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 *sage* RNAi embryos have a phenotype similar to *sens* and that *sage* 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: $sens^{E2}$, *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 $hkb^{AI7\Delta2}$ allele was generated in our laboratory. All other stocks were from the Bloomington Stock Center.

w^{1118} 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 devitellenized 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 anti-β-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′-ATGACGGATCAACTGCTGAGCTCCA-3′, 5′-CGCTCCCCAA-TATCGTTGCCA-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 w^{1118} 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* (Cronmiller and Cummings, 1993). The in vitro translated DA protein, labeled using ^{35}S-methionine, migrated in an SDS gel at about 80 kDa, which agrees with the previously documented molecular weight of DA (Cronmiller 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 $sens^{E2}$ 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 $sens^{E2}$ 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, $sens^{E58}$ and $sens^{I235}$, as well as for transheterozygotes of $sens^{E2}$ and a deficiency for *sens*, suggesting that $sens^{E2}$ behaved as an amorph in our studies. The phenotype seen in the $sens^{E2}$ mutant

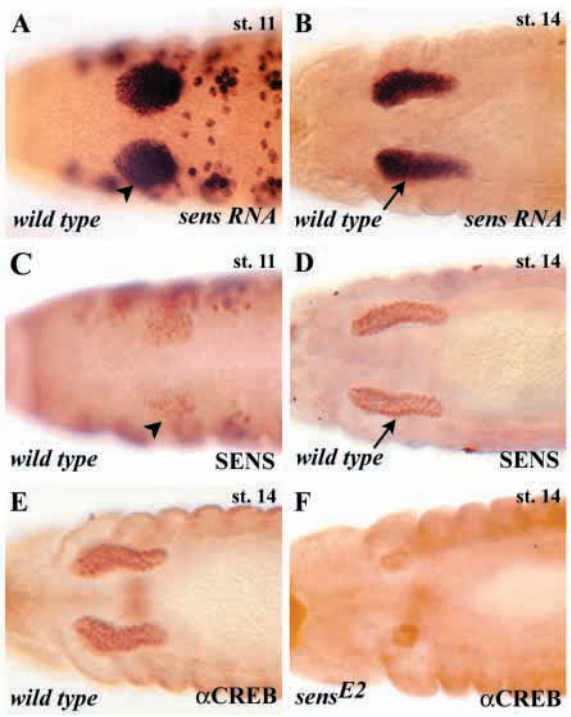


Fig. 1. *sens* expression in the embryonic salivary glands. All views are ventral and embryos are oriented with anterior to the left. (A-D) *sens* mRNA is expressed in the salivary placodes at stage 11 (arrowheads) and later in the salivary glands at stage 14 (arrows). (E,F) The salivary glands in *sens* mutant embryos at stage 14 are smaller than those of wild-type embryos.

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; Vaessin et al., 1994). By contrast, *da* expression is ubiquitous in the early embryo and is upregulated in the salivary glands of older embryos (Cronmiller 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

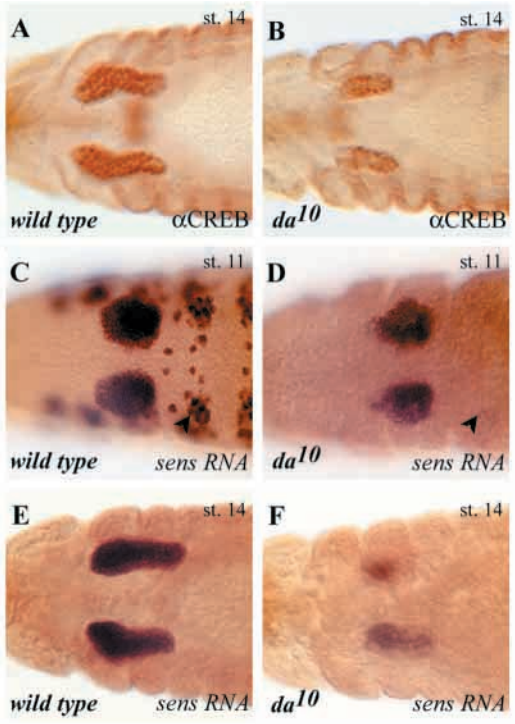


Fig. 2. Regulation of *sens* by *daughterless* (*da*) in salivary glands. (A,B) da^{10} embryos had small salivary glands compared with wild type. (C,D) *sens* mRNA is expressed in the salivary placodes of da^{10} embryos at stage 11. The PNS expression of *sens* is missing in da^{10} embryos (arrowhead). (E,F) *sens* expression is markedly reduced at later stages in the salivary glands of da^{10} embryos compared with wild-type embryos.

