Senseless and Daughterless confer neuronal identity to epithelial cells in the Drosophila wing margin

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The basic helix-loop-helix (bHLH) proneural proteins Achaete and Scute cooperate with the class I bHLH protein Daughterless to specify the precursors of most sensory bristles in Drosophila. However, the mechanosensory bristles at the Drosophila wing margin have been reported to be unaffected by mutations that remove Achaete and Scute function. Indeed, the proneural gene(s) for these organs is not known. Here, we show that the zinc-finger transcription factor Senseless, together with Daughterless, plays the proneural role for the wing margin mechanosensory precursors, whereas Achaete and Scute are required for the survival of the mechanosensory neuron and support cells in these lineages. We provide evidence that Senseless and Daughterless physically interact and synergize in vivo and in transcription assays. Gain-of-function studies indicate that Senseless and Daughterless are sufficient to generate thoracic sensory organs (SOs) in the absence of achaete-scute gene complex function. However, analysis of senseless loss-of-function clones in the thorax implicates Senseless not in the primary SO precursor (pI) selection, but in the specification of pI progeny. Therefore, although Senseless and bHLH proneural proteins are employed during the development of all Drosophila bristles, they play fundamentally different roles in different subtypes of these organs. Our data indicate that transcription factors other than bHLH proteins can also perform the proneural function in the Drosophila peripheral nervous system.

KEY WORDS: Neurogenesis, Proneural genes, PNS, Sensory organs, bHLH proteins

INTRODUCTION
The body of an adult Drosophila is decorated with hundreds of sensory organs (SOs) that allow the animal to process information from the environment. The development of these organs has served as a model system to identify and characterize novel molecular players required for neurogenesis (Jan and Jan, 1993). Most organs perform a mechanosensory function, including the SOs on the thorax (macro- and microchaetae), and those with stout and slender bristles at the anterior wing margin (AWM). Others serve a chemosensory function, like the organs with recurved bristles at the AWM (Hartenstein and Posakony, 1989). Each mechanosensory organ is composed of a shaft and a socket cell visible from the outside, and a neuron and a sheath cell underneath the cuticle. The development of the adult SOs can be roughly divided into the following steps (Hartenstein and Posakony, 1989; Jan and Jan, 1993). The potential to become a sensory precursor is first refined from a ‘proneural cluster’ (Cubas et al., 1991; Skeath and Carroll, 1991) – a group of cells expressing low levels of proneural proteins – to a primary SO precursor (pI), which expresses high levels of proneural proteins. This process of pI selection is mediated via Notch-mediated lateral inhibition (Heitzler and Simpson, 1991; Lai, 2004; Schweisguth, 2004; Simpson, 1990), and the resulting accumulation of proneural proteins specifies pI cells as neural precursors. Each pI then undergoes an asymmetric division to generate a pIIa and a pIIb. The pIIa will further divide to generate the shaft and socket cells. The pIIb will give rise to a glial cell, which migrates away and undergoes apoptosis in some lineages (Gho et al., 1999), and the pIIb, which will divide asymmetrically to generate neuron and sheath cells (Posakony, 1994). Finally, these cells differentiate to form the adult SO.

The neural commitment of pIs results from the function of evolutionarily conserved proneural genes. All known proneural genes encode bHLH-type transcription factors, which are expressed before and during pI specification, and are necessary and sufficient to generate SOs in the ectoderm (Cubas et al., 1991; Ghysen and Dambly-Chaudiere, 1989; Romani et al., 1989; Skeath and Carroll, 1991). Tissue-specific bHLH proneural proteins like Achaete (Ac) and Scute (Sc) (Villares and Cabrera, 1987) form functional heterodimers with the ubiquitously expressed bHLH protein Daughterless (Da) (Caudy et al., 1988), and bind E-box sequences in target enhancers to activate transcription (Murre et al., 1989). As the expression of Ac and Sc usually stops before pIs undergo asymmetric divisions (Modolell, 1997), it is thought that, by activating the expression of a host of ‘neural-specific genes’ in pI cells, proneural genes coordinate the genetic program that governs the entire SO development (Reeves and Posakony, 2005). One of these targets is senseless (sens), which encodes a zinc-finger transcription factor (Jafar-Nejad et al., 2003; Nolo et al., 2000). Loss and gain of sens function result in loss and gain of SOs in flies (Nolo et al., 2000). Sens functions as a binary switch during the selection of the AWM chemosensory pIs (Jafar-Nejad et al., 2003). Specifically, in proneural clusters that will give rise to chemosensory pIs, low levels of Sens repress ac and sc expression in epidermal cells, but high levels activate proneural gene expression in presumptive pIs and thereby contribute to the selection and specification of sensory precursors. However, the molecular mechanism of the loss of mechanosensory bristle subtypes in sens clones is not known.

