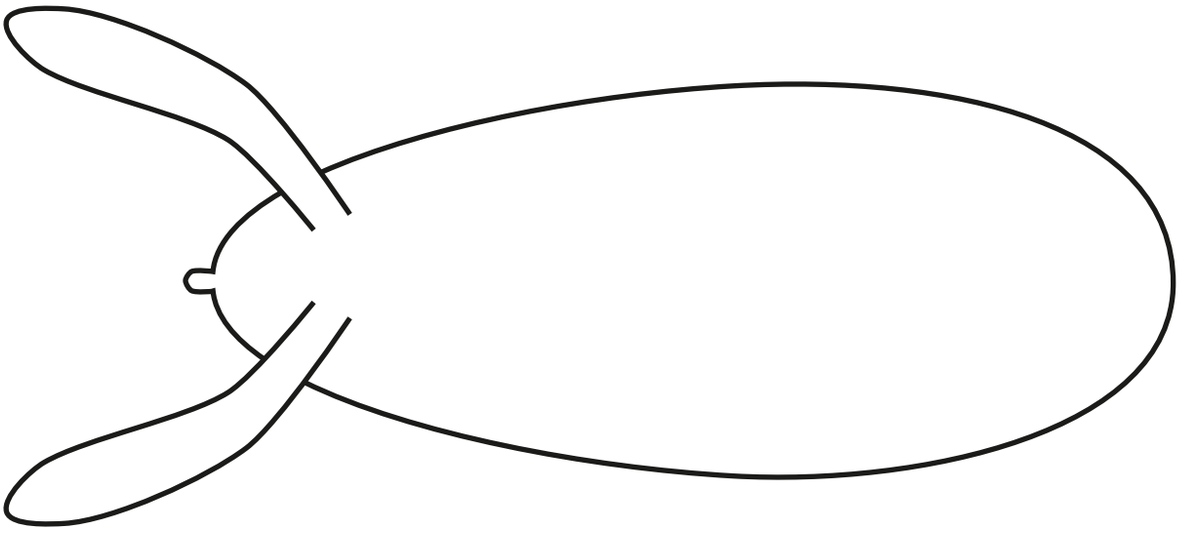
DISS. ETH Nr. 20065

The Role of Autophagy During *Drosophila* Oogenesis



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The Role of Autophagy During *Drosophila* Oogenesis

ABHANDLUNG
zur Erlangung des Titels

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Abstract

Autophagy is an evolutionarily conserved lysosomal degradation process promoting cell survival under several stresses, such as starvation. Thereby, unneeded cytoplasmic components are broken down to reuse the resulting molecules for essential processes. In small eukaryotes such as yeast, this is the major task of autophagy. However, in multicellular organisms, autophagic degradation is involved in many processes, as for example in immunity and cellular remodeling. Furthermore, autophagy is also implicated in several human pathophysiologies, including neurodegeneration and cancer. However, in most cases, the function and regulation of autophagy during development is still unclear, and new roles of autophagy are frequently revealed, highlighting the impact of autophagy research. This thesis provides novel findings that will help to clarify two major unsolved issues in the field of autophagy: The physiological implication of autophagy and the pathways guiding this function.
The major regulator of autophagy is the insulin/TOR signaling pathway. In *Drosophila*, insulin/TOR signaling influences the size and structure of ovaries, and nutrient depletion induces autophagy in the fat body, but the role of nutrient signaling and autophagy during oogenesis remains unknown. By establishing *Drosophila* ovaries as a novel model system to investigate autophagy, this work demonstrates that autophagy is induced by starvation in ovarian germ cells (GCs) as well as in surrounding somatic follicle cells (FCs), which depends on a functional autophagic machinery. Furthermore, it is here shown that the insulin/TOR pathway controls autophagy in these tissues. Additionally, the analysis of chimeric flies indicates that autophagy is not required in GCs during oogenesis, but that loss of autophagy in FCs leads to developmental defects. This mechanism depends on the cellular context, and defects are only present in a situation where FCs are mutant for autophagy, but GCs are wildtype. Closer analysis of eggs covered by autophagy deficient FCs reveals several defects that have previously been described for flies exhibiting mutations in classical cell-cell signaling pathways. Egg morphogenesis depends on a tightly linked communication between FCs and GCs, and the regulation of cell-cell signaling pathways has been demonstrated to involve

endocytosis and endosomal trafficking. Since autophagy and endosomal pathways are known to intersect in order to target vesicles for lysosomal degradation, this work examines whether autophagy modulates cell-cell signaling pathways. The posterior FCs that are important for cell-cell signaling events and egg differentiation, are here identified as the cell subpopulation responsible for autophagy dependent oogenesis defects. Furthermore, I present evidence that suggests that loss of autophagy modulates Notch signaling in FCs.

This work establishes an important function for autophagy during oogenesis and reveals a novel implication for autophagy in the modulation of cell-cell signaling pathways, thus contributing to the understanding of the role and regulation of autophagy in animal development.

Zusammenfassung

Autophagie (Autophagozytose) ist ein zellulärer Abbauprozess, bei welchem nicht mehr benötigte zelleigene Bestandteile in Lysosomen verdaut werden, um die daraus resultierenden Moleküle erneut für lebenswichtige Vorgänge verwenden zu können. Dieser Prozess kann durch zellulären Stress, zum Beispiel Hunger, entstehen und stellt eine evolutionär sehr alte Funktion dar, die zum Überleben der Zelle beiträgt. In einzelligen Eukaryoten, wie zum Beispiel der Hefe, ist dies die Hauptaufgabe der Autophagie. In mehrzelligen Organismen ist Autophagie ausserdem für viele weitere Prozesse, wie zum Beispiel die Immunantwort oder die Umwandlung von Larvengewebe in die Adultform von Insekten von Bedeutung. Des weiteren spielt Autophagie auch bei vielen Krankheiten wie Krebs oder Neurodegenerative Erkrankungen eine zentrale Rolle.

Auch heute noch werden der Autophagie immer wieder neue Funktionen zugeordnet, und die Rolle und Regulierung während der Entwicklung ist nicht vollständig geklärt. Dies zeigt, wie wichtig es ist Autophagie weiter zu erforschen.

Die Ergebnisse dieser Dissertation werden dazu beitragen, zwei wesentliche Fragen im Forschungsfeld der Autophagie - die physiologische Rolle der Autophagie und deren Regulation - zu klären.

Autophagie wird hauptsächlich durch den Insulin/TOR Signalweg gesteuert, welcher in der weiblichen Taufliege (*Drosophila*) zum Beispiel auch für die Grösse der Eierstöcke (Ovarien) verantwortlich ist. Zudem wird Autophagie unter Nahrungsknappheit im Fettkörper der Fliege aktiviert. Ob jedoch die Auslösung von Autophagie durch Hungergefühl auch in den Ovarien eine Rolle spielt ist noch nicht bekannt.

Deshalb wurden im Rahmen dieser Doktorarbeit die Ovarien der Taufliege als Modell zur Untersuchung der Autophagie etabliert. Es konnte gezeigt werden, dass Nahrungsentzug Autophagie in der Keimbahn, wie auch in den somatischen Follikelzellen auslöst, welches vom Vorhandensein bestimmter Autophagie-Gene abhängt. Des weiteren konnte beschrieben werden, dass Autophagie in den Ovarien durch den Insulin/TOR Signalweg reguliert wird.

Darüber hinaus zeigte die Untersuchung von chimären Fliegen, dass Autophagie in der Keimbahn nicht für die Entwicklung der Eier benötigt wird, dass jedoch der Verlust von Autophagie in den Follikelzellen Entwicklungsstörungen verursacht. Dies ist abhängig vom zellulären Zusammenhang, denn Entwicklungsstörungen wurden nur beobachtet wenn die Follikelzellen mutant sind für Autophagiegene, die Keimzellen jedoch den Wildtyp aufweisen. Weitere Untersuchungen zeigten, dass diese Eier Phänotypen besitzen, wie sie bereits für Fliegen beschrieben wurden welche eine fehlerhafte zelluläre Kommunikation haben. Die Morphogenese von Eiern ist abhängig von der zellulären Kommunikation zwischen der Keimbahn und den Follikelzellen und es wurde gezeigt, dass die Regulierung dieser interzellulären Signalwege auch Endozytose und endosomalen Transport beinhaltet. Da bekannt ist, dass sich Vesikel aus autophagie- und endosomalen Transportwegen für den Abbau in Lysosomen schneiden, liegt es nahe, dass Autophagie auch in der Modulation von interzellulären Kommunikationswegen eine Rolle spielen könnte. Dies wurde hier untersucht. Dabei konnten posteriore Follikelzellen, welche wichtig für interzelluläre Kommunikation und Differenzierung sind, als diejenigen Zellen identifiziert werden, welche für die durch fehlende Autophagie verursachten Entwicklungsdefekte verantwortlich sind. Zudem wurde gefunden, dass Autophagie eine Rolle in der Modulation des Notch-Signalweges in Follikelzellen spielt.

Diese Dissertation offenbart eine wichtige Aufgabe der Autophagie während der Eientwicklung in *Drosophila* und deckt einen neuen Bereich auf, in welchem Autophagie eine Rolle spielt: Die Modulierung von zellulären Kommunikationswegen. Dies trägt zum Verständnis der Rolle und Regulierung der Autophagie während der Entwicklung bei.

I Introduction

1.1 Prelude on autophagy - history and basics

Only about 10 years ago, the word ‘autophagy’ has been a widely unknown term. Although it was already introduced at a conference in 1963 by Christian de Duve (Klionsky, 2008) and the first article was printed in 1967, showing that glucagon can induce autophagy in the rat liver (Deter and De Duve, 1967), only very few experts knew the meaning of the word at that time (FIG. 1.1). Remarkably, this did not change over 30 years until Yoshinori Ohsumi cloned the first autophagy gene, the yeast *autophagy-related gene 1* (*ATG1*) (Matsuura *et al.*, 1997). Now, just at the beginning of this year, the 35th autophagy gene was given a name (Nazarko *et al.*, 2011). But lets go little back again. In 2007, when I read the descriptions for open positions within the ‘Life Science Zürich Graduate School’, I came across the catchy project title ‘The role of autophagy and growth control during *Drosophila* oogenesis’. My very first performance towards my PhD title started exactly in that moment when I typed into ‘Google’: a-u-t-o-p-h-a-g-y.

So what is autophagy? In an interview with Daniel Klionsky, Christian de Duve described how he invented the term: “I was in a word-coining mood and proposed the terms “endocytosis” and “exocytosis” for the processes they now designate. I also distinguished the “heterophagic” (eating others) and “autophagic” (eating self) functions of lysosomes and suggested the name “autophagic vacuoles” for Novikoff ’s cytolysomes (which would have been a perfectly apt term).” (Klionsky, 2008). In fact, autophagy is an intracellular, lysosomal degradation process occurring in all eukaryotic cells from yeast to mammals. Under normal induction, as for example during cellular stresses such as starvation, unnecessary cytosolic components are digested and recycled to promote cell survival.

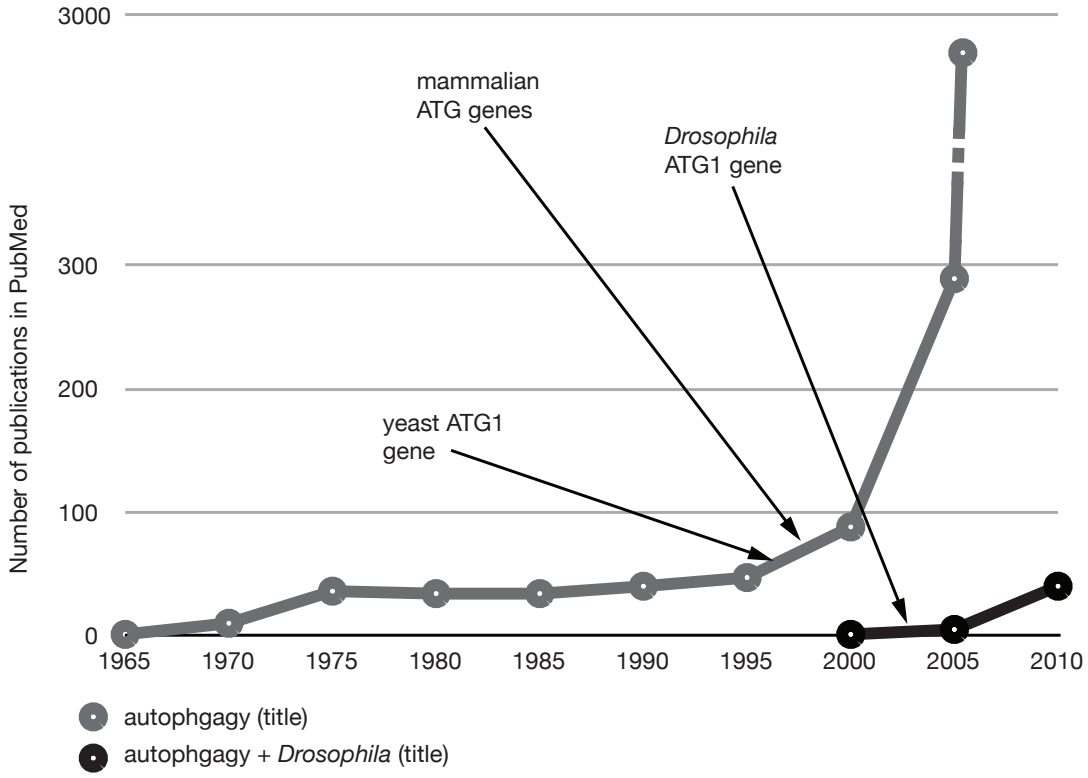

FIGURE 1.1 Increase of autophagy research. The grey graph displays the number of papers retrieved by a Pubmed search for autophagy (title only). A 30 year 'gap' between the discovery and the dramatical increase shows the importance of the discovery of the *ATG* genes. Only since 2001 but with growing interest has autophagy research been applied to *Drosophila*.

However, autophagy can also lead to programmed cell death (PCD, type II). Furthermore, autophagy is also needed throughout normal development, it plays a role in innate and adaptive immunity, lifespan extension and many human pathophysiologies, such as neurodegeneration, metabolic disorders and cancer (Mizushima *et al.*, 2008). Three main autophagy types can be distinguished by the means of cargo delivery: chaperone-mediated autophagy, microautophagy and macroautophagy (Yen and Klionsky, 2008) (FIG. 1.2). Chaperon-mediated autophagy (CMA) is the only form of autophagy that does not involve membrane rearrangements and exists only as a selective way (FIG. 1.2 C). In the current understanding, soluble cytosolic proteins that share the CMA-targeting motif are recognized by the chaperone heat-shock cognate 70 (HSC70). HSC70 binds to the lysosome-associated membrane protein type 2A (LAMP2A) that is associated with a translocation complex at which the proteins are unfolded and directly translocated into the lysosomal lumen (Cuervo, 2011).

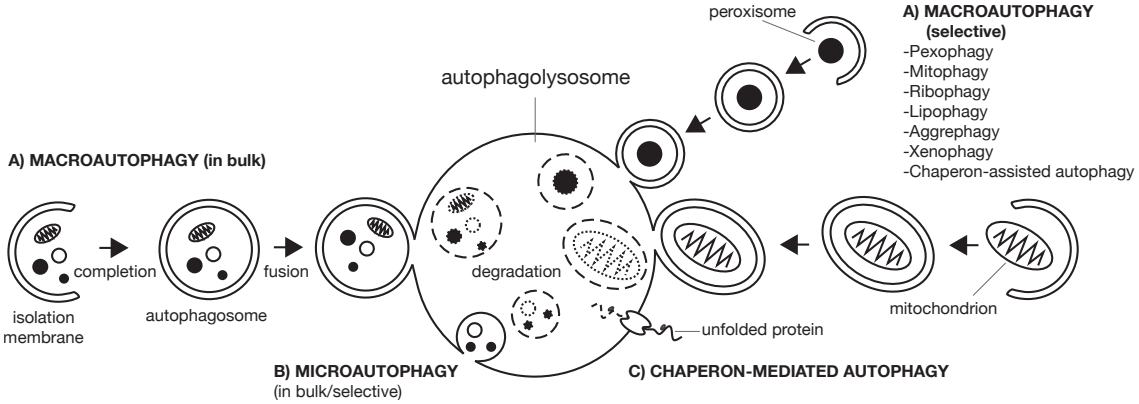

FIGURE 1.2 Types of autophagy. Usually, 'autophagy' designates the most widely studied form of autophagy: Macroautophagy **(A)**. Bulk cytoplasm or damaged organelles are selectively and non-selectively enclosed by a double-membrane organelle (autophagosome) that after completion fuses with the lysosome to degrade the cargo. In a faster form, called Microautophagy **(B)**, cytosolic components are translocated by direct invagination of the cytosolic membrane. Chaperon-mediated autophagy **(C)** uses a translocation complex to transport unfolded proteins to the lysosomal lumen for degradation.

Microautophagy is by far the least understood process. Like CMA, cytosolic components are internalized directly at the lysosomal or late endosomal membrane (FIG. 1.2 B). However, no translocation complex is involved and the non-selective, or chaperon delivered cargo is sequestrated through direct invagination of the lysosomal membrane, which forms vesicles that pinch off into the lysosome (Klionsky *et al.*, 2007; Cuervo, 2011).

During macroautophagy (FIG. 1.2 A), portions of cytoplasm (non-selective) or damaged organelles (selective, *e.g.* mitophagy for mitochondria) are enclosed in characteristic double-membraned vesicles that subsequently fuse with lysosomes to break down the cargo and recycle essential molecules for protein synthesis or energy production (Yang and Klionsky, 2010a). Macroautophagy is the best characterized autophagic pathway and the one this thesis will focus on. For simplicity hereafter I will use 'autophagy' to refer to macroautophagy.

1.2 Regulation of autophagy

With its multiple cellular functions, precise regulation of autophagy is highly important since uncontrolled autophagy - too much or too little - can be deleterious. Several signaling pathways influence autophagy, but most intersect at “the central regulator of autophagy”, the target of rapamycin (TOR) complex 1 (TORC1). Under nutrient rich conditions, active TORC1 phosphorylates autophagy-related (ATG) proteins, which prevents the association with other ATG proteins and leads to inhibition of autophagy (Neufeld, 2009).
In the following chapter, I will shortly summarize the current knowledge about the core machinery of autophagy in yeast, dissect differences between the most widely used model organisms for autophagy (yeast, *Drosophila*, and mammals), and stress pathways regulating autophagy, in particular the insulin/TOR pathway.

1.2.1 The core ATG machinery in yeast

Autophagy was initially detected by electron microscopy in mammals (see 1.1), but the breakthrough was achieved with systematic screens in yeast that provided insights into its molecular regulation (Tsukada and Ohsumi, 1993). This led to the discovery of the most important players in autophagy regulation, the ATG proteins. In the last 15 years since the first ATG protein (ATG1) was cloned (Matsuura *et al.*, 1997), 35 ATG proteins could be described. The “core” machinery includes, among others, only about 15 ATG proteins and is divided into major functional steps that are essential for autophagy in all eukaryotic cells and guided by groups of distinct ATG complexes: autophagosome induction or initiation (ATG1 complex), nucleation and elongation (phosphatidylinositol (PI) 3-kinase (PI3K) group), expansion and elongation (ATG12-ATG5-ATG16 complex and ATG8 conjugation system) and recycling (ATG2-ATG9-ATG18 complex) (Nakatogawa *et al.*, 2009).
The central role of autophagy regulation is undertaken by TOR. By phosphorylation, TORC1 inhibits the interaction between ATG1 and ATG13 that is essential for the onset of autophagy. However, under nutrient-poor conditions, TORC1 is inactive, ATG13 is rapidly dephosphorylated, binds to

ATG1 and both proteins recruit a ternary complex consisting of ATG17, ATG29 and ATG31, which might function as a scaffolding structure to mobilize multiple ATGs to the phagopore-assembly site (PAS) (Kamada *et al.*, 2000; Kawamata *et al.*, 2008). Assembling proteins and lipids at the PAS is the initial step of nucleation. It has been shown that activation of the PI3K complex I consisting of ATG6, ATG14, Vps34 (vacuolar protein sorting 34, the only PI3 kinase in yeast) and Vps15 is essential for nucleation. Some ATG proteins, *e.g.* ATG18, can bind phosphatidylinositol 3-phosphate (PI3P), which is produced by PI3K and enriched at sites where autophagosomes form (Noda *et al.*, 2010; Obara and Ohsumi, 2011). ATG18 in turn can bind ATG2, and both proteins are essential for the shuttling of ATG9, the only membrane spanning protein, between autophagosomal membrane sources and the PAS (Reggiori *et al.*, 2004). Also, the ATG1 complex, particularly ATG17, might be involved in the dynamics and the membrane supply accomplished by ATG9 (Sekito *et al.*, 2009).

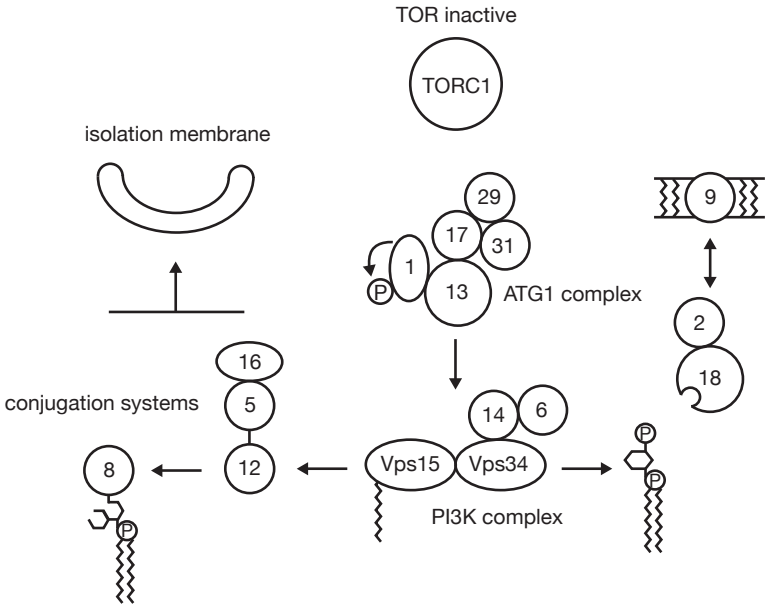

FIGURE 1.3 The core machinery in yeast. Inactivation of TORC1 leads to dephosphorylation of the ATG1 complex to initiate autophagy. For vesicle nucleation (recruitment of lipids and other ATG proteins) the PI3K complex needs to be active, whereas vesicle expansion depends on two ubiquitin-like systems: ATG12-ATG5 and ATG8-PE. Both conjugation complexes are bound to the autophagosomal membrane. ATG9 may act in the retrograde transport of other ATGs *e.g.* ATG18-ATG2.

In order to form the autophagosomal membrane, two ubiquitin-like conjugation systems are required. ATG12, which gets conjugated to ATG5, and ATG8, that is conjugated to phosphatidylethanolamine (PE) (Geng and Klionsky, 2008). Similar to the canonical ubiquitin system, the ATG12 protein is activated by the formation of a thioester bond with the E1-like enzyme, ATG7. Thereafter, it is

transferred to the E2-like enzyme ATG10 and finally conjugated to ATG5. The ATG12-ATG5 complex further interacts with ATG16 to form a tetrameric complex. This supports the lipidation of the second conjugation system, ATG8-PE, as well as its subcellular localization as an E3-like enzyme (Geng and Klionsky, 2008). Under normal conditions, ATG8 exists as a soluble precursor in the cytosol and is transferred to the autophagosomal membrane upon autophagy induction to support membrane expansion. Therefore, the cystein protease ATG4 cleaves ATG8, and two E1- and E2-like proteins (ATG7 and ATG3, respectively) mediate the conjugation of ATG8 to PE. Due to the protease activity of ATG4, ATG8-PE conjugation is reversible and ATG8 can be liberated from the autophagosomal membrane (Geng and Klionsky, 2008). Lipidation and also synthesis of ATG8 are activated under starvation conditions, thus allowing for an elegant way to follow autophagy progression (Klionsky *et al.*, 2007). After completion of the autophagosome, prior to the autophagosome-lysosome fusion, some of the ATG coat proteins are retrieved from the membrane, a process that again involves ATG2, ATG9 and ATG18 (Yorimitsu and Klionsky, 2005). Finally, the autophagosomal cargo gets degraded by the acidic hydrolases of the lysosome and essential molecules such as amino acids are recycled and reused for important cellular processes (Yorimitsu and Klionsky, 2005).

1.2.2 Differences in the yeast, mammalian and *Drosophila* system

After the discovery of several ATG proteins and insights into their function in yeast (see 1.2.1), autophagy-related proteins were also detected in mammals and *Drosophila*. As autophagy is a highly conserved process in all eukaryotes, the majority of ATG proteins are maintained in higher organisms (TABLE 1.1). Nevertheless, important differences in role and regulation exist. As mentioned above, TORC1 negatively regulates autophagy under well-fed conditions by blocking the ATG1 complex. However, the inhibition pathway varies among eukaryotes (Chang and Neufeld, 2010). In contrast to the yeast model, in which hyperphosphorylation of ATG13 by TORC1 inhibits interaction with an active ATG1 (Kamada *et al.*, 2000), the *Drosophila* serine-threonine ATG1 kinase is constitutively bound to ATG13 (Chang and Neufeld, 2009). Equally, the mammalian ATG1 ortholog Unc-51-like kinase (ULK1 and the closely related ULK2) interact with mammalian ATG13 and associated proteins FIP200 (the mammalian homolog of ATG17) and ATG101, regardless of TORC1 activity

(FIG. 1.4 A) (Hosokawa *et al.*, 2009). However, under starvation conditions, yeast ATG13 gets dephosphorylated by one or several phosphatases, leading to association with ATG1, upregulation of the kinase activity of ATG1, and autophagy induction (Nakatogawa *et al.*, 2009). Likewise, the *Drosophila* and mammalian ATG1-ATG13 and ULK1-ATG13 complexes, respectively, are no longer phosphorylated by TORC1 but ATG1 (ULK1) still phosphorylates itself and ATG13, which causes initiation of autophagy (FIG. 1.4 B).

TABLE 1.1 The core proteins for autophagy

Drosophila	CG	chr.	yeast	mammals	function	biochem. function
ATG1	10967	2L	ATG1	ULK1, 2	induction	Ser/Thr kinase
ATG2	1241	3L	ATG2	ATG2A, B	recycling	ATG2-ATG9 complex
Aut1	6877	3L	ATG3	ATG3	expansion	E2-like enzyme, conjugates PE to ATG8
ATG4	4428	2L	ATG4	ATG4A-D	expansion	Cysteine protease cleaves ATG8 C-terminus
ATG5	1643	X	ATG5	ATG5	expansion	ATG5-ATG12-ATG16 complex
ATG6	5429	3R	ATG6	BECN1 (Beclin1)	nucleation	Vps34 complex, Bcl-2 interacting
ATG7	5489	2R	ATG7	ATG7	expansion	E1-like enzyme, activates ATG8 and ATG12
ATG8a	32672	X	ATG8	GABARAP, LC3	expansion	Ubiquitin-like protein, conjungated to PE
ATG9	3615	2R	ATG9	ATG9A, B	recycling	Integral membrane protein, interacts with ATG2
CG12821	12821	2R	ATG10	ATG10	expansion	E2-like enzyme, conjugates ATG5 and ATG12
ATG12	10861	3L	ATG12	ATG12	expansion	Ubiquitin-like protein, conjungated to ATG5
ATG13	7331	3R	ATG13	ATG13	induction	ATG1 complex
CG11877	11877	3R	ATG14	ATG14(L)/Barkor	nucleation	Vps34 complex
CG31033	31033	3R	ATG16	ATG16L1, L2	expansion	ATG5-ATG12-ATG16 complex
CG1347	1347	3R	ATG17	FIP200	induction	ATG1 complex
ATG18	7986	3L	ATG18	WIPI-1, 2, 3, 4	recycling	Peripheral membrane protein, PI3P binding
CG7053	7053	X	-	ATG101	induction	ATG1 complex, ATG13 binding
Vps34	5373	2R	Vps34	Vps34	nucleation	PI3 kinase
Vps15	9746	3R	Vps15	p150	nucleation	Vps34 complex
CG6116	6116	2L	Vps38	UVRAG	nucleation	Vps34 complex

Nucleation of the autophagosomal membrane requires the activation of the PI3K complex. There is only one PI3K in yeast, which is present in two complexes important for either autophagy (complex I) or the vacuolar protein sorting (Vps) pathway (complex II, contains Vps38 instead of ATG14, see FIG. 1.3) (Noda *et al.*, 2010; Obara and Ohsumi, 2011). In contrast, *Drosophila* and mammals possess three classes of PI3K that function in autophagy. The established class I PI3K and its product phosphatidylinositol (3,4,5)-triphosphate (PI(3,4,5)P3) are known to inhibit autophagy though activation of

the TOR pathway (Chang and Neufeld, 2010). However, the *Drosophila* and mammalian class III PI3K complex consisting of Vps34 (PI3K), mammalian p150 or *Drosophila* ird1 (homologues of Vps15) and mammalian Beclin-1 (BECN1, homolog of *Drosophila* and yeast ATG6) corresponds to the yeast PI3K complex I and positively regulates autophagy (Simonsen and Tooze, 2009; Zirin and Perrimon, 2010).

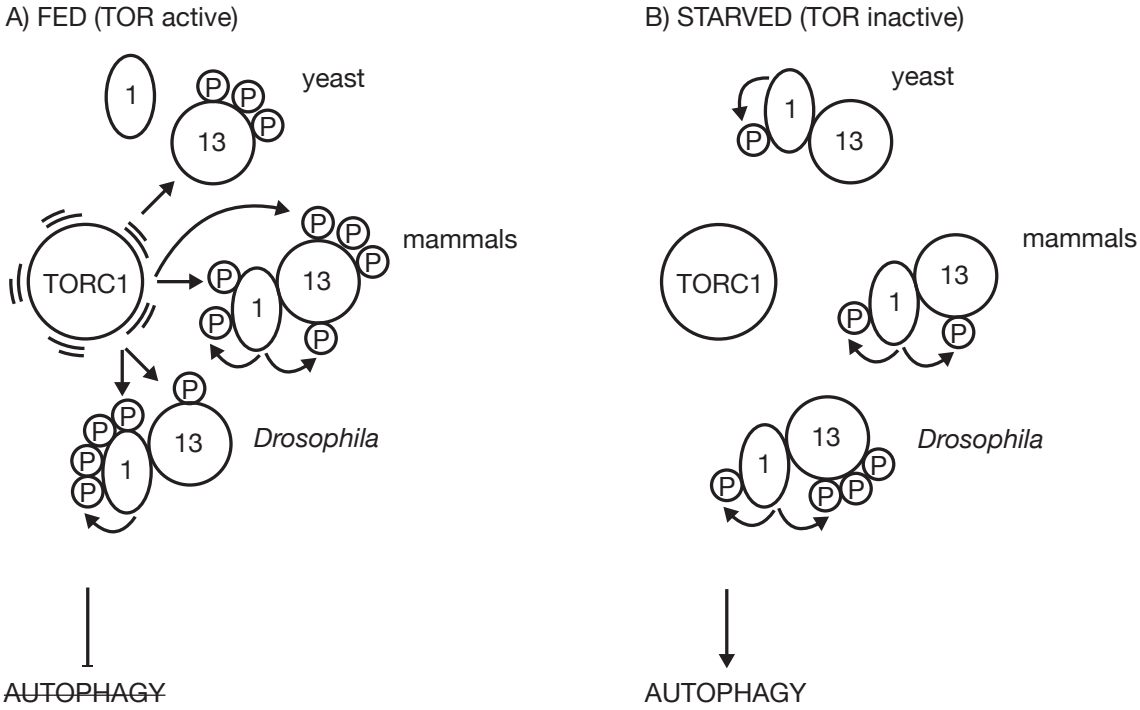

FIGURE 1.4 The ATG1 complex is differently regulated in eukaryotes. (A) Active TORC1 inhibits the induction of autophagy in all models by phosphorylation of the ATG1-ATG13 complex. In mammals and *Drosophila*, this complex is present under fed and starved conditions but highly phosphorylated ATG13 (mammals) and ATG1 (*Drosophila*) proteins inhibit the activity of the complex under fed conditions. **(B)** Starvation leads to rapid dephosphorylation of the proteins and autophagy induction. In yeast, phosphorylation of ATG13 by TORC1 prevents complex formation with ATG1 **(A)**, and only under starvation, when TORC1 is inactive, the proteins bind to each other and initiate autophagy **(B)**. For simplicity, all proteins were named after the corresponding yeast nomenclature and other complex members (ATG17, ATG29, ATG31; see FIG. 1.3) were hidden.

To make things even more complicated, the mammalian Vps34-p150-BECN1 complex can be associated with different combinations of proteins: ATG14L (ATG14-like protein), Ambra1 (activating molecule in Beclin1-regulated autophagy), UVRAG (ultraviolet irradiation resistance-associated gene, the ortholog to Vps38) and Rubicon (RUN domain and cysteine-rich domain containing, Beclin1-interacting protein), depending on the cellular context and

function (Simonsen and Tooze, 2009). Although orthologs of UVRAG, ATG14L and Rubicon also exist in *Drosophila*, it is not yet solved how they function in the PI3K-III complex (Chang and Neufeld, 2010).

As in yeast, vesicle formation and expansion in mammals is mediated by the two ubiquitin-like systems ATG5-ATG12 and ATG8-PE. All the yeast components (see 1.2.1 and TABLE 1.1) have mammalian homologs that function in a similar manner (Yang and Klionsky, 2010b). There are at least eight homologues of ATG8 in mammals that are all essential for autophagy but act differently at early or late stages of autophagosome maturation (Weidberg *et al.*, 2010). Likewise, *Drosophila* homologues exist for all the proteins required in the yeast conjugation systems (TABLE 1.1) although there are only two ATG8 proteins (ATG8a, ATG8b), which both localize at autophagosomes and might act redundantly in autophagy (Scott *et al.*, 2004). Mammalian ATG9 (mATG9) is not as extensively studied as in yeast, but it also travels from the Golgi network and late endosomes to peripheral sites overlapping with autophagosomes upon autophagy induction. Recent studies showed that p38 mitogen-activated protein kinase (MAPK) acts as a negative regulator of mATG9 in autophagy, suggesting a mechanism by which nutrients could control autophagy (Webber and Tooze, 2010). The only indication for a role of ATG9 in *Drosophila* autophagy arises from a study about immunity against vesicular stomatitis virus in *Drosophila* Schneider-2 (S2) cells where interfering ribonucleic acid (RNAi) mediated knock-down of ATG9 led to an increase of the infection due to the resulting defect in autophagy (Shelly *et al.*, 2009).

In summary, most of the ATG proteins and autophagic core processes are highly conserved among eukaryotes, with *Drosophila* serving as an intermediate between yeast and mammals. Studies in all of the three species contribute equally to a better understanding of autophagy dynamics.

1.2.3 The central role of the TOR complex in autophagy regulation

Induction of autophagy and suppression of growth in response to stresses, such as starvation is essential for survival in all eukaryotic cells. The target of rapamycin (TOR), a serine/threonine protein kinase downstream of the insulin signaling pathway, is the central regulator of multiple cellular responses to nutrient and growth factor signals. Insulin, or *Drosophila* insulin-like peptides (Dilps) are secreted in response to food uptake and trigger an intracellular

signaling cascade that leads to the metabolization of nutrients, which in turn results in growth in cell size and number and inhibition of autophagy (Jung *et al.*, 2010). In the *Drosophila* canonical insulin signaling pathway (FIG. 1.5), activation of the insulin receptor causes phosphorylation of chico, the homolog of mammalian insulin receptor substrates (IRS), providing a binding site for PI3K. Active PI3K produces PIP3 at the plasma membrane, which recruits protein kinase B (PKB, or Akt) that inhibits the GTPase-activating tuberous sclerosis protein complex (TSC1/TSC2), leading to a stabilization of the Ras homologue enriched in brain (Rheb) GTPase, which finally activates TORC1. The tumor suppressor phosphatase and tensin homolog (PTEN) catalyzes the reverse reaction from PIP3 to PIP2. Active TORC1 stimulates growth by turning on protein translation via p70 ribosomal protein S6 kinase (S6K) and inhibiting autophagy by regulating ATG1 and PI3K complexes (Oldham and Hafen, 2003; Chang *et al.*, 2009).

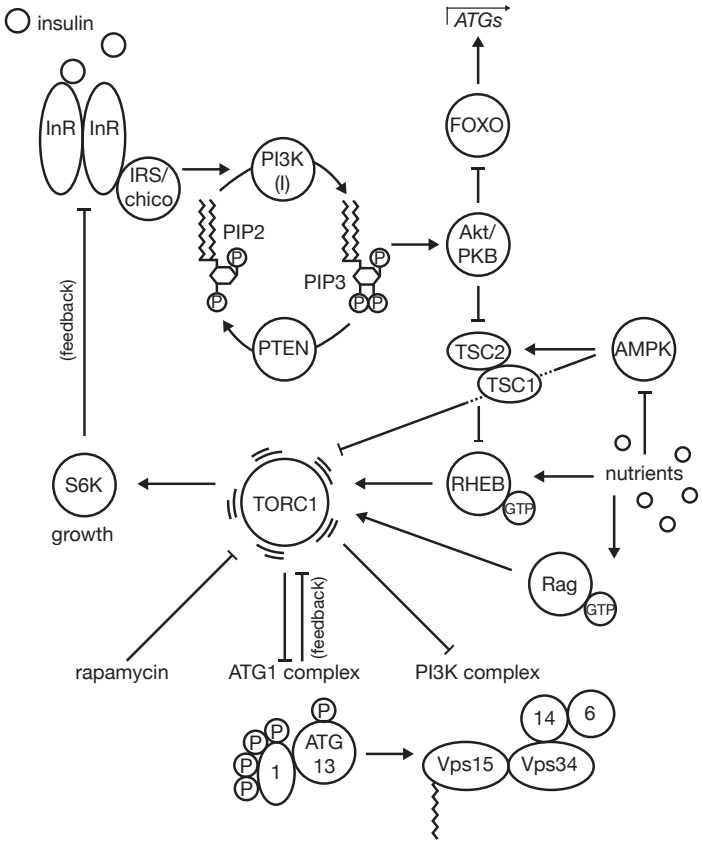

FIGURE 1.5 Canonical insulin signaling and the central role of TORC1. Binding of insulin to its receptor or direct sensing of nutrients triggers a signaling cascade that activates TORC1. Active TORC1 inhibits autophagy by blocking the ATG1 and PI3K (III) complexes and supports growth by stimulating ribosomal S6 kinase (S6K) to upregulate translation. Sensing of nutrients and energy also directly regulates the GTPases Rheb and Rag and the AMP activated kinase (AMPK), which in turn activate TORC1 directly or indirectly. Active protein kinase B (PKB/Akt) inhibits the transcription factor FOXO, which otherwise can upregulate the transcription of *ATG* genes. Negative feedback loops ensure basal levels of autophagy and protect from excessive growth under nutrient rich conditions. The drug rapamycin is able to block TORC1, leading to growth inhibition and initiation of autophagy.

Abbreviations: InR, insulin receptor; IRS, insulin receptor substrate; PI3K, phosphatidylinositol (PI) 3-kinase; PIP2, PI 2-phosphate; PIP3, PI 3-phosphate; PTEN, phosphatase and tensin homolog; TSC, tuberous sclerosis protein complex, FOXO, Forkhead box subgroup O; GTP, Guanosine-5'-triphosphate; Vps, vacuolar protein sorting

Originally, TOR was found in a screen for yeast colonies that could grow on rapamycin, a secondary metabolite produced by bacteria found on the Easter Island (Rapa Nui) with cytostatic activity. TOR is active in two distinct complexes (TORC1 and TORC2) that are both conserved in higher eukaryotes, but only TOR in TORC1 can be targeted by rapamycin and thus is responsible for the control of growth and autophagy (Loewith, 2011). Although TOR is regulated by the nutrient sensitive insulin signaling, there is also a faster, direct way for TOR to recognize a surplus or lack of energy, upon which it switches between promoting and suppressing growth or autophagy. Two independent screens, a RNAi screen in *Drosophila* S2 cells and a proteomic approach in mammalian embryonic kidney cells found Rag GTPase proteins as mediators for the amino-acid signal to TORC1 (Kim *et al.*, 2008; Sancak *et al.*, 2008). Before, Rheb GTPase was thought to respond to amino acids but this is now under debate (Dennis *et al.*, 2011; Kim and Guan, 2011). Another branch of the insulin/TOR pathway regulates autophagy via *Drosophila* FOXO, a member of the Forkhead box subgroup O (FOXO) transcription factors, which is also needed for stress resistance (Chang and Neufeld, 2010). It has been shown that in aging muscle cells, the basal level of autophagy can be sustained by FOXO overexpression, which is due to an increased expression of *ATG* genes in these cells (Demontis and Perrimon, 2010). In addition, adenosine monophosphate (AMP) activated protein kinase (AMPK) signaling feeds into TORC1 to control autophagy. AMPK is activated under starvation or hypoxia, when energy levels decrease and AMP levels increase and cells suffer from low energy stress. In order to suppress superfluous energy consumption for growth, AMPK directly phosphorylates TSC2 and the TORC1 subunit raptor, thus inhibiting TORC1 activity (Shaw, 2009). In order to obtain a full circle, negative feedback loops from the ATG1 complex to TORC1 and from S6K to IRS/chico ensure basal levels of autophagy for the removal of aggregated or damaged organelles, and protect from accelerated growth under nutrient rich conditions (Chang *et al.*, 2009; Chang and Neufeld, 2010).

1.2.4 Other pathways that regulate autophagy

With its multiple inputs, the most prominent regulator of autophagy clearly is TORC1. But there are also other pathways that have been shown to influence autophagic activity, although the complete regulation of autophagy is complex

and far from being understood. One of these other regulators is the Jun-N-terminal kinase (JNK) signaling pathway, which is a ubiquitous and versatile stress sensor in metazoans, stimulated by various factors (*e.g.* heat, UV irradiation, starvation, inflammation) (FIG. 1.6). These stimuli trigger a kinase cascade, involving members of the JNK kinase kinase family (JNKK), which then phosphorylate and activate a member of the mitogen-activated protein kinase kinase (MKK) family that finally activates JNK. Multiple nuclear and cytoplasmic targets, mostly transcription factors, can be regulated by JNK, including AP-1 (Jun/Fos heterodimers) and FOXO (Weston and Davis, 2007). Thus, FOXO is regulated by both, the insulin pathway, in particular PKB/Akt (see 1.2.3), and the JNK pathway, and is modulating *ATG* gene expression (Chang and Neufeld, 2010; Demontis and Perrimon, 2010). In particular, expression of *Beclin-1* (*ATG6* in *Drosophila*, see TABLE 1, FIG 1.5) has shown to be upregulated by JNK in human cell lines and in the *Drosophila* gut (Li *et al.*, 2009; Wu *et al.*, 2009).

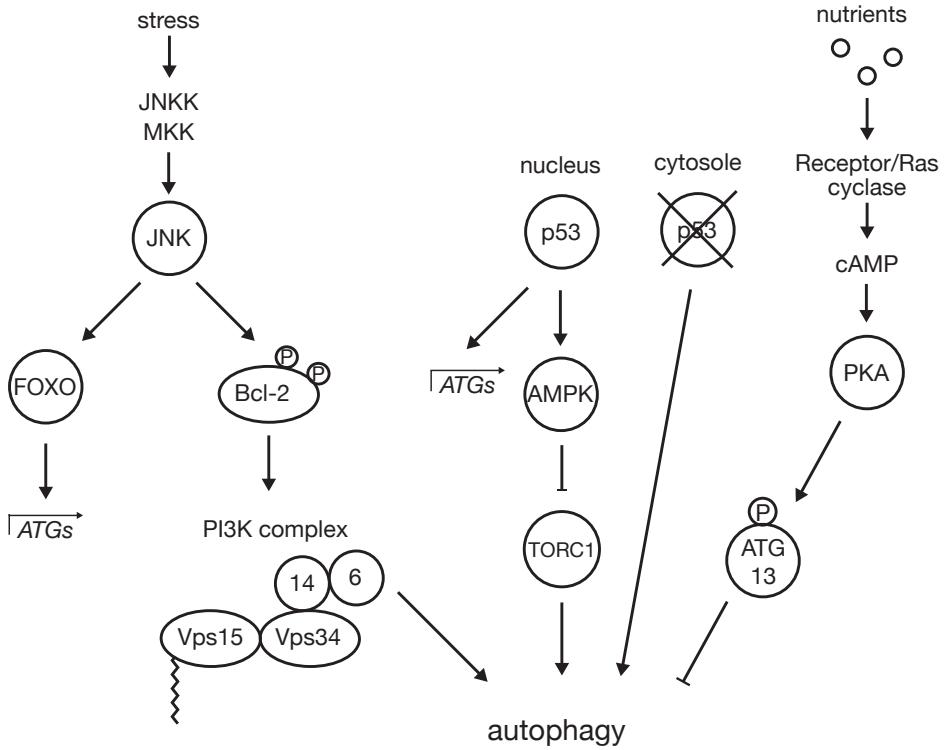

FIGURE 1.6 Other pathways regulating autophagy. Besides TOR, also other pathways have been shown to influence autophagic activity. The JNK pathway regulates FOXO activated transcription of *ATG* genes and association of Bcl-2 with Beclin-1/ATG6. The tumor suppressor p53 controles autophagy via transcription of DRAM, activation of AMPK and cytosolic loss of p53. In yeast, cAMP activated PKA inhibits accumulation of ATG13 at the PAS. Abbreviations: AMPK, AMP activated kinase; Bcl-2, B-cell lymphoma 2; cAMP, cyclic adenosine monophosphate; FOXO, Forkhead box subgroup O; JNK, Jun-N-terminal kinase; JNKK, JNK kinase; MKK, mitogen-activated protein kinase kinase; PKA, cAMP-dependent protein kinase; PI3K, phosphatidylinositol 3-kinase; Vps, vacuolar protein sorting.

