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Fork head and Sage maintain a uniform and patent salivary gland lumen through regulation of two downstream target genes, *PH4αSG1* and *PH4αSG2*

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(Fkh) is required to block salivary gland apoptosis, internalize salivary gland precursors, prevent expression of duct genes in secretory cells and maintain expression of *CrebA*, which is required for elevated secretory function. Here, we characterize two new Fkh-dependent genes: *PH4αSG1* and *PH4αSG2*. We show through in vitro DNA-binding studies and in vivo expression assays that Fkh cooperates with the salivary gland-specific bHLH protein Sage to directly regulate expression of *PH4αSG2*, as well as *sage* itself, and to indirectly regulate expression of *PH4αSG1*. *PH4αSG1* and *PH4αSG2* encode α-subunits of resident ER enzymes that hydroxylate prolines in collagen and other secreted proteins. We demonstrate that salivary gland secretions are altered in embryos missing function of *PH4αSG1* and *PH4αSG2*; secretory content is reduced and shows increased electron density by TEM. Interestingly, the altered secretory content results in regions of tube dilation and constriction, with intermittent tube closure. The regulation studies and phenotypic characterization of *PH4αSG1* and *PH4αSG2* link Fkh, which initiates tube formation, to the maintenance of an open and uniformly sized secretory tube.

KEY WORDS: *Drosophila*, Fork head (Fkh), Prolyl-4-hydroxylase, Sage, Salivary gland, Tube morphogenesis

INTRODUCTION

The *Drosophila* embryonic salivary gland is an ideal model for studies of tube formation and organ specialization (reviewed by Andrew et al., 2000; Bradley et al., 2001; Abrams et al., 2003). Salivary gland formation requires the homeotic transcription factors Sex combs reduced (Scr), Extradenticle (Exd) and Homothorax (Hth); in loss-of-function mutants for any one of the corresponding genes, salivary glands fail to form. Moreover, ectopic expression of Scr leads to formation of additional salivary glands (Panzer et al., 1992; Andrew et al., 1994). Expression of *Scr* and *hth* and nuclear localization of Exd disappear shortly after the gland initiates morphogenesis (Henderson and Andrew, 2000), suggesting that genes specifying the salivary gland fate are not directly involved in terminal differentiation.

Several transcription factor genes are expressed in the early salivary gland under the control of Scr, Exd and Hth, including *fkh*, which encodes a Fox family winged-helix transcription factor homologous to mammalian Foxa2 (HNF3β) and *C. elegans* PHA-4 (Weigel et al., 1989a; Weigel et al., 1989b; Horner et al., 1998; Kalb et al., 1998). In the embryo, Fkh controls apical constriction of the salivary cells as they invaginate to form tubes and promotes salivary cell survival by inhibiting apoptosis (Myat and Andrew, 2000). Fkh also prevents the expression of duct-specific genes in the secretory region of the gland (Kuo et al., 1996; Haberman et al., 2003), maintains its own expression (Zhou et al., 2001) and maintains expression of *CrebA*, a bZip transcription factor required to upregulate expression of secretory pathway component genes (Abrams and Andrew, 2005). In prepupal larvae, Fkh activates expression of the *sgs* glue genes, which encode secreted proteins that allow the pupae to adhere to a substratum (Lehmann and Korge, 1996; Mach et al., 1996). Thus, Fkh is required throughout gland development.

To learn more about the range of activities Fkh has in the salivary gland and to understand the mechanisms of Fkh gene regulation, we identified and characterized two downstream targets with Fkh-dependent expression: *PH4αSG1* and *PH4αSG2*, referred to as *SG1* and *SG2*. We show that Fkh works with Sage, a salivary gland-specific bHLH protein, to regulate expression of *SG2* directly as well as expression of *sage* itself. Surprisingly, we show that although *SG1* is also regulated by Fkh and Sage, its regulation by these proteins is indirect.

SG1 and *SG2* encode homologues of the α-subunits of ER enzymes that hydroxylate proline residues in collagen, a major component of the extracellular matrix, as well as other secreted proteins (Kivirikko and Pihlajaniemi, 1998). As the *Drosophila* collagen genes are not expressed in the embryonic salivary gland (Le Parco et al., 1986; Knibiehler et al., 1990; Yasothornsrikul et al., 1997; Chartier et al., 2002), these enzymes must hydroxylate proline residues in other secreted or transmembrane proteins. Consistent with this idea, apical salivary gland secretions were altered in embryos missing *SG1* and *SG2*; secretory content was reduced in volume, was more electron dense and was less fibrillar when assessed by transmission electron microscopy. The loss of secretory volume correlated with regions of tube dilation, constriction and closure.

MATERIALS AND METHODS

Whole-mount in situ hybridization and immunohistochemistry

cDNAs RE03865 (*fkh*), RE59356 (*sage*), CK02267 (*SG1*) and LP12267 (*SG2*) were used for in situ hybridization using standard methods (Lehmann and Tautz, 1994). Immunohistochemistry was carried out using Biotin-conjugated horse-radish peroxidase (HRP) for light microscopy (Reuter et al., 1990) and the following primary antibodies: αSG1 (1:15,000, this work), αSG2 (1:8,000, this work), αβgal (1:5000; Promega; Madison, WI) and αFkh (1:1000). Biotin-conjugated secondary antibodies were used at a dilution of 1:500 and signal was amplified using the Vectastain kit (Vector Laboratories; Burlingame, CA). β-gal staining of all reporter constructs from

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the same gene were carried out at the same time with approximately the same volume of embryos (~100 μl) and were reacted for exactly 3 minutes to prevent variations in signal resulting from differences in embryo volume, antibody variation and reaction time differences. Immunohistochemistry for confocal microscopy was carried out using the following antibodies: αKDEL (1:500; Stressgen, San Diego, CA), αSG1 (1:5,000), αSG2 (1:1,000), αβgal (1:500; Promega; Madison, WI), αCrb [1:30; Drosophila Hybridoma Studies Bank (DHSB); Iowa City, Iowa], αCrebA (1:500) (Andrew et al., 1997), αSpectrin (1:1; DHSB), $\alpha\beta_H$spectrin (1:100) (Thomas and Kiehart, 1994) and αCatenin (1:250) (Oda et al., 1994). Fluorescently tagged Alexa-secondary antibodies were used at a dilution of 1:500 (Molecular Probes; Eugene, OR).

Antiserum production

Antiserum was generated in rat to an N-terminal fragment of SG1 and in rabbit to an N-terminal fragment of SG2 (Covance; Denver, PA). The details of cloning, protein production and purification are available upon request.

Fkh protein purification and electrophoretic gel mobility shift assays

The region encoding the DNA-binding domain (DBD) of Fkh was amplified from genomic DNA by PCR and subcloned into the expression vector pProEx (Invitrogen; Carlsbad, CA). Recombinant protein was induced by the same method used for antiserum production (contact authors for details) and purified under denaturing conditions by affinity purification with Ni-NTA agarose beads, according to the manufacturer's protocol (Qiagen, Germany). The resulting protein preparation, which was reasonably pure (a single visible band on Coomassie stained SDS-PAGE), was concentrated ~10× using a Vivaspin concentrator (Vivascience; Hannover, Germany) to a final concentration of 1 μg/μl and stored at 4°C until use.

Plus- and minus-strand oligonucleotides corresponding to the putative Fkh-binding sites upstream of SG1 and SG2 (see Figs 2 and 3 for sequences) were synthesized by the Johns Hopkins Biosynthesis and Sequencing Facility. The plus-strand oligos were kinase-labeled according to the manufacturer's protocol (Invitrogen; Carlsbad, CA) and annealed to the corresponding minus strand by boiling the samples for 5 minutes and cooling them to room temperature. Unlabelled double-stranded oligos were similarly prepared for competition experiments. Binding reactions were carried out in DNA-binding buffer (20 mM HEPES, pH 6.8; 40 mM KCl), 1 mM DTT, 10% glycerol, 2 μg of Fkh DBD protein and 11.9 pmol of labeled oligonucleotide probe. Poly dIdC (1.4 μg) was added as a nonspecific binder to each reaction. Cold competitor oligos were added last (at 15×, 30× and 60× the concentration of the labeled oligos) and the reactions were incubated for 20 minutes on ice. Binding reactions were run on 7% polyacrylamide gels (50 mM HEPES pH 6.8) for 6 hours at 10 mA at 4°C and prepared for autoradiography by standard methods.

