RESEARCH ARTICLE

Specification of the endocrine primordia controlling insect moulting and metamorphosis by the JAK/STAT signalling pathway

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Abstract

The corpora allata and the prothoracic glands control moulting and metamorphosis in insects. These endocrine glands are specified in the maxillary and labial segments at positions homologous to those forming the trachea in more posterior segments. Glands and trachea can be homeotically transformed into each other suggesting that all three evolved from a metamerically repeated organ that diverged to form glands in the head and respiratory organs in the trunk. While much is known about tracheal specification, there is limited information about corpora allata and prothoracic gland specification. Here we show that the expression of a key regulator of early gland development, the snail gene, is controlled by the Dfd and Scr Hox genes and by the Hedgehog and Wnt signalling pathways that induce localised transcription of upd, the ligand of the JAK/STAT signalling pathway, which lies at the heart of gland specification. Our results show that the same upstream regulators are required for the early gland and tracheal primordia specification, reinforcing the hypothesis that they originated from a segmentally repeated organ present in an ancient arthropod.

Author summary

The main endocrine organs controlling insect moulting and metamorphosis are the corpora allata and the prothoracic glands. Genetic experiments in Drosophila melanogaster suggested that, despite their extremely different morphology and function, the corpora allata and the prothoracic glands are homologous to the respiratory trachea. All three organs derive from a primordium arising at similar locations along the cephalic and trunk segments, they activate common developmental genes using the same cis-regulatory elements, and can be transformed into each other by modifying Hox expression. One key difference between glands and trachea is that the endocrine primordia activate the Epithelial to Mesenchymal inducer gene snail. Using the snail gland specific enhancer as a proxy for gland formation, we show that the glands are specified by the same inputs specifying the trachea. These include the JAK/STAT, the Hedgehog and Wingless signalling pathways.
pathways as well as inputs from the Hox genes. These observations support the hypothesis that during arthropod evolution, a metamerically repeated organ diverged to give rise to endocrine glands in the head and respiratory organs in the trunk segments.

Introduction

Arthropods are characterised by the presence of an external skeleton that protects them from injury but also constrains their growth during development. This problem is solved by a dedicated endocrine system controlling the periodic moulting of the exoskeleton. Two glands control the process of larval moulting and metamorphosis in insects: the corpora allata (CA), which secrete Juvenile Hormone; and the prothoracic glands (PG), which secrete Ecdysone [1]. In holometabolous insects, secretion of both of these hormones to the haemolymph induces the larva moulting into a larger larva, while secretion of Ecdysone alone induces metamorphosis [2]. Similar endocrine glands secreting hormones related to those produced by the CA and the PG have been identified in crustaceans, indicating that this system has an ancient evolutionary origin [1,3,4].

Analysis of development in Drosophila melanogaster showed that the CA and the PG primordia are specified in the lateral ectodermal cells of the maxillary and the labial segment respectively, at homologous locations to those giving rise in more posterior segments to the fly’s respiratory organs [5]. During early development, the CA and the PG primordia exhibit a similar behaviour to that of the tracheal primordia, with the epithelium invaginating to form small sacks of cells resembling tracheal pits. However, while the tracheal primordia maintain an epithelial organization throughout development, the gland cells soon experience an Epithelial to Mesenchymal Transition (EMT) induced by snail (sna) gene expression [5]. Following Snail activation, the CA and the PG coalesce into a single primordium that migrates across four segments until it reaches the dorsal part of the first abdominal segment (A1). This migration is guided by several intermediate landmarks that serve as “stepping stones” during their long-range migration (Fig 1A) [6]. Once in A1, the CA/PG primordium fuses ventrally to the corpora cardiaca, an independent endocrine organ of mesodermal origin [7,8], and dorsally to the contralateral primordium, giving rise to a ring structure encircling the anterior aorta. Therefore, the mature ring gland is a composite endocrine organ formed by three different glands, two of ectodermal origin (the CA and the PG) and one of mesodermal origin, the corpora cardiaca [1,6].

Despite their different morphology and function, the CA and the PG have several characteristics in common with the trachea. First, the CA and the PG are specified in the cephalic lateral ectoderm at homologous positions to those forming the tracheal primordia in the trunk segments. Second, all three organs express the gene encoding the transcription factor Ventral veinless (Vvl) activated through the same enhancer (vvl1+2). Third, ectopic expression of the Deformed (Dfd) or the Sex combs reduced (Scr) Hox genes can transform tracheal primordia cells into gland cells and, conversely, the ectopic activation of trunk Hox genes can transform the gland primordia into trachea. These observations led to the proposal that the CA, the PG and the trachea arose from a metamerically repeated ancient structure that evolved divergently in each segment giving rise to three completely different organs [5]. This hypothesis has been reinforced by functional studies performed in the Oncopeltus hemipteran insect [9].

In comparison to the extensive knowledge we have of the mechanisms specifying the Drosophila tracheae [10–20], little is known about CA and PG specification. The first signs of CA and PG specification are noticeable when these primordia start expressing the sna gene [5].
Fig 1. Localisation of the specific corpora allata and prothoracic snail cis regulatory elements. (A) Scheme of a st16 embryo representing the CA and PG in green, the corpora cardiaca (CC) in red and the aorta and heart in blue. The migratory route followed by the three gland primordia towards their

Wild type

snail RNA

snai^Arg^R2

snai^Arg^R2; snail-gfp BAC rescue

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Snail is a zinc-finger transcription factor conserved in vertebrates where its function has also been associated to the induction of EMT [21–23]. Apart from its function in the endocrine primordia, Snail is also required for the formation of the mesoderm [24,25]. The sna-rg-GFP reporter gene, made with a 1.9 kb sna cis-regulatory element, is the earliest known specific marker for the CA and the PG primordia [5]. sna-rg-GFP expression is first activated at the beginning of organogenesis (st11), after the two gland primordia have just invaginated in the maxillary and labial segments, and its expression is maintained throughout embryonic gland development (Fig 1G and 1H). Thus, sna expression is a CA and PG specific marker comparable to what trh expression is for the trachea. Both genes encode transcription factors labelling the respective primordia at the earliest stages of development and both genes are required for the development of the organs where they are activated. Therefore, finding the upstream regulators of sna-rg expression should help uncovering the mechanisms required for gland specification. Moreover, the comparison of the gene network activating sna expression in the gland with that activating trh expression in the trachea will allow us to confirm if both organs share similar upstream regulators as would be expected if they shared a common evolutionary origin.

