Common origin of insect trachea and endocrine organs from a segmentally repeated precursor

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Running title: Common origin of trachea and endocrine organs

Highlights:

* We describe the developmental origin of the two main insect endocrine organs

* We show the gene-network required for the development of insect endocrine organs

* We introduce a novel model to study EMT and concerted cell migration

* We uncover one of the most extreme cases of evolutionary organ divergence

Summary:

Segmented organisms have serially repeated structures [1] **that become specialized in some segments [2]. We show that the *Drosophila corpora allata*, the prothoracic glands and the trachea have a homologous origin and can convert into each other. The tracheal epithelial tubes develop from ten trunk placodes [3, 4]; homologous ectodermal cells in the maxilla and labium, form the *corpora allata* and the prothoracic glands. The early endocrine and trachea gene networks are similar, with STAT and Hox genes inducing their activation. The initial invagination of the trachea and the endocrine primordia is identical, but activation of Snail in the glands induces an Epithelial to Mesenchymal Transition after which the *corpora allata* and prothoracic gland primordia coalesce and migrate dorsally joining the *corpora cardiaca* to form the ring gland. We propose that the Arthropod ectodermal endocrine glands and the respiratory organs arose through an extreme process of divergent evolution from a metameric repeated structure.**

Results and discussion

The endocrine control of moulting and metamorphosis in insects is regulated by the *corpora allata* (*ca*) and the *prothoracic glands* (*pg*), which secrete Juvenile Hormone and Ecdysone respectively [5]. In diptera, these glands and the *corpora cardiaca* (*cc*) fuse during development to form a tripartite endocrine organ called the ring gland (Fig.1A). While the corpora cardiaca is known to originate from the migration of anterior mesodermal cells, the origin of the other two ring gland components is unclear [6, 7].

The tracheae have a completely different structure consisting of a tubular network of polarized cells [4]. The tracheae are specified in the second thoracic to the eighth abdominal segments (T2-A8) by the activation of *trachealess* (*trh*) and *ventral veinless* (*vvl*) [4, 8-12].

We isolated the enhancers controlling *trh* and *vvl* in the tracheal primordia and showed they are activated by JAK/STAT signalling [13]. While the *trh* enhancers are restricted to the tracheal primordia in the T2 to A8 segments, the *vvl1+2* enhancer is also expressed in cells at homologous positions in the maxilla (Mx), labium (Lb), T1 and A9 segments in a pattern reproducing the early transcription of *vvl* (Fig.1C-E). The fate of these non-tracheal *vvl* expressing cells was unknown, but it was shown that ectopic *trh* expression transforms these cells into tracheae [14]. To identify their fate we made *vvl1+2-EGFP* and *mCherry* constructs. Although the *vvl1+2* enhancer drives expression transiently [13], the stability of the EGFP and mCherry proteins labels these cells during development. We observe that while the T1 and A9 patches remain in the surface and integrate with the embryonic epidermis, the patches in the Mx and Lb invaginate just as the tracheal primordia do (Fig.2N-N'). Next the Mx and Lb patches fuse and a group of them undergo an Epithelial to Mesenchymal Transition (EMT)

initiating a dorsal migration towards the anterior of the aorta where they integrate into the ring gland (Fig.2A-I and Fig.1F). To find out what controls the EMT we studied the expression of the *snail* (*sna*) gene, a key EMT regulator [15]. Besides its expression in the mesoderm primordium, we found that *sna* is also transcribed in two patches of cells that become the migrating primordium (Fig. S1A). Using *sna* BACs with different *cis*-regulatory regions [16-18] we localized the enhancer activating *sna* in the ring gland primordium (*sna-rg*). A *sna-rg-GFP* construct labels the subset of Mx and Lb *vvl1+2* expressing cells that experience EMT and migrate to form the ring gland (Fig.2B-I, Movies S1-S2). Staining with *seven-up* (*svp*) and *spalt* (*sal*) [also known as *salm*] [19, 20] markers, that label the *ca* and the *pg* respectively (Fig.1B,G), shows that the *sna-rg-GFP* cells form these two endocrine glands (Fig.2K',M'). The *sna-rg-GFP* expressing cells in the Mx activate *svp* (Fig.2J) and those in the Lb activate *sal* (Fig.2L) before they coalesce, indicating that the *ca* and *pg* are specified in different segments before they migrate.

To test if Hox genes, the major regulators of antero-posterior segment differentiation [21], participate in gland morphogenesis we stained *vvl1+2-GFP* embryos and found that the Mx *vvl1+2* primordium expresses Deformed (Dfd) and the Lb primordium Sex combs reduced (Scr) while the T1 primordium expresses very low levels of Scr (Fig.3A). *Dfd* mutant embryos lack the *ca*, while *Scr* mutant embryos lack the *pg* (Fig.S2). Dfd and Scr expression in the gland primordia is transient suggesting they control their specification. Consistently, in *Dfd, Scr* double mutant embryos *vvl1+2* is not activated in the Mx and Lb patches (Fig.3B) and the same is true for *vvl* transcription. In these mutants the *sna-rg-GFP* expression is almost absent (Fig.3C) and the *ca* and *pg* do not form. In each case Dfd controls the expression of the Mx patch and Scr of the Lb patch.

We tested the capacity of different Hox genes to rescue the ring gland defects of *Scr, Dfd* double mutants (Fig.3D,G,K,O and Fig.S3). Induction of *Dfd* with the *sal-Gal4* line in these mutants, restores the expression of *vvl1+2* (Fig.3G) and *sna-rg-GFP* in the Mx and the Lb. However, in contrast to the wild type (Fig.2J-M), both segments form a *ca* as all cells express Svp (Fig.3E-F). Similarly, induction of *Scr* also restores the *vvl1+2* (Fig.3K) and *sna-rg-GFP* expression but both primordia form a *pg* as they activate Sal and *phantom* [22], an enzyme required for ecdysone synthesis (Fig.3I-J). The capacity of both Dfd and Scr to restore *vvl* expression regardless of the segment, made us test if other Hox proteins could have the same function. Induction of *Antennapaedia (Antp)*, *Ultrabithorax (Ubx)*, *abdominal-A (abd-A)* or *Abdominal-B (Abd-B)* restores *vvl1+2* expression in the Mx and Lb, but these cells form tubes instead of migratory gland primordia (Fig.3L-O and Fig.S3). These cephalic tubes are trachea as they do not activate *sna-rg* (Fig.S3E'), express Trh and their nuclei accumulate Tango (Tgo), a maternal protein that is only translocated to the nucleus in salivary glands and tracheal cells [23], indicating that the trunk Hox proteins can restore *vvl* expression in the Mx and Lb, but induce their transformation to trachea.

To investigate if *vvl* and *trh* expression is normally under Hox control in the trunk, we focused on Antp that is expressed at high levels in the tracheal pits (Fig.S3J). In double *Dfd, Antp* mutant embryos (Fig.S3K-K') *vvl1+2* is maintained in the Lb where Scr is present, while the Mx, T1 and T2 patches are missing. In T3-A8 *vvl1+2* expression, although reduced, is present probably due to the expression of Ubx, Abd-A and Abd-B in the posterior thorax and abdomen. Thus, Antp regulates *vvl* expression in the tracheal T2 primordium. Surprisingly, in *Dfd, Antp* double mutants, Trh and Tgo are maintained in the T2 tracheal pit (Fig.S3K), indicating that although Hox genes can activate ectopic *trh* expression, in the tracheal primordia they may be acting redundantly

with some other unidentified factor, explaining why the capacity of Hox proteins to specify trachea had not been reported previously.