Fly strains

fkh^{6} *H99* (Myat and Andrew, 2000) and *fkh*^{P261} (M. M. Myat and D.J.A., unpublished) were used for *fkh* regulation studies. *Df(3R)Exel6216* was generated by Exelixus (Parks et al., 2004). Mutant and deficiency lines were maintained over a third chromosome balancer (*TM3* or *TM6B*) carrying *Ubx-lacZ* or *twi-GFP* constructs to allow identification of homozygous mutants by the absence of *lacZ* hybridization, βgal staining or GFP fluorescence. *fkh-GAL4* (Henderson and Andrew, 2000) or *tubulin-GAL4* (Lee and Luo, 1999) were used to drive expression of UAS constructs.

Generation of reporter gene and expression constructs

The details for subcloning the open reading frames (ORF) for *sage*, *SG1* and *SG2* into pUAST (Brand and Perrimon, 1993), and for subcloning the salivary gland enhancers for all three genes into the Casper β-gal vector (Thummel et al., 1988) are available upon request. Mutations in candidate binding sites were made by site directed mutagenesis using the QuikChange XL Kit or QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene; Cedar Creek, TX).

The reporter gene and expression constructs were introduced into the germline of w^{1118} flies (Spradling and Rubin, 1982). The insertions were mapped to individual chromosomes genetically by following the w^{+} eye color marker using marked balancer chromosomes.

RESULTS

Fork head is required for the salivary gland expression of SG1 and SG2

We previously discovered a complex of ten genes encoding prolyl 4-hydroxylase α subunits, six of which showed tissue-specific embryonic expression (Abrams and Andrew, 2002). Two of the genes, *SG1* and *SG2*, were specifically expressed in the salivary glands beginning at stage 11 and continuing through embryogenesis and larval stages (Fig. 1A, B; data not shown). To learn which early transcription factors regulate *SG1* and *SG2*, we examined expression of the two genes in embryos mutant for the salivary gland genes, *fkh*, *CrebA* and *huckebein* (*hkb*). Although *CrebA* and *hkb* had no effect on expression, *SG1* and *SG2* mRNA disappeared in *fkh* mutants (Fig. 1A,B; data not shown). As with the mRNAs, SG1 and SG2 proteins, which localize to the ER (Fig. 1E), were not detected in the *fkh* mutants (Fig. 1D; data not shown). Thus, salivary gland expression of *SG1* and *SG2* requires Fkh.

To begin to identify sequences required for *SG1* and *SG2* expression, we generated transgenic lines carrying reporter gene constructs with ~1 kb of upstream DNA, including a small region of the 5′ end of the ORF, fused to *lacZ* (Fig. 2A and 3A; *SG1 972-lacZ* and *SG2 885-lacZ*). As with the endogenous genes, *lacZ* expression (measured by βgal staining) from all of the transgenic lines carrying each reporter gene construct was observed in the salivary gland beginning at stage 11 and continuing through embryogenesis (Fig. 2B-D; Fig. 3B-D). Variable expression of βgal was also observed in other cells; for example, expression of βgal was seen in the embryonic hemocytes with one *SG1 972-lacZ* line (Fig. 2B-D,H,I) and in a subset of gut endodermal cells with all the *SG2 885-lacZ* lines (Fig. 3B-D,H). Salivary gland staining for both the *SG1* and *SG2* constructs disappeared in *fkh* mutants (Fig. 2I, Fig. 3I), although the hemocyte-specific staining with the *SG1 972-lacZ* line was still observed (Fig. 2I).

To test whether Fkh regulation of *SG1* and *SG2* is direct, we first asked if Fkh binds in vitro to the sequences in each enhancer that match published core Fkh-binding sites (Lehmann and Korge, 1996; Mach et al., 1996; Lehmann et al., 1997). Fkh bound strongly to site 'd' in the *SG1 972* enhancer, as determined by electrophoretic mobility shift assays (EMSA) (Fig. 2A, part a). This consensus-binding site maps ~100 bp upstream of the ATG start codon. Weak and potentially non-specific binding was also observed with site 'b', which maps ~230 nucleotides upstream (Fig. 2A, part b, left gel; increased amounts of unlabeled site 'b' DNA did not decrease binding to labeled site 'd'). Double-stranded oligos corresponding to the two remaining consensus Fkh-binding sequences in the *SG1 972* enhancer were not bound by Fkh (sites 'a' and 'c') and did not compete for binding to site 'd' by EMSA (data not shown). Fkh bound to three out of the four consensus binding sequences in the *SG2 885* enhancer. DNA fragments corresponding to sites 'f' and 'h' were bound strongly by Fkh (Fig. 3A, parts a,b, left gels), whereas site 'g' showed moderate binding and did not compete well with the strong binding site 'h' (data not shown). The in vitro Fkh binding sites in *SG2* map between 120 and 170 nucleotides upstream of the start codon.

To test if regulation of *SG1* and *SG2* is through the in vitro Fkh-binding sites, we mutated the *SG1* and *SG2* Fkh-binding sites in each reporter gene construct. Double-stranded DNA fragments corresponding to these mutations did not compete for in vitro binding of Fkh by EMSA (Fig. 2A, part a; Fig. 3A, parts a,b, right gels; data not shown). Surprisingly, expression of βgal from *SG1 972 fkh-lacZ* (sites 'b' and 'd' mutated) showed little change in salivary gland expression (Fig. 2E-G). Expression of βgal from *SG2*

885 fkh-lacZ (sites 'f', 'g' and 'h' mutated) was distinct from wild-type *SG2 885-lacZ*; early expression was reduced and later expression was variable (Fig. 3E-G). These results indicate that expression of *SG1* is unlikely to be controlled directly by Fkh and that expression of *SG2* is only partially directly dependent on Fkh.

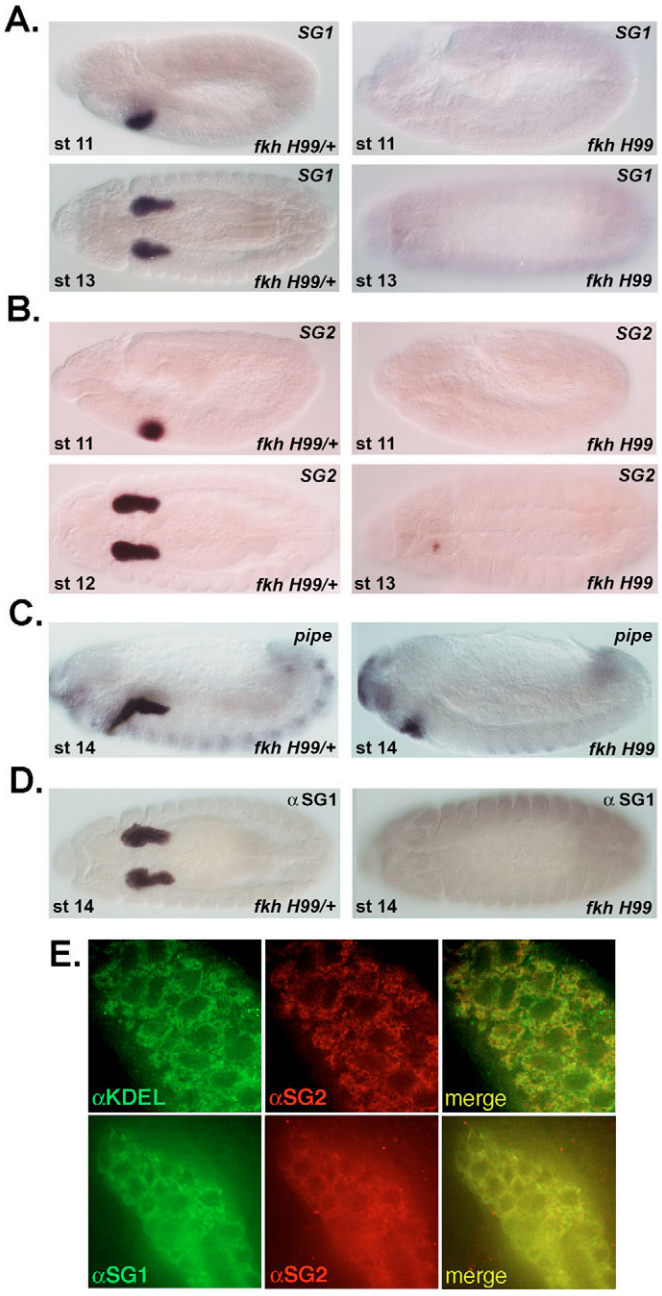

Fig. 1. Fkh regulates expression of *SG1* and *SG2*. (**A**,**B**) Expression of *SG1* and *SG2* RNA and (**D**) SG1 protein was absent in *fkh* mutants. (**C**) Expression of *pipe* was unaffected by loss of *fkh*. (**E**) SG2 (red) colocalized with the ER marker KDEL (green) and SG1 (green) colocalized with SG2 (red) (top and bottom panels, respectively). We examined expression of genes with a *fkh* null mutation in the background of the *H99* deficiency, which removes the pro-apoptotic genes; this background allows salivary gland cell survival in a *fkh* mutant (Myat and Andrew, 2000). Expression of *pipe*, *SG1* and *SG2* were the same in *fkh* mutants that also did not carry the *H99* deficiency.

