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RESEARCH ARTICLE

SoxNeuro and Shavenbaby act cooperatively to shape denticles in the embryonic epidermis of *Drosophila*

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ABSTRACT

During development, extracellular signals are integrated by cells to induce the transcriptional circuitry that controls morphogenesis. In the fly epidermis, Wingless (Wg)/Wnt signaling directs cells to produce either a distinctly shaped denticle or no denticle, resulting in a segmental pattern of denticle belts separated by smooth, or 'naked', cuticle. Naked cuticle results from Wg repression of *shavenbaby* (*svb*), which encodes a transcription factor required for denticle construction. We have discovered that although the *svb* promoter responds differentially to altered Wg levels, Svb alone cannot produce the morphological diversity of denticles found in wild-type belts. Instead, a second Wg-responsive transcription factor, SoxNeuro (SoxN), cooperates with Svb to shape the denticles. Co-expressing ectopic *SoxN* with *svb* rescued diverse denticle morphologies. Conversely, removing *SoxN* activity eliminated the residual denticles found in *svb* mutant embryos. Furthermore, several known Svb target genes are also activated by SoxN, and we have discovered two novel target genes of SoxN that are expressed in denticle-producing cells and that are regulated independently of Svb. We conclude that proper denticle morphogenesis requires transcriptional regulation by both SoxN and Svb.

KEY WORDS: Wingless, Wnt, Shavenbaby, Ovo, SoxNeuro, Denticle, Morphogenesis, *Drosophila melanogaster*

INTRODUCTION

Information from signaling pathways must be interpreted and modulated for the proper execution of cellular events during development. Much of developmental biology research has focused on the signaling pathways that govern cell fate specification. However, this approach reveals only what the upstream signals are, not what factors integrate and implement this activity to orchestrate downstream morphogenetic events. Understanding the bridge from signal to pattern will ultimately reveal how cells take proper form during development.

Pattern in developing tissues is generated by highly organized cell movements and morphogenetic rearrangements (Pilot and Lecuit, 2005). For example, differentiation of epithelial cells often features cell shape changes that are driven by the actin-based cytoskeleton. Such processes include the formation of denticles, hairs or sensory bristles in *Drosophila* (Appel et al., 1993) and the stereocilia of the vertebrate inner ear (Tilney et al., 1992). To understand how these developmental events are fine-tuned requires an *in vivo* system where signaling pathways and their output can be readily analyzed.

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Denticle formation in the ventral epidermis of the *Drosophila* embryo provides an excellent system in which to answer such questions (reviewed by Bejsovec, 2013). Denticles are hook-like structures produced by ventral epidermal cells during late embryogenesis (Fig. 1A). They form in a stereotyped pattern as actin-based protrusions that extend from the apical side of the cell and become coated with chitinous cuticle. These provide traction for the crawling larva after the embryo hatches. Denticles are organized in segmentally repeating units known as belts. Each belt is composed of six rows of cells, where each row produces a denticle of specific size, shape and polarity (Fig. 1B,C). The denticle belts are separated by regions of 'naked cuticle', which are secreted by cells that do not produce any actin-based protrusions. The naked cuticle region is specified by high-level Wg signaling (Bejsovec and Martinez Arias, 1991; Noordermeer et al., 1992), which mediates repression of the transcription factor *shavenbaby* (*svb*; *ovo* – FlyBase) (Payre et al., 1999) in a striped pattern (Fig. 1D-F). Svb is a zinc-finger transcription factor (Mevel-Ninio et al., 1995; Andrews et al., 2000; Delon et al., 2003) that activates a multitude of genes involved in actin organization, extracellular matrix secretion, or cuticle deposition (Chanut-Delalande et al., 2006). Svb has been considered the primary effector of denticle morphogenesis, integrating inputs from multiple signaling pathways and converting them to structural output.

The cytoskeletal processes that form denticles begin with polarized actin filaments that accumulate and acquire shape over a period of ~2 h (Dickinson and Thatcher, 1997; Price et al., 2006; Walters et al., 2006; Bejsovec and Chao, 2012). During this time, proteins involved in cytoskeletal reorganization, many of which are transcriptionally regulated by Svb, begin to localize to the denticles. For example, Svb directs the expression of *forked*, which encodes an actin-bundling protein that functions in denticle and dorsal hair formation (Chanut-Delalande et al., 2006) as well as in adult bristle formation (Grieshaber and Petersen, 1999). The genes *singed* (Cant et al., 1994) and *shavenoid* (Ren et al., 2006) are also controlled by Svb, and function during embryogenesis in actin dynamics and denticle formation. Other proteins that function in cytoskeletal dynamics, such as Diaphanous, Enabled and Arp2/3, are not influenced by Svb activity but localize to denticles and aid in their construction. Human homologs of these factors promote an analogous process to construct stereocilia in the developing ear; their loss of function results in physical defects that can lead to hearing impairment and deafness (reviewed by Chanut-Delalande et al., 2012).

Here we explore how the morphological diversity of denticles within the belt is generated. Wg activity is required for the diversification of denticles, in a process that is less clearly understood than the Wg-mediated specification of naked cuticle. In the absence of *wg* gene function, not only is the naked cuticle component of the pattern lost, but also the denticles produced are reduced to a single type of denticle, similar to those in row 5

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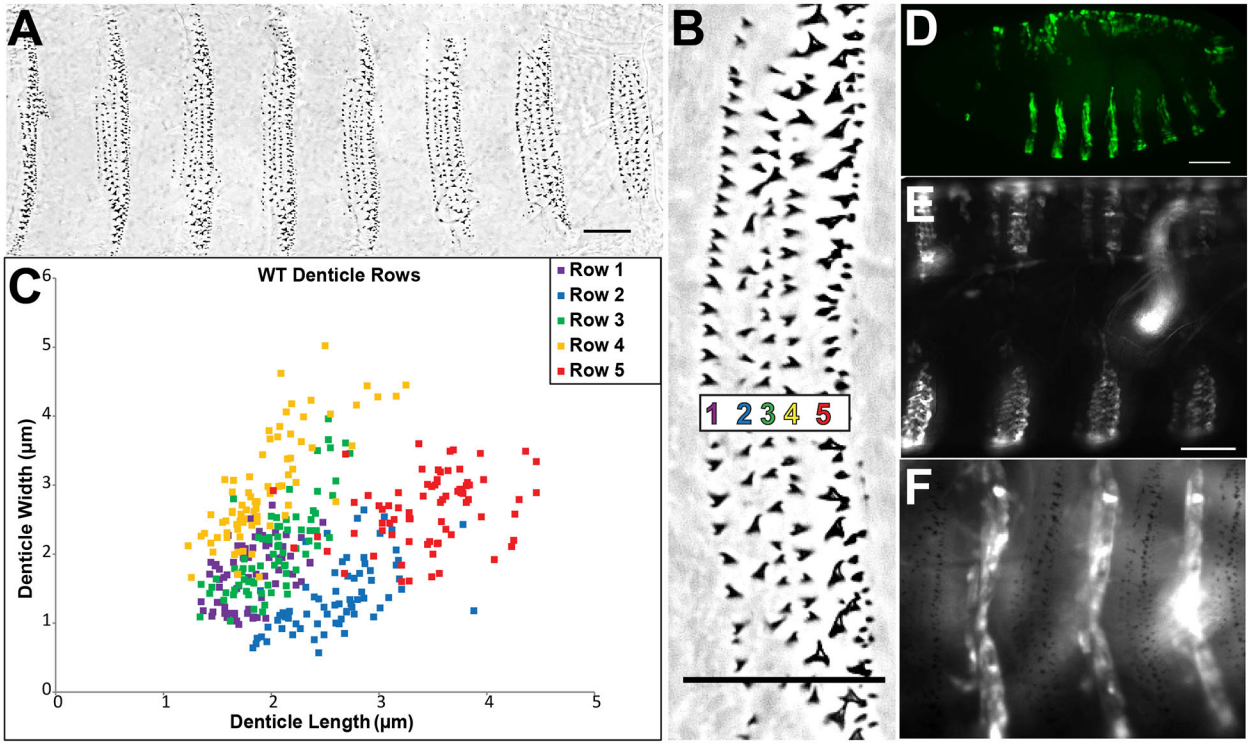