To find out what are the mechanisms inducing CA and PG specification we have analysed how snail expression is activated in the primordia of these organs. We show that the Wnt and Hh pathways determine the antero-posterior segmental location where the sna-rg enhancer is activated. This is achieved indirectly through the localised transcriptional activation of the upd gene, which encodes a ligand activating the JAK/STAT signalling pathway. We show STAT directly activates sna expression in the glands and propose that the Hox input required for activating sna expression is mediated indirectly.

**Results**

**sna expression in the CA and PG primordia is activated by a single cis-regulatory region**

Expression of the snail gene in the corpora allata (CA) and the prothoracic gland (PG) primordia is key for their specification and development [5]. To test if the sna-rg cis-regulatory region previously described is the only element activating snail expression in the CA and the PG primordia, we created snaΔrgR2, a deletion generated with the CRISPR-Cas9 system using specific single guide RNAs (Figs 1B and S1 and Materials and Methods). RNA *in situ* hybridization reveals snaΔrgR2 embryos lack sna expression in the CA and PG primordia while maintaining it in other organs (Fig 1C–1F).

Embryos homozygous for snaΔrgR2 or heterozygous for this deletion over the sna1 null allele are not viable. These embryos develop a normal mesoderm with the only obvious phenotypic
defect being the almost complete degeneration of the CA and the PG primordia (Fig 1G–1J). Embryo lethality and gland development are fully restored by a sna-GFP BAC construct [26], revealing that snaΔrgR2 lethality is due to the sna deletion (Fig 1K–1M).

These results prove that the snaΔrgR2 deletion inactivates the only regulatory region driving sna expression in the CA and PG gland primordia, allowing us to use sna-rg-GFP reporter expression as a proxy to discover the upstream trans regulatory elements involved in sna transcription and CA and PG specification.

**Requirement of the Wnt signalling pathway for gland specification**

The vvl and sna genes are co-expressed in the CA and the PG, but the expression of sna in the gland primordia does not depend on Vvl function [5], suggesting that both genes may respond to similar upstream regulatory cues in the gland region. As tracheal vvl expression expands in wingless (wg) mutants [20], we tested if sna-rg spatial activation is also restricted through the Wnt signalling pathway. In wgCX4 or in wgen11 homozygous mutant embryos sna-rg-GFP expression in the maxilla and the labium appears duplicated at st11 (Fig 2A and 2B). The duplicated primordia form in cells normally expressing Wg and are located at the same dorso-ventral position where the endogenous primordium of that segment forms. The ectopic and the normal sna-rg expressing cells become migratory coalescing into a single larger gland primordium, suggesting the ectopic cells form functional gland primordia, although this expanded primordium cannot reach the embryo’s dorsal side due to the general defects in wg mutants.

Ectopic UAS-wg expression driven in the maxilla and labium with the sal-Gal4 driver eliminates sna-rg reporter expression (Fig 2C). This repression is mediated through the Wnt canonical pathway as sna-rg-GFP expression is also eliminated by ectopic expression of an activated form of Armadillo (UAS-ArmS10, Fig 2D) [27]. Surprisingly, we found that while sna-rg expression is normal in embryos homozygous for the pan2 zygotic null allele of dTCF [a.k.a. Pangolin [28,29]], the DNA binding protein downstream of the Wg signalling pathway (Fig 2E), double mutant wgCX4, pan2 embryos lack the ectopic gland primordia but not the endogenous ones (Fig 2F). These results suggest that Arm-dTCF can prevent sna-rg expression in Wg expressing cells but it does not affect the formation of the endogenous gland primordia which are out of Wg signalling range.

**Requirement of the Hedgehog (Hh) signalling pathway for gland specification**

It has been reported that vvl expression in the tracheal primordia is strongly reduced in hh mutants [12]. The Hh and Wnt signalling pathways cross-regulate in the trunk epidermal cells where Hedgehog signalling is required for maintenance of wg expression in the adjacent ectodermal cells of the anterior compartment, and Wg signalling is required for the maintenance of hh and engrailed (en) expression in the posterior compartment [30]. As a result of this cross-regulation, wg, en and hh mutant embryos have similar phenotypes in the trunk ventral ectodermal segments [31]. However, in the cephalic region, where the glands are specified, such cross regulation does not occur, with Engrailed expression being maintained in the posterior segments of the maxilla and the labium in the absence of wg function [32]. To study the effect of Hh signalling on gland development, we analysed hhAC and enE homozygous mutant embryos and found an almost complete absence of sna-rg expression (Fig 3B and 3C). Engrailed activates hh expression in the posterior compartment, from where secreted Hh induces the pathway in neighbouring cells. The final target is the Cubitus interruptus (Ci) protein that can act either as a transcriptional activator or as a repressor depending on the
Fig 2. Wnt pathway requirement for gland specification. (A-F) st11 gland primordia double labelled with sna-rGFP in green, and in red the wg expressing cells with a wg<sup>en11</sup> reporter line (A-B) or the vvl expressing cells with a vvl<sup>1+2-mCherry</sup> reporter line (C-F). (A) Control sna-rGFP wg<sup>en11-lacZ/+</sup> embryo. sna-rGFP labels specifically the CA and the PG cells invaginating at the anterior end of the maxillary and labial segments respectively. (B) In wg<sup>en11</sup> homozygous mutants, ectopic patches of sna-rGFP expression appear on the wingless expressing cells (arrows) in the maxilla and labium at the same dorso-ventral positions as the endogenous ones. (C) Wg ectopic expression driven in the maxillary and labial segments with spalt-Gal4 results in the downregulation of sna-rGFP expression (asterisks). Downregulation of vvl<sup>1+2</sup> cephalic expression is also observed. (D) Ectopic expression of activated Armadillo results in sna-rGFP downregulation (asterisks). (E) pan<sup>2</sup>(dTcf) null mutants present normal sna-rGFP expression and Engrailed (blue) stripe expression is maintained. (F) Wg<sup>CX4</sup> pan<sup>2</sup> double mutant embryos do not present ectopic endocrine primordia. Embryo in panel F is also stained with anti-Wg to recognise the homozygous wg mutants. Scale bar 50 μm.

