Original Article

Stage-specific regulation of programmed cell death during oogenesis of the medfly *Ceratitis capitata* (Diptera, Tephritidae)

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ABSTRACT In the present study, we describe novel features of programmed cell death in developing egg chambers occurring during mid- and late-oogenesis of the medfly *Ceratitis capitata*. During mid-oogenesis, the spontaneously degenerated egg chambers exhibit typical characteristics of apoptotic cell death. Their nurse cells contain fragmented DNA and fragmented actin, as revealed by TUNEL assay and immunolabelling, respectively. *In vitro* caspase activity assays and immunostaining procedures demonstrated that the atretic egg chambers acquired high levels of caspase activity. Distinct features of autophagic cell death were also observed during *C. capitata* mid-oogenesis, as revealed by the monodansylcadaverine staining approach and ultrastructural examination performed by transmission electron microscopy. Additionally, atretic egg chambers exhibit an upregulation of lysosomal proteases, as demonstrated by a procathepsin L immunolabelling procedure. At the late stages of *C. capitata* oogenesis, apoptosis and autophagy coexist, manifesting cell death features that are similar to the ones mentioned above, being also chaperoned by the involvement of an altered cytochrome c conformational display. We propose that apoptosis and autophagy operate synergistically during *C. capitata* oogenesis for a more efficient elimination of the degenerated nurse cells and abnormal egg chambers.

KEY WORDS: *apoptosis, autophagy, Ceratitis capitata, nurse cell, programmed cell death*

Introduction

The egg chamber, or follicle, is the structural and functional unit of insect ovary. The egg chambers are formed in the anterior region of the ovary and they move towards the posterior pole as they develop during oogenesis. Thus, each ovariole contains an array of developmentally ordered egg chambers. In the meroistic polytrophic ovarioles of *Drosophila melanogaster* and *Ceratitis capitata*, each egg chamber consists of 16 germ line cells (15 nurse cells and 1 oocyte), surrounded by a monolayer of somatic epithelial cells, called follicle cells (Trougakos and Margaritis, 2002; Berg, 2005). The nurse cells support the development of the oocyte providing organelles, proteins and maternal RNAs to the oocyte through the ring canals (Mahajan-Miklos and Cooley, 1994; Trougakos and Margaritis, 2002). The development of the egg chamber has been divided into several discrete stages (fourteen according to King, 1970; twenty according to Margaritis, 1985; 1986).

Programmed cell death is an evolutionary conserved and genetically regulated process, where superfluous cells undergo self-destruction by activation of a cell suicide program (Danial and Korsmeyer, 2004). There are two major types of programmed cell death, apoptotic cell death (or type 1) and autophagic cell death (or type 2), both of which are defined by distinct morphological criteria. Apoptotic cell death is mainly characterized by nuclear condensation (pyknosis) and DNA fragmentation (karyorhexis), without major ultrastructural changes of cytoplasmic organelles. In the autophagy-mediated cell death, the major morphological feature is the accumulation of autophagic vacuoles in the cytoplasm (Bursch, 2001; 2004; Lockshin and Zakeri, 2004). Apoptosis involves the regulated activation of caspases, a family of cysteine proteases that specifically recognize and cleave critical cellular target proteins, such as nuclear lamins and other structural

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Abbreviations used in this paper: FC, follicle cells; MDC, monodansylcadaverine stain; NC, nurse cell; NN, nurse cell nucleus; OC, oocyte.

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components, ultimately leading to cell death (Kornbluth and White, 2005). On the other hand, caspases do not seem to be required for the execution of the autophagic cell death program (Bursch, 2001; Gozuacik and Kimchi, 2004), although both types of programmed cell death have been previously reported to coexist (Clarke, 1990; Martin and Baehrecke, 2004). In both cases, the dying cells are efficiently phagocytosed by neighboring epithelial cells, or surrounding phagocytes (Krieser and White, 2002).

As it has been demonstrated before, the process of pro-grammed cell death is activated and implemented during Diptera germarium, mid- and late-oogenesis (Drummond-Barbosa and Spradling, 2001; Nezis *et al.*, 2000; 2001; 2002; 2003; 2005; 2006a; 2006b; 2006c, for reviews see McCall, 2004; Baum *et al.*, 2005). Follicular atresia, a distinct pattern of developmentally regulated cell death, has been sporadically observed during Diptera mid-oogenesis (Giorgi and Deri, 1976; Nezis *et al.*, 2000; 2003; 2006a; Uchida *et al.*, 2004), or massively induced in response to (a) nutritional deprivation, (b) ecdysone signalling malfunction, (c) treatment with chemotherapeutic drugs (i.e. etoposide, or staurosporine) and (d) ectopic death of follicle cells in *Drosophila* (Buszczak *et al.*, 1999; Chao and Nagoshi, 1999; DeLorenzo *et al.*, 1999; Soller *et al.*, 1999; Nezis *et al.*, 2000; Terashima and Bownes, 2004). The atretic egg chambers contain degenerated nurse cells, mainly characterized by condensed chromatin, fragmented DNA and disorganized actin cytoskeleton network (Buszczak *et al.*, 1999; Chao and Nagoshi, 1999; DeLorenzo *et al.*, 1999; Soller *et al.*, 1999; Nezis *et al.*, 2000; 2003; 2006a). Nurse and follicle cell death programs are also required for the normal maturation of the developing follicles during the late stages of *Drosophila*, *Bactrocera oleae* and *C. capitata* oogenesis (Cavaliere *et al.*, 1998; Foley and Cooley, 1998; Nezis *et al.*, 2000; 2001; 2002; 2003; 2006b; 2006c, for reviews see McCall, 2004; Baum *et al.*, 2005).

In the present study, we demonstrate, for the first time, that apoptosis and autophagy function together during mid- and late-oogenesis of the medfly *C. capitata*. Apoptosis in mid-oogenesis exhibits caspase activation that is tightly associated with DNA fragmentation and actin cytoskeleton disorganization. Interest-ingly, this apoptotic process seems to be implemented through a cytochrome c-independent mechanism. On the other hand, au-tophagy is mainly characterized by the formation of autophagosomes and the production of cathepsin-like proteases that are likely related to the fragmentation of the yolk spheres. During late-oogenesis, apoptosis is executed through a limited caspase activation program, chaperoned with a modified cyto-chrome c conformational display. At the end of apoptosis, autoph-agy strongly facilitates the more efficient clearance of the nurse cell remnants by the adjacent follicle cells.

