Development 135, 2301-2309 (2008) doi:10.1242/dev.017053

Timing of Wingless signalling distinguishes maxillary and antennal identities in *Drosophila melanogaster*

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The *Drosophila* adult head mostly derives from the composite eye-antenna imaginal disc. The antennal disc gives rise to two adult olfactory organs: the antennae and maxillary palps. Here, we have analysed the regional specification of the maxillary palp within the antennal disc. We found that a maxillary field, defined by expression of the Hox gene *Deformed*, is established at about the same time as the eye and antennal fields during the L2 larval stage. The genetic program leading to maxillary regionalisation and identity is very similar to the antennal one, but is distinguished primarily by delayed prepupal expression of the ventral morphogen Wingless (Wg). We find that precociously expressing Wg in the larval maxillary field suffices to transform it towards antennal identity, whereas overexpressing Wg later in prepupae does not. These results thus indicate that temporal regulation of Wg is decisive to distinguishing maxillary and antennal organs. Wg normally acts upstream of the antennal selector *spineless* (*ss*) in maxillary development. However, mis-expression of Ss can prematurely activate *wg* via a positive-feedback loop leading to a maxillary-to-antenna transformation. We characterised: (1) the action of Wg through *ss* selector function in distinguishing maxillary from antenna; and (2) its direct contribution to identity choice.

KEY WORDS: Wingless, Eye-antenna disc, Spineless, Temporal regulation, Maxillary palps

INTRODUCTION

Most of the external cuticle of the adult fruit fly *Drosophila melanogaster* derives from epithelial structures called imaginal discs (Morata, 2001). The eye-antennal (E-A) imaginal disc gives rise to the major head sensory organs (eyes, ocelli, antennae and maxillary palps) (Haynie and Bryant, 1986), making it a model for the development of multiple organs from a composite rudiment. From the second larval instar onwards, the complementary expression domains of the transcription factors *eyeless* (*ey*)/*twin of eyeless* (*toy*) and *cut* (*ct*) define fields that ultimately yield the eye and antenna, respectively (Dominguez and Casares, 2005; Kenyon et al., 2003). Axial organisation of the antenna employs a canonical genetic cascade typical of ventral appendages, analogous to the one described for legs (Brook et al., 1996). This cascade starts with the expression of the homeobox selector gene *engrailed* in the posterior compartment, where it specifies posterior cell fate and activates transcription of the *hedgehog* (*hh*) gene. Hedgehog protein secreted by posterior cells diffuses across the AP boundary to nearby anterior cells, where it activates the transcription of target genes, including *decapentaplegic* (*dpp*/TGF-β) anterodorsally and *wingless* (*wg*/Wnt) anteroventrally (Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). Mutual antagonism between these two morphogen growth factors is necessary to activate expression of the homeodomain transcription factor Distal-less (Dll) in the centre of the disc, thereby setting the proximodistal (PD) axis (Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). Activation of *Dll* in the antennal disc leads to the co-expression of Dll and the TALE homeodomain protein Homothorax (*hth*; the *Drosophila* Meis1 homolog), whereas they do not overlap in the leg disc (Dong et al., 2001). Two lines of argument indicate that *Dll* and *hth* jointly specify antennal fate. First, haploinsufficient alleles of *Dll* or mitotic clones of *hth*$^{-}$ cells can transform antennae into legs (Casares and Mann, 1998; Dong et al., 2000; Dong et al., 2001). Second, ectopic co-expression of *Dll* and *hth* can cause transformations to antenna (Dong et al., 2000; Dong et al., 2002). *Dll* and *hth* are thus required, respectively, for the specification of the distal versus the proximal domains of ventral discs but also for the specification of antennal fate (Abu-Shaar and Mann, 1998; Dong et al., 2000; Dong et al., 2002; Wu and Cohen, 1999). They regulate multiple target genes during antennal development that function in specifying antennal structures and/or repressing leg development (Casares and Mann, 1998; Dong et al., 2000; Dong et al., 2002). One of these targets is *spineless* (*ss*), which encodes a b-HLH-PAS protein homologous to the mammalian Aryl Hydrocarbon Receptor (Duncan et al., 1998). *ss* is considered to be an antennal selector gene as the antennae of *ss*$^{-}$ adults are transformed to distal legs while the mis-expression of Ss induces ectopic antennal structures in adult legs (Dong et al., 2002; Duncan et al., 1998; Struhl, 1982). *Ss* controls distal antennal differentiation at least in part via the activation of its target genes *distal antenna* (*dan*) and *distal antenna related* (*dan-r*) that encode nuclear 'pipsqueak' motif proteins (Emerald et al., 2003). Ectopic expression of *dan* or *dan-r* causes a partial transformation of distal leg towards antenna, whereas loss of both functions has the opposite effect (Emerald et al., 2003; Suzanne et al., 2003). They are thus considered to be effector genes of antennal identity.

Though the antennal imaginal disc has often been treated as a single cellular field giving rise to the adult antenna, it has long been known that the second olfactory organ of the adult head – the maxillary palp – also originates from the antennal disc (Haynie and Bryant, 1986). A maxillary (Mx) primordium that gives rise to the adult maxillary palp emerges from the antennal disc as a localised outgrowth in early pupae (Jurgens and Hartenstein, 1993). However, very little has been described concerning the developmental origins of the Mx palp, nor of the genetic program leading to its final form. Two of the antennal determinant genes, *ss* and *Dll*, are known to be required for Mx development, as loss of

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Accepted 7 May 2008

their functions leads to adults with reduced or deleted Mx structures, respectively (Cohen and Jurgens, 1989; Duncan et al., 1998). Consistent with these mutant phenotypes, both *Dll* and *ss* are expressed at the pupal stage in the Mx primordium of the antennal disc (Duncan et al., 1998; Panganiban, 2000). Contrary to the antenna, recent work places *Dll* downstream of *ss* in the maxillary field (Emmons et al., 2007). Additionally, the homeotic genes *proboscipedia* (*pb*; HoxA2/B2) and *Deformed* (*Dfd*; Hox A4-D4) are both expressed in the Mx primordium (Benassayag et al., 2003; Diederich et al., 1991) and required there, as adult Mx palps are deleted by mitotic Dfd^{-} clones, and reduced in pb^{-} homozygotes (Merrill et al., 1987; Pultz et al., 1988). As ectopic *pb* expression transforms distal antennae into Mx palps, *pb* is considered to be a Mx selector gene (Cribbs et al., 1995). The reciprocal transformation of Mx into antennae can be observed when *ss* or *wg* are mis-expressed in Mx cells (Duncan et al., 1998; Johnston and Schubiger, 1996). Thus, the antennal and Mx organs appear to be homologous structures that emerge from the same imaginal disc, share key selector genes involved in their specification and both contribute to adult olfactory function.

In this paper, we address the issue of regional specification of the Mx field within the antennal disc. We find that the Mx field is defined by Deformed expression from the second larval instar onwards. The program for Mx regionalisation that emerges here is a temporally deferred version of the antennal program, owing to the delayed expression of the ventral signal Wg in the prepupal Mx field. We show that precocious *wg* expression in this tissue is sufficient to transform Mx to antenna, indicating that the delayed Wg expression in the Mx primordium is crucial in distinguishing antennal and Mx identity. Finally, our analysis reveals that Wg acts through *ss* in the maxillary field, but *wg* can also influence organ identity independently of the *ss* selector function.

