senseless is necessary for the survival of embryonic salivary glands in Drosophila

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Summary

Apoptosis in developing Drosophila embryos is rare and confined to specific groups of cells. We explain how one organ, salivary glands, of Drosophila embryos avoids apoptosis. senseless (sens), a Zn-finger transcription factor, is expressed in the salivary primordium and later in the differentiated salivary glands. The regulation of sens expression in the salivary placodes is more complex than observed in the embryonic PNS. We have shown that sens expression is initiated in the salivary placodes by fork head (fkh), a winged helix transcription factor. The expression of sens is maintained in the salivary glands by fkh and by daughterless (da), a bHLH family member. In this study, we have identified sage, a salivary-specific bHLH protein as a new heterodimeric partner for da protein in the salivary glands. In addition, our data suggest that senseless RNAs embryos have a phenotype similar to sens and that senseless is necessary to maintain expression of sens in the embryonic salivary glands. Furthermore, we show that in the salivary glands, sens acts as an anti-apoptotic protein by repressing reaper and possibly hid.

Key words: Embryonic salivary glands, senseless, Lyra, Apoptosis, reaper, hid, Drosophila

Introduction

Programmed cell death or apoptosis is an integral part of development of all higher organisms and is used to remove obsolete cells in the organism. As the cells undergo programmed cell death, they show stereotypical changes that include cellular condensation, DNA fragmentation and formation of apoptotic bodies (Kerr et al., 1972). In the Drosophila embryo, apoptosis is observed in the head and at the segmental boundaries to define the segmental grooves in the embryo (Abrams et al., 1993). Three closely linked Drosophila genes, reaper, hid (W – FlyBase) and grim, have been identified as activators of apoptosis. The overexpression of these genes causes ectopic cell death, while removal of the region encoding these three genes prevents cell death in the embryo (Chen et al., 1996; Grether et al., 1995; White et al., 1994; White et al., 1996). In addition, other players involved in the cell death pathway in vertebrates such as the caspases and inhibitors of apoptosis (IAPs) (Vaux and Korsemeyer, 1999), have been found in Drosophila (reviewed by Abrams, 1999; Bangs et al., 2000).

We are beginning to understand the transcriptional regulation of the three upstream cell death genes, reaper, hid and grim. Steroid hormone signaling activates reaper and hid expression during histolysis of larval salivary glands and midgut (Jiang et al., 1997). Drosophila p53 has been shown to activate reaper in response to irradiation but not in response to developmental cell death in the embryo (Brodsky et al., 2000). The Hox gene Deformed can directly activate reaper expression at the segmental boundaries in the maxillary segment in the embryo (Lohmann et al., 2002). The only known negative regulator is the Ras-MAPK pathway. It represses hid and allows the survival of cells in the Drosophila eye (Kurada and White, 1998).

Though apoptosis is an essential part of development, there are many tissues that do not show any cell death during embryogenesis. The lack of cell death in these tissues can be ascribed to lack of activators of the apoptotic pathway or the presence of repressors of apoptosis in these tissues. One of the tissues that does not show any programmed cell death in the embryo is the embryonic salivary gland (Myat and Andrew, 2000). The salivary glands are derived from 80-100 cells of the ventral ectoderm in parasegment 2 of the embryo. Previous studies have shown that the expression of the homeotic gene Sex combs reduced (Scr) in parasegment 2 is necessary for specification of the salivary primordium (Panzer et al., 1992). Scr is expressed in the entire ectoderm of parasegment 2, including the cells that will form the salivary placodes. Embryos mutant for Scr lack salivary glands and overexpression of Scr in other parasegments of the embryo can lead to ectopic salivary gland formation (Panzer et al., 1992). Ventrally, the two salivary placodes are separated by two rows of cells that give rise to the salivary ducts. The ventral extent of the salivary placodes is specified by EGFR signaling that occurs in the cells closest to the ventral midline of the embryo (Kuo et al., 1996). As germband retraction proceeds, the cells of the salivary placodes invaginate to form the salivary glands (reviewed by Bradley et al., 2001).

One of the Scr-induced transcription factors that is crucial for salivary gland formation is fork head (fkh). fkh, which encodes a winged helix transcription factor, is expressed in the
salivary placodes beginning at embryonic stage 10 and continues to be expressed in the salivary glands throughout embryonic and larval development (Weigel et al., 1989b). fkh is necessary for many aspects of salivary morphogenesis, including the distinction between salivary gland and duct primordia, invagination of the placodes and survival of salivary placode cells (Kuo et al., 1996; Weigel et al., 1989a). In fkh mutant embryos, salivary placodes do not invaginate and undergo apoptosis as the germband retracts (Myat and Andrew, 2000; Weigel et al., 1989a).