Results

The nurse cells of the atretic egg chambers evince both apoptotic and autophagic cell death features during *C. capitata* mid-oogenesis checkpoint.

The development of the egg chamber of the medfly *C. capitata* is divided into 14 distinct stages (Mouzaki and Margaritis, 1991; Nezis *et al.*, 2003; Trougakos and Margaritis, 2002). In the present study, we thoroughly examined the developmental stages

7-13 (Fig. 1). We have previously shown that egg chambers during *C. capitata* mid-oogenesis degenerate in low frequency. These sporadically produced egg chambers are characterized by abnormal morphology and loss of their cellular compartmental-ized organization and integrity compared to the normal ones of the same developmental stages (Nezis *et al.*, 2003). We have further analyzed these abnormal egg chambers and found that their nurse cells contain condensed chromatin and fragmented DNA (Figs. 2 A-C). In order to unveil the alterations of actin cytoskel-eton network, we applied an immunolabelling approach using the anti-fractin antibody as a specific probe. During apoptosis, the beta actin cytoskeletal component is specifically cleaved by activated caspases. This cleavage results in the release of approximately 130 amino acid residues (~ 15 kDa) from the carboxyl-terminus of the beta actin subunit, generating a trun-cated 32 kDa amino-terminal fragment of actin, widely known as fractin (Yang *et al.*, 1998; Rossiter *et al.*, 2000). All the abnormal egg chambers tested are characterized by intense anti-fractin immunostaining signals mainly in the nurse cell cluster, directly indicating the generation of the 32 kDa truncated actin fragment (Fig. 2D and data not shown).

Using the rhodamine-conjugated substrate of active caspases Red-VAD-FMK, we clearly demonstrate the occurrence of highly activated caspases in all the atretic egg chambers tested (Fig. 2E). Moreover, through an immunolabelling approach, using the CM1 antibody that specifically recognizes an activated form of the *Drosophila* caspase Drice (Laundrie *et al.*, 2003; Peterson *et al.*, 2003), we detect positive staining signals in the degenerating nurse cell cluster, indicating the presence of activated caspase-3-like (Drice, or Drice-like) proteases (Fig. 2 F,G). On the contrary, no positive signal for activated caspases could ever be observed
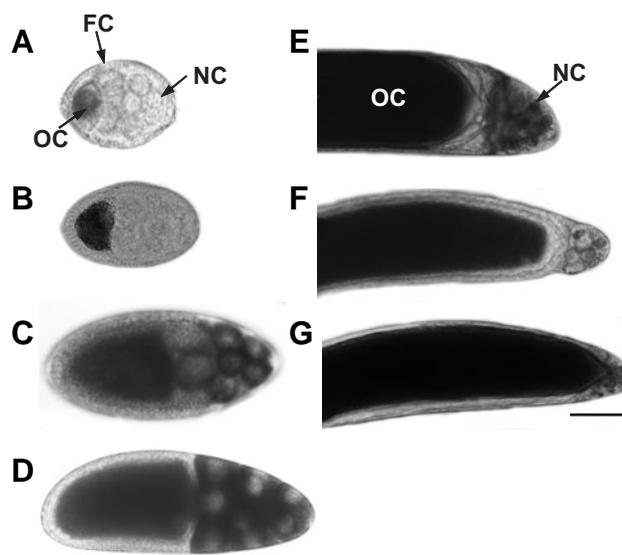
Fig. 1. The development of the egg chamber during oogenesis of the medfly *C. capitata*. *The general morphology of representative egg chambers at the developmental stages 7-13 is illustrated in light micro-graphs* **(A-G)**. *The size of the egg chamber increases gradually up to the late stages of oogenesis.* **(A)** *stage 7,* **(B)** *stage 8,* **(C)** *stage 9,* **(D)** *stage 10,* **(E)** *stage 11,* **(F)** *stage 12 and* **(G)** *stage 13. FC, follicle cells; NC, nurse cells and OC, oocyte. Scale bars: (A-D) 50 μm; (E-G) 100 μm.*
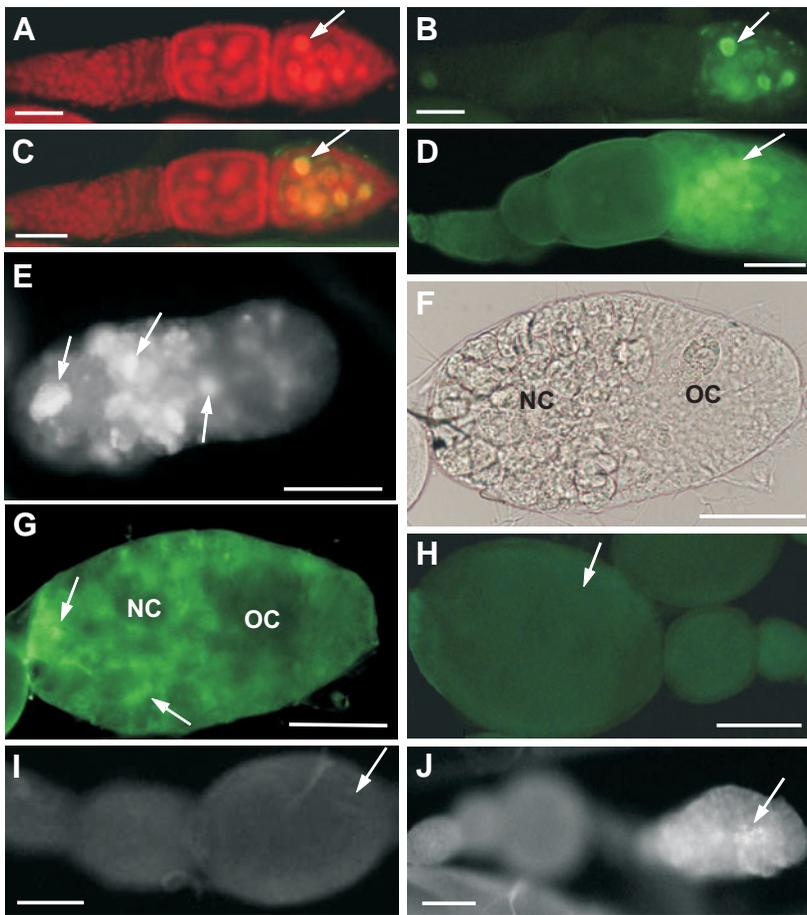
Fig. 2. Spontaneously induced programmed cell death during *C. capitata* mid-oogenesis exhibits typical apoptotic features, such as DNA fragmentation, formation of a truncated beta actin fragment and activation of a caspase repertoire program. (A-C) *Fluorescent micrographs of a representative* C. capitata *atretic egg chamber during mid-oogenesis after propidium iodide staining* **(A)** *and TUNEL assay* **(B)**. **(C)** *Merge of the (A) and (B) images. Intense signals (respective arrows) are clearly observed in the nurse cell nuclei (NN) of stage 7-8 egg chambers and not in previous developmental stages.* **(D)** *Fluorescent micrograph of an ovariole after immunostaining with the anti-fractin antibody. The intense positive signals observed in the nurse cells (arrow) of stage 7-8 egg chamber demonstrate the presence of fragmented actin. Note the absence of detectable signal in the previous developmental stages.* **(E)** *Red-VAD-FMK strong staining of stage 7-8 atretic egg chambers reveals the presence of active caspases in the degenerating nurse cell cluster (arrows).* **(F-H)** *Immunostaining with the CM1 antibody evinces the activation of the caspase family member Drice (or Drice-like proteins) in the nurse cells of a representative stage 7-8 atretic egg chamber (G, arrows). (F) Phase contrast light micrograph of (G). (H) No staining can be observed in a normal egg chamber of the same stage (arrow).* **(I)** *Fluorescent micrograph after an immunolabelling approach using the 2aG8 anti-cytochrome c monoclonal antibody. No traceable staining could ever be observed in all the tested atretic egg chambers (arrow).* **(J)** *Fluorescent micrograph after immunostaining reactions using the anti-Dronc antibody. The stage 7-8 atretic egg chamber exhibits positive signal (arrow). NC, nurse cells and OC, oocyte. Scale bars: 50 μm.*

