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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 pIIIb, 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

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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 pI progeny that is independent of pI 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 genetics

We used the following *Drosophila* strains in this study: (1) *Canton-S*, (2) *y w* (3) $sc^{10\text{-}1}$, (4) *w* dsh^3 *FRT19A/FM7*, (5) *Df(1)260-1,* y^-*/FM7*, (6) *Df(1)sc-B57,* sn^3*/FM6* (The Bloomington Stock Center), (7) *hs-FLP tub-GAL4 UAS-*GFP^{nls} (Wang and Struhl, 2005), (8) *UAS-*$sens^{C12}$, (9) *UAS-*$sens^{C5}$, (10) *UAS-*$sens^{C6}$, (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)*mwh*^{1} $sens^{E2}$ *FRT80B/TM6B*, (15) da^3 ck^{13} *FRT40A/CyO*, (16) *Df(1)260-1,* y^- sn^3 *FRT19A/FM7*, (17) *Df(1)sc-B57,* sn^3 *FRT19A/FM7*, (18) *hs-FLP tub-GAL80 FRT19A; act-GAL4 UAS-CD8::GFP/CyO*, (19) *hs-FLP tub-GAL4 UAS-*GFP^{nls}*; tub-GAL80 M(3)67C FRT80B/TM6B*, (20) *y w Ubx-FLP; ubi-GFP FRT80B*, (21) *y w hs-FLP;* y^+ ck^{13} *FRT40A/CyO* (this study), (22) *UAS-P35* (Huh et al., 2004), (23) wg^{CX4} *FRT40A/CyO* (gift from G. Mardon, Baylor College of Medicine), (24) *UAS-*da^{45}, (25) *UAS-*da^{52} (Cadigan et al., 2002), (26) *UAS-*TCF^{DN} (van de Wetering et al., 1997), (27) *Eq-GAL4 /TM6B* (Pi et al., 2001), (28) $sca^{109\text{-}68}$*-GAL4* (Frise et al., 1996), (29) *C96-GAL4* (Gustafson and Boulianne, 1996), (30) *dpp-GAL4* (Staehling-Hampton et al., 1994), and (31) *A101-lacZ/TM3,* Sb^1 (Huang et al., 1991). da^3 (Cronmiller and Cummings, 1993), $sens^{E2}$ (Nolo et al., 2000), Wg^{CX4} (Bejsovec and Wieschaus, 1993) and dsh^3 (Perrimon 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-βGal (1:1000; Promega), mouse α-Da (1:50) (Cronmiller 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 pI selection

To understand the molecular basis of SO formation at the AWM, we first revisited the WM phenotype in $sc^{10\text{-}1}$ 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 $sc^{10\text{-}1}$ 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 $sc^{10\text{-}1}$ compared with wild-type males (Fig. 1E), suggesting a minor role for Ac and Sc in AWM