We examine the role of senseless (sens; Ly – FlyBase) in salivary gland development. Like fkh, sens, which encodes a Zn-finger transcription factor, is expressed in the salivary glands. The Zn-finger motifs in SENS show homology to the Zn finger domains of mammalian GFI-1 protein and to the PAG-3 protein of C. elegans. SENS binds to the GFI-1 consensus sequence and potentially acts as a transcriptional repressor (Nolo et al., 2000).

Previous work has illustrated the role for sens in neuronal development. It is expressed in the sensory organ precursors in the embryonic peripheral nervous system, as well as the wing and eye antennal imaginal discs. sens has been shown to be necessary and sufficient for neuronal fate specification. Embryos mutant for sens show loss of ES and Ch neurons in the peripheral nervous system (Nolo et al., 2000). In the wing imaginal discs, loss of sens also results in loss of neuronal fate. In addition, sens is important for specification of R8 cell fate in the eye ommatidia by preventing rough from being expressed in the R8 precursors (Frankfort et al., 2001). Moreover, ectopic expression of sens in the ectodermal imaginal cells can make these cells take on a neuronal fate (Nolo et al., 2000; Nolo et al., 2001). Thus, sens appears to be primarily expressed in cells fated to adopt a neuronal fate and is necessary for them to maintain their neuronal identity.

However, embryonic salivary glands are an exception. Although the cells in the salivary glands are not neuronal, they do express sens throughout embryonic development (Nolo et al., 2000) (this paper). Despite the expression of sens, the cells of the salivary placodes maintain their ectodermal character and do not adopt a neuronal fate. This led us to ask two questions: what are the genes that activate sens expression in the salivary glands, and what role does sens play in the morphogenesis of the embryonic salivary glands, a non-neuronal tissue?

Our data demonstrate that both the regulation and downstream effectors of sens show significant differences between the PNS and the salivary glands. Although DA:bHLH heterodimers stimulate sens transcription in both tissues, this complex is not needed to start the expression of sens during salivary development. Instead, fkh expression in the salivary placodes initiates sens expression. Then SAGE, a bHLH protein, acts as a novel DA partner to maintain sens expression. Furthermore, we find that sens functions as an anti-apoptotic protein in the salivary glands by preventing the expression of reaper and hid. By blocking these proapoptotic genes, sens allows survival of the salivary gland cells.

Materials and methods

**Drosophila stocks**

The following mutants and transgenic stocks were used in this study: sens^al^, UAS-sens C5, UAS-sens C8 and UAS-sens C12 (Nolo et al., 2000); pR-11-lacZ (Brodsky et al., 2000); and Df(3L)XR38 (Peterson et al., 2002). The hkb^Al2^ allele was generated in our laboratory. All other stocks were from the Bloomington Stock Center. w^{118} flies were used as wild-type controls for all the experiments.

**Immunocytochemistry**

Embryos were collected on molasses/agar plates and dechorionated using 50% bleach. These embryos were then fixed in a 1:1:2 mixture of PBS, 10% formaldehyde (Polysciences) and heptane (Sigma) for 30 minutes at room temperature. Embryos were devitellinized using methanol (Sigma) and stored in methanol at 4°C prior to immunostaining. Embryos were incubated overnight at 4°C with one or a combination of the following antibodies: rat anti-CREB (1:5000) (Andrew et al., 1997), rabbit anti-FKH (1:3000), rabbit antiβ-galactosidase (1:1000, Vector Laboratories) and guinea pig anti-SENSELESS (1:1000) (Nolo et al., 2000). The secondary antibodies used to detect these primary antibodies include biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories), biotinylated goat anti-rat IgG (1:200, Vector Laboratories) and biotinylated goat anti-guinea pig IgG (1:200, Vector Laboratories). These conjugates were then detected using the Vectastain ABC kit (Vector Laboratories), followed by incubation with 0.5 mg/ml diaminobenzidine and 0.06% hydrogen peroxide. The embryos were then cleared with methyl salicylate and photographed using the Nomarski optics on the Leica DMRB microscope.

For fluorescent staining, embryos were incubated with secondary antibodies conjugated to either Alexa 488 or Alexa 546 (1:500, Molecular Probes) after the primary antibody incubation. The embryos were then cleared in 50% glycerol, followed by 70% glycerol in PBS containing 2% n-propyl gallate (Sigma) and visualized using the Zeiss 510 confocal microscope.

