

Notch signaling and the determination of appendage identity

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The Notch signaling pathway defines an evolutionarily conserved cell–cell interaction mechanism that throughout development controls the ability of precursor cells to respond to developmental signals. Here we show that Notch signaling regulates the expression of the master control genes *eyeless*, *vestigial*, and *Distal-less*, which in combination with homeotic genes induce the formation of eyes, wings, antennae, and legs. Therefore, Notch is involved in a common regulatory pathway for the determination of the various *Drosophila* appendages.

appendage induction | *eyeless* | *vestigial* | *Distal-less*

How organ identity is determined is one of the fundamental questions in developmental biology. In *Drosophila* the imaginal discs, the primordia of the trunk, and the appendages of the adult fly provide a unique system to study the determination of organ identity. The imaginal disc cells maintain their disc-specific determination from the time they are established during embryogenesis until they finally differentiate during metamorphosis. Recently several *Drosophila* genes have been identified that are capable of inducing organogenesis when expressed ectopically. The most striking example being *eyeless* (*ey*), the homologue of *Pax-6* in vertebrates, which is capable of inducing ectopic eyes on wings, legs, and antennae (1, 2). *Vestigial* (*vg*) when expressed ectopically can induce wings and halteres in other regions of the body plan than the thorax (3, 4). It therefore seems useful to consider these genes as master control genes for the morphogenesis of these organs (5, 6). To understand organogenesis, we have to elucidate the regulatory mechanisms of these master control genes.

The Notch signaling pathway defines an evolutionarily conserved cell–cell interaction mechanism, which throughout development controls the ability of precursor cells to respond to developmental signals (7–9). Given the fundamental role of the Notch pathway during development, we were interested to explore the role of Notch signaling in the determination of disc identity. In wing morphogenesis, Notch signaling triggers the expression of *vg*, a control gene for wing and haltere formation, along the dorso-ventral boundary of the wing disc (3, 10). In eye morphogenesis of *Drosophila*, Notch signaling participates in cell fate decisions during photoreceptor differentiation (11) and in dorso-ventral patterning (12, 13). A proneural function also has been suggested (14), but the function of Notch at earlier stages of eye development has not been examined. Here we show that Notch signaling regulates *ey* expression during eye induction. Significantly it also is involved in the determination of other *Drosophila* appendages, i.e. wings, antennae, and legs, and regulates the respective control genes *vg* (3) and *Distal-less* (*Dll*) (15). We conclude that Notch signaling is involved in a common regulatory pathway for the determination of the identity of the various *Drosophila* appendages.

Materials and Methods

Histochemistry and *in Situ* Hybridization. For immunohistochemistry, staged larvae were dissected in cold PBS and fixed in PEM

(100 mM Pipes, pH 6.9/2 mM MgSO₄/1 mM EGTA/4% formaldehyde) for 25 min on ice. After washing with PBT (PBS containing 0.3% Triton X-100), blocking was performed in PBTB (PBS containing 0.3% Triton X-100 and 5% BSA) for 2 h at 4°C. Antibody staining was performed by using as primary antibodies mouse anti- β -galactosidase (Promega) at 1:1,000, rat anti-embryonic lethal abnormal visual system (ELAV) (16) at 1:20, rat anti-EY (17) at 1:300, Mouse anti-DLL (18) at 1:10, and rabbit anti-VG (19) at 1:200 overnight at 4°C. Immunofluorescent detection was performed by using dichlorotriazinyl amino-fluorescein (DTAF) and Cy3-conjugated donkey anti-IgGs (Jackson ImmunoResearch). After washing with PBTB, discs were dissected in PBS and mounted in Vectashield (Vector Laboratories). The preparations were analyzed on a Zeiss Axiohot microscope equipped for epifluorescence.

β -galactosidase staining was performed as described (20). For cuticle preparations, adults were dissected in PBS, mounted in Faure's mounting medium. For *in situ* hybridization, the probes were labeled with digoxigenin-dUTP and detected by digoxigenin antibodies (Boehringer).