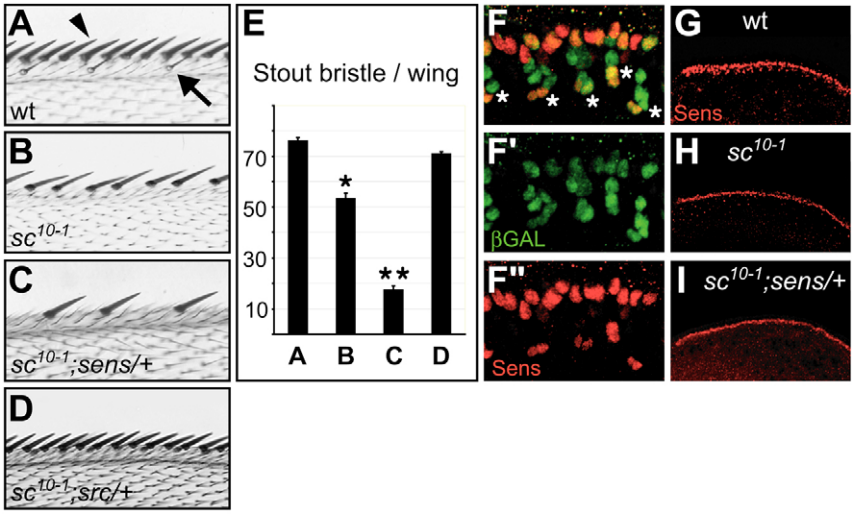

Fig. 1. A requirement for *ac* and *sc* in AWM mechanosensory organ development after pI selection. (**A-E**) *ac*, *sc* and *sens* regulate the number of AWM stout bristles. Close-up views of the AWM of (A) *Canton-S* (wild type, wt), (B) $sc^{10\text{-}1}$/*Y*, (C) $sc^{10\text{-}1}$*/Y;* $sens^{E2}$/+ and (D) $sc^{10\text{-}1}$*/Y; src*/+ are shown. src, *sens* genomic rescue construct; these flies have three copies of the wild-type *sens* gene. Arrow and arrowhead in A point to a chemosensory bristle and a stout bristle, respectively. (E) Quantification of the number of stout bristles in A-D; 10-14 wings were quantified for each genotype. One-way ANOVA with Scheffe error protection indicates that the difference between $sc^{10\text{-}1}$/*Y* and $sc^{10\text{-}1}$*/Y; src*/+ is not statistically significant. Moreover, a *t*-test for independent samples shows that $sc^{10\text{-}1}$/*Y* (*) and $sc^{10\text{-}1}$*/Y;* $sens^{E2}$/+ (**) are significantly different from all of the other genotypes (P<0.0001). Error bars indicate s.e.m. (**F-F″**) Double-staining of the AWM of an 8- to 10-hour APF *A101-lacZ* pupa for β-Gal (green) and Sens (red) indicates colocalization of the two proteins in the mechanosensory pIs and the internal cells of the presumptive chemosensory organs (asterisks in F). (**G-I**) Sens staining of the AWM of (G) *A101-lacZ* (wild type), (H) $sc^{10\text{-}1}$/*Y* and (I) $sc^{10\text{-}1}$*/Y;* $sens^{E2}$/+ pupae at 12-14 hours APF does not show a significant difference in the number of mechanosensory pIs between these genotypes. Note the absence of chemosensory clusters in H and I.

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 $sc^{10\text{-}1}$ 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 $sc^{10\text{-}1}$ 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 $sc^{10\text{-}1}$ 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 *neuralized* locus that drives *lacZ* expression in pIs and their progeny (Blair, 1993; Huang et al., 1991). At 2 hours after puparium 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, $sc^{10\text{-}1}$ and $sc^{10\text{-}1}$*; 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 $sc^{10\text{-}1}$ 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 mechano- and chemosensory organs stain strongly at this stage (Fig. 2A). In contrast to the modest AWM bristle loss in the $sc^{10\text{-}1}$ adults, the number of neurons is severely reduced in $sc^{10\text{-}1}$ pupae (Fig. 2B). In addition, removal of one copy of *sens* virtually eliminates the neurons that persist in $sc^{10\text{-}1}$ AWM, although a few socket cells are still present in the $sc^{10\text{-}1}$*; 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.