Fig. 1. Denticles display a stereotyped morphological diversity. (A) Ventral epidermal cells of wild-type (WT) *Drosophila* embryos secrete a segmental pattern of denticle belts. In this and all subsequent images, anterior is to the left unless otherwise noted. (B) Each WT belt consists of six rows of cells, each producing a characteristic size, shape and polarity of denticle. (C) Denticle diversity in the anterior five rows (each row is color coded) was quantified by measuring length and base width. (D,E) *7.3-Gal4*, controlled by the *svb* proximal promoter element, drives *UAS-GFP* expression in segmental stripes in embryos (D; anti-GFP antibody staining). These stripes coincide with the denticle-producing cells in freshly hatched larvae (E; live-imaged GFP, lateral view with dorsal up). (F) *wg-Gal4*-driven *UAS-GFP* shows that the *wg* expression domain lies beneath the naked cuticle portion of the segmental pattern (live-imaged GFP, ventral view). Scale bars: 50 µm.

(Nusslein-Volhard et al., 1984, 1985; Bejsovec and Wieschaus, 1993). This diversification cannot be explained by Svb modulation alone. Although *svb* is necessary and sufficient for denticle formation, ectopically expressed *svb* is unable to recapitulate the denticle morphologies characteristic of the wild-type belt (Payre et al., 1999). This suggested that additional factors are required for the formation of specific denticle shapes. We have discovered that a second transcription factor, encoded by *SoxNeuro* (*SoxN*), is necessary and sufficient for denticle formation, and its ectopic co-expression with *svb* rescues denticle morphology to more closely approximate wild-type structures. These observations, along with the previously described antagonistic relationship of SoxN with the Wg pathway (Chao et al., 2007), suggest that SoxN might act at the interface of signaling pathway and effector output during denticle morphogenesis.

RESULTS

svb promoter elements respond differentially to altered Wg

Wg signaling generates denticle diversity in the ventral epidermis between 4 and 6 h after egg laying, and then specifies naked cuticle from 6 to ∼10 h (Bejsovec and Martinez Arias, 1991). We have recently found that Wg is also required at later developmental stages for proper denticle morphogenesis (Bejsovec and Chao, 2012). These findings suggested that Wg activity in denticle-producing cells might titrate Svb levels, where different threshold levels of Svb would direct the diverse morphologies within a belt. To test this idea, we obtained several well-characterized *svb* enhancer constructs that drive expression in ventral denticle-producing cells as well as in dorsal trichome-forming cells (McGregor et al., 2007; Frankel et al., 2010). The *7.3* 'proximal' enhancer, which is located close to the *svb* transcription start site (McGregor et al., 2007), drives expression in all of the ventral denticle-producing cells (Fig. 1D,E), whereas enhancers located more distal to the *svb* start site drive expression in subsets of the overall *svb* domain. The *DG3* enhancer promotes expression in a single row of cells at the posterior edge of each belt (Fig. 2A), in roughly the position where row 5 denticles will form in the mature epidermis. This expression domain expanded slightly when Wg activity was partially reduced in wg^{PE2} mutant embryos (Fig. 2B). The wg^{PE2} missense allele produces a protein that is distributed normally across the segment and is sufficient to generate denticle diversity, but cannot bind the Frizzled 2 receptor strongly enough to specify naked cuticle (Hays et al., 1997; Moline et al., 2000). Expansion of *DG3* enhancer-driven expression was greater but still not uniform when *wg* activity was removed completely in wg^{ts} mutant embryos at restrictive temperature (Fig. 2C). By contrast, the *E3* enhancer drives two stripes of expression in each denticle-producing belt: an anterior stripe that roughly prefigures where rows 1 and 2 will form, and a more posterior stripe at row 5 (Fig. 2D). This more complex pattern of ventral expression suggests that there might be multiple inputs into the activity of this enhancer. Indeed, we found that the two stripes of *E3*-driven expression respond differently to varying levels of Wg signaling. Reduced Wg activity in wg^{PE2} embryos allows the anterior stripe to expand in an anterior direction (Fig. 2E) into cells that produce diverse denticle types at a position where naked cuticle would form in a wild-type embryo. We observe a similar effect when wg^{ts} embryos are cultured at permissive temperature for the first 5 h of development (Fig. S1A,B), which generates denticle

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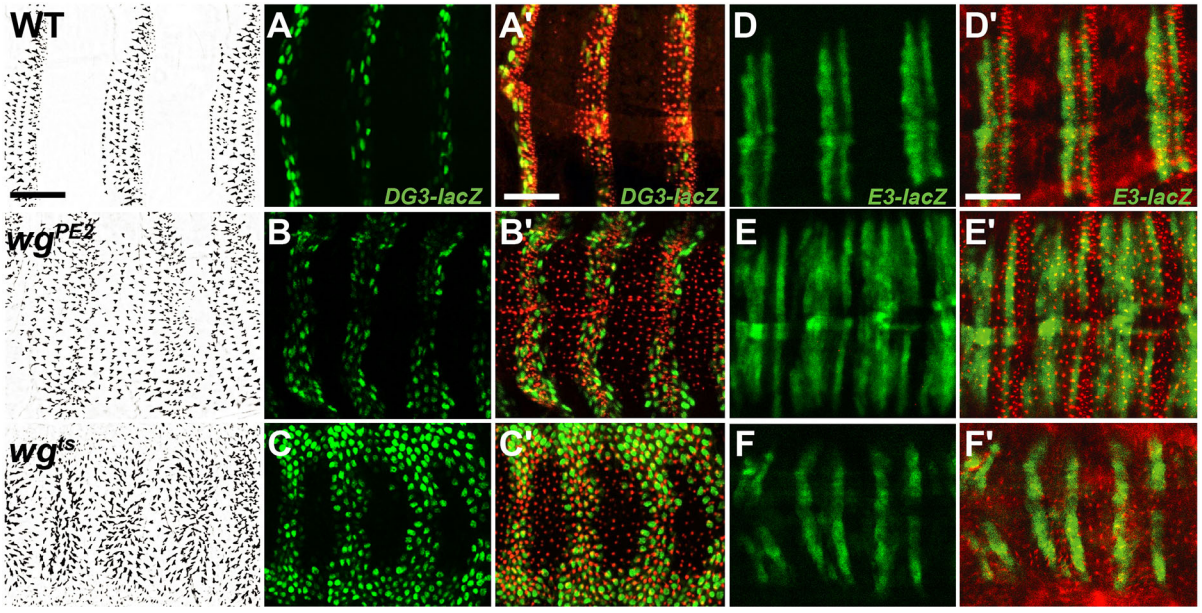

Fig. 2. *svb* promoter elements exhibit sensitivity to Wg activity. (A) The *svb DG3* enhancer drives *lacZ* expression (green) in a subset of posterior denticle-producing cells in each belt. Rhodamine phalloidin (red in A′-F′, which show merges) highlights developing denticles in embryos 14 to 16 h after egg laying. (B) wg^{PE2} embryos have partial Wg function, producing normal denticle diversity but no naked cuticle. In these mutants, *DG3* expression expanded to a width of three to four rows of cells. (C) wg^{ts} embryos at restrictive temperature (25°C) lose both naked cuticle and denticle diversity. In these mutants, *DG3* enhancer expression expanded more extensively, particularly along the ventrolateral surfaces. (D) The *svb E3* enhancer drives expression in a pair of unequal stripes underlying each WT segmental belt. (E) In wg^{PE2} mutants, each anterior stripe of the pair expanded in an anterior direction, into cells that show increased denticle diversity when cuticle is secreted. (F) *wg* loss of function produced the opposite effect on *E3*-driven expression: no change in the anterior stripe, and a slight expansion of the posterior stripe so that it was equal in width to the anterior stripe. Scale bars: 50 µm.

diversity without naked cuticle specification (Bejsovec and Martinez Arias, 1991). The opposite effect was seen when *wg* function was eliminated more completely; the anterior *E3* stripe was unchanged while the posterior stripe expanded in width to match the anterior stripe, both in wg^{ts} mutants cultured at restrictive temperature (Fig. 2F) and in wg^{CX4} null mutants (Fig. S1C). Thus, the *DG3* and *E3* promoter elements of *svb* are sensitive to Wg activity level.

