ABSTRACT

Title of thesis: IMPLICATION OF MATRIX METALLOPROTEINASES ONE AND TWO IN AUTOPHAGIC CELL DEATH IN THE *DROSOPHILA* SALIVARY GLAND

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Programmed cell death plays an important role in normal development. Defects in this process contribute to cancer. Matrix metalloproteinases (MMPs), zinc-dependant endopeptidases that cleave components of the extracellular matrix, are among the multiple protease types implicated in cell death.

Here I provide evidence that MMPs function in *Drosophila* salivary gland cell death. Misexpression of the MMP inhibitor *timp* inhibited timely salivary gland cell death, while misexpression of *mmp2* induced premature salivary gland cell death. *mmp* RNA interference was inconclusive because salivary gland persistence observed at 28°C was similar to *fkh-GAL4* negative controls. MMPs and caspases might have an additive effect, since misexpression of *timp* and the caspase inhibitor *p35* together enhanced salivary gland persistence compared to either *timp* or *p35* misexpression

alone. I also provide descriptive confocal microscopy of wild-type salivary glands using α-Spectrin and the polarity marker Crumbs which suggest that polarity is lost during salivary gland cell death.

IMPLICATION OF MATRIX METALLOPROTEINASES ONE AND TWO IN AUTOPHAGIC CELL DEATH IN THE *DROSOPHILA* SALIVARY GLAND

by

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“No, no, you're not thinking, you're just being logical.” (Niels Bohr)

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INTRODUCTION

Cell death is a normal component of development. It is required in the formation of structures such as fingers and toes[1], deleting structures such as the tadpole's tail[2], controlling cell numbers such as the excess cells generated in the developing nervous system[3], and eliminating abnormal cells such as lymphocytes with auto-immune potential[4].

Schweichel and Merker defined three types of physiological cell death in 1973: heterophagy (now known as apoptosis), autophagic cell death and non-lysosomal cell death[5]. Of these, apoptosis and autophagic death are the most common, and differ in their degradation mechanisms. While individual apoptotic cells are consumed by phagocytes prior to degradation by the phagocyte lysosome, groups of dying autophagic cells contain the lysosomal machinery necessary for much of their own degradation. Dying autophagic cells form double-membraned structures called autophagosomes around organelles and other cellular components. Autophagosomes then fuse with lysosomes where their contents are degraded by lysosomal hydrolases[6].

Proteases play an important role in programmed cell death. The core apoptosis machinery was first discovered in *Caenorhabitis elegans* and includes the cell death effector CED-3, a protease that is homologous to the mammalian caspase family[7]. Many other proteolytic molecules such as matrix metalloproteinases (MMPs), the ubiquitin proteasome system and lysosomal proteases have been implicated in cell death, and the role of MMPs in cell death is the goal of this study. In 1962, Gross and Lapiere showed that the regressing tadpole's tail produced diffusible endopeptidases

that could degrade collagen gels *in vitro*[8]. These enzymes are synthesized as inactive zymogens requiring subsequent activation[9]. Structurally similar enzymes were found in numerous species, and in 1995 they were grouped together to form the novel family of matrix metalloproteinases, or MMPs[10].

The MMP family is a sub-group of the metzincin superfamily of metallo-proteinases. The metzincins are characterized by a methionine below the catalytic site and a conserved zinc-binding motif at the catalytic site. The twelfth and last amino acid in the zinc-binding motif is family-specific: the serralysin family of bacterial enzymes has a proline, the astacins (ex: Bone Morphogenetic Protein 1) a glutamic acid, the ADAMs (a desintegrin and metalloproteinase) an aspartic acid, and the MMPs a serine[10].

The human genome encodes 23 MMPs and four Tissue Inhibitors of Metalloproteases (TIMPs)[11]. Three MMPs are known in *Caenorhabditis elegans*[12], two in *Drosophila melanogaster*[13-15], and one in *Arabidopsis thaliana*[16]. Although MMPs can cleave many extracellular matrix proteins, individual substrate specificities have been hard to identify due to overlapping MMP functions in the redundant mammalian systems that have been most studied.

MMPs are classified into eight categories based on variations in structure, and consist of an N-terminal pre-domain, a pro-domain, a catalytic domain and a C-terminal hemopexin domain (**Fig.1**). The pre-domain is cleaved after directing synthesis to the endoplasmic reticulum (ER) to target the protein for secretion, and the pro-domain maintains enzyme latency until its cleavage after secretion[17]. The zinc-binding catalytic domain controls cleavage specificity by its substrate-

recognition sites as well as the active site itself[18]. The hemopexin domain, joined by a hinge to the catalytic domain, can specify MMP localization (e.g. with either a transmembrane domain or a GPI anchor) and enhance substrate specificity. In addition, it has also been shown that the N-terminal domain of TIMPs bind directly to the catalytic site of MMPs, whereas the C-terminal domain interacts with the hemopexin domain regulating MMP inhibition. TIMPs are secreted and can reversibly inhibit MMPs[17].

Regulation of MMP activity occurs at numerous levels including: transcription, proteolytic activation, pericellular proteolysis, inhibition by TIMPs, and clearance. Most is known about transcription regulation. Diverse stimuli such as cell stress, changes in cell shape, phorbol esters, integrin-derived signals, and extracellular matrix proteins (including MMP-specific substrates) can regulate MMP gene expression[17]. MMP transcription depends on the organization of *mmp* promoters, notably the position of *cis*-regulatory elements such as transcription factor ETS-binding sites[19], p53-binding sites[20] and activator protein-1 (AP-1) sites, which bind *c-fos* and *c-jun* proto-oncogene products activated by cytokines and growth factors[17]. In addition, the expression level of specific *mmp* genes can be either reduced or increased by genetic variation, such as SNP variation: for example, the 2G allele of a human *mmp1* SNP is associated with ovarian cancer[21]. Proteolytic activation is another regulatory step, where the pro-domain is cleaved from MMPs. This is typically initiated either by active MMPs or other proteases after secretion. Some MMPs, however, including human MMP11 and transmembrane MMPs, contain a furin-activation domain allowing intracellular activation by this serine protease[22].

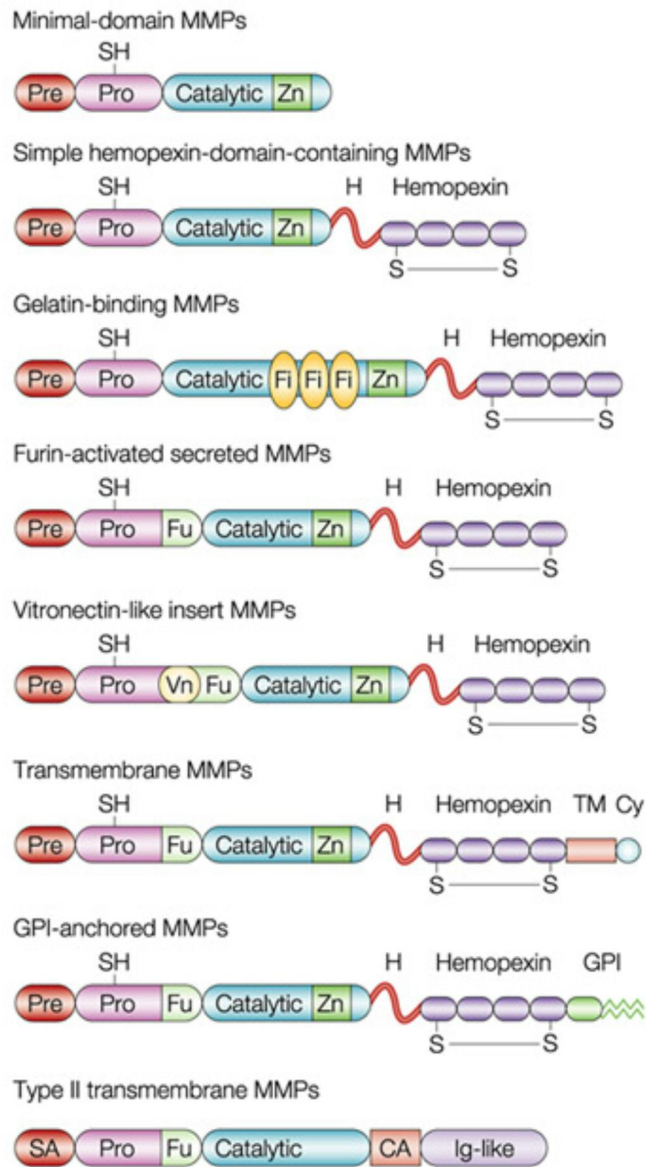

Fig.1: Eight structural categories of MMPs [11]

Few MMP substrates have been identified *in vivo*, although many have been tested *in vitro. In vitro,* MMP11 cleaves IGF-binding proteins, which indirectly increase IGF[23], and α1-proteinase inhibitor, which releases a cleavage product promoting tumor growth[24]. MMP11 also cleaves α2-macroglobulin, whereas MMP19 cleaves collagens I and IV, fibronectin, gelatin I, tenascin and casein[17]. Methods for identifying MMP targets are emerging and include yeast two-hybrid screens with the MMP-specific hemopexin domain as bait[25] and phage-display libraries[26]. *In vivo* studies generally involved mice mutant for specific *mmp* genes, although the redundant function of other *mmp* genes complicates analyses. One *in vivo* study showed that MMP9 releases a fragment from collagen IV that is a functional angiogenesis inhibitor named Tumstatin[27].