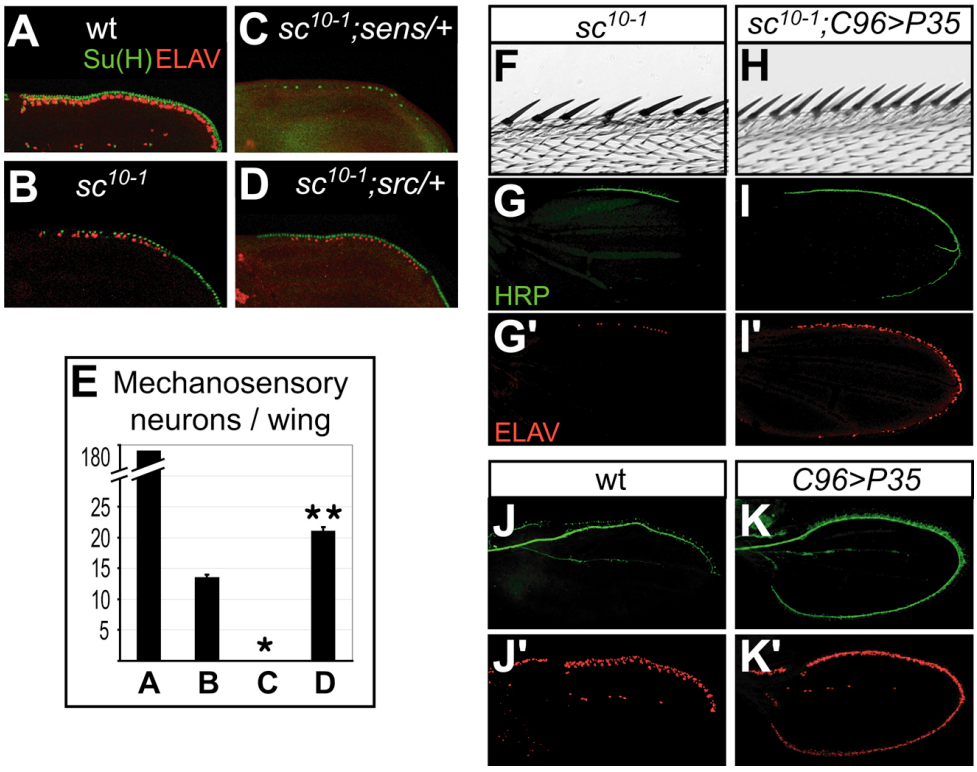

Fig. 2. Ac and Sc promote the survival of the AWM mechanosensory lineages. (**A-E**) Loss of *ac* and *sc* results in a dramatic decrease in the number of AWM mechanosensory neurons. Double-staining of the AWM of (A) *y w* (wild type), (B) $sc^{10\text{-}1}/Y$, (C) $sc^{10\text{-}1}/Y$*; sens*^{E2}/+ and (D) $sc^{10\text{-}1}/Y$*; src*/+ pupae at 24 hours APF for Su(H) (green) and Elav (red) indicates that the loss of neurons and socket cells in a $sc^{10\text{-}1}$ 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 $sc^{10\text{-}1}$ is less than 10% of wild type, and is significantly different from $sc^{10\text{-}1}/Y$*; sens*^{E2}/+ (*P<0.0001) and $sc^{10\text{-}1}/Y$*; src*/+ (**P<0.005). Error bars indicate s.e.m. (**F-K′**) Overexpression of the anti-apoptotic protein P35 in the WM rescues the $sc^{10\text{-}1}$ stout SO phenotype. (F,H) Close-up views of the AWM from a $sc^{10\text{-}1}/Y$ (F) and a $sc^{10\text{-}1}/Y$*; C96-GAL4 UAS-P35*/+ (H) fly; (G,G′,I,I′,J-K′) wings of 36- to 42-hour APF pupae doubly stained with anti-HRP (green) and anti-Elav (red). Comparison of $sc^{10\text{-}1}/Y$ (G,G′) and $sc^{10\text{-}1}/Y$*; C96-GAL4 UAS-P35*/+ (I,I′) indicates that neurons undergo apoptosis in $sc^{10\text{-}1}$ 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 $sc^{10\text{-}1}$ 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; Jafar-Nejad and Bellen, 2004; Nolo et al., 2000; Wallis et al., 2003; Yucel et al., 2003), we examined whether blocking apoptosis rescues the $sc^{10\text{-}1}$ mechanosensory bristle loss. We find that overexpression of the baculovirus anti-apoptotic protein P35 (Hay et al., 1994) in the wing margin restores $sc^{10\text{-}1}$ 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 pIs or their progeny in the AWM.