Increasing levels of Svb affect denticle length but not morphology

The differential responsiveness of *svb* enhancers led us to test whether different levels of Svb might specify distinct denticle shapes. We therefore developed an assay for denticle formation. The *wg-Gal4* line drives ectopic *UAS* transgene expression in a row of one to two cells that would be fated to produce naked cuticle (Fig. 1F). Thus, any denticles formed in this ectopic location are the direct result of transgene activity. *wg-Gal4*-driven *UAS-svb* (*wg>svb*) produced a stripe of ectopic denticles that are long and thin, lacking the widened base that is characteristic of denticles within wild-type belts (Fig. 3A). This defective morphology has been observed previously when *UAS-svb* was driven with a variety of *Gal4* drivers (Payre et al., 1999). We measured the length and width of these ectopic denticles and compared them with wild-type denticle measurements: *wg>svb* denticles consistently fell below the normal width of any denticle in the wild-type belt (Fig. 1C, Fig. 3B). This observation suggests that either higher levels of Svb than are produced by this system are required for proper denticle shape, or that additional factors are required.

To differentiate between these two possibilities, we generated fly lines that carry multiple copies of the *UAS-svb* transgene. Driving multiple *UAS-svb* transgenes with *wg-Gal4* produced denticles that are on average 13.9% longer than those produced by a single transgene (Fig. 3C,D). The additional length of these *wg>svb;svb* denticles shows that Gal4 levels are not limiting for driving both *UAS* transgenes at high levels. However, these longer ectopic denticles were thin and lacked the widened base (Fig. 3D), and thus were similar in shape to those produced by a single transgene. We conclude that Svb transcription factor output controls denticle length, but alone is not sufficient to produce normal denticle shape.

Co-expression of *SoxN* and *svb* rescues ectopic denticle morphology

Because higher levels of Svb did not rescue ectopic denticle morphology, we suspected that *wg-Gal4*-expressing cells might lack additional factor(s) necessary for proper denticle morphogenesis. We had previously isolated mutations in *SoxN* as suppressors of *wg* loss-of-function phenotypes, and we demonstrated that this Sox class transcription factor plays a role in downregulating Wg pathway activity (Chao et al., 2007). *SoxN* is expressed in the bands of cells that produce denticle belts, and its activity is required for establishing the proper expression domain of *svb* (Overton et al., 2007). There is also a feedback loop whereby Svb is necessary for the maintenance of *SoxN* expression (Overton et al., 2007). When we examined *SoxN* in our denticle assay system we found that, like *svb*, it was able to promote ectopic denticle formation. *wg>SoxN* ectopic denticles were fewer in number and were shorter than those produced by ectopic *svb* (Fig. 3E,F). On average, the *wg>SoxN* ectopic denticles also had wider bases than *wg>svb* denticles, raising the possibility that SoxN activity controls base width whereas Svb controls length. Thus, the combined activities of the transcription factors might be necessary for shaping denticles properly.

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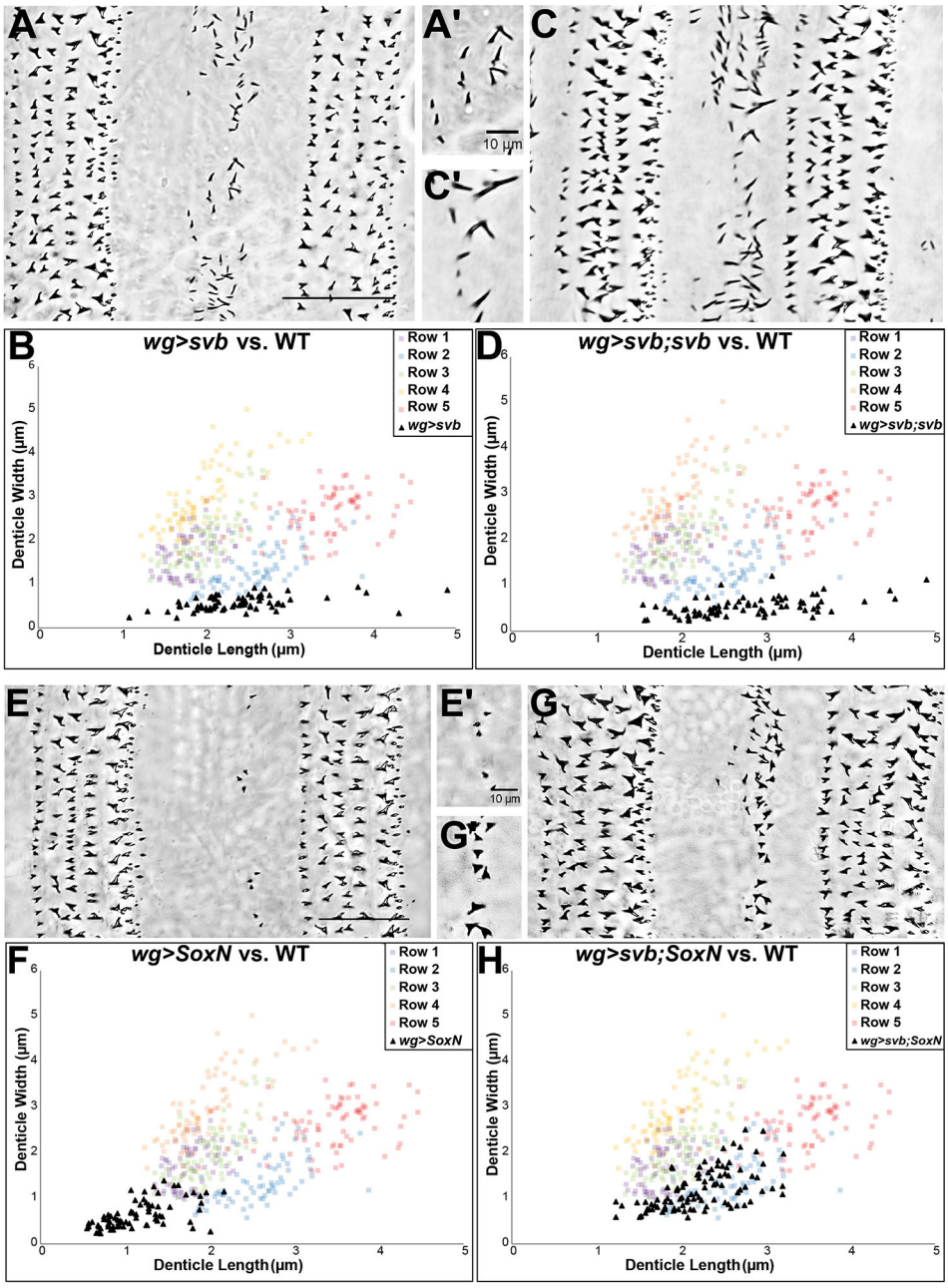

Fig. 3. Ectopic *svb* alters denticle length but not shape, whereas co-expression with *SoxN* rescues shape. (A) *wg-Gal4* drives ectopic expression of *UAS-svb* in what would normally be naked cuticle. This ectopic expression was sufficient to form denticles. (A′,C′,E′,G′) Higher magnification views from A,C,E,G. (B) Measurements of ectopic denticles (black triangles) lie outside the range of wild-type denticle measurements (light-colored boxes): the *wg>svb* denticles are narrower at the base than WT, although their lengths fall mostly within the range of WT lengths (mean=2.44, s.d.=0.72). (C) Increasing the dose of Svb, through the expression of two *UAS-svb* transgenes, yielded longer denticles without rescuing shape. (D) Measurements of these ectopic denticles (black triangles) shifted the plots along the *x*-axis (length, mean=2.78, s.d. =0.72) without altering the *y*-axis values (width). (E) *UAS-SoxN* was sufficient to form small, blunted denticles when ectopically expressed with the *wg-Gal4* driver. (F) SoxN-induced ectopic denticles are of reduced length and width compared with WT denticles. (G) Co-expression of *UAS-SoxN* with *UAS-svb* produced ectopic denticles that more closely resembled WT than did those produced by either transgene alone. (H) The length and width of these ectopic denticles significantly overlap the range of sizes found in a WT denticle belt. Scale bars: 50 µm, except 10 µm in A′,C′,E′,G′.