**In situ hybridization**

Whole-mount in situ hybridization was performed as described by Tautz and Pfeifle (Tautz and Pfeifle, 1989) with modifications (Harland, 1991) using antisense digoxigenin-labeled probes. The signal was visualized using nitro blue tetrazolium and BCIP as substrates for alkaline phosphatase. Following the in situ hybridization, the embryos were immunostained for β-galactosidase as described above in the immunocytochemistry protocol. The embryos were rinsed and cleared in 50% glycerol followed by 70% glycerol and photographed using Nomarski optics on the Leica DMRB microscope.

**TUNEL staining**

After fixation, embryos were immunostained with rabbit anti-FKH and rabbit anti-β-Galactosidase, followed by a fluorescent detection using goat anti-rabbit IgG-Alexa 488 (Molecular Probes, 1:500). The immunostained embryos were then processed for TUNEL staining as described below. Embryos were treated with 4 μg/ml of proteinase K for 5 minutes at room temperature and then postfixed with 1:1 mixture of 10% formaldehyde and PBS. TUNEL staining was performed using the Intergen Apoptag Kit. Briefly, the embryos were incubated with equilibration buffer for 1 hour, followed by an overnight incubation with terminal deoxynucleotidyl transferase (TdT) at 37°C. To detect TUNEL staining, the embryos were incubated with rhodamine-conjugated anti-digoxigenin antibody at 4°C overnight. The embryos were then cleared with 50% glycerol, followed by 2% n-propyl gallate in 70% glycerol and imaged using the Zeiss Confocal microscope.

**RNA interference**

The primers used to PCR amplify sage from genomic DNA were: 5’-ATGACGGAATCAACTGCTGAGCTCCA-3’, 5’-CGCTCCCCAA-TATCGTGGCC-3’. The genomic sage fragment (845 bp) was then cloned into pBluescript and used to make dsRNA for RNA
interference using the protocol described by Kennerdell and Carthew (Kennerdell and Carthew, 1998). The dsRNA as well as injection buffer was injected into pre cellularization w1118 embryos. The embryos were aged overnight at 18°C. They were then fixed with 1:1:2 mixture of PBS, 10% formaldehyde and heptane for 30 minutes and manually devitellenized. The devitellenized embryos were then immunostained as described above.

**GST pulldown**
The *sage* cDNA was obtained using an embryonic cDNA library as a template for PCR. The primers were as shown above but with different restriction sites (*Sal*I and *Nco*I) flanking the primers to facilitate cloning into pGEX. The PCR fragment was about 800 bp and was ligated into the pGEM Teasy vector (Promega) and then inserted into a *Sal*I-*Nco*I cut pGEX-2TKN vector (modified version of pGEX vector from Amersham Biosciences) and transformed in BL-21 cells. SAGE protein is expected to be around 30 kDa and GST-SAGE was found to be around 60 kDa. We also made a truncated version of SAGE that lacks the C-terminal bHLH domain (SAGE Δ) that was around 50 kDa. The GST-conjugated proteins were bound to GST-agarose beads overnight. The beads were rinsed and stored at 4°C.

The Promega WGA-in vitro transcription and translation kit was used to make DA protein from pBS-da (Crommiller and Cummings, 1993). The in vitro translated DA protein, labeled using 35S-methionine, migrated in an SDS gel at about 80 kDa, which agrees with the previously documented molecular weight of DA (Crommiller and Cummings, 1993).

For the GST-pulldown assay, DA was incubated with beads coupled with GST-alone, GST-SAGE or GST-SAGE Δ overnight at 4°C. The beads were washed and eluted with 20 mM glutathione. The eluates were electrophoresed on a denaturing gel and visualized by autoradiography.

**Results**

*sens* is expressed in the embryonic salivary glands

*sens* is necessary for the specification of sensory organ precursors in the PNS of the embryo (Nolo et al., 2000). The only other embryonic expression of *sens* is in the developing salivary glands (Nolo et al., 2000) (Fig. 1). In situ hybridization showed that *sens* mRNA is first expressed in the dorsal cells of the salivary placodes at stage 11 of embryogenesis. As the embryo undergoes germ band retraction, *sens* mRNA expression expands to include all the cells of the salivary placodes, but is excluded from the salivary duct precursors (Fig. 1A). We observed a similar expression pattern of SENS protein, though the protein is not expressed at high levels in the ventral part of the salivary placodes (Fig. 1C). Though *sens* mRNA and protein disappear from the embryonic PNS by stage 13, both continue to be expressed in the embryonic and larval salivary glands (Fig. 1B,D and data not shown).