While carrying out these experiments, we observed the appearance of several neurons in the PWM of the $sc^{10\text{-}1}$ pupae upon P35 overexpression (Fig. 2I,I′). 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,J′). 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 $sc^{10\text{-}1}$ male flies (data not shown), the proneural 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 deficiencies that remove the whole *ASC* (*ac*, *sc*, *lethal of scute* and *asense*) and examined the AWM bristles. As shown in Fig. 3A, lack of the *ASC* is compatible with bristle formation, although many of the mutant bristles show abnormal morphology. Although this observation is in agreement with previous data on the role of *asense* in SO differentiation, it precludes *asense* from substituting for the proneural role of *ac* and *sc* in the AWM.

The other known tissue-specific bHLH proneural genes, *atonal* and *amos*, are not normally expressed in the wing margin (Goulding et al., 2000; Jarman et al., 1993). However, it has been shown that bHLH proteins can repress the expression of one another in the vertebrate spinal neural tube (Gowan et al., 2001), suggesting that *amos* 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-

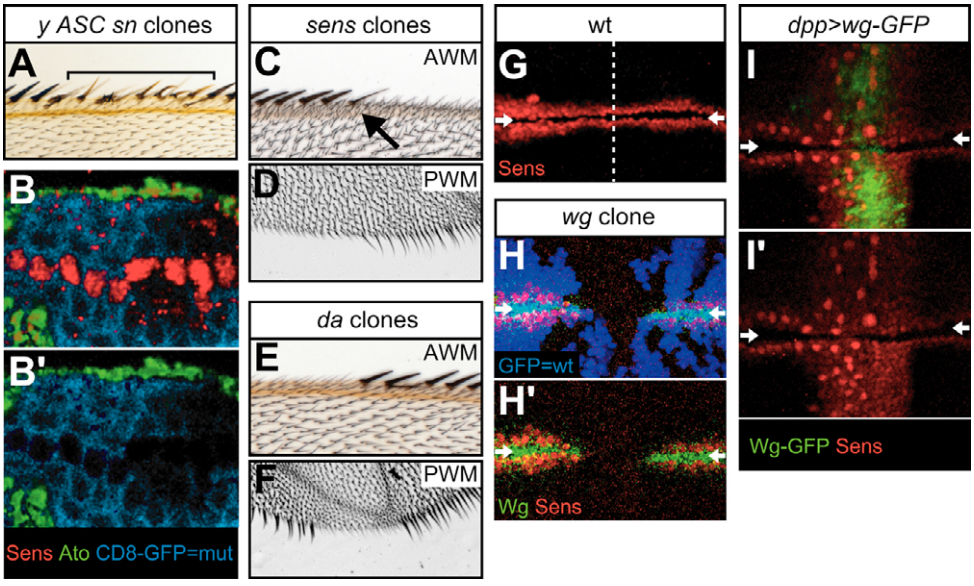

Fig. 3. *sens* and *da* are required for AWM mechanosensory and PWM bristle formation. (**A**) A MARCM clone of *Df(1)260-1* in the AWM. The mutant bristles are *yellow* and *singed*. (**B**,**B′**) A MARCM clone of *Df(1)260-1* in the wing of an 8- to 10-hour APF pupa stained for Sens (red) and Atonal (green). CD8-GFP (blue) marks the membranes of the mutant cells. Note that the accumulation of Sens in mechanosensory pIs 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 pIs. (**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^3 clones lack AWM (E) and PWM (F) bristles. The mutant tissues are marked by *crinkled*. (**G-I′**) *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^{CX4} 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* requires 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$^{109\text{-}68}$-GAL4* driver generates 17.1±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.