Ac and Sc are the proneural proteins for the majority of the adult external SOs (Garcia-Bellido and Santamaria, 1978; Rodriguez et al., 1990). However, it has been reported that the
mechanosensory organs of the AWM and the non-innervated bristles of the posterior wing margin (PWM) are not affected by the loss of Ac and Sc function (Garcia-Bellido and Santamaria, 1978; Jack et al., 1991). Here, we report that sens and da provide the pIs of the AWM mechanosensory organs and PWM bristles with neural identity. In addition, we identify a novel role for Ac and Sc in the survival of the mechanosensory pl progeny that is independent of pl selection and specification. Gain-of-function experiments in the thorax indicate that Da and Sens are able to generate ectopic SOs in mitotic clones of the ac-sc complex (ASC). However, unlike in the wing margin (WM), sens function is not required for the selection or specification of the pIs of the thoracic SOs in wild-type animals. Altogether, our data indicate that Sens and the bHLH proteins Ac and Sc serve clearly different functions during the development of mechanosensory organs of the thorax and the WM.

MATERIALS AND METHODS
Fly strains and genotypes
We used the following Drosophila strains in this study: (1) Canton-S, (2) y w (3) sc10-1/Y, (4) w dsh1 FRT19A/FM7, (5) Df1 lsc-B57, snr/FM6 (The Bloomington Stock Center), (7) hs-FLP tub-GAL4 UAS-GFP (Wang and Struhl, 2005), (8) UAS-sensC12, (9) UAS-sensC5, (10) UAS-sensC6, (11) y w; src (sens rescue construct) (Nolo et al., 2000), (12) UAS-Wg::GFP, (13) UAS-CD8::GFP hs-FLP; tub-GAL80 FRT40A; tub-GAL4/TM6B (gift from S. Cohen, EMBL), (14) hmrB sensD FRT80B/TM6B, (15) da+ c13 FRT40A/CyO, (16) Df1 lsc-B57, snr/FM7, (17) Df1 lsc-B57, snr/FM7, (18) hs-FLP tub-GAL80 FRT40A; act-GAL4 UAS-CD8::GFP/CyO, (19) hs-FLP tub-GAL4 UAS-GFP5A; tub-GAL80 M3/67C FRT80B/TM6B, (20) y w Ubx-FLP; ubi-GFP FRT80B, (21) y w hs-FLP; y+ c13 FRT40A/CyO (this study), (22) UAS-P15 (Huh et al., 2004), (23) wgF3 FRT80A/CyO (gift from G. Mandon, Baylor College of Medicine), (24) UAS-da52 (Cadigan et al., 2002), (26) UAS-TCE286 (van de Wetering et al., 1997), (27) Eq-GAL4/TM6B (Pi et al., 2001), (28) sc10-1/Y GAL4 (Friske et al., 1996), (29) C96-GAL4 (Gustafson and Boulianne, 1996), (30) dpp-GAL4 (Staehling-Hampton et al., 1994), and (31) A101-lacZ/TM3, Sb1 (Huang et al., 1991), da+ (Crommier and Cummings, 1993), sensC2 (Nolo et al., 2000), Wg-CX4 (Bejsovec and Wieschaus, 1993) and dsh1 (Perimmon and Mahowald, 1987) are null alleles. All crosses were set at 25°C. To generate mitotic clones either Ubx-FLP was used, which generates rather large clones in the thorax, or hs-FLP, in which case first instar larvae of the desired genotype were heat shocked for 1 hour at 37°C. See Table S1 in the supplementary material for the full genotypes of flies used in clonal analysis.

Immunohistochemistry and imaging
Dissections and antibody staining were performed using standard protocols. The following antibodies were used: rabbit α-Amos (1:2000) (Goulding et al., 2000), rabbit α-Atonal (1:5000) (Jarman et al., 1994), mouse α-bGal (1:1000; Promega), mouse α-Da (1:50) (Crommier and Cummings, 1993), mouse anti-Elav (1:200; DSHB) (Robinow and White, 1991), rabbit α-HRP (1:1000; Jackson Laboratories) (Jan and Jan, 1982), mouse α-P35 (1:1000) (Huh et al., 2004), rabbit α-Prospero (1:1000) (Justice et al., 2003), rabbit α-Sc (1:200) (Skeath and Carroll, 1991), guinea pig α-Sens (1:1000) (Nolo et al., 2000), Rat α-Su(H) (1:2000) (Gho et al., 1996), mouse α-Wg (1:10; DSHB) (Brook and Cohen, 1996), Cy3- and Cy5-conjugated secondary antibodies (1:500; Jackson Laboratories). Images were captured using a LSM510 confocal microscope, processed using Amira 3.1 and Adobe Photoshop 7.0, and assembled using Adobe Illustrator 10.0.

Cell culture, transcription assays and GST pull-down experiments
The S2 cell transfections, luciferase assays and the GST pull down were performed as described previously (Jafar-Nejad et al., 2003).