*sens* mutant embryos have small salivary glands

As *sens* is essential in other tissues, we examined its role in salivary morphogenesis. Embryos mutant for *sens*Δ2 had small salivary glands, about half to a third the size of normal salivary glands (Fig. 1E,F). In addition, the salivary glands of stage 16 *sens*Δ2 embryos were smaller than those in stage 13 embryos, suggesting that the loss of cells may be progressive. Similar phenotypes were obtained for two other alleles, *sens*Δ58 and *sens*Δ235 as well as for transheterozygotes of *sens*Δ2 and a deficiency for *sens*, suggesting that *sens*Δ2 behaves as an amorph in our studies. The phenotype seen in the *sens*Δ2 mutant salivary glands can be rescued by overexpressing *sens* in the embryo (data not shown), indicating that the observed phenotype is due to the lack of *sens* function in the salivary primordium.

*sens* expression in the salivary glands is dependent on bHLH proteins

Because the salivary glands are the only non neural tissue in the embryo to express *sens*, we were curious to see how different the regulation of *sens* transcription is in this tissue. In the PNS, DA forms heterodimeric complexes with proneural bHLH proteins. These complexes are necessary for both the initiation and maintenance of *sens* expression in the sensory organ precursors (Nolo et al., 2000). The proneural genes *achaete*, *scute*, *lethal of scute*, *asense* and *atonal* are mainly expressed in the proneural clusters and are absent from the salivary placodes (Brand et al., 1993; Cabrera et al., 1987; Jarman et al., 1993; Romani et al., 1987; Vaessen et al., 1994). By contrast, *da* expression is ubiquitous in the early embryo and is upregulated in the salivary glands of older embryos (Crommiller and Cummings, 1993) (V.C. and S.K.B., unpublished), suggesting that *da* might be involved in regulating the expression of *sens* in the salivary placodes. If so, *da* mutants would have a salivary phenotype similar to *sens* mutants. In confirmation of this hypothesis, salivary glands in *da* mutants were smaller than in wild-type embryos (Fig. 2A,B). In situ hybridization showed that the levels of *sens* mRNA (and protein, data not shown) were dramatically reduced in the salivary glands of *da* mutants.
sage, a salivary-specific bHLH gene, is necessary for regulating sens

Although known DA partners are not expressed during salivary development, a genome-wide survey for genes encoding bHLH proteins (Moore et al., 2000) identified sage, a gene whose expression is salivary gland-specific in the embryo (Moore et al., 2000). The expression of sage in the salivary placodes is first observed at stage 10, the stage at which the first Scr targets begin their salivary expression (Fig. 3A). sage continues to be expressed in the salivary glands throughout embryogenesis (Fig. 3B) and into larval development (Li and White, 2003). Scr-mutant embryos lack salivary glands and do not express sage (data not shown). Double stranded RNA interference was used to test whether sage is required for salivary gland development. Forty percent of the embryos injected with sage dsRNA, showed small salivary glands, compared with 10% for the injection buffer control (Fig. 3C,D). We also found that SENS levels were reduced by sage dsRNA injection (Fig. 3G,H). These observations indicate that sage is required for regulation of sens in the salivary glands. SENS expression does initiate in the absence of sage, as it does in da mutant embryos.

It has been suggested that class II bHLH proteins, the class that includes SAGE, can heterodimerize with DA (Ledent and Vervoort, 2001). To test whether SAGE indeed forms a complex with DA, we used a GST pulldown assay with 35S-DA protein and GST-SAGE. DA protein bound to GST-SAGE but not GST alone (Fig. 3I). In addition, DA did not bind to a truncated SAGE that lacked the C-terminal bHLH domain (data not shown). These observations show that DA can partner with SAGE in vitro and suggest that SAGE and DA form a complex in vivo to regulate the expression of sens in the salivary glands.

In the sensory organ precursors of the PNS, sens is necessary to maintain the expression of the proneural genes (Nolo et al., 2000). Similarly, we find that sage RNA is decreased in sens mutants (Fig. 3B,F), suggesting a positive feedback loop...
between sens and sage. However, expression of da appears to be unaffected in sens mutants (data not shown).