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pathway’s activation state. In the absence of Hh, Ci is cleaved giving rise to a protein repressing the transcription of its direct targets [33]. Conversely, in the presence of Hh, the pathway’s activation prevents Ci’s cleavage, giving rise to a transcriptional activator [34].

We find that in ci$^{94}$ null embryos sna-rg-GFP is expressed in its normal pattern (Fig 3D), indicating Ci is not a necessary activator of sna expression in the glands. We also found that in double en$^{E}$, ci$^{94}$ mutant embryos the sna-rg-GFP expression is recovered compared to en$^{E}$ embryos (Fig 3 compare panel C with E), indicating that the Ci repressor form prevents sna-rg activation. To confirm this, we expressed UAS-Ci76, the repressor isoform of Ci [33], with the sal-Gal4 line and found this causes an almost complete absence of sna-rg expression (asterisk marks the approximate position where stage 13 migrating primordia should be expected). (G) Overexpression of the Ci activator isoform with sal-Gal4 results in an expansion of sna-rg expression. (H) Double wg$^{EXA}$, hh$^{AC}$ mutant embryos lack sna-rg expression (asterisks). (I) Model summarising regulatory interactions between the Wnt and Hh pathways and the sna-rg enhancer (The interactions represented are not assumed to be direct). Panels D–E were also stained with anti-Ci, panel E with anti-En and panel H with anti-Wg to identify the mutant embryos. All embryos are at st11 except F and G which are at st13 and st12. Scale bar 50 μm.

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The above data fit a model where sna-rg expression is under negative regulation, either directly or indirectly, mediated by the Wnt and Hh signalling pathways (Fig 3I). Although Ci repression of sna-rg activity should be relieved by Hh signalling anteriorly and posteriorly to the En expressing cells, the Wnt parallel repressive function prevents sna-rg activation in Wg expressing cells restricting the formation of the CA and PG primordia to the most anterior cells of the maxillary and labial segments.

Regulation of Upd ligand expression by the Wg and Hh pathways

Previously we showed that JAK/STAT signalling is required for sna-rg expression [5]. To find out if the Wg and Hh signalling pathways regulate sna indirectly via JAK/STAT signalling, we reanalysed the spatio-temporal activation of upd in wild type and mutant embryos, paying special attention to the maxillary and labial segments where the gland primordia are specified. In st9 wild type embryos, upd is expressed in segmental stripes immediately posterior to the Engrailed expressing cells (S2 Fig). This pattern of transcription evolves to form a transient antero-posterior lateral stripe that rapidly resolves at early stage 11 into two patches of expression in the maxilla and labium corresponding to the sites where the CA and PG glands form (Figs 4C and S2E). Expression analysis of 10xSTAT-GFP, a reporter that is universally activated in cells where the JAK/STAT pathway is active [35,36] confirms JAK/STAT signalling activation at st10 and 11 in the CA and PG primordia (Fig 4A–4B). Although upd is transcribed in both primordia, we noticed that expression of both upd RNA and the 10XSTAT-GFP reporter is more transient in the CA than in the PG primordium (Fig 4A–4D).

We next analysed if the Wnt and the Hh pathways affect upd transcription in the gland primordia. In hhAC null embryos, we find that the transient upd expression in the CA and PG primordia disappears (Fig 4E), while in wgCGT mutants upd RNA expression expands (Fig 4F). We also found that ectopic expression of the activator Ci protein results in a non-fully penetrant expansion of upd expression in stage 10 embryos (Fig 4H–4I). These results suggest that the effects on sna-rg expression caused by mutations affecting the Wnt and Hh signalling pathways are mediated indirectly through the JAK/STAT signalling pathway.

Possible cross-regulation between Hox, wg, hh and upd in the maxillary and labial segments

Development of the CA and PG and normal expression of the sna-rg reporter in the maxilla and the labium require Dfd and Scr function [5], therefore we studied if there are any cross-regulatory interactions among the genes involved in gland primordia specification.

We first analysed wg and en mutant embryos and found that the expression of Dfd and Scr is not significantly affected (S3A–S3F Fig). Similarly, neither En nor Wg expression is affected in Dfd Scr mutant embryos (S3G–S3J Fig), discarding a possible interaction between Dfd and Scr and the Wnt/Hh signalling pathways. In contrast, we found that the transient expression of upd transcription in the CA and PG primordia almost disappears in Dfd Scr mutant embryos (Fig 4G), indicating that the Hox proteins can regulate JAK/STAT signalling as previously shown for Abd-B [37]. These results indicate that the Hox, and the Wnt/Hh pathways indirectly mediate the regulation of the sna-rg enhancer through their modulation of upd expression and JAK/STAT signalling activation.

