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The *Drosophila* HMG-domain proteins SoxNeuro and Dichaete direct trichome formation via the activation of *shavenbaby* and the restriction of Wingless pathway activity

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Trichomes are cytoplasmic extrusions of epidermal cells. The molecular mechanisms that govern the differentiation of trichome-producing cells are conserved across species as distantly related as mice and flies. Several signaling pathways converge onto the regulation of a conserved target gene, *shavenbaby* (*svb*, *ovo*), which, in turn, stimulates trichome formation. The *Drosophila* ventral epidermis consists of the segmental alternation of two cell types that produce either naked cuticle or trichomes called denticles. The binary choice to produce naked cuticle or denticles is affected by the transcriptional regulation of *svb*, which is sufficient to cell-autonomously direct denticle formation. The expression of *svb* is regulated by the opposing gradients of two signaling molecules – the epidermal growth factor receptor (Egfr) ligand Spitz (Spi), which activates *svb* expression, and Wingless (Wg), which represses it. It has remained unclear how these opposing signals are integrated to establish a distinct domain of *svb* expression. We show that the expression of the high mobility group (HMG)-domain protein SoxNeuro (SoxN) is activated by Spi, and repressed by Wg, signaling. SoxN is necessary and sufficient to cell-autonomously direct the expression of *svb*. The closely related protein Dichaete is co-regulated with SoxN and has a partially redundant function in the activation of *svb* expression. In addition, we show that SoxN and Dichaete function upstream of Wg and antagonize Wg pathway activity. This suggests that the expression of *svb* in a discreet domain is resolved at the level of SoxN and Dichaete.

KEY WORDS: SoxNeuro, Dichaete, *shavenbaby*, *Drosophila* epidermal growth factor receptor (Der)- and Wingless (Wg)-pathway activities, Epidermal differentiation

INTRODUCTION

The *Drosophila* ventral epidermis is the classic model system in which to study the mechanisms that regulate the specification of two distinct cell types: cells that secrete cuticle with trichomes (denticles) and cells that secrete naked cuticle. Many studies have focused on the patterning mechanisms that govern the establishment of alternating fields of denticle-producing versus smooth cells (Fig. 1, cartoon) (for reviews, see Hatini and DiNardo, 2001; Sanson, 2001). These studies have identified the transcription factor *shavenbaby* (*svb*) as the most downstream target of the signaling cascades that pattern the ventral epidermis (Payre et al., 1999). Recent studies have shown that *svb* is necessary and sufficient to direct denticle formation in a cell-autonomous manner by regulating the expression of genes whose products are involved in epidermal cell-shape remodeling (Chanut-Delalande et al., 2006). In each segment of the ventral epidermis, *svb* is expressed in a discreet domain of six rows of cells. The spatial limits of this domain reflect the inputs of Der- and Wg-pathway activities on the transcriptional regulation of *svb* expression: Der pathway activity stimulates and Wg pathway activity represses *svb* expression (Szuts et al., 1997).

The expression of *svb* in a discreet and invariable domain poses a question that appears in many developmental contexts: how do individual cells integrate opposing extrinsic information such that the response of the cell is non-stochastic and invariable? Circumstantial evidence suggests that *svb* is not a direct target gene of the Wg signaling cascade. Our study shows that the high mobility group (HMG)-domain protein SoxNeuro (SoxN) has a dual role in the establishment and maintenance of a discreet domain of *svb* expression. First, SoxN expression is activated by Der- and repressed by Wg-pathway activities. SoxN is necessary for the expression of *svb* and is sufficient to cell-autonomously activate *svb* expression even in the presence of high levels of Wg signaling. Hence, SoxN represents a molecular link between these signaling cascades and the expression of *svb*. Moreover, we show that *svb* is required for the maintenance of but not for the establishment of the late epidermal SoxN expression. This indicates a reciprocal regulatory relationship between *svb* and SoxN. Second, many vertebrate Sox proteins have been shown to antagonize Wg pathway activity. We present evidence that this function is conserved in *Drosophila* SoxN. Hence, the spatial limits of both SoxN expression and Wg pathway activity are determined by a negative-feedback loop. These results suggest that the expression of *svb* in a discreet domain is resolved at the level of SoxN. Furthermore, we show that a closely related HMG-domain protein, Dichaete, is co-regulated with SoxN and has a redundant, albeit weaker, function in the activation of *svb* expression and in the restriction of Wg pathway activity.

MATERIALS AND METHODS

Fly stocks

Oregon R flies were used as the wild-type stock. The EMS-induced alleles $SoxN^{U6\text{-}35}$ and $SoxN^{GA1192}$ have been described previously (Buescher et al., 2002; Overton et al., 2002). $SoxN^{U6\text{-}35}$ contains a C to T transition that results in a nonsense codon at amino acid position 133; $SoxN^{GA1192}$ contains a C to T transition that results in nonsense codon at amino acid position 172. The putative translation products of both mutant alleles do not contain the HMG domain. The following *Gal4* driver lines and *UAS*-constructs were used:

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scaGal4 (which drives expression in the ventral ectoderm from early stage 9 onward) and *wgGal4* were obtained from the Bloomington Stock Center. *enGal4* (Brand and Perrimon, 1993), *armVP16Gal4* (Sanson et al., 1996) was kindly provided by J. P. Vincent (MRC National Institute for Medical Research, London, UK); *UAS-SoxN* (Overton et al., 2002). *pangolin*^{2} (synonymous with *dTCF*^{2}) (van de Wetering et al., 1997) and *wg*^{cx4} (Baker, 1988) were obtained from the Bloomington Stock Center. *D*^{r72} was kindly provided by S. Russell (Soriano and Russell, 1998). *UAS-Egfr*^{Act}, *UAS-der*^{DN} and *UAS-wgHA* were kindly provided by J. Bateman and J. Ng (both from King's College London, London, UK), respectively. The amorphic allele *svb*^{1} was kindly provided by F. Payre (Payre et al., 1999). *UAS-SoxN-YFP* was generated by Gateway cloning of the complete *SoxN* coding sequence into the pTVW vector.