In addition to transcriptional regulation of *ATG* genes, JNK is also responsible for the post-translational regulation of the B-cell lymphoma 2 (Bcl-2) protein, which was originally identified as an oncogene. JNK mediated phosphorylation of Bcl-2 disrupts its binding to pro-apoptotic BH3 domain containing proteins such as Bax, but also binding to the PH3 domain of Beclin-1/ATG6 in a stress-time dependent manner. A short period of stress and low Bcl-2 phosphorylation levels allow the dissociation from Beclin-1/ATG6 to promote autophagy and survival, whereas prolonged stress leads to maximum phosphorylation levels and disruption of the Bcl-2-Bax complex, inducing caspases and apoptosis (Levine *et al.*, 2008; Wei *et al.*, 2008). Another signaling pathway well known to be implicated in apoptosis and tumor suppression involves p53. On one hand, p53 can activate AMPK (see 1.2.3), which triggers autophagy via inhibition of TORC1 and transcriptional activation of the damage-regulated modulator of autophagy (DRAM). On the other hand, the sole loss of cytoplasmic p53 itself also induces autophagy, possibly also through a TOR-dependent mechanism (Eisenberg-Lerner *et al.*, 2009). Another sensor for environmental changes, which is considered to act in parallel to TOR for the regulation of nutrient dependent changes and autophagy, is the cAMP (cyclic adenosine monophosphate) dependent protein kinase (PKA) pathway. It seems that the influence of PKA on autophagy is strongly species dependent (Chen and Klionsky, 2011), however, studies on yeast clearly show that PKA directly phosphorylates ATG13 to inhibit the association with the PAS. Since this does not effect the ATG13-ATG1 interaction, and because the inhibition of PKA and TOR leads to an even faster autophagic response, the authors conclude that phosphorylation at different sites of ATG13 inhibits autophagy cooperatively, namely the association of ATG1-ATG13 by TOR and the localization of ATG13 to the PAS by PKA (Stephan *et al.*, 2009).

In summary, multiple screens and epistasis experiments have led to a wide coverage of known proteins functioning in and interacting with autophagy. The high conservation of all ATG proteins and their regulators implies an evolutionary very old and important role of autophagy for cellular growth and survival.

1.3 Autophagy in health and disease

Autophagy is a commonly used, multifunctional process in all organisms. It provides an internal source of nutrients for energy generation under low food conditions, prevents cellular damage by replacing outdated organelles, digests toxic aggregated proteins and pathogens and along with apoptosis, it degenerates unneeded structures during cellular remodeling and programmed cell death. In this chapter, I will illustrate the roles of developmental and starvation induced autophagy on examples in different model systems, shortly summarize the interplay of autophagy and cell death, and explain the role of autophagy in several human diseases.

1.3.1 Autophagy during animal development

It is clear from chapter 1.2 that autophagy is mainly regulated via nutrient sensing pathways in order to protect the cell from low energy stress. However, autophagy is also needed during normal development, *e.g.* for the turnover of damaged organelles or in cellular remodeling, and in many cases autophagy is indispensable for survival. In *Drosophila*, *ATG2* and *ATG18* homozygous mutations are larval lethal, but *ATG1* and *ATG13* homozygous mutants survive until late pupal stages although all mutants show a strong inhibition of autophagy. In contrast, *ATG7* homozygous mutant flies and *ATG8* hypomorphic mutants are viable, even though having defects in autophagy. These mutants are also more sensitive to starvation, accumulate neuronal aggregates and are short-lived (Scott *et al.*, 2004; Juhasz *et al.*, 2007a; Simonsen *et al.*, 2008; Chang and Neufeld, 2009). The first evidence that autophagy is needed during normal *Drosophila* development came from a study about the steroid hormone 20-hydroxyecdysone (ecdysone) which is upregulated at late larval stages to trigger metamorphosis (Riddiford, 1993). During the massive cellular reorganization from larval to adult tissues, autophagy is used in midgut structures, salivary glands and the fat body in order to get rid of or replace these tissues with the future adult cells (Lee and Baehrecke, 2001; Lee *et al.*, 2002; Rusten *et al.*, 2004; Berry and Baehrecke, 2007). Most likely, autophagy is indispensable in metamorphosis and that is why most *ATG* mutants die before or during pupal stages. However, the survival of *ATG7* and *ATG8*

mutants suggests that either some of the *ATG* genes are redundant or undertake different or specialized roles during development.

In adult flies, developmental autophagy has only been reported during oogenesis. Transmission electron microscope studies revealed autophagosomes during late oogenesis, when dying nurse cells pass on their nutrients to the growing oocyte (Velentzas *et al.*, 2007). Similar studies have also shown that developmental cell death in the germarium and during mid-oogenesis depends on autophagy (Nezis *et al.*, 2009). However, Nezis *et al.* suggest that mutations in *ATG* genes lead to the persistence of the inhibitor of apoptosis protein (IAP) Bruce, reduced deoxyribonucleic acid (DNA) fragmentation and caspase activation in late oogenesis, implying that autophagy controls the activation of programmed apoptotic cell death in these cells (Nezis *et al.*, 2010). This is contrary to studies on developmental salivary gland cell death where it has been shown that caspases are active in *ATG* mutant salivary gland cells, and a combined autophagy-apoptosis suppression decreases salivary gland degradation (Berry and Baehrecke, 2007). This indicates that regulation of developmental autophagy might be context or organ specific (see also 1.3.2 below).

Conversely, nutrient recycling under starvation by autophagy is a response conserved in all organisms and organs and is essential to survive serious nutrient stress in wildlife. The group of Y. Ohsumi first described the massive accumulation of autophagosomes in yeast after nitrogen starvation. Under well fed conditions, autophagy is almost undetectable in yeast, but nitrogen depletion rapidly triggers a cell differentiation process called sporulation where autophagy is involved in bulk protein degradation for cellular remodeling (Takeshige *et al.*, 1992). Similarly, autophagy is inducible by starvation in the larval *Drosophila* fat body, an organ analogous to the mammalian liver, and during oogenesis at two nutrient status checkpoints, the germarium and mid-oogenesis (Scott *et al.*, 2004; Hou *et al.*, 2008).

In mice, most organs and muscles and in particular the liver also strongly activate autophagy in response to starvation (Mizushima *et al.*, 2004). However, even under nutrient rich conditions, autophagy is detectable in those tissues, indicating important roles in basal autophagy or during development (Mizushima *et al.*, 2004). Indeed, the earliest autophagic event detectable in mammals happens already shortly after fertilization of the oocyte and might be important for amino acid supply or the degradation of unnecessary proteins in order to facilitate remodeling. Oocyte-specific *ATG5*$^{-/-}$ knock out mice that lack the maternally inherited ATG5 protein die at the four- to eight-cell stages

(Tsukamoto *et al.*, 2008). Conventional *ATG5*$^{-/-}$ knock out mice survive early embryogenesis due to maternal ATG5 protein, but die neonatal within one day (Kuma *et al.*, 2004). Directly after birth, autophagy is actively induced in all tissues except the brain in order to survive the starvation period between the end of placenta feeding and the first own meal. In *ATG* knock out neonates, amino-acid levels in plasma and tissue are decreased, implying that autophagy is important to control the availability of nutrients in this early neonatal starvation period (Kuma *et al.*, 2004).

Autophagy is also involved later in mammalian development in the differentiation of erythrocytes (erythropoiesis) and lymphocytes via selective removal of mitochondria, the so-called mitophagy (see 1.1, FIG. 1.2). During erythropoiesis, a series of differentiation steps from the release of the bone marrow to the mature red blood cell, the later erythrocyte will lose its nucleus and all its organelles in order to replace it with haemoglobin molecules. Recent studies suggest that mitophagy is an important mechanism during this erythrocyte maturation. Mice that lack *ATG7* specifically in haematopoetic cells selectively accumulate damaged mitochondria, which leads to premature cell death, severe anemia, and finally organismal death after 8-14 weeks of age. These mice also have a significant decrease in T- and B-lymphocytes with accumulating mitochondria in T-cells (Mortensen *et al.*, 2010). In addition, autophagy also plays a role in the remodeling of differentiating adipocytes. During *in vitro* differentiation of mouse embryonic fibroblasts (MEFs) into adipocytes, massive autophagy could be detected. In contrast, primary *ATG5*$^{-/-}$ MEFs that lack autophagy were significantly delayed in this differentiation process, and neonatal *ATG5*$^{-/-}$ and *ATG7*$^{-/-}$ mice possess fewer adipocytes and are leaner than wild type newborns (Baerga *et al.*, 2009; Zhang *et al.*, 2009). Finally, autophagy was also reported in the mammalian ovary. During folliculogenesis, a fraction of follicles mature and are ovulated, but most undergo atresia, a hormonally controlled cell death process (Kaipia and Hsueh, 1997). This has been associated with the induction of apoptosis, but the involvement of autophagy was also speculated (Duerrschmidt *et al.*, 2006). In a recent publication, immunostaining for LC3 (yeast ATG8) showed that the autophagic machinery is present in rat follicular cells during all developmental stages, whereas no LC3 expression was detected in the oocytes. In addition, the onset of apoptosis and autophagy showed the same pattern in a rat model for follicular development, which suggests that autophagy is induced in follicle cells during atresia (Choi *et al.*, 2010).

Following the enthusiasm about apoptosis research and the high interest in autophagy, an increasing number of studies focused on the interplay of both pathways. Although to date, energy supply, organelle turnover, and remodeling are considered the most important roles of autophagy, the function of autophagy in programmed cell death and the interplay with apoptosis is attracting more and more attention. In contrast to necrosis, which is the third and most unorganized way to die for a cell, autophagy and apoptosis are both well controlled. During apoptotic cell death, the nucleus is condensing, DNA is cleaved, cells are fragmented by caspases and wrapped into apoptotic bodies that get eaten up by specialized phagocytic cells without an inflammatory response (Edinger and Thompson, 2004). Many of the apoptotic regulators are also shared by autophagy (see 1.2.4), and context specific regulation has been observed (Eisenberg-Lerner *et al.*, 2009). Due to these findings, three interaction models for both pathways have been described (FIG. 1.7). First, both pathways lead independently, although coordinately, to cell death. As a consequence, one program can take over if the other is defective or inhibited. Second, apoptosis leads to death, whereas autophagy favors survival and thus acts as an antagonist to block cell death. And third, autophagy supports apoptosis, for example by providing energy, or is participating in the regulation of apoptosis (*e.g.* IAP Bruce) (Eisenberg-Lerner *et al.*, 2009).

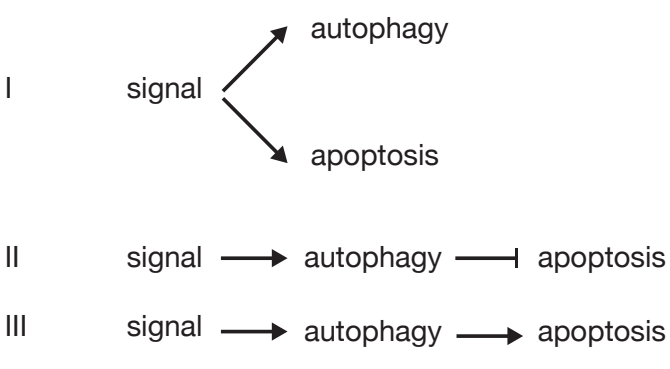

FIGURE 1.7 Interplay of autophagy and apoptosis. Depending on the trigger, organs or model systems, the interaction of autophagy and apoptosis to induce cell death varies. Apoptosis and autophagy can act independently to induce cell death (I), or autophagy supports survival and inhibits apoptosis-induced cell death (II). Alternatively, autophagy facilitates apoptosis in order to kill the cell (III).

The initial observation that linked both pathways was that Bcl-2, well known as an antiapoptotic protein, could also inhibit autophagy by binding Beclin-1/ATG6 (see also 1.2.4) (Pattingre *et al.*, 2005; Levine *et al.*, 2008). Since then,

many other examples for a cross-talk between both pathways have been described. For example, the core autophagic protein ATG5 (see 1.2.1) can be cleaved by calpains upon apoptosis stimulation, thus forming a truncated product that induces apoptosis rather than autophagy (Yousefi *et al.*, 2006). This is also true for ATG4 whose caspase-3 dependent cleaved version stimulates autophagy-independent apoptosis (Betin and Lane, 2009). However, autophagy can also inhibit apoptosis, which has been shown in mammalian cells where autophagy is able to degrade active caspase-8 (Hou *et al.*, 2010). In *Drosophila*, cooperation of autophagy and apoptosis has been described during metamorphosis in ecdyson induced cellular remodeling of midgut and salivary gland cells (Lee *et al.*, 2002; Gorski *et al.*, 2003; Martin and Baehrecke, 2004). In salivary glands, combined inhibition of both pathways decreased degradation, whereas in midgut cells, the collective inhibition did not result in a further delay of midgut removal, indicating that here, different regulatory mechanism control cell death (Berry and Baehrecke, 2007; Denton *et al.*, 2009). Similar to salivary glands, developmental elimination of *Drosophila* neuronal stem cells also depends on both apoptosis and autophagy, and only simultaneous inhibition promotes long-term neural stem cell survival (Siegrist *et al.*, 2010). In addition, mid- and late-oogenesis cell death in *Drosophila* ovaries shows apoptotic as well as autophagic characteristics (Velentzas *et al.*, 2007). An RNAi based screen on the ability of apoptosis-related genes to regulate starvation-induced autophagy detected six candidates, among others IAP Bruce and the effector caspase Dcp-1. During oogenesis, inhibition of autophagy in dying egg chambers led to a decrease in apoptotic cell death, and loss of Bruce resulted in an increase of both, apoptosis and autophagy (Hou *et al.*, 2008). Later, it was demonstrated that degradation of Bruce via autophagy triggers apoptotic cell death, presenting the third possible interacting mechanisms of autophagy and apoptosis (Nezis *et al.*, 2010). In addition, it has recently been shown that mitochondrial dynamics, Bcl-2 family members and autophagic proteins regulate nurse cell death in *Drosophila*, and that follicle cells are important for the uptake of nurse cell material for degradation (Tanner *et al.*, 2011).

From the previous chapters it is becoming clear that autophagy is a pivotal process, and it is perhaps not surprising that defects in autophagy can evoke fatal diseases. Certainly, the recent increase of autophagy research is partially due to the role of autophagy in several human diseases, including cancer, neurodegeneration, infections, muscle disorders and heart or liver diseases (Mizushima *et al.*, 2008). Precise knowledge about the regulation and function of autophagy will open novel opportunities to develop therapies against these disorders. Once more, the tumor suppressor gene *Beclin-1/ATG6* played a pioneering role, in this case to understand the mechanism between autophagy and cancer. The monoallelic deletion of *Beclin-1/ATG6* is responsible for tumorigenesis in numerous human breast, ovarian, and prostate cancers, which could be linked to the loss of autophagy in these cells (Liang *et al.*, 1999). Heterozygous mutant *Beclin-1/ATG6* mice show decreased autophagy and an accelerated rate of spontaneous tumor development, which could also be observed in *ATG4c* deficient mice. Furthermore, other components of the Beclin-1/ATG6- complex like UVRAG or Ambra1 (see 1.2.2) also have tumor suppressive characteristics (Mizushima *et al.*, 2008). In contrast to this tumor suppressor function, autophagy can also support tumorigenesis by providing energy for survival and growth of solid tumors, making it a challenging drug target that has to be treated differently at varying stages of cancer development (Mizushima *et al.*, 2008).

Autophagy has a consistently positive role in bestowing an organisms longevity via removal of damaged cellular components and organelles, thereby limiting the production of oxygen species and stress. In adult *Drosophila*, *ATG* gene expression levels decrease as flies age, leading to the accumulation of insoluble ubiquitinated protein aggregates in muscular and neuronal tissue (Demontis and Perrimon, 2010). Also, *ATG7* or *ATG8* mutant flies as well as *ATG7* mutant mice have a reduced lifespan and increased aggregation of ubiquitinated proteins, indicating a role for autophagy in anti-aging (Komatsu *et al.*, 2006; Juhasz *et al.*, 2007a; Simonsen *et al.*, 2008; Demontis and Perrimon, 2010). Given that starvation, or rather caloric restriction, which is reduced food uptake without malnutrition, is the key behavior in order to extend lifespan, it is not surprising that autophagy has anti-aging effects.

Another age-, but also disease-dependent consequence of accumulated protein aggregates is neurodegeneration. The severity of diseases such as Alzheimer's, Huntington's or Parkinson’s disease usually correlates with the

accumulation of misfolded proteins and, as in cancer, autophagy primarily seems to play a protective role by degrading these aggregates. For example, expression of ATG8 in fly brains resulted in reduced age-dependent protein aggregation (Simonsen *et al.*, 2008). Furthermore, induction of autophagy via TOR inhibition attenuates huntingtin accumulation and neurodegeneration in *Drosophila* and mice models of Huntington disease (Ravikumar *et al.*, 2004). However, inefficient lysosomal clearance resulting through the block of autophagy can also lead to the production and accumulation of toxic aggregated peptides in immature autophagosomes, thus accelerating progression of Alzheimer's disease (Yu *et al.*, 2005).

In addition, autophagy does not only degrade damaged organelles but also unwanted intracellular organisms like bacteria and viruses, a process called xenophagy (see 1.1) (Deretic and Levine, 2009). A *Drosophila* study revealed for the first time that an intracellular pattern recognition receptor of the innate immune system can recognize and deliver *Listeria* bacteria to autophagosomes, which prevented bacterial growth and promoted survival after infection (Yano *et al.*, 2008). A similar process was also found in humans where the protein NDP52 functions as a receptor that detects and binds *Salmonella* and LC3 at the same time in order to deliver it to autophagosomes (Thurston *et al.*, 2009). However, since insects lack the adaptive immune response, the involvement of autophagy in major histocompatibility complex (MHC) class I and II antigen presentation could so far only be shown in mammalian cell lines (Paludan *et al.*, 2005; English *et al.*, 2009).

Finally, autophagy also seems to contribute to follicular atresia in mammals (see 1.3.1), where excessive cell death causes sterility, and autophagy is also associated with chemotherapy-induced premature menopause and other fertility disorders (Krysko *et al.*, 2008).

Taken together, misregulation of autophagy goes hand in hand with serious diseases and therefore drug-induced therapies that direct autophagy would be beneficial. However, a better understanding of the regulation of the autophagic machinery is needed in order to find specific ways to manipulate autophagy.

1.4 *Drosophila melanogaster* as a model organism to study autophagy

As mentioned before, the fruit fly *Drosophila melanogaster* provides an intermediate between yeast and mammals concerning the conservation of the autophagic machinery. Various advantages make *Drosophila* a particularly good candidate to study autophagic processes. *Drosophila* has a very short life cycle (FIG. 1.8), is small and easy to maintain but features a physiology comparable to mammals. For example, the ovaries of *Drosophila* are true homologous to the human reproductive system, and both are highly sensitive to starvation (Thomson *et al.*, 2010). As in yeast, powerful genetic tools are available for *Drosophila* and in many cases, single orthologs of *ATG* genes allow for non-redundant research. A huge database collects any genetic data (www.flybase.org), mutant flies for almost all genes are available (www.flystocks.bio.indiana.edu) and numerous RNAi lines have been generated (www.stockcenter.vdrc.at, www.shigen.nig.ac.jp/fly/nigfly) that allow for downregulation of genes in a tissue-specific manner. Moreover, autophagy in *Drosophila* is not only stress inducible, but also occurs during distinct developmental steps, as for instance during tissue remodeling in pupal stages and in late stages of oogenesis (Yin and Thummel, 2005; Bass *et al.*, 2009). Thus, *Drosophila* serves as an outstanding model to investigate the physiological role of developmental and stress induced autophagy *in vivo*.
In the following section, I will shortly describe the life cycle of a fly, explain different organs used to study autophagy, and in more detail illustrate the role of autophagy during *Drosophila* oogenesis.

1.4.1 Life cycle of *Drosophila*

The complex life cycle of holometabolous insects like *Drosophila* takes about 9 days, starts with a fertilized egg, which is approximately 0.5 mm long, and ends with the adult fly that is about 2.5 mm long. After the egg is fertilized and laid, rapid nuclear divisions without cell division form a syncytium. After nine divisions, the nuclei move to the periphery to form a blastoderm, membranes

grow in, and some specialized cells, the pole cells, move to the posterior end. These will later give rise to the germline and are easily distinguishable, making them suitable for germline manipulations. Also, the borderless syncytium is an advantage for genetic manipulations, since all molecules can diffuse it is easy to accomplish transgenesis. The worm-like larva hatches one day after fertilization and within five days and two molts it has reached the critical weight for pupariation. During metamorphosis, most larval tissues are degraded and replaced by adult structures including wings and legs that derive from imaginal discs already present in the larva. Finally, the adult fly hatches from its pupal case and will be fertile within 12 hours (Wolpert, 2002).

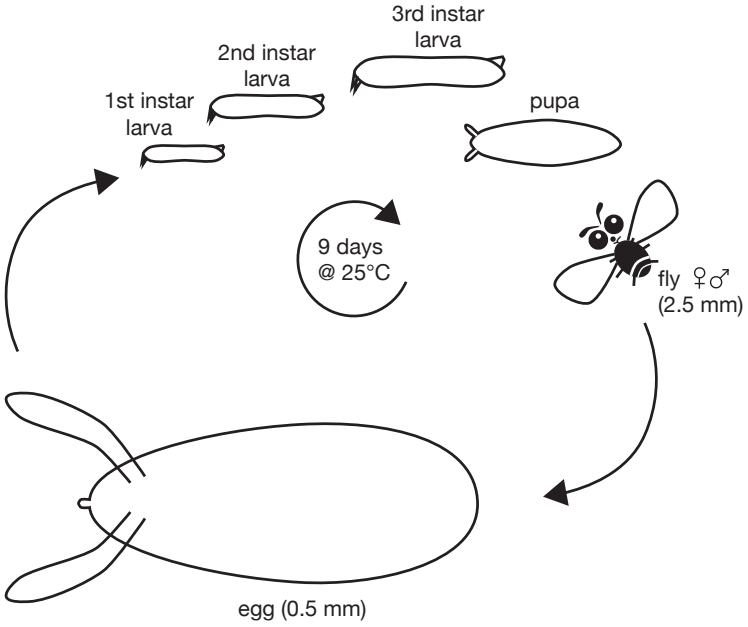

FIGURE 1.8 Life cycle of *Drosophila melanogaster*. It takes *Drosophila* 9 days from one generation to the next. Within one day, the fertilized oocyte develops into a multicellular, segmented and proper defined larva that has only two things in mind: feeding and growing. After two molts, the final sized third instar larva pupariates and completely remodels from the larval worm-like structure to an adult fly. The young adult hatches as a virgin but within 12 hours, oogenesis is switched on and eggs can be fertilized.

1.4.2 Autophagy research in *Drosophila*

Autophagy research in *Drosophila* was initiated by studies about developmental and starvation induced autophagy in the salivary gland and the larval fat body and since then, outstanding research in autophagy has mainly been achieved in these two organs (Lee and Baehrecke, 2001; Juhasz *et al.*, 2003; Rusten *et al.*, 2004; Scott *et al.*, 2004). The larval life is characterized by eating and enormous mass accumulation, and the majority of nutrients are stored in a single-cell thick tissue, named fat body, which is analogous to the

mammalian liver. Prior to metamorphosis, the larva stops feeding and begins searching for a pupation site. During this developmental starvation period, required nutrients are made accessible by autophagy in fat body cells. Interestingly, autophagy can also be triggered artificially by larval starvation at any time point during development, making it a useful tool to study the regulation of autophagy (Scott *et al.*, 2004). During metamorphosis, a peak of the hormone ecdyson induces autophagy-dependent remodeling and degradation of larval tissues including the fat body and salivary glands (see 1.3.1) (Lee and Baehrecke, 2001; Juhasz *et al.*, 2003; Rusten *et al.*, 2004). Salivary glands, also well known for their huge polytene chromosomes, served in a number of transcriptomic and proteomic studies as a model to identify novel regulators of metamorphosis. By comparing younger larval salivary glands with those of wandering or pupariating larva that already activated the cell death program, many new factors involved in the degradation of salivary glands could be revealed. Among others, several autophagy genes including *ATG5* and *Beclin-1/ATG6*, apoptotic genes such as the effector caspase *Dcp-1*, the DIAP1 inhibitor *reaper,* and *Bcl-2* family members, and proteins involved in growth control like the hippo-pathway kinase Warts were found to be upregulated (Gorski *et al.*, 2003; Lee *et al.*, 2003; Martin *et al.*, 2007). Subsequent work showed that Warts regulates autophagy in a class I PI3K dependent manner (Dutta and Baehrecke, 2008). A similar screen on degenerating larval fat body tissue further identified up-regulation of ATG and lysosomal genes, but also down-regulation of mitochondrial and chaperon encoding genes, including the protein FKBP39 that later was shown to inhibit autophagy *in vivo* by regulating the transcription factor FOXO (Juhasz *et al.*, 2007b). Furthermore, in a proteomics approach comparing proteins from normal versus starved fat bodies, the lipid desaturase Desat1 was identified to be required for starvation-induced autophagy (Köhler *et al.*, 2009). As studies in these *Drosophila* tissues continued, more insights could be gained regarding the role and regulation of autophagy. This is exemplified by the remarkable findings that TOR is necessary and sufficient to suppress autophagy (Scott *et al.*, 2004), that S6K acts in a negative feedback loop to limit detrimental autophagy, the discovery that ecdyson signaling induces autophagy via down regulation of class I PI3K (Rusten *et al.*, 2004), and that cooperation of autophagy and apoptosis are needed for salivary gland cell death (Berry and Baehrecke, 2007). However, the role and regulation of

autophagy seems to be highly dependent on the environmental context and tissue used. For example, autophagy and apoptosis display a varying interplay during different stages of oogenesis (see 1.4.5). Further, autophagy is required for midgut cells to shrink during larval midgut cell death, however, *ATG7* mutant cells in the gut are still able to shrink and activate autophagy, in contrast to *ATG7* mutant fat body cells (E. Baehrecke, pers. communication and (Juhasz *et al.*, 2007a; Ryoo and Baehrecke, 2010)). Therefore, to fully understand the regulation of autophagy it is extremely important to study this process under diverse conditions and in different tissues.

1.4.3 The females gem: *Drosophila* ovaries

Another *Drosophila* organ that came into focus for autophagy research is the female reproductive system, the ovaries. Initially, oogenesis was studied for its role in embryo patterning, but it has since been used for many more aspects, *e.g.* stem cell research, cell cycle control, role of mRNA localization, cell death regulation and many more. There are several aspects that make it an especially useful tool for cell biological and developmental research: The *Drosophila* ovaries are the largest organ with the oocyte being the largest single cell, making it easily accessible for microscopic analyses and live imaging. Every stage of development and almost every cellular process is present within this single organ, from the first division of a stem cell until the fully developed mature egg. Two different tissues, the somatic follicle cells and the germline, are useful for comparisons and studies of differentiation and communication. And last but not least, the ovaries are not essential for the survival of a fly, allowing all types of tricky manipulations.
The ovaries are present as a pair in the abdomen of a female fly (FIG. 1.9 A). Each is made up of approximately 18 ovarioles, which are strings of continuously developing single egg chambers (FIG. 1.9 B). Based on their morphology, the egg chambers have been divided into 14 stages whereby stage 1 - 6 refer to early mitotic stages, 7 - 10 to endocyclic mid-oogenesis stages and 11 - 14 to late stages (Cummings *et al.*, 1971). At the anterior tip of each ovariole is the germarium, the structure where germline and somatic stem cells reside. Two to three germline stem cells divide asymmetrically to produce another stem cell and a daughter cell, which begins differentiation.

After four incomplete divisions of the daughter cell, a 16-cell cyst forms that is interconnected via cytoplasmic bridges (ring canals) for nutrient transport. Only one of these 16 cells will be selected as the oocyte, while the other 15 become polyploid nurse cells that produce nutrients for the growing oocyte.

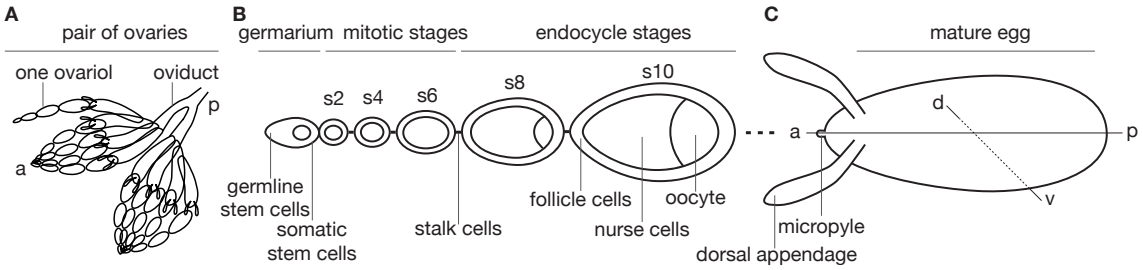

FIGURE 1.9 *Drosophila* oogenesis (A) In the abdomen of a female fly, a pair of ovaries is connected via the oviduct with the outside and contains approximately 18 ovarioles. **(B)** Ovarioles are continuously developing egg chambers starting anteriorly (a) with the germarium and ending posteriorly (p) with the mature egg, a process that is divided into 14 stages (s). Somatic and germline stem cells reside in the germarium and repeatedly divide to form a 16-cell cyst that subsequently differentiates into nurse cells and the oocyte surrounded by a monolayer of follicle cells and interconnected by stalk cells. **(C)** The mature egg has a well defined polarization with the dorsal appendages, important for respiration, and the micropyle, necessary for fertilization, on the dorsal-anterior side.

Every cyst will be enveloped by a monolayer of somatic follicle cells and interconnected via stalk cells. The follicle cells are important for separating the eggs, synthesizing yolk, for egg shell production and axis determination. The axes of each ovariole (anterior - germarium, posterior - mature egg) are carried throughout development and their determination is essential for correct patterning of the egg. Polarization is achieved by a number of cell-cell signaling events between the germline and the follicle cells as well as among the follicle cells themselves that involve well-conserved signaling cascades such as Delta-Notch, Janus kinase/signal transducer and activator of transcription (JAK/STAT), and epidermal growth factor receptor (EGFR) signaling (see 1.5). Any failure of these pathways can be fatal for the embryo and leads to a disrupted egg shell or malformed extra-embryonic structures like the dorsal appendages or the micropyle, which are important for respiration or fertilization, respectively (FIG. 1.9 C) (Roth and Lynch, 2009).

1.4.4 Building up *Drosophila* ovaries - signaling pathways in an eggshell

Several differentiation and symmetry-breaking steps during *Drosophila* oogenesis are tightly regulated by multiple cell-cell signaling cascades (Roth and Lynch, 2009). Maturation of the egg starts with the asymmetric division of a germline stem cell and subsequent divisions of the daughter cell to form a 16-cell germline cyst. Division and selection of the oocyte completes in the germarium in region 2a and at the transition to region 2b, where the somatic stem cells are located at fixed opposing positions and begin to migrate between the cysts (FIG. 1.10 A). In region 3, when follicle cells (FCs) largely cover the cyst, some FCs at the border between region 2b and 3 start to specialize into stalk and polar cells and begin to separate the cysts. The differentiation of stalk and polar cells establishes the long axis of the egg chamber and thus the future anterior-posterior (A/P) axis of the egg and the positioning of the oocyte at the posterior end. This is also the first of several interactions between the somatic FCs and the germline and requires Delta-Notch and JAK-STAT signaling (Roth and Lynch, 2009). Notch is a transmembrane receptor expressed only in the FCs and activated by the germline-derived ligand Delta or the FC expressed ligand Fringe (Fng) (Roth, 2001). Notch undergoes a set of proteolytic cleavages, resulting in the release of the Notch intracellular domain (NICD) that can bind to nuclear effectors and activates the transcription of downstream targets (Schweisguth, 2004). In germarium region 3, Delta is expressed by the germline cyst and activates Notch in the surrounding FCs. Additional expression of Fng in anterior polar/stalk precursor cells enhances Notch activation, which causes the precursor cells to differentiate into polar cells (FIG. 1.10 A) (Roth, 2001). Polar cells are the only cells that express unpaired (upd), the ligand that activates JAK/STAT signaling. Binding of upd to its predimerised receptor causes the receptor-associated JAK kinase to phosphorylate itself and the receptor to create a binding site for STAT. Phosphorylated STAT dimerises and translocates into the nucleus to induce target gene expression (Arbouzova and Zeidler, 2006). Since Notch activated cells repress the translocation of transcription factor STAT to the nucleus, the upd ligand can only reach cells with no or low levels of Notch signaling. Activation of JAK/STAT thus induces anterior polar/stalk precursor cells to differentiate into stalk cells and lateral FCs to differentiate into main body FCs (Assa-Kunik *et al.*, 2007) (FIG. 1.10 A).

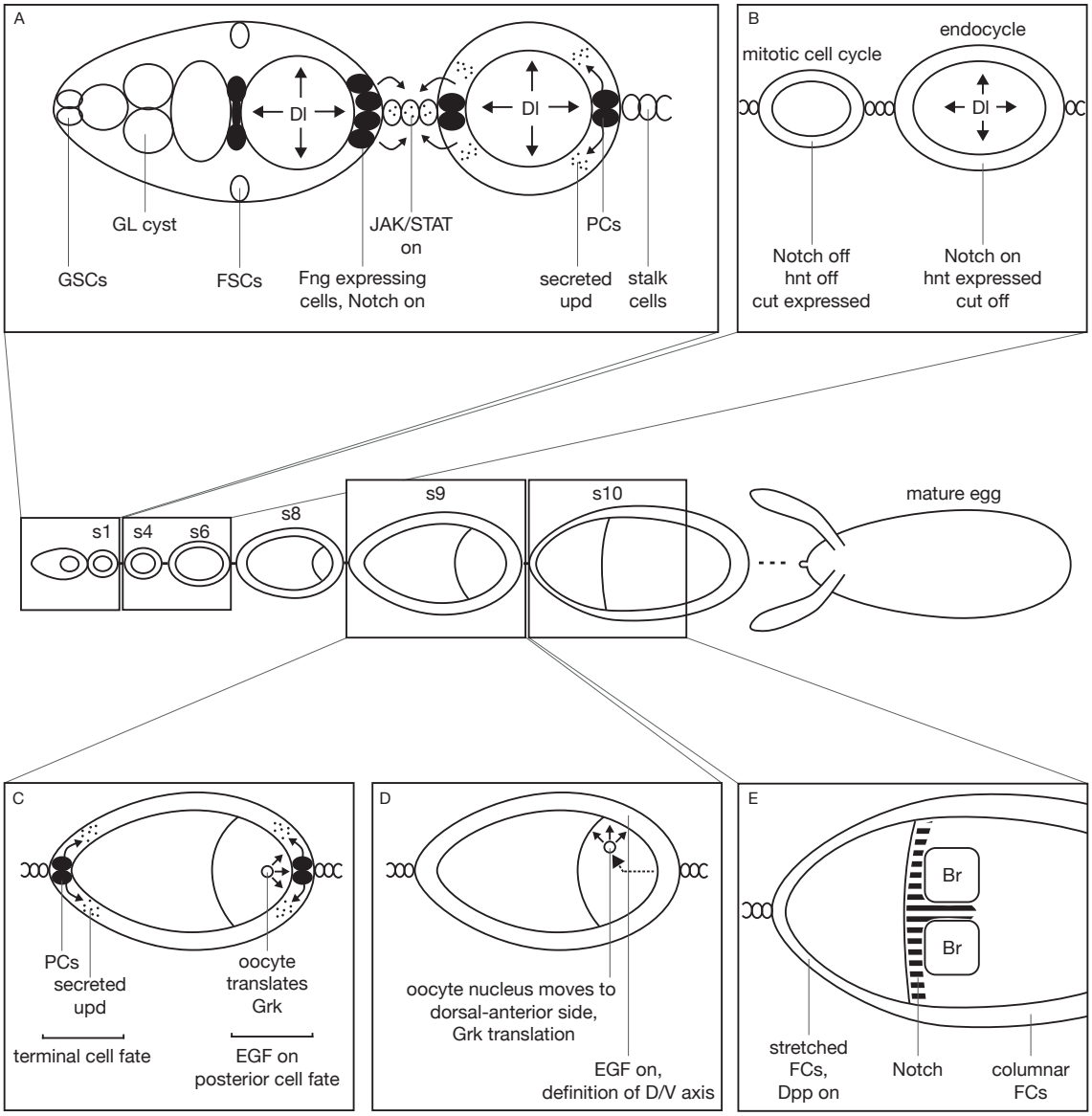

FIGURE 1.10 Communication within *Drosophila* oogenesis. Multiple signaling pathways are active several times during oogenesis to ensure correct egg patterning. **(A)** In the germarium, follicular stem cells (FSCs) divide to provide an epithelial sheet to surround the germ line (GL) cysts. These FCs encounter high levels of Delta (Dl) expressed by the GL. In addition, Fringe (Fng) expression in the precursors of stalk and polar cells (PC) enhances Notch (N) signaling resulting in PC fate. FCs that do not contact the germline have low levels of N activation and differentiate into stalk cells. PCs express upd, which diffuses to adjacent FCs and activates JAK/STAT signaling in cells with low levels of N activation (stalk cells). Main body FCs that express high levels of N are resistant to JAK/STAT activation. **(B)** Between stage 2 and 5, Dl is not expressed, N is inactive, *cut* is expressed and the FCs divide mitotically. By stage 6/7, a second round of Dl expression in the GL activates N in the FCs that consequently switch from the mitotic cycle to endocyclic DNA replication. This terminates *cut* expression and activates *hnt*. **(C)** Upd secretion by the PCs defines a terminal cell fate. By stage 9, the oocyte accumulates Gurken (Grk) protein in the posterior corner, which activates epidermal growth factor receptor (EGFR) signaling in neighboring FCs that subsequently assume a posterior cell fate. **(D)** The posterior FCs send an unknown signal back to the oocyte, which results in microtubuli reconstruction and movement of the oocyte nucleus to a lateral-anterior position. A second round of Grk activates EGFR signaling in flanking FCs and leads to the definition of the dorso-ventral (D/V) axis. **(E)** At stage 10, the cells that form the dorsal appendages (DA) are defined by EGFR signaling and a gradient of Decapentaplegig (Dpp) expression in stretched cells. Broad gets restricted to two patches of future DA roof cells, and the midline cells experience a third round of N activation. Anterior is to the left and posterior to the right.

Since these signaling events only happen in the anterior region of the older region 3 cyst, differentiated stalk cells directly contact the FCs of the younger cyst, inducing expression of the cell adhesion molecule cadherin in these cells. Cadherin expression is also elevated in the oocyte, causing a preferential location at the posterior end (Gonzalez-Reyes and St Johnston, 1998). After the germline is completely surrounded by FCs, interconnected by stalk cells, and the AP axis is established, the remaining cysts differentiate into nurse cells (NCs) and supply the oocyte with nutrients, proteins, and mRNAs. As the oocyte grows, the FCs undergo 8 to 9 mitotic devisions until stage 6/7 in order to maintain a continuous epithelial sheet. Multiple pathways are active during these mitotic stages such as Wingless, Hedgehoge and JNK, but the exact role of these pathways is still not completely clear (Klusza and Deng, 2010). When the egg reaches stage 6/7, another round of germline-to-FC interaction via the Delta-Notch pathway induces the switch from a mitotic to an endoreplication program (M/E switch). Again, Delta is expressed by germ cells (GCs) and activates Notch in adjacent FCs (FIG. 1.10 B). Notch activates the transcriptional repressor Hindsight (Hnt), which terminates mitosis and promotes the M/E switch through down regulation of the transcription factor Cut (Sun and Deng, 2007). Interestingly, TOR mutant FCs, for unknown reasons, cannot undergo the M/E switch and remain mitotic (LaFever *et al.*, 2010). Within these stages, the egg chamber also changes from a rather round form to an ellipsoidal structure with a pair of polar cells at the anterior and posterior end. Upd secretion by the polar cells only reaches the most distal FCs that therefore assume a terminal FC fate, which is further refined by the gradual secretion of upd that specifies border, stretched, and centripetal FCs, each with a specific function for the mature egg such as the generation of dorsal appendages (DAs) or the micropyle (FIG. 1.10 C). This pattern is mirror-symmetric until Gurken (Grk) protein, translated by the oocyte, accumulates in the posterior-most part of the oocyte and activates EGFR signaling in the terminal FCs next to it (FIG. 1.10 C). Grk, a transforming growth factor α (TGFα) like ligand, activates dimerization and auto-phosphorylation of the EGF receptor, which in turn activates several signal transduction cascades including MAPK that finally define the signal receiving cells as posterior FCs (Xi *et al.*, 2003). Between stages 7 and 9, an unknown signal from the newly defined posterior FCs to the oocyte (back signaling) triggers a reconstruction of the microtubuli (MT) network where the posterior MT organization center disappears and MTs send their plus ends from the anterior and lateral sites into the oocyte (González-Reyes *et al.*, 1995; Chang *et al.*, 2011). Specific mRNAs like *bicoid* and *oscar* are localized at the anterior and posterior poles, important

for the later pattern of the head and thorax, or defining the site of the pole plasma assembly, respectively (Chang *et al.*, 2011). Along with MT reorganization, the oocyte nucleus moves from the posterior side to an asymmetrical anterior position, which will be defined as the future dorsal side of the egg chamber by a second round of grk/EGFR signaling from the oocyte to the overlaying FCs (FIG. 1.10 D). The activation of EGFR signaling in the dorsal FCs inhibits expression of the sulfotransferase pipe, which sulfonates vitellin-membrane components on the ventral side. This information is stored until the egg is fertilized, and the modification results in the production of the Spätzle ligand that activates the Toll receptor on the ventral side, which finally triggers the nuclear gradient of the transcription factor Dorsal and patterning of the dorso-ventral (D/V) axis (Schupbach, 2009). The second peak of EGFR signaling does not only define the D/V axis, but also initiates a special patterning of the FCs that later leads to the formation of DAs. Together with a gradient of the secreted morphogen Decapentaplegig (Dpp), a TGF-β family member, which is expressed in the flat layer of stretched FCs that cover the anterior part of the egg in late mid-oogenesis stages, the molecules define two groups of cells, the FC midline and two patches follicle roof cells lateral to the midline. This leads to a restricted expression pattern of the initially uniformly expressed transcription factor Broad (Br) to the two patches of future roof cells (FIG. 1.10 E). In addition, Notch is activated in stage 10 egg chambers in a dorsal-anterior T-shape and is essential for the correct differentiation of roof- and floor cells, corresponding to the future outer and inner layers of the DAs (Berg, 2008).

Taken together, three classical pathways are used repeatedly throughout oogenesis to establish the A/P and D/V axes and specialize FCs in their function: Delta-Notch, EGFR and JAK-STAT. Thus, *Drosophila* oogenesis provides an excellent system to investigate principles of these conserved signaling pathways as well as new modulators and the mechanism by which cells choose their fate.

1.4.5 Autophagy and cell death in *Drosophila* ovaries

The ovaries are the largest organ of the fly, making up the majority of the weight difference between females and males, which is as large as ~30%.

Thus, egg production is a highly energy consuming job that strongly depends on the availability of food, or more precisely protein, but also on sex peptide and hormone signaling. Nutrient deprived flies immediately shut down the costly egg production to ensure survival of the animal. Premature eggs stop developing and die by programmed cell death in oder to retrieve essential nutrients. However, even under nutrient rich conditions, sporadic cell death occurs at so called 'checkpoints' in order to ensure proper egg development. Interestingly, flies mutant for components of the insulin pathway are sterile and also block oogenesis under nutrient rich conditions (Drummond-Barbosa and Spradling, 2001).

