

Transcriptional Control of Epidermal Cell Shape in the *Drosophila* Embryo

by

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Dissertation submitted in partial fulfillment of  
the requirements for the degree of Doctor  
of Philosophy in the University Program in  
Genetics and Genomics of  
in the Graduate School  
of Duke University

2017

ABSTRACT

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## Abstract

During development, morphogenetic processes require the integration of numerous signals to properly shape cells and tissues. These signals are interpreted by cells to induce the precise transcriptional circuitry that controls morphogenesis. In the fly embryonic epidermis, the fly Wnt, *wingless (wg)*, generates naked cuticle zones that separate the ventral denticle belts by repressing expression of *shavenbaby (svb)*, which encodes a transcription factor required for denticle formation. What is not known is how Wg and Svb interact to produce the stereotyped diversity of denticle shapes within each belt. One possibility is that graded levels of Svb, responding to graded levels of Wg signaling, determine denticle shape. Indeed, we found that the *svb* promoter responds differentially to altered Wg activity levels. However, artificially increasing the levels of ectopic *svb* does not produce morphologically distinct denticles, suggesting that additional factors are involved. We have discovered that a second Wg-responsive transcription factor, encoded by *SoxNeuro (SoxN)*, cooperates with Svb to shape the denticles. Co-expressing *SoxN* with *svb* is sufficient to rescue the morphology of denticles in an ectopic location. Furthermore, embryos that lack Svb activity retain the ability to produce a small number of rounded, reduced ventral denticles, due to SoxN activity. We found that *svb; SoxN* double mutant embryos secrete cuticles that completely lack ventral denticles and dorsal hairs. We also found that a group of known Svb target genes belonging to the zona pellucida family of proteins are differentially

activated by SoxN. Finally, we discovered two novel target genes of SoxN, *CG16885* and *CG30101*, which are expressed in denticle-producing cells and which are regulated independently of Svb. SoxN was shown previously to down-regulate Wg signaling and to promote expression of *svb*. Here, we propose that SoxN acts with Svb, in an additional, direct role, to promote denticle morphogenesis.

## Acknowledgements

There are many people that I would like to thank for their help and encouragement through this process. The majority of the work presented here is published as “SoxNeuro and Shavenbaby act cooperatively to shape denticles in the embryonic epidermis of *Drosophila*” (*Development* 144(12):2248-2258). This work would not have been possible without the initial characterization of SoxNeuro by Anna Chao, Amy Bejsovec, and Whitney Jones. My committee consisting of Dr. Dave Sherwood, Dr. Pelin Volkan, and Dr. Debby Silver has been encouraging and extremely helpful in my research efforts.

I would like to acknowledge my previous mentors that prepared me for graduate school. My first scientific mentor is Dr. Stuart Tsubota at SUNY College at Brockport. He introduced me to *Drosophila melanogaster* and the endless possibilities that it presents as a model system. The thesis project that I conducted in his lab sparked my excitement for science and set me off on my curiosity driven journey. I would next like to thank Dr. Michael Welte at the University of Rochester. Michael hired me as a technician after I finished my Master’s, and helped me refine my techniques and helped me begin to think more like a scientist. Michael’s excitement for science is genuine and contagious. His role as a mentor was instrumental in my progress and has impacted me greatly. Lastly, I would like to thank Dr. Amy Bejsovec at Duke University. Amy has been a tremendous mentor, and more of a guide in my journey through graduate school. She is the perfect mentor for me, and I feel grateful for having

been in her lab for the last six years. Like Michael, she exhibits an excitement and curiosity that is impossible to ignore. These attributes made graduate school a time of growth, success, and fun.

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# **1. Introduction to Patterning and Morphogenesis in the Ventral Epidermis of the Drosophila Embryo**

## ***1.1 Development of the embryonic epidermis in Drosophila***

Information from signaling pathways must be interpreted and modulated for the proper execution of cellular events during development. Much of developmental biology 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. A common epithelial cell shape that requires cytoskeletal reorganization is flattening, the increase in apical and basal surface and shortening of the lateral edges. This process is seen during wing formation in insects and during epiboly (Fristrom, 1988). The converse process, columnarization is less common but occurs in follicle cells during *Drosophila* oogenesis (Zarnescu and Thomas, 1999). Furrow formation, or invagination is a more complex form of cell shape change that

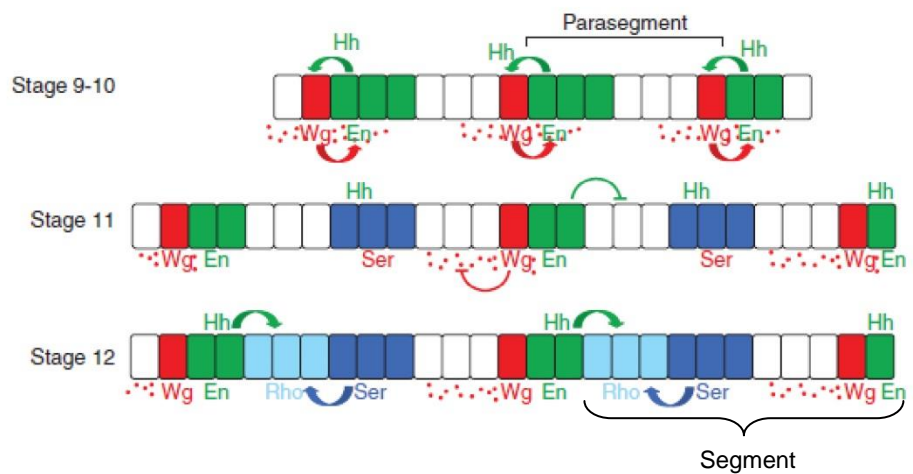
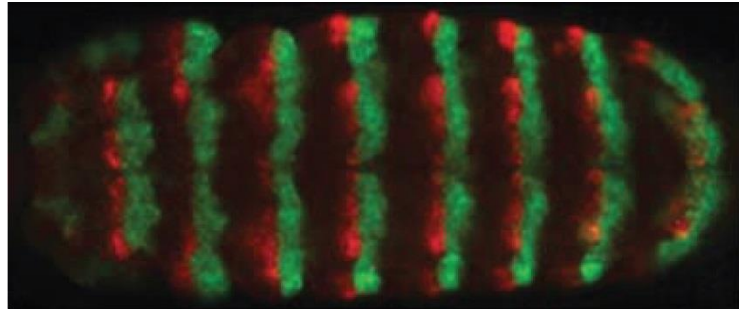
occurs during development. This process requires bending of epithelial sheets and functions in the organization of the endoderm and mesoderm during gastrulation in many animals (Keller, 1981; Leptin and Grunewald, 1990; Anstrom, 1992).

Other actin-mediated morphogenetic 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 morphogenetic consequences can be readily analyzed. The *Drosophila* embryo has provided a powerful genetic system that has contributed significantly to our understanding of tissue patterning and cell morphogenesis.

Embryogenesis in *Drosophila* is a process that takes approximately 24 hours from fertilization to hatching. The patterning of the anterior/posterior axis begins with maternally deposited *bicoid* (*bcd*) RNA that is localized at the anterior pole of the embryo (Berleth et al., 1988). Diffusion from the anterior tip creates a concentration gradient of the Bicoid transcription factor, which then elicits differential transcriptional activity in nuclei along the anterior/posterior axis. This results in the activation of gap genes which also encode transcription factors. Together with the Bicoid gradient, these gene activities are responsible for segmentation of the embryo, activating pair rule

genes that are expressed in a more refined pattern in alternating segments. The activity of the pair rule genes defines the parasegments (Martinez-Arias and Lawrence, 1985) and leads to the activation and establishment of segment polarity gene expression. The primary genes of this group, *wingless* (*wg*), *hedgehog* (*hh*), and *engrailed* (*en*) are responsible for patterning the epidermis of the embryo. In embryonic stages 9-10, *wg* expression occurs in a single row of cells and the Wg protein is secreted, and signals across the parasegment boundary to the posteriorly adjacent row of cells where it promotes and maintains *en* expression (Fig 1.1). En encodes a transcription factor, which activates *hh* expression in this posterior row of cells in each segment. Hh protein is a secreted growth factor that signals back to the anterior, *wg*-expressing row of cells. This feedback maintains the expression of *wg* and *en*, establishing the organizing center for each epidermal segment (DiNardo et al., 1988; Martinez Arias et al., 1988; Hidalgo and Ingham, 1990; Bejsovec and Martinez Arias, 1991). The signaling of each pathway becomes polarized at the parasegment boundary at stage 11, (van den Heuvel et al., 1989; Gonzalez et al., 1991; Gritzan et al., 1999) antagonizing the expression of *Serrate* (*Ser*), a Notch ligand, which is only expressed in the middle of each parasegment (Alexandre et al., 1999; Sanson et al., 1999). At stage 12, Wg expression represses *rhomboid* (*rho*), a modulator of EGF signaling, while Hh and Notch activity contribute to the establishment of this expression block

(Alexandre et al., 1999) (Fig 1.1). The signaling domains in the epidermis established at this time are essential for the determination of cell fate.



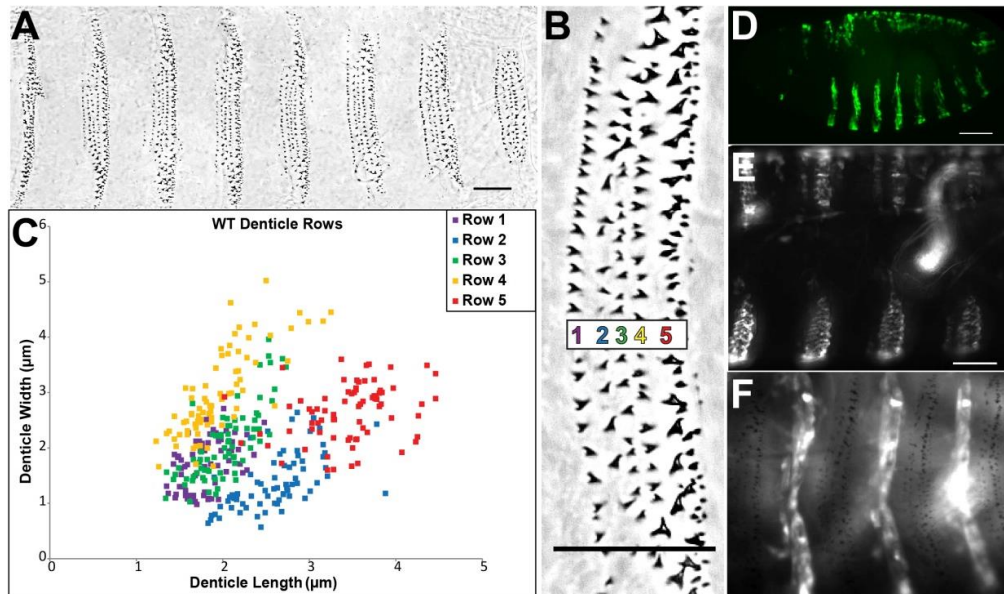
**Figure 1.1 Establishment of signaling domains and pattern by Wg in the epidermis.**

Signaling from both Wg (red) and Hh/En (green) define the parasegmental boundaries. Embryos are positioned anterior to the left, posterior to the right. Modified from (Swarup and Verheyen, 2012).

## **1.2 *Shavenbaby* governs cell fate in the epidermis**

A major cell fate decision in the epidermis is whether or not a cell will produce a denticle. Denticles are hook-like structures produced by ventral epidermal cells during late embryogenesis (Fig. 1.2A). 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. The mature structures provide traction for the crawling larva after the embryo hatches. Denticles are organized in segmentally repeating units, known as belts. Each belt is comprised of six rows of cells where each row produces a denticle with specific size, shape, and polarity (Fig. 1.2B,C). The denticle belts are separated by regions of “naked cuticle”, 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 represses expression of *ovo/shavenbaby (svb)* (Payre et al., 1999) in a striped pattern (Fig. 1.2D-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. While the *Wg* pathway represses transcription of *svb*, the EGF/DER pathway positively regulates *svb* transcription (Payre et al., 1999).

Therefore, upstream signals from these pathways dictate the transcriptional output of *svb*.



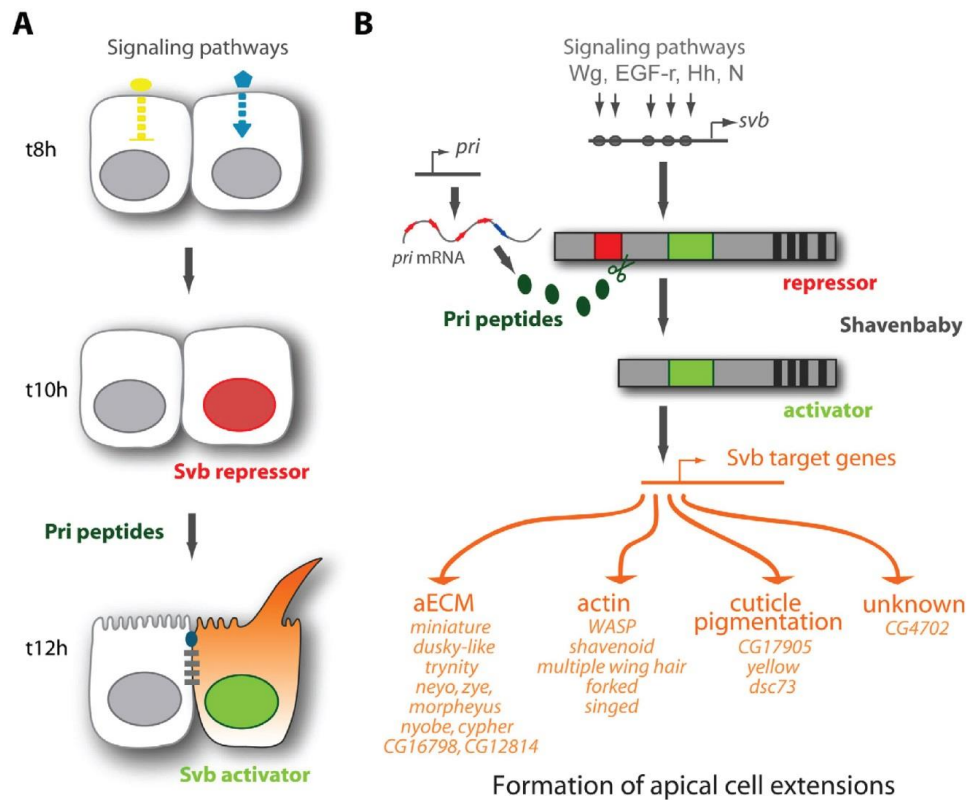
**Figure 1.2 Denticles display a stereotyped morphological diversity.**

(A) Ventral epidermal cells of wild-type (WT) embryos secrete a segmental pattern of denticle belts. In this and all subsequent images, anterior is to the left and scale bars are 50  $\mu\text{m}$  unless otherwise noted. (B) Each WT belt consists of 6 rows of cells, each producing a characteristic size, shape, and polarity of denticle. (C) Denticle diversity was quantified by measuring length and base-width, and plotting the values for each row in a different color. *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).

The *ovo/svb* locus is a complex region that has two distinct functions corresponding to different regulatory regions, which produce distinct polypeptides. The Ovo protein is necessary for the differentiation and survival of the female germline (Oliver et al., 1987) and is involved in germline sex determination (Oliver et al., 1990). The Svb protein is responsible for the morphogenesis of denticles and dorsal hairs in the embryonic epidermis (Mevel-Ninio et al., 1995). Embryos that lack *svb* gene product lose the majority of denticles and dorsal hairs, yet a number of blunted denticles remain in the posterior of each ventral belt (Payre et al., 1999). Conversely, ectopic expression of *svb* is sufficient to form denticles. These results identify Svb as a determinant of cell fate in the ventral epidermis. The pattern of dorsal hairs is also specified by *svb* expression. *Drosophila* species exhibit variation in this dorsal pattern, which is dictated in a species-specific manner by a number of cis-regulatory elements controlling expression of *svb* in a subset of cells (reviewed in Stern and Frankel, 2013).

To make matters even more complex, there is an additional factor, *polished-rice* (*pri*) that is required for denticle formation through its action on Svb. The *pri* polycistronic transcript encodes numerous small peptides of 11-32 amino acids in length (Kondo et al., 2007). Translation of the full-length *svb* transcript produces a repressive form of Svb, which has an amino-terminal extension that is cleaved by Pri to convert it to an activator (Kondo et

al., 2010). This provides a temporal control over the onset of denticle morphogenesis, because expression of *pri* is triggered by an ecdysone pulse in stage 14 of embryogenesis (Chanut-Delalande et al., 2014). These events precede the activation of effector molecules that will construct and shape denticles.



**Figure 1.3 Regulation of denticle morphogenesis through Pri and Svb.**

(A) Signaling converges on epidermal cells to determine cell fate through the activation of Svb in a repressor form. Pri converts Svb to its activator form, allowing downstream target activation. (B) The *svb* regulatory region directs transcription in the appropriate cells. Pri peptides of 11-32 amino acids in length cleave the N-terminal portion of Svb converting it to its activator form. Svb activates genes that interact with the extracellular matrix, actin remodeling genes, cuticle pigmentation and others. Figure from (Chanut-Delalande et al., 2012).