Clonal Analysis. *Su(H)* mutant clones were induced by using the flippase (FLP/FRT) technique in larvae of the genotype *w* HSF1p; *Su(H)*SF8 FRT40A/*N-myc* FRT40A (21).

Results

Opposite Effects of Inhibition and Activation of Notch Signaling on Eye Morphogenesis. The intracellular domain of the truncated Notch receptor reflects a constitutively activated state (*Notch* activated, *N^{act}*) and the extracellular domain of the truncated receptor mimics loss-of-function phenotypes representing a dominant negative form (*Notch dominant negative*, *N^{dm}*) (11, 22). To examine the role of Notch signaling in early eye development, we expressed these truncated forms in the early eye imaginal disc, using the *GAL4* system (23) with the eye-specific enhancer of the *ey* gene (24). This eye-specific enhancer induces target gene expression in the eye primordia of the embryo and maintains expression throughout eye morphogenesis. In contrast to *ey* expression in the wild-type eye-antennal disc, the enhancer-driven reporter gene expression is not down-regulated in the differentiating cells posterior to the morphogenetic furrow but it extends all over the eye disc and into the area of the antennal disc where the rostral membrane is going to be formed (24). However, the expression in the antennal disc is quite variable from disc to disc. Consistent with previous loss of Notch function studies (25, 26), crossing *ey enhancer-GAL4* (*ey-GAL4*) flies to a stock carrying *N^{dm}* under an upstream-activating sequence for

Abbreviations: EY, *eyeless*; DLL, *Distal-less*; VG, *vestigial*; ELAV, embryonic lethal abnormal visual system; UAS, upstream-activating sequence.

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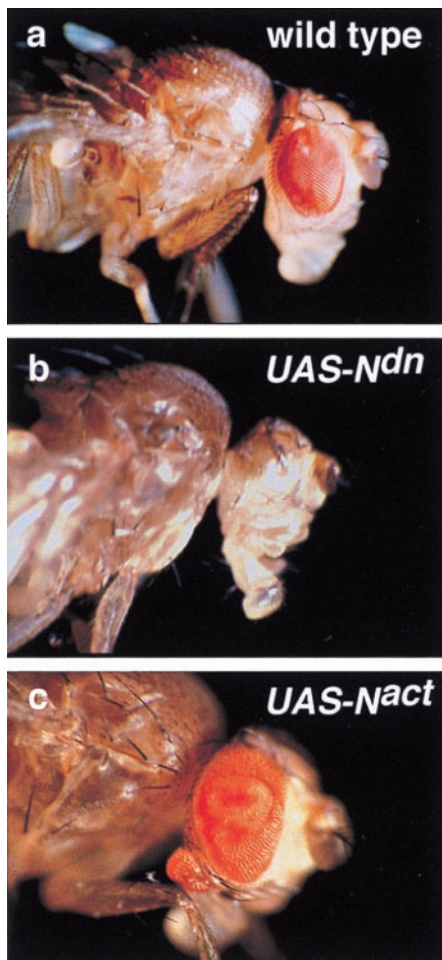


Fig. 1. Eye reduction versus hyperplasia and ectopic eye induction by the inhibition and the activation of *Notch* signaling driven by *ey-GAL4*. (a) Wild-type fly head. (b) *UAS-N^{dn} ey-GAL4* fly lacking eye. (c) *UAS-N^{act} ey-GAL4* fly shows hyperplasia of the eye and an ectopic eye on the rostral membrane of the head.

GAL4 (UAS-N^{dn}) results in a strongly reduced eye phenotype in all transheterozygous flies similar to that of the *ey²* mutant (Fig. 1*b*). Inhibition of *Notch* signaling by misexpression of *Hairless (H)* and dominant negative forms of *Delta (Dl)* and *Serrate (Ser)* also leads to a reduction or complete absence of the eye (data not shown).

Activation of *Notch* signaling by crossing *ey-GAL4* flies to a *UAS-N^{act}* line leads to significant pupal lethality, but all transheterozygotes that escape lethality show hyperplasia of the eyes with a significant increase in the number of facets (Fig. 1*c*). The disc overgrowth is found in all eye discs of *ey-GAL4 UAS-N^{act}* larvae, consistent with a role of *Notch* signaling in growth control of the eye imaginal disc. Furthermore, about 16% of the escapers (19/119) formed ectopic eyes on the rostral membrane of the head, which is derived from the antennal disc (Fig. 1*c*).