**fkh is necessary for initiation of sens expression in the salivary glands**

Although da and sage are necessary for maintaining sens expression, initiation of sens in the salivary placodes did not depend on either of these genes. As sens expression in the salivary placodes initiates at stage 11, later than primary Scr target genes, we thought sens might be indirectly activated by Scr through one of these primary targets. As expected, we found that sens expression is absent in Scr mutant embryos. sens expression was unchanged in embryos mutant for several Scr-regulated early transcription factors such as huckebein (Fig. 4C), tracheless and eyegone (data not shown). However, fkh mutant embryos show a complete absence of sens expression in the salivary placodes and never express sens at the later stages. The expression of sens in the PNS is unaffected in these mutants (Fig. 4B). da and sage RNAs were unchanged at stages 10 and 11 in fkh mutants, indicating that the lack of sens is not due to the effects on da or da expression. There was a slight reduction in sage RNA at stage 12 (data not shown), which may be due to the positive feedback loop between sens and sage in the salivary placodes. Thus, sens expression in the salivary placodes is initiated by fkh and is maintained at high levels throughout embryogenesis by da and sage.

**Small salivary glands in sens mutants are not due to improper cell fate specification**

The smaller salivary glands in sens mutants have fewer cells than normal salivary glands (Fig. 1E,F). One possible explanation for the phenotype is that fewer cells are specified to be the salivary placodes in sens mutants and these smaller placodes would lead to smaller salivary glands. In addition, if the remaining cells of the salivary primordium were converted to duct precursors, then the salivary ducts might be larger in diameter or longer than normal. However, in sens embryos, the salivary placodes appear to be normally specified and are similar to wild-type placodes in size and cell numbers (Fig. 5A,B). In addition, salivary ducts in sens mutants were no different from wild-type salivary ducts (Fig. 5C,D). Thus, the salivary glands of sens mutants appear to be reduced in size after the normal number of cells has been specified.

**Salivary glands in sens mutants undergo apoptosis**

The normal cell fate specification and the progressive loss of cells in sens mutant salivary glands led us to test for cell death as the glands develop. TUNEL staining was used to detect apoptotic cells in wild-type and sens mutant embryos. In contrast to wild-type salivary glands, there were a number of TUNEL-positive cells in the salivary glands of sens mutant embryos (Fig. 6A,B). Most of the apoptotic cell death in the sens mutant salivary glands happened during stages late 12 through 14. Few apoptotic cells were seen in salivary glands of older embryos (data not shown). Consistent with the normal size of the placodes in sens mutants, apoptosis did not begin until after the cells have invaginated.

As reaper, hid and grim are the upstream genes known to be activated in response to apoptotic signals (Chen et al., 1996; Grether et al., 1995; White et al., 1994), we examined their expression in the developing salivary glands of wild-type and sens mutant embryos. In situ hybridization showed that in wild-type embryos, hid and grim are not expressed at any stage in the developing salivary glands. reaper, however, shows a weak expression near the dorsal posterior edge of the salivary placode but no expression later in the salivary gland. In sens mutant embryos, expression of reaper was markedly upregulated (Fig. 6D,E), and hid was expressed in the invaginating salivary glands (Fig. 6H,I). reaper and hid expression was observed in stage 12-13 salivary glands but disappears from older salivary glands. Expression of grim was unaffected (data not shown). Thus, induction of proapoptotic genes reaper and hid precedes the apoptotic cell death in the salivary gland cells of sens mutant embryos.

**sens is necessary for the survival of cells in the embryonic salivary glands**

The apoptosis observed in sens mutants may be a result of other problems with the salivary glands or may be due to the anti-apoptotic role of sens in the salivary glands. These two
expression in these rescued glands, it appears that the cells on the medial side of the salivary glands did not express reaper and were probably the cells that survive to form the small salivary glands in sens mutant embryos.

Another method used to rescue cell death and to test the genetic interaction between sens and pro apoptotic genes is to make double mutants between sens and Df(3L)H99, a large deficiency that removes reaper, hid, and grim (White et al., 1994). Ninety-three percent of the embryos mutant for both sens and Df(3L)H99 show normal size salivary glands compared with 0% of embryos mutant for sens alone (Fig. 7B,C, Fig. 8). There were some abnormalities in late stage, double-mutant embryos that included curving and kinking of the salivary glands, but these morphogenetic defects were also observed in embryos mutant for Df(3L)H99 alone. Thus, there is a genetic interaction between sens and the cell death genes within the Df(3L)H99 – reaper, hid and grim. As grim is unaffected in sens-mutant salivary glands, sens must interact with reaper or hid or both. To determine which, we made sens hid and sens reaper double mutants. The sens reaper double mutants were examined as homozygotes as well as in trans with sens Df(3L)H99 double mutants. Both reaper and hid mutations were able to partially suppress sens-mediated cell death in the salivary glands (Fig. 7E,F). However, the suppression of sens phenotype by hid was both qualitatively and quantitatively weaker than that by rpr (Fig. 8).