Fkh is required for expression of Sage

The above experiments suggest that although Fkh is required for *SG1* and *SG2* expression, its role is likely to be at least partly indirect and through other Fkh-dependent transcription factors. *sage*, which encodes a bHLH transcription factor expressed in only the salivary gland, is an attractive candidate for contributing to *SG1* and *SG2* activation (Fig. 4A) (Moore et al., 2000; Chandrasekaran and Beckendorf, 2003). *sage* is first detected during embryonic stage 10, about the same time as *fkh* expression is first observed, and continues to be expressed in the salivary gland through the remainder of embryogenesis (Fig. 4A) and throughout larval life (Li and White, 2003). In *fkh* mutants, initial expression of *sage* was unaltered; however, *sage* expression disappeared during embryonic stage 12 and was undetectable by embryonic stage 13 (Fig. 4B). Thus, Fkh is required to maintain but not initiate *sage* expression.

To determine if Fkh directly controls *sage* expression, we built a *sage* reporter gene construct by fusing 1.2 kb of *sage* DNA, containing upstream sequences and spanning the translation start site, to a *lacZ* reporter (Fig. 5A). Expression from independent transformant lines carrying this construct (*Sage 1.2-lacZ*) was salivary-gland-specific, in a pattern consistent with expression of endogenous *sage* (Fig. 5B,C). In *fkh* mutants, a notable reduction in βgal expression was observed with this construct, indicating that it includes a Fkh-dependent salivary gland enhancer (Fig. 5D,E). The 1.2 kb fragment contains a single consensus Fkh-binding site (Fig. 5A, blue oval and sequences), which maps ~230 nucleotides upstream of the start codon and matches 16/16 nucleotides of one of the two strongest in vitro Fkh binding sites in the *SG2 885* enhancer (site 'h'; Fig. 3). To test if Fkh works through this site to maintain *sage* expression, we established transgenic fly lines carrying the *sage* reporter gene construct in which the Fkh site was mutated. βgal expression from all four *Sage 1.2 fkh-lacZ* lines was reduced compared with expression from lines carrying the wild-type version of the construct (Fig. 5F,G). Thus, the Fkh-binding site in the *sage* enhancer is required for wild-type levels of expression, but the relatively high level of expression that persists suggests that other factors must also contribute.

The wild-type *sage* reporter gene construct also contains five consensus bHLH-binding sites (CANNTG; Fig. 5A). To test whether *sage* auto-regulates through these sites, we created transgenic lines carrying mutated versions of the reporter construct in which either only the bHLH consensus sites were mutated (*Sage 1.2 bHLH-lacZ*) or both the bHLH sites and Fkh site were mutated (*Sage 1.2 fkh bHLH-lacZ*). *Sage 1.2 bHLH-lacZ* had reduced βgal expression compared with levels from the wild-type construct (compare Fig. 5H,I with Fig. 5B,C). *Sage 1.2 fkh bHLH-lacZ* lines expressed βgal to levels seen with the wild-type construct in *fkh* mutants (compare Fig. 5J,K with Fig. 5D,E). These findings suggest that both Fkh and a bHLH protein directly activate the robust levels of *sage* expression observed in the wild-type salivary gland. Although we cannot definitively identity the bHLH protein involved, we favor the idea that Sage autoactivates via the bHLH binding sites.

Sage and Fkh control high level SG2 expression

To determine whether Sage activates expression of *SG1* or *SG2*, we would ideally examine their expression in *sage* loss-of-function mutants, but such mutations have not been reported and the deficiency that removes *sage* also removes *neuralized*, a neurogenic gene required to form non-neuronal ectodermal derivatives in the ventral region of the embryo, including salivary

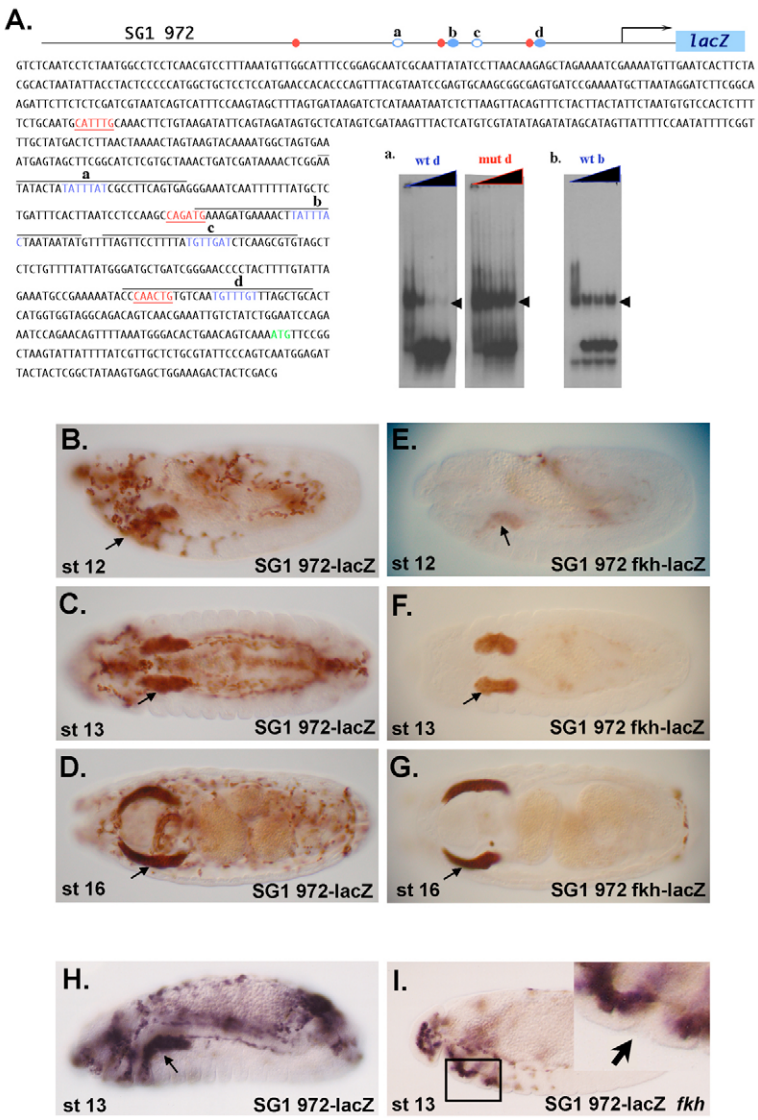

Fig. 2. Regulation of *SG1* by Fkh is indirect. (**A**) 972 nucleotides of sequence upstream and spanning the translation start site (green letters) of *SG1* were sufficient to direct Fkh-dependent salivary gland expression of the *lacZ* reporter gene. Only two of the four putative Fkh-binding sites in the 972 nucleotide sequence (b and d, blue sequence and filled blue ovals) were bound by Fkh protein in vitro and competed for binding (Aa, Ab; arrowheads indicate P^{32}-labelled oligos, the migration of which in the gel has been slowed owing to Fkh binding; the triangles on the top of each gel indicate relative concentrations of cold competitor). Whereas unlabelled wild-type d oligos competed well for binding with labeled wild-type d, the mut d oligo did not compete (compare left and right gels in Aa, arrowheads). Wild-type b oligos competed less well than did wild-type d for binding but better than mut d (compare gel in Ab with gels in Aa, arrowheads). Three consensus bHLH binding sites (CAXXTG) are present in the 972 nucleotide SG1 enhancer (red sequences and red filled circles). (**B-D**) Wild-type versions of the SG1 972-lacZ construct gave high level βgal expression in the salivary gland (arrows) and variable expression in other tissues (non-salivary gland staining in B-I corresponds to hemocytes). (**E-G**) Mutations in the two in vitro Fkh-binding sites (b and d) did not affect the salivary gland expression of the reporter constructs. (**H**,**I**) Expression of the SG1 972-lacZ construct was absent in salivary glands (arrows) of *fkh* mutants, although expression in other tissues (hemocytes) was unaffected (here, embryos were stained in the presence of NiCl, causing the reaction substrate to turn purple/black instead of red/brown, as in the embryos in B-G).

glands (Hartenstein et al., 1992). Therefore, we asked if expression of Sage in new places induces ectopic expression of either *SG1* or *SG2,* using a ubiquitously expressed *tubulin*-GAL4 to drive expression of a wild-type *sage* cDNA (*tub-GAL4:UAS-sage*). In these embryos, both *SG1* and *SG2* mRNAs were expressed at ectopic sites. In addition to its normal salivary gland expression, *SG1* mRNA was detected in paired ectodermal stripes within each segment, as well as in the foregut and hindgut during stage 11 (Fig. 6A, part a). At later stages, *SG1* mRNA was detected in the foregut, hindgut and Malpighian tubules. Ectopic *SG2* mRNA was detected in regions of the foregut and hindgut, and in the Malpighian tubules (Fig. 6Ab). Thus, Sage can activate expression of *SG1* and *SG2* in new places, making it a likely regulator for both genes. Importantly, the ectopic domains of *SG1* and *SG2* expression were limited to cells that also express Fkh (Fig. 6A, part c), suggesting that Fkh may also regulate an essential co-factor for Sage, potentially a bHLH partner protein.