The first cell death checkpoint is located within region 2 of the germarium, probably to ensure that the correct number of FCs is given to envelope the germline cyst. This regions stains positive for apoptotic as well as autophagic markers during normal development, but this is highly elevated under starved conditions (Drummond-Barbosa and Spradling, 2001; Hou *et al.*, 2008; Nezis *et al.*, 2009). This indicates that maintenance of the germline and the follicle stem cells is directly regulated by the insulin/TOR signaling pathway, but whether insulin/TOR also regulates autophagic cell death in the germarium remains to be shown (LaFever and Drummond-Barbosa, 2005; LaFever *et al.*, 2010). Similar observations were made at a second check point during mid-oogenesis at stage 7 to 8, directly before vitellogenesis (the deposition of yolk into the oocyte). Degenerating mid-stage egg chambers display markers for autophagy, and starved *ATG* mutant germline eggs are impaired to activate autophagy, but also DNA fragmentation, denotive for apoptosis (Velentzas *et al.*, 2007; Hou *et al.*, 2008; Nezis *et al.*, 2009). Apoptotic cell death in most *Drosophila* tissues, including the salivary glands, is regulated by the canonical apoptosis pathway that includes proteins like Reaper, Hid or Grim in order to inhibit IAPs such as Bruce. Surprisingly, this seems not to be the case for mid-oogenesis cell death since these proteins are not required for mid-stage egg chamber degradation, thus leaving room for alternative cell death pathways, such as autophagy (Lee *et al.*, 2003; Peterson *et al.*, 2007). Indeed, germline mutants of the effector caspase Dcp-1 display a block in mid-oogenesis cell death resulting in egg chambers with degenerated follicle cells and persisting germline (Laundrie *et al.*, 2003). The caspase Dcp-1 was shown to be involved in the regulation of autophagy, and activity of Dcp-1 is sufficient to induce autophagy during mid-oogenesis even under fed conditions (Hou *et al.*, 2008; Kim *et al.*, 2010). In addition to cell death in the germarium and during mid-oogenesis, which is mostly regulated through environmental conditions, specific nurse cell death

during late stages of oogenesis is necessary for normal maturation of the egg. Further, FC death of specialized polar FCs occurs during normal development and is important for correct axis specification and formation of the micropyle (Pritchett *et al.*, 2009). After the oocyte has reached its final size, nurse cells are no longer needed for feeding and die. Nurse cell death initiates before dumping, a process characterized by actin fiber bundles that stretch from the nurse cells to the oocyte and transport all the remaining cytoplasm and nutrients to support the oocyte for the last time. After dumping, the nurse cell nuclei and other remnants get fragmented and finally die. Similar to mid-oogenesis cell death, canonical cell death proteins like Reaper and Grim are not required for nurse cell death, although surprisingly, caspases like Dronc, Drice and Dcp-1 participate in late oogenesis cell death. However, mutants of apoptotic genes only show few persisting nurse cell nuclei, proposing that other cell death pathways can take over (Baum *et al.*, 2007; Peterson *et al.*, 2007). Just recently it was shown that autophagy indeed is activated in dying nurse cells, and that genetic inhibition of autophagy in the germline prevents DNA fragmentation, leading to persisting nurse cell nuclei (Nezis *et al.*, 2010). Furthermore, developmental cell death is not restricted to nurse cells, but also affects FCs, which die at two points during oogenesis. After chorion deposition in late oogenesis, FCs undergo cell death in a similar way as nurse cells during mid and late oogenesis, showing the appearance of autophagic structures and condensed chromatin, but unexpectedly, no DNA fragmentation, which suggests a mechanism independent of caspases (Nezis *et al.*, 2006). It is known that excessive DNA replication might cause cancer, thus in most cells, rereplication of DNA activates cell death and triggers caspases (Hook *et al.*, 2007; Mehrotra *et al.*, 2008). However, nurse cells are polyploid and FCs switch during mid-oogenesis to an endoreplication cycle in order to produce plenty of yolk and chorion. It has been demonstrated that proapoptotic gene promoters are especially silenced in endocycling cells, thus repressing apoptosis and protecting the cell, which might be the reason for the occurrence of alternative cell death pathways such as autophagy during oogenesis (Mehrotra *et al.*, 2008). During earlier stages of oogenesis, polar cells, a specialized subtype of FCs at the anterior and posterior end of each egg chamber, are required for the differentiation of border cells and for the formation of the micropyle. Initially, polar cells exist as a small cluster of cells, but by mid-oogenesis, extra cells are eliminated, leaving exactly two polar cells at each side of the egg. So far, this elimination is the only ovarian cell death that uses the canonical cell death pathway including Hid, Dronc and Drice and where autophagy has not been

observed (Khammari *et al.*, 2010). However, the selection of polar cells occurs prior to the endocycle switch, before apoptosis is repressed.

Taken together, autophagy has become a clear alternative to canonical caspase mediated cell death during *Drosophila* oogenesis, but still very little is known about the regulation, upstream activators, the restriction to distinct cells, and the physiological role of autophagy besides cell clearance. The establishment of the ovaries as a model for autophagy serve to further investigate these issues.

1.5 Rationale for the PhD project

As outlined in the previous chapters, autophagy plays important roles during eukaryotic development, as well as in a variety of diseases. However, genetic analyses to examine the regulation of autophagy, although excessively done in yeast, are still rare in metazoans. Likewise, studies focusing on the physiological function of autophagy, on organ specific roles for autophagy or on independent functions of *ATG* genes are still represented in minority. The manifold opportunities of genetic manipulations and the advantage of having several cellular processes within one organ make the *Drosophila* ovaries an excellent tool for studying the role and regulation of autophagy.

This PhD project was motivated by the observation that flies mutant for components of the insulin signaling pathway have underdeveloped eggs that lack vitellogenic stages and are sterile (Bohni *et al.*, 1999; Stocker *et al.*, 2003; Richard *et al.*, 2005; Werz *et al.*, 2009), a phenotype that is similarly observed in the ovaries of nutrient deprived flies (Drummond-Barbosa and Spradling, 2001). Despite its role in growth control, insulin/TOR signaling is also crucial for the control of autophagy. In the *Drosophila* fat body, autophagy serves to provide nutrients during starvation, but it remained unclear if autophagy also plays a role in other nutrient responding organs, such as the ovaries (Rusten *et al.*, 2004; Scott *et al.*, 2004). Notably, starvation is able to induce programmed cell death during *Drosophila* oogenesis, however, the interconnection to autophagy was not investigated (Drummond-Barbosa and Spradling, 2001; Velentzas *et al.*, 2007).

Since autophagy is known to serve as a nutrient source under starvation conditions, autophagy may adopt an essential role in the response to nutrient depletion in the ovaries as well. The aim of this PhD project was to establish the ovaries as a model organ to study autophagy and to understand how nutrient-dependent growth regulation is related to autophagy. This enabled us to investigate the physiological role of autophagy during oogenesis.

Insights into the organ specific regulation of autophagy are crucial to improve the understanding of the role that autophagy plays in health and disease. This will be important in order to develop novel therapies based on the manipulation of autophagy.

II Results

2.1 Autophagy in *Drosophila* ovaries is induced by starvation and is required for oogenesis

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Autophagy in *Drosophila* ovaries is induced by starvation and is required for oogenesis

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Autophagy, an evolutionarily conserved lysosome-mediated degradation, promotes cell survival under starvation and is controlled by insulin/target of rapamycin (TOR) signaling. In *Drosophila*, nutrient depletion induces autophagy in the fat body. Interestingly, nutrient availability and insulin/TOR signaling also influence the size and structure of *Drosophila* ovaries, however, the role of nutrient signaling and autophagy during this process remains to be elucidated. Here, we show that starvation induces autophagy in germline cells (GCs) and in follicle cells (FCs) in *Drosophila* ovaries. This process is mediated by the ATG machinery and involves the upregulation of *Atg* genes. We further demonstrate that insulin/TOR signaling controls autophagy in FCs and GCs. The analysis of chimeric females reveals that autophagy in FCs, but not in GCs, is required for egg development. Strikingly, when animals lack *Atg* gene function in both cell types, ovaries develop normally, suggesting that the incompatibility between autophagy-competent GCs and autophagy-deficient FCs leads to defective egg development. As egg morphogenesis depends on a tightly linked signaling between FCs and GCs, we propose a model in which autophagy is required for the communication between these two cell types. Our data establish an important function for autophagy during oogenesis and contributes to the understanding of the role of autophagy in animal development.
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Autophagy, a conserved degradation process, serves as an energy reserve in response to starvation, but also has critical roles in cellular remodeling during development, immunity and cancer.[1] The central regulator of autophagy is the target of rapamycin (TOR), a downstream kinase of the insulin/insulin-like growth factor (IGF) signaling pathway (IIS).[2]

In *Drosophila*, IIS/TOR signaling regulates autophagy in the fat body,[3,4] but it remains unclear whether autophagy is also important in other nutrient-responding organs. The *Drosophila* ovaries are of special interest, as starvation inhibits ovarian development[5] and mutations in IIS components lead to defects in oogenesis and female sterility.[6–9] These findings raise the question whether IIS/TOR signaling controls autophagy during oogenesis.

Notably, starvation induces programmed cell death (PCD) during *Drosophila* oogenesis in the germarium, in nurse cells (NCs) and follicle cells (FCs),[5] and increases caspase activity during mid-oogenesis.[10] At later stages, NCs also undergo developmental PCD necessary to complete oogenesis. So far, primarily the implication of apoptosis has been investigated. Only recent reports show that autophagy occurs in the germarium, during mid-oogenesis and in dying NC. Interestingly, inhibition of *Atg* genes prevents DNA fragmentation, suggesting that autophagy and apoptotic cell death are connected.[11,12] However, the regulatory mechanisms underlying these processes and the contribution of different ovarian cell types (GCs and FCs) are still unknown.

This motivated us to examine the crosstalk between autophagy and nutrient signaling during *Drosophila* oogenesis. We show that starvation induces autophagy in both GCs and FCs. Surprisingly, autophagy is specifically required in FCs, and oogenesis is unaffected when both GCs and FCs are autophagy deficient. This suggests that the incongruity between an autophagy-deficient soma and an autophagy-competent germline is responsible for the oogenesis defect. Consequently, we hypothesize that autophagy is required for proper communication between these two cell types.

Results

Starvation induces autophagy in *Drosophila* FCs and GCs. Nutrient deprivation affects *Drosophila* ovary size and egg production, and induces PCD in GCs and FCs.[5] Thus, we tested whether autophagy is induced by starvation during *Drosophila* oogenesis using lysotracker (LTR). Upon starvation, LTR accumulated in region 2a/2b of the germarium and in stage 1–8 GCs (Figures 1a and a′, Supplementary Figure 1). However, LTR staining in the germarium was also visible under fed conditions (Figure 1a, Supplementary Figure 1B), but increased during starvation. Further, LTR-positive structures accumulated within FCs in stage 1–8 ovaries on starvation (Figures 1b and b′), whereas in later stages, FCs displayed starvation-independent LTR staining (Supplementary

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Keywords: autophagy; *Drosophila*; oogenesis; starvation; insulin/TOR
Abbreviations: Atg, autophagy-related; DA, dorsal appendage; FCs, follicle cells; FRT, flp recombinase target; GCs, germ cells; GLCs, germline clones; gs, grandchildless; hs, heatshock; IGF, insulin-like growth factor; IIS, insulin/insulin-like growth factor signaling; LTR, lysotracker-red; NCs, nurse cells; PCT, pole cell transplantation; PCD, programmed cell death; PCOS, polycystic ovary syndrome; RFP, red fluorescent protein; Rheb, Ras homologue enriched in brain; TEM, transmission electron microscopy; TOR, target of rapamycin; WB, western blot; WT, wild type
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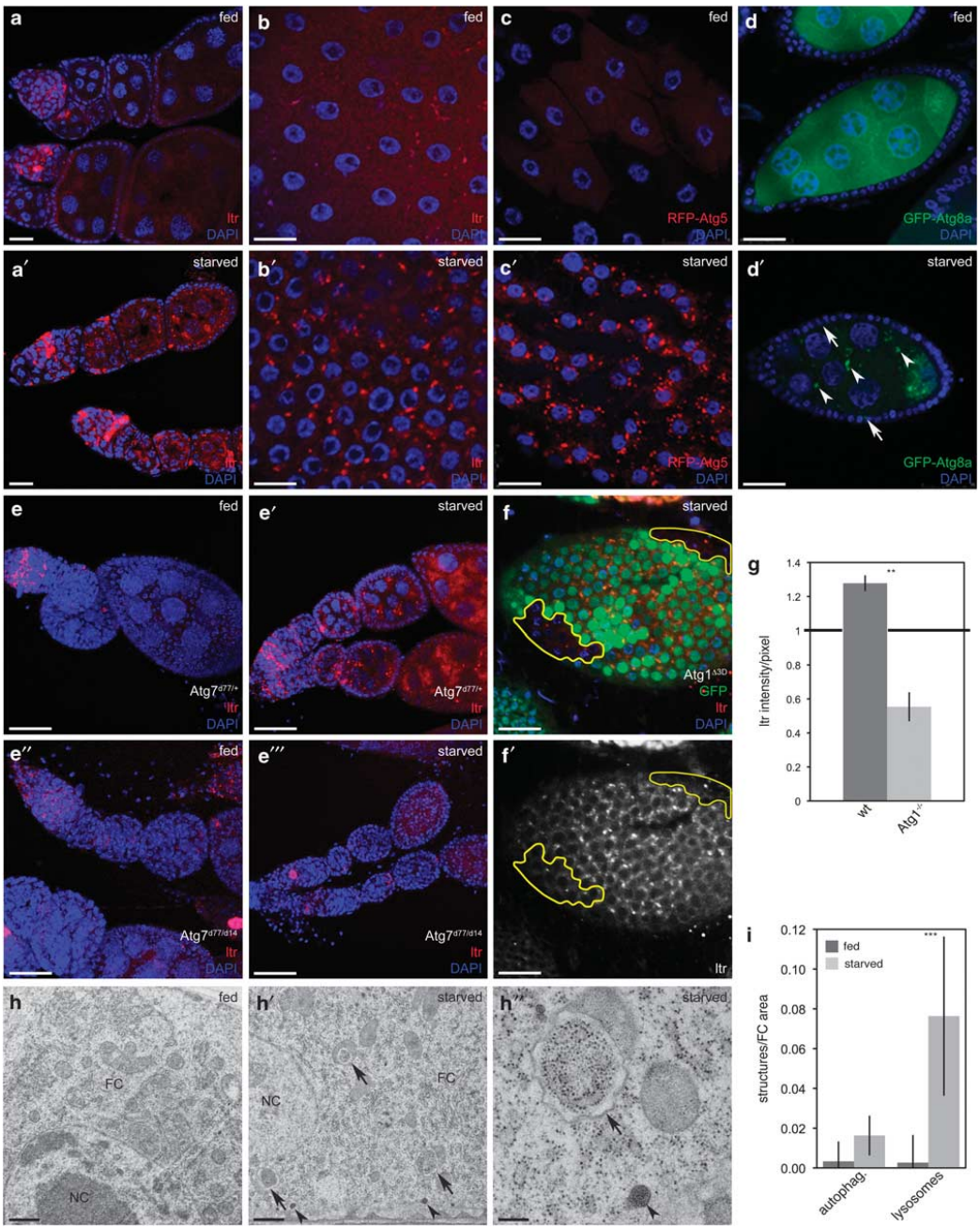
Figures 1G and G′). As reported previously,[11] we also detected high levels of LTR staining in dying egg chambers (Supplementary Figures 1F and F′), whereas the staining of healthy eggs was generally more subtle, but concentrated to distinct punctae. Thus, we focused our analyses on healthy egg chambers.



To confirm these results, we established transgenic flies expressing fluorescently tagged dAtg5 and dAtg8a proteins (Supplementary Figure 2). Starvation resulted in the formation of punctuate structures in GCs and FCs during mid-oogenesis in flies expressing UASp-GFP-dAtg8 (Figures 1d and d′), and equivalent structures were observed in FCs of flies expressing the soma-specific UASt-RFP-Atg5 (Figures 1c and c′).

Further, transmission electron microscopy (TEM) analyses revealed that lysosomes and autophagosomes are only occasionally found in FCs of fed flies (Figure 1h), whereas starvation increased the abundance of lysosomes and double membrane-bound vesicles containing undigested cytoplasmic material, indicative of autophagosomes (Figures 1h′ and h″, quantification 1i).

To confirm that these observations are truly autophagy dependent, we examined ovaries mutant for *Atg7*. Flies lacking *Atg7* are viable, but unable to induce autophagy.[13] Starvation induced LTR staining in the ovaries of *Atg7* heterozygous control flies, but not in *Atg7* homozygous mutant flies (Figures 1e′, and e‴). We further used the FLP/FRT system to induce FC clones homozygous mutant for *Atg1*, a kinase essential for autophagy.[14] Although WT cells accumulated LTR-positive structures upon starvation, autophagy induction was impaired in neighboring clones lacking *Atg1* (Figures 1f and f′, quantification 1g), demonstrating that ovarian autophagy requires functional ATG signaling.

Starvation induces dAtg8 conversion and *Atg* gene expression in *Drosophila* ovaries. To monitor changes in cleaved dAtg8-II as an indicator of autophagy induction,[15] we generated a *Drosophila* Atg8 antibody. An upregulation of dAtg8-II protein in the ovaries was already detectable 6 h after starvation, while the levels of dAtg8-I remained unchanged (Figures 2a and a″). Both dAtg8-I and dAtg8–II were completely vanished in protein extracts from larvae expressing UAS-dAtg8-RNAi, confirming antibody specificity (Figure 2a′). Consistently, the dAtg8 antibody detected punctuated structures in stage 8 FCs upon nutrient depletion (Figure 2a‴). Thus, starvation induces dAtg8 conversion and the accumulation of dAtg8-positive autophagosomes in *Drosophila* ovaries.

Several reports reveal that autophagy induction was accompanied by increased Atg gene expression,[16–18] thus, we investigated *Atg* gene expression in *Drosophila* ovaries by quantitative real-time PCR. All genes examined showed a slight, but significant upregulation upon starvation (Figure 2b). These molecular readouts further confirm that starvation induces autophagy in *Drosophila* ovaries.

IIS/TOR controls ovarian autophagy. In *Drosophila*, autophagy is regulated by IIS/TOR signaling in the fat body[3,4] and salivary glands.[19] Ovarian development is strongly affected by nutrient availability,[5] and mutants in IIS/TOR pathway components are sterile,[6–9] suggesting that IIS/TOR signaling also regulates autophagy during oogenesis. Therefore, we investigated whether inhibition of TOR by rapamycin is able to mimic starvation-induced autophagy in the ovaries. Injection of RAD (a rapamycin derivative) into the female abdomen led to small ovaries lacking vitellogenic stages (Figure 3b), whereas control injection did not affect ovarian development and egg production (Figure 3a). RAD-treated females were fully viable, but produced 80 and 98% less offspring on day 1 and 2 after injection, respectively, compared with controls. LTR staining was dramatically increased in FCs and GCs of RAD-treated ovaries (Figures 3a′–b″), which was comparable with starvation-induced autophagy (Figures 1a–b′, Supplementary Figure 1), indicating that nutrient deprivation and TOR inhibition act on the same autophagic mechanism in *Drosophila* ovaries.

Alternatively, to test whether activation of IIS/TOR signaling was sufficient to suppress starvation-induced autophagy, we generated FC clones expressing Rheb, an upstream activator of TOR.[4,8,20] Notably, FCs overexpressing Rheb lacked LTR staining even under starvation (Figures 3c and c′, quantification 3d). Thus, IIS/TOR signaling controls starvation-induced autophagy in a cell-autonomous manner in the ovaries, and is sufficient to inhibit autophagy even under starvation.

Autophagy is required for FC development. As starvation triggers autophagy in FCs and GCs, the questions remain whether autophagy is essential for oogenesis, and whether autophagy is required in the FCs or GCs. To answer these issues, we created chimeric animals lacking *Atg* gene function in either the germline or the somatic FCs.

First, we generated germline chimeras by pole cell transplantations (PCT).[21] Chimeric ovaries composed from an *Atg1* hemizygous germline and WT FCs were defective in autophagy, as starvation did not induce LTR staining in the mutant GCs, but in the enveloping WT FCs (Figures 4a″ and a‴). This demonstrates that autophagy was induced in the chimeras, but only in WT tissue, and confirms the necessity of *Atg1* for starvation-induced autophagy. In starved sibling control chimeras, in which the GCs inherited the chromosome balancers, LTR-positive structures emerged in WT FCs and GCs (Figures 4a and a′). Surprisingly, *Atg1* germline chimeras developed functional ovaries, and their egg-laying behavior and hatching rates were indistinguishable from sibling control chimeras, albeit the offspring developed with a delay of 2 days (Figure 4c). When the *Atg1* germline chimeras were crossed with *Atg1* heterozygous males, the resulting *Atg1* homozygous mutant animals died in late larval

Figure 1 Starvation induces autophagy in *Drosophila* FCs and GCs. (**a** and **b**) LTR staining is increased in germaria, GCs (**a′**) and in stage 8 FCs (**b′**) upon starvation. (**c** and **d**) RFP-dAtg5 accumulates upon starvation in stage 8 FCs (**c′**) and GFP-dAtg8a in FCs (arrows) and GCs (arrowheads) (**d′**). (**e**–**e‴**) *Atg7* mutants fail to induce autophagy. (**f** and **f′**) *Atg1* mutant FC clones (marked by the lack of GFP) do not induce LTR staining. (**g**) LTR intensity/pixel of *Atg1* mutant clones normalized to heterozygous cells. (**h**–**h″**) TEM images depict an accumulation of autophagosomes (arrows) and lysosomes (arrowheads) in starved FCs. (**i**) TEM quantification of FCs from fed *versus* starved flies ($n = 2$). Only healthy egg chambers were considered for the analysis. Scale bars: (**a**, **a′**, **d**, **d′**, **f** and **f′**) 20 μm, (**b**–**c′**) 10 μm, (**e**–**e‴**) 50 μm, (**h** and **h′**) 500 nm, (**h″**) 200 nm. Error bars show S.D. of the mean, $^{***}P < 0.001$, $^{**}P < 0.01$.Genotypes: (**a**–**b** and **h**–**h′**) *y w*, (**c**) *da-Gal4/UAS-RFP-dAtg5*, (**d**) *da-Gal4/UASp-dAtg8a*, (**e**) $Atg7^{d14}/Atg7^{d77}$, $Atg7^{d14}$, (**f**) *hs flp/+*; $Atg1^{\Delta 3D}$ *FRT80B/FRT80B-UbiGFP*

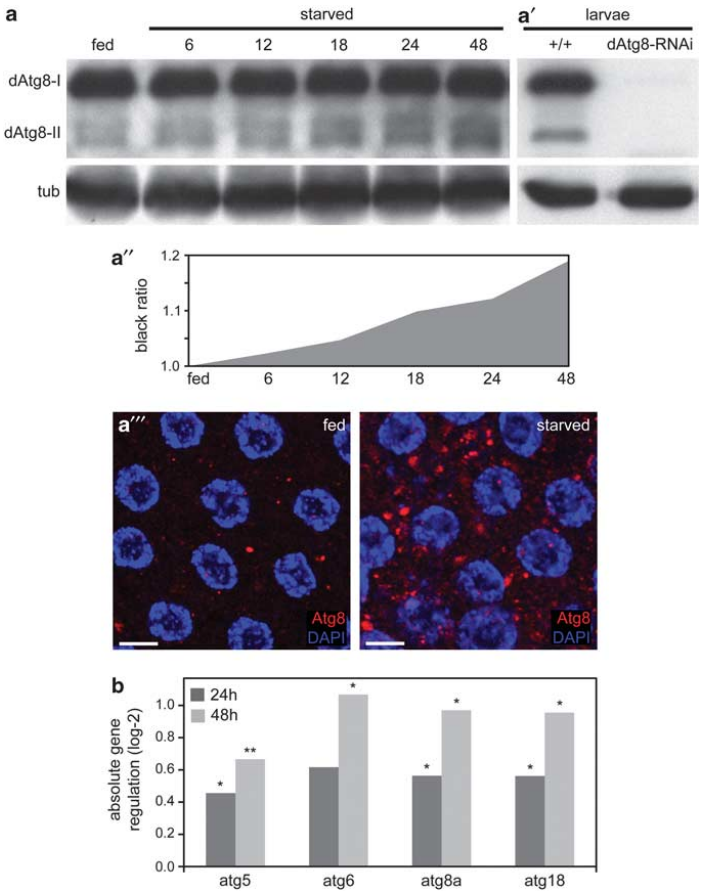


Figure 2 Starvation induces dAtg8 conversion and *Atg* gene expression in *Drosophila* ovaries. (**a**) Western blot (WB) showing the increase of dAtg8-II in a starvation time course. (**a′**) Expression of dAtg8 is diminished in larvae ubiquitously expressing *dAtg8a-RNAi.* Tubulin served as loading control. (**a″**) Quantification of Atg8-II WB signals measured as grey values using ImageJ. Rising grey values represent the increase of dAtg8-II in **a**. (**a‴**) Accumulation of dAtg8 labeled autophagosomes in starved stage 8 FCs. (**b**) Quantitative real-time PCR of ovary RNA samples from fed flies (reference expression level), 24 and 48 h starved flies. $n = 5$; *P*-values: $^{*}P < 0.05$, $^{**}P < 0.01$. Scale bars: (**a‴**) 10 μm. Genotypes: (**a**, **a‴** and **b**) *y w*, (**a′**) *y w, UAS-dAtg8-RNAi, da-Gal4*

stages, similar to *Atg1* homozygous mutants derived from heterozygous mothers.[14] To further verify that autophagy is redundant in GCs for proper oogenesis, we created germline mosaics for *Atg13*. Knockout of *Atg1* or *Atg13* results in a similar defect in autophagy.[22] Accordingly, *Atg13* mutant GCs were defective in autophagy as monitored by the lack of LTR staining (Figures 4b″ and b‴), however, the chimeras were fully fertile with normal egg-laying behavior and hatching rates (Figure 4c). Further, we did not detect any defects in egg chamber development or egg morphology in *Atg1* or *Atg13* germline chimeras. It was recently reported that *Atg1* GLCs show a partial disruption of developmental NC death.[23] We occasionally observed persisting NC nuclei in stage-14 eggs; however, these events occurred with a low frequency in both the *Atg1* germline chimeras as well as in control siblings (4 *versus* 1.3%, respectively). *Atg13* germline mosaics did not show disruption of NC death, thus we conclude that developmental NC death is not affected in *Atg* germline chimeras. This indicates that autophagy in GCs is not required for egg development.

To analyze the function of autophagy in FCs, we created mosaics in which only the FCs were homozygous mutant for *Atg1*, whereas the GCs were heterozygous. First, we made use of flies carrying the *Apc* mutation that disrupts FC function, leading to flaccid eggs lacking dorsal appendages (DAs) and anterior chorion structures (see Materials and Methods). The removal of *Apc* by irradiating $+/Apc$ control larvae restored FC function,[24] resulting in females producing eggs with normal-looking DAs and embryonic cuticle (Figure 4d), and larvae hatched and developed to adults.

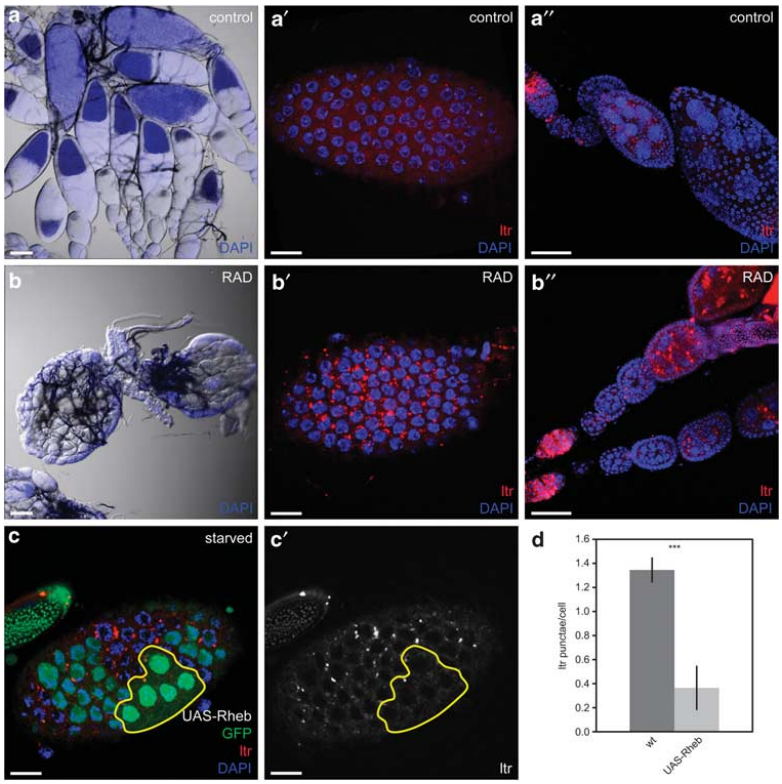


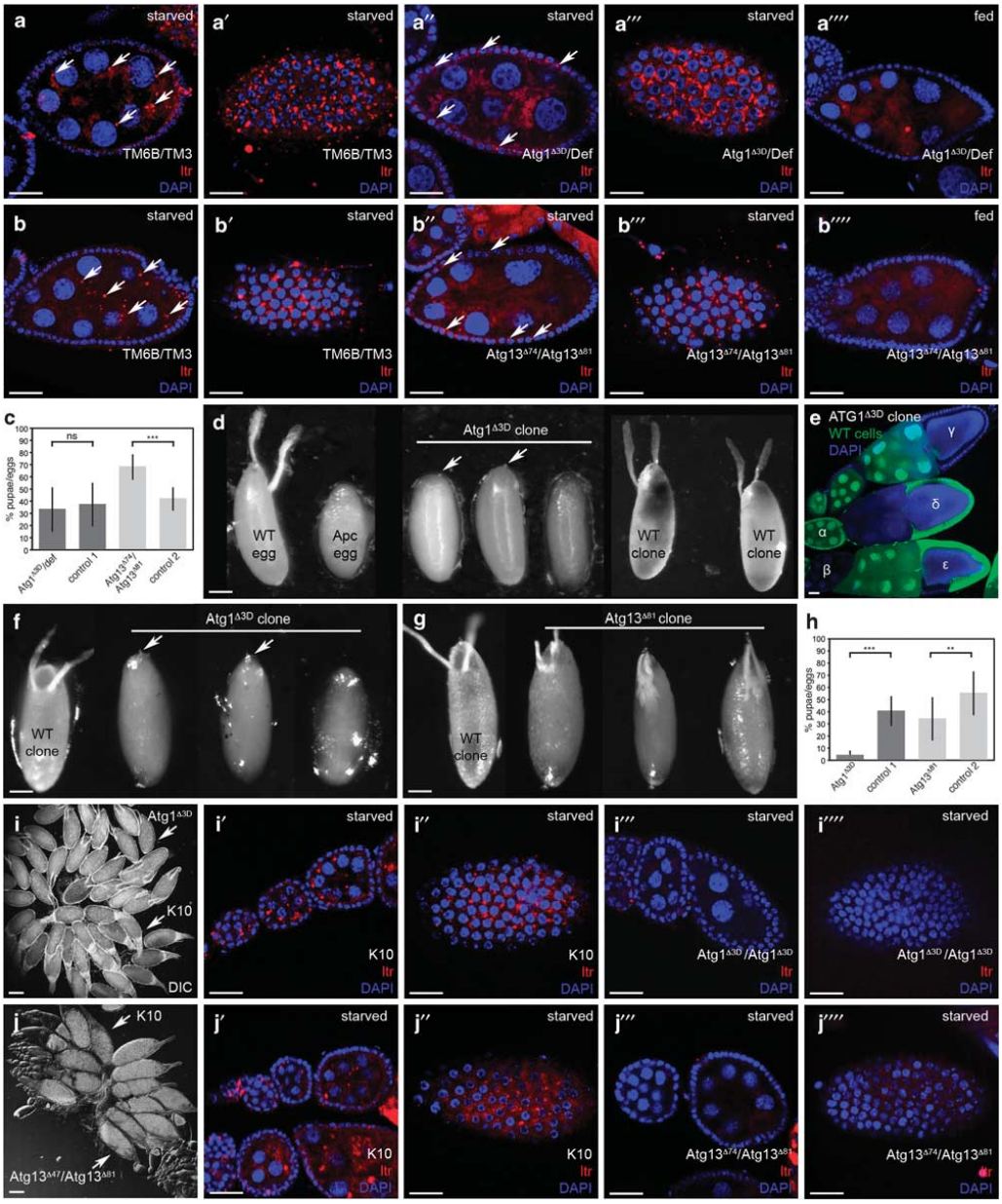
Figure 3 IIS/TOR signaling controls autophagy in *Drosophila* ovaries. (**a**–**b**″) Injection of RAD leads to small ovaries lacking vitellogenic stages (**b**) and a strong accumulation of autophagolysosomes in FCs (**b**′) and GCs (**b**″). Control ovaries are of normal size (**a**) and barely show LTR staining in FCs (**a**′) or GCs (**a**″). (**c** and **c**′) Generation of stage 7 FC clones overexpressing Rheb using the flp-out-Gal4/UAS method results in cells with high (strong GFP signal) and low (weak GFP signal) transgene expression. Only cells with bright GFP signals and enlarged nuclei (as an indication of enhanced cell size due to Rheb overexpression) were considered for the analyses. (**d**) Quantification of LTR staining in Rheb overexpressing clones compared with WT cells. Error bars show S.D. of the mean, $n=8$, $^{***}P<0.001$. Scale bars: (**a** and **b**) 100 μm, (**a**′, **b**′, **c** and **c**′) 10 μm, (**a**″ and **b**″) 50 μm. Genotypes: (**a**–**b**) *y w*, (**c**) *hs flp*/+; *act4CD24Gal4 UAS-GFP/UAS-Rheb*$^{EP50.084}$

In contrast, females resulting from *Atg1*$^{\Delta 3D}$/*Apc* irradiated larvae containing *Atg1*$^{\Delta 3D}$/*Atg1*$^{\Delta 3D}$ FC clones deposited non-typical *Apc* eggs that were non-flaccid, but contained short and rudimentary DAs. In these eggs, embryonic cuticle never appeared, and no larvae hatched (Figure 4d), suggesting that *Atg1* function is essential in FCs of *Drosophila* ovaries.

To confirm this, we created FC clones homozygous for *Atg1* using the heatshock (hs)-flp/FRT system. Hs-induced mitotic recombination resulted in *Atg1* homozygous mutant FC clones (identified by the lack of GFP) in 69% of the egg chambers with most of them being mosaic (Table 1, Figure 4e). Flies containing *Atg1* mutant FC clones laid very few eggs that resembled those generated by irradiation, lacking DAs and embryonic cuticle (Figure 4f), and only 5% of the eggs hatched (Figure 4h). Quantification revealed that 89% of the eggs laid by females containing *Atg1* mutant FC clones exhibited DA defects; consequently, only 11% of the chimeric eggs hatched (Supplementary Figure 3). Defective DA formation was only observed in 15% of the eggs containing control clones, and 58% of the control eggs hatched (Supplementary Figure 3). Given that 15% of the control eggs showed egg defects, the frequency of the egg phenotype that is solely due to the *Atg1* deletion (74%) is in accordance with the frequency of FC clones observed in the *Atg1* chimeras (69%), suggesting that almost every egg chamber containing *Atg1* mutant FC clones resulted in defective eggs. These data confirm the requirement of *Atg1* gene function in the FCs for proper oogenesis.

However, *Atg7* mutants, although clearly autophagy-defective, did not exhibit a severe oogenesis phenotype. Eggs derived from *Atg7* homozygous mutants showed a slight reduction in hatching rates compared with heterozygous controls (74 and 85%, respectively) with 18% of the eggs displaying eggshell defects, suggesting that *Atg7* mutations have only minor effects on egg development. This may implicate that the defect in egg development caused by the lack of *Atg1* is not due to autophagy, but an alternate function of Atg1. To verify that the observed phenotype is not restricted

to Atg1, we created hs-induced FC clones mutant for *Atg13*. Clone induction was equally effective as for *Atg1*, with 84% of the egg chambers containing *Atg13* homozygous mutant FC clones (Table 1). However, the phenotype was somewhat weaker, as we detected fewer eggs with DA defects (42%, see Supplementary Figure 3, Figure 4g) and 34% of the eggs hatched (Figure 4h, Supplementary Figure 3). The weaker effect of the *Atg13* deletion is consistent with the observation



that the lethality associated with *Atg13* is less severe than for *Atg1*,[22] and may be explained by differences in protein perdurance. Nevertheless, the similarity in phenotypes observed for *Atg1* and *Atg13* mutations confirm that autophagy in FCs is necessary for proper egg development.

Although this strongly suggests that the observed phenotype depends on autophagy, the question remains why *Atg7* mutants do not show oogenesis defects, although autophagy is clearly disrupted in their FCs (Figures 1e–e‴). Apparently, the two conditions, whole animal *versus* mosaic, must constitute distinctive situations in which the *Atg* deficiency is interpreted differently. Although both GCs and FCs are autophagy defective in *Atg7* mutants, the chimeras lack *Atg* gene function only in the FCs. This led us to propose that inputs from the cellular environment may affect the outcome of the autophagic signal. Consequently, we created chimeras lacking *Atg* genes specifically in the ovaries (in both GCs and FCs) by larval ovary transplantation experiments. We used host larvae carrying the *fs(1)K10* mutation leading to eggs containing a mass of chorionic material instead of two DAs present in WT eggs,[25,26] therefore, the transplanted ovary can be distinguished from *K10/K10* host ovaries by the appearance of DAs (Figures 4i and j). Strikingly, although autophagy was clearly disrupted in *Atg1* or *Atg13* homozygous mutant ovaries as monitored by the lack of LTR staining in both GCs and FCs (Figures 4i‴, i⁗, j‴ and j⁗), the mutant ovaries developed normally and gave rise to offspring with hatching rates comparable with those of the germline chimeras (37 and 64%, respectively). The respective *K10/K10* ovary from the same animal displayed normal LTR staining in both GCs and FCs, confirming that autophagy was induced in the chimeric animals, but only in the host tissues (Figures 4i′, i″, j′ and j″). Further, we did not observe any defects in egg chamber development or DA formation in *Atg1* or *Atg13* mutant ovaries. This indicates that egg development is unaffected when both ovarian cell types are autophagy deficient, which is in accordance with *Atg7* mutant flies not showing an oogenesis phenotype. This suggests that the oogenesis defect in the FC chimeras may be caused by an incompatibility between the mutant soma and a WT-like germline. As oogenesis requires a tight coordination of germline and soma,[27] it is tempting to speculate that signaling between these tissues is dysfunctional if FCs are autophagy defective.

Discussion

This work establishes the *Drosophila* ovaries as an attractive model system to study autophagy. Starvation induces autophagy in FCs and GCs of *Drosophila* ovaries under the control of IIS/TOR signaling. Notably, IIS/TOR signaling affects various processes during *Drosophila* oogenesis. For example, overexpression of activated protein kinase B (Akt) disrupts the deposition of the NC cytoplasm into the oocyte (NC dumping).[28] Furthermore, eliminating GCs modulates IIS, leading to prolonged lifespan and reproduction.[29,30] IIS also mediates ovarian stem cell proliferation in response to nutrients.[5]

Referring this to the mammalian system, the role of IIS during oogenesis is of special interest regarding one of the most common endocrine disorders, the polycystic ovary syndrome (PCOS). PCOS represents the most prevalent cause of anovulatory infertility characterized by large numbers of immature follicles. Remarkably, PCOS is often associated with type 2 diabetes and impaired IIS.[31] Interestingly, apoptosis regulators are upregulated in patients affected

Table 1 Frequency of HS-FLP induced FC clones

	Percentage of FC clones				Percentage of GC clones		
Genotype	**Entire clone**	**Mosaic clone**	**Total**	**No clone**	**Entire clone**	**No clone**	**Total number of egg chambers counted**
$Atg1^{\Delta 3D}$	22 ± 4	48 ± 13	69 ± 9	31 ± 9	7 ± 7	93 ± 7	236+157 (n = 2)
FRT80iso	13 ± 2	51 ± 8	64 ± 5	36 ± 9	13 ± 5	87 ± 5	134+130 (n = 2)
$Atg13^{\Delta 81}$	38 ± 0	46 ± 9	84 ± 4	16 ± 9	30 ± 1	70 ± 1	40+127 (n = 2)
FRT82iso	51 ± 4	25 ± 6	76 ± 5	24 ± 10	40 ± 0	60 ± 0	48+60 (n = 2)

Figure 4 *Atg1* and *Atg13* are required for FC development. (**a–c**) GC mosaics. Starvation induces autophagy (monitored by LTR staining) in GCs (**a** and **b**) and FCs (**a′** and **b′**) of control *TM6B/TM3* GC chimeras, whereas GC chimeras homozygous mutant for *Atg1* (**a″** and **a‴**) or *Atg13* (**b″** and **b‴**) display LTR staining only in WT FCs, which is not seen in fed control GC chimeras (**a⁗** and **b⁗**). Quantification of offspring shows similar hatching rates as control *TM6B/TM3* females (**c**). (**d–h**) FC mosaics. (**d**) X-ray induced FC clones. Shown are a normal egg deposited by a WT fly, a flaccid *Apc* mutant egg, missing DAs and anterior chorion structures, and eggs with *Atg1* mutant FC clones, missing DAs, but showing a micropyle (arrows). Generation of WT FC clones in *Apc/+* animals completely rescued the *Apc* phenotype. Typical examples of the resulting WT-like eggs are shown (WT clone). (**e–h**) Heat-shock induced clones. (**e**) Hs-flp induced mitotic recombination results in ovaries comprising WT (α) or completely mutant (β) egg chambers, egg chambers with all FCs mutant (γ), all GCs mutant (δ) or mosaic FCs (ε). (**f**) Eggs with *Atg1* mutant clones lack DAs, but feature a micropyle (arrows). (**g**) Eggs with *Atg13* mutant clones show variable phenotypes with reduced DAs. (**h**) Hatching rate of eggs containing *Atg1* or *Atg13* mutant FC clones. (**i** and **j**) Ovarian chimeras generated by larval ovary transplantations. After implantation, both *K10* and *Atg1* (**i**) or *Atg13* (**j**) mutant ovaries are attached to the oviduct. Upon starvation, autophagy (monitored by LTR staining) is induced in GCs (**i′** and **j′**) and FCs (**i″** and **j″**) of *K10* control ovaries, whereas *Atg* mutant ovaries are unable to induce autophagy (**i‴–j⁗**). Error bars show S.D. of the mean, ***$P<0.001$, **$P<0.01$. Scale bars: (**a–b⁗**, **e**, **i′–i⁗** and **j′–j⁗**) 20 μm, (**d**, **f** and **g**) 100 μm, (**i** and **j**) 250 μm. Genotypes: (**a–a⁗**) donor: $Atg1^{\Delta 3D}$-*FRT80B/TM6B*, *Df(3L)BSC613/TM3*, host: $Tm2^{gs}/Tm2^{gs}$, (**b–b⁗**) donor: $Atg13^{\Delta 81}$/*TM6B*, $Atg13^{\Delta 74}$/*TM3*, host: $Tm2^{gs}/Tm2^{gs}$, (**d**) w^{1118}, *Fs(3)Apc/+*, *Fs(3)Apc*/$Atg1^{\Delta 3D}$-*FRT80B*, (**f** and **e**) *FRT80isogenic/FRT80-UbiGFP* (WT clone), $Atg1^{\Delta 3D}$-*FRT80B/FRT80-UbiGFP*, (**g**) *FRT82isogenic/FRT82-UbiGFP* (WT clone), $Atg13^{\Delta 81}$-*FRT82/FRT82-UbiGFP*, (**i** and **j**) donor: $Atg1^{\Delta 3D}/Atg1^{\Delta 3D}$, $Atg13^{\Delta 74}/Atg13^{\Delta 81}$ host: *fs(1)K10/fs(1)K10*

by PCOS,[32] suggesting a role for PCD in the onset of the disease. Thus, alterations in IIS could lead to a disregulation of ovarian autophagy, which might be implicated in the development of polycystic ovaries. Further investigations will reveal whether modulation of autophagy in *Drosophila* leads to PCOS-like phenotypes.

During *Drosophila* oogenesis, several cell death checkpoints have been reported. Despite the developmental PCD of NCs, starvation induces egg chamber degeneration within the germarium and during mid-oogenesis, suggesting that dying egg chambers respond to the environmental status that is monitored before investing energy into egg production.[5] Interestingly, NC death, normally initiated at stage 10, is observed already at stage 8 under starvation, suggesting that those cells respond to nutrient availability as well.[33,34] Although these reports focused mainly on apoptotic cell death, the fact that PCD during mid-oogenesis strictly requires the caspase Dcp-1, which is nonessential for most other death pathways in the fly, suggests the existence of a non-redundant death mechanism during mid-oogenesis.[33]

Here, we show that starvation induces autophagy in the germarium and GCs during mid-oogenesis. This is consistent with recent publications indicating that autophagy contributes to PCD in the ovary.[11,12] Interestingly, this process is regulated by Dcp-1,[11] suggesting that apoptosis and autophagy coordinate the progress of oogenesis.

Surprisingly, we find that autophagy is not required for germline development. This is in accordance with *Atg7* mutant flies being fertile.[13] However, other *Atg* mutant phenotypes in *Drosophila* suggest a role for autophagy during development. Flies mutant for *Atg1* are pupal lethal,[4] and *Atg1* germline clones (GLCs) achieved using the Ovo^D technique show reduced DNA fragmentation and a partial disruption in NC death.[11,12] In the present study, we generated germline chimeras using the PCT technique where, in contrast to the Ovo^D system, a germline completely mutant for a certain gene is generated in a WT background. This technique excludes any perdurance and maternal contribution. Unlike the GLCs generated using Ovo^D, the transplanted pole cells are hemizygous mutant for the gene of interest, which excludes second site lethal effects. Further, PCT results in true germline chimeras without affecting the somatic cells, while the generation of GLC in the Ovo^D system also induces FC clones, which may interfere with the mutant phenotype. In germline chimeras generated by PCT, we do not observe any egg chamber defects and conclude that autophagy is not required in the germline. The discrepancy between our and the recently published data concerning reduced NC death could be explained by the different experimental setups and their limitations mentioned above.

However, as autophagy is induced upon starvation in GCs, the question remains whether oogenesis depends on autophagy in GCs when nutrients are limited. Further studies will reveal whether autophagy-deficient ovaries develop normally under such conditions.

Moreover, we demonstrate that starvation induces autophagy in FCs, confirming that FCs are involved in controlling the nutritional status to ensure germline development. Further, autophagy in FCs is essential for proper oogenesis. Thus, what could be the function of FCs during egg development, and how could autophagy contribute to this process?