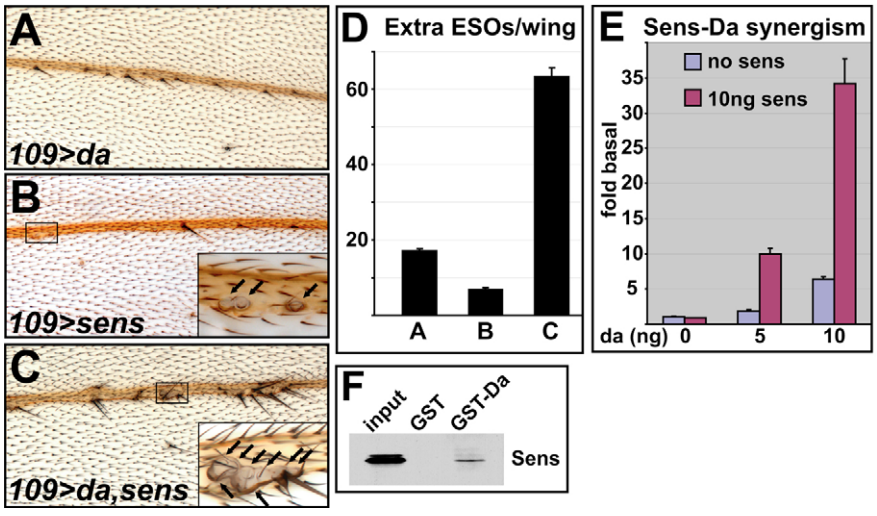

Fig. 4. Da and Sens synergize in vitro and in vivo, and bind to each other. (**A-D**) Co-expression of Da and Sens in the wing results in a synergistic increase in the number of ectopic SOs generated. The third wing vein region of (A) *sca$^{109\text{-}68}$-GAL4/UAS-da^{52}*, (B) *sca$^{109\text{-}68}$-GAL4 UAS-sens^{C12}* and (C) *sca$^{109\text{-}68}$-GAL4 UAS-sens^{C12}/UAS-da^{52}* flies are shown. In D, the number of extra SOs are quantified for each genotype ($n\geqslant17$). Arrows in insets indicate the extra campaniform (dome-shaped) sensilla. (**E**) Sens strongly increases the level of *ac-luciferase* transcription induced by Da. (**F**) In vitro-translated Sens can be pulled-down using GST-Da, but not with GST alone. Western blotting with an anti-Sens antibody was used for detection.