Immunostaining of eye-antennal discs of *ey-GAL4 UAS-lacZ UAS-N^{act}* larvae using an ELAV antibody to identify the differentiating photoreceptor cells and a β -galactosidase antibody to monitor the *N^{act}* protein shows that both the strong hyperplasia of the eye disc and ectopic eye formation in the antennal disc correlate with the expression of *N^{act}* (Fig. 2*a* and *b*). However, the time window for expression of the truncated receptors is critical. Transheterozygotes in which either *N^{dn}* or *N^{act}* were driven by the *glass* promoter *GMR-GAL4*, which drives expres-

sion in all cells posterior to the furrow only (27), showed only a mild phenotypic effect. As reported previously (11, 22), *N^{dn}* results in a roughening of the eye, whereas *N^{act}* produces a polished eye phenotype (data not shown). Therefore, the timing of *Notch* signaling is of crucial importance.

Notch Signaling Regulates *eyeless* Expression. The reduced eye phenotype caused by expression of *N^{dn}* and the induction of ectopic eyes by the expression of *N^{act}* are similar not only to loss-and-gain mutants of *ey* but also resemble two other mutations acting downstream (17) in the *ey* developmental pathway, *eyes absent (eya)* (28) and *dachshund (dac)* (29). Furthermore, a second *Pax-6* gene of *Drosophila*, *twin of eyeless (toy)*, was found to be an upstream regulator of *ey* capable of inducing ectopic eyes by inducing *ey* (30). To determine the epistatic relationship of *Notch* to those genes, we studied the effect of *N^{dn}* on ectopic eye induction by *ey* and *toy*.

A *dpp*-enhancer *GAL4* line (31) (*dpp-GAL4*) was crossed to flies carrying both *UAS-N^{dn}* and *UAS-ey* or alternatively to *UAS-N^{dn}* and *UAS-toy*. Transheterozygotes from both crosses exhibited ectopic eyes on legs and wings in all flies (data not shown). The size of the ectopic eyes were similar to those of the transheterozygous controls *dpp-GAL4 UAS-ey* and *dpp-GAL4 UAS-toy*, respectively, suggesting that *Notch* acts upstream of *ey* and *toy*. Double immunostaining of eye-antennal discs from transheterozygous *ey-GAL4 UAS-N^{act}*, *UAS-lacZ* using an anti-EY antibody to reveal EY protein and anti- β -galactosidase antibody to indirectly reveal *N^{act}* demonstrated that *ey* expression is induced in all eye discs by the activation of *Notch* signaling. Moreover, strong ectopic expression of EY protein was observed (Fig. 2*d*). The ectopic expression pattern of EY corresponds to that of *lacZ* reflecting the expression of *N^{act}* protein (Fig. 2*c*). Analysis of *ey* expression by *in situ* hybridization indicates that *ey* is induced at the transcriptional level (data not shown). Similarly, ectopic expression of *toy* also was induced in the antennal discs of *ey-GAL4 UAS-N^{act}* larvae (data not shown). Thus, activation of *Notch* signaling can induce *toy* and *ey* expression in antennal discs. Expression of *N^{act}* also correlates with the ectopic induction of photoreceptor cells as revealed by ELAV staining (Fig. 2*a* and *b*).

Notch activation of *ey* and *toy* depends on the downstream effector of *Notch*, *Suppressor of Hairless (Sh)* (32), because *Su(H)* mutant clones (21) generated anterior to the morphogenetic furrow in the eye fail to produce adult structures (data not shown), in agreement with a requirement of *Notch* signaling during eye morphogenesis.

