Caspases function in autophagic programmed cell death in *Drosophila*

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Summary

Self-digestion of cytoplasmic components is the hallmark of autophagic programmed cell death. This auto-degradation appears to be distinct from what occurs in apoptotic cells that are engulfed and digested by phagocytes. Although much is known about apoptosis, far less is known about the mechanisms that regulate autophagic cell death. Here we show that autophagic cell death is regulated by steroid activation of caspases in *Drosophila* salivary glands. Salivary glands exhibit some morphological changes that are similar to apoptotic cells, including fragmentation of the cytoplasm, but do not appear to use phagocytes in their degradation. Changes in the levels and localization of filamentous Actin, α-Tubulin, α-Spectrin and nuclear Lamins precede salivary gland destruction, and coincide with increased levels of active Caspase 3 and a cleaved form of nuclear Lamin. Mutations in the steroid-regulated genes *βFTZ-F1*, *E93*, *BR-C* and *E74A* that prevent salivary gland cell death possess altered levels and localization of filamentous Actin, α-Tubulin, α-Spectrin, nuclear Lamins and active Caspase 3. Inhibition of caspases, by expression of either the caspase inhibitor p35 or a dominant-negative form of the initiator caspase Dronc, is sufficient to inhibit salivary gland cell death, and prevent changes in nuclear Lamins and α-Tubulin, but not to prevent the reorganization of filamentous Actin. These studies suggest that aspects of the cytoskeleton may be required for changes in dying salivary glands. Furthermore, caspases are not only used during apoptosis, but also function in the regulation of autophagic cell death.

Key words: Autophagic cell death, Apoptosis, Caspases, Salivary glands, *Drosophila*

Introduction

Programmed cell death plays an important role in the maintenance of animal homeostasis by eliminating unwanted cells and tissues, sculpting developing structures, controlling cell numbers and removing abnormal cells (Baehrecke, 2002; Jacobson et al., 1997). Apoptosis and autophagic cell death are the two most prominent morphological forms of cell death that occur during animal development (Clarke, 1990; Schweichel and Merker, 1973). Apoptotic cells exhibit specific morphological and biochemical changes, including the condensation and margination of chromatin to the nuclear membrane, DNA fragmentation, blebbing, formation of apoptotic bodies and exposure of the lipid phosphatidylserine on the outer leaflet of the plasma membrane (Fadok et al., 1992; Kerr et al., 1972; Wyllie, 1980). Although less is known about autophagic cell death, DNA fragmentation occurs, and autophagic vacuoles form that are used for destruction of cytoplasm (Lee and Baehrecke, 2001; Schweichel and Merker, 1973). Apoptosis and autophagic cell death differ in several ways, and the clearest distinction appears to be the mechanisms that are used for degradation of the dying cell. During apoptosis, the dying cell is engulfed by and degraded in the lysosome of a phagocyte. By contrast, cells undergoing autophagic cell death internalize cytoplasmic components into vacuoles that are targeted to the lysosome of the dying cell for degradation. Until recently, it was believed that apoptosis and autophagic cell death were controlled by different mechanisms. However, studies of *Drosophila* salivary gland cell destruction indicate that some of the genes that function during apoptosis also function in autophagic cell death (Lee and Baehrecke, 2001).

Steroids are important regulators of programmed cell death in animals (Baehrecke, 2000; Distelhorst, 2002; Evans-Storm and Cidlowski, 1995). During *Drosophila* development, the steroid 20-hydroxyecdysone (ecdysone) triggers cell death of many larval tissues, including the larval salivary glands (Jiang et al., 1997). Elevation of the ecdysone titer 12 hours after puparium formation (apf) triggers autophagic cell death in the salivary gland, and this tissue is completely destroyed by 16 hours apf (Jiang et al., 1997). Ecdysone is bound by its heterodimeric receptor that is encoded by *EcR* and *usp*, and acts in combination with the competence factor *βFTZ-F1* (*ftz-f1* – FlyBase) to regulate transcription of the primary response genes *E93* (*Eip93F* – FlyBase), *BR-C* (*br* – FlyBase) and *E74* (*Eip74EF* – FlyBase) (Broadus et al., 1999; Woodard et al., 1994). Salivary glands fail to die in animals with mutations in *βFTZ-F1*, *E93*, *BR-C* and *E74A*, and exhibit altered transcription of the secondary response cell death genes *rpr*, *hid* (*W* – FlyBase), *ark*, *dronc* (*Nc* – FlyBase) and *drice* (*Ice* – Flybase) during autophagic cell death (Broadus et al., 1999;

Jiang et al., 2000; Lee et al., 2003; Lee et al., 2002b; Lee et al., 2000; Restifo and White, 1992).

The *Drosophila* genome contains cell death genes that have been conserved between nematodes and humans (Aravind et al., 2001; Vernooy et al., 2000). Although the *Drosophila* cell death genes *reaper* (*rpr*), *head involution defective* (*hid*), *grim* and *sickle* appear novel (Chen et al., 1996; Christich et al., 2002; Grether et al., 1995; Srinivasula et al., 2002; White et al., 1994; Wing et al., 2002), the proteins they encode contain limited but significant N-terminal sequence identity with the mammalian proteins Omi/Htra2 and Smac/Diablo (Wu et al., 2000; Wu et al., 2001). These conserved N-terminal sequences interact with the *Drosophila* inhibitor of apoptosis DIAP1 (Hay et al., 1995), and this interaction is similar to the interaction of Omi/Htra2 and Smac/Diablo with mammalian IAPs (Wu et al., 2000; Wu et al., 2001). The interaction of Rpr, Hid, Grim and Sickle with DIAP1 relieves inhibition of caspase proteases that then cleave substrates (Goyal et al., 2000; Wang et al., 1999). Seven caspases have been identified in *Drosophila*, including Dronc, Dredd, Dream/Strica, Decay, Daydream/Damm, Drice and Dcp-1 (Chen et al., 1998; Dorstyn et al., 1999a; Dorstyn et al., 1999b; Doumanis et al., 2001; Fraser and Evan, 1997; Harvey et al., 2001; Song et al., 1997). Dronc, Dredd and Dream/Strica are initiator caspases that can be activated in the presence of the homolog of mammalian Apaf1 Ark/Dark/dApaf1/Hac-1 (Kanuka et al., 1999; Rodriguez et al., 2002; Rodriguez et al., 1999; Zhou et al., 1999). Once active, the initiator caspases activate the effector or executioner caspases, including Decay, Daydream/Damm, Drice and Dcp-1, that in turn cleave cell substrates during programmed cell death.