in the normal egg chambers during mid-oogenesis (Fig. 2H). Additionally, immunostaining reactions using the anti-Dronc polyclonal antibody demonstrates a stage-specific (7-8) accumulation of the Dronc-like caspase in the nurse cell cluster of the atretic egg chambers (Fig. 2J). In order to identify whether cytochrome c plays any role in the observed caspase activation program, we applied a specific immunostaining approach using the monoclonal antibody 2aG8 (Mueller and Jemmerson, 1996). This particular antibody has been previously shown to recognize an altered and exposed epitope of cytochrome c, which is specifically unmasked during programmed cell death in *Drosophila melanogaster* egg chambers (Varkey *et al.*, 1999). As illustrated

in Fig. 2I, we were not able to obtain any detectable anti-cytochrome c immunoreaction signal in any of the atretic egg chambers tested (n = 250), suggesting the absence of the cytochrome c involvement (or at least the display, or unmasking of the respective epitope) in the execution of the nurse cell cluster apoptotic death during mid-oogenesis.

To answer the question whether autophagy occurs during mid-

Fig. 3. Programmed cell death during *C. capitata* mid-oogenesis manifests typical features of autophagy. (A) *Monodansylcadaverine (MDC) staining of an atretic stage 7-8 egg chamber. The positive strong signals reflect the presence of autophagic vacuoles (arrows).* **(B)** *A normally developed stage 7-8 egg chamber (arrow), with a prominent absence of autophagosome formation (MDC negative staining).* **(C)** *Immunostaining with an anti-procathepsin L specific antibody, revealing a strong and punctate staining pattern in the abnormal egg chamber during mid-oogenesis (arrows).* **(D)** *Transmission electron micrograph of an atretic stage 7-8 egg chamber, illustrating the presence of degenerated yolk spheres (arrows).* **(E)** *Transmission electron micrograph of an abnormal stage 7-8 egg chamber demonstrating the occurrence of large autophagosomes (arrows). They contain numerous vesicles of different sizes and multi-lamellar membranes. Scale bars: (A-C) 50 μm; (D) 1 μm; (E) 10 μm.*
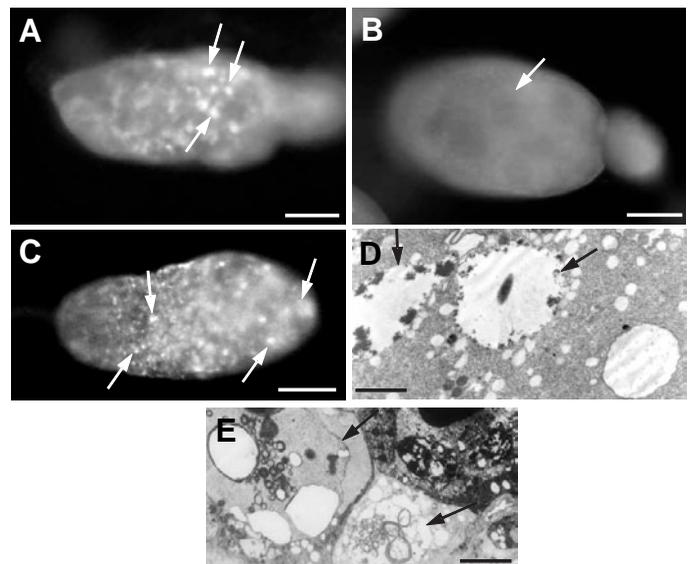
oogenesis of *C. capitata*, we first used monodansylcadaverine staining for tracing the autophagic vacuoles. Several positive signals are clearly observed in the nurse cell cluster of all the abnormal egg chambers tested (Fig. 3A), while the physiologically developed ones are completely devoid of any detectable staining (Fig. 3B). Interestingly, an immunostaining approach using the anti-procathepsin L polyclonal antibody, raised against the *Sarcophaga peregrine* protein family member (Uchida *et al.*, 2001), clearly reveals an intense and punctate staining pattern all over the non-compartmentalized atretic egg chamber (Fig. 3C).

The examination of the ultrastructural morphology of the sporadically produced atretic egg chambers discloses the occurrence of several degenerated yolk spheres with fragmented and degraded contents (Fig. 3D). Moreover, the atretic egg chambers are characterized by presence of large autophagosomes that are scattered in all the areas of the abnormal egg chamber, containing numerous vesicles and multi-lamellar membranes of various sizes (Fig. 3E and data not shown).

Ovarian nurse cells of Ceratitis capitata *during late-oogenesis exhibit both apoptotic and autophagic cell death*

It was previously shown that the nurse cells of *C. capitata* egg chambers normally degenerate during the late stages of oogenesis (Nezis *et al.*, 2003). Using the acridine orange staining, we demonstrate that during the developmental stages 12 and 13 all the nurse cell nuclei become apoptotic and contain fragmented DNA (Fig. 4 C,E), while no DNA fragmentation event can be detected at stage 11 (Fig. 4A).

