Death. *Current Biology* **17**, 1–11.
55. **Sena, L. A. and Chandel, N. S.** (2012). Physiological Roles of Mitochondrial Reactive Oxygen Species. *Mol Cell* **48**, 158–167.
56. **Seo, B. J., Yoon, S. H. and Do, J. T.** (2018). Mitochondrial dynamics in stem cells and differentiation. *Int J Mol Sci*.
57. **Shadel, G. S. and Horvath, T. L.** (2015). Mitochondrial ROS Signaling in Organismal Homeostasis. *Cell* **163**, 560–569.
58. **Spradling, A. A. C.** (1993). *Developmental genetics of oogenesis. The Development of Drosophila melanogaster*.
59. **Spradling., A. C.** (1993). Developmental genetics of oogenesis. In The Development of Drosophila Melanogaster, .
60. **Taguchi, N., Ishihara, N., Jofuku, A., Oka, T. and Mihara, K.** (2007). Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission. *Journal of Biological Chemistry*.
61. **Tanner, E. A., Blute, T. A., Brachmann, C. B., McCall, K., Abdelwahid, E., Yokokura, T., Krieser, R. J., Balasundaram, S., Fowle, W. H., White, K., et al.** (2011). Bcl-2 proteins and autophagy regulate mitochondrial dynamics during programmed cell death in the Drosophila ovary. *Development* **138**, 327–38.
62. **Theodosiou, N. A. and Xu, T.** (1998). Use of FLP/FRT system to study Drosophila development. *Methods: A Companion to Methods in Enzymology* **14**,.
63. **van der Bliek, A. M., Shen, Q. and Kawajiri, S.** (2013). Mechanisms of mitochondrial fission and fusion. *Cold Spring Harb Perspect Biol* **5**,.
64. **Verstreken, P., Ly, C. v., Venken, K. J. T., Koh, T. W., Zhou, Y. and Bellen, H. J.** (2005). Synaptic mitochondria are critical for mobilization of reserve pool vesicles at Drosophila neuromuscular junctions. *Neuron* **47**,.
65. **Vincow, E. S., Merrihew, G., Thomas, R. E., Shulman, N. J., Beyer, R. P., MacCoss, M. J. and Pallanck, L. J.** (2013). The PINK1-Parkin pathway promotes both mitophagy and selective respiratory chain turnover in vivo. *Proc Natl Acad Sci U S A*.
66. **von Stockum, S., Nardin, A., Schrepfer, E. and Ziviani, E.** (2016). Mitochondrial dynamics and mitophagy in Parkinson's disease: A fly point of view. *Neurobiol Dis* **90**,.
67. **Wang, Z. H., Clark, C. and Geisbrecht, E. R.** (2016). Drosophila clueless is involved in Parkin-dependent mitophagy by promoting VCP-mediated Marf degradation. *Hum Mol Genet* **25**,.

68. **Wu, W., Tian, W., Hu, Z., Chen, G., Huang, L., Li, W., Zhang, X., Xue, P., Zhou, C., Liu, L., et al.** (2014). ULK1 translocates to mitochondria and phosphorylates FUNDC1 to regulate mitophagy. *EMBO Rep* **15**,.
69. **Xie, T. and Spradling, A. C.** (1998). decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. *Cell* **94**,.
70. **Yamamoto, M., Kensler, T. W. and Motohashi, H.** (2018). The KEAP1-NRF2 system: A thiol-based sensor-effector apparatus for maintaining redox homeostasis. *Physiol Rev* **98**,.
71. **Yamano, K. and Youle, R. J.** (2013). PINK1 is degraded through the N-end rule pathway. *Autophagy* **9**,.
72. **Yan, D., Neumüller, R. A., Buckner, M., Ayers, K., Li, H., Hu, Y., Yang-Zhou, D., Pan, L., Wang, X., Kelley, C., et al.** (2014). A regulatory network of Drosophila germline stem cell self-renewal. *Dev Cell* **28**,.
73. **Youle, R. J. and Narendra, D. P.** (2011). Mechanisms of mitophagy. *Nat Rev Mol Cell Biol*.

Figure 1

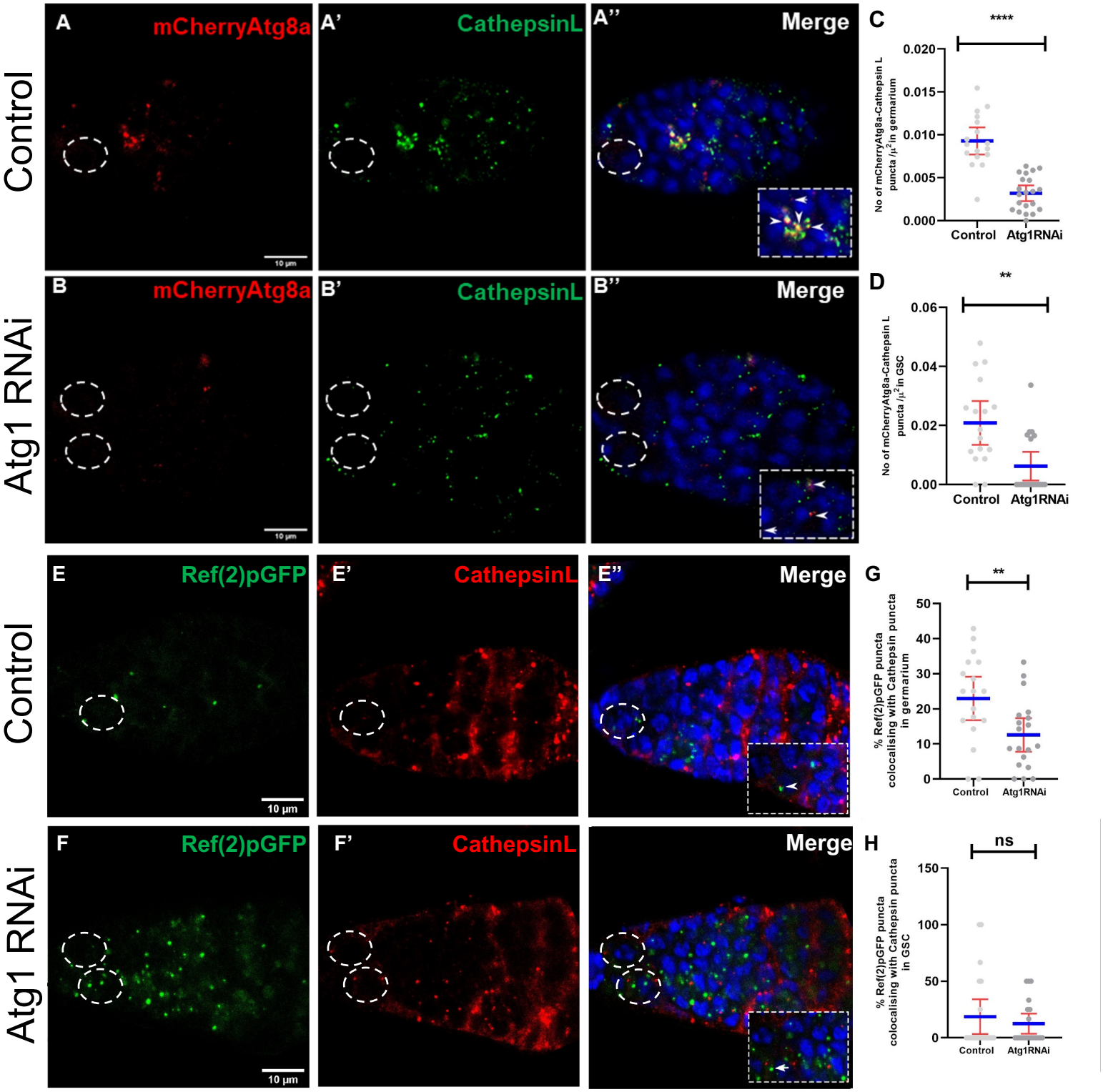

Control
Atg1 RNAi
A
mCherryAtg8a
A'
CathepsinL
A''
Merge
B
mCherryAtg8a
B'
CathepsinL
B''
Merge
C
****
No of mCherryAtg8a-Cathepsin L puncta /μ² in germarium
0.020
0.015
0.010
0.005
0.000
Control
Atg1RNAi
D
**
No of mCherryAtg8a-Cathepsin L puncta /μ² in GSC
0.06
0.04
0.02
0.00
Control
Atg1RNAi
E
Ref(2)pGFP
E'
CathepsinL
E''
Merge
10 μm
G
**
% Ref(2)pGFP puncta colocalising with Cathepsin puncta in germarium
50
40
30
20
10
0
Control
Atg1RNAi
F
Ref(2)pGFP
F'
CathepsinL
Merge
10 μm
H
ns
% Ref(2)pGFP puncta colocalising with Cathepsin puncta in GSC
150
100
50
0
Control
Atg1RNAi