RESULTS
Ac and Sc are required for normal development of the AWM mechanosensory organs, but not for pl selection
To understand the molecular basis of SO formation at the AWM, we first revisited the WM phenotype in sc10-1/Y mutants, which lose the function of Ac and Sc (Campuzano et al., 1985). Fig. 1A shows part of the AWM of a wild-type male Drosophila, with a dorsal row of spaced chemosensory bristles (arrow) and a medial uninterrupted row of stout mechanosensory bristles (arrowhead). As reported previously, the AWM of sc10-1/Y males lacks chemosensory bristles but still contains mechanosensory bristles (Garcia-Bellido and Santamaria, 1978; Jack et al., 1991) (Fig. 1B). However, quantification of the number of stout bristles indicates a small yet statistically significant reduction in sc10-1/Y compared with wild-type males (Fig. 1E), suggesting a minor role for Ac and Sc in AWM...
mechanosensory bristle formation. As sens has been shown to be expressed in pIs and to genetically interact with proneural genes during the development of the SO precursors (Jafar-Nejad et al., 2003; Nolo et al., 2000; Quan et al., 2004), we tested whether modifying the sens dosage alters bristle number in the AWM. We find that removal of one copy of sens in sc10-1 males results in a very severe decrease in the number of stout bristles (Fig. 1C,E). Conversely, adding an extra genomic copy of wild-type sens restores the number of stout bristles of the sc10-1 flies to near wild-type numbers (Fig. 1D,E). Neither removing nor adding a copy of sens shows a bristle phenotype in an otherwise wild-type background (data not shown). Therefore, our observations indicate an important role for sens during stout bristle formation, and an accessory role for ac and sc. This is different from thoracic SOs, which are completely lost in the absence of ac and sc function.

The stout bristle phenotype of sc10-1 flies could arise from a failure in pI selection and specification, from defective differentiation, or from cell death after pI formation. To distinguish between these alternatives, we stained pupal wings for Sens, which is expressed in the precursors of all SOs examined so far (Frankfort et al., 2004; Jafar-Nejad et al., 2003; Nolo et al., 2000). We first established that Sens marks AWM mechanosensory organ pIs by double-labeling A101-lacZ pupae for Sens and β-Gal. A101-lacZ is an enhancer trap inserted in the neutralized locus that drives lacZ expression in pIs and their progeny (Blair, 1993; Huang et al., 1991). At 2 hours after pupariation formation (2 hours APF), high levels of Sens colocalize with β-Gal in chemosensory organ pIs, and lower levels of Sens are detected in two broad bands that extend along the whole WM (see Fig. S1 in the supplementary material). Around 8-10 hours APF, when the mechanosensory pIs are being specified (Hartenstein and Posakony, 1989), Sens is strongly expressed in these cells, as evidenced by its colocalization with β-Gal (Fig. 1F-F'). At this time point, the chemosensory pIs have already undergone their asymmetric divisions and formed sensory clusters, whose internal cells are marked by Sens at this stage. Staining of pupal wings at 12-14 hours APF with an anti-Sens antibody does not show a significant difference in the number of AWM mechanosensory precursors among wild-type, sc10-1 and sc10-1; sens+/– pupae (Fig. 1G-I). Note that one row of Sens positive cells that are lost in the mutant wings are the chemosensory clusters, which depend on ac and sc. These observations indicate that a defect in pI formation does not account for the sc10-1 stout bristle loss, and that another proneural gene is required for the specification of the stout SOs.

We next examined whether the progeny of mechanosensory pIs are properly formed during pupal development. We stained 24-hour APF pupal wings with antibodies raised against Elav (Robinow and White, 1991) and Su(H) (Gho et al., 1996), which mark neurons and socket cells, respectively. In wild-type pupae, neurons and socket cells of both mecano- and chemosensory organs stain strongly at this stage (Fig. 2A). In contrast to the modest AWM bristle loss in the sc10-1 adults, the number of neurons is severely reduced in sc10-1 pupae (Fig. 2B). In addition, removal of one copy of sens virtually eliminates the neurons that persist in sc10-1 AWM, although a few socket cells are still present in the sc10-1; sens+/– pupae (Fig. 2C,E). This indicates that ac, sc and sens are required for proper pI progeny development at the AWM. Taken together, the data indicate that ac and sc are not required for the selection, specification and division of the mechanosensory pIs at the AWM. However, they contribute to the normal development of the pI progeny.

**Fig. 2. Ac and Sc promote the survival of the AWM mechanosensory lineages.** (A-E) Loss of ac and sc results in a dramatic decrease in the number of AWM mechanosensory neurons. Double-staining of the AWM of (A) y w (wild type), (B) sc10-1/Y, (C) sc10-1/Y; sens+/– and (D) sc10-1/Y; src+/– pupae at 24 hours APF for Su(H) (green) and Elav (red) indicates that the loss of neurons and socket cells in a sc10-1 background is quite sensitive to sens gene dosage. Quantification of the number of mechanosensory neurons in A-D is shown in E. Five wings were analyzed for each genotype. The number of neurons in sc10-1 is less than 10% of wild type, and is significantly different from sc10-1/Y; sens+/– (*, P<0.0001) and sc10-1/Y; src+/– (**P<0.005). Error bars indicate s.e.m. (F-K') Overexpression of the anti-apoptotic protein P35 in the WM rescues the sc10-1 stout SO phenotype. (F-H) Close-up views of the AWM from a sc10-1/Y (F) and a sc10-1/Y; C96-GAL4 UAS-P35+ (H) fly; (G,G'; I,I') wings of 36- to 42-hour APF pupae doubly stained with anti-HRP (green) and anti-Elav (red). Comparison of sc10-1/Y (G,G') and sc10-1/Y; C96-GAL4 UAS-P35+/– (I,I') indicates that neurons undergo apoptosis in sc10-1 flies. Note the extra neurons in the PWM (compare with J). Also, unlike the PWM of a y w (wild-type) wing (J,J'), which is devoid of neurons, a C96-GAL4 UAS-P35+/– wing (K,K') is lined with cells that express neuronal markers.
Ac and Sc suppress apoptosis in the AWM mechanosensory lineages