Drosophila MMP1 and MMP2 are more closely related to human MMPs than to each other, suggesting an ancient divergence[15]. Comparison of human and fly MMP protein sequences indicates MMP1 resembles human MMP19 and 28 (furin[1]-activated and secreted), while MMP2 resembles human MMP11 (also furin-activated and secreted). Both have predicted GPI anchor sequences[14, 15], suggesting membrane association. *mmp1* has two splice forms. Northern blots of total RNA have shown that *mmp1.f1* is only present in early pupae, whereas *mmp1.f2* (henceforth *mmp1*) and *mmp2* are present at multiple stages throughout *Drosophila* life. *timp* RNA is present at all the stages when MMPs are expressed, although TIMP tissue distribution has not been studied. MMP1 is mainly expressed in the migrating dorsal epithelium during dorsal closure and in the migrating primordia of the adult trachea. *mmp2* is expressed

[1] Furin is a proprotein convertase of the secretory pathway and is localized in the trans-golgi network.

in a variety of tissues, notably in wing imaginal discs and the morphogenetic furrow of the developing eye disc. TIMP has been shown to inhibit both MMPs *in vivo*[15].

Drosophila MMP mutants have been created by P element excision and ethylmethane sulfonate (EMS) mutagenesis[15]. Homozygous *mmp1* mutants have tracheal defects with broken dorsal trunks and few survive to puparium formation, while homozygous *mmp2* mutants survive until pupation and possess defects in larval midgut histolysis, suggesting MMP2 is required for destruction of this tissue[15]. *mmp1* and *mmp2* double mutants survive embryogenesis but die as larvae.

The *Drosophila* larval salivary gland is a good model to study programmed cell death because of its well-studied morphology and genetic regulatory pathway [28]. The salivary gland epithelium undergoes synchronized cell death that is triggered by the steroid 20-hydroxy-ecdysone (ecdysone) 10 to 12 hours after puparium formation (APF) and destruction is complete by 16 hours APF[29]. At 12 hours APF, salivary gland cells are cubic in shape and contain large vacuoles. The cells then become round and the gland appears “bumpy”. By 14 hours APF, these large vacuoles fragment and the nuclei separate from the cytoplasm. At 15 hours APF, the cytoplasm undergoes blebbing and 16 hours APF only cellular debris remains[30, 31].

Ecdysone triggers larval salivary gland programmed cell death by activation of a transcription regulatory hierarchy [28]. The ecdysone receptor complex, formed from the nuclear ecdysone receptor (EcR) and Ultraspiracle (USP) [32-34], binds ecdysone and induces transcription of the “early” regulatory genes, including *Broad-Comp*lex (*BR-C*), *E74A* and *E93*[35-37]. Absence of the competence factor βFTZ-F1 prevents transcription of these early genes[38, 39]. The BR-C, E74A and E93 transcription factors

regulate the "late" core cell death genes *reaper* (*rpr*), *head involution defective* (*hid*), *Drosophila Apaf1-related killer* (*dark*) and the caspases *dronc* and *drice*[40, 41]. Rpr and HID, like their Smac/Diablo mammalian counterpart, interact with the *Drosophila* inhibitor of apopotosis DIAP1 to lift caspase inhibition[42]. Dronc, one of the initiator caspases, is activated in the presence of Dark and cleaves effector caspases such as Drice[43]. Thus activated, these proteases are responsible for cleaving specific cell substrates leading to death[31].

Salivary gland cell death has both autophagic and apoptotic characteristics. Autophagic vacuoles are present in dying salivary glands 13h to 14h APF[30], and autophagy (Atg) gene transcription is induced by ecdysone[44]. In addition, the apoptotic caspase-induced characteristics of DNA fragmentation and cytoplasmic blebbing are seen during death[30]. Caspase activity is required, as overexpression of the baculovirus pan-caspase inhibitor p35 prevents DNA fragmentation, caspase-dependent changes in nuclear Lamin and Tubulin degradation, and cell death[29, 31]. Salivary glands disappear in four to six hours. This rapid organ destruction is probably a critical part of metamorphosis, in which the body undergoes massive transformation in four to five days. The utilization of several death processes is perhaps necessary to achieve such a rapid and efficient degradation.

In contrast to these cellular mechanisms of cell death, the morphology of the dying salivary gland resembles the death process of tissues called anoikis. This form of epithelium and endothelium-specific cell death is triggered by the separation of epithelial cells from their basal lamina and their subsequent loss of cell polarity[45]. Anoikis defects have been found to promote cancer[46, 47]. Cells destined for anoikis

which have lost contact with their basal lamina may survive if they have acquired one or more hallmarks of cancer[48], such as a limitless replicative potential or a cell death evasion potential. These cells are thus potentially metastatic since they are mobile and self-sufficient. The mechanisms of anoikis are not fully understood, although it seems cytoskeletal rearrangement is required. MMPs may function as the effectors separating cells from their basement membrane in the salivary gland model, since they are upregulated at the time of cell death in this tissue[44].

At the onset of cell death 12 hours APF, *timp* RNA is downregulated while *mmp1* and *mmp2* RNAs are upregulated[44], suggesting that MMPs might participate in salivary gland cell death. Are MMPs involved in salivary gland cell death? If so, how do they contribute to cell death? Here I investigate MMP loss of function and MMP gain of function animals to determine their role in salivary gland cell death.

MATERIALS AND METHODS

Fly strains

uas-timp, *uas-mmp1.f1*, *uas-mmp1.f2*, *uas-mmp2*, and 8 recessive *mmp* mutants maintained in combination with the balancer CyO were obtained from Dr A. Page-McCaw. The FRT strain carrying the double *mmp1Q112** and *mmp2W307** mutants was obtained from Dr. Y.N. Jan.

Whole animal histology

Drosophila of various genotypes were staged either 6, 12, 20 or 24 hours after puparium formation (APF) at either 18°C, 25°C or 28°C. The pupal cases were partially removed, and the animals were fixed in 5% acetic acid, 4% formaldehyde, 1% glutaraldehyde and 80% ethanol for 16 hours at 4°C, embedded in paraffin, sectioned, stained in Weigert's Hematoxylin and Pollack Trichrome, and analyzed using a Zeiss Axiophot II microscope (Zeiss, Oberkochen, Germany).

Salivary gland immunohistochemistry and confocal microscopy

Wild-type Canton S white prepupae were staged to 8h, 12h, 12.5h, 13h, or 13.5h APF at 25°C. Salivary glands were dissected in Phosphate Buffered Saline (PBS), fixed in 4% paraformaldehyde and heptane for 20 minutes at room temperature. The salivary glands were blocked in PBS with 1% Bovine Serum Albumin and 0.1% Triton-X (PBSBT) and incubated with primary antibodies for 16 hours at 4°C. Antibodies against α-Spectrin[49] and Crumbs[50] were obtained from the Developmental

Studies Hybridoma Bank, University of Iowa, Iowa, USA, and Dr. D. Branton, respectively. After washing in PBSBT four times during 30 minutes, salivary glands were incubated with fluorescent secondary antibodies for two hours at room temperature then washed again in PBSBT four times during 30 minutes, and stained with the nuclear dye TOTO-3. TOTO-3 and secondary antibodies were purchased from Molecular Probes, Carlsbad, California, USA. Finally, they were mounted in three-dimensional chambers on glass slides with VectaShield from Vector Laboratories, Burlingame, California, USA, and visualized with a Zeiss Axiovert 100M confocal microscope (Zeiss, Oberkochen, Germany).

