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An essential role for the *Drosophila Pax2* homolog in the differentiation of adult sensory organs

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SUMMARY

The adult peripheral nervous system of *Drosophila* includes a complex array of mechanosensory organs (bristles) that cover much of the body surface of the fly. The four cells (shaft, socket, sheath, and neuron) which compose each of these organs adopt distinct fates as a result of cell-cell signaling via the Notch (N) pathway. However, the specific mechanisms by which these cells execute their conferred fates are not well understood. Here we show that *D-Pax2*, the *Drosophila* homolog of the vertebrate *Pax2* gene, has an essential role in the differentiation of the shaft cell. In flies bearing strong loss-of-function mutations in the *shaven* function of *D-Pax2*, shaft structures specifically fail to develop. Consistent with this, we find that D-Pax2 protein is expressed in all cells of the bristle lineage during the mitotic (cell fate specification) phase of bristle development, but becomes sharply restricted to the shaft and sheath cells in the post-mitotic (differentiative) phase. Two lines of evidence described here indicate that *D-Pax2* expression and function is at least in part downstream of cell fate specification mechanisms such as N signaling. First, we find that the lack of late D-Pax2 expression in the socket cell (the sister of the shaft cell) is controlled by N pathway activity; second, we find that loss of *D-Pax2* function is epistatic to the socket-to-shaft cell fate transformation caused by reduced N signaling. Finally, we show that misexpression of *D-Pax2* is sufficient to induce the production of ectopic shaft structures. From these results, we propose that *D-Pax2* is a high-level transcriptional regulator of the shaft cell differentiation program, and acts downstream of the N signaling pathway as a specific link between cell fate determination and cell differentiation in the bristle lineage.

Key words: *Drosophila melanogaster*, Neurogenesis, Sensory organ development, *shaven* (*sv*), *Pax* genes, Differentiation, Morphogenesis

INTRODUCTION

The mechanosensory bristles on the body surface of adult *Drosophila* constitute the majority of the adult peripheral nervous system (PNS). The development of these organs takes place during late larval and early pupal stages and involves a well-studied sequence of events. A sensory organ precursor (SOP) cell produces, via a fixed lineage, the four cells that make up the mature bristle organ (Fig. 1; Hartenstein and Posakony, 1989). The lineage overall includes three pairs of sister cells, the pIIA and pIIB secondary precursors; the tormogen and trichogen, which produce the external socket and shaft structures, respectively; and the thecogen (sheath or glial cell) and neuron. Alternative fates are allocated to the members of each sister cell pair through the coupling of intrinsic and extrinsic mechanisms (for review, see Posakony, 1994). The intrinsic mechanisms include the asymmetric distribution of the Numb protein at each division. For example, Numb is localized to one side of the SOP; the subsequent division of this cell partitions Numb exclusively to the pIIB daughter (Rhyu et al., 1994). The extrinsic mechanisms involve cell-cell communication via the Notch (N) signaling pathway (for review, see Greenwald, 1998; Kimble and Simpson, 1997). The N protein is a transmembrane receptor; interaction of the N extracellular domain with the transmembrane ligands Delta (Dl) or Serrate stimulates cleavage of the receptor's intracellular domain (N^{IC}), which is then transported to the nucleus (Lecourtois and Schweisguth, 1998; Schroeter et al., 1998; Struhl and Adachi, 1998). There, it interacts directly with the transcription factor Suppressor of Hairless [Su(H)] (Fortini and Artavanis-Tsakonas, 1994; Furukawa et al., 1992; Schweisguth and Posakony, 1992; Tamura et al., 1995), and the $Su(H)/N^{IC}$ complex activates transcription of target genes, including members of the *Enhancer of split* Complex (Bailey and Posakony, 1995; Jarriault et al., 1995; Lecourtois and Schweisguth, 1995). In the bristle lineage, N-Dl signaling between sister cells promotes the fates of pIIA, tormogen and thecogen (Fig. 1; Posakony, 1994). Their respective sister cells (pIIB, trichogen, neuron) adopt alternative fates by virtue of being resistant to reciprocal N signals, at least in part because

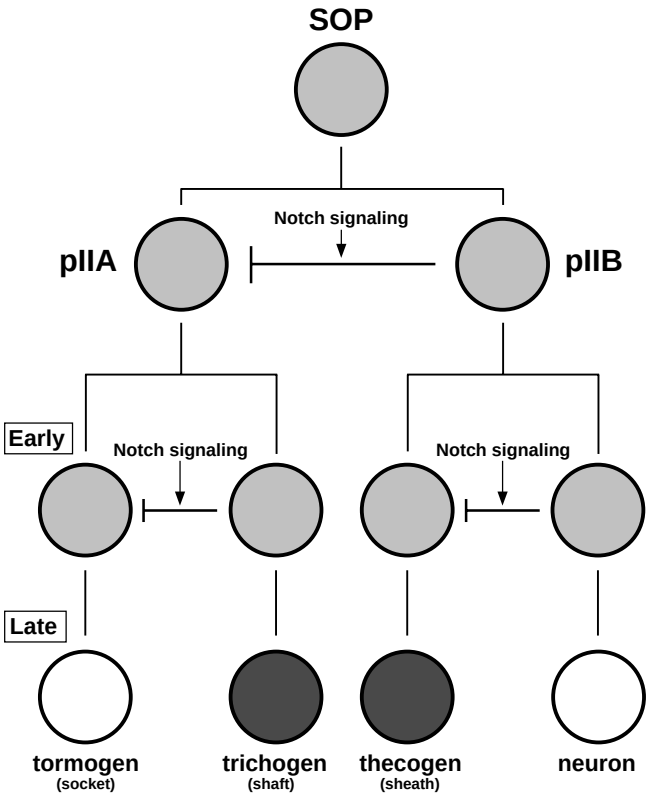

Fig. 1. Schematic representation of the adult mechanosensory bristle lineage and of the role of N signaling in bristle cell fate specification (adapted from Posakony, 1994). At each division, the cell fate asymmetry of the daughter cells requires the activity of the N signaling pathway, such that the cell on the left in each pair is prevented by this signal from adopting the same fate as its sister cell on the right. The signal itself is bidirectional, with each cell both sending and receiving. The pIIA secondary precursor, the tormogen, and the thecogen are responsive to the N-mediated signals sent by their sister cells, but pIIB, the trichogen, and the neuron are made resistant to the reciprocal signal by N pathway antagonists such as Numb and Hairless. The early pattern of D-Pax2 protein accumulation in this lineage is marked by light shading, the late pattern (bottom) by dark shading appearing only in the trichogen and thecogen.

of the asymmetric distribution of Numb, which acts as an antagonist of N activity (Frise et al., 1996; Guo et al., 1996; Posakony, 1994; Rhyu et al., 1994). Although the mechanisms involved in cell fate specification in the adult bristle lineage have been fairly intensively studied, relatively little is known about how these cell fates are executed once they are assigned.

The genetic locus *shaven* (*sv*) was originally identified by Bridges through a spontaneous mutation on the fourth chromosome that conferred a bristle loss phenotype (see Lindsley and Zimm, 1992). Using light microscopic analysis of *shaven-naked* (sv^n) flies, Lees and Waddington (1942) described variable defects in bristle shaft development that were not accompanied by corresponding reductions in socket structures. They also noted both similarities and differences between *sv* mutant phenotypes and those conferred by loss-of-function mutations of *Hairless* (*H*). *H* is now known to be a direct antagonist of N signaling (Bang et al., 1995; Bang and Posakony, 1992; Lyman and Yedvobnick, 1995; Schweisguth and Posakony, 1994); the H protein binds to Su(H) and interferes with its DNA-binding activity (Brou et al., 1994). Lees and Waddington (1942) demonstrated a strong phenotypic interaction between *sv* and *H*, such that double mutants exhibit a large number of 'double socket' macrochaetes and microchaetes. Since these pioneering studies, little developmental genetic analysis of the *sv* locus has been carried out.

Recently, Fu and Noll (1997) cloned *D-Pax2*, the *Drosophila* homolog of the vertebrate *Pax2* gene, and identified it as the gene *sparkling* (*spa*), a fourth-chromosome locus that had previously been mapped very close to *sv* (Hochman, 1971). *spa* mutants display a rough eye phenotype marked by defects in ommatidial assembly and in cone and pigment cell development. Further study showed that the *spa* and *sv* functions correspond to the same transcription unit, and that the existing mutants of each type carry lesions in separate transcriptional enhancers that drive *D-Pax2* expression specifically in the eye (*spa*) or in sensory organs (*sv*) (Fu et al., 1998).