MATERIALS AND METHODS

The 180° rotation of the eye-antennal disc during development results in an inversion of dorsal and ventral cells. To avoid confusion, we employ the convention that dorsal cells are those that express *dpp*.

Drosophila strains and transgenic lines

The strain used as wild type in this study was *w*. Reporter genes used were *dpp-lacZ* BS3.0 (Blackman et al., 1991) and *ptc-lacZ* (Zhang and Kalderon, 2000). The *ss* null allele $ss^{D115.7}$ (ss^{-}) is described by Duncan et al. (Duncan et al., 1998). For targeted mis-expression, *ptc-GAL4* driver line (Hinz et al., 1994) was combined with responder constructs *UAS-ss* (Duncan et al., 1998) or *UAS-*wg^{ts} M7-2.1 (Wilder and Perrimon, 1995). The fly strains employed for clonal analysis were: (1) *w; FRT82B*, (2) *hs-FLP*, *w; FRT82B Ub-GFP*, *M(3)RpS3/TM6B, Hu Tb*, (3) *y w hs-FLP*; *FRT82B* $pygo^{130}$*/TM6B, Hu Tb*, (4) *y hs-FLP*; *FRT42D Ub-GFP/Cyo*, and (5) *y w; FRT42D* Dll^{SA1}*/SM5-TM6B, Hu Tb*. Stocks specifically constructed for this work were: (1) *ptc-GAL4;* ss^{-}*/TM6B, Hu Tb,* (2) *UAS-*wg^{ts}*,* ss^{-}*/TM6B, Hu Tb*, (3) *hs-FLP; ptc-GAL4; FRT82B* $pygo^{130}$*/TM6B, Hu Tb*, and (4) *UAS-ss; FRT82B Ub-GFP RpS3/TM6B, Hu Tb.*

Conditions for temporal wg^{ts} activation

After crossing *ptc-GAL4* and *UAS-*wg^{ts} flies, egg lays were collected for 24 hours, and then adults were removed to fresh medium. Development is slowed for this genotype (Johnston and Schubiger, 1996). Developing animals were maintained at 25°C for 1 day (~L1), 2 days (~L2), 3 days (~early L3), 4 days (~mid L3), 5 days (~late L3) or 6 days (~early pupa), then shifted to 18°C to activate *wg*. Dan staining was performed on imaginal discs from late L3 larvae or from pupae, depending on the timing of *wg* activation. For molecular analysis the wg^{gof} and wg^{gof}, ss^{-} larvae (*ptc-GAL4/+; FRT82B* ss^{-}*/UAS-*wg^{ts}, ss^{-}) were shifted to 18°C during L3 stage, after 4 or 5 days of development.

Clonal analysis

Clones were generated using the FLP/FRT system (Xu and Rubin, 1993).

For lineage analysis, Minute-enhanced mitotic clones were induced in *hsFLP;FRT82B/FRT82BUb-GFP, M(3)RpS3* animals by a single 30-minute heat shock at 38°C (condition where there is one clone per antennal disc) after about 1 (L1), 2 (L2), or 3 (L3) days of development.

In the other experiments, clones were generated by a single 1-hour heat shock at 37°C during the first or second larval instar, then dissected and stained in late third instar or in pupal discs: in *hs-Flp; FRT82B* $pygo^{130}$*/FRT82B Ub-GFP, M(3)RpS3* animals for *pygo* mutant clones; in *hs-Flp; ptc-GAL4/UAS-ss; FRT82B* $pygo^{130}$*/FRT82B Ub-GFP, M(3)RpS3* (ss^{gof}, $pygo^{-}$) for *pygo* mutant clones overexpressing *ss*; or in *hs-FLp; FRT42D* Dll^{SA1}*/FRT42D Ub-GFP* for *Dll* mutant clones.

Immunocytochemistry and antibodies

Larvae or white pupae were prepared for immunofluorescence essentially as described by Agnes et al. (Agnes et al., 1999). All incubations were performed without agitation to avoid damaging the pupal tissues. Primary antibodies used were: mouse anti-Cut 2B10 (1/200), concentrated anti-Inv 4D9 (1/20) and anti-Wg 4D4 (1/200) from the Developmental Studies Hybridoma Bank (DSHB); mouse anti-Dll, 1/500 (I. Duncan); rabbit polyclonal anti-Dfd, 1/250 (T. Kaufman); rabbit anti-Hth, 1/250 (N. Azpiazu); rat polyclonal α-Dan, 1/300 (S. Cohen); guinea pig anti-Ss, 1/1000 (L. Jan and Y. N. Jan); and rabbit anti-βGal, 1/5000 (Cappel, Promega). Mounted discs were viewed using a Zeiss LSM410 or a Leica TCS SP2 confocal microscope.

Adult cuticle analysis

Flies of interest were stored in ethanol until dissection. Detailed examinations of dissected heads mounted in Hoyer's medium were performed by light microscopy on a Zeiss Axiophot.

RESULTS

Distinct antennal and maxillary territories are established early in larval development

The antennal disc gives rise to two distinct olfactory organs: the antenna and the maxillary palp which is derived from a small bud-like primordium in the pre-pupal disc (Jurgens and Hartenstein, 1993). Two Hox genes required for Mx differentiation, *proboscipedia* (*pb*) and *Deformed* (*Dfd*) (Merrill et al., 1987; Pultz et al., 1988), are expressed in the Mx territory: Pb is initiated in the pre-pupal Mx primordium and Dfd is present in L3 (Benassayag et al., 2003; Diederich et al., 1991). We therefore examined expression of the Dfd protein as a potential molecular marker to follow maxillary development within the antennal disc. Dfd already accumulates in maxillary cells in early L2 larvae, the period at which segregation of the eye and antennal territories occurs (Kenyon et al., 2003), in a pattern complementary to the antennal field marker Cut (Fig. 1A). This pattern of exclusion between Cut and Dfd then persists both in the disc proper and in the peripodial membrane through L3 (Fig. 1B), in early pupae (Fig. 1C), and into metamorphosis, where Dfd remains in the Mx palps of a pupal head after fusion of the eye-antennal and labial discs (Fig. 1D, arrows). We conclude that Dfd marks a Mx field that is present in the antennal disc from the L2 larval stage onwards into pupal development.