### **1.3 Conserved mechanisms of apical morphogenesis**

The cytoskeletal processes that form denticles begin with polarized actin filaments that accumulate and acquire shape over a period of roughly 2 hours (Dickinson and Thatcher, 1997). An actin meshwork begins to accumulate in the apical region of the presumptive denticle-producing cells. These diffuse actin pools condense and sharpen over time, localizing to the posterior edge of the cell. Microtubules begin to accumulate at the base of the denticle precursor and within its actin core (Price et al., 2006). During denticle morphogenesis, proteins involved in cytoskeletal reorganization, many of which are transcriptionally regulated by Svb, begin to localize to the denticle precursors. For example, Svb directs the expression of *forked (f)*, 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 (sn)* (Cant et al., 1994) and *shavenoid (sha)* (Ren et al., 2006) are also controlled by Svb, and function during embryogenesis in actin dynamics and denticle formation. Other proteins that control cytoskeletal dynamics, such as Diaphanous, Enabled, and Arp2/3, are not influenced by Svb activity but localize to developing denticles and aid in their construction (Price et al., 2006). Svb regulation is not relegated only to effectors involved in actin dynamics, it also directs the transcription of proteins that localize to the membrane and organize the extracellular matrix. The most well-characterized example of this

is the group of zona pellucida domain (ZPD)-containing genes that require Svb for their expression and that are differentially localized within the denticle structure (Chanut-Delalande et al., 2006; Fernandes et al., 2010). ZPD proteins have been shown to contribute to cell shape remodeling in a wide variety of systems (reviewed in Plaza et al., 2010). Though these proteins belong to this same group based on homology, each shows a distinct localization and non-redundant role. The ZPD protein Miniature (M) localizes to the medial region of denticles in the endocuticle layer, the thickest layer of cuticle immediately overlying the cell. Dusky-like (Dyl) localization is restricted to the tip of denticles, while Zyn and Trinity localize to the denticle base. ZPD proteins are found in other systems as well, exhibiting similar functions. The CUT genes in *C. elegans* encode ZPD proteins that are components of the cuticle. These genes are expressed in hypodermal cells that produce the alae, bilateral elaborations of the cuticle (Sebastiano et al., 1991; Muriel et al., 2003; Sapio et al., 2005). Defects in these genes result in malformation or absent alae. ZPD proteins also function in the morphogenesis of kidney cells. The kidney duct contains columnar  $\alpha$  cells with apical microvilli, and flattened, irregularly-shaped  $\beta$  cells. Polymers of the ZPD protein hensin form to convert cells from the  $\beta$  form to the  $\alpha$  form (Al-Awqati, 2008). These examples illustrate a common mechanism with shared effectors in the differentiation of epithelial cells.

The morphogenesis of apical protrusions is critical to many developmental processes. In many cases, the apical surfaces of cells act as the barrier between the environment and underlying tissue. Many cells develop specialized structures essential for their function, and defects in the development of these structures underlie many diseases. Cilia are apical structures with microtubules at their core that are present in nearly every cell in the body (Marshall and Nonaka, 2006). They function to transduce signals from the environment to the cell and regulate processes such as differentiation, proliferation, and gene expression (Oh and Katsanis, 2012). Defects in cilia formation result in ciliopathies that can lead to retinal degeneration, renal disease, and skeletal dysplasias (Lin et al., 2003; Beales et al., 2007; Murga-Zamalloa et al., 2009). Microvilli are finger-like apical protrusions that contain an organized actin core (Bretscher and Weber, 1978). They are well known for their role in nutrient absorption in the intestine, but are thought to have a much more diverse functional capacity in general processes of cell physiology (Lange, 2011). Proteins such as sodium channels and glucose transporters localize to tips to facilitate microvillar function (Cau et al., 1985; Orci et al., 1989). Microvillus inclusion disease is caused by defects in structure of the apical surface of enterocytes. It is associated with atrophies of microvilli or microvilli inclusions, leading to diarrhea and dehydration (Cutz et al., 1989). Thus, these specialized structures are extremely important for basic cellular function as well as human

health. Uncovering the genes involved in their development and function will lead to a better understanding of cell biology and disease.

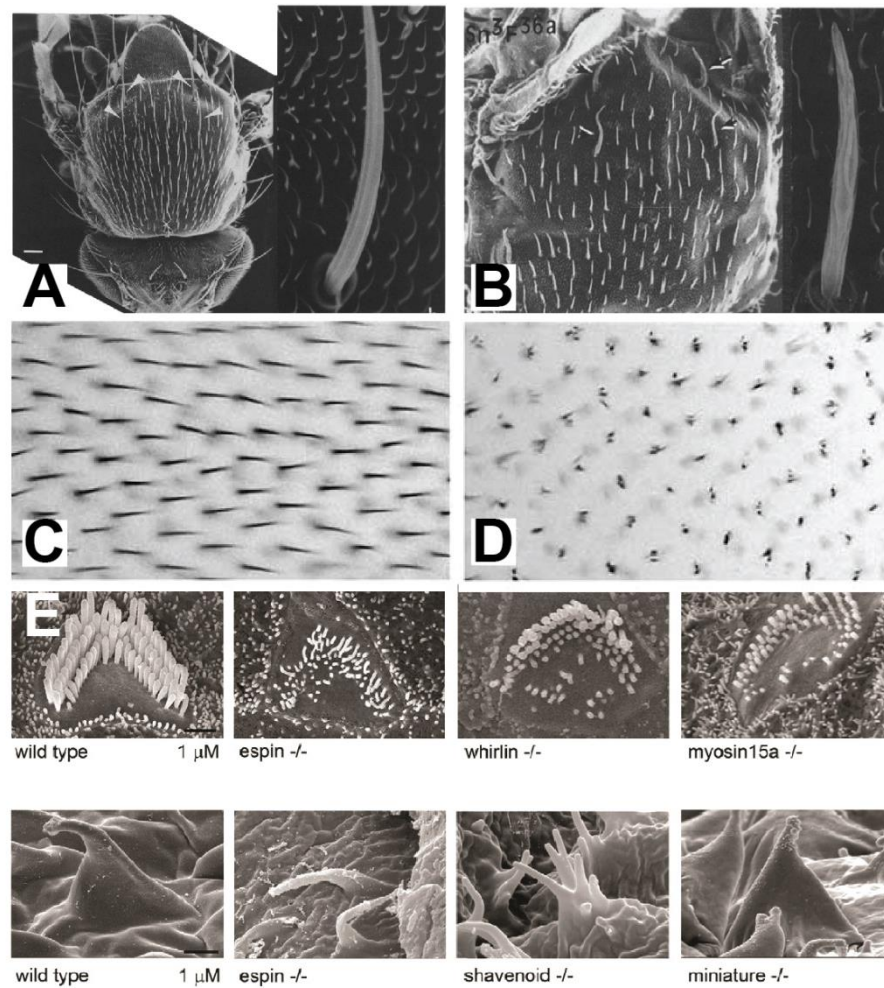
The fruit fly provides other examples of actin-based epithelial extensions, such as adult sensory bristles and wing hairs that are useful models for studying cell morphogenesis. Many of the same effector molecules that participate in denticle morphogenesis are also required for bristle and/or wing hair formation. Bristles serve as chemoreceptors and mechanoreceptors in the adult fly. Like denticles, numerous bundles of cross-linked actin filaments extend from the base towards the tip of the bristle. In *sn* mutant bristles, the number of actin bundles is not affected but the bundles are smaller and flattened (Tilney et al., 1995) and the structural integrity of the actin bundles is affected (Cant et al., 1994). *f* mutant bristles also exhibit smaller actin bundles, while the *f,sn* double mutant bristles lack actin filament bundles completely and the bristles are shorter and highly distorted (Fig. 1.4A,B). Another *Svb* target gene, Wiskott-Aldrich Syndrome proteins (*wsp*) interacts with Arp2/3 and is required for the development and formation of sensory bristles (Tal et al., 2002).

Wing hairs have been used extensively as a model to study cell polarity, but they also exhibit similarities to denticles and bristles in the molecular basis of their construction. Wing hairs are constructed from both actin filaments and microtubules, which cooperate in the morphogenesis and

stabilization of the hair. The product of the Svb target *sha* localizes to both wing hairs and bristles and interacts with the actin cytoskeleton (Ren et al., 2006). Mutations in *sha* result in the delay of hair morphogenesis, with some cells forming no hairs or smaller hairs (Fig. 1.4C,D).

Although *Drosophila* is a strong genetic system in which to identify and study genes involved in epithelial morphogenesis, other model systems can also provide information on the morphogenesis of apical structures. The stereocilium is an actin-based apical projection in the vertebrate ear that is required for hearing. The loss or breakdown of structural integrity leads to deafness. Many of the genes necessary for stereocilium development and maintenance have been discovered in mouse models. Mutations in these genes result in defects that have been shown to lead to progressive hearing loss in mice (Sekerikova et al., 2011; Furness et al., 2013). These genes, such as *Espin* and *Eps8(L2)* have human homologs that exhibit similar function. Not surprisingly, many of the effector molecules which have been discovered in stereocilium morphogenesis function in a manner similar to those involved in producing denticles (Figure 1.4E). Protein-truncating mutations in the human homolog of *dia*, *DFNA1*, are associated with familial deafness (Lynch et al., 1997) . The vertebrate homolog to *f*, *espin*, localizes to stereocilia tips and is necessary for stereocilium stability (Bartles et al., 1998). *Espin* is found at high levels (Sekerikova et al., 2006) along with the

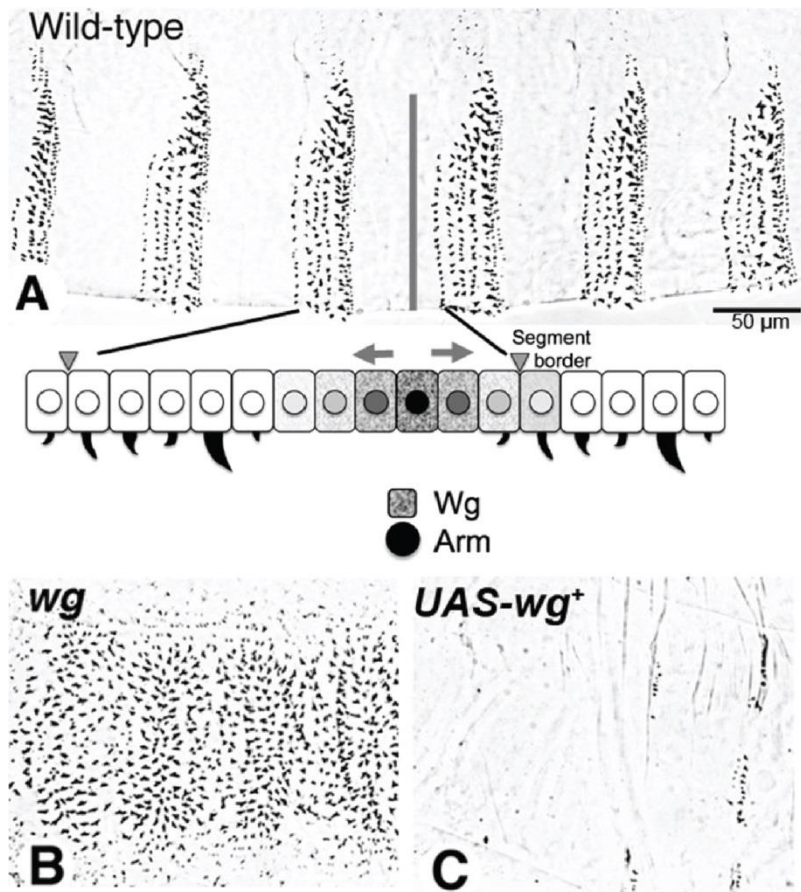
vertebrate *sn*, fascin, in the developing stereocilia (Shin et al., 2010). The abundance of these cross linkers is thought to provide stabilization and promote elongation of the actin bundles. Though many of these effectors have been identified and implicated, the transcription factor(s) that regulate these genes has yet to be identified. However, there has been some insight into the upstream components required for specifying the specialized cells that produce stereocilia. The transcription factor MATH1 is necessary for specification of the auditory hair cells that develop stereocilia, though the target genes activated by MATH1 have not been identified (Bermingham et al., 1999). The specification of auditory hair cells also requires signaling from the FGF, Notch, and Hedgehog pathways (reviewed in Kelly and Chen, 2009). Thus stereocilia not only show similarities in their structure and in the genes required for their construction, but also in the signaling inputs that specify the fates of the cells that produce these structures. Investigating denticle morphogenesis, particularly focusing on how differences in shape can be achieved, may contribute to our understanding of how defects in these other morphological processes are manifested.



### Figure 1.4 Conserved mechanisms of apical extensions

(A) SEM image of WT bristles on the adult thorax. (B) Mutations in *f* and *sn* result in bristles that are shorter than WT and distorted. Modified from (Tilney et al., 1995). (C) Light micrograph of WT wing hairs on adult wings, proximal is to the left and distal is to the right. Each cell produces a single distally-oriented hair. (D) A strong allele of *sha* causes almost all of the cells in this region of the wing to form several small hairs. Modified from (Ren et al., 2006). (E) SEM images showing cochlear outer hair cells in wild type mice and mice mutant for *espin*, *whirlin* and *myosin 15a*; pictures adapted from (Manor et al., 2011; Sekerkova et al., 2011). SEM images of denticles mutant for the homologous effector genes *espin* (*f*), *sha*, and *m*. All show defects in morphology. Modified from (Chanut-Delalande et al., 2012).

For my thesis project I was interested in the question of 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 part of the pattern lost, but also the denticles produced are reduced to a single type of denticle, similar to those in row 5 (Fig. 1.5) (Nusslein-Volhard, 1984; Nusslein-Volhard et al., 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*) acts cooperatively with *svb* to shape denticles. *SoxN* is expressed in the denticle producing cells in the ventral epidermis and is necessary for the proper morphogenesis of denticles. Loss of *SoxN* results in denticles with a defective morphology along with a slight expanse of naked cuticle. It is also sufficient for denticle formation when it is expressed in regions of naked cuticle. These denticles are different in appearance from both WT and *svb* ectopically-induced denticles, as they are significantly shorter.



**Figure 1.5 Wg loss of function and hyperactive phenotypes**

(A) WT cuticle pattern of the ventral epidermis consists of denticle belts separated by expanses of naked cuticle. Wg is secreted from a single row of cells (grey vertical line) activating the pathway in the adjacent rows. (B) *wg*-loss-of-function embryos lose all naked cell fate and exhibit the “lawn of denticles” phenotype. Denticle diversity is also lost in these embryos. (C) Hyperactivation of *wg* in the epidermis results in the specification of naked cuticle in all ventral cells. Modified from (Bejsovec, 2013).

However, we find that its ectopic co-expression with *svb* rescues denticle morphology to more closely approximate wild-type structures. Our lab has shown that SoxN suppresses the activity of Wg acting at the level of the transcription factor Tcf to regulate downstream target genes (Chao et al., 2007). Additionally, SoxN is necessary for the proper expression pattern of *svb* and is repressed by Wg (Overton et al., 2007). The results from these previous studies suggested that SoxN simply acts as an intermediate between Wg and *svb*, but our current work indicates a more direct contribution from SoxN. These collective observations suggest that SoxN may act at the interface of signaling pathway and effector output during denticle morphogenesis.

## **2. Regulatory elements of *svb* respond to altered Wg activity and cells respond to increasing levels of *svb*.**

### ***2.1 Introduction***

Gene regulation during development requires both temporal and spatial control. This is executed through the integration of signaling cues at the regulatory region of genes to control transcription. The best studied cis-regulatory elements are enhancers and promoters (Levine, 2010) that contain binding sites where transcription factors are recruited to activate genes (Ong and Corces, 2011). In multicellular organisms with multiple cell types, gene expression must be restricted to specific groups of cells at specific times during development. This regulation often is mediated through the activity of multiple enhancers. Variation in enhancer sequences is thought to be the primary cause of morphological divergence between species (Carroll, 2008; Stern and Orgogozo, 2008).

One of the best examples of the evolution of morphological traits has been characterized in *Drosophila*. The pattern of fine dorsal hairs on first instar larvae exhibits minor variation between species, but is completely replaced by naked cuticle in *Drosophila sechelia* (Sucena and Stern, 2000). The cause of this variation was mapped entirely to changes at the *ovo/svb* locus. The expression of *svb* correlates with the differences seen in the dorsal hair pattern, suggesting that differences in the regulatory region or enhancers of *svb* were responsible

(Sucena et al., 2003). These enhancers were identified and tested with reporter constructs; their combined expression pattern copies the pattern of dorsal hairs. While these reporters primarily show expression in the dorsal epidermis, several of the enhancer constructs are also expressed in a subset of cells in the ventral denticle belts (McGregor et al., 2007). The presence of these enhancers may not only affect which cells express *svb* and when, but could also have an effect on the levels of Svb produced in each cell (Banerji et al., 1981).