To investigate in detail *D-Pax2*'s role in adult sensory organ development, we first found it necessary to generate several new *sv* loss-of-function alleles, all of which are stronger than the existing sv^n, *shaven-depilate* (sv^{de}), and sv^{35a} alleles (Lindsley and Zimm, 1992). In addition to characterizing their external adult phenotypes, we have used these strong mutants to analyze the developmental basis of the observed bristle defects, using a variety of cell type-specific markers for the sensory organ lineage. We have also defined the temporal and spatial patterns of D-Pax2 protein accumulation in the developing microchaete field of the pupal notum. We have examined the relationship between *D-Pax2* expression and function in the bristle lineage and the process of cell fate specification by the N signaling pathway. Finally, we have investigated the phenotypic consequences of misexpressing *D-Pax2* in developing sensory organs. All of our analyses indicate that one key role of *D-Pax2* is the control of trichogen (shaft) cell differentiation, and we propose that *D-Pax2* acts as an important bridge between the allocation of cell fate via the N pathway and the execution of that fate. The dynamic D-Pax2 expression pattern, along with other circumstantial evidence, suggests that *D-Pax2* has additional functions in sensory organ development, including potential roles in cell fate determination and sheath cell differentiation.

MATERIALS AND METHODS

Drosophila stocks and crosses

Flies were cultured on standard yeast-cornmeal-molasses-agar medium at 25°C in a humidified chamber. For mutations and chromosomes not specifically described below, see Lindsley and Zimm (1992) or FlyBase (1998).

D-Pax2 (*sv*) mutant stocks

The *D-Pax2* mutant alleles used here were maintained as follows:

w^{1118}; sv^n
w^{1118}; $sv^{de}/Tp(2;4)ey^D$
w^{1118}; $sv^6/Tp(2;4)ey^D$
w^{1118}; $sv^7/Tp(2;4)ey^D$
w^{1118}; sv^8/ci^D

sv^n and sv^{de} both show more severe adult bristle defects in *trans* to *Df(4)G*, which is deficient for the *D-Pax2* locus, than as homozygotes. The new alleles sv^6, sv^7 and sv^8 (see below), which as homozygotes are viable as late as the pharate adult stage, are embryonic lethal in *trans* to *Df(4)G*. By this criterion, all of the *D-Pax2* mutant alleles used here represent loss-of-function mutations.

A101 staining

Pupae from a cross of w^{1118}*; A101/TM3;* sv^7*/Tp(2;4)*ey^D to w^{1118}*; A101/TM3;* sv^8*/Tp(2;4)*ey^D were selected.

A1-2-29 staining

Pupae from a cross of w^{1118}*; A1-2-29/CyO;* sv^7*/Tp(2;4)*ey^D to w^{1118}*; A1-2-29/CyO;* sv^8*/Tp(2;4)*ey^D were selected.

D-Pax2 antibody staining

w^{1118} was used as the wild-type strain. For double staining with A101, larvae and pupae from a cross of w^{1118} to *A101/TM6B* were used. For anti-D-Pax2 staining of *D-Pax2* mutants, *sv* homozygotes (sv^6) or transheterozygotes (sv^7/sv^8) were selected from crosses of the stocks listed above.

Loss of *H* function

To produce a highly expressive microchaete 'double socket' phenotype, we made use of the hypomorphic allele H^{21} (also known as HC23; Bang et al., 1991; Bang and Posakony, 1992).

Overexpression of *H*

Pupae from the stock w^{1118}*; P[*w^+*, Hs-H]-3* (Bang and Posakony, 1992) and selected pupae from the stock w^{1118}*; P[*w^+*, Hs-H]-3;* sv^{de}*/Tp(2;4)*ey^D were subjected to heat-shock treatment (see below).

Misexpression of *D-Pax2*

For misexpression of *D-Pax2* using the GAL4/UAS system (Phelps and Brand, 1998), flies of the genotype w^{1118}*; P[*w^+*, UAS-D-Pax2]* (see below) were crossed to either of two GAL4 driver strains, *w; P[*w^+*, hs-GAL4]* (Brand et al., 1994) or MS 1096 (Capdevila and Guerrero, 1994; Milán et al., 1998). From the first cross, aged pupae were subjected to heat-shock treatment (see below), with pupae from the parent stocks serving as controls.

EMS mutagenesis screen for new *sv* alleles of *D-Pax-2*

The design of a strategy for the recovery of strong *sv* loss-of-function alleles of *D-Pax2* was based on the following observations. A new mutation on the X chromosome designated *P29* (J. K. and J. W. P., unpublished results) displays a dominant bristle loss phenotype affecting almost exclusively the postorbital bristles. This mutation is homo- and hemizygous viable and interacts with existing *sv* mutant alleles and with *Df(4)G*, in the following manner. *P29/+; Df(4)G/+* and *P29/Y; Df(4)G/+* flies exhibit extensive bristle loss on the abdomen, a phenotype not observed with either *P29/+* or *Df(4)G/+* flies (J. K. and J. W. P, unpublished observations). We reasoned that placing mutagenized fourth chromosomes in the *P29* heterozygous background could allow the recognition of strong *sv* loss-of-function alleles in an F_1 screen. w^{1118}*/Y* adult male flies bearing an isogenized fourth chromosome were fed 40 mM ethylmethanesulfonate (EMS; Lewis and Bacher, 1968) in 5% sucrose overnight (approx. 16 hours) and then mated to w^{1118} *P29; P454-1* virgin females. *P454-1* is a homozygous viable w^+ P-element insertion on the fourth chromosome (Roseman et al., 1995), which permitted unambiguous identification of flies bearing the mutagenized fourth chromosome in the F_2 generation. Potential *sv* mutants were selected from the F_1 progeny based on the abdominal bristle loss phenotype. The potential mutant fourth chromosomes were then balanced over ci^D and tested in a secondary screen by placing them in *trans* to *Df(4)G*, sv^n, and sv^{de}. In two separate runs, a total of approximately 19,800 mutagenized genomes were screened in this way. Four new confirmed *sv* alleles of *D-Pax2* (sv^5, sv^6, sv^7, and sv^8) were recovered; all were classified as such on the basis of their chromosomal segregation and the adult *sv* phenotypes or lethality they exhibited in the secondary tests (see Results).

Generation of *UAS-D-Pax2* transgenic fly lines

A 2.8 kb *Eco*RI-*Xba*I fragment of the previously described *spa* cDNA clone cpx1 which includes the entire protein coding region and 0.25 kilobases (kb) of its 3′ untranslated region (Fu and Noll, 1997) was ligated into the cloning site of the pUAST vector (Brand and Perrimon, 1993). Several independent *P[*w^+*, UAS-D-Pax2]* transgenic lines were established following P element-mediated transformation of w^{1118} recipients (Rubin and Spradling, 1982). A homozygous viable transgene insertion on the third chromosome was used for all experiments reported here.

Heat-shock treatment

For induction of *H* overexpression, pupae bearing a *Hs-H* transgene were subjected at 24 hours after puparium formation (APF) to three 1-hour exposures at 37°C separated by 90-minute intervals of recovery at 25°C (Bang and Posakony, 1992). Aged pupae (12-24 hours APF) bearing the *hs-GAL4* driver were subjected to a 37°C heat shock for 1 hour.

Scanning electron microscopy (SEM)

Scanning electron microscopy was carried out as described by Bang et al. (1991).

Immunohistochemistry

Primary antibodies used in this study include mouse anti-β-galactosidase (Promega), diluted 1:400; mAb 22C10 (Zipursky et al., 1984) hybridoma supernatant (a gracious gift from S. Benzer; also Developmental Studies Hybridoma Bank, University of Iowa), diluted 1:100; S12 (rabbit anti-BarH1; Higashijima et al., 1992) polyclonal antiserum (a gracious gift from T. Kojima), diluted 1:100; mAb 9F8A9 (mouse anti-Elav; O'Neill et al., 1994) hybridoma supernatant (Developmental Studies Hybridoma Bank, University of Iowa), diluted 1:100; and rabbit anti-D-Pax2 polyclonal antiserum (Fu and Noll, 1997), diluted 1:50. Nota or imaginal discs were dissected from pupae or larvae in PBS, fixed in 4% paraformaldehyde in PBS for 15-30 minutes at room temperature, and washed several times in PBS containing 0.1% Triton X-100 (PBS-Tx). Tissue was then incubated overnight at 4°C with antibodies in PBS-Tx + 2% BSA.

Enzymatic detection

Tissue was extensively washed in PBS-Tx and then incubated with appropriate secondary reagents – horseradish peroxidase- or alkaline phosphatase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG – for 3 hours at room temperature. Tissue was extensively washed with PBS-Tx and incubated in 0.1 M Tris (pH 7.5) containing 0.6 mg/ml diaminobenzidine and 0.03% H_2O_2 (for horseradish peroxidase staining) or in 0.1 M Tris (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$ containing 0.33 mg/ml NBT and 0.16 mg/ml BCIP (for alkaline phosphatase staining). Staining reactions were continued for 30 minutes to 2 hours, until control tissue showed clear patterns. Reactions were stopped by extensive washing with PBS-Tx. Stained tissue was dehydrated and mounted in Epon.