The sna-rg reporter is not expressed in Df(1)os1A embryos (Fig 5A and 5B). To test if generalised Upd expression in the maxilla and labium can activate sna-rg independently of other upstream positive or negative inputs, we induced UAS-upd with either the sal-Gal4 or the arm-Gal4 lines. We observe that these embryos have expanded sna-rg expression along the antero-posterior axis in the maxillary and labial segments (Fig 5C). Analysis of Sal expression, which
Fig 4. JAK/STAT pathway requirement for gland specification. (A-B) Wild type embryos carrying the sna-rg-mCherry (red) and 10xSTAT-GFP (green) reporter genes. (A) At st10, embryos present patches of green staining in the maxillary and labial segments at the position where the gland primordia will become activated. Weak sna-rg expression starts to appear. (B) At st11, when sna-rg-mCherry is strongly activated, 10xSTAT-GFP expression has faded from the maxilla while is maintained in the labium. Note that 10xSTAT-GFP expression is also activated in the tracheal primordia. (C-I) upd mRNA in situ hybridization in st10-st11 embryos. (C) A wild type early st11 embryo showing upd mRNA in the maxillary and labial segments is restricted to two patches where the CA and the PG form. (D) At late st11, upd mRNA expression only remains in the PG (asterisk marks the position where the CA primordium is located). (E) hh null mutants lose maxillary and labial upd transcription (asterisks), which remains in the tracheal primordia. (F) In wg null mutants, upd transcription is extended in the maxillary and labial segments (arrows), as well as expanding...
labels the PG primordium [5] shows that Upd ectopic expression induces a moderate expansion of the CA primordia while resulting a much larger increase of the PG primordium (Fig 5D and 5E). This expansion occurs mostly in the antero-posterior axis from cells where the Hh and the Wnt pathways are normally blocking sna-rg expression, while expansion is less noticeable in the dorso-ventral axis. This indicates that most of the antero-posterior intrasegmental inputs provided by the segment polarity genes converge on Upd transcription but that the dorso-ventral information is registered downstream of Upd.

We finally tested if activation of UAS-upd with the sal-Gal4 driver line can rescue sna-rg activation in Dfd Scr mutant embryos. We found that the residual levels of GFP observed in sna-rg Dfd Scr mutant embryos are not increased in sal-Gal4 UAS-upd sna-rg Dfd Scr embryos (Fig 5F and 5G), indicating that besides regulating upd expression, the Hox input has further requirements for gland formation.

Therefore, localised Upd expression defines the antero-posterior intrasegmental localisation of the CA and PG primordia, but other signals besides STAT must be controlling the dorso-ventral and the cephalic sna activation either directly at the sna-rg enhancer level or through unknown intermediate regulators.

Analysis of the direct regulation of sna-rg enhancer by STAT

To find out if the Hox and STAT inputs regulate sna expression directly, we searched for putative binding sites in the cis-regulatory region of sna-rg. To facilitate the bioinformatic analysis we dissected the 1.9 kb sna-rg regulatory element down to a 681bp fragment we call R2P2 (S1A–S1E Fig). The sna-rg-R2P2-GFP reporter construct drives high levels of expression in the CA and PG and its expression is even more specific as it lacks the low levels of GFP expression observed in the haemocytes and neurons of the larger sna-rg-GFP reporter.

Computational JASPAR analysis [38] of the 681bp R2P2 sequence identified three putative Hox-Exd-Hth and three putative STAT binding sites (Fig 6A). Further subdivision of sna-rg-R2P2 in two halves shows that neither the A1 nor the A2 half drive embryonic expression (Fig 6C and 6D). Reporters containing A1 fused to either the proximal part of A2 (the sna-rg A1+A2prox-GFP reporter containing a single STAT site) or to the distal part of the A2 element (the sna-rg A1+A2dist-GFP reporter containing two STAT sites) recovered ring gland expression (Fig 6E and 6F). The recovery of expression when A1 is fused to either fragment, both containing STAT binding sites, made us wonder if the lack of expression of the A1 fragment is due to the absence of STAT binding sites. To test this hypothesis, we added to A1 a 20bp fragment that contains a single functional STAT site taken from an unrelated gene [the vvl1+2 enhancer of the ventral veinless gene [20]] creating the sna-rg A1+STAT reporter. We find that A1+STAT drives expression in both the maxilla and the labium and that this depends on JAK/STAT signalling, as mutation of the STAT-binding site abolishes expression in the sna-rg A1+STATmut reporter (Fig 6G and 6H). Taken together, these experiments show that the presence of functional STAT binding sites is required for sna activation in the CA and PG primordia and that the 300bp A1 fragment can interpret the segmental cephalic positional information, suggesting that the Hox-Exd-Hth site located in A1 could mediate Dfd and Scr input to the enhancer.
Fig 5. Epistatic relationship between JAK/STAT, Wnt, Hh and Hox inputs over sna-regulation. (A-C) Embryos expressing \textit{sna-reg-GFP} (green) and \textit{vvl1+2-mCherry} (red) stained with anti-En (blue). (A) \textit{sna-reg} and \textit{vvl1+2} expression in st11 control.
embryos. Panels (A') and (A'') show each channel separately to appreciate the co-expression of both markers in the gland primordia. (B) Df(1)os1A embryos show an almost complete downregulation of sna-rg and vvl1+2 expression from the CA and PG (asterisks). (C) Ectopic Upd expression driven with sal-Gal4 induces ectopic sna-rg and vvl1+2 expression in the gnathal segments, which for sna-rg is more pronounced in the labium than in the maxilla. Note that in the maxillary segment Upd can induce ectopic dorsal vvl1+2 but not sna-rg expression, this is expected as Dfd only induces sna-rg ventrally in the maxilla. (D-E) sna-rg-GFP embryos stained with anti-GFP (green) and anti-Sal (red). In control embryos (D) Sal labels the PG primordium but not the CA. In arm-Gal4 embryos ectopically expressing Upd (E) the PG is more expanded than the CA as shown by the number of cells co-expressing Sal and GFP. (F-G) sna-rg-GFP expression (green) in st13 Dfd Scr mutant embryos (F), or Dfd Scr mutant embryos after ectopic Upd expression driven with the sal-Gal4 line (G) showing that Upd activation is not sufficient to rescue gland formation in Dfd Scr mutants. In Dfd Scr mutant embryos (F), although the gland primordia become apoptotic, residual GFP expression indicates that there must exist Hox independent inputs activating the sna-rg enhancer. Embryos in (F-G) are also stained with anti-Scr to recognise the homozygous mutants. Scale bars 50 μm.