The sensitivity of the sc^{10.1} AWM phenotype to sens dosage strongly suggests cooperation between Ac, Sc and Sens during mechanosensory lineage development. As sens and its vertebrate homolog growth factor independent 1 (Gfi1) (Zweidler-Mckay et al., 1996) have been shown to prevent apoptosis in several contexts (Chandrasekaran and Beckendorf, 2003; Grimes et al., 1996; Jafari-Nejad and Bellens, 2004; Nolo et al., 2000; Wallis et al., 2003; Yucel et al., 2003), we examined whether blocking apoptosis rescues the sc^{10.1} mechanosensory bristle loss. We find that overexpression of the baculovirus anti-apoptotic protein P35 (Hay et al., 1994) in the wing margin restores sc^{10.1} stout bristles to wild-type numbers (Fig. 2F,H; data not shown). Moreover, the neuronal loss is also efficiently rescued, as evidenced by the staining patterns of Elav and the neuronal membrane marker HRP (Jan and Jan, 1982) (Fig. 2G-I′). These observations indicate that ac and sc, as well as sens, promote the survival of mechanosensory pls or their progeny in the AWM.

While carrying out these experiments, we observed the appearance of several neurons in the PWM of the sc^{10.1} pupae upon P35 overexpression (Fig. 2L′). This is different from the phenotype in wild-type wings, where all SOs and nerves reside in the anterior compartment (Palka et al., 1983) (Fig. 2J′). The adult non-innervated bristles at the PWM do not normally contain a sheath cell, a neuron or a socket cell, although presumptive socket cells are found in early pupae along the PWM (Hartenstein and Posakony, 1989) (data not shown). As shown in Fig. 2K,K′, inhibition of apoptosis in the wing margin of wild-type pupae results in the generation of a large number of neurons at the PWM. These neurons are able to send out axons, which grow along the PWM towards the distal end, where they merge with the marginal nerve that runs along the AWM towards the thorax (Fig. 2K,K′) (Palka et al., 1983). These data indicate that PWM bristles do have the potential to generate neurons and send out axons, but that they are normally non-innervated because the neurons or their precursors undergo apoptosis (Blair, 1992; Lawrence, 1966). It should be noted that as these non-innervated bristles are not lost in sc^{10.1} male flies (data not shown), the pronuclear gene for the PWM bristles is unknown.

sens and da are required for the specification of the AWM mechanosensory and PWM bristle precursors

Our data indicate that rather than playing the proneural role for the AWM mechanosensory organs, Ac and Sc provide a differentiation/survival signal during the development of these bristles. One of the candidates for the AWM proneural gene is asense, another bHLH gene in the ASC that has previously been shown to be required for AWM mechanosensory bristle development (Brand et al., 1993; Dominguez and Campuzano, 1993). To explore if, in the absence of Ac and Sc function, asense can assume a proneural role for the stout bristles, we generated marked mitotic clones of Df(1)260-1/AWM phenotype to dosage sc^{10.1}D (Fig. G-I′). Overexpression of Wg-GFP (green) along the AP boundary (Fig. I,I′) provides the expression of one another in the vertebrate spinal neural tube (Gowan et al., 2001), suggesting that anos and/or atonal might be ectopically expressed upon removal of the ASC and serve as the proneural gene(s) for the AWM mechanosensory organs. We therefore stained mitotic clones of the ASC in the AWM with anti-

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**Fig. 3. sens and da are required for AWM mechanosensory and PWM bristle formation.** (A) A MARCM clone of Df(1)260-1 in the AWM. The mutant bristles are yellow and singed. (B,B′) A MARCM clone of Df(1)260-1 in the wing of an 8- to 10-hour APF pupa stained for Sens (red) and Atonal (green). CD8-GFP (blue) marks the membranes of the mutant cells. Note that the accumulation of Sens in mechanosensory pls is not affected by the loss of the ASC. However, despite the background staining of Ato along the physical margin due to the high gain used in the scans, no Ato staining is detected in mechanosensory pls. (C,D) AWM (C) and PWM (D) bristles are lost in sens clones. The mutant tissue in C is marked with multiple wing hairs (mwh). Note that upon loss of stout bristles, a chemosensory bristle (arrow) is misplaced to the stout row, as reported previously (Couso et al., 1994). (E,F) da^{+} clones lack AWM (E) and PWM (F) bristles. The mutant tissues are marked by crinkled. (G-H′) sens expression is activated by Wingless in the WM. All panels show the central part of the wing pouch in third instar wing imaginal discs. Arrows point to the WM; the dashed line depicts the anteroposterior (AP) boundary of the wing disc; anterior to the left. (G) Sens expression in a y w (wild type) wing disc. (H,H′) Loss of Wg (green) and Sens (red) expression in a Wg^{Cxd} clone. The mutant tissue lacks GFP (blue). Note that even in the heterozygous tissue flanking the mutant clone, Sens expression is decreased, indicating that sens required very high levels of Wingless signaling for proper expression. (I,I′) Overexpression of Wg-GFP (green) along the AP boundary induces Sens (red) expression.
Atonal and anti-Amos antibodies (Jarman et al., 1994; zur Lage et al., 2003). Although Sens is strongly expressed in mutant mechanosensory pIs that lack the ASC, we could not detect Atonal or Amos staining in these cells (Fig. 3B,B; data not shown). We therefore conclude that another gene or set of genes is required to provide the AWM mechanosensory organs with neuronal identity.

