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Specification of cell fates within the salivary gland primordium

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Abstract

The *Drosophila* salivary gland is a simple tubular organ derived from a contiguous epithelial primordium, which is established by the activities of the homeodomain-containing proteins Sex combs reduced (SCR), Extradenticle (EXD), and Homothorax (HTH). EGF signaling along the ventral midline specifies the salivary duct fate for cells in the center of the primordium, while cells farther away from the source of EGF signal adopt a secretory cell fate. EGF signaling works, at least in part, by repressing expression of secretory cell genes in the duct primordium, including *fork head (fkh),* which encodes a winged-helix transcription factor. FKH, in turn, represses *trachealess (trh),* a duct-specific gene initially expressed throughout the salivary gland primordium. *trh* encodes a basic helix–loop–helix PAS-domain containing transcription factor that has been proposed to specify the salivary duct fate. In conflict with this model, we find that three genes, *dead ringer (dri), Serrate (Ser),* and *trh* itself, are expressed in the duct independently of *trh.* Expression of all three duct genes is repressed in the secretory cells by FKH. We also show that SER in the duct cells signals to the adjacent secretory cells to specify a third cell type, the imaginal ring cells. Thus, localized EGF- and Notch-signaling transform a uniform epithelial sheet into three distinct cell types. In addition, *Ser* directs formation of actin rings in the salivary duct.

Keywords: Boundary formation; *dead ringer*; *fork head*; Imaginal ring; *Notch*; Salivary duct; Salivary gland; *Serrate*; *trachealess*

Introduction

The larval salivary gland of *Drosophila melanogaster* is a simple tubular organ composed of three cell types (Bradley et al., 2001). The large secretory cells, which secrete digestive enzymes and glue proteins, are arranged into two unbranched epithelial tubes. At the proximal ends of the secretory tubes are the small imaginal ring cells, which will form the adult salivary gland during metamorphosis. The imaginal ring connects the secretory organ to the salivary duct, a Y-shaped tube comprised of a common duct, which opens into the mouth, and two individual ducts, which connect the common duct to the imaginal rings. Both the duct and secretory cells are polytenized to accommodate the metabolic needs of these large cells, whereas the imaginal ring cells remain small and diploid. All three salivary gland cell types originate from a single contiguous layer of ectoderm on the ventral surface of parasegment two (PS2) (Andrew et al., 2000). The cells invaginate and undergo extensive movements to aquire their final shapes and positions within the embryo (Bradley et al., 2001).

The salivary gland cell fate is specified in PS2 by the locally expressed homeotic gene *Sex combs reduced (Scr)* (Panzer et al., 1992), along with two more generally expressed genes, which encode the essential cofactors Extradenticle (Exd) and Homothorax (Hth) (Henderson and Andrew, 2000). Salivary gland specification is repressed in the dorsal ectoderm of PS2 by the TGF β-like signaling molecule Decapentaplegic (DPP), restricting the salivary glands to the ventral ectoderm (Panzer et al., 1992; Henderson et al., 1999). The salivary gland fate is also repressed in regions posterior to PS2 by the zinc finger protein encoded by *teashirt (tsh)* and the homeotic protein encoded by *Abdominal-B (Abd-B)* (Andrew et al., 1994).

EGFR ligands secreted by the cells of the ventral midline specify the most ventral salivary gland cells to adopt the salivary duct fate, as opposed to the secretory cell fate. EGF-signaling works, at least in part, by repressing expression of several secretory cell-specific genes, including *fork*

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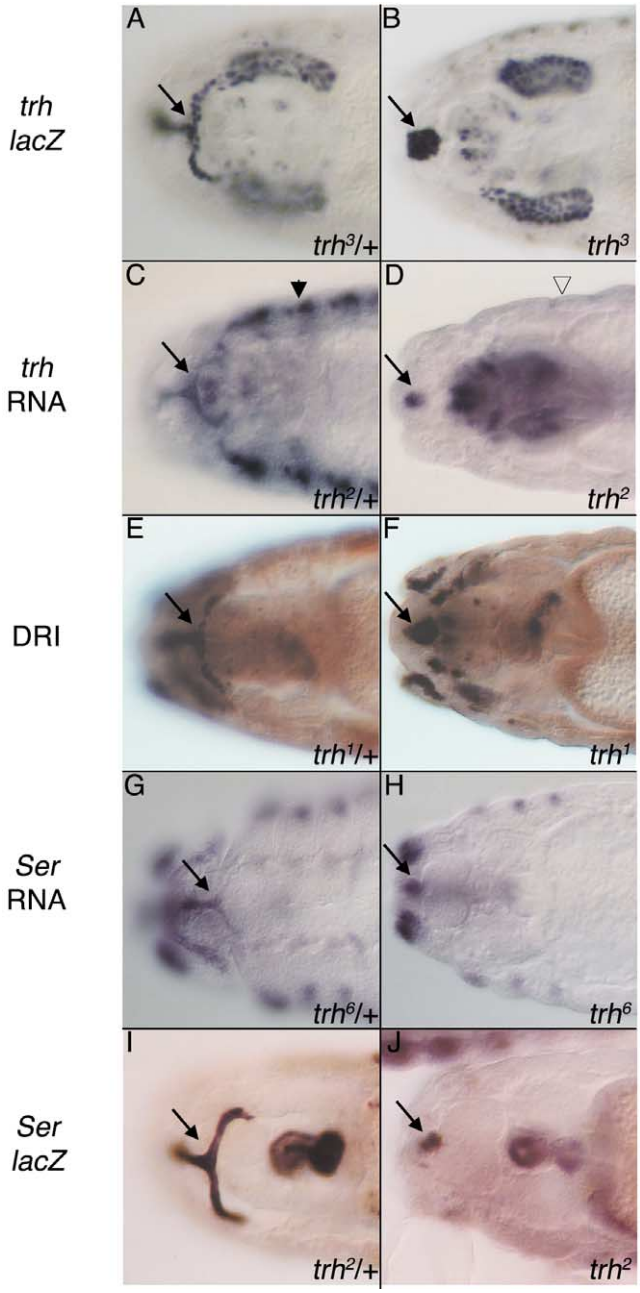