Fluorescence detection

Tissue was prepared as described above. After incubation with primary antibodies, tissue was washed extensively with PBS-Tx and then incubated with Oregon Green-conjugated goat anti-rabbit IgG and Texas Red-conjugated goat anti-mouse IgG (Molecular Probes).

RESULTS

Adult bristle phenotypes associated with *D-Pax2* loss-of-function alleles

Three new *sv* loss-of-function alleles of *D-Pax2* (sv^6, sv^7, sv^8) were recovered in an EMS mutagenesis screen (see Materials and Methods). Homozygotes of all three can survive to pharate adulthood (see below) and display profound bristle defects over the entire body surface. We examined these mutant phenotypes by SEM and compared them to those conferred by the previously identified alleles sv^n and sv^{de} (Fig. 2; see Lees and Waddington, 1942). All *sv* mutants show bristle shaft abnormalities of varying severity. These defects are present in all areas of the body, but we have concentrated our analysis on the macrochaetes and microchaetes of the notum. Common microchaete defects include stunted or misshapen shafts (primarily sv^n and sv^{de}; Fig. 2B,C,F,G) and empty sockets (sv^{de}, sv^6, sv^7, and sv^8; Fig. 2G,H,K and data not shown). Macrochaetes exhibit these same phenotypes (Fig. 2B-D) and occasionally other rarer defects as well, such as double sockets (all mutant alleles; Fig. 2F,G,J) and odd shaft-like structures with no apparent socket (sv^6 and sv^7; Fig. 2L). Individual macrochaetes exhibit certain defects more frequently than others. For example, sv^{de} flies often have an empty socket at the position of the anterior dorsocentral (aDC) macrochaete (Fig. 2C,G), but are much more likely to have a double socket at the posterior dorsocentral (pDC) macrochaete position (Fig. 2G). The socketless shaft-like structures of sv^6 also appear specifically at the aDC position and not the pDC position.

The mutant alleles of *D-Pax2* we have studied can be ordered on the basis of the severity of the microchaete phenotype they confer, as follows: $sv^6 = sv^7 = sv^8 > sv^{de} > sv^n$. The three new alleles give almost complete microchaete shaft loss, whereas sv^{de} flies exhibit a variable number of stunted shafts and sv^n flies only slightly misshapen shafts. This ranking is preserved when lethality is considered. Most sv^6 and sv^7 homozygotes die before the wandering larva stage, some die at the wandering larva stage, and the remaining few die as pharate adults. A somewhat higher percentage of sv^8 homozygotes survive to the pharate adult stage. Almost all sv^{de} homozygotes eclose, but are uncoordinated and die within a few days. sv^n flies are fully homozygous viable. However, there is some phenotypic complexity within this allelic order, as sv^n macrochaetes are more affected than sv^{de} macrochaetes. In addition, sv^n/sv^{de} transheterozygotes do not display an intermediate phenotype, but instead show a weaker phenotype than that of either homozygote.

In contrast to flies mutant for the *sv* function of *D-Pax2*, *spa* mutants exhibit various eye defects, including improper ommatidial arrangement, absence of lens material, and necrotic pits, but do not show significant bristle abnormalities (Fu and Noll, 1997; Lindsley and Zimm, 1992). Of the five *sv* alleles analyzed here, only sv^6, sv^7, and sv^8 confer any eye defects; these include mild roughening of the eye due to fused ommatidia, and occasional scarring of the lens (data not shown). These alleles interact very weakly with spa^{pol} (Fu and Noll, 1997; Lindsley and Zimm, 1992). Therefore, *spa* function is largely intact in these new *sv* mutants, and

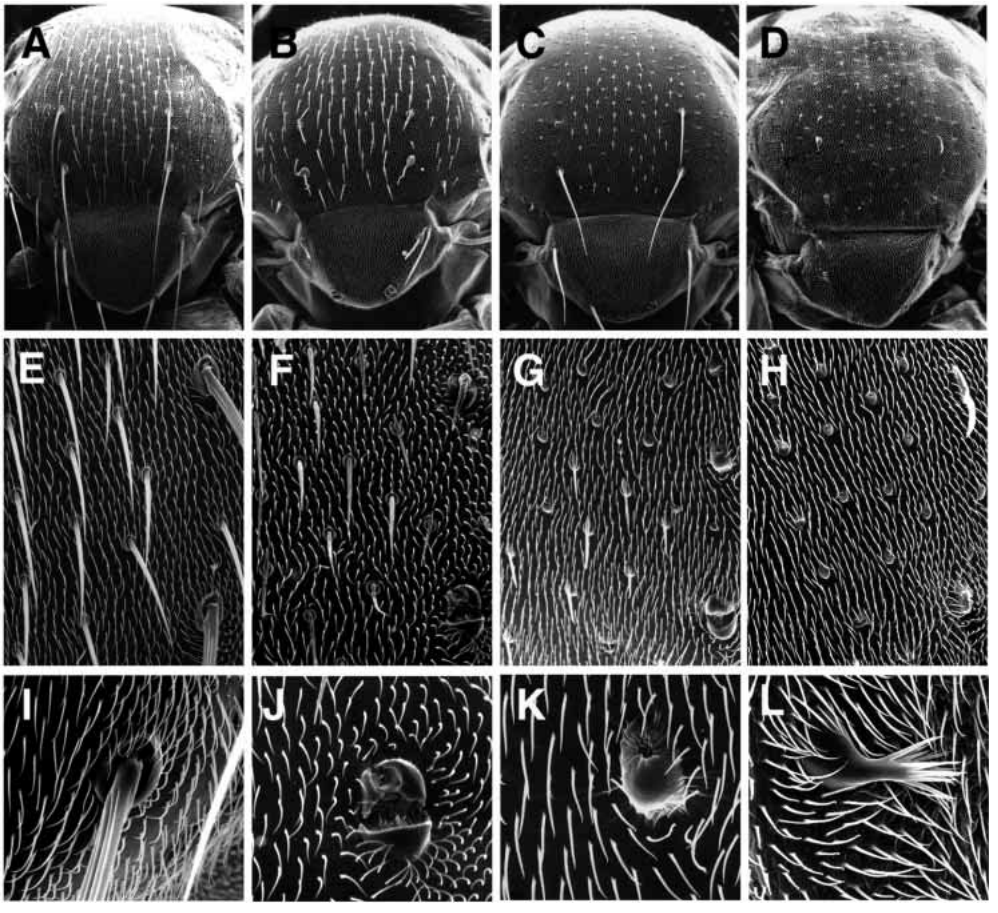

Fig. 2. Phenotypic effects of loss of *D-Pax2* function. Increasingly severe failure of bristle shaft differentiation is depicted in scanning electron micrographs of adult or pharate adult nota of w^{1118}; sv^n (B,F,J), w^{1118}; sv^{de} (C,G,K), and w^{1118}; sv^6 (D,H,L) flies in comparison to wild-type (w^{1118}) nota (A,E,I). Anterior is toward the top. (A-D) Full dorsal thorax; (E-H) closer view of the dorsocentral region of the notum; (I-L) various macrochaete structures from this region at higher magnification. Of the new *D-Pax2* mutant alleles described here, the phenotype of only sv^6 is shown; however, sv^7, sv^8, and the various transheterozygous combinations of these three all exhibit external adult phenotypes that closely resemble those of sv^6 homozygotes.

none of the mutations are null for all functions of the *D-Pax2* locus.