To test this hypothesis, we co-expressed *UAS-svb* and *SoxN* with *wg-Gal4* in our denticle formation assay. Expression of both transcription factors together dramatically rescued the morphology of ectopic denticles, particularly the widened base that is highly characteristic of most wild-type denticles (Fig. 3G). Comparing length and base width values with those of wild-type denticles revealed that these ectopic denticles fell within the range of wild-type denticle measurements (Fig. 3H). Furthermore, these ectopic denticles exhibited diverse morphologies. The majority were similar to row 2 denticles, with a minority resembling the denticles of rows 1 or 3.

Because our denticle formation assay involved the expression of transgenes in the endogenous *wg* expression domain, it was possible that high-level Wg signaling in these cells might somehow block the ability of Svb to direct proper denticle morphogenesis. Since SoxN downregulates Wg pathway activity, the co-expression of *SoxN* might then reduce Wg activity, removing its putative block on Svb and indirectly altering denticle shape. To test this possibility, we used a different *Gal4* driver line to drive expression outside of the *wg* expression domain. The *engrailed* (*en*)-*Gal4* transgene is expressed in the two rows of epidermal cells posterior to the *wg*-expressing cells: the row of naked cuticle cells just anterior to the denticle belts and the first row of denticle-producing cells (DiNardo et al., 1985). Driving *UAS-svb* in the *en* domain produced ectopic denticles similar in shape and size to those produced with *wg-Gal4* (Fig. S2A). Likewise, *en-Gal4*-driven co-expression of *svb* and *SoxN* rescued denticle morphology, just as *wg-Gal4*-driven co-expression did (Fig. S2B). Because distance from Wg production did not alter ectopic denticle morphology, it is likely that the ability of SoxN to rescue morphology is at least partially independent of its ability to suppress Wg activity.

To further explore the interplay of Svb and SoxN in driving denticle morphogenesis, we examined loss-of-function mutant

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phenotypes. *SoxN* null mutations result in a slight decrease in the denticle belt expanse and some defects in denticle morphology (Chao et al., 2007; Overton et al., 2007) (Fig. 4A). By contrast, *svb* loss-of-function mutations show a more severe loss of both ventral denticles (Fig. 4B) and dorsal trichomes (Payre et al., 1999). Some denticles remain in the most posterior rows, namely rows 5 and 6, of each belt; these remaining denticles have been described as 'atrophied' or 'blunted' (Payre et al., 1999). Coincidentally, immunolocalization of SoxN in wild-type embryos suggests that it is present at higher levels in rows 5 and 6 of the denticle-producing cells (Overton et al., 2007). Thus, SoxN activity might account for the denticles remaining in *svb* mutants. If so, loss of both *svb* and *SoxN* together would be predicted to block denticle formation completely. We found this to be the case: *svb;SoxN* double-mutant embryos produce uniformly naked cuticle (Fig. 4C), suggesting that the atrophied structures displayed by *svb* mutants result from SoxN activity.

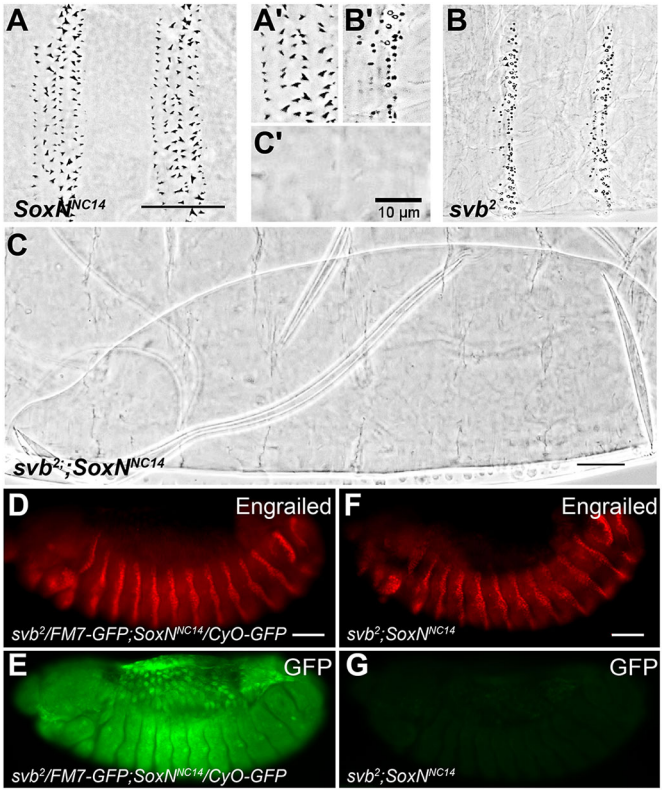

Fig. 4. Loss of both Svb and SoxN results in embryos that lack denticles. (A) *SoxN* mutants produce defective denticle morphologies and a slight excess of naked cuticle. (B) *svb* mutants produce excess naked cuticle but retain small, blunted denticles in posterior rows of each belt. (C) *svb;SoxN* double mutants completely lack ventral denticles, showing a fully penetrant uniform naked cuticle phenotype [6.21% (21/338) of total fertilized embryos, which is close to the expected 6.25% frequency of double-mutant embryos]. (A′-C′) Higher magnification views from A-C. (D-G) This phenotype was not due to excessive Wg signaling, as *engrailed* (*en*) target gene expression was not altered. (D) Anti-En antibody staining was detected in two rows of cells posterior to the *wg*-expressing cells in each segment of 10 h embryos. (E) Heterozygous siblings were identified by their expression of GFP from tagged balancer chromosomes. (F) En expression was not expanded in embryos that were homozygous mutant for both *svb* and *SoxN*, as verified by the absence of GFP fluorescence from the tagged balancer chromosomes (G). This pattern would be expected to expand to four rows of cells in a *wg* gain-of-function situation (Noordermeer et al., 1992; Pai et al., 1997). Scale bars: 50 µm, except 10 µm in A′-C′.

An alternative explanation for this all-naked phenotype is that Wg signaling might be hyperactivated in the absence of *svb* and *SoxN*. High and uniform expression of *wg* throughout the epidermis results in a comparable loss of all ventral denticles, although such embryos also show reduced body size and severe head defects (Noordermeer et al., 1992), which we do not observe. We examined the expression of *en*, a known Wg target gene (DiNardo et al., 1988; Ingham et al., 1988) that expands in expression in embryos with hyperactivated Wg signaling (Noordermeer et al., 1992; Pai et al., 1997). We found that *en* expression did not expand in *svb;SoxN* double mutants (Fig. 4F,G), as compared with their heterozygous siblings (Fig. 4D,E). Thus, the naked cuticle phenotype shown by *svb;SoxN* double mutants was not due to hyperactivity of Wg signaling, but rather to a downstream requirement for both transcription factors in driving denticle formation.