Activation of *Notch* Signaling in an *eyeless* Mutant Background Induces Ectopic Antennae. Consistent with the finding that *ey* acts downstream of *Notch*, the expression of *N^{act}* in an *ey²* or *ey^R* hypomorphic mutant background generates eyes of a reduced size. Approximately 72% (63/88) of the *ey-GAL4 UAS-N^{act}; ey²* flies that survived were found to have reduced eyes and about 15% of these flies (13/88) had both a reduced original and a reduced ectopic eye. However, in addition to ectopic eyes *N^{act}* also induced ectopic antennae in 25% (22/88) of these flies on the side of the head that is derived from the eye disc. Many of the induced ectopic antennae were complete with all three antennal segments and the arista (Fig. 3*a*). Similar results also were obtained with *ey^R*, the other hypomorphic allele (data not shown), but no ectopic antennae were found in *ey-GAL4 UAS-N^{act} ey⁺* flies (Fig. 1*c*), indicating that *Notch* signaling induces antenna formation in a loss-of-function *ey* mutant background.

Because *Distal-less* in combination with *extradenticle (exd)* and *homothorax (hth)* specifies the antennae (33, 34), we monitored DLL expression in the eye discs that are capable of forming ectopic antennae. In wild-type larvae, DLL protein is expressed in the antennal but not in the eye disc (Fig. 4*g*). In all of the tested

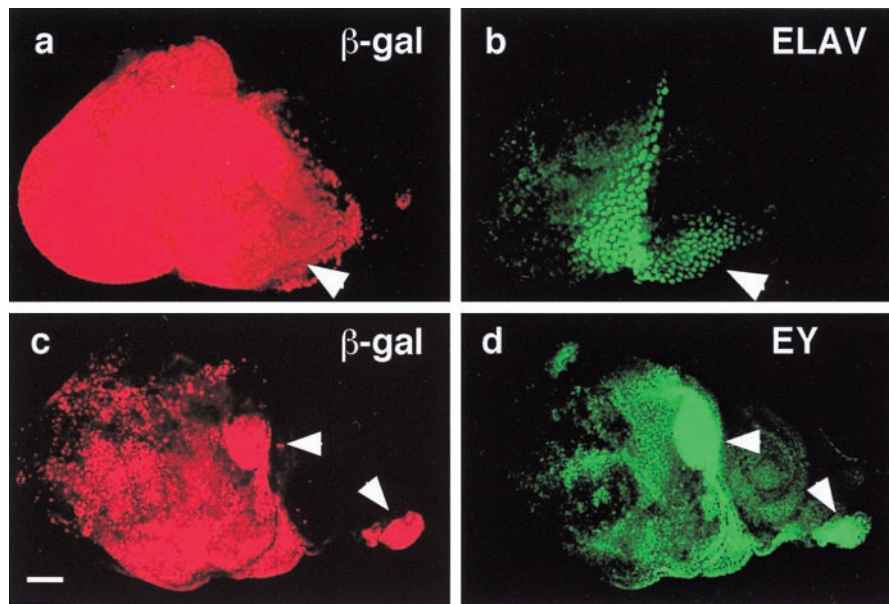


Fig. 2. Induction of ectopic photoreceptor cells and *eyeless* by Notch signaling. (a) Anti- β -galactosidase (β -gal) antibody staining of a *UAS-N^{act} UAS-lacZ ey-GAL4* eye-antennal disc. Activation of the *lacZ* reporter reflects the distribution of constitutively activated Notch protein. Arrowhead indicates hyperplastic portion. (b) Immunostaining of same disc as in a with antibody against the neuronal marker ELAV. In the hyperplastic portion (arrowhead) ectopically induced photoreceptors. (c) Anti- β -galactosidase antibody staining of a *UAS-N^{act} UAS-lacZ ey-GAL4* eye antennal disc. Activation of the *lacZ* reporter reflects the distribution of *N^{act}* protein. Arrowheads indicate areas of strong *lacZ* expression. (Bar indicates 50 μ m.) (d) Immunostaining of same disc as in c with antibody against EY. Ectopic EY expression is induced in the areas of strong *lacZ* expression (arrowheads).

discs (30/30) in *ey-GAL4 UAS-N^{act} ey²* animals that form ectopic antennae from the eye disc, significant DLL expression was detected ectopically (Fig. 4 a and b). By contrast, no ectopic expression of DLL was detected in the eye discs of *ey²* control larvae (Fig. 4 c and d). In 14 of 30 *ey-GAL4 UAS-N^{act} ey²* larvae additional ectopic expression of DLL in a few cells of the antennal disc (Fig. 4 e and f) was observed. This indicates that *Notch* signaling directly or indirectly induces ectopic expression of *Dll* in the eye-antennal disc, leading to the ectopic induction of antennae.