Fig. 1. At least three genes are expressed in the salivary duct independently of *trh*. Expression of β-gal from a *trh* enhancer trap (A, B), *trh* RNA (C, D), DRI (E, F), *Ser* RNA (G, H), and β-gal from a transgene driven by a fragment of the *Ser* enhancer (I, J) in *trh* heterozygotes (A, C, E, G, I) and homozygotes (B, D, F, H, J) is shown. Arrows indicate the salivary duct cells. The filled arrowhead in (C) indicates expression of *trh* in the trachea, which is absent in the *trh* mutant in (D) (open arrowhead). At least two *trh* alleles were examined for expression of each gene.

head (fkh), which encodes a winged-helix transcription factor (Kuo et al., 1996). In EGF pathway mutants, *fkh* and other secretory cell genes are expressed in all salivary gland cells instead of just the lateral cells that normally give rise to the secretory organ (Bradley et al., 2001). Other genes, including *trachealess (trh),* which encodes a basic helix–loop–helix PAS domain-containing transcription factor, are not repressed by EGF-signaling and are expressed in the duct precursors. In fact, *trh* is initially expressed in all salivary gland cells, and is subsequently repressed in the secretory cells by FKH (Isaac and Andrew, 1996). A model has been proposed in which TRH specifies the salivary duct fate by activating downstream salivary duct genes, and FKH specifies the secretory cell fate by simultaneously repressing *trh* and activating secretory cell-specific genes (Kuo et al., 1996). This model is supported by the profound phenotypes caused by the loss of *fkh* and *trh;* secretory and duct cells fail to invaginate and form tubes in *fkh* and *trh* mutants, respectively (Weigel, 1989; Isaac and Andrew, 1996). This model fails to account for the fact that many genes are expressed in the secretory cells independently of *fkh* (Bradley et al., 2001), and the same may also be true of *trh* in the duct cells. Little is known about the specification of the third salivary gland cell type, the imaginal ring cells, although

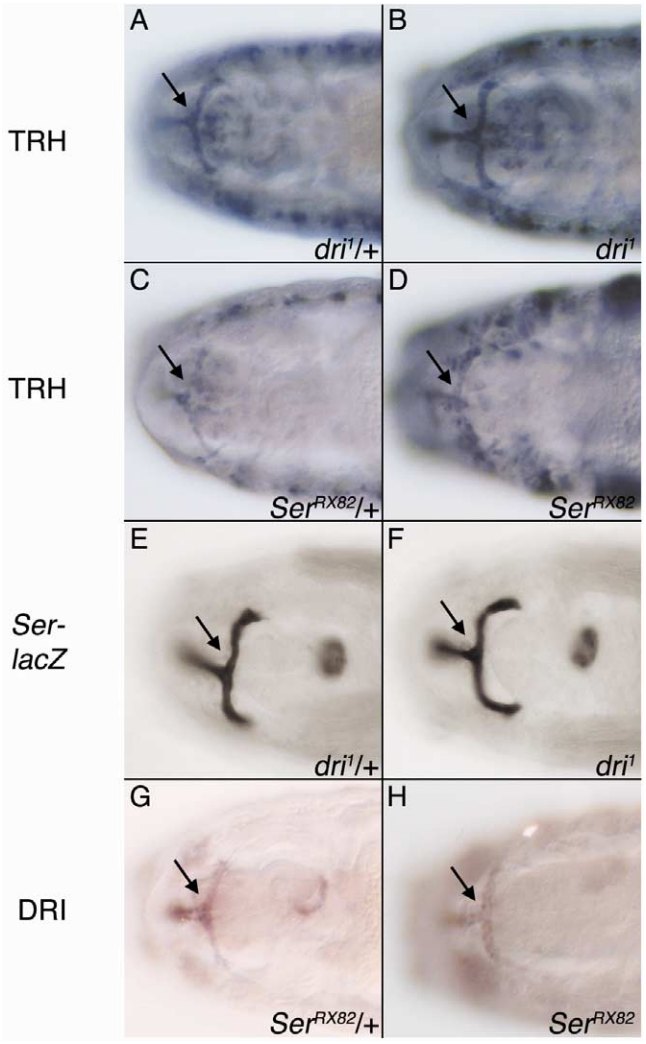


Fig. 2. Three genes are expressed in the salivary duct independently of each other. Expression of TRH (A–D), *Ser-lacZ* (E, F), and DRI (G, H) in *dri* heterozygotes (A, E), *dri* homozygotes (B, F), *Ser* heterozygotes (C, G), and *Ser* homozygotes (D, H) at embryonic stage 15 is shown. Arrows indicate the salivary duct.