Figure 2

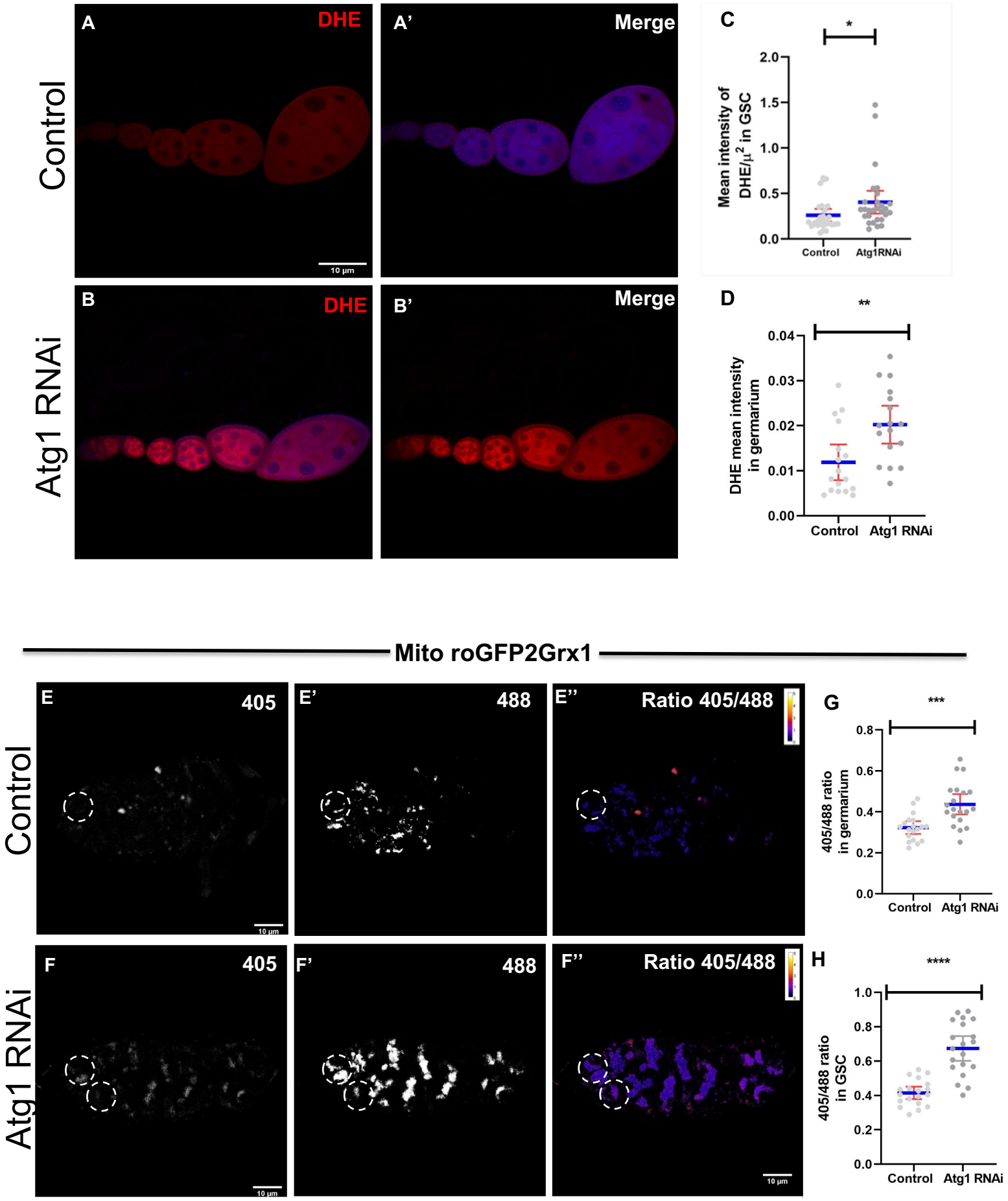

Control
Atg1 RNAi
A
DHE
A'
Merge
B
DHE
B'
Merge
10 µm
C
*
Mean intensity of DHE/µ2 in GSC
2.0
1.5
1.0
0.5
0.0
Control
Atg1RNAi
D
**
DHE mean intensity in germarium
0.04
0.03
0.02
0.01
0.00
Control
Atg1 RNAi
Mito roGFP2Grx1
E
405
E'
488
E''
Ratio 405/488
F
405
F'
488
F''
Ratio 405/488
G
***
405/488 ratio in germarium
0.8
0.6
0.4
0.2
0.0
Control
Atg1 RNAi
H
****
405/488 ratio in GSC
1.0
0.8
0.6
0.4
0.2
0.0
Control
Atg1 RNAi

Figure 3

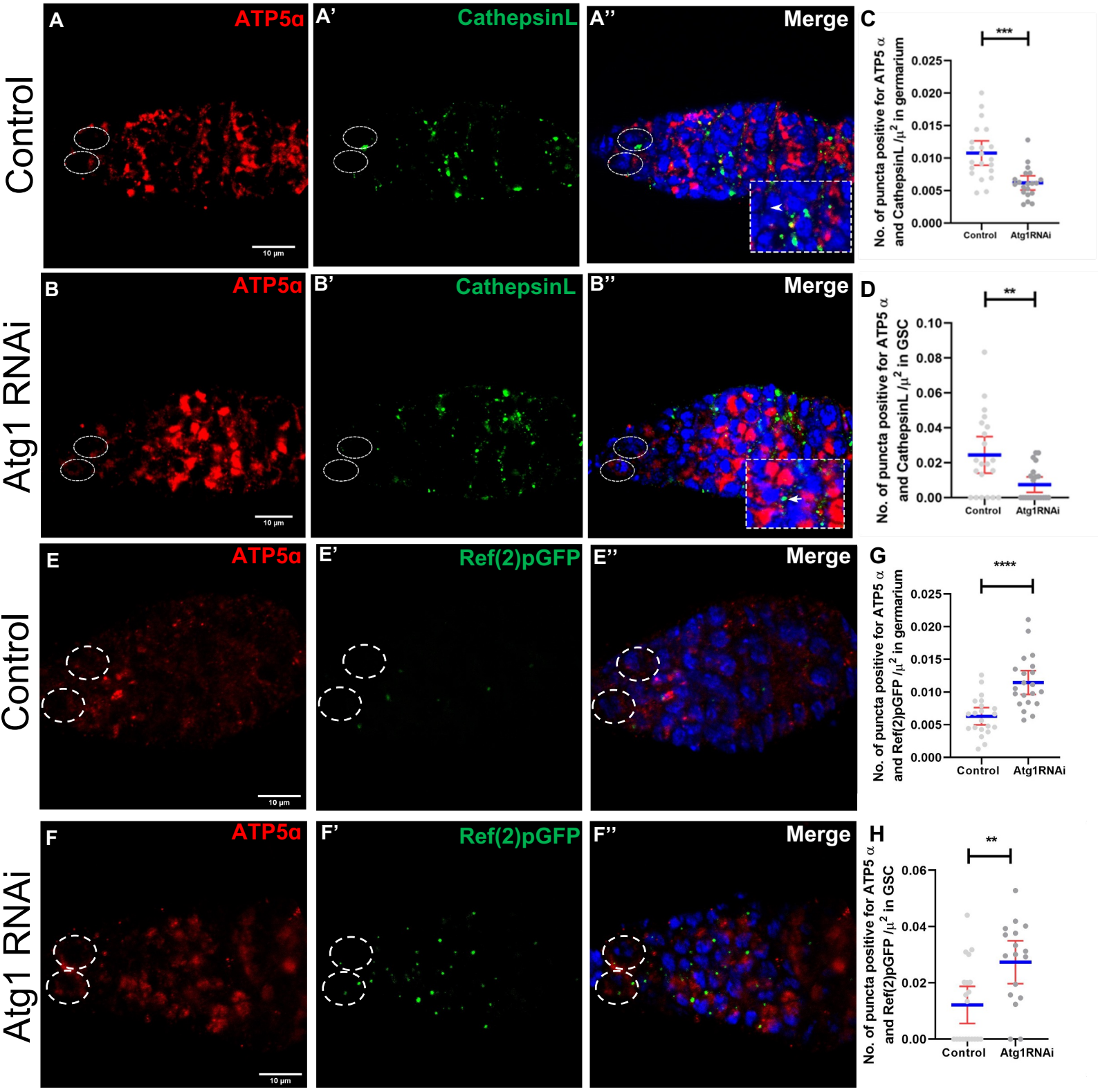

Control
Atg1 RNAi
Control
Atg1 RNAi
A
ATP5α
A'
CathepsinL
A''
Merge
B
ATP5α
B'
CathepsinL
B''
Merge
E
ATP5α
E'
Ref(2)pGFP
E''
Merge
F
ATP5α
F'
Ref(2)pGFP
F''
Merge
10 μm
C
***
No. of puncta positive for ATP5 α and CathepsinL /μ2 in germarium
0.025
0.020
0.015
0.010
0.005
0.000
Control
Atg1RNAi
D
**
No. of puncta positive for ATP5 α and CathepsinL /μ2 in GSC
0.10
0.08
0.06
0.04
0.02
0.00
Control
Atg1RNAi
G
****
No. of puncta positive for ATP5 α and Ref(2)pGFP /μ2 in germarium
0.025
0.020
0.015
0.010
0.005
0.000
Control
Atg1RNAi
H
**
No. of puncta positive for ATP5 α and Ref(2)pGFP /μ2 in GSC
0.06
0.04
0.02
0.00
Control
Atg1RNAi