We studied *sna* null mutants to find out its requirement for ring gland development, but their aberrant gastrulation [24, 25] precluded analyzing specific ring gland defects. To investigate *sna* function in the gland primordia we rescued the *sna* mutants with the *sna-squish BAC* [16] that drives normal Sna expression except in the ring gland (Fig.S1B). These embryos have a normal gastrulation and activate the *sna-rg-GFP*, however the gland primordia degenerate and disappear (Fig.4B). To block apoptosis we made these embryos homozygous for the H99 deficiency that removes three apoptotic inducers [26]. In this situation, the *ca* and *pg* primordia invaginate and survive but they do not undergo EMT (Fig.4C). As a result the gland primordia maintain epithelial polarity, do not migrate and form small pouches that remain attached to the epidermis (Fig.4D).

Vvl is required for tracheal migration [8, 9, 27]. In *vvl* mutant embryos *sna-rg-GFP* expression is activated but the cells degenerate (Fig.4E). In *vvl* mutant embryos also mutant for H99, the primordia undergo EMT and migrate up to the primordia coalescence, however the later dorsal migration does not progress (Fig.4F-G).

We have shown that the *ca* and *pg* develop from *vvl* expressing cephalic cells at positions where other segments form trachea, suggesting that they could be part of a segmentally repeated structure that is modified in each segment by the activity of different Hox proteins. As the cephalic primordia are transformed into trachea by ectopic expression of trunk Hox (Fig.3L-N), we tested if the trachea primordia could form gland cells. Ectopic expression of *Dfd* with *arm-Gal4* results in the activation of *sna-rg-GFP* on the ventral side of the tracheal pits (not shown). These *sna-rg-GFP* expressing cells also express *vvl 1+2*, Trh and have nuclear Tgo showing that they

conserve tracheal characteristics. These *sna-rg-GFP* positive cells do not show EMT and keep associated to the ventral anterior tracheal branch. The strength of ectopic *sna-rg-GFP* expression increases when ectopic Dfd is induced in *trh* mutant embryos (Fig.3P). However migratory behaviours in the *sna-rg-GFP* cells are only observed if Dfd is coexpressed with Sal (Fig.3Q-T). Thus *sal* is expressed several times in the gland primordia, first at st9-10 repressing trunk Hox expression in the cephalic segments (Fig.S3G), and second from st11 in the prothoracic gland. It is uncertain if *sal* requirement for migration is linked to the first function or if it represents an additional role.

Our results show that the endocrine ectodermal glands and the respiratory trachea develop as serially homologous organs in *Drosophila*. The identical regulation of *vvl* in the primordia of trachea and gland by the combined action of the JAK/STAT pathway and Hox proteins (Fig.3, Fig.S3 and S4) could represent the vestiges of an ancestral regulatory network retained to specify these serially-repeated structures; while the activation of Sna for gland development, and Trh and Tgo for trachea formation could represent network modifications recruited later by specific Hox proteins during the functional specialization of each primordium (Fig.4H). This hypothesis or alternative possibilities should be confirmed by analyzing the expression of these gene networks in various arthropod species. The diversification of glands and respiratory organs must have happened before the split of Insects and Crustaceans as there is a correspondence between the endocrine glands in both Classes, with the *corpora cardiaca* corresponding to the pericardial organ; the *corpora allata* to the Mandibular organ; and the prothoracic gland to the Y-gland [5, 28, 29]. Despite their divergent morphology, a correspondence between the Insect trachea and the Crustacean gills can also be made, as both respiratory organs coexpress *vvl* and *trh* during their

organogenesis [30]. Divergence between endocrine glands and respiratory organs may have occurred when the evolution of the arthropod exoskeleton required solving two simultaneous problems: the need to moult to allow growth and the need of specialized organs for gas exchange.

Experimental Procedures

Constructs generated for this work:

A polylinker with EcoRI and XbaI restriction sites at the ends was synthesized (Sigma) and digested with EcoRI and XbaI. A pCaSpeR.lacZ.NLS was digested with EcoRI and XbaI, releasing the polylinker, the hs43 promoter and the lacZ.NLS that was substituted with the new polylinker creating (pCaSpeR polylinker) where a PH membrane domain from Phospholipase C delta digested with BamHI-XbaI, was cloned creating (pCaSpeR-PH). Next mCherry, was PCR amplified from pCS2+ vector, with BamHI/BglII tailed primers. A sequenced mCherry PCR product was digested with BamHI-BglII and cloned into pCaSpeR-PH BamHI digested (restriction site included in polylinker). Next, the hs43 promoter digested with BamHI-BglII from pCaSpeR.NLS-LacZ, was cloned into BamHI, completing the plasmid pCaSpeR-mCherry-PH.

pCaSpeR-EGFP-PH, was constructed in a similar way using EGFP from the pCS2+ vector to create a pCaSpeR-EGFP-PH plasmid.

The *sna*-rg enhancer was amplified by PCR from *Drosophila* genomic DNA and cloned into pGEMTeasy using the following primers:

Upper *sna*-rg primer: 5'-ACCAAACCAGAACTCCAGACC-3'

Lower *sna*-rg primer: 5'- GCTTGGGTTTTTCGTTTTCAG-3'

Finally, the enhancer was subcloned into pCaSpeR-EGFP-PH to create a reporter plasmid. The *vvl 1+2* enhancer [13] was cloned from pGEMT-easy to pCaSpeR-mCherry-PH and pCaSpeR-EGFP-PH reporter plasmids. Both plasmids where transformed into *Drosophila* by the *Drosophila* Consolider-Ingenio 2007 transformation platform (Spain).

Fly stocks

The following mutant alleles and transgenic lines from the *Drosophila* Bloomington stock collection where used:

Dfd^{4} ; Dfd^{l6}, Scr^{4} ; Df(3L)H99; Df(1)os1A; Dfd^{l6} , $Antp^{7}$; P{HZ}svp^{3}; vvl^{GA3}; trh^{8}; sna^{1}; sna^{18}; P{UAS-Dfd.B}W4; P{UAS-Scr.M}EE2; P{UAS-Antp.Mb}W1; P{UAS-myr-mRFP}. UAS-sal and *Df (2)5 sal^{-} $salr^{-}$* were obtained from R. Barrio, and *phm-GAL4>CD8::GFP* line [31] from Marco Milán. The *sna-lacZ* 6kb reporter and *sna-GFP BAC* collection was obtained from A. Stathopoulos and M. Levine. The following stocks from our laboratory where used: *UAS-Ubx; UAS-AbdBm* [32]; the reporter *vvl1+2-lacZ*; and the enhancer trap lines *sal-GAL4* 459.2 and *arm-Gal4* (Bloomington stock number 1560).

Primary antibodies: Chicken anti-gfp (Abcam); rabbit anti-gfp (Invitrogen); rat anti-RFP (Chromotek); rabbit or mouse anti-ßGal (Promega) and Chicken anti-ßGal (Abcam); rat anti-Trh (J. Casanova); rabbit anti-Sal (R. Barrio); rabbit anti-Svp (R.