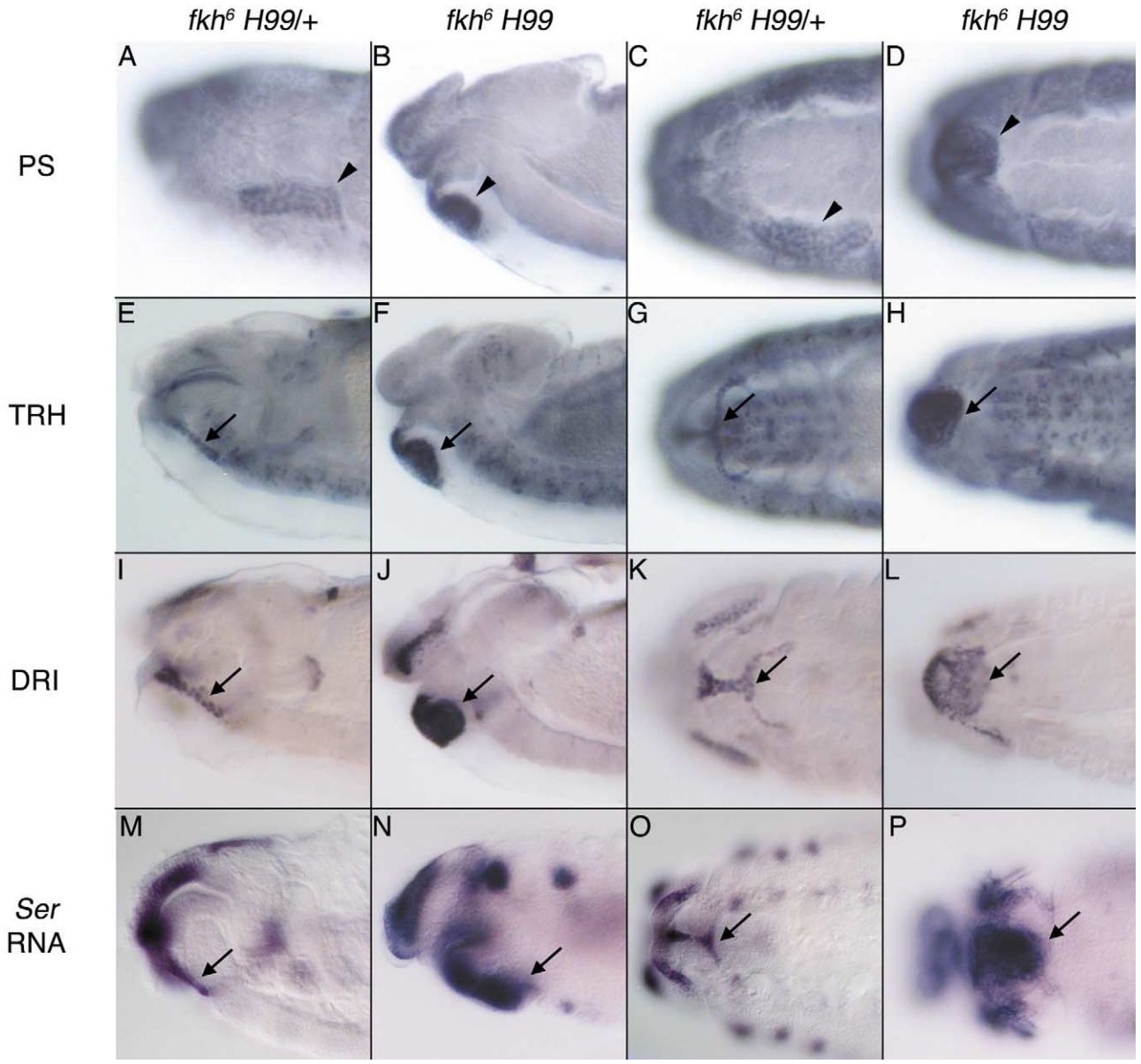


Fig. 3. *fkh* represses expression of several salivary duct genes in the secretory cells. Expression of PS (A–D), TRH (E–H), DRI (I–L), and *Ser* RNA (M–P) in *fkh*^{6} *H99* heterozygous (A, C, E, G, I, K, M, O) and homozygous embryos (B, D, F, H, J, L, N, P) is shown. Arrowheads indicate secretory cells (A–D). Arrows indicate duct cells (E, G, I, K, M, O) or the cluster of uninvaginated duct and secretory cells (F, H, J, L, N, P). Lateral views are shown in the left two columns, and ventral views are shown in the right two columns. All embryos are at embryonic stage 15.

nuclear transplantation experiments have shown that they arise from the same precursors as the duct and secretory cells (Harbecke et al., 1996).

Notch signaling is an evolutionarily conserved pathway that specifies cell fates throughout development, controlling such diverse processes as follicle cell polytenization, sensory organ specification, and cell cycle arrest at the wing margin (Johnston and Edgar, 1998; Artavanis-Tsakonas et al., 1999; Deng et al., 2001). The Notch receptor is a 300-kDa single-pass transmembrane protein that is activated by either of two ligands, Delta (DL) or Serrate (SER), both of which are also single-pass transmembrane proteins. Since both the ligand and receptor are transmembrane proteins, Notch signaling involves only local interactions between adjacent cells (Artavanis-Tsakonas et al., 1999). Notch receptor activation causes multiple proteolytic cleavages of the Notch receptor, releasing the intracellular domain from the plasma membrane and allowing it to move into the nucleus, where it interacts with Suppressor of Hairless (SuH) to direct expression of several target genes, including *mastermind* and the genes of the *Enhancer of split* complex (Ye and Fortini, 2000). Notch signaling can play a role in multiple steps of the same process. For instance, Notch is required for several steps of myogenesis, including specification of muscle progenitors and patterning of muscle formation through signals from the ectoderm, and for every

cell fate decision involved in sensory organ formation (Fuerstenberg and Giniger, 1998; Artavanis-Tsakonas et al., 1999).

Notch signaling often mediates boundary formation, in which signaling between two fields of cells specifies a new cell type between them. Some boundary cells are signaling centers, such as the midline cells of the eye imaginal disc. The midline secretes an unknown signal that directs the ommatidia in each half of the eye to rotate ninety degrees in the opposite direction from the ommatidia in the other half (Choi and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). Other boundaries are physical barriers that form structures later in development, such as the joints of the leg (de Celis et al., 1998; Bishop et al., 1999; Rauskolb and Irvine, 1999). In the wing imaginal disc, Notch signaling between the dorsal and ventral cells specifies the wing margin, which is a physical structure as well as a signaling center. The wing margin cells will form the bristles at the wing edge and secrete the signaling molecule Wingless (WG), which directs growth and gene expression throughout the disc (Couso et al, 1995; Diaz-Benjumea and Cohen, 1995; de Celis et al., 1996).

Here, we focus on the mechanisms of cell fate determination within the salivary gland primordium. We show that TRH does not control expression of all duct genes as was previously suggested (Kuo et al., 1996) and that FKH independently represses all tested duct genes in the secretory primordium. We also find two roles for the Notch ligand SER. Duct-specific SER specifies the imaginal ring, a boundary cell population between the duct and secretory primordia, and also directs formation of actin rings in the salivary duct.

Materials and methods

Drosophila strains

The following alleles were used: Ser^{RX82} and Ser^{RX106} (Thomas et al., 1991), Ser^{5A29} (Harding et al., 1995), $Df(Ser)^{+82f24}$ (Wiellette and McGinnis, 1999), *Ser* II-4.0-*lacZ* (Bachmann, 1998), dri^{-1} (Shandala et al., 1999), trh^{1}, trh^{2}, trh^{3}, and trh^{6} (Isaac and Andrew, 1996), fkh^{6}, and *Df(3R)H99* (described in Flybase, http://flybase.bio.indiana.edu/).