Cloning of RNAi constructs

Two recombinant plasmids (snapback RNAi constructs) were constructed in which antiparallel and complementary sequences of either *mmp1* (CG4859, scaffold number AE003464) or *mmp2* (CG1794, scaffold number AE003832) were separated by an intervening non-complementary sequence [51]. These sequences were placed under the control of a UAS GAL4-binding site sequence in a P-element vector [52]. This design allows expression in the presence of GAL4, and enables the formation of a RNA hairpin and initiates RNA interference. DNA fragments were amplified from either wild-type Canton S genomic DNA, cDNA fragments, or reverse transcribed and amplified from wild-type Canton S RNA at 13.5h APF when *mmp1* expression peaks[44].

mmp1 primer sequences:

5’ CGGAATTCACATGACAAATAGACGAGCC 3’ forward (EcoRI)

5’ GGGGTACCTGCCGTTCTTGTAGGTGAAC 3’ reverse (KpnI)

5’ GCTCTAGACACATGACAAATAGACGAGCC 3’ reverse (XbaI)

5’ GGGGTACCTTGAAGACGGGTTCAAAGC 3’ forward (KpnI)

mmp2 primer sequences:

5’ GGAACAATCCAAGCAACAATC 3’ forward (EcoRI)

5’ ACATAATACTCGCGACCTGG 3’ reverse (XhoI)

5’ GGAACAATCCAAGCAACAATC 3’ reverse (KpnI)

5’ CGTATACAGCATCCACCTTG 3’ forward (XhoI)

All restriction enzymes were purchased from New England Biolabs, Beverly, Massachusetts, USA. Individual PCR fragments were first inserted into the TOPO® pCR2.1 vector (Invitrogen, Carlsbad, California, USA) and then individually subcloned into the pUAST vector. Plasmids were transformed into SURE® competent cells (Stratagene, La Jolla, California, USA). DNA was isolated using QIAfilter™ plasmid maxi kit (Qiagen, Hilden, Germany). Transgenic flies containing these P-elements were obtained following standard procedures [53, 54] by Best Gene Inc. (http://www.thebestgene.com/) for *mmp1* and Duke University Model Systems Genomics (http://www.biology.duke.edu/model-system/FlyShop/index.html) for *mmp2*. Six *mmp1* and two *mmp2* insertions were then mapped using the balancer strain *w-; CyO/ScO; TM2/TM6B*, and crossed to flies expressing GAL4 controlled by the salivary gland-specific promoter Forkhead. Animals for all eight insertions were were processed for salivary gland histology (see above).

RESULTS

Function of MMPs in salivary glands

To test the hypothesis that MMPs function in salivary gland cell death, I mis-expressed *timp* in salivary glands by crossing *forkhead promoter-GAL4* to *uas-timp* in order to maintain elevated TIMP levels. *timp*-expressing flies were aged to 12 and 24 hours APF at 25°C, embedded in paraffin, sectioned and examined by light microscopy. None of the negative controls, parental lines of either *uas-timp* (n=10) or *forkhead promoter-GAL4* (n=10) crossed to wild-type Canton S, had salivary glands 24 hours APF. By contrast, 80% of *timp*-expressing animals had persistent salivary glands 24 hours APF (n=61), eight hours after salivary glands normally die. Of these animals with salivary glands, 20% had intact salivary glands, and 80% were fragmented and partly degraded (**Fig.2**). These data indicate that MMP inhibition promotes salivary gland survival.

It has been suggested that TIMP might function in an MMP-independent manner in mammalian cells[55]. Therefore it is preferable to test if MMP loss-of-function prevents salivary gland cell death. I analyzed 26 different *mmp1* and *mmp2* loss-of-function mutant genotypes for salivary gland cell persistence 24h APF. These genotype combinations were created from 4 recessive *mmp1* and 4 recessive *mmp2* mutants obtained from Dr. A. Page-McCaw. *mmp1 P(K04809)* contains a P-element insertion, *mmp1*^{2} is a deletion of the catalytic sequence, and *mmp2Df(2R)Uba1-Mmp2* is a deletion allele of the 5' region of *mmp2* and the upstream gene *uba1*[15]. The other 5 alleles are point mutations described in **Fig.3**.

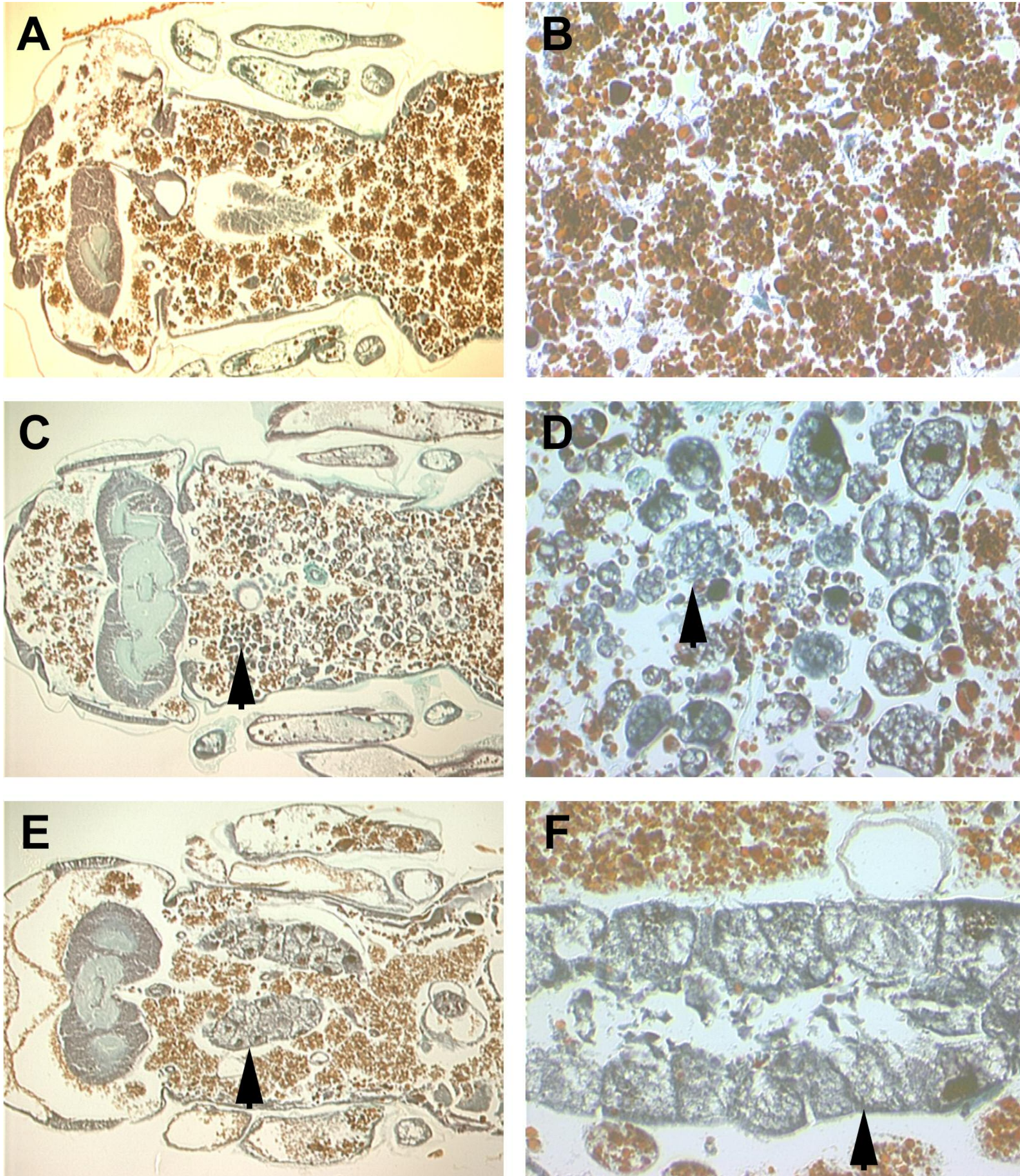

Fig.2: *Timp* misexpression causes salivary gland persistence 24h APF. Stained sections of paraffin-embedded pupae aged to 24 hours. *Uas-timp* crossed to C^s has no salivary glands 24h APF (A and B), whereas *uas-timp* crossed to *fkh-GAL4* possess salivary gland fragments (C and D), or intact salivary glands (E and F). Arrows either indicate salivary glands or salivary gland fragments. Panels B, D and F are magnifications (from the same or a similar animal) of panels A, C and E.