SoxN is sufficient to form denticles independently of Svb

We have shown that *SoxN* is sufficient to produce denticles when ectopically expressed and appears to be responsible for the atrophied denticles present in *svb* mutants. However, because of the feedback interactions between SoxN and *svb*, we wondered to what extent SoxN could promote denticle formation independently of *svb*. We drove high and uniform expression of *UAS-SoxN* in the epidermis of *svb* mutants using the *E22c-Gal4* line. *E22c>SoxN* in *svb* mutants resulted in the production of ventral denticles with the atrophied morphology (Fig. 5A,B). These atrophied denticles were arranged in a segmental pattern reminiscent of denticle belts, with fewer denticles in the region where naked cuticle would normally form. This suggests the presence of additional pattern information that is independent of Svb and SoxN. We also observed the restoration of some trichomes on the dorsal surface (Fig. 5C,D). This was surprising because dorsal trichomes had been definitively connected with *svb* activity (Sucena and Stern, 2000) and dorsal epidermal cells do not normally express *SoxN* (Cremazy et al., 2000). Thus, SoxN appears able to substitute for Svb in dorsal element formation, suggesting that *svb* and *SoxN* have some shared target genes.

SoxN differentially activates ZPD genes

We set out to identify genes that might be subject to Svb and SoxN co-regulation. We first tested whether any known Svb target genes were also responsive to SoxN. A major class of Svb targets are the Zona Pellucida domain (ZPD)-containing proteins that associate with the apical extracellular matrix of cuticle (Fernandes et al., 2010). Each ZPD protein localizes to a specific compartment within the developing denticle, with non-redundant functions in sculpting denticle shape. The well-studied ZPD gene *miniature* (*m*) was activated robustly by ectopic expression of *svb* (Chanut-Delalande et al., 2006) (Fig. 6A). We found that ectopic expression of *SoxN* did not activate ectopic *m* expression (Fig. 6B), nor did it contribute to the strength of *m* expression when co-expressed with *svb* (Fig. 6C). Therefore, SoxN does not appear to play a role in *m* regulation. Furthermore, this indicated that any Svb that is coincidentally induced by ectopic SoxN through the feedback loop is not sufficient to drive *m* target gene activation.

Next we tested ZPD candidates that were found to have residual expression in *svb* mutants, such as *nyobe* (*nyo*). In *svb* mutants, *nyo* expression levels were reduced substantially, but some expression remained in the posterior rows of denticle-producing cells (Fernandes et al., 2010). We found that expressing either *svb* or *SoxN* alone was insufficient to activate detectable expression of *nyo* (Fig. 6D,E). Even doubling the amount of ectopic *svb* was

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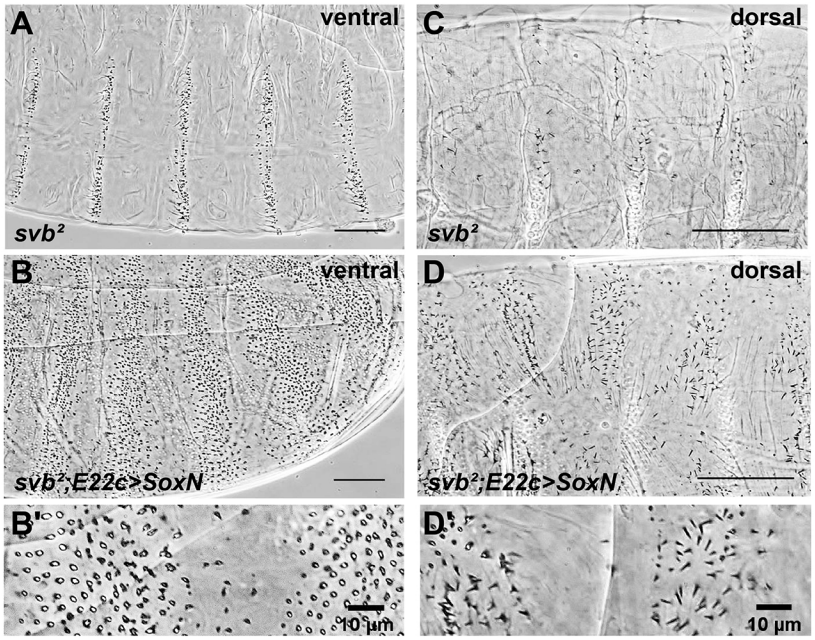
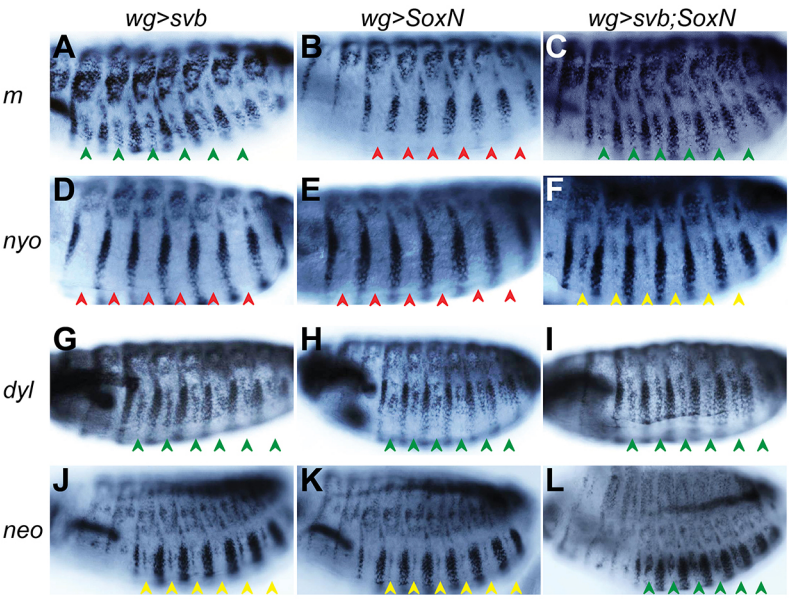


Fig. 5. SoxN is sufficient to form denticles independently of Svb. (A) In *svb* mutants, a small number of blunted denticles were retained in cells that comprise rows 5 and 6 within each belt. (B) Uniform epidermal *UAS-SoxN* expression, driven by *E22c-Gal4*, increased the number of blunted ventral denticles. (C,D) Most dorsal trichomes were lost in *svb* mutant embryos (C), but some were restored by ectopic *SoxN* expression (D). (B′,D′) Higher magnification views of B,D. Note that the pattern of dorsal trichomes and blunted denticles shows segmental modulation (B,D) even though Svb is absent and SoxN is produced ubiquitously. Scale bars: 50 µm, except 10 µm in A′-C′.

insufficient to activate *nyo* (Fig. S3). By contrast, co-expression of *svb* and *SoxN* together activated moderate *nyo* expression (Fig. 6F). Thus, *nyo* requires input from both Svb and SoxN for proper expression.

In examining other ZPD gene products, we discovered two Svb targets that can be activated by either Svb or SoxN. The gene *dusky-like* (*dyl*) was activated when *svb* was expressed ectopically (Fig. 6G), but was also detected strongly when *SoxN* alone was



M

	wg>svb	wg>SoxN	wg>svb;SoxN
m	Strong	None	Strong
nyo	None	None	Moderate
dyl	Strong	Strong	Strong
neo	Moderate	Moderate	Strong

Fig. 6. SoxN differentially activates ZPD genes. (A-L) Whole-mount *in situ* hybridization of 14-16 h embryos. Arrowheads indicate the *wg* domain where transgenes were expressed ectopically; the color describes the relative strength of gene activation: red, none; yellow, moderate; green, strong. (A) *UAS-svb*, driven ectopically with *wg-Gal4*, was sufficient to activate *m* expression. (B) Ectopic *UAS-SoxN* did not activate *m* expression. (C) Level of *m* expression when both transcription factors were ectopically expressed was no higher than that of *UAS-svb* alone. (D-F) *nyo* expression was not activated through the expression of either *svb* (D) or *SoxN* (E) alone, but was activated when they were co-expressed (F). (G-I) *dyl* expression was activated at substantial levels by either *svb* (G) or *SoxN* (H), and was not driven to higher levels by the co-expression of *svb* and *SoxN* (I). (J-L) *neo* expression was detected at low levels when *svb* (J) or *SoxN* (K) alone was ectopically expressed, but showed much stronger expression when both *svb* and *SoxN* were expressed together (L). (M) Summary of the contribution of each transcription factor to target gene activation.