Cripps); rabbit anti-aPKC (Santa Cruz); rabbit anti-Dfd (T. Kaufman); anti-DIG-AP (Roche) and from the Hybridoma Bank we obtained mouse anti-Tgo; mouse anti-Fas II; mouse anti-Scr; mouse anti-AbdB; mouse anti-Ubx (FP3.38) and mouse anti-Antp.

Secondary antibodies: The following Invitrogen antibodies were used: anti-mouse A488; anti-mouse A555; anti-mouse A647; anti-chicken A488; anti-Goat A647; anti-Rabbit A488; anti-Rabbit A555; anti-Rabbit A647 and anti-Rat A555.

RNA probes: In situ RNA probes: *snail* RE35237 and *phantom* RE03155. Riboprobes were made using DIG RNA Labeling Kit (Roche).

Microscopy:

In vivo time-lapse microscopy was done using a Leica DMI 6000 SP5 MP-AOBS inverted confocal microscope. A Leica SPE Confocal Microscope was used for multiple antibody stainings and in situ/antibody stainings. Images were processed using ImageJ and Imaris (7.6).

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Figure legends

Figure 1. Ring gland structure and primordia origin. (A) Frontal view reconstruction of a late embryo ring gland showing the position of the three independent glands: The *corpora allata* (green) expresses *svp*, the prothoracic glands (red) *phantom*, and the *corpora cardiaca* (blue) is labelled by FasII. (B) Dorsal view of a late embryo labelled with *sal-Gal4* driving *UAS-RFP* and *svp-lacZ*. The ring gland is localized anterior to the aorta (note that *svp* also labels some cardiomyocites in the aorta and heart (yellow asterisk). (C) At extended germ band *vvl1+2-EGFP* labels the tracheal primordia in T2-A8 as well as cells in more anterior and posterior segments. (D) Expression of the Trh protein in the ten tracheal primordia as well as the salivary gland. (E) Double staining of *vvl1+2* and Trh. (F) Frontal view of a ring gland in the process of fusing dorsally stained with FasII and *vvl1+2-EGFP*, the *corpora allata* and the prothoracic glands express *vvl1+2* showing their ectodermal origin, while the *corpora cardiaca* does not. (G) Ring gland z-section of a *sal-Gal4 UAS-RFP, svp-lacZ* embryo stained with RFP, anti-bgal and FasII. Images from this series where used for the reconstruction shown in (A). White scale bar 50μm, yellow scale bars 10μm.

Figure 2. Formation of the *corpora allata*, prothoracic glands and tracheae. (A-I) Embryos double stained to detect the *vvl1+2-mCherry* marking gland and trachea (red), and the *sna-rg-EGFP* (green) marking the corpora allata and prothoracic glands. (A) Early st10 embryos express *vvl1+2* in a segmentally repeated pattern. (B) In st11 embryos *vvl1+2* cells invaginate in all segments except in T1 and A9. In the Mx and Lb a subgroup of *vvl1+2* cells activate *sna-rg-EGFP*. (C) Tracheal primordia from T2 to A8 start branching at the time when the *sna-rg-EGFP* cells experience the Epithelial to

Mesenchymal Transition. (D) Dorsal migration of the coalesced *ca/pg* primordium, all migrating cells express *sna-rg-EGFP* while non-snail expressing *vvl1+2* cells stay behind. (E-G) Close-up of the Mx and Lb segments before (E-F) and after (G) the two gland primordia coalesce. Note that there are two different coalescence processes: one at st11 when the *vvl1+2* expressing cells invaginating from the Mx and Lb come together (F); another, at st13 when the migrating *sna-rg-EGFP* cells coalesce (G). (H) Dorsal view of the *sna-rg-EGFP* cells at the time when the prothoracic glands meet the *corpora cardiaca* (unstained in this figure). (I) Dorsal view of the ring gland as the contralateral *corpora allata* primordia fuse (comparable frontal view in Fig.1F-G). (J-M) Specification of the *corpora allata* and prothoracic glands. (J) *svp-lacZ, sna-rg-EGFP* embryos double stained with anti-ßgal and anti-GFP show that the *corpora allata* is specified in the Mx prior to coalescence and maintains *svp* expression until the ring gland is formed (K-K'). (L) *sna-rg-EGFP* cells double stained using anti-Spalt to show that the prothoracic gland is specified in the Lb before coalescence. (M-M') Embryo expressing *sal-Gal4* showing that this line drives expression specifically in the prothoracic gland. (N-N´) Embryos double stained for *vvl1+2* and aPKC show that both the gland primordia (N) and the trachea primordia (N´) maintain epithelial polarity during invagination. (O-O') In the Mx and Lb segments only *vvl1+2* cells expressing *sna-rg-EGFP* experience EMT and lose apical aPKC; in contrast, *vvl1+2* cells never lose apical polarity in the trachea (P). Asterisk in g and o labels *vvl1+2* cells that do not activate *sna* and maintain epithelial characteristics. White scale bar 50μm, yellow scale bars 10μm, purple scale bars 25μm. (See also Fig. S1 and Movies S1-S2).

Figure 3. Hox genes control the specification of *corpora allata*, prothoracic glands and trachea. (A) Ventral view of a *vvl1+2*-EGFP embryo stained with anti-Dfd (blue)

and anti-Scr (red). (B-C) *Dfd, Scr* double mutant embryos fail to activate *vvl1+2* in the Mx and Lb (B, asterisks) and have very low levels of sna-rg (C). (D,G,K,O) *Dfd, Scr* double mutants expressing GFP or different Hox genes with the *sal-Gal4* line that drives early expression in the Mx, Lb and T1 segments (D). (D-D´) Expression of GFP does not rescue *vvl1+2* expression (red) in *Dfd, Scr* mutant embryos. Expression of Dfd (G), Scr (K) or Antp (O) rescues *vvl1+2* expression in both Mx and Lb (asterisks). (E-F) Embryos rescued with Dfd produce ring glands composed exclusively of *corpora allata* cells as judged by Svp expression. (H-H") Wild type ring glands express *phm* only in the subset of *sna-rg* cells forming the prothoracic gland. (I-I") Embryos rescued with Scr produce ring glands composed exclusively of prothoracic gland cells as judged by *phm* or (J) *sal-Gal4* expression in all the *sna-rg* cells. (L) *Dfd, Scr* embryo rescued with Antp show nuclear Tgo in the cephalic segments. (M-M") Close up of an embryo of the same genotype as in (L) showing the *vvl1+2* primordium in the Mx is transformed towards trachea as it expresses Tango. (N-N') *Dfd, Scr* embryo rescued with Antp induces expression of Trh in the head indicating they are forming trachea. (P) Ectopic expression of Dfd in the ectoderm using the *arm-Gal4* line induces robust expression of *sna-rg-EGFP* in the trunk segments of *trh* mutant embryos. (Q-T) Embryos labelled with *vvl1+2 mCherry* and expressing Dfd and Sal with the *arm-Gal4* line induce the expression of *sna-rg-EGFP* in a ventral subset of *vvl1+2* cells (Q); the *sna-rg*-EGFP cells become motile (R-R') migrating dorsally (S-T). Note that despite the UAS-Scr line (K) expressing lower amounts of protein than other Hox lines used (G,O), these are sufficient to rescue *vvl1+2* expression. White scale bars 50μm, yellow scale bars 10μm. (See also Fig. S2 and S3 S4).