Wg signaling generates denticle diversity in the ventral epidermis between 4 and 6 hours after egg-laying, and then specifies naked cuticle from 6 to roughly 10 hours (Bejsovec and Martinez Arias, 1991). We have recently found that Wg has an additional role in denticle morphogenesis at later developmental stages (Bejsovec and Chao, 2012). A genetic interaction was discovered between *wg* and the non-muscle myosin VIIa, *crinkled* (*ck*) (Kiehart et al., 2004; Bejsovec and Chao, 2012). Mutations in *ck* modify denticles in the *wg* loss-of-function mutants, eliminating the sharply hooked ends and giving the denticles a "melted" appearance. Using a temperature-sensitive *wg* allele (*wg<sup>ts</sup>*) in a *ck* background, the temporal requirement for proper denticle shaping was tested. Shifting embryos to the restrictive temperature at 9.5 hours resulted in misshapen denticles. This revealed a late-stage requirement for Wg in the denticle-producing cells. Because Wg is known to repress *svb* expression, we examined Svb target genes for a role in the defects seen in the *wg,ck* embryos. Mutations

in *miniature (m)* and *f* result in defective denticle morphologies that, when combined with the *ck* mutation, resemble the *wg,ck* phenotype. This was an odd result, since presumably in *wg* loss of function embryos, Svb and therefore its targets will be at higher levels. This suggested that these targets are required at precise levels, and that any alterations during morphogenesis will disrupt denticle shape. Consistent with this idea, the overexpression of *m* in the epidermis in a *ck* mutant background also results in defective denticles. We see a similar result with the overexpression of *svb* in a *ck* mutant using *E22cGal4*. 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. Here we test these hypotheses by taking advantage of *svb* enhancer reporter constructs that are expressed in the ventral epidermis. We observe how their expression is affected by changes in Wg activity and investigate whether altering *svb* levels can affect denticle morphology.

## **2.2 Experimental Procedures**

### **2.2.1 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 minutes in 4% formaldehyde and PEM buffer (0.1 M PIPES, 1 mM EDTA, 2 mM MgSO<sub>4</sub>, 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 3 times in fresh ethanol. Rhodamine phalloidin (Molecular Probes/ThermoFisher) and anti-beta galactosidase antibody (Promega) both were used at 1:500. Anti-beta galactosidase antibody from the Developmental Studies Hybridoma Bank (University of Iowa) was used at 1:50 on embryos that were devitellinized using methanol instead of ethanol. Secondary antibodies (Jackson ImmunoResearch) were used at 1:500. Images were captured on a Zeiss 710 confocal microscope.

### **2.2.2 *Drosophila melanogaster* strains and culturing**

*w*<sup>1118</sup> 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 and D. Stern. The *wg*<sup>ts</sup> and *wg*<sup>PE2</sup> alleles are described in (Baker, 1988) and (Bejsovec and Wieschaus, 1995) respectively. All other stocks were obtained from the Bloomington Stock Center. Two different *UAS-svb* transgenes, one on chromosome II and one on chromosome III were used in

ectopic activation. In experiments where a single copy of *svb* is expressed, the transgene inserted on chromosome II was used. Both transgenes were crossed into the same background for dual expression experiments (*wg>svb;svb*). 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 in (Jones and Bejsovec, 2005).

### **2.2.3 Quantification of denticle morphology**

Cuticle images were captured with a SPOT camera 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. A minimum of 5 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.

## 2.3 Results

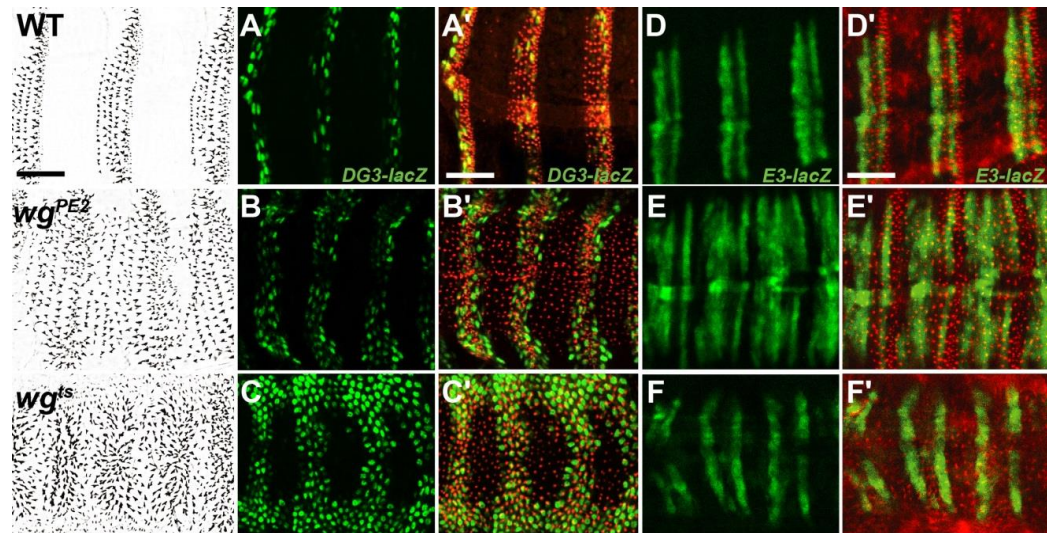
### 2.3.1 *svb* enhancers exhibit sensitivity to Wg activity

To test our hypothesis that Wg activity sets precise Svb threshold levels, 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* transcriptional start site (McGregor et al., 2007; Bejsovec, 2013), drives expression in all of the ventral denticle-producing cells (Fig. 1.2D, 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. 2.1A), 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<sup>PE2</sup>* mutant embryos (Fig. 2.1B). The *wg<sup>PE2</sup>* missense allele produces a protein that is distributed normally across the segment and is sufficient to generate denticle diversity, but cannot bind the Dfz2 receptor strongly enough to specify naked cuticle (Hays et al., 1997; Moline et al., 2000). Expansion of *DG3* enhancer expression was greater but still was not uniform when *wg* activity was removed completely in *wg<sup>ts</sup>* mutant embryos at restrictive temperature (Fig. 2.1C). This result was somewhat expected based on previous experiments showing expanded *svb* expression in a *wg* loss of function embryo.

Yet, the observation that expression is limited to a subset of cells suggests spatial restriction that may be independent of Wg. The presence or absence of additional factors/signals may account for this.

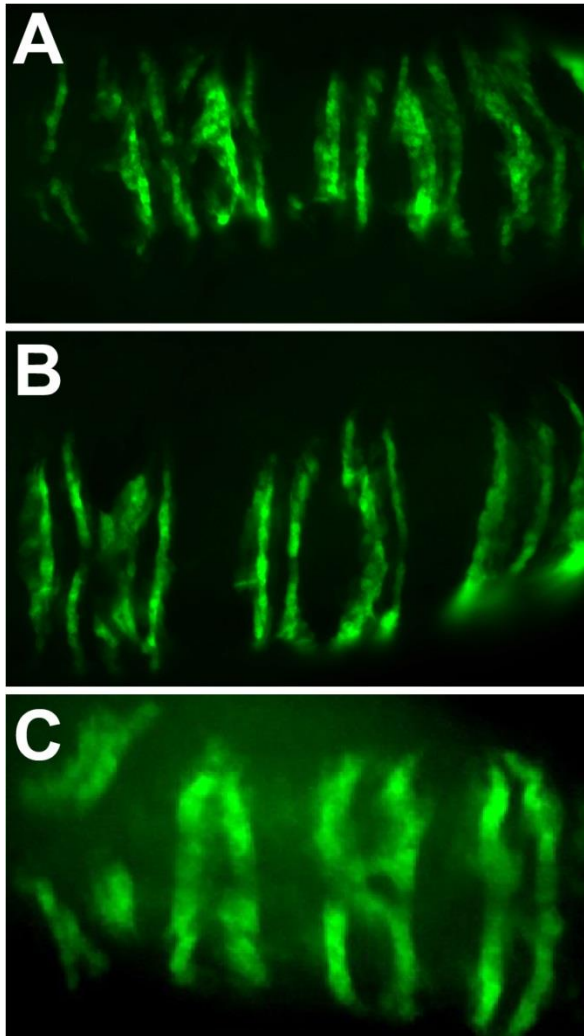
By contrast, the *E3* enhancer drives two stripes of expression in each denticle-producing belt: an anterior stripe that roughly prefigures where rows 1 and 2 will form and a more posterior stripe at row 5 (Fig. 2.1D). This more complex pattern of ventral expression suggests that there may be multiple inputs into the activity of this enhancer. Indeed, we found that the two stripes of *E3* expression respond differently to varying levels of Wg signaling. Reduced Wg activity in *wg<sup>PE2</sup>* mutant embryos allows the anterior stripe to expand in an anterior direction (Fig. 2.1E), 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<sup>ts</sup>* mutant embryos are cultured at permissive temperature for the first 5 hours of development (Fig. 2.2A,B), which generates denticle 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<sup>ts</sup>* mutants cultured at restrictive temperature (Fig. 2.1F) and in *wg<sup>CX4</sup>* null mutants (Fig. 2.2C). Thus both the *DG3* and *E3* promoter elements of *svb* are sensitive to Wg activity level, with the *E3*

element showing a complex response where the anterior stripe of expression appears to be activated, rather than repressed, by low levels of Wg signaling.



**Figure 2.1 *shavenbaby* 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. (A' - F') Rhodamine phalloidin (red) highlights developing denticles in embryos 14 to 16 hours after egg-laying. (B) *wg<sup>PE2</sup>* embryos have partial Wg function, producing normal denticle diversity but no naked cuticle. In these mutants, *DG3* expression expanded to a width of 3-4 rows of cells. (C) *wg<sup>ts</sup>* 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 segmental belt. (E) In *wg<sup>PE2</sup>* 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* 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.



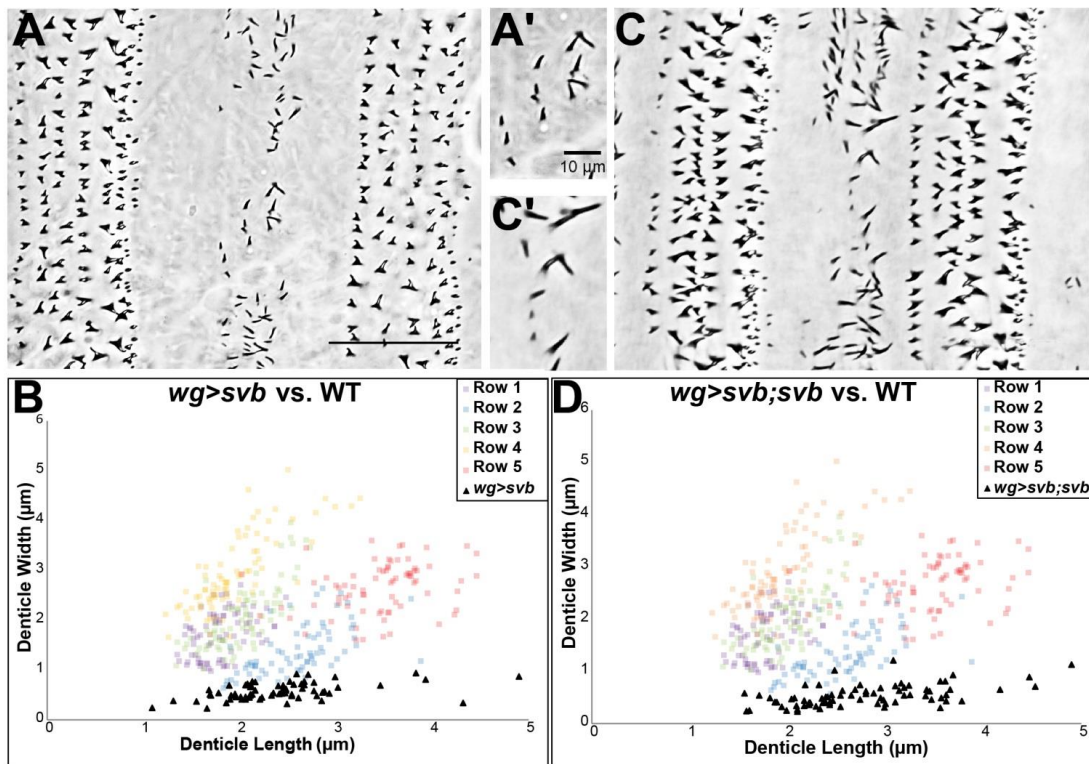
**Figure 2.2 E3-lacZ promoter elements in *wg* mutant embryos.**

(A) and (B) Two different *wg<sup>ts</sup>* mutant embryos that were cultured at permissive temperature until 5 hours after egg-laying, to allow generation of denticle diversity, and then shifted to restrictive temperature to remove later Wg function. Both show expansion of the anterior stripe of *E3* expression similar to that observed in the partial-function *wg<sup>PE2</sup>* mutant embryos. Thus the anterior stripe correlates with regions of the denticle belt where diverse denticle types are being specified. (C) *E3* expression in the RNA null mutant, *wg<sup>CX4</sup>*, does not show expansion of the anterior stripe, but instead shows expansion of the posterior stripe so that it matches the width of the anterior stripe. This mutant phenotype is indistinguishable from the *wg<sup>ts</sup>* mutant at restrictive temperature.

### 2.3.2 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. 1.2F). 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. 2.3A). This defective morphology has been previously observed when *UAS-svb* was driven with a variety of different *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. 1.2C,2.3B). 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. 2.3C,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 lack the widened base (Fig. 2.3D), and thus were similar in shape to those produced by a single transgene. We conclude that Svb transcription factor output controls denticle length, but Svb alone is not sufficient to produce normal denticle shape.



**Figure 2.3: Increasing ectopic *svb* alters denticle size but not 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': higher magnification views from A and C respectively; in this and all subsequent **insets**, scale bars are 10  $\mu\text{m}$ ). (B) Measurements of these ectopic denticles (black triangles) lay outside the range of wild-type denticles (light-colored boxes): the *wg>svb* denticles were narrower at the base than wild-type, although their lengths fell mostly within the range of WT lengths (mean = 2.44, std. dev. = 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, std. dev. = 0.72) without altering the y-axis values (width).

## **2.4 Discussion**

Regulatory regions containing multiple enhancers allow for a high degree of temporal and spatial regulation during development. The upstream factors that bind to these regions are expressed in subsets of cells at specific developmental stages to produce the specificity of gene expression. The networks responsible for this regulation can be very complex, and in most cases it is not known precisely how their workings culminate in the highly organized expression patterns observed. Determining the regulators, and the manner in which each regulatory element responds to changes in the network, provides the foundation for elucidating the dynamics of such a complex system and its developmental results.

Because Wg is secreted, it is possible that the cells that comprise the denticle belts receive differing amounts of signal. The signal transduced converges on the different enhancers to induce or repress transcriptional activity. In many instances enhancers can have equivalent or strongly overlapping expression patterns that may provide robustness against changes in the environment (Barolo, 2012). However, integration of several enhancers may result in synergistic activity. An example of this occurs in *Drosophila* with the expression of *brinker* (*brk*). Brk is a transcriptional repressor that is negatively regulated by Decapentaplegic (Dpp) signaling (Kirkpatrick et al., 2001). Dpp functions as a morphogen and represses *brk* in a concentration-dependent

manner. The *brk* gene contains multiple regulatory modules that integrate the information from Dpp to generate the final expression pattern, and the presence of multiple enhancers can help boost expression levels (Yao et al., 2008).

As seen in Fig 2.1, the enhancers of *svb* display expression in a subset of cells within the denticle belts. The *DG3* enhancer is expressed in a single row of cells in the posterior of each belt, while *E3* is expressed in two separate rows of cells. The *DG3* enhancer shows a progressive expansion with a decrease in Wg activity – from a single row of cells to a wider expression block. We found that the *svb E3* enhancer element showed differential responsiveness to Wg signaling, where low levels of signaling in the *wg<sup>PE2</sup>* 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. These differences in response are presumably due to the binding site differences between regulatory modules or the presence/absence of regulatory factors in those cells. Analyzing these enhancers for putative binding sites may uncover new factors that function in *svb* regulation.

The possibility of additional inputs into *svb* regulation requires revisiting the basic model. The central premise of epidermal patterning is the repression of *svb* by Wg. The involvement of Wg in this process has been clearly shown, yet there has been no evidence of direct repression of *svb* by Wg. The general view

is that Wg signaling transcriptionally activates target genes through the binding of Tcf to gene promoters (Barolo and Posakony, 2002). There are few examples of Wg/Wnt activation directly repressing genes through Tcf binding. These consist of the gene *stripe* in the *Drosophila* epidermis (Piepenburg et al., 2000), *dpp* in the fly leg imaginal disc (Theisen et al., 2007) and the gene for E-cadherin, *CDH1*, in mice (Jamora et al., 2003). Little is known about the mechanism of Wg repression or how some target genes are specified to turn on versus off. Known examples of direct repression occur through the traditional Tcf binding site (CTTTGWWS W=A/T, S=C/G), therefore differences in sequence cannot account for the on versus off question. In the case of *dpp* repression, Brk is required, making it possible that additional factors are responsible for this specificity. In at least one case, however, sequence does control this difference. The gene *Ugt36Bc* in *Drosophila* contains novel Tcf binding sites (AGAWAW, W=A/T) that are required for repression (Blauwkamp et al., 2008). Replacement of these sites with the traditional Tcf binding sequence causes activation in the presence of Wg signal. The identification of other such novel sites is difficult since the sequence is not long enough to provide the information needed for a stringent search. The presence of either kind of Tcf-binding sequence has not yet been found in the *svb* regulatory regions, raising the possibility that Wg-mediated repression occurs through an intermediate. Such indirect repression occurs in the *Drosophila* midgut, where high levels of Wg activate *teashirt*, which then represses *Ubx* transcription (Waltzer et al., 2001). In *Xenopus* development as well, the

endoderm is patterned by differential Wnt signaling. The homeotic gene *hhex* is required for pancreatic development and is indirectly repressed by Wnt through Vent2 (McLin et al., 2007). Each of these possible modes of repression needs to be further investigated to deepen our understanding of *svb* regulation in *Drosophila* and how it promotes epidermal patterning.