Phenotypic analysis

Cuticles of first-instar larvae were prepared as described (Buescher et al., 2004).

Immunohistochemistry

Embryos were collected, fixed and immunostained as previously described (Yang et al., 1997). Primary antibodies were polyclonal mouse anti-SoxN (1:1000) (Buescher et al., 2002); polyclonal rabbit anti-Dichaete (1:1000) (Nambu and Nambu, 1996); monoclonal mouse anti-Engrailed/Invected (also known as Engrailed, En) (4D9; developed by C. Goodman). The Engrailed/Invected hybridomas were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development (NICHD) and maintained by the University of Iowa, Iowa City, IA. Histochemical detection was performed with Jackson ImmunoResearch HRP-conjugated secondary antibodies and visualized by the glucose-oxidase-DAB-nickel method, as described (Shu et al., 1988). Fluorescent secondary antibodies were obtained from Jackson ImmunoResearch (Cy3- and FITC-conjugated anti-mouse and anti-rabbit antibodies).

In situ hybridization

RNA in situ hybridization was carried out as described previously (Tautz and Pfeifle, 1989). For the generation of the *svb* RNA probe, a *svb*-specific DNA fragment of approximately 1.5 kb length was amplified from genomic DNA using PCR and subsequently used as a template for in vitro transcription with T7 RNA polymerase.

RESULTS

SoxN expression in the ventral epidermis is regulated by Der- and Wg-pathway activities

The wild-type ventral larval cuticle exhibits a segmentally re-iterated pattern of six rows of denticles separated by stretches of naked cuticle (Fig. 1A,A′). We have previously reported that *SoxN* mutant animals show an aberrant cuticle phenotype (Buescher et al., 2002). The present study was performed using two independent, putatively amorphic alleles of *SoxN*, which have essentially identical mutant phenotypes: *SoxN*^{GA1192} (Buescher et al., 2002) (also see Materials and methods) and *SoxN*$^{U6\text{-}35}$ (Overton et al., 2002). *SoxN* homozygous mutant embryos die late in embryogenesis, after the secretion of cuticle. The *SoxN* mutant phenotype is characterized by a moderate loss of denticles and a corresponding increase of naked cuticle (Fig. 1B,B′). To understand the role of SoxN in the formation of denticles, we first examined the expression pattern of SoxN. During embryogenesis, SoxN protein expression undergoes dynamic changes (Buescher et al., 2002; Cremazy et al., 2000). To determine the precise boundaries of SoxN expression within segments, we double-stained wild-type embryos with anti-SoxN and anti-En, the latter providing a stable landmark throughout embryogenesis. From stage 5 to late stage 8, SoxN is expressed throughout the ventral ectoderm (Fig. 1C) (Buescher et al., 2002; Cremazy et al., 2000; Overton et al., 2002). From stage 9 onwards, SoxN protein decays; this is first observed in a narrow stripe just