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expressed ectopically (Fig. 6H). There was no discernible difference in *dyl* expression when both factors were ectopically co-expressed (Fig. 6I). By contrast, the gene *neo* was activated weakly when either *svb* or *SoxN* alone was expressed (Fig. 6J,K), but was expressed strongly when both genes were expressed together (Fig. 6L). Thus, we have discovered four classes of Svb downstream targets: those that are activated exclusively by Svb; those that require input from both Svb and SoxN to be activated; those that are activated by either Svb or SoxN with apparently equal efficacy; and those that can be activated by either Svb or SoxN but require both for optimal expression (Fig. 6M).

SoxN activates epidermal genes independently of Svb

We next tested whether SoxN could direct the expression of epidermal target genes independently of Svb. We used an image search tool in Fly Express (http://www.flyexpress.net/) to find genes that exhibit a pattern of expression similar to that of *SoxN*. We identified two uncharacterized genes, *CG16885* and *CG30101*, that met this criterion. These genes are expressed solely in the ventral epidermis, in cells that prefigure the denticle belts. Ectopic expression of *SoxN*, but not of *svb*, activated *CG16885* expression (Fig. 7A,C), indicating that it is a downstream target gene of SoxN. *CG16885* had been previously tested and eliminated as a potential *svb* target via *in situ* hybridization, as its expression did not change in *svb* loss-of-function embryos (Kondo et al., 2010) nor in embryos in which *svb* was knocked down with RNA interference (Fig. 7E). Thus, *CG16885* expression is not regulated by Svb. A second gene, *CG30101*, had not been previously tested for regulation by Svb. We found that ectopic expression of *SoxN* was sufficient to activate *CG30101* (Fig. 7B) and that ectopic expression of *svb* failed to activate *CG30101* expression above background levels (Fig. 7D). As with *CG16885*, driving uniform embryonic epidermal expression of *svb* RNAi produced no discernible change in *CG30101* expression (Fig. 7F), indicating that Svb does not regulate *CG30101*. Under the same conditions, *svb* RNAi phenocopies *svb* null embryos and results in the robust loss of *m* expression (Fig. 7G). Thus, we have identified two epidermal target genes that can be activated by SoxN without input from Svb.

Consistent with these results, both *CG16885* and *CG30101* require input from SoxN for their normal expression during development. We hand-selected *SoxN* homozygous mutant embryos by absence of GFP signal from a tagged balancer chromosome, and performed *in situ* hybridization to detect *CG16885* or *CG30101* expression. The expression of *m* was also tested as a control. In *SoxN* mutants, *m* expression was not affected in the dorsal epidermis, although there appeared to be somewhat weaker expression than normal in the ventral epidermis (Fig. 7J,M). This is likely to be due to the feedback loop whereby SoxN boosts *svb* expression in ventral, but not dorsal, cells. Ventral *CG16885* expression was significantly reduced in *SoxN* mutants compared with their heterozygous siblings (Fig. 7H,K). Expression of *CG30101* was also diminished in *SoxN* mutant embryos, although not as dramatically as *CG16885* (Fig. 7I,L). Together, these results suggest that SoxN is necessary and sufficient for the proper expression of *CG16885* and *CG30101* during development of the embryonic epidermis.

Predicted SoxN binding sites can be identified in target genes

The transcriptional regulation of these SoxN target genes and SoxN-Svb co-targets is likely to be mediated directly. Genome-wide binding patterns and expression profiling of SoxN using DamID

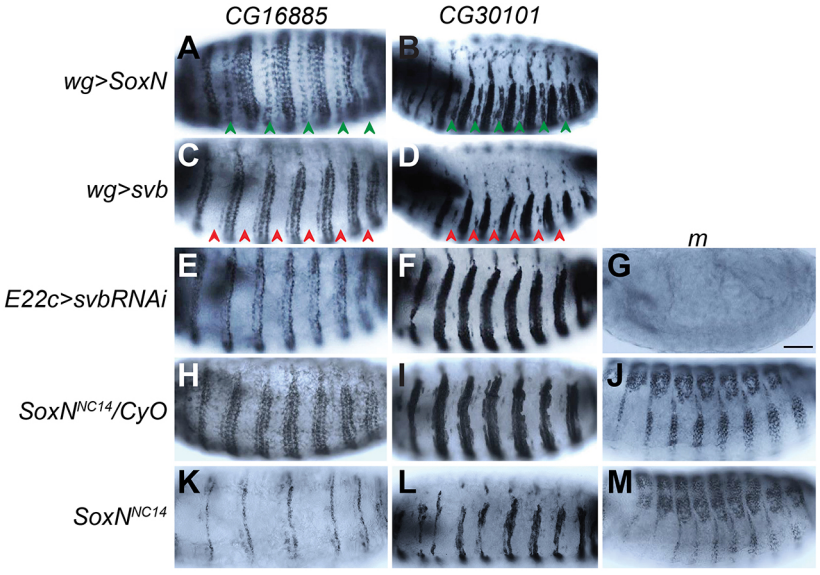

Fig. 7. SoxN activates *CG16885* and *CG30101* independently of Svb. (A-D) Ectopic *SoxN*, driven by *wg-Gal4*, activated expression of *CG16885* (A) and *CG30101* (B), whereas ectopic *UAS-svb* was insufficient to activate expression of either *CG16885* (C) or *CG30101* (D). (E,F) Knockdown of *svb* in the epidermis, using *E22c-Gal4* to drive uniform expression of *UAS-svbRNAi*, did not affect expression of *CG16885* (E) or *CG30101* (F). (G) *svb* RNAi substantially reduced the expression of the known Svb target *m*, indicating that it was effective at reducing Svb activity. (H-M) Both *CG16885* and *CG30101* require input from SoxN. *SoxN*^{NC14} homozygous mutant embryos were hand sorted for the absence of the GFP-tagged balancer chromosome, and probed for *CG16885*, *CG30101* or *m* expression (*n*>100 homozygous mutant embryos for each). Heterozygous *SoxN*/*CyO twist>GFP* siblings, sorted for the presence of GFP, were stained as controls, and showed wild-type patterns of gene expression (H-J). Normal *CG16885* (H) expression levels were reduced in *SoxN* mutant embryos (K), with some residual expression in cells that would produce denticle rows 5 and 6. Normal *CG30101* expression levels (I) showed a less dramatic, but still substantial, reduction in homozygous *SoxN* embryos (L). Wild-type pattern of *m* expression (J) was not changed in *SoxN* homozygous mutants (M), although ventral expression was somewhat reduced. Presumably this was due to the partial dependence of ventral *svb* expression on SoxN activity; dorsal *m* expression (top) appeared normal. Scale bar: 50 µm.