To test whether the exclusive patterns of Dfd and Cut markers reflect separate cell populations, we examined growth-enhanced wild-type mitotic clones that touch the Dfd/Cut limit (in the posterior antennal disc; see Fig. 1B,C). In the conditions used, most antennal discs contained one or no clones. Most clones induced from L2 onwards were restricted to the maxillary (12/33; Fig. 1E) or the posterior antennal territory (12/33; Fig. 1F). Another class of clones circumnavigates the posterior antenna to include maxillary and anterior antennal cells (6/33; not shown), consistent with previous observations (Morata and Lawrence, 1979). Only 3/33 L2-induced

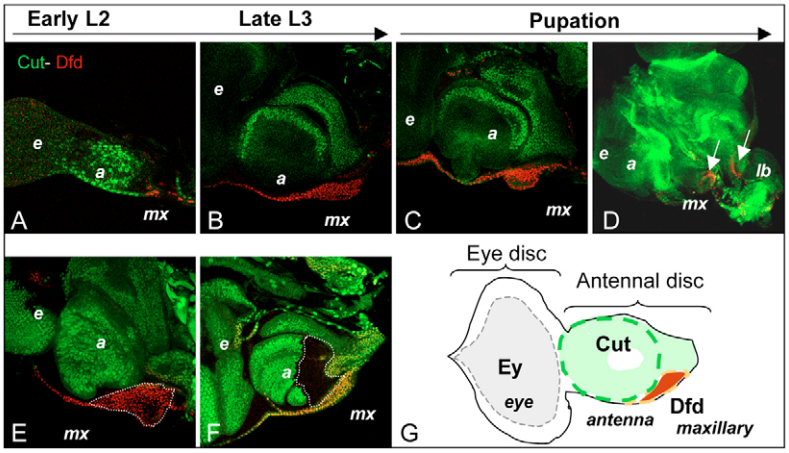


Fig. 1. Separate antennal and maxillary fields. (**A-D**) Exclusive expression domains of Dfd (red) and Cut (green) proteins in eye-antennal discs of (A) an early L2 larva; (B) a late L3 larva; (C) a prepupa; and (D) a pupal head after fusion of two eye-antennal and two labial (lb) discs (maxillary fields marked by arrows in D). (**E**,**F**) Clonal restriction established between antennal and maxillary territories in L2 is reflected by clones restricted either to the maxillary field (E;18/33) or to the posterior antennal field (F;12/33). Mitotic clones identified by the absence of GFP (broken lines) were situated relative to the maxillary territory identified by anti-Dfd (red). (**G**) Schematic representation of the eye-antennal disc, with its eye (grey), antennal (green) and maxillary (red) fields expressing Eyeless, Cut and Deformed proteins, respectively. Antennal discs are oriented with posterior towards the right and dorsal towards the bottom; e, a and mx indicate eye, antenna and maxillary primordia, respectively.

clones encompassed both posterior antennal and maxillary cells, while a significantly larger proportion of clones induced in L1 larvae did (18/59 clones). This difference indicates that the expression domains detected with Dfd and Cut markers in early L2 reflect the establishment of a clonal restriction between Mx and antennal fields.

The Mx organ employs a temporally delayed version of the antennal program

Very little is known about how the Mx organ is patterned. We therefore examined the expression of known participants of the genetic cascade common to ventral appendages, and directly compared expression of these markers between the adjacent Mx and antennal territories of the same imaginal disc (Fig. 2).

Initial anteroposterior specification involves expression of *engrailed/invected* (*en/inv*) that determines posterior cell identity and activates transcription of the *hedgehog* (*hh*) morphogen there. In L2 larvae, the single group of cells expressing En/Inv/Hh proteins in the antennal disc is restricted to the antennal territory, as it does not overlap with Dfd-expressing cells (Fig. 2A,B; data not shown). Unexpectedly, the Hh transcriptional target *patched* (*ptc*) is activated on both sides of these posterior antennal cells, bordering them in adjacent anterior antennal cells but also in the maxillary field (Fig. 2F,G; as seen by a *ptc-lacZ* transgene that recapitulates normal *ptc* expression). The same result was obtained with antisera directed against Ptc protein or the activator form of the Hh target protein Cubitus interruptus (Ci) (not shown). These data strongly suggest that both antennal and maxillary cells receive and transduce diffusible Hh signal from a common antennal source at this stage. The first evidence for AP compartmentalisation of the maxillary field is detected in early L3 larvae, when En/Inv co-expression with Dfd is observed (Fig. 2C, arrowhead), and is clearly detectable in late L3 larvae and in pre-pupae (arrowhead in Fig. 2D,E). This zone of En-expressing cells largely excludes the pre-existing stripe of *ptc-lacZ* expression during L3 and pupal stages (arrowhead Fig. 2H-J).

Concerning DV axis organisation, *dpp-lacZ* and *wg* appear simultaneously in the antennal territory in early L2 (Fig. 2K). This expression in two adjacent wedges is maintained through L2, L3 and into pupal stages (Fig. 2L-O). Organisation of the PD axis is not yet noted in early L2 larvae, where Hth is uniformly distributed across the antennal disc, while Dll is absent (Fig. 2P). Immediately following the onset of *dpp* and *wg* expression during L2, *Dll* (Fig. 2Q) and *dachshund* (*dac*; not shown) are activated, while Hth retracts from the centre of the disc (Fig. 2P,Q) giving rise during L3 to distinct domains defined by Dll alone, joint Dll/Hth and Hth alone (Fig. 2R-S) (Dong et al., 2001).

In the maxillary field, *ptc* expression in late L2 (Fig. 2G) is not accompanied by *dpp* or *wg* (Fig. 2L). *dpp-lacZ* expression in the maxillary territory appears in early L3 larvae (Fig. 2M). By contrast, Wg is absent throughout L3 development (Fig. 2M,N), and only appears nearly 2 days later at the L3/pupal transition, in anterior maxillary cells, adjacent to (and exclusive of) those expressing Dpp (Fig. 2O). Mitotic hh^{-} clones confirmed that this maxillary *wg* expression is *hh* dependent (not shown). In prepupae, Dll appears in a group of Mx cells centred on the Dpp-Wg junction that largely overlaps Hth there (inset, Fig. 2T). Dac is not detected in the Mx primordium (not shown). These data, summarised at the bottom of Fig. 2, indicate that the maxillary region deploys a program similar to the antennal program but delayed by the late appearance of Wg.

Timing of *wg* signalling defines maxillary versus antennal identity

Temporally regulated Wg thus might play a key role in distinguishing the genetic programs leading to maxillary and antennal fates. One described consequence of mis-expressing Wg is the transdetermination of maxillary palps to antennae (Johnston and Schubiger, 1996). We re-examined this effect of Wg on Mx/Ant identity, paying particular attention to the temporal activity of Wg. A conditionally active Wg^{ts} protein that is secreted at 18°C but not at 25°C (Gonzalez et al., 1991; Wilder and Perrimon, 1995), was driven by *ptc-Gal4* for varied times and durations at 18°C (Fig. 3; see also Materials and methods). Mx-to-Ant transformations were scored by two criteria: (1) expression of antennal markers, especially Dan, in the Mx territory of the E-A disc (Fig. 3A), and (2) appearance of identifiable adult antennal tissue, notably aristae, in place of Mx palps (Fig. 3B, arrow).

ptc-Gal4>UAS-wg^{ts} animals that develop at 25°C eclose as normally patterned adults (not shown). Animals raised at 18°C from embryogenesis onwards all died before L2. By contrast, shifting from 25 to 18°C during larval development yielded a Mx-to-Ant transformation whose frequency was strongly influenced by timing (see table in Fig. 3). When the passage to 18°C was carried out in L1 larvae, we observed significant numbers of transformed larvae/pupae and adults (28% of Dan-expressing discs, 53% of adults with Mx-to-Ant transformation). The penetrance of the transformation was markedly enhanced when the permissive temperature was installed later, in L2 (63% and 85%), early L3 larvae (73% and 90%) or mid-L3 (83% and 89%), then maintained until adult eclosion. Thus, the most efficient conditions leading to Mx-to-Ant transformation were those where Wg was over-expressed concomitantly with Dpp in Mx cells (see Fig. 3).