To learn if Sage could contribute directly to expression of *SG1* or *SG2*, we further mutated the *SG1 972-lacZ* and *SG2 885-lacZ* reporter constructs to disrupt all of the bHLH-binding sites to which Sage is predicted to bind (*SG1 972 bHLH-lacZ* and *SG2 885 bHLH-lacZ*; Figs 2, 6). The bHLH-binding site mutations were also combined with mutations in the Fkh-binding sites (*SG1 972 fkh bHLH-lacZ* and *SG2 885 fkh bHLH-lacZ*; Fig. 2, Fig. 6B,C). The mutations in the bHLH-binding sites in *SG1 972 bHLH-lacZ* had very little, if any, effect on the pattern or levels of βgal expression when compared with the wild-type construct (compare Fig. 6B, parts a-a″ with Fig. 2B-D). Similarly, lines carrying the *SG1* construct with mutations in both the Fkh and bHLH sites (*SG1 972 fkh bHLH-lacZ*) had levels of expression comparable with the wild-type construct (compare Fig. 6B, part b with Fig. 2B-D). These findings suggest several possibilities: (1) Fkh/Sage regulation of *SG1* is direct and sites that do not bind on their own in vitro bind in vivo, perhaps through cooperative binding; (2) Fkh/Sage regulation of *SG1* is redundant with another Fkh/Sage-dependent transcription factor(s); or (3) Fkh/Sage regulation of *SG1* is largely indirect and through an unidentified Fkh/Sage-dependent transcription factor(s), a possibility we strongly favor.

βgal expression from the construct containing the *SG2* enhancers with mutations in the consensus bHLH-binding sites (*SG2 885 bHLH-lacZ*) was somewhat reduced and variable at later stages compared with expression of the wild-type construct (*SG2 885-lacZ*; Fig. 6Ca). βgal expression was nearly absent with the construct in which both the Fkh and consensus bHLH sites were mutated (*SG2*

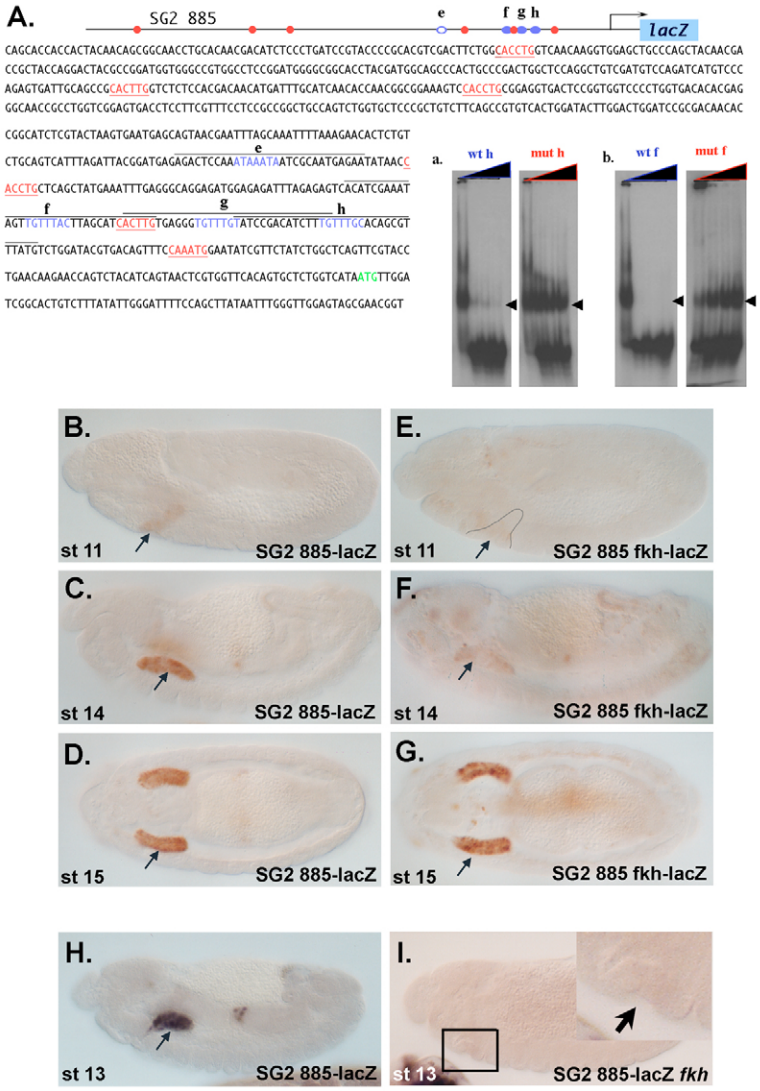

Fig. 3. Regulation of *SG2* by Fkh is direct. (**A**) 885 nucleotides of sequence upstream and spanning the translation start site of *SG2* (green letters) was sufficient to direct Fkh-dependent salivary gland expression of the *lacZ* reporter gene. Although the 885 nucleotide sequence contains four putative Fkh-binding sites (A, blue ovals and blue sequences), only three of the sites (f,g,h, blue filled ovals) were bound by Fkh protein in vitro or competed for binding (Aa, Ab). Whereas both unlabelled wild-type h and wild-type f compete well for binding with P^{32}-labelled wild-type h, neither mut h nor mut f compete for binding (compare left to right gels in Aa and Ab). Adding cold competitor in all cases reduced the amount of labeled oligo that failed to enter the gel, making it appear as if the addition of mutant oligos increased the specific binding of Fkh to the labeled oligo, especially on the gel on the right with the mut site f competitor. Six consensus bHLH binding sites (CAXXTG) are present in the 885 nucleotide SG2 enhancer (A, red circles and red sequences). (**B-D**) Embryos carrying a wild-type SG2 885-lacZ construct had βgal expression in the salivary glands (arrows) beginning at stage 11 and continuing through embryogenesis. (**E-G**) Embryos carrying the SG2 885 fkh-lacZ constructs with mutations in the three Fkh in vitro binding sites (f,g,h) had reduced salivary gland expression at early stages (arrow indicates the area outlined in black, which is the unstained salivary gland) and variable expression at later stages (arrows in F,G). (**H**,**I**) The salivary gland expression (arrows) of the SG2 885-lacZ construct visible in wild type (H) was absent in the salivary glands of *fkh* mutants (I). Embryos in H,I were stained in the presence of NiCl.

885 fkh bHLH-lacZ; Fig. 6C, part b). These findings indicate that *SG2* expression is controlled directly by a combination of Fkh and a bHLH protein, most probably Sage.

SG1 and SG2 are required for maintaining the salivary gland lumena

To determine which aspects of Fkh (and Sage) function *SG1* and/or *SG2* mediate, we examined Crumbs (Crb) staining in the salivary glands of *Df(3R)Exel6216* embryos; Crb spans the apical membranes of epithelial cells, with high levels in a domain just apical to the adherens junctions, allowing examination of lumen morphology. *Df(3R)Exel6216* removes most of the genes in the PH4α 99F complex, including *SG1* and *SG2* (Fig. 7A). In embryos stage 15 and older, defects in the apical surfaces of the secretory cells were consistently observed; specifically, the lumena were irregular with regions where intense Crb staining appeared to span across the lumen (Fig. 7B). Confocal imaging of salivary glands stained with different combinations of nuclear (CrebA), apical (Crb, β_HSpectrin), subapical (αCatenin) and basolateral markers (αSpectrin) revealed gross irregularities in the salivary gland lumena in 100% of deficiency embryos embryonic stage 15 and older (Fig. 7C). The lumena had regions of expansion and constriction, as well as regions where the tubes appeared closed [Fig. 7C (asterisks); data not shown]. Confocal sections revealed that the small lumena were often surrounded by Crb and β_HSpectrin staining (Fig. 7C, middle right panels; data not shown), again suggesting that the tubes are occluded. Three-dimensional projections of salivary glands stained for CrebA and Crb revealed lumena of uniform diameter in late stage wild-type salivary glands that were never observed in the deficiency embryos; instead, the lumena from deficiency embryos were always irregular, with regions of both dilation and constriction (Fig. 7D). Cell polarity and other aspects of gland morphology were not significantly altered, although the mutant glands displayed some level of curvature and shape irregularities, probably owing to the changes in cell contact at the apical surfaces.