(Fig. 2E,F), suggesting that DA regulates *sens* in the both the PNS and salivary gland. However, unlike the PNS, salivary gland *sens* expression initiates in the absence of *da* (Fig. 2C,D).

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 ^{35}S-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

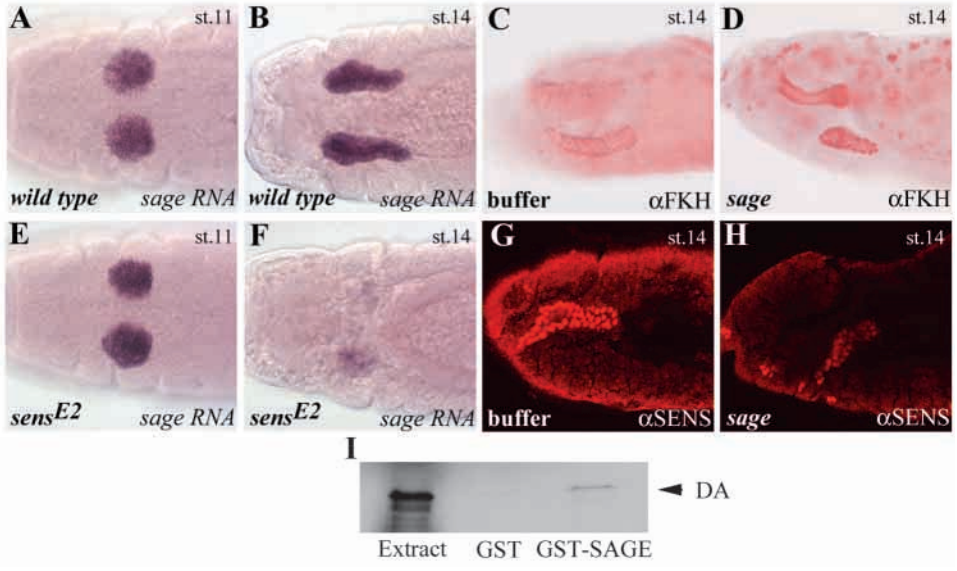


Fig. 3. *sage*, a salivary-specific bHLH protein, regulates *sens*. (A,B) *sage* is first expressed in the salivary placodes at stage 10 and continues to be expressed in the salivary glands of stage 14 wild-type embryos. (C,D) The embryos injected with *sage* dsRNA showed smaller salivary glands compared with embryos injected with injection buffer. (E,F) *sage* mRNA is expressed at normal levels in the salivary placodes of $sens^{E2}$ embryos but is reduced during later stages in the salivary glands of $sens^{E2}$ embryos compared with wild-type embryos (compare F with B). (G,H) SENS levels, although present, are reduced in *sage* dsRNA-injected embryos when compared with embryos injected with injection buffer. (I) GST pulldown showing that DA can bind SAGE. Lane 1, in vitro transcription translation extract without the GST fusion proteins; lane 2, GST + DA; lane 3, GST-SAGE + DA. The arrowhead indicates the band corresponding to DA. DA shows strong binding to GST-SAGE but not to GST alone.

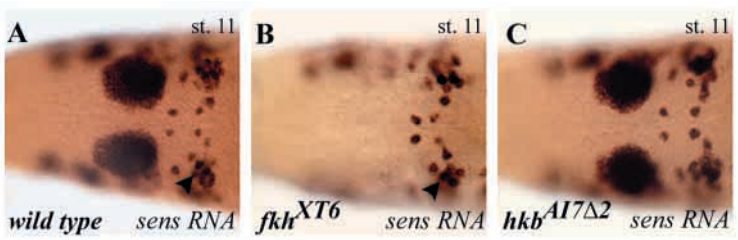


Fig. 4. *fork head* is necessary for the initiation of *sens* expression in the placodes. (A-C) *sens* mRNA expression is observed in the salivary placodes of wild-type (A) and $hkb^{AI7\Delta2}$ (C) embryos but not in fkh^{XT6} (B) embryos. The arrowhead (B) indicates the continued expression of *sens* in the PNS neurons in fkh^{XT6} embryos.