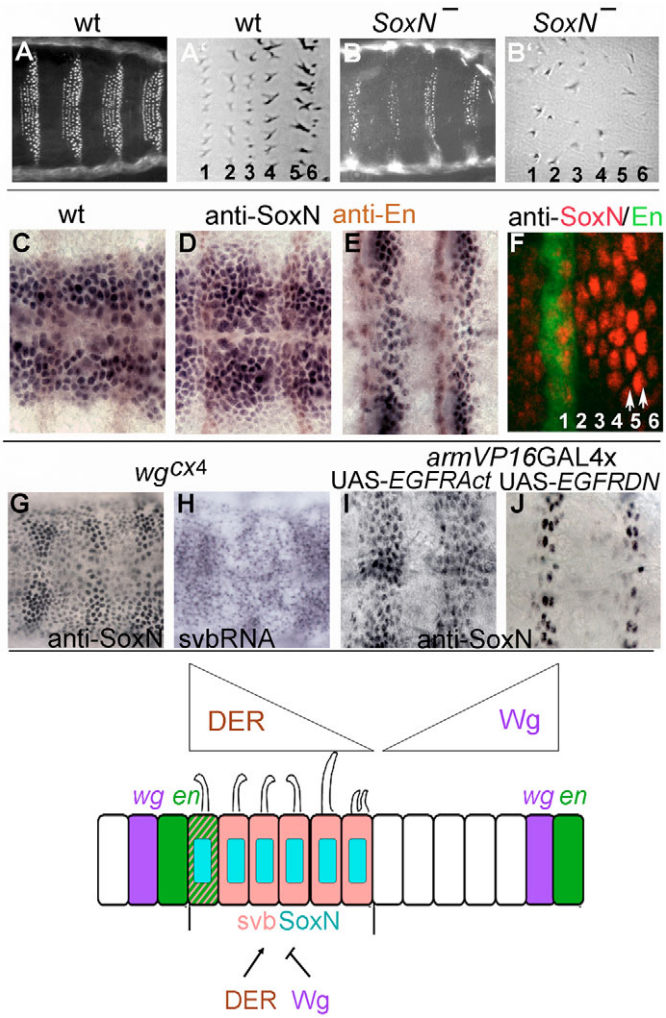

Fig. 1. Cuticle phenotype and expression pattern of *SoxN*. (**A-B′**) *SoxN* mutants have an aberrant cuticle phenotype. (A) Wild type (wt); cuticle of first instar larva. (A′) Wild type; single denticle belt. (B) *SoxN*^{GA1192} mutant; cuticle of first instar larva. (B′) *SoxN*^{GA1192} mutant; single denticle belt. Numbers (1-6) represent the six rows of denticles. (**C-J**) Expression pattern of *SoxN* and its regulation by signaling pathway activities. (C-E) Wild-type embryos double-stained with anti-SoxN (black) and anti-En (brown). (C) Stage 8; SoxN protein expression is ubiquitous. (D) Stage 9; SoxN expression decays in a narrow stripe just anterior to the En domain. (E) Stage 13; SoxN expression is restricted to segmental stripes, each with a width of six cells. (F) Stage 15 *enGal4-UAS-taulacZ* embryos double-stained with anti-SoxN (red) and anti-β-galactosidase (green, detecting En expression). The anterior-most row of SoxN-expressing cells (1) corresponds to the posterior row of En-expressing cells. Arrows, notice that SoxN expression in the two posterior-most rows (5,6) is higher than in the anterior four rows (1-4). (G) Staining of stage-15 *wg*^{cx4} mutant embryos with anti-SoxN; notice that SoxN expression is derepressed as compared with wild type. (H) Staining of stage-15 *wg*^{cx4} embryos with an *svb*-specific RNA probe; notice that the expression patterns of *SoxN* and *svb* in *wg*^{cx4} embryos are highly similar. (I) Staining of stage-15 *armVP16Gal4-UAS-Egfr*^{Act} embryos with anti-SoxN; notice that SoxN expression expands posteriorly by two to three rows of cells. (J) Staining of stage-15 *armVP16Gal4-UAS-Egfr*^{DN} embryos with anti-SoxN; notice that SoxN expression is reduced. Cartoon: schematic representation of the ventral epidermis with the expression patterns of the *wg*, *en*, *svb* and *SoxN* genes. *SoxN* and *svb* are co-expressed in six rows of cells, which differentiate to produce denticles. *SoxN* and *svb* expression is stimulated by Der- and repressed by Wg-pathway activities.

anterior to the En domain, so that, at late stage 9, SoxN protein remains present in the entire epithelium with the exception of the Wg expression domain (Fig. 1D). After stage 9, SoxN protein expression further decays throughout the segment. From early stage 12 onwards, SoxN expression is re-initiated in segmental stripes of 6-cell width at the lateral periphery of the ventral epidermis. Subsequently, these stripes expand along the dorsoventral (D/V) axis and, from stage 13 onwards, span the ventral epidermis. SoxN remains expressed in 6-cell-wide stripes until the end of embryogenesis (Fig. 1E). However, we noticed that, after stage 13, the level of SoxN expression is further upregulated in the two posterior-most rows of cells (Fig. 1F, arrows). En and SoxN stripes partially overlap so that the anterior-most row of SoxN expression co-localizes with the posterior row of En expression (Fig. 1F). These results indicate that, during the embryonic stages in which epidermal cell fate is specified, SoxN expression is restricted to those cells that will differentiate to produce denticles. Most notably, the *SoxN* and *svb* expression domains completely coincide (Fig. 1, cartoon).

The expression domain of *svb* is established by the Der- and Wg-pathway activities (Szuts et al., 1997). This prompted us to examine whether the late expression of SoxN is regulated by the same signaling cascades. In *wg* null mutant embryos (wg^{cx4}) SoxN expression did not decay after stage 8 and remained ubiquitous until the end of embryogenesis (Fig. 1G). In fact, the aberrant expression patterns of *SoxN* and *svb* in *wg* mutant embryos were very similar (compare Fig. 1G with 1H). However, derepression of SoxN in a *wg* mutant background did not result in uniform levels of SoxN expression throughout the ventral epidermis. Instead, the level of ectopic SoxN expression remained lower than that of SoxN within its endogenous domain, suggesting that the establishment of wild-type levels of SoxN expression might require additional stimulating input(s). Onset and location of SoxN expression in epidermal stripes suggests that the activation of the Der pathway via its ligand Spi might stimulate SoxN expression. Activation of the Der pathway throughout the ventral epidermis with the *armadillo* (*arm*)-*VP16* driver (*armVP16Gal4-UAS-*$Egfr^{Act}$) indeed resulted in a posterior expansion of SoxN expression by two to three rows of cells (Fig. 1I). Conversely, reduction of Der pathway activity throughout the ventral epidermis (*armVP16Gal4-UAS-*$Egfr^{DN}$) resulted in a strong loss of SoxN expression (Fig. 1J). Taken together, our data indicate that the late expression of SoxN in epidermal stripes is positively regulated by Der- and negatively regulated by Wg-pathway activities. Hence, the co-expression of *SoxN* and *svb* reflects their regulation by the same signaling cascades.