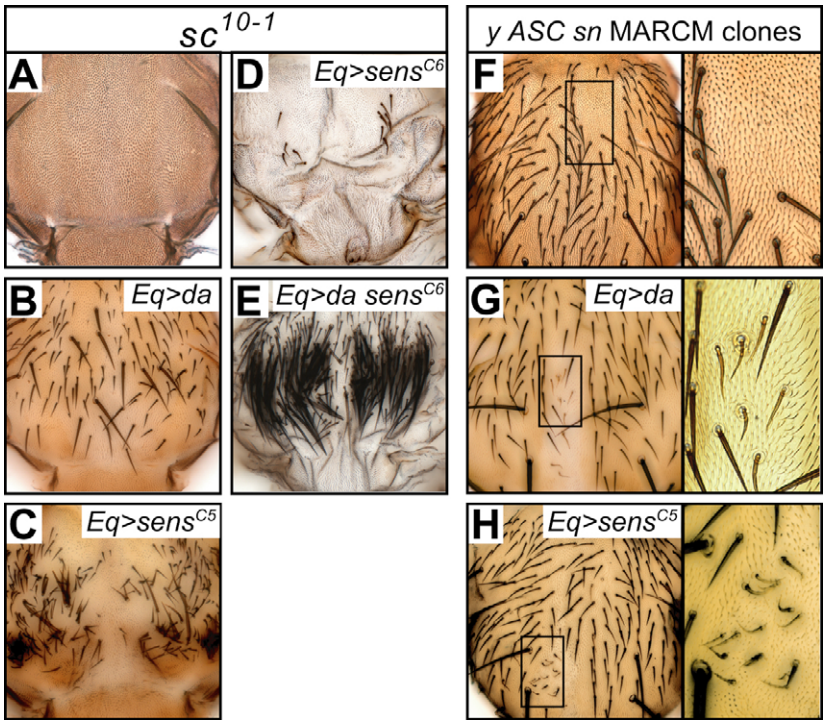

Fig. 5. Overexpression of Sens and Da in the thorax can generate bristles in *ASC* clones. (**A**) Loss of Ac and Sc leads to a loss of all bristles in the thorax. (**B**,**C**) Overexpression of (B) Da or (C) Sens using *Eq-GAL4* results in the formation of numerous bristles in a sc^{10-1} background. Note that overexpression of Sens in the midline region causes some thorax closure defects and abnormal midline morphology. (**D**) Overexpression of Sens using a moderate *UAS-sens* transgene is able to induce a few SOs in a sc^{10-1} background. (**E**) Co-expression of Sens and Da results in the generation of a large number of SOs in sc^{10-1} flies. Note that because flies used in D and E were taken out of the pupal case as pharate adults, their cuticle is somewhat wrinkled. (**F-H**) MARCM clones of *Df(1)260-1*. Mutant bristles are *yellow* and *singed*. In clones of the *ASC* in the thorax, which are normally devoid of bristles (F), overexpression of (G) Da or (H) Sens induces SO formation.

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 pI 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 sc^{10-1} males (Fig. 5A,B). Similarly, driving a strong *UAS-sens* transgene with *Eq-GAL4* can induce multiple bristles in a sc^{10-1} 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 ($UAS\text{-}sens^{C6}$) only generates a few extra SOs in sc^{10-1} males (Fig. 5D), the number of SOs generated in a sc^{10-1} background by the co-expression of Da and Sens is much larger than that induced by *UAS-da* or $UAS\text{-}sens^{C6}$ alone (Fig. 5B,D,E). However, it is possible that overexpression of *da* or *sens* in sc^{10-1} 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 pI formation in the absence of *sens* function

We next sought to determine whether *sens* and *da* require the function of one another for pI 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 pI 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 pI progeny but not the pI 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

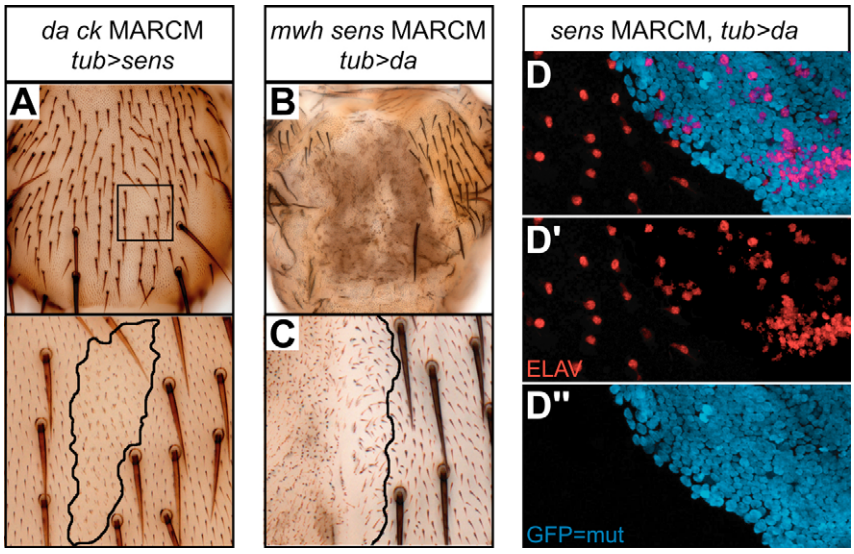

Fig. 6. Da is able to induce pI formation in the absence of Sens function. (**A**) A MARCM *da*^{3} clone that overexpresses *sens* using *tub-GAL4*. Note the absence of microchaetae in the mutant clone (the closed line in the close-up view), which is marked by *crinkled*. (**B**,**C**) Low (B) and high (C) magnification views of two MARCM clones of *sens*^{E2} that overexpress *da* using *tub-GAL4*. No microchaetae are formed in the clone, which is marked by *mwh*. (**D-D″**) Overexpression of *da* in a MARCM *sens* clone in the thorax using *tub-GAL4* results in the generation of numerous additional Elav^{+} cells (red), often clustered. Note that the neurons are usually well spaced in the wild-type tissue. The image is from a 36-hour APF pupa. GFP (blue) marks the nuclei of the *sens* mutant cells.