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), *trachealess* 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 *sage* 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

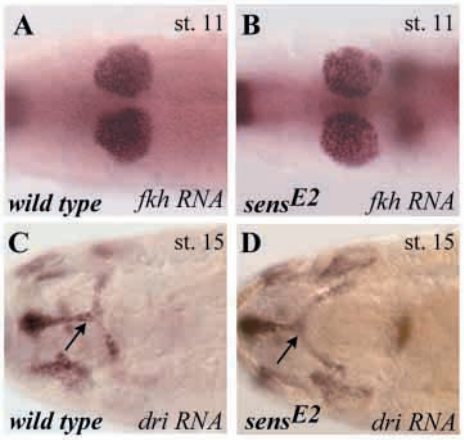


Fig. 5. $sens^{E2}$ embryos have normal placodes and salivary ducts. (A,B) The salivary placodes in stage 11 $sens^{E2}$ embryos are identical to those in wild-type embryos. (C,D) In addition, the salivary ducts (arrow) in $sens^{E2}$ embryos as visualized by duct marker, *dead ringer*, are similar in length and diameter to wild-type embryos.

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

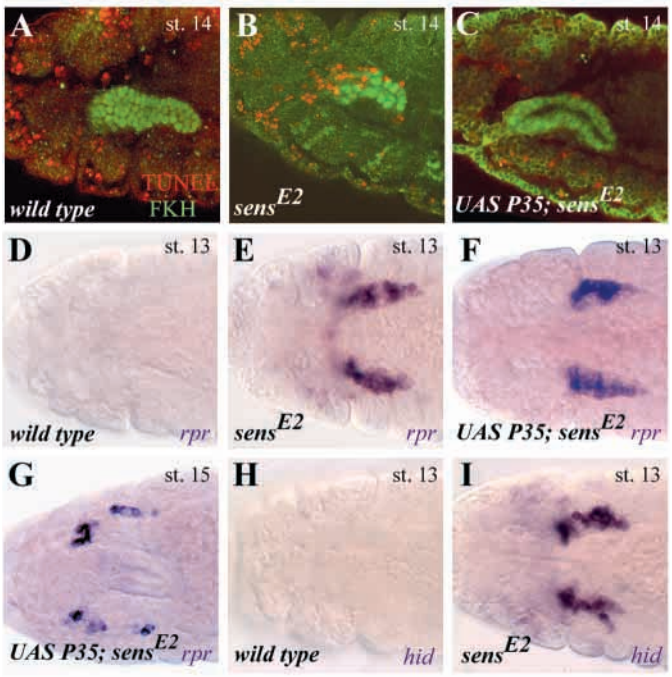


Fig. 6. Apoptosis in salivary glands of *sens*^{E2} embryos. (A-C) TUNEL staining in the salivary glands showed no apoptotic cells in the salivary glands of wild-type embryos. However, *sens*^{E2} embryos showed presence of a large number of apoptotic cells that are absent in *arm-GAL4: UAS-P35; sens*^{E2} embryos. Note the longer salivary glands in the *arm-GAL4: UAS-P35; sens*^{E2} embryos compared with *sens*^{E2} embryos. (D-G) *reaper* mRNA is not expressed in the salivary glands of stage 13 wild-type embryos but is upregulated in *sens*^{E2} as well as *arm-GAL4: UAS-P35; sens*^{E2} embryos. In *sens*^{E2} embryos, *reaper* RNA disappears from the salivary glands at stage 14 (data not shown), whereas it continues to be expressed in the salivary glands of stage 15 *arm-GAL4: UAS-P35; sens*^{E2} embryos (G). (H,I) Expression of *hid* is also induced in the salivary glands of *sens*^{E2} embryos when compared with wild-type embryos.

possibilities can be differentiated by examining the salivary glands in *sens* mutant embryos where the cell death pathway has been blocked. If the cell death in the *sens* mutant salivary glands is a secondary effect, blocking cell death would not lead to rescue of *sens* phenotype, whereas if *sens* functions in the cell death pathway, then the rescue of cell death in *sens* mutants should result in normal morphogenesis of salivary glands. One of the methods to rescue embryonic cell death is by expressing an anti-apoptotic protein P35 in the embryo using the UAS-GAL4 system. P35 is a baculovirus protein that is homologous to the IAPs and has been shown to rescue apoptotic cells in wide variety of organisms including *Drosophila* embryos and other *Drosophila* tissues (Hay et al., 1994; Rabizadeh et al., 1993; Sugimoto et al., 1994; Xue and Horvitz, 1995). When we expressed P35 ubiquitously in *sens* mutant embryos using *arm-GAL4*, the salivary glands were normal in size and went through normal morphogenesis (Fig. 6C, see Fig. 8). The rescued salivary glands also showed normal expression of late salivary gland markers such as *slalom*, indicating that they were functioning normally (data not shown). However, most of the cells in these glands continued to show high expression of *reaper* at stage 13 and later (Fig. 6F,G). Based on the *reaper* 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.