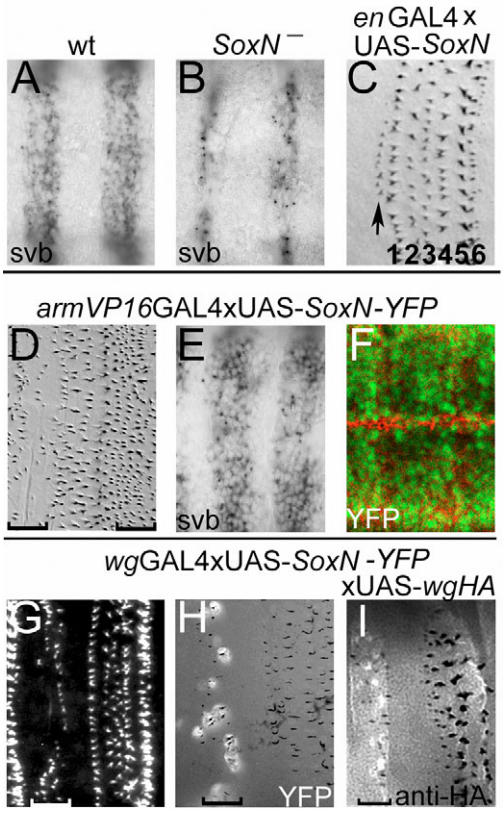

Fig. 2. SoxN is necessary and sufficient to activate *svb* expression/direct denticle formation. (**A**,**B**) *svb* RNA expression in stage-14 wild-type (wt; A) and $SoxN^{GA1192}$ (B) embryos. (**C**) Ectopic denticle formation in an *enGal4-UAS-SoxN* larva. The arrow indicates ectopic denticle formation in the anterior row of the En-expressing stripe. Rows are labeled 1-6. (**D**,**E**) Misexpression of *SoxN* with the *armVP16Gal4* driver results in ectopic denticle formation (indicated by brackets; D) in those areas where ectopic expression of *svb* occurs (E); note that *svb* is expressed in broad stripes but is not ubiquitous. (**F**) Anti-GFP staining (green) of stage-12 *armVP16Gal4-UAS-SoxN-YFP* embryos; note that *armVP16Gal4* drives *SoxN* expression in broad stripes but is not ubiquitous. The red staining indicates the ventral midline. (**G-I**) Misexpression of *SoxN* with the *wgGal4* (*wgGal4-UAS-SoxN-YFP*) driver results in ectopic denticle formation. (G) Ectopic denticle formation in *wgGal4-UAS-SoxN-YFP* larvae. (H) Cuticle and anti-YFP staining of stage-17 *wgGal4-UAS-SoxN-YFP* embryos; note that SoxN directs ectopic denticle formation in a cell-autonomous manner. (I) Cuticle and anti-HA staining of stage-17 *wgGal4-UAS-SoxN-UAS-wg* embryos; note that concomitant misexpression of *SoxN* and *wg* results in ectopic denticle formation in a cell-autonomous manner.

SoxN is necessary and sufficient to activate the expression of *svb* downstream of the Der- and Wg-pathway activities

Our observations raise the issue as to whether the expression of *SoxN* and *svb* are regulated in parallel by the Der- and Wg-pathway activities, or whether Spi and Wg might regulate the expression of *SoxN*, which could in turn activate the expression of *svb*. To address this, we first examined whether loss of *SoxN* results in a loss of *svb* expression. In stage-14 *SoxN* mutant embryos, we found that *svb* expression was strongly reduced compared with wild-type, indicating that SoxN is necessary for the expression of *svb* (Fig. 2A,B). To determine whether SoxN is sufficient to cause ectopic *svb* expression/denticle formation, we used a number of *Gal4* lines that drive expression in different parts of the ventral epidermis, and observed that misexpression of SoxN resulted in ectopic denticle formation. For example, misexpression of SoxN in the anterior row of the En stripe (*enGal4-UAS-SoxN*) resulted in ectopic denticle formation within this row of cells (Fig. 2C). Misexpression of SoxN with the *armVP16Gal4* driver (*armVP16Gal4-UAS-SoxN-YFP*) resulted in ectopic denticle formation in most of the ventral epidermis except for in narrow stripes anterior to the row 1 denticles. In these regions, ectopic denticle formation remained sparse. The ectopic denticle formation reflected the ectopic expression of *svb* in broadened segmental stripes, which were separated by narrow gaps with little or no *svb* expression (Fig. 2E). Although the *armVP16Gal4* driver has been described as directing the expression of transgenes in a ubiquitous manner, staining of *armVP16Gal4-UAS-SoxN-YFP* embryos with anti-GFP indicated that this was not

the case. Instead, we observed YFP-immunoreactivity in broad stripes, which were separated by narrow stripes with little or no YFP (Fig. 2F). This suggests that the ectopic expression of *svb*/ectopic denticle formation in *armVP16Gal4-UAS-SoxN-YFP* embryos reflects the distribution of SoxN-YFP.

To examine whether the SoxN-mediated formation of ectopic denticles is strictly cell-autonomous, we misexpressed *SoxN-YFP* with the *wgGal4* driver. We observed two to three rows of ectopic denticles in *wgGal4-UAS-SoxN-YFP* larvae (Fig. 2G). Ectopic denticle formation was found be restricted to those cells that showed YFP-immunoreactivity (Fig. 2H), indicating that SoxN directs the formation of denticles in a cell-autonomous manner. Vertebrate Sox proteins have been shown to antagonize Wnt pathway activity by sequestering nuclear β-catenin (see below) (Akiyama et al., 2004; Haremaki et al., 2003; Mansukhani et al., 2005; Zorn et al., 1999). To determine whether the SoxN-mediated formation of denticles within the *wg* expression domain might be due to a reduction of Wg pathway activity, we co-expressed *wg* together with SoxN (*wgGal4-UAS-SoxN-YFP-UAS-wgHA*). We observed ectopic denticle formation in cells that expressed high levels of *wg*, as shown by anti-hemagglutinin (anti-HA) staining (Fig. 2I). These results indicate that SoxN activates the expression of *svb* downstream of Wg pathway activity.