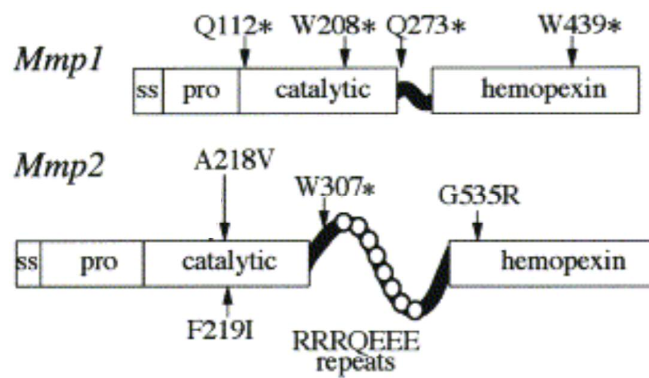

Fig.3: MMP point mutations[15]

Tables 1 and **2** describes *mmp* mutant genotypes aged to 24 hours APF at 25°C, embedded in paraffin, sectioned and examined by light microscopy. *Mmp* mutants were either unfit for analysis because of either developmental delays or lethality prior to pupation, or survived without any abnormal salivary gland phenotype. I could only analyze the weakest alleles, since the strongest alleles all die as larvae[15]. This might explain why salivary glands do not survive. *mmp1Q273** homozygous mutants were the least defective genotype possessing salivary glands 24 hours APF (**Fig.4**). Of the nine *mmp1Q273** pupae staged, four had persistent salivary glands, and of these four, two did not head evert, providing clear evidence of developmental arrest prior to the stage salivary glands die. Although previous work suggests that MMP1 might be specifically implicated in head eversion[15, 56], this phenotype could also indicate that *mmp1Q273** mutants are developmentally delayed or arrest development just prior to this event. MMP mutant salivary gland cell death phenotypes could therefore not be reliably analyzed using this approach.

Since loss-of-function analyses of MMP mutants is complicated by pleiotropy that causes developmental arrest, I attempted to silence *mmp1* and *mmp2* expression in salivary glands by tissue-specific RNA interference. None of the *mmp1* RNAi animals (n=30) or parental controls *forkhead promoter-GAL4* (n=10) and *uas-mmp1* (n=10) crossed to Canton S had persistent salivary glands at 24 hours APF at 25°C. By contrast, at 20 hours APF at 28°C, all *mmp1* RNAi animals had pieces of persistent salivary glands (n=10), as did 70% of parental control *forkhead promoter-GAL4* crossed to Canton S (n=10, **Fig.5**). Since animals develop more rapidly when raised at 28°C, 20h APF at 28°C is considered equivalent to 24h APF at 25°C.

Table 1: ***Mmp*** **mutant heterozygous and homozygous combinations 24h APF.**
[a] Example of a heterozygous genotype: all other *mmp1* and *mmp2* heterozygous mutants had similar phenotypes (data not shown). Heterozygous animals were analyzed by dissection for salivary gland persistence. [b] Subset of animals with salivary gland persistence and developmental defects 24h APF.

genotypes	parents crossed (pairs)	n	salivary gland persistence	developmental defects
Mmp2A218V [a] / *CyO*	30	6	0	0
*Mmp1Q112** / *Mmp1Q112**	30	3	1	1[b]
*Mmp1Q273** / *Mmp1Q273**	30	9	4	2[b]
$Mmp1^2$ / $Mmp1^2$	30	2	0	0
Mmp1P(K04809) / *Mmp1P(K04809)*	30	3	1	1[b]
Mmp2A218V / *Mmp2A218V*	30	5	0	0
Mmp2F219I / *Mmp2F219I*	30	2	0	0
*Mmp2W307** / *Mmp2W307**	30	2	0	0
Mmp2Df(2R)Uba1-Mmp2 / *Mmp2Df(2R)Uba1-Mmp2*	30	2	0	0

Table 2: ***Mmp* mutant heteroallelic and double trans-heteroallelic combinations 24h APF.** [a] Subset of animals with salivary gland persistence and developmental defects 24h APF. [b] Larval-stage and older progeny were not seen, indicating lethality.

genotypes	parents crossed (pairs)	n	salivary gland persistence	developmental defects
Mmp2Df(2R)Uba1-Mmp2 / Mmp2F219I	30	13	0	0
$Mmp1^2$ + / + Mmp2A218V	30	ND[b]	-	-
Mmp1Q112* + / + Mmp2A218V	30	3	0	0
Mmp1Q273* + / + Mmp2A218V	30	1	1	1[a]
$Mmp1^2$ + / + Mmp2F219I	30	ND[b]	-	-
Mmp1Q112* + / + Mmp2F219I	30	ND[b]	-	-
Mmp1Q273* + / + Mmp2F219I	30	3	0	0
Mmp1P(K04809) + / + Mmp2F219I	30	2	0	0
$Mmp1^2$ + / + Mmp2W307*	30	1	1	1[a]
Mmp1Q112* + / + Mmp2W307*	30	1	0	0
Mmp1Q273* + / + Mmp2W307*	30	3	2	2[a]
Mmp1P(K04809) + / + Mmp2W307*	30	1	0	0

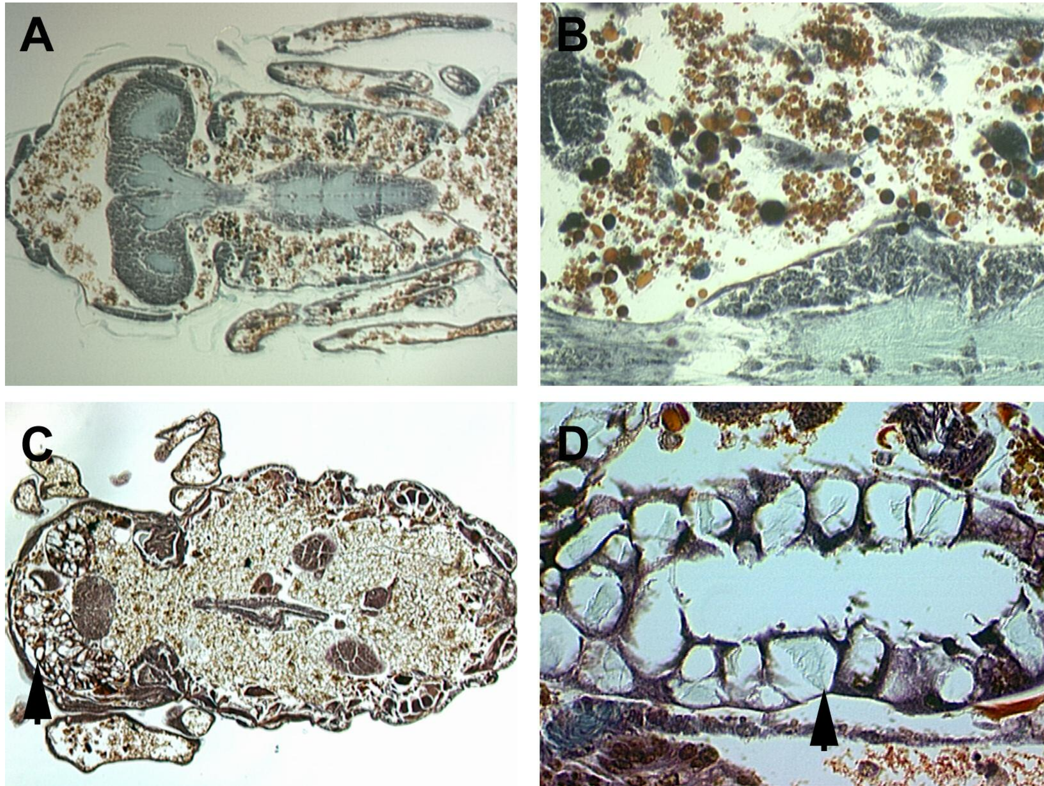

Fig.4: *Mmp1Q273 mutants cause salivary gland persistence 24h APF but are developmentally delayed.** Stained sections of paraffin-embedded pupae aged to 24 hours. (A and B) C^s controls. (C) Mutant pupae are not head everted, and still have larval muscle, signs of global delayed development. (D) Salivary glands are intact. Arrows either indicate salivary glands or salivary gland fragments. Panels B and D are magnifications (from the same or a similar animal) of panels A and C.