Figure 4

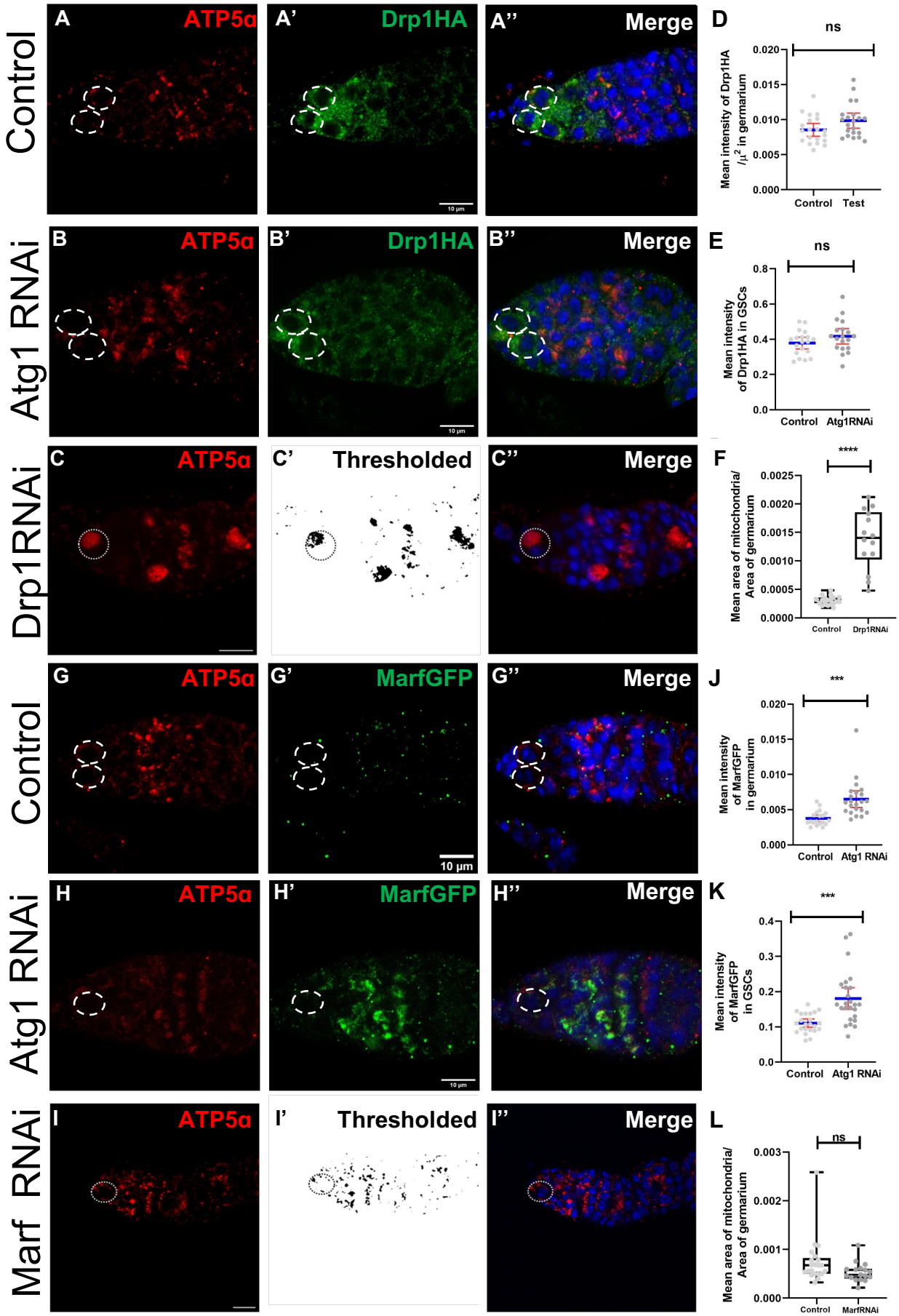
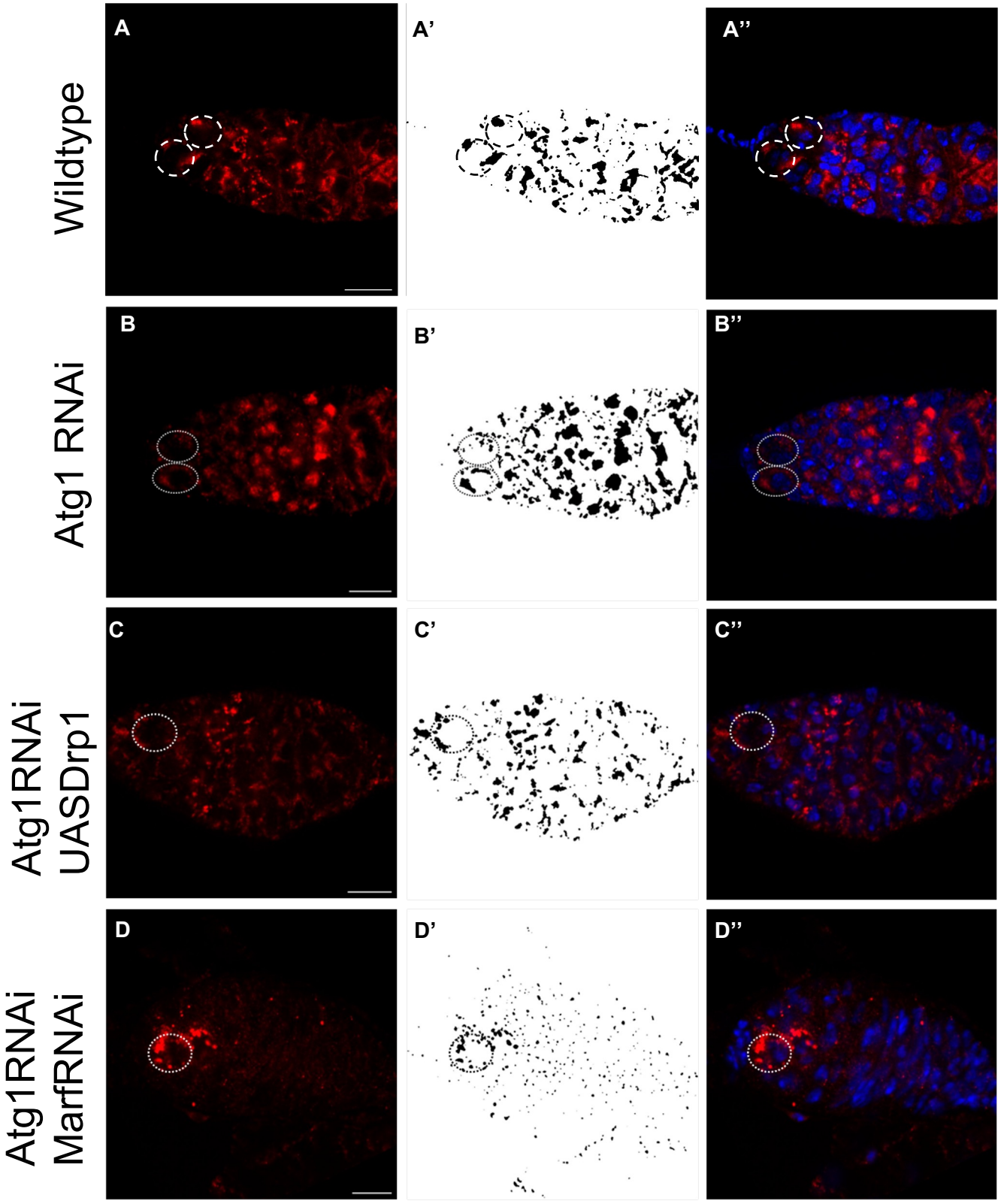