Expression of D-Pax2 protein in developing sensory organs

We used an anti-D-Pax2 antiserum (Fu and Noll, 1997) to examine the temporal and spatial pattern of D-Pax2 protein accumulation in the pupal notum during microchaete development (Fig. 3). D-Pax2 expression is first apparent in the SOP nucleus before division (14 hours after puparium formation (APF); Fig. 3A). After SOP division (16 hours APF), D-Pax2 is present at similar levels in the nuclei of the two daughter cells, the secondary precursors pIIA and pIIB (Fig. 3B). Following the completion of the pIIA and pIIB divisions (18 hours APF), all four cells of the microchaete lineage express D-Pax2 at fairly comparable levels, though one cell (the presumptive trichogen; see below) is regularly distinguishable at this stage by its slightly elevated accumulation of the protein (Fig. 3C). Subsequently, the pattern of D-Pax2 expression is refined (Fig. 3D-G) so that by 32 hours APF, D-Pax2 protein is present in only two cells, one containing a large polyploid nucleus (either the trichogen or the tormogen; Hartenstein and Posakony, 1989) and one containing a small nucleus (either the neuron or thecogen) (Fig. 3G). By double-labeling nota with anti-D-Pax2 antibody (brown) and the neuron/shaft marker mAb 22C10 (blue) (Hartenstein and Posakony, 1989; Zipursky et al., 1984), we were able to identify unambiguously the two cells that express D-Pax2 at 32 hours APF (Fig. 3H,I). We find that microchaete neurons (clearly identifiable by their 22C10-positive cell bodies and axons) are D-Pax2-negative, but are positioned adjacent to small, D-Pax2-positive nuclei in 22C10-negative cells (Fig. 3H), which by inference are thecogens. This interpretation is confirmed by a second double-labeling experiment using anti-Elav (a specific nuclear marker for post-mitotic neurons; O'Neill et al., 1994; Robinow and White, 1988) and anti-D-Pax2 antibodies at 32 hours APF. In both macrochaetes (Fig. 3H, inset) and microchaetes (not shown), the small nucleus that labels with anti-D-Pax2 (green) is clearly distinct from the small nucleus that labels with anti-Elav (red). We also observe that microchaete and macrochaete trichogen cells, definitively identified by the presence of 22C10 reactivity in the shaft structure (Hartenstein and Posakony, 1989), encompass large D-Pax2-positive nuclei (Fig. 3I). Thus, the two bristle cells that exhibit specific nuclear expression of D-Pax2 at 32 hours APF are the thecogen and trichogen.

These results (summarized in Fig. 1) distinguish two phases of D-Pax2 expression in the bristle lineage: an early stage, in which the protein appears at similar levels in the SOP and all of its progeny as cell division proceeds (light shading); and a late stage, in which D-Pax2 expression is restricted to the postmitotic trichogen (shaft) and thecogen (sheath) cells (dark shading).

Since the earliest D-Pax2 expression in the bristle lineage is observed in the SOP itself, we were interested in obtaining a better definition of the time at which the SOP acquires D-Pax2 positivity. Wing imaginal discs from late third-instar larvae and 0-, 2-, and 4-hour APF pupae were examined for expression of an early SOP marker (the *neuralized* enhancer trap insertion A101; Bellen et al., 1989; Huang et al., 1991; Price et al., 1993) and of D-Pax2 protein (Fig. 4). We found that, in late third-instar discs with virtually complete A101 SOP patterns, D-Pax2 is expressed in only a small number of SOPs corresponding to the earliest-developing sensory organs (Fig. 4A; Huang et al., 1991). These include the pDC, posterior scutellar (pSC), and anterior postalar (aPA) macrochaete positions, as well as the L3-2 and giant sensillum of the radius (GSR) campaniform sensilla sites (Huang et al., 1991). At this stage, only the SOPs corresponding to the distalmost chemosensory organs of the anterior wing margin show any D-Pax2 positivity (Fig. 4A), consistent with the distal-to-proximal progression by which these precursors acquire A101 expression. As wing disc development progresses in early pupae, additional SOPs acquire D-Pax2 expression as they mature (Fig. 4B,C), in a pattern that correlates well with the order in which they express A101. By 4 hours APF, all A101-positive SOPs are also D-Pax2-positive (Fig. 4D). These observations establish that D-Pax2 protein accumulates to detectable levels relatively late during SOP development, just before the division of the SOP.

Finally, we examined D-Pax2 protein expression in pupal nota from *D-Pax2* mutants (Fig. 5). Both sv^8 homozygotes (data not shown) and sv^7/sv^8 transheterozygotes show apparently normal D-Pax2 accumulation in the microchaete field at 20 hours APF, relatively soon after the completion of the bristle cell divisions (Fig. 5A,B). However, by 32 hours APF, D-Pax2 protein is present in these mutants at greatly reduced levels in all four cells (Fig. 5D,E). sv^6 homozygotes already exhibit very low levels of D-Pax2 protein at 20 hours APF (Fig. 5C), and none is detectable by 32 hours APF (Fig. 5F).

Bristle cell development in *D-Pax2* mutants

Various markers capable of distinguishing the cells of the bristle lineage were used to investigate microchaete development in pupal nota of *D-Pax2* mutants (Fig. 6). In particular, we were interested in determining the cellular basis of the uniform loss of shaft structures observed in strong *sv* genotypes. Although sv^6, sv^7 and sv^8 exhibit identical external phenotypes, sv^6 mutants accumulate the lowest levels of D-Pax2 protein in developing microchaetes (see Fig. 5). Therefore, nota from sv^6 homozygotes were analyzed with the antibody reagents mAb 22C10 and anti-BarH1 (see below). However, for analyses involving the enhancer trap markers A101 and A1-2-29, we used nota from sv^7/sv^8 transheterozygotes, because this allelic combination gives the best survival to early pupal stages in these enhancer trap backgrounds.

To investigate whether the shaft loss phenotype results from the loss of the trichogen cell (e.g., a defect in the bristle lineage, or the death of the trichogen), we again made use of the A101 enhancer trap marker. A101 drives β-galactosidase expression in the nucleus of the SOP (see Fig. 4), its two daughter cells, and the four terminal cells of the bristle organ (Bellen et al., 1989; Huang et al., 1991). We find that pupal notum A101 expression in a strong *D-Pax2* loss-of-function background (sv^7/sv^8) is apparently unchanged from that in a wild-type background (Fig. 6A,E). At 24 hours APF, when all microchaete precursors have completed their divisions, four A101-positive cells are observed at each microchaete position in the mutant (Fig. 6E), though adult nota of the same genotype display almost no microchaete shafts (see for comparison Fig.

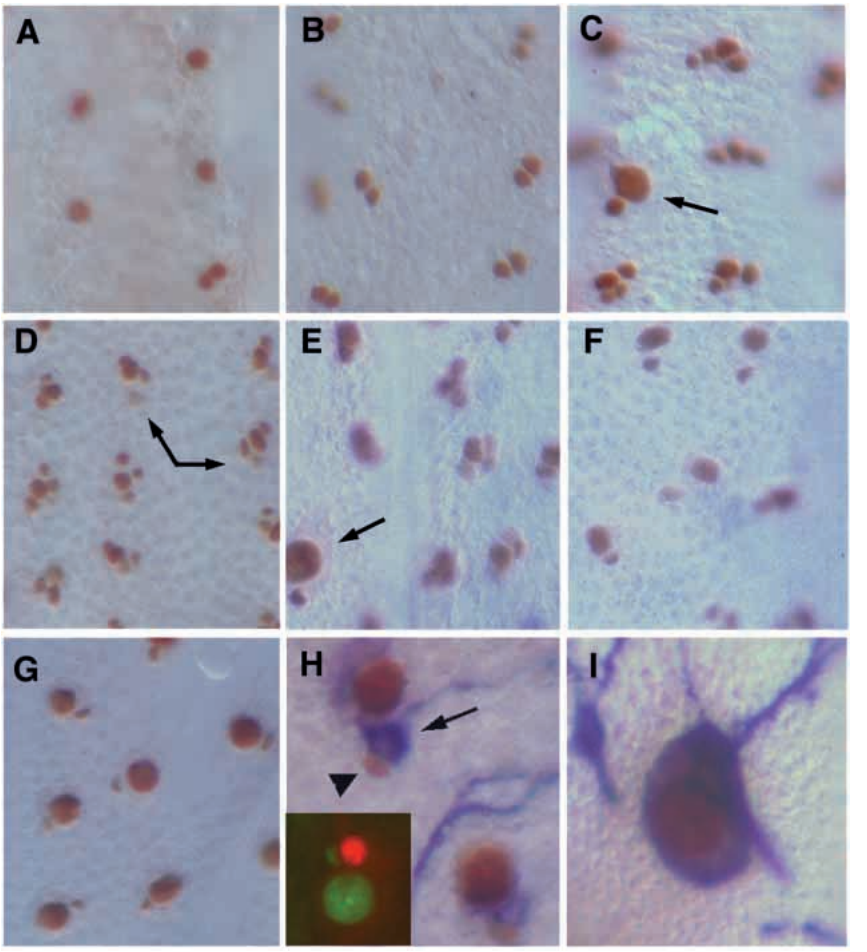

Fig. 3. Pattern of D-Pax2 protein accumulation in pupal nota. (A-G) Time course of D-Pax2 expression during early pupal development; (H,I) cell-type specificity of late D-Pax2 expression. Wild-type (w^{1118}) pupae were dissected at various times after puparium formation (APF); nota were incubated with anti-D-Pax2 primary antiserum, followed by an HRP-conjugated (brown staining in A-I) or a fluorescent (green in H inset) secondary reagent. (A-C) Early (mitotic phase) D-Pax2 expression pattern in developing microchaetes at (A) 14 hours APF, (B) 16 hours APF, and (C) 18 hours APF. (D-G) Progressive refinement to the late pattern of D-Pax2 expression in post-mitotic microchaetes at (D) 20 hours APF, (E) 24 hours APF, (F) 28 hours APF, and (G) 32 hours APF. Nota in H and I (32 hours APF) were also incubated with mAb 22C10 (blue color) to identify the neuron and shaft cell (Hartenstein and Posakony, 1989); macrochaete shown in inset to H is from a notum incubated at 32 hours APF with both anti-D-Pax2 (green) and anti-Elav (red, a specific nuclear marker for the neuron). Arrows in C and E point to earlier-developing macrochaetes that as expected have already fully resolved to the late pattern of expression. Joined arrows in D point to tormogen cells in which the loss of D-Pax2 expression is already quite apparent. Arrowhead in H points to the thecogen nucleus; arrow points to the neuron cell body.