Embryo immunohistochemistry and in situ hybridization

The Pasilla (PS) (Sheshiah et al., 2001) and TRH antisera (Henderson et al., 1999) have been previously described and were used at dilutions of 1:5000 and 1:500, respectively. The mouse monoclonal β-galactosidase antibody was obtained from Promega Corp. (Madison, WI, USA) and was used at a dilution of 1:5000. The DRI antiserum was raised against a construct described in Gregory et al. (1996). DRI was produced in BL21(DE3) cells (Novagen, Madison, WI, USA) induced with 1 mM IPTG for 8 h. Protein was purified in inclusion bodies as described (Rio et al, 1986). Rat polyclonal antiserum was raised (Covance Research Products, Princeton, NJ, USA) against 1 mg of the renatured inclusion body protein. The antiserum was used at dilutions of 1:20,000 to 1:30,000. Embryo fixation and staining were performed as described (Reuter et al., 1990). Embryonic RNA was detected by whole-mount in situ hybridization as described by Lehmann and Tautz (1994). All embryos were visualized and photographed on a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY, USA) by using Nomarski optics and Kodak print film (Eastman Kodak, Rochester, NY, USA).

Dissection and staining of second instar salivary glands and trachea

Embryos were collected overnight and aged 2 days until larvae had reached second instar. Tissues were dissected in 0.7% NaCl, rinsed twice for 5 min, and fixed in 1 × PBS, 3.7% formaldehyde for 30 min. Tissues were washed three times for 5 min in 0.7% NaCl before staining. To visualize F-actin accumulation, the glands or trachea were stained for 20 min in 2 unit/ml Texas-Red-X phalloidin (Molecular Probes, Eugene, OR, USA) in 0.7% NaCl. Nuclei were visualized by staining for 5 min in 2 μg/ml Hoechst 33258 or by staining for 10 min in 0.1 nM SYTOX dye (Molecular Probes, Eugene, OR, USA) in 0.7% NaCl. After staining, tissues were washed three times and then cleared and mounted in 70 or 85% glycerol. Fluorescent images were obtained on a Zeiss Axiophot with 20 × and 40 × objectives using Kodak print film. Confocal microscopy was performed on an Ultraview LCI laser spinning disc microscope (Perkin Elmer, Wellesley, MA, USA). Three-dimensional reconstructions were performed by using Volocity (Improvision, Coventry, England).

Results

Three genes are expressed in the salivary duct independently of trh

In *trh* mutants, salivary duct cells fail to invaginate and remain clustered on the embryo surface (Fig. 1A and B) (Isaac and Andrew, 1996). Based on this phenotype and the loss of expression of other duct genes in *trh* mutant embryos, it has been proposed that *trh* is required to establish salivary duct identity (Kuo et al., 1996). Based on this model, expression of all duct genes would be dependent on *trh,* even *trh* itself. Indeed, *trh* activity is required to maintain *trh* expression in the trachea (Wilk et al., 1996). We asked if *trh* is required to maintain its own expression in the salivary duct as well. In embryos mutant for two EMS *trh* alleles, trh^{1} and trh^{2}, *trh* RNA was absent from the trachea at late stages (Fig. 1C and D, and data not shown). How-

ever, *trh* RNA was still observed at approximately wild-type levels in the salivary duct cells, indicating that *trh* does not autoregulate in the salivary duct as it does in the trachea.

Two other genes, *dead ringer* (*dri;* also known as *retained*) and *Serrate (Ser),* are expressed to high levels in the salivary duct. *dri* encodes an ARID-box transcription factor whose role in the salivary duct has not yet been determined (Gregory et al., 1996). *Ser* encodes a ligand for the *Notch* receptor (Fleming et al., 1990; Thomas et al., 1991), whose role in this tissue is also unknown (Kuo et al., 1996). Expression levels of both *dri* and *Ser* were unaffected in *trh* mutants. DRI protein was present in the uninvaginated salivary duct cells that remain on the surface of *trh* mutants (Fig. 1E and F). Similarly, both *Ser* RNA and β-galactosidase expressed under the control of a *Ser* enhancer (*Ser-lacZ*) (Bachmann, 1998) were expressed in salivary duct cells in *trh* mutants (Fig. 1G–J). Thus, *trh* is neither required for its own expression nor for the expression of at least two other salivary duct genes.

Since dri and *Ser* are expressed independently of *trh,* we asked whether there is any regulatory relationship among the three genes. *trh* expression was not altered in embryos mutant for *dri* or *Ser* (Fig. 2A–D). Similarly, *Ser* expression was not altered in *dri* mutants, and DRI expression was not altered in *Ser* mutants (Fig. 2E–H). Thus, all three genes are expressed in the salivary duct independently of the other two.

fkh represses expression of duct genes in the secretory cells

trh is initially expressed throughout the salivary gland, in both duct and secretory cell primordia, but becomes restricted to the duct cells by *fkh* (Isaac and Andrew, 1996; Kuo et al., 1996). The model proposed by Kuo et al. (1996) suggests that FKH acts through repression of *trh* to limit expression of all duct genes to only the ventral preduct portion of the salivary gland primordium. Since we have shown that expression of at least three genes is *trh*-independent, it was unclear how their expression is limited to the duct. We tested whether or not expression of the *trh*-independent duct genes is affected by FKH. Since salivary gland cells undergo apoptosis in *fkh* mutants (Myat and Andrew, 2000), we performed the experiments in the background of the *H99* deficiency, which blocks apoptosis by removing the apoptosis-activating genes *hid, grim,* and *reaper* (White et al., 1994). As in *fkh* mutants alone, all salivary gland cells remained on the surface of the embryo in *fkh H99* embryos. In these embryos, secretory cells expressed the secretory marker Pasilla (PS) (Fig. 3A–D) and TRH was expressed in all salivary gland cells (Fig. 3E–H). Similarly, expression of both DRI and *Ser* expanded into the secretory cells of *fkh H99* embryos, suggesting that *fkh* is required to prevent secretory cell expression of multiple duct genes independently (Fig. 3I–P). Expression of all three genes was also observed throughout the salivary gland primordium of *fkh* mutants without the *H99* deficiency, demonstrating that the observed expression profiles were not affected by the *H99* deficiency (data not shown). Also, expression of all of these genes was unchanged in *H99* homozygous embryos, further indicating that the changes in gene expression are due to *fkh* (data not shown).

Ser mutants have abnormal duct morphology

Given the role of *trh* in salivary duct morphogenesis, what is the role of the two TRH-independent salivary duct genes? Staining of *dri* mutants with the duct markers TRH, *Ser,* or CRB (Fig. 2B and F, and data not shown) did not reveal any overt morphological changes from wild-type embryos. Staining of *Ser* mutants with DRI revealed only a subtle, partially penetrant defect, where the distal ends of the individual ducts were slightly enlarged (data not shown). Differences between *Ser* and wild-type embryos in the distal ends of the salivary ducts were more apparent with staining for cytoplasmic *Ser-lacZ,* which revealed that the ends of the individual ducts were splayed in the region where they contacted the secretory cells (Fig. 4).