Figure 4. Function of Sna and Vvl in the ring gland. (A) Wild type *ca* and *pg* primordia labelled with *sna-rg-GFP* at st11. (B) The *ca* and *pg* become apoptotic and disintegrate when *sna* is removed specifically from the primordia. (C) When apoptosis is blocked from *ca* and *pg* primordia that do not express Sna, the cells do not go into EMT and remain attached to the surface without losing apical aPKC (purple). (D) *ca* and *pg* primordia not expressing Sna do not migrate and the primordia remain separated at late stages. (D') Close up of the pg in (D) showing that the primordium's apical side integrates with the neighbouring epithelial cells. (E) In *vvl* mutant embryos the ring gland primordia also become apoptotic. (F-G) *vvl* mutant embryos where apoptosis is prevented by the deletion of the apoptotic inducers. The ca and pg invaginate and go into EMT losing apical aPKC. Although both primordia coalesce (G) dorsal migration does not progress. (H) Schematic representation of the early ectodermal endocrine and respiratory primordia specification gene-network. In both networks Hox and STAT induce the expression of the early transcription factors. While all Hox genes can induce *vvl* expression only Dfd and Scr induce *sna* expression, while trunk Hox genes induce Trachealess expression. Sal represses trunk Hox expression in the head (H) preventing *trh* expression. White scale bar 50μm, yellow scale bars 10μm (See also Fig. S4).

Supplemental information

Figure S1. Related to Figure 2. sna expression in the corpora allata and prothoracic gland. (A) *In situ sna* mRNA expression during embryogenesis. At blastoderm (st4) *sna* is activated in the mesoderm primordium, this expression soon disappears with *sna* becoming activated at st11 in specific cells including the ca and pg primordia (asterisks) before coalescence. Expression of *sna* is maintained during dorsal migration (st13) and ceases just before dorsal fusion (red asterisks show wing and haltere *sna* expression appearing as ring gland expression fades away at st15). Schematic representation of the genomic *sna* region including part of the neighbouring *Tim17b2* gene, and the constructs and BAC elements used to localize the *sna*-ring gland enhancer. (C) Summary of ring gland activity of the different constructs. The Full upstream BAC and the BAC element deleting both distal and proximal shadow mesoderm enhancers maintain ring gland expression. The sna SQUISH BAC lacks ring gland expression but is capable of rescuing the germ band abnormalities of *sna* null mutant embryos without rescuing *sna* function in the ring gland. These data and the expression of a 6kb *sna-lacZ* reporter gene in the ring gland primordia, locates the region controlling *sna* expression in the ring gland to a 1.9 kb fragment. (D) Expression of the *sna-rg* enhancer drives strong levels of EGFP in the *corpora allata* and the prothoracic glands from st11 up to larval stages.

Figure S2. Related to Figure 3. Hox gene requirement for *corpora allata* and prothoracic gland development. Dorsal views of late *svp-lacZ* embryos stained with anti-ßGAL (green) and a *phm* RNA probe (purple) to show the *corpora allata* and prothoracic gland in wild type (A), *Dfd* (B) or *Scr* mutant embryos (C). In the dorsal

region focused here, *svp-lacZ* also labels four cardiomyocites per segment in the aorta. In these stainings there is some unspecific labelling of the tracheal lumen. In *Dfd* mutants the corpora allata do not form (B) but the prothoracic glands (B') develop normally. In *Scr* mutants the prothoracic glands do not form (C) but the corpora allata reach their normal destination (C'). Yellow scale bars 10μm.

Figure S3. Related to Figure 3. Capacity of Hox genes to activate tracheal and ring gland markers. (A) In a *Dfd, Scr* double mutant embryo, ectopic expression of Ubx (blue) with a *sal-Gal4* line rescues expression of *vvl1+2* (red) in the Mx and Lb (asterisks in A') and also induces ectopic Trh expression (purple in the close up shown in B). (C) Ectopic Abd-B expression (green) also rescues *vvl1+2* expression (red in C and C´) in the Mx and Lb (asterisks in C') and induces ectopic Trh expression (purple in D). (E) Ectopic Ubx expression rescues *vvl1+2* expression but does not induce *sna-rg-EGFP (*E' asterisks). (F) In a wild type embryo, ectopic Antp expressed with *sal-Gal4* induces anterior Trh expression in T1 and Mx but does not eliminate the *sna-rg* expression driven by cephalic Hox genes (asterisks). (G-H) In *sal* mutant embryos there is ectopic Ubx expression (G) correlating with the formation of ectopic trachea observed in these embryos as shown by nuclear Tgo localization (H). (I) Ectopic expression of Ubx with *sal-Gal4* induces trachea formation in T1 but not in the Mx and Lb segments probably due to endogenous Dfd and Scr expression in these segments (compare with B). (J) *vvl1+2-EGFP* embryos costained with Antp (red) show high Antp expression in the T2 tracheal pit and moderate levels in T1. (K-K') In *Dfd, Antp* double mutant embryos *vvl1+2* expression (red) is reduced from the Mx, T1 and T2 segments correlating with the domains where Dfd and Antp proteins should be expressed. High levels of *vvl1+2* expression are maintained in the Lb where Scr is

expressed. Intermediate levels of *vvl1+2* expression are observed in T3-A5, which is the domain of Ubx and Abd-A expression. High levels of *vvl1+2* are present in the Abd-B expression domain. Tgo nuclear localization (green in K) is not affected in the T2 tracheal primordium despite the absence of Antp indicating that there is another mechanism to maintain Trh/Tgo expression in trunk segments besides the Hox proteins. White scale bars 50μm, purple scale bars 25μm.

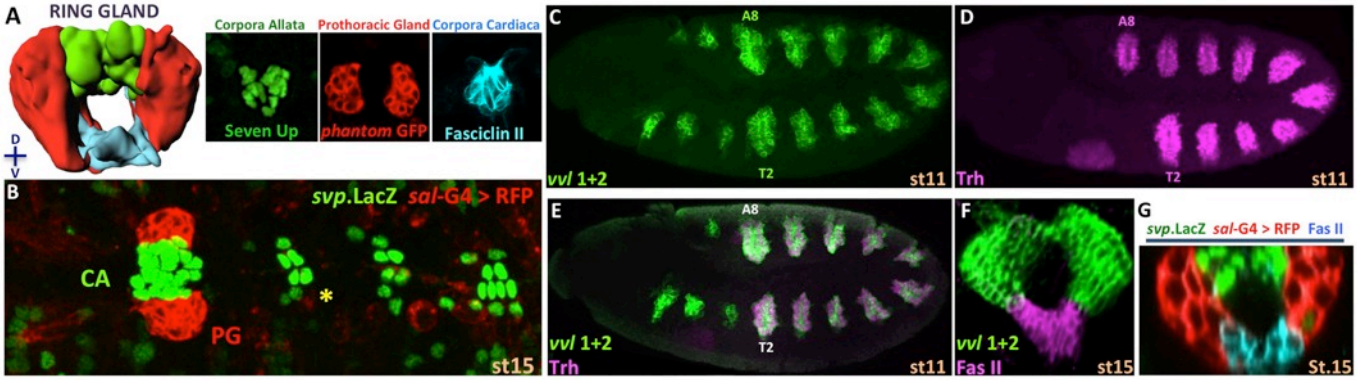
Figure S4. Related to Figure 4. JAK/STAT signalling requirement for Sna and Vvl expression in the ring gland and tracheae primordia. (A) Wild type embryo expressing *sna-rg* (green) and *vvl1+2* (red in A'). (B) *Df(1)os1A* embryos lacking all three upd ligands required for JAK/STAT activation[33] show reduced *sna-rg* expression (asterisks in B) and *vvl1+2* expression (red in B´). White scale bars 50μm.