Activation of Notch Signaling Combined with the Ectopic Expression of Antennapedia Induces Ectopic Wings and Legs on the Head. The observation that *N^{act}* can induce both ectopic eyes and, in a

specific genetic background, antennae led us to consider the possibility that *Notch* signaling also might induce the formation of other appendages in a different genetic context. To test this hypothesis, we combined the activation of *Notch* signaling with ectopic expression of *Antennapedia (Antp)*. The latter is known to determine the identity of the second thoracic segment (T2) (35, 36), which on the dorsal side gives rise to a pair of wings and on the ventral side to a pair of second legs. For this purpose, transgenic flies of the constitution *ey-GAL4 UAS-N^{act} UAS-Antp* were generated. About 26% (17/65) of the flies escaping pupal lethality were found to have ectopic wings on the head (Fig. 3b). Almost all ectopic wing structures consisted of dorsal and ventral wing blades bordered by bristles of the wing margin (double and

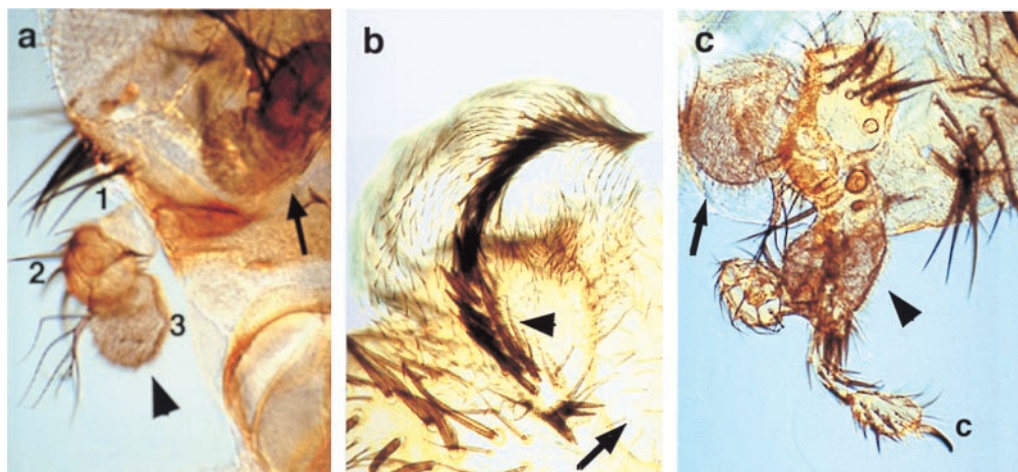


Fig. 3. Induction of ectopic antennae, wings, and legs in different genetic contexts. (a) Induction of an ectopic antenna (arrowhead) on the side of the head in *UAS-N^{act} ey-GAL4 ey²* mutant fly. 1, 2, and 3: first, second, and third antennal segments, respectively. Arrow indicates normal antenna. (b) Induction of ectopic wing structures (arrowhead) in the eye region (arrow) by activation of Notch signaling and the simultaneous ectopic expression of *Antennapedia (Antp)*. Wing blade and triple row of wing marginal bristles are induced in a *UAS-N^{act} UAS-Antp ey-GAL4* fly replacing parts of the original eye. (c) Partial transformation of the ectopic antenna (arrowhead) into leg structures with a claw (c). Arrow indicates original antenna.