To test for any potential cell fate changes at the ends of the individual ducts in *Ser* mutants, we analyzed expression of several salivary gland markers. By coimmunofluorescence with *Ser*-lacZ, we found that the cells at the duct ends still expressed DRI and did not express the secretory cell markers dCREBA and PS (data not shown). Thus, the change in duct morphology is likely not due to a change in duct cell fate. We also did not detect a change in staining for the phosphorylated form of histone H3, indicating that loss of *Ser* did not cause a change in cell proliferation (data not shown).

Ser mutants have no salivary gland imaginal ring

Ser transcripts are first detected in the duct cell primordia during embryonic stage 11, when most of the salivary gland precursors are still on the embryo surface (Kuo et al., 1996). *Notch,* the gene encoding the receptor for SER, is transiently upregulated in the secretory primordia prior to invagination (Kidd et al., 1989). At this stage, the cells are arranged so that SER could signal to the approximately 10–11 secretory cells in direct contact with either side of the duct primordium. The enlarged distal ends of the ducts of late stage *Ser* mutants suggest that the duct cells are either in contact with more cells or with larger cells than in wild-type. Thus, SER could be signaling to the adjacent secretory cells to either undergo programmed cell death or to be smaller than the other secretory cells. If the role of *Ser* signaling in this system is to induce programmed cell death, we expect that blocking programmed cell death would give the same phenotype as observed in *Ser* mutants. Staining with duct markers revealed that the ends of the individual ducts were normal in *H99*-deficient embryos (data not

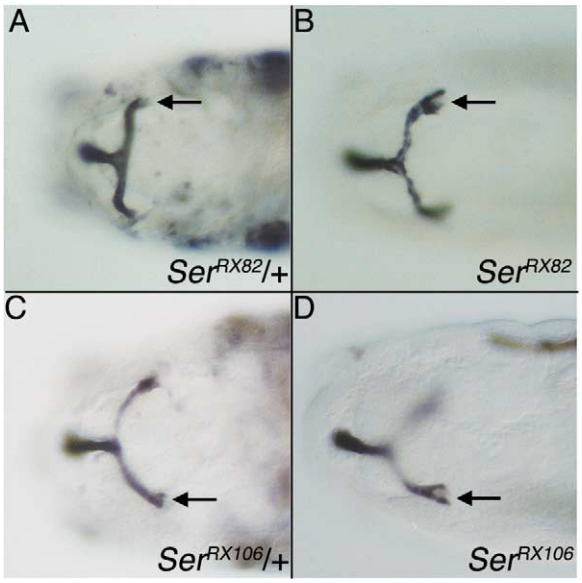


Fig. 4. The distal ends of the individual ducts of *Ser* mutants are enlarged. Expression of *lacZ* driven by a 4-kb fragment of the *Ser* enhancer in *Ser* heterozygotes (A, C) and homozygotes (B, D) is shown. Arrows indicate the ends of the individual ducts.

shown), indicating that the *Ser* phenotype is probably not due to a defect in apoptosis.

Another explanation for the enlarged distal ends of the ducts in *Ser* mutant embryos is that the cells they contact are larger than in wild-type embryos. In the mature larval salivary gland, the ends of the duct are in direct contact with the salivary gland imaginal ring. While the duct and secretory cells are large and polytenized at larval stages, the imaginal ring cells are small and diploid (Bradley et al., 2001). If, in *Ser* embryos, the imaginal cells were absent or transformed into secretory cells, the duct connected to them would have to spread wider to make a complete connection. Unfortunately, adequate markers for the embryonic salivary gland imaginal ring are not currently available. Moreover, *Ser* mutant larvae do not survive to the third larval instar stage, where the imaginal ring population is easily distinguished from the duct and secretory cell populations by the dramatic differences in nuclear size. Nonetheless, differences in nuclear size can be distinguished as early as the second instar stage in wild-type larvae, and *Ser* mutants do survive to the second larval instar. Although the *Ser* mutant larvae are smaller and have smaller cells than wild-type larvae at the same stage, distinctions between the different salivary gland cell populations were readily observed.

To test for the presence of the imaginal ring in *Ser* mutants, we dissected the salivary glands from wild-type and *Ser* mutant second instar larvae and stained with Hoechst or Sytox to visualize nuclei. In wild-type and *Ser* heterozygous glands, the diploid imaginal ring nuclei were clearly present as small nuclei positioned between the polytenized duct and secretory nuclei (Fig. 5A and C). In *Ser* homozygous glands, the diploid nuclei were absent and all salivary gland nuclei were large and polytenized (Fig. 5B and D). We also simultaneously stained second instar salivary glands with Hoechst or Sytox and Texas Red-conjugated phalloidin to visualize the cortical F-actin of the salivary gland cells. Confocal optical sections of wild-type and *Ser* heterozygous glands revealed small imaginal nuclei surrounded by a tight ring of actin, demonstrating the smaller size of the imaginal cells compared with the duct and secretory cells (Fig. 5E and F). *Ser* homozygous glands are missing these small cells (Fig. 5G and H). Thus, the imaginal ring cells are missing in second instar salivary glands of *Ser* mutants, potentially accounting for the enlarged distal tips of the individual ducts observed in late embryonic stages.