We further analyzed the apoptotic features of the developing egg chambers, using an antibody staining approach for fragmented actin (fractin). At stage 11, no traceable signal for fragmented actin could be observed in all the egg chambers tested (Fig. 4B). On the contrary, fragmented actin immunostaining reactions were clearly detected during stages 12 and 13 in the nurse cell cluster (Fig. 4 D,F).

To elucidate the potential implication of cytochrome c in the implementation of the nurse cells apoptotic program, we applied immunolabelling with the anti-cytochrome c monoclonal antibody 2aG8 (Mueller and Jemmerson, 1996) at the developing *C. capitata* egg chambers. No staining was revealed in all three compartments of a representative stage 10 egg chamber (Fig. 5A). On the contrary, during the next developmental stage 11 the nurse cell cluster is characterized by an intense anti-cytochrome c immunostaining, which is mainly dispersed in all the respective nurse cell cytoplasmic areas (Fig. 5C). Even though this staining pattern is maintained unaffected during stage 12 (Fig. 5E), during the next developmental stage 13 several bright and distinct specks are clearly observed in the nurse cell compartment (Fig. 5G). Thus, it is very likely that cytochrome c constitutes an essential signalling component for the effective execution of the *C. capitata* nurse cells apoptotic program.

A similar staining pattern can be disclosed during *C. capitata* late-oogenesis, using the anti-Dronc polyclonal antibody. A diffused anti-Dronc immunostaining signal is primarily detected during stage 11 mainly in the respective cytoplasmic areas of the nurse cell compartment (Fig. 5D). This dispersed staining profile remains unaffected during the next developmental stage 12 (Fig. 5F), while the stage 13 is mainly characterized by a more focalized signal (Fig. 5H). On the other hand, all stage 10 egg chambers are completely devoid of any immunostaining reaction in all three compartments (oocyte, nurse cells and follicle cells) (Fig. 5B).

In order to illuminate the actual participation and contribution of active caspases in the cell death program of *C. capitata* nurse cells, we applied an active caspase *in situ* assay, using the Red-VAD-FMK fluorescent inhibitor as the specific detecting probe. As it is clearly illustrated in Fig. 6A, no positive signals for active caspases could ever be detected in any area of the stage 10 egg chambers. Similar negative results were also obtained using the CM1 polyclonal antibody that specifically recognizes an activated form of the *Drosophila* caspase family member Drice (Peterson *et al.*, 2003) (Fig. 6B). During the next developmental stage 11, Red-VAD-FMK positive signals for active caspases are clearly detected in the nurse cell cluster, especially in the nurse cells adjacent to the oocyte (Fig. 6C), while no traceable CM1 immunostaining reaction is observed in any of the egg chamber compartments (Fig. 6D). In contrast, the following stage 12 is mainly characterized by distinct signals produced after the applica-
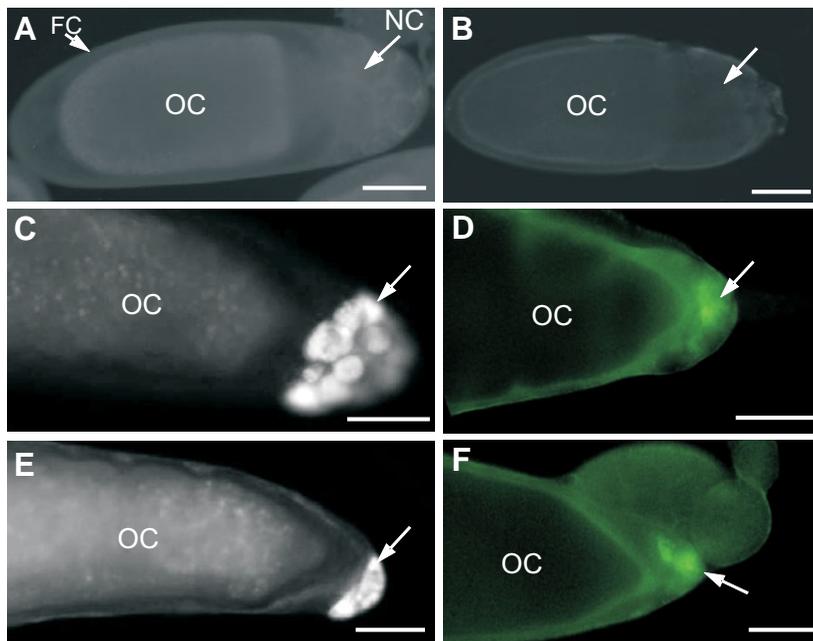
Fig. 4. Programmed cell death during *C. capitata* late-oogenesis exhibits typical apoptotic features, such as DNA fragmentation and fragmented actin. (A,C,E) *Stage-specific DNA fragmentation, as revealed by an acridine orange staining approach.* **(B,D,F)** *Developmentally regulated fractin production, as it is disclosed by an anti-fractin immunostaining.* **(A,B)** *Stage 11 egg chambers. Observe the complete absence of traceable positive signals for both acridine orange staining (A) and anti-fractin immunolabelling (B), all over the egg chamber.* **(C,D)** *Stage 12 egg chambers, exhibiting intense positive signals exclusively produced in the nurse cell cluster after both acridine orange staining (C) and anti-fractin immunolabelling (D) (respective arrows).* **(E,F)** *During stage 13, strong positive signals for both applied staining approaches can be observed, respectively (arrows). NC, nurse cells; FC, follicle cells; OC, oocyte. Scale bars: 100 μm.*
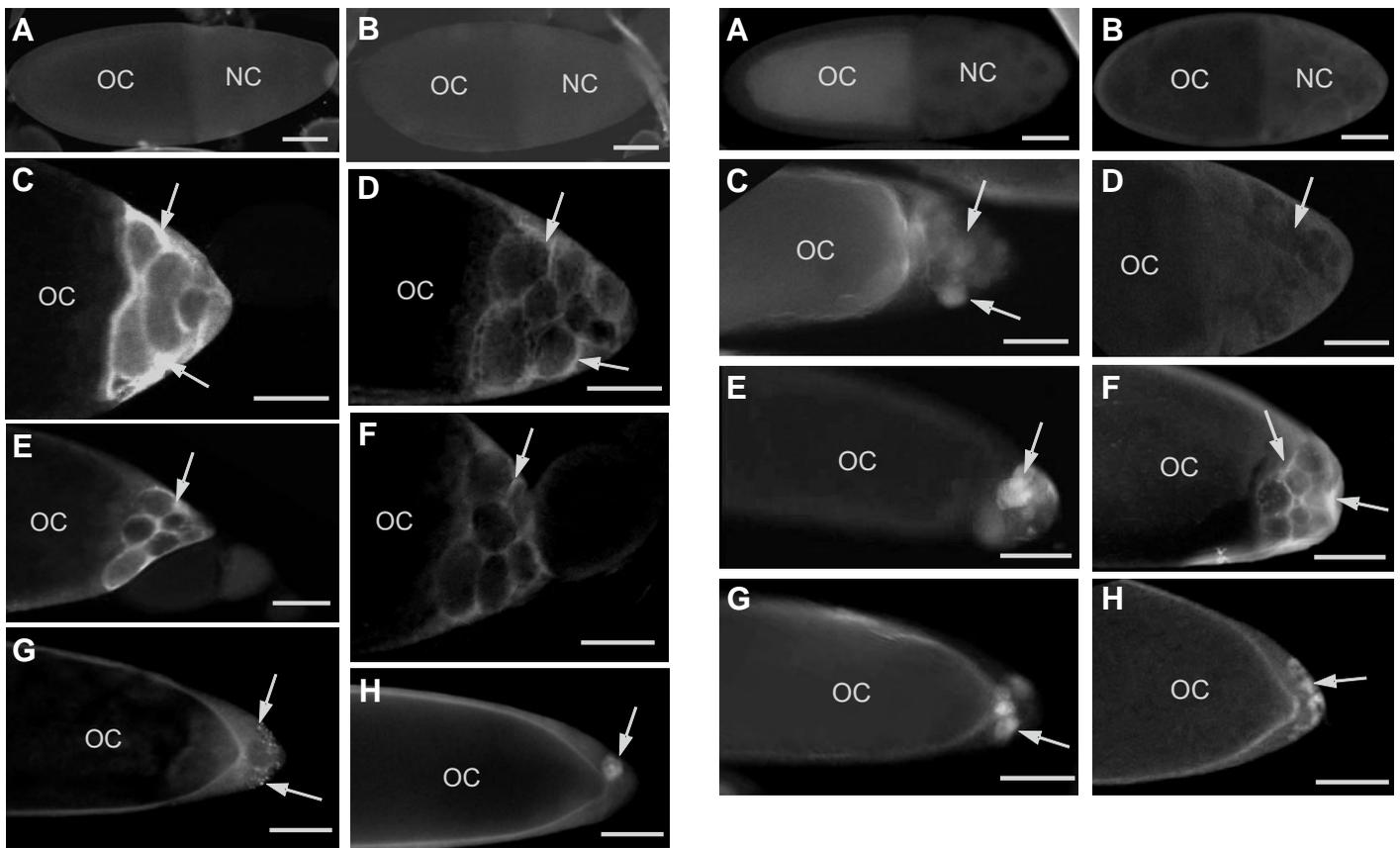
Fig. 5 (Left Column). Late stage egg chambers of *C. capitata* exhibit the presence of an altered cytochrome c conformational display and upregulation of Dronc-like proteins. *Confocal and conventional light micrographs after immunostaining of developing egg chambers with the 2aG8 anti-cytochrome c monoclonal antibody* **(A, C, E, G)** *and an anti-Dronc antibody* **(B, D, F, H). (A,B)** *Stage 10 egg chambers, showing no detectable staining for cytochrome c and Dronc, respectively.* **(C,D)** *During stage 11, intense diffused staining can be clearly observed in the nurse cell cytoplasm for both cytochrome c and Dronc-like proteins, respectively (arrows). A similar pattern is also obtained during the following stage 12* **(E,F)***,(see respective arrows). During stage 13, the obtained immunostaining respective pattern for either cytochrome c or Dronc-like caspases becomes more focalized* **(G,H)***, (see arrows). The staining observed at the oocyte periphery is due to autofluorescence of chorion. NC, nurse cells and OC, oocyte. Scale bars: 100 μm.*

