Notopleural Mutations Enhance Defects In Imaginal Disc Epithelial Morphogenesis And Macrochete Elongation Associated With Mutations in the Stubble-Stubbloid Locus

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NOTOPLEURAL MUTATIONS ENHANCE DEFECTS IN IMAGINAL DISC EPITHELIAL MORPHOGENESIS AND MACROCHETE ELONGATION ASSOCIATED WITH MUTATIONS IN THE STUBBLE-STUBBLOID LOCUS

by

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ABSTRACT

The *Stubble-stubbloid* locus encodes a transmembrane serine protease (Stubble) necessary for the proper formation of sensory bristles, and the morphogenesis of leg and wing epithelia. Genetic and cell biological analysis indicate a role for Stubble in actin cytoskeletal dynamics and cell shape changes in developing epithelia and bristles. Previously reported genetic interactions between Stubble and the Rho1 signaling pathway suggest Stubble influences actin cytoskeleton dynamics in developing imaginal discs through interactions with the Rho1 pathway. This work will discuss a genetic screen conducted to further investigate the role of Stubble in bristle and imaginal disc morphogenesis. From 50,000 EMS-mutagenized chromosomes 12 enhancers of the recessive *sbd*\textsuperscript{201} allele were identified, including 6 new *sbd* alleles. Consistent with the current understanding of genetic interactions regulating imaginal disc morphogenesis, mutations in two Rho1 pathway genes, *zipper* (2 alleles) and *Rho1*, were isolated. Additionally, three new mutant enhancers of *sbd*\textsuperscript{201} were isolated, one of which has been identified as an allele of the cadherin gene *Dacshous*, another as an allele of the muscle myosin heavy chain gene, and the last as an allele of *Notopleural (Np)*. Dominant and recessive mutations in the *Stubble* locus interact with the *Np* allele identified in this screen, in regards to both limb and bristle development, respectively. Mutations in the *Np* locus were first identified in 1936, but this locus remains poorly characterized and has never been cloned. The genetic and phenotypic characterization of *Np* will be discussed along with experiments that have mapped the position of the *Np* locus to a 50kb region at the border of the 44F12, 45A1 cytological regions.
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CHAPTER ONE: INTRODUCTION

Epithelial morphogenesis is a fundamental process that is essential for development and homeostasis in metazoan animals. Epithelia are sheets of cells that undergo tissue-specific developmental programs which induce changes in the shape of component cells and the membrane as a whole. This restructuring of epithelia is referred to as epithelial morphogenesis. Epithelial morphogenesis is induced by global hormone signaling and local paracrine signaling, which precisely coordinate the activity of intracellular regulators of cell shape. After epithelial morphogenesis occurs, subsequent specialization and differentiation of cells within the epithelium results in the formation of adult structures.

Epithelia define organs, forming boundaries between tissues and between the animal and the external environment. They are essential for maintaining gradients, aiding in nutrient absorption during digestion, are key barriers to desiccation, and play essential roles in wound healing. Numerous oncogenic progressions are associated with the mis-regulation of epithelial cells, leading to excess unregulated growth (tumorogenesis), collective migration, and the eventual loss of epithelial identity (metastasis). Though epithelial morphogenesis is critically important for both animal development and human health, many aspects of the regulation of epithelial morphogenesis are not well understood. For example: 1) What are the mechanisms that trigger changes in cell shape required for epithelial morphogenesis? 2) How do cells within an epithelium interact with their local environment, specifically the surrounding extracellular matrix, to enact gross changes in tissue morphology? 3) How do global extracellular signals and intracellular signals coordinate to effect tissue-specific changes in cell shape and epithelial
structure? In the following pages I will present the results of a forward genetic screen that was conducted with the goal of contributing to our understanding of the coordination of global extracellular signals and intracellular signals during cell shape change in epithelial morphogenesis.