Given the strong expression of Sens in AWM mechanosensory pIs, even in ASC clones (Fig. 1F,F’, Fig. 3B), and the role proposed for sens in early steps of pI specification (Jafar-Nejad et al., 2003; Nolo et al., 2000), we examined whether sens is required for WM bristle formation. As shown in Fig. 3C, sens clones in the AWM are devoid of mechanosensory bristles. In addition, the non-innervated bristles of the PWM are lost in the absence of sens function (Fig. 3D), in agreement with our observation that Sens is expressed in wild-type PWM bristle precursors (see Fig. S2 in the supplementary material). Together, the data suggest a proneural role for sens in the specification of the precursors of mechanosensory organs and non-innervated bristles of the WM. To rule out the possibility that loss of WM bristles in the absence of sens function is due to apoptosis, we used the MARCM (Lee et al., 2000) system to overexpress P35 in sens clones, and found that P35 is unable to rescue the neurons and support cells in sens clones (see Fig. S3 in the supplementary material). This shows that loss of Sens cannot be overcome by suppressing cell death in WM mechanosensory lineages and provides further evidence for a proneural role for sens.

Because none of the known tissue-specific bHLH proneural proteins seems to be involved in the specification of these precursors, we investigated whether Da is required for this process. We generated clones of a null da allele and observed that both AWM and PWM bristles are lost in da clones (Fig. 3E,F). These observations provide strong evidence that Da and Sens cooperate to specify the precursors of the WM SOs.

**Wingless (Wg) signaling is necessary and sufficient to induce sens expression in the wing**

In all cases reported so far, sens is a downstream target of Ac and Sc in bristle precursors (Frankfort et al., 2004; Jafar-Nejad et al., 2003; Nolo et al., 2000). However, expression of sens at the AWM and PWM (Fig. 3G) is independent of ac, sc and da function (Jafar-Nejad et al., 2003). It has been suggested that Wg signaling at the WM is responsible for sens expression (Parker et al., 2002). To test this, we stained wing imaginal discs harboring clones of a null wg allele for Wg and Sens, and observed that Sens expression in the WM is lost in wg clones (Fig. 3H,H’). Also, ectopic expression of Wg-GFP along the anteroposterior boundary of the wing imaginal disc results in a broad ectopic domain of sens expression (Fig. 3I,I’). Finally, we find that Sens expression in the WM is lost in mitotic clones of the essential Wg signaling component dishevelled (Klingensmith et al., 1994), and also upon misexpression of a dominant-negative form of the Wg transducer Tcf (van de Wetering et al., 1997) (data not shown). Therefore, in agreement with a previous report (Parker et al., 2002), these observations place sens downstream of Wg in the WM, unlike in other tissues where sens is activated by proneural proteins.

**Sens and Da synergize in vivo and in transcription assays, and physically interact**

It has previously been shown that co-expression of Sens with Ac or Sc results in a synergistic increase in the number of ectopic bristles generated in transgenic flies (Frankfort et al., 2004; Jafar-Nejad et al., 2003; Nolo et al., 2000). As our data indicate that Sens and Da, but not other proneural proteins, are required to specify AWM mechanosensory and PWM non-innervated bristle precursors, we wondered whether a synergistic relationship also exists between Da and Sens. Overexpression of da using the sca-H11032, GAL4 driver generates 17±0.5 extra SOs along the third wing vein (Fig. 4A,D; n=29). A weak UAS-sens transgene produces 7.0±0.5 extra SOs, preferentially of the dome-shaped, campaniform sensilla type, along the third wing vein (Fig. 4B,D; n=25). Co-expression of da and sens generates 63.5±2.1 SOs composed of both bristles and campaniform sensilla in the same region (Fig. 4C,D; n=17). We conclude that sens and da synergize to promote pI formation.