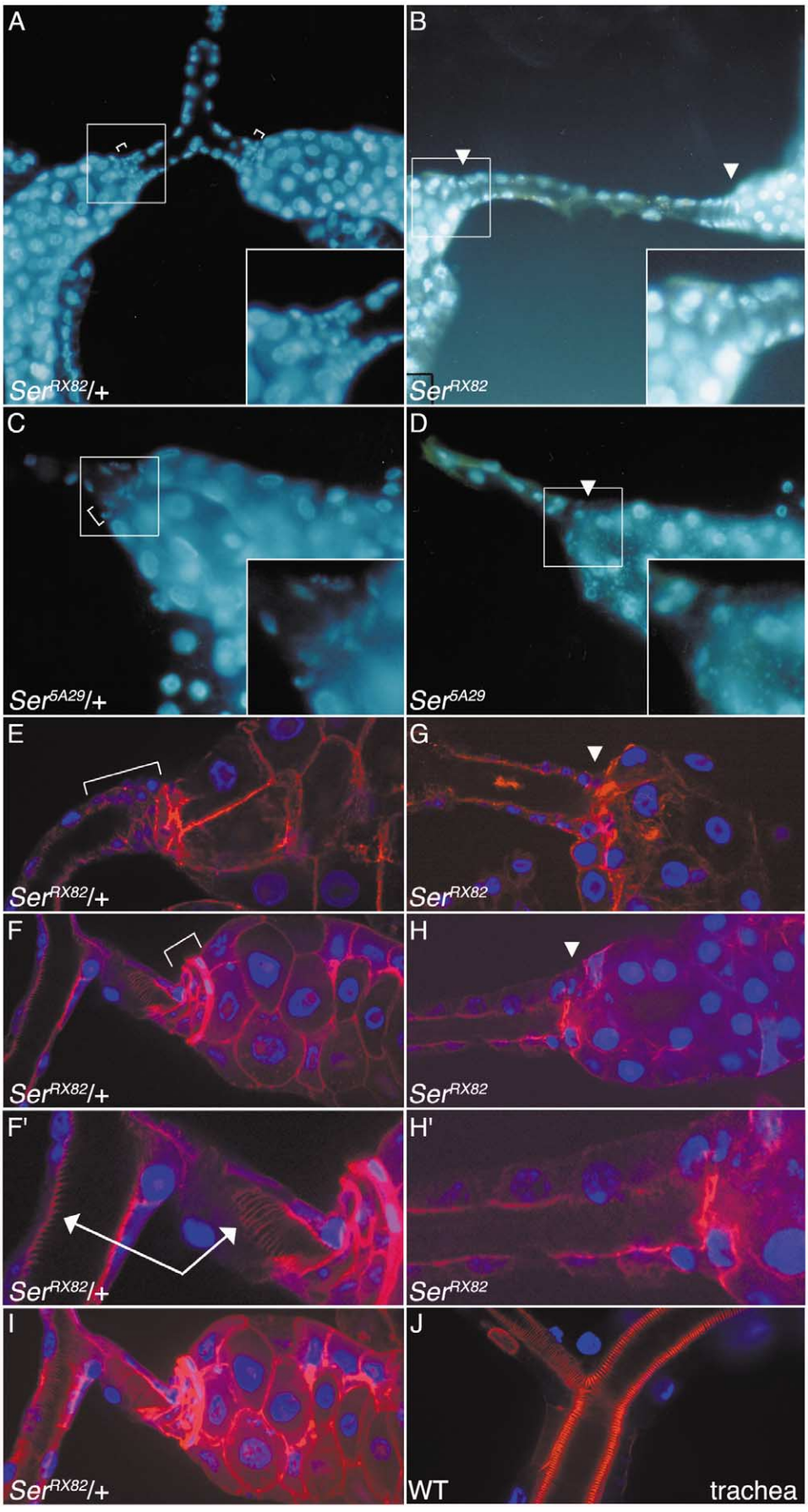
Ser directs formation of actin rings in the salivary duct

Phalloidin staining of second instar salivary glands revealed a unique organization of F-actin in the salivary ducts. While faint levels of cortical actin were observed in salivary duct cells, intense phalloidin staining was observed in bands that formed regular rings around the lumen of the salivary duct and imaginal ring (Fig. 5F′). Three-dimensional reconstruction of the salivary duct revealed that these actin rings encircled the entire salivary duct and imaginal ring of wild type and *Ser* heterozygous glands (Fig. 5I). These actin rings were missing from the ducts of *Ser* homozygous second instar glands (Fig. 5H′). The trachea had similar phalloidin-staining rings in every branch examined (Fig. 5J), although the rings in the trachea were unaffected by mutations in *Ser* (data not shown).

Discussion

trh is not the primary determinant of duct cell fate

Previous reports have proposed that *trh* is the primary gene responsible for salivary duct cell specification (Kuo et al., 1996). This model was based on two observations: that *trh* maintains its own expression, and that other duct genes depend on *trh* for their expression (Kuo et al., 1996; Wilk et al., 1996). We have found that both of these observations are incorrect. While *trh* does autoregulate in the trachea, it does not regulate itself in the salivary duct (Fig. 1C and D). Also, both *Ser* and *dri* are expressed in the duct cells independently of *trh* (Fig. 1E–J). Thus, *trh* is not the primary determinant of duct cell fate. Instead, our findings support an earlier model in which *trh* is required for the morphogenesis of the tubes that comprise the salivary duct, in keeping with its role in the trachea and filzkörper (Isaac and Andrew, 1996). In all three of these tissues, the primordial cells fail to invaginate and form tubular organs, although other tissue-specific markers are still expressed. Thus, we expect that, in the salivary duct, TRH regulates expression of genes required for tube morphogenesis, as has


A
Ser^RX82/+
B
Ser^RX82
C
Ser^5A29/+
D
Ser^5A29
E
Ser^RX82/+
G
Ser^RX82
F
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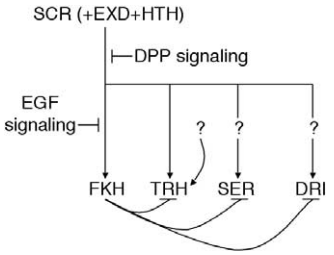

Fig. 6. *fkh* restricts expression of several genes to the salivary duct. SCR with its cofactors EXD and HTH direct expression of several salivary gland genes, including *fkh*, and other secretory cell genes, as well as the duct genes *trh*, *Ser*, and *dri*. Based on the time when *Ser* and *dri* transcripts first appear, their transcription may not be directly activated by SCR/EXD/HTH; an unknown intermediate molecule(s) may be involved, indicated by "?". DPP signaling represses expression of all salivary gland genes. EGF signaling represses expression of *fkh* and other secretory cell genes in the duct cells. FKH then represses *trh*, *Ser*, and *dri* expression in the secretory cells. Although this model states that FKH represses expression of duct genes, it does not rule out a role for additional secretory cell genes in this process.

been shown for the trachea (Ohshiro and Saigo, 1997; Zelzer and Shilo, 2000). Indeed, *btl*, which encodes the FGF-receptor required for tracheal branch migration, is a TRH target in both the trachea and salivary duct, although its role in the salivary duct is unclear, since the loss of *btl* does not overtly affect salivary gland formation (Ohshiro and Saigo, 1997; D.D.I. and D.J.A., unpublished results).