Dying salivary gland cells contain autophagic vacuoles, but also appear to use apoptosis genes, including caspases. Expression of the caspase inhibitor p35 blocks salivary gland cell death and DNA fragmentation (Jiang et al., 1997). Furthermore, the apoptosis genes *rpr*, *hid*, *ark*, *dronc* and *drice* are induced just before autophagic cell death (Dorstyn et al., 1999a; Jiang et al., 1997; Jiang et al., 2000; Lee et al., 2003; Lee et al., 2002b; Lee et al., 2000). These data suggest similarities between autophagic cell death and apoptosis, but little is known about how salivary gland cells are degraded. Salivary glands appear to have the lysosomal machinery that is required to degrade their own organelles, yet it is not clear whether they require the assistance of phagocytes. Although salivary gland cells also appear to use genes that are considered part of the core apoptotic machinery, including caspases, the morphology of these cells and cells undergoing apoptosis are distinct. Although caspase function is required for DNA fragmentation in dying salivary glands (Lee and Baehrecke, 2001), it is not known if these proteases cleave similar substrates in cells that die by apoptotic and autophagic cell death.

Here we show that salivary gland destruction exhibits some similarities to apoptosis, including blebbing and fragmentation of cells. Changes in the levels of proteins, including nuclear Lamins and α-Tubulin, are regulated by caspase cleavage of these substrates. By contrast, filamentous Actin appears to reorganize in a caspase-independent manner prior to degradation. Animals with mutations in *βFTZ-F1*, *E93*, *BR-C* and *E74A* possess altered levels of active caspase, and altered levels of structural proteins during salivary gland autophagy.

Expression of either the caspase inhibitor p35 or a dominant-negative form of the initiator caspase Dronc is sufficient to prevent changes in nuclear Lamins and α-Tubulin, but not in filamentous Actin. Combined, these studies indicate that caspases and cytoskeleton reorganization are needed to drive the process of salivary gland autophagic cell death.

Materials and methods

Salivary gland histology

Wild-type Canton S *Drosophila* were maintained at 25°C and aged to different stages following puparium formation. For paraffin sections and light microscopy, whole pupae were fixed, embedded, sectioned, and either stained with Gill's Hematoxylin and Pollack Trichrome or analyzed using the TUNEL method, as previously described (Lee and Baehrecke, 2001), and examined using a Zeiss Axiophot II microscope. For examination of thin sections by electron microscopy, whole pupae were fixed for 4 hours in 4% paraformaldehyde, 8 hours in 3% glutaraldehyde/0.2% tannic acid in 0.1 M Mops buffer (pH 7.0) at room temperature, and 16 hours in 3% glutaraldehyde/1% paraformaldehyde in 0.1 M Mops buffer (pH 7.0) at 4°C, post fixed in 1% osmium tetroxide for 1 hour, embedded in Spurr's resin, sectioned and analyzed using a Zeiss EM 10 transmission electron microscope.

Antibody staining

Antibodies against α-Spectrin (Dubreuil et al., 1987), Lamin Dm0 ADL84 (Stuurman et al., 1995), α-Tubulin 3A5 (Piperno and Fuller, 1985), Croquemort (Franc et al., 1996) and active Drice (Yoo et al., 2002) were obtained from Drs D. Branton, P. A. Fisher, M. T. Fuller, N. C. Franc and B. A. Hay, respectively. Antibodies against cleaved Caspase-3 (Asp175) and cleaved Lamin (Asp230) were obtained from Cell Signaling Technology (Beverly, MA). Rhodamine Phalloidin, TOTO-3 and secondary antibodies were purchased from Molecular Probes (Eugene, OR).

Wild-type Canton S, and *βFTZ-F1* (*βFTZ-F1¹⁷/βFTZ-F1¹⁹*), *BR-C* (*rbp⁵/Y*), *E74A* (*E74Aᴾ[neo]/Df(3L)st-81k19*) and *E93* (*E93¹/Df(3R)93Fˣ²*) mutant salivary glands were dissected from animals staged relative to puparium formation at 25°C, fixed in 4% paraformaldehyde/heptane for 20 minutes at room temperature, blocked in phosphate buffered saline containing 1% BSA and 0.1% Triton-X (PBSBT), and incubated with primary antibodies for 16 hours at 4°C. The *βFTZ-F1*, *BR-C*, *E74A* and *E93* mutants that were used in this study and their general defects in salivary gland cell death have been previously described (Broadus et al., 1999; Lee and Baehrecke, 2001; Lee et al., 2000; Restifo and White, 1992). Following incubation with primary antibodies, salivary glands were washed for 2 hours in PBSBT, incubated with appropriate secondary antibodies for two hours at room temperature, washed for another 2 hours in PBSBT at room temperature, incubated in 0.5 μl TOTO-3 in 1 ml PBSBT for 10 minutes at room temperature, and washed in PBSBT for an additional hour at room temperature. For rhodamine Phalloidin staining of filamentous Actin, salivary glands were fixed in 16% paraformaldehyde, incubated in 1% Triton-X in PBS, and incubated in 5 μl of rhodamine Phalloidin in 100 μl PBS for 20 minutes at room temperature, as previously described (Frydman and Spradling, 2001). Salivary glands were mounted in Vectashield (Vector Laboratories) and examined using a Zeiss Axiovert 100 M confocal microscope. The confocal microscope settings and length of exposure were identical in all analyses.

Expression of p35 and dominant-negative Dronc in salivary glands

To express p35 in salivary glands, *y*, *w*; *UAS-p35/UAS-p35* males were crossed to *y,w, fkh-GAL4/y,w, fkh-GAL4* virgin females. Progeny of this cross were aged to 24 hours after puparium formation (apf) and

stained for expression of nuclear Lamin Dm0, Croquemort, α-Tubulin, α-Spectrin and filamentous Actin, as described above. To examine the impact of expressing dominant-negative Dronc in salivary glands, transgenic males containing either dominant-negative *UAS-Dronc C318A* (Meier et al., 2000) or *UAS-Dronc C318G* (Quinn et al., 2000) were crossed to virgin females of either the salivary gland GAL4 promoter strain *y,w, fkh-GAL4/y,w, fkh-GAL4* or the heat-inducible promoter strain *y,w, hs-GAL4/y,w, hs-GAL4*. Controls consisted of dominant-negative Dronc transgenic strains that were not crossed to a GAL4 driver strain. As the dominant-negative Dronc C318A strain had a higher level of persistent salivary glands in preliminary studies, all data that are reported were derived from this strain. Progeny derived from the *fkh-GAL4* and *UAS-Dronc C318A* cross were aged to 24 hours apf, and analyzed for salivary gland persistence. Progeny from derived from the *hs-GAL4* and *UAS-Dronc C318A* cross were heat-shocked for 30 minutes at 37°C at 11, 13 and 16 hours apf, and analyzed for salivary gland persistence. Persistent salivary glands were stained for expression of nuclear Lamin Dm0, Croquemort, α-Tubulin, α-Spectrin and filamentous Actin as described above.