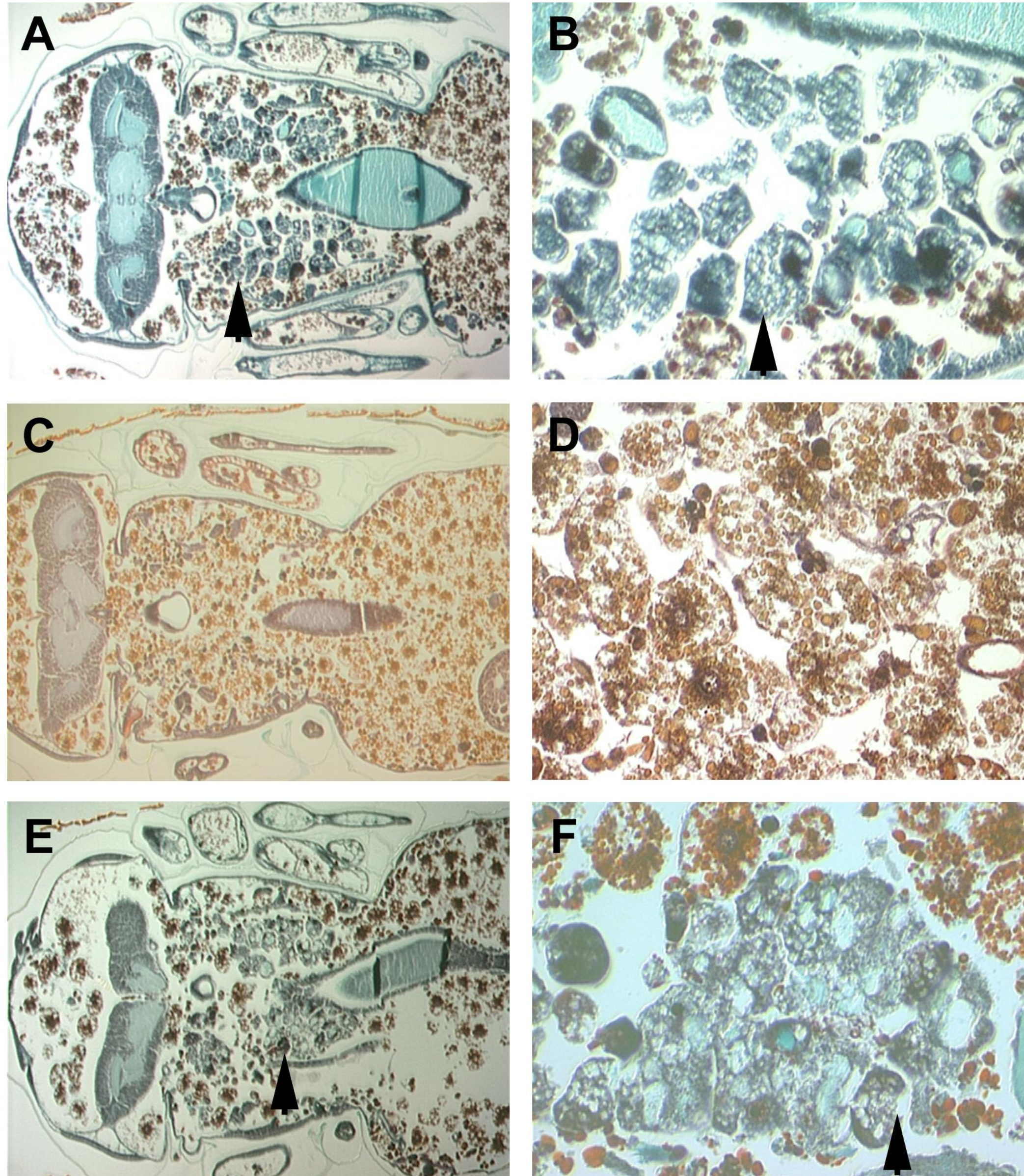

Fig.5: *Mmp1 RNAi* causes salivary gland persistence 20h APF at 28°C but so does *fkh-GAL4* crossed to C^s. Stained sections of paraffin-embedded pupae raised at 28°C and aged to 20 hours. *Fkh-GAL4* crossed to C^s has small salivary glands fragments (A and B) and *uas-mmp1RNAi* crossed to C^s has no gland persistence (C and D), whereas *uas-mmp1RNAi* crossed to *fkh-GAL4* show large gland fragments (E and F). Arrows indicate glands or gland fragments. Panels B, D and F are magnifications (from the same or a similar animal) of panels A, C and E.

Since controls also had defects in degradation, the analysis of MMP1 function in salivary glands based on this RNA interference experiment is inconclusive.

Similar to RNAi studies of *mmp1*, none of the *mmp2* RNAi negative controls, including parental lines *uas-mmp2* RNAi (n=10) and *forkhead promoter-GAL4* (n=10) both crossed to Canton S, had salivary glands 24 hours APF at 25°C. By contrast, 12.7% of *mmp2* RNAi animals had persistent salivary gland fragments 24 hours APF (n=55, **Fig.6**). In the hope of strengthening the phenotype, I raised and staged flies at 28°C, since Gal4 is suspected to be more active at this higher temperature. This temperature increased salivary gland persistence to 70% of *mmp2* RNAi - expressing animals 24 hours APF (n=10). Elevated temperature also led to 70% persistence of fragments in negative controls of *forkhead promoter-GAL4* crossed to Canton S (n=10). These fragments are in the last stages of disintegration, while *mmp2* RNAi glands are more intact (**Fig.7**).

In order to complement loss-of-function analyses of MMPs, I misexpressed *mmp1* and *mmp2* in salivary glands to determine if they were sufficient to induce death. None of the *uas-mmp2* negative controls, including parental lines *uas-mmp2* (n=10) and *forkhead promoter-GAL4* (n=10) both crossed to Canton S showed abnormal salivary gland morphology or premature cell death 6 hours APF at 25°C. 75% of *mmp2*–expressing animals had abnormal gland morphology that appear to prematurely degrade (n=29, **Fig.8**). These data indicate that ectopic expression of *mmp2* is sufficient to induce premature cell death of salivary glands.

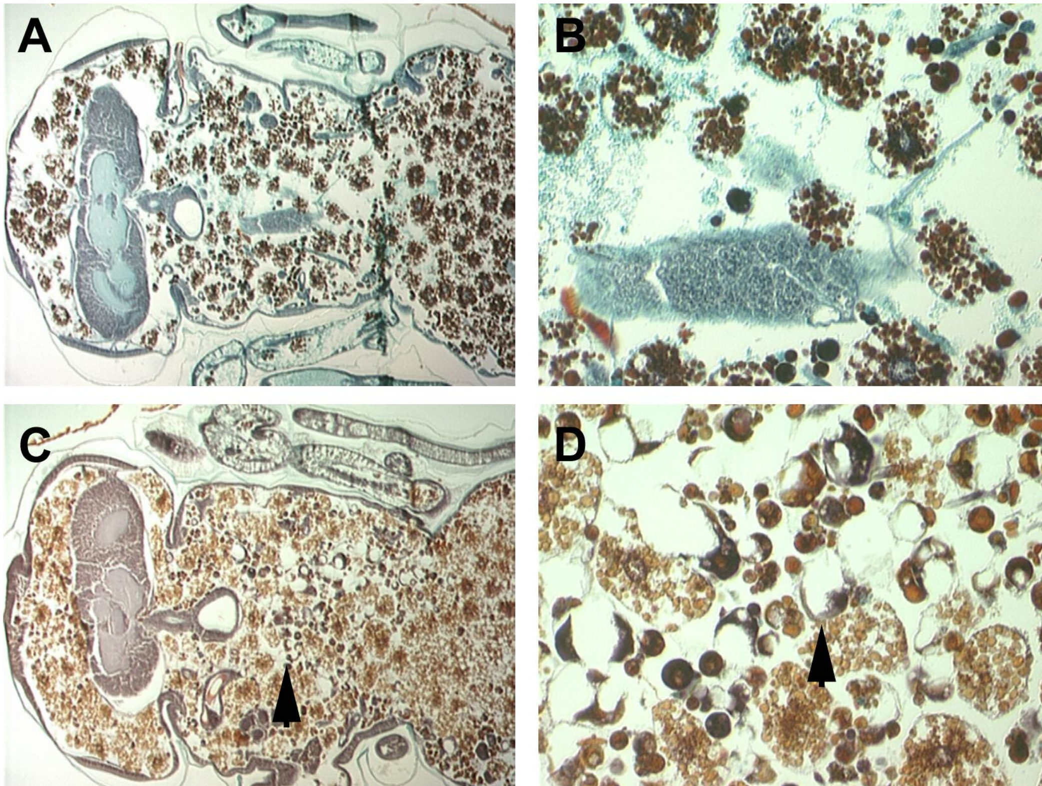

Fig.6: ***Mmp2 RNAi*** **causes weak salivary gland persistence 24h APF at 25°C.** Stained sections of paraffin-embedded pupae raised at 25°C and aged to 24 hours. *Uas-mmp2RNAi* crossed to C^s has no gland persistence (A and B), whereas *uas-mmp2RNAi* crossed to *fkh-GAL4* show small gland fragments (C and D). Arrows indicate glands or gland fragments. Panels B and D are magnifications (from the same or a similar animal) of panels A and C.