**Epithelia**

Epithelia are critical for the development, form, and function of animal organs, literally shaping animals and their component parts. Though the variety of structures formed by epithelia is truly astounding in its diversity, the basic components that define epithelia are universal. Epithelia are sheets of cells, in close apposition, that exhibit apical-basal polarity. Epithelia typically are covered on their apical surface by an extracellular matrix, and are collectively anchored to a basement membrane. Cells within an epithelium exhibit lateral associations to adjacent cells some of which allow the exchange of cytoplasmic components. Other lateral associations are necessary to maintain the structural integrity of the epithelial sheet. While each cell contains its own actin cytoskeletal scaffold that determines its individual shape, the tight association of epithelial cells allows force to be distributed across membranes. Collective changes in cell shape and the mechanical forces these changes exert across membranes are responsible for major changes in epithelial shape, orientation, and location (reviewed in SCHOCK and PERRIMON, 2002). Though there are some differences in the structure of vertebrate and invertebrate epithelial cells, the components that contribute to the regulation of cell shape change are similar in all important ways (VON KALM et al. 1995). All cells contain an apical surface that allows cell-type specific exchange with a lumen. The apical surface is covered in a tissue-specific extracellular matrix that is a complex microenvironment, as well as a
medium for paracrine signaling. Cells that will undergo apical constriction contain a sub-apical belt composed of actin and myosin. Adherens junctions, composed primarily of cadherins, are found on the lateral surfaces basal to the actin-myosin contractile belt. Adherens junctions are essential for maintaining the integrity of epithelia and communicating mechanical forces across the cells. The basal surfaces of cells are directly associated with a basement membrane, primarily through integrins. Basal membranes themselves typically consist of type IV collagen, fibronectin, laminin and proteoglycans (reviewed in FESSLER and FESSLER, 1989; VON KALM et al., 1995). Collectively, the ability of epithelia to respond to signaling from the extracellular environment, intercellular signaling between cells within an epithelial layer, and stimuli generated by mechanical force, permits complex morphological change during development.

Four general sequential steps required for an epithelium to undergo morphogenetic change have been schematically defined by SCHOCK and PERRIMON (2002).

1) The expression of transcription factors causes a set of epithelial cells to differentiate and become competent to both receive and respond to extracellular stimuli that elicit changes in cell shape (LEPTIN et al. 1995).

2) Some or all of the differentiated cells are stimulated by an external signal, and the signal is subsequently propagated within individual cells. (MONTELL et al. 2001; ODA and TSUKITA, 2001).

3) Upon stimulation and propagation of the signal, specific cellular responses are induced including remodeling in the actin cytoskeleton and modification in cell-cell adhesion properties, leading to reconfiguration of the shape and structure of the epithelium (GUMBINER, 1996).
Following changes in cell shape and epithelial configuration, specific cells within the epithelium go on to proliferate, differentiate or die, and thereby the process of epithelial morphogenesis is completed (CONLON and RAFF, 1999; VAUX and KORSMEYER, 1999).

Overview of the broad biological context of this study

In this study, we are primarily interested in the mechanism of cell shape change, as it is one of the most fundamental processes associated with epithelial morphogenesis. The most common and widespread regulators of the cytoskeletal dynamics responsible for cell shape change are members of the Rho family of monomeric GTPases (SCHOCK and PERRIMON, 2002). Drosophila epithelia, the system used in this study, are especially well suited for investigating the function of Rho family GTPases in epithelial morphogenesis. In the Drosophila embryo, the Drosophila Rho homolog, Rho1, has been shown to be required for cell shape changes in a number of morphogenetic processes. These morphogenetic processes include cytokinesis, cellularization, ventral furrow formation, gastrulation, dorsal closure, and head involution, as well as the invagination of the midgut during early organogenesis (reviewed in SETTLEMAN, 2001). All of these processes result from a similar mechanism, the Rho-induced contraction of the apical actin-myosin belt leading to cellular constriction. This mechanism is reiterated extensively in metazoan development and required for the proper formation and function of many animal epithelia (DAWES-HOANG et al. 2005).

Many developmental processes studied in Drosophila closely parallel processes that contribute to normal mammalian development and homeostasis. For example, the process of dorsal closure in Drosophila, as a mechanism, is highly similar to the process of wound healing.
in mammals, and depends on the coordinated activities of three Rho family proteins (Rac, Cdc42 and Rho1; NOBES and HALL, 1999 and references there in). Additionally, the basic process of epithelial morphogenesis through actin-myosin dependent changes in cell shape appears to play a central role in a number of pathologies, such as invasive metastatic cancers. Thus, it is crucial to understand the intracellular and global signaling mechanisms that stimulate or suppress the local activation of Rho signaling that leads to the actin cytoskeleton contraction necessary for such epithelial morphological changes to occur.