Control
A
ATP5α
A'
Drp1HA
A''
Merge
D
ns
Mean intensity of Drp1HA /μ2 in germarium
0.020
0.015
0.010
0.005
0.000
Control
Test
Atg1 RNAi
B
ATP5α
B'
Drp1HA
B''
Merge
E
ns
Mean intensity of Drp1HA in GSCs
0.8
0.6
0.4
0.2
0.0
Control
Atg1RNAi
Drp1RNAi
C
ATP5α
C'
Thresholded
C''
Merge
F
****
Mean area of mitochondria/ Area of germarium
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
Control
Drp1RNAi
Control
G
ATP5α
G'
MarfGFP
G''
Merge
10 μm
J
***
Mean intensity of MarfGFP in germarium
0.020
0.015
0.010
0.005
0.000
Control
Atg1 RNAi
Atg1 RNAi
H
ATP5α
H'
MarfGFP
H''
Merge
K
***
Mean intensity of MarfGFP in GSCs
0.4
0.3
0.2
0.1
0.0
Control
Atg1 RNAi
Marf RNAi
I
ATP5α
I'
Thresholded
I''
Merge
L
ns
Mean area of mitochondria/ Area of germarium
0.003
0.002
0.001
0.000
Control
MarfRNAi

Figure 5


Wildtype
Atg1 RNAi
Atg1RNAi UASDrp1
Atg1RNAi MarfRNAi
A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''

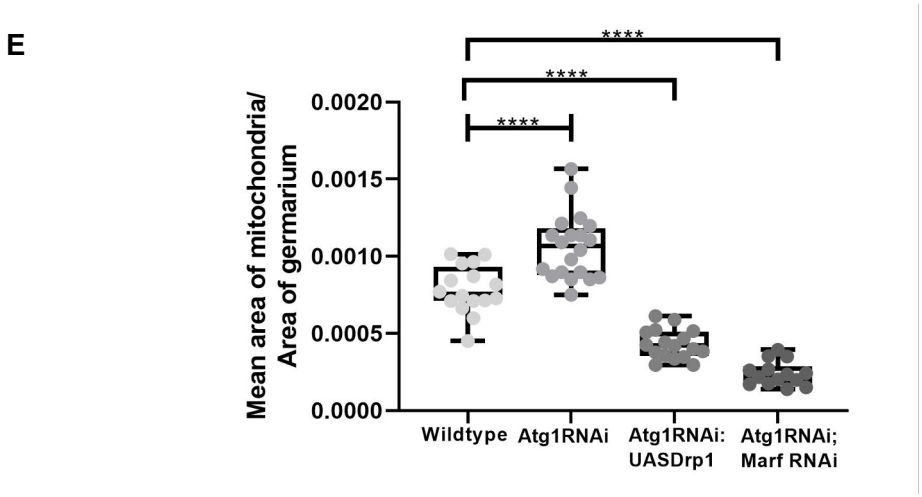

E
Mean area of mitochondria/ Area of germarium
0.0020
0.0015
0.0010
0.0005
0.0000
****
****
****
Wildtype
Atg1RNAi
Atg1RNAi: UASDrp1
Atg1RNAi; Marf RNAi

Figure 6

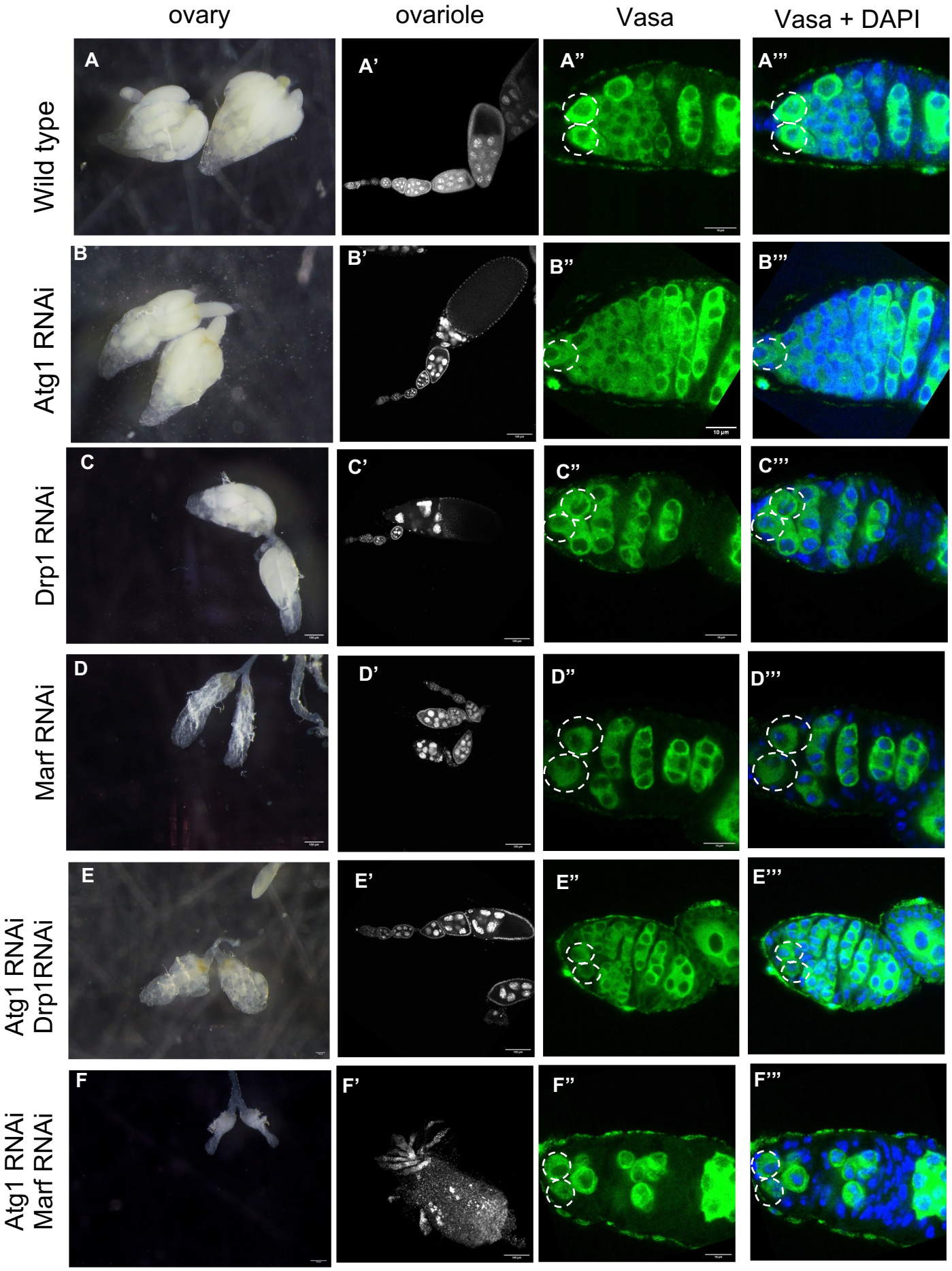
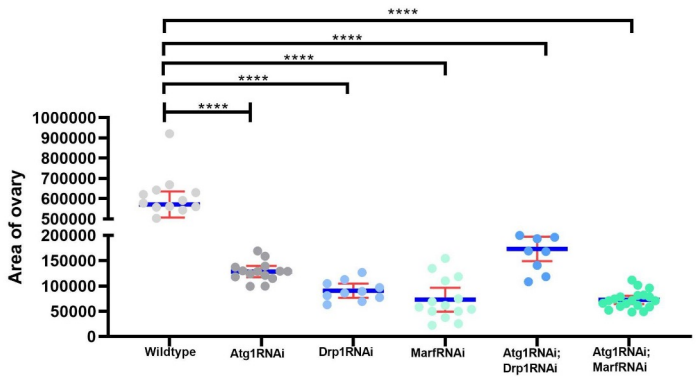
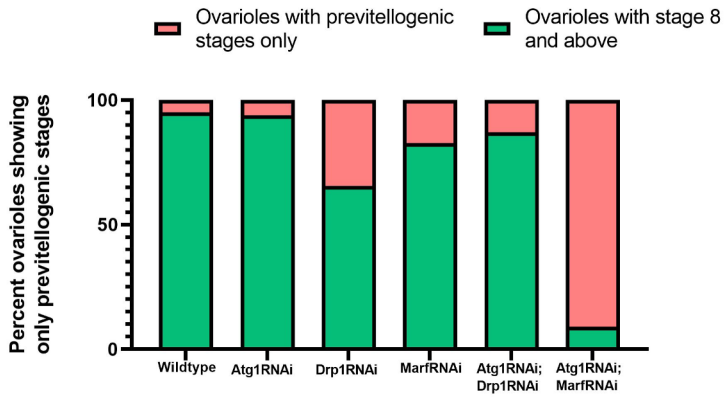

ovary
ovariole
Vasa
Vasa + DAPI
Wild type
Atg1 RNAi
Drp1 RNAi
Marf RNAi
Atg1 RNAi
Drp1RNAi
Atg1 RNAi
Marf RNAi
A
A'
A''
A'''
B
B'
B''
B'''
C
C'
C''
C'''
D
D'
D''
D'''
E
E'
E''
E'''
F
F'
F''
F'''