Fig. 6 (Right Column). Limited caspase activation occurs in a stage-specific manner during *C. capitata* late-oogenesis. *Confocal and conventional light micrographs of representative egg chambers after Red-VAD-FMK staining* **(A, C, E, G)** *and an immunolabelling approach using the CM1 antibody* **(B, D, F, H). (A,B)** *Late stage 10 representative egg chambers showing the complete absence of caspase activity for both applied staining approaches.* **(C,D)** *Positive signals for the Red-VAD-FMK staining reagent are detected in the nurse cell cluster during stage 11 (C), while no traceable staining can be observed for an activated Drice (or Drice-like) caspase (D) (arrows).* **(E-H)** *Nurse cell compartment-specific staining reactions for both Red-VAD-FMK* **(E,G)** *and CM1* **(F,H)** *labelling approaches are revealed during stage 12 (E,F) and stage 13 (G,H) of* C. capitata *oogenesis (arrows). NC, nurse cells and OC, oocyte. Scale bars: 100 μm.*

tion of either the Red-VAD-FMK or CM1 labelling approach, as it is demonstrated in Fig. 6 E,F, respectively. During the developmental stage 13, the obtained, from both techniques, positive reactions are focalized in size, producing a more distinct and punctate staining pattern (Fig. 6 G,H).

In order to examine whether an autophagic cell death process is essentially involved during *C. capitata* late-oogenesis, we used monodansylcadaverine staining for tracing the occurrence of autophagic vacuoles. Stage 10 and 11 egg chambers do not appear to develop any positive staining signal (Fig. 7A and data not shown). On the contrary, during the following stages 12 and 13 punctate discrete monodansylcadaverine staining patterns are distinguished in the nurse cells of the developing egg cham-

bers (Fig. 7 C,E). When the ultrastructural organization of the stage 12 and 13 egg chambers was examined, several autophagosomes, containing numerous vesicles, membranes and dense masses, are readily observed in the degenerated nurse cells (Fig. 7 G,H and data not shown). These autophagosomes are mainly located in close proximity to the actin bundles (Fig. 7G).