In addition, although Df(3L)H99 rescues the salivary glands of sens mutants, it does not rescue the PNS neurons as monitored by 22C10 staining (data not shown). Thus, the interaction between sens and genes involved in the cell death pathway is unique to the salivary glands.

**sens represses the transcription of reaper in the salivary glands**

Previous studies have demonstrated that the 11 kb region upstream from the reaper transcription start site is responsive to apoptotic signals (Brodsky et al., 2000). We used flies carrying 11 kb of reaper promoter fused to β-galactosidase (Rpr-11-lacZ) to test whether sens represses the reporter activity. In wild type embryos, Rpr-11-lacZ drives very low expression in the dorsal posterior part of the salivary placode and some expression in the invaginated portion of the salivary gland at stage 12 (Fig. 9A′,A″). There is very low expression observed in later embryos (Fig. 9C′,C″). In sens mutant embryos, the expression of the Rpr-11-LacZ fragment in the salivary placodes is dramatically increased and expression remains elevated throughout embryogenesis (Fig. 9B′,B″,D′,D″). These results indicate that sens directly or indirectly represses the expression of reaper in the normal salivary glands.

**Expression of sens does not rescue the salivary gland phenotype of fkh mutant embryos**

Like sens mutant embryos, the salivary placodes of fkh mutants express reaper and hid and undergo extensive apoptosis (Myat and Andrew, 2000). As sens expression is activated by fkh and sens represses reaper and hid, we tested whether the cell death observed in fkh mutants was due to lack of sens in the salivary glands. However, ubiquitous expression of sens was not sufficient to rescue cell death in fkh mutant embryos (Fig.
7G,H). Thus, in addition to sens, fkh appears to control other regulators of apoptosis in the embryonic salivary glands.

**Discussion**

**fkh induces initial sens expression in the developing salivary glands**

sens is predominantly expressed in the proneural clusters in the PNS and imaginal discs where it is induced by proneural bHLH genes (Nolo et al., 2000; Frankfort et al., 2001). Therefore, the expression of sens in a non-neuronal tissue such as the salivary glands was interesting. Our results show that regulation of sens in the salivary glands is more complicated than in the proneural tissues.

sens expression in the salivary glands can be divided into two parts: initiation and maintenance. We find that sens is initiated in the salivary placodes in response to fkh, one of the initial set of salivary genes that are directly activated by Scr at the beginning of stage 10 (4.3 hours AEL) (Panzer et al., 1992; Weigel et al., 1989b). sens expression begins about an hour later and may be directly regulated by fkh. There are FKH binding sites present at the 3’ end of sens and a fragment carrying these sites is sufficient to recapitulate the expression in the salivary glands (V.C. and S.K.B., unpublished) (Nolo et al., 2000).

As sens is a fkh target and because both sens and fkh embryos show extensive salivary apoptosis, we thought that apoptosis in fkh mutants might be caused by lack of sens. Because rescuing cell death in fkh mutants does not rescue normal morphogenesis (Myat and Andrew, 2000), our model was that sens normally protects salivary cells from cell death, and other fkh target genes direct the cell

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**Fig. 7.** The phenotype of sens is suppressed by cell death genes in Df(3L)H99. (B,C) The small salivary glands in sensE2 embryos (B) are rescued in embryos that are also mutant for Df(3L)H99 (C). Note that the salivary glands of Df(3L)H99 embryos are similar to wild type (compare A with D). (E,F) The small salivary glands in sensE2 embryos are partially rescued in embryos mutant for sensE2 and either reaper (Df(3L)XR38; E) or hid (W5014; F). (G,H) The salivary glands in fkhX76 mutants are not rescued by overexpression of UAS-sens driven by arm-GAL4. (A-F) Salivary glands visualized using an antibody to FKH. (G,H) An antibody against β-galactosidase is used to visualize the enhancer trap N33 that is expressed in the salivary glands.