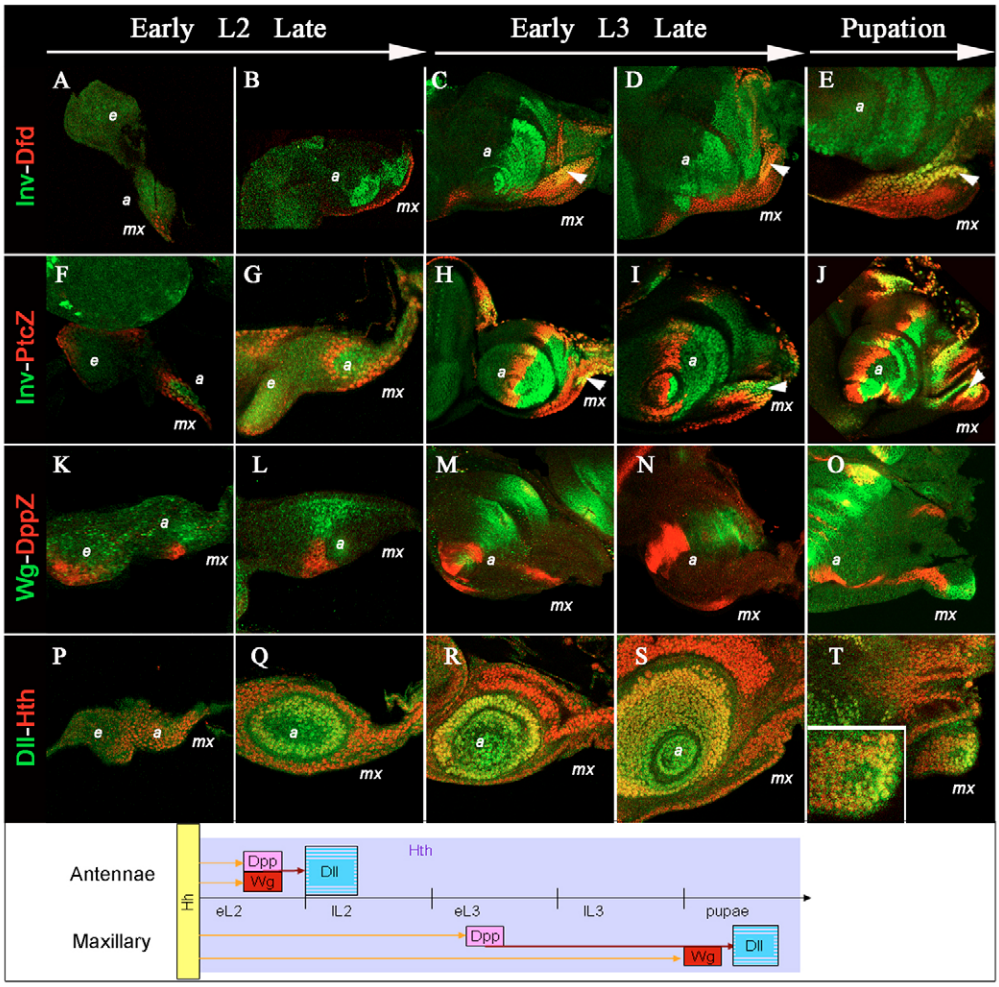


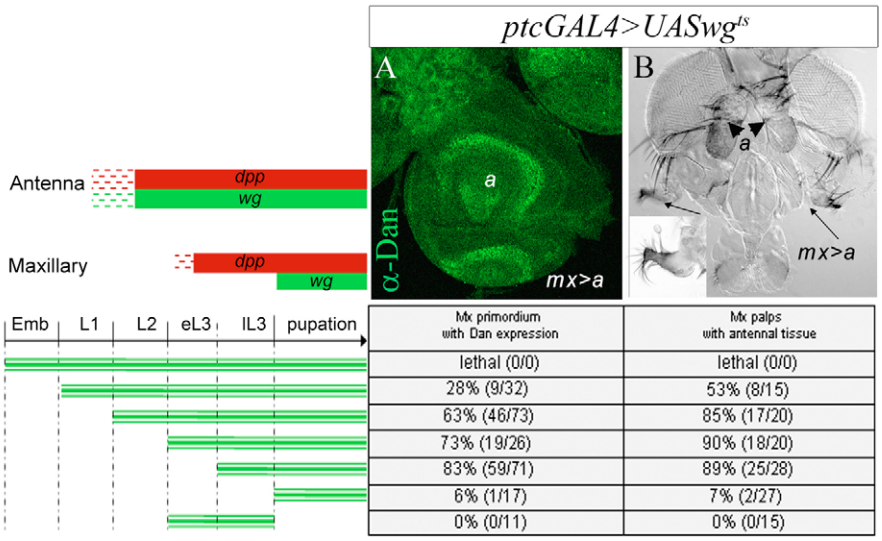
Fig. 2. Axial patterning of the antennal disc during larval and pupal development. Representative images with several marker combinations are presented for early-mid L2 (A,F,K,P), late L2 (B,G,L,Q), early L3 (C,H,M,R), late L3 larvae (D,I,N,S) and early pupae (E,J,O,T). (**A-E**) Inv (green) and Dfd (red) expression. (**F-J**) Inv/En (green) and *ptc-lacZ* (ptcZ, red) expression. Arrowheads mark maxillary Inv-expressing cells in C-E,H-J. (**K-O**) Wg (green) and *dpp-lacZ* (dppZ, red) expression. (**P-T**) Dll (green) and Hth (red) expression. Dll is co-expressed with Hth in both antennal (S) and maxillary primordia (T, inset). The results are summarised at the bottom in which Hth is indicated with a purple background. eL2, early L2; lL2, late L2; eL3, early L3; lL3, late L3. Antennal discs are oriented with posterior towards the right and dorsal towards the bottom; e, a and mx indicate eye, antenna and maxillary primordia, respectively.

Temperature shifts to 18°C of pre-pupae produced almost no transformation (6% and 7%). This result shows that overexpression of the Wg morphogen in pre-pupae is not sufficient to direct Mx-to-Ant transformation, and indicates that precocious Wg activation is a crucial initiator of Mx-to-Ant transformation. We infer that the rare individuals obtained with Mx-to-Ant transformation under this condition have probably been subjected to Wg overexpression during the late L3 stage, in light of the 24-hour egg-lay periods employed (see Materials and methods). Finally, when transgenic Wg was expressed solely during the L3 larval stage, no transformed individuals were obtained (0/26), suggesting that continuous expression of Wg and/or a precise spatial pattern of ectopic expression are required for efficient transformation. These results were confirmed using the flip-out technique to generate new sources of Wg that do not depend on the *ptc* promoter. Wg-expressing clones (act>y+>wg) induced by hsFlp in second instar larvae were sufficient to trigger aristal formation on adult Mx palps and ectopic Dan expression in the L3 larval Mx field (not shown). Taken together, these results indicate that temporal control of *wg* activity in pre-pupae versus larvae is crucial to distinguishing the Mx-specific developmental program from its antennal counterpart.