This study is part of ongoing research investigating *Rho1* activity in the epithelial morphogenesis of *Drosophila* leg and wing imaginal discs whose metamorphic development is largely dependent on changes in cell shape. In the following sections I review Rho proteins and features of their activity pertinent to epithelial morphogenesis, then I will discuss the structure and development of *Drosophila* imaginal discs. Finally I discuss the structure and function of the *Drosophila* protease Stubble, a putative Rho activator known to be required for proper cell shape changes during imaginal disc development.

**Rho GTPases are essential intracellular regulators**

A Rho family member was first identified as a Ras GTPase homolog in *Aplasia* in 1985 and also identified in yeast soon after (MAUDELE and AXEL, 1985; MAUDELE *et al.* 1987). Interest in the Rho family of proteins increased dramatically in 1993 when serum starved fibroblast cells were shown to form focal adhesions and actin stress fibers in a Rho-dependent manner in response to the application of serum (RIDLEY and HALL, 1992). The importance of Rho proteins was further highlighted by experiments demonstrating that cellular transformations induced by the oncogene *dbl* were the result of its direct association with, and activation of, Rho
proteins (HART et al. 1994). Over the next ten years it was shown that, in addition to modulating changes in the actin cytoskeleton, Rho GTPases are also essential for the regulation of cell size, cell proliferation, apoptosis, apical-basal cell polarity, planar polarity, cell adhesion, cell migration, membrane trafficking, transcription, tumor suppression and tumorogenesis (reviewed in VAN AELST and D’SOUZA-SHOREY, 1997; NOBES and HALL, 2000; GOMEZ DEL PULGAR et al. 2005).

Rho family proteins are small molecular switches that integrate external stimuli to induce dynamic changes in the actin cytoskeleton and regulate gene expression. Rho proteins receive and transmit signals through direct, protein-protein interactions at critical junctions in signal transduction pathways. All monomeric, guanine nucleotide binding proteins (G-proteins or GTPases), are considered members of the Ras superfamily, which includes the Ras, Rho, Rab, Ser/Arf, and Ran families (TAKAI et al. 2001). Rho family members can be distinguished from other members of the Ras superfamily (e.g., Ras and Ran) by the presence of a 13-residue insert that forms a compact alpha-helix. Rho family proteins (e.g. Rho, Cdc42, and Rac) can be distinguished from each other on the basis conserved sequences in the loop structures of their GTPase domains. (VETTER and WITTINGHOFER, 2001) The Drosophila genome contains only one Rho homolog, Rho1, which makes this system particularly amenable to loss of function genetic analysis. The human genome contains three Rho homologs, RhoA, RhoB, and RhoC, which have independent, as well as overlapping functions. For the sake of clarity, I refer to all Rho homologs (i.e. RhoA, RhoB, RhoC and Rho1) collectively as Rho proteins or simply Rho, and will use specific names when discussing a particular homolog, such as Rho1.
Figure 1. Regulation of Rho signaling

Extracellular stimuli activate RhoGEFs, which promote the exchange of GDP for GTP and activate Rho. Active Rho stimulates any variety of effectors (small green arrow). RhoGAPs catalyze the hydrolysis of GTP, and return Rho to an inactive, GDP-bound state. Inactive, GDP-bound Rho can be removed from the cell membrane and shuttled to the cytosol by RhoGDIs, which prevents further activation of Rho.
Small G-proteins contain GTP/GDP binding-GTP hydrolyzing domains (G-domains) that inefficiently hydrolyze GTP and act as molecular switches, being active when bound to GTP, and inactive when bound to GDP (Figure 1). GTP bound Rho maintains a high-energy conformation that permits direct interactions with down-stream effectors. Interactions with effectors or regulators destabilize the Rho-GTP association and induce the hydrolysis of GTP, which leaves the Rho protein bound to GDP and in the inactive or “off” state (FRAME and BRUNTON, 2002; MAESAKI et al. 1999). Rho family proteins are regulated by three classes of proteins, GTPase activating proteins (GAPs), GTPase dissociation inhibitors (GDIs), and guanyl-nucleotide exchange factors (GEFs) (reviewed in BOGUSKI and MCCORMICK, 1993). The primary negative-regulators of Rho protein activity are GAPs and GDIs. GAPs accelerate GTP hydrolysis and stabilize GDP binding, while GDIs stabilize GDP binding and, more significantly, complex with Rho proteins to translocate them to the cytosol, away from activating complexes located at the cell membrane (TAKAI et al. 2001). Rho is activated by GEFs, which catalyze the exchange of GDP for GTP in response to extracellular signals. (SCHMIDT and HALL, 2002).