FCs have a fundamental role during oogenesis. The patterning of FCs into discrete subtypes is crucial for egg development, as specialized FC sub-populations guide various steps during oogenesis. Eggshell morphogenesis further depends on the migration of different FC sub-populations to form a columnar epithelium over the oocyte, the micropyle and the DAs. FCs also secrete the chorion, a multilayered structure surrounding the oocyte essential for embryonic survival.[27,35] Interestingly, autophagy in FCs seems to be tightly associated with the spatial pattern of chorion synthesis, as autophagic death occurs at the anterior pole of the egg chamber where chorion formation is first completed.[36]

Notably, autophagy deficiency only affects oogenesis in a cellular context where FCs are mutant for *Atg* genes and GCs are WT. On the basis of this incompatibility, we hypothesize that dysfunctional signaling between soma and germline may be responsible for the oogenesis phenotype. For example, a signal arising in the WT germline may not be processed correctly in the mutant FCs and thus disrupts egg development. Alternatively, autophagy-deficient FCs may be incapable of generating a signal required in the GCs or necessary for the differentiation of specific FC sub-populations. However, if both cell types are deficient in autophagy, the absence of such a signal prevents a false interpretation by the other cell type, and egg development occurs normally. This model may be applied to explain the lack of oogenesis defects in *Atg7* mutant flies.

Thus, what are the signals during oogenesis that require autophagy? Egg development depends on signaling between GCs and FCs and between sub-populations of FCs. Three signaling pathways are involved in these processes: Notch, EGFR and Jak/STAT.[27] Notch is required for proliferation, differentiation and migration of FCs.[27] Interestingly, loss of the cysteine protease *Atg4* modulates Notch signaling in *Drosophila*,[37] thus, it is tempting to speculate that impaired *Atg* signaling may lead to malfunction of the Notch receptor to affect cell fate determination during oogenesis. The identification of the signaling pathway affected by the loss of autophagy in the FCs will shed light on the yet unsolved issue on which pathways are controlled by autophagy during the development of higher organisms.

Although the lethality associated with many *Atg* mutations in *Drosophila* indicates a fundamental role for autophagy during development, the function of some *Atg* genes is dispensable for fly development. Thus, some *Atg* genes may function redundantly, or other mechanisms compensate for autophagy deficiencies during development. Alternatively, given that certain *Atg* mutations have cell-context specific effects, there could be factors that determine specificity. Our findings on the incompatibility between autophagy-deficient soma and autophagy-competent germline demonstrate that the generation of chimeras is crucial to elucidate the tissue-specific function of a gene in a context relevant to physiology and development.

Our data clearly indicate that autophagy is indispensable for oogenesis. The understanding of molecular events regulating PCD in the fly ovary is still incomplete, and the communication

of death signals between FCs and GCs remains to be defined. The present study suggests that the nutrient response of FCs and GCs implies crosstalk between these two tissues. Further studies will aid to understand the fundamentals underlying this cell communication.

Materials and Methods

***Drosophila* maintenance, starvation and stocks.** Flies were raised on standard yeast/cornmeal agar at 25°C. Four-day-old females were starved on 10% sucrose agar at 25°C for 24 h if not otherwise stated.

D. melanogaster stocks used: *y w, w*^{1118} (controls), *Atg7*$^{\Delta 14}$, *Atg7*$^{\Delta 77}$, *Atg7*$^{\Delta 4}$, *Atg1*$^{\Delta 3D}$, *Atg13*$^{\Delta 74}$ and *Atg13*$^{\Delta 81}$ (kindly provided by T. Neufeld),[4,13,22] *Atg8-RNAi* 43096 (VDRC, Vienna, Austria), *UAS-Rheb*$^{50.084}$, *Fs(3)Apc*,[24] *Tm2*^{gs},[38] *fs(1)K10*,[39] *Df(3L)BSC613, FRT80-UbiGFP, FRT82-UbiGFP* and *Act*$<$ *CD2*$<$ *Gal4 UAS-GFP* (Bloomington *Drosophila* Stock Center, Indiana University, IN, USA).

Transgenic flies. *dAtg5* (5′-CAC CAT GGC CCA CGA CCG CGA G-3′; 5′-AAC ATC CTT GTA GTC CAC CGA-3′) and d*Atg8a* (5′-CAC CAT GAA GTT CCA ATA CAA GGA-3′; 5′ GTT AAT TTT GGC CAT GCC G-3′) coding regions were PCR amplified and cloned into pTGW and pPGW vectors (Carnegie Institution, WA, USA) to express the transgenes either in the soma (UASt-RFP-Atg5) or in both the soma and the germline (UASp-GFP-Atg8). Constructs were injected into *y w* embryos for transformation according to standard procedures. Three transgenic lines on two different chromosomes were established and tested for each construct.

LTR assay, tissue preparation and confocal microscopy. Ovaries were dissected in PBS, incubated for 1 min in 100 μM Lysotracker red DND-99 (Invitrogen, Molecular Probes, Basel, Switzerland) to label acidic organelles including autolysosomes, washed three times in PBS and fixed in 4% paraformaldehyde for 20 min. Ovaries were embedded in mounting medium with DAPI (Vectashield, Vector Laboratories, Inc., Burlingame, CA, USA) and images were obtained using a confocal microscope (Leica, Wetzlar, Germany, DM5500Q, TCS-SPE; objective lenses: Leica, 20 $\times$ (0.70), 40 $\times$ (1.15), 63 $\times$ (1.30); acquisition software: LAS AF v.2.0.1, Leica, Wetzlar, Germany) at room temperature and edited using Adobe Illustrator and Photoshop CS4.

Transmission EM. Ovaries were fixed for 4 h in 2% glutaraldehyde, 1% osmium tetroxide in 0.1 m cacodylate buffer, and postfixed for 4 h in 2% osmium tetroxide. After dehydration in an acetone series, ovaries were embedded in Spurr. Sections (50 nm) were stained with uranyl acetate and lead citrate on Formvar/Carbon covered copper grids (Quantifoil, Jena, Germany) and viewed on a transmission EM (Morgani 268, FEI Europe, Eindhoven, Netherlands). Quantification of the autophagic area was performed on ovaries from two different flies for each condition. In total, 15–20 randomly chosen FCs were photographed at $\times$ 4000 magnification, and autophagic structures and lysosomes were counted. Autophagic structures were scored according to their morphology, comprising all structures that contained recognizable cytosolic material.

Antibody generation, western blotting and immunofluorescence. Rabbits were immunized with the dAtg8 peptide: H_2N-MKFQYKEEHAFEKRR-CONH$_2$ (Eurogentec, Seraing, Belgium). The serum was double affinity purified and specificity of the antibody was shown on WB (Figure 2).

For WBs, twenty ovaries per time point or alternatively, five third instar larvae were extracted in lysis buffer (120 mM NaCl, 50 mM Tris-HCl, 20 mM NaF, 1 mM Benzamidine, 1 mM EDTA, 6 mM EGTA, 15 M NA_4P2O_7, 1% Nonidet P-40) containing protease inhibitors. Proteins were separated on a 12% SDS-PAGE gel and blotted onto Nitrocellulose (Hybond ECL, GE Healthcare, Uppsala, Sweden). Primary antibodies were applied overnight at 4°C: anti-dAtg8 1 : 1000, anti-tubulin (T-9026, Sigma-Aldrich, Buchs, Switzerland) 1 : 10000 and secondary antibodies for 2 h at RT: anti-rabbit-HRP 1 : 10000 (Jackson ImmunoResearch Europe Ltd., Suffolk, UK), anti-mouse-HRP 1 : 10000 (Jackson ImmunoResearch Europe Ltd.). For quantification of WB signals, Image J software (National Institutes of Health, Bethesda, MD, USA) was used to calculate the grey values of Atg8-II bands in fed *versus* starved conditions. Grey values of ovaries from fed flies were set as one.

For immunofluorescence, ovaries were fixed for 20 min in 4% PFA in 1 : 1 PBS/Heptan, dehydrated by methanol series and blocked with 2% normal donkey serum in PBS supplemented with 0.1% Triton X-100 and 1% DMSO. Primary antibody dilution was applied overnight at 4°C (anti-dAtg8, 1 : 500), secondary antibody for 2 h at RT (anti-rabbit-TexasRed 1 : 200, Jackson ImmunoResearch Europe Ltd.).

RNA purification and quantitative real-time PCR. Total RNA from 40 ovaries per time point was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). 2 μg RNA was reverse transcribed using SuperScriptIII reverse transcriptase (Invitrogen, Basel, Switzerland), following the manufacturer's protocol. Runs were performed in duplicates for five different biological replicates with a Rotor-Gene 6000 cycler (Corbett, Qiagen, Hilden, Germany) and SYBR Green Master Mix (Roche, Basel, Switzerland) and melting curve analyses were performed. Data were analyzed using REST (relative expression software tool) and Microsoft Excel software. Relative expression ratios were normalized to *rpl23* and *actin5c*, which showed no significant expression difference between fed and starved ovaries. mRNA levels of the respective genes of fed flies served as reference levels.

RAD treatment. RAD (Novartis, Basel, Switzerland) was dissolved in ethanol and diluted to 100 μM with Robb's minimal saline (2.6 mM NaCl, 2.0 mM KCl, 0.5 mM Glucose, 0.06 mM MgSO4. 7H2O, 0.06 mM MgCl2. 6H2O, 0.05 mM CaCl2, 0.1 mM Na2HPO4, 0.018 mM KH2PO4, pH 6.75)

RAD solution (0.2 μl per fly) or control solution containing ethanol at the same dilution was injected into the ventral mid-lateral part of the abdomen and ovaries were analyzed after 24 h. For offspring analyses, females were transferred to fresh vials every day and the number of offspring counted.

Pole cell transplantation. Pole cells (embryonic germline precursor cells) from *Atg1 or Atg13* hemizygous mutant donor embryos were transplanted into host embryos derived from females homozygous for *Tm2*^{gs}, a *grandchildless* (*gs*) type of mutation.[38] As there is no germline in the *gs*-derived embryos, ovaries of *gs*-females are rudimentary and contain only the mesodermal components. Donor embryos were generated by crossing *Atg1*$^{\Delta 3D}$*/TM6B* females with *Df(3L)BSC613*/*TM3* males or *Atg13*$^{\Delta 74}$*/TM6B* females with *Atg13*$^{\Delta 81}$*/TM3* males. Pole cells were collected from single blastoderm-stage donor embryos and transplanted into 2–3 host blastoderm stage embryos.[21] Eclosing females were mated with WT males to determine the genotype of the progeny before LTR analysis. Germline chimeras with *Atg1*$^{\Delta 3D}$*/TM3, Df(3L)BSC613/TM6B* or *Atg13*$^{\Delta 74}$*/TM3, Atg13*$^{\Delta 81}$*/TM6B* as well as *TM3/TM6B* germline cells served as internal controls. Three independent experiments with a total of 11 (*Atg1*) or 14 (*Atg13*) mutant germline chimeras and 16 (*Atg1*) or 23 (*Atg13*) control sibling females were performed.

X-ray irradiation. *Atg1*$^{\Delta 3D}$*/Fs(3)Apc* late third instar larvae were X-ray irradiated for the induction of mitotic recombination (10 Gy; 110 kV, 1 mm Al filter, 0.31 Gy/min). *Apc* disrupts the function of anterior FCs, leading to the degeneration of almost all the egg primordia, with few developing to flaccid eggs lacking DAs and anterior chorion structures. *Apc* does not affect the function of the GCs. Removal of *Apc* through mitotic recombination restores FC function and allows the development of offspring from the mosaic egg primordia.[24]

Eclosing *Atg1*$^{\Delta 3D}$*/Fs(3)Apc* females were mated with WT males in single vials and egg production was analyzed every day for 12 days, a time period required to identify $\geq$95% of the mosaics. As controls, + */Fs(3)Apc* larvae were irradiated and analyzed. 17 *Atg1*$^{\Delta 3D}$*/Fs(3)Apc* or 77 + */Fs(3)Apc* mosaics deposited at total of 44 or 253 non-Apc eggs, respectively, indicating that the two types of mosaics produced non-Apc eggs with a similar frequency.

FLP induced FC clones. The FLP/FRT recombination method was used to generate FC clones. FC clones overexpressing *UAS-Rheb* were achieved by heatshocking 4-day-old females for 20 min at 34°C. FC clones mutant for *Atg1* or *Atg13* were generated by heatshocking flies of the genotypes *FRT80-Atg1*$^{\Delta 3D}$*/FRT80-UbiGFP* or *FRT82-Atg13*$^{\Delta 81}$*/FRT82-UbiGFP* for 1 h at 37°C during larval development on five consecutive days. Resulting adults were mated with WT males in single vials and egg production was monitored every day for 5 days for egg laying analysis. Laid eggs were photographed, counted and kept on 25°C until hatching. Pupae and offspring were counted.

Larval ovary transplantation. For larval ovary transplantations,[25] one mutant ovary dissected from either *Atg1*$^{\Delta 3D}$ homozygous or *Atg13*$^{\Delta 74}$*/Atg13*$^{\Delta 81}$ larvae was transplanted into *fs(1)K10* homozygous host larvae. Host females were mated with WT males in single vials for identification of the egg genotype and egg laying analysis. Host females with *Atg* mutant eggs were starved and stained with LTR.

Conflict of interest

The authors declare no conflict of interest.

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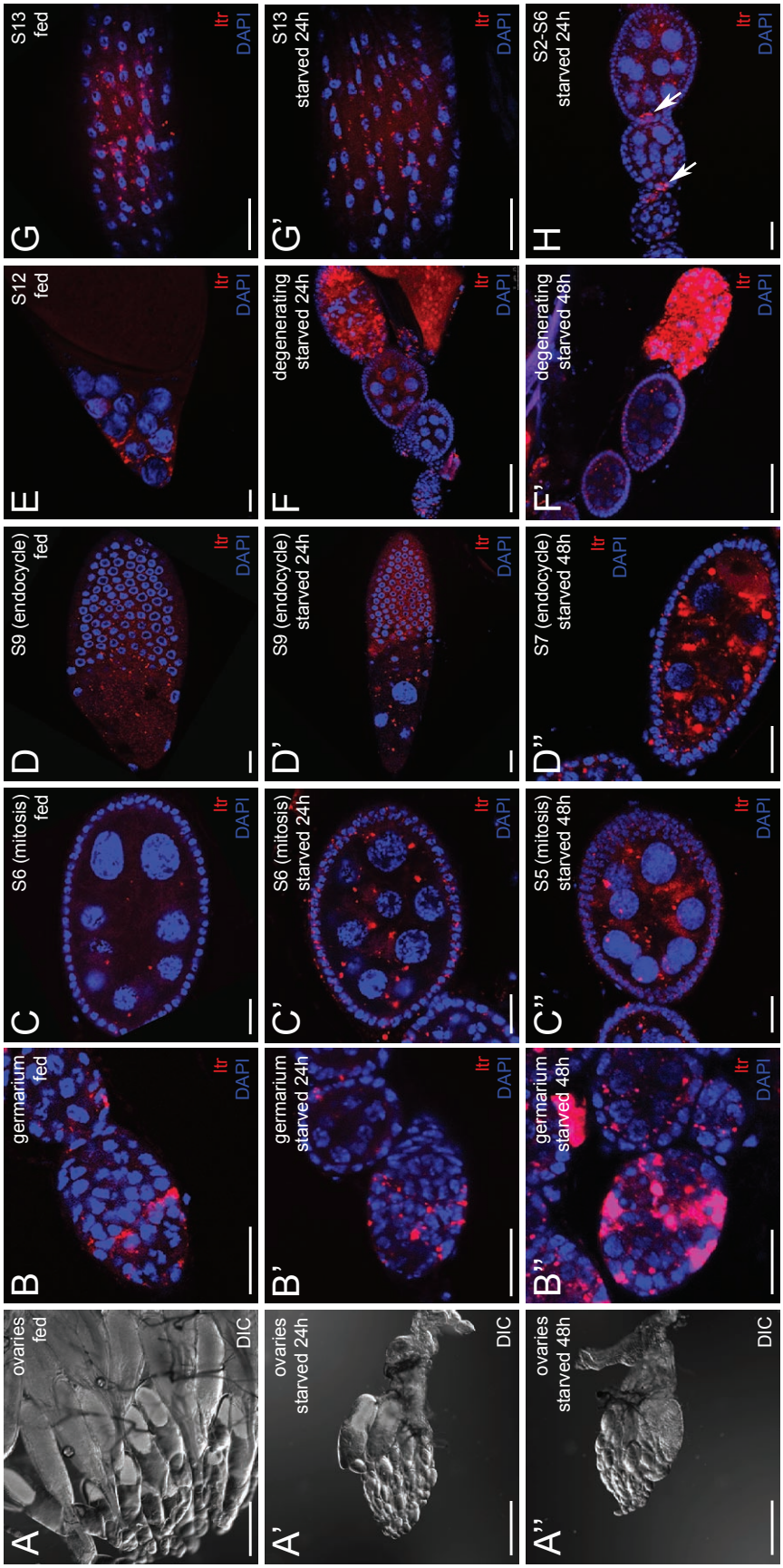
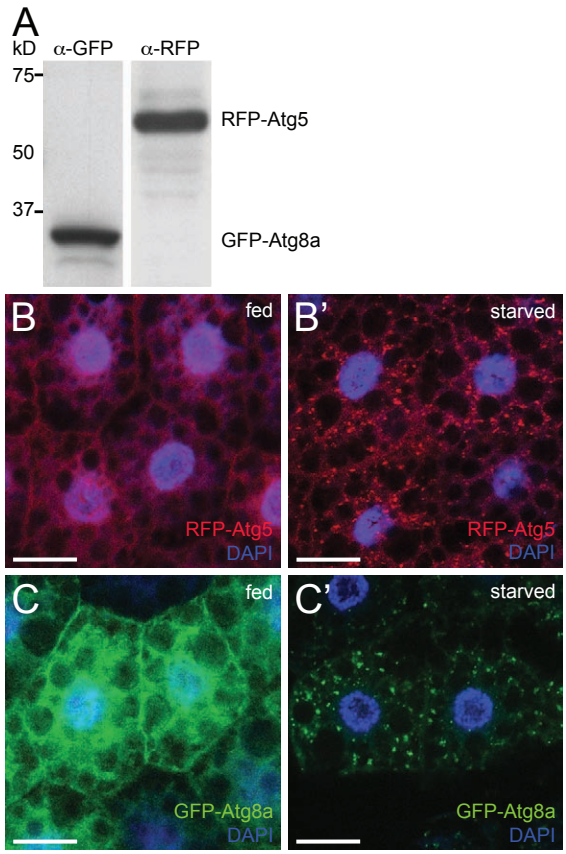
1. Melendez A, Neufeld TP. The cell biology of autophagy in metazoans: a developing story. *Development* 2008; **135**: 2347–2360.
2. Neufeld TP. TOR-dependent control of autophagy: biting the hand that feeds. *Curr Opin Cell Biol* 2010; **22**: 157–168.
3. Rusten TE, Lindmo K, Juhasz G, Sass M, Seglen PO, Brech A *et al.* Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway. *Dev Cell* 2004; **7**: 179–192.
4. Scott RC, Schuldiner O, Neufeld TP. Role and regulation of starvation-induced autophagy in the Drosophila fat body. *Dev Cell* 2004; **7**: 167–178.
5. Drummond-Barbosa D, Spradling AC. Stem cells and their progeny respond to nutritional changes during Drosophila oogenesis. *Dev Biol* 2001; **231**: 265–278.
6. Bohni R, Riesgo-Escovar J, Oldham S, Brogiolo W, Stocker H, Andruss BF *et al.* Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. *Cell* 1999; **97**: 865–875.
7. Richard DS, Rybczynski R, Wilson TG, Wang Y, Wayne ML, Zhou Y *et al.* Insulin signaling is necessary for vitellogenesis in Drosophila melanogaster independent of the roles of juvenile hormone and ecdysteroids: female sterility of the chico1 insulin signaling mutation is autonomous to the ovary. *J Insect Physiol* 2005; **51**: 455–464.
8. Stocker H, Radimerski T, Schindelholz B, Wittwer F, Belawat P, Daram P *et al.* Rheb is an essential regulator of S6K in controlling cell growth in Drosophila. *Nat Cell Biol* 2003; **5**: 559–565.
9. Werz C, Kohler K, Hafen E, Stocker H. The Drosophila SH2B family adaptor Lnk acts in parallel to chico in the insulin signaling pathway. *PLoS Genet* 2009; **5**: e1000596.
10. Peterson JS, Barkett M, McCall K. Stage-specific regulation of caspase activity in drosophila oogenesis. *Dev Biol* 2003; **260**: 113–123.
11. Hou YC, Chittaranjan S, Barbosa SG, McCall K, Gorski SM. Effector caspase Dcp-1 and IAP protein bruce regulate starvation-induced autophagy during Drosophila melanogaster oogenesis. *J Cell Biol* 2008; **182**: 1127–1139.
12. Nezis IP, Lamark T, Velentzas AD, Rusten TE, Bjorkoy G, Johansen T *et al.* Cell death during *Drosophila melanogaster* early oogenesis is mediated through autophagy. *Autophagy* 2009; **5**: 298–302.
13. Juhasz G, Erdi B, Sass M, Neufeld TP. Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila. *Genes Dev* 2007; **21**: 3061–3066.
14. Scott RC, Juhasz G, Neufeld TP. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. *Curr Biol* 2007; **17**: 1–11.
15. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T *et al.* LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 2000; **19**: 5720–5728.
16. Gorski SM, Chittaranjan S, Pleasance ED, Freeman JD, Anderson CL, Varhol RJ *et al.* A SAGE approach to discovery of genes involved in autophagic cell death. *Curr Biol* 2003; **13**: 358–363.
17. Juhasz G, Puskas LG, Komonyi O, Erdi B, Maroy P, Neufeld TP *et al.* Gene expression profiling identifies FKBP39 as an inhibitor of autophagy in larval Drosophila fat body. *Cell Death Differ* 2007; **14**: 1181–1190.
18. Zinke I, Schutz CS, Katzenberger JD, Bauer M, Pankratz MJ. Nutrient control of gene expression in Drosophila: microarray analysis of starvation and sugar-dependent response. *EMBO J* 2002; **21**: 6162–6173.
19. Berry DL, Baehrecke EH. Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila. *Cell* 2007; **131**: 1137–1148.
20. Saucedo LJ, Gao X, Chiarelli DA, Li L, Pan D, Edgar BA. Rheb promotes cell growth as a component of the insulin/TOR signalling network. *Nat Cell Biol* 2003; **5**: 566–571.
21. Illmensee K, Mahowald AP. Transplantation of posterior polar plasm in Drosophila. Induction of germ cells at the anterior pole of the egg. *Proc Natl Acad Sci USA* 1974; **71**: 1016–1020.
22. Chang YY, Neufeld TP. An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. *Mol Biol Cell* 2009; **20**: 2004–2014.
23. Bass BP, Tanner EA, Mateos San Martin D, Blute T, Kinser RD, Dolph PJ *et al.* Cell-autonomous requirement for DNaseII in nonapoptotic cell death. *Cell Death Differ* 2009; **16**: 1362–1371.
24. Erdelyi M, Szabad J. Isolation and characterization of dominant female sterile mutations of Drosophila melanogaster. I. Mutations on the third chromosome. *Genetics* 1989; **122**: 111–127.
25. Beadle GW, Ephrussi B. Ovary transplants in Drosophila melanogaster: meiosis and crossing-over in superfemales. *Proc Natl Acad Sci USA* 1937; **23**: 356–360.
26. Wieschaus E, Marsh JL, Gehring W. fs(1)K10, a germline-dependent female sterile mutation causing abnormal chorion morphology in Drosophila melanogaster. *Wilhelm Roux's Arch Dev Biol* 1978; **184**: 75–82.
27. Poulton JS, Deng WM. Cell-cell communication and axis specification in the Drosophila oocyte. *Dev Biol* 2007; **311**: 1–10.
28. Cavaliere V, Donati A, Hsouna A, Hsu T, Gargiulo G. dAkt kinase controls follicle cell size during Drosophila oogenesis. *Dev Dyn* 2005; **232**: 845–854.
29. Clancy DJ, Gems D, Harshman LG, Oldham S, Stocker H, Hafen E *et al.* Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. *Science* 2001; **292**: 104–106.
30. Flatt T, Min KJ, D'Alterio C, Villa-Cuesta E, Cumbers J, Lehmann R *et al.* Drosophila germ-line modulation of insulin signaling and lifespan. *Proc Natl Acad Sci USA* 2008; **105**: 6368–6373.
31. Dunaif A, Thomas A. Current concepts in the polycystic ovary syndrome. *Annu Rev Med* 2001; **52**: 401–419.
32. Avellaira C, Villavicencio A, Bacallao K, Gabler F, Wells P, Romero C *et al.* Expression of molecules associated with tissue homeostasis in secretory endometria from untreated women with polycystic ovary syndrome. *Hum Reprod* 2006; **21**: 3116–3121.
33. Buszczak M, Cooley L. Eggs to die for: cell death during Drosophila oogenesis. *Cell Death Differ* 2000; **7**: 1071–1074.
34. Terashima J, Bownes M. Translating available food into the number of eggs laid by Drosophila melanogaster. *Genetics* 2004; **167**: 1711–1719.
35. Berg CA. The Drosophila shell game: patterning genes and morphological change. *Trends Genet* 2005; **21**: 346–355.
36. Nezis IP, Stravopodis DJ, Margaritis LH, Papassideri IS. Programmed cell death of follicular epithelium during the late developmental stages of oogenesis in the fruit flies Bactrocera oleae and Ceratitis capitata (Diptera, Tephritidae) is mediated by autophagy. *Dev Growth Differ* 2006; **48**: 189–198.
37. Thumm M, Kadowaki T. The loss of Drosophila APG4/AUT2 function modifies the phenotypes of cut and Notch signaling pathway mutants. *Mol Genet Genomics* 2001; **266**: 657–663.
38. Erdelyi M, Michon AM, Guichet A, Glotzer JB, Ephrussi A. Requirement for Drosophila cytoplasmic tropomyosin in oskar mRNA localization. *Nature* 1995; **377**: 524–527.
39. Galanopoulos VK, Orr W, Szabad J, Kafatos FC. Genetic analysis of chorion formation in Drosophila melanogaster: I. The effects of one somatic-specific and seven germ-line-specific mutations. *Dev Genet* 1989; **10**: 87–97.

Supplementary information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)



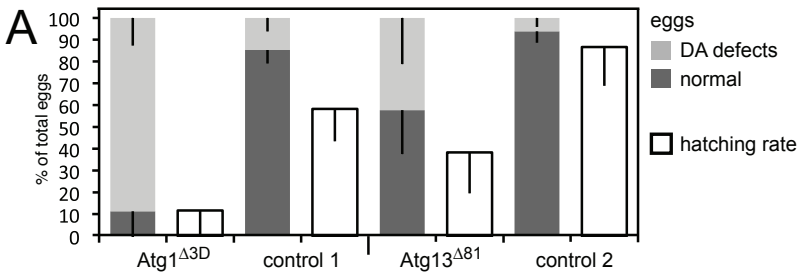
Suppl. FIG.1 Starvation-induced autophagy during oogenesis
Starvation of *Drosophila* females leads to degeneration of stage 8 and 9 egg chambers (F, F'), causing smaller ovaries containing only few vitellogenic and mature eggs (A-A''). Lack of nutrients also results in an accumulation of autolysosomes (marked by LTR) in the germarium (B-B''), in FCs during mitosis stages 2 to 6 (C-C''), in FCs during endoreplication stages 7 to 9 (D-D'') and in anterior and posterior FCs (H), most likely representing polar cells. Despite starvation-induced, also developmental autophagy exists in degenerating NCs at stage 12 to 13 (E) and mature FCs at stage 13 (G, G'). Scale bars: (A-A'') 200µm, (B-B'', C- C'', D- D'', E, H) 20 µm, (F, F', G, G' 50µm). Genotypes: *y w*

Suppl. FIG.2 Expression and functionality of fluorescently-tagged Atg proteins



(A) Western blot of *Drosophila* Schneider-2 cells, expressing GFP-tagged dAtg8 and RFP-tagged dAtg5, respectively, under the control of actin-Gal4. **(B-C)** Starvation of animals expressing theses markers induces the formation of punctuated structures in *Drosophila* fat body cells. Genotypes: (B) *da-GAL4/UAS-RFP-dAtg5, da-GAL4/UASp-dAtg8a*

Suppl. FIG. 3 Percentage of defective eggs and hatch rates of eggs containing hs-flp induced FC clones



(A) Shown are percentages of eggs displaying DA defects (light grey bars) and normal eggs (dark grey bars) and the hatch rates from these eggs (open bars). Approximately 10 females per genotype were monitored over 4 days. Error bars show SD of the mean. Genotypes: *Atg1$^{\Delta 3D}$-FRT80B/FRT80-UbiGFP, FRT80isogenic/FRT80-UbiGFP* (control 1), *Atg13$^{\Delta 81}$-FRT82/FRT82-UbiGFP, FRT82w+y+/FRT82-UbiGFP* (control 2). Statistic analyses of hatching rates are provided in figure 4H.

2.2 Lack of autophagy in *Drosophila* follicle cells causes egg chamber defects and modulates Notch signaling

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2011, manuscript in preparation

Abstract

Regulation of cell-cell signaling pathways has been demonstrated to involve endocytosis and endosomal trafficking. Furthermore, autophagy and endosomal pathways are known to intersect in order to target vesicles for lysosomal degradation. However, the role of autophagy in the modulation of cell-cell signaling pathways remains to be investigate. We previously showed that autophagy in follicle cells (FCs), but not in germ cells (GCs), is required for egg development in *Drosophila*, and that this depends on the cellular context. Here, we demonstrate that the lack of autophagy in FCs causes severe egg chamber defects and that autophagy is especially required in the posterior FCs. We could further show that loss of autophagy modulates Notch signaling in the FCs and propose a model in which autophagy is important for correct receptor activation. These findings reveal a novel function for autophagy in the modulation of endocytosed receptors and will contribute to the understanding of Notch pathway regulation.

Introduction

Autophagy is a tightly regulated intracellular lysosomal degradation process occurring in all eukaryotic cells from yeast to mammals. Under normal induction, as for example during cellular stress, unnecessary cytosolic components are recycled to promote cell survival. However, autophagy can also lead to programmed cell death and is needed throughout normal development. Furthermore, it plays a role in immunity, lifespan extension and many human pathophysiologies, such as neurodegeneration and cancer (Chen and Klionsky, 2011).

In *Drosophila*, it has been shown that autophagy is crucial during metamorphosis in the remodeling of larval tissues such as the fatbody and salivary glands and that starvation can induce autophagy in nutrient responding organs, *e.g.* the fatbody and the ovaries (Rusten *et al.*, 2004; Scott *et al.*, 2004; Berry and Baehrecke, 2007; Barth *et al.*, 2011). Nutrient depletion induces autophagy at several “check points” during *Drosophila* oogenesis. The first check point in region 2b in the germarium stains positive for autophagic as well as apoptotic markers during normal development but this is highly elevated under starvation conditions (Drummond-Barbosa and Spradling, 2001; Hou *et al.*, 2008; Nezis *et al.*, 2009; Barth *et al.*, 2011). Similar observations

have also been made for the second check point during mid-oogenesis. At this stage, degenerating egg chambers display markers for autophagy and eggs having a *ATG* mutant germline are impaired to activate autophagy, but also DNA fragmentation, which is denotive for apoptosis (Peterson *et al.*, 2003; Velentzas *et al.*, 2007; Hou *et al.*, 2008; Nezis *et al.*, 2009).

In addition to starvation-induced autophagy, developmental autophagy has also been reported in germ cells (GCs) and follicle cells (FCs) during oogenesis. Late stage FCs undergo cell death after chorion deposition, showing the appearance of autophagic structures and condensed chromatin, but no DNA fragmentation, which suggests a mechanism independent of caspases (Nezis *et al.*, 2006; Barth *et al.*, 2011). It has also been shown that developmental cell death in the germarium region 2 and during mid-oogenesis, as well as the nurse cell death occurring in late oogenesis depend on autophagy (Velentzas *et al.*, 2007; Nezis *et al.*, 2009). Recently, Nezis *et al.* demonstrated that autophagosomal markers accumulate in dying stage 13 nurse cells and that egg chambers mutant for *ATG1*, *ATG13* and *Vps34* in the germline showed no DNA fragmentation, but persisting nurse cells nuclei (PNCN), thus suggesting that autophagy is required in the germline (Nezis *et al.*, 2010). This is contrary to our observations that *ATG* mutant GCs give rise to normal eggs without the appearance of PNCNs. In contrast, we show that *ATG* gene deficiency in the FCs lead to defective eggs, indicating that autophagy is necessary in the FCs, but not the GCs, to support proper egg development (Barth *et al.*, 2011). Accordingly, we suggested that these varying findings are due to the different methods used. Nezis et al. used the flippase recognition target (FLP-FRT) mediated Ovo^D technique (Nezis *et al.*, 2010), which produces mutant GCs, but also clones of mutant FCs, whereas in the pole cell transplantation experiments, GCs are mutant but FCs are completely wild-type (WT) (Barth *et al.*, 2011). Thus, the defect in nurse cell nuclei clearance observed in the Ovo^D experiments may be due to the lack of autophagy in the FCs rather than in the GCs. Interestingly, autophagy deficiency only affects oogenesis in a cellular context in which FCs are mutant for *ATG* genes and GCs are WT, indicating that a dysfunctional signaling between soma and germline may be responsible for the oogenesis defects (Barth *et al.*, 2011).

Several classical signaling pathways during oogenesis are shared between the GCs and FCs and are essential for cell differentiation and axis specification (Poulton and Deng, 2007). For example, Gurken protein translated by the oocyte activates epidermal growth factor receptor (EGFR) signaling in the adjacent terminal FCs, defining them as posterior FCs (González-Reyes et al., 1995). In turn, a yet unknown signal from the newly defined posterior FCs to

the oocyte (back signaling) triggers a reconstruction of the microtubuli network, causing the oocyte nucleus to move from the posterior side to an asymmetrical anterior position, which subsequently will be defined as the dorsal side of the egg chamber by a second round of Gurken/EGFR signaling from the oocyte to the overlaying FCs (González-Reyes *et al.*, 1995; Chang and Neufeld, 2010). Furthermore, Gurken signaling also guides dorsal migration of the border cells (Duchek and Rorth, 2001). On the other hand, signaling of the germline expressed ligand Delta to the Notch receptor expressed by FCs leads to differentiation of polar cells in early stages, a switch from the mitotic to an endoreplication program during mid-oogenesis and the correct differentiation of dorsal appendage (DA) roof and floor cells in late oogenesis (López-Schier and Johnston, 2001; Ward *et al.*, 2006; Assa-Kunik *et al.*, 2007). For both pathways, EGFR and Delta-Notch, it has been shown that endocytosis and endosomal trafficking is required within ligand and/or receptor presenting cells for activation, regulation and degradation of the signal (Yamamoto *et al.*, 2010; Avraham and Yarden, 2011). Since it has been shown that loss of the cysteine protease *ATG4* modulates Notch signaling in the *Drosophila* wing, and that fatbody cells mutant for the phosphatidylinositol 3-kinase Vps34 accumulate Notch, it is tempting to speculate that impaired autophagy may lead to dysregulation of endosomal trafficking of Notch or other receptors (Thumm and Kadowaki, 2001; Juhasz *et al.*, 2008).

In this study, we extend our analyses on the role of autophagy in the FCs during oogenesis. We provide further evidence for the necessity of autophagy in the somatic FCs and the involvement of autophagy in the modulation of cell-cell signaling pathways. By using the FRT-FLP method, we show that FCs mutant for *ATG* genes exhibit several phenotypes similar to mutants with defects in the classical cell-cell signaling pathways. Furthermore, we could designate specific FC subpopulations that are involved in the autophagy-dependent control of egg development by using spatially restricted interfering RNA (RNAi) mediated knock down. Finally, we provide evidence for the involvement of autophagy in the modulation of the Notch signaling pathway.

These results reveal a novel function of autophagy and open exciting opportunities to examine the influence of autophagy on receptor/ligand regulation. As Notch plays important roles in tissue differentiation and tumorigenesis, the understanding of its signal modulation will be of special value.

(I) Ovaries lacking *ATG1* in the FCs exhibit multiple egg chamber defects

During *Drosophila* oogenesis, small changes can disturb the precise control of egg development, leading to misshaped egg chambers and malformed mature eggs. Accordingly, by generation of *ATG1* mutant FC clones with the FLP-FRT method, multiple egg chamber defects were observed. The most prominent phenotype were egg chambers with an abnormal number of germline cysts. Many of the eggs showed more than the usual 16 cell cysts normally observed in WT egg chambers (FIG. 1 A, A') and was represented in 33% of the egg chambers containing *ATG1* mutant FC clones (4% in control WT clones), whereas a reduction in cyst number compared to WT eggs was observed less frequently (FIG. 1 B, B'). Eggs presenting more germline cysts are composed of two fused egg chambers (compound egg chambers), where the mutant FCs have not migrated between the germline cysts. In some cases, single egg chambers within one ovariole showed a wrong orientation (FIG. 1 C, C') or featured two oocytes (FIG. 1 D, D') and various ovarioles were lacking stalk cells that interconnect the single egg chambers (FIG. 1 A, A', E, E'). Many stage 14 egg chambers also displayed persisting nurse cell nuclei (PNCN) (FIG. 1 H-I, arrowheads), a phenotype that was previously described for *ATG* mutant germline clones achieved with the Ovo^D system (TABLE 1) (Nezis *et al.*, 2010). However, in stage 14 eggs with an *ATG* mutant germline generated by pole cell transplantations, PNCNs could not be detected (TABLE 1) (Barth *et al.*, 2011). Thus, we suggest that *ATG* mutant FC clones, which are also induced using the Ovo^D method (SUPPL. FIG. 1), are responsible for the presence of PNCNs in those eggs. In fact, both heat-shock FLP induced *ATG* mutant FC clones (which also generate occasional germline clones, see Barth et al., 2011), and e22c-FLP induced *ATG* mutant FC clones (which exclusively lead to FC clones, see Duffy et al., 1998) produce PNCNs (TABLE 1). Interestingly, PNCNs are found in both situations, in eggs with an *ATG* mutant or WT germline, as long as the FCs are autophagy deficient (data not shown). Additionally, compound egg chambers (HS-FLP induced 6%, control WT clones 0%, and e22c-FLP induced 17%, control WT clones 7%) and PNCNs (TABLE 1) could also be observed by generation of *ATG13* mutant FC clones and by clone induction using e22c-FLP. Furthermore, we have previously shown that mature eggs with

ATG1 and ATG13 mutant FC clones often display missing, shortened or malformed DAs (FIG. 1 F, G, arrowheads) (Barth *et al.*, 2011). Taken together, this selection of phenotypes obtained through the generation of *ATG1* mutant clones solely in the FCs in combination with our published data, where an imbalance of autophagy between GCs and FCs causes egg chamber defects, strongly suggests a role for autophagy in the FCs of *Drosophila* ovaries.

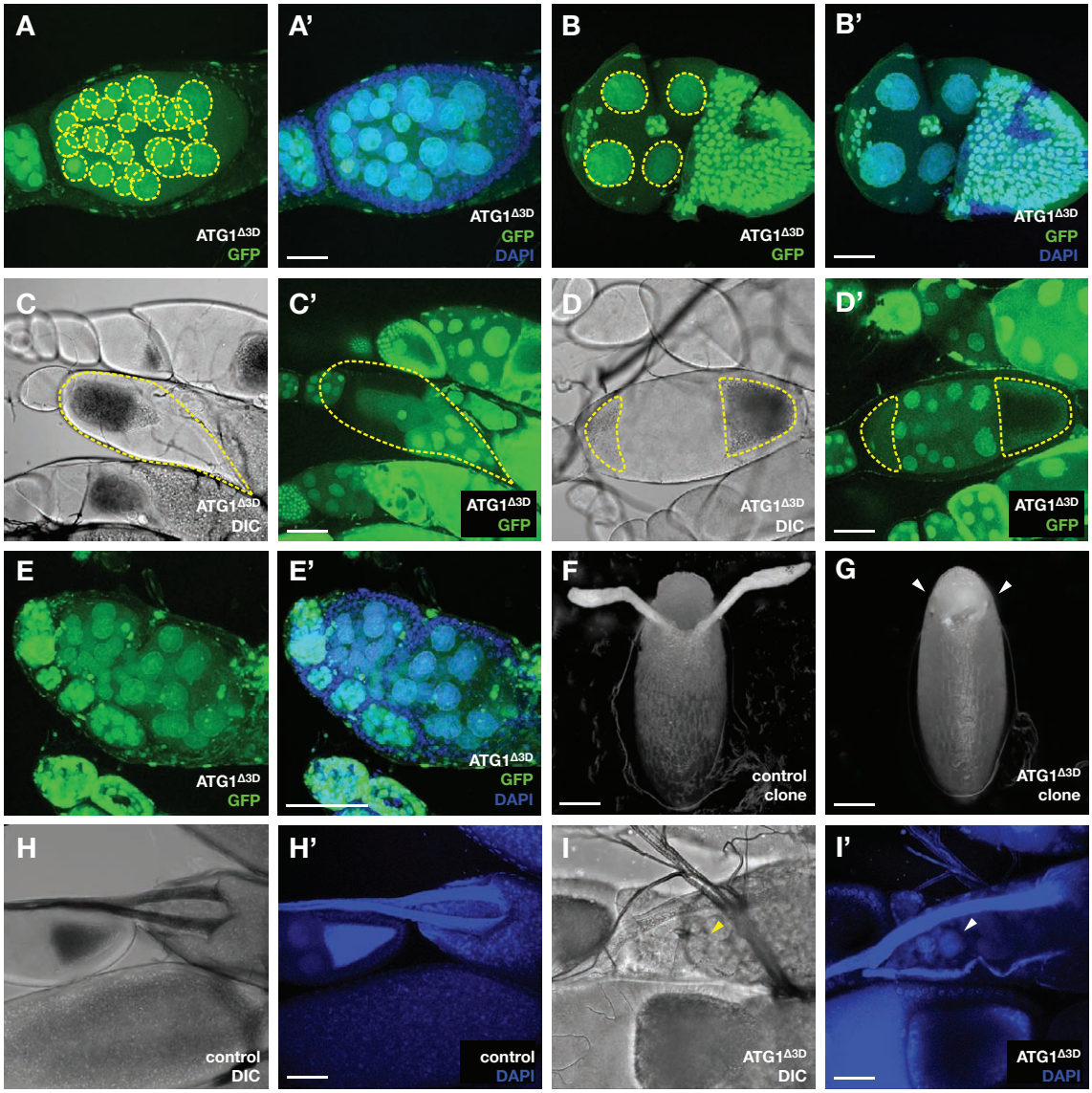

FIGURE 1. Lack of *ATG1* in the FCs affects proper egg development. Heat shock flippase (HS-FLP) mediated generation of FC clones mutant for *ATG1* (marked by the lack of GFP) caused a wide array of severe defects during oogenesis. We often observed compound eggs with more than 16 cysts (outlined in yellow) **(A, A')** and ovarioles that lack stalk cells between the egg chambers **(E, E')**. Some eggs had less than the 16 germline cyst normally present in WT eggs (outlined in yellow) **(B, B')**. Less frequently, inverted eggs containing the oocyte in the wrong position (outlined in yellow) **(C, C')** or eggs with two oocytes (outlined in yellow) were observed **(D, D')**. Mature eggs regularly lack DAs (arrowheads) **(F, G)**. Stage 14 egg chambers often contain PNCN (arrowheads) **(I, I')**, which are already degenerated in control eggs with WT clones **(H, H')**. Anterior is to the left, posterior to the right, except F, G: anterior to the top, posterior to the bottom, dorsal to the front. Scale bar: 50 μm Genotypes: A-E, G and I: *hs flp/+; ATG1$^{\Delta 3D}$ FRT80B/FRT80B-UbiGFP*. F and H: *hs flp/+; FRT80Biso/FRT80B-UbiGFP*.

FCs are important for axis specification and patterning of the egg, and functional cell-cell signaling between the different tissues and cells is crucial for proper development (Poulton and Deng, 2007).

We could previously show that autophagy deficiency only affects oogenesis in a cellular context in which FCs are mutant for *ATG* genes and GCs are WT (Barth *et al.*, 2011). Therefore we hypothesize that autophagy could be implicated in the modulation of signal transduction pathways required for oogenesis. Interestingly, many of the phenotypes described here are observed in mutants of the classical signaling pathways that are needed for cell differentiation and axis specification: Delta-Notch, JAK/STAT and EGFR. For example, egg chambers with Notch mutant FC clones lack stalk cells and have encapsulation defects, resulting in compound egg chambers with more than the normal number of cysts (López-Schier and Johnston, 2001). Fused egg chambers as well as mislocalization of the oocyte are also observed in mutants of the JAK/STAT and EGFR pathway (Goode *et al.*, 1996; McGregor *et al.*, 2002). In addition, defects in several signaling pathways result in misshaped egg chambers and malformed dorsal appendages (Berg, 2005). For example, egg chambers mutant for Slimb, a *Drosophila* Skp, Cullin, F-box containing (SCF) complex member targeting proteins for proteasomal degradation show several of the above described phenotypes and Slimb has been implicated in the regulation of the Dpp and Notch pathways (Muzzopappa and Wappner, 2005; Matsumoto *et al.*, 2011). However, we did not observe the generation of ectopic polar cells, a phenotype described for the deregulation of the hedgehoge (hh) pathway (data not shown) (Forbes *et al.*, 1996).