In addition, the generation of autophagic vacuoles is escorted by a distinct immunostaining profile obtained through the usage of the anti-procathepsin L polyclonal antibody that has been raised against the *Sarcophaga peregrine* protein family member (Uchida *et al.*, 2001). As it is illustrated in Fig. 7D, a representative stage 12 egg chamber is mainly characterized by punctate staining
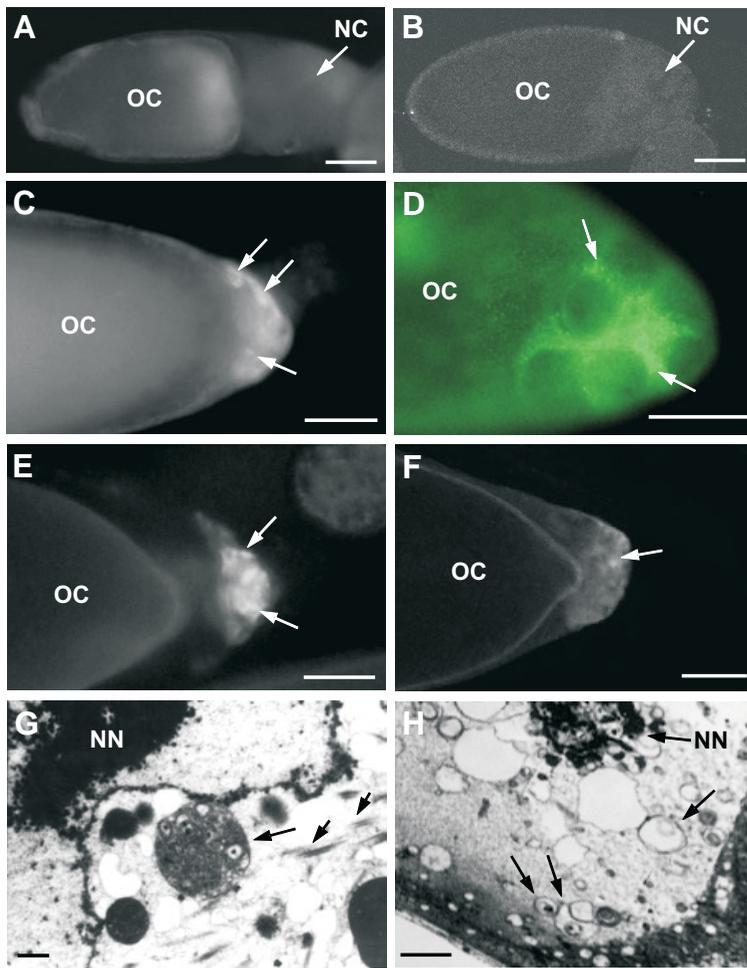
Fig. 7. Programmed cell death during *C. capitata* late-oogenesis is characterized by typical features of autophagy and upregulation of lysosomal enzymes. (A,C,E) *Monodansylcadaverine (MDC) staining and* **(B,D,F)** *anti-procathepsin L immunolabelling.* **(A,B)** *During stage 11, no traceable positive signal can be observed after either MDC staining (A), or anti-procathepsin L immunolabelling (B).* **(C-F)** *During the following developmental stages 12 **(C, D)** and 13 **(E, F)**, distinct strong MDC (C,E) and anti-procathepsin L (D,F) staining patterns are clearly distinguished in the degenerated nurse cell cluster (respective arrows).* **(G)** *Transmission electron micrograph illustrating the occurrence of an autophagosome (arrow) in a nurse cell during stage 12. The particular autophagosome is located in close proximity to actin bundles (small arrows).* **(H)** *Transmission electron micrograph demonstrating the occurrence of several autophagosomes (arrows) in a nurse cell during stage 13 (NN-arrow: nurse cell nuclear remnants). NC, nurse cells; NN, nurse cell nuclei and OC, oocyte. Scale bars: (A-F) 100 μm; (G) 1 μm; (H) 5 μm.*

restricted to the nurse cell compartment. During the following stage 13, the nurse cells seem to contain intense anti-procathepsin L immunostaining signals (Fig. 7F). On the other hand, all the analyzed stage 11 egg chambers are devoid of any detectable staining in all three compartments (Fig. 7B).

Discussion

Programmed cell death occurs sporadically during mid-oogenesis and normally during late-oogenesis and results in the degeneration of the nurse cell cluster in *Ceratitis capitata* (Nezis *et al.*,

2003). The efficient execution of this cell death program involves chromatin condensation, DNA fragmentation and phagocytosis of the cellular debris by adjacent follicle cells (Nezis *et al.*, 2003). In the present study, we reveal for the first time that apoptotic and autophagic cell death coexist during the above developmental stages.

During mid-oogenesis, the nurse cells of the spontaneously degenerated egg chambers contain fragmented DNA and fragmented beta actin, as well. The pancaspase VAD-FMK substrate assay clearly reveals that the caspase repertoire program is highly activated at these atretic egg chambers. Moreover, a distinct caspase-3-like (Drice, or Drice-like) protein is also activated, as it is demonstrated by the CM1 immunolabelling approach. Recent studies in *Drosophila* have shown that caspases and their inhibitors play a significant role in the execution of the mid-oogenesis cell death. Over-expression of an active form of the effector caspase Dcp-1 in the ovary results in the degeneration of egg chambers during mid-oogenesis (Peterson *et al.*, 2003). This phenotype is also characterized by high levels of the active caspase Drice and can be suppressed by over-expression of the apoptotic inhibitor DIAP-1 (Peterson *et al.*, 2003). Additionally, flies defective for Dcp-1 display a lack of sporadically degenerated stage 7-8 egg chambers (Laundrie *et al.*, 2003; Nezis *et al.*, 2005). Thus, it is reasonable to assume that the *C. capitata* homologous respective members of Dcp-1 and Drice caspases are responsible for the execution of the sporadically observed cell death program during mid-oogenesis. Interestingly, homologous caspase family members have been shown to exist in other Diptera species, like the mosquito *Anopheles stephensi* (Abraham *et al.*, 2004). The regulation of mid-oogenesis cell death in *Drosophila* is mainly controlled by the proper balance between juvenile hormone and ecdysone (Soller *et al.*, 1999; Buszczak *et al.*, 1999). Interestingly, the *C. capitata* homologous member of the ecdysone receptor, CcEcR, is expressed at its highest levels during mid-oogenesis (Verras *et al.*, 2002). Additionally, in mosquitoes there is a significant increase in the levels of ecdysone following a blood meal, which allows for the onset of vitellogenesis (Raikhel *et al.*, 2002). Thus, it is very likely that ecdysone and juvenile hormone levels can also control whether an egg chamber survives or perishes during *C. capitata* mid-oogenesis. Recent studies in *Drosophila* revealed that the gene for Dronc caspase is significantly upregulated after 20E-hydroxy-ecdysone treatment, or poor nutrition that results in degeneration of the egg chambers during mid-oogenesis (Terashima and Bownes, 2005). The upregulation and accumulation of the Dronc-like protein we observed herein is in absolute agreement with the above results.