Rho, Rac and Cdc42, the three best-characterized Rho family homologs, collectively have over 60 identified target effectors. This diversity of interactors is unparalleled in G proteins and is strong indicator of the biological significance of Rho family G proteins (NOBES and HALL, 2000). Rho proteins control the actin cytoskeleton and cell shape change by regulating two major processes: actin filamentation and actin-myosin contraction. Rho promotes actin filamentation by binding and activating effectors, Diaphanous (Dia) and Rho kinase (RHOK) (Figure 2). Dia acts by directly stimulating actin filamentation, while RHOK acts by down-regulating inhibitors of actin filamentation (TOMINGA et al. 2000; COPELAND and
TREISMAN 2002). Specifically, RHOK activates LIM kinase (LIMK), which phosphorylates and thereby deactivates cofilin. Active cofilin destabilizes filamentous actin, and acts in opposition to the ubiquitous stabilizer of actin filamentation, profilin (SOMOGYI and RORTH, 2004).

Rho stimulates actin-myosin contraction through three signaling mechanisms downstream of RHOK that promote the activation of the myosin regulatory light chain (MRLC; Figure 3). Phosphorylated MRLC complexes with myosin heavy chain and the myosin essential light chain to induce actin-myosin contraction (reviewed in LANDSVERK and EPSTEIN, 2005). RHOK can promote actin myosin contraction by directly phosphorylating MRLC, by phosphorylating and activating myosin light chain kinase (MLCK) which subsequently activates MRLC, or by phosphorylating and inactivating myosin phosphatase (MYPT), a negative regulator of MRLC (AMANO et al. 1996; KAYUZA et al. 2001; MUZINO et al. 2002:). Thus, RHOK regulates actin-myosin contraction by interacting with activators and negative regulators of this process.
Rho activates *Diaphanous*, which enhances profilin mediated F-actin filamentation. Rho also stimulates RHOK which acts through LIMK to inhibit the de-stabilization of F-actin by cofilin (Modified from SOMOGYI and RORTH, 2004.).
RHOK promotes actin myosin contraction by activating myosin light chain kinase (MLCK). MLCK phosphorylates the myosin regulatory light chain (MRLC), and inhibits myosin phosphatase (MYPT), which de-phosphorylates the myosin regulatory light chain. Phosphorylated MRLC combines with the myosin essential light chain (MELC) and non-muscle myosin heavy chain to induce actin myosin contraction.

Figure 3. RHOK induces actin myosin contraction
The current understanding of the mechanisms of activation of Rho homologs remains limited compared to knowledge of their diversity of interactions and ubiquitous involvement in essential cell biological processes. The only known direct activators of Rho signaling are G-protein coupled receptors (GPCRs) (SEASHOLTZ et al. 1999). All GPCRs are seven pass transmembrane receptors that receive extracellular stimuli and activate intracellular heterotrimeric G-proteins. The alpha subunit of several types of heterotrimeric G-proteins have been shown to activate RhoGEFs. The association between G-protein signaling and RhoGEFs is further supported by the observation that several RhoGEFs contain Regulator of G-protein-like (RGSL) motifs that specifically interact with the alpha subunit of heterotrimeric G-proteins (BRAGA, 2002; SEASHOLZ et al. 1999; WHITEHEAD et al. 2001). Rac and Cdc42 homologs have been shown to be activated by cadherins and receptor tyrosine kinases, however, the mechanism of activation, direct or indirect, is unknown. Activation of Rho by cadherins and receptor tyrosine kinases has not been reported (BRAGA, 2002). It is not clear if other mechanisms of Rho activation remain to be identified. Additionally, little is known about the mechanisms that instigate the movement of inactive Rho from the cytoplasm to the membrane where Rho associates with membrane-bound activators, and what triggers the release of Rho from cytosolic GDIs at the cell membrane (DERMARDIROSSIAN and BOKOCH, 2005). The regulation of Rho may be more complex than current models depict, and mounting evidence indicates that signals modulating Rho family protein activity may act on all components of their regulation, including their localization within the cell and the rate at which they cycle between inactive and active forms. Thus, it is likely that the regulators of Rho activity are themselves highly regulated (SYMONS and SETTLEMAN, 2001).
One of the most important mechanisms of Rho protein activity that remains to be elucidated is the regulation of their temporal and spatial activities. Many common developmental processes, such as epithelial morphogenesis through apical constriction, as well as basic cellular activities (e.g., cytokinesis), are dependent on Rho activity, and a better understanding of the mechanisms that modulate Rho proteins temporally and spatially is a critical priority. When considered in the context of human health, the question of identifying extracellular factors that regulate intracellular Rho activity is of particular relevance. Misregulation of Rho is implicated in a number of pathologies, including hypertension, neurological disorders, erectile dysfunction, and cancers (GOMEZ del PULGAR et al. 2005). Over 20 types of cancers have been associated with misregulation of Rho proteins, including pancreatic, breast, skin, colorectal, testicular, thyroid, and prostate cancer. (GOMEZ del PULGAR et al. 2005). Despite the number of cancers that have been associated with the misregulation of Rho family proteins, and in contrast to Ras (another GTPase known to be involved in cancer progression), there are no reports of oncogenic, positive or negative activating mutations in Rho (RIHET et al. 2001; ANZAR et al. 2004). It is probable that any somatic mutations in Rho that significantly disrupt its function are either cell-lethal or prevent cell division. However, several RhoGEFs, including *diablo* (*dbl*), are considered oncogenes and somatic mutations in such genes are correlated with a number of cancers (VAN AELST and D'SOUZA-SCHOREY, 1997). Clearly, the regulation of Rho proteins is of equal importance in maintaining homeostasis as it is in development, which makes any advancement in our understanding of how Rho proteins are specifically activated in time and space of critical interest.