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

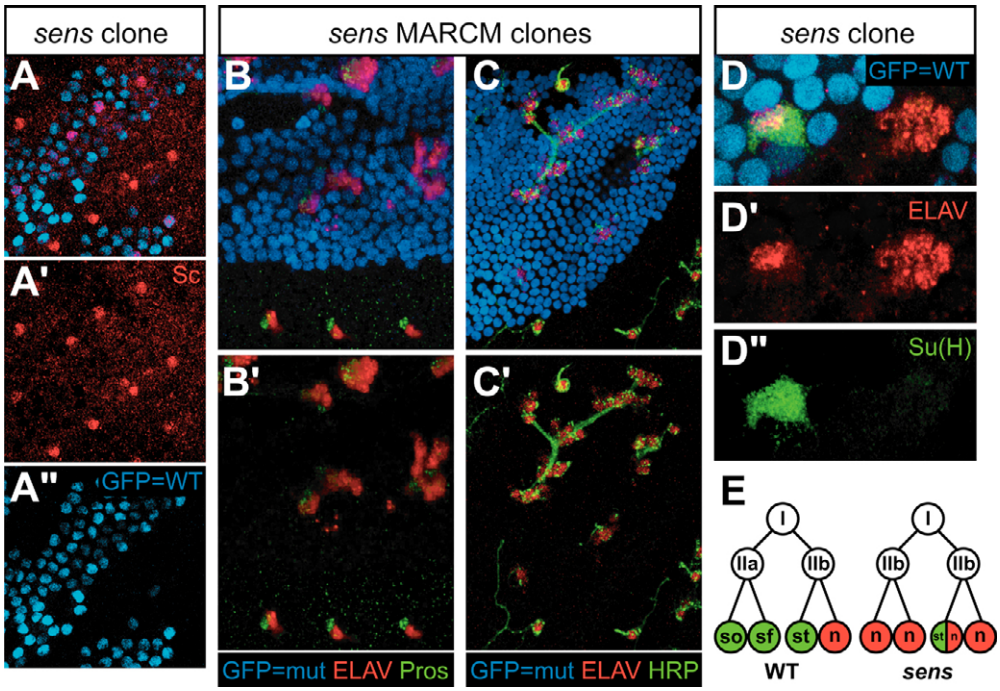

Fig. 7. *sens* regulates cell-fate specification in the microchaetae pI progeny. (**A-A″**) Sc expression is restricted to single cells in a *sens* clone. Shown is a *sens*^{E2} clone in a 12-hour APF pupal thorax stained for Sc (red). GFP (blue) marks the wild-type tissue. (**B**,**B′**) A MARCM clone 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 *sens*^{E2} (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 pI selection and specification. However, it does regulate several key aspects of SO development, including proper cell fate determination of the pI 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 (Garcia-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 (Ghysen 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 (Garcia-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 pIs 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 pIs 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 pIs 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 pI 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 AWM 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 pI selection. The more severe loss of neurons compared with support cells associated with the loss of Ac and Sc in $sc^{10\text{-}1}$ 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 $sc^{10\text{-}1}$ 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 $sc^{10\text{-}1}$ 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 pI 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 pI 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 pIs. We were therefore surprised to find that microchaetae pI 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 pIs, as well as for timely downregulation of *phyllopod* expression in epidermal cells. This suggests a dual role for Sens in pIs 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 pI selection is not disrupted. We now present evidence that microchaetae pIs are indeed selected in *sens* clones and that they divide to generate progeny. However, the mutant pIs exhibit an abnormal division pattern, and we observe a pIIa-to-pIIb 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 pIs are selected.