2D,H). Moreover, we have found that four A101-positive microchaete cells remain present at least as late as 32 hours APF (data not shown). These results suggest that microchaete SOPs execute a normal series of divisions in *D-Pax2* mutants, and indicate that the *sv* shaft loss phenotype is not due to a reduction in the number of SOP progeny cells.

To assess the status of the trichogen cell following the precursor divisions and during the differentiative phase, we made use of the enhancer trap marker A1-2-29 (Bier et al., 1989). A1-2-29 expresses β-galactosidase exclusively in the tormogen and trichogen cell nuclei (Fig. 6B; Hartenstein and Posakony, 1990), and is a relatively late marker for these cells. Its expression begins well after the bristle cell divisions have occurred (in the microchaete lineage, at approximately 28-30 hours APF). We found that at 32 hours APF, expression of A1-2-29 at microchaete positions in sv^{7}/sv^{8} nota is consistently different from that in wild-type nota (Fig. 6B,F). At most

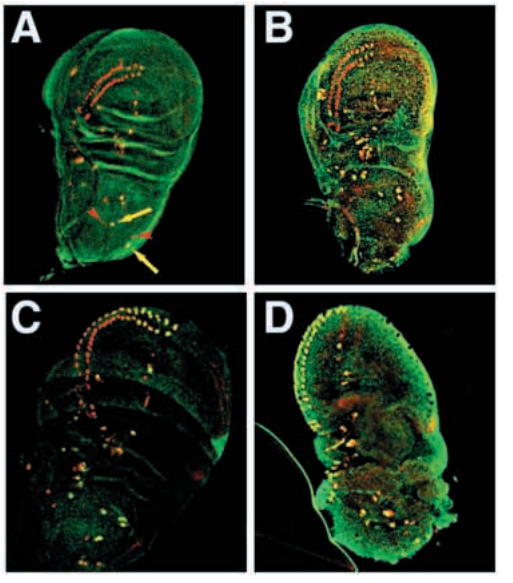

Fig. 4. D-Pax2 protein accumulates late in SOP development. Wing imaginal discs (genotype w^{1118}*; A101/+*) from (A) late third-instar larvae, (B) white prepupae, (C) pupae at 2 hours APF, (D) pupae at 4 hours APF were dissected and double-labeled with anti-β-galactosidase (to detect A101 expression, red) and anti-D-Pax2 (green) primary antibodies, followed by fluorescent secondary antisera. Nuclei of cells expressing both A101 and D-Pax2 are yellow. By late third instar (A), the A101 SOP pattern is virtually complete (Huang et al., 1991), but D-Pax2 is detectably expressed in the SOPs of only the earliest-developing sensory organs. For example, D-Pax2 is clearly present in the posterior dorsocentral and posterior scutellar macrochaete SOPs (yellow arrows in A) but is absent from the later-emerging anterior dorsocentral and anterior scutellar macrochaete SOPs (red arrowheads). As disc development proceeds (B-D), D-Pax2 appears in increasing numbers of SOPs until its expression pattern completely overlaps the A101 pattern (D).

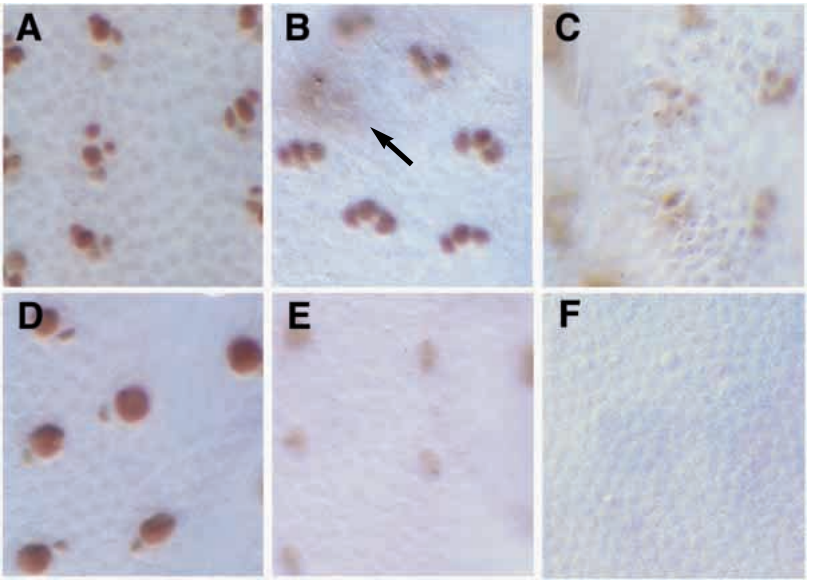

Fig. 5. Expression of D-Pax2 protein in microchaete cells of *D-Pax2* mutants. Pupal nota of (A,D) wild type (w^{1118}), (B,E) w^{1118}; sv^7/sv^8, (C,F) w^{1118}; sv^6, stained with anti-D-Pax2 antiserum. (A-C) 20 hours APF, (D-F) 32 hours APF. A relatively normal early pattern of D-Pax2 protein accumulation is preserved in sv^7/sv^8 (B) but not in sv^6 (C), while the late stage of D-Pax2 expression is markedly reduced or absent in sv^7/sv^8 (E) and sv^6 (F). Arrow in B points to a macrochaete within the field of microchaetes that similarly shows loss of late D-Pax2 expression.

positions, only one cell is clearly labeled by the anti-β-galactosidase antibody (Fig. 6F). Occasionally, an adjacent cell exhibits moderate staining, which may correspond to the few stunted bristle shafts that appear on sv^7/sv^8 adult nota. Because A101 marks four cells in sv^7/sv^8 nota, the presence of only one A1-2-29-positive cell is not due to the loss of the trichogen or tormogen. Instead, one of these cells (which we deduce to be the trichogen, given the relatively normal morphology of *D-Pax2* mutant sockets; see Fig. 2) fails to express properly a late marker normally associated with it. This observation suggests that the *D-Pax2* shaft loss defect results from a failure of normal trichogen cell differentiation.

The defects in shaft morphogenesis in *D-Pax2* mutants, along with the expression pattern of D-Pax2 protein, point to an essential role for *D-Pax2* function in the trichogen cell. However, D-Pax2 is also expressed strongly in the thecogen cell. To assess the status of this cell in *D-Pax2* mutant genotypes, we made use of the S12 antiserum, which is directed against the BarH1 homeodomain protein (Higashijima et al., 1992). In wild-type microchaetes at 32 hours APF, S12 labels the small thecogen cell nucleus strongly and gives weak nuclear staining of the three other cells of the lineage (Fig. 6C). We find that in sv^6 mutants, a single small nucleus is likewise stained strongly by the anti-BarH1 antiserum (Fig. 6G). In addition, one or more of the other nuclei is labeled more intensely than in wild type. We have not identified which cell this corresponds to, but from its size it is almost certainly the tormogen or trichogen. In contrast to what is observed uniformly in the microchaete field, scutellar macrochaete positions occasionally lack strongly labeled BarH1-positive nuclei, possibly indicating a defect in the specification or differentiation of the thecogen cell. Nevertheless, our results overall show that