To confirm STAT’s binding sites requirement, we mutated all three putative sites on the R2P2 fragment generating the sna-rg-R2P2 STATmut construct expressing simultaneously the LifeActin-GFP and nuclear Histone-RFP reporter markers (Materials and Methods). Comparing its expression to the sna-rg-R2P2-eGFP-PH, we find that although mutating the three STAT binding sites completely abolishes the reporter’s expression in the PG, surprisingly, it does not eliminate its expression from the CA, where its activation is only slightly delayed (Fig 6I and 6J). The CA expression of the sna-rg-R2P2 STATmut construct still depends on upd activity as it disappears in Df(1)os1A embryos lacking all Upd ligands (Fig 6K). These results indicate that either there is a cryptic STAT site in the enhancer by Hox proteins

Analysis of the regulation of sna-rg enhancer by Hox proteins

To test genetically the requirement of the Hox proteins and their cofactors for sna activation, we studied sna-rg expression in mutants for Dfd Scr and for the Hox cofactor hth [39]. In Dfd Scr mutant embryos few cells activate sna-rg-GFP expression at st11 (S4A Fig) and those that do soon acquire an apoptotic aspect (Fig 5F), confirming Hox requirement for gland development. Similarly, in hthP2 mutants, sna-rg expression almost disappears (S4B Fig). As described above, there are three JASPAR predicted Hox-Exd-Hth putative binding sites present in the R2P2 fragment. We first mutated the sites located in sna-R2P2 closer to the STAT binding sites in the A2 region and found that the expression of the mutated construct was almost identical to the wild type fragment. The dispensability of these two Hox-Exd-Hth sites for sna activation in the maxilla and labium is further confirmed by the strong expression driven by the snaA1+A2prox-GFP reporter that lacks these two sites (Fig 6E). Although the snaA1-A2prox reporter construct is slightly derepressed in the cephalic region, it is still active in the CA and PG primordia, indicating that, if there is any direct requirement for Hox activation, this would be mediated by the Hox-Exd-Hth site located in fragment A1. This site has a class 2 sequence (TGACAAAT) that has been shown by SELEX-seq analysis to bind preferentially Dfd and Scr proteins in complex with the Exd-Hth cofactors [[40] and S4C Fig]. We mutated in snaA1-A2prox this class 2 site to (TGATCAAAT) which is not detected in vitro by any Hox-Exd protein complex and found the embryos maintain robust expression in the CA and the PG suggesting the enhancer is not a direct Hox target (S4D' Fig). To confirm this, we also mutated the putative class 2 site in snaA1+A2prox changing its affinity to class 1 or class 3 Hox proteins. Such changes have been shown to affect the spatial expression of vvl1+55, a reporter construct directly regulated by the Dfd and Scr proteins and as result only active in the maxilla and the labium. While mutating in vvl1+55 the class 2 site towards a class 3 site conferring affinity for either Antp, Ubx, Abd-A and Abd-B protein in complex with Exd strongly activates
Fig 6. Direct regulation of sna-rg by STAT. (A) Representation of the minimal sna-rg R2P2 subfragments indicating the location of the putative STAT (pink crosses) and Hox-Exd-Hth DNA binding sites (black boxes). Mutated STAT binding sites are represented.
with a red X over the pink cross. (B) sna-rg R2P2-GFP expression. (C) No GFP expression is observed in A1 nor in A2 constructs (D). Gland expression is observed when A1 is joined to the A2 proximal half (E) or when A1 is joined to the A2 distal half (F). (G) A1 fused to a 20bp from fragment the vvl1+2 enhancer containing a functional STAT binding site. (H) A1 fused to the same 20bp fragment where the STAT binding site has been mutated. (I-K) Embryos carrying both the sna-rg-R2P2-GFP-PH (green) and the sna-rg-R2P2-STATmut Histone2B-RFP-GFP-PH (red and green) constructs. Red nuclear expression is not observed at early stage 11 (I) but can be detected later exclusively in the CA (J). Df(1)os1A embryos lacking all Upd ligands (K) do not express sna-rg-R2P2-STATmut mCherry-GFP-PH. Black arrows in (E-G) point to the CA/PG gland primordia, red arrows to ectopic expression outside the glands. Scale bars 50 μm.

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the enhancer in the trunk, and mutating the sequence towards a class 1 site that confers affinity for the Lab protein activates the enhancer in the intercalary segment where Labial is expressed [19]; equivalent mutations of the class 2 site in the A1+A2prox fragment did not modify significantly the spatial expression of the reporter, which remains expressed mostly in the maxilla and labium (S4E–S4G Fig), further supporting that sna is not directly activated by the Hox-Exd-Hth complex in the endocrine glands.