In *wg* null mutant embryos, *svb* is expressed throughout the ventral epidermis and the resulting cuticle is covered by a lawn of denticles (Fig. 3A) (Payre et al., 1999). Our results suggest that ectopic denticle formation in *wg* mutants might result from the derepression of *SoxN*, which, in turn, activates the expression of *svb*. Removal of SoxN function in a *wg* mutant background resulted in cuticles with few or no ectopic denticles, indicating that ectopic denticle formation in *wg* mutants is, at least in part, dependent on SoxN (Fig. 3B). We confirmed this result by analyzing the function of SoxN in the absence of *pangolin* (*pan*, *dTCF*), the nuclear effector of Wg signaling (van de Wetering et al., 1997). pan^2 mutant larvae were covered by a lawn of denticles (Fig. 3C). Reduction of SoxN function (pan^2*;SoxN/+*) resulted in fewer ectopic denticles, whereas removal of all SoxN function (pan^2*;SoxN*) resulted in a *SoxN* mutant cuticle phenotype (Fig. 3D,E). Hence, SoxN acts genetically downstream of *wg* to activate the expression of *svb*.

As shown above, SoxN expression is activated by Der pathway activity. This suggests that SoxN might act downstream of Spi to activate *svb*. To confirm this hypothesis we analyzed the epistatic relationships of the Der pathway, SoxN and *svb*. Previous studies had shown that activation of the Der pathway throughout the ventral epidermis results in the formation of ectopic denticles (Payre et al., 1999; Szuts et al., 1997). We confirmed this result using the *scabrous* (*sca*)-*Gal4* driver to misexpress an activated form of the epidermal growth factor receptor (Egfr) (*scaGal4-UAS-*$Egfr^{Act}$). *scaGal4* drives the expression of transgenes throughout the ventral epidermis from stage 9 onwards. Activation of the Der pathway throughout the ventral epidermis resulted in the formation of three to four rows of ectopic denticles posterior to the wild-type row-6 denticles (Fig. 3F). Removal of SoxN function in embryos misexpressing $Egfr^{Act}$ (*SoxN;scaGal4-UAS-*$Egfr^{Act}$) strongly reduced ectopic denticle formation, indicating that the ability of Der pathway activity to direct denticle formation is at least in part dependent on SoxN function (Fig. 3G).

Taken together, our results show that SoxN directs the formation of denticles downstream of Der- and Wg-pathway activities and upstream of *svb*. Hence, SoxN links the Der- and Wg-signaling cascades with *svb*, expression of which itself is necessary and sufficient to direct denticle formation.

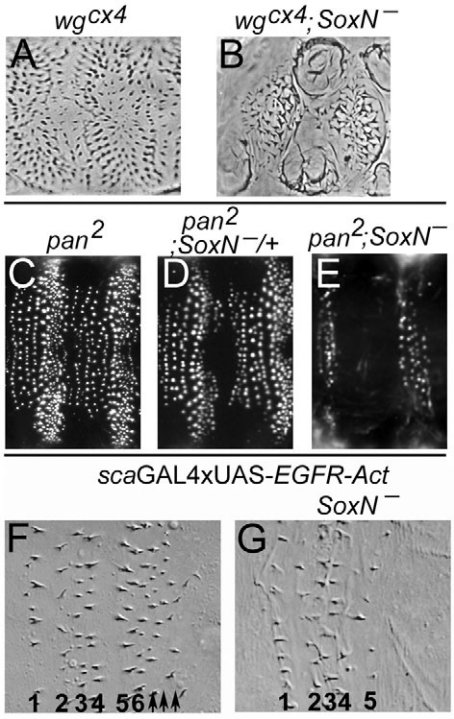

Fig. 3. SoxN acts downstream of Der- and Wg-pathway activities to direct denticle formation. (**A**) Cuticle of a *wg* null mutant larva. (**B**) Cuticle phenotype of a *wg;*$SoxN^{U6\text{-}35}$ double-mutant larva; notice that removal of SoxN function largely rescues the *wg* mutant phenotype. (**C-E**) Removal of SoxN function rescues the *pan* mutant phenotype. (C) pan^2 mutant cuticle. (D) pan^2*;*$SoxN^{U6\text{-}35}$*/+* cuticle. (E) pan^2*;*$SoxN^{U6\text{-}35}$ cuticle. (**F**) Cuticle of an *scaGal4-UAS-*$Egfr^{Act}$ larva; note the ectopic denticles posterior to the wild-type row-6 denticles (indicated by arrows). (**G**) Cuticle of an *scaGal4-UAS-*$Egfr^{Act}$*;*$SoxN^{GA1192}$ larva; note that ectopic denticle formation is largely suppressed.

svb function is necessary for the maintenance of epidermal SoxN expression

The expression of SoxN in epidermal stripes of 6-cell width is established by stage 13 and is maintained until the end of embryogenesis. As shown above, the establishment of the SoxN expression domain results from the opposing activities of the Der and Wg pathways. However, the maintenance of SoxN expression until the end of embryogenesis might depend on additional factors. From late stage 13 onwards, *SoxN* and *svb* are co-expressed. Taking into account that Svb is a transcription factor, it is conceivable that Svb acts to support the late expression of SoxN. To analyze whether Svb plays a role in the maintenance of SoxN expression, we stained svb^1 mutant embryos with anti-SoxN antibody. At embryonic stage 13, SoxN expression in wild-type and svb^1 mutant embryos showed no appreciable differences, indicating that *svb* function is not required for the establishment of the SoxN expression domain (Fig. 4A,B). This result is consistent with our observation that the epidermal expression of SoxN precedes and is necessary for the expression of *svb*. However, in stage-16 svb^1 mutant embryos, the expression of SoxN was strongly reduced as compared with wild-type embryos, indicating that *svb* function is necessary for the maintenance of SoxN expression (Fig. 4C,D). Taken together, our results demonstrate a reciprocal regulatory relationship between