Our experiments with ectopic expression of *svb* suggest that expression levels do play a role in shaping the denticles. We observed that increasing *svb* levels results in longer denticles. Thus, the ability of *svb* enhancers to respond to altered Wg may result in different levels of *svb* that generate structural differences in each denticle row. However this cannot account for the overall shaping of each denticle row because the ectopic-*svb* denticles lack the widened base that we see within wild-type denticle belts. This suggests that there are additional components of the system that are required for the intricate shaping of denticles. Additional transcription factors that are expressed in the denticle-producing cells may contribute to the process of denticle morphogenesis.

### **3. SoxN Contributes to Denticle Morphogenesis**

#### ***3.1 Introduction***

Enhancers that direct the activation of genes typically contain clusters of transcription factors binding sites. The binding of multiple transcription factors can specify the expression domain of target genes with a high degree of transcriptional precision (Small et al., 1992; Halfon et al., 2000). For example, the overlapping expression of both activators and repressors defines the striped patterns of gene expression observed during segmentation of the fly embryo (Stanojevic et al., 1991). When multiple transcriptional activators are expressed in overlapping domains, this combinatorial effect fine-tunes enhancer activity and ultimately cell fate.

Our observation that *svb* enhancers are differentially sensitive to Wg activity supports the idea that transcription factor levels may be a major determinant of denticle shape. We propose that each row in the denticle belt expresses *svb* at a different level in response to differing levels of Wg activity. However, Svb activity alone cannot account for denticle shaping; the denticles that result from ectopically expressed *svb* lack the typical wide base that is found in WT denticles. This suggests that additional factors are expressed within the denticle belts, and contribute to denticle morphogenesis. In addition to simply being expressed in denticle producing cells, factors that show interactions with *svb* or with the Wg pathway would represent good candidates. The transcription

factor SoxNeuro (SoxN) meets these criteria as the possible missing link: it is expressed in denticle producing cells; it is negatively regulated by Wg; and it directs *svb* expression.

The Sox family of proteins consists of a family of transcriptional regulators that contain a high-mobility-group (HMG) DNA binding domain closely related to the mammalian testis determining factor SRY (Gubbay et al., 1990; Sinclair et al., 1990). Sox transcription factors are conserved in all metazoans and function in a number of developmental processes from eye development (Kamachi et al., 1998) to stem cell maintenance (Avilion et al., 2003). SoxN is related to the vertebrate SoxB group of genes that consists of *Sox1*, *Sox2* and *Sox3*. The HMG domain of SoxN shares 95% similarity with the SoxB genes (Wegner, 1999). In the *Drosophila* embryo, the expression pattern of SoxN is primarily observed in the presumptive neuroectoderm and ventral nerve cord. However, from stage 12 until the end of embryogenesis, SoxN is expressed in the presumptive denticle producing cells (Cremazy et al., 2000).

Our lab isolated a SoxN mutation in a screen looking for suppressors of *wg* mutant phenotypes. Null mutations in SoxN slightly rescue the patterning defects observed in a hypomorphic *wg*<sup>NE2</sup> allele (Chao et al., 2007). On its own, loss of SoxN is embryonic lethal and results in an increased expanse of naked cuticle and defects in denticle morphology. Ectopic expression of SoxN throughout the epidermis results in ectopic production of denticles in the naked

zone and defects in dorsal segmental patterning. Our epistasis experiments place SoxN function at the level of Tcf, the Wg-responsive transcription factor that activates target genes (van de Wetering et al., 1997; Brunner et al., 1997). The *Drosophila* SoxN protein is able to suppress vertebrate Wnt-activated gene expression, showing a conserved mechanism of antagonism.

In addition to its role as a Wg suppressor, SoxN is also negatively regulated by Wg and positively regulates *svb* (Overton et al., 2007). In *wg* loss of function embryos the expression of *SoxN* expands throughout the ventral epidermis in a fashion similar to *svb*. *svb* expression is strongly reduced in *SoxN* loss of function embryos, suggesting that SoxN positively regulates *svb*. Conversely, SoxN expression is established normally in *svb* loss of function embryos, but decays during later stages of development. This suggests that *svb* is necessary for the maintenance of SoxN expression. These data taken together position SoxN as a prime candidate to be an additional transcription factor that promotes denticle morphogenesis.

## **3.2 Experimental Procedures**

### **3.2.1 *Drosophila melanogaster* strains and culturing**

$w^{1118}$  flies were used as wild-type controls, to match the genetic background of transgenic stocks. *UAS-SoxN* was a gift from S. Russell. The *SoxN<sup>NC14</sup>* allele was generated via 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 in (Jones and Bejsovec, 2005). In the case of *svb<sup>2</sup>* and *SoxN<sup>NC14</sup>*, hatching rates were calculated after outcrossing to remove balancer chromosomes. Cuticles of all progeny, unhatched embryos as well as hatched larvae, were examined.

### **3.2.2 Quantification of denticle morphology**

Cuticle images were captured with a SPOT camera 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  $\mu\text{m}$  of the most ventral region were used. A minimum of 5 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.

### **3.2.3 Embryo preparation and imaging**

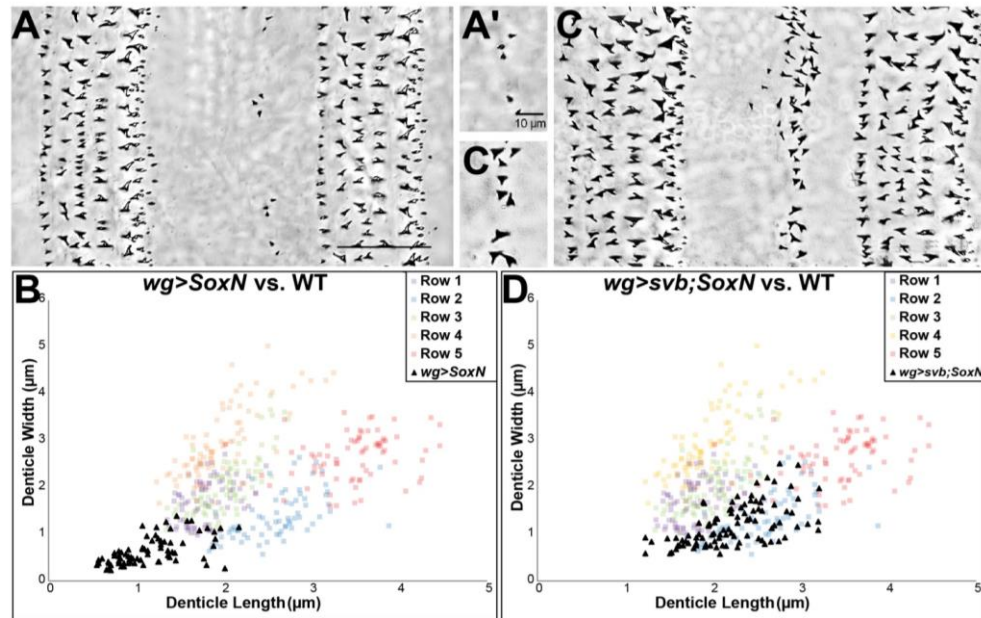
Embryos were collected and aged to the specified developmental time. For immunostaining, embryos were dechorionated in bleach and fixed for 20 minutes in 4% formaldehyde and PEM buffer (0.1 M PIPES, 1 mM EDTA, 2 mM MgSO<sub>4</sub>, pH 6.9). For actin filament visualization, vitelline membranes were removed by vigorously shaking in a 1:1 mixture of ethanol and heptane and washing 3 times in fresh ethanol. Rhodamine phalloidin (Molecular Probes/ThermoFisher) and anti-beta galactosidase antibody (Promega) both were used at 1:500. Anti-En antibody from the Developmental Studies Hybridoma Bank (University of Iowa) was used at 1:50 and anti-GFP antibody (EMD Millipore) was used at 1:500, on embryos that were devitellinized using methanol instead of ethanol. Secondary antibodies (Jackson ImmunoResearch) were used at 1:500. Images were captured on a Zeiss 510 confocal microscope. To examine cuticles, eggs were allowed to develop for 24 hours at 25°C, dechorionated with bleach, and mounted in Hoyer's medium. Images were captured with SPOT camera (Diagnostic Instruments) on a Zeiss Axioplan microscope, and were processed with SPOT imaging.

### **3.3 Results**

#### **3.3.1 Expressing *SoxN* with *svb* rescues ectopic denticle morphology**

Because increasing the 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. When we examined *SoxN* in our denticle assay system, we found that *SoxN*, like *svb*, 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. 3.2A,B). On average, the *wg>SoxN* ectopic denticles also had wider bases than *wg>svb* denticles, raising the possibility that *SoxN* activity controls base width while *Svb* controls length. Thus the combined activities of these two transcription factors might be necessary for shaping denticles properly.

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. 3.2C). 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. 3.2D). Furthermore, these ectopic denticles exhibited diverse morphologies. The majority were similar to row 2 denticles, with lower numbers that resemble the denticles of rows 1 or 3.

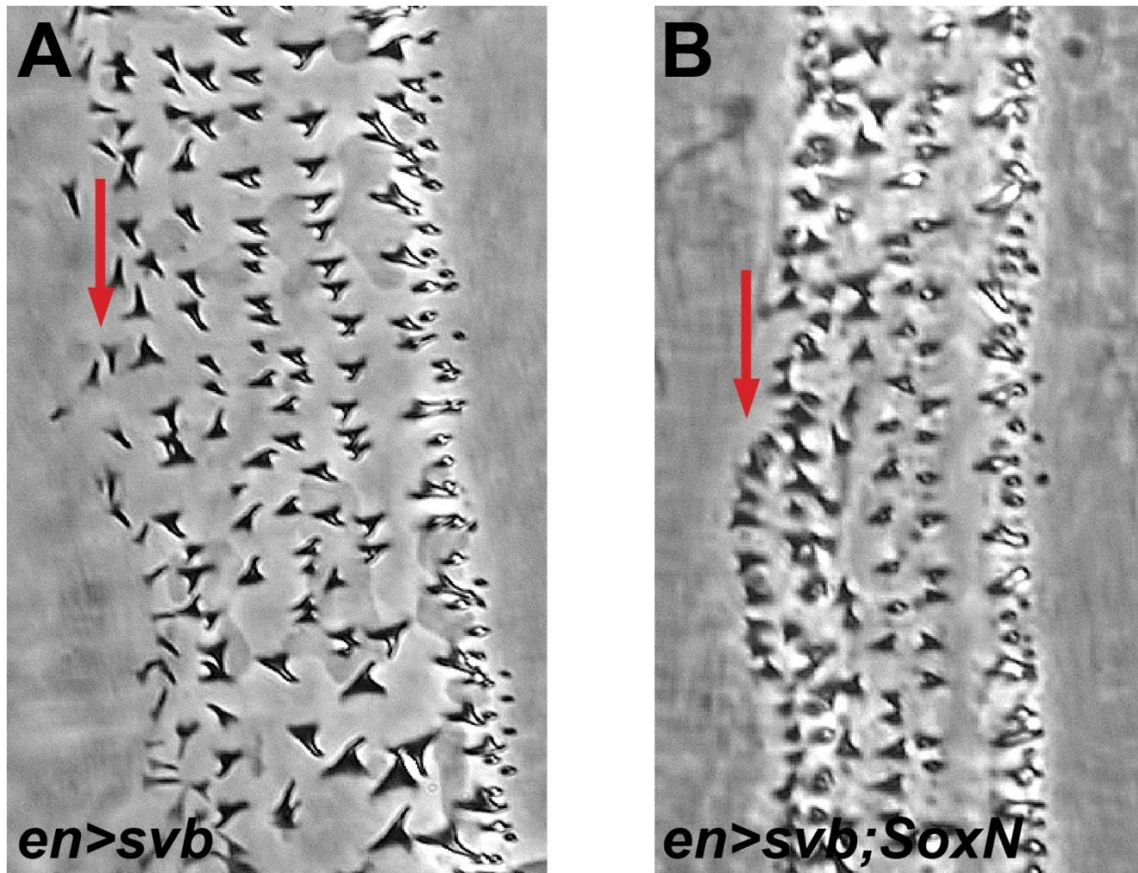


**Figure 3.1 Co-expressing *SoxN* with *svb* rescues ectopic denticle shape**

(A) *UAS-SoxN* was sufficient to form small, blunted denticles when ectopically expressed with the *wg-Gal4* driver. A', C': higher magnification views from A and C respectively (B) *SoxN*-induced ectopic denticles were reduced in both length and width compared with wild-type denticles. (C) Coexpression of *UAS-SoxN* with *UAS-svb* produced ectopic denticles that more closely resembled wild-type than did those produced by either transgene alone. (D) Length and width of these ectopic denticles significantly overlap the range of sizes found in a WT denticle belt.

### 3.3.2 Distance from Wg production did not alter ectopic denticle morphology

Because our denticle formation assay expressed transgenes in the endogenous *wg* expression domain, it was possible that high level Wg signaling in these cells might somehow block Svb's ability to direct proper denticle morphogenesis. Since SoxN down-regulates 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-Gal4* (*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. 3.3A). Likewise, *en-Gal4*-driven co-expression of *svb* and SoxN rescued denticle morphology, just as *wg-Gal4*-driven co-expression did (Fig. 3.3B). Because distance from Wg production did not alter ectopic denticle morphology, it is likely that SoxN's ability to rescue morphology is at least partially independent of its ability to suppress Wg activity.

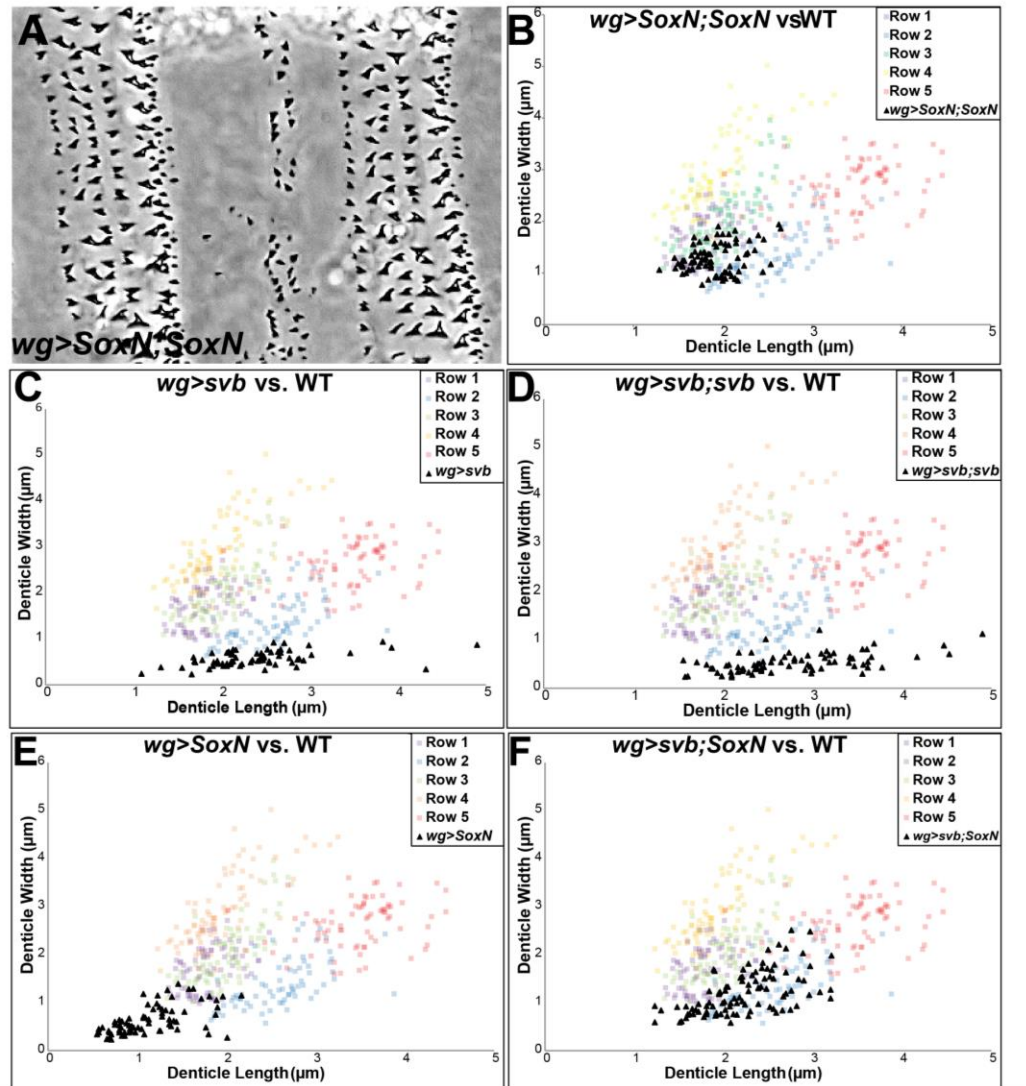


**Figure 3.2 Distance from *wg* production does not affect ectopic denticle rescue**

(A) The morphologies of ectopic denticles produced by *en-Gal4* driven *svb* were similar to those produced by *wg-Gal4* driven *svb*, indicating that overlap of *svb* expression with *wg* expression cannot account for the defective morphology of ectopic denticles. (B) Driving both *UAS-svb* and *SoxN* with *en-Gal4* rescued ectopic denticle morphology. Red arrows indicate rows of cells that form ectopic denticles.