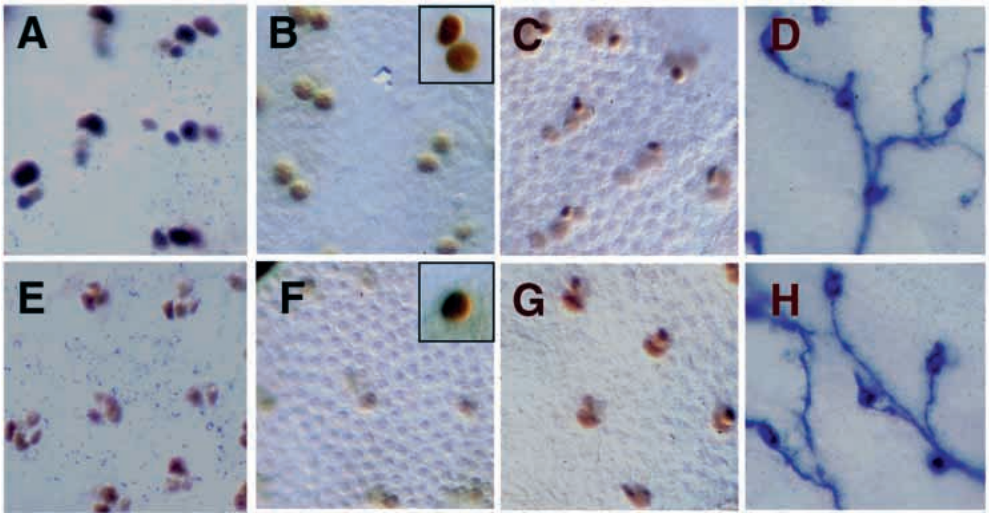

Fig. 6. Bristle cell development in *D-Pax2* mutants. Wild-type (A-D) and *D-Pax2* mutant (E-H) pupal nota were examined for expression of various bristle cell identity markers in developing microchaetes. (A,E) In 24 hour APF pupal nota of w^{1118}; *A101/TM3* (A) and w^{1118}; *A101/TM3;* sv^7/sv^8 (E), expression of A101 (all cells of the lineage) was detected with anti-β-galactosidase (brown); these preparations were also labeled with anti-D-Pax2 (blue). Only *D-Pax2*$^+$ nota show significant expression of D-Pax2 at this time (see Fig. 5). (B,F) In 32 hour APF pupal nota of w^{1118}; *A1-2-29/A1-2-29* (B) and w^{1118}; *A1-2-29/A1-2-29;* sv^7/sv^8 (F), expression of A1-2-29 (trichogen and tormogen) was detected with anti-β-galactosidase (brown). Insets show closer views of representative microchaete positions. (C,G) In 32 hour APF pupal nota of wild-type (w^{1118}) (C) and w^{1118}; sv^6 (G), expression of BarH1 protein (thecogen) was detected with the S12 antiserum. (D,H) In 32 hour APF pupal nota of wild-type (w^{1118}) (D) and w^{1118}; sv^6 (H), the neuron and trichogen are revealed by mAb 22C10.

thecogen cells are generally present and express a normal specific marker in the strong, though not null, *D-Pax2* mutant genotypes we have studied.

Finally, we examined the status of microchaete sensory neurons in sv^6 mutants, using mAb 22C10. Because this reagent marks the neuron's axonal and dendritic projections as well as its soma (Fig. 6D), it is useful for establishing not only the presence of the neuron but also its proper morphogenesis. 22C10 staining of sv^6 pupal nota at 32 hours APF reveals a normal field of microchaete neurons, with axons extended and fasciculated along typical pathways (Fig. 6D,H). As with the anti-BarH1 antiserum, 22C10 detects occasional abnormalities in scutellar macrochaete neurons. The posterior scutellar macrochaete position sometimes shows no 22C10-positive neuron, whereas the anterior scutellar macrochaete often displays two 22C10-stained cell bodies with what appear to be fasciculating axonal projections (data not shown). Again, although loss of *D-Pax2* function can evidently give rise to defects in neuronal specification or differentiation, the vast majority of bristle sensory neurons appear normal in the genotypes we have studied.

Lack of late D-Pax2 expression in the tormogen cell is controlled by Notch pathway activity

We have described above our observation that D-Pax2 protein is expressed strongly in the trichogen cell at 32 hours APF, while it is undetectable in the tormogen cell at the same stage. Given the essential role of the N cell-cell signaling pathway in the trichogen/tormogen cell fate decision, we were interested in determining the relationship between the activity of this pathway and the extreme asymmetry in late D-Pax2 expression in these two cells. For this purpose we took advantage of the strong sensitivity of the trichogen and tormogen cell fates to the level of activity of *H* (Bang et al., 1991; Bang and Posakony, 1992), a key negative regulator of the N pathway (Bang et al., 1995; Bang and Posakony, 1992; Brou et al., 1994; Lyman and Yedvobnick, 1995). We examined the pattern of D-Pax2 protein accumulation in genetic backgrounds representing either a loss or a gain of *H* function (Fig. 7). In H^{21} homozygous adults, almost all microchaete bristles exhibit a 'double socket' phenotype (Fig. 7B) that results from the conversion of the shaft cell to a socket cell fate (Bang et al., 1991), due to inappropriate N pathway activity in that cell (Bang et al., 1995; Lyman and Yedvobnick, 1995). When H^{21} pupal nota are stained by anti-D-Pax2 antibody at 32 hours APF, the small D-Pax2-positive thecogen cell nucleus is evident, but staining is not detected in the converted trichogen cell (Fig. 7E). The converse result is obtained when *H* is misexpressed under the control of a heat-shock promoter early during microchaete differentiation. This treatment inhibits N pathway activity and yields a 'double shaft' phenotype by converting the tormogen cell to a trichogen cell fate (Fig. 7C; Bang and Posakony, 1992). Pupal nota from such heat-treated *Hs-H* transgenic flies show three D-Pax2-expressing cells at 32 hours APF, one with a small nucleus and two with large nuclei (Fig. 7F). We interpret these as the thecogen, the trichogen, and the converted tormogen, respectively. Taken together, our results indicate that the activity of the N signaling pathway controls the absence of late (32 hours APF) D-Pax2 expression in the wild-type tormogen (socket) cell.

Loss of *D-Pax2* function is epistatic to the effect of reduced Notch signaling on the expression of the shaft cell fate

The finding that the activity of the N signaling pathway is an essential determinant of the late pattern of D-Pax2 protein accumulation in the trichogen (D-Pax2$^+$) and tormogen (D-Pax2$^-$) cells prompted us to examine further the functional relationship of the N pathway and *D-Pax2* in the expression of the trichogen cell fate. We again made use of altered *H* activity as a means of modulating N pathway activity in the bristle lineage. As described above, overexpression of *H* after the completion of the microchaete cell divisions results in the expression of the shaft cell fate by both the normal trichogen and the transformed tormogen (see Fig. 7C; Bang and Posakony, 1992); that is, reduction of N pathway function promotes the expression of the shaft differentiation program. Loss of *D-Pax2* function has an oppositely directed effect: the stronger *D-Pax2* mutant genotypes we have studied (sv^{de}, sv^6, sv^7, sv^8, and combinations thereof) cause a broad failure of shaft development (see Fig. 2) while permitting the normal or nearly normal expression of the other bristle cell fates (see Figs 2, 6). We investigated the epistatic relationship between excess *H* activity and loss of *D-Pax2* function by combining these two conditions and observing the effect on shaft differentiation (Fig. 8). We find that homozygosity for even the comparatively weak allele sv^{de} (Fig. 8B; see Fig. 2C,G,K) effectively suppresses the 'double shaft' phenotype of heat-treated *Hs-H* transgenic flies (Fig. 8A,C). Thus, in the combined genotype large territories devoid of external bristle structures are observed (Fig. 8C). We interpret this effect as follows. Excess *H* activity alone causes both the normal trichogen and the transformed 'tormogen' to adopt the shaft fate, but reduction of *D-Pax2* function prevents the expression of this fate by either cell, so no external cuticular structures are produced by the double mutant bristles. In other words, loss of *D-Pax2* activity is epistatic to the effects of reduced N pathway function on the expression of the shaft differentiation program. This result strongly suggests that *D-Pax2* acts at least in part 'downstream' of the N-dependent trichogen/tormogen cell fate decision in the bristle lineage, and plays a major role in the differentiation phase of shaft cell development.