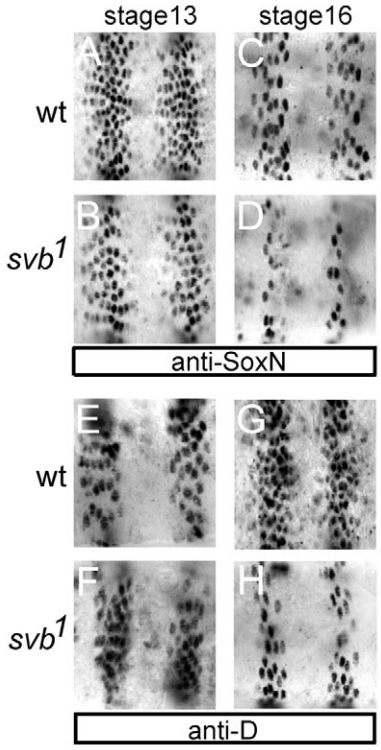

Fig. 4. *svb* function is necessary for the maintenance of epidermal SoxN and Dichaete expression. (**A-H**) Anti-SoxN stainings (A-D) and anti-Dichaete (anti-D) stainings (E-H) of wild-type (WT) and *svb*^{1} mutant embryos. Wild-type (A) and *svb*^{1} mutant (B) stage-13 embryos; note that there is no appreciable difference between wild-type and *svb*^{1} mutant embryos, indicating that *svb* is not required for the establishment of the SoxN expression domain. Wild-type (C) and *svb*^{1} mutant (D) stage-16 embryos. (D) Notice the severe reduction of SoxN expression, indicating a role for *svb* in the maintenance of SoxN expression. Wild-type (E) and *svb*^{1} mutant (F) stage-13 embryos; notice that there is no appreciable difference between wild-type and *svb*^{1} mutant embryos, indicating that *svb* is not required for the establishment of the Dichaete expression domain. Wild-type (G) and *svb*^{1} mutant (H) stage-16 embryos. (H) Notice the reduction in Dichaete expression, indicating a role for *svb* in the maintenance of Dichaete expression.

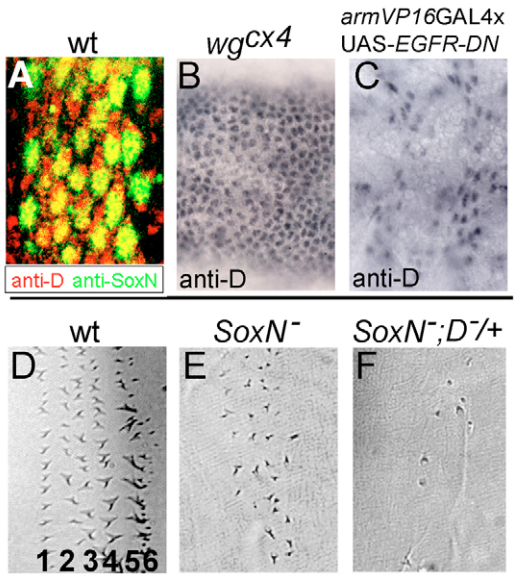

Fig. 5. Dichaete is co-expressed and co-regulated with SoxN and is required for denticle formation. (**A**) Double-staining of wild-type (wt) stage-15 embryos with anti-SoxN (green) and anti-Dichaete (anti-D; red); notice the complete overlap between SoxN and Dichaete expression (yellow). (**B**) Staining of stage-15 *wg*^{cx4} mutant embryos with anti-D; notice that Dichaete is ectopically expressed. (**C**) Staining of stage-15 *armVP16Gal4-UAS-Egfr*^{DN} embryos with anti-D; notice the reduction in Dichaete expression. (**D**) Wild-type cuticle. (**E**) *SoxN*$^{U6\text{-}35}$ mutant cuticle. (**F**) *SoxN*$^{U6\text{-}35}$*;D/+* mutant cuticle.

SoxN and *svb*: SoxN is required for the establishment of the expression of *svb*; in turn, *svb* is necessary to maintain the expression of *SoxN*.

The HMG-domain protein Dichaete has a partially redundant function in the formation of denticles