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and ChIP techniques (Ferrero et al., 2014; Carl and Russell, 2015) have shown that SoxN can bind in regulatory regions of *nyo*, *CG16885* and *CG30101*. Likewise, Svb binding sites have been identified in enhancers that control downstream Svb target genes, including *nyo* (Menoret et al., 2013). Locating functional binding sites for Svb and SoxN in *nyo* regulatory regions will help us to understand how these two transcription factors might cooperatively activate the target gene. We performed a computational search for SoxN binding consensus sequences in *nyo*, as well as in *CG16885* and *CG30101*, and identified a number of predicted binding sites (Fig. 8A, asterisks; Table S1). In *nyo*, none of the putative SoxN binding sites that we found falls within the previously identified enhancer region (Fig. 8A, orange bar). All are in introns upstream or downstream of the Svb-binding enhancer element, although one site is located within 1 kb of this enhancer. Since the previously defined region may represent a minimal core enhancer, additional flanking sequences might be required to achieve the robust output required during development or during times of variation in environmental

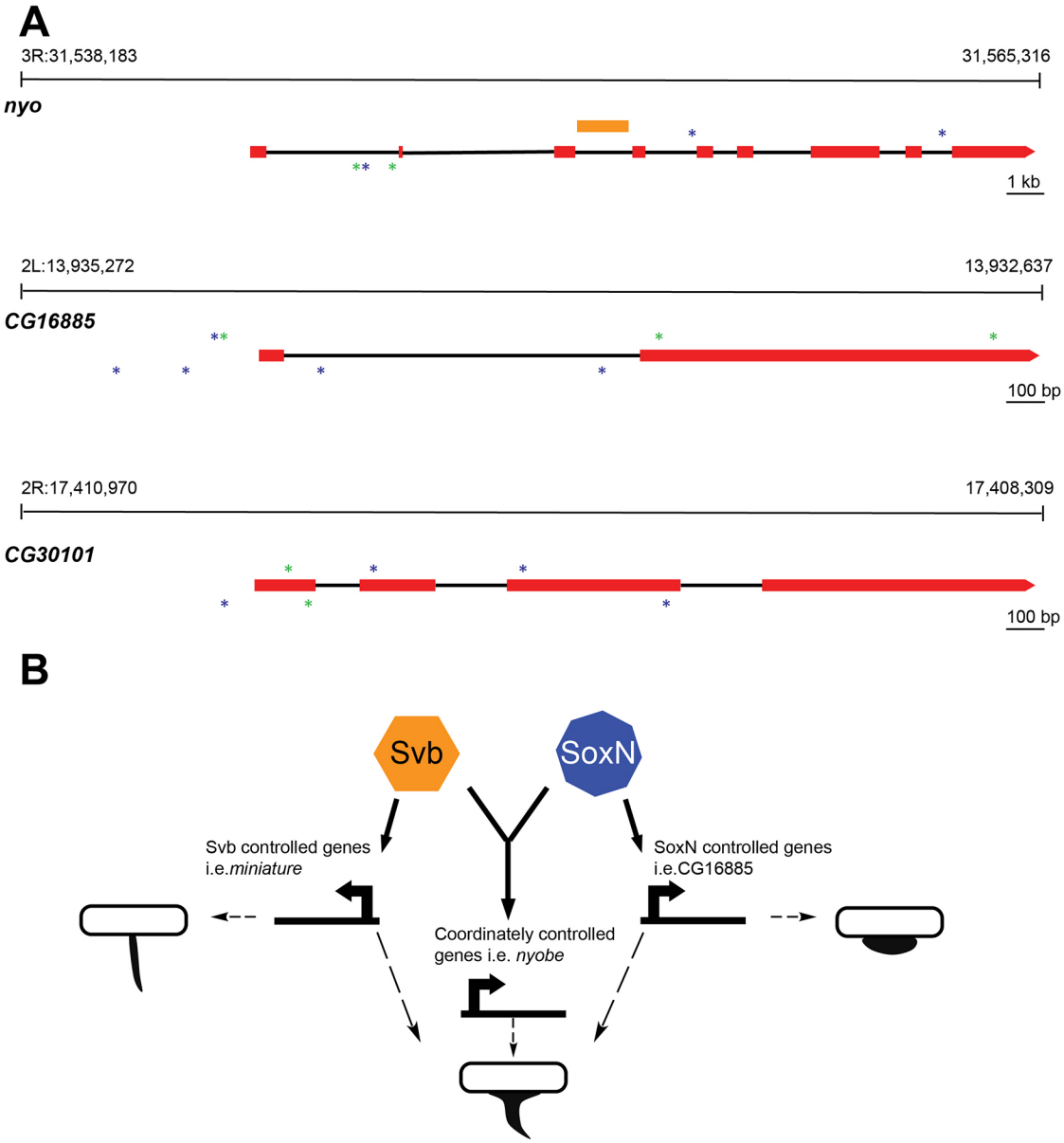

Fig. 8. Predicted SoxN binding sites in *nyo*, *CG16885*, and *CG30101* lead to a model for denticle morphogenesis. (A) The upstream and coding region for *nyo*, *CG16885*, and *CG3010* showing the positions of putative binding sites (asterisks) as determined by FIMO. Asterisk above the gene indicates binding to the plus strand; asterisk below indicates binding to the minus strand. All binding sites shown are high confidence hits: $P<0.0001$ for *nyo* and $P<0.001$ for *CG16885* and *CG30101* (data are summarized in Table S1). Orange bar over the fourth intron in *nyo* represents a previously identified functional enhancer activated by Svb (Menoret et al., 2013). *CG16885* and *CG3010* also contained a number of predicted binding sites for SoxN in their upstream regulatory regions. Green asterisks indicate those predicted sites that are conserved between *D. melanogaster* and *D. yakuba*; some of the sites are also conserved in more distantly related *Drosophila* species (Table S1). (B) Model of the proposed epidermal role of transcription factors Svb and SoxN during denticle formation. Svb promotes the expression of a myriad of genes involved in actin reorganization and cuticle biosynthesis. Genes such as *m* are activated solely through *svb* expression and collectively these genes direct the formation of long, thin denticles. SoxN promotes the expression of genes such as *CG16885* that direct the formation of a wide base but that are insufficient to cause elongation of the denticle. Both transcription factors are required to activate some genes, such as *nyo*. The output of these shared genes, in combination with the output of genes that Svb and SoxN activate independently, produces the balance of gene products required for proper denticle elongation and shaping.

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conditions, such as temperature fluctuation (Ludwig et al., 2011). Therefore, it is possible that this enhancer element may extend to the nearby predicted SoxN binding site. Alternatively, several independent regulatory elements, some of which contain SoxN binding sites, might be integrated to control expression of *nyo*.

Like *nyo*, the sequences surrounding and within both *CG16885* and *CG30101* contain a number of computationally predicted SoxN binding sites (Fig. 8A, Table S1). The *CG16885* sequence contains several sites in the region immediately upstream of the transcription start site, as well as within the intron and second exon. The sites found in *CG30101* all reside in exons, except for one located upstream of the start site. Several of the predicted SoxN binding sites in *nyo*, *CG16885*, and *CG30101* are strongly conserved in other *Drosophila* species (Fig. 8A, green asterisks), suggesting that these sites might be functional in the observed transcriptional control. We do not yet know whether the *CG16885* and *CG30101* gene products play a role in shaping denticles, but both show similarities to cuticular proteins in a number of other insects. As with the ZPD family of proteins (Jazwinska and Affolter, 2004), the protein sequences of these SoxN targets predict a signaling peptide at the N-terminus, which may localize each to the membrane or to the extracellular matrix. No other predicted domains were detected by BLAST (Altschul et al., 1990) in either protein. Further investigation of this novel class of proteins might provide mechanistic insight into denticle morphogenesis.