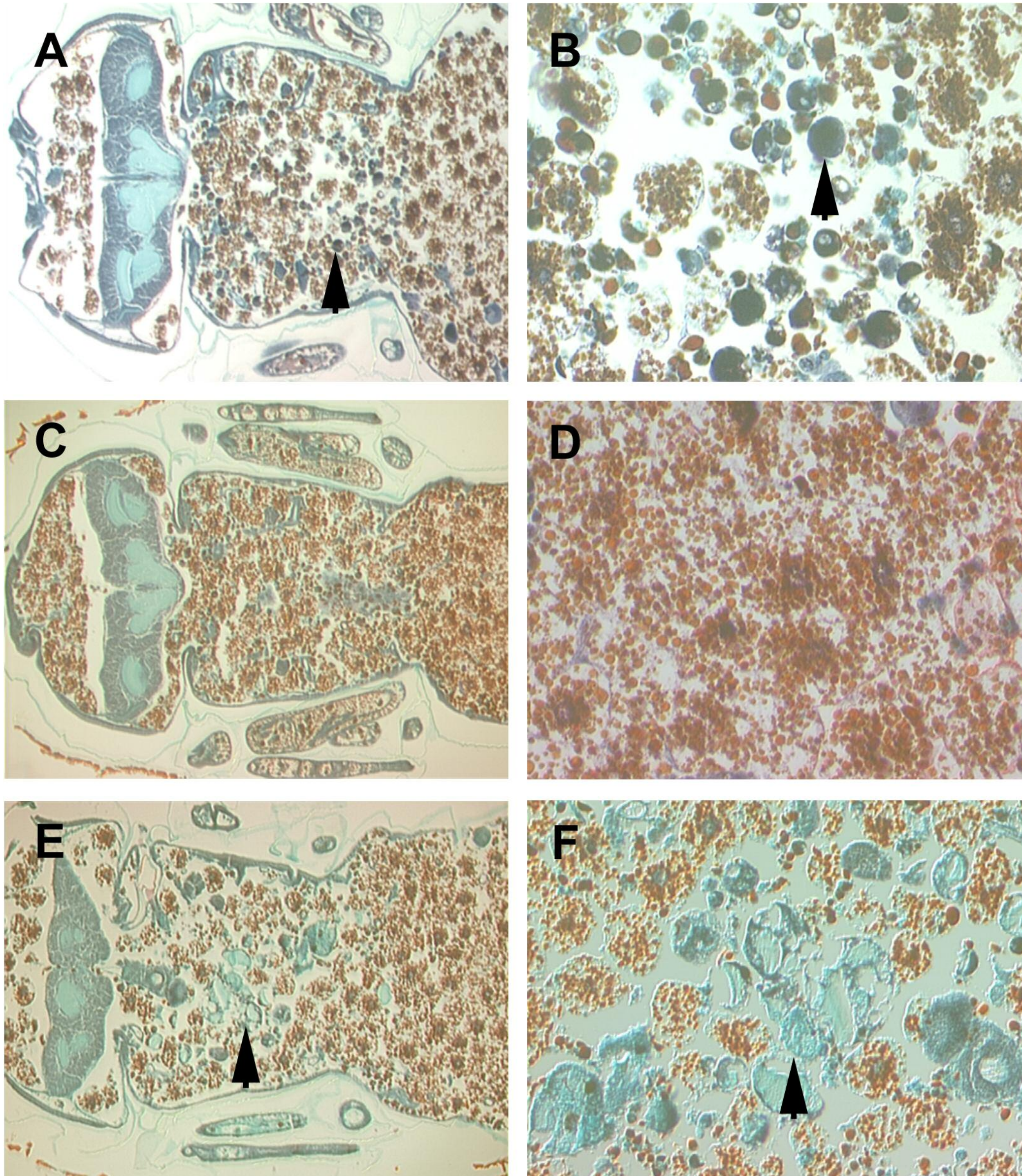

Fig.7: *Mmp2 RNAi* causes salivary gland persistence 24h APF at 28°C. Stained sections of paraffin-embedded pupae raised at 28°C and aged to 24 hours. *Fkh-GAL4* crossed to C^s has small salivary glands fragments (A and B) and *uas-mmp2RNAi* crossed to C^s has no gland persistence (C and D), whereas *uas-mmp2RNAi* crossed to *fkh-GAL4* show large gland fragments (E and F). Arrows indicate glands or gland fragments. Panels B, D and F are magnifications (from the same or a similar animal) of panels A, C and E.

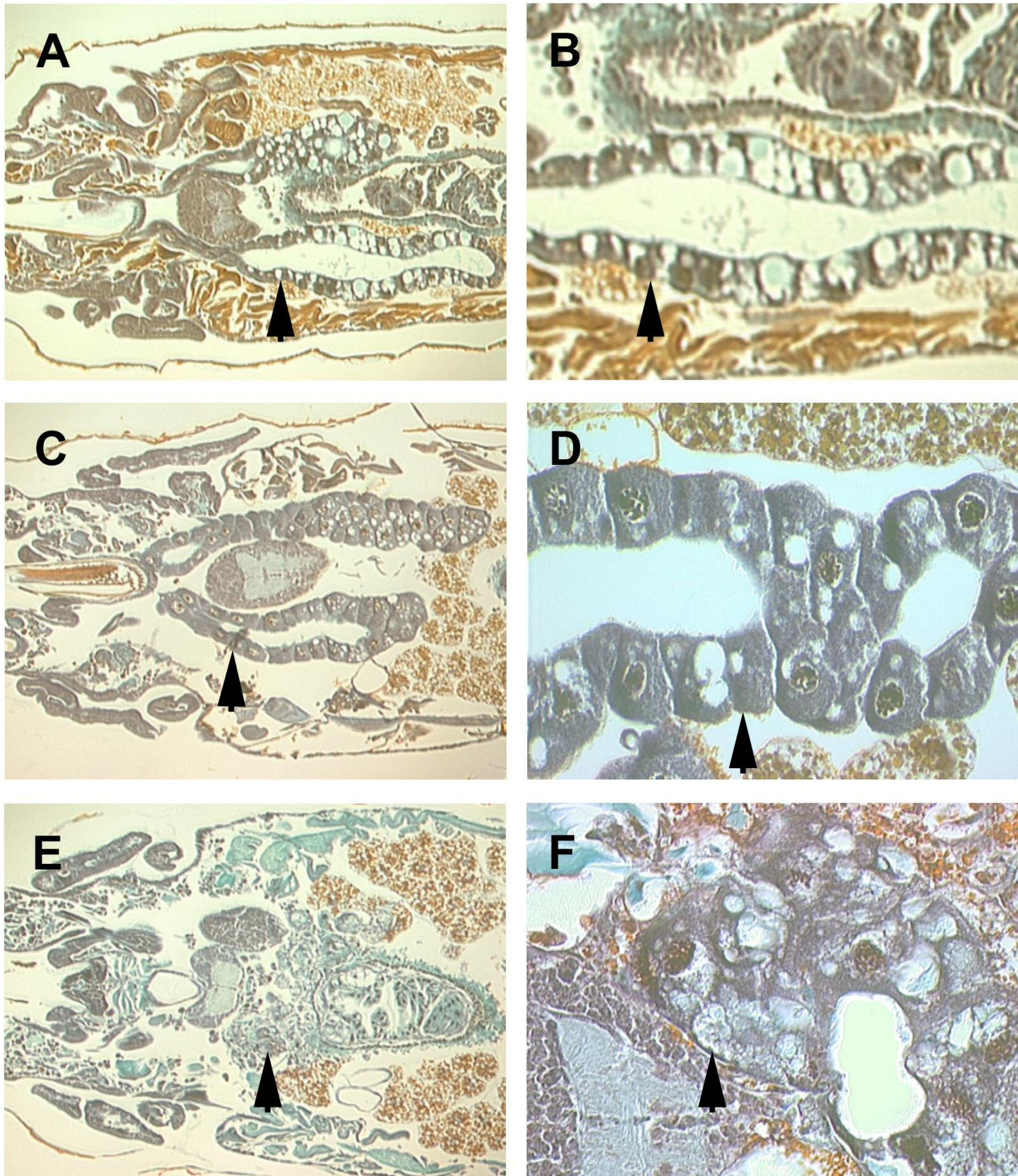

Fig.8: *Mmp2* misexpression causes early salivary gland degradation 6h APF. Stained sections of paraffin-embedded aged to 6 hours. *Fkh-GAL4* crossed to C^s has intact salivary glands (A and B) and so does *uas-mmp2* crossed to C^s (C and D), whereas *uas-mmp2* crossed to *fkh-GAL4* shows disorganized glands (E and F). Arrows indicate glands or gland fragments. Panels B, D and F are magnifications (from the same or a similar animal) of panels A, C and E.