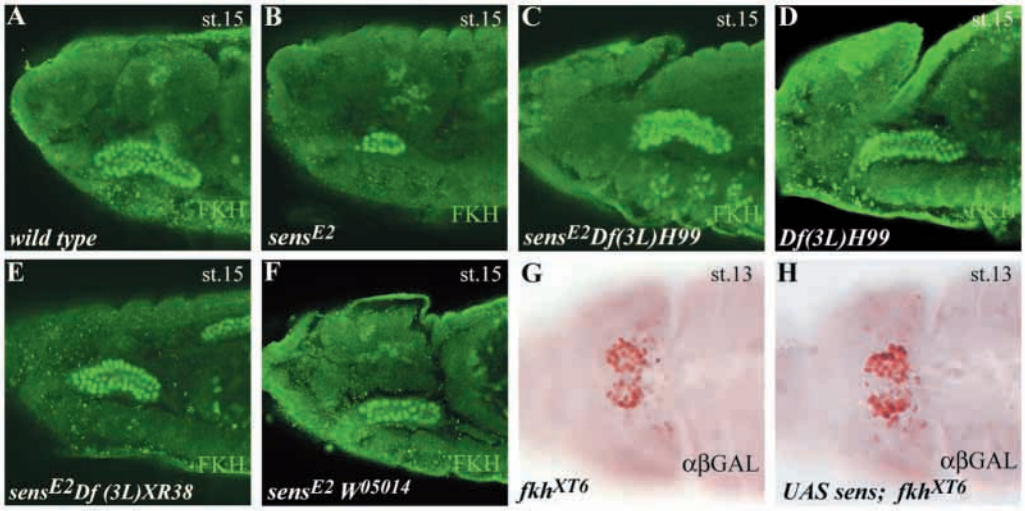


Fig. 7. The phenotype of *sens* is suppressed by cell death genes in *Df(3L)H99*. (B,C) The small salivary glands in $sens^{E2}$ embryos (B) are rescued in embryos that are also mutant for *Df(3L)H99* (C). Note that the salivary glands of *Df(3L)H99* embryos are similar to wild type (compare A with D). (E,F) The small salivary glands in $sens^{E2}$ embryos are partially rescued in embryos mutant for $sens^{E2}$ and either *reaper* (*Df(3L)XR38*; E) or *hid* (W^{05014}; F). (G,H) The salivary glands in fkh^{XT6} 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.

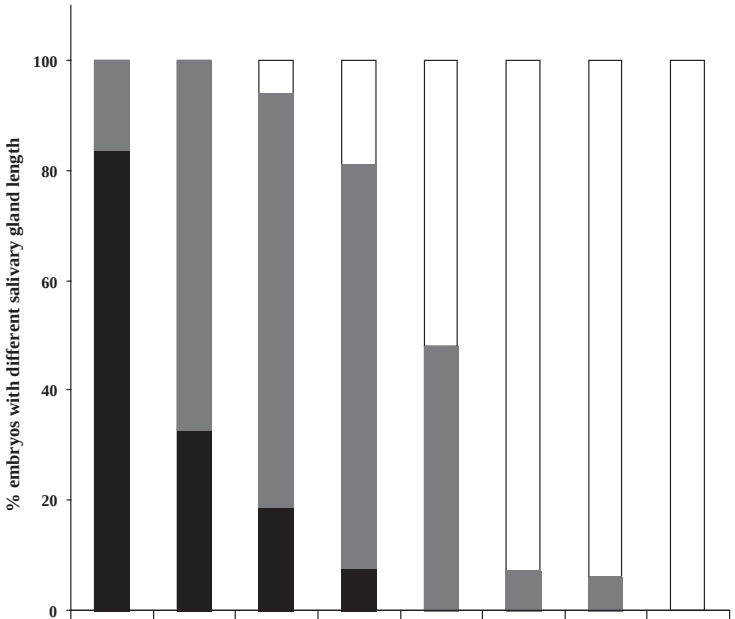
7G,H). Thus, in addition to *sens*, *fkh* appears to control other regulators of apoptosis in the embryonic salivary glands.