*Drosophila* imaginal disc development is an excellent model system for investigating the temporal and spatial regulation of *Rho1* activity, and how this activity modulates changes in cell
shape required for epithelial morphogenesis. *Drosophila* leg and wing imaginal discs are composed of immature epithelia that undergo a rapid transformation into adult organs. In this system Rho1 activity induces cell shape change in a discrete group of cells (those comprising the imaginal epithelia), at a specific time in development (pupation, the onset of metamorphosis). This is accomplished by the coordination of Rho activation and tissue-specific signaling components expressed within imaginal disc cells in response to the global regulatory hormone ecdysone. Indeed, imaginal discs isolated from late third instar larva are fully capable of undergoing epithelial morphogenesis when appropriately cultured and provided with exogenous ecdysone indicating that additional exogenous signals are not required for imaginal disc morphogenesis (FRISTROM and FRISTROM, 1993). The simplicity of this system allows us to investigate, *in vivo*, the elements required for a highly tissue-specific coordinated contraction of cells enacted by a single temporal hormonal cue.

**Imaginal Disc Development: Steroid hormone regulated epithelial morphogenesis**

*Drosophila* leg and wing imaginal discs originate as invaginations in the embryonic epidermis that are fully separated into distinct sack-like structures at the beginning of larval life. These epithelial monolayers grow by mitotic division throughout larval life until the third, and final larval instar, when a transient pulse of ecdysone causes leg and wing imaginal disc cells to cease dividing and initiate morphogenesis (FRISTROM and FRISTROM, 1993). At this developmental point, leg and wing imaginal discs have grown in size and cell number, and the folded, columnar imaginal epithelium can be discerned on one side of the disc, and the flat, squamous peripodial epithelium on the other. The imaginal and peripodial epithelia are continuous and oriented with their apical surface facing the internal lumen of the disc. Prior to
metamorphosis the imaginal epithelium is concentrically folded, and overlapping boundaries of transcription factor expression have defined cell fates across its topography. In leg discs, expression of the *dachshund* gene specifies the regions that will become the tibia, femur, and tarsi of the leg. (ABU-SHAAR and MANN, 1998). Prior to metamorphosis, the apical surface of cells that comprise the presumptive femur, tibia, and tarsi are highly anisometric in shape, being long in the circumferential direction and narrow along the proximal distal axis. During pupariation, the anisometric cells change shape in response to the ecdysone signal and become isometric. This coordinated change in cell shape across the epithelium in turn leads to a remodeling of tissue shape (Figure 4). The process of elongation of the leg imaginal disc into a tube by cell shape change occurs rapidly, over a span of six hours, and transforms the imaginal leg tissue from an immature epithelium to an adult organ that is reconfigured and repositioned in order to complete development (FRISTROM and FRISTROM, 1993; VON KALM *et al.* 1995). This process provides a unique opportunity to investigate mechanisms and effectors required for epithelial morphogenesis. Many attributes of this system have previously been elucidated. Transcription factors required for specifying cells fated to undergo the shape changes that drive epithelial morphogenesis have been identified, and the process has been thoroughly characterized on a cellular level (FRISTROM and FRISTROM, 1993; VON KALM *et al.* 1995). Several important observations come from pioneering experiments carried out *in vitro* where imaginal discs isolated from late third instar larvae were cultured through to elongation. The process of *Drosophila* metamorphosis is absolutely dependent on ecdysone, and it has been shown that without the application of exogenous ecdysone cultured discs do not elongate (FRISTROM and FRISTROM, 1993). Thus, ecdysone is required to trigger imaginal disc morphogenesis.
Figure 4. Changes in cell shape during leg imaginal disc elongation.