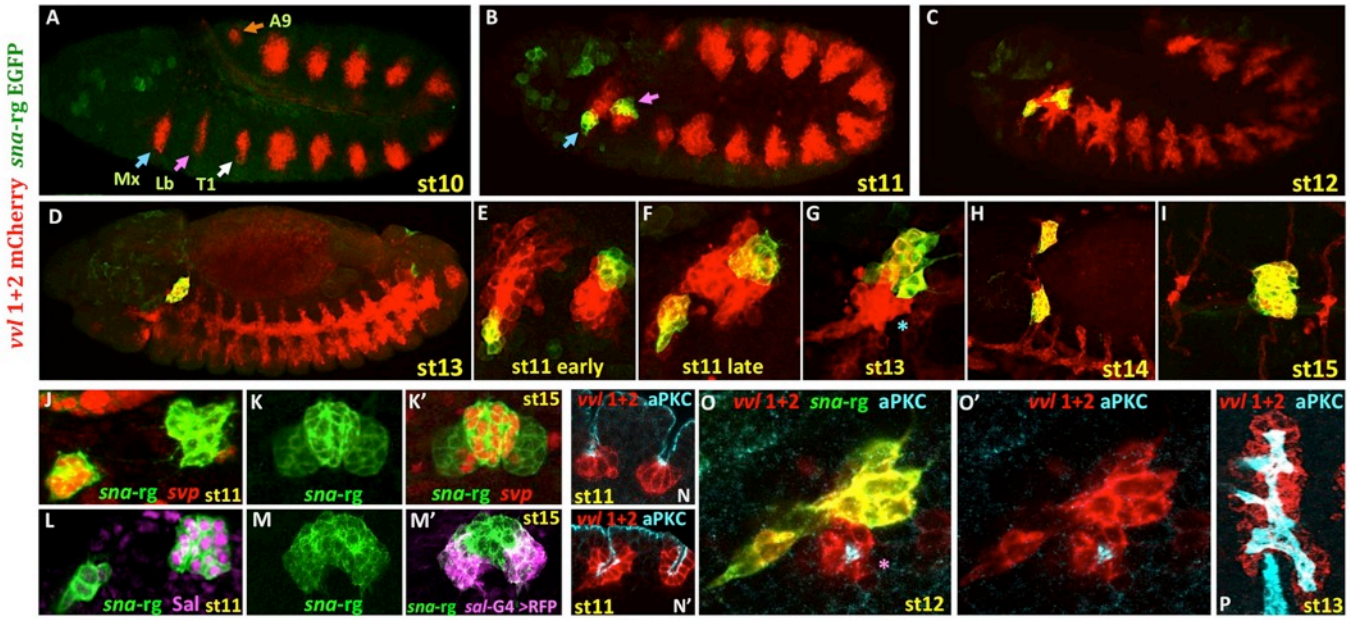
Supplementary movie 1. Primordia coalescence and dorsal migration.

Lateral view of st11 to st14 *sna-rg-EGFP* homozygous embryos showing the coalescence and dorsal migration of the *corpora allata* and the prothoracic gland. Scale bar 50μm.

Supplementary movie 2. Ring gland dorsal fusion.

Dorsal view of st14 to st15 *sna-rg-EGFP* homozygous embryos showing the fusion of the contralateral *corpora allata*. Note the active formation of filopodia. Scale bar 10μm.


A
RING GLAND
D
V
Corpora Allata
Prothoracic Gland
Corpora Cardiaca
Seven Up
phantom GFP
Fasciclin II
B
svp.LacZ sal-G4 > RFP
CA
PG
*
st15
C
A8
vvl 1+2
T2
st11
D
A8
Trh
T2
st11
E
A8
vvl 1+2
Trh
T2
st11
F
vvl 1+2
Fas II
st15
G
svp.LacZ sal-G4 > RFP Fas II
St.15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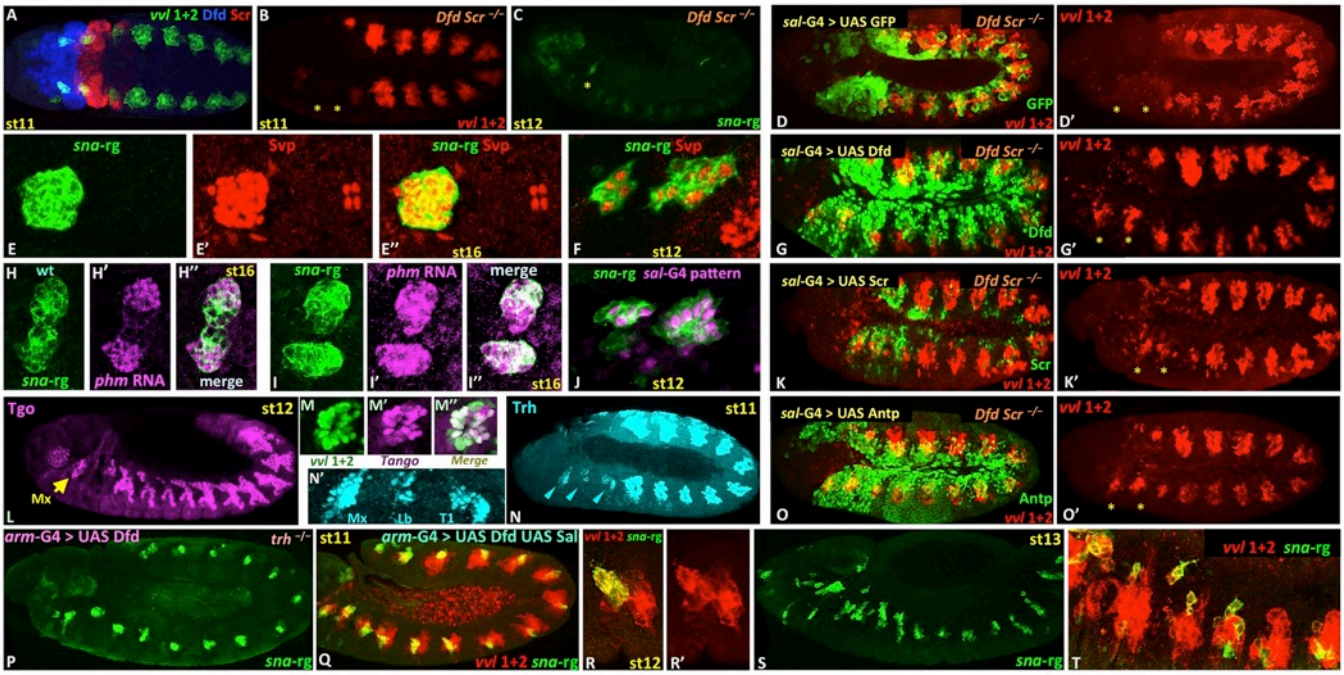
A
A9
Mx
Lb
T1
st10
B
st11
C
st12
D
st13
E
st11 early
F
st11 late
G
st13
H
st14
I
st15
J
sna-rg svp st11
K
sna-rg
K'
st15
sna-rg svp
vvl 1+2 aPKC
st11
N
L
sna-rg Sal st11
M
sna-rg
M'
st15
sna-rg sal-G4 >RFP
vvl 1+2 aPKC
st11
N'
O
vvl 1+2 sna-rg aPKC
st12
O'
vvl 1+2 aPKC
vvl 1+2 aPKC
P
st13