wg signalling controls maxillary *ss* expression

Mis-expressing Wg is sufficient to provoke a Mx-to-Ant transformation. However, the same transformation is induced on mis-expressing Spineless (Ss) antennal selector protein under *ptc-Gal4* control (Duncan et al., 1998). We therefore compared the temporal and spatial maxillary expression of Wg and Ss. In the wild-type Mx territory, both Wg and Ss are absent in L3 (Fig. 2M,N; not shown), then appear simultaneously there at the L3-prepupal transition (Fig. 4A), followed in prepupae by Dll activation in a subset of *ss*-expressing cells (Fig. 4B). Mis-expressed Wg induces a precocious Mx expression of Ss and Dll (Fig. 4C; not shown). Conversely, mis-expressing Ss (*ptc-Gal4>UAS-ss*) resulted in precocious Wg accumulation in L3 larvae that is first detected in the band of *ptcGal4>ss*-expressing cells (not shown) and then resolved to an antennal-like wedge pattern (Fig. 4D). This *ss*-dependent Wg activation starts in early L3 larvae, concomitant with and adjacent to endogenous *dpp* expression (not shown), and leads to earlier activation of Dll protein in the Mx field (not shown). These gain-of-function experiments strongly underline the capacities of Wg and Ss for mutual activation. Furthermore, both Wg and Ss direct a remarkably rapid reorganisation of the Mx territory towards an antennal primordium during L3 (Fig. 4C,D), though Wg seems more potent in this respect.

We therefore examined the relationship between *wg* and *ss* using loss-of-function mutations. Both affect maxillary development, as ss^{-} homozygotes and adults deficient for *wg* signalling harbour reduced palps (Duncan et al., 1998) (and not shown). In ss^{-} mutant pupae, Wg and Dll proteins accumulate at roughly normal levels in the Mx primordium (Fig. 4E,F). Conversely, when *wg* signalling was abolished via mitotic clones that eliminate the obligatory



Mx primordium with Dan expression	Mx palps with antennal tissue
lethal (0/0)	lethal (0/0)
28% (9/32)	53% (8/15)
63% (46/73)	85% (17/20)
73% (19/26)	90% (18/20)
83% (59/71)	89% (25/28)
6% (1/17)	7% (2/27)
0% (0/11)	0% (0/15)

Fig. 3. Temporally regulated *wg* expression distinguishes maxillary and antennal differentiation programs. (Top left) A schematic representation shows the normal temporal programs for *dpp* (red) and *wg* (green) expression in antennal and maxillary territories. (**A**,**B**) larval imaginal discs and adult heads harbouring mx-to-antennal transformations (mx>a). (Bottom left) The timing of *wg* mis-expression (see Materials and methods) is indicated by the striped green lines under the time bar. Each corresponds to a line in the results table, bottom right, indicating the effects of temporally regulated Wg mis-expression scored on Dan expression and on the adult mx>a transformation. Antennal discs are oriented with posterior towards the right and dorsal towards the bottom; a and mx indicate antenna and maxillary primordia, respectively.

pathway element *pygopus* (Belenkaya et al., 2002), Ss (Fig. 4G) and Dll (not shown) were absent from mutant *pygo*$^{-}$ maxillary cells. The cell-autonomous loss of *ss* expression in *pygo*$^{-}$ maxillary cells indicates that *wg* signalling is required for *ss* activation there. Although we have not directly tested the role of *dpp* signalling, these loss-of-function results support a model where *dpp*/*wg* signalling acts upstream of *Dll* and *ss* in the maxillary palp, as for other ventral appendages. Furthermore, in Mx *Dll*$^{-}$ mutant clones, Ss is expressed normally (Fig. 4H). This suggests that, contrary to the antenna, Mx *ss* expression is independent of *Dll*.

In normal Mx development *wg* activates *ss*; the gain-of-function experiments further reveal that the Ss selector can activate *wg* via a positive-feedback loop. Together, these observations suggest that the transformation of maxillary-to-antenna induced by Ss may likewise require *wg* and that Wg is a central player in distinguishing Mx from antennal differentiation.

wg controls the maxillary-versus-antenna identity choice via *ss*-dependent and -independent activities

The complex interactions revealed by the preceding results led us to examine the relationship of *wg* and *ss* in maxillary/antennal development through functional tests of epistasis. In *ss*$^{-}$ mutants, antennae are transformed to distal legs (Duncan et al., 1998) and maxillary palps are reduced relative to wild type (compare Fig. 5A$_1$, 5A$_2$), whereas *ptc*-directed Wg^{ts} (*wg*^{gof}) induces a highly penetrant Mx-to-Ant transformation (Fig. 3B). Most double-mutant *wg*^{gof}; *ss*$^{-}$ adults harbour a large nondescript maxillary outgrowth (Fig. 5A$_3$), but in some the Mx was replaced by a tarsus culminating in two distal claws (Fig. 5A$_4$). That the maxillary field can be re-directed by Wg to a leg in the *ss*$^{-}$ condition suggests that mis-expressed Wg has reorganized the maxillary region into a permissive 'pre-antennal' environment whose transformation to antenna is blocked by the absence of *ss* activity (see Fig. 5G). We therefore examined the molecular organisation of *wg*^{gof}; *ss*$^{-}$ larval E-A discs, using Dfd and Dan as Mx and antennal markers, respectively (Fig. 5B). Singly, *wg*^{gof} (Fig. 3A, Fig. 5C) and *ss*^{gof} (Fig. 5D) induced a similar reorganisation of the Mx region to antenna, visible in L3 larvae by retraction of Dfd and de novo Dan and Dac accumulation (Fig. 5C,D; not shown). By contrast, in *wg*^{gof}; *ss*$^{-}$ larvae, Dan was not observed in either the antennal or the maxillary field (Fig. 5E), correlating with the non-antennal adult Mx outgrowth (Fig. 5A$_{3,4}$). This indicates that mis-expressed Wg requires normal *ss* function to induce Dan and the subsequent antennal program. However, Dfd was reliably retracted from the budding 'maxillary' region of the same *wg*^{gof}; *ss*$^{-}$ animals (Fig. 5E, arrowhead), similar to *wg*^{gof} alone (Fig. 5C). This retraction of Dfd indicates that Wg signalling can reorganise the Mx field independently of Ss.

In the reciprocal test, *pygo*$^{-}$ clones were used to remove Wg signalling activity from ss^{gof} tissues. No adults were obtained under these conditions, but Minute-enhanced *pygo*$^{-}$ clones could be obtained in the antennal-maxillary region of *ss*^{gof} larvae (Fig. 5F,F′). As described above, *pygo*$^{-}$ clones lead to inactivation of *ss* (Fig. 4G) and its target gene *dan* (not shown). In *ss*^{gof}; *pygo*$^{-}$ double mutant cells, Dan was activated only in the narrow band of cells expressing Ss under the *ptc*-Gal4 driver but not in the wider concentric rings typical of the Mx-to-antennal transformation (compare Fig. 5F with 5D), whereas normal Dfd expression persisted throughout the Mx field (Fig. 5F′). Ss protein is thus sufficient to induce Dan expression but requires *wg* signalling activity to achieve the Mx-to-Ant transformation, as seen by tissue morphology, molecular reorganisation of Dan-expressing regions and persistence of Dfd. Taken together, these results indicate that Wg provides a crucial input to the program initiated by Ss, but can also contribute to identity choice independently of Ss.