### 3.3.3 Increased SoxN levels changes denticle shape

The expression of multiple copies of *svb* results in denticles longer than denticles produced by a single copy. This result shows that epidermal cells respond to an increase in transcription factor levels. Additionally, the expression of both *svb* and *SoxN* rescues the ectopic denticle morphology, suggesting that each factor controls different aspects of denticle shape. To determine what effects increasing *SoxN* levels would have on denticle shape, I expressed two copies of *UAS-SoxN* using *wg-Gal4*. This produced ectopic denticles both longer and wider than did expressing a single copy of *SoxN*. These ectopic denticles were similar in size to WT rows 1 and 3 (Fig 3.3). This result suggests that cells respond to increased *SoxN* levels as they do to increased *svb*. However, in the case of extra *SoxN* these ectopic denticles exhibit WT length and width.



**Figure 3.3 Increasing SoxN levels also rescues denticle morphology.**

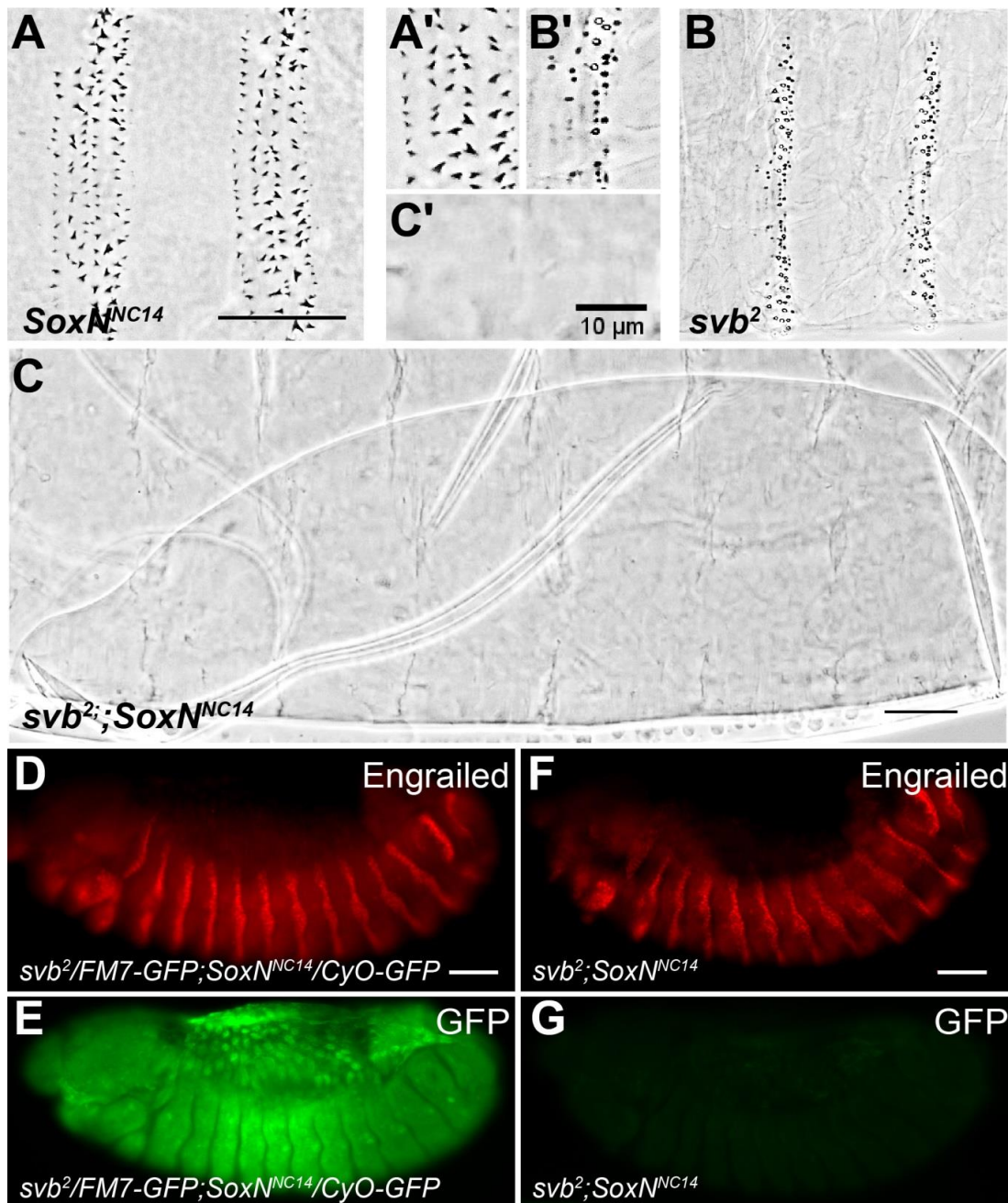
(A) Increasing SoxN levels (*wg>SoxN;SoxN*) rescues the ectopic denticle morphology. (B) Expression of two copies of *SoxN* produces denticles that are similar in size and shape to row 1 and row 3 WT denticles. (C-F) Ectopic denticle plots are from figures 2.3 and 3.1 for reference.

### 3.3.4 Loss of *svb* and *SoxN* results in embryos that lack denticles

To further explore the interplay of *Svb* and *SoxN* in driving denticle morphogenesis, we examined loss of function mutant 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. 3.5A). By contrast, *svb* loss of function mutants show a more severe loss of both ventral denticles (Fig. 3.5B) and dorsal trichomes (Payre et al., 1999). Some denticles remain in the most posterior rows, 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. 3.5C), suggesting that the “atrophied” structures displayed by *svb* mutants result from *SoxN* activity.

An alternative explanation for this all-naked phenotype is that *Wg* signaling might be hyperactivated in the absence of *svb* and *SoxN*. High 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 in the *svb;SoxN* double mutants. We examined expression of *en*, a known Wg target gene (DiNardo et al., 1988; Ingham et al., 1988) whose expression expands 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. 3.5F,G) compared to their heterozygous siblings (Fig. 3.5D,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.

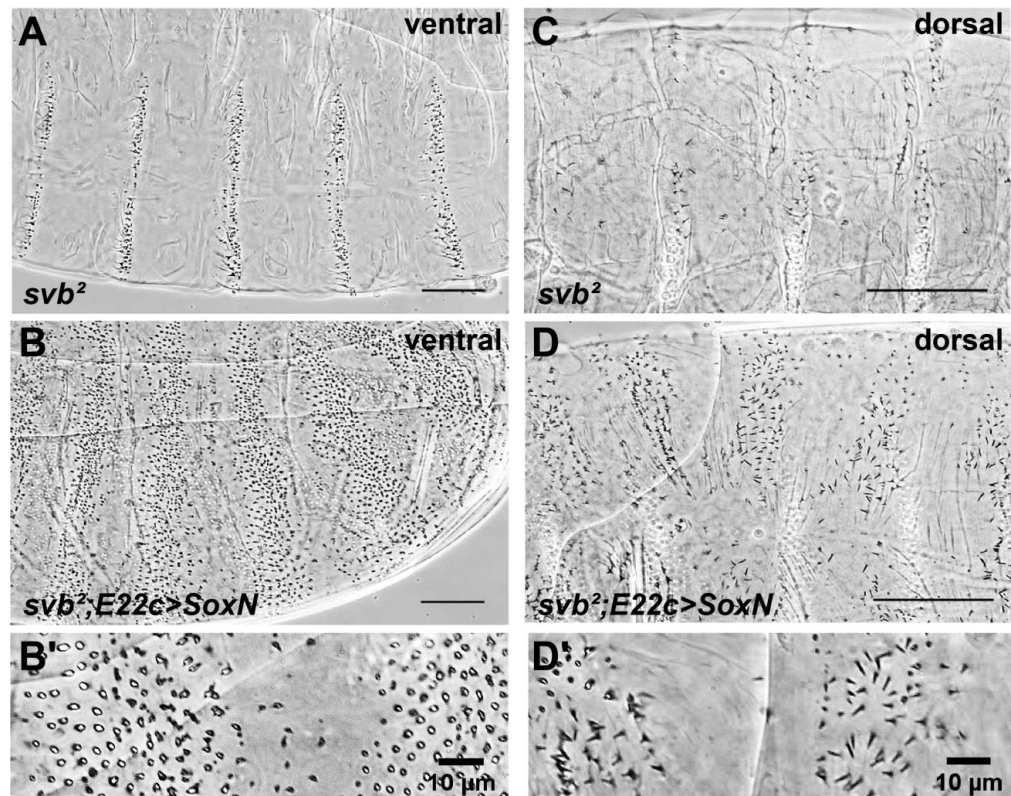


**Figure 3.4: Loss of *svb* and *SoxN* result in embryos that completely lack denticles.**

*SoxN* mutants produced defective denticle morphologies and a slight excess of naked cuticle. A'-C', higher magnification views from A-C. (B) *svb* mutants produced excess naked cuticle but retained small, blunted denticles in posterior rows. (C) *svb;SoxN* double mutants completely lacked ventral denticles, showing a fully penetrant uniform naked cuticle phenotype (21/338 total fertilized embryos = 6.21%, close to the expected 6.25% frequency of doubly mutant embryos). (D-G) This phenotype was not due to excessive Wg signaling, as *engrailed* 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-hour-old 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*, verified by 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)

### 3.3.5 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 uniform expression of *UAS-SoxN* in the epidermis of *svb* mutants, using the *E22c-Gal4* line. *E22c>SoxN* in *svb* mutants resulted in production of ventral denticles with the “atrophied” morphology (Fig. 3.6A,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. 3.6C,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.



**Figure 3.5 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. 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. Segmental modulation in the pattern of dorsal trichomes and blunted denticles can be observed (B, D) even though *Svb* is absent and *SoxN* is produced ubiquitously.

### **3.4 Discussion**

The findings presented in this chapter modify the previous model of denticle morphogenesis. The notion had been that *Svb* is the single key factor that functions in producing and shaping denticles, and that all patterning information converges upon *svb* to direct transcription of effector genes. We show here that this is not the case. The transcription factor *SoxN* contributes to denticle morphogenesis in combination with *Svb*. Co-expression of *svb* and *SoxN* rescues ectopic denticle morphology suggesting that both factors are required for proper denticle shaping.

We also show that *SoxN* is capable of producing denticles in the absence of *svb*. Although ectopic *SoxN* is sufficient to form denticles, this might have been an indirect result via *svb* activation. If such were the case, we would not expect to observe any denticles when expressing *SoxN* in the absence of *svb*. Our experiments show that *SoxN* is able to produce denticles in the absence of *svb*. Interestingly the resulting denticles do not protrude significantly, but take on a rounded appearance. These denticles are similar to those present in *svb* loss of function embryos. Consistent with this observation, we showed that the denticles in *svb* null embryos are the result of *SoxN* activity; embryos lacking both *svb* and *SoxN* are completely devoid of denticles. These experiments collectively suggest that *SoxN* plays an important role in controlling denticle base shape/width.

Changing the levels of each transcription factor had an effect on their control of denticle shape. As with *svb*, increased dosage of *SoxN* changes the cellular response. In the case of increased *SoxN*, ectopic denticles are wider and longer than those produced by action of a single transgene. These ectopic denticles resemble denticle shapes typical of rows 1 and 3 in WT belts. This is an interesting observation because expressing a single copy *svb* and *SoxN* together produces denticles similar in size to row 2 of WT belts. Thus we find support for the idea that differences in transcription factor levels may be responsible for the diversity in shape and size between WT rows.

The target genes of *Svb* have been well-studied and give us some insight into how the transcription factor functions in morphogenesis. Genes involved in actin bundling, cross-linking and organization are activated to produce the apically protruding structure of the denticle (Chanut-Delalande et al., 2006). Studies have been conducted that investigate *SoxN* target genes, though these focused specifically on the CNS or were performed in whole embryos (Ferrero et al., 2014; Carl and Russell, 2015). It is possible that some genes that function in denticle morphogenesis may be regulated by *SoxN* in both the CNS and epidermis, but we would expect to see some differences in target genes between tissues. Investigating targets of *SoxN* that are exclusive to the epidermis may reveal details of the specific role that *SoxN* plays in denticle shaping and how it impacts denticle width.

## 4. SoxN Activates Epidermal Target Genes Cooperatively with Svb and Independently

### 4.1 Introduction

Developmental programs are governed by the interactions between transcription factors and the target genes they regulate. Epidermal differentiation in the *Drosophila* embryo requires the collective activity of various effector molecules, primarily activated by *svb*. The identification of *svb* target genes has provided a mechanistic understanding of denticle morphogenesis. Svb triggers the expression of genes that function in the polymerization and bundling of actin filaments (reviewed in Chanut-Delalande et al., 2012) as well as cuticle proteins (Andrew and Baker, 2008) and pigmentation enzymes (Chanut-Delalande et al., 2006). Identifying targets that might be activated by Svb in combination with SoxN or by SoxN exclusively will help us gain a better understanding of the precise shaping of denticles and how the diverse morphologies of different rows are established.

The first study designed to discover effectors involved in denticle formation used known gene expression patterns from the Berkeley *Drosophila* Genome Project (Tomancak et al., 2002) to identify genes that are expressed in a striped pattern in the ventral epidermis of the embryo (Chanut-Delalande et al., 2006). These were then tested for altered expression in *svb* mutant embryos. This study identified *m*, *f*, *sn*, *sha*, and *wsp* as target genes of *svb*. Independent

mutations in these genes exhibit specific defects in denticle shape. Combinations of these mutations result in more severe defects, suggesting that each gene plays a direct role in denticle formation. Each of these proteins localizes to a specific subcellular region of the denticle, which presumably relates to their function in denticle construction. Epidermal expression of these genes is either completely lost or severely reduced in the absence of *svb*. Interestingly, the ectopic expression of *svb* (*wg>svb*), is sufficient to strongly activate only *sha* and *sn*. Ectopic *svb* results in only weak *wsp* activation, and in *f* expression that is barely detectable. This result was the first suggestion that additional factors may be required for target gene activation.

A similar study was performed looking at a group of genes known as the Zona Pellucida Domain (ZPD) gene family (Fernandes et al., 2010). These genes encode proteins that interact between the apical membrane and the extracellular matrix. As with the previously mentioned study, loss of *svb* results in the complete loss or diminished expression of each gene. Loss of each gene results in specific defects in denticle morphology, suggesting that these targets have non-redundant functions. Unlike the previously mentioned study, the ability of ectopic *svb* to activate each of these genes was not tested.

Genome-wide searches for SoxN target genes have been performed as well. Some of the *svb* target genes mentioned above were identified in these studies. This suggests that either both SoxN and Svb are capable of activating

the same gene independently, or they are both required to direct transcription. Presumably, SoxN also regulates epidermal genes independently of Svb. I explore these possibilities in this chapter.

## **4.2 Experimental Procedures**

### **4.2.1 In situ hybridization**

Hybridizations were performed as described in (Wilk et al., 2010). Anti-sense probes were made from cloned PCR products of genomic DNA, in the range of 700 base pairs – 1 kilobase. Primers encompassing the genomic region to be probed contain different restriction sites on each primer. Vectors were linearized for in vitro transcription to produce anti-sense probes.

### **4.2.2 Binding site prediction**

Potential SoxN binding sites were identified by entering target gene sequences from Flybase (Gramates et al., 2017) into 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  $p$  values  $< 10^{-3}$ .