**Fig. 8.** Graphical representation of the rescue of sens phenotype by cell death genes. The graph shows the percentage of embryos with particular salivary gland lengths in the different genotypes and the table below shows the numbers represented in the graph. The lengths of the salivary glands are assessed by comparing them with the length of the ectodermal thoracic segments. The numbers in parenthesis are the number of embryos for each condition. Asterisk indicates the percentage of embryos with salivary glands that did not invaginate completely. Double asterisks indicate that all the embryos in this category had salivary glands that were two segments long.
using RNAi that absence of sage family members to heterodimerize with DA. We have shown our results are the first to demonstrate the ability of Mesp of the Mesp family (Moore et al., 2000; Peyrefitte et al., 2000). Although FKH can initiate expression of sens in the salivary placodes, it partners with the proteins of the ACHAETE-SCUTE Complex or with ATONAL to regulate sens expression (Nolo et al., 2000).)

** movements and shape changes needed to form the salivary gland. However, the apoptosis of the salivary placodes in fkh mutants could not be rescued by ubiquitous expression of sens. There are two explanations for this result. The first possibility is that we did not overexpress sens at high enough levels to overcome cell death. However, we do not believe that to be the case because we used the same arm-GAL4:UAS-sens combination to rescue the sens phenotype. Furthermore, arm-Gal4:UAS-P35 rescues cell death in sens mutants. Thus, we favor the second possibility, that loss of fkh leads to multiple proapoptotic changes, only one of which is the failure to activate sens.

** da and sage maintain sens expression in the salivary glands**

Although FKH can initiate expression of sens in the salivary placodes, we show that both DA and SAGE are required for high level sens expression at later stages. DA is also known to control the expression of sens in the PNS. There, it partners with the proteins of the ACHAETE-SCUTE Complex or with ATONAL to regulate sens expression (Nolo et al., 2000). For sens regulation in the salivary primordium, we have identified a new DA partner, SAGE, which belongs to the bHLH proteins of the Mesp family (Moore et al., 2000; Peyrefitte et al., 2000). Our results are the first to demonstrate the ability of Mesp family members to heterodimerize with DA. We have shown using RNAi that absence of sage leads to a decrease in the size of the glands and a reduction in levels of SENS. In turn, SENS appears to positively regulate the levels of sage mRNA in the salivary glands. The existence of this positive feedback loop leads to the question of which protein, SAGE or SENS, is the true antagonist of apoptosis in the salivary glands. The presence of sage mRNA in sens mutants sheds some light on this issue. In sens mutants, high levels of Rpr-11-lacZ are induced at stage 12, in the salivary placodes. At this stage, sens mutant embryos still express sage and da mRNA in the placodes at normal levels (Fig. 3E). Reduction in sage mRNA is not observed until stages 13-14, by which time the salivary glands of sens mutants are already reduced in size (Fig. 3F). These results indicate that sens, not sage, is necessary to maintain the survival of the salivary gland cells.

** Fig. 10. The epistatic relationships in the salivary glands and in the peripheral nervous system. In the salivary glands, Scr activates fkh and sage. fkh is necessary for the initiation (I) of sens expression. sage, together with da, is necessary for the maintenance (M) of sens expression. In turn, sens is necessary to maintain the expression of sage in the salivary glands via a positive feedback loop. sens represses reaper and hid and prevents cell death. By contrast, in the peripheral nervous system, achaete-scute complex genes (AS-C) and da are needed together to initiate and maintain sens expression (Nolo et al., 2000). By a feedback loop similar to that in the salivary glands, sens amplifies the expression of the AS-C genes. Downstream from sens, it represses E(spl) transcription and is necessary for neuronal cell fate determination.**

** sens acts as an anti-apoptotic factor in the salivary glands**

Our data indicate that sens acts as a survival factor in the
embryonic salivary glands. This anti-apoptotic effect of sens is tissue specific. Although sens is expressed in the embryonic PNS and the imaginal discs, sens is not necessary for preventing apoptosis in these tissues. Instead, in the embryonic PNS and imaginal discs, sens is necessary for cell fate specification (Nolo et al., 2000; Frankfort et al., 2001). Absence of sens in the embryonic PNS does cause massive apoptosis of the sensory organ precursors. However, blocking cell death in the PNS of sens mutants does not rescue the normal number of neurons expressing neuronal markers such as 22C10, indicating that in the PNS, cell death is a secondary effect of aberrant cell fate. In addition, in the wing and eye imaginal discs, sens is needed for cell fate specification but does not appear to be involved in the apoptotic pathway. Thus, not only are there differences in sens regulation between neuronal and non neuronal tissues, but the role of sens and genes that are regulated by sens also differ between the PNS and the salivary glands. Accordingly, genes regulated by sens in the PNS do not appear to be regulated in the salivary glands. In the PNS and imaginal discs, sens regulates the Notch-Delta signaling pathway by altering the expression of Delta and Enhancer of split genes (Nolo et al., 2000; Nolo et al., 2001). We observed that the expression of Delta, E(spl) or other Notch pathway components are not altered in sens mutant salivary glands (V.C. and S.K.B., unpublished).