TABLE 1. Quantification of persisting nurse cell nuclei comparing different methods used

	persisting NC nuclei (PNCN)			
Genotype	PCT	*Ovo^{D}*	*HS-FLP* induced	*e22c* induced
ATG1$^{\Delta 3D}$	3.7 +/- 3.6	62.2 +/- 5.9*	41.2 +/- 2.9	57.2 +/- 6.8
control	1.2 +/- 2.2	5.4 +/- 2.2*	11.0 +/- 2.2	5.9 +/- 1.0
ATG13$^{\Delta 74}$	0 +/- 0	60 +/- 4.6*	36.5 +/- 2.9	24.4 +/- 4.2
control	0 +/- 0	5.4 +/- 2.2*	15.4	1.5 +/- 2.2

Total number of egg chambers counted: PCT, *ATG1$^{\Delta 3D}$*: 144; control: 132; *ATG13$^{\Delta 74}$*: 89; control: 79 .HS-FLP, *ATG1$^{\Delta 3D}$*: 171; control: 269; *ATG13$^{\Delta 74}$*: 153; control: 13. e22c-FLP: *ATG1$^{\Delta 3D}$*: 56; control: 84; *ATG13$^{\Delta 74}$*: 93; control: 86. * Representative results from Nezis et al., 2010, control is *w^{1118}*. Abbreviations: PCT, pole cell transplantation; HS-FLP, heat-shock flippase.

(II) Specialized FC subpopulations are responsible for the autophagy-dependent DA defects

The phenotypes observed in egg chambers with *ATG1* mutant FCs point to an essential function of autophagy in the modulation of a signal transduction pathway during ovary development. As the performance and impact of many signaling pathways is restricted to specific FC subpopulations, we decided to localize the FC type that is responsible for the defects observed in eggs containing *ATG* mutant FCs. Spatially defined GAL4 driver lines to express RNAi against different *ATG* genes were used to knock down autophagy (Brand and Perrimon, 1993). In order to verify the specific activity of the GAL4 lines in certain FC subpopulations, we first documented the expression pattern using a UAS-GFP construct (FIG. 2). The broadest pattern, showing expression in nearly all FCs from the somatic follicular stem cells until late oogenesis, was observed using the e22c-GAL4 driver line (Duffy *et al.*, 1998) (FIG. 2 A and I). Slow border cell (slbo) -GAL4 is especially expressed in the border cells, a group of migratory cells that travels together with the anterior polar cells to the oocyte in stage 9 eggs and later forms the micropyle (Montell, 2003). Slbo-GAL4 is also expressed in stretched and columnar FCs at the dorsal anterior side (centripetal FCs) and the posterior end. It can be detected already in posterior FCs at early stage 9 when border cells at the anterior tip start to express slbo (Rorth *et al.*, 1998) (FIG. 2 B and I). Fru-GAL4 (fruitless-GAL4, also 168-GAL4) is expressed in the interconnecting stalk cells, in anterior and posterior FCs starting from stage 6, in border cells, stretched FCs and in very posterior columnar FCs (FIG. 2 C and I). The c355-GAL4 driver is expressed from stage 7 onwards in all cells including border, stretched and columnar cells, but not in polar cells (Manseau *et al.*, 1997) (FIG. 2 D and I). c306-GAL4 drives expression in stalk cells, weak in anterior FCs, stronger in posterior FCs, border cells, stretched cells and columnar FCs similar to slbo (Manseau *et al.*, 1997) (FIG. 2 E and I). The 109-30-GAL4 driver is expressed only in stalk cells and in the stalk precursor cells in the germarium (Hartman *et al.*, 2010) (FIG. 2 F and I). Unpaired (upd) -GAL4 (Bai and Montell, 2002) drives expression exclusively in polar cells (FIG. 2 G and I), a pair of specialized FCs at the anterior and posterior end of the egg chamber that function as organizer cells (Montell, 2003). Eyeless (ey) -GAL4 served as control and is not expressed in the ovaries (FIG. 2 H and I).

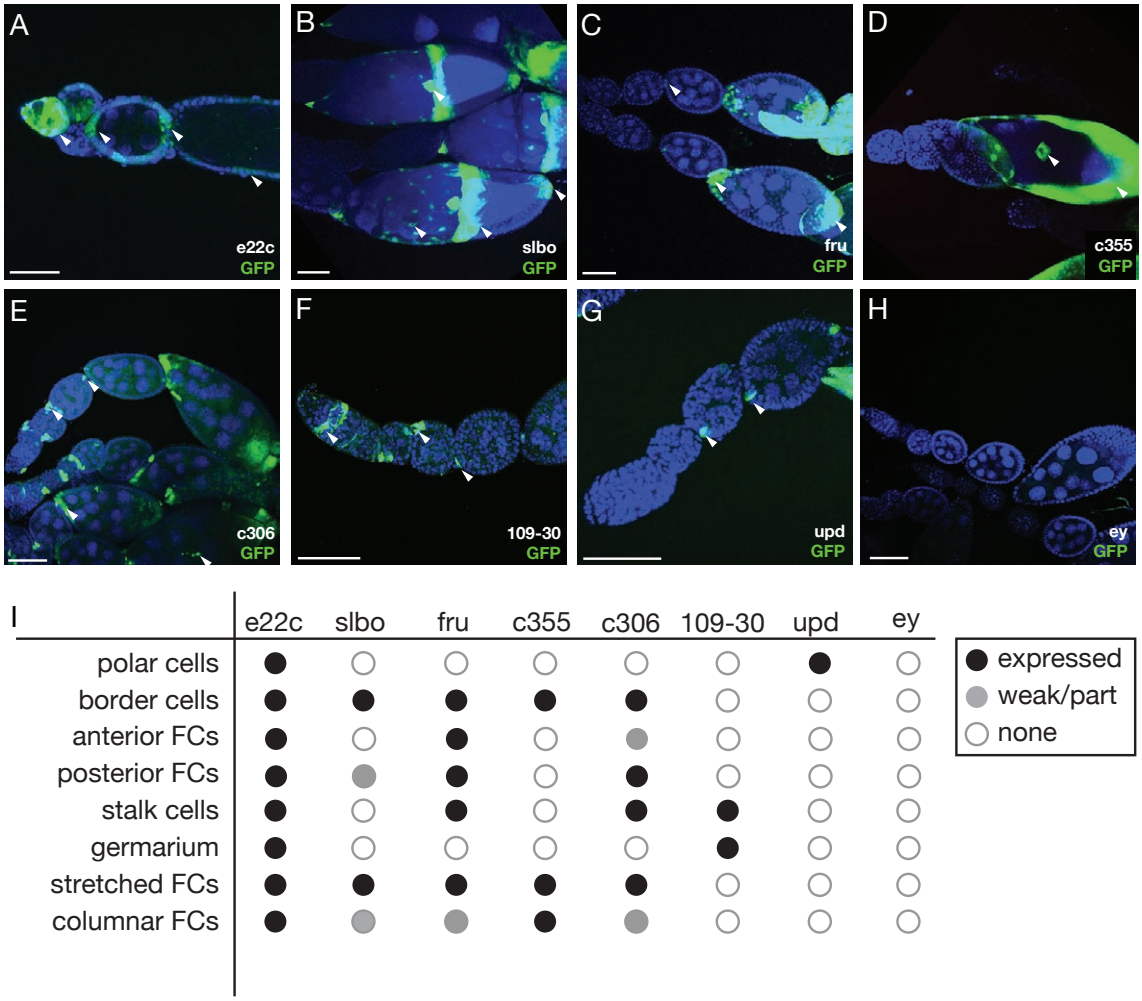

FIGURE 2. GFP expression pattern of different GAL4 driver lines. A) e22c is expressed in the follicular stem cells and thus in all FCs, albeit patchy. **B)** Slbo is strongly expressed in border cells, in stretched FCs and in columnar FCs at the dorsal anterior side (centripetal FCs) and the posterior end. It is also expressed in posterior FCs at early stage 9 when border cells at the anterior tip start to express slbo. **C)** Fru-GAL4 (168-GAL4) is expressed in stalk cells, in anterior and posterior FCs from stage 6/7 on, in border cells, stretched FCs and in the very posterior columnar FCs. **D)** c355 is expressed from stage 7 onwards in all cells including border, stretched, and columnar cells, but not in polar cells. **E)** c306 is expressed in stalk cells, weakly in anterior FCs, stronger in posterior FCs, border cells, stretched cells and columnar FCs, similar to slbo. **F)** 109-30 is expressed in some cells in the germarium and in stalk cells. **G)** Upd is exclusively expressed in the polar cells. **H)** ey-GAL4 served as control and is not expressed in the ovaries. **I)** Summary of the expression patterns of the GAL4 lines used. Anterior/posterior FCs corresponds to the stages 6/7 to early 9, stretched and columnar to stages 9 till stage 10/11. Anterior is to the left, posterior to the right. Scale bar: 50 *µ*m. Genotypes: A: *e22c-GAL4/ UAS-GFP,* B: *slbo-GAL4/UAS-GFP,* C: *fru(168)-GAL4/UAS-GFP,* D: *c355-GAL4/+; UAS-GFP/+,* E: *c306-GAL4/+; UAS-GFP/+,* F: *109-30-GAL4/UAS-GFP,* G: *upd-GAL4/+; UAS-GFP/+,* H: *ey-GAL4/UAS-GFP*.

As the most persistent phenotype of eggs containing *ATG* mutant FC are malformed, shortened or missing dorsal appendages (DAs) (FIG. 1 G) (Barth *et al.*, 2011), we scored the frequency of this phenotype as a readout for the effect of *ATG* knock down in certain FC subpopulations. The e22c-GAL4 driver

is expressed in virtually all FCs and as expected, this comprehensive expression pattern led to the most severe DA defects, resulting in 44% eggs with missing or malformed DAs after expression of ATG1-RNAi (FIG. 3 A and C). Also, expression of ATG4-RNAi (36%), and ATG5-RNAi (13%) with the e22c-GAL4 driver resulted in significantly more eggs with DA defects when compared to control eggs (lacZ-RNAi, 3%) (FIG. 3 A and B, C', C"). Expression of ATG8-RNAi led to pupal lethality probably due to expression of the e22c-GAL4 driver in other tissues during development and the strength of the RNAi line used (FIG. 3 A). We obtained a slightly weaker DA phenotype by expression of ATG1-RNAi with slbo-GAL4 (27%) or fru-GAL4 (34%) (FIG. 3 A and D, E), and as for e22c-GAL4, the expression of ATG4-RNAi with slbo-GAL4 (24%) and fru-GAL4 (18%) was less severe than for ATG1-RNAi (FIG. 3 A and D', E'). Expression of ATG5-RNAi with slbo-GAL4 resulted in a minor number of defective eggs, however, the severity of DA defects was comparable to those obtained with the other ATG-RNAi lines (FIG. 3 D").

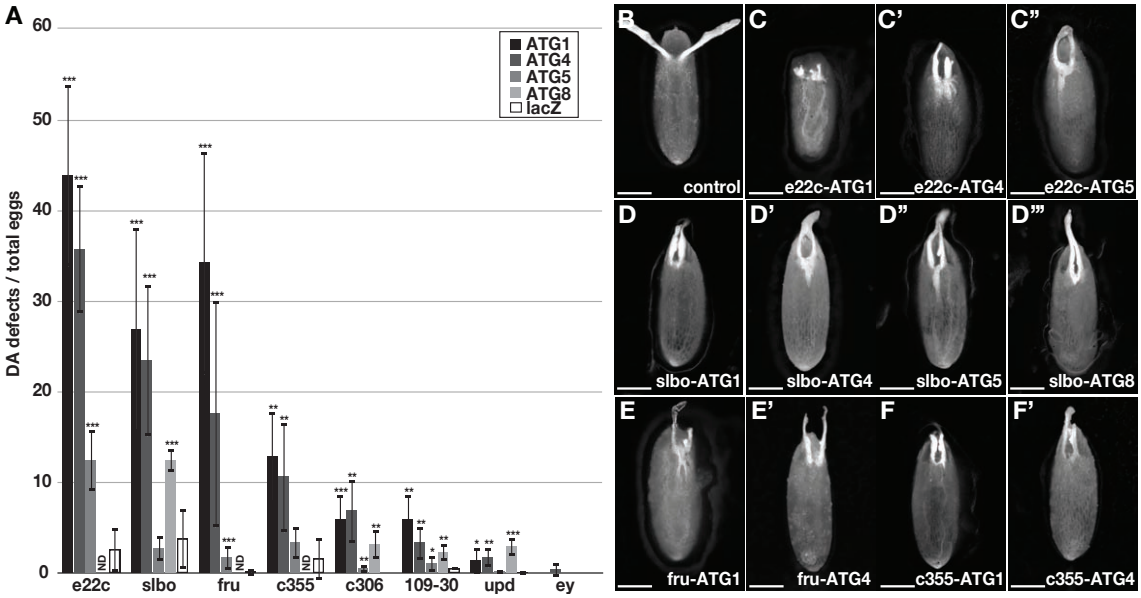

FIGURE 3. Inhibition of autophagy in certain FC subtypes causes differently pronounced phenotypes. Quantification of DA defects in eggs expressing *ATG1, ATG4, ATG5* and *ATG8* RNAi with GAL4 driver lines that are expressed in certain FC subpopulations. Downregulation of *ATG* gene expression with the broad e22c-GAL4 driver, which is expressed in all FCs from the follicular stem cell until late stages, causes the most severe DA defects **(A and C-C")**. Knocking down *ATG* genes with slbo-GAL4 and fru-GAL4 causes similar strong phenotypes **(A and D-E')**. Overexpression of ATG RNAi with c355-GAL4, c306-GAL4, 109-30-GAL4 and upd-GAL4 only generated a low percentage of eggs with defective DAs **(A and F, F').** Similarly, the controls (UAS-lacZ, ey-GAL4) only occasionally showed defective eggs **(A and B)**. A: Error bars show S.D. of the mean, *P*-values: **P*<0.05, ***P*<0.01, ****P*<0.001. B-F': Anterior is to the top, posterior to the bottom, dorsal to the front. Scale bar: 100 μm. Genotypes: GAL4 driver see FIG. 2, ATG-RNAi lines see SUPPL. FIG. 2.

Expression of ATG8-RNAi with fru-GAL4 was again lethal for the flies, but expression with slbo-GAL4 led to significant DA defects (13%) (FIG. 3 A). c355-GAL4 driven expression of ATG1- and ATG4-RNAi still led to a defect DA rate of 13% and 11%, respectively (FIG. 3 A and F, F'), but expression of all other ATG-RNAi lines with the remaining GAL4 drivers (c355-, c306-, 109-30-, and upd-) generally resulted in rates of eggs with defective DAs below 7% (FIG. 3 A). Control expression of all RNAi constructs with ey-GAL4, which is not expressed during oogenesis, showed no DA defects (FIG. 2 I and H, FIG. 3 A). In general, it seemed that ATG1-RNAi causes the strongest phenotypes, followed by ATG4-, ATG8- and ATG5-RNAi. Expression of lacZ-RNAi with the FC-GAL4 lines (FIG. 3 A, white bars) and expression of ATG-RNAi using ey-GAL4 (FIG. 3 A) as a control only occasionally showed defective DAs.

In order to examine the efficiency of ATG-RNAi expression on the progression of autophagy, we induced clones in the larval fatbody expressing the ATG-RNAi constructs using the FLP-out/GAL4 technique (SUPPL. FIG. 2) (Ito *et al.*, 1997). The fat body of *Drosophila*, a nutrient storage organ, rapidly reacts to starvation with the induction of autophagy, which can be easily monitored by lysotracker (LTR) staining (Scott *et al.*, 2004). Under fed conditions, LTR staining is diffuse, but accumulates in dots under starvation conditions (SUPPL. FIG. 2 A-B'). In fat body cell clones expressing the different RNAi lines, autophagy was inhibited as visualized by a strong reduction in LTR dots under starvation when compared to surrounding WT cells, indicating that the applied RNAi lines effectively knocked down *ATG* gene expression (SUPPL. FIG. 2 C-F').

In summary, inhibition of autophagy in follicular subgroups showed the strongest effect with the e22c-, slbo- and fru-GAL4 driver. All those driver lines are expressed in border cells, posterior FCs and the stretched and columnar cells in later stages, and only some of the drivers are expressed in polar cells, stalk cells, the anterior FCs, and the germarium. None or only minor DA defects were observed with upd-GAL4, which is exclusively expressed in polar cells, indicating that the polar cells are not responsible for the phenotypes seen with e22c-, slbo- and fru-GAL4. Further, expression solely in the stalk cells and stalk precursor cells in the germarium with the 109-30-GAL4 driver did not cause strong defects. Weak expression in the anterior FCs is driven by c306-GAL4, however the c306-GAL4 driver led only to a low percentage of eggs with defective DAs. In addition, slbo-GAL4 is not expressed in anterior FCs, which also excludes the anterior FCs to be the cause for the DA phenotype. Expression in border cells and the stretched and columnar cells is also driven

by c355- and c306-GAL4, but using these drivers only results in minor DA defects. Surprisingly, c306-Gal4, which has a similar expression pattern as fru-GAL4, only shows weak DA defects. An explanation could be that c306-GAL4, although strongly expressed in stalk, border, and posterior cells in later stages, is only slightly expressed in terminal cells at earlier stages (FIG. 2 E). Taken together, the posterior FCs are the only FC subpopulation that shows a common expression pattern by e22c-, slbo- and fru-GAL4 and is thus likely to be involved in generating the DA defect.

Several signaling pathways are important in posterior FCs. Until stage 5 during oogenesis, the oocyte grows and the FCs are still dividing to cover the egg. The two polar cell pairs express the JAK/STAT ligand upd at both poles, leading to a mirror-symmetric pattern of terminal FCs. Within stage 6/7, EGFR and Delta/Notch signaling pathways start to determine the egg. The oocyte translates Gurken protein at the posterior side, which activates the EGFR receptor in adjacent FCs, causing them to adopt a posterior cell fate (Roth and Lynch, 2009). Also in stage 6, the germline starts to express Delta, the ligand that activates Notch signaling in the surrounding FCs, which leads to a switch from the mitotic cell cycle to an endoreplication cycle (Sun *et al.*, 2008). These findings suggest that either EGFR or Notch signaling might be affected by the loss of autophagy, and thus causes the generation of compound eggs and malformed DAs.

(III) Autophagy modulates Notch signaling in *Drosophila* follicle cells

Signaling between the oocyte and the somatic FCs determines the body axes during *Drosophila* oogenesis. The discrete patterning of the FCs along this axis, which is achieved by Delta-Notch and EGFR signaling, is important for the establishment of anterior-posterior polarity (Keller Larkin *et al.*, 1999). *Drosophila* ovaries containing *ATG1* mutant FCs show compound egg chambers and lack stalk cells. In addition, expression of ATG-RNAi in posterior FCs leads to malformed DAs (see above). Since Notch pathway mutants show encapsulation defects and lack stalk cells (López-Schier and Johnston, 2001), and we did not detect alterations in the EGFR pathway (data not shown), we hypothesize that Notch signaling might be altered in autophagy deficient FCs.

Notch signaling activity can be studied by monitoring the expression of two downstream target genes, Cut and Hindsight (Hnt). The transcription factor Cut is expressed during oogenesis in all FCs, beginning in the germarium until

stage 6. Concomitant with the cell-cycle switch, Notch pathway activation downregulates Cut expression and Cut protein vanishes (SUPPL. FIG. 3 A, B) (Sun and Deng, 2005). Notch signaling also leads to expression of another transcription factor, Hnt, which can be detected in FCs from stage 6 onwards (SUPPL. FIG. 3 A, C) (Sun and Deng, 2007). We tested the involvement of autophagy in the modulation of Notch signaling by inducing FC clones mutant for *ATG* genes and performed immunostainings against the downstream targets Cut and Hnt. In FCs mutant for *Notch*, Cut is not downregulated and remains expressed beyond stage 6 (FIG. 4 A-A"). Conversely, stage 6 *ATG1* and *ATG13* mutant FCs display weaker Cut staining compared to surrounding WT cells (FIG. 4 B-C"). However, the modulation of Cut expression is only seen in stage 6 egg chambers, suggesting an earlier downregulation of Cut in autophagy deficient cells rather than a complete inhibition. FC clones in egg chambers of earlier or later stages displayed Cut stainings comparable to surrounding WT cells (data not shown). On the other hand, *Notch* mutant FCs fail to upregulate Hnt after the cell cycle switch (FIG. 4 D-D"), however, *ATG1* and *ATG13* mutant FC clones display precocious or stronger upregulation of Hnt (FIG. 4 E-F") compared to WT FCs. Thus, *ATG* deficient FC clones display the opposite phenotype of FC clones mutant for *Notch*, suggesting that Notch signaling might be enhanced by the lack of autophagy. Endocytic internalization and trafficking has been shown to be essential for γ-secretase cleavage of Notch, resulting in the release of the Notch intracellular domain (NICD), which is able to translocate to the nucleus to activate the transcription of Notch target genes (Vaccari *et al.*, 2008). In fact, mutants that increase endosomal retention of the Notch receptor, *e.g.* mutants for the endosomal sorting complex required for transport (ESCRT), show enhanced Notch activity (Vaccari *et al.*, 2008). We propose that an absence of autophagy might lead to a pause in the normally rapid endosomal processing of internalized Notch, which in turn leads to pronounced NICD cleavage and enhanced Notch activity.

FIGURE 4. Autophagy modulates Notch signaling in *Drosophila* FCs

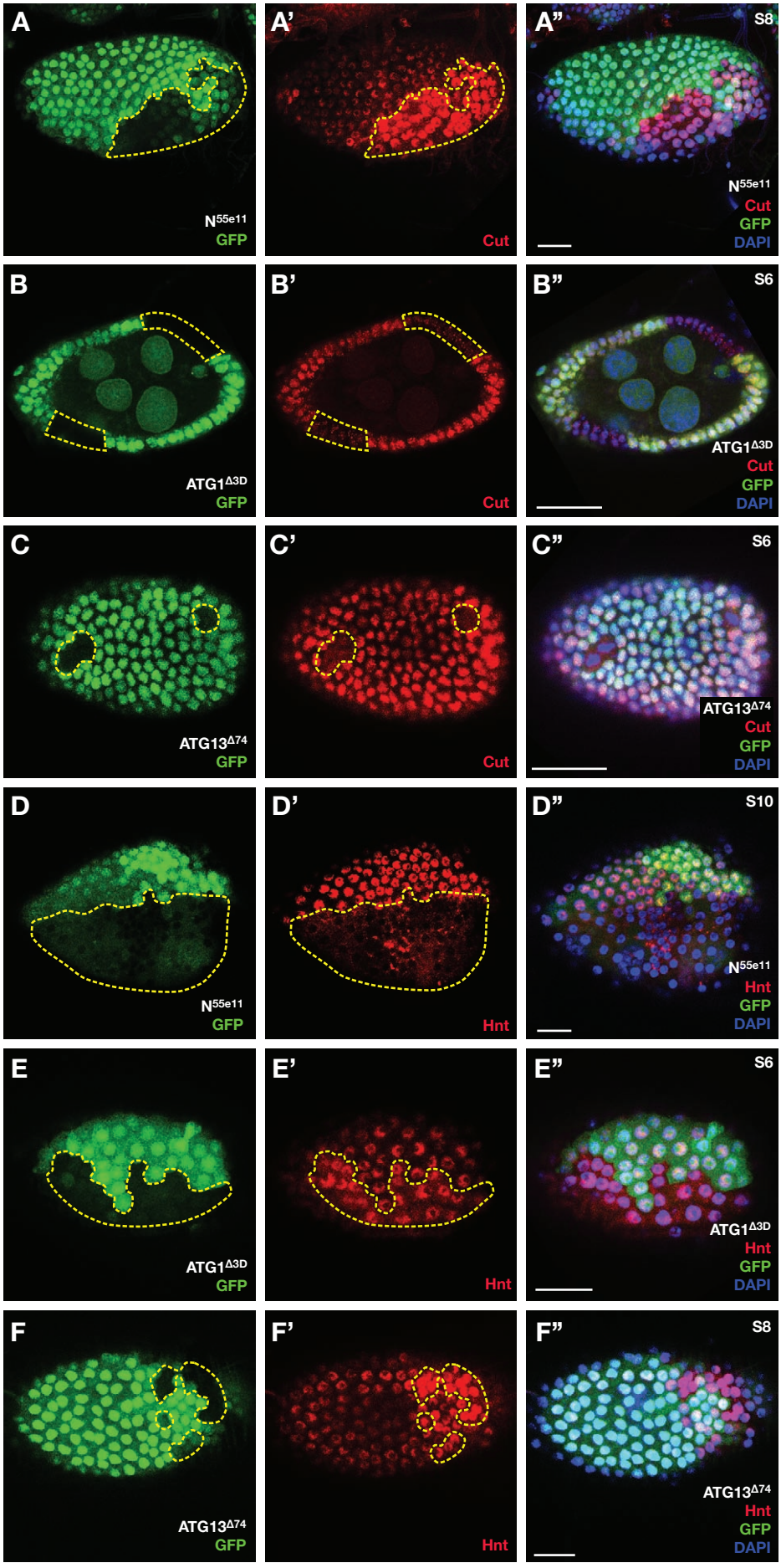

FIGURE 4. Autophagy modulates Notch signaling in *Drosophila* FCs. (A) In HS-FLP induced FC clones mutant for *Notch* (marked by the lack of GFP, outlined in yellow), Cut is not downregulated post stage 6 but is continuously expressed. In contrast, *ATG1* **(B)** or *ATG13* **(C)** mutant FCs show a precocious downregulation of Cut compared to WT cells. **(D)** Under normal conditions, the expression of Hindsight (Hnt) is upregulated by Notch signaling, which is not accomplished in cells mutant for *Notch* (marked by the lack of GFP, outlined in yellow). In FC clones mutant for *ATG1* **(E)** or *ATG13* **(F)**, Hnt upregulation occurs earlier or stronger than in the surrounding WT cells. Anterior is to the left, posterior to the right. Scale bar: 20 µm. Genotypes: A, D: *hs flp FRT19A-UbiGFP/N^{55e11} FRT19A.* B, E: *hs flp/+; ATG1$^{\Delta 3D}$ FRT80B/FRT80B-UbiGFP.* C, F: *hs flp/+; ATG13$^{\Delta 74}$ FRT82/FRT82-UbiGFP*

Our previous findings indicated that autophagy is especially required in FC during oogenesis, and that this is dependent on the cellular context, since oogenesis is only affected when FCs are mutant and GCs are WT (Barth *et al.*, 2011). Here, we show that autophagy deficiency in the FCs causes severe egg chamber defects. We further demonstrate that autophagy is presumably required in posterior FCs, and that defective autophagy leads to modulation of the Notch signaling pathway. This finding is especially relevant since dysregulation of Notch has been implicated in tumorigenesis (Ranganathan *et al.*, 2011).

Generation of *ATG* deficient FCs leads to a wide range of phenotypes, many of which have been observed in mutants of various cell-cell signaling pathways. Mutants of the Notch signaling pathway display compound egg chambers and lack stalk cells (López-Schier and Johnston, 2001), and EGFR and JAK/STAT pathway mutants display fused egg chambers as well as mislocalization of the oocyte (Goode *et al.*, 1996; McGregor *et al.*, 2002). Interestingly, it has been reported that the loss of the cysteine protease ATG4 modulates Notch signaling (Thumm and Kadowaki, 2001).

Using two readouts for Notch signaling during oogenesis, Cut and Hnt, we could show that loss of autophagy in *ATG1* and *ATG13* mutant FC clones modulates Notch signaling. This modulation is only visible in stage 6 of oogenesis, in which the Notch pathway is switched on by the expression of Delta in the germline, suggesting that Notch deregulation caused by the lack of autophagy can be rapidly compensated in later stages. It has been shown that endocytosis and endocytic trafficking regulate Notch activity and that retention of Notch in endosomal vesicles accelerates γ-secretase cleavage and intensifies Notch signaling (Vaccari *et al.*, 2008). Recently, the *Drosophila* UV-resistance associated gene (UVRAG), which has been implicated in autophagy and endocytosis, was shown to regulate Notch receptor endocytosis and subsequent degradation (Lee *et al.*, 2011). The authors show that UVRAG mutant cells are impaired in activating autophagy, but assume that defects in endocytosis rather than autophagy are responsible for Notch deregulation. However, the authors also suggest that UVRAG is required for endocytic trafficking or subsequent targeting of Notch to lysosomes. Furthermore, loss of Vps34, which is required for autophagy induction and progression, but also for endocytosis, results in the accumulation of Notch (Juhasz *et al.*, 2008). It is feasible that the strong phenotype observed in UVRAG and Vps34 mutants is a combination of deregulated endocytic trafficking and autophagosomal receptor

degradation, whereas the sole loss of autophagy has only a minor or temporary impact on degradation and can rapidly be compensated by other mechanism, *e.g.* direct fusion of endosomes with lysosomes without the involvement of the autophagic machinery. However, intersections between the endosomal and autophagy pathways have long been described (Gordon and Seglen, 1988; Liou *et al.*, 1997). In addition, *ESCRT* mutants show a ligand independent activation of Notch signaling, which might result from altered endosomal trafficking and endosomal accumulation, and ESCRT is also required for autophagy (Rusten *et al.*, 2007; Vaccari *et al.*, 2008). Thus, several proteins are implicated in both autophagy and endosomal receptor sorting.

In *ATG1* mutant FCs, Cut and Hnt expression is inversely regulated compared to *Notch* loss-of-function clones, which suggests an activation of the signal. This is in accordance with UVRAG, Vps34 or ESCRT mutants, where Notch signaling is also increased (Juhasz *et al.*, 2008; Vaccari *et al.*, 2008; Lee *et al.*, 2011). However, compound egg chambers and the lack of stalk cells are phenotypes known for Notch mutants, whereas constitutive active Notch signaling leads to longer stalk cells (Larkin *et al.*, 1996), a phenotype that is not observed in ovaries with *ATG* mutant FCs. Nevertheless, since modulation of Notch signaling is only observed in stage 6 egg chambers, it is possible that either this dysregulation is not strong enough to cause severe gain of function Notch phenotypes, or that autophagy has no impact on Notch signaling during the differentiation of stalk cells in early oogenesis. Although we did not observe modulations in EGFR signaling (data not shown), the possibility remains that autophagy has stage or cell type specific functions in the modulation of other cell-cell signaling pathways that could also cause the observed oogenesis defects. The EGF receptor is also regulated by endocytosis and endosomal trafficking (Sorkin and Goh, 2009), thus autophagy might also be involved in EGFR receptor degradation. Moreover, FC mutants for the SCF complex protein Slimb also lack stalk cells and show dorsal appendage (DA) defects (Muzzopappa and Wappner, 2005) and SCF complex family members have been implicated in the targeting of the Notch receptor for degradation (Matsumoto *et al.*, 2011). This could hint to a common mechanism of Notch degradation failure leading to DA defects. However, eggs mutant for Slimb in the FCs also show ectopic polar cells, which we did not observe in *ATG* mutant FCs. In addition, using the upd-GAL4 driver line to knock-down ATG-RNAi in polar cells, we did not observe DA defects. Using other GAL4-driver lines, we could show that autophagy deficiency especially in posterior FCs leads to DA defects. Differentiation of posterior FCs starts at stage 6 where Notch is activated and the FCs switch from a mitotic to an endocycle program. The

secretion of upd by the polar cells patterns terminal cell fates and Gurken translation in the posterior corner of the oocyte defines the posterior cell fate (Roth and Lynch, 2009). Thus, all three pathways are active in posterior FCs. We recently demonstrated that the requirement of autophagy in FCs depends on a cellular context, since DA defects are only seen in eggs with *ATG* mutant FCs and WT GCs (Barth *et al.*, 2011). Because secretion of upd and activation of JAK/STAT signaling in neighbouring FCs is a GC independent signaling process (Xi *et al.*, 2003), and since we did not observe DA defects by ATG-RNAi expression in polar cells, we exclude autophagy dependent modulation of JAK/STAT from causing DA defects. EGFR signaling is activated in posterior FCs upon Gurken translation by the oocyte and movement of the oocyte nucleus to a lateral-anterior position requires an unknown back-signaling by the FCs (Deng and Bownes, 1998; Roth and Lynch, 2009). Thus, it could be possible that autophagy deficient FCs are impaired in transmitting the signal back to the oocyte. However, egg chambers with ATG deficient FCs always displayed normal Gurken signaling and movement of the oocyte to the designated places in the oocyte. Moreover, we would expect to see the same phenotype in a situation where both, FCs and GCs are mutant, since autophagy deficiency in the GCs would not be able to further modulate the deregulated back-signaling. Consequently, we also exclude modulation of EGFR signaling from causing the observed DA defects. Notch is activated in FCs by a signal from the germline and both signaling partners, receptor and ligands, are transmembrane proteins that are regulated by internalization and endosomal trafficking (Nichols *et al.*, 2007). Interestingly, mutants defective for endocytosis show abnormal trafficking of Delta and reduced Notch signaling activity (Parks *et al.*, 2000). Thus, it could be possible that in a situation where both tissues are mutant for *ATG* genes, the lack of autophagy also modulates endocytic processing of Delta in the germline, which leads to reduced ligand signaling that is able to compensate the increased activity in autophagy deficient FCs. Indeed, it has been shown that liquid facets (lqf), the *Drosophila* homologue of Epsin, which is required for endocytosis of Delta, also has defects in autophagy (Overstreet *et al.*, 2003; Csikos *et al.*, 2009). However, it seems that the interplay of autophagy and Notch signaling is dependent on the strength of the signal present since we only observed a modulation of Notch signaling in autophagy deficient cells right after the pathway has been switched on.

As mentioned before, generation of *ATG* deficient FCs displays a wide range of phenotypes, including persisting nurse cell nuclei (PNCN). During late oogenesis, nurse cells (NCs) transport their cytoplasm to the oocyte

('dumping') and undergo programmed cell death (displaying apoptotic and autophagic markers), which is necessary for normal maturation of the egg (Velentzas *et al.*, 2007; Nezis *et al.*, 2010). It has been shown that germline clones (GLCs) mutant for *ATG* genes induced by the Ovo^D technique display PNCN, indicating that NC death requires a functional autophagic machinery in the germline (Nezis *et al.*, 2010). However, using the pole cell transplantation technique we could show that *ATG* mutant germline clones do not exhibit PNCN (Barth *et al.*, 2011). Using the e22c-FLP method, which does not affect the germline (Duffy *et al.*, 1998), we could now demonstrate that generation of *ATG* mutant FC clones likewise produces PNCN as Ovo^D induced *ATG1* GLCs. It could be possible that autophagy is similarly important for NC death in the GCs as in the FCs. However, we could demonstrate that GLC induction using the Ovo^D system also induces FC clones, which could be an explanation for PNCN in *ATG* deficient GLCs. Since surrounding FCs act as non-professional phagocytes and take up remnants of the dying NCs during mid- and late oogenesis NC death (Giorgi and Deri, 1976; Nezis *et al.*, 2000), it is likely that autophagy deficiency leads to an incomplete clearance of inclusion bodies and thus PNCN. In addition, PNCN are found in both situations, in eggs with a *ATG* mutant or WT germline, as long as the FCs are autophagy deficient, which indicates a phenotype independent of the incompatibility model and the other oogenesis defects caused by *ATG* mutations in FCs.

In summary, our work shows that autophagy is critical in *Drosophila* FCs and has the ability to modulate the Notch signaling pathway. This opens novel possibilities of endosomal receptor regulation and might be relevant for studies concerning cancer treatment. Notably, the situation in a tumor resembles our experimental set up in which an imbalance between WT and mutant tissue assigns a fate to a certain cell type. The dysregulation of autophagy may represent an advantage and therefore affect carcinogenesis.

Autophagy and endocytosis equally represent relevant inputs for lysosomal degradation but the interplay of both pathways is still poorly understood. Further studies will be required to clarify whether autophagy is indeed involved in the endocytic regulation of ligands and receptors in cell-cell signaling pathways.

Drosophila maintenance and stocks.

Flies were raised on standard yeast/cornmeal agar at 25 °C. *Drosophila melanogaster* stocks used: *ATG1$^{\Delta 3D}$ FRT80B*, *ATG5-RNAi*, *ATG13$^{\Delta 74}$ FRT82* (kindly provided by T. Neufeld) (Scott *et al.*, 2004; Chang *et al.*, 2009). *ATG1$^{\Delta 3D}$ FRT80B-UbiGFP* (recombined from *ATG1$^{\Delta 3D}$*, T.N.). *ATG1-RNAi* (GD16133), *ATG4-RNAi* (KK107317), *ATG8-RNAi* (KK109654), *lacZ-RNAi*, (VDRC, Vienna, Austria) . *e22c-GAL4 UAS-FLP;FRT80-UbiGFP*, *e22c-GAL4 UAS-FLP;FRT82-UbiGFP* (Duffy *et al.*, 1998) (kindly provided by T. Schüpbach). *fru-GAL4* (168-GAL4) (kindly provided by A.-M. Pret). *upd-GAL4* (kindly provided by S. Noselli) (Bai and Montell, 2002). *c306-GAL4* (3743) (Manseau et al., 1997), *c355-GAL4* (3750) (Manseau *et al.*, 1997), *109-30-GAL4* (7023) (Hartman *et al.*, 2010), *slbo-GAL4* (6458), *ey-GAL4*, *UAS-GFP, N^{55e11} FRT19A* (28813), *FRT19-UbiGFP, FRT80B-UbiGFP*, *FRT82-UbiGFP, FRT80iso, FRT82iso, FRT80 w+, y w* (Bloomington *Drosophila* Stock Center, Indiana University, IN, USA). *Ovo^{D}-FRT8*0 (kindly provided by P. Gallant/P. Rorth).

LTR assay, starvation, tissue preparation, immunostainings and microscopy

For LTR assays, early L3 larva were starved for 2h in 10% sucrose in PBS solution. Fatbody tissue was dissected in PBS, incubated for 1 min in 100 mM Lysotracker red DND-99 (Invitrogen, Molecular Probes, Basel, Switzerland) to label acidic organelles including autolysosomes, washed three times in PBS and live imaged using a confocal microscope (see below). Ovaries were dissected in PBS, fixed in 4% paraformaldehyde (PFA) for 20 min, embedded in mounting medium with DAPI (Vectashield, Vector Laboratories, Inc., Burlingame, CA, USA). Ovaries for immunostainings were prepared as described elsewhere (Barth *et al.*, 2011). Primary antibodies used: mouse anti-Hnt (1:100), mouse anti-Cut (1:100) (Developmental Studies Hybridoma Bank, IA, USA). Secondary antibody: Cy3 anti-mouse (1:300) (GE Healthcare, Germany). Images were obtained using a confocal microscope (Leica, Wetzlar, Germany, DM5500Q, TCS-SPE; objective lenses: Leica, 20x (0.70), 40x (1.15), 63x (1.30); acquisition software: LAS AF v.2.0.1, Leica, Wetzlar, Germany) and a

digital microscope (Keyence, Osaka, Japan, VHX-1000D; objective lens: VH-Z100R 100x-1000x zoom lens) at room temperature and edited using Adobe Illustrator and Photoshop CS5.

Generation of mosaic tissues

The FLP/FRT recombination method was used to generate FC, germline and fatbody clones. Heat-shock induced FC clones mutant for *ATG1*, *ATG13*, or *Notch* were generated by placing the flies of the genotypes *FRT80B-ATG1$^{\Delta 3D}$/FRT80B-UbiGFP*, *FRT82-ATG13$^{\Delta 74}$/FRT82-UbiGFP* or *FRT19A-N^{55e11}/FRT19A-UbiGFP* for 1 h at 37 °C during larval development on day 2, 3 and 4 after egg laying. For *e22c-GAL4 UAS-FLP* induced clones, flies were crossed with *FRT80B-ATG1$^{\Delta 3D}$* or *FRT82-ATG13$^{\Delta 74}$* and dissected 4 days after hatching. Germline *Ovo^{D}* clones were induced by heat shock (HS) as described in Nezis et al. (2010). Fatbody FLP out clones were achieved though HS independent induction as described in (Britton *et al.*, 2002).

Egg quantification

Adult females with the appropriate phenotype were mated with WT males in single vials and eggs with intact and defect DAs were quantified every day for 4 consecutive days for egg laying analysis. For each genotype and independent experiment, the eggs of 5 individual females were counted, n=3.

Acknowledgements

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DA	dorsal appendage
Dl	Delta
EGFR	epidermal growth factor receptor
ESCRT	endosomal sorting complex required for transport
ey	eyeless
FC	follicle cell
FLP-FRT	flippase recognition target
fru	fruitless
GC	germ cell
GLC	germline clone
hh	hedgehoge
Hnt	hindsight
HS	heat shock
lqf	liquid facets
LTR	lysotracker
NC	nurse cell
NICD	Notch intracellular domain
PFA	paraformaldehyde
PNCN	persisting nurse cell nuclei
RNAi	interfering ribonucleic acid
SCF	Skp, Cullin, F-box complex
S.D.	standard deviation
slbo	slow border cell
upd	unpaired
UVRAG	UV-resistance associated gene
WT	wild type

Assa-Kunik, E., I.L. Torres, E.D. Schejter, D.S. Johnston, and B.-Z. Shilo. 2007. *Drosophila* follicle cells are patterned by multiple levels of Notch signaling and antagonism between the Notch and JAK/STAT pathways. Development. 134:1161-1169.

Avraham, R., and Y. Yarden. 2011. Feedback regulation of EGFR signalling: decision making by early and delayed loops. Nat Rev Mol Cell Biol. 12:104-117.

Bai, J., and D. Montell. 2002. Eyes absent, a key repressor of polar cell fate during *Drosophila* oogenesis. Development. 129:5377-5388.

Barth, J.M., J. Szabad, E. Hafen, and K. Kohler. 2011. Autophagy in *Drosophila* ovaries is induced by starvation and is required for oogenesis. Cell Death Differ. 18:915-924.

Berg, C. 2005. The *Drosophila* shell game: patterning genes and morphological change. Trends Genet. 21:346-355.

Berry, D., and E. Baehrecke. 2007. Growth arrest and autophagy are required for salivary gland cell degradation in *Drosophila*. Cell. 131:1137-1148.

Brand, A., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 118:401-415.

Britton, J.S., W.K. Lockwood, L. Li, S.M. Cohen, and B.A. Edgar. 2002. *Drosophila*'s insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. Dev Cell. 2:239-249.

Chang, Y., G. Juhasz, P. Goraksha-Hicks, A. Arsham, D. Mallin, L. Muller, and T. Neufeld. 2009. Nutrient-dependent regulation of autophagy through the target of rapamycin pathway. Biochem Soc Trans. 37:232-236.

Chang, Y.Y., and T.P. Neufeld. 2010. Autophagy takes flight in *Drosophila*. FEBS Lett. 584:1342-1349.

Chen, Y., and D.J. Klionsky. 2011. The regulation of autophagy - unanswered questions. J Cell Sci. 124:161-170.

Csikos, G., M. Lippai, T. Lukacsovich, G. Juhasz, L. Henn, M. Erdelyi, P. Maroy, and M. Sass. 2009. A novel role for the *Drosophila* epsin (lqf): involvement in autophagy. Autophagy. 5:636-648.

Deng, W.M., and M. Bownes. 1998. Patterning and morphogenesis of the follicle cell epithelium during *Drosophila* oogenesis. Int J Dev Biol. 42:541-552.

Drummond-Barbosa, D., and A. Spradling. 2001. Stem cells and their progeny respond to nutritional changes during *Drosophila* oogenesis. Dev Biol. 231:265-278.

Duchek, P., and P. Rorth. 2001. Guidance of cell migration by EGF receptor signaling during *Drosophila* oogenesis. Science. 291:131-133.

Duffy, J.B., D.A. Harrison, and N. Perrimon. 1998. Identifying loci required for follicular patterning using directed mosaics. DEVELOPMENT-CAMBRIDGE-. 125:2263-2271.

Forbes, A.J., H. Lin, P.W. Ingham, and A.C. Spradling. 1996. hedgehog is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in *Drosophila*. Development. 122:1125-1135.

Giorgi, F., and P. Deri. 1976. Cell death in ovarian chambers of *Drosophila melanogaster*. J Embryol Exp Morphol. 35:521-533.

González-Reyes, A., H. Elliott, and D. St Johnston. 1995. Polarization of both major body axes in *Drosophila* by gurken-torpedo signalling. Nature. 375:654-658.

Goode, S., M. Morgan, Y.P. Liang, and A.P. Mahowald. 1996. Brainiac encodes a novel, putative secreted protein that cooperates with Grk TGF alpha in the genesis of the follicular epithelium. Dev Biol. 178:35-50.

Gordon, P.B., and P.O. Seglen. 1988. Prelysosomal convergence of autophagic and endocytic pathways. Biochem Biophys Res Commun. 151:40-47.

Hartman, T.R., D. Zinshteyn, H.K. Schofield, E. Nicolas, A. Okada, and A.M. O'Reilly. 2010. *Drosophila* Boi limits Hedgehog levels to suppress follicle stem cell proliferation. J Cell Biol. 191:943-952.

Hou, Y., S. Chittaranjan, S. Barbosa, K. McCall, and S. Gorski. 2008. Effector caspase Dcp-1 and IAP protein Bruce regulate starvation-induced autophagy during *Drosophila melanogaster* oogenesis. J Cell Biol. 182:1127-1139.

Ito, K., W. Awano, K. Suzuki, Y. Hiromi, and D. Yamamoto. 1997. The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells (vol 124, pg 761, 1997). Development. 124:U2-U2.

Juhasz, G., J.H. Hill, Y. Yan, M. Sass, E.H. Baehrecke, J.M. Backer, and T.P. Neufeld. 2008. The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in *Drosophila*. J Cell Biol. 181:655-666.

Keller Larkin, M., W.M. Deng, K. Holder, M. Tworoger, N. Clegg, and H. Ruohola-Baker. 1999. Role of Notch pathway in terminal follicle cell differentiation during *Drosophila* oogenesis. Dev Genes Evol. 209:301-311.