Protein extraction and western blot analysis

Wild-type Canton S salivary glands were dissected from animals staged 8, 10, 12 and 14 hours apf, homogenized in 0.1% glycerol, 2% SDS, 0.125% 1M Tris (pH 6.8), 0.05% β-mercaptoethanol, and 0.05% Bromo-phenol blue, and boiled for 5 minutes at 100°C. Equal amounts of total protein extracts were separated by electrophoresis on 12% acrylamide gels, and either transferred to 0.45 µm Immobilon-P membranes (Millipore), or duplicate gels of identical extracts were assessed for equal loading and integrity by Coomassie blue staining (Bio-Rad). Membranes were blocked in 10% non-fat milk in PBS with 1% Tween 20 for 1 hour at 37°C, incubated in primary antibody for 16 hours at 4°C, washed in PBS containing 1% Tween 20 at room temperature, incubated with the appropriate HRP-conjugated secondary antibody for 1 hour at 37°C, washed and developed using ECL detection reagents 1 and 2 (Amersham) at 25°C, and exposed to film.

Results

Dying salivary gland cells exhibit some characteristics of apoptosis

Dying salivary gland cells contain autophagic vacuoles, suggesting that these cells are capable of degrading themselves (Lee and Baehrecke, 2001). However, it is not clear what happens to these cells in the late stages of destruction. To address this issue, wild-type Canton S animals were aged either 12, 14, 14.5 or 15 hours after puparium formation (apf), embedded in paraffin, then sectioned and stained (Fig. 1). Salivary glands have large vacuoles and intact nuclei 12 hours apf (Fig. 1A; *n*=10 animals/stage). By 14 hours apf, vacuoles are smaller in size and nuclei appear to have separated from the cytoplasm (Fig. 1B; *n*=12 animals/stage). This separation is more apparent by 14.5 hours apf, and is accompanied by blebbing and separation of cytoplasmic fragments (Fig. 1C,D; *n*=12 animals/stage). At 15 hours apf, cellular debris is scattered throughout the area where salivary glands were located (Fig. 1E; *n*=10 animals/stage).

To determine the nature of the cellular debris as salivary glands are destroyed, the TUNEL assay was used to distinguish salivary gland nuclei containing fragmented DNA from possible cytoplasmic fragments and possible phagocytes that had engulfed dying cell corpses that contain fragmented DNA (Fig. 2). TUNEL-positive salivary gland nuclei begin to
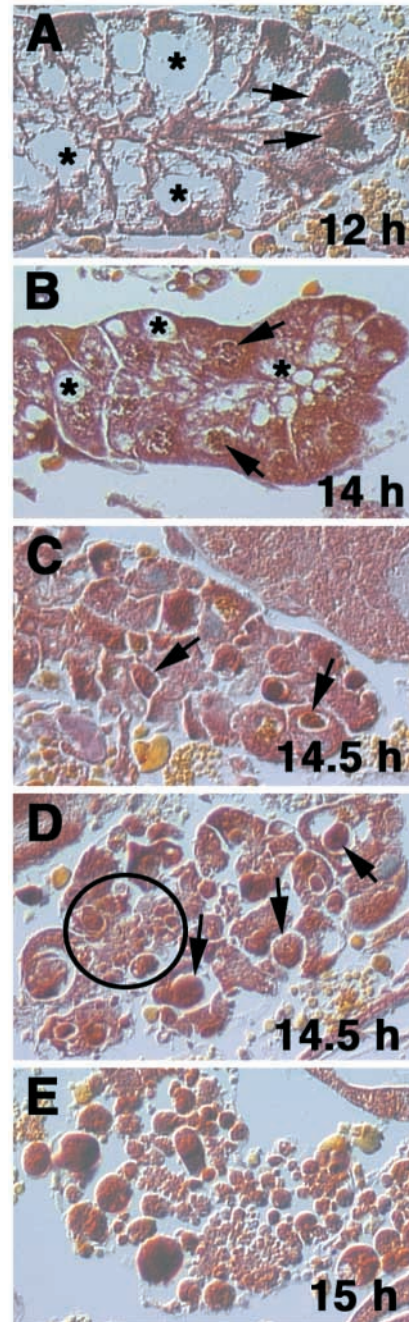

Fig. 1. Salivary gland cells fragment as they die. (A) Twelve hour apf salivary glands have large vacuoles (asterisks) and nuclei (arrows). (B) Vacuoles (asterisks) are smaller in salivary glands of 14 hour apf animals, and nuclei (arrows) begin to separate from the cytoplasm. (C-E) Salivary glands rapidly degenerate 14.5 hours to 15 hours apf. (C,D) Nuclei (arrows) and cytoplasm of salivary glands separate 14.5 hours apf, and fragments of cytoplasm (enclosed in circle) are present. (E) By 15 hours apf, fragments of cellular debris are scattered throughout the area that was occupied by salivary glands. All images are at the same magnification.

separate from the cytoplasm 14 hours apf (Fig. 2A; *n*=13 animals/stage). At 15 and 16 hours apf, TUNEL-positive salivary gland nuclei and TUNEL-negative structures that are presumably fragments of cytoplasm are present (Fig. 2B,C; *n*=22 animals/stage). Differentially stained cells that lack TUNEL-staining also appear when most of the salivary gland degradation has occurred, but it is unclear whether these cells are phagocytes, based on this resolution. Transmission electron microscopy (TEM) analyses of salivary glands isolated 14.5 hours apf revealed the separation of nuclei from the cytoplasm (Fig. 2D; *n*=15 animals/stage). Furthermore, membranous structures resembling smooth endoplasmic reticulum are present in the balls of cellular debris, indicating that these are spheres of cytoplasm (Fig. 2E; *n*=15 animals/stage). Although

differentially stained cells were observed at very late stages of salivary gland degradation (Fig. 2B,C), no obvious phagocytes containing cell fragments were observed using TEM (data not shown; *n*=many grids of sections from 15 animals staged 14.5-15 hours apf).