(a-d) Micrographs of prepupal leg discs. In (c) and (d) apical confocal sections are stained with phalloidin to visualize filamentous actin and cell boundaries. (a) A leg at the beginning of the prepupal period. (b) A leg after 6 hours of prepupal development. During the transition from (a) to (b) the proximal segments (bracketed) have narrowed circumferentially and elongated while the distal segments have remained relatively unchanged. (c and d) Surface (apical) views of the segments indicated by arrows in (a) and (b). Cell boundaries stain strongly because of the presence of an apical belt of actin associated with the adherens junction. Note that the apical ends of the cells change shape from anisometric (c) to isometric (d) as the disc elongates. (e) A schematic illustration of the relationship between change in cell shape and change in tissue shape. (f) An apical view of an elongated leg stained for non muscle myosin, which, like actin, localizes to the apical belt. Figure and caption taken from von Kalm et al. 1995.
The changes in cell shape that drive leg disc elongation are dependent on actin filamentation and a restructuring of the actin cytoskeleton. Exposure to cytochalasins, compounds which inhibit actin filamentation, blocks elongation in cultured imaginal discs, and this effect is reversed upon the removal of cytochalasins (FRISTROM et al. 1975). Several lines of evidence have demonstrated that extracellular proteolytic activity also plays a key role in imaginal disc elongation. Leg disc elongation occurs in vivo over a span of six hours, and roughly 12-18 hours in vitro (FRISTROM and FRISTROM, 1993). Exposing cultured discs to the serine protease trypsin was shown to greatly increase the rate of leg disc elongation, reducing this period in vitro to several minutes and leading to abnormally long and thin legs (FEKETE et al. 1975). Conversely, protease inhibitors block elongation (SHUBIGER et al. 1989). These experiments demonstrate that extracellular proteolysis is required for imaginal disc elongation. Interestingly, exposure to exogenous proteases alone is not sufficient to induce leg disc elongation in the absence of ecdysone, or when F-actin filamentation is blocked by cytochalasin-B (FEKETE et al. 1975). These data demonstrate that ecdysone is required for the induction of imaginal disc elongation, and that elongation is dependent on the coordination of changes in the actin cytoskeleton with extracellular proteolytic activity.

Genetic experiments have made major contributions to our understanding of imaginal disc morphogenesis. These experiments have capitalized on a characteristic malformed leg and wing phenotype exhibited in animals carrying mutations inhibiting their ability to execute cell shape changes during development (Figure 5; BEATON et al. 1988; KISS et al. 1988; CONDIC et al. 1991; FRISTROM and FRISTROM, 1993; VON KALM et al. 1995). Malformed legs typically exhibit shorter, bent, and thickened femurs, tibia that may also be twisted or bowed, and occasional defects in tarsal segments. Wings exhibit a crumpled phenotype, wing blisters,
and frequently fail to expand. Using this phenotype, several Rho signaling pathway genes required for cell shape change, including \textit{Rho1}, \textit{RhoGEF2}, \textit{RHOK}, and \textit{non-muscle myosin heavy chain (zip)}, have been shown to be required for leg and wing imaginal disc morphogenesis (GOTWALS and FRISTROM 1991; HALSELL \textit{et al.} 2000; BAYER \textit{et al.} 2003; WARD \textit{et al.} 2003). Mutations in these Rho pathway genes cause embryonic lethality in homozygotes, but many doubly heterozygous combinations, such as \textit{Rho1} and \textit{zip}, display a highly penetrant malformation leg and wing phenotype. This provides strong evidence that these loci have a central role in generating the cell shapes changes that drive leg and wing elongation.