Parallel to their in vivo synergy, Sens and proneural proteins have been shown to synergize in S2 cell transcription assays (Jafar-Nejad et al., 2003). To examine if Sens can transcriptionally synergize with Da, we performed transcription assays in Drosophila S2 cells using an ac-luciferase construct that contains multiple E-boxes and a Sens-binding site as a reporter. As shown in Fig. 4E, while Sens alone does not affect luciferase expression, it significantly increases the expression induced by Da, providing further evidence for synergy between the two proteins. Moreover, GST pull-down experiments indicate that Da and Sens physically interact (Fig. 4F). Altogether, these data support a model in which sens and da cooperate to specify bristle precursor cells in the WM via the transcriptional activation of key target genes.
Overexpression of sens or da in the thorax results in SO formation in the absence of the ASC

Our data indicate that the precursors of the AWM mechanosensory organs and PWM bristles are specified via the function of the sens and da proneural genes. Also, unlike thoracic pIs in which the Ac and Sc proneural proteins directly activate sens transcription (Jafar-Nejad et al., 2003), sens expression in the WM is controlled by Wg. Accordingly, we hypothesized that in precursors of thoracic bristles, the primary task of proneural proteins with respect to providing neural identity to ectodermal cells might be to upregulate Sens, which will then specify the pl fate together with Da. If this hypothesis is correct, one would predict that, if expressed at sufficiently high levels, sens and da should be able to substitute for the function of ac and sc. Indeed, overexpression of da in the thorax using Eq-GAL4 generates numerous bristles in sc10-1 males (Fig. 5A,B). Similarly, driving a strong UAS-sens transgene with Eq-GAL4 can induce multiple bristles in a sc10-1 background, in agreement with a previous report (Pi et al., 2004) (Fig. 5C). Of note, Sens and Da synergize to promote bristle formation even in the absence of ac and sc function: although overexpression of Sens with Eq-GAL4 using a moderate transgene (UAS-sensC6) only generates a few extra SOs in sc10-1 males (Fig. 5D), the number of SOs generated in a sc10-1 background by the co-expression of Da and Sens is much larger than that induced by UAS-da or UAS-sensC6 alone (Fig. 5B,D,E). However, it is possible that overexpression of da or sens in sc10-1 flies induces the expression of ASense, which then substitutes for the function of Ac and Sc. To address this issue, we overexpressed da or sens in MRACM clones of two small deficiencies that lack the ASC, Df(1)260-1 and Df(1)sc-B57 (see Materials and methods). As shown in Fig. 5F, thoracic ASC clones are devoid of bristles. However, overexpression of da (Fig. 5G) or sens (Fig. 5H) results in the formation of bristles in these clones. Staining with anti-Sens antibody (Nolo et al., 2000) indicates that even in the absence of ASC function, overexpression of da is sufficient to induce high levels of Sens in single cells, the presumptive pIs (see Fig. S4 in the supplementary material). Together, these data indicate that not only do Sens and Da endow the WM epidermal cells with neural identity in the wild-type context, but their WM proneural function can also be replicated in the thoracic bristle lineages in overexpression studies.

Overexpression of da is able to induce pl formation in the absence of sens function

We next sought to determine whether sens and da require the function of one another for pl specification in thorax. Analysis of MARCM da clones that overexpress sens indicates that Sens cannot generate bristles in the absence of da function (Fig. 6A). Similarly, no microchaetae are formed in MARCM sens clones in which da is overexpressed, although the cuticle in these clones is abnormal (Fig. 6B,C). These observations suggest that sens and da require the function of one another in order to generate extra bristles in the thorax. However, staining of the clones with the anti-Elav antibody shows that the similarity between the adult phenotypes of sens and da clones – namely, the loss of adult bristles – is misleading: although no Elav+ cells are observed upon overexpression of sens in da clones (data not shown), a large number of neurons are formed in MARCM clones of sens in which da is overexpressed (Fig. 6D-D’). These data indicate that although sens requires da to induce pl formation in overexpression studies, high levels of da can efficiently generate pIs in sens clones. However, these pIs do not generate shaft or socket cells, only extra neurons.

The above observations suggest that sens may be required for proper cell fate determination of the pl progeny but not the pl itself. By contrast, we have previously proposed that sens is involved in specifying microchaetae pIs, based on the severe loss of thoracic bristles in adult sens clones and the high level expression of sens in the pIs of these bristles (Jafar-Nejad et al., 2003). To clarify these discrepancies and further dissect the function of sens in
microchaetae development, we first stained pupae harboring sens clones with an anti-Sc antibody at 12 hours APF, when the single microchaetae pIs are being selected (Hartenstein and Posakony, 1989). As shown in Fig. 7A–A', single cells accumulate Sc in sens clones, providing strong evidence that sens function is not required for microchaetae pI selection. Although these pIs are able to divide, there is a delay in their division compared with wild-type pIs (data not shown). The loss of shaft and socket structures in adult sens clones on the thorax (Nolo et al., 2000) indicates that the sens– pIs develop highly aberrantly. Indeed, the staining of sens clones for Elav and the sheath cell marker Prospero (Justice et al., 2003) shows that sens mutant sensory clusters contain multiple neurons and an occasional sheath cell (Fig. 7B, B'). The mutant neurons are capable of sending out axons, as indicated by rather thick HRP+ extensions that connect the mutant neuronal clusters (Fig. 7C, C'). Also, staining sens clones with an anti-Su(H) antibody shows that, unlike wild-type microchaetae clusters, which contain one neuron and one socket cell, more than 98% of the mutant microchaetae clusters lack a socket cell (Fig. 7D–D'; n=200). Together, these data indicate a gain of neurons in sens mutant sensory clusters at the expense of the support cells, which strongly suggests a pIIa-to-pIIb transformation and also a sheath-to-neuron transformation later in the lineage (Fig. 7E). Moreover, inhibition of apoptosis via overexpression of P35 fails to restore shaft and socket cells in sens mutant tissue.