DISCUSSION

In this paper, we have analysed the regional specification of the antennal and the maxillary fields within the composite eye-antennal disc. This analysis shows that (1) a maxillary field leading to the adult Mx palp emerges in second instar larvae; (2) the specification and formation of the Mx primordium occurs in pre-pupae, i.e. much later than for other ventral appendages, via a program similar to that used to specify antennae; and (3) delayed Wg activation in the Mx field plays a crucial role, through *ss* or independently, in specifying Mx identity. We conclude that the temporal regulation of Wg is crucial in establishing Mx identity.

The antennal imaginal disc is divided early into distinct but communicating antennal and maxillary territories

Much previous attention for the eye-antennal disc has been directed towards the separation into eye and antennal fields (Dominguez and Casares, 2005; Kenyon et al., 2003; Kumar and Moses, 2001).

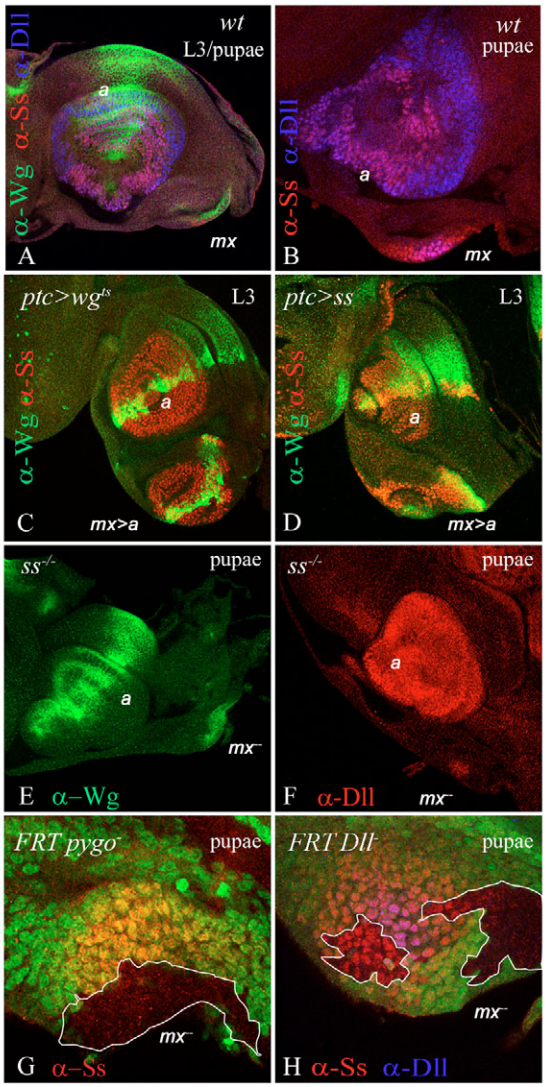


Fig. 4. *wg* and *ss* are functionally linked in maxillary/antennal programs. (**A**) In wild-type L3/pupal transition, Wg (green) and Ss (red), but not Dll (blue), proteins are detected in maxillary (mx) cells. (**B**) In wild type, Dll is first detected in the mx primordium of white pre-pupae. (**C**,**D**) Both *wg* and *ss* induce a maxillary-to-antennal transformation through a mechanism involving reciprocal activation. (C) In wg^{gof} L3 larvae (*ptc-GAL4>UAS-*wg^{ts} shifted to 18°C at 5 days AEL), Ss (red) is precociously induced on the band of Wg-expressing cells (green) within the neo-antennal territory (mx>a). (D) In ss^{gof} (*ptc-GAL4>UAS-ss*) L3 larvae, the mx>a transformation is associated with precocious Wg expression (green) overlying the band of Ss-expressing cells (red) in the mx field. (**E**,**F**) In ss^{-} mutant pupae, normal expression is observed for Wg (green) or Dll (red) proteins. (**G**) Clone of $pygo^{-}$ cells (no green GFP marker, outlined) where nuclear Ss protein (red) is cell-autonomously absent from mutant maxillary cells. (**H**) Clone of Dll^{-} cells [no green GFP marker (outlined) and no Dll (blue)] where nuclear Ss protein (red) is present in mutant maxillary cells. (E-H) These genotypes lead to reduction or loss of adult Mx palps (mx^{-}). Antennal discs are oriented with posterior towards the right and dorsal towards the bottom; a and mx indicate antenna and maxillary primordia, respectively.