Larkin, M.K., K. Holder, C. Yost, E. Giniger, and H. Ruohola-Baker. 1996. Expression of constitutively active Notch arrests follicle cells at a precursor stage during *Drosophila* oogenesis and disrupts the anterior-posterior axis of the oocyte. Development. 122:3639.

Lee, G., C. Liang, G. Park, C. Jang, J.U. Jung, and J. Chung. 2011. UVRAG is required for organ rotation by regulating Notch endocytosis in *Drosophila*. Dev Biol. 356:588-597.

Liou, W., H.J. Geuze, M.J. Geelen, and J.W. Slot. 1997. The autophagic and endocytic pathways converge at the nascent autophagic vacuoles. J Cell Biol. 136:61-70.

López-Schier, H., and D. Johnston. 2001. Delta signaling from the germ line controls the proliferation and differentiation of the somatic follicle cells during *Drosophila* oogenesis. Genes & Development. 15:1393.

Manseau, L., A. Baradaran, D. Brower, A. Budhu, F. Elefant, H. Phan, A.V. Philp, M. Yang, D. Glover, K. Kaiser, K. Palter, and S. Selleck. 1997. GAL4 enhancer traps expressed in the embryo, larval brain, imaginal discs, and ovary of *Drosophila*. Dev Dyn. 209:310-322.

Matsumoto, A., I. Onoyama, T. Sunabori, R. Kageyama, H. Okano, and K.I. Nakayama. 2011. Fbxw7-dependent degradation of Notch is required for control of "stemness" and neuronal-glial differentiation in neural stem cells. J Biol Chem. 286:13754-13764.

McGregor, J.R., R. Xi, and D.A. Harrison. 2002. JAK signaling is somatically required for follicle cell differentiation in *Drosophila*. Development. 129:705.

Montell, D.J. 2003. Border-cell migration: the race is on. Nature Reviews Molecular Cell Biology. 4:13-24.

Muzzopappa, M., and P. Wappner. 2005. Multiple roles of the F-box protein Slimb in *Drosophila* egg chamber development. Development. 132:2561-2571.

Nezis, I., T. Lamark, A. Velentzas, T. Rusten, G. Bjorkoy, T. Johansen, I. Papassideri, D. Stravopodis, L. Margaritis, H. Stenmark, and A. Brech. 2009. Cell death during *Drosophila* melanogaster early oogenesis is mediated through autophagy. Autophagy. 5:298-302.

Nezis, I., D. Stravopodis, L. Margaritis, and I. Papassideri. 2006. Programmed cell death of follicular epithelium during the late developmental stages of oogenesis in the fruit flies Bactrocera oleae and Ceratitis capitata (Diptera, Tephritidae) is mediated by autophagy. Dev Growth Differ. 48:189-198.

Nezis, I.P., B.V. Shravage, A.P. Sagona, T. Lamark, G. Bjorkoy, T. Johansen, T.E. Rusten, A. Brech, E.H. Baehrecke, and H. Stenmark. 2010. Autophagic degradation of dBruce controls DNA fragmentation in nurse cells during late *Drosophila* melanogaster oogenesis. The Journal of Cell Biology:1-12.

Nezis, I.P., D.J. Stravopodis, I. Papassideri, M. Robert-Nicoud, and L.H. Margaritis. 2000. Stage-specific apoptotic patterns during *Drosophila* oogenesis. Eur J Cell Biol. 79:610-620.

Nichols, J.T., A. Miyamoto, and G. Weinmaster. 2007. Notch signaling--constantly on the move. Traffic. 8:959-969.

Overstreet, E., X. Chen, B. Wendland, and J.A. Fischer. 2003. Either part of a *Drosophila* epsin protein, divided after the ENTH domain, functions in endocytosis of delta in the developing eye. Curr Biol. 13:854-860.

Parks, A.L., K.M. Klueg, J.R. Stout, and M.A. Muskavitch. 2000. Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. Development. 127:1373-1385.

Peterson, J., M. Barkett, and K. McCall. 2003. Stage-specific regulation of caspase activity in *Drosophila* oogenesis. Dev Biol. 260:113-123.

Poulton, J., and W. Deng. 2007. Cell-cell communication and axis specification in the *Drosophila* oocyte. Dev Biol. 311:1-10.

Ranganathan, P., K.L. Weaver, and A.J. Capobianco. 2011. Notch signalling in solid tumours: a little bit of everything but not all the time. Nat Rev Cancer. 11:338-351.

Rorth, P., K. Szabo, A. Bailey, T. Laverty, J. Rehm, G.M. Rubin, K. Weigmann, M. Milan, V. Benes, W. Ansorge, and S.M. Cohen. 1998. Systematic gain-of-function genetics in *Drosophila*. Development. 125:1049-1057.

Roth, S., and J.A. Lynch. 2009. Symmetry breaking during *Drosophila* oogenesis. Cold Spring Harbor Perspect Biol. 1:a001891.

Rusten, T., K. Lindmo, G. Juhasz, M. Sass, P. Seglen, A. Brech, and H. Stenmark. 2004. Programmed autophagy in the *Drosophila* fat body is induced by ecdysone through regulation of the PI3K pathway. Dev Cell. 7:179-192.

Rusten, T.E., T. Vaccari, K. Lindmo, L.M.W. Rodahl, I.P. Nezis, C. Sem-Jacobsen, F. Wendler, J.-P. Vincent, A. Brech, D. Bilder, and H. Stenmark. 2007. ESCRTs and Fab1 regulate distinct steps of autophagy. Current Biology. 17:1817-1825.

Scott, R.C., O. Schuldiner, and T.P. Neufeld. 2004. Role and regulation of starvation-induced autophagy in the *Drosophila* fat body. Dev Cell. 7:167-178.

Sorkin, A., and L.K. Goh. 2009. Endocytosis and intracellular trafficking of ErbBs. Exp Cell Res. 315:683-696.

Sun, J., and W.-M. Deng. 2005. Notch-dependent downregulation of the homeodomain gene cut is required for the mitotic cycle/endocycle switch and cell differentiation in *Drosophila* follicle cells. Development. 132:4299-4308.

Sun, J., and W.-M. Deng. 2007. Hindsight mediates the role of notch in suppressing hedgehog signaling and cell proliferation. Developmental Cell. 12:431-442.

Sun, J., L. Smith, A. Armento, and W.-M. Deng. 2008. Regulation of the endocycle/gene amplification switch by Notch and ecdysone signaling. The Journal of Cell Biology. 182:885-896.

Thumm, M., and T. Kadowaki. 2001. The loss of *Drosophila* APG4/AUT2 function modifies the phenotypes of cut and Notch signaling pathway mutants. Mol Genet Genomics. 266:657-663.

Vaccari, T., H. Lu, R. Kanwar, M.E. Fortini, and D. Bilder. 2008. Endosomal entry regulates Notch receptor activation in *Drosophila melanogaster*. J Cell Biol. 180:755-762.

Velentzas, A.D., I.P. Nezis, D.J. Stravopodis, I.S. Papassideri, and L.H. Margaritis. 2007. Mechanisms of programmed cell death during oogenesis in *Drosophila virilis*. Cell Tissue Res. 327:399-414.

Ward, E., X. Zhou, L. Riddiford, C. Berg, and H. Ruohola-Baker. 2006. Border of Notch activity establishes a boundary between the two dorsal appendage tube cell types. Developmental Biology. 297:461-470.

Xi, R., J.R. McGregor, and D.A. Harrison. 2003. A gradient of JAK pathway activity patterns the anterior-posterior axis of the follicular epithelium. Developmental Cell. 4:167-177.

Yamamoto, S., W.-L. Charng, and H.J. Bellen. 2010. Endocytosis and Intracellular Trafficking of Notch and Its Ligands. 92.

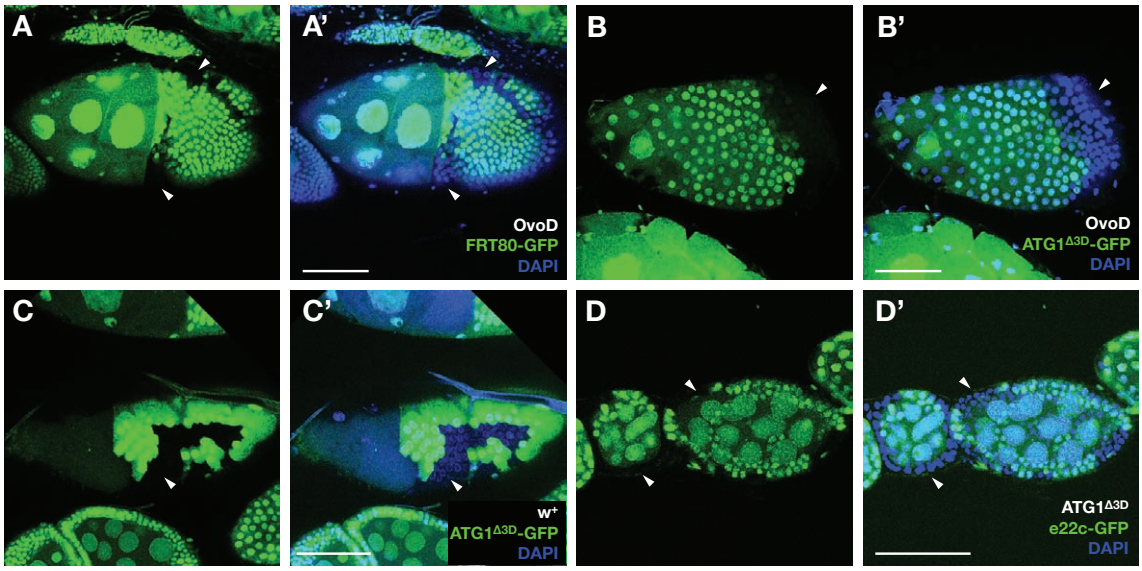


FIGURE S1. Generation of FC clones by different techniques. (A-B') Generation of germline clones (GLCs) using the HS-FLP FRT *Ovo^{D}* technique induces a complete mutant germline since GCs homozygous for the dominant female sterile mutation *Ovo^{D}* die. However, mutant clones are also induced in the somatic tissue where the mutation is not lethal. Thus, eggs with a mosaic FC epithelium occur and develop (A-B', arrowheads). The *Ovo^{D}* chromosome is marked by the lack of GFP. **(C, D')** For comparison, HS-FLP induced FC clones (*ATG1* mutant clones are marked with GFP) (C, C') and e22c-GAL4 UAS-FLP induced FC clones (mutant clones are marked by the lack of GFP) are shown (D, D'). Anterior is to the left, posterior to the right. Scale bar: 50 µm. Genotypes: A: *hs flp/+; Ovo^{D} FRT80B/FRT80B-UbiGFP*, B: *hs flp/+; Ovo^{D} FRT80B/Atg1Δ3D FRT80B-UbiGFP*, C: *hs flp/+; w^{+} FRT80B/Atg1Δ3D FRT80B-UbiGFP*, D: *hs flp/+; e22c UAS-FLP; FRT80B-UbiGFP/Atg1Δ3D FRT80B-UbiGFP.*

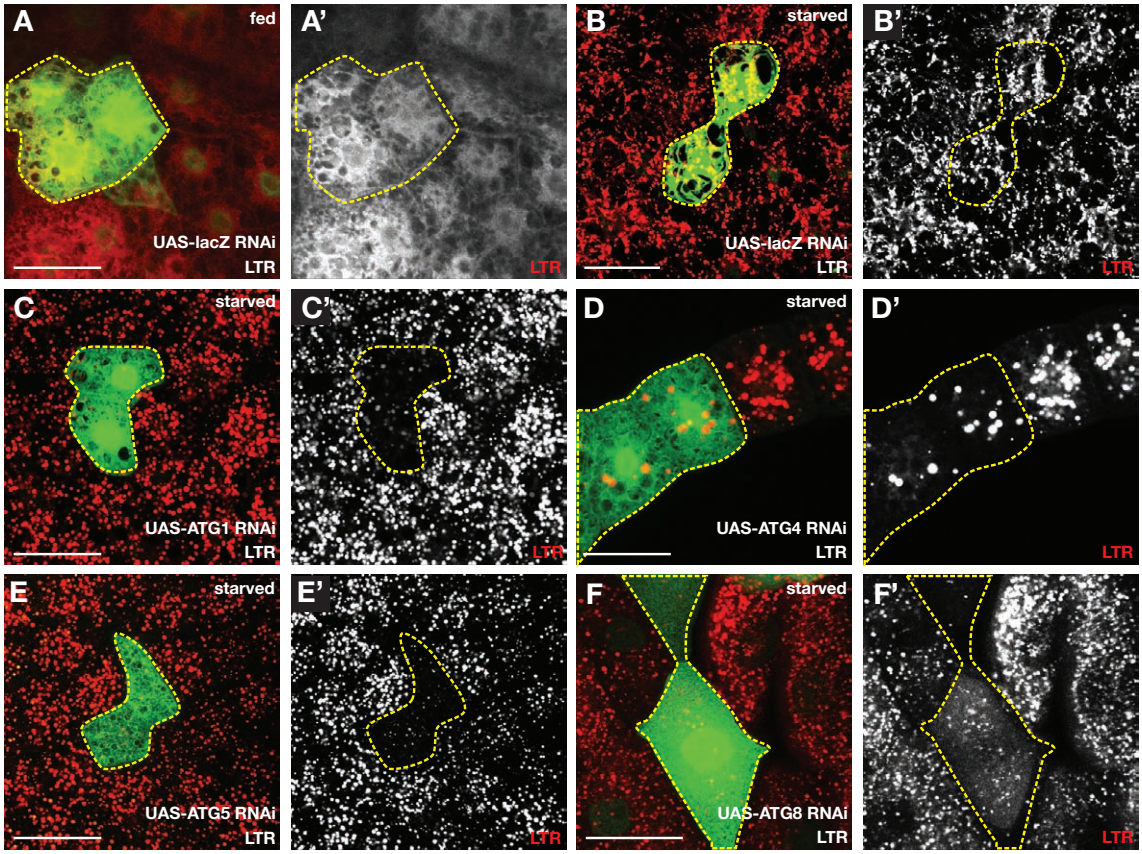


FIGURE S2. Autophagic activity is reduced in ATG-RNAi treated cells.
LTR staining of fat bodies expressing ATG-RNAi constructs in clones induced with the FLP-out/ GAL4 system. **(A, A')** Under well-fed conditions, LTR staining is ubiquitously distributed in wild type (WT) cells and cells expressing control lacZ-RNAi (marked with GFP). **(B, B')** Under starvation, control lacZ-RNAi expressing cells accumulate LTR positive dots as in surrounding WT cells. **(C-F')** Expression of ATG1-RNAi inhibits the formation of LTR positive dots compared to surrounding WT cells **(C, C')**. The same is seen for ATG4-RNAi **(D, D')**, ATG5-RNAi **(E, E')** and ATG8-RNAi **(F, F')**. Scale Bar: 50 µm. Genotypes: A, B: *hs flp/UAS-lacZ^{RNAi};;act>CD2>GAL4 UAS-GFPnls/+,* C: *hs flp/+;UAS-ATG1^{RNAi}/+;act>CD2>GAL4 UAS-GFPnls/+,* D: hs flp/+;UAS-ATG4^{RNAi}/+;act>CD2>GAL4 UAS-GFPnls/+, E: hs flp/UAS-ATG5^{RNAi};;act>CD2>GAL4 UAS-GFPnls/+, F: *hs flp/+;UAS-ATG8^{RNAi}/+;act>CD2>GAL4 UAS-GFPnls/+.*

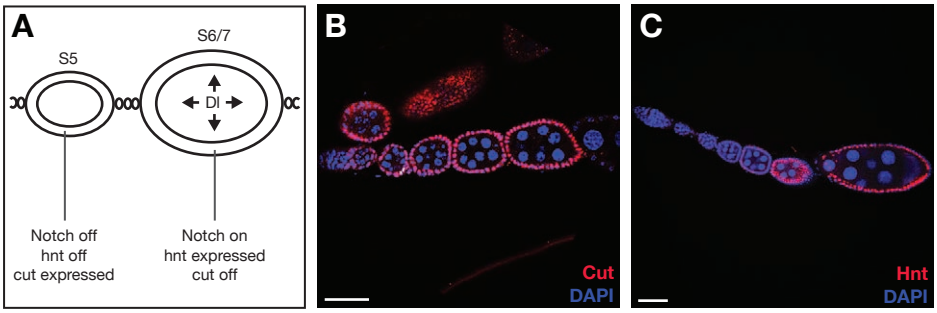


FIGURE S3. Expression pattern of the Notch signaling targets Cut and Hnt. (A) Schematic representation of Notch signaling activity. Until stage 5, Delta (Dl) is not expressed by the germline, Notch is not activated in FCs, and Cut is expressed whereas Hnt is absent. By stage 6, Dl is expressed by the germline and activates Notch in FCs, Cut is downregulated, and Hnt is expressed **(B)** Expression of Cut starting in the germarium and continuing until stage 6. **(C)** Expression of Hnt is absent in early stages but expression is activated by stage 6. Anterior is to the left, posterior to the right. Scale bar: 50 µm. Genotypes: *y w.*

2.3 Does autophagy modulate cell-cell signaling pathways during *Drosophila* oogenesis?

As mentioned in section 1.4, the elaborate structure of the *Drosophila* egg is the result of several classical signaling pathways that are essential for cell differentiation, pattern formation and axis specification during oogenesis and are often shared between GCs and FCs (Poulton and Deng, 2007). Since autophagy deficiency only affects oogenesis in a cellular context where FCs are mutant for *ATG* genes and GCs are WT (see 2.1), it is tempting to speculate that a dysfunctional signaling between soma and germline may be responsible for the oogenesis defects. In order to identify the cell-cell signaling pathway that might be modulated by autophagy, I analyzed the phenotypes of ovaries containing *ATG1* mutant FCs, determined the FC subpopulations causing these defects (see 2.2) and examined the involvement of the main cell-cell signaling pathways during oogenesis: EGFR, JAK-STAT and Delta-Notch.
The following chapter contains mostly preliminary and additional data from studies that were performed in order to determine the signaling pathway that is possibly regulated by autophagy to affect egg development.

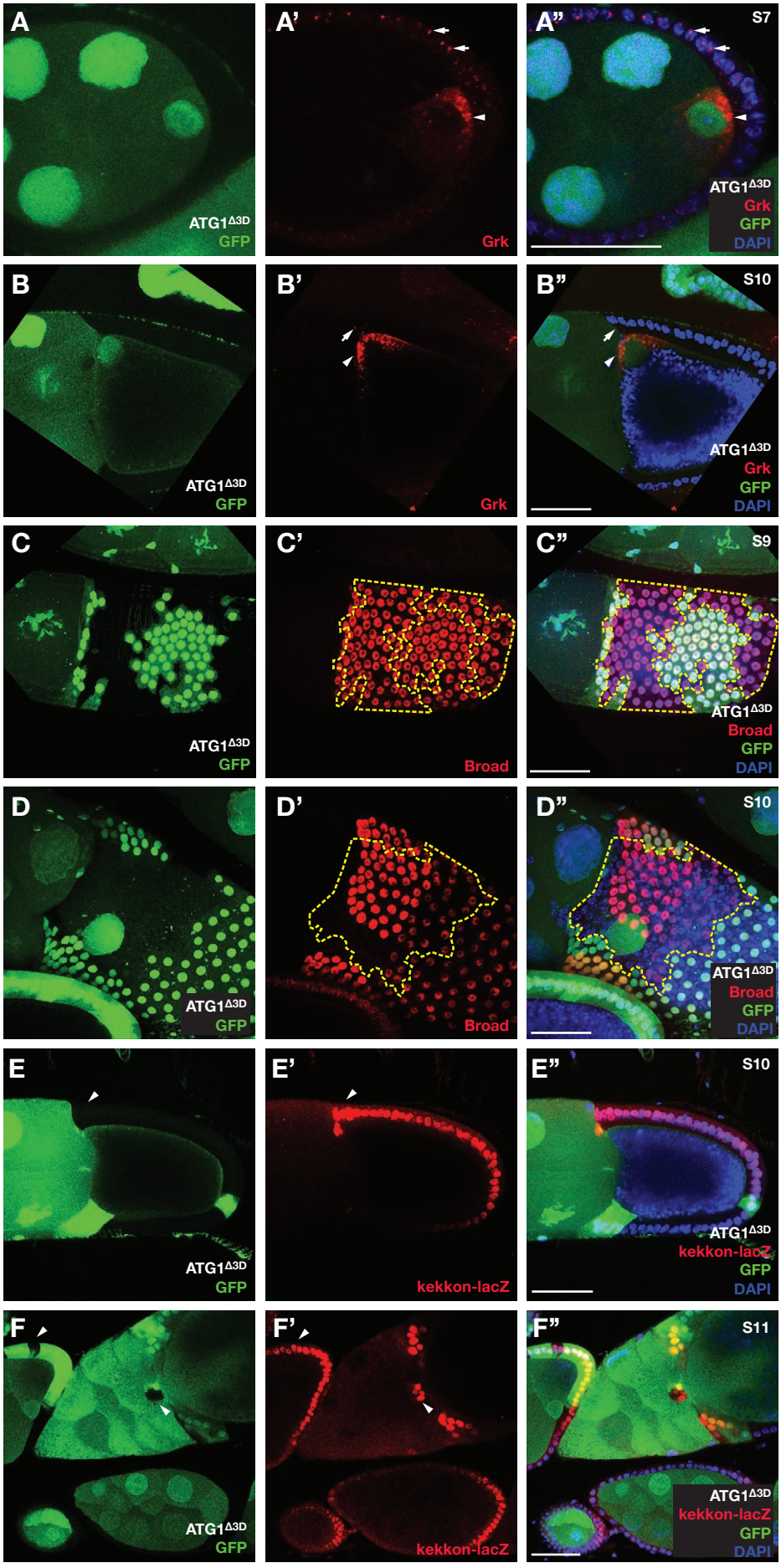
2.3.1 Epidermal growth factor receptor (EGFR) signaling

During *Drosophila* oogenesis, two phases of locally defined EGFR signaling define first the posterior, and later the dorsal FCs (Xi *et al.*, 2003; Schupbach, 2009). In both cases, Grk protein is translated by the oocyte and activates EGFR signaling in adjacent FCs (see FIG. 1.10). Subsequent receptor dimerization and autophosphorylation activates signaling cascades that control gene expression and cell behavior such as differentiation or migration (Avraham and Yarden, 2011).

After EGFR signaling has defined the posterior cells, an unknown signal is send back to the oocyte, causing a reorganization of the microtubule network and movement of the oocyte nucleus to an asymmetrical anterior position (González-Reyes *et al.*, 1995; Chang *et al.*, 2011). Assuming that autophagy regulates the transduction of the EGFR signaling cascade in posterior FCs, the back-signaling and subsequent movement of the oocyte nucleus should be impaired in autophagy deficient FCs. Therefore, *ATG1* mutant FCs were generated using the FRT/FLP method, and the distribution of Grk protein was visualized. In stage 6/7 egg chambers, which are completely covered by *ATG1* mutant FCs and thus defective for autophagy, distribution of Grk protein to the posterior side of the oocyte appeared normal (FIG. 2.3.1 A-A" arrowheads). Further, movement of the nucleus to the asymmetrical anterior side, and the second round of Grk translation in later stages of eggs containing *ATG1* mutant FCs took place as in WT eggs (FIG. 2.3.1 B-B" arrowheads). At these stages, Grk is secreted by the oocyte and activates EGFR in terminal and dorsal FCs. Grk uptake by the FCs is not affected through the lack of autophagy since *ATG1* deficient FCs display Grk staining similar to WT cells (FIG. 2A-B', arrows). Occasionally, eggs with *ATG1* mutant FCs display compound egg chambers, having more than the normal 16 germline cysts and two oocytes (see chapter 2.1). Those oocytes lie at opposing positions in the egg chamber, but both secrete Grk in an anterodorsal distribution (FIG. 2.3.2 A-A" arrowheads), and Grk is also taken up normally by the FCs (FIG. 2.3.2 A-A" arrows). Co-staining of Grk and dATG8, which labels autophagosomes under starved conditions, showed no colocalization of the proteins, indicating that autophagy is most likely not involved in the processing of Grk (FIG. 2.3.2 B-B"). Taken together, *ATG* deficiency in the FCs does not affect Grk localization, the release of Grk from the oocyte, and the uptake into FCs. Further, Grk in the FCs does not seem to be processed by the autophagic machinery.

The second round of EGFR signaling leads to a very restricted target gene expression and the formation of specialized FCs that will later form the dorsal appendages (DAs). The initial EGFR activating signal is followed by feedback loops that activate an increasingly complex set of gene expression patterns (Avraham and Yarden, 2011). Three genes that are expressed upon Grk/EGFR signaling are *Rhomboid (Rho)*, *Broad (Br)* and *kekkon (kek)*, amongst others. The protease Rho and the transcription factor Broad are expressed in neighboring and non-overlapping FC groups that later form the floor (lower part) and the roof (upper part) of the DAs, respectively. The transmembrane protein kekkon is expressed in response to EGFR signaling in an anterodorsal manner and acts in a feedback loop to repress EGFR signaling (Ghiglione *et al.*, 1999; Yakoby *et al.*, 2008; Zartman *et al.*, 2009). Since eggs having *ATG* mutant FCs display vigorously malformed or missing DAs, autophagy may modulate EGFR signaling in later stages during DA formation. To test this hypothesis, HS-FLP induced *ATG1* FC clones were generated and EGFR signaling readouts were used, such as an antibody against the Broad protein and the enhancer trap line BB142, in which lacZ is expressed under the control of the *kekkon* promoter (Schupbach and Roth, 1994; Pai *et al.*, 2000). In stage 9, Broad is uniformly expressed in all FCs overlying the oocyte, but gets restricted by stage 10 to two patches of future roof cells. In both stages, clones of *ATG1* mutant FCs showed no difference in Broad expression and distribution when compared to neighboring WT cells (FIG. 2.3.1 C-D"). Likewise, the expression of kekkon in *ATG1* mutant dorsal FCs (marked by the absence of GFP, arrowhead) overlying the oocyte nucleus of stage 10 egg chambers (FIG. 2.3.1 E-E") and in FCs of stage 11 egg chambers where DA begin to form (FIG. 2.3.1 F-F") appeared normal compared to WT cells (GFP positive), suggesting that autophagy does also not affect EGFR signaling in those stages.
Taken together, these results indicate that EGFR signaling in the FCs is not modulated by the lack of autophagy.

FIGURE 2.3.1 EGFR signaling in *ATG1* mutant FC clones



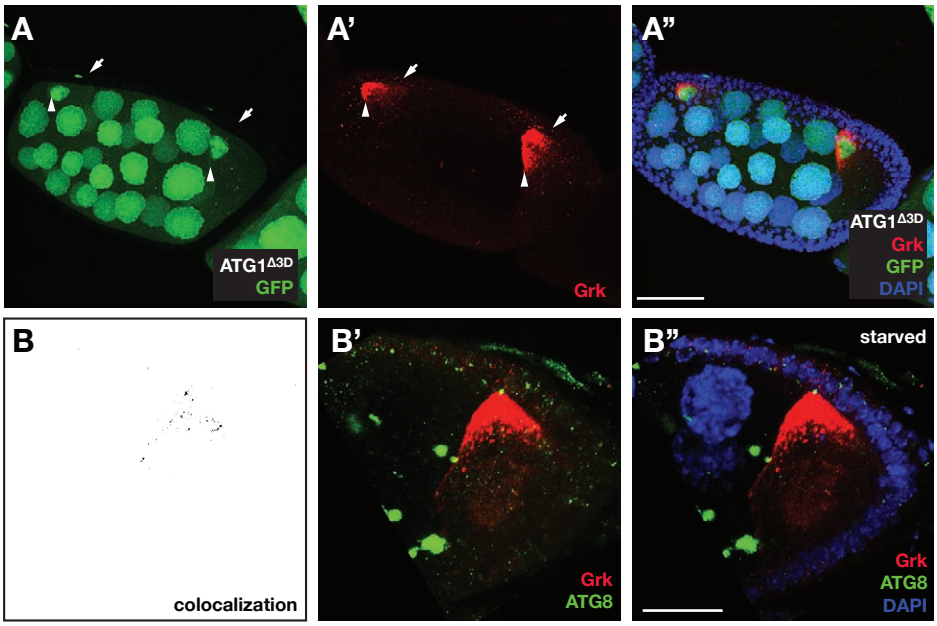


FIGURE 2.3.2 Grk protein localization in *ATG1* mutant FC clones. (A-A") HS-FLP mediated generation of *ATG1* FC clones occasionally leads to compound egg chambers, featuring more than the normal 16 germline cysts and two oocytes secreting Grk protein (arrowheads), which is taken up by FCs (arrows). **(B-B")** Co-staining of Grk protein and dATG8 under starved conditions. The colocalization pattern **(B)** displays that Grk protein, which is taken up by FCs, is not enclosed in ATG8 positive autophagosomes. Anterior is always to the left, posterior to the right. Scale bar: A: 50 µm; B: 20 µm. Genotypes: A: *ATG1$^{\Delta 3D}$-FRT80B/FRT80-UbiGFP.* B: *y w*

FIGURE 2.3.1 EGFR signaling in *ATG1* mutant FC clones. HS-FLP induced *ATG1* clones (marked by the lack of GFP) were examined for different read-outs of EGFR signaling. **(A, B)** Gurken (Grk) protein (stained in red) is translated by the oocyte and activates the EGF receptor in adjacent FCs. Normal accumulation of Grk in the posterior corner of the oocyte (arrowhead) and uptake of Grk by FCs (arrows) are seen in stage 7 egg chambers (A-A"), as well as after the move of the nuclei to the anterior-dorsal side in stage 10 eggs (B-B"). **(C, D)** The transcription factor Broad is expressed in all stage 9 oocyte-associated FCs and no difference is seen between WT and *ATG1* mutant FCs (outlined in yellow) (C-C"). By stage 10, Broad gets repressed in midline FCs and all other FCs except the two patches of future roof cells, which is equally seen in WT and *ATG1* mutant FCs (outlined in yellow) (D-D"). **(E, F)** In eggs containing *ATG1* mutant FCs, a normal distribution of kekkon (kek, stained in red) expression is seen in FCs overlying the nucleus in stage 10 eggs (E-E") and also in stage 11 eggs in cells that later form the dorsal appendages (F-F"). Anterior is to the left, posterior to the right. In B and E, dorsal is to the top. Scale bar: 50 µm. Genotypes: A-D: *ATG1$^{\Delta 3D}$-FRT80B/FRT80-UbiGFP*. E, F: *P[w^{+} lac-Z]BB142* (=kekkon-lacZ); *ATG1$^{\Delta 3D}$-FRT80B/FRT80-UbiGFP*.

During oogenesis, the polar cells are the only cells that express and secrete unpaired (upd), the ligand that activates JAK/STAT signaling. This is important for stem cell maintenance, induction of stalk cell fate, definition of terminal FC fates and determination of border cell fate. Upd secretion and JAK/STAT activation takes place solely in FCs, without germline communication (Xi *et al.*, 2003). However, Notch signaling is needed for polar cell induction, which is activated by the ligand Delta that is expressed in the germline (Assa-Kunik *et al.*, 2007). Additionally, in early oogenesis, polar cells exist as a small cluster of cells, but by mid-oogenesis, extra cells are eliminated through programmed cell death, leaving exactly two polar cells (Khammari *et al.*, 2010). Loss of polar cells, an increase in the number of polar cells, or ectopically expressed polar cells can cause fused egg chambers and reorientation of the oocyte (McGregor *et al.*, 2002), resulting in compound or inverted eggs as shown in chapter 2.2 FIG. 1 A, C and E.

To determine whether the lack of autophagy in FCs affects JAK/STAT signaling, the polar cell fate and localization were assayed using antibodies to the cell adhesion molecule Fasciclin III (FasIII), which is specifically expressed in polar cells from stage 3 to 10 of oogenesis (Ruohola *et al.*, 1991). Expression of FasIII is active in polar cells and in immature FCs of WT eggs during early oogenesis, but is limited to the polar cell pairs in later stages (McGregor *et al.*, 2002). FasIII is also highly abundant during early stages of normal and compound eggs containing *ATG1* mutant FCs (marked by the lack of GFP, arrowheads) and is solely expressed in polar cells during later stages (arrows) (FIG. 2.3.3 A-A"). Extra polar cells die until mid-oogenesis by programmed cell death, which can also be seen in polar cells mutant for *ATG1* (FIG. 2.3.3 B-B", arrow). This results in exactly two pairs of polar cells at the anterior and posterior poles of WT as well as *ATG1* mutant FCs (FIG. 2.3.3 C-D", arrowheads). Polar cells also act as organizers that define border cell fate. By stage 9, the pair of anterior polar cells migrates together with a cluster of border cells to the nurse cell-oocyte interface where it will later be important for the formation of the micropyle (Montell, 2003). In egg chambers consisting of *ATG* mutant FCs, cell migration defects were never observed, and expression of ATG-RNAi specifically in the polar cells using the upd-GAL4 driver did not lead to DA defects nor a defective micropyle (see 2.1 and 2.2). In conclusion, polar cell fate, localization and formation of the micropyle, and consequently also JAK/STAT signaling, is not affected by the loss of autophagy in FCs.

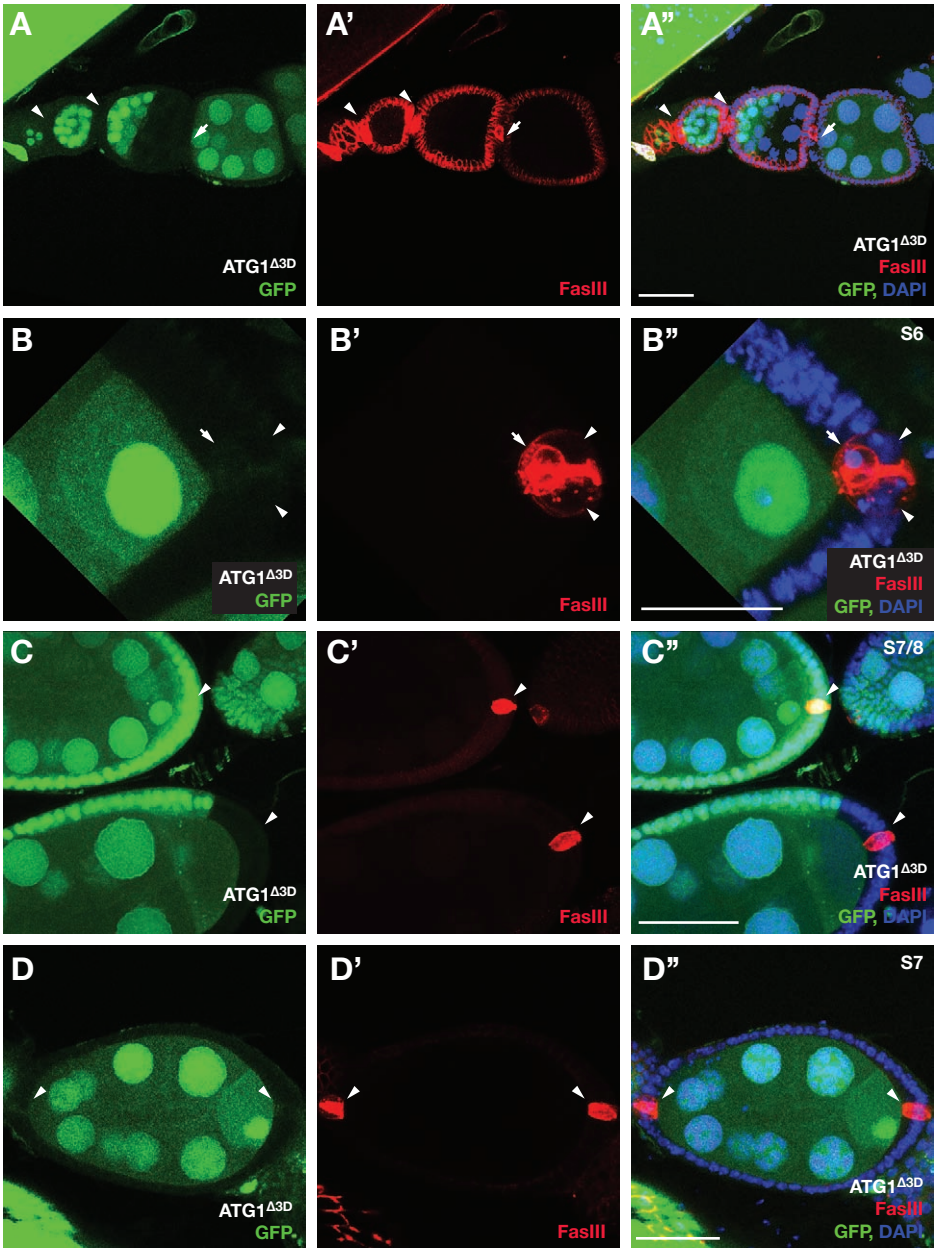


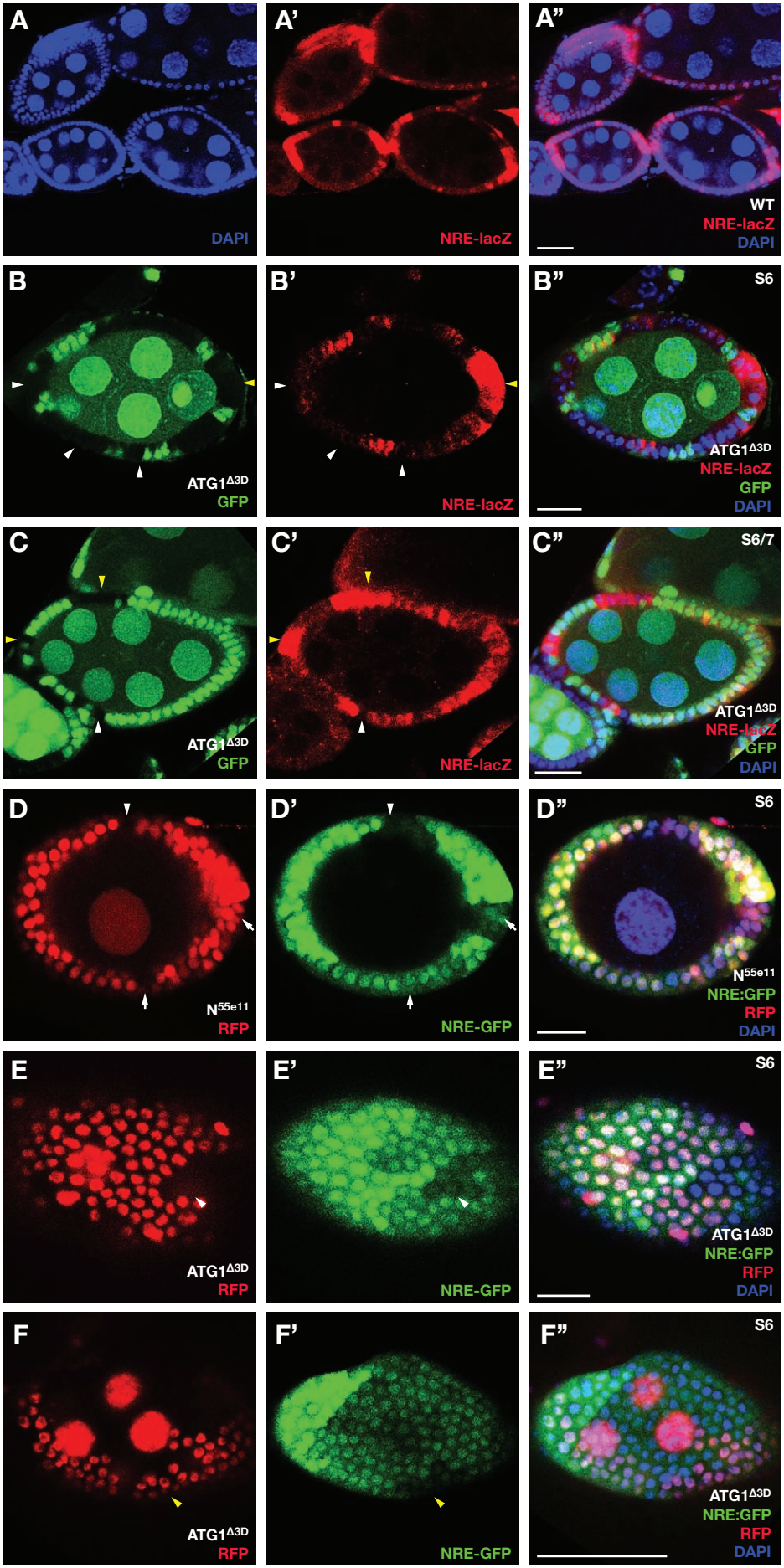
FIGURE 2.3.3 JAK/STAT signaling is not affected in *ATG1* mutant FCs. Polar cells were marked using an antibody against Fascilin III (FasIII) in eggs containing HS-FLP induced *ATG1* mutant clones. **(A)** During early stages, FasIII is expressed in all FCs (arrowheads) but gets restricted to the polar cell pairs in later stages (arrow). Regular staining of early polar cell clusters and polar cell pairs is seen in normal and compound egg chambers containing *ATG1* mutant FCs (marked by the lack of GFP). **(B)** Polar cell clusters are reduced to two cells by programmed cell death. In a stage 6 egg with *ATG1* mutant FCs, the pair of polar cells is seen (arrowheads) and a third polar cell having condensed chromatin and inclusion bodies indicative of cell death (arrow). **(C, D)** After the reduction of polar cell number, pairs of polar cells are seen at the posterior side of WT (C, upper arrowhead) and *ATG1* mutant FCs (C, lower arrowhead), but also at the anterior and posterior sides of eggs (arrowheads) that are completely surrounded by *ATG1* mutant FCs (D). Anterior is always to the left, posterior to the right. Scale bars: A, C, D: 50 µm. B: 20 µm. Genotype: *ATG1$^{\Delta 3D}$-FRT80B/FRT80-UbiGFP*

The third pathway that is possibly modulated by autophagy during oogenesis is Delta-Notch signaling. By using two readouts for Notch signaling, the transcription factors Hnt and Cut, we demonstrated that loss of autophagy in *Drosophila* FCs modulates Notch signaling (see 2.2). In the following section, I show additional preliminary results that require further evaluation, but might provide extra hints concerning the mechanism by which autophagy may modulate Notch signaling.

In *Drosophila* ovaries, the ligand of the Notch receptor is Delta, a transmembrane protein expressed in GCs. Delta activates Notch recurrently during oogenesis, thus leading to the differentiation of polar cells, the switch from a mitotic to an endocyclic programm, and the differentiation of DA roof- and floor cells (Sun and Deng, 2005; Assa-Kunik *et al.*, 2007; Sun and Deng, 2007; Berg, 2008). The activated Notch receptor undergoes a set of proteolytic cleavages, resulting in the release of the Notch intracellular domain (NICD) that enters the nucleus and attaches directly to DNA-binding proteins to turn on the transcription of different downstream targets (Schweisguth, 2004). In *Drosophila*, this DNA-binding protein is called Suppressor of Hairless (Su(H)) (Lecourtois and Schweisguth, 1997; Furriols and Bray, 2000). Coupling of Su(H) binding sites with lacZ or GFP sequences provides a reporter (Notch responsive element (NRE); NRE-lacZ, NRE:GFP) for Notch signaling activity (Krejci *et al.*, 2009; Saj *et al.*, 2010). In order to investigate whether autophagy deficiency is able to modulate Notch signaling, these two reporter constructs were used in eggs containing *ATG1* mutant FC clones generated by the HS-FLP method. The NRE construct is driving lacZ expression in FCs from stage 6 on in accordance with the onset of Delta expression and Notch activation in FCs (FIG. 2.3.4 A-A").

FIGURE 2.3.4 Modulation of NRE in *ATG1* mutant FCs. The Notch response element (NRE), based on Su(H) binding sites, was used to visualize Notch activity in FCs. **(A)** NRE-lacZ is expressed in a patchy pattern upon stage 6 in FCs. **(B and C)** HS-FLP induced *ATG1* FC clones (marked by the lack of GFP) display two opposing stainings of the NRE signal when compared to adjacent WT cells (marked by GFP): An increase of the signal (yellow arrowheads) and decrease (white arrowheads). **(E and F)** Both phenotypes could also be observed with a NRE-GFP construct (WT cells are marked by RFP expression, *ATG1* mutant cells lack RFP). **(D)** The NRE signal is lost in *Notch* mutant FCs (marked by the lack of RFP, arrowheads) and appears also patchy in WT cells (arrows). Anterior is always to the left, posterior to the right. Scale bars: 20 µm. Genotypes: A: *y w;NRE-lacZ*, B, C: *hs flp/+;NRE-lacZ/CyO;ATG1$^{\Delta 3D}$-FRT80B/FRT80-UbiGFP*, D: *hs flp FRT19A-UbiGFP/N^{55e11} FRT19A;NRE:GFP/+,* E, F: *hs flp/+;NRE:GFP/CyO; ATG1$^{\Delta 3D}$-FRT80B/FRT80-UbiGFP.*

FIGURE 2.3.4 Modulation of NRE in *ATG1* mutant FCs



However, the expression of the NRE reporter construct is patchy and generally more pronounced in terminal FCs (FIG. 2.3.4 A'). NRE-lacZ staining in *ATG1* mutant FCs (marked by the lack of GFP) leads to two opposing results, an attenuation (white arrowheads) and an increase (yellow arrowheads) of the signal in comparison to neighboring WT cells (FIG. 2.3.4 B-C"). The differences do not correlate with the A/P or D/V axes and could not be assigned to different developmental stages. Neither do clones and stainings overlap merely by coincidence, because in many cases, NRE-lacZ expression corresponds tightly to clonal boundaries. Since lacZ visualization involves sometimes difficult immunostaining procedures, we verified the results by using another NRE reporter coupled to GFP (Saj *et al.*, 2010). The expression of Notch activated GFP is equally patchy as lacZ expression (FIG. 2.3.4 D'-F') and similarly represents either attenuation of the NRE:GFP reporter (white arrowhead) in *ATG1* mutant cells (marked by the lack of RFP) (FIG. 2.3.4 E-E"), or an increase in NRE:GFP (yellow arrowhead) (FIG. 2.3.4 F-F"). As a control, Notch mutant clones were induced (FIG. 2.3.4 D-D"). Most FCs mutant for Notch displayed a loss of the NRE-GFP signal when compared to adjacent WT cells (arrowhead). However, although increase of NRE-GFP in *Notch* mutant FCs was not observed, some FCs exhibited a patchy NRE:GFP expression, which was not correlated to clonal boundaries (FIG. 2.3.4 D-D", arrows). Again, the differences did not correlate with the A/P axis and since all eggs examined were between stage 6/7 of oogenesis, the developmental stage is unlikely to influence the opposing results of the NRE signal. Furthermore, NRE expression was modulated by both single cell clones (FIG. 2.3.4 C-D") and larger clones (FIG. 2.3.4 C-C", E-E"), thus excluding clonal size as a factor for the fluctuating NRE signal. However, different contributions of specialized FC subpopulations (*e.g.* posterior, stretched, centripetal FCs) to NRE modulation can not be ruled out, since the differentiation status of the FCs is hardly distinguishable without specific markers for these cells. In general, the quantification of the NRE reporter seems difficult due to the patchy expression seen in FCs.