Fig. 7. sens regulates cell-fate specification in the microchaetae pI progeny. (A–A') Sc expression is restricted to single cells in a sens clone. Shown is a sensE2 clone in a 12-hour APF pupal thorax stained for Sc (red). GFP (blue) marks the wild-type tissue. (B, B') A MARCM clone of sens in the thorax 24–30 hours APF stained for Elav (red) and Prospero (green). Note the multiple neurons and an occasional sheath cell in mutant clusters. (C, C') A MARCM clone of sens in the thorax around 28–30 hours APF, stained for Elav (red) and HRP (green). Because of the presence of several neurons in each cluster, some axonal tracts in the mutant tissue are thicker than their wild-type counterparts. (D–D') Microchaetae pI progeny undergo cell fate transformation in sens clones. A wild-type (left) and a sensE2 (right) sensory cluster is shown at 24–26 hours APF, stained for Elav (red) and Su(H) (green). Note that the mutant cluster contains multiple neurons but no socket cells. (E) Simplified model of the microchaetae lineages in wild-type and sens animals. Note that in addition to the pIIa-to-pIIb transformation, many mutant clusters also exhibit a sheath-to-neuron transformation in the pIIb progeny. so, socket; sf, shaft; st, sheath; n, neuron.
MARCM sens clones, further supporting a fate change (data not shown). In summary, these observations indicate that unlike the WM mechanosensory bristles for which Sens plays a proneural role, in the microchaetae lineage sens is not required for pl selection and specification. However, it does regulate several key aspects of SO development, including proper cell fate determination of the pl progeny.

**DISCUSSION**

In 1978, García-Bellido and Santamaria reported that ac and sc are required for the generation of the majority of the *Drosophila* bristles (García-Bellido and Santamaria, 1978). The large body of work that followed this discovery led to the realization that Ac and Sc are members of the bHLH proneural (Glysen and Dambly-Chaudiere, 1989; Romani et al., 1989) protein family, which are involved in early steps of neurogenesis in flies and vertebrates (Bertrand et al., 2002; Hassan and Bellen, 2000). Later, two other bHLH genes, atonal and amos, were shown to play the proneural role for almost all SOs that did not depend on Ac and Sc function (Goulding et al., 2000; Huang et al., 2000; Jarman et al., 1993; Jarman et al., 1994), with the notable exception of the WM mechanosensory bristles (García-Bellido and Santamaria, 1978; Jack et al., 1991). Here we show, based on multiple lines of evidence, that Sens plays the proneural role for these bristles: sens expression in the WM begins before the selection of mechanosensory pl's in a proneural cluster (see Fig. S1 in the supplementary material), similar to other proneural proteins (Cubas et al., 1991; Skeath and Carroll, 1991); sens expression is upregulated in presumptive pl's and is downregulated in ectodermal cells (Fig. 1, see also Figs S1, S2 in the supplementary material), just like ac and sc expression is refined to pl's in thoracic proneural clusters (Cubas et al., 1991; Skeath and Carroll, 1991); loss and gain of sens function result in loss and gain of SOs in the wing (Figs 3, 4); and Sens synergizes with the Da protein in vivo and in transcription assays, and binds Da in a GST pull-down assay (Fig. 4). Unexpectedly, overexpression of the anti-apoptotic protein P35 in the WM results in the generation of a large number of neurons along the PWM, uncovering the neural identity of the PWM bristle precursors. Similar to the AWM, the expression pattern and loss-of-function phenotype of sens in the PWM indicate a proneural role for sens for the PWM bristles as well. However, the neural potential of the PWM bristles is not realized in the wild-type situation because of apoptosis of the pl progeny, providing an example of the role of apoptotic machinery in diversifying the various sensory lineages, as recently highlighted by Lai and Orgogozo (Lai and Orgogozo, 2004). In summary, Sens satisfies all the genetic and developmental criteria for being a proneural protein for the WM bristles, and is the only zinc finger protein shown to play a proneural role in SO development in flies.

As for other proneural proteins, the proneural function of Sens requires the function of Da. Da serves as the binding partner for the bHLH proneural proteins to bind E-box sequences (Huang et al., 2000; Jarman et al., 1993; Murre et al., 1989) and is also able to bind DNA as homodimers (Jafar-Nejad et al., 2003; Murre et al., 1989). No function has been assigned to Da homodimers in *Drosophila*, largely because of the identification of tissue-specific bHLH proteins in most contexts in which Da functions. In the WM mechanosensory precursors, however, none of the known tissue-specific bHLH proneural proteins is expressed, suggesting a proneural role for Da homodimers. One might argue that there is probably an unknown dimerization partner for Da in these sensory precursors, and we cannot exclude this possibility. However, two groups have independently identified all *Drosophila* genes encoding bHLH proteins using database searches of the complete *Drosophila* genome (Moore et al., 2000; Peyrefitte et al., 2001) and none of the newly identified bHLH proteins are predicted to be a transcriptional activator of the Ac-Sc or Atonal families (Moore et al., 2000). Also, none of these genes shows an embryonic expression pattern compatible with a proneural function for the CNS (Moore et al., 2000; Peyrefitte et al., 2001). Because we find that da is required for mechanosensory organ formation, and as it can efficiently generate bristles in the absence of ASC, we propose that Da homodimers cooperate with Sens to endow neural identity to PWM mechanosensory organs and PWM bristle precursors. The physical interaction of these two proteins and the strong transcriptional synergy between them strongly favors a role in activating key target genes in SO development.