Figure 6

G



H



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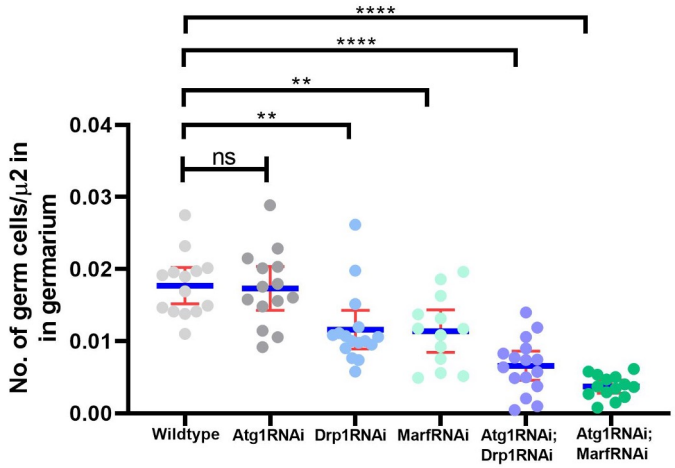
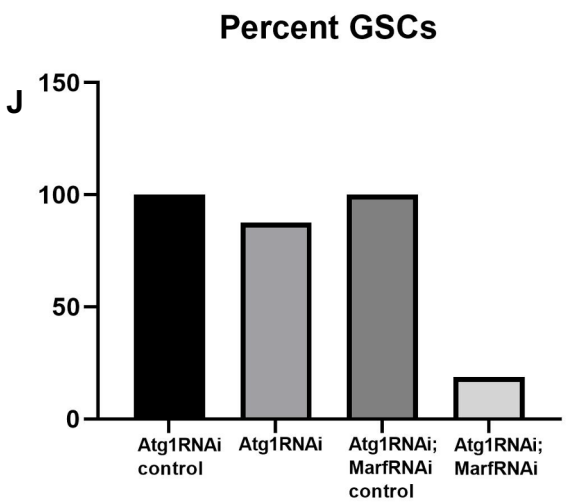
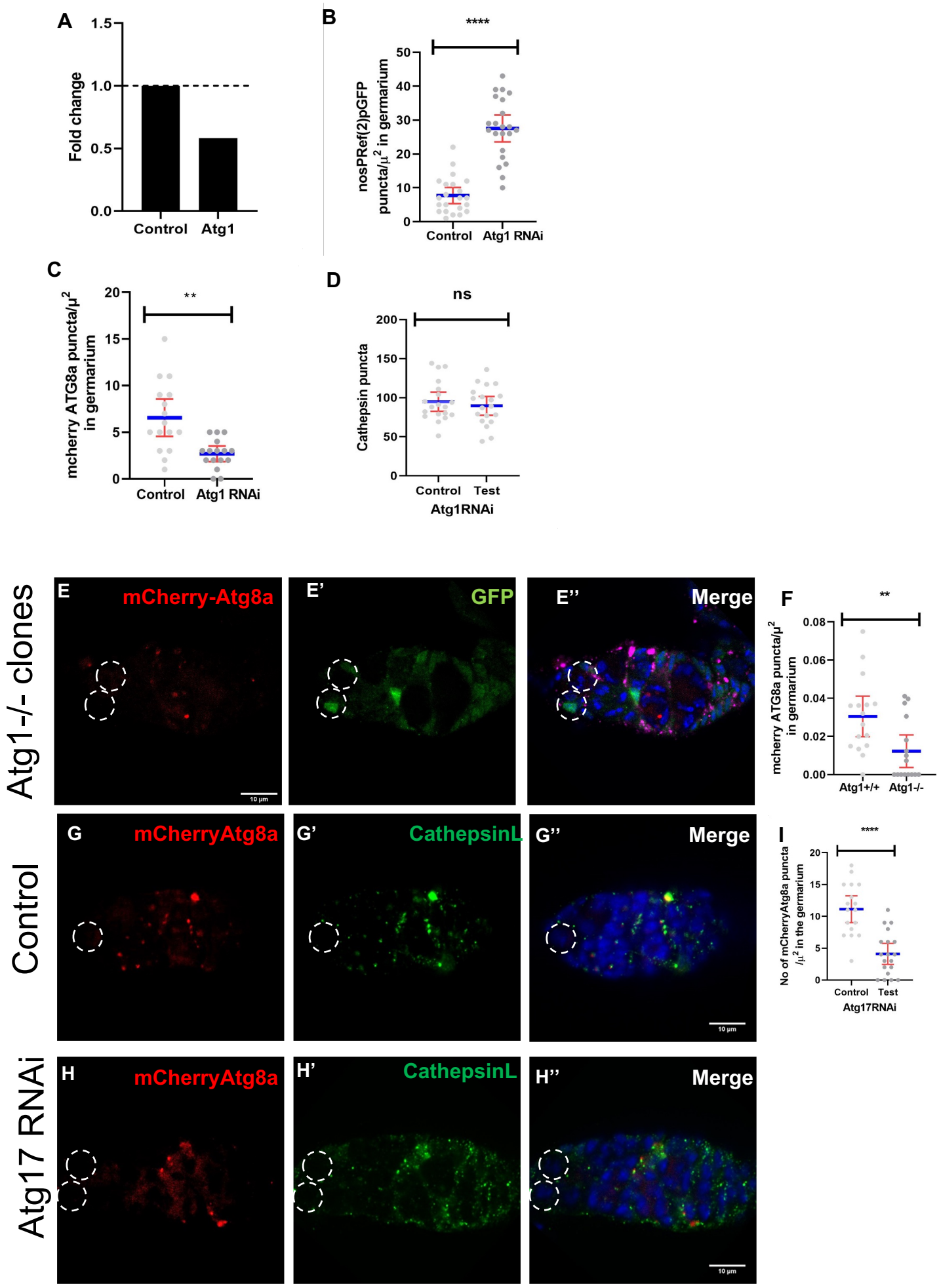