As described above, loss of *SoxN* does not cause a complete loss of *svb* expression and denticle formation. Likewise, in *SoxN;wg* and *pan;SoxN* double mutants, some denticles are still formed. This suggests that SoxN cannot be the only factor that activates *svb* expression/denticle formation. Dichaete, an HMG-domain protein closely related to SoxN, has been shown previously to be expressed in the ventral epidermis and to have a partially redundant function in the establishment of neural fate (Buescher et al., 2002; Overton et al., 2002). We wondered whether Dichaete might also have a function in the activation of *svb* expression/denticle formation. Previous studies have shown that, during the embryonic stages 5 to 9, Dichaete expression partially overlaps that of SoxN (Overton et al., 2002). We sought to determine whether there is any overlap between SoxN and Dichaete expression during later stages of embryonic development. Double-staining of wild-type embryos with anti-SoxN and anti-Dichaete antibodies revealed that SoxN and Dichaete co-localized in the ventral epidermis from stage 12 until the end of embryogenesis (Fig. 5A). Furthermore, like SoxN, Dichaete expression was negatively regulated by Wg pathway activity: in *wg* null mutant embryos (*wg*^{cx4}), Dichaete was derepressed and found throughout the ventral epidermis, although its expression levels were not uniform (Fig. 5B). In addition, we found that Dichaete expression is positively regulated by Der pathway activity: misexpression of *Egfr*^{Act} (*armVP16Gal4-UAS-Egfr*^{Act}) resulted in an expansion of Dichaete expression; whereas a reduction of Der pathway activity (*armVP16Gal4-UAS-der*^{DN}) caused a partial loss of Dichaete expression (Fig. 5C and data not shown). As shown above, during the late stages of embryogenesis, the maintenance of *SoxN* expression depends on *svb*. To determine whether the late expression of Dichaete requires *svb* function, we stained *svb*^{1} mutant embryos with the anti-Dichaete antibody. At embryonic stage 13, no significant difference was observed between wild-type and *svb*^{1} mutant embryos (Fig. 4E,F). However, at stage 16, Dichaete expression was found to be reduced in *svb*^{1} mutant embryos as compared with wild type (Fig. 4G,H). This indicates that *svb* is required for the maintenance but not for the establishment of the Dichaete expression domain. Taken together, these results show that, from mid-embryogenesis onwards, SoxN and Dichaete are co-regulated and, as a result, co-expressed.

Previous studies have shown that loss of Dichaete results in an aberrant cuticle pattern (Nambu and Nambu, 1996). However, these defects mainly reflect the early function of Dichaete in segmentation rather than a specific function in the regulation of *svb* expression. To

determine whether Dichaete has a more specific function in regulating the expression of *svb*/denticle formation, we lowered the level of Dichaete function in a *SoxN* mutant background (*SoxN;Dichaete/+*). We observed that this change strongly enhanced the *SoxN* mutant phenotype, because denticle formation was nearly completely abolished (Fig. 5D-F). This indicates that Dichaete has a function in the regulation of *svb* expression that is revealed in the absence of *SoxN*. This conclusion is supported by the observation that misexpression of Dichaete in the ventral epidermis results in ectopic denticle formation (P.M.O. and S. Russell, unpublished results).

SoxN and Dichaete antagonize Wg pathway activity in the ventral epidermis

The results described above do not provide much insight into the issue of how opposing extrinsic information is integrated such that a sharp posterior border of *svb* expression is achieved. Instead, our results shift the problem from the regulation of *svb* expression to the regulation of its activators – SoxN and Dichaete. However, additional regulatory functions of SoxN and Dichaete might provide a solution to this problem. Experimental evidence in vertebrate systems has shown that members of the Sox protein family negatively regulate the activity of the Wg pathway in different developmental contexts (Akiyama et al., 2004; Haremaki et al., 2003; Mansukhani et al., 2005; Zorn et al., 1999). Sox proteins were shown to reduce Wg signaling by sequestering nuclear β-catenin and preventing its binding to TCF factors. Nuclear β-catenin acts as a transcriptional co-activator at Wg target genes by associating with dTCF (Cadigan and Nusse, 1997). A possible role for *Drosophila* Sox proteins in antagonizing Wg signaling has not been explored so far.

This prompted us to examine whether SoxN and Dichaete might have a function in restricting Wg pathway activity. Due to the function of SoxN and Dichaete in the regulation of *svb* expression, analysis of the cuticle phenotype, the classic read-out for Wg signaling, does not provide a suitable model. Instead, we analyzed the expression of the *en* gene, which is positively regulated by Wg signaling. During the embryonic stages 9 and 10, the maintenance of En expression in epithelial stripes requires Wg signaling and the width of the En stripe is a read-out of the strength of Wg signaling. Staining of late-stage-10 *SoxN* mutant embryos revealed an expansion of the En stripe from the wild-type 2-cell width to a 3- to 4-cell width at the ventral midline (compare Fig. 6A with 6C). This observation demonstrates that, in *SoxN* mutant embryos, Wg pathway activity is increased. Conversely, misexpression of *SoxN* (*armVP16Gal4-UAS-SoxN*) resulted in a partial decay of *en* expression from stage 10 onwards, indicating a reduction in Wg pathway activity (Fig. 6B). These results show that SoxN negatively regulates Wg pathway activity in the ventral epidermis. To determine whether Dichaete can antagonize Wg pathway activity, we reduced Dichaete function in a *SoxN* mutant background (*SoxN;D/+*) and examined late-stage-10 embryos with the anti-En antibody. We observed an expansion of the En stripe from a 3- to 4-cell width in *SoxN* single mutant embryos to a 5- to 6-cell width in *SoxN;D/+* embryos at the ventral midline (Fig. 6D). This result indicates that Dichaete and SoxN have redundant functions in restricting Wg pathway activity.