Our data also reveal that Ac and Sc promote the survival of the WM mechanosensory neurons and support cells independently of pl selection. The more severe loss of neurons compared with support cells associated with the loss of Ac and Sc in sc10-1 suggests either that the neurons (or their precursors) are more sensitive to the lack of ac and sc function, or that the loss of support cells is secondary to the neuronal death, as reported previously for another insect (Garcia-Bellido and Santamaria, 1978). The observation that adding or removing one copy of wild-type sens strongly modifies the sensory lineage apoptosis observed in sc10-1 animals indicates that, in addition to a proneural function, Sens also plays an anti-apoptotic role in these cells; this is in agreement with many reports on the role of sens and its homologues in mammals and *C. elegans* in preventing apoptosis (Jafar-Nejad and Bellen, 2004). It is interesting to note that although Ac and Sc are not detected in the PWM by antibody staining (Cubas et al., 1991; Skeath and Carroll, 1991), P35 overexpression rescues many more neurons in the PWM of wild-type flies than in sc10-1 animals (Fig. 2). This indicates a requirement for Ac and Sc in these cells.

During the third instar larval period, low levels of Sens are expressed in the proneural clusters along the AWM that will give rise to the pl cells of the AWM chemosensory bristles. Using in vivo and in vitro assays, we have previously shown that low levels of Sens repress, and high levels of Sens activate, ac and sc expression in these proneural clusters, and thereby that Sens is involved in pl selection (Jafar-Nejad et al., 2003). Given the similar low-level expression of Sens in thoracic microchaetae proneural clusters and the severe loss of microchaetae in adult sens clones, we had hypothesized that Sens also functions during proneural upregulation and in the selection of the microchaetae pl's. We were therefore surprised to find that microchaetae pl selection does not require Sens function. A recent report by Pi and colleagues (Pi et al., 2004) presented data on the function of the adaptor protein Phyllopod and its relationship with Sens in microchaetae development. On the one hand, Sens was shown to be required for the function of Phyllopod in the pl's, as well as for timely downregulation of phyllopod expression in epidermal cells. This suggests a dual role for Sens in pl's and surrounding epidermal cells, in agreement with the binary switch model (Jafar-Nejad et al., 2003). On the other hand, phyllopod expression could still be upregulated in single cells in sens mutant clones, suggesting that pl selection is not disrupted. We now present evidence that microchaetae pl's are indeed selected in sens clones and that they divide to generate progeny. However, the mutant pl's exhibit an abnormal division pattern, and we observe a pIfa-to-pIb transformation, as evidenced by a gain of neurons at the expense of support cells. These data indicate that Sens regulates several aspects of microchaetae precursor development after the pl's are selected.
In summary, the normal development of all adult bristles in flies relies on the function of Ac and Sc, Da and Sens. Our data indicate that despite the structural and functional similarities between various adult bristles, Sens functions at four distinct steps in different lineages (Table 1). First, in the WM mechanoreceptor and non-innervated lineages, very high levels of Wingless induce the expression of Sens, which assumes a true proneural role and specifies SO fate independently of the typical proneural proteins Ac and Sc. Second, in the WM chemo-sensory lineages, for which ac and sc are the proneural genes, Sens is required for pl selection, as it represses proneural gene expression in ectodermal cells and activates proneural gene expression in presumptive pIs (Jafar-Nejad et al., 2003). Third, even though gain-of-function studies show that Sens is able to induce pl formation in the thorax in the absence of Ac and Sc function, it normally plays a later role in specification of the pIIa versus the pIIb of microchaetae lineages. Fourth, Sens is required for the survival of the pl progeny in the WM mechanosensory lineages. We also find that ac and sc prevent apoptosis in this lineage independently of pl specification. Finally, our data suggest that a typical Da heterodimeric complex is not required during the formation of the WM mechanosensory and non-innervated bristle pIs. Hence, the cooperation between the same group of genes is adapted in different ways to ensure the proper development of various SOs.

The Sens homolog Gfi1 plays important roles in several developmental processes, including inner ear hair cell development (Wallis et al., 2003), hematopoietic stem cell self-renewal rate (Hock et al., 2004), intestinal cell fate specification (Shroyer et al., 2005) and neutrophil differentiation (Hock et al., 2003; Karsunky et al., 2004), intestinal cell fate specification (Shroyer et al., 2005) and neutrophil differentiation (Hock et al., 2003; Karsunky et al., 2004), intestinal cell fate specification (Shroyer et al., 2005). Molecular genetics of the achaete-scute gene complex of D. melanogaster. Cell 40, 327-338.


Hock, H., Hamblen, M. J., Rooke, H. M., Traver, D., Bronson, R. T. and Cameron,