Changes in protein levels and localization are associated with increased caspase activity during autophagic cell death

Salivary glands undergo dynamic morphological changes during autophagic cell death (Lee and Baehrecke, 2001) (Figs 1, 2). These changes in cell shape and organization may be controlled by the modification of structural protein organization, and/or by the activity of proteases, including caspases. As structural proteins have been implicated as substrates for caspases (Cryns and Yuan, 1998), we examined

Fig. 2. Salivary glands have several characteristics of apoptosis, and appear to degrade without the assistance of phagocytes. (A-C) TUNEL assay was used to distinguish nuclei that contain fragmented DNA from cytoplasmic fragments and possible phagocytes. (A) Salivary glands possess TUNEL-positive nuclei (arrows) 14 hours apf. (B) TUNEL-positive nuclei (arrows) are surrounded by TUNEL-negative structures that are presumably fragments of salivary gland cytoplasm 15 hours apf, as well as by a small number of unidentified cells (asterisks) that did not contain obvious cell corpses. (C) At 16 hours apf, few TUNEL-positive structures are present in the location where salivary glands reside prior to destruction, and unidentified cells (asterisks) are present, but do not contain obvious dead cell corpses. (D) Transmission electron micrograph of salivary glands 14.5 hours apf, showing the separation of the nucleus (n) from the cytoplasm (c). (E) Membranous structures reminiscent of smooth endoplasmic reticulum are present in some of the balls of cytoplasm (c). Images in A-C are the same magnification. Scale bars: in D, 10 μm; in E, 1 μm.
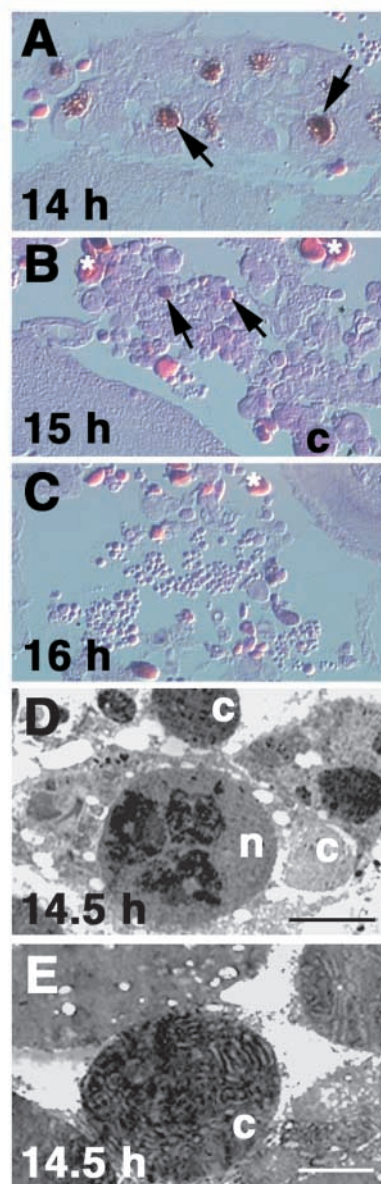
changes in the localization and abundance of structural proteins during cell death. Wild-type Canton S animals were staged either 8, 10, 12 or 14 hours apf, salivary glands were dissected, fixed and stained with antibodies against α-Tubulin, α-Spectrin, nuclear Lamin DmO or Croquemort, and rhodamine phalloidin was used to label filamentous Actin (Fig. 3; *n*=many cells from 20 animals/stage/stain). The localization of both α-Tubulin and filamentous Actin changes, from a network spread across the cytoplasm of salivary gland cells 8 hours apf to a less evenly distributed and somewhat clustered localization in the case of filamentous Actin 10 hours apf (Fig. 3). By 12 hours apf, α-Tubulin is difficult to detect, and filamentous Actin is extremely clustered in the cytosoplasm and spread along the cortical region of the cell. By 14 hours apf, α-Tubulin is absent and filamentous Actin is difficult to detect. Although α-Spectrin remains present and associated with the cortex, its levels also appeared to decrease between 12 and 14 hours apf in some dying salivary gland cells. Nuclear Lamins are also present 8 hours apf, and decrease in expression in 10- to 12-hour apf salivary glands. This change initially appears as spots associated with the chromosomes, and Lamins are difficult to detect by 14 hours apf. At the stage when nuclear Lamins are nearly absent in salivary glands, Croquemort is initially detected in the cytoplasm, making this protein an excellent marker for late stage cytoplasmic changes.

Nuclear Lamins have been implicated as caspase substrates (McCall and Steller, 1998; Rao et al., 1996). Therefore, we examined whether changes in nuclear Lamin DmO levels and localization are associated with the expression of active caspases and cleavage of nuclear Lamins (Fig. 4; *n*=many cells from 22 animals/stage/stain). Active caspase-3/Drice protein levels increase in the cytoplasm as nuclear Lamin DmO decreases in abundance 10 to 12 hours apf, and this is the time when the cleaved form of nuclear Lamin appears in the cytoplasm. By 14 hours apf, the levels of Lamin DmO are low or absent, and active caspase-3/Drice and cleaved Lamins are abundant in the cytoplasm (Fig. 4; *n*=many cells from 46 animals/stage/stain).

The association of protein changes with increases in active caspase levels suggests that proteolysis by caspases occurs during salivary gland autophagic cell death. It is possible that changes in structural protein localization in the absence of proteolysis are also important for proper cell degradation to occur. Protein was extracted from salivary glands of wild-type animals that were staged 8, 10, 12 and 14 hours apf, and equal amounts of protein/stage were separated by electrophoresis and analyzed by western blot to assess quantitative changes in proteins. Western blots were incubated with antibodies against Lamin and Actin (Fig. 5). Full-length 76 kD Lamin is cleaved to 45 kD 10 hours apf, which is consistent with the expected size based on the location of the caspase cleavage site in Lamin, and the timing of changes in immunolocalization of this protein (Fig. 4). This 45 kD cleavage product is also present 12 and 14 hours apf. By contrast, the full-length 42 kD Actin protein is present in equal quantity until 12 hours apf, and is absent by 14 hours, with no detectable cleavage product. The difference in the timing of the degradation of nuclear Lamins and Actin is intriguing, and suggests the possibility that the changes in filamentous Actin localization observed 10 hours apf (Fig. 3) are not due to proteolysis of Actin by

caspases, but may be caused by reorganization of Actin, followed by proteolysis 14 hours apf.