A role for FKH as a master regulator of secretory cell fates has been rejected by multiple groups (Bradley et al., 2001; Zhou et al., 2001). We propose a model where the salivary gland fate and the distinction between duct and secretory fate within the primordium is initiated by the coordinate system provided by the early patterning genes (Fig. 6). SCR/EXD/HTH in the absence of DPP- and EGF-signaling specifies the secretory cell fate, and SCR/EXD/HTH in the absence of DPP-signaling and in the presence of EGF-signaling specifies the salivary duct fate. As a consequence of this combinatorial system for cell fate specification, multiple different genes are activated in the different salivary gland cell types. It is the combined activities of these downstream genes that make secretory cells different from duct cells. Moreover, since *Scr* and *hth* expression disappears from the salivary gland quite early (Henderson and Andrew, 2000), the downstream target genes must maintain as well as elaborate on these cell fate decisions.

fkh suppresses duct gene expression in the secretory cells

fkh has many roles in secretory cell development. FKH prevents secretory cell apoptosis, mediates apical constriction during invagination, regulates its own expression, maintains expression of *dCREBA*, and regulates expression of the ecdysone-stimulated glue genes *sgs3* and *sgs4* (Weigel, 1989; Lehmann and Korge, 1996; Mach et al., 1996; Myat and Andrew, 2000). We found that FKH represses expression of all tested duct genes in the secretory cells (Fig. 6). In *fkh* mutants, *trh*, *Ser*, and *dri* are expressed throughout the salivary gland primordium in both duct and secretory cells (Fig. 3). It is unclear whether FKH directly regulates duct gene expression or regulates expression through some currently unidentified upstream activator(s). The 4-kb *Ser* salivary duct enhancer used in these studies contains several potential FKH binding sites, indicating that FKH repression of *Ser* could be direct (data not shown). FKH repression of duct gene expression suggests a role for FKH in reinforcing the secretory cell fate. FKH is required to maintain the distinction between duct and secretory primordia that is initially established by EGF-signaling (Fig. 7). First, EGF-signaling initiates the distinctions between duct and secretory cells by blocking expression of secretory-specific genes in the duct primordium. Then, the genes whose duct expression is blocked by EGF-signaling, specifically *fkh*, maintain this distinction by repressing duct gene expression and maintaining their own expression, thus sharpening the boundaries between duct and secretory primordia by interpreting the gradient of EGF-signaling into a binary cell fate decision.

Ser specifies the salivary gland imaginal ring

The gradient of EGF signal from the ventral midline initiates early differences in duct versus secretory cell populations. The boundary then becomes more firmly established by FKH. We propose that the salivary gland imaginal ring cells are then specified at the boundary between the duct and secretory cells in the salivary gland primordium (Fig. 7). While our assay for imaginal ring specification analyzes salivary glands 2 days after embryogenesis (Fig. 5), two lines of evidence suggest that imaginal ring specification occurs during embryogenesis. *Ser* is expressed in the salivary duct cells beginning at embryonic stage 11 (Kuo et al., 1996), when the duct cells and the adjacent secretory cells are still on the surface of the embryo. NOTCH, the receptor for SER, is transiently upregulated in the secretory cells at stage 11 (Kidd et al., 1989). Thus, at this stage, the gland has high-level expression of ligand in the duct primordia and high-level expression of the receptor in adjacent secretory cells and, therefore, is when signaling

Fig. 5. The salivary glands of second instar *Ser* larvae have no imaginal ring. Hoechst staining of *Ser* heterozygous (A, C) and homozygous (B, D) glands is shown. Confocal optical sections of Sytox (blue) and Texas Red-Phalloidin (red) staining of Ser^{RX82} heterozygous (E, F, F′) or homozygous (G, H, H′) glands and wild type trachea (J) is shown. Brackets indicate the imaginal ring. Arrowheads indicate where the missing imaginal ring would normally be. Arrows indicate the actin rings in the salivary duct. A three-dimensional reconstruction of confocal sections (I) reveals that actin rings encirle the entire salivary duct lumen.