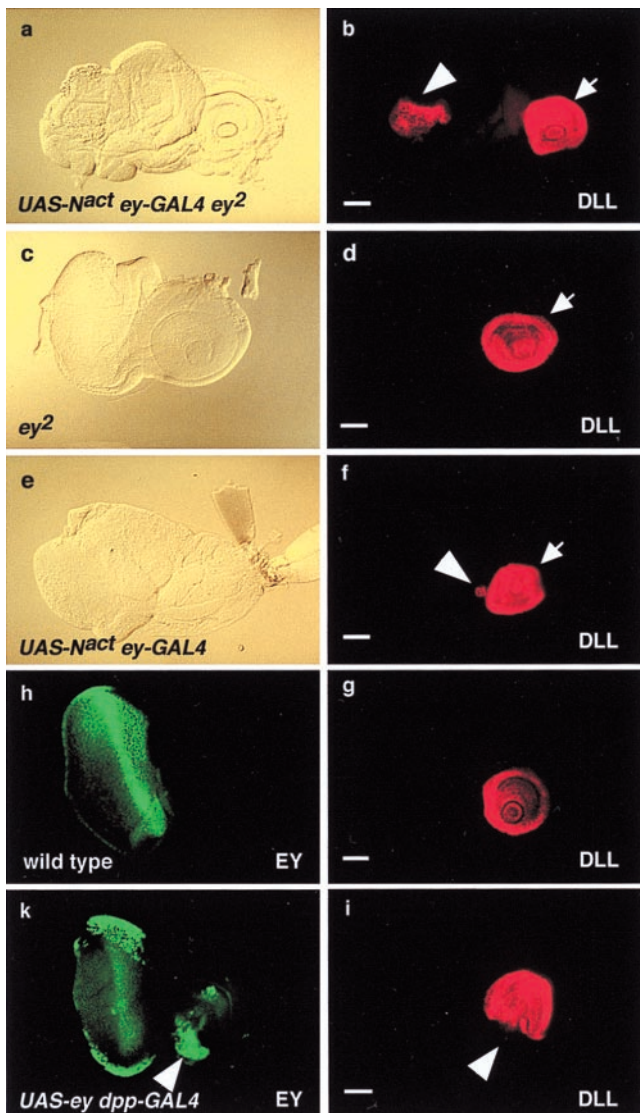


Fig. 4. Ectopic induction of *Distal-less* in *eyeless²* mutant eye-antennal discs by the activation of *Notch* signaling driven by *ey-GAL4*. (a) Bright-field micrograph of an eye-antennal disc of a *UAS-N^{act} ey-GAL4 ey²* larva. Note the hyperplasia of the eye disc. (b) Immunostaining of same disc as in a with antibody against DLL. Ectopic DLL expression is induced in the eye disc (arrowhead). Arrow indicates original DLL expression in the antennal disc. (c) Bright-field micrograph of an eye-antennal disc of a *ey²* larva. The eye disc is reduced. (d) Immunostaining of the same disc as in c with antibody against DLL. DLL expression is confined to the antennal disc. (e) Bright-field micrograph of an eye-antennal disc of a *UAS-N^{act} ey-GAL4* larva. (f) Immunostaining of the same disc as in e with antibody against DLL. Ectopic DLL expression is induced in a few cells of the antennal disc (arrowhead). Arrow indicates original DLL expression in the antennal disc. (g) Wild-type expression of DLL in eye-antennal disc. (h) Wild-type expression of EY in eye-antennal disc. (i) Immunostaining of same disc as in k with an antibody against DLL. Arrowhead indicates the repression of DLL in the region of ectopic EY expression. (k) Immunostaining of an eye-antennal disc of *UAS-ey dpp-GAL4* larva with antibody against EY. Arrowhead indicates the ectopic expression of EY in the antennal disc. Posterior is to the left and dorsal is up. (Bars = 50 μ m.)

triple row), but lacking wing veins. In contrast, wing structures induced by the ectopic expression of *vg*, the wing margin is not formed (3), suggesting that *Notch* signaling and *Antp* are acting upstream of *vg*. Furthermore, about 17% (11/65) of these flies showed ectopic leg structures induced by secondary transforma-