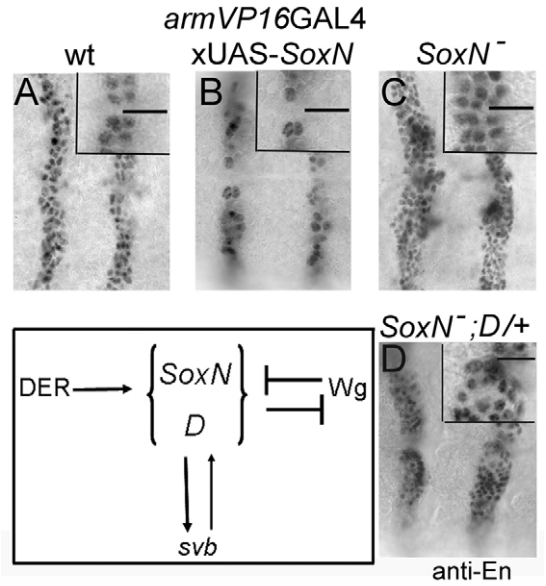

Fig. 6. SoxN and Dichaete negatively regulate Wg pathway activity. (**A-D**) Anti-En stainings of late-stage-10 embryos. Insets show higher magnifications of the En expression around the ventral midline. Black lines indicate the ventral midline. (A) Wild type (wt). (B) *armVP16Gal4-UAS-SoxN*; notice the reduction in En expression. (C) $SoxN^{U6\text{-}35}$; notice the increase in En expression. (D) $SoxN^{U6\text{-}35}$;*D/+*; notice that the increase in En expression is stronger than in *SoxN* single mutants. Cartoon: regulatory interactions between the Der- and Wg-pathway activities, SoxN, Dichaete (denoted by D) and *svb*. SoxN and Dichaete expression is stimulated by Der- and repressed by Wg-pathway activity. SoxN and Dichaete negatively regulate Wg pathway activity and activate the expression of *svb*. *svb* is not required for the establishment of the epidermal SoxN and Dichaete expression domains. However, *svb* is necessary for the maintenance of SoxN and Dichaete expression during the late stages of embryogenesis.

DISCUSSION

In the embryonic ventral epidermis of *Drosophila*, two alternative cell fates are specified: smooth cells and trichome-producing cells. These binary cell fates are distinguished by the expression of *svb*, the most-downstream effector of epidermal morphogenesis. *svb* is necessary and sufficient to cell-autonomously direct trichome formation (Payre et al., 1999). The expression of *svb* is regulated by the opposing gradients of two signaling molecules: Spi, which activates, and Wg, which represses, *svb* expression. *svb* is expressed in segmentally reiterated, epidermal stripes, which invariantly encompass six rows of cells. This raises the question of how is opposing extrinsic information integrated to establish a distinct domain of *svb* expression with a sharp posterior border?

In this study, we demonstrate that the HMG-domain proteins SoxN and Dichaete represent a molecular link between the expression of *svb* and the upstream Der- and Wg-signaling cascades. We show that SoxN and Dichaete are expressed in the ventral epidermis at the time when epidermal cell fates are specified. The late phase of SoxN and Dichaete expression is stimulated by Der- and repressed by Wg-pathway activity. These regulatory mechanisms result in the expression of SoxN and Dichaete in those six rows of cells within each abdominal segment that differentiate to produce trichomes. SoxN and, to a lesser extent, Dichaete, are necessary and sufficient to activate the expression of *svb*. Furthermore, our results show that the well-described repression of *svb* by Wg is due to the repression of SoxN, which, in turn, results in the loss of *svb* activation. Likewise, the Spi-mediated activation of *svb* expression relies on the activation of SoxN, which, in turn, activates *svb*. This indicates that the competition of Der- and

Wg-pathway activities for the specification of trichome-producing versus smooth cell fates is resolved at the level of SoxN and Dichaete.

These results do not provide much insight into the issue of how opposing extrinsic information is integrated such that a sharp posterior border of *svb* expression is achieved. Instead, they raise the question of how is a sharp posterior border of SoxN and Dichaete expression established/maintained? Our findings suggest that this is achieved by a combination of negative- and positive-feedback loops (Fig. 6, cartoon). First, we provide evidence that SoxN and Dichaete negatively regulate Wg pathway activity. This negative-feedback loop provides a likely mechanism for the establishment and maintenance of a sharp posterior border of SoxN and Dichaete expression. The issue arises of how robust this system might be in the face of fluctuating levels of Wg pathway activity. The efficiency with which SoxN and Dichaete restrict Wg pathway activity will crucially rely on the levels of SoxN and Dichaete protein. In this context, it is noteworthy that the levels of SoxN protein, but not Dichaete, are several-fold higher in the two posterior-most rows of the SoxN stripe compared with the anterior four rows (Fig. 1F). The regulatory mechanisms that underlie the different levels of SoxN expression are currently unclear. Second, we provide evidence that the maintenance of SoxN and Dichaete expression is supported by a positive-feedback loop: *svb*, the expression of which is activated by SoxN and Dichaete, is itself required for the maintenance of SoxN and Dichaete expression (Fig. 6, cartoon). Together, these mechanisms contribute to an invariant read-out of cell identity from opposing Der- and Wg-pathway activities.

In *Drosophila*, SoxN and Dichaete are necessary and sufficient to activate the expression of *svb*, which in turn directly regulates the expression of genes involved in trichome morphogenesis (Chanut-Delalande et al. 2006). Is a function in hair formation of the Sox proteins conserved in other species, including vertebrates? A previous study has shown that the mouse Sox9 protein is required for the differentiation of hair-producing epidermal cells and acts genetically downstream of sonic hedgehog pathway activity (Vidal et al., 2005). This study did not address whether Sox9 regulates the expression of *movo1* (*Ovol1*), the mouse ortholog of *svb* (Dai et al., 1998). Nevertheless, the demonstrated roles of SoxN, Dichaete and Sox9 raise the exciting question of do Sox proteins have an essential function in the activation of an epidermal differentiation program that is conserved across species as distantly related as mice and flies?

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Note added in proof

A study by Chao et al. confirms our observation that SoxN controls Wg signaling (Chao et al. , 2007). This study suggests that SoxN does not antagonize Wg signaling by sequestering *armadillo*.

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