To determine if the loss of *SG1* and/or *SG2* causes the abnormal salivary gland lumen morphology, we created embryos that were homozygous for the deficiency but also carried the salivary gland specific *fkh*-GAL4 driver in trans to UAS-*SG1* or UAS-*SG2*. Staining with Crb antibody revealed variable rescue of the salivary gland (data not shown). Confocal analysis of embryos from the same crosses simultaneously stained for either SG1 or SG2 and for Crb revealed a direct correlation between detectable expression of the proteins and normal lumen morphology. In embryos in which we could not detect either SG1 or SG2 protein from the *fkh*-GAL4 driven UAS-*SG1* or UAS-*SG2* constructs, the lumena had the same

defects observed in the homozygous deficiency embryos (Fig. 7E, middle panels). In embryos of the same genotype in which we could detect either SG1 or SG2 protein, lumen morphology appeared normal (Fig. 7E, bottom panels). Thus, expression of either gene is sufficient to rescue the deficiency phenotypes, suggesting that the SG1 and SG2 are interchangeable, consistent with their high degree of sequence homology (36.2% identity, 53.5% similarity).

To gain insight into the *Df(3R)Exel6216* phenotype, salivary glands were imaged by transmission electron microscopy (TEM). TEMs of wild-type stage 16/17 salivary glands showed relatively uniformly sized lumena with apparent narrowing only in the regions where the salivary glands normally curve (Fig. 8A-A‴). By contrast, TEMs of salivary glands from the deficiency embryos revealed gross lumenal irregularities, with small lumena surrounded by large regions where the tubes appeared to be closed (Fig. 8B-B‴,C-C′). Given the irregular lumenal morphologies observed in the deficiency embryos with confocal microscopy, it is likely that more lumena are present in these glands than is suggested by the TEMs; it is simply the irregularity of the lumen that limits the amount captured in a single thin section. High-magnification TEM images revealed structures characteristic of adherens junctions (AJs) adjacent to the small lumena (arrowheads in Fig. 8C′). These junctions could be newly formed complexes created when cells that were originally separated by the lumen came into direct contact.

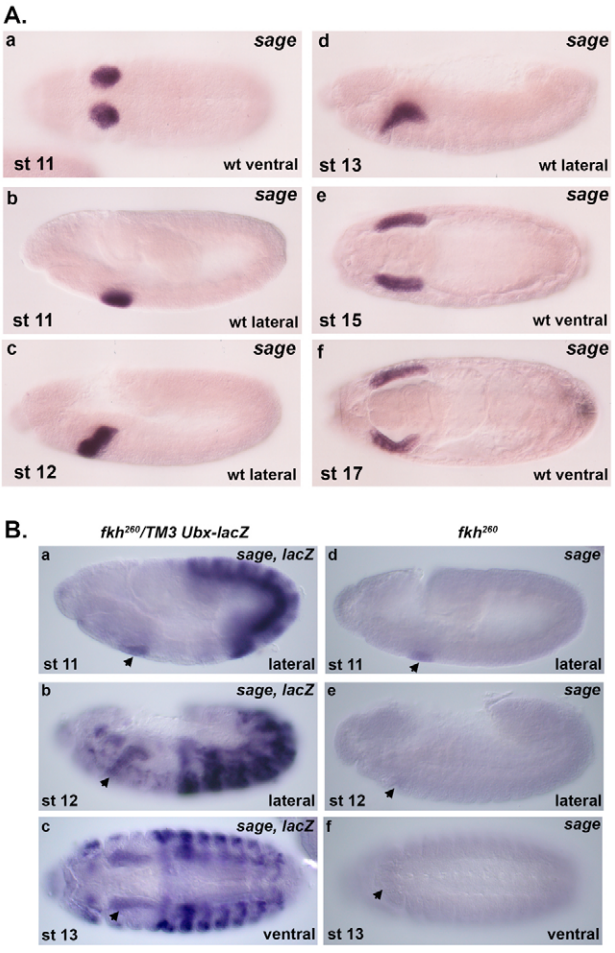

Fig. 4. *sage* is expressed specifically in the salivary gland and its later expression requires Fkh. (**A**) *sage* was detected in the salivary glands of wild-type embryos beginning at the placode stage through the end of embryogenesis. (**B**) *sage* expression after stage 12 was absent in *fkh* mutants. Compare staining indicated by arrows in left panels in the *fkh* heterozygotes, which also show *lacZ* expression from the *TM3 Ubx-lacZ* balancer chromosome (a-c), with staining indicated by arrows in the *fkh* mutants in the right panels (d-f). Salivary glands do not invaginate in *fkh* mutants (d-f).

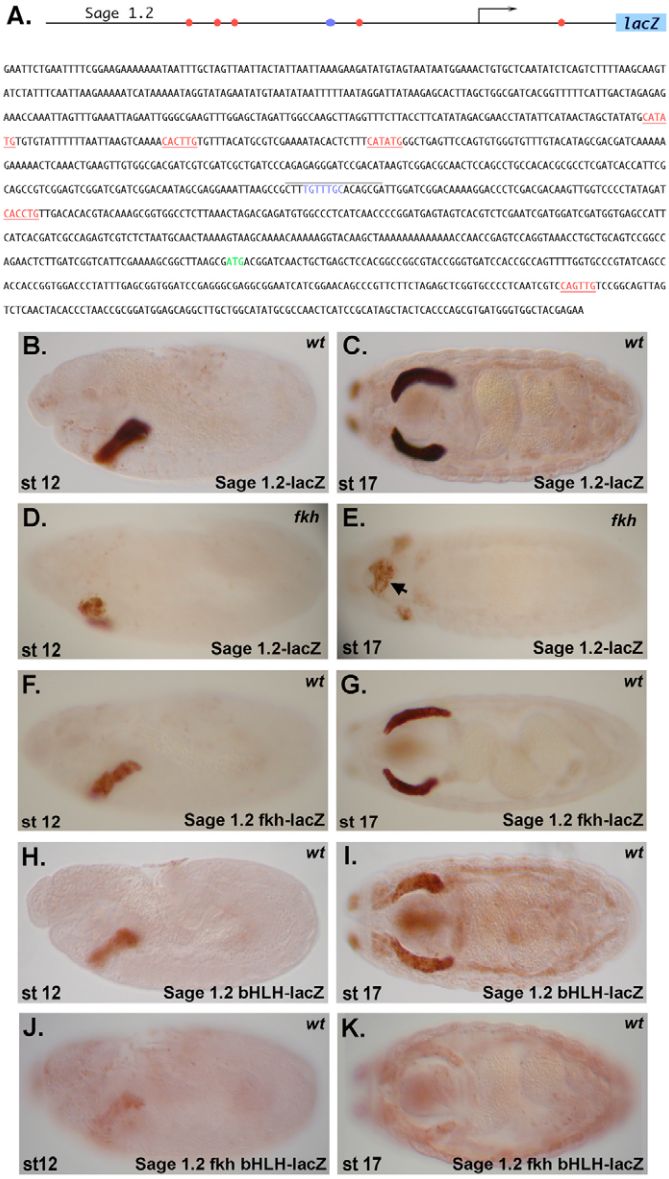

Fig. 5. *sage* is directly regulated by Fkh and Sage. (**A**) 1.2 kb of sequence upstream and spanning the translation start site (green letters) of *sage* is sufficient to direct Fkh-dependent salivary gland expression of the *lacZ* reporter gene. This sequence contains five consensus bHLH-binding sites (red circles and sequences) and one Fkh-binding site (blue oval and sequence). (**B**,**C**) The wild-type Sage 1.2-lacZ construct gave robust salivary gland expression both early and late. (**D**,**E**) Expression of the wild-type Sage 1.2-lacZ was diminished in *fkh* mutant salivary glands, which do not invaginate. (**F**,**G**) Mutations disrupting either the Fkh site or (**H**,**I**) the bHLH sites resulted in reduced salivary gland expression of βgal. (**J**,**K**) Loss of both Fkh and bHLH sites resulted in reduced expression of the reporter gene to levels observed in *fkh* mutants (compare salivary gland expression of βgal in J and K with D and E).