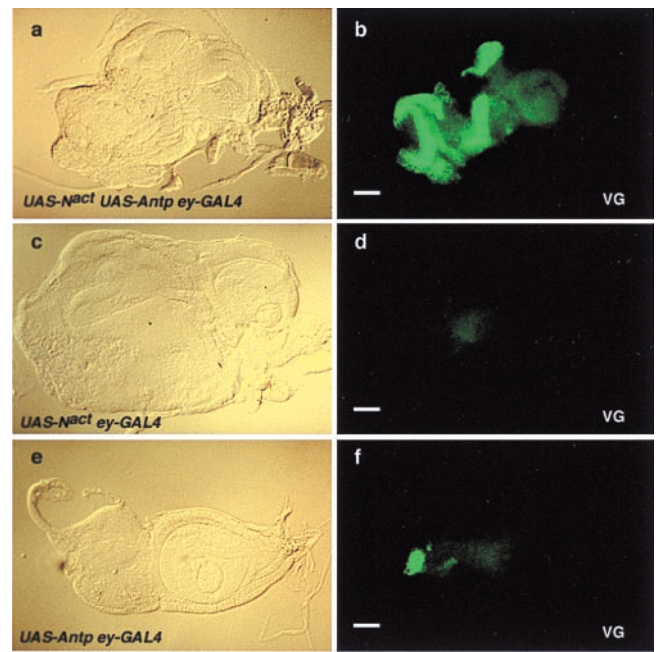


Fig. 5. Ectopic induction of *vestigial* in the eye discs by the activation of *Notch* signaling and the simultaneous ectopic expression of *Antennapedia* driven by *ey-GAL4*. (a) Bright-field micrograph of an eye-antennal disc of a *UAS-N^{act} UAS-Antp ey-GAL4* larva. Note hyperplasia of the eye disc. (b) Immunostaining of the same disc as in a with antibody against VG. Ectopic VG expression is induced in the eye disc. Note hyperplasia of the eye disc. (c) Bright-field micrograph of an eye-antennal disc of a *UAS-N^{act} ey-GAL4* larva. (d) Immunostaining of the same disc as in c with antibody against VG. No VG expression is detected. (e) Bright-field micrograph of an eye-antennal disc of a *UAS-Antp ey-GAL4* larva. (f) Immunostaining of the same disc as in e with antibody against VG. Ectopic VG expression is induced in a small region of the eye disc. Posterior is to the left and dorsal is up. (Bars = 50 μ m.)

tion of the ectopic antennal tissue into leg structures (e.g., arista into tarsus) (Fig. 3c). The *ey-GAL4 UAS-Antp* control flies did not show any ectopic wing structures, but they clearly exhibited reduced eyes, suggesting that the ectopic expression of *Antp* partially represses *ey* in the eye discs of these animals. An additional 10% (7/71) of these flies showed a transformation of the original antenna to leg structures. On the heads of *ey-GAL4 UAS-N^{act}* flies, no wing nor leg structures were found (Fig. 1c). Therefore, activation of *Notch* signaling when combined with the ectopic expression of *Antp* driven by *ey-GAL4* is capable of inducing wing and leg structures on the head.

In wild-type larvae, the *vg* gene is expressed in the wing but not in the eye disc (19). By contrast, in *ey-GAL4 UAS-N^{act} UAS-Antp* animals in which ectopic wing structures are induced in the eye disc all of the tested eye discs (25/25) showed significant ectopic expression of VG protein (Fig. 5 a and b), whereas no ectopic expression of VG was detected in *ey-GAL4 UAS-N^{act}* control larvae (Fig. 5 c and d). However, *ey-GAL4 UAS-Antp* larvae showed VG expression in a small region of the eye discs in seven of 11 tested discs (Fig. 5 e and f), consistent with a synergistic effect of endogenous *Notch* activity with ectopic *Antp* expression on VG expression. It therefore appears that activation of *Notch* signaling in context of *Antp* expression induces *vg* expression in the eye discs and that there are synergistic effects between *Notch* signaling and *Antp* expression. Kim *et al.* (3) have shown that the *Notch* signaling pathway is used to specifically activate the boundary enhancer of the *vg* gene necessary for dorso-ventral wing formation. The same enhancer also may be used for ectopic formation of the wing, a point that

has to be investigated further. A dorso-ventral boundary also is established by Notch in the eye disc that controls growth and polarity in the *Drosophila* eye (13).