## 4.3 Results

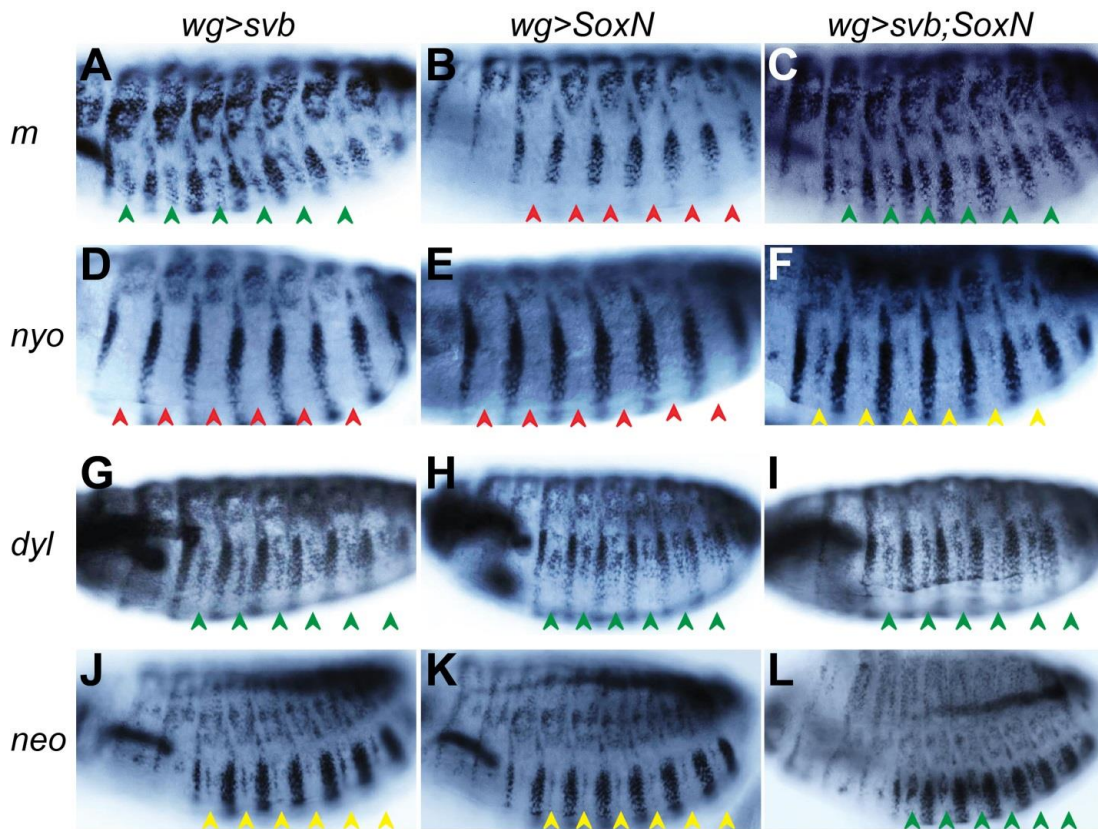
### 4.3.1 SoxN differentially activates ZPD genes

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 the denticle shape. The well-studied ZPD gene, *miniature (m)*, was activated robustly by ectopic expression of *svb* (Chanut-Delalande et al., 2006) (Fig. 4.1A). We found that ectopic expression of *SoxN* did not activate ectopic *m* expression (Fig. 4.1B), nor did it contribute to the strength of *m* expression when co-expressed with *svb* (Fig. 4.1C). 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 neither *svb* nor *SoxN* alone was sufficient to activate detectable

expression of *nyo* (Fig. 4.1D,E). Even doubling the amount of ectopic *svb* was insufficient to activate *nyo* (Fig. 4.2B). By contrast, co-expression of *svb* and *SoxN* together activated moderate *nyo* expression (Fig. 4.1F). 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. 4.1G), but was also detected strongly when *SoxN* alone was expressed ectopically (Fig. 4.1H). There was no discernible difference in *dyl* expression when both factors were ectopically co-expressed (Fig. 4.1I). By contrast, the gene *neo* was activated weakly when either *svb* or *SoxN* alone was expressed (Fig. 4.1J,K), and was expressed strongly when both genes were expressed together (Fig. 4.1L). 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 (summarized in Fig. 4.1M).

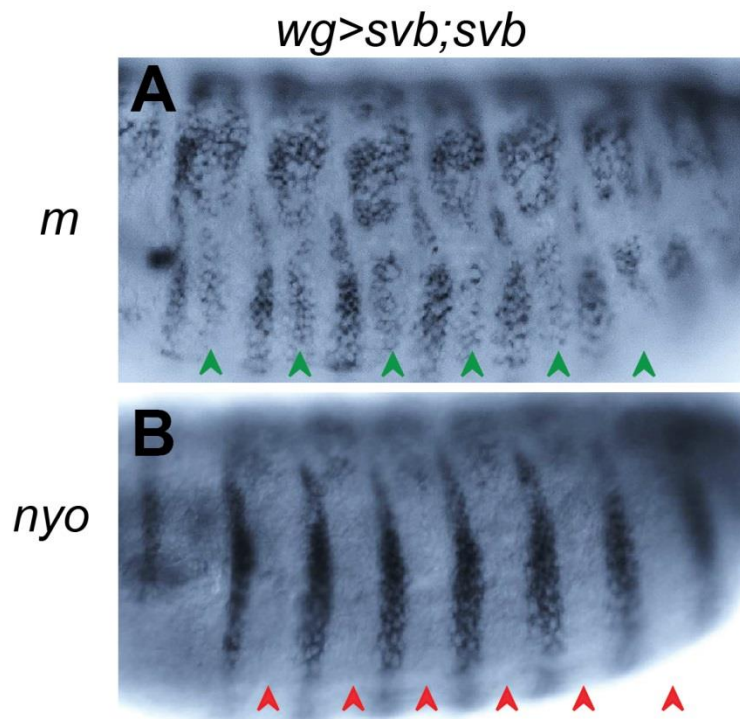


**M**

	<i>wg&gt;svb</i>	<i>wg&gt;SoxN</i>	<i>wg&gt;svb;SoxN</i>
<i>m</i>	Strong	None	Strong
<i>nyo</i>	None	None	Moderate
<i>dyl</i>	Strong	Strong	Strong
<i>neo</i>	Moderate	Moderate	Strong

#### Figure 4.1: SoxN differentially activates ZPD genes

(A) Whole mount in situ hybridization of 14- to 16-hour-old embryos showed that *UAS-svb*, driven ectopically with *wg-Gal4*, was sufficient to activate *m* expression. Arrows in panels A - L indicate the *wg* domain where transgenes were expressed ectopically. Color of arrow describes the relative strength of gene activation (red = none, yellow = moderate, green = strong). (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. *nyo* expression was not activated through the expression of either *svb* (D) or *SoxN* (E) alone, but was activated when they were coexpressed (F). *dyl* was activated at substantial levels by either *svb* (G) or *SoxN* (H), and was not driven to higher levels of expression by the co-expression of *svb* and *SoxN* (I). *neo* expression was detected at low levels when *svb* (J) or *SoxN* (K) alone was ectopically expressed, and showed much stronger expression when both *svb* and *SoxN* were expressed together (L). (M) Summary of each transcription factor's contribution to target gene activation.



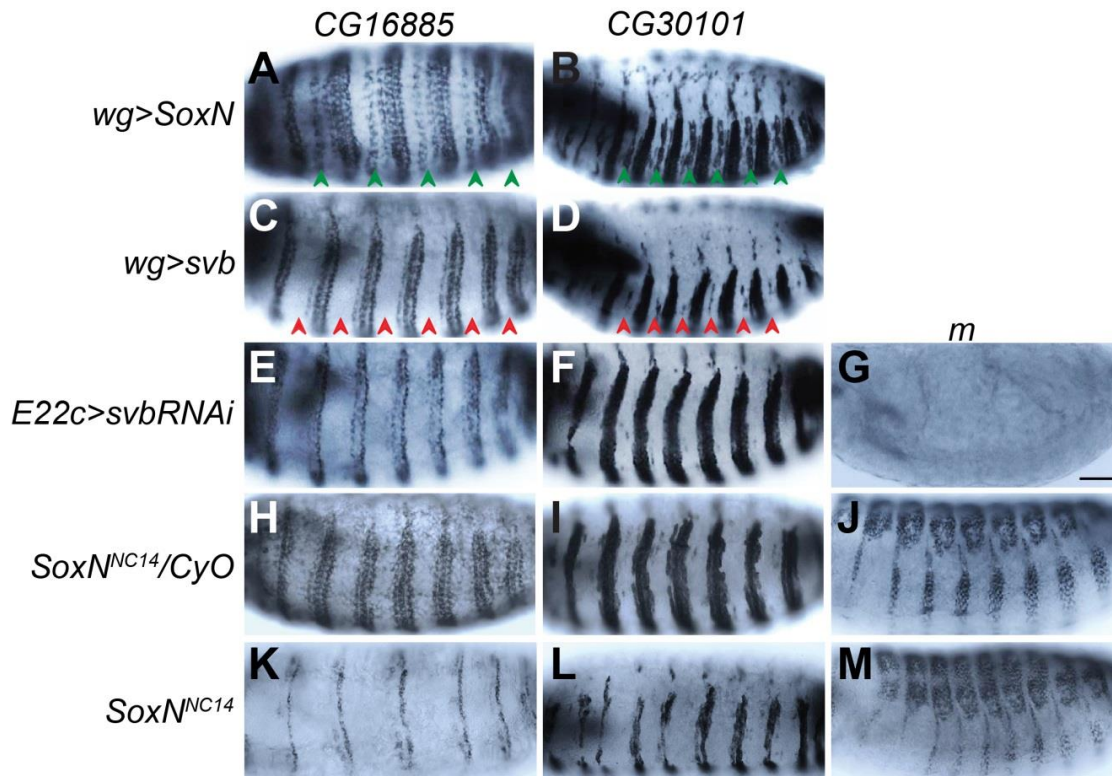
**Figure 4.2. Doubling the *svb* transgene dose does not increase target gene expression.**

(A) *miniature* RNA in situ hybridization in embryos with *wg-Gal4* driving two *UAS-svb* transgenes show same level of ectopic *m* induced (green arrows) as observed with single transgene (Fig. 3.1A). (B) *nyobe* RNA in situ hybridization in embryos with *wg-Gal4* driving two *UAS-svb* transgenes shows no ectopic expression induced (red arrows), similar to what is observed with single transgene (Fig. 3.1D).

### 4.3.2 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. We found that ectopic expression of *SoxN*, but not of *svb*, activated *CG16885* expression (Fig. 4.3A,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 where *svb* was knocked down with RNA interference (Fig. 4.3E). 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 4.3B) and that ectopic expression of *svb* failed to activate *CG30101* expression above background levels (Fig. 4.3D). As with *CG16885*, driving uniform embryonic epidermal expression of *svb RNAi* produced no discernible change in *CG30101* expression (Fig. 4.3F), 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. 4.3G). Thus we have identified two epidermal target genes that can be activated by SoxN without input from Svb.

Consistent with these results, both genes require input from SoxN for their normal expression during development. We hand-selected *SoxN* homozygous mutant embryos by absence of Green Fluorescent Protein (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, while there appeared to be somewhat weaker expression than normal in the ventral epidermis (Fig. 4.3J,M). This is likely due to the feedback loop where SoxN boosts *svb* expression in ventral, but not dorsal, cells. We found that ventral *CG16885* expression was significantly reduced in *SoxN* mutants compared with their heterozygous siblings (Fig. 4.3H,K). Expression of *CG30101* was also diminished in *SoxN* mutant embryos, although not as dramatically as was *CG16885* (Fig. 4.3I,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.



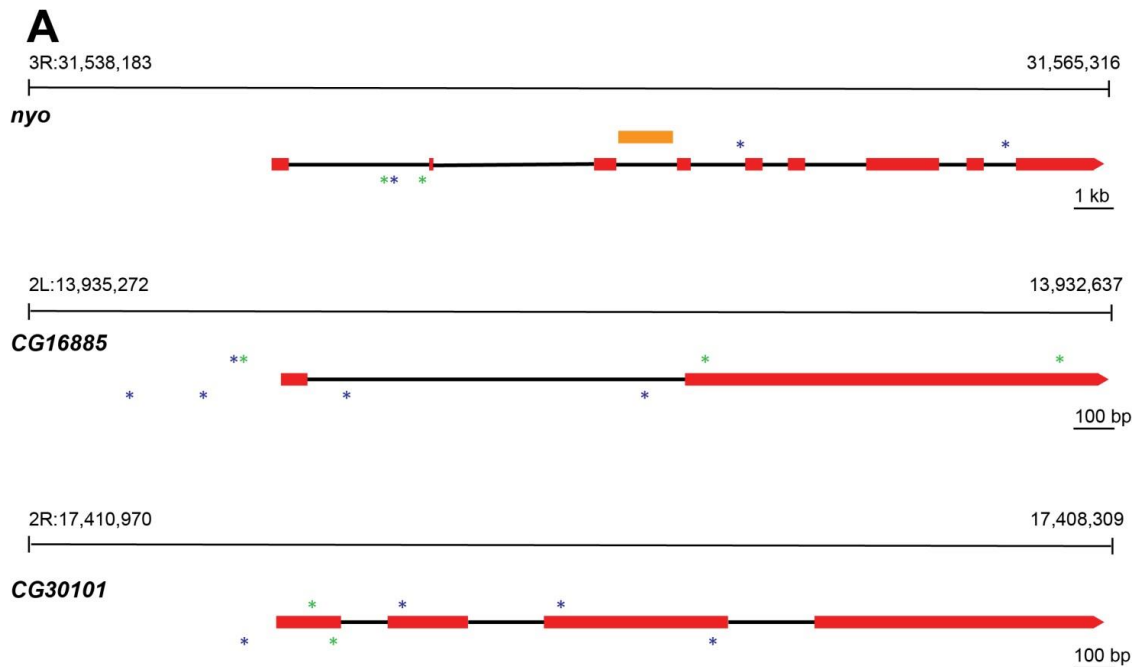
**Figure 4.3. SoxN activates CG16885 and CG30101 independently of Svb.** Ectopic SoxN, driven by *wg-Gal4*, activated expression of CG16885 (A) and CG30101 (B), whereas ectopic *UAS-svb* was insufficient to activate expression of either CG16885 (C) or CG30101 (D). Knockdown of *svb* in the epidermis, using *E22c-Gal4* to drive uniform expression of *UAS-svb-RNAi*, 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<sup>NC14</sup> 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 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 (at top) appeared normal.

### 4.3.3 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 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 *CG16885* and *CG30101*, and identified a number of predicted binding sites (asterisks in Fig. 4.4A; Table 1). In *nyo*, none of the putative SoxN binding sites we found fall within the previously identified enhancer region (orange bar in Fig. 4.4A). All are in introns either upstream or downstream of the Svb-binding enhancer element, although one site is located within one kilobase of this enhancer. Since the previously defined region may represent a minimal core enhancer, additional flanking sequences may be required to get the robust output required during development or during times of variation in nature (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, may 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. 4.4A; Table 1). The *CG16885* sequence contains several sites in the region immediately upstream of the transcriptional start site, as well as within the intron and second exon. The sites found in *CG30101* all reside in exons except for one that is 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 (indicated by green asterisks in Fig. 4.4A), suggesting that these sites might be functional in the observed transcriptional control.



**Figure 4.4. Predicted SoxN binding sites in *nyo*, *CG16885*, and *CG30101* lead to a model for denticle morphogenesis.**

(A) Schematic diagram of upstream and coding region for *nyo* containing putative binding sites (asterisks) determined using FIMO (Find Individual Motif Occurrences). Asterisk above the gene indicates binding to the (+) strand; asterisk below indicates binding to (-) strand. All binding sites shown are high confidence hits:  $p$ -value  $< 10^{-4}$  for *nyo* and  $< 10^{-3}$  for *CG16885* and *CG30101* (data are summarized in Table 1). Yellow bar over the fourth intron in *nyo* represents a functional enhancer activated by *svb* (Menoret et al., 2013). *CG16885* and *CG30101* 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 1).

**Table 4.1. List of predicted SoxN binding sites in *nyo*, CG16885 and CG30101**

Gene	Binding Site Coordinates	p-value	Coordinate Sequence	Conserved Species
CG16885	2L:13932099..13932109	5.13E-05	GCGAACAATTA	
	2L:13932090..13932100	2.70E-04	GCAAACAATGT	
	2L:13934471..13934481	2.95E-04	TGGAACAATGT	sechellia, simulans
	2L:13933769..13933779	2.95E-04	ACGCACATCGC	sechellia, simulans, yakuba, erecta
	2L:13935173..13935183	3.82E-04	CTGTACAATTA	
	2L:13934919..13934929	5.74E-04	ACGGACAGTGT	sechellia, simulans, yakuba
	2L:13933923..13933933	8.40E-04	CCCAACATTGC	sechellia, simulans
	2L:13934972..13934982	8.40E-04	GTGAACACTCG	sechellia, simulans
	2L:13932682..13932692	8.91E-04	CTGTACTACTCG	sechellia, simulans, yakuba, erecta
	2L:13935488..13935498	9.06E-04	ACTAACACTTC	
CG30101	2R:17410142..17410152	3.52E-04	GGAACAATTA	sechellia, simulans, yakuba, erecta, ananasse
	2R:17409197..17409207	4.01E-04	CCGTACACCGT	sechellia, simulans
	2R:17410109..17410119	4.72E-04	CTCAACAATGC	sechellia, simulans, yakuba, erecta, ananasse, pseudoobscura, persimilis, willistoni, mojavensis, virilis, grimshawi
	2R:17410435..17410445	7.25E-04	GCGTACATTCA	
	2R:17409565..17409575	8.18E-04	CTCAACAATGT	
	2R:17409948..17409958	9.32E-04	CGCCACAATGG	
<i>dusky-like</i>	3L: 4308522..4308532	4.07E-05	ATGAACAATGG	
	3L: 4311650..4311660	9.43E-05	ACGCACAGTTC	simulans
<i>neo</i>	3R:29825746..29825756	5.60E-05	CCGTACAATTA	sechellia, simulans
<i>nyobe</i>	3R:31547625..31547635	5.72E-05	CGGCACAATGG	simulans, yakuba
	3R:31547719..31547729	5.99E-05	GCGAACAATAA	simulans
	3R:31549064..31549074	8.78E-05	ACAAACAATGA	simulans, yakuba, erecta
	3R:31559928..31559938	9.05E-05	ACGCACAGCGC	sechellia, simulans
	3R:31564141..31564151	9.67E-05	GCGAACAACCA	sechellia, simulans

Coordinates of each putative binding site are listed and are relative to the genomic sequence in Flybase *D. melanogaster* (R6.13). Binding sites are ranked by p-value.