Mutations in steroid-regulated genes impact protein changes and caspase activation during autophagic cell death

Animals with mutations in the ecdysone regulated genes βFTZ-F1, E93, BR-C and E74A have salivary glands that persist past the time when wild-type salivary glands are destroyed (Broadus et al., 1999; Lee and Baehrecke, 2001; Restifo and White, 1992). Each of these mutants appears to be arrested at different stages of cell destruction (Lee and Baehrecke, 2001), enabling the examination of how βFTZ-F1, E93, BR-C and E74A impact protein changes and caspase activation during autophagic cell death. Salivary glands were dissected from mutants aged to 24 hours apf (8 hours after salivary glands are destroyed in wild-type animals) and stained with antibodies against either α-Tubulin, α-Spectrin, nuclear Lamin DmO or Croquemort; rhodamine phalloidin was used to label filamentous Actin (Fig. 6; *n*=many cells from 20 animals/genotype/stain). βFTZ-F1 mutant salivary glands are arrested prior to the onset of most changes in these proteins (see Fig. 3 for comparison with wild-type), although filamentous Actin is observed in clumps in the cytoplasm indicating that some protein changes progress to slightly later stages. E93 mutant salivary gland cells possess α-Tubulin and α-Spectrin protein levels and localization that are similar to those observed in 10- to 12-hour apf salivary gland cells. However, filamentous Actin localization has changed to a late stage that is similar to 12 to 14 hour apf wild-type, and decreased levels of Lamin DmO and the presence of Croquemort protein indicates that several protein changes have occurred that are normally associated with cell death. BR-C mutant salivary gland cells possess abnormal structural protein changes that we never observe in wild-type cells. For example, α-Tubulin and α-Spectrin staining do not overlap, and α-Spectrin is present in a wider area than expected. This is

interesting as α-Spectrin remains present in the cortical region of the cell in BR-C mutants, even though these cells appear to lack plasma membranes (Lee and Baehrecke, 2001). In addition, filamentous Actin appears speckled in the cytoplasm of BR-C mutants. E74A mutant salivary gland cells progress to a late stage in cell death, as filamentous Actin and nuclear Lamin staining are weak, and Croquemort is present, similar to 14 hour apf wild-type cells. The only exceptions are that α-Tubulin and α-Spectrin are both present, and that there are fragments of α-Spectrin staining in the cytoplasm, which are consistent with the apparent presence of membrane fragments in E74A mutant salivary glands (Lee and Baehrecke, 2001).

The differences in the distribution of proteins in βFTZ-F1, E93, BR-C and E74A mutant salivary glands may indicate that differences in caspase activation occur in these animals. Therefore, we examined the distribution of active Caspase-3/Drice, nuclear Lamin DmO and cleaved nuclear Lamin in βFTZ-F1, E93, BR-C and E74A mutants (Fig. 7, *n*=many
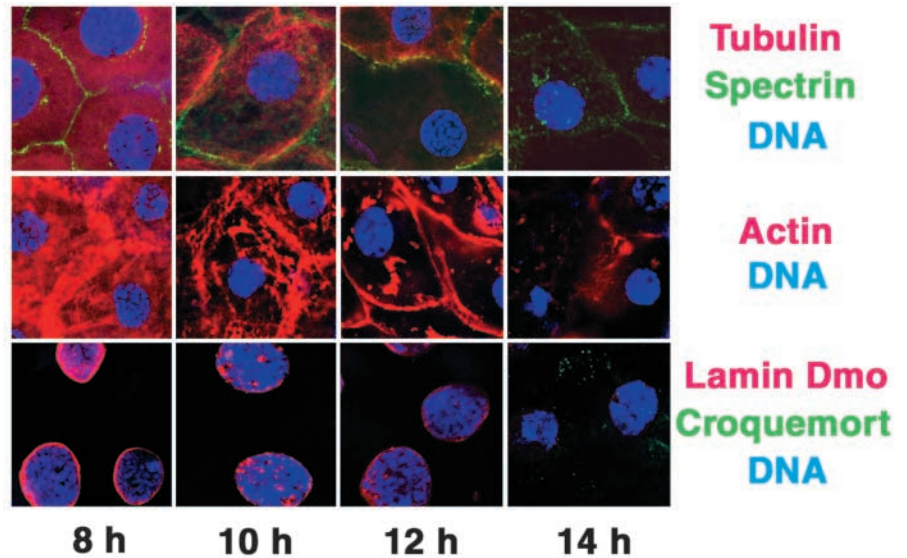
Fig. 3. Structural proteins exhibit dynamic changes during salivary gland autophagic cell death. Salivary glands of staged wild-type Canton S animals stained with antibodies against α-Tubulin (red) and α-Spectrin (green) (top panels), with rhodamine phalloidin to visualize filamentous Actin (red; middle panels), and with antibodies against nuclear Lamin DmO (red) and Croquemort (green) (lower panels). Nuclei are labeled with TOTO-3 (blue) in all images.
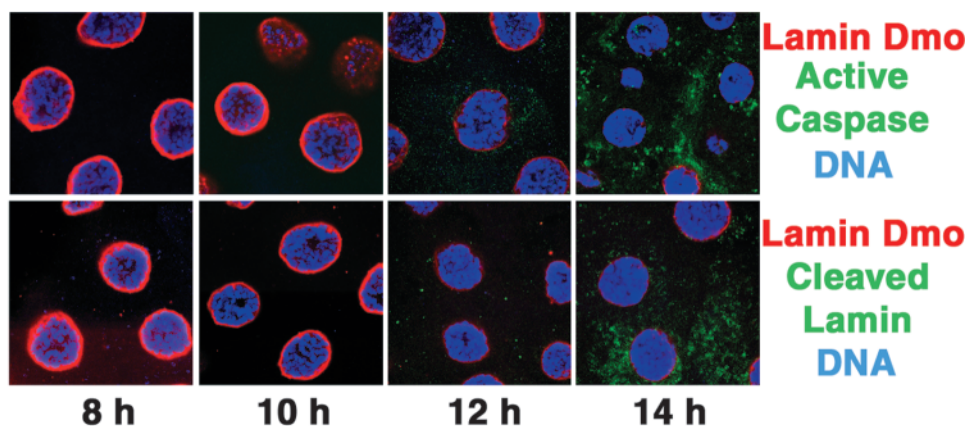
Fig. 4. Changes in nuclear Lamins are associated with increased levels of active caspase. Salivary glands from wild-type Canton S were dissected and stained with antibodies against: nuclear Lamin DmO (red) and active Caspase-3/Drice (green) (top panels); and nuclear Lamin DmO (red) and cleaved nuclear Lamin (green) (bottom panels). Nuclear Lamin DmO is present 8 and 10 hours apf, but decreases by 12 hours apf. At this stage, active Caspase-3/Drice and cleaved nuclear Lamin are present in the cytoplasm; levels of these increase by 14 hours apf.
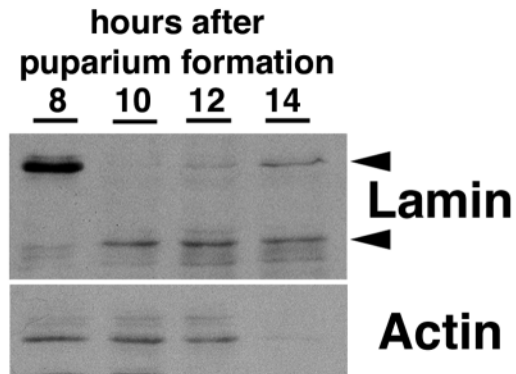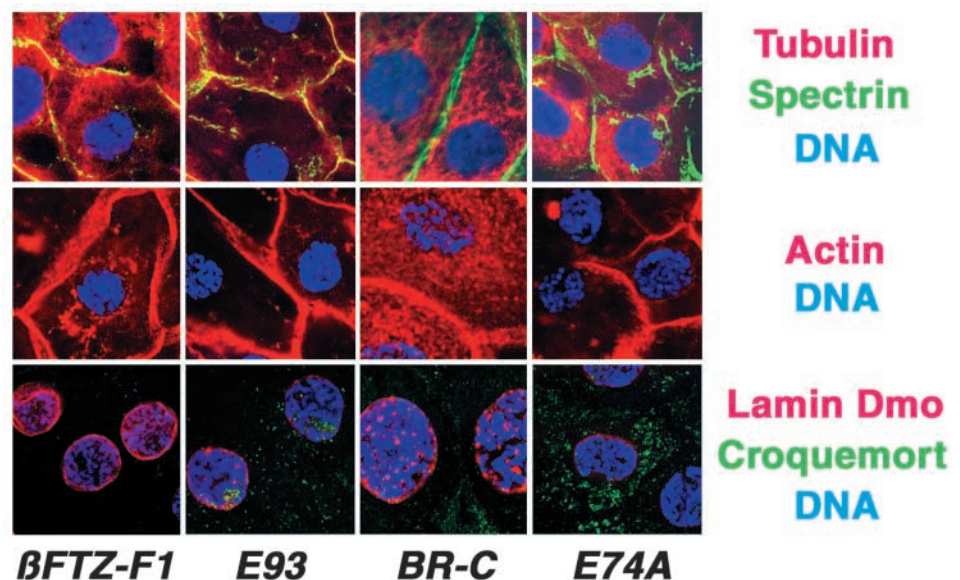
Fig. 5. Changes in the localization of Lamins coincide with their degradation, whereas Actin changes localization prior to degradation. Western blot analysis of wild-type salivary gland protein extracts show that full-length 76 kD Lamin is cleaved to 45 kD (the expected size following caspase cleavage) starting at 10 hours apf. By contrast, full-length Actin persists through 12 hours apf, but is not detected in extracts 14 hours apf.