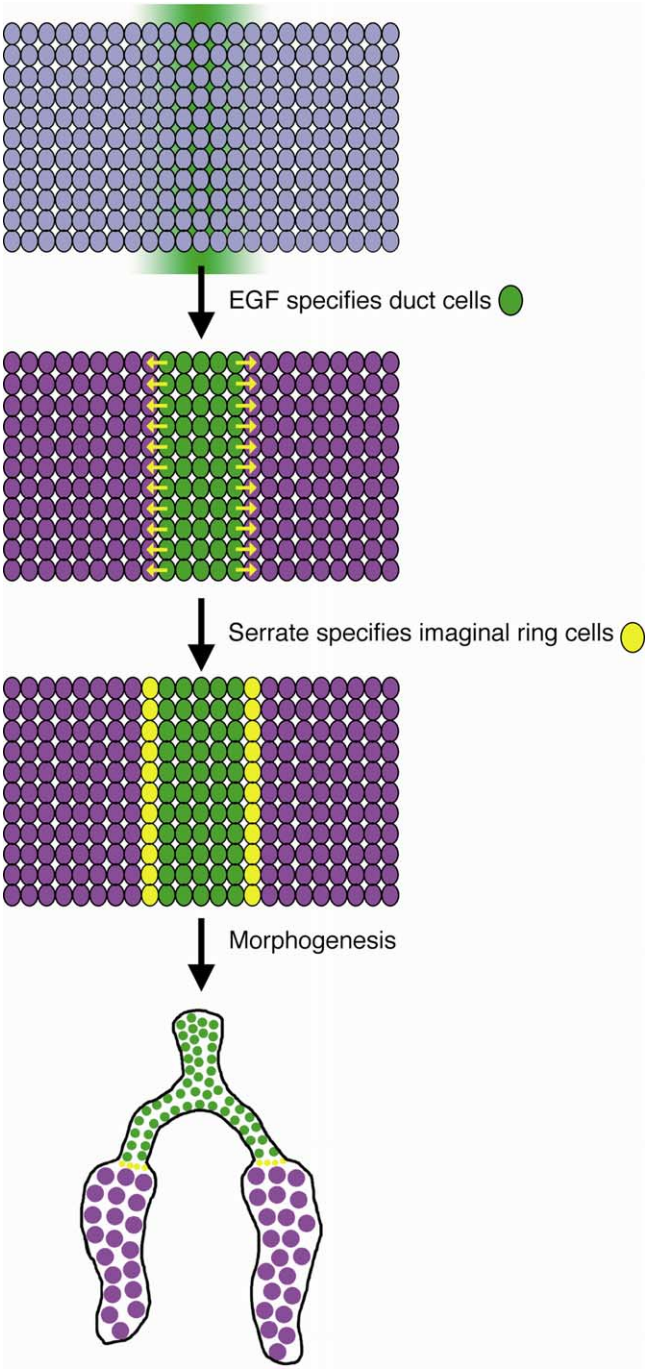

Fig. 7. The salivary gland primordium is divided into three cell types through the action of two signaling pathways. The salivary gland primordium is exposed to EGF ligand along the ventral midline (green box), distinguishing the salivary duct cells (green circles) from the secretory cells (purple circles). The duct cells express SER, and SER in the cells at the boundary between duct and secretory cells (yellow arrows) directs the secretory cells at the boundary to become imaginal ring cells (yellow circles). The cells then invaginate and migrate to form the various tubes of the mature salivary gland.

is likely to occur. Furthermore, the salivary ducts of *Ser* mutants have abnormal distal ends that can be observed in late stage embryos (Fig. 4), indicating that a defect in salivary gland formation has already occurred. While we were unable to assay for imaginal ring formation in the embryo due to a lack of markers for imaginal ring cells, the evidence suggests that *Ser* acts during embryogenesis to specify the imaginal ring. Nonetheless, we cannot rule out the possibility that SER specifies the imaginal ring at later embryonic stages when the salivary gland has internalized and when the salivary gland cells are in their final relative positions.

We were unable to directly test the role of NOTCH in imaginal ring specification because of the other role NOTCH plays in the ventral ectoderm. In *Notch* mutants, all the cells of the ventral ectoderm adopt a neuronal fate, as opposed to an epithelial fate, and salivary glands do not form due to an absence of epithelial precursors (Hartenstein et al., 1992). This role for *Notch* in protecting salivary gland cells from becoming neuronal appears to continue even after the salivary gland is specified. In mutants carrying the *Ser* allele *Beaded of Goldschmidt (BdG),* which encodes a secreted dominant-negative form of *Ser* (Hukriede and Fleming, 1997), salivary gland cells are missing, even though *BdG* is not expressed in or near the salivary gland until the salivary duct cells are specified (data not shown).

Since SER directs secretory cells to adopt the imaginal ring fate, we expected that ectopic SER would transform more secretory cells into imaginal ring cells. However, ectopic expression of *Ser* in secretory cells did not have any discernable effect on salivary glands (data not shown). We attribute this lack of phenotype to the observation that Notch signaling in the embryo depends on relative levels of ligand instead of absolute or threshold levels. Overexpression of *Ser* using a heat-shock promoter has no effect on wild-type cuticles, but overexpression of *Ser* in *Ser* mutants causes malformations of the mouth hooks and gut (Wiellette and McGinnis, 1999). Similarly, overexpression of *Ser* in the secretory cells may fail to disrupt the relative levels of Notch signaling in the salivary gland, resulting in a wild-type gland.

SER-mediated specification of the salivary gland imaginal ring is another example of a role for Notch signaling mediating boundary formation, the process of specifying a new cell type between two adjacent groups of cells by intercellular signaling. However, specification of the imaginal ring is different than the Notch-mediated boundary formation that occurs in imaginal discs. In the eye and wing imaginal discs, a boundary is formed at the interface between *Dl* and *Ser*-expressing cells. Similarly, both ligands are also involved in joint specification in the leg discs (reviewed in Irvine and Rauskolb, 2001). In contrast, only SER appears to be involved in specification of the imaginal ring. This situation is analogous to that of the embryonic hindgut, where DL is the only ligand required to specify the boundary cells in the large intestine (Fusse and Hoch, 2002; Iwaki and Lengyel, 2002). Thus, boundary formation in the salivary gland and hindgut represent a new class of boundary formation mediated by unidirectional Notch signaling.

SER regulates actin organization in the salivary duct

The salivary duct and trachea have regularly spaced actin rings encircling their lumena (Fig. 6). These actin rings are unlike actin structures described in other tissues, suggesting that they may be part of unique structures found only tubular tissues. The rings in the trachea may be associated with the taenidia, a series of epithelial folds along the lumen of the trachea that are postulated to give the trachea strength and flexibility (Manning and Krasnow, 1993). Both the actin rings and the taenidia appear to corkscrew around the lumen of the trachea. Such structures could also give the salivary duct the strength and flexibility it needs to carry secretory products to the larval mouth.

SER is required for actin rings to form in the salivary duct. While Notch signaling usually affects cell fate decisions, it has been shown to control cellular behaviors without affecting cell fate. Notch signaling directs neurons to arrest or retract neurites, a process involving changes in the actin cytoskeleton (Berezovska et al., 1999; Sestan et al., 1999). Also, Delta1 increases the cohesiveness and reduces the motility of cultured human keratinocytes (Lowell and Watt, 2001). Thus, SER could regulate the cytoskeleton of salivary duct and imaginal ring cells independently of cell fate specification. Though we cannot rule out the possibility that the loss of actin rings is a secondary effect of the general growth defects observed in *Ser* mutants, a role in actin ring formation would explain the prolonged expression of *Ser* in the salivary duct after imaginal ring specification. The fact that *Ser* does not control actin ring formation in the trachea correlates with the observation that *Ser* is only expressed in a subset of the trachea, while actin rings were found in every tracheal branch examined. However in the salivary gland, SER appears to control both the imaginal ring fate and the cytoskeleton of the salivary duct.

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Note added in proof. Subsequent to the acceptance of the final manuscript, the *Tsp74F* gene was shown by the Berkeley Drosophila Genome Project (http://www.fruitfly.org) to be expressed in the salivary gland imaginal ring ("adult salivary primorida") in stage 16 embryos. Expression of this transcript is not observed in the region of the imaginal ring in Ser^{RX82} homozygous embryos, further supporting the model that imaginal ring cells are not specified in the absence of Ser function.

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