## 4.4 Discussion

After discovering that both Svb and SoxN function to shape denticles, we next examined how their activity affected downstream gene expression. Svb target genes have been well-studied and participate in a number of different functional aspects of denticle morphogenesis. Uncovering the genes regulated by SoxN may shed light on the mechanism by which SoxN acts to shape the denticle, with particular control over the width of the base. The cooperativity of these transcription factors will also aid in our understanding of transcriptional regulation.

In the case of *nyo*, we see that both transcription factors are required for activation. This result may suggest that these proteins act in a complex as cofactors to activate transcription. It is often thought that cofactors are bound to adjacent DNA sequences, and interact directly to reinforce each other's binding. However, it is possible that more indirect mechanisms of cooperativity are at work here. Some transcription factors exhibit indirect interactions through common transcriptional regulators resulting in a synergistic effect; such is the case with the activation of the gene *IFN $\beta$* . In response to viral infection, *IFN $\beta$*  activation requires the activity of several transcription factors that interact and form a complex through the recruitment of the common co-activator p300 (Merika et al., 1998). Binding of transcription factors can also induce DNA bending which can increase the affinity of other transcription factors to bind-or formation of a

multi protein complex (Falvo et al., 1995; Panne et al., 2007). There is substantial evidence of HMG transcription factors, such as SRY (Ferrari et al., 1992), HMG1 (Pil et al., 1993), and Sox2 (Scaffidi and Bianchi, 2001), functioning as DNA-bending proteins.

Determining the function of both *CG16885* and *CG30101* are of obvious interest. We do not yet know whether the *CG16885* and *CG30101* gene products play a role in shaping denticles, but both genes 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 signal peptide at the amino-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 may provide mechanistic insight into denticle morphogenesis.

Our investigation for SoxN activated target genes was successful in the identification of *CG16885* and *CG30101*. However, since we have yet to determine the function of these genes, their identification does not contribute to a mechanistic understanding of denticle morphogenesis. We can continue to use the same approach by looking at more genes that exhibit similar expression patterns to SoxN and that are not controlled by *svb*. We can also comb through the list of genes from the genome-wide analyses of SoxN binding. Though these

studies were performed in other tissues, we can look for genes that are known to be expressed in epidermal stripes or to be involved in processes such as cytoskeletal remodeling. Also, genes such as cadherins, which were found in the SoxN target gene studies, exert many of their effects through cytoskeletal interactions (Niessen et al., 2011). These genes may be of interest and would add a novel mechanism of denticle morphogenesis. These studies have also shown SoxN binding in genes coding for chitin deacetylase and cuticular proteins. Genes involved in cuticle hardening and pigmentation are known Svb targets, suggesting that these are good candidates for epidermal targets of SoxN. Testing for SoxN activation of these genes will add more to our understanding of the regulation of epidermal differentiation.

## 5. Summary and conclusions

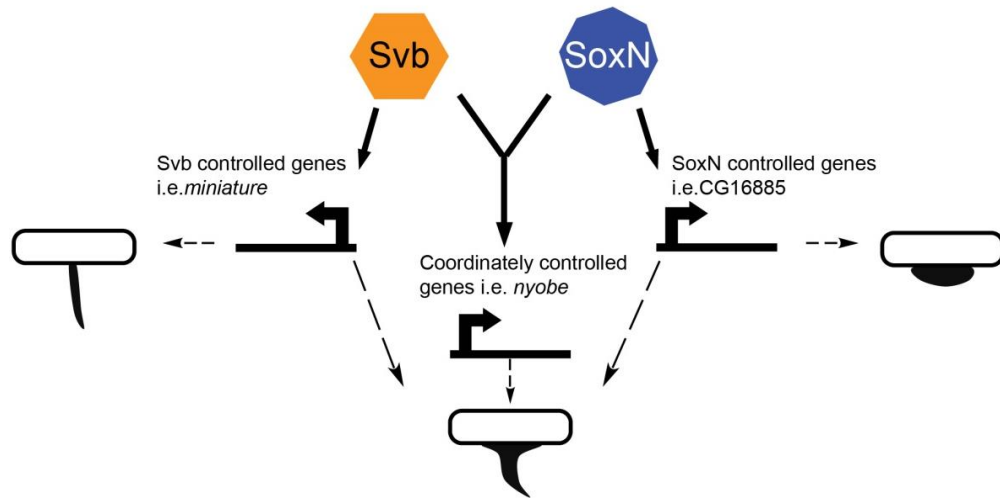
The primary goal of this study was an attempt to answer a general question of developmental biology as well as a more specific question in *Drosophila* embryogenesis. First, how are morphological events that occur during development transcriptionally regulated and properly executed? Many of the signaling pathways that are responsible for morphogenetic events have been identified, but how these signals specify cellular events needs to be better studied. Second, how are the stereotyped, diverse morphologies of denticles produced by the embryonic epidermis specified? Loss of *Wg* results in the loss of denticle diversity, but how does *Wg* signaling function in this capacity? In addition, how does a single transcription factor, *Svb*, produce these various shapes within the denticle belts? Our results provide insight into these fundamental questions in developmental biology.

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 in 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. 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 precise levels for each protein may be critical to the final shape assumed. Our finding that *Svb* and *SoxN* differentially activate ZPD genes, and other targets, raises the possibility that each row of cells within a belt may experience slightly different levels of the gene products that control cell shape. Whether this differential regulation is specific to the ZPD group of genes or extends to other known *Svb* targets is an interesting question for future studies.

Indeed, our experiments suggest that *Svb* and *SoxN* may be responsible for two different aspects of denticle construction. We have shown here that increased levels of *Svb* produced longer denticles, without altering their overall shape. Thus *Svb* may 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 where *Svb* controls genes that specify denticle length whereas *SoxN* controls genes that regulate base circumference (Fig. 5.1). 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 may be an upper limit to 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. 1.5B) where almost all have the size and shape of row 5 denticles, the large denticles at the posterior of the wild-type belt (Fig. 1.2B).



**Figure 5.1 Model depicting the proposed epidermal role for each transcription factor 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 that of genes they activate independently, produces the balance of gene products required for proper denticle elongation and shaping.

In addition to *Svb* and *SoxN*, one or more as-yet uncharacterized factors may modulate denticle size and shape, because 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 may 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.

*SoxN*'s role as a suppressor of *Wg* may be important for the denticle producing cells. The negative feedback on *Wg* signaling may help to refine the level of *Wg* response within the denticle belt rows. *SoxN* suppresses *Wg* signal at the level of target gene transcription, but the details of mechanism are not yet understood. The experiments that our lab has performed were unable to detect

interactions between SoxN and Tcf or beta-catenin in HEK293T cells. Repeating these co-immunoprecipitation experiments with embryos, particularly protein extracts from denticle producing cells, might help establish whether such interactions occur. It is well documented that Sox proteins interact with Tcf and beta-catenin to modulate Wnt-responsive transcription (reviewed in Kormish et al., 2010). Wg suppression by SoxB proteins has been observed from sea urchins (Kenny et al., 1999) to humans (Chen et al., 2008) through their interaction with beta-catenin. However, it is possible that SoxN binding increases the affinity of Tcf and Groucho, the transcriptional co-repressor of Wg targets (Cavallo et al., 1998) without direct physical interaction. Determining how SoxN's action regulates Wg signaling is important because, as with mutations in Wg pathway components, the deregulation of Sox genes has been associated with various cancers (Dong et al., 2004). There is also evidence of Sox transcription factors modulating oncogenic Wnt target genes (Sinner et al., 2007). Thus elucidating the mechanism of Wg suppression by SoxN will add to our understanding of signal modulation that may have health implications.

The transcription factor Dichaete (D) is an HMG transcription factor with similarity to SoxN that is also expressed in the ventral epidermis. D exhibits some redundancy in establishing neuronal cell fates with SoxN (Buescher et al., 2002; Overton et al., 2002). It will be interesting to determine how much this redundancy extends to the epidermis and denticle morphogenesis. Preliminary

experiments that I have performed show that the ectopic expression of *D* is not sufficient to form denticles. Also, the co-expression of *D* with *svb* does not rescue denticle morphology. Ectopic expression of *D* is not sufficient for the activation of either *CG16885* or *CG30101*. Yet, like SoxN, *D* is negatively regulated by Wg activity and antagonizes Wg signaling (Overton et al., 2007). Mutations in *D* show cuticle defects and enhance the mutant SoxN phenotype (Nambu and Nambu, 1996). Two different studies have investigated *D* target genes in the CNS, with one looking specifically at the redundancy in gene targets with SoxN (Shen et al., 2013; Ferrero et al., 2014). Although we found no evidence that *D* plays a role in regulating transcription of effector genes, it might participate in the regulatory network governing epidermal differentiation. An investigation of *D* target genes in the CNS identified numerous components of the Wnt and EGF pathways (Shen et al., 2013). Since both of these pathways participate in the regulation of *svb*, it is possible that these regulatory interactions would also affect epidermal cells. It will be interesting to determine whether these pathway genes are regulated by *D* within the epidermis, and whether SoxN exhibits redundancy in their regulation.

The functions of the novel SoxN target genes that we have identified in this work need to be investigated further. Although there is currently no experimental data describing *CG16885* and *CG30101*, there is predictive data that suggests a role for these genes in epidermal patterning. The STRING database uses known and predictive associations to compile protein networks in

a number of organisms including *Drosophila* (Franceschini et al., 2013). Analysis of *CG16885* and *CG30101* predicts interactions with cuticular proteins such as Cpr92F, Cpr97Eb, and Cpr100A. Each of these proteins contains a predicted signal peptide along with a chitin binding domain, which is a conserved domain found in insect larval cuticles (Rebers and Riddiford, 1988). Another predicted interacting protein, Serpentine, is a putative chitin deacetylase (Luschnig et al., 2006). In arthropods, the deacetylation of chitin in vitro results in decreased density of chitin fibrils (Cho et al., 2000) and is thought to affect the orientation and structure of the fibrils (Neville, 1975). These predicted interactor genes, along with other uncharacterized predicted interactors, exhibit similar expression patterns. These genes should be investigated to determine whether or not their loss of function results in defects in denticle morphology. Additionally, these predicted interactor genes may be targets of SoxN. Their expression could be assayed in *SoxN* mutants and *SoxN* gain of function embryos to test for regulatory effects. Since many of these proteins are similar in sequence, it is possible that they are functionally redundant. Thus it may be necessary to examine their loss of function in multiple mutant combinations, which may reveal phenotypes not exhibited by each individual mutant.

The data presented here suggest that transcription factor levels affect denticle shape. We propose that the balance of Svb and SoxN in each row specifies the denticle morphology. Rows in which denticles are longer would

have increased levels of Svb, whereas rows in which denticles have wider bases would have increased levels of SoxN. Increasing levels of each transcription factor may proportionately increase target gene expression. This has been demonstrated with NF- $\kappa$ B in target genes with clustered binding sites (Giorgetti et al., 2010). In this scenario, differences in effector molecule levels, the output from Svb and SoxN target genes, would ultimately be responsible for denticle shape. Alternatively, there may be a critical threshold for each transcription factor that needs to be achieved in order to transcriptionally activate certain target genes (Fiering et al., 1990). In this case, some denticle rows would express target genes exclusive from other rows. Row specific expression is seen with the gene *stripe*, which encodes a transcription factor that is expressed in rows 2 and 5 of the denticle belts (Dilks and DiNardo, 2010). It is possible that both of these scenarios occur within the denticle producing cells. Investigating these possibilities as well as how transcription factor levels are established will provide valuable insight into the transcriptional regulation of development.

A precise balance of protein levels may 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 critically on specific levels 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 may help elucidate the forces that shape stereocilia in the human inner ear, and that go awry in some hereditary forms of deafness.

## Appendix A: Testing the role of Sloppy-paired in Wg-mediated repression

As discussed in Chapter 2, it is possible that the Wg repression of *svb* is indirect, acting through an intermediate repressor. Expression of the *sloppy paired* (*slp*) locus is activated by Wg signaling and encodes a known transcriptional repressor. In early stages of development, Slp functions as a repressor in the anterior edge of the most anterior stripe of several pair-rule genes (Andrioli et al., 2004) and represses *en* and *hh* (Cadigan et al., 1994b). Thus we judged Slp to be a good candidate for an intermediate between Wg signaling and Svb repression.

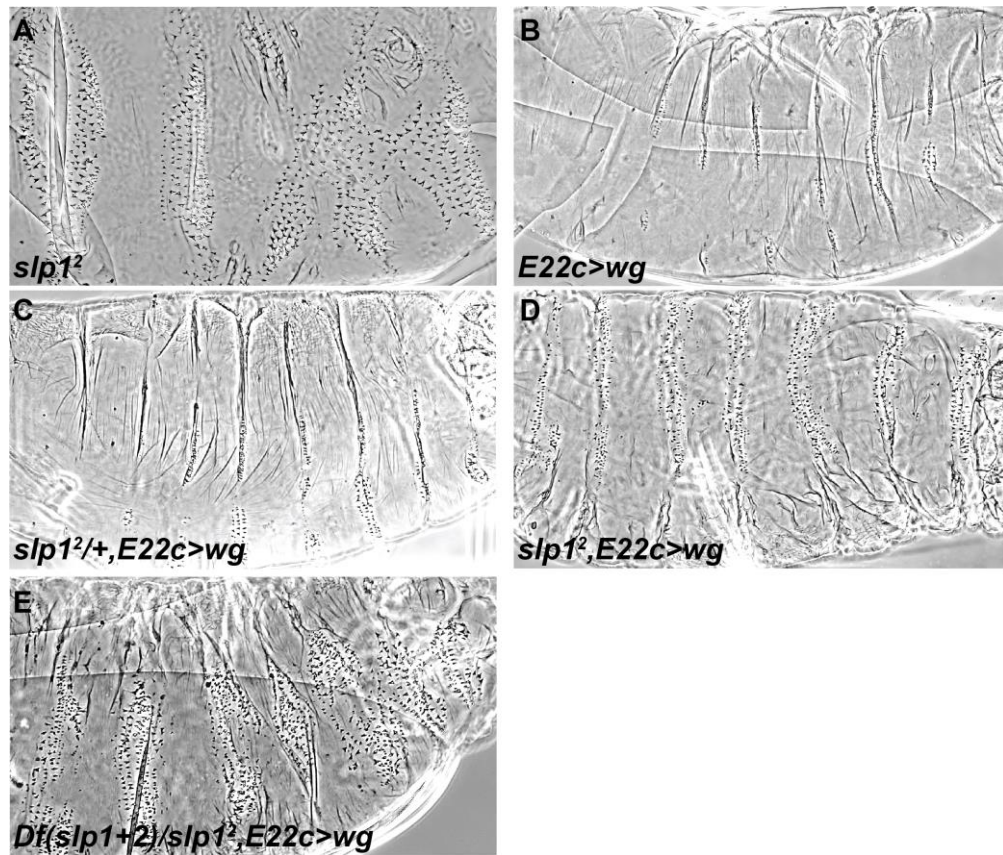
The *slp* locus contains two genes, *slp1* and *slp2*, that are involved in segmentation (Grossniklaus et al., 1992). Each gene encodes a forkhead transcription factor with functional redundancy (Hacker et al., 1992; Cadigan et al., 1994a). Loss of the entire *slp* locus produces a lawn of denticles phenotype similar to that observed in *wg* null embryos (Cadigan et al., 1994a). Single mutations in either *slp1* or *slp2* result in loss of naked cuticle, producing fusions of the more posterior denticle belts (Fig 6.1A). I set out to test whether Slp may function in *svb* repression, but there are two difficulties with this approach: the functional redundancy of *slp1* and *slp2*, and their requirement at earlier stages in development. To avoid these difficulties, I used ectopic expression to test whether *slp* acted downstream of Wg. Hyperactivation of *wg* in the epidermis results in the loss of all denticles, producing uniform naked cuticle (Fig

1.6B,6.1B). If this naked cuticle requires activation of *slp* which then represses *svb*, then *wg* hyperactivation in a *slp* mutant embryo should not result in the loss of denticles. On the other hand, if Wg signaling directly represses *svb* expression, then the loss of *slp* should have little to no effect on the loss of denticles/ uniform naked cuticle phenotype.