Misexpression of *D-Pax2* results in the development of ectopic shaft structures

It is clear from the results presented thus far that *D-Pax2* function is essential for normal expression of the shaft cell fate. We were interested as well in determining whether *D-Pax2* has the capacity to promote shaft differentiation in a cell in which it is not normally expressed. To address this question, we misexpressed *D-Pax2* using the *GAL4/UAS* system (Brand and Perrimon, 1993). A *UAS-D-Pax2* transgenic fly line (see Materials and Methods) was crossed to various *GAL4* driver lines. Many such combinations result in embryonic lethality; however, two combinations permit survival to the pharate adult stage and proved informative (Fig. 9). *hs-GAL4/UAS-D-Pax2* flies are normal when raised continuously at 25°C. Heat shocks (37°C) as brief as 15 minutes applied to this genotype in early pupal stages (12-24 hours APF) are lethal, usually well before the pharate adult stage. Nevertheless, a small percentage of pupae heat shocked at 24 hours APF develop to pharate adults and can be examined for bristle abnormalities. All of these

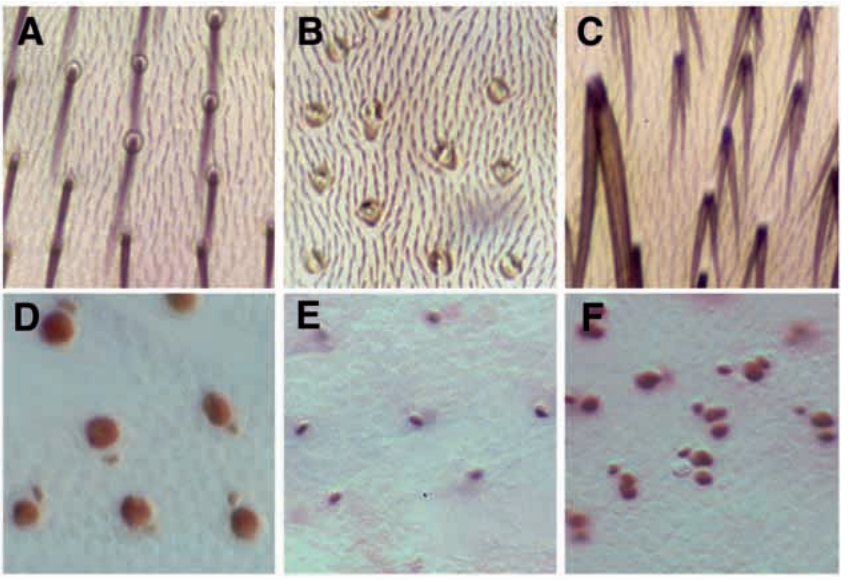

Fig. 7. Changes in D-Pax2 expression in response to altered N signaling in the bristle lineage. (A-C) Light micrographs of cuticle preparations of adult nota; (D-F) D-Pax2 expression in notum microchaetes at 32 hours APF, detected with anti-D-Pax2 antiserum. (A,D) Wild type (w^{1118}); (B,E) H^{21}/H^{21}; (C,F) w^{1118}; $P[w^+, Hs\text{-}H]\text{-}3$. Nota in C and F are from animals subjected to the heat-shock regimen described in Materials and Methods.

animals display some bristles on the notum (Fig. 9A) and head (Fig. 9C) that have extra shaft structures and lack sockets. Most commonly, two smaller shafts are observed flanking a more normal-sized central shaft (Fig. 9C). On the notum, approximately one-third of the microchaetes are affected; on the head, particular macrochaete positions (e.g., the orbital bristles) consistently display this phenotype. MS 1096, an enhancer trap insertion in the *dLMO* gene (Milán et al., 1998), drives GAL4 expression in the dorsal compartment of the wing pouch of the third-instar wing disc (Capdevila and Guerrero, 1994). Using a *UAS-lacZ* reporter gene, we have observed that MS 1096 also drives expression weakly in broad central domains in the pupal notum at 12-24 hours APF, and more strongly in one or two large cells at microchaete positions from 20 to 32 hours APF (data not shown). *MS 1096/+; UAS-D-Pax2/+* animals die as pharate adults, have grossly malformed wings, and exhibit bristle abnormalities. In particular regions of the notum (Fig. 9B) and head (Fig. 9D), bristles with extra shaft structures and lacking sockets are observed. The microchaetes in the rows nearest to the dorsocentral macrochaetes, as well as specific head macrochaete positions, are consistently affected in this way. As with the *hs-GAL4* driver, the microchaetes display a common 'three shaft' phenotype with a full-size shaft flanked by two smaller structures (Fig. 9B).

These results demonstrate the capacity of misexpressed *D-Pax2* to promote ectopic expression of a recognizable shaft differentiation program.

DISCUSSION

In this study we have extended the pioneering work of Lees and Waddington (1942) to show that *D-Pax2*, a member of the family of genes encoding Pax transcription factors (Fu et al., 1998), plays a major role in the normal development of *Drosophila* adult sensory organs. Specifically, *D-Pax2* function is required for the proper differentiation of the trichogen or shaft cell. In strong *D-Pax2* (*sv*) loss-of-function mutants, the trichogen cell arises as usual in the bristle lineage but fails to produce the characteristic shaft structure (this paper; Lees and Waddington, 1942). Our results indicate that *D-Pax2* acts downstream of the N signaling pathway as a high-level transcriptional regulator of the shaft cell differentiation program.

Pax genes have been implicated in a variety of cell fate determination and differentiation processes (see for review Dahl et al., 1997; Noll, 1993). The mouse *Pax2* homolog plays an important role in kidney and CNS development (Burrill et al., 1997; Torres et al., 1995; Urbánek et al., 1997), and is also necessary for normal ear and eye development (Otteson et al., 1998; Torres and Giráldez, 1998). As noted in the Introduction, *sv* corresponds to the same gene as *spa* (Fu et al., 1998), which is necessary for the development of both cone and primary pigment cells in the *Drosophila* eye (Fu and Noll, 1997). Therefore, like its mammalian counterpart, *D-Pax2* is required for the development of multiple sensory systems. In each case, it governs the differentiation and morphogenesis of specific cell types in complex organs.

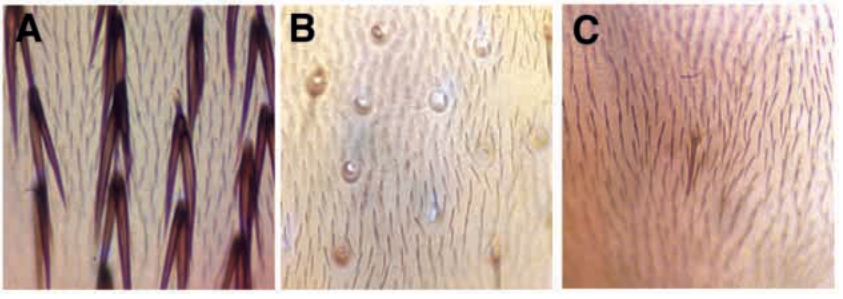

Fig. 8. Epistatic interaction between gain of *H* function and loss of *D-Pax2* function. Shown are adult cuticle preparations from flies of the genotypes w^{1118}; $P[w^+, Hs\text{-}H]\text{-}3$ (A), w^{1118}; sv^{de} (B), and w^{1118}; $P[w^+, Hs\text{-}H]\text{-}3$; sv^{de} (C). In A and C, overexpression of *H* was induced at 24 hours APF via the heat-shock regimen described in Materials and Methods.

D-Pax2 is essential for differentiation of the trichogen (shaft) cell

Several lines of evidence presented here indicate that *D-Pax2* has a critical function in trichogen cell differentiation after cell fate determination in the bristle lineage. First, weaker *D-Pax2* loss-of-function mutants (sv^n, sv^{de}) display reduced, malformed shafts (Lees and Waddington, 1942), while our stronger loss-of-function alleles (sv^6, sv^7, sv^8) cause a complete lack of shaft structures, with only empty sockets remaining (Fig. 2). The finding that these stronger alleles confer a highly expressive shaft loss phenotype distinct from a 'double socket' phenotype (indicative of a trichogen-to-tormogen cell fate transformation; Bang et al., 1991; Lees and Waddington, 1942) allows us to discern a role for *D-Pax2* in shaft differentiation separate from its possible function in trichogen cell fate specification (see below). Secondly, the pattern of D-Pax2 protein accumulation refines from general expression in all four cells during the period of cell division (the time of cell fate specification) to tightly restricted expression in only the postmitotic, differentiating trichogen and thecogen cells (Figs 1, 3). Moreover, we have shown that the late asymmetry in D-Pax2 expression in the trichogen (D-Pax2$^+$) and tormogen (D-Pax2$^-$) is subservient to N pathway-mediated cell fate specification (Fig. 7). When N signaling in the lineage is enhanced by reducing *H* activity, the resulting trichogen-to-tormogen cell fate transformation is accompanied by a loss of late D-Pax2 protein accumulation in the transformed cell. Conversely, when N pathway activity is antagonized by increasing *H* function, the transformation of the tormogen to a second trichogen is mirrored by late D-Pax2 protein accumulation in this cell. Thus, one outcome of the normal N pathway-mediated trichogen/tormogen cell fate decision is the post-mitotic loss of D-Pax2 expression from the tormogen cell, and its continuation at a high level in the trichogen, consistent with a differentiative role for D-Pax2 in the latter cell. Thirdly, loss of *D-Pax2* function is epistatic to the 'double shaft' phenotype resulting from reduced N pathway activity (Fig. 8), again strongly suggesting that *D-Pax2* acts at least in part 'downstream' of the trichogen/tormogen cell fate decision. Finally, the enhancer trap insertion A1-2-29, a specific post-mitotic marker for both trichogen and tormogen cells, is expressed in only a single cell (the tormogen) in the microchaete lineage in *D-Pax2* mutants (Fig. 6). This is in contrast to both wild-type microchaetes and mutant microchaetes in which the trichogen cell has been transformed to a second tormogen (Schweisguth and Posakony, 1994), where two A1-2-29-positive cells are observed. Thus, although the presumptive trichogen cell is present in *D-Pax2* mutants, as demonstrated by its expression of A101, it does not express a late marker normally associated with it. This result is again consistent with our interpretation that *D-Pax2* is essential for trichogen cell differentiation, as distinct from fate specification.