The TEMs also revealed a decrease in apically secreted material and a notable increase in the electron density of the secretions (Fig. 8A-C, asterisks). This difference in secretory volume and staining intensity was also observed in thick sections of glands stained with toluidine blue (data not shown). In addition, the lumenal material in the wild-type glands had a more 'fibrillar' appearance than that of the deficiency embryos (compare Fig. 8A‴ with 8B‴ and 8C′), although the electron density of the secretions in the deficiency animals could make such fibrillar structure difficult to discern. Interestingly, the number, size and electron density of the secretory granules found in the apical regions of the salivary gland cells from the wild-type and deficiency embryos were not different (compare Fig. 8A″ with 8B″ and 8C), suggesting that the altered properties of the secretory content arose post-secretion. The correlation between altered lumenal content and irregularities in lumen size and morphology suggests a role for apical secretions in maintaining a uniformly sized and open salivary gland tube.

DISCUSSION

Fkh regulation of salivary gland genes

The diverse activities of Fkh support a major role for this protein in controlling many of the tissue specific functions of the salivary gland. Indeed, the long list would support a model wherein Fkh could be viewed as an organ-specifying gene, much like the role proposed for its *C. elegans* homologue, PHA-4, in pharynx development (Gaudet and Mango, 2002). Nonetheless, our studies reveal notable differences in the roles of the two genes in organ formation. PHA-4 is proposed to directly regulate expression of all pharynx-specific genes (Gaudet and Mango, 2002), whereas Fkh is required for the expression of only about one-third of the salivary gland-specific genes tested so far (>200 genes; E. Grevengoed and D.J.A., unpublished). Moreover, many downstream genes are indirect targets of Fkh. For example, although Fkh is required for high-level expression of 34 secretory pathway component genes, its role in their activation is largely indirect through maintenance of

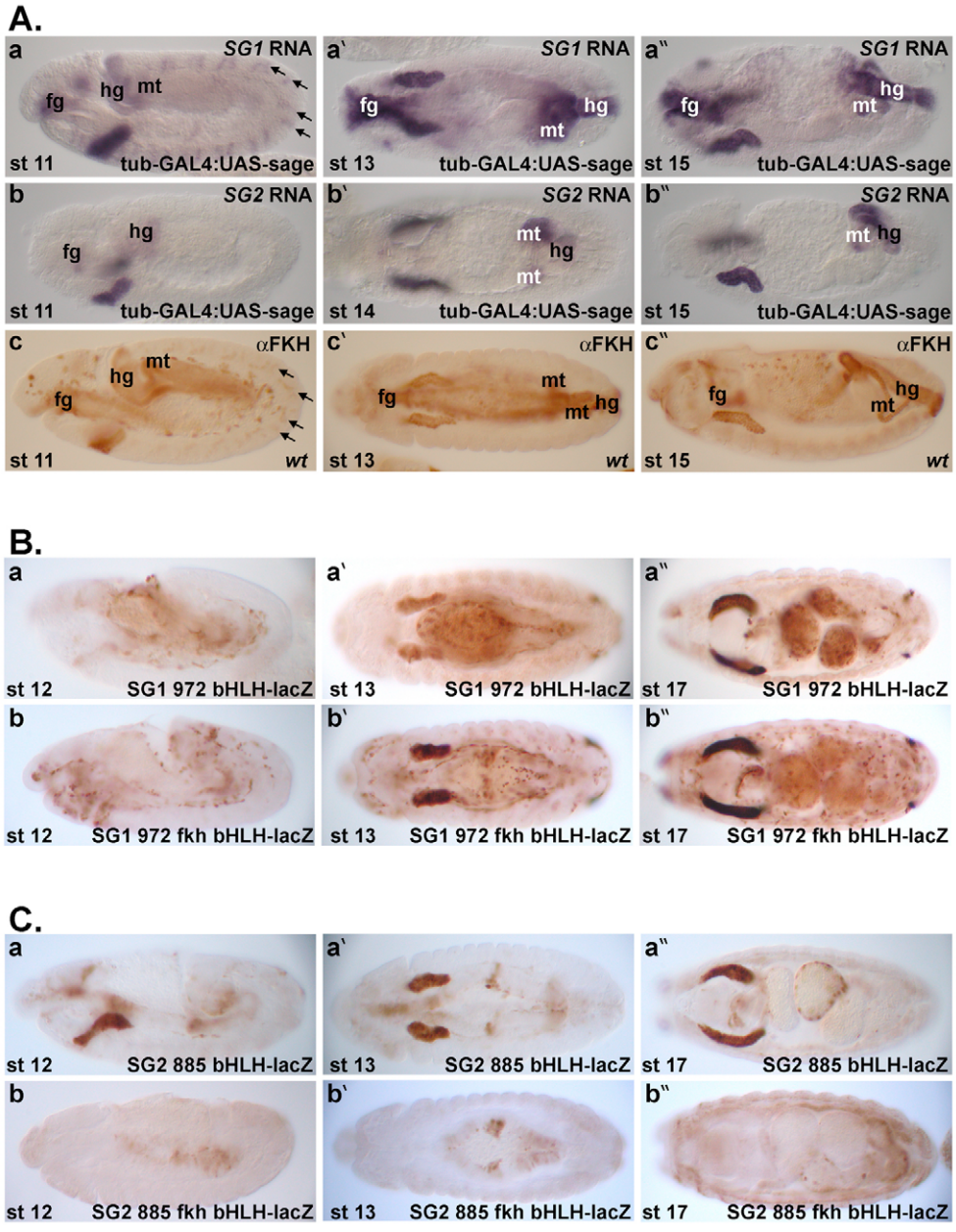

Fig. 6. *SG2* is directly regulated by Fkh and Sage, whereas *SG1* is not. (**A**) Global expression of *sage*, achieved by driving UAS-*sage* with a *tub*-GAL4 driver, resulted in ectopic expression of both *SG1* and *SG2* in tissues outside the salivary gland, notably in the foregut (fg), hindgut (hg) and Malphigian tubules (mt) (Aa-a″, Ab-b″). (Aa) *SG1* was also expressed in two rows of cells in each segment during stage 11 (arrows). (Ac-c″) The tissues that expressed ectopic *SG1* and *SG2* also express Fkh protein, including faint expression in two rows of cells in each segment during stage 11 (Ac, arrows). (**B**) Mutations in the bHLH binding sites (Ba-a″) or the bHLH and Fkh-binding sites (Bb-b″) in the SG1 972 enhancer did not affect expression of the *lacZ* reporter. (**C**) Mutations in the bHLH binding sites (Ca-a″) or the bHLH and Fkh-binding sites (Cb-b″) in the SG2 885 enhancer resulted in either reduction or complete loss of salivary gland expression of the *lacZ* reporter, respectively.