Previously, different ATG-RNAi lines were effectively used to knock-down autophagy in FCs (see 2.2). Here, the daughterless (da) -GAL4 driver was used to express ATG1- and ATG5-RNAi in FCs as another way to assess NRE activity on ATG deficient cells. Since da-GAL4 expression is restricted to random FC groups, virtually a clonal situation with WT and RNAi expressing cells can be obtained. ATG1-RNAi expression (marked by concomitant RFP expression) again led to attenuation (white arrowheads) as well as an increase (yellow arrowheads) of NRE:GFP signal when compared to neighboring WT

cells (FIG. 2.3.5 A-B"). However, the cellular boundaries of RNAi expressing FCs are not as clear as clonal boundaries in *ATG1* mutant FC clones and expression often appeared weak, as judged by the RFP signal. Since GAL4 expression is temperature dependent (Duffy, 2002), ATG5-RNAi expressing flies were transferred to 29°C for 6 days to maximize expression levels. Although boundaries became more clear and expression of ATG5-RNAi was enhanced, an up- (white arrowheads) and downregulation (yellow arrowheads) of the NRE:GFP signal in ATG5-RNAi treated cells (FIG. 2.3.5 C-E") was still observed. Again, the patchy expression of the NRE reporter impedes the convincing analysis of the data. Due to the variable expression of the reporter constructs in the FCs, NRE does not seem to represent a reliable readout for Notch activity in the ovaries.

The same pattern, an in- or decrease of the NRE reporters in autophagy deficient FCs was observed in all situations, however, the alterations in NRE activation are difficult to explain. Both NRE constructs are based on Su(H) binding sites for the NICD, which results from cleavages of the whole Notch receptor, and are mostly present in the nucleus. Thus, the loss of the Notch receptor in Notch mutant clones is expected to cause an absence of NRE positive signal. Indeed, Notch mutant FC clones do not express NRE-GFP. We proposed that a modulation of Notch signaling through autophagy is caused by modified endosomal trafficking or retarded lysosomal degradation of the Notch receptor (see 2.2). Thus, NRE-GFP should be enhanced in ATG mutant clones. Indeed, this is observed in many cases when the NRE reporters are used. However, the patchy expression of the NRE reporter construct does not allow for conclusive statements about Notch activity in these experiments. On the contrary, the results obtained with two other Notch readouts (Cut and Hnt, see chapter 2.2) displayed consistent outcomes. Equal modulation of the Cut and Hnt readouts in autophagy deficient FCs carrying two independent *ATG* mutations, *ATG1* and *ATG13*, corroborate the hypothesis of an involvement of autophagy in the modulation of the Notch signaling pathway.

The NRE:GFP reporter was originally designed for a wing-based assay facilitating screening of RNAi lines for novel Notch regulators (Saj *et al.*, 2010). In order to examine the influence of autophagy on Notch signaling in systems other than ovaries, this assay was adopted to express ATG-RNAi lines in the larval wing imaginal discs. During wing development, Notch signaling is active at the D/V boundary of wing imaginal discs, displayed by NRE:GFP expression (FIG. 2.3.6 A and B, B').

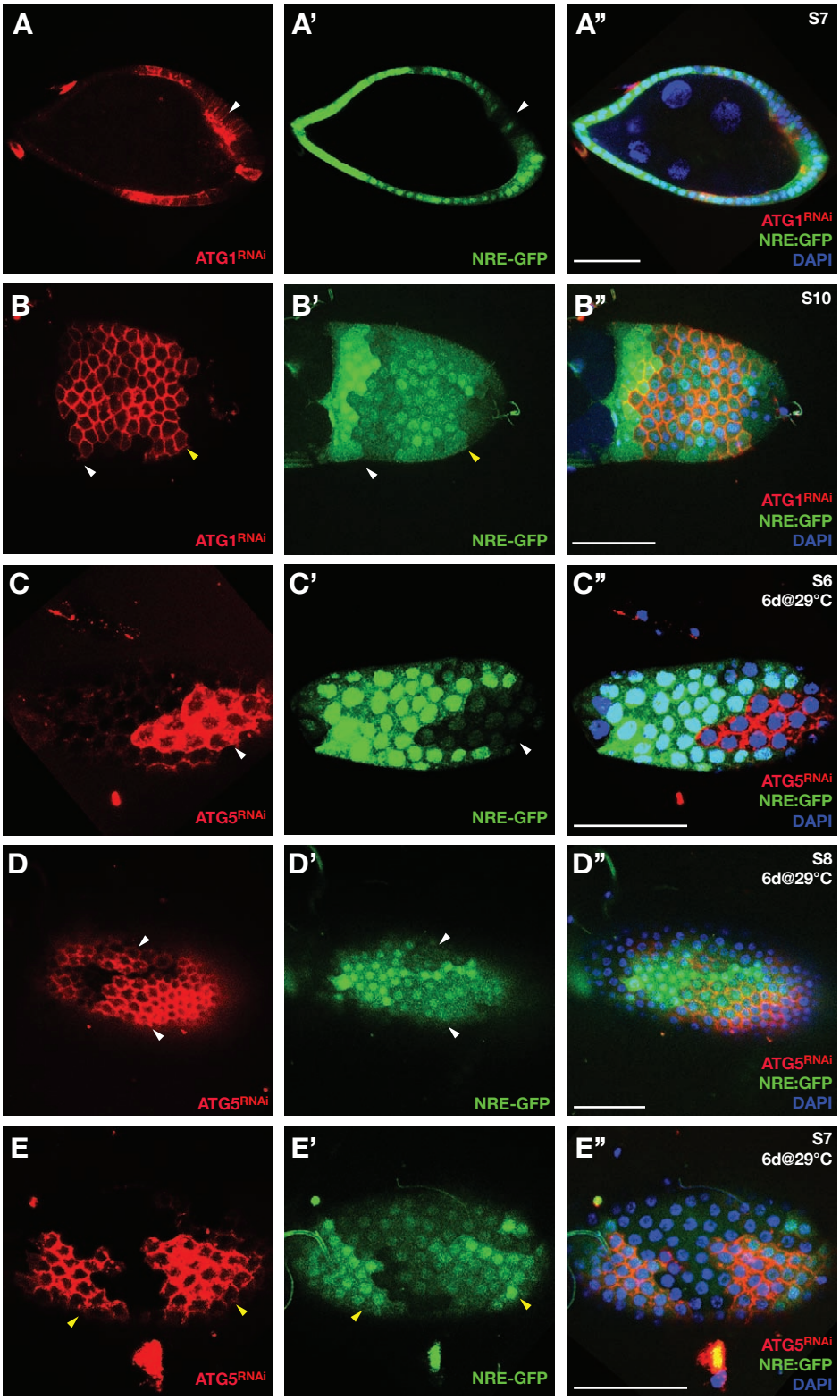


FIGURE 2.3.5 Modulation of NRE by expression of ATG-RNAi in FCs. The NRE:GFP was used to visualize Notch activity in FCs. **(A and B)** NRE-GFP (marked in green) can be modulated in two fashions by expression of ATG1-RNAi (marked by concomitant expression of RFP): An increase of the signal (yellow arrowheads) and a decrease (white arrowheads). **(C-E)** The same alterations can be seen after expression of ATG5-RNAi at higher temperatures. Anterior is always to the left, posterior to the right. Scale bar 50 µm. Genotypes: A, B: *ATG1^{RNAi}/NRE:GFP;*, C-E: *ATG5^{RNAi}/+; NRE:GFP/+;.*

By using engrailed (en) -GAL4 as a driver line, RNAi expression can be targeted to the posterior half of the wing imaginal disc, leaving the anterior side WT as an internal control (FIG. 2.3.6 A and B, B'). Expression of Su(H)-RNAi (marked by RFP) as a control resulted in the expected loss of NRE:GFP signal in the posterior compartment (FIG. 2.3.6 C, C'), whereas overexpression of Delta led to the induction of secondary D/V boundaries along the A/P axis (FIG. 2.3.6 D, D', arrowheads). Expression of ATG1-, ATG4- and ATG5-RNAi (FIG. 2.3.6 E-G), as well as overexpression of ATG1 (FIG. 2.3.6 H-H') in the posterior compartment did not significantly alter the NRE:GFP signal compared to the anterior WT side. However, this might be due to a very strong Notch signal in the wing imaginal discs, since a modulation of Cut and Hnt by the lack of autophagy (see 2.2) could only be observed in oogenesis stages right after Notch is switched on. In addition, it has been shown that autophagy has organ specific functions (E. Baehrecke, pers. communication) and it might be possible that Notch is not modulated by autophagy in the wing imaginal discs.
Since defective Notch signaling is marked by wing margin patterning defects resulting in wing 'notches', the wings of adult flies were also investigated by expressing ATG-RNAi under the control of en-GAL4. Expression of Notch-RNAi induces notches at the wing margins in the posterior half of the wing as a result of insufficient Notch activation at the D/V boundary, whereas WT flies have smoothly rounded posterior wing boundaries (FIG. 2.3.6 A' and I, J). In contrast, expression of ATG1- and ATG5-RNAi led to slightly larger posterior wing compartments (FIG. 2.3.6 K, L, arrowheads). Because Notch activity is involved in cell proliferation (Baonza and Garcia-Bellido, 2000), this would suggest an upregulation of Notch signaling and is in accordance with the results obtained for Cut and Hnt regulation (see 2.2). However, ATG deficient cells also have a growth advantage and are enlarged under starved conditions, which could also cause the increase in wing size (Scott *et al.*, 2007).
In summary, the analysis of NRE activation in cells that are deficient for autophagy did not reveal a consistent and clear picture concerning the modulation of Notch signaling. In contrast, the results of the Notch signaling readouts Cut and Hnt (see 2.2) clearly point to an upregulation of the Notch pathway by the loss of autophagy. An explanation could be that the regulation of Cut and Hnt expression is a direct downstream effect of Notch signaling activity in *Drosophila* FCs, whereas the NRE reporter represents binding sites of the Su(H) DNA-binding transcription factor, which only mediates the expression of downstream genes. It has been shown that activation of specific Notch target genes requires further transcriptional activators and even that

Notch can regulate cellular processes independent of Su(H) (Furriols and Bray, 2001; Fiuza and Arias, 2007). Thus, the FC specific readouts Cut and Hnt seem to be more reliable and consistent than the results of the NRE reporter, mainly due to the difficulties associated with the NRE reporter discussed above.

FIGURE 2.3.6 Modulation of NRE by ATG-RNAi expression in *Drosophila* imaginal wing discs.

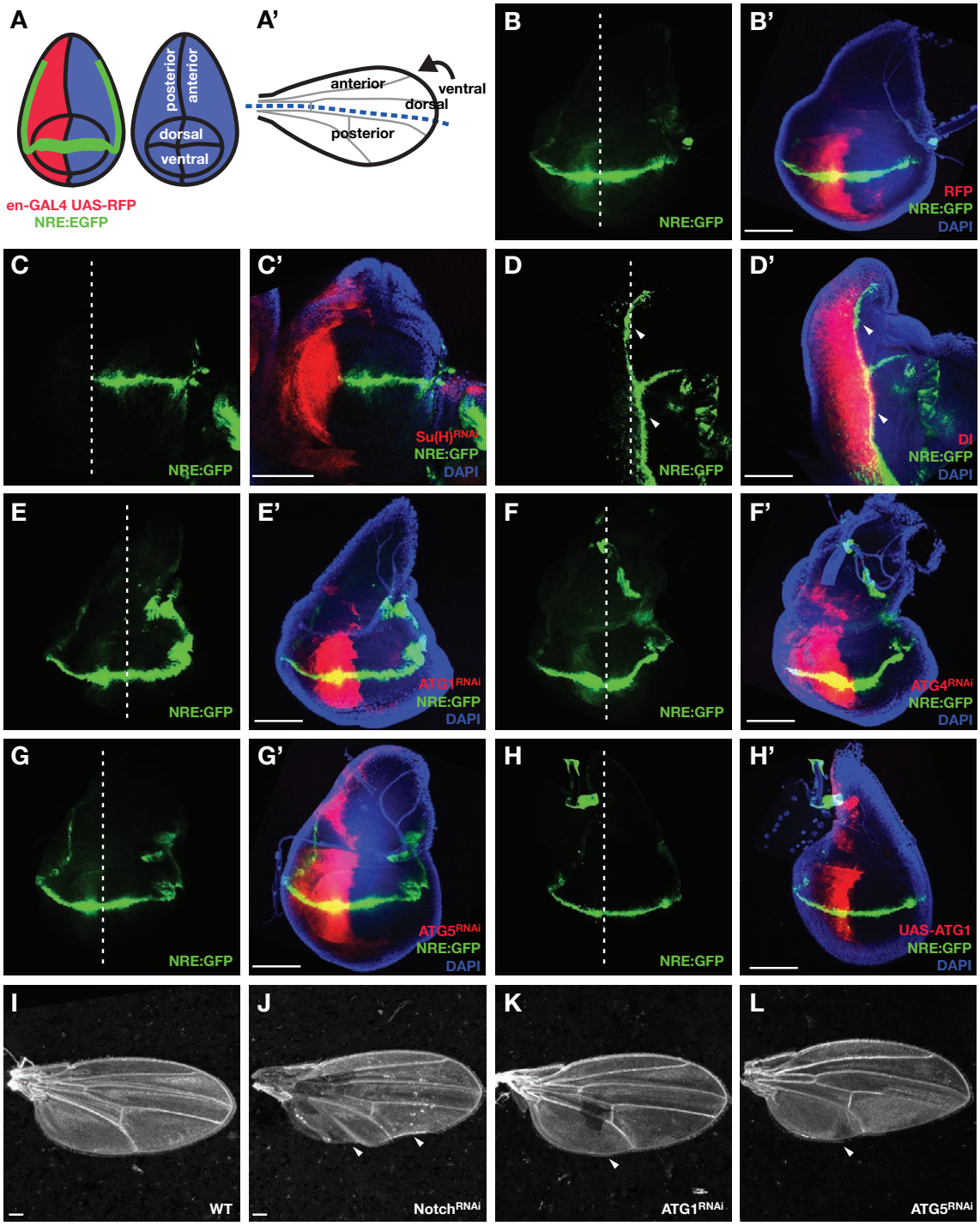


FIGURE 2.3.6 Modulation of NRE by ATG-RNAi expression in *Drosophila* wing imaginal discs. (A) Model of NRE reporter expression in the wing imaginal discs. Notch is activated at the D/V boundary resulting in NRE-GFP expression (green). **(B)** Engrailed (en) -GAL4 drives expression in the posterior compartment, visible by RFP co-expression (red). The anterior compartment stays WT (A/P boundary in dotted line). **(C)** Expression of Su(H)-RNAi resulted in the loss of the NRE signal. **(D)** Delta (Dl) expression induced a second D/V boundary at the A/P axis (arrowheads). **(E-H)** Expression of ATG-RNAi or overexpression of ATG1 did not visibly change the NRE signal. **(I, J)** Expression of Notch-RNAi by en-GAL4 causes wing 'notches' in the posterior wing compartment. **(K, L)** ATG-RNAi expression led to a slight increase of the posterior wing area (arrowheads). Wing imaginal discs: Posterior is to the left, anterior to the right, dorsal to the top, ventral to the bottom. Wings: Anterior is to the top, posterior to the bottom, dorsal on the front, ventral at the back. Scale bars: 100 µm. Genotypes: B: *;en-GAL4 UAS-RFP NRE:GFP*/+, C: *en-GAL4 UAS-RFP NRE:GFP*/+;*Su(H)^{RNAi}*/+, D: *en-GAL4 UAS-RFP NRE:GFP*/+;*UAS-Dl*/+, E, K: *en-GAL4 UAS-RFP NRE:GFP/ATG1^{RNAi}*, F: *en-GAL4 UAS-RFP NRE:GFP/ATG4^{RNAi}*, G, L: *ATG5^{RNAi}*/+;*en-GAL4 UAS-RFP NRE:GFP*/+;, H: *en-GAL4 UAS-RFP NRE:GFP*/+;*UAS-ATG1^{6B}*/+, I: *;en-GAL4 UAS-RFP NRE:GFP*/+, J: *en-GAL4 UAS-RFP NRE:GFP/Notch^{RNAi}*

III Discussion

Autophagy, a tightly regulated degradation process occurring in all eukaryotic cells from yeast to mammals, has been implicated in immunity, lifespan extension and many human pathophysiologies, such as neurodegeneration and cancer (Chen and Klionsky, 2011). Especially for cancer therapies, it will be crucial to assign pathways that are regulating autophagy, as autophagy plays a dual role in cancer, acting both as tumor suppressor and protector of cancer cells, depending on the cellular context and the stage of tumorigenesis (Chen and Karantza, 2011). In addition, autophagy was shown to be required during normal development, for example for the turnover of aged organelles and for cellular remodeling (Baehrecke, 2002; Rabinowitz and White, 2011). However, in many cases, the role of autophagy during development is still unclear and new pathway components implicated in regulating autophagy are repeatedly discovered, highlighting the relevance of autophagy research. This thesis provides novel findings that will help to clarify two major unsolved issues in the field of autophagy: The physiological role of autophagy and the regulatory pathways governing this function.

Here, I aimed to study the physiological role of autophagy during development using the *Drosophila* ovaries as a model system. We could demonstrate that autophagy is induced by starvation in germ cells (GCs) as well as in surrounding somatic follicle cells (FCs) of the ovaries, and that this depends on a functional ATG machinery. Furthermore, the insulin/TOR pathway is shown to control autophagy induction in these tissues. We additionally found that autophagy is not required in GCs during oogenesis, but that loss of autophagy in FCs leads to developmental defects. This depends on the cellular context, and defects are only present in a situation where FCs are mutant for autophagy, but GCs are WT. Furthermore, we could assign the posterior FCs as the cell subpopulation causing the oogenesis defects. Finally, evidence is presented for a modulation of the Notch signaling pathway by autophagy in *Drosophila* FCs, which might be responsible for the egg chamber

defects. This is to date the first direct indication for an involvement of autophagy in regulating the Notch pathway. Notch signaling plays critical roles during development in cell-fate decisions and tissue patterning, and is found to be dysregulated in a variety of cancers (Bolos *et al.*, 2007). Thus, the discovery of a regulatory link between autophagy and Notch signaling may open novel opportunities for cancer therapies.
As detailed results are discussed in the single chapters, in the following part I will focus on a short discussion of the main findings of this thesis, point to unanswered questions and explain new techniques that could solve these issues.

3.1 Physiological role of autophagy during *Drosophila* oogenesis

It has long been known that starvation induces autophagy in mammals (Ashford and Porter, 1962; Deter and De Duve, 1967) and it was previously shown that nutrient deprivation induces autophagy in the *Drosophila* fatbody (Scott *et al.*, 2004). However, there was no evidence that starvation is also able to induce autophagy in other nutrient responding organs. In addition, it has been shown that starvation affects ovary size and egg production and induces programmed cell death in *Drosophila* ovaries (Drummond-Barbosa and Spradling, 2001), suggesting a physiological role for autophagy during *Drosophila* oogenesis.
To study this putative role, we established the *Drosophila* ovaries as a model system. Since molecular readouts were limited to lysotracker assays (Scott *et al.*, 2004), fluorescently tagged marker proteins expressed in both the soma and the germline were generated, as well as a *Drosophila* ATG8 antibody to reveal that autophagy is induced upon starvation in GCs and somatic FCs during *Drosophila* oogenesis. By using *ATG* mutant flies and tissue, and measuring *ATG* gene expression levels, we could also show that starvation-induced autophagy depends on a functional ATG machinery. During the course of this study, two other groups examined the relationship between autophagy and apoptosis using *Drosophila* ovaries as a model system and found that starvation induces autophagy in the germarium and in dying mid-stage egg chambers (Hou *et al.*, 2008). Further, cell death during early oogenesis was shown to be mediated through autophagy, and it was demonstrated that autophagy controls DNA fragmentation during late oogenesis (Nezis *et al.*,

2009; Nezis *et al.*, 2010). Both studies are in accordance with our results (Barth *et al.*, 2011) and confirm that autophagy is induced by starvation in *Drosophila* ovaries.
Together, these findings establish the *Drosophila* ovaries as a novel model system to study the physiological role of autophagy.

Given the multiple roles of autophagy in both health and disease, this process must be tightly regulated, since too much or too little autophagy can be fatal for the cell. The growth regulatory insulin/TOR signaling pathway also controls autophagy in the fat body and in salivary glands of *Drosophila* (Rusten *et al.*, 2004; Scott *et al.*, 2004; Berry and Baehrecke, 2007). By either blocking or activating insulin/TOR signaling with the drug rapamycin or through overexpression of Rheb, respectively, we could show that insulin/TOR signaling also controls starvation induced autophagy in the *Drosophila* ovaries. Interestingly, mutants of the insulin/TOR pathway display only premature eggs and are sterile (Bohni *et al.*, 1999; Stocker *et al.*, 2003; Richard *et al.*, 2005; Werz *et al.*, 2009), suggesting a role for nutrient signaling during oogenesis. In the mammalian system, impaired insulin signaling was found to be associated with the polycystic ovary syndrom (PCOS), which is accompanied by an upregulation of apoptosis regulators (Dunaif and Thomas, 2001; Avellaira *et al.*, 2006). Thus, alterations in insulin signaling could lead to dysregulation of autophagy, which might be implicated in the development of PCOS. The results of this doctoral work provide the basis for further studies on insulin/TOR dependent autophagy regulation, including investigations to reveal whether modulations of autophagy by insulin signaling can lead to PCOS.
TOR controls autophagy by direct phosphorylation of ATG proteins, and in addition to the insulin pathway, several other pathways such as the energy measuring AMPK pathway, merge at TOR (Shaw, 2009). Although the regulation of autophagy by TOR has been studied extensively, questions concerning basic functions remain. For example, it is still unclear how TOR is activated by amino acids. It has been reported that amino acids regulate the nucleoide status of small GTPases, the RAG proteins, which can activate TOR (Kim *et al.*, 2008; Sancak *et al.*, 2008). However, the exact mechanism of TOR activation as well as the mechanism by which amico acids activate Rags are still unknown. Another open question is the role of TOR dependent phosphorylation of ATG proteins, since activation of ATG proteins by TOR, and association of ATG1 and ATG13 differ between the yeast, *Drosophila*, and mammalian systems and the hierarchy of phosphorylations is still confusing (Kamada *et al.*, 2000; Chang and Neufeld, 2009; Hosokawa *et al.*, 2009). For

example, it is still unclear whether the phosphorylation of ATG13 is essential for ATG1 in order to be active, and contradictory results in yeast and *Drosophila* systems are described (Chang and Neufeld, 2010; Kamada *et al.*, 2010). Furthermore, the targets of the ATG1 complex and the exact function of this complex remain to be elucidated. It has been proposed that ATG1 could have other functions besides autophagy; however, there is no data on the involvement of ATG1 in other pathways so far. Therefore, the identification of both ATG1/ATG13 interaction partners, and of the direct substrates of ATG1 are needed to clarify theses issues. Immunoprecipitation assays and subsequent tandem mass spectrometry (MS/MS) analyses could be employed to reveal proteins bound to the ATG1 complex. Further, the development of novel methods to capture and detect phosphorylation sites using MS-based techniques will allow the discovery of proteins phosphorylated by ATG1 (Bodenmiller and Aebersold, 2010). These studies will give valuable insights into the proteins and the respective signaling pathways that are controlled by the autophagic machinery. Finally, as mentioned before, excessive autophagy can be deleterious for the cell, thus an effective mechanism to downregulate autophagy is essential for survival (Klionsky and Nemchenko, 2011). However, most regulatory components known to date are kinases, which suggests that the corresponding phosphatases remain to be identified. Genome-wide proteomic phosphatase screening, making use of the RNAi collection existing for *Drosophila* (http://stockcenter.vdrc.at), could be used to answer this issue. Also, the biochemical context underlying insulin/TOR control is still poorly characterized and systematical interactome studies are currently carried out to identify novel network components (Glatter *et al.*, 2011, manuscript accepted at Mol Syst Biol.). Since ovaries have been established as a model for autophagy, they would be suitable organs to investigate these questions. The availability of large amounts of tissue-specific biological material would be of great advantage for proteomic studies.

Drosophila presents an ideal model system since it offers a genetic tractability compared to yeast (*e.g.* loss-of-function alleles, RNAi), yet is less complex than the mammalian system (*e.g.* humans posses four orthologues of yeast ATG8). Specifically for the ovaries, elegant methods exist to create mosaics in which only the germline or somatic cells are homozygous for a mutation, thus allowing the analysis of otherwise lethal genes and the investigation of functions in a cellular context. In addition, *Drosophila* oogenesis presents various developmental processes within one organ, and autophagic events can be investigated simultaneously in different cell types. Multiple sensitive cell-cell interacting mechanisms during several developmental stages within the same

animal may allow to discover subtle affects of gene manipulations. Also, novel tissue culture methods will allow the *ex-vivo* analysis of ovaries (He *et al.*, 2011; Systems Biology of the *Drosophila* wing, http://www.systemsx.ch/). Ovaries are especially suited for *ex-vivo* analyses since they are a self-contained organ, are simple to dissect and possess large cells that are easy to visualize. In culture, nutrient supply can be tightly controlled and live imaging allows to study the dynamics of autophagic processes under different stimuli.

Having established the *Drosophila* ovaries as a model system to study autophagy, this study investigated whether autophagy is required in GCs or FCs for normal egg development. Tools to generate germ line clones mutant for specific genes have been developed (Chou and Perrimon, 1992) and homozygous viable *ATG* mutants exist (Juhasz *et al.*, 2007a). However, using the Ovo^{D} technique, generation of clones in other tissues *e.g.* the FCs, might distort the result. Furthermore, in viable autophagy mutants (*e.g.* ATG7), it was shown that autophagy can adopt organ specific functions, making it difficult to validate results (E. Baehrecke, pers. communication). Therefore, we generated chimeric flies lacking ATG function in either the GCs, FCs or the entire ovary using the pole cell transplantation, FC irradiation, and ovary transplantation techniques, respectively (FIG. 3.1). The advantages of the pole cell transplantation method are the lack of perdurance and maternal contribution. In addition, transplanted pole cells are hemizygously mutant for the gene of interest, excluding second site lethal effects. The ovary transplantation technique is a unique method to create a chimeric animal where only the ovaries are mutant in an otherwise wilt-type fly, thus permiting to dissect the physiological role of lethal genes in the ovaries. The application of these techniques allowed the creation of specific chimeras and showed that autophagy deficiency in the germline and the entire ovary did not affect egg development, whereas eggs containing *ATG* mutant FCs resulted in lethality and produced dorsal appendage defects. These findings suggested that the incompatibility between autophagy-competent GCs and autophagy-deficient FCs leads to defective egg development (FIG. 3.1). Egg morphogenesis depends on a tightly linked signaling between FCs and GCs, thus, we proposed a model in which autophagy is required for the communication between these two cell types. The lack of autophagy might modulate a signal in the FCs, leading to a wrong response, *e.g.* defective development (FIG. 3.1 C), which does not happen in a situation where both tissues are mutant, because the corresponding cell-cell signaling may be modulated equally in FCs and GCs (FIG. 3.1 A). In a situation where only the GCs are mutant for autophagy, the signaling is not modulated in the FCs and the lack of *ATG*

genes in the GCs does not affect development and survival (FIG. 3.1 B). This data establish an important function for autophagy during oogenesis and contribute to the understanding of the role of autophagy in animal development.

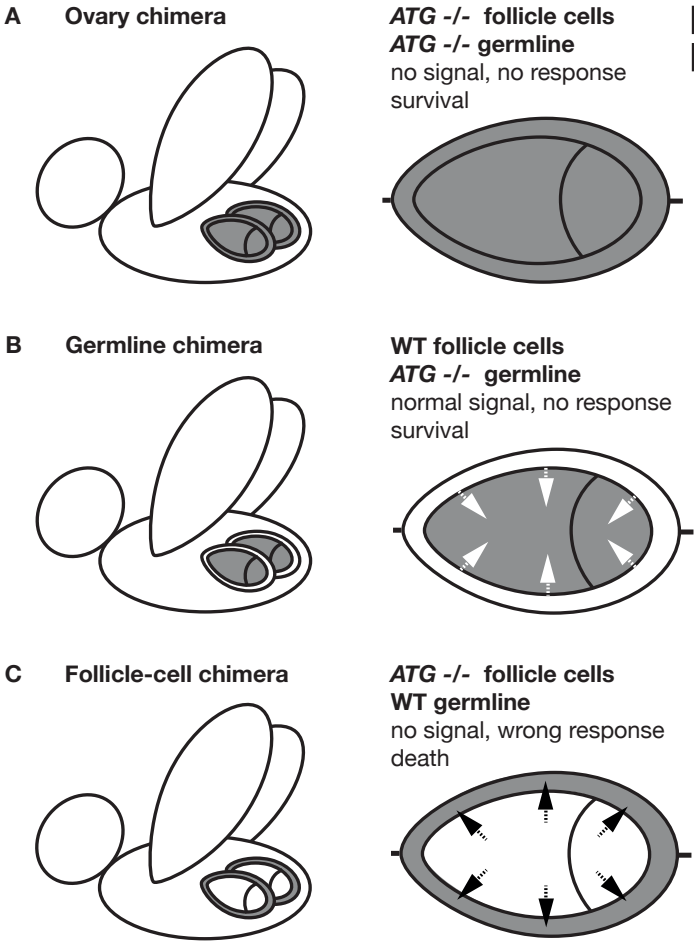


FIGURE 3.1 Incompatibility model. Mosaic animals in which only a certain tissue is mutant for *ATG* genes allows the analysis of autophagy function in a multicellular context. **(A)** Ovarian chimeras comprised of a WT body (white) and *ATG* mutant FCs and GCs (grey). **(B)** In germline chimeras, all somatic cells are WT, including the ovarian FCs, and only GCs are *ATG* mutant. In both situations, lack of autophagy did not cause developmental defects. **(C)** In FC chimeras, only FCs are mutant for *ATG* genes. This situation may lead to an incompatibility with the surrounding WT tissue (the GCs and the fly body) to cause developmental defects. Abbreviations: WT wild type.

3.2 Notch as a novel pathway regulated by autophagy

Motivated by the results showing that autophagy is required in the *Drosophila* FCs in a cellular context, I became interested to reveal the underlying mechanism that causes this incompatibility. Three evolutionarily conserved signaling pathways are shared between the GCs and FCs during oogenesis and are essential for cell differentiation and axis specification: Delta-Notch, EGFR and JAK/STAT (Poulton and Deng, 2007). Importantly, all three signaling pathways are highly implicated in cancer development, thus novel insights in

the modulation of these pathways is of great value (Constantinescu *et al.*, 2008; Mitsudomi and Yatabe, 2010; Ranganathan *et al.*, 2011).

For the EGFR and Delta-Notch pathways, it has been shown that endocytosis and endosomal trafficking is required within ligand and/or receptor presenting cells for activation, regulation and degradation of the signal (Yamamoto *et al.*, 2010; Avraham and Yarden, 2011), and intersections between the endosomal and autophagy pathways have long been described (Gordon and Seglen, 1988; Liou *et al.*, 1997). Furthermore, UVRAG, Vps34, and ESCRT, all proteins required for endosomal sorting and trafficking, have also been implicated in autophagic regulation (Rusten *et al.*, 2007; Juhasz *et al.*, 2008; Lee *et al.*, 2011). In addition, it has been proposed that *ATG4* modulates Notch signaling in the *Drosophila* wing (Thumm and Kadowaki, 2001). These reports suggest that autophagy might be implicated in the endosomal regulation of receptor/ligand signaling.

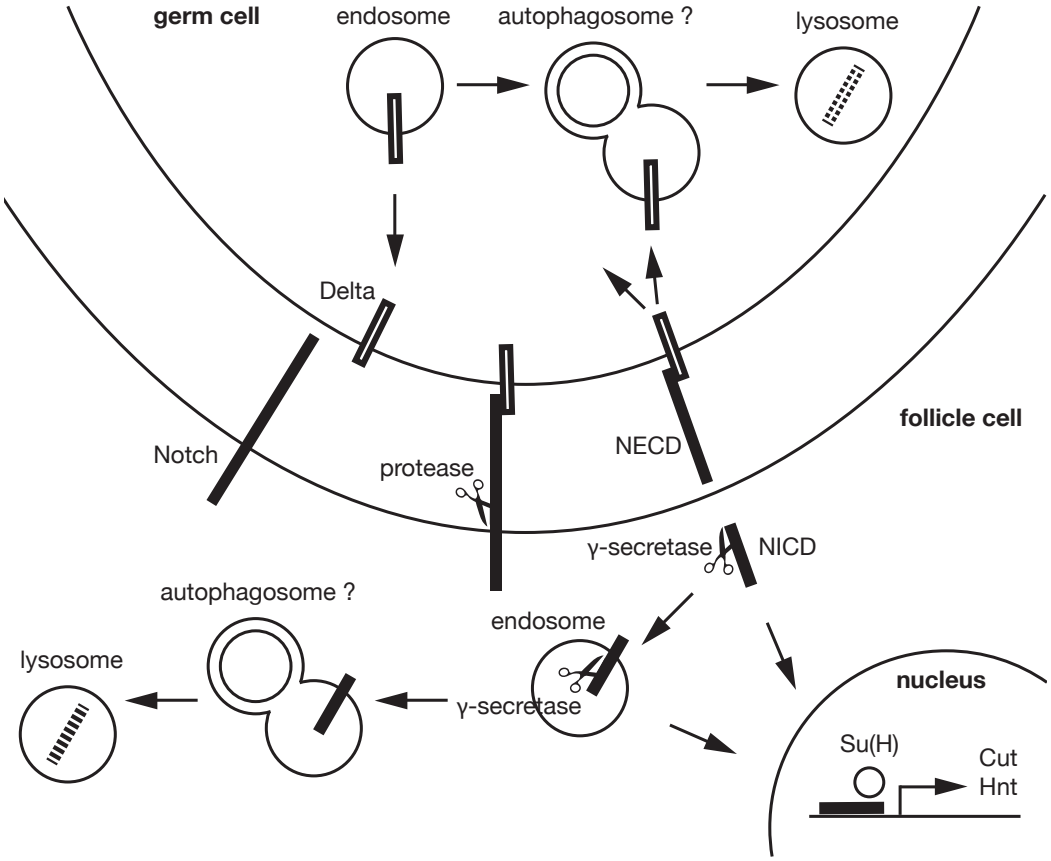


FIGURE 3.2 Activation of Notch signaling. Notch is activated by binding of the ligand Delta, expressed in GCs. Delta can be internalized before and/or after Notch activation and degraded, possibly also via autophagy. Activated Notch undergoes several cleavages by proteases. γ-secretase cleavage occurs at the plasma membrane and in endosomes and releases the Notch intracellular domain (NICD) from the Notch extracellular domain (NECD). NICD translocates to the nucleus and activates, together with co-activators (*e.g.* Su(H)), the transcription of Notch target genes (*e.g.* Cut, Hnt). NICD signaling is terminated by lysosomal degradation and possibly also by autophagic degradation.

This doctoral work demonstrated that the generation of *ATG* mutant FCs leads to several egg chamber defects, many of which are consistent with defects obtained by the modulation of the above mentioned signaling pathways. Further, using spatially restricted GAL4 driver lines to express RNAi against *ATG* genes in specific subpopulations of FCs, we could show that autophagy deficiency, especially in the posterior FCs, causes egg chamber defects. Finally, this study revealed that two specific Notch signaling readouts in *Drosophila*, Cut and Hnt, are modulated in the FCs by the loss of autophagy. Both readouts are inversely modulated compared to the Notch loss-of-function situation, suggesting an increase of Notch activity due to the lack of autophagy. Taken together, these results point to an involvement of autophagy in the regulation of the Notch signaling pathway. As mentioned above, dysregulation of Notch has been implicated in tumorigenesis, but autophagy also plays a considerable role in tumorigenesis (Rosenfeldt and Ryan, 2011). Therefore, gaining insights into a possible regulation of Notch signaling by autophagy may provide novel opportunities for cancer therapies. However, many issues remain unanswered. It has been shown that retention of Notch in endosomal vesicles accelerates γ-secretase cleavage and intensifies Notch signaling (Vaccari, 2008), thus we propose that the lack of autophagy leads to a delayed or reduced degradation of the Notch intracellular domain (NICD), responsible for signal transduction, thereby extending and enhancing Notch signaling. However, this hypothesis needs to be confirmed. First, if the lack of autophagy leads to enhanced Notch signaling, does hyperactivation of autophagy, *e.g.* by the expression of *ATG* genes, result in a decrease of Notch signaling? This could be investigated by using the FLP-out technique to overexpress UAS-ATG1 in FC clones and determine the expression of Notch reporters. However, the existing UAS-ATG1 construct was shown to induce cell death, and overexpression is lethal in many cases, thus a new construct under the endogenous promotor will have to be generated and tested for further studies. In addition, a consequent question arising from this experiment would be, if hyperactivation of autophagy and an decreased Notch activity could be rescued by the block of endosome/lysosome fusion.

Furthermore, if Notch is degraded by autophagy, autophagosomal markers and the NICD should be detectable in the same compartments. This could be revealed with a functional GFP labelled Notch (Kawahashi and Hayashi, 2010) or immunostainings for the NICD and fluorescently tagged ATG proteins or dATG8 antibodies. In addition, several markers exist for different endosomal compartments (Vaccari *et al.*, 2008) and could be used to test their overlap with autophagosomes and/or the NICD. It has been shown that UVRAG, Vps34, and

ESCRT mutants are defective in autophagy and all three exhibit enhanced Notch signaling activity (Rusten *et al.*, 2007; Juhasz *et al.*, 2008; Vaccari *et al.*, 2008; Lee *et al.*, 2011). Therefore, it would be interesting to examine whether the Notch signaling phenotype of these mutants can be rescued in FCs by an activation of autophagy or alternatively by activation of other degradation pathways. It might indeed be possible that other degradation pathways quickly compensate for the Notch modulating role of autophagy, which is suggested by the observation that Notch signaling is only modulated in a very restricted time frame by the loss of autophagy, namely stage 6, right after Notch signaling is switched on. Actually, it has been shown that ubiquitin-dependent degradation by SCF complex family members regulates Notch activity (Matsumoto *et al.*, 2011). Thus, autophagy and proteasomal degradation could cooperatively degrade the NICD and consequently compensate for each other. Concomitant inhibition of both systems could clarify this issue.

Another question arises from the proposed incompatibility model. If the modulation of the Delta-Notch signaling causes the incompatibility between wild-type GCs and *ATG* mutant FCs, it should be possible to rescue the egg chamber defects by the loss of Delta signaling from the GCs. To substantiate this theory, a combined experiment of larval ovary and pole cell transplantations, in which a chimeric fly with *Delta* mutant GCs and *ATG* mutant FCs is generated, could be performed. However, both methods are technically challenging, and a large number of chimeras will be required for conclusive quantifications.

Furthermore, some of the egg chamber defects obtained by *ATG* mutant FCs are not solely correlated with phenotypes for defective Notch signaling. Therefore, the contribution of other signaling pathways can not be completely excluded. For example, dysregulation of Decapentaplegic (Dpp) signaling also causes dorsal appendage defects (Shravage *et al.*, 2007). A dpp-lacZ construct, antibody stainings for the Dpp signal transducer pMAD or expression of the direct Dpp target gene, dad-lacZ could help to clarify this consideration. In addition, since EGFR signaling is also regulated via receptor endocytosis and degradation (Sorkin and Goh, 2009), the methods used could have failed to detect subtle changes in EGFR signaling. More sensitive readouts, *e.g.* antibody labeling for diphosphorylated ERK, might answer this question.

In summary, this doctoral work shows that autophagy is critical in *Drosophila* FCs and has the ability to modulate the Notch signaling pathway. This opens novel possibilities of endosomal receptor regulation and might be relevant for studies concerning cancer treatment.

Yeast genetics have allowed to make significant progress in identifying the molecular machinery of autophagy. However, the understanding of the physiological roles of autophagy has lagged behind these advances and it still remains a challenge to identify autophagic components and regulatory mechanism that are unique to higher eukaryotes. Autophagy has been implicated in various diseases, thus it is important to fill these gaps of knowledge. *Drosophila* provides a useful model, combining a simple system with low genetic redundancy, tractable to genetic modifications and analyses, but with enough complexity and similarity to human physiology to allow disease-relevant studies of processes such as neurodegeneration, tumorigenesis, and development.

Until recently, genetic studies were limited to examine an “organism minus one gene” by mutational analysis. However, upcoming systems biology approaches allow to analyze global changes at a cell- and genome-wide level. Novel techniques as DNA or RNA microarrays, whole transcriptome shotgun sequencing (RNA-seq) or proteomic profiling by mass spectrometry and advanced life cell imaging are now available for high-throughput analyzes. Using the *Drosophila* ovaries in combination with these approaches, the understanding of the physiological role of autophagy and discovery of novel regulators could provide significant progress and open various possibilities to continue and expand the current research field of autophagy. Since the ovaries are the largest organ of the fly, enough biological sample can easily be obtained for large-scale analysis, and fly proteome data is available (www.peptideatlas.org). Also, the enormous size of ovarian cells make the ovaries especially suitable for life-cell imaging, which could also be combined with ovary *ex-vivo* culturing. In addition, ovarian cell lines are available allowing also for *in-vitro* approaches. Furthermore, the various advantages of the fly as a complex multicellular organism provides the opportunity to analyze ATG function in various tissues and under different cellular conditions to further elucidate the physiological function of autophagy. In contrast to RNAi experiments in cell cultures, the fly offers the opportunity to analyze true mutants in a physiological system.

The unique possibility to combine systems biology approaches and multi gene analysis using genome-wide association studies in natural variations with single gene analyses in a tissue-specific context will contribute to answer fundamental but still unsolved questions concerning the physiological role of autophagy and the network controlling this process.

IV Materials & Methods

Materials and methods are described in detail within sections 2.1 and 2.2. Some additional information of material and methods used in section 2.3 are described below.

4.1 Materials and Methods described in chapter 2.1

Drosophila maintenance, *Drosophila* stocks, starvation assay (flies), generation of transgenic flies, LTR assay (ovaries), tissue preparation, confocal microscopy, transmission EM, antibody generation, western blotting, immunofluorescence, RNA purification, quantitative real-time PCR, RAD treatment, pole cell transplantation, X-ray irradiation, FLP induced FC clones, larval ovary transplantation

4.2 Materials and Methods described in chapter 2.2

Drosophila maintenance, *Drosophila* stocks, LTR assay (fatbody), starvation assay (larva), tissue preparation, immunostainings, microscopy, generation of mosaic tissues, egg quantification

4.3 Materials and Methods used in chapter 2.3

4.3.1 *Drosophila* stocks

Flies were raised on standard yeast/cornmeal agar at 25 °C. *D. melanogaster* stocks used: *ATG1$^{\Delta 3D}$ FRT80B, ATG5-RNAi* (kindly provided by T. Neufeld) (Scott *et al.*, 2004). *N^{55e11} FRT19A (28813), FRT19-UbiGFP, FRT80B-UbiGFP* (Bloomington *Drosophila* Stock Center, Indiana University, IN, USA). *ATG1-RNAi* (GD16133) (VDRC, Vienna, Austria). *NRE-lacZ* (kindly provided by S. Bray) (Krejci *et al.*, 2009). *en-GAL4 UAS-myr-RFP NRE:EGFP, Su(H)-RNAi*, *Notch-RNAi*, *UAS-Dl* (kindly provided by G. Merdes) (Saj *et al.*, 2010). *P[w^{+} lac-Z]BB142 (kekkon-lacZ) (kindly provided by T. Schüpbach)* (Schupbach and Roth, 1994; Pai *et al.*, 2000).