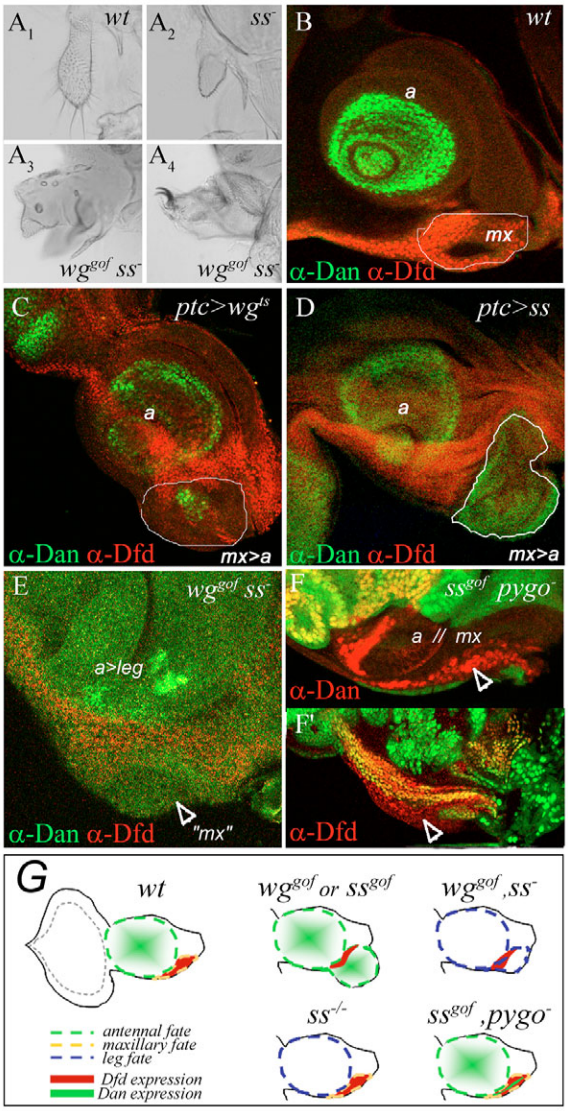


Fig. 5. Wg acts upstream of Ss and independently of it in transforming maxillary identity to antennal. (**A_1-A_4**) Light-microscope images of adult maxillary palps. (A_1) Wild-type maxillary palps with their bordering row of bristles. (A_2) ss^{-} null homozygote shows strongly reduced maxillary palps. (A_3) wg^{gof}; ss^{-} double mutants (shifted to 18°C at 5 days AEL) generally have an enlarged structure, unlike either wg^{gof} neo-antennae or ss^{-} stubs. These structures are positioned higher on the head, like neo-antennae (as seen in Fig. 3B). (A_4) Same genotype as in A_3, but with the maxillary palp replaced by distal leg with its distinctive claws. (**B-E**) Dan (green) and Dfd (red) expression in L3 E-A of (B) a wild-type antennal disc, (C) a wg^{gof}-induced transformation (*ptc-GAL4>UAS-*wg^{ts} shifted to 18°C at 5 days AEL), (D) a ss^{gof}-induced transformation (*ptc-Gal4>UAS-ss*), (E) a wg^{gof}; ss^{-} disc (shifted to 18°C at 5 days AEL) where Dfd (red) is excluded from the maxillary territory (arrowhead). (**F**,**F′**) In ss^{gof} discs harbouring large $pygo^{-}$ clones (no GFP), Dan (red) is expressed in a narrow band of maxillary cells (F, arrowhead) and Dfd expression (red) is maintained (F′, arrowhead). (**G**) Schematic summary of results. Antennal discs are oriented with posterior towards the right and dorsal towards the bottom; a and mx indicate antenna and maxillary primordia, respectively.

However, how the antennal region gives rise to both the antenna and the maxillary palp has not been addressed. We found that expression of Dfd (Mx) and Cut (Ant) define clonally separate antennal and maxillary fields of the antennal disc established by L2, at roughly the same time as the eye-antennal demarcation. Morata and Lawrence (Morata and Lawrence, 1979) showed that adult clones induced as late

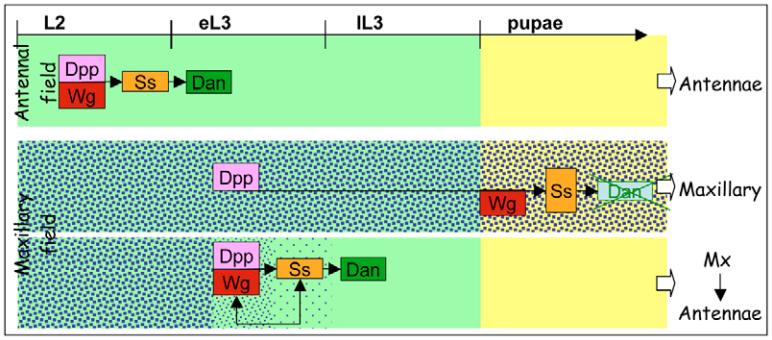


Fig. 6. Summary model comparing features of antennal (first line) and maxillary (second line) programs, and indicating their changes in the maxillary-to-antennal transformation (third line). Larval L2, early L3 (eL3), late L3 (lL3) and pupal stages are indicated at the top. Stages permissive to Dan expression in maxillary territory are in green; refractory are in yellow. The stippled background indicates Dfd expression associated with maxillary development.

as third instar larvae can encompass the Mx palp and anterior antennal cells. The clonal boundary we found in imaginal discs, separating the maxillary field defined by Dfd expression from posterior antennal cells, is compatible with their conclusion. The antenna is constructed through a program initiated by En and relayed by Hh. Maxillary and antennal cells appear to share this Hh source during L2, as (1) En/Hh is limited to antennal cells at this stage (Fig. 2A,B), (2) this antennal Hh source informs cells on both sides, as seen by *ptc* expression (Fig. 2F,G), and (3) mitotic hh^- clones show that Mx *ptc* and *wg* expression is dependent on antennal *hh* signalling (not shown).

The maxillary program is a temporally divergent version of the antennal program

The antennal and maxillary fields of the E-A imaginal disc deploy similar programs to construct the adult olfactory organs. Both fields use Hh-mediated activation of *wg* and *dpp* signals, show extensive Hth/Dll overlap and accumulate Ss transcription factor. The Mx proximodistal axis is defined by a distal and a proximal domain marked by *Dll* and *hth*, respectively, but without a medial Dac-expressing domain. However, the relationships between *Dll* and *ss* differ in the maxillary field compared with the antenna: (1) Ss can be detected before Dll in Mx at the L3/pupal transition (Fig. 4A,B), and is expressed in cells that do not express Dll (Fig. 4B); (2) Ss expression is maintained in Dll^- mutant Mx clones (Fig. 4H); and mis-expressed *ss* can induce Dll (not shown). These results concur with the conclusion of Emmons et al. (Emmons et al., 2007) that *ss* contributes to *Dll* activation in the Mx primordium. However, their conclusion is supported by a delayed pupal onset of Dll in ss^- animals compared with normal activation in late L3. We have not been able to observe this delay in ss^- mutants because with our culture conditions and reference wild-type stock, we first detect Dll about 1-2 hours after the L3/pupal stage (Fig. 4B).

The most flagrant divergence between the antennal and maxillary programs resides in the timing for deployment of key signalling pathways and transcription factors: larval for the antenna and pupal for the maxillary organ. The central players common to the antennal and Mx programs show specific and divergent timelines as illustrated in Fig. 2. In the antennal territory, the Hh targets genes *ptc*, *dpp* and *wg* are activated co-temporally, whereas in the maxillary field *dpp* is delayed by roughly 12 hours, and *wg* for more than 2 days. The late creation of the *dpp*/*wg* interface presumably explains the late *Dll* and *ss* activation, and thus the delay in a clear morphological maxillary primordium until the beginning of the pupariation.

Temporal wg regulation is central to distinguishing Max/Ant identity

In the Mx field, we observed that the onsets of *dpp* (in L3 larvae) and *wg* (in pre-pupae) were temporally uncoupled. Re-synchronising *wg* and *dpp* in the Mx field by precociously expressing Wg (Fig. 3) redirected Mx toward a neo-antennal program deploying *ss*, *dan, Dll* and *dac* (Fig. 4C, Fig. 3A; not shown). Conversely, Ss mis-expression leading to a Mx-to-antennal transformation is also correlated with premature expression of Wg next to endogenous *dpp* at the maxillary AP boundary (Fig. 4D and not shown). Taken together, these experiments thus indicate that the timing of Wg expression coincides with the choice of primordial fate: maxillary versus antenna (Fig. 3, model in Fig. 6). We can conclude that regulating temporal *wg* expression rather than Wg dose is crucial to this decision, as Wg overexpression in pupae does not provoke a Mx-to-antennal transformation (Fig. 3). Thus, the developmental choice between two alternative identities is dependent upon the time at which the instructive *dpp*/*wg* couple acts: larval for antenna, later in pre-pupae for maxillary (Fig. 2; model in Fig. 6).

The delayed onset of *wg* expression in the Mx primordium presumably depends on transcriptional regulation mediated by regulatory DNA elements. A recent paper from Pereira et al. identified *wg* 3′ flanking regulatory sequences directing larval reporter gene expression in dorsal or in ventral imaginal discs (Pereira et al., 2006). None of their lines, including those that are expressed in the larval antenna, showed detectable expression in the pupal maxillary primordium (not shown). This indicates that maxillary directed *wg* expression results from a spatiotemporal regulation distinct from the antenna. A central landmark of the larval/pupal transition, the hormonal surge triggering metamorphosis, might also be important in activating, or de-repressing, maxillary *wg* expression. Ecdysone-responsive cis-regulatory elements have been reported in the proneural gene *atonal* required for sensory organ formation (Niwa et al., 2004).