Table 1. The roles of *ac* and *sc* and *sens* in the development of the adult external SOs

	Thoracic microchaetae	WM chemoreceptors	WM mechanoreceptors
ac and *sc*	Proneural	Proneural	Survival
sens	pI progeny fate specification	pI selection and specification	Proneural Survival

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 chemosensory lineages, for which *ac* and *sc* are the proneural genes, Sens is required for pI 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 pI 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 pI progeny in the WM mechanosensory lineages. We also find that *ac* and *sc* prevent apoptosis in this lineage independently of pI 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., 2002). Moreover, Gfi1 has an oncogenic potential (Moroy, 2005) and has been implicated in several human diseases, such as hereditary neutropenia (Person et al., 2003), spinocerebellar ataxia type 1 (Tsuda et al., 2005) and small cell lung carcinoma (Kazanjian et al., 2004). Therefore, given the structural and functional similarities between Gfi1 and Sens (Jafar-Nejad and Bellen, 2004), further analysis of the various aspects of Sens function in *Drosophila* SO development will continue to help unravel the mechanisms of Gfi1 function in health and disease.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/9/1683/DC1

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Table S1. Complete genotypes of the animals used in some images in this study

Figure	Genotype
3A-B′, 5F	*Df(1)260-1, y^- sn^3 FRT19A/hs-FLP tub-GAL80 FRT19A; act-GAL4 UAS-CD8::GFP/+*
3C, 7A-A″, 7D-D″	*y w Ubx-FLP; ubi-GFP FRT80B/mwh^1 $sens^{E2}$ FRT80B*
3D	*y w hs-FLP; ubi-GFP FRT80B/$sens^{E2}$ FRT80B*
3E,F	*y w hs-FLP; y^+ ck^{13} FRT40A/da^3 ck^{13} FRT40A*
3H,H′	*y w Ubx-FLP; ubi-GFP FRT40B /wg^{CX4} FRT40A*
5B	*sc^{10-1}/Y; Eq-GAL4 UAS-da^{45}/+*
5C	*sc^{10-1}/Y; Eq-GAL4 UAS-$sens^{C5}$/+*
5D	*sc^{10-1}/Y; Eq-GAL4 UAS-$sens^{C6}$/+*
5E	*sc^{10-1}/Y; Eq-GAL4 UAS-da^{45}/UAS-$sens^{C6}$*
5G	*Df(1)260-1, y^- sn^3 FRT19A/hs-FLP tub-GAL80 FRT19A; UAS-CD8::GFP/+; Eq-GAL4 UAS-da^{45}/+*
5H	*Df(1)260-1, y^- sn^3 FRT19A/hs-FLP tub-GAL80 FRT19A; UAS-CD8::GFP/+; Eq-GAL4 UAS-$sens^{C5}$/+*
6A	*UAS-CD8::GFP hs-FLP; tub-GAL80 FRT40A/da^3 ck^{13} FRT40A; tub-GAL4/UAS-$sens^{C5}$*
6B-D″	*hs-FLP tub-GAL4 UAS-GFP^{nls}; UAS-da^{52}/+; tub-GAL80 M(3)67C FRT80B/ mwh^1 $sens^{E2}$ FRT80B*
7B-C″	*hs-FLP tub-GAL4 UAS-GFP^{nls}; tub-GAL80 M(3)67C FRT80B/mwh^1 $sens^{E2}$ FRT80B*
S3	*hs-FLP tub-GAL4 UAS-GFP^{nls}; UAS-P35/+; tub-GAL80 M(3)67C FRT80B/mwh^1 $sens^{E2}$ FRT80B*
S4	*Df(1)sc-B57, sn^3 FRT19A/hs-FLP tub-GAL80 FRT19A; act-GAL4 UAS-CD8::GFP/UAS-da^{52}*