cells from 10 animals/genotype/stain). Active caspase and cleavage of Lamin are not detected in the salivary glands of *βFTZ-F1* mutant animals. *E93* mutant salivary glands possess low levels of active caspase and cleaved Lamin, and retain substantial amounts of Lamin DmO. In *BR-C* mutant salivary glands, Lamin DmO is localized in spots, low levels of active caspase associate with the nucleus, and cleaved Lamin is not detected. *E74A* mutants progress to a late stage with little Lamin DmO staining, and with high levels of active caspase-3/Drice and cleaved Lamin being present in the cytoplasm. These results indicate that the differences in the cell morphology of *βFTZ-F1, E93, BR-C* and *E74A* mutant

salivary glands may be caused by the amount of caspase activity in these mutants.

Inhibition of caspases alters protein changes that occur during autophagic cell death

Expression of the caspase inhibitor p35 is sufficient to prevent salivary gland destruction and DNA fragmentation, although inhibition of caspases does not prevent changes in vacuolar structures in these cells (Lee and Baehrecke, 2001). As transcription of the initiator caspase *dronc* immediately precedes salivary gland cell death (Lee et al., 2000), we tested whether expressing a dominant-negative form of Dronc is sufficient to prevent salivary gland cell death. In animals that express a dominant-negative Dronc C318A transgene under the control of a heat-inducible promoter, 22% of the pupae had persistent salivary glands 24 hours apf (*n*=383), whereas no salivary gland cells persisted in control animals containing the same transgene that were not heat-shocked. Similar analyses using the salivary gland-specific fkh-GAL4 driver resulted in 10% of the animals having persistent salivary glands (*n*=122). As in studies of p35, expression of dominant-negative Dronc is sufficient to prevent DNA fragmentation in persistent salivary glands (Fig. 8A; *n*=8), even though DNA is fragmented in midguts. In addition, expression of dominant-negative Dronc does not prevent vacuolar changes in the cytoplasm of persistent salivary glands (Fig. 8A).

To determine which proteins changes are regulated by caspases during salivary gland autophagic cell death, p35 and dominant-negative Dronc were expressed in salivary glands prior to cell death changes and analyzed 24 hours apf (Fig. 8B; *n*=many cells from 8 animals/transgene/stain). When p35 is expressed, α-Tubulin and α-Spectrin are localized in a pattern that is similar to that of 8-hour apf salivary gland cells (see Fig. 3 to compare to wild-type cells), indicating that caspases are

Fig. 6. Animals with mutations in *βFTZ-F1, E93, BR-C* and *E74A* have altered levels and localization of structural proteins. Salivary glands were dissected from mutants 24 hours apf, and analyzed for the levels and localization of: α-Tubulin (red) and α-Spectrin (green) (top panels); filamentous Actin (red) (middle panels); and nuclear Lamin DmO (red) and Croquemort (green) (lower panels). The α-Tubulin network is present in the salivary glands of all of these mutants, but it appears to be at slightly lower levels in the cytoplasm of *E93* mutants. α-Tubulin appears to be absent near the cortex in *BR-C* and *E74A* mutant animals, given the lack of overlap with α-Spectrin staining. α-Spectrin is present along the cortex in *βFTZ-F1, E93* and *BR-C*, and in fragments in the cytoplasm of *E74A* mutant salivary glands. Filamentous Actin is aggregated in clumps in the cytosol, and is expressed along the cortical region of salivary gland cells in *βFTZ-F1, E93, BR-C* and *E74A* mutant animals, although differences clearly exist in the localization of this protein in the different mutants. Nuclear Lamin DmO is present and is evenly expressed in association with the nuclear membrane in *βFTZ-F1* mutant salivary gland cells, is expressed at a lower level with punctate spots in *E93* and *BR-C* mutant salivary glands, and is difficult to detect in *E74A* mutant salivary gland cells. Croquemort is not detectable in *βFTZ-F1* mutant salivary glands, is expressed at a lower level in *E93* and *BR-C* salivary glands, and is highly expressed in *E74A* mutant salivary gland cells.
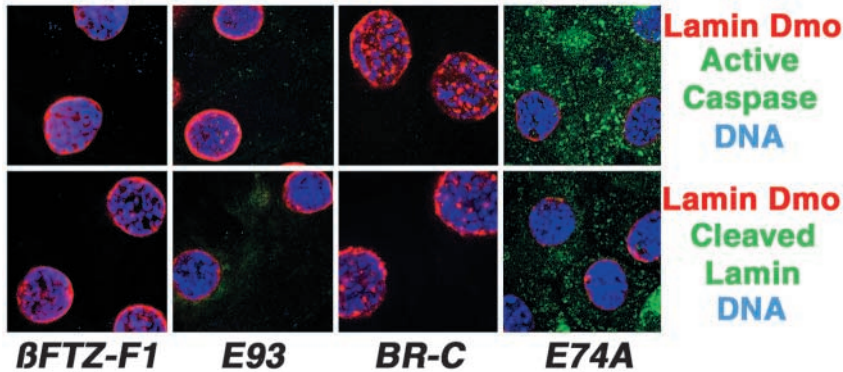
Fig. 7. Mutations in *βFTZ-F1*, *E93*, *BR-C* and *E74A* impact caspase activation during autophagic cell death. Salivary glands were dissected from mutants 24 hours apf, and either analyzed for abundance and localization of nuclear Lamin DmO (red) and active Caspase-3/Drice (green) (top panels), or nuclear Lamin DmO (red) and cleaved nuclear Lamin (green) (bottom panels). Active Caspase-3/Drice and cleaved nuclear Lamins are absent in *βFTZ-F1* salivary glands, are present at extremely low levels in *E93* and *BR-C* salivary gland cells, and are expressed at elevated levels in *E74A* mutant salivary gland cells.