A decisive role for Wg in organ identity

The similar Mx-to-ant transformations induced by Wg or Ss indicate that both are key players in distinguishing Mx from antennal identity. In normal Mx development, the onsets of nuclear Ss selector protein and diffusible Wg growth factor were temporally indissociable (Fig. 4A). Our results from loss-of-function experiments place *wg* signalling upstream of *ss* in the Mx primordium, as abolishing *wg* signal cell-autonomously silences *ss* (Fig. 4G), whereas *wg* is still expressed in the Mx primordium of a ss^- mutant (Fig. 4E). However, the gain-of-function experiments show that *ss* can also activate *wg* by an autoregulatory loop (Fig. 4D). This suggests that *wg* provides an obligatory input in distinguishing Mx from Ant identities.

On confronting wg^{gof} (Mx-to-ant) with loss of *ss* (stunted Mx), the resulting structures were unlike either (Fig. 5A), suggesting that Ss and Wg act at the same level. Importantly, mis-expressed Wg can reorganize the Mx territory in the absence of Ss. In most cases, this yields an undefined outgrowth (Fig. $5A_3$) and occasionally gives rise to a distal clawed leg in place of the Mx palp (Fig. $5A_4$). We infer

that the Mx-to-leg transformation seen for mis-expressed Wg in conjunction with *ss*$^{-}$ reflects a Mx primordium that has already been reoriented toward a pre-antennal environment by the action of Wg (Fig. 5G). Accordingly, Dfd is seen to retract from the maxillary field of *wg*^{gof}*; ss*$^{-}$ larvae, as well as in the *wg*^{gof} alone (Fig. 5E,C). Wg is thus unable to activate Dan without *ss* but can confer antennal patterning characteristics correlated with the absence of Dfd. Conversely, *ss* is in any case sufficient to activate Dan, but without Wg it cannot induce Dfd retraction from the Mx field (Fig. 5F,F′). Thus, the absence of Dfd is not a simple consequence of Dan activation in the Mx field and reflects Wg activity. Taken together, these various results indicate that Wg can contribute to a tissue reorganisation involved in identity choice. Recent studies have implicated regulation of signalling pathways as an important element in identity choice, as *dpp* signalling regulated by *Ultrabithorax* in the haltere and by *vestigial* in the wing helps distinguish between these homologous structures (Crickmore and Mann, 2006; de Navas et al., 2006; Makhijani et al., 2007; Mohit et al., 2006).

The present work demonstrates that the timing of *wg* expression is involved in distinguishing maxillary from antennal organs. Wg is well known as a potent mitogen, and we cannot exclude that its effect on tissue-reorganisation and identity occurs at the level of cell proliferation (Kenyon et al., 2003; Serrano and O'Farrell, 1997). Nevertheless, as *wg* exerts distinct effects on proliferation and patterning in the developing wing (Neumann and Cohen, 1996), these two aspects of *wg* function appear to be separable. Similarly, Wg acts in specifying the wing primordium independently of its DV axis specification (Ng et al., 1996), and in distinguishing the eye primordium from the dorsal head vertex (Royet and Finkelstein, 1997). The novel role of *wg* regulation in maxillary specification, where changing the temporal framework for this single signalling output incites maxillary cells to reorganise as an antennal organ, leads us to consider that the Wg morphogen acts not only as a DV axial factor but also as an identity determinant.

Appendage diversification

Appendage identity results from an interplay of regionalising signals and selector transcription factors such as Hox genes. No Hox selector gene is expressed in the antennal field, where identity has been attributed to the instructive quality of co-expressed Dll and Hth (Dong et al., 2000). Downstream Hth/Dll targets include *ss* and its own targets, *dan* and *dan-related*, which are required for antennal differentiation (Emerald et al., 2003; Suzanne et al., 2003). The Mx primordium also possesses the configuration thought to procure antennal identity: co-expression of Hth and Dll associated with Ss expression in the Dll-expressing cells (Fig. 2T, Fig. 4B). However, Ss-expressing cells of the antenna activate *dan*, whereas Mx cells expressing Ss do not, raising the question what constitutes a cellular context refractory to *dan* activation? One possible explanation would be that the presence of Hox proteins in the Mx territory impedes *dan* expression there. The Mx region expresses two Hox proteins, Dfd and Pb, which might distinguish maxillary versus antennal identity. However, *pb*$^{-}$, *Dfd*$^{-}$ or double mutant *pb*$^{-}$ *Dfd*$^{-}$ Mx primordia are not transformed to antenna and *dan* is not expressed there (not shown). These Hox selectors are not responsible for repressing *dan* expression in the Mx region, and thus they do not control the crucial steps distinguishing maxillary versus antennal programs. We propose a model where the pupal application of an antennal-like program in the Mx primordium prevents *ss*-dependent *dan* activation there. This hypothesis is supported by the fact that precociously expressing Wg in the same territory is associated with premature Ss expression that induces Dan there. That *dan* can be activated in larval maxillary territory but not in pre-pupae suggests the existence of a larval competent stage (Fig. 6, green background) that is terminated in prepupae refractory to the same signal (Fig. 6, yellow background). Our working model supposes that Dfd contributes to elaborating Mx competence, and proposes that the primary signal of Mx specification is the delayed Wg expression in the prepupal stage (refractory to *dan* activation). Conversely, earlier maxillary expression of Wg in the competent stage of *dan* activation permits re-organisation toward antennal identity (Fig. 6, Mx-to-Ant).

A driving force in metazoan evolution may have been the diversification of regulatory paradigms for controlling morphogen activities to create distinct appendages. The delayed initiation of Wg uncoupled from *dpp* in the maxillary primordium is an unexpected situation for a ventral appendage, and suggests that strategies for uncoupling *dpp* from *wg* may be important for diversifying developmental outcomes (Fig. 6). Our dissection of a novel program leading to a ventral appendage reveals that temporal regulation of signalling molecules may contribute to organ identity in as yet unexplored ways that help to create appendage diversity.

We thank our colleagues in Toulouse for helpful discussions, especially Muriel Boube, Henri-Marc Bourbon, Jean Deutsch, Yacine Graba, Jim Mahaffey and Alain Vincent for their critical readings of the manuscript. We acknowledge the colleagues who provided us with mutant stocks or antibodies: I. Duncan, T. Kaufman, N. Azpiazu, S. Cohen, L. Jan, Y. N. Jan and E. Sanchez-Herrero. Monoclonal antibodies directed against Wg (4D4, developed by S. M. Cohen), Cut (2B10, developed by G. M. Rubin) and Invected (4D9, developed by C. S. Goodman) proteins were obtained from the Developmental Studies Hybridoma Bank. We thank Bruno Savelli, Brice Ronsin and Alain Jauneau for their help with the confocal microscope. This work benefited from the support of the Centre National de Recherche Scientifique (CNRS) and grants from the Association pour la Recherche sur le Cancer (ARC) and the Agence Nationale de Recherche (ANR NT05-3-42540). G.L. was supported by graduate fellowships from the French Ministère de l'Education Nationale et de la Recherche and the Association pour la Recherche sur le Cancer (ARC).

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