The anti-apoptotic role of sens, though tissue specific, appears to be conserved through evolution. Previous studies have shown that Gfi1, the vertebrate ortholog of sens can prevent apoptosis by repressing Bax (Grimes et al., 1996b). Furthermore, Gfi1 knockout mice show increased apoptosis in the inner ear neurons (Wallis et al., 2003). The C. elegans homolog, PAG-3 mutant animals shows increased apoptosis but it is not clear if the apoptosis is a consequence of improper cell fate specification (Cameron et al., 2002).

Gfi1 has been shown to be a transcriptional repressor (Zweidler-Mckay et al., 1996). SENS lacks the SNAG repressor domain that is present in Gfi1 (Grimes et al., 1996a; Nolo et al., 2000). Therefore, sens could be either a repressor or an activator in Drosophila. Previous studies have shown that sens represses the expression of rough in the eye imaginal discs and of E(spl) in the PNS (Frankfort et al., 2001; Nolo et al., 2000; Nolo et al., 2001). We have shown that sens can repress Rpr-11-lacZ in normal salivary glands, perhaps acting directly as a repressor or indirectly by inducing the expression of another repressor. By contrast, sens is necessary for maintaining the expression of proneural genes in the PNS and sag in the salivary primordium. There are potential SENS-binding sites upstream or downstream of hid, sge and the proneural genes, as well as in the 11 kb promoter of reaper, suggesting that sens could be directly regulating all these genes. Further studies will be needed to understand whether sens acts as both a transcriptional repressor or activator and whether it requires specific co-factors for these distinct functions.

Why is there a need for sens in the embryonic salivary glands?
The need for sens in the developing salivary gland specifically to prevent cell death raises the question of why these cells need special protection. We suggest two possibilities, one related to the initial specification of the salivary placodes and the other related to cell cycle and cytoskeletal rearrangements required for proper morphogenesis.

It is possible that the salivary gland cells are at risk of death because of similarities between these cells and cells in other segments that are fated to die. The involvement of homeotic genes might provide a common theme between the salivary placode cells and other apoptotic cells. Deformed induces apoptosis at the boundary of the mandibular and maxillary lobes by activating reaper (Lohmann et al., 2002). Similarly Abdominal B (Abd-B) can activate reaper at the boundaries of abdominal segments A6/A7 and A7/A8 (Lohmann et al., 2002). It is therefore possible that Scr, which is needed to specify the salivary primordium, can bind and activate reaper in the labial segment. In support of this hypothesis, low levels of reaper are expressed in the dorsal posterior part of the salivary placode. Removal of the sens repression would then reveal the presence of a strong activator of reaper transcription. Interestingly, though Deformed and Abd-B are expressed throughout their respective segments, apoptosis is limited to the boundaries, indicating the presence of activators at the boundaries or repressors in the rest of the segment. In parasegment 2, sens might be an analogous repressor, antagonizing Scr induction of reaper and hid in the salivary primordium.

Alternatively, this predisposition to apoptosis might be due to intrinsic aspects of salivary gland morphogenesis that occur after the cells are specified. The salivary placode is unique in that its cells exit the mitotic cell cycle early, at cycle 15 rather than at cycle 16 as the rest of the epidermis does. Shortly thereafter, as the cells are invaginating into the embryo, they are the first cells to enter the endoreplication cycle. Furthermore, they are the only cells that appear to enter endoreplication from G2 instead of G1 (Smith and Orr-Weaver, 1991). We imagine that these unusual changes in cell cycle or the simultaneous occurrence of cell cycle changes and cytoskeletal rearrangements in the invaginating salivary placodes might sensitize checkpoints that have the potential to cause apoptosis. Consistent with this idea, the small piece of the reaper promoter that contains the p53 response element is active in wild type salivary glands (Brodsky et al., 2000), suggesting that p53 may be induced in the salivary primordium and push these cells to the brink of cell death. If so, sens would be necessary to counter p53 and prevent strong induction of reaper throughout the salivary placodes.

In either scenario, the induction of reaper and hid would result in apoptosis of the salivary primordium. Therefore, the presence of sens to repress proapoptotic genes is crucial for the survival of the salivary glands during embryogenesis.

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