involved in regulating changes in these proteins. Similarly, nuclear Lamin DmO levels and localization appear similar to that in 8-hour apf salivary glands. However, the cytoplasm of these cells has clearly progressed to a later stage as Croquemort is expressed (we never observe high levels of both Lamin DmO and Croquemort in wild-type cells). Although expression of p35 blocks several protein changes, filamentous Actin localization is similar to later stages in dying salivary glands, suggesting that the changes in Actin structure may not be dependent on caspase function. This is consistent with the differences in the timing of changes in nuclear Lamins and filamentous Actin based on quantitative western blot studies (Fig. 5). Salivary glands that survive following the expression of dominant-negative Dronc exhibit limited changes in α-Tubulin, α-Spectrin, and nuclear Lamin DmO (Fig. 8B). However, Croquemort expression is observed, indicating that changes in the cytoplasm have occurred. Expression of dominant-negative Dronc was not sufficient to prevent changes in filamentous Actin, consistent with the possibility that some changes in proteins during autophagic cell death of salivary glands are not regulated by caspases.
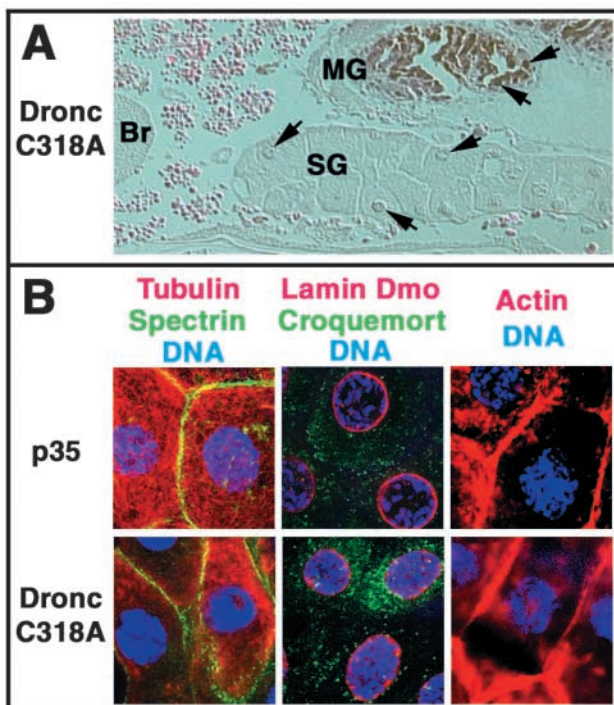
Discussion

Apoptosis and autophagic cell death share some morphological characteristics

Studies of dying salivary glands indicate that autophagic cell death has both similarities to and differences from apoptosis. Several markers of apoptosis, including nuclear staining by Acridine Orange and DNA fragmentation, appear 2 hours following the rise in steroid hormone that triggers salivary gland cell death (Jiang et al., 1997). The cytoplasm and membranes of salivary gland cells bleb and fragment as these cells degrade (Figs 1, 2), and these are characteristics that are common to apoptotic cells (Kerr et al., 1972). These common morphologies raise the questions of what distinguishes apoptosis from autophagic cell death, and what common and different regulatory mechanisms mediate these forms of cell killing.

The mechanisms of cell degradation and removal appear to provide the clearest distinction between apoptosis and autophagic cell death. In salivary glands, dynamic changes in vacuole structure immediately precede their demise, and such changes have not been reported in apoptotic cells (Kerr et al., 1972; Schweichel and Merker, 1973). Within one hour of salivary gland DNA degradation, large vacuoles appear to break into smaller vacuoles, and this occurs within 2 hours of complete tissue destruction (Lee and Baehrecke, 2001) (Fig. 1). As these large vacuoles fragment, smaller but distinct vacuoles accumulate near the plasma membrane, and

Fig. 8. Inhibition of caspases prevents protein changes and salivary gland cell death. (A) The TUNEL assay was used to distinguish nuclei that contain fragmented DNA from nuclei with intact DNA. Animals staged 24 hours apf that express the dominant-negative form of Dronc C318A possess persistent salivary glands (SG) that lack vacuoles, but have nuclei with intact DNA (arrows), even though the DNA of the larval midgut (MG) is fragmented (dark stain, arrows). The brain (Br) is indicated to assist with orientation. (B) Expression of p35 and dominant-negative Dronc C318A prevent changes in the expression of α-Tubulin (red) and α-Spectrin (green), shown in the left panels, and nuclear Lamin DmO (red) (middle panels). By contrast, expression of p35 and dominant-negative Dronc C318A did not prevent the increase in abundance of Croquemort (green in the middle panels) in the cytoplasm, even though nuclear Lamin DmO (red) is expressed in the same cell, indicating that some cytoplasmic changes are not regulated by caspases. Similarly, filamentous Actin (red in the right panels) changes proceed and resemble 12-hour apf wild-type salivary gland cells, even though expression of p35 and dominant-negative Dronc C318A prevent the death of these cells.

autophagic vacuoles containing components of the cytoplasm, including mitochondria, are formed. Salivary gland cells then begin to fragment, and nuclei and components of the cytoplasm then disperse within the haemocoel (Fig. 2). Although we can not exclude a role for phagocytes in autophagic cell death, salivary gland cells proceed to late stages of degradation without the assistance of phagocytes. It has been suggested that phagocytes may play a secondary role in the removal of cellular debris towards the end of autophagic cell death (Schweichel and Merker, 1973), and our results are consistent with this possible conclusion. The presence of autophagic vacuoles in degrading salivary glands further indicates that autophagic cells use their own lysosomes for degradation, whereas apoptotic cells depend on phagocytes for the bulk degradation of long-lived cellular proteins.