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



	sens (61)	*sens hid* (33)	*sens rpr* (47)	*sens rpr sens H99* (26)	*arm-GAL4; UAS-P35 sens* (29)	*sens H99* (29)	*Df(3L)H99* (16)	*wild type* (65)
Normal (2.5 segments)	0	0	6	19	52	93	94	100
1 to 2 segments	16	67	75	73	48 **	7 *	6 *	0
less than 1 segment	84	33	19	8	0	0	0	0

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.

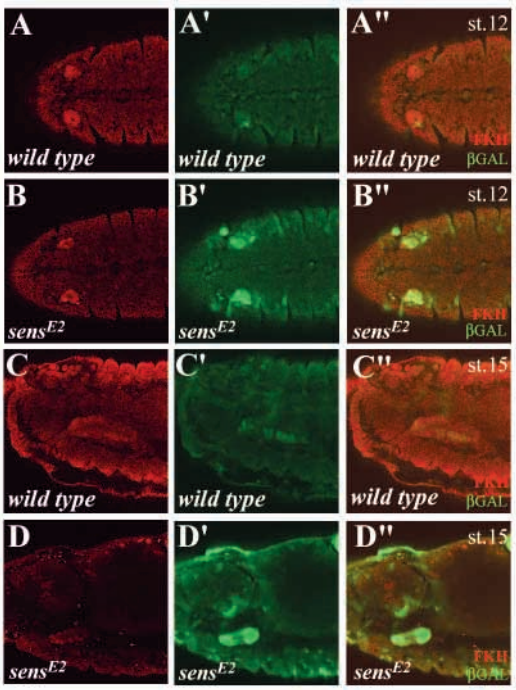


Fig. 9. *sens* represses the expression of a *reaper* reporter in the salivary glands. (A,A′,A″) In wild type embryos, at stage 12 the *Rpr-11-lacZ* expression (green) is observed in the dorsal posterior cells of the salivary placodes. (C,C′,C″) At later stages, expression of *Rpr-11-lacZ* in barely above background levels throughout the salivary glands. (B,B′,B″,D,D′,D″) By contrast, $sens^{E2}$ embryos show marked upregulation of *Rpr-11-lacZ* expression in the salivary placodes and in the invaginated portion of the salivary glands at stage 12 and continue to show high expression at later stages in the salivary glands.

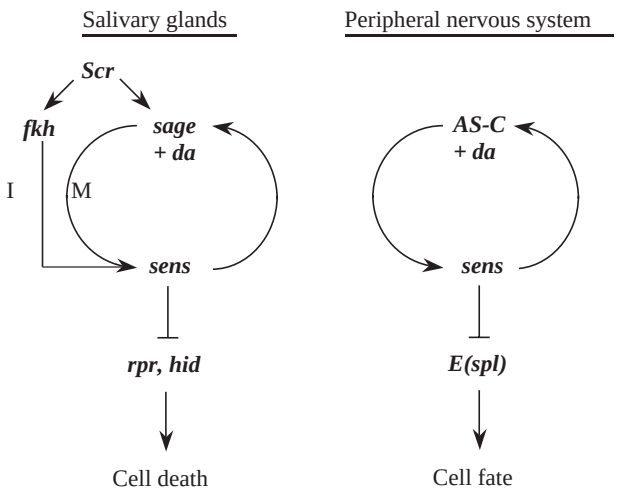


Fig. 10. The epistatic relationships in the salivary glands and in the peripheral nervous system. In the salivary glands, *Scr* activates *fkh* and *sage*. *fkh* is necessary for the initiation (I) of *sens* expression. *sage*, together with *da*, is necessary for the maintenance (M) of *sens* expression. 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.

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.

A summary of these regulatory interactions and a comparison with the regulation in the peripheral nervous system is provided in Fig. 10. A similar circuit controls the regulation of expression of Gfi1, the vertebrate ortholog of *sens*, in the inner ear cells of mice. The bHLH protein Math1 (Atoh1 – Mouse Genome Informatics), a homolog of *atonal*, is necessary to maintain Gfi1 mRNA, but not for its initiation in the inner ear cells. It would be interesting to examine if *fkh* family members are involved in this case to initiate the Gfi1 expression. However, the feedback regulation of *sens* onto *sage* or proneural genes (this paper) (Nolo et al., 2000) is not observed between Gfi1 and Math1 (Wallis et al., 2003).

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* mutants also 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 *sage* in the salivary primordium. There are potential SENS-binding sites upstream or downstream of *hid*, *sage* 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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