Misexpression of isoform 1 or 2 of MMP1 both induced a prolonged third larval instar stage followed by death as larvae at 25°C and 18°C (data not shown). These data indicate that elevated levels of MMP1 are toxic to animals, and may suggest that salivary glands are required for proper development.

Interaction between caspases and MMPs

Caspases are required in salivary gland cell death: the apoptosis genes *rpr, hid, dark, dronc* and *drice* are induced just before autophagic cell death[40, 41, 44, 57] and expression of the pan-caspase inhibitor p35 and a dominant-negative form of *dronc* blocks salivary gland cell death and DNA fragmentation[29, 31]. In addition, loss-of-function mutations in *dark* and *dronc* appear to delay salivary gland cell death[58, 59, 60].

I created a line expressing both *p35* and *timp*. At 24h APF at 25°C, salivary glands in these animals (78% salivary gland persistence, n=9) are morphologically more intact than in animals expressing either *p35* alone (71% salivary gland persistence, n=7) or *timp* alone (80% salivary gland persistence, n=61). These qualitative differences in salivary gland morphology suggest caspases and MMPs function in an additive manner that leads to cell death (**Fig.9**).

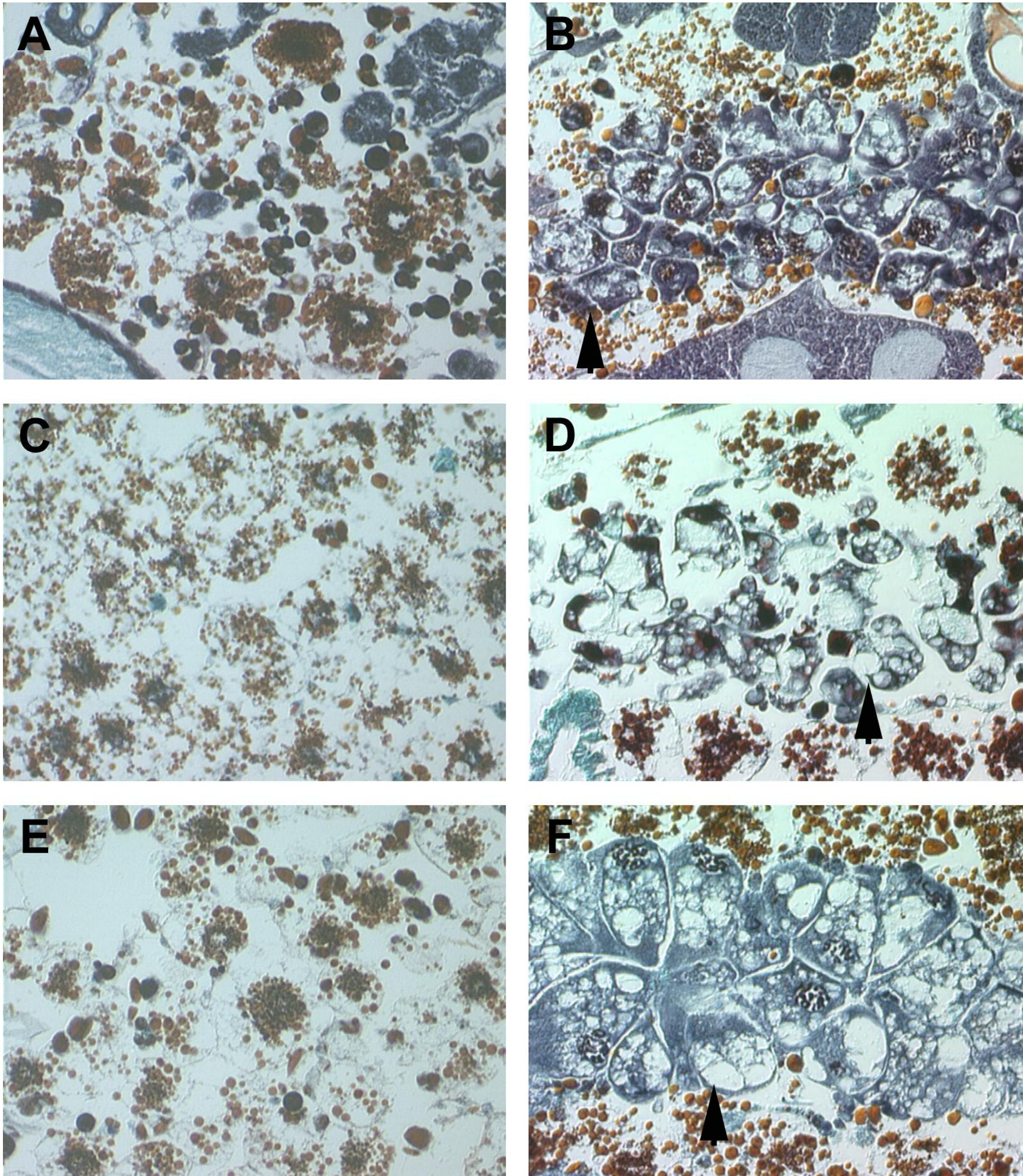

Fig.9: Combined *p35* and *timp* misexpression causes salivary glands to appear more intact than *p35* or *timp* misexpression alone 24h APF at 25°C. Stained sections of paraffin-embedded pupae aged to 24 hours. *Uas-p35* (A), *uas-timp* (C), and *uas-p35; uas-timp* (E) have no salivary glands 24h APF. *Uas-p35* crossed to *fkh-GAL4* (B) and *uas-timp* crossed to *fkh-GAL4* (D) possess salivary gland fragments whereas *uas-p35; uas-timp* crossed to *fkh-GAL4* (F) shows intact salivary glands. Arrows either indicate salivary glands or salivary gland fragments.

Markers of wild-type salivary gland cell death

No markers exist to detect changes in cellular MMP activity in the context of developing cells. As a first step towards determining if cell detachment and loss of cell polarity can be used as markers of MMP activity during cell death, I monitored loss of cell polarity during salivary gland cell death in wild-type Canton S using confocal microscopy. I performed antibody stains against the apical transmembrane polarity marker Crumbs (red), and the cortical plasma membrane-associated protein Spectrin (green). Nuclei were stained with TOTO3 (blue). At 8 and 12 hours APF (**Fig.10 A and B**), cells are organized into a cube-shaped, polarized epithelium, where Crumbs is localized at the apical surface near the salivary gland lumen. As cell death progresses to 13.5h APF (**Fig.10 C and D)**, cells elongate, staining is less efficient, and Crumbs diffuses into the cytoplasm and the lumen. Discs Large, a basolateral polarity marker, also diffuses 13h APF (data not shown). These data suggest that polarity is lost during salivary gland cell death.

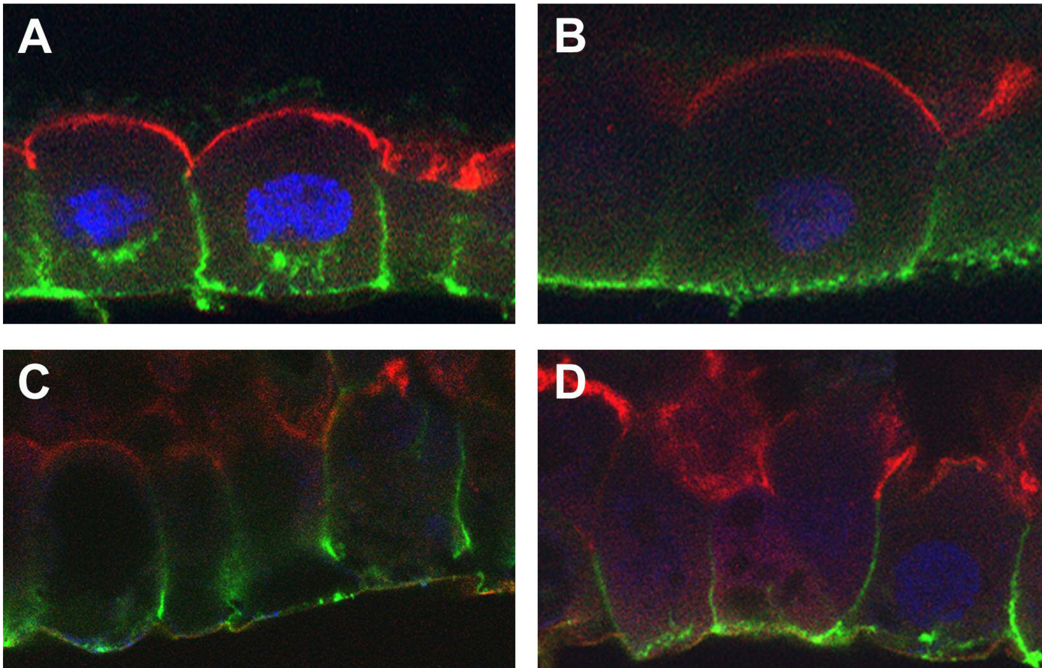

Fig.10: Changes in cell shape and polarity during salivary gland cell death in *C*^{s}. Confocal microscopy of wild-type salivary glands stained with anti-Spectrin (green), anti-Crumbs (red) and TOTO3 (blue). Timepoints are 8h APF (A), and a timeline spanning 12h to 13.5h APF (B, C and D).