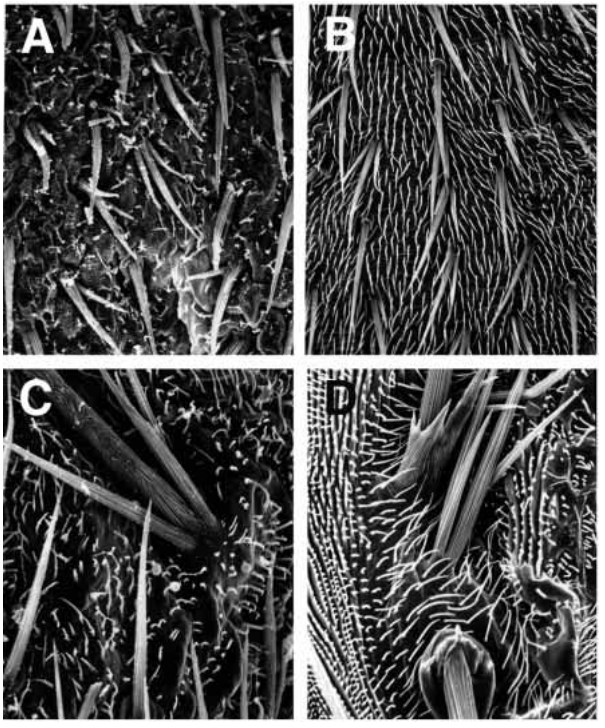

Fig. 9. Induction of ectopic bristle shaft structures by *D-Pax2* misexpression. Shown are scanning electron micrographs of the cuticle of pharate adults bearing one copy of a *UAS-D-Pax2* transgene in combination with one copy of the following GAL4 drivers: (A,C) *hs-GAL4*; (B,D) MS 1096 (see text). Flies bearing the *hs-GAL4* driver were subjected to heat shock (37°C) for 1 hour at 24 hours APF.

D-Pax2 can direct the formation of ectopic shaft structures

Loss-of-function phenotypes show clearly that *D-Pax2* gene function is necessary for normal shaft cell development. Further insight into *D-Pax2*'s place in the shaft differentiation hierarchy can be gained by asking whether mis- or overexpression of *D-Pax2* can promote the production of ectopic shaft structures. We find that both general expression of *UAS-D-Pax2* (driven by *hs-GAL4*) and more specific expression under the control of the MS 1096 driver cause the differentiation of extra shaft structures at the expense of a normal socket structure (Fig. 9). It appears that the 'triple shaft' structures we observe (a larger central shaft flanked by two smaller shafts) consist of a combination of the normal shaft produced by the trichogen and two ectopic shaft structures arising from the 'tormogen' cell. These results indicate that *D-Pax2* is capable of ectopically inducing the program required for elaboration of a bristle shaft structure, and thus evidently occupies a relatively high position in the regulatory hierarchy that controls this process.

Relationship between D-Pax2 expression and Notch signaling

The pattern of late, postmitotic D-Pax2 expression in the adult mechanosensory bristle lineage (in the trichogen and thecogen) is very intriguing in its relationship to N signaling (see Fig. 1). We have shown here that perturbations of N signaling that lead to cell fate symmetry on the trichogen/tormogen side of the lineage (either 'double shaft' or 'double socket' phenotypes) result in appropriate alterations in the late pattern of D-Pax2 protein accumulation (Fig. 7). Similarly, we have recently found that inhibition of N pathway activity sufficient to transform the thecogen cell to a sensory neuron fate leads to a loss of D-Pax2 expression in this cell (J. K., E. C. Lai, and J.

W. P., unpublished observations). Thus, it seems that the pattern of late D-Pax2 expression on both sides of the bristle lineage is dependent, either directly or indirectly, on N signaling. Paradoxically, however, the two cells that express D-Pax2 at the late stage occupy opposite positions with respect to the directionality of N signaling (see Fig. 1; Posakony, 1994). It is the resistance of the presumptive trichogen cell to N signaling which allows it to adopt its fate, whereas it is the responsiveness of the thecogen cell to N signaling that inhibits its adoption of the neural cell fate. Viewed another way, N signaling is evidently responsible for the late expression of D-Pax2 in the thecogen, but for its absence in the tormogen, the corresponding N-responsive cell on the other side of the lineage. How can we explain these oppositely directed responses to the same signal? It is likely that the developmental history of each cell in the lineage helps define how it responds to new signals. For example, the fates of both the thecogen and the tormogen cells depend on their sensitivity to N signaling. However, the thecogen is a daughter of a cell that was resistant to N signaling one generation earlier, whereas the tormogen descends from a cell that was responsive to N signaling. Thus, past regulatory events may influence the manner in which a cell responds to new signals.

Does *D-Pax2* have a role in thecogen development?

We have shown here that D-Pax2 is expressed at a high level in the post-mitotic thecogen cell. Nevertheless, even in the relatively strong *D-Pax2* loss-of-function mutants we have generated, the thecogen generally continues to express normal markers of this cell type, including A101 (Fig. 6A,E) and BarH1 (Fig. 6C,G). Infrequent exceptions are evident at certain macrochaete positions, where anti-BarH1 sometimes fails to detect the thecogen nucleus. Staining with an antibody directed against another nuclear thecogen cell protein, Prospero, produced similar results (data not shown). These findings indicate that the thecogen is not strongly affected by the severe *D-Pax2* loss-of-function conditions we have tested, but it must be noted that we have yet to identify a true *D-Pax2* null allele (see below). If thecogen cell specification and/or differentiation are comparatively resistant to loss of *D-Pax2* function, a null allele may be required to uncover a role for the gene in these processes.

Does *D-Pax2* participate in cell fate specification in the bristle lineage?

While the work presented here defines a clear role for *D-Pax2* in the differentiation of the shaft cell, circumstantial evidence suggests that the gene may also function in sensory organ cell fate determination. First, the early expression pattern of D-Pax2 is certainly consistent with such a role. D-Pax2 protein accumulation is first detectable in the SOP just before its division and is present in all four cells of the lineage during the period when cell fates are determined (Figs 1, 3, 4). Second, certain rare phenotypic effects of the *D-Pax2* loss-of-function genotypes we have examined are reminiscent of those caused by mutations in genes with a clearer role in cell fate specification. For example, 'double sockets' resembling those of *H* hypomorphs, indicative of a trichogen-to-tormogen cell fate transformation (Bang et al., 1991; Lees and Waddington, 1942), are observed infrequently at specific bristle positions in *D-Pax2* mutants (Fig. 2; see Toney and Thompson, 1980). Moreover, quadruple socket structures occur occasionally on the abdomens of sv^6 flies, suggesting that *D-Pax2* may be important in the pIIA/pIIB secondary precursor decision as well as in the trichogen/tormogen decision. Finally, the finding of Lees and Waddington (1942) that sv^n strongly enhances the *H* 'double socket' phenotype is again suggestive of a role for *D-Pax2* in trichogen cell fate specification.

To address this issue definitively, it will be necessary to make use of a null allele of *D-Pax2*. We have been unable to recover such an allele even after several different EMS and P element-mobilization mutagenesis screens. We have been able to generate relatively small chromosomal deficiencies which remove *D-Pax2* and surrounding loci (J. K. and J. W. P., unpublished results; Fu et al., 1998), but these mutations are embryonic lethal. Because *D-Pax2* resides on the fourth chromosome, somatic mosaic analysis with these deletion mutants is difficult at best. Efforts to obtain a null mutation in the locus are continuing, using novel mutagenesis screens.

Regardless of the outcome of these future studies, the strong loss-of-function mutations described here have enabled us to analyze in detail a key role of *D-Pax2* in controlling the execution of the bristle shaft differentiation program. From our results, we propose that *D-Pax2* is a high-level transcriptional regulator of this program, and acts downstream of the N pathway as a specific link between cell fate determination and cell differentiation in a sensory organ lineage.

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