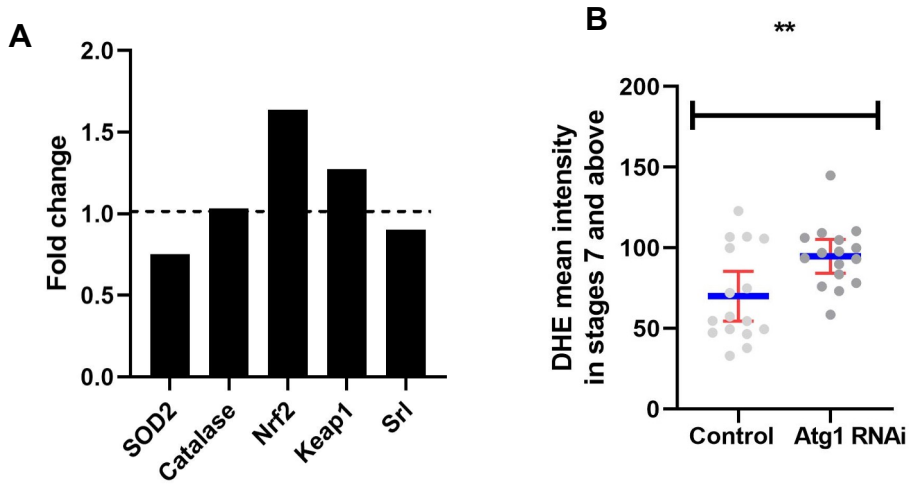
J



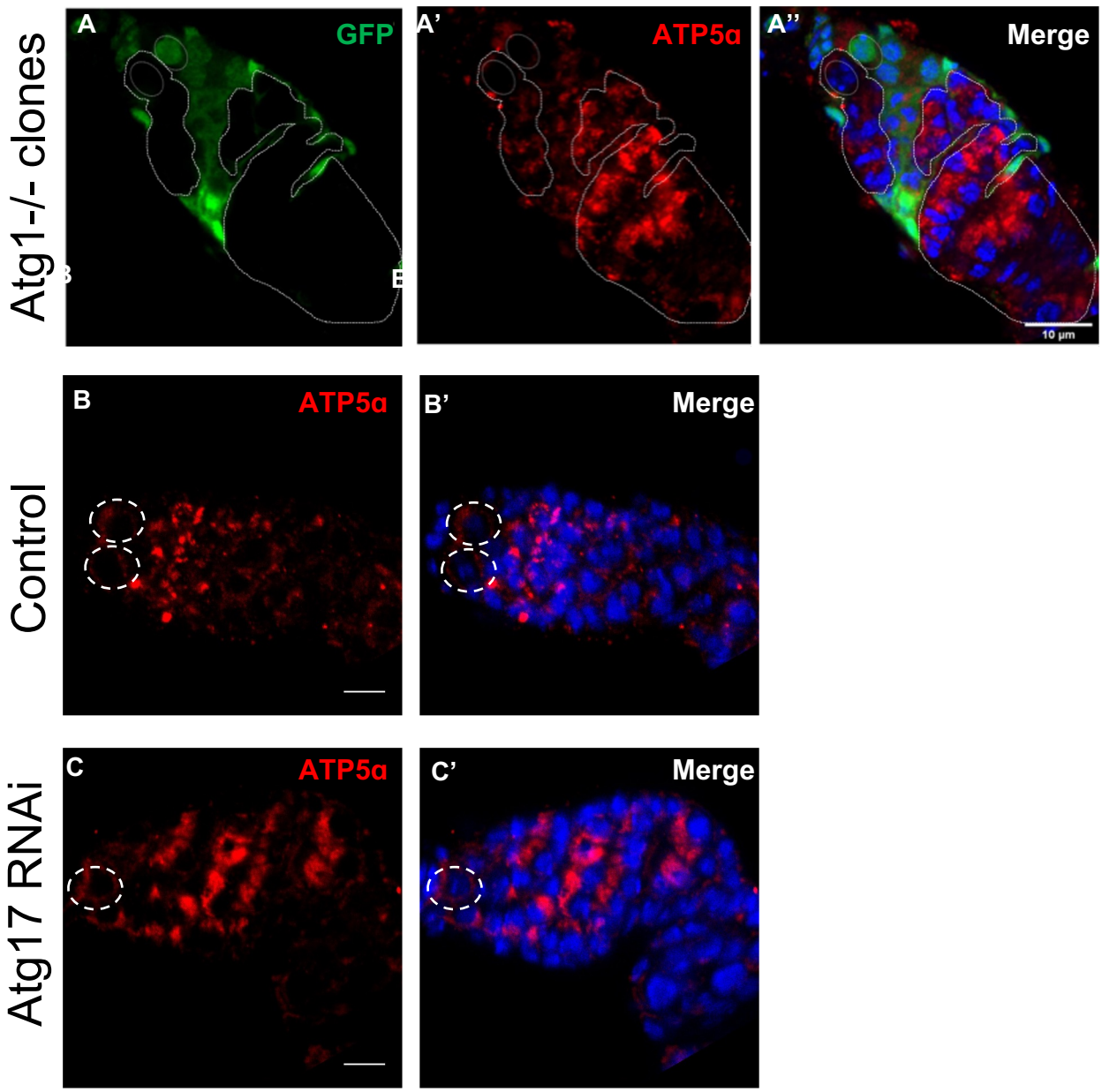
Supplementary Figure 1



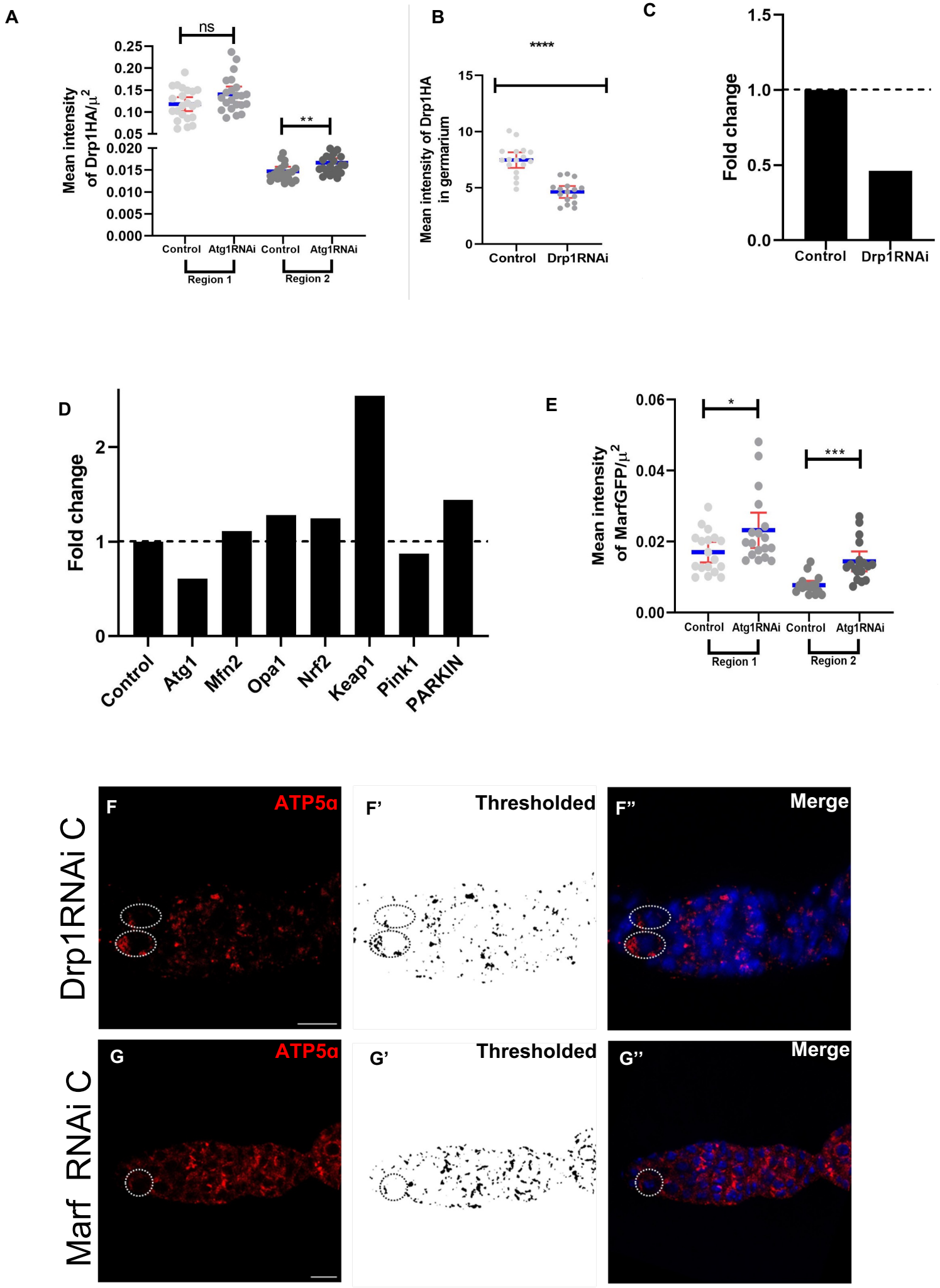
Supplementary Figure 2



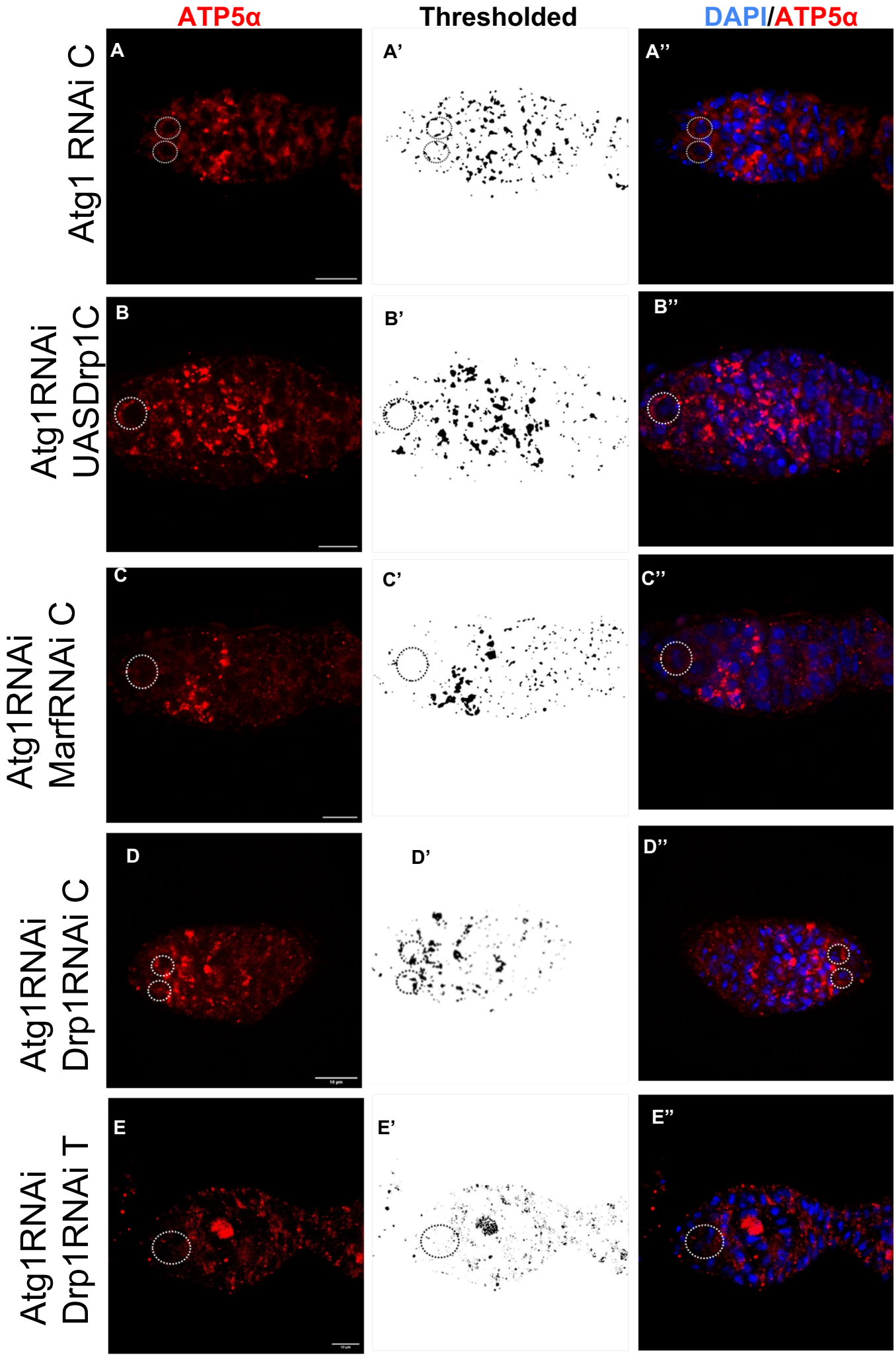