Maintenance of the Actin cytoskeleton may be necessary for autophagic cell death

Dying cells exhibit dynamic changes in cell shape (Kerr et al., 1972; Schweichel and Merker, 1973). The changes in cell organization that occur during salivary gland cell death are likely to be controlled by the modification of structural protein organization. Indeed, dynamic changes in the abundance and localization of filamentous Actin, α-Tubulin, α-Spectrin and nuclear Lamins precede the death of salivary glands (Fig. 3). At least two possible explanations exist for how such changes in protein expression are regulated in dying cells. One possibility is that proteases cleave structural proteins (Cryns and Yuan, 1998), and that this results in the changes in cell shape. Alternatively, changes in cell shape could be regulated by the assembly of cytoskeletal proteins, such as filamentous Actin, through signaling that is mediated by small GTPases (Coleman and Olson, 2002).

Proteolysis and changes in the assembly of the cytoskeleton both appear to be involved in the regulation of changes that occur during autophagic cell death of salivary glands. Although caspases play an important role in the cell death of salivary glands, several lines of evidence suggest that some changes in the structure of the cytoskeleton may occur in a caspase-independent manner. First, whereas changes in filamentous Actin localization occur in synchrony with changes in proteins such as nuclear Lamins that are cleaved by caspases (Fig. 3), changes in Actin protein levels are delayed by 4 hours (Fig. 5). Second, mutations in steroid-signaling genes, such as βFTZ-F1, that prevent expression of active caspase-3 and cleavage of nuclear Lamins do not prevent changes in filamentous Actin localization (Fig. 6). Third, although inhibition of caspases by expression of either p35 or a dominant-negative form of Dronc is sufficient to prevent changes in nuclear Lamins and α-Tubulin, these inhibitors are not sufficient to block changes in filamentous Actin (Fig. 8). These data are further supported by the observation that numerous small GTPases increase their expression immediately prior to salivary gland cell death (Lee et al., 2003). Although previous studies have suggested that changes in the Actin cytoskeleton are required for autophagic cell death (Bursch et al., 2000; Jochova et al., 1997), the failure to distinguish between cytoskeleton proteolysis and rearrangement has made it difficult to interpret the potential significance of maintenance of the cytoskeleton during cell death.

Several possibilities exist to explain why the cytoskeleton is maintained during cell death. The cytoskeleton could be used to restrict the subcellular location and activity of pro-apoptotic regulators of the Bcl-2 family, and activation of apoptosis (Puthalakath et al., 1999; Puthalakath et al., 2001). This mechanism seems unlikely during salivary gland cell death because the Actin cytoskeleton is maintained after caspase-dependent cleavage of substrates, including nuclear Lamins (Figs 3-5, 8). Alternatively, the Actin cytoskeleton could be maintained as a substrate to localize proteins, membranes and vacuoles within the cell. Intracellular trafficking plays an important role in autophagy, as membrane-bound cytoplasmic components (autophagic vacuoles) are transported to the lysosome for degradation (Baehrecke, 2003; Klionsky and Emr, 2000; Ohsumi, 2001). As we observe autophagic vacuoles at stages after caspase activation and cleavage of substrates such as nuclear Lamins (Lee and Baehrecke, 2001) (Figs 3-5), it is possible that the Actin cytoskeleton is maintained to enable transport of vacuoles to lysosomes.

Caspases are required for autophagic cell death

Studies of salivary glands indicate that caspases play an important role in their autophagic cell death. The caspase-encoding genes *dronc* and *drice* show an increase in their transcription following the rise in steroid that triggers salivary gland autophagic cell death (Lee et al., 2003; Lee et al., 2002a; Lee et al., 2000). This increase in caspase transcription corresponds to the increase in active caspase protein levels and in the cleavage of substrates such as nuclear Lamins in dying salivary glands (Figs 4, 5). Mutations in the steroid-regulated *βFTZ-F1*, *E93* and *BR-C* genes, which prevent salivary gland cell death, exhibit little or no active Caspase-3/Drice expression, and have altered α-Tubulin, α-Spectrin and nuclear Lamin expression in salivary glands (Figs 6, 7). Although *E74A* mutants prevent salivary gland cell death, they have elevated Caspase-3/Drice levels and degraded nuclear Lamins (Fig. 7). Although these data are consistent with the partially degraded morphology of *E74A* mutant salivary glands (Lee and Baehrecke, 2001), it remains unclear what factor(s) *E74A* may regulate that are required for normal cell death. However, our data indicate that *βFTZ-F1*, *E93* and *BR-C* play a crucial role in determining caspase levels in dying salivary gland cells, and this is supported by the impact of these genes on the transcription of *dronc* (Lee et al., 2002a). Significantly, inhibition of caspases by expression of either p35 or dominant-negative Dronc is sufficient to prevent DNA fragmentation, changes in nuclear Lamins and α-Tubulin, and death of salivary glands (Jiang et al., 1997; Lee and Baehrecke, 2001) (Fig. 8).

The morphologies of dying cells indicate that apoptosis and autophagy are distinct. However, the difference between these cells becomes less apparent when one considers characteristics that were previously considered to be specific to apoptosis. Clearly, markers such as DNA fragmentation, expression and function of caspases, and cleavage of caspase substrates can exist in cells that possess the morphology of autophagic cell death. As expression of dominant-negative Dronc is sufficient to block caspase-dependent changes in salivary glands, our studies also indicate that the mechanism for caspase activation during autophagic cell death is similar to apoptosis during *Drosophila* development; the initiator caspase Dronc regulates the activation of the executioner caspase Drice and cleavage of

cell substrates (Yu et al., 2002). It is surprising how little is known about the activity of caspases in developing animals, as these proteases have been a subject of substantial investigation. Recent studies indicate that caspases do not only function during autophagic and apoptotic cell death, but that they are also used to degrade proteins during the differentiation of sperm in *Drosophila* (Arama et al., 2003). Studies of salivary glands indicate that the distinction between apoptosis and autophagic cell death may be more subtle than their morphology suggests, and raise the question of what makes these cells look so different. Restriction of caspase activity within compartments of the dying cell may provide one possible explanation, but it is also possible that other mechanisms of proteolysis occur during autophagic cell death. This possibility is supported by the large increase in transcription of non-caspase proteases just before cell death of salivary glands (Lee et al., 2003), and by the fact that, unlike apoptotic cells that require phagocyte lysosomes, salivary gland cells appear to degrade themselves through autophagy. Future studies should provide important insights into the similarities and differences in the mechanisms that regulate apoptosis and autophagic programmed cell death.

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