In *ey-GAL4 UAS-N^{act} UAS-Antp* ectopic legs also are induced on the head (Fig. 3c), which is accompanied by DLL expression in 21 of 21 tested eye discs (data not shown). In contrast, no DLL expression is detected in eye discs of *ey-GAL4 UAS-Antp* larvae (data not shown), which is in agreement with the adult phenotype of these animals.

In view of the above observations we propose that the effects of Notch signaling on the various appendages depend on the context provided by control genes such as *ey* and *Antp*. In the eye primordia, Notch signaling induces *ey* expression, which induces a cascade of downstream genes leading to eye morphogenesis. In conjunction with *Antp*, Notch signaling induces *vg*, leading to wing formation. At low levels of *ey* expression, Notch signaling induces *Dll*, leading to antenna morphogenesis. In the case of the leg, Notch also induces *Dll* expression that in conjunction with *Antp* leads to leg formation (15).

Discussion

Combinatorial Genetic Interactions Specify the Identity of the Various Appendages. Segmental identity is specified by the homeotic genes that are active in a particular combination in each segment. Within a given segment, the appendages are specified by a different set of subsidiary control genes; the eyes are specified by *ey*, the wings and haltere by *vg*; the legs by *Dll* and the antennae by *Dll* in combination with *extradenticle* (*exd*) and *homothorax* (*hth*) (33, 34). Our results indicated that they all are regulated by Notch signaling, sharing the same cell signaling pathway, which raises the possibility that the appendage specificity is provided by a combinatorial interaction between Notch and the homeotic and the subsidiary control genes.

The repression of one control gene by the expression of another seems to be a widespread mechanism to ensure that the developmental pathways are mutually exclusive so that the formation of intermediary cell types is prevented. Similar to the repression of *ey* by *Antp*, we found that *ey* directly or indirectly represses *Dll*. In hypomorphic *ey* mutants, the activation of Notch signaling leads to ectopic expression of *Dll* in the eye disc, suggesting that *ey* might repress *Dll* in the wild-type eye disc. In *dpp-GAL4 UAS-ey* transheterozygous flies EY is expressed on the ventral side of the posterior half of the antennal discs under the control of the *dpp*-enhancer (Fig. 4k), whereas DLL is not detectable in this area (Fig. 4i). A similar mutually exclusive expression is found in the leg discs of these flies (data not shown), suggesting that *ey* represses *Dll* expression.

Based on these findings, we propose a model to explain the difference between the eye and antennal pathway starting from a common signaling mechanism. Notch signaling induces the expression of both *ey* and *Dll*. However, in the eye primordia *ey* represses *Dll* and induces eye morphogenesis. By contrast, in the antennal disc *ey* is repressed by a repressor, resulting in *Dll* expression that confers antennal (ventral appendage) specificity. Two of the possible candidates for the repressor are the homeobox genes *exd* and *hth*, because both *exd*⁻ and *hth*⁻ mutant clones in the rostral membrane region of the antennal disc can result in ectopic eye development, which presumably is caused by derepression of *ey* (37, 38). Both *exd* and *hth* also may function in conjunction with *Dll* serving as corepressors.

The Fundamental Role of Notch Signaling in Development and Evolution. In this study, we have shown that Notch signaling regulates *ey* expression at the early stages of eye morphogenesis. By analogy to the *Drosophila* paradigm, it is therefore likely that the expression of *Pax-6* may be regulated by Notch signaling, given the extraordinary conservation of Notch function from ascidians (39) to mammals (40). Notch signaling seems to participate in dorso-ventral patterning of the *Drosophila* wing (3, 10) and eye (12, 13) and also in the vertebrate limb (41, 42). Thus our study raises the possibility that Notch is involved in the control of both vertebrate and invertebrate appendage formation. In describing the developmental role of Notch (7), it has been proposed that Notch signaling modulates the ability of individual precursor cells to respond to developmental signals, whether differentiation, proliferation, or apoptotic cues. The present study extends the fundamental role of Notch by indicating that the implementation of entire developmental programs leading to appendage formation and organogenesis may be controlled by Notch activity.

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