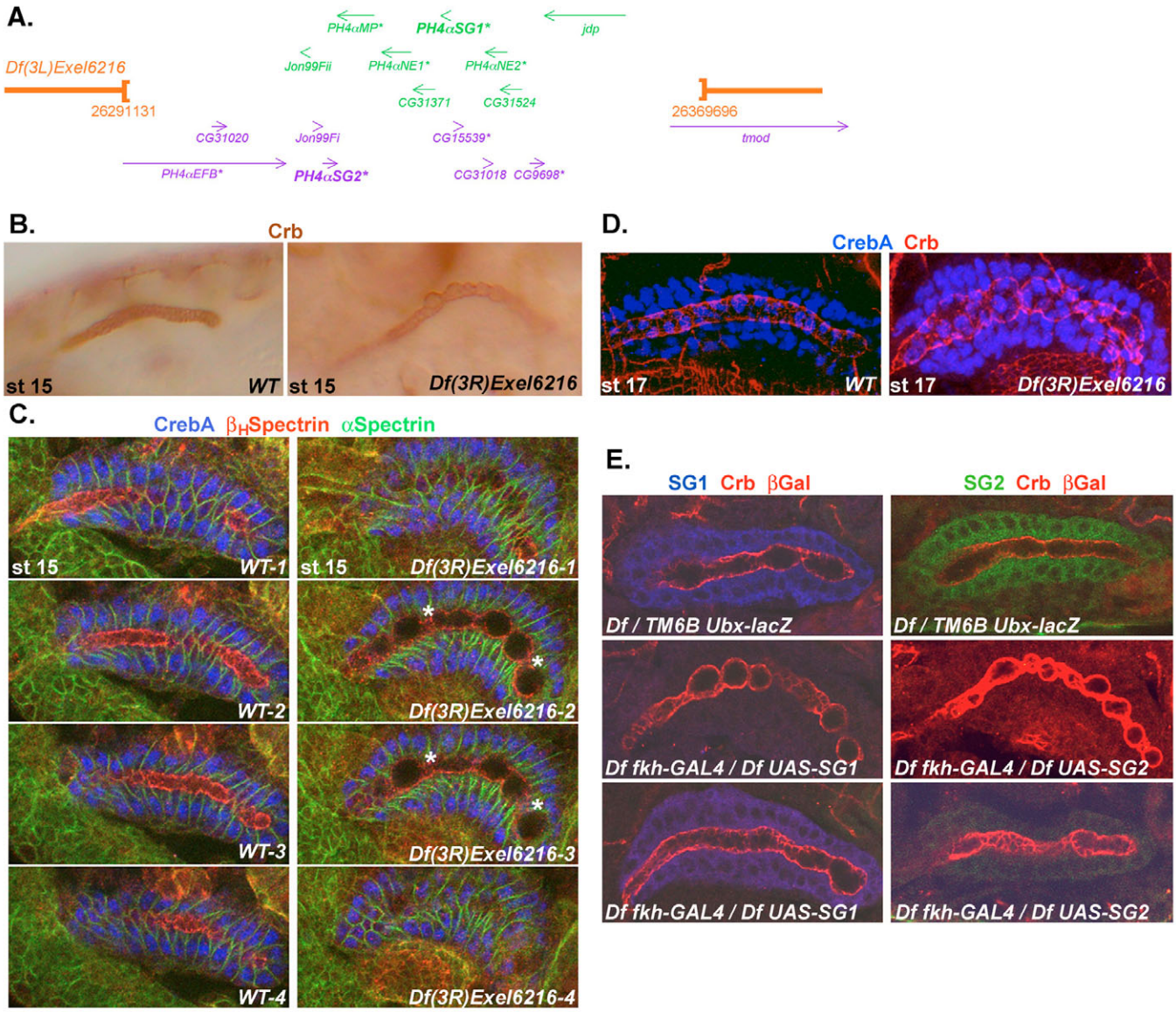

Fig. 7. Loss of *SG1* and *SG2* results in regions of salivary tube closure. (**A**) *Df(3R)Exel6216* deletes only 16 genes, eight of which are the previously characterized PH4α genes (asterisks), including *SG1* and *SG2* (Abrams and Andrew, 2002). (**B**) *Df(3R)Exel6216* salivary glands have abnormal lumenal morphology. (**C**) Confocal images of salivary glands stained with CrebA (blue), β_HSpectrin (red) and αSpectrin (green) revealed regions of tube dilation, constriction and apparent closure (asterisks) in the *Df(3R)Exel6216* embryos (the images –1 to –4 are different focal planes from the same glands). (**D**) Composite images of ~50-80 0.3 μm sections of salivary glands stained with CrebA (blue) and Crb (red) revealed uniform lumen diameters wild-type embryos (left panel) but variable lumenal diameters in the *Df(3R)Exel6216* embryos (right panel). (**E**) Expression of either *SG1* (left panels) or *SG2* (right panels) driven by a *fkh*-GAL4 driver rescues the salivary gland lumenal irregularities of *Df(3R)Exel6216* embryos. Although all of the embryos of the genotypes shown in the lower two sets of panels in E were expected to express either SG1 or SG2, expression of both proteins was variable, with undetectable expression in some embryos (middle panels). Detectable SG1 and SG2 expression correlated with rescue of the lumenal defects (lower panels). *Df(3R)Exel6216* is balanced over *TM6B, Ubx-lacZ*, allowing us to distinguish the heterozygous from homozygous deficiency embryos by also staining with an α-βgal antiserum. βgal staining (red) can be seen in the heterozygous embryos in the top panels in E near the distal end of the salivary gland.

CrebA, which is more directly involved in the expression of these genes (Abrams and Andrew, 2005). As observed with the secretory pathway genes, more than half of the Fkh targets require Fkh only for maintenance, not initiation (E. Grevengoed and D.J.A., unpublished), suggesting that regulation is mediated by Fkh-dependent downstream transcription factors. Even with *SG1* and *SG2*, which absolutely require Fkh for all stages of expression, only *SG2* appears to be regulated directly by Fkh. Fkh regulation of *SG1* appears to be through an intermediate, currently unidentified transcription factor, even though the *SG1* enhancer contains two sites that bind Fkh protein in vitro. Indeed, detailed enhancer analyses have revealed only a small number of direct transcriptional targets of Fkh, including both *sage* and *CrebA*, which encode transcription factor genes that, in turn, either function downstream of or in parallel with Fkh to regulate gene expression. Thus, although Fkh functions as a key regulator of salivary gland development, it does so in collaboration with other early expressed transcription factors.

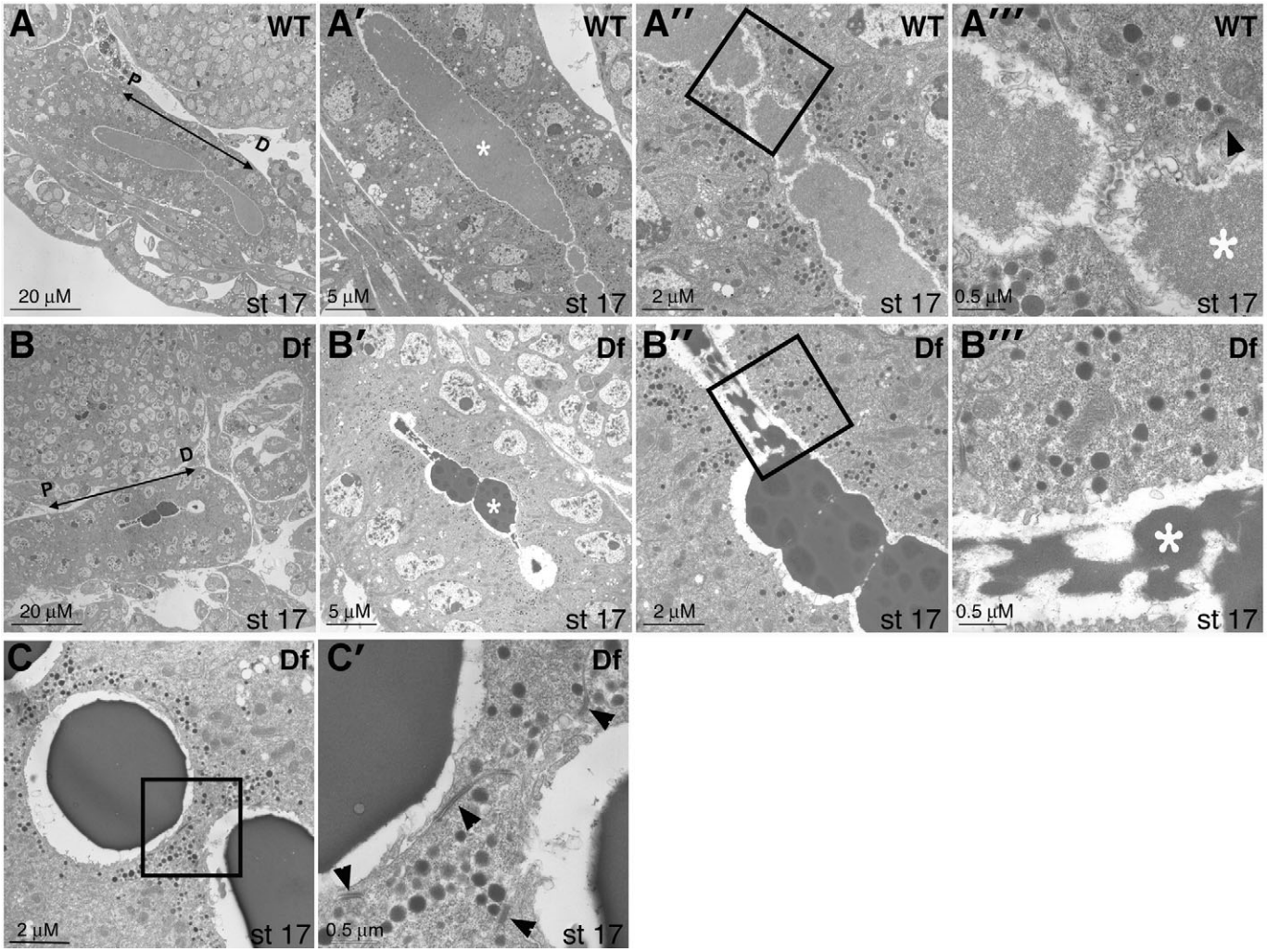

Fig. 8. Loss of *SG1* and *SG2* affects the quality and quantity of lumenal secretory content. (**A-C**) TEM analysis of wild-type (Oregon R) and *Df(3R)Exel6216* salivary glands revealed differences in both the volume and morphology of apical secretions. (A-A‴) Thin sections of wild-type salivary gland cells revealed uniform apical cell surfaces surrounding a relatively large lumenal space filled with fibrillar matrix material (asterisks). (B-B‴) Thin sections of *Df(3R)Exel6216* salivary glands revealed irregular and small lumenal spaces containing matrix of increased density (asterisks) and large regions where the apical cell surfaces appear to meet. (C,C′) New adherens junctions (AJs) appear to have formed at the contact site of cells arising from opposite sides of the lumen of *Df(3R)Exel6216* salivary glands (arrowheads indicate AJs). Secretory vesicle number and morphology were similar in wild-type and *Df(3R)Exel6216* salivary glands, as were other aspects of salivary gland cell morphology. A-A‴, B-B‴ and C-C′ are images from the same gland showing increased magnification from left to right; in some cases the angle of the image is changed slightly from panel to panel. Boxed regions in A″, B″ and C correspond to magnified images shown in A‴, B‴ and C′, respectively. *Df(3R)Exel6216* was balanced over *TM3, twi-GFP*, allowing us to select homozygous deficiency embryos prior to fixation.