To conduct this test, I recombined the *slp1<sup>2</sup>* null allele onto a chromosome II carrying *E22c-Gal4*, and separately onto a chromosome II carrying *UAS-wg*. Since both transgenes and the *slp* locus are located on chromosome II, crossing the *E22c-Gal4* driver line to the *UAS-wg* line will produce progeny where all of the embryos that overexpress *wg* must also be homozygous mutant for *slp1*. I first crossed the *slp1<sup>2</sup>,UAS-wg* potential recombinants to *E22c-Gal4* to verify the presence of *UAS-wg*. Although these embryos are only heterozygous for the *slp1<sup>2</sup>* mutation, they produced noticeably more denticles than *E22c>wg* embryos (Fig 6.1C). Thus simply reducing the dose of Slp by half compromises the ability of Wg to promote naked cuticle cell fate. I then crossed the two recombinant lines to produce *slp1<sup>2</sup>* homozygous mutants that hyperactivate *wg*. These embryos showed an enhanced ability to form denticle belts (Fig 6.1D). While these denticles did not form the uniform lawn associated with the *slp* mutant phenotype, this may be due to the presence of *slp2*. Therefore I recombined the *E22c-Gal4* transgene onto a chromosome II bearing a deficiency that removes both *slp1* and *slp2*, *Df(slp1+2)*. Unfortunately, embryos homozygous for this

deficiency die prior to full cuticle secretion, which makes scoring cuticle pattern difficult. I therefore crossed this recombinant line to *slp1,UAS-wg* to produce Wg-overexpressing embryos with only a single copy of *slp2*. These *Df(slp1+2), E22c/slp1,UAS-wg* embryos with half the dose of *slp2*, produce even larger denticle belts with some fusion due to loss of naked cuticle separating the belts (Fig 6.1E). These experiments strongly suggest that the Wg-mediated specification of naked cuticle requires some aspect of *slp* activity.

As mentioned earlier, there may be additional inputs into the regulatory region of *svb*. The *slp* genetic experiments show that removal of *slp* impacts the ability of Wg to specify naked cuticle. The modENCODE project aimed to discover the functional elements in the genomes of model organisms, including transcription factor binding sites (Celniker et al., 2009). Using both ChIP-chip and ChIP-seq experiments, Slp1 binding sites throughout the *Drosophila* genome were detected. Two Slp1 binding sites were found in the regulatory region of *svb*. The first is in the proximal 7.3 enhancer that is expressed in the entire denticle belt; the second is in the overlap region shared between the *DG2/DG3* enhancers. The presence of these binding sites allows us to propose a simple model where Wg signaling activates *slp* expression, which produces the repressor that controls *svb* expression.



**Figure 6.1 Loss of *slp* suppresses the *Wg* hyperactivation phenotype**

(A) Loss of *slp1* function results in denticle belt fusion. (B) Expressing *wg* throughout the epidermis using *E22Gal4* results in the loss of denticles and the complete specification of naked cell fate. (C) Embryos heterozygous for the *slp1*<sup>2</sup> allele results in a slight increase in denticles when expressing *wg* with *E22cGal4*. (D) Epidermal expression of *wg* in the absence of *slp1* function results in denticle belt formation. (E) Epidermal expression of *wg* in the absence of *slp1* and a single copy of *slp2* results in expanded denticle belts and loss of some naked cuticle.

## **Appendix B: Detailed protocols**

### ***7.1 Step-by-step instructions for measuring denticles using FIJI***

Open up images in FIJI and duplicate (Ctrl+Shift+D) as to not alter the original image. Select “Image” tab, then “Adjust”, followed by “Threshold” (Ctrl+Shift+T). Select threshold that is best suited for desired image. I used the “Default” setting for all of the denticle measurements. Objects in image that you want to measure are colored in red at this point. Select the “Analyze” tab and “Select Measurements” to choose any default measurements that FIJI is able to determine i.e. area or perimeter. Then proceed to “Analyze Particles” under the same tab. This will set the minimum and maximum limits to pixel quantity. Limits used were typically 40-Infinity. Click on “Show” droplist and select “Outlines” to produce a drawing of your image. After selecting “Ok”, your image will show the objects that FIJI was able to determine and each will have a number. The drawing will show all of the outlined objects with their corresponding numbers. Save the “Results” window as an Excel file, as well as the drawing and threshold image. You will use the threshold image to measure denticles. Prior to taking measurements, set scale accordingly (images used for measuring denticles were all 63X). After setting proper scale, use the “\*Straight\*” selection on the tool bar, and draw lines from one end of denticle base to the other and choose “select scale” to record the measurement in micrometers. To measure length, draw line from the tip of denticle to the midpoint of the base and view under “select scale”.

Record these measurements along with the corresponding image and number.  
Only denticles within 75 $\mu$ m of the midline were measured. A minimum of 70 denticles were measured to calculate averages.

## **7.2 In Situ Hybridization**

### **Cloning of probe template**

Optimal probe length is between 700-1000 bp. Probes were designed against the largest exons within the gene of interest. Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) was utilized for primer design. After choosing primer pairs, restriction sites were added to the ends of each primer, along with an extra 6 bases. The first 12 bases of the forward primers are TGCTTAGAATTC, the last 6 being the EcoRI recognition sequence. The first 12 bases of the reverse primer are TAAGCAAAGCTT, the last 6 being the HindIII recognition sequence. Primers were diluted to 100µm stock and 10µm working solution. PCR reactions were set up with the following volumes:

- 12.5µl of 2X Taqman master mix
- 1µl DNA template (*w1118*)
- 1µl forward primer
- 1µl reverse primer
- 9.5µl ddH<sub>2</sub>O

Used PCR cycle labelled "Probe PCR"

- |                    |   |
|--------------------|---|
| 1.) 95°C           | 34 cycles from step 2-4                   |
| 2.) 95°C – 30 sec  | * Optimum annealing temperature should be |
| 3.) 60°C* – 30 sec | determined prior using gradient function  |
| 4.) 72°C – 1 min   |   |

5.) 72°C – 3 min

6.) 12°C – infinite

PCR reactions were observed on 0.7% agarose gels (40ml TAE, 0.28g agarose, 2µl Sybr safe). Run only 5µl, save remainder to recover for cloning. After confirming PCR products of the correct size and specificity, proceed to DNA isolation. Isolation can be done using GE or Qiagen following their protocols.

The isolated PCR products along with the vector (in this case pBluescript (pBS)) can now be digested with EcoRI and HindIII. The EcoRI digest is performed first (double digests can be performed simultaneously if using same buffer). The following volumes were used:

- 5µl of DNA/PCR product                      Incubate at 37°C for 1 hour
- 2µl of EcoRI                                      Inactivate at 65°C for 20 minutes
- 2µl of 10X EcoRI Buffer
- 11µl ddH<sub>2</sub>O

Followed by HindIII digest; add these volumes to previous digests:

- 1µl HindIII                                      Incubate at 37°C for 1 hour
- 2.3µl 10XNEBuffer 2                      Inactivate at 80°C for 20 minutes

Ligation of PCR product and linearized pBS vector is performed with 2 different ratios of insert:vector with the following volumes:

- 3.5µl of insert                      - 7µl of insert                      Incubate at 16°C overnight
- 1µl of vector                              - 1µl of vector
- 1µl of T4 DNA ligase              - 1µl of T4 DNA ligase
- 1µl of 10X ligase buffer              - 1µl of 10X ligase buffer

Put the required number of tubes of blue/white competent cells on ice to thaw (I used XL-1 cells) along with the same number of cuvettes. Warm 1ml of SOC media per transformation at 37°C. Add 1µl of ligation to a tube of cells and tap with finger to mix. Add cell/ligation mix to cuvette (50µl) being careful not to introduce air bubbles. Electroporate using Micropulser; select “Bacteria”, “Time” followed by “Pulse”. Add 1ml of SOC to cuvette and return cells to original tube. Incubate the cells in the 37°C shaker for 1 hour (tape to test tube rack attached to shaker). Place the required number (2 plates per inoculation) of antibiotic plates (in this case LB-Amp) at 37°C to warm up for 30 minutes. After they are warm, add to each plate 40µl of Xgal and 40µl of IPTG. Store plates in a dark space (drawer) for remainder of the inoculation. For each sample apply 75µl to a plate and 200µl to another and spread cells. Incubate plates overnight at 37°.

Pick white colonies with pipette tip and place in test tube with 4ml LB and 4µl 100mg/ml ampicillin. Place at a slant in test tube rack in shaker at 37°C overnight. \*Perform this step at the end of the day.

Isolate plasmids the following morning, following Qiagen kit instructions. Perform digest to confirm insert; I used *HinDIII* because it was plentiful and digests well. I followed the same digest conditions previously mentioned. Run samples on gel against linearized pBS as a control. Sequencing of your clones will also confirm successful insert. Quantify the concentration of plasmid using the Nanodrop.

The successful clones must be linearized for the *in vitro* transcription reaction. The orientation of the insert is known because I used two different restriction sites. The correct enzyme must be chosen (*EcoRI* vs. *HinDIII*), so that the probe produced is the anti-sense. Refer to FlyBase for gene orientation. Ideally, you would digest up to 10µg of DNA, but you can use as low as 5µg. Digest volumes depend on your plasmid concentration. Perform the digest with a total volume of 50µl and let it proceed overnight. Run 5µl of digest on gel to confirm linearization. Precipitate the linearized clones the next day. To the digests, add:

- 5µl of 0.2mg/ml glycogen                      Incubate at -20°C for 1 hour
- 5µl 3M NaOAc (1/10 total volume)
- 125µl of cold 100% ethanol (2.5X total volume)

Spin samples at 4°C for 20 minutes at max speed, remove supernatant. Add 100µl of cold 70% ethanol and spin for 10 minutes at 4°C at max speed.



Spin probes down for 20 minutes at max speed at 4°C and remove supernatant.

Add 100µl of cold 70% ethanol and spin for 10 minutes at 4°C at max speed.

Remove supernatant being careful not to disturb pellet. Allow pellet to air dry, then resuspend in 25µl of DEPC water. Determine concentrations using Nanodrop.

### **Embryo fixation**

Embryo collection is the same as our lab would normally perform; stage and time appropriately. Prior to the fixation prepare fresh 40% formaldehyde:

- 0.368 paraformaldehyde                      Place in heat block at 80°C until in solution
- 1ml DEPC water
- 1.4µl NaOH

Remove embryos from plate and move to mesh basket. Wash embryos with water and dechorionate with bleach. Wash away bleach with water and move embryos to scintillation vial with 1ml heptane:1ml 10% formaldehyde in PBS (750µl PBS, and 250µl of fresh 40% formaldehyde). \*Use PBS exclusive for RNA/in situ experiments. Place scintillation vial on shaker for 20 minutes. In the meantime prepare tubes of 1:1 heptane/methanol at 1 ml. Remove formaldehyde/PBS and add extra heptane to make embryo removal easier. Transfer embryos to 1:1 heptane/methanol and shake vigorously for 1 minute. Transfer embryos to a new tube and wash 3X in methanol. Store at -20°C.

## **Embryo preparation for hybridization**

\*Prepare fresh 40% formaldehyde prior to this section. All wash volumes are 500 $\mu$ l, on nutator at room temperature unless otherwise noted.

Wash the embryos in fresh methanol for 5 minutes. Rehydrate embryos in 1:1 PBS 0.1% Tween/methanol for 5 minutes. Hydrate in PBS Tween for 5 minutes, 2X. Post-fix embryos in PBS Tween/4% formaldehyde for 20 minutes. Wash embryos in PBS Tween for 2 minutes, 3X. Dilute a stock solution of 20mg/ml proteinase K in PBS Tween to 5.34 $\mu$ g/ml (1 $\mu$ l of stock in 3.5ml PBS Tween). Add diluted proteinase K to embryos and incubate embryos for 13 minutes, agitating embryos with pipette every 4 minutes. Place tube with embryos on ice for 1 hour. Remove proteinase K and wash embryos with 2mg/ml glycine for 2 minutes, 3X. Rinse embryos 2X in PBS Tween to remove glycine. Post-fix embryos in PBS Tween/4% formaldehyde for 20 minutes. Rinse embryos in PBS Tween 5X. Rinse embryos with a 1:1 mixture of PBS Tween and RNA hybridization buffer. Replace with 100% RNA hybridization buffer.

## **Hybridization**

Boil RNA hybridization buffer solution for 5 minutes in heat block (500 $\mu$ l per tube). Cool tubes on ice for 5 minutes. Remove hybridization buffer from embryos and replace with cooled RNA hybridization buffer. Incubate samples in water bath at 56°C for 2 hours.

Prepare probe-specific solution by diluting 50ng of probe into 100µl of RNA hybridization buffer. (Prior to this step I make a working stock of probes at 50ng/ml, so I will only need to add 1µl to buffer). Approximately 15 minutes prior to the end of pre-hybridization, heat the diluted probe to 80°C for 3 minutes. Cool probes on ice for 5 minutes. Remove buffer from embryos and add cooled probe, making sure that embryos move around when the solution is added. Hybridize overnight for 12-16 hours at 56°C.

Pre-warm the following solutions at 56°C (400µl per wash, at 56°C):

- RNA hybridization buffer (enough for 2 washes)
- 3:1 RNA hybridization buffer/PBS Tween
- 1:1 RNA hybridization buffer/PBS Tween
- 1:3 RNA hybridization buffer/PBS Tween
- PBS Tween (enough for 4 washes)

Remove probe and store at -80°C (can be used up to 2 more times). Wash with pre-warmed hybridization buffer for 15 minutes 2X. Wash with 3:1 hybridization buffer/PBS Tween for 15 minutes. Wash with 1:1 hybridization buffer/PBS Tween for 15 minutes. Wash with 1:3 hybridization buffer/PBS Tween for 15 minutes. Wash with pre-warmed PBS Tween for 5 minutes, 4X. Cool embryos to room temperature.

## Probe detection

Prepare PBS Tween/1% milk solution. Wash embryos for 10 minutes in PBS Tween/1% milk for 10 minutes at room temperature on nutator. Dilute 1 $\mu$ l of anti-digoxigenin-AP (anti-DIG) antibody in 1ml of PBS Tween/1% milk (1:1000). Incubate embryos in 1:1000 anti-DIG for 2 hours at room temperature on nutator.

Wash embryos in PBS Tween/1% milk for 5 minutes on nutator, 3X. Wash in PBS Tween for 20 minutes at room temperature on nutator, 4X. Prepare AP developing solution (enough for 2 washes (500 $\mu$ l) per sample and 750 $\mu$ l per tube for final reaction).

- DEPC water
- 10X alkaline phosphatase (AP) buffer
- Tween 20 (0.1%)

Wash each sample in AP buffer for 5 minutes on nutator, 2X. Add 15 $\mu$ l of NBT/BCIP for every 750 $\mu$ l of AP buffer/reaction to make AP developing solution (i.e. 2 reactions = 1.5 ml AP buffer and 30 $\mu$ l NBT/BCIP). Add 750 $\mu$ l of AP developing solution to each tube of embryos. Cover in foil and place on nutator at room temperature. Periodically check embryos for signal development. Stop reaction with 3 changes of PBS Tween. Rinse samples 6X in 100% ethanol. Rinse in PBS Tween. Mount embryos in 100 $\mu$ l of 70% glycerol.

### **7.3 SoxN binding site prediction**

This requires the data from SELEX experiments which was provided to me by Dr. Raluca Gordan. Determine the sequence that you want to investigate for binding sites. Calculate the frequencies of each base in the sequence that you will test. Open the SoxN.txt motif file with the SELEX data and change the “background letter frequencies” to your calculated frequencies. The format of the file is described here: <http://meme-suite.org/doc/examples/sample-dna-motif.meme>. The sequences need to be in FASTA format. Enter the sequence and attach the motif file into FIMO: <http://meme-suite.org/tools/fimo>. You can select the confidence interval depending on how stringent you would like it to be. The sequence of any hits will be listed along with their associated p-values.

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## Biography

Nicholas Philip Rizzo was born on March 9, 1983 in Rochester, NY to Natalie Rizzo and Jose Rodriguez. He obtained an associate's degree in liberal arts from Monroe Community College in 2005. Afterwards he moved to SUNY College at Brockport to finish his bachelor's degree (2006) in biological sciences and remained for his master's degree (2008) in biological sciences. For his master's thesis he worked in the lab of Dr. Stuart Tsubota on the project "Defining the Limits and Mapping the Control Sequences of the Gene, *enhancer of rudimentary*, in *Drosophila melanogaster*". Part of this work was published in 2007 (Roykhman et al.) and later in 2016 (Tsubota et al.). In the summer of 2008 he went to the University of Rochester to work as a technician in the lab of Dr. Michael Welte. He spent 3 years in this position and was a part of 2 different publications (Yu et al. 2011 and Arora et al. 2016) while conducting an independent research project "The role of dappled/wech in the regulation of lipid-droplet transport". From here he applied for graduate school programs and was accepted in to the Duke Cell and Molecular Biology program in the fall of 2011. He joined the lab of Dr. Amy Bejsovec and produced a thesis titled "Transcriptional control of epidermal cell shape in the *Drosophila* embryo". This work was published in *Development* and titled: "SoxNeuro and Shavenbaby act cooperatively to shape denticles in the embryonic epidermis of *Drosophila*." He defended his thesis on July 24, 2017.