Supplementary figure 3



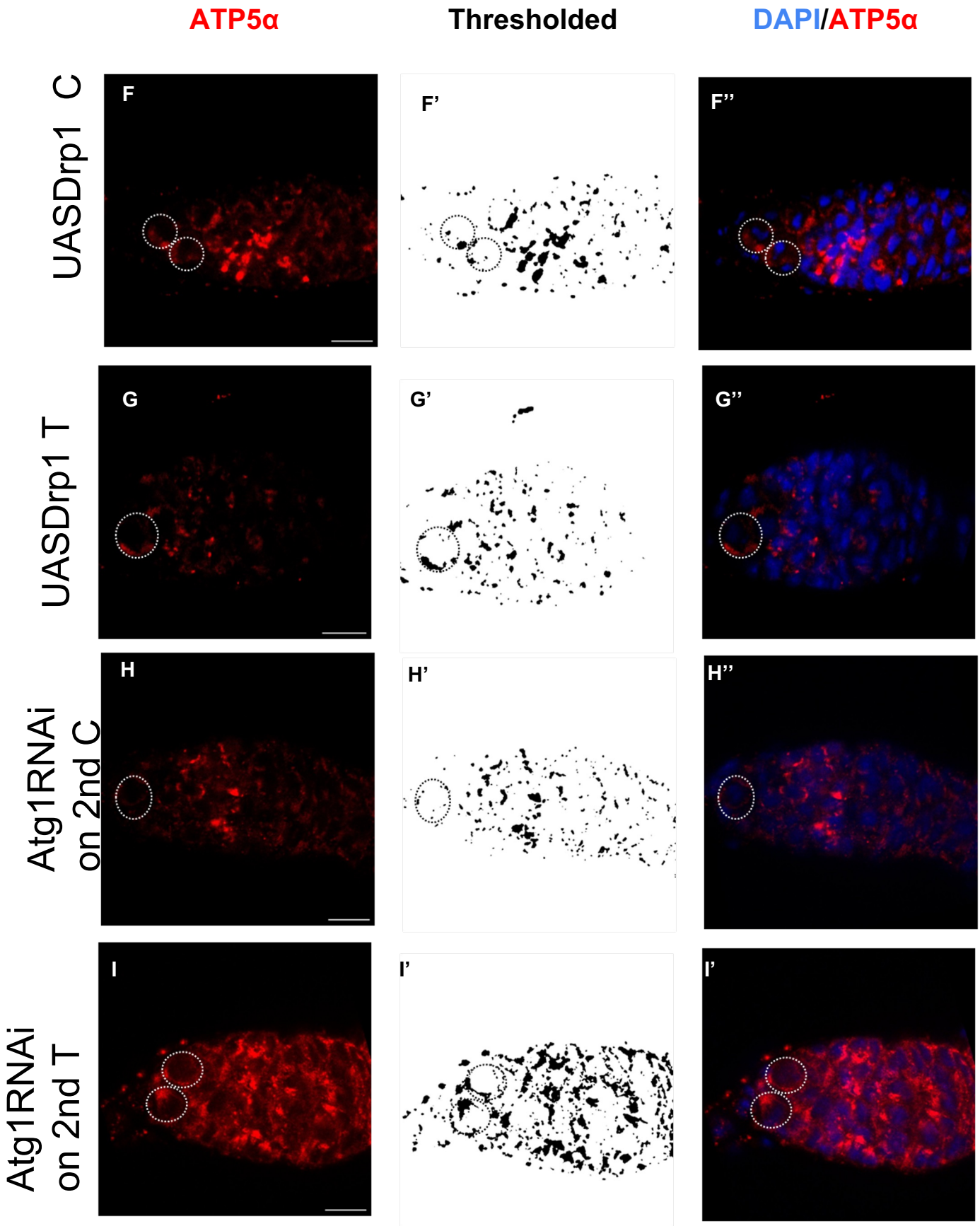
Supplementary Figure 4

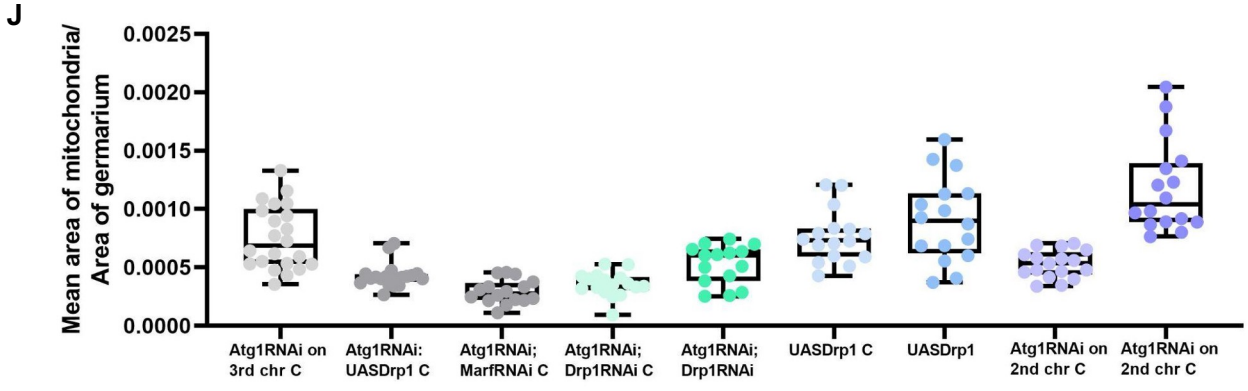


Supplementary figure 5

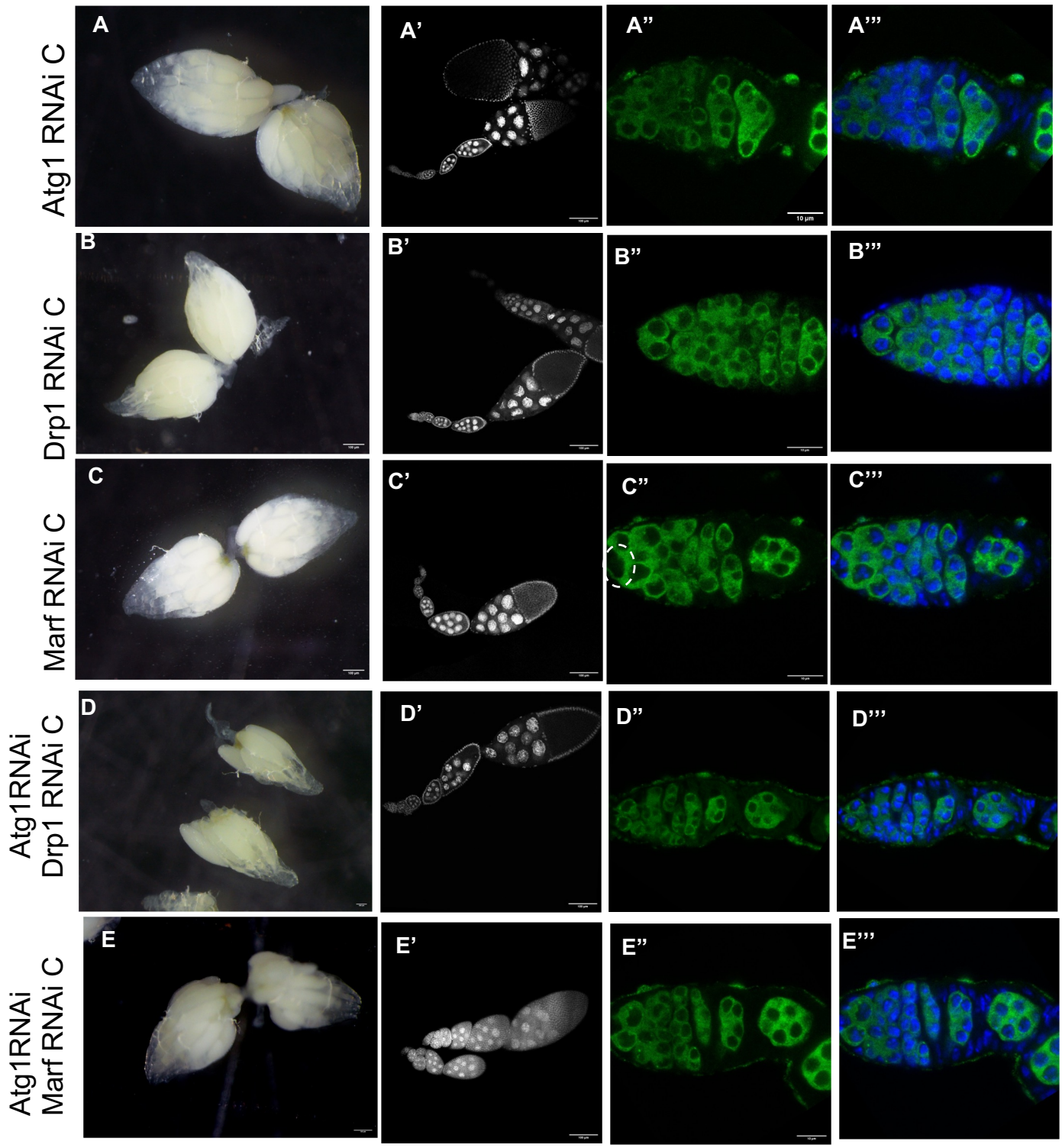


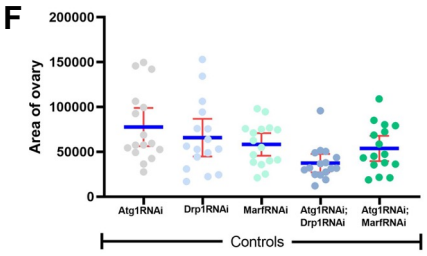