A
vvl 1+2 Dfd Scr
st11
B
Dfd Scr -/-
st11
vvl 1+2
C
Dfd Scr -/-
st12
sna-rg
sal-G4 > UAS GFP
Dfd Scr -/-
GFP
vvl 1+2
D
vvl 1+2
D'
sna-rg
E
Svp
E'
sna-rg Svp
E''
st16
sna-rg Svp
F
st12
sal-G4 > UAS Dfd
Dfd Scr -/-
Dfd
vvl 1+2
G
vvl 1+2
G'
H wt
sna-rg
H'
phm RNA
H''
st16
merge
sna-rg
I
phm RNA
I'
merge
I''
st16
sna-rg sal-G4 pattern
J
st12
sal-G4 > UAS Scr
Dfd Scr -/-
Scr
vvl 1+2
K
vvl 1+2
K'
Tgo
st12
Mx
L
M
vvl 1+2
M'
Tango
M''
Merge
N'
Mx
Lb
T1
Trh
st11
N
sal-G4 > UAS Antp
Dfd Scr -/-
Antp
vvl 1+2
O
vvl 1+2
O'
arm-G4 > UAS Dfd
trh -/-
sna-rg
P
st11
arm-G4 > UAS Dfd UAS Sal
vvl 1+2 sna-rg
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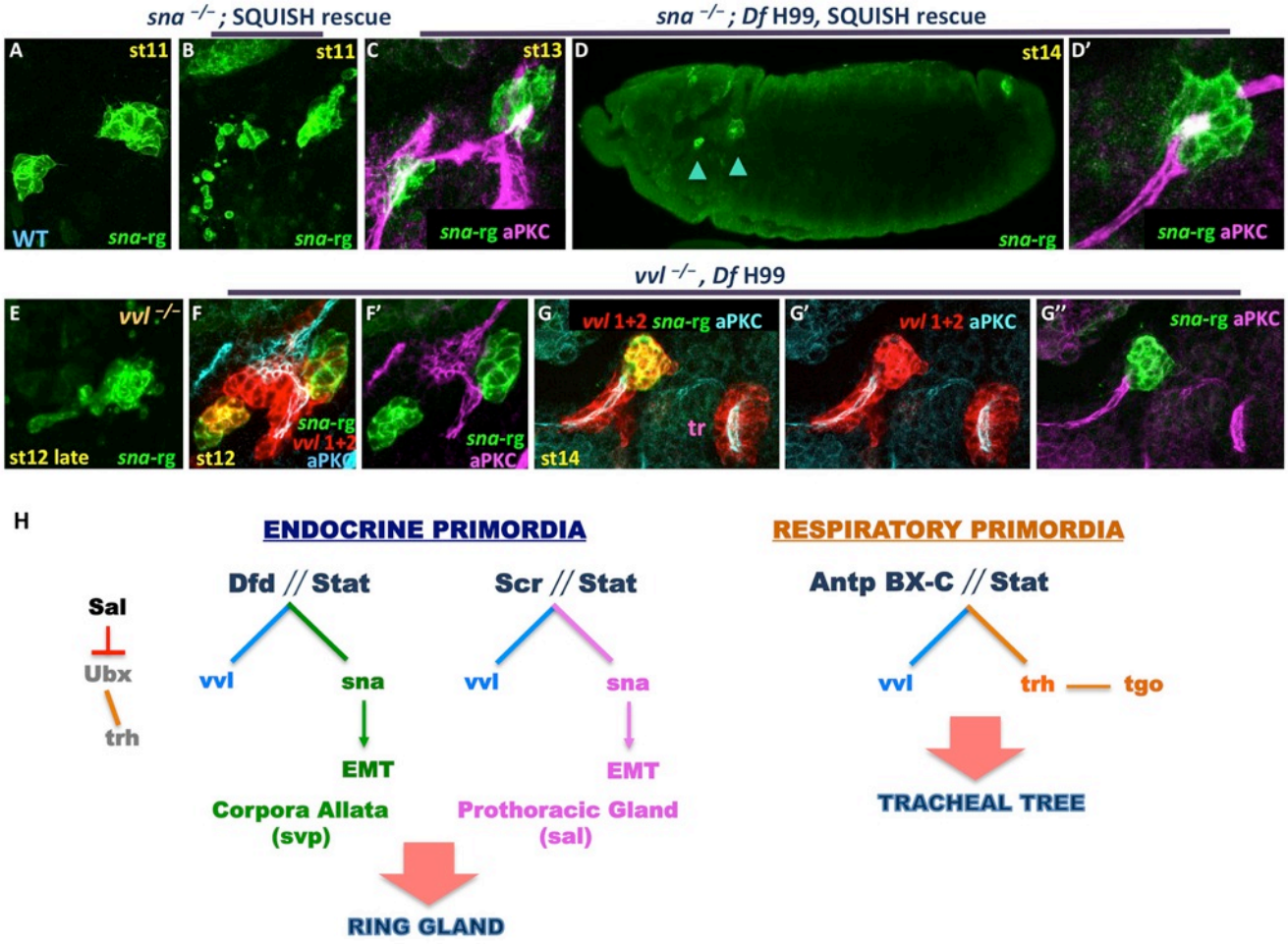
R
st12
R'
st13
sna-rg
S
vvl 1+2 sna-rg
T