DISCUSSION

The *Drosophila* embryonic cuticle provides a powerful system in which to study the genetic control of tissue patterning. Elegant studies have revealed that regulation of Svb controls dorsal and ventral cuticle patterning in drosophilids (reviewed by Delon and Payre, 2004; Stern and Frankel, 2013) through its activation of numerous effector molecules (Chanut-Delalande et al., 2012). Yet whether and how Svb activity might control the row-specific shapes of ventral denticles has been unclear. The group of ZPD proteins regulated by Svb, some of which we have shown here are also regulated by SoxN, are targeted to specific apical regions and are required to maintain contact between the cell membrane and the cuticle (Fernandes et al., 2010). Although these studies noted no difference in ZPD protein levels among the rows of denticle-producing cells, it remains possible that subtle differences, below the level of detection, might play some role in denticle diversity. We found that the *svb E3* enhancer element shows differential responsiveness to Wg signaling, whereby low levels of signaling in the wg^{PE2} mutant correlated with both expansion of the *E3* anterior stripe and increased denticle diversity. This suggests that *svb* expression, and hence its transcriptional targets, may be exquisitely sensitive to graded levels of Wg activity in the wild-type embryonic epidermis. Various ZPD proteins have been shown to act in cell shape remodeling in other systems (Roch et al., 2003; Sapio et al., 2005), where a balance of the precise levels of each protein may be critical to the final shape adopted. Our finding that Svb and SoxN differentially activate ZPD genes, and other targets, raises the possibility that each row of cells within a belt might experience slightly different levels of the gene products that control cell shape.

Indeed, our experiments suggest that Svb and SoxN may be responsible for two different aspects of denticle construction. We have shown that increased levels of Svb produce longer denticles, without altering their overall shape. Thus, Svb might activate a battery of genes that promote lengthening of the actin-based protrusions that form denticles. Conversely, expression of *SoxN* alone produced denticles with a short, blunted structure. Only when *SoxN* was co-expressed with *svb* did the ectopic denticles acquire a widened base that more closely matched the wild-type shape. These results lead us to propose a model whereby Svb controls genes that specify denticle length whereas SoxN controls genes that regulate base circumference (Fig. 8B). Varying the levels of each transcription factor with respect to the other could account for the varying length versus width that characterizes each denticle row in the wild-type belt. However, there might be an upper limit to the response in denticle-producing cells. In *wg* loss-of-function mutants, both Svb and SoxN are expressed at very high, uniform levels (Payre et al., 1999; Overton et al., 2007). This extreme overproduction of Svb and SoxN corresponds with a lawn of denticles (Fig. 2C) where almost all have the size and shape of row 5 denticles, which are the large denticles at the posterior of the wild-type belt (Fig. 1B).

In addition to Svb and SoxN, one or more as yet uncharacterized factors might modulate denticle size and shape, since we observe some segmental denticle diversity when *SoxN* is expressed uniformly in a *svb* mutant embryo. We propose that the independent or combined effects of Svb and SoxN, and possibly other factors, set a specific expression level for a given target gene, and that this precise level of target gene output within a denticle row contributes to the final shape of the denticle produced. The set point for Svb and SoxN levels might be achieved by response to the graded distribution of secreted Wg, and/or of other signals within each epidermal segment, to produce the diverse denticle morphologies in each belt. Consistent with this idea, *svb in situ* hybridization images give the impression of higher mRNA accumulation in the more posterior rows of denticle-secreting cells (Payre et al., 1999), and the same effect has been noted for SoxN antibody staining (Overton et al., 2007). These posterior row cells, which produce the largest denticles, are the furthest from the stripe of Wg-producing cells in each segment (Fig. 1F) and so would experience the least repression from Wg signal transduction.

A precise balance of protein levels might be a common theme for Sox class transcription factors, and may be relevant to cell shape control in other systems. The activity of the SoxN mammalian homolog Sox2 has been shown to depend on specific levels of the protein in embryonal carcinoma cells and embryonic stem cells (Boer et al., 2007), as well as in cochlear cells (Kempfle et al., 2016), which produce the stereocilia. Further characterization of the role of SoxN in shaping *Drosophila* denticles might help elucidate the forces that shape stereocilia in the human inner ear, and that go awry in some hereditary forms of deafness.

MATERIALS AND METHODS

Drosophila melanogaster strains and culturing

w^{1118} flies were used as wild-type controls, to match the genetic background of transgenic stocks. The *UAS-svb*, *7.3-Gal4*, and *svb-lacZ* lines were gifts from F. Payre (Paul Sabatier University, Toulouse, France) and D. Stern (HHMI Janelia Research Campus, USA). The wg^{ts} and wg^{PE2} alleles are described by Baker (1988) and Bejsovec and Wieschaus (1995), respectively. *UAS-SoxN* was a gift from S. Russell. The $SoxN^{NC14}$ allele was generated by EMS mutagenesis (Chao et al., 2007). The *UAS-svbRNAi* line was obtained from the Vienna Drosophila Resource Center. All other mutations, balancer chromosomes and *Gal4* lines were obtained from the Bloomington Stock Center. Flies were reared on cornmeal-agar-molasses and embryos were collected on apple juice agar plates; all were cultured at 25°C. Cuticle preparations were performed as described (Jones and Bejsovec, 2005). In the case of svb^2 and $SoxN^{NC14}$, hatching rates were calculated after outcrossing to remove balancer chromosomes. Cuticles of all progeny, i.e. unhatched embryos as well as hatched larvae, were examined.

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Quantification of denticle morphology

Cuticle images were captured with a SPOT camera (Diagnostic Instruments) and were processed using FIJI software (Schindelin et al., 2012). For wild-type embryos, denticle belts in abdominal segments 4 and 5 were used for measurements. For both wild-type and ectopic denticles, only denticles within 75 µm of the most ventral region were used. At least five different animals were used to collect measurements, with over 70 denticles scored for each plot. Width was measured at the base of each denticle and length was measured as the distance from the midpoint of the base to the tip.

Embryo preparation and imaging

Embryos were collected and aged to the specified developmental time. For immunostaining, embryos were dechorionated in bleach and fixed for 20 min in 4% formaldehyde in PEM buffer (0.1 M PIPES, 1 mM EDTA, 2 mM $MgSO_4$, pH 6.9). For actin filament visualization, vitelline membranes were removed by vigorously shaking in a 1:1 mixture of ethanol and heptane and washing three times in fresh ethanol. Rhodamine phalloidin (Molecular Probes/ThermoFisher) and anti-β-galactosidase antibody (Promega, Z3781) were both used at 1:500. Anti-En antibody (Developmental Studies Hybridoma Bank, University of Iowa) was used at 1:50 and anti-GFP antibody (EMD Millipore, MAB3580) was used at 1:500 on embryos that were devitellinized using methanol instead of ethanol. Secondary antibodies (Jackson ImmunoResearch, 115-165-003, 111-225-144, 111-165-144) were used at 1:500. Images were captured on a Zeiss 510 confocal microscope. To examine cuticles, eggs were allowed to develop for 24 h at 25°C, dechorionated with bleach and then mounted in Hoyer's medium. Images were captured with SPOT camera (Diagnostic Instruments) on a Zeiss Axioplan microscope, and were processed with SPOT imaging.

In situ hybridization

Hybridizations were performed as described (Wilk et al., 2010). Antisense probes were made from cloned PCR products of genomic DNA in the range 700 bp to 1 kb. Primers encompassing the genomic region to be probed contain different restriction sites on each primer (Table S2). Vectors were linearized for *in vitro* transcription to produce antisense probes. The antisense probe against *m* was made from a 930 bp *Bam*HI fragment of the cDNA clone RE53556, obtained from the Drosophila Genomics Resource Center (stock no. 9261).

Binding site prediction

Potential SoxN binding sites were identified by entering target gene sequences from FlyBase (Gramates et al., 2017) into Find Individual Motif Occurrences (FIMO) (Grant et al., 2011). The SoxN motif was downloaded from the TRANSFAC database, generated from HT-SELEX data (Nitta et al., 2015). Matches were filtered out with a threshold of $P<0.001$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.P.R., A.B.; Methodology: N.P.R., A.B.; Validation: N.P.R., A.B.; Formal analysis: N.P.R., A.B.; Investigation: N.P.R., A.B.; Writing - original draft: N.P.R.; Writing - review & editing: A.B.; Visualization: N.P.R., A.B.; Supervision: A.B.; Project administration: A.B.; Funding acquisition: A.B.

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Supplementary information

Supplementary information available online at
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