Supplementary Figure 5

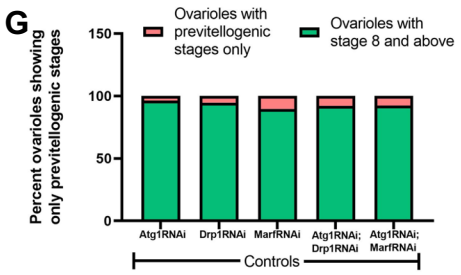


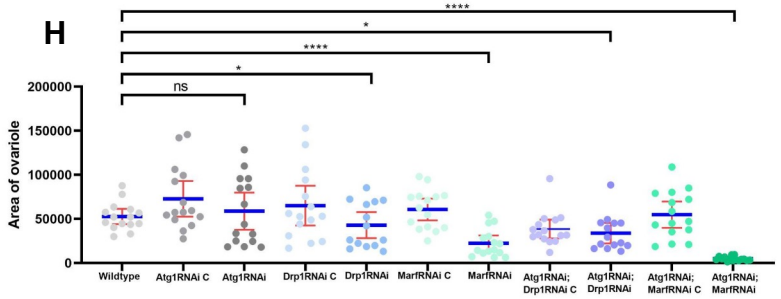
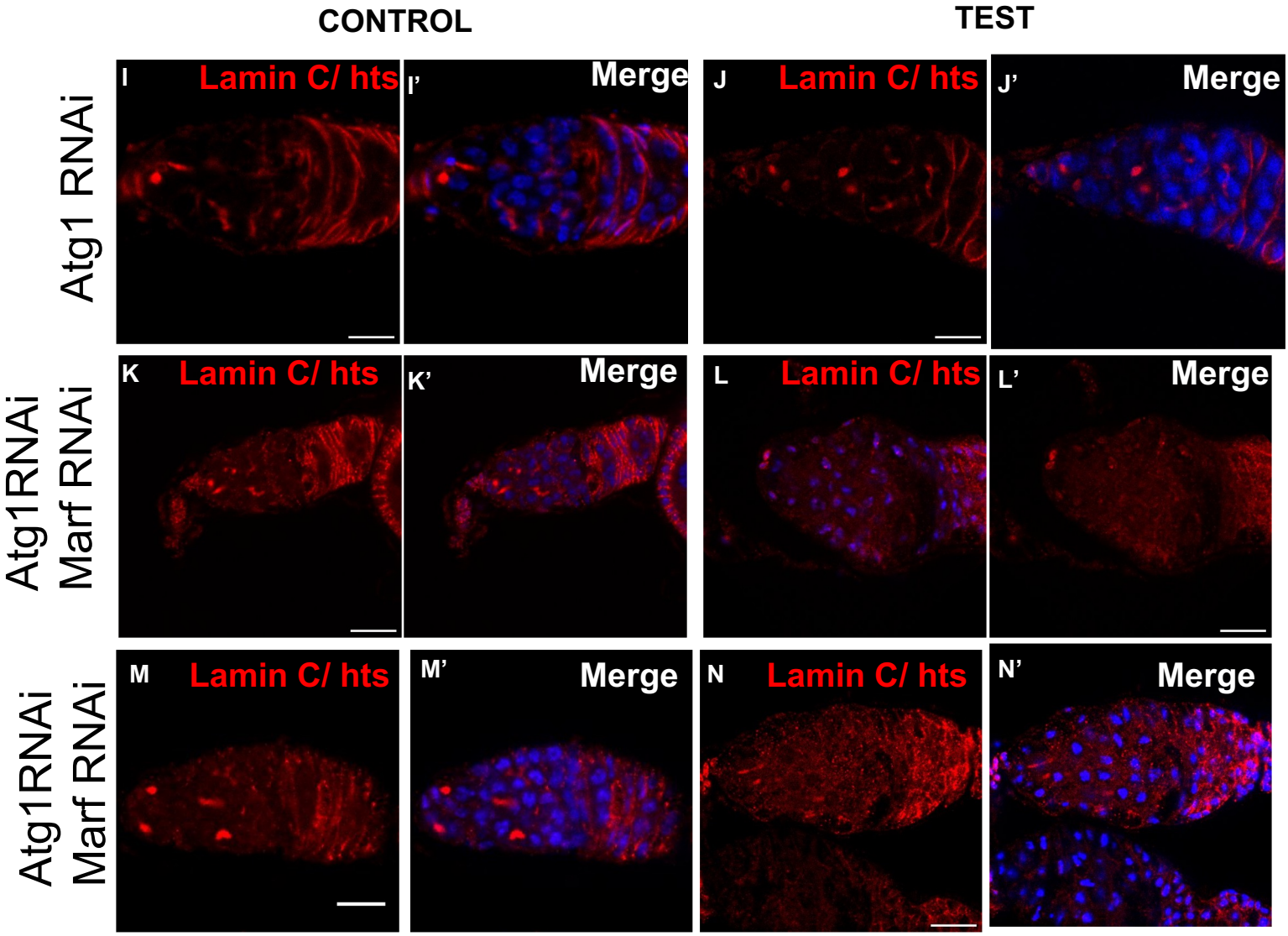


Supplementary Figure 6





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