DISCUSSION

MMP1 and MMP2 function in cell death

At the onset of cell death 12 hours APF, *timp* RNA is downregulated while *mmp1* and *mmp2* RNAs are upregulated[44]. These data suggest that MMPs might function in salivary gland cell death. To test this hypothesis, I misexpressed *timp* in the salivary gland by crossing *forkhead promoter-GAL4* to *uas-timp.* Misexpressing *timp* causes salivary gland persistence in 80% of animals 24h APF at 25°C. These data indicate that downregulation of *timp* is required for salivary gland cell death, but do not directly show that MMPs are required for salivary gland cell death. By contrast, misexpression of *mmp2* in salivary glands causes premature degradation in 75% of salivary glands 6h APF at 25°C in animals that are not killed prior to this stage. This indicates that expression of *mmp2* alone is sufficient to induce salivary gland cell death.

Heterozygous *mmp* mutants have been shown to survive without defects, while the majority of homozygous *mmp1* and *mmp2* mutants die during larval and pupal development, respectively[15]. Our study confirms that *mmp* mutants die during these stages, but does not find differences in the timing of death between *mmp1* and *mmp2* mutant animals. It is also intriguing that double trans-heterozygous mutants (with one allele mutant for *mmp1* and one mutant for *mmp2*) have a lethality similar to or greater than that of homozygous *mmp1* and *mmp2* mutants 24h APF. This suggests that MMP1 and MMP2 may have at least partly redundant functions. Previous work has shown, however, that *mmp2* is required for larval gut histolysis, but not *mmp1*.

This argues that MMP1 and MMP2 functions are not interchangeable[15]. More animals need to be observed to further analyze these findings.

To complement gain-of-function approaches, salivary-gland-specific RNA interference of *mmp1* and *mmp2* was attempted because *mmp* mutants had pleiotropic defects and died before they could be evaluated for altered salivary gland cell death. Several pieces of evidence suggest that MMPs function in salivary gland cell death: A) *mmp1* RNAi animals have a slightly higher percentage of salivary gland persistence compared to their *fkh-GAL4* controls crossed to C^s at 28°C, and *mmp2* RNAi animals have more intact salivary glands than their *fkh-GAL4* controls crossed to C^s; B) *uas-mmp* RNAi controls crossed to C^s never have salivary gland persistence; and C) mis-expression of *mmp2* causes premature salivary gland cell death, while *fkh-GAL4* controls crossed to C^s have intact salivary glands 6h APF. However both *mmp1* RNAi and *mmp2* RNAi animals and their *fkh-GAL4* controls crossed to C^s show relatively similar percentages of salivary gland persistence at 28°C. RNA interference experiments are therefore inconclusive.

Overall, these results suggest that MMP2 functions in salivary gland cell death. MMPs are known to play a critical role in numerous physiological cell death processes involving extra-cellular matrix remodeling, such as bone formation in mice[61] and larval intestinal epithelium apoptosis in *Xenopus laevis*[62]. In addition, MMPs have been implicated in human endothelial cell anoikis by cleaving integrins bound to the endothelial basement membrane[63]. Recently *Drosophila* MMP1 has been shown to coimmunoprecipitate with and perhaps cleave the cell-adhesion molecule Ninjurin in S2 cell culture[64]. MMPs might play a similar role in the salivary

gland by remodeling the extra-cellular matrix leading to cell death. Major limitations to existing published work are the functional redundancy of MMPs in mammals, the lack of definite genetic experimentation in other models, and thus the scarcity of reliable information on *in vivo* MMP activity in physiological processes. Studying MMPs in the *Drosophila* salivary gland circumvents these limitations and could prove that MMPs are necessary and sufficient for programmed cell death in this tissue.

Additional experiments are required to clearly determine if MMPs function in salivary gland cell death. First, it is possible that the RNA interference carried out in these experiments was only partially efficient. Decreased *mmp* RNA levels in Northern blot hybridization analyses of *mmp* RNAi animals would indicate that RNA interference is effective, by comparison with *uas-mmp* RNAi controls crossed to C^s. Second, I designed flies combining *mmp1* and *mmp2* RNAi which could be used to determine if salivary gland persistence increases 24h APF at 25°C compared to *fkh-GAL4* controls crossed to C^s. Third, it would be ideal to investigate flies that are mosaic for an *mmp1Q112** and an *mmp2W307** mutation using the Mosaic Analysis with a Repressible Cell Marker (MARCM) method[65]. The creation of these mosaic animals would circumvent the pleiotropic defects present in homozygous mutants. In salivary glands, *mmp1Q112** and *mmp2W307** double mutant cells expressing GFP could be analyzed starting from 12h APF at 25°C, and compared to their wild-type neighbors undergoing cell death. Changes in cell shape and polarity could be evaluated by performing antibody stains against Spectrin and Crumbs using confocal

microscopy, and caspase activity could be analyzed by TUNEL assay and active caspase-3 antibody stains.

Interaction between caspases and MMPs

Caspases are necessary for salivary gland cell death[29, 31]. If MMPs also function in salivary gland cell death, the requirement for two protease systems and their relationship is critical to resolve. The morphologically additive effect of combining *p35* and *timp* expression suggests that MMPs and caspases may both function in salivary gland cell death, and that they may act in parallel pathways. Little is known about the potential interactions between MMPs and caspases. However it appears from other models that different protease types may indeed function independently. In the human breast cancer cell line MCF-10A, for example, caspase-dependent apoptosis as well as caspase-independent autophagic cell death can be separately triggered to induce acini lumen formation[66]. In the salivary gland, several lines of evidence indicate that such pathways are kept distinct spatially and temporally in order to fulfill different functions. Caspase-dependent changes include DNA fragmentation as well as Tubulin and nuclear Lamin cleavage[31], whereas MMPs are expected to localize at the cell-matrix boundary and induce changes in basement membrane contact and indirectly on cell polarity[64]. In addition, *caspases* are upregulated at 10h APF just before the onset of cell death, while *mmps* are upregulated at 12h APF[44]. The activation of independent proteolytic pathways in the salivary gland is perhaps necessary to achieve rapid and efficient tissue degradation.

To formally explore the potential relationship between MMPs and caspases, it is

possible to test whether they function in a common or in parallel pathways. One useful experiment would involve misexpressing MMPs in the salivary glands of either *dronc* or *dark* loss-of-function mutants. To complement this, flies misexpressing *dronc* in the salivary glands of *mmp1Q273** mutants would test the reciprocal genetic relationship. Misexpression of either *mmp2* or *dronc* separately cause premature cell death[62] (**Fig.8**). If either *mmp* or *caspase* mutants suppress this ectopic cell death phenotype, we will then conclude that MMPs and caspases function in a common pathway. If *mmp* or *caspase* mutants fail to alter *mmp2* and *dronc* misexpression phenotypes, we will then conclude that MMPs and caspases work in parallel pathways to induce cell death.

Conclusion

I have described how cells change from a cubic shape to a round shape as they die in the *Drosophila* larval salivary gland, and how cell polarity is disrupted. Cytoskeletal changes also occur, partly through caspase activity[31]. I therefore propose that an anoikis-like cell death mechanism occurs in the salivary gland, and that MMPs, upregulated at the time of cell death[44], are the effectors separating cells from their basal lamina.

Future work is needed to understand the role of MMPs in salivary gland cell death, the substrates cleaved by MMPs, and the relationship between MMPs and caspases. This study suggests that MMPs function in the death of salivary glands during development.

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