sna -/- ; SQUISH rescue
sna -/- ; Df H99, SQUISH rescue
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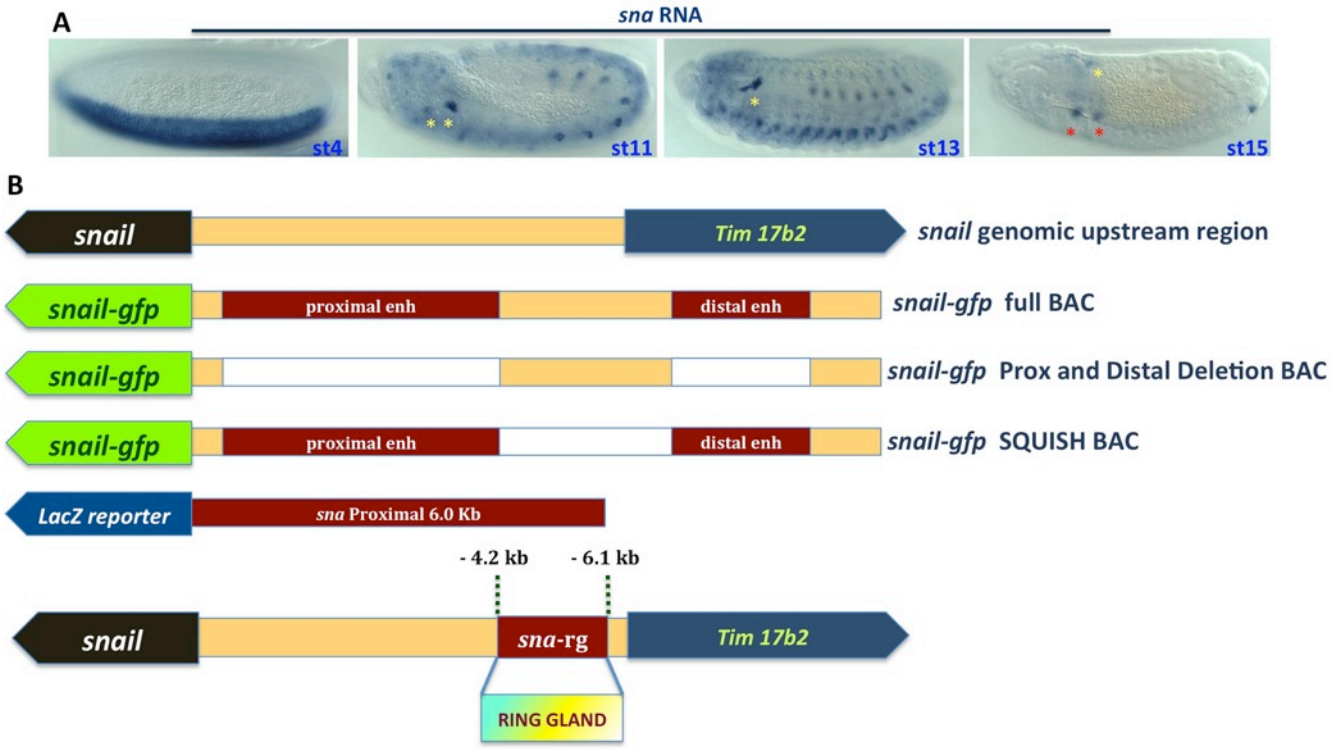
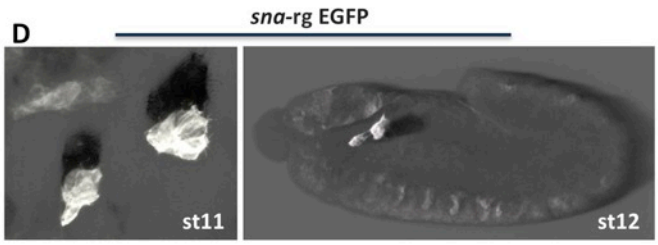
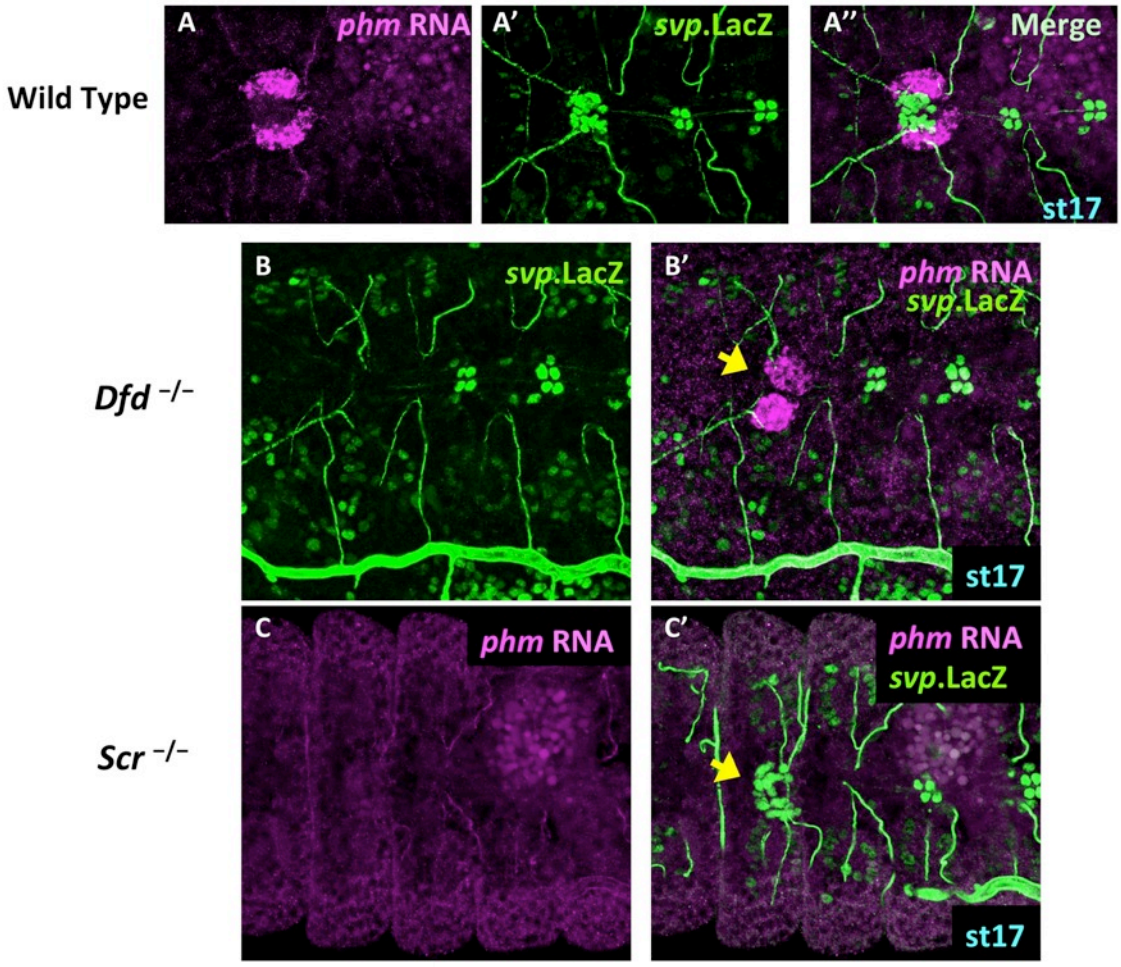
B st11 sna-rg
C st13 sna-rg aPKC
D st14 sna-rg
D' sna-rg aPKC
vvl -/- , Df H99
E vvl -/- st12 late sna-rg
F st12 sna-rg vvl 1+2 aPKC
F' sna-rg aPKC
G vvl 1+2 sna-rg aPKC st14 tr
G' vvl 1+2 aPKC
G'' sna-rg aPKC
H
ENDOCRINE PRIMORDIA
RESPIRATORY PRIMORDIA
Sal
Ubx
trh
Dfd // Stat
vvl
sna
EMT
Corpora Allata (svp)
Scr // Stat
vvl
sna
EMT
Prothoracic Gland (sal)
RING GLAND
Antp BX-C // Stat
vvl
trh
tgo
TRACHEAL TREE



C

Gene Construct	Ring Gland Expression
sna-gfp Full BAC	Positive
sna-gfp Proximal and Distal Deletion BAC	Positive
sna-gfp SQUISH BAC	Negative
sna Proximal 6.0 Kb LacZ	Positive