**Discussion**

**Intrasegmental specification of the CA and the PG**

We have found that the Wnt, the Hh and the JAK/STAT signalling pathways contribute to the specification of the CA and the PG in the maxillary and labial segments. Our results indicate that the Hh and the Wnt pathways act indirectly by negatively regulating the spatial activation of the Upd ligand (Fig 7). Engrailed activation of hh transcription in the posterior compartment of the cephalic segments leads to Hh diffusion to the neighbouring cells in the maxilla and labium. Hh pathway activation prevents the formation of the Ci repressor protein allowing the activation of upd transcription at both sides of the posterior compartment. However, anterior to the engrailed stripe, Wnt pathway activation prevents upd transcription. As a result of the combined Hh and Wnt inputs, upd can be briefly transcribed at stage 11 in two localised ectodermal patches from where it induces JAK/STAT signalling, which activates sna transcription in the CA and the PG primordia through the sna-rg enhancer.

sna transcription in the endocrine primordia is mediated through a 681bp non-redundant cis-regulatory element located 5.439bp upstream of the sna transcription unit. The sna-rg-R2P2 enhancer contains a STAT 3n and two STAT 4n binding sites conforming to the canonical sequence TTCNNN(N)GAA [41,42]. Deletion from the R2P2 enhancer of a 278bp fragment containing all three putative STAT binding sites results in a complete loss of expression that can be regained by adding a single STAT 4n binding site from an unrelated gene, demonstrating STAT’s direct involvement in sna regulation. It is interesting to note that the position where we inserted the new STAT site is on the opposite end to where the endogenous STAT sites are located, indicating there is flexibility on STAT protein localisation with respect to other transcriptional regulators binding to the enhancer, something also noticed for the vvl1 +2 STAT-regulated cis-regulatory element [20].

The activation of Upd in the gland primordium at st11 is very transient while sna transcription is maintained at least until st16 of embryogenesis, indicating that JAK/STAT signalling is only required for sna’s initial activation in the CA and PG primordia but not for its maintenance. Maintenance of the sna-rg-R2P2 enhancer must be achieved by other elements of the gland gene-network induced by STAT or the Hox proteins. The existence of such a maintenance mechanism could explain why in wild type embryos, after mutating all three canonical STAT sites in the sna-rg-R2P2 STATmut reporter, the expression is still maintained in the CA: In these wild type embryos the endogenous gland gene-network is still functioning, activating the proposed ring gland maintenance mechanism that would be able to act on the sna-rg-R2P2
**STATmut** reporter even if it lacked the early STAT input. Although we cannot completely discard that the expression from the *sna-rg* enhancer in wild type embryos uncovers upstream factors mediating CA and PG specification. *upd* transcription is repressed directly or indirectly by both Ci and Arm-dTCF. Hh signalling from the posterior compartment releases the Ci repressive activity over *upd* transcription. Dfd and Scr proteins prevent the gland primordia from entering into apoptosis and also up-regulate Upd expression. Activated STAT induces *sna* transcription by direct binding to the *sna-rg* enhancer. Besides the Hox proteins, an additional factor (question mark) also contributes to *upd* expression in the maxilla and labium. This additional factor could be the activated form of the Ci protein or another factor still to be defined.

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**Regulatory similarities between CA, PG and tracheal specification**

The CA, the PG and the tracheae have been proposed to originate by the divergence of an ancient serially repeated organ present in an arthropod ancestor [5]. If this was the case, specification of all three organs would be expected to be under similar upstream regulation. Using the early activation of *sna* transcription in the gland primordia through the *sna-rg* enhancer as a proxy for their specification, we found that the CA and PG primordia require the same signalling pathways controlling the specification of the tracheal primordia. JAK/STAT pathway activity is key for the activation of *vvl* and *trh* in the trachea and also for *sna* in the glands.
Moreover, direct STAT binding to a tracheal specific early enhancer is required for vvl activation and here we show the direct STAT binding is also required for sna specific expression in the glands. Similarly, the Wnt pathway that is required for restricting vvl and trh expression to the tracheal primordium is also restricting the spatial expression of sna in the cephalic segments, although the sna-rg ectopic activation observed in wg mutants is less pronounced than that observed for vvl and trh in the trachea, that in some instances results in a continuous tracheal pit stretching from T2 to A8 due to the fusion of the primordia in neighbouring segments [12,19,20].

Previous work has reported the requirement of Hh for vvl expression in the trachea and for tracheal branch specification [12,43]. Here we also find that Hh and En are required in the gland primordia, although we find this requirement to be more pronounced in the glands than in the trachea judging from the almost complete disappearance of sna-rg expression. We also found that in hh mutants, upd expression disappears in gland primordia but not in tracheal cells, supporting the idea that Hh requirement is stronger in glands than in trachea.

Ectopic trunk Hox protein expression can activate both vvl and trh tracheal expression in the head. Hox requirement for vvl expression is fundamental both in the glands and in the tracheal primordia being controlled via direct DNA binding sites [19]. Although anterior Antp, Ubx, Abd-A or Abd-B Hox expression can also induce ectopic trh activation, it is unclear if this is mediated through direct Hox binding to the DNA regulatory sites [5]. Similarly, the Dfd and Scr Hox proteins are required for gland formation and their ectopic expression can induce ectopic sna-rg activation in the tracheal cells [5]. Our results indicate that Hox requirement for sna activation may be indirect, as mutating the putative Hox binding sites in the enhancer does not affect its expression. The CA and the PG primordia co-express sna-rg-GFP and Dfd and Scr briefly during st11 at the very early specification stage, with Hox expression becoming undetectable in the glands when they initiate migration [5]. Our observation that the transient upd expression in the gland primordia is affected in hh, wg and Dfd Scr mutant embryos, suggests that these pathways regulate sna expression in the ring gland indirectly through the activation of JAK/STAT signalling.

Another interesting similarity between glands and trachea is that, although ectopic Hox gene expression can ectopically induce sna-rg and trh outside their normal domain, the lack of Hox expression does not completely abolish their endogenous expression, indicating that in both cases a second positive input can compensate for the absence of Hox mediated activation. Our results suggest that, at least in the glands, this redundant input could be provided by the activating Ci form (Figs 3G and 4I), but further analysis to confirm this possibility and discard alternative sna-rg activators should be performed.