In atretic follicles of the mosquito *Culex pipiens pallens*, ovarian cathepsin-like proteinases, which have been accumulated within the developing oocyte primarily for embryogenesis, are activated to digest yolk proteins and other cellular substrate structures (Uchida *et al.*, 2001). Additionally, the atretic follicles of the olive fruit fly *Bactrocera oleae* contain several fragmented yolk spheres (Nezis *et al.*, 2006a). The above observation is very similar to the one we describe herein in degenerating egg chambers of *C. capitata*. In the present study, anti-procathepsin L

immunolabelling shows intense punctate staining in the atretic egg chambers. We could speculate that ovarian cathepsin-like proteinases are specifically activated in *C. capitata* abnormal egg chambers in order to digest yolk proteins and other cellular substrate structures. It has been also previously reported that cathepsins are implicated in the activation of caspases (Bursch, 2001; Chwieralski *et al.*, 2006) Thus, it is likely that the degeneration of the egg chambers during mid-oogenesis is also regulated by a stage-specific activation of distinct endogenous ovarian proteinases.

Autophagy is defined as the process by which a portion of the cytosol and organelles are sequestered in a random manner by isolation membranes. Such an isolation membrane engulfs portions of the cytoplasm and forms a double-membrane-layered initial autophagic vacuole, or autophagosome, that fuses with a lysosome and generates the autolysosome, where the cellular components of the vacuole are degraded (Bursch, 2001; 2004; Lockshin and Zakeri, 2004; Muller *et al.*, 2004; Juhasz and Sass, 2005). In multicellular organisms, autophagy was integrated to cell death events in post-mitotic cells in order to cope with the mass of cytoplasm needed to be degraded in a short period of time, thereby lowering the exigency for macrophages, or epithelial cells, to clear up the corpses (Lockshin and Zakeri, 2004). During mid-oogenesis of *C. capitata*, the sporadically degenerated egg chambers contain large autophagosomes, demonstrating the stage-specific activation of a distinct autophagic cell death program. It is very likely that the cellular contents of the nurse cells and oocyte compartment of the atretic egg chambers are partially degraded and lysed inside the generated autophagosomes. Thus, the follicle cells that are committed to phagocytose and wipe up the nurse cell and oocyte remnants (Nezis *et al.*, 2003) have less work to fulfill. We propose that an autophagic cell death program strongly supports the follicular epithelium phagocytotic activities for a rapid and more efficient clearance of the cellular debris in the atretic egg chambers during *C. capitata* mid-oogenesis.

During the late stages of oogenesis the nurse cells exhibit fragmented DNA and actin, an altered cytochrome c conformational display (differential epitope unmasking) and a caspase repertoire activation program. We have previously demonstrated that the actin cytoskeleton network is reorganized during late-oogenesis in *C. capitata* (Nezis *et al.*, 2003), commencing by developmental stage 11 when actin bundles are formed. The formation of these bundles is unlikely associated with a caspase-mediated actin cleavage, since at this particular stage (11) no staining for fragmented actin can be detected, in all the tested egg chambers. The autophagosomes observed in the degenerating nurse cells during late-oogenesis are mainly located in close proximity to actin bundles. Actin cytoskeleton integrity has been previously shown to play an essential role in the execution of autophagic cell death, while the sequestration of cytoplasmic structural components involves the action of intermediate filaments, as well (Bursch *et al.*, 2000; Bursch, 2004). Thus, we suggest that the caspase-independent actin bundles formation is necessary for the implementation of autophagic cell death during *C. capitata* late-oogenesis.

In *Drosophila* egg chambers, the anti-cytochrome c monoclonal antibody 2aG8 has been proposed to recognize an altered configuration of the protein and specifically labels dying cells (Varkey *et al.*, 1999). Through utilization of the particular antibody, we were able to reveal an altered cytochrome c conformational display that was initially detected in the nurse cells of stage 11 egg chambers, exhibiting a rather diffuse cytoplasmic staining. In the same stage egg chambers, we observed an upregulation of Dronc (or Dronc-like) caspase family member that is tightly associated with the activation of caspase repertoire program, as this is clearly revealed by the VAD-FMK fluorescent inhibitor assay. We propose that during the developmental stages 11 and 12 cytochrome c likely activates the Dronc (or Dronc-like) caspase family member, which, in turn, is able to trigger the Drice (or Drice-like) proteolytic activities that are likely associated with the efficient implementation of the *C. capitata* nurse cell death program. Caspase activation during late-oogenesis in *C. capitata* seems to occur in limited levels, compared to mid-oogenesis since the level and localization of activity differ among the various developmental stages. This finding completely corroborates previously reported results in *Drosophila* (Peterson *et al.*, 2003), suggesting that the careful and limited caspase activation in dying nurse cells may prevent the destruction of the connected oocyte.

Interestingly, in contrast to what has been observed during late-oogenesis, the mid-oogenesis nurse cells apoptosis seems to be a cytochrome c-independent process, even though an alternative implication of the cytochrome c signalling component can not be excluded. This differential contribution of cytochrome c in the execution of mid- and late-oogenesis apoptotic programs comprises a novel and important issue that needs further exploration and enlightenment.

Distinct features of a nurse cell autophagic death can be detected during the late developmental stages of *C. capitata* oogenesis, as it is clearly evidenced by the presence of several autophagosomes and the accumulation of the procathepsin L family member. It has been previously reported that cathepsin-like proteinases are implicated in the activation of the caspase repertoire program (Bursch, 2001; Chwieralski *et al.*, 2006). The contribution and critical role of lysosomal enzymes in the execution of mid- and late-oogenesis respective apoptotic programs comprise novel interesting issues that await further exploration.

In conclusion, our data contribute to the elucidation of the mechanisms of programmed cell death during oogenesis in the medfly *C. capitata*. We propose that during mid-oogenesis the cell death program, specifically implemented in atretic egg chambers, causes accumulation and activation of Dronc, or Dronc-like proteins independently from cytochrome c involvement. Drice, or Drice-like proteins are subsequently activated, likely inducing DNA fragmentation events and fractin generation that is tightly associated with the actin cytoskeleton disorganization. In parallel, several autophagosomes are formed in order to lower the exigency of the adjacent follicle cells to clear up the nurse cell corpses. During late-oogenesis, the cell death stimuli cause an alteration in the cytochrome c conformational display that is likely implicated in the Dronc, or Dronc-like caspase activation. The subsequently activated Drice, or Drice-like caspases cause DNA fragmentation and alterations in the actin cytoskeleton network. In parallel, autophagosomes are formed in order to efficiently support the adjacent follicle cells to absorb the nurse cell corpses. We propose that after completion of the execution phase of apoptosis, autophagy helps for

the clearance of the apoptotic corpses from the adjacent follicular epithelium.