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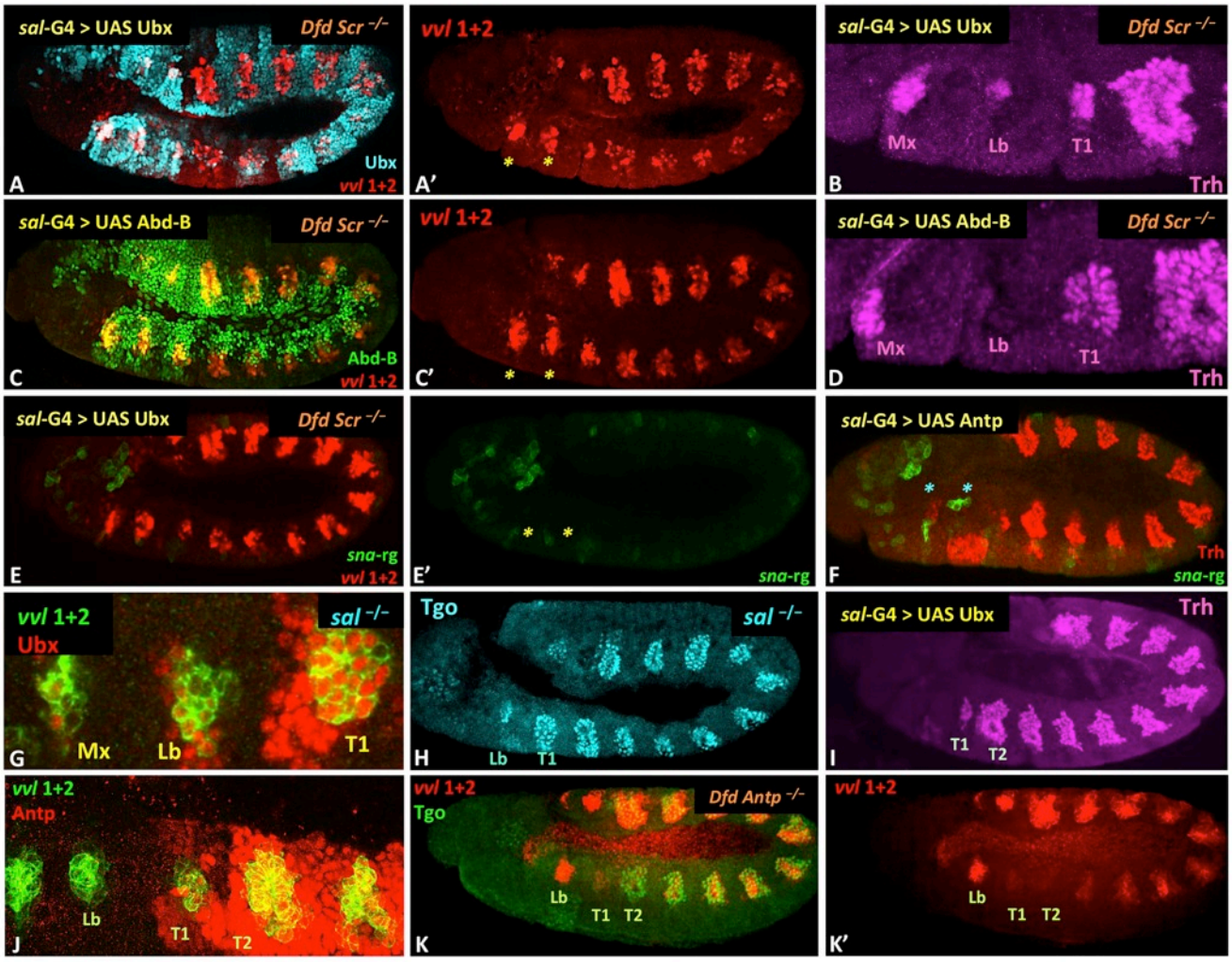
A'
svp.LacZ
A''
Merge
st17
Dfd -/-
B
svp.LacZ
B'
phm RNA
svp.LacZ
st17
Scr -/-
C
phm RNA
C'
phm RNA
svp.LacZ
st17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Dfd Scr -/-
Ubx
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A
vvl 1+2
A'
sal-G4 > UAS Ubx
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Mx
Lb
T1
Trh
B
sal-G4 > UAS Abd-B
Dfd Scr -/-
Abd-B
vvl 1+2
C
vvl 1+2
C'
sal-G4 > UAS Abd-B
Dfd Scr -/-
Mx
Lb
T1
Trh
D
sal-G4 > UAS Ubx
Dfd Scr -/-
sna-rg
vvl 1+2
E
sna-rg
E'
sal-G4 > UAS Antp
Trh
sna-rg
F
vvl 1+2
Ubx
sal -/-
G
Mx
Lb
T1
Tgo
sal -/-
H
Lb
T1
sal-G4 > UAS Ubx
Trh
I
T1
T2
vvl 1+2
Antp
J
Lb
T1
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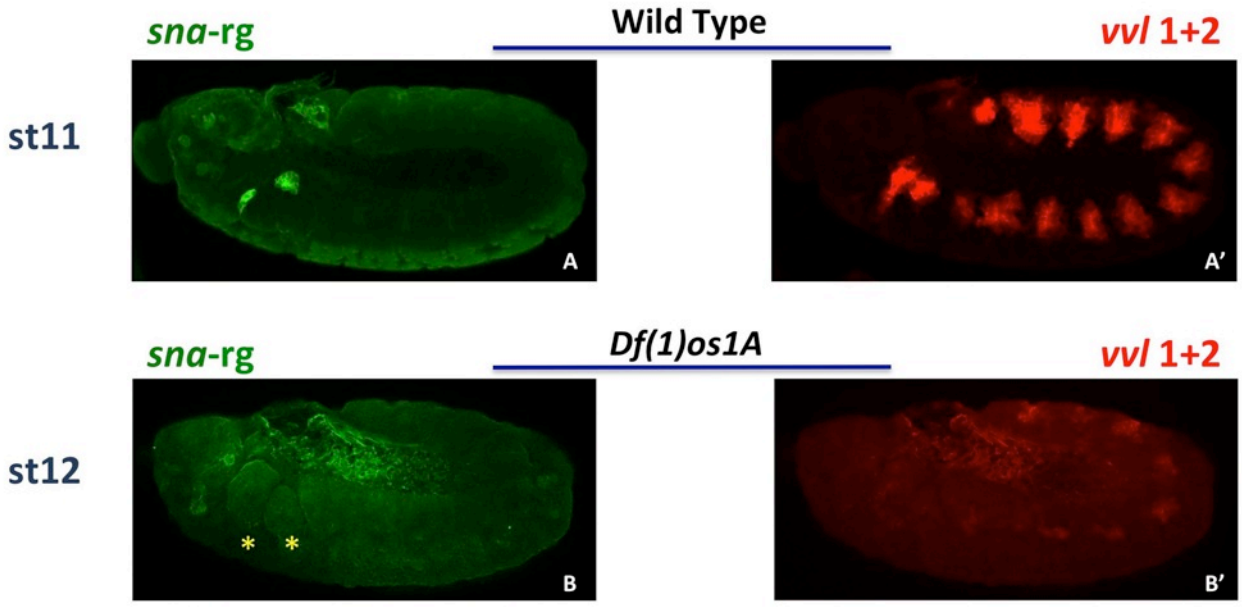
Wild Type
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st11
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A'
Df(1)os1A
sna-rg
vvl 1+2
st12
B
B'