**Differences between CA and PG specification**

Development of the CA and the PG requires Sna activation, which in both primordia is regulated by the same enhancer. Also, both primordia are specified in the lateral ectoderm of the maxilla and the labium in cell clusters expressing vvl [5]. Despite these similarities, the position occupied by both primordia with respect to the vvl patch of expression is different. The CA is specified in the most ventral cells of the vvl maxillary patch and the PG is specified in the most dorsal cells of the vvl labial patch (Fig 5A). This suggests that despite their sharing of Hh, Wnt and JAK/STAT pathway regulation, the expression in each primordium must also have differential regulation. We have been unable to separate a CA enhancer from a PG enhancer by dissecting the sna-R2P2 cis-regulatory module into smaller fragments, suggesting that any gland specific binding sites in the enhancer are probably interspersed with the shared ones. The only case in which we were able to affect expression in the PG without affecting the CA was after
mutating the three STAT binding sites. Our results indicate sna expression in the CA is under direct STAT regulation (Fig 6C, 6G and 6H), as it is in the PG. The persistence of expression in the CA of the sna-rg-R2P2 STATmut reporter gene can be explained by the existence of differing expression maintenance factors in the CA and in the PG. Gland specific transcription factors like Seven-up (Svp) in the CA and Spalt (Sal) in the PG have been described that are expressed in the early gland primordia when they start their migration [5,44,45]. Future studies will help to discover if these or other gland specific factors are responsible for controlling the maintenance of sna-rg enhancer as well as the slightly different dorso-ventral positions where each gland is specified.

Our analysis of snail activation in the CA and PG shows that these glands and the trachea share similar upstream regulators, reinforcing the hypothesis that both diverged from an ancient segmentally repeated organ. In Drosophila melanogaster the CA and the PG primordia experiment a very active migration after which they fuse to the corpora cardiaca forming the ring gland [6]. This differs from more basal insects where the CA fuses to the corpora cardiaca but not to the PG, and from the Crustacea where the three equivalent glands are independent of each other [2–4,46]. As the mechanisms we here describe relate to the early specification of the glandular primordia in Drosophila, it will be interesting to investigate if the equivalent genes are also involved in the endocrine gland specification of more distant arthropods.

Materials and methods

Fly stocks

Flies were reared at 25˚C on standard Drosophila medium. The following mutant alleles and transgenic lines were used: wg\textsuperscript{xst}; CyO, wg\textsuperscript{en11}–lacZ; hh\textsuperscript{AC}; Df(2)en\textsuperscript{E}; ci\textsuperscript{94}; pan\textsuperscript{2}; Df(1)os1A; Dfd\textsuperscript{16}; Scr\textsuperscript{4}; Dfd\textsuperscript{16} Scr\textsuperscript{4} AbdB\textsuperscript{MB}; hth\textsuperscript{P2}; 10xSTAT–GFP; arm–Gal4; sal–Gal4; UAS–arm\textsuperscript{S10}; UAS–upd; UAS–wg; UAS–ci\textsuperscript{76}; UAS–ci\textsuperscript{PKA} [34] and sna–GFP BAC [26]. The following stocks previously generated in our laboratory were used: sna–rg–GFP; sna–rg–mCherry; sna–rgR2P2–GFP; vvl\textsuperscript{1+2}–mCherry.

Generation of sna\textsuperscript{ArgR2} deletion

The snail-rg R2 enhancer was removed by CRISPR-Cas9 site-directed deletion. Two flanking sna-rg sgRNAs (S1 Table) where cloned into the directed insertion vector pCDF4 [47] and the constructs injected into the 25C (#B25709) or the 68A (#B25710) landing sites using the phiC31 standard method at the Drosophila Consolider-Ingenio 2007 Transformation platform, CBMSO/ Universidad Autónoma de Madrid. Germ line deletions were induced by combining the above sna-rg sgRNA transgenes with nos-Cas9 [47]. Putative heterozygous mutant/If males were individually crossed to sna\textsuperscript{1}/CyO females to identify mutations lethal over the null sna\textsuperscript{1} allele. Lethal alleles were tested by PCR to detect the generation of a deletion and the exact nature of the deletion was confirmed by sequencing using the FwdSeq snaCRISPR and RvsSeq snaCRISPR primers (S1 Table and S1F Fig).

Immunostaining

Eggs laid overnight at 25˚C or 29˚C were dechorionated with 50% household bleach for 3 minutes and fixed with formaldehyde 4% and heptane in a 1:1 proportion for 20 minutes at room temperature. After removing the heptane, methanol was added and shaken vigorously to remove the vitelline membrane, embryos were stored for up to two weeks in PBS Tween 0.1%. The following primary antibodies were used: rabbit anti-GFP (Invitrogen, 1:300); rat anti-RFP (Chromotek, 1:200); mouse anti-β-Gal (Promega, 1:1000); mouse anti-En (Hybridoma Bank,
Fluorescent images were obtained with Leica SPE confocal microscope and processed with ImageJ and Photoshop. Light microscopy samples were visualized in a Zeiss Axioplan microscope.

**In situ hybridization**

155BS-upd from Doug Harrison and RE35237 from BDGP cDNAs were used to generate upd and sna RNA probes using the DIG RNA Labeling Kit (Roche). Secondary biotinylated antibody against mouse (1:200, Jackson ImmunoResearch) was used for double detection of RNA and protein.

**Constructs**

snaR2P2 subdivision avoided breaking sequence blocks conserved among different Drosophila species genomes (http://genome.ucsc.edu/). Fragments A1, A2 and A1+A2proximal were amplified by PCR using the following oligos: sna-rg for(KpnI) and snaR2P2B3rev; SnaR2P2B4for and sna-rg R2P2 rev (KpnI); or sna-rg for(KpnI) and snaR2P2B4rev respectively (S1 Table). Fragments were cloned into pGEMt-easy (Promega #A1360) and sequenced.