When we began our analysis of Fkh regulation of SG1 and SG2, we looked for every potential direct binding site within the *SG1* and *SG2* enhancers using data from three different studies (Lehmann and Korge, 1996; Mach et al., 1996; Lehmann et al., 1997), which revealed a seven bp core consensus Fkh-binding site. Although we found four such consensus sites within each enhancer, not all of the sites were bound by Fkh protein in vitro. Three sites showed strong binding (‘d’, ‘f’ and ‘h’), two sites showed moderate (‘g’) or weak (non-specific) binding (‘b’) and three sites were not bound at all (‘a’, ‘c’ and ‘e’). Subsequently, Takiya et al. (Takiya et al., 2003) reported Fkh DNA binding to be strongly influenced by negative cooperativity among neighboring bases. Our binding data are consistent with their findings. The three strong Fkh-binding sites in the *SG1* (‘d’) and *SG2* (‘f’ and ‘h’) enhancers matched the optimal sequences for binding determined by Takiya et al. (Takiya et al., 2003) in all positions (Table 1). The moderate Fkh-binding site (‘g’) had a C in position 11, which was shown to reduce binding affinity. The weak Fkh-binding site (‘b’) had an inhibitory T_9A_{10} dinucleotide, and the three sites that did not bind Fkh at all (‘a’, ‘c’ and ‘e’) each had an unfavorable A_7T_8 dinucleotide as well as nucleotides unfavorable for binding at positions 9 and/or 11. Our demonstration that the Fkh-binding sites within both the *SG2* and *sage* enhancers are required for their full level salivary gland expression indicates a direct correlation between in vitro studies and site occupancy in vivo; sites that function in vivo are bound by the Fkh protein in vitro. Our studies of *SG1* regulation, however, indicate that not all sites bound by Fkh protein in vitro are necessary in vivo as *SG1* reporter gene expression was unaltered when the Fkh sites were disrupted.

Table 1. Comparison of Fkh-binding site matrix derived from in vitro studies by Takiya et al. (Takiya et al., 2003) to the sites tested in this study

Position	Gene	1	2	3	4	5	6	7	8	9	10	11
Matrix consensus		N	T	G/A	T	T	T/G	G/A G_7 (unfavorable) A_7 (unfavorable)	A/C/T A_8 T_8	A/T T_9 (inhibitory)	N A_{10}	A/T
Site d	*SG1*	A	T	G	T	T	T	G	T	T	T	A
Site f	*SG2*	T	T	G	T	T	T	A	C	T	T	A
Site h	*SG2*	T	T	G	T	T	T	G	C	A	C	A
Site g	*SG2*	G	T	G	T	T	T	G	T	A	T	C
Site b	*SG1*	T	T	A	T	T	T	A	C	T	A	A
Site e	*SG2*	T	T	A	T	T	T	A	T	T	T	G
Site a	*SG1*	A	T	A	T	T	T	A	T	C	G	C
Site c	*SG1*	A	T	G	T	T	G	A	T	C	T	C

Maintaining a uniform salivary gland lumen

Functional analysis of *SG1* and *SG2* suggests a role for apical secretion in the maintenance of uniform open salivary gland tubes. This finding supports studies in the *Drosophila* trachea, demonstrating the importance of apical secretions during tracheal remodeling (Jazwinska et al., 2003; Araujo et al., 2005; Devine et al., 2005; Tonning et al., 2005). Jazwinska et al. (Jazwinska et al., 2003) showed that two apical proteins, Piopio (Pio) and Dumpy (Dp), are required during secondary branch formation. In this process, cells that are arrayed side-by-side in a multicellular tube rearrange to an end-to-end configuration to form unicellular tubes, while maintaining tube integrity with uniform lumenal space. In *pio* or *dp* mutants, tracheal cells detach from the main tracheal artery just as they complete their rearrangements to form unicellular tubes. The authors suggest that this occurs because the formation of the autocellular junctions of the unicellular tubes continues to completion instead of stopping at the point where the cells contact their proximal neighbors. The authors further suggest that Pio and Dp contribute to an apical ECM that prevents reduction in lumen diameter as the secondary branches form. A role for an apical ECM in maintaining tube diameter has also been discovered in the multicellular tubes of the dorsal trunk (Araujo et al., 2005; Devine et al., 2005; Tonning et al., 2005). Genetic or pharmacological disruptions in chitin synthesis lead to regions of tube constriction and dilation. Tonning et al. (Tonning et al., 2005) showed the existence of a transient chitin network in the tracheal lumen that ensures that as the different segments of the dorsal trunk fuse to form a continuous tube, uniform tube diameter is maintained.

Our studies demonstrate a correlation between apical ECM volume/morphology and lumen size uniformity, even in tubes not undergoing extensive cell rearrangements. The phenotypes of *SG1/SG2*-deficient salivary glands suggest that the apical ECM has both a barrier function that prevents cells from contacting one another and closing the tube, as well as a scaffolding function that prevents lumenal dilation. Similar defects were observed with mutations in *pasilla* (*ps*), which encodes a splicing factor homologous to the mammalian proteins Nova1 and Nova2 (Seshaiah et al., 2001). At the TEM level, *ps* mutants exhibit a decrease in secreted lumenal content and a reduction in the number and size of apical secretory granules. Although, as a splicing factor, Pasilla must be acting indirectly to affect secretion levels, its phenotype demonstrates a direct correlation between secretory volume and lumen size uniformity, a correlation supported by the defects in *SG1/SG2*-deficient glands. As *SG1* and *SG2* encode enzymes that could modify proteins in apical secretions, their role in this process is likely to be more direct.

The apical matrix of wild-type glands forms a fibrillar network structure that is not apparent in salivary glands of embryos missing *SG1* and *SG2*. This phenotype suggests that protein modification by the SG1 and SG2 prolyl-4-hydroxylases (PH4s) alters the character of secreted apical proteins to allow them to form fibrillar structures that maintain an expanded network of ECM. A role for prolyl hydroxylation in the formation of fibrillar collagen has been known for decades (for a review, see Myllyharju and Kivirikko, 2004). Although canonical collagens are not expressed in the *Drosophila* salivary gland, a large number of uncharacterized genes encoding secreted proteins that contain the Pro-X-Gly repeats exist, which could be substrates for SG1/SG2 prolyl hydroxylation. Collagens are the major protein components of the ECM, where they serve key structural roles as exemplified by mutations in the human genes that lead to fragile bones, bone and joint deformities, as well as fragile skin and blood vessels (for a review, see Myllyharju and Kivirikko, 2004). A ‘structural’ role for a collagen-related protein(s) in the apical matrix of salivary glands is consistent with our observations. Interestingly, formation of collagen fibrils occurs post-secretion, where enzymes outside the cell remove the propeptides from procollagen to allow fibrillar collagen formation. Similarly, the fibrillar nature of the lumenal secretions of the wild-type *Drosophila* salivary gland is not visible in the subapical secretory vesicles, suggesting that the fibrillar structures found in the apical matrix also form post-secretion. We propose that the denser apical matrix with reduced volume is the basis for the defects observed in *SG1/SG2*-deficient salivary glands. In areas where there is little to no apical content, the opposing sides of the tubes meet to either close or form very small lumena lined with small apical surfaces and closely arrayed adherens junctions. The similarity of the *SG1/SG2* deficiency phenotypes to those seen with mutations affecting the *Drosophila* trachea suggests the potential for shared mechanisms for maintaining lumen size uniformity in epithelial tubes.

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