4.3.2 Tissue preparation, immunostainings and microscopy

Ovaries and wing imaginal discs were dissected in PBS, fixed in 4% paraformaldehyde (PFA) for 20 min, embedded in mounting medium with DAPI (Vectashield, Vector Laboratories, Inc., Burlingame, CA, USA). Ovaries for immunostainings were prepared as described elsewhere (Barth *et al.*, 2011). Immunostainings with β-Galactosidase antibodies (lacZ stainings) were prepared as described in Barth et al. (2010) without Methanol dehydration.
Primary antibodies used: Mouse anti-β-Galactosidase (1:300) (Z378A, Promega, WI, USA), rabbit anti-dATG8 (1:200) (Barth *et al.*, 2011), mouse anti-Gurken (1:50) (1D12), mouse anti-Fasciclin III (7G10), mouse anti-Broad-core (1:100) (25E9.D7), (Developmental Studies Hybridoma Bank, IA, USA). Images were obtained as described in chapter 2.2.

List of abbreviations

A/P	anterior-posterior
Ambra1	activating molecule in Beclin1-regulated autophagy
AMP	adenosine monophosphate
AMPK	AMP activated kinase
ATG	autophagy-related
ATG14L	ATG14-like protein
Bcl-2	B-cell lymphoma 2
BECN1	Beclin-1
Br	Broad
cAMP	cyclic adenosine monophosphate
CMA	chaperon-mediated autophagy
D/V	dorso-ventral
DA	dorsal appendage
da	daughterless
Dilps	*Drosophila* insulin-like peptides
Dl	Delta
DNA	deoxyribonucleic acid
Dpp	Decapentaplegic
DRAM	damage-regulated modulator of autophagy
e.g.	for example (*exempli gratia*)
ecdysone	20-hydroxyecdysone
EGFR	epidermal growth factor receptor
en	engrailed
ESCRT	endosomal sorting complex required for transport
ey	eyeless
FasIII	Fasciclin III
FC	follicle cells
FLP-FRT	flippase recognition target
Fng	Fringe
FOXO	Forkhead box subgroup O
fru	fruitless
FSC	follicular stem cell
GABARAP	γ-aminobutyric acid type A receptor associated protein
GC	germ cell
GFP	green fluorescence protein
GL	germ line
GLC	germline clone
Grk	Gurken
gs	grandchildless
GTP	Guanosine-5'-triphosphate

hh	hedgehoge
Hnt	hindsight
HS	heatshock
HSC	70 heat-shock cognate
IAP	inhibitor of apoptosis protein
IGF	insulin/insulin-like growth factor
IIS	insulin/insulin-like growth factor signaling
InR	insulin receptor
IRS	insulin receptor substrates
JAK	Janus kinase
JNK	Jun-N-terminal kinase
JNKK	JNK kinase kinase
kek	kekkon
LAMP2A	lysosome-associated membrane protein type 2A
lqf	liquid facets
LTR	lysotracker-red
M/E switch	mitotic to endoreplication cycle switch
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblasts
MHC	major histocompatibility complex
MS/MS	tandem mass spectrometry
MT	microtubuli
N	Notch
NC	nurse cell
NECD	Notch extracellular domain
NICD	Notch intracellular domain
NRE	Notch responsive element
PAS	phagopore-assembly site/pre-autophagosomal structure
PC	polar cells
PCD	programmed cell death
PCOS	polycystic ovary syndrom
PE	phosphatidylethanolamine
PFA	paraformaldehyde
PI	phosphatidylinositol
PI(3,4,5)P3	phosphatidylinositol (3,4,5)-triphosphate
PI3K	phosphatidylinositol 3-kinase
PI3P	phosphatidylinositol 3-phosphate
PKA	cAMP-dependent protein kinase
PKB (Akt)	protein kinase B
PNCN	persisting nurse cell nuclei
PTEN	phosphatase and tensin homolog
RFP	red fluorescent protein
Rheb	Ras homologue enriched in brain
Rho	Rhomboid
RNA	ribonucleic acid

RNAi	ribonucleic acid interference
Rubicon	RUN domain and cysteine-rich domain containing
S2	*Drosophila* Schneider-2 cells
S6K	ribosomal S6 kinase
SCF	Skp, Cullin, F-box complex
S.D.	standard deviation
slbo	slow border cell
STAT	signal transducer and activator of transcription
Su(H	Suppressor of Hairless
TEM	transmission electron microscopy
TGFα	transforming growth factor α
TOR	target of rapamycin
TORC1	target of rapamycin complex 1
TORC2	target of rapamycin complex 2
TSC1	tuberous sclerosis protein complex1
TSC2	tuberous sclerosis protein complex2
ULK1	Unc-51-like kinase
upd	unpaired
UVRAG	ultraviolet irradiation resistance-associated gene
Vps	vacuolar protein sorting
WT	wild type

List of figures

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2006, October 3-6, Turin, Italy. Prion Meeting 2006. Poster: Conserved roles of vertebrate prion proteins; E. Málaga-Trillo, GP Solis, L Luncz, JMI Barth, Y Schrock and CAO Stuermer

List of publications

Lack of autophagy in *Drosophila* follicle cells causes egg chamber defects and modulates Notch signaling. Barth JM, Hafen E, Köhler K., 2011, in preparation.

Autophagy in *Drosophila* ovaries is induced by starvation and is required for oogenesis. Barth JM, Szabad J, Hafen E, Köhler K. Cell Death Differ. 2011 Jun;18(6):915-24. Epub 2010 Dec 10.

A combined proteomic and genetic analysis identifies a role for the lipid desaturase Desat1 in starvation-induced autophagy in *Drosophila.* Köhler K, Brunner E, Guan XL, Boucke K, Greber UF, Mohanty S, Barth JM, Wenk MR, Hafen E. Autophagy. 2009 Oct;5(7):980-90. Epub 2009 Oct 22.

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References

Systems Biology of the *Drosophila* wing, http://www.systemsx.ch/. WingX - Systems Biology of the *Drosophila* wing, *in-vitro* culture. *In* http://www.wingx.ch/research/subgroups/in_vitro_culture/.

Arbouzova, N.I., and M.P. Zeidler. 2006. JAK/STAT signalling in Drosophila: insights into conserved regulatory and cellular functions. *Development*. 133:2605.

Ashford, T.P., and K.R. Porter. 1962. Cytoplasmic components in hepatic cell lysosomes. *J Cell Biol*. 12:198-202.

Assa-Kunik, E., I.L. Torres, E.D. Schejter, D.S. Johnston, and B.-Z. Shilo. 2007. Drosophila follicle cells are patterned by multiple levels of Notch signaling and antagonism between the Notch and JAK/STAT pathways. *Development*. 134:1161-1169.

Avellaira, C., A. Villavicencio, K. Bacallao, F. Gabler, P. Wells, C. Romero, and M. Vega. 2006. Expression of molecules associated with tissue homeostasis in secretory endometria from untreated women with polycystic ovary syndrome. *Hum Reprod*. 21:3116-3121.

Avraham, R., and Y. Yarden. 2011. Feedback regulation of EGFR signalling: decision making by early and delayed loops. *Nat Rev Mol Cell Biol*. 12:104-117.

Baehrecke, E.H. 2002. How death shapes life during development. *Nat Rev Mol Cell Biol*. 3:779-787.

Baerga, R., Y. Zhang, P.H. Chen, S. Goldman, and S. Jin. 2009. Targeted deletion of autophagy-related 5 (atg5) impairs adipogenesis in a cellular model and in mice. *Autophagy*. 5:1118-1130.

Baonza, A., and A. Garcia-Bellido. 2000. Notch signaling directly controls cell proliferation in the Drosophila wing disc. *Proc Natl Acad Sci U S A*. 97:2609-2614.

Barth, J.M., J. Szabad, E. Hafen, and K. Kohler. 2011. Autophagy in Drosophila ovaries is induced by starvation and is required for oogenesis. *Cell Death Differ*. 18:915-924.

Bass, B.P., E.A. Tanner, D. Mateos San Martín, T. Blute, R.D. Kinser, P.J. Dolph, and K. McCall. 2009. Cell-autonomous requirement for DNaseII in nonapoptotic cell death. *Cell Death Differ*. 16:1362-1371.

Baum, J.S., E. Arama, H. Steller, and K. McCall. 2007. The Drosophila caspases Strica and Dronc function redundantly in programmed cell death during oogenesis. *Cell Death Differ*. 14:1508-1517.

Berg, C.A. 2008. Tube formation in Drosophila egg chambers. *Tissue Eng Part A*. 14:1479-1488.

Berry, D., and E. Baehrecke. 2007. Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila. *Cell*. 131:1137-1148.

Betin, V.M., and J.D. Lane. 2009. Caspase cleavage of Atg4D stimulates GABARAP-L1 processing and triggers mitochondrial targeting and apoptosis. *J Cell Sci*. 122:2554-2566.

Bodenmiller, B., and R. Aebersold. 2010. Quantitative analysis of protein phosphorylation on a system-wide scale by mass spectrometry-based proteomics. *Methods Enzymol*. 470:317-334.

Bohni, R., J. Riesgo-Escovar, S. Oldham, W. Brogiolo, H. Stocker, B. Andruss, K. Beckingham, and E. Hafen. 1999. Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. *Cell*. 97:865-875.

Bolos, V., J. Grego-Bessa, and J.L. de la Pompa. 2007. Notch signaling in development and cancer. *Endocr Rev*. 28:339-363.

Chang, C.W., D. Nashchekin, L. Wheatley, U. Irion, K. Dahlgaard, T.G. Montague, J. Hall, and D. St Johnston. 2011. Anterior-Posterior Axis Specification in Drosophila Oocytes: Identification of Novel bicoid and oskar mRNA Localization Factors. *Genetics*. 188:883-896.

Chang, Y., G. Juhasz, P. Goraksha-Hicks, A. Arsham, D. Mallin, L. Muller, and T. Neufeld. 2009. Nutrient-dependent regulation of autophagy through the target of rapamycin pathway. *Biochem Soc Trans*. 37:232-236.

Chang, Y., and T. Neufeld. 2009. An Atg1/Atg13 complex with multiple roles in TOR-mediated autophagy regulation. *Mol Biol Cell*. 20:2004-2014.
Chang, Y.Y., and T.P. Neufeld. 2010. Autophagy takes flight in Drosophila. *FEBS Lett*. 584:1342-1349.
Chen, N., and V. Karantza. 2011. Autophagy as a therapeutic target in cancer. *Cancer Biol Ther*. 11:157-168.
Chen, Y., and D.J. Klionsky. 2011. The regulation of autophagy - unanswered questions. *J Cell Sci*. 124:161-170.
Choi, J.Y., M.W. Jo, E.Y. Lee, B.K. Yoon, and D.S. Choi. 2010. The role of autophagy in follicular development and atresia in rat granulosa cells. *Fertil Steril*. 93:2532-2537.
Chou, T., and N. Perrimon. 1992. Use of a yeast site-specific recombinase to produce female germline chimeras in Drosophila. *Genetics*. 131:643-653.
Constantinescu, S.N., M. Girardot, and C. Pecquet. 2008. Mining for JAK-STAT mutations in cancer. *Trends Biochem Sci*. 33:122-131.
Cuervo, A.M. 2011. Chaperone-mediated autophagy: Dice's 'wild' idea about lysosomal selectivity. *Nat Rev Mol Cell Biol*. 12:535-541.
Cummings, M.R., N.M. Brown, and R.C. King. 1971. The cytology of the vitellogenic stages of oogenesis in Drosophila melanogaster. 3. Formation of the vitelline membrane. *Z Zellforsch Mikrosk Anat*. 118:482-492.
Demontis, F., and N. Perrimon. 2010. FOXO/4E-BP signaling in Drosophila muscles regulates organism-wide proteostasis during aging. *Cell*. 143:813-825.
Dennis, M.D., J.I. Baum, S.R. Kimball, and L.S. Jefferson. 2011. Mechanisms involved in the coordinate regulation of mTORC1 by insulin and amino acids. *J Biol Chem*. 286:8287-8296.
Denton, D., B. Shravage, R. Simin, K. Mills, D.L. Berry, E.H. Baehrecke, and S. Kumar. 2009. Autophagy, not apoptosis, is essential for midgut cell death in Drosophila. *Curr Biol*. 19:1741-1746.
Deretic, V., and B. Levine. 2009. Autophagy, immunity, and microbial adaptations. *Cell Host Microbe*. 5:527-549.
Deter, R.L., and C. De Duve. 1967. Influence of glucagon, an inducer of cellular autophagy, on some physical properties of rat liver lysosomes. *J Cell Biol*. 33:437-449.
Drummond-Barbosa, D., and A. Spradling. 2001. Stem cells and their progeny respond to nutritional changes during Drosophila oogenesis. *Dev Biol*. 231:265-278.
Duerrschmidt, N., O. Zabirnyk, M. Nowicki, A. Ricken, F.A. Hmeidan, V. Blumenauer, J. Borlak, and K. Spanel-Borowski. 2006. Lectin-like oxidized low-density lipoprotein receptor-1-mediated autophagy in human granulosa cells as an alternative of programmed cell death. *Endocrinology*. 147:3851-3860.
Duffy, J.B. 2002. GAL4 system in Drosophila: a fly geneticist's Swiss army knife. *Genesis*. 34:1-15.
Dunaif, A., and A. Thomas. 2001. Current concepts in the polycystic ovary syndrome. *Annu Rev Med*. 52:401-419.
Dutta, S., and E.H. Baehrecke. 2008. Warts is required for PI3K-regulated growth arrest, autophagy, and autophagic cell death in Drosophila. *Curr Biol*. 18:1466-1475.
Edinger, A.L., and C.B. Thompson. 2004. Death by design: apoptosis, necrosis and autophagy. *Current opinion in cell biology*. 16:663-669.
Eisenberg-Lerner, A., S. Bialik, H.U. Simon, and A. Kimchi. 2009. Life and death partners: apoptosis, autophagy and the cross-talk between them. *Cell Death Differ*. 16:966-975.
English, L., M. Chemali, J. Duron, C. Rondeau, A. Laplante, D. Gingras, D. Alexander, D. Leib, C. Norbury, R. Lippe, and M. Desjardins. 2009. Autophagy enhances the presentation of endogenous viral antigens on MHC class I molecules during HSV-1 infection. *Nat Immunol*. 10:480-487.
Fiuza, U.M., and A.M. Arias. 2007. Cell and molecular biology of Notch. *J Endocrinol*. 194:459-474.
Furriols, M., and S. Bray. 2000. Dissecting the mechanisms of suppressor of hairless function. *Dev Biol*. 227:520-532.
Furriols, M., and S. Bray. 2001. A model Notch response element detects Suppressor of Hairless-dependent molecular switch. *Current Biology*. 11:60-64.

Geng, J., and D.J. Klionsky. 2008. The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. *EMBO Rep*. 9:859-864.

Ghiglione, C., K.L. Carraway, 3rd, L.T. Amundadottir, R.E. Boswell, N. Perrimon, and J.B. Duffy. 1999. The transmembrane molecule kekkon 1 acts in a feedback loop to negatively regulate the activity of the Drosophila EGF receptor during oogenesis. *Cell*. 96:847-856.

Glatter, T., R. Schittenhelm, O. Rinner, K. Roguska, A. Wepf, M. Juenger, K. Köhler, I. Jevtov, H. Choi, A. Schmidt, A. Nesvizhskii, H. Stocker, E. Hafen, R. Aebersold, and M. Gstaiger. 2011, manuscript accepted at Mol Syst Biol. Modularity and hormone sensitivity of the *Drosophila melanogaster* insulin/target of rapamycin (InR/TOR) interaction proteome.

González-Reyes, A., H. Elliott, and D. St Johnston. 1995. Polarization of both major body axes in Drosophila by gurken-torpedo signalling. *Nature*. 375:654-658.

Gonzalez-Reyes, A., and D. St Johnston. 1998. The Drosophila AP axis is polarised by the cadherin-mediated positioning of the oocyte. *Development*. 125:3635-3644.

Gordon, P.B., and P.O. Seglen. 1988. Prelysosomal convergence of autophagic and endocytic pathways. *Biochem Biophys Res Commun*. 151:40-47.

Gorski, S., S. Chittaranjan, E. Pleasance, J. Freeman, C. Anderson, R. Varhol, S. Coughlin, S. Zuyderduyn, S. Jones, and M. Marra. 2003. A SAGE approach to discovery of genes involved in autophagic cell death. *Curr Biol*. 13:358-363.

He, L., X. Wang, and D.J. Montell. 2011. Shining light on Drosophila oogenesis: live imaging of egg development. *Curr Opin Genet Dev*.

Hook, S.S., J.J. Lin, and A. Dutta. 2007. Mechanisms to control rereplication and implications for cancer. *Curr Opin Cell Biol*. 19:663-671.

Hosokawa, N., T. Hara, T. Kaizuka, C. Kishi, A. Takamura, Y. Miura, S. Iemura, T. Natsume, K. Takehana, N. Yamada, J.L. Guan, N. Oshiro, and N. Mizushima. 2009. Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol Biol Cell*. 20:1981-1991.

Hou, W., J. Han, C. Lu, L.A. Goldstein, and H. Rabinowich. 2010. Autophagic degradation of active caspase-8: a crosstalk mechanism between autophagy and apoptosis. *Autophagy*. 6:891-900.

Hou, Y., S. Chittaranjan, S. Barbosa, K. McCall, and S. Gorski. 2008. Effector caspase Dcp-1 and IAP protein Bruce regulate starvation-induced autophagy during Drosophila melanogaster oogenesis. *J Cell Biol*. 182:1127-1139.

Juhasz, G., G. Csikos, R. Sinka, M. Erdelyi, and M. Sass. 2003. The Drosophila homolog of Aut1 is essential for autophagy and development. *FEBS Lett*. 543:154-158.

Juhasz, G., B. Erdi, M. Sass, and T. Neufeld. 2007a. Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila. *Genes Dev*. 21:3061-3066.

Juhasz, G., J.H. Hill, Y. Yan, M. Sass, E.H. Baehrecke, J.M. Backer, and T.P. Neufeld. 2008. The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in Drosophila. *J Cell Biol*. 181:655-666.

Juhasz, G., L. Puskas, O. Komonyi, B. Erdi, P. Maroy, T. Neufeld, and M. Sass. 2007b. Gene expression profiling identifies FKBP39 as an inhibitor of autophagy in larval Drosophila fat body. *Cell Death Differ*. 14:1181-1190.

Jung, C.H., S.-H. Ro, J. Cao, N.M. Otto, and D.-H. Kim. 2010. mTOR regulation of autophagy. *FEBS letters*.

Kaipia, A., and A.J. Hsueh. 1997. Regulation of ovarian follicle atresia. *Annu Rev Physiol*. 59:349-363.

Kamada, Y., T. Funakoshi, T. Shintani, K. Nagano, M. Ohsumi, and Y. Ohsumi. 2000. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J Cell Biol*. 150:1507-1513.

Kamada, Y., K. Yoshino, C. Kondo, T. Kawamata, N. Oshiro, K. Yonezawa, and Y. Ohsumi. 2010. Tor directly controls the Atg1 kinase complex to regulate autophagy. *Mol Cell Biol*. 30:1049-1058.

Kawahashi, K., and S. Hayashi. 2010. Dynamic intracellular distribution of Notch during activation and asymmetric cell division revealed by functional fluorescent fusion proteins. *Genes Cells*. 15:749-759.

Kawamata, T., Y. Kamada, Y. Kabeya, T. Sekito, and Y. Ohsumi. 2008. Organization of the pre-autophagosomal structure responsible for autophagosome formation. *Mol Biol Cell*. 19:2039-2050.
Khammari, A., F. Agnes, P. Gandille, and A.M. Pret. 2010. Physiological apoptosis of polar cells during Drosophila oogenesis is mediated by Hid-dependent regulation of Diap1. *Cell Death Differ*. 18:793-805.
Kim, E., P. Goraksha-Hicks, L. Li, T.P. Neufeld, and K.L. Guan. 2008. Regulation of TORC1 by Rag GTPases in nutrient response. *Nat Cell Biol*. 10:935-945.
Kim, J., and K.L. Guan. 2011. Amino acid signaling in TOR activation. *Annu Rev Biochem*. 80:1001-1032.
Kim, Y.I., T. Ryu, J. Lee, Y.S. Heo, J. Ahnn, S.J. Lee, and O. Yoo. 2010. A genetic screen for modifiers of Drosophila caspase Dcp-1 reveals caspase involvement in autophagy and novel caspase-related genes. *BMC Cell Biol*. 11:9.
Klionsky, D.J. 2008. Autophagy revisited: a conversation with Christian de Duve. *Autophagy*. 4:740-743.
Klionsky, D.J., A.M. Cuervo, and P.O. Seglen. 2007. Methods for monitoring autophagy from yeast to human. *Autophagy*. 3:181-206.
Klionsky, D.J., and A. Nemchenko. 2011. There is more to autophagy than induction: regulating the roller coaster. *Autophagy*. 7:801-802.
Klusza, S., and W.-M. Deng. 2010. At the crossroads of differentiation and proliferation: Precise control of cell-cycle changes by multiple signaling pathways in Drosophila follicle cells. *BioEssays : news and reviews in molecular, cellular and developmental biology*.
Köhler, K., E. Brunner, X.L. Guan, K. Boucke, U.F. Greber, S. Mohanty, J.M.I. Barth, M.R. Wenk, and E. Hafen. 2009. A combined proteomic and genetic analysis identifies a role for the lipid desaturase Desat1 in starvation-induced autophagy in Drosophila. *Autophagy*. 5:980-990.
Komatsu, M., S. Waguri, T. Chiba, S. Murata, J. Iwata, I. Tanida, T. Ueno, M. Koike, Y. Uchiyama, E. Kominami, and K. Tanaka. 2006. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature*. 441:880-884.
Krejci, A., F. Bernard, B.E. Housden, S. Collins, and S.J. Bray. 2009. Direct response to Notch activation: signaling crosstalk and incoherent logic. *Sci Signal*. 2:ra1.
Krysko, D.V., A. Diez-Fraile, G. Criel, A.A. Svistunov, P. Vandenabeele, and K. D'Herde. 2008. Life and death of female gametes during oogenesis and folliculogenesis. *Apoptosis*. 13:1065-1087.
Kuma, A., M. Hatano, M. Matsui, A. Yamamoto, H. Nakaya, T. Yoshimori, Y. Ohsumi, T. Tokuhisa, and N. Mizushima. 2004. The role of autophagy during the early neonatal starvation period. *Nature*. 432:1032-1036.
LaFever, L., and D. Drummond-Barbosa. 2005. Direct control of germline stem cell division and cyst growth by neural insulin in Drosophila. *Science*. 309:1071-1073.
LaFever, L., A. Feoktistov, H.-J. Hsu, and D. Drummond-Barbosa. 2010. Specific roles of Target of rapamycin in the control of stem cells and their progeny in the Drosophila ovary. *Development*. 137:2117-2126.
Laundrie, B., J.S. Peterson, J.S. Baum, J.C. Chang, D. Fileppo, S.R. Thompson, and K. McCall. 2003. Germline cell death is inhibited by P-element insertions disrupting the dcp-1/pita nested gene pair in Drosophila. *Genetics*. 165:1881-1888.
Lecourtois, M., and F. Schweisguth. 1997. Role of suppressor of hairless in the delta-activated Notch signaling pathway. *Perspect Dev Neurobiol*. 4:305-311.
Lee, C.Y., and E.H. Baehrecke. 2001. Steroid regulation of autophagic programmed cell death during development. *Development*. 128:1443-1455.
Lee, C.Y., E.A. Clough, P. Yellon, T.M. Teslovich, D.A. Stephan, and E.H. Baehrecke. 2003. Genome-wide analyses of steroid- and radiation-triggered programmed cell death in Drosophila. *Curr Biol*. 13:350-357.
Lee, C.Y., B.A. Cooksey, and E.H. Baehrecke. 2002. Steroid regulation of midgut cell death during Drosophila development. *Dev Biol*. 250:101-111.
Lee, G., C. Liang, G. Park, C. Jang, J.U. Jung, and J. Chung. 2011. UVRAG is required for organ rotation by regulating Notch endocytosis in Drosophila. *Dev Biol*. 356:588-597.
Levine, B., S. Sinha, and G. Kroemer. 2008. Bcl-2 family members: dual regulators of apoptosis and autophagy. *Autophagy*. 4:600-606.

Li, D.D., L.L. Wang, R. Deng, J. Tang, Y. Shen, J.F. Guo, Y. Wang, L.P. Xia, G.K. Feng, Q.Q. Liu, W.L. Huang, Y.X. Zeng, and X.F. Zhu. 2009. The pivotal role of c-Jun NH2-terminal kinase-mediated Beclin 1 expression during anticancer agents-induced autophagy in cancer cells. *Oncogene*. 28:886-898.

Liang, X.H., S. Jackson, M. Seaman, K. Brown, B. Kempkes, H. Hibshoosh, and B. Levine. 1999. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature*. 402:672-676.

Liou, W., H.J. Geuze, M.J. Geelen, and J.W. Slot. 1997. The autophagic and endocytic pathways converge at the nascent autophagic vacuoles. *J Cell Biol*. 136:61-70.

Loewith, R. 2011. A brief history of TOR. *Biochem Soc Trans*. 39:437-442.

Martin, D., B. Balgley, S. Dutta, J. Chen, P. Rudnick, J. Cranford, S. Kantartzis, D. DeVoe, C. Lee, and E. Baehrecke. 2007. Proteomic analysis of steroid-triggered autophagic programmed cell death during Drosophila development. *Cell Death Differ*. 14:916-923.

Martin, D.N., and E.H. Baehrecke. 2004. Caspases function in autophagic programmed cell death in Drosophila. *Development*. 131:275-284.

Matsumoto, A., I. Onoyama, T. Sunabori, R. Kageyama, H. Okano, and K.I. Nakayama. 2011. Fbxw7-dependent degradation of Notch is required for control of "stemness" and neuronal-glial differentiation in neural stem cells. *J Biol Chem*. 286:13754-13764.

Matsuura, A., M. Tsukada, Y. Wada, and Y. Ohsumi. 1997. Apg1p, a novel protein kinase required for the autophagic process in Saccharomyces cerevisiae. *Gene*. 192:245-250.

McGregor, J.R., R. Xi, and D.A. Harrison. 2002. JAK signaling is somatically required for follicle cell differentiation in Drosophila. *Development*. 129:705.

Mehrotra, S., S.B. Maqbool, A. Kolpakas, K. Murnen, and B.R. Calvi. 2008. Endocycling cells do not apoptose in response to DNA rereplication genotoxic stress. *Genes Dev*. 22:3158-3171.

Mitsudomi, T., and Y. Yatabe. 2010. Epidermal growth factor receptor in relation to tumor development: EGFR gene and cancer. *FEBS J*. 277:301-308.

Mizushima, N., B. Levine, A.M. Cuervo, and D.J. Klionsky. 2008. Autophagy fights disease through cellular self-digestion. *Nature*. 451:1069-1075.

Mizushima, N., A. Yamamoto, M. Matsui, T. Yoshimori, and Y. Ohsumi. 2004. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol Biol Cell*. 15:1101-1111.

Montell, D.J. 2003. Border-cell migration: the race is on. *Nature Reviews Molecular Cell Biology*. 4:13-24.

Mortensen, M., D.J. Ferguson, M. Edelmann, B. Kessler, K.J. Morten, M. Komatsu, and A.K. Simon. 2010. Loss of autophagy in erythroid cells leads to defective removal of mitochondria and severe anemia in vivo. *Proc Natl Acad Sci U S A*. 107:832-837.

Nakatogawa, H., K. Suzuki, Y. Kamada, and Y. Ohsumi. 2009. Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nat Rev Mol Cell Biol*. 10:458-467.

Nazarko, V.Y., T.Y. Nazarko, J.C. Farre, O.V. Stasyk, D. Warnecke, S. Ulaszewski, J.M. Cregg, A.A. Sibirny, and S. Subramani. 2011. Atg35, a micropexophagy-specific protein that regulates micropexophagic apparatus formation in Pichia pastoris. *Autophagy*. 7:375-385.

Neufeld, T.P. 2009. TOR-dependent control of autophagy: biting the hand that feeds. *Current opinion in cell biology*.

Nezis, I., T. Lamark, A. Velentzas, T. Rusten, G. Bjorkoy, T. Johansen, I. Papassideri, D. Stravopodis, L. Margaritis, H. Stenmark, and A. Brech. 2009. Cell death during Drosophila melanogaster early oogenesis is mediated through autophagy. *Autophagy*. 5:298-302.

Nezis, I., D. Stravopodis, L. Margaritis, and I. Papassideri. 2006. Programmed cell death of follicular epithelium during the late developmental stages of oogenesis in the fruit flies Bactrocera oleae and Ceratitis capitata (Diptera, Tephritidae) is mediated by autophagy. *Dev Growth Differ*. 48:189-198.

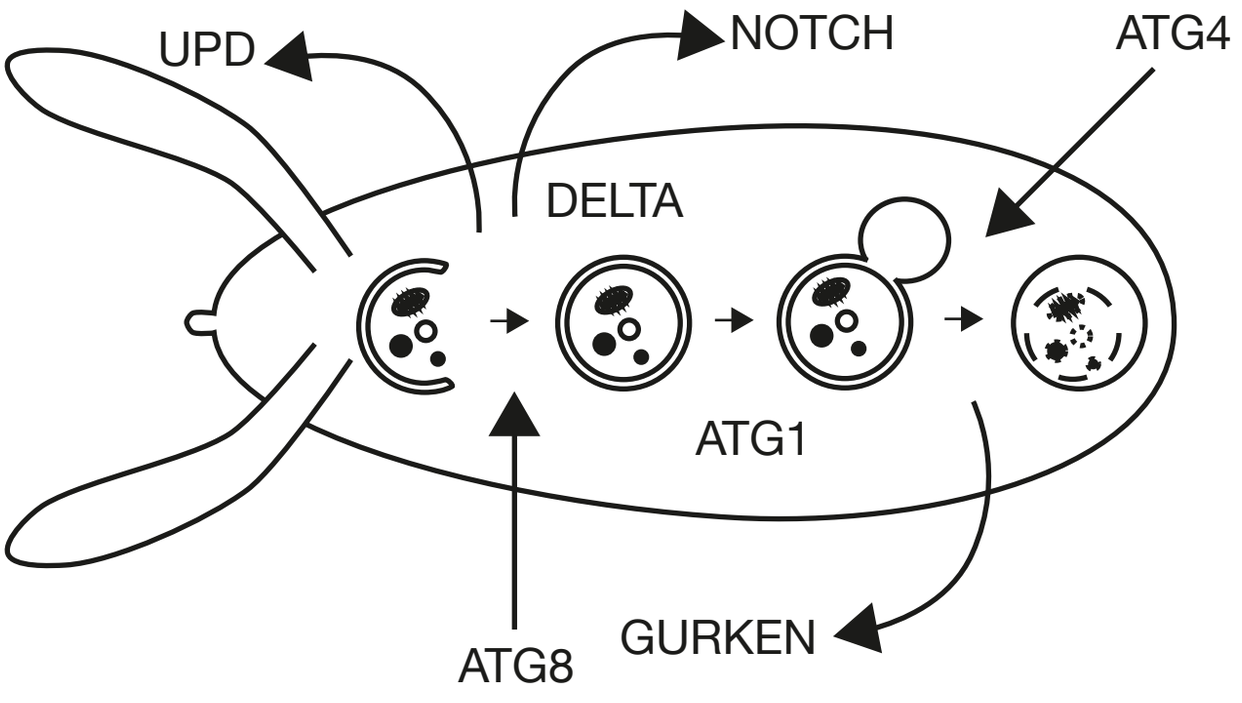
Nezis, I.P., B.V. Shravage, A.P. Sagona, T. Lamark, G. Bjorkoy, T. Johansen, T.E. Rusten, A. Brech, E.H. Baehrecke, and H. Stenmark. 2010. Autophagic degradation of dBruce controls DNA fragmentation in nurse cells during late Drosophila melanogaster oogenesis. *The Journal of Cell Biology*:1-12.

Noda, T., K. Matsunaga, N. Taguchi-Atarashi, and T. Yoshimori. 2010. Regulation of membrane biogenesis in autophagy via PI3P dynamics. *Semin Cell Dev Biol*. 21:671-676.

Obara, K., and Y. Ohsumi. 2011. PtdIns 3-Kinase Orchestrates Autophagosome Formation in Yeast. *J Lipids*. 2011:498768.
Oldham, S., and E. Hafen. 2003. Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. *Trends Cell Biol*. 13:79-85.
Pai, L.M., G. Barcelo, and T. Schupbach. 2000. D-cbl, a negative regulator of the Egfr pathway, is required for dorsoventral patterning in Drosophila oogenesis. *Cell*. 103:51-61.
Paludan, C., D. Schmid, M. Landthaler, M. Vockerodt, D. Kube, T. Tuschl, and C. Munz. 2005. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science*. 307:593-596.
Pattingre, S., A. Tassa, X. Qu, R. Garuti, X.H. Liang, N. Mizushima, M. Packer, M.D. Schneider, and B. Levine. 2005. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell*. 122:927-939.
Peterson, J.S., B.P. Bass, D. Jue, A. Rodriguez, J.M. Abrams, and K. McCall. 2007. Noncanonical cell death pathways act during Drosophila oogenesis. *Genesis*. 45:396-404.
Poulton, J., and W. Deng. 2007. Cell-cell communication and axis specification in the Drosophila oocyte. *Dev Biol*. 311:1-10.
Pritchett, T.L., E.A. Tanner, and K. Mccall. 2009. Cracking open cell death in the Drosophila ovary. *Apoptosis*. 14:969-979.
Rabinowitz, J.D., and E. White. 2011. Autophagy and metabolism. *Science*. 330:1344-1348.
Ranganathan, P., K.L. Weaver, and A.J. Capobianco. 2011. Notch signalling in solid tumours: a little bit of everything but not all the time. *Nat Rev Cancer*. 11:338-351.
Ravikumar, B., C. Vacher, Z. Berger, J.E. Davies, S. Luo, L.G. Oroz, F. Scaravilli, D.F. Easton, R. Duden, C.J. O'Kane, and D.C. Rubinsztein. 2004. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat Genet*. 36:585-595.
Reggiori, F., K. Tucker, P. Stromhaug, and D. Klionsky. 2004. The Atg1-Atg13 complex regulates Atg9 and Atg23 retrieval transport from the pre-autophagosomal structure. *Dev Cell*. 6:79-90.
Richard, D., R. Rybczynski, T. Wilson, Y. Wang, M. Wayne, Y. Zhou, L. Partridge, and L. Harshman. 2005. Insulin signaling is necessary for vitellogenesis in Drosophila melanogaster independent of the roles of juvenile hormone and ecdysteroids: female sterility of the chico1 insulin signaling mutation is autonomous to the ovary. *J Insect Physiol*. 51:455-464.
Riddiford, L.M. 1993. Hormone receptors and the regulation of insect metamorphosis. *Receptor*. 3:203-209.
Rosenfeldt, M.T., and K.M. Ryan. 2011. The multiple roles of autophagy in cancer. *Carcinogenesis*. 32:955-963.
Roth, S. 2001. Drosophila oogenesis: coordinating germ line and soma. *Curr Biol*. 11:R779-781.
Roth, S., and J.A. Lynch. 2009. Symmetry breaking during Drosophila oogenesis. *Cold Spring Harbor Perspect Biol*. 1:a001891.
Ruohola, H., K.A. Bremer, D. Baker, J.R. Swedlow, L.Y. Jan, and Y.N. Jan. 1991. Role of neurogenic genes in establishment of follicle cell fate and oocyte polarity during oogenesis in Drosophila. *Cell*. 66:433-449.
Rusten, T., K. Lindmo, G. Juhasz, M. Sass, P. Seglen, A. Brech, and H. Stenmark. 2004. Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway. *Dev Cell*. 7:179-192.
Rusten, T.E., T. Vaccari, K. Lindmo, L.M.W. Rodahl, I.P. Nezis, C. Sem-Jacobsen, F. Wendler, J.-P. Vincent, A. Brech, D. Bilder, and H. Stenmark. 2007. ESCRTs and Fab1 regulate distinct steps of autophagy. *Current Biology*. 17:1817-1825.
Ryoo, H.D., and E.H. Baehrecke. 2010. Distinct death mechanisms in Drosophila development. *Curr Opin Cell Biol*. 22:889-895.
Saj, A., Z. Arziman, D. Stempfle, W. van Belle, U. Sauder, T. Horn, M. Dürrenberger, R. Paro, M. Boutros, and G. Merdes. 2010. A combined ex vivo and in vivo RNAi screen for notch regulators in Drosophila reveals an extensive notch interaction network. *Developmental Cell*. 18:862-876.

Sancak, Y., T.R. Peterson, Y.D. Shaul, R.A. Lindquist, C.C. Thoreen, L. Bar-Peled, and D.M. Sabatini. 2008. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science*. 320:1496-1501.
Schupbach, T. 2009. Developmental biology: Pipe's smoking guns. *Curr Biol*. 19:R548-550.
Schupbach, T., and S. Roth. 1994. Dorsoventral patterning in Drosophila oogenesis. *Curr Opin Genet Dev*. 4:502-507.
Schweisguth, F. 2004. Regulation of notch signaling activity. *Curr Biol*. 14:R129-138.
Scott, R., G. Juhasz, and T. Neufeld. 2007. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. *Curr Biol*. 17:1-11.
Scott, R., O. Schuldiner, and T. Neufeld. 2004. Role and regulation of starvation-induced autophagy in the Drosophila fat body. *Dev Cell*. 7:167-178.
Sekito, T., T. Kawamata, R. Ichikawa, K. Suzuki, and Y. Ohsumi. 2009. Atg17 recruits Atg9 to organize the pre-autophagosomal structure. *Genes Cells*. 14:525-538.
Shaw, R.J. 2009. LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. *Acta Physiol (Oxf)*. 196:65-80.
Shelly, S., N. Lukinova, S. Bambina, A. Berman, and S. Cherry. 2009. Autophagy is an essential component of Drosophila immunity against vesicular stomatitis virus. *Immunity*. 30:588-598.
Shravage, B.V., G. Altmann, M. Technau, and S. Roth. 2007. The role of Dpp and its inhibitors during eggshell patterning in Drosophila. *Development*. 134:2261-2271.
Siegrist, S.E., N.S. Haque, C.H. Chen, B.A. Hay, and I.K. Hariharan. 2010. Inactivation of both Foxo and reaper promotes long-term adult neurogenesis in Drosophila. *Curr Biol*. 20:643-648.
Simonsen, A., R.C. Cumming, A. Brech, P. Isakson, D.R. Schubert, and K.D. Finley. 2008. Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila. *Autophagy*. 4:176-184.
Simonsen, A., and S.A. Tooze. 2009. Coordination of membrane events during autophagy by multiple class III PI3-kinase complexes. *J Cell Biol*. 186:773-782.
Sorkin, A., and L.K. Goh. 2009. Endocytosis and intracellular trafficking of ErbBs. *Exp Cell Res*. 315:683-696.
Stephan, J.S., Y.Y. Yeh, V. Ramachandran, S.J. Deminoff, and P.K. Herman. 2009. The Tor and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to control autophagy. *Proc Natl Acad Sci U S A*. 106:17049-17054.
Stocker, H., T. Radimerski, B. Schindelholz, F. Wittwer, P. Belawat, P. Daram, S. Breuer, G. Thomas, and E. Hafen. 2003. Rheb is an essential regulator of S6K in controlling cell growth in Drosophila. *Nat Cell Biol*. 5:559-565.
Sun, J., and W.-M. Deng. 2005. Notch-dependent downregulation of the homeodomain gene cut is required for the mitotic cycle/endocycle switch and cell differentiation in Drosophila follicle cells. *Development*. 132:4299-4308.
Sun, J., and W.-M. Deng. 2007. Hindsight mediates the role of notch in suppressing hedgehog signaling and cell proliferation. *Developmental Cell*. 12:431-442.
Takeshige, K., M. Baba, S. Tsuboi, T. Noda, and Y. Ohsumi. 1992. Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. *J Cell Biol*. 119:301-311.
Tanner, E.A., T.A. Blute, C.B. Brachmann, and K. Mccall. 2011. Bcl-2 proteins and autophagy regulate mitochondrial dynamics during programmed cell death in the Drosophila ovary. *Development*. 138:327-338.
Thomson, T.C., K.E. Fitzpatrick, and J. Johnson. 2010. Intrinsic and extrinsic mechanisms of oocyte loss. *Mol Hum Reprod*. 16:916-927.
Thumm, M., and T. Kadowaki. 2001. The loss of Drosophila APG4/AUT2 function modifies the phenotypes of cut and Notch signaling pathway mutants. *Mol Genet Genomics*. 266:657-663.
Thurston, T.L., G. Ryzhakov, S. Bloor, N. von Muhlinen, and F. Randow. 2009. The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. *Nat Immunol*. 10:1215-1221.
Tsukada, M., and Y. Ohsumi. 1993. Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. *FEBS Lett*. 333:169-174.

Tsukamoto, S., A. Kuma, M. Murakami, C. Kishi, A. Yamamoto, and N. Mizushima. 2008. Autophagy is essential for preimplantation development of mouse embryos. *Science*. 321:117-120.
Vaccari, T., H. Lu, R. Kanwar, M.E. Fortini, and D. Bilder. 2008. Endosomal entry regulates Notch receptor activation in Drosophila melanogaster. *J Cell Biol*. 180:755-762.
Velentzas, A.D., I.P. Nezis, D.J. Stravopodis, I.S. Papassideri, and L.H. Margaritis. 2007. Mechanisms of programmed cell death during oogenesis in Drosophila virilis. *Cell Tissue Res*. 327:399-414.
Webber, J.L., and S.A. Tooze. 2010. New insights into the function of Atg9. *FEBS Lett*. 584:1319-1326.
Wei, Y., S. Sinha, and B. Levine. 2008. Dual role of JNK1-mediated phosphorylation of Bcl-2 in autophagy and apoptosis regulation. *Autophagy*. 4:949-951.
Weidberg, H., E. Shvets, T. Shpilka, F. Shimron, V. Shinder, and Z. Elazar. 2010. LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. *EMBO J*. 29:1792-1802.
Werz, C., K. Kohler, E. Hafen, and H. Stocker. 2009. The Drosophila SH2B family adaptor Lnk acts in parallel to chico in the insulin signaling pathway. *PLoS Genet*. 5:e1000596.
Weston, C.R., and R.J. Davis. 2007. The JNK signal transduction pathway. *Curr Opin Cell Biol*. 19:142-149.
Wolpert, L. 2002. Principles of development. Oxford University Press, Oxford. xxv, 542 p. pp.
Wu, H., M.C. Wang, and D. Bohmann. 2009. JNK protects Drosophila from oxidative stress by trancriptionally activating autophagy. *Mech Dev*. 126:624-637.
Xi, R., J.R. McGregor, and D.A. Harrison. 2003. A gradient of JAK pathway activity patterns the anterior-posterior axis of the follicular epithelium. *Developmental Cell*. 4:167-177.
Yakoby, N., J. Lembong, T. Schüpbach, and S.Y. Shvartsman. 2008. Drosophila eggshell is patterned by sequential action of feedforward and feedback loops. *Development*. 135:343.
Yamamoto, S., W.-L. Charng, and H.J. Bellen. 2010. Endocytosis and Intracellular Trafficking of Notch and Its Ligands. 92.
Yang, Z., and D.J. Klionsky. 2010a. Eaten alive: a history of macroautophagy. *Nat Cell Biol*. 12:814-822.
Yang, Z., and D.J. Klionsky. 2010b. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol*. 22:124-131.
Yano, T., S. Mita, H. Ohmori, Y. Oshima, Y. Fujimoto, R. Ueda, H. Takada, W.E. Goldman, K. Fukase, N. Silverman, T. Yoshimori, and S. Kurata. 2008. Autophagic control of listeria through intracellular innate immune recognition in drosophila. *Nat Immunol*. 9:908-916.
Yen, W.L., and D.J. Klionsky. 2008. How to live long and prosper: autophagy, mitochondria, and aging. *Physiology (Bethesda)*. 23:248-262.
Yin, V.P., and C.S. Thummel. 2005. Mechanisms of steroid-triggered programmed cell death in Drosophila. *Semin Cell Dev Biol*. 16:237-243.
Yorimitsu, T., and D. Klionsky. 2005. Autophagy: molecular machinery for self-eating. *Cell Death Differ*. 12 Suppl 2:1542-1552.
Yousefi, S., R. Perozzo, I. Schmid, A. Ziemiecki, T. Schaffner, L. Scapozza, T. Brunner, and H.U. Simon. 2006. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nat Cell Biol*. 8:1124-1132.
Yu, W.H., A.M. Cuervo, A. Kumar, C.M. Peterhoff, S.D. Schmidt, J.H. Lee, P.S. Mohan, M. Mercken, M.R. Farmery, L.O. Tjernberg, Y. Jiang, K. Duff, Y. Uchiyama, J. Naslund, P.M. Mathews, A.M. Cataldo, and R.A. Nixon. 2005. Macroautophagy--a novel Beta-amyloid peptide-generating pathway activated in Alzheimer's disease. *J Cell Biol*. 171:87-98.
Zartman, J.J., J.S. Kanodia, L.S. Cheung, and S.Y. Shvartsman. 2009. Feedback control of the EGFR signaling gradient: superposition of domain-splitting events in Drosophila oogenesis. *Development*. 136:2903-2911.
Zhang, Y., S. Goldman, R. Baerga, Y. Zhao, M. Komatsu, and S. Jin. 2009. Adipose-specific deletion of autophagy-related gene 7 (atg7) in mice reveals a role in adipogenesis. *Proc Natl Acad Sci U S A*. 106:19860-19865.
Zirin, J., and N. Perrimon. 2010. Drosophila as a model system to study autophagy. *Semin Immunopathol*. 32:363-372.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