Fragment A1+A2distal was done using a modified protocol based on [48]. Template plasmid pGEMt-easy::SnaRGRP2 was amplified using primers SnaR2P2B3rev and SnaR2P2B5for flanking the deleted region. Before transformation, the PCR product was incubated 2h at 37˚C with DpnI restriction enzyme, polynucleotide kinase PNK, ligase and ligase buffer 2X.

pGEMt-easy::SnaRG-A1, pGEMt-easy::SnaRG-A2 and pGEMt-easy::SnaRG-A1+A2proximal were digested with Asp718 and subcloned into pCasper-PH-eGFP; pGEMt-easy::SnaRG-A1+A2distal were digested with NotI and subcloned into pCasper-PH-eGFP.

The pCASPER-PH-eGFP::A1+STAT-WT and pCASPER-PH-eGFP::A1+STAT-mut constructs were done using a pair of annealed oligos: statS1-NotI-for and statS1-NotI-rev; or statS1mut-NotI-for and statS1mut-NotI-rev respectively. Annealed oligos containing the STAT binding site and overhang NotI sites were cloned into pCASPER-PH-eGFP::SnaRGR2P2A1 or pCASPER-PH-eGFP::SnaRGR2P2A2 NotI-digested.

The sna-rg-R2P2 STATmut enhancer was made by performing a PCR mutagenesis in two steps: a first PCR round with the Fwd sna-rg + R2P1 Rvs OP 92E mut primers and with the Fwd SD 92E OP mut + Rvs sna-rg R2P2 92E mut primers (S1 Table). The two resulting PCR amplicons containing overlapping sequences, were mixed and used as a template to be amplified with the external primers: Fwd sna-rg BamHI + Rvs sna-rg R2P2 92E mut BamHI, to generate the final snail-R2P2 STATmut enhancer fragment, which was subcloned into the BamHI site from a pCaSpeR modified version to simultaneously express H2B-mRFP-P2A-LifeActinGFP.

**Hox and STAT binding site mutagenesis**

The putative class II (TGACAAAT) Hox binding site located at position 222–229 of A1+A2proximal was mutated to either erase the Hox site or to modify its affinity towards another Hox binding class site as described in [40]. Mutations were induced according to [48] using the following oligos: sna mut1 for and sna mut1 rev; sna mut2 for and sna mut2 rev; sna mut3 for and sna mut3 rev or sna mutNull for and sna mutNull rev.
The STAT binding site located at 504–513 (TTCCAATGAA) was mutated using the same method, with Sna mutSTAT for and Sna mutSTAT rev oligos.

Supporting information

S1 Fig. Isolation of a minimal sna-rg enhancer. (A) Scheme of DNA elements tested indicating the location of the STAT binding sites. The sna-rg R2 reporter comprises the cis-regulatory sequence deleted in the CRISPR-Cas9 sna<sup>ArgR2</sup> mutation. Expression at st11 of the full sna-rg-GFP (B), sna-rg R1-GFP (C), sna-rg R2-GFP (D), and sna-rg R2P2-GFP (E) constructs. (F) Scheme showing the fragment deleted in the sna<sup>ArgR2</sup> mutation indicating the sequence of the sgRNAs used and the sequences flanking the deletion. (TIF)

S2 Fig. Dynamic upd expression in wild type embryos. Whole mount in situ RNA expression in 5-7h wild type embryos. Right panels show close ups of the maxillary and labial segments. (A) At st9 upd is expressed in stripes, located posterior to the En stripe (B). (C) At st10 there is a transient upd anteroposterior stripe running along the lateral ectoderm. (D) Close up of a st10 embryo focusing at the maxillary and labial segments. (E-E') At early st11 lateral ectoderm expression is detected in the gland primordia and in the tracheal pits. (F-F') At late st11 upd expression disappears from the CA and is detected in the PG primordium and in the invaginating trachea. Panels (B-D) show embryos double stained with anti-En (brown). Scale bars 50 μm. (TIF)

S3 Fig. Dfd, Scr, En and Wg expression in wild type and mutant embryos. Dfd expression in (A) control heterozygous or in (B) wg<sup>CX4</sup> and (C) en<sup>E</sup> homozygous embryos. Scr expression in (D) control heterozygous or in (E) wg<sup>CX4</sup> and (F) en<sup>E</sup> homozygous embryos. En expression in (G) control heterozygous or (H) Dfd<sup>16</sup> Scr<sup>4</sup> embryos. Wg expression in (I) control heterozygous or (J) Dfd<sup>16</sup> Scr<sup>4</sup> embryos. Images show lateral views of the mandibular to the T1 segment in st11 embryos. Scale bars 50 μm. (TIF)

S4 Fig. Hox regulation of the sna-rg enhancer. Expression of sna-rg-R2P2 minimal enhancer in a Dfd Scr double mutant (A) or in a hth<sup>P2</sup> mutant (B). (C) Hox binding site modifications introduced in the sna-rg A1+A2proximal constructs beside a heat map indicating their SELEX-seq DNA-binding affinity preferences. (D) Embryo expressing simultaneously a sna-rg-mCherry construct (D” red) and sna-rg A1+A2proxHoxNull-GFP (D’ green) where the putative DNA binding site has been changed to a sequence not recognised by Hox-Exd-Hth in vitro. The expression of the sna-rg A1+A2proxHoxNull-GFP construct in the ring gland is not affected by the putative binding site mutation as well as maintaining the ectopic ventral expansion normal to a sna-rg A1+A2prox reporter. (E) sna-rg A1+A2prox strong class3Hox-GFP. (F) sna-rg A1+A2prox strong class2Hox-GFP. (G) sna-rg A1+A2prox strong class1Hox-GFP. Mutant embryos in (E-G) show strong expression in the maxilla and labium and the expression in other segments is barely affected. Panel C is modified from [40]. Embryos in (A) are also stained with anti-Scr to recognise the homozygous mutants. Scale bars 50 μm. (TIF)

S1 Table. Oligo sequences used in this study. (DOCX)
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