Materials and Methods

Ceratitis capitata *culturing*

C. capitata (Diptera, Tephritidae) adult insects were kept in a 25ºC culture room, fed on standard diet (10% yeast, 40% sugar in water) and slightly etherized before dissection. Dissections were carried out in cold Ringer's solution and ovaries were separated into single ovarioles. Staging of the developing egg chambers during oogenesis was accomplished according to Margaritis (1986), Mouzaki and Margaritis (1991) and Nezis *et al.* (2001; 2003).

Acridine orange staining

Ovaries were dissected in Ringer's solution and separated into individual egg chambers. The egg chambers were then incubated with 1.6 µM acridine orange in Ringer's solution for 5 min in the dark. Subsequently, they were washed for 5 min in Ringer's solution and immediately mounted onto vidal glass slides in fresh Ringer's solution. Elapsed time from dissection to the end of the viewing was restricted to 20 min. Samples were examined using a Nikon Eclipse TE 2000-S fluorescent microscope.

Double staining with propidium iodide and TUNEL assay

Egg chambers were fixed in PBS containing 4% formaldehyde plus 0.1% Triton X-100 for 25 min. After three washes in PBS for 5 min each, the egg chambers were incubated with PBS containing 20 µg/ml proteinase K for 15 min. Then, they were washed three times in PBS for 5 min each and incubated with the TUNEL reaction mixture for 3 hours at 37°C in the dark. Next, the egg chambers were washed three times in PBS for 5 min each, subsequently incubated with PBS containing 800 µg/ml RNaseA for 60 min and finally stained with 700 ng/ml propidium iodide in PBS for 15 min. At the end of the procedure, the egg chambers were washed three times in PBS, mounted in anti-fading mounting medium and viewed using a Nikon Eclipse TE 2000-S fluorescent microscope.

Active caspase *in situ* assay

Ovaries were dissected in Ringer's solution and the separated individual egg chambers were examined for the presence of active caspases using the Red-VAD-FMK detection kit (rhodamine-conjugated inhibitor of active caspases) (Oncogene Research Products, San Diego, USA). After the egg chambers were briefly rinsed with PBS, they were immediately transferred into the reaction mixture and incubated for 30 min at 25ºC in the dark. The treated egg chambers were subsequently rinsed three times with wash buffer, washed twice with PBS and finally put on a glass slide. The specimens were immediately observed under a Nikon Eclipse TE 2000-S fluorescent microscope using the appropriate filters.

Immunohistochemical procedures

Ovaries were dissected in Ringer's solution and separated into individual egg chambers. The egg chambers were fixed in PBS containing 4% formaldehyde for 20 min. After fixation, the samples were washed with PBS and subsequently incubated in PBS containing 0.1% Triton X-100 for 10 min. After the egg chambers were extensively washed with PBS, they were incubated in PBS containing 20 mM glycine and 1.5% BSA for 45 min. The primary antibodies used in the present study are given and commented as following: (a) the anti-fractin polyclonal antibody (Chemicon International, USA) was utilized at a dilution of 1:1000, (b) the mouse anti-cytochrome c monoclonal antibody of the 2aG8 clone was kindly obtained by Professor Ron Jemmerson (University of Minnesota, USA) and used at a concentration of 22 µg/ml, (c) the rabbit CM1 polyclonal antibody was supplied by BD Biosciences Pharmingen (USA) and used at a dilution of 1:1000, (d) the rabbit anti-Dronc polyclonal antibody that recognizes both the precursor (50 kDa) and the processed (39 kDa) form of the *Drosophila* caspase family member Dronc (Waldhuber *et al.*, 2005) was provided by

Professor Claudia Petritsch (Ludwig-Maximilian-University Munich, Germany) and utilized at a dilution of 1:200 and (e) the rabbit anti-procathepsin L (*Sarcophaga peregrine*) polyclonal antibody was supplied by Professor Koichi Homma (Faculty of Pharmaceutical Sciences, University of Tokyo, Japan) and used at a final dilution of 1:1000. After incubation with the respective primary antibody, the treated egg chambers were washed four times with PBS-T (PBS containing 0.1% Triton X-100 and 0.5% BSA). The secondary antibodies, conjugated with either FITC or rhodamine, were purchased from Santa Cruz Biotechnology Inc. (California, USA). Finally, the egg chambers were mounted in antifading mounting medium and viewed using a Nikon EZ 2000 confocal laser scanning microscope, or a TE 2000-S inverted fluorescent microscope. Routine procedures applied as controls to demonstrate the specificity of the immunostaining protocols used were: (i) the usage of the respective pre-immune serum instead of the antibody serum and (ii) the omission of the respective primary antibody. In all cases, no immunoreactivity could ever be observed.

Monodansylcadaverine staining for autophagic vacuoles

Monodansylcadaverine (MDC) staining was performed as following: dissected ovaries were briefly rinsed with PBS and immediately transferred into a PBS solution containing 0.05 mM MDC (Fluka, Sigma-Aldrich, Germany) for 10 min at 25ºC in the dark (Biederbick *et al.*, 1995; Nezis *et al.*, 2006b). The treated ovaries were washed four times with PBS and immediately observed under a Nikon Eclipse TE 2000-S fluorescent microscope using the appropriate filters.

Transmission electron microscopy

C. capitata egg chambers were processed for conventional transmission electron microscopy as following: ovaries were dissected in Ringer's solution and fixed in a PBS solution containing 1% glutaraldehyde and 1% OsO_4, pH 6.2, for 60 min at 4ºC. After three washes in distilled water for 5 min each at 4ºC, the ovaries were stained *en bloc* overnight in 0.5% uranyl acetate at 4ºC in the dark. The specimens were then dehydrated through a graded series of ethanol concentrations, infiltrated in propylene oxide and embedded in Epon-Araldite (Fullam Inc., New York, USA). Ultrathin sections were mounted on uncoated copper grids, stained with uranyl acetate and lead citrate and subsequently observed using a Philips EM 300 electron microscope.

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