How is the Rho pathway activated to induce the coordinated cell shape changes required for imaginal disc elongation? Ecdysone is the temporal signal that triggers metamorphosis in \textit{Drosophila}, including imaginal disc morphogenesis. Ecdysone, however, acts primarily on the level of gene regulation, while Rho pathway components are ubiquitously expressed and not ecdysone inducible (THUMMEL 2002; BAYER \textit{et al.} 2003; WARD \textit{et al.} 2003). Several ecdysone inducible genes required for leg disc morphogenesis have been identified, including the \textit{broad} locus, a master regulator of metamorphosis. The \textit{broad} locus (\textit{br}) encodes a family of ecdysone inducible transcription factors, which, collectively, are required for the induction of metamorphosis. Animals homozygous for null \textit{br} alleles do not pupariate and die after a protracted third larval instar. The allele \textit{br}^{l} is a weak hypermorph that in male hemizygotes and female homozygotes exhibits short and wide, or broad, wings (KISS \textit{et al.} 1988). Transheterozygous combinations of \textit{br}^{l} with null \textit{br} alleles result in severe wing defects and second and third leg malformations (KISS \textit{et al.} 1988). Another ecdysone inducible gene, the \textit{Stubble-stubbloid (Sb-sbd)} locus was found to act as a strong second-site non-complementer of \textit{br}^{l} with regards to the malformed leg and wing phenotype (BEATON \textit{et al.} 1988; GOTWALS
and FRISTROM, 1991). Although the *broad* locus encodes a family of transcription factors, it does not regulate the transcription of *Sb-sbd* (D'AVINO and THUMMEL, 1998). Thus, the basis of the genetic interaction between *broad* and *Sb-sbd* is not understood. It is currently thought that one or several *br*-regulated genes must act along with the Stubble protein to execute imaginal disc morphogenesis (WARD et al. 2003). A mutagenesis screen carried out by Gotwals and Fristrom (1991) for enhancers of *br¹¹⁷* identified a *sbd* allele, *sbd²⁰¹*, and an allele of non-muscle myosin heavy chain, *zip⁴⁰⁴*, providing the first evidence that the early ecdysone responsive genes *br* and *Sb-sbd* coordinate with the Rho pathway during imaginal disc morphogenesis. Later screens, as well as reverse genetic approaches, demonstrated that multiple components of the *Rho¹* signaling hierarchy, notably *Rho¹* and *RhoGEF²* interact with both *Sb-sbd* and *br*, demonstrating a clear link between these two pathways (BAYER et al. 2003; WARD et al. 2003; CHEN et al. 2004).
Figure 5. Malformed legs and wings resulting from failures in cell shape change.

(A and D) wild-type leg and wing morphology. Note the long, slender shape of a normal femur and tibia. Mildly (B and F) and severely (C and G) malformed third legs and wings taken from an animal of the genotype $Rho1^{E3.10/++; Sb^{63b}/+}$. Note the abnormally short and thick femur and tibia and indentation in the femur (G) and crumpled wing (C). Images and text are based on Bayer et al. 2003.
Table 1: Genes associated with the malformed leg phenotype in the mutant condition

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein product</th>
<th>Referencea</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rho1 Pathway Genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rho1</em></td>
<td>Small GTPase</td>
<td>HALSELL <em>et al.</em> 2000</td>
</tr>
<tr>
<td><em>Zipper</em></td>
<td>Nonmuscle myosin II heavy chain</td>
<td>GOTWALS and FRISTROM 1991</td>
</tr>
<tr>
<td><em>DRhoGEF2</em></td>
<td>Rho1-specific guanine nucleotide exchange factor</td>
<td>HALSELL <em>et al.</em> 2000</td>
</tr>
<tr>
<td><em>DRho kinase</em></td>
<td>Rho1 activated kinase</td>
<td>BAYER <em>et al.</em> 2003</td>
</tr>
<tr>
<td><em>Lim Kinase</em></td>
<td>RhoK activated kinase</td>
<td>CHEN <em>et al.</em> 2004</td>
</tr>
<tr>
<td><em>Blistered</em></td>
<td>Drosophila serum response factor</td>
<td>GOTWALS and FRISTROM 1991</td>
</tr>
<tr>
<td><em>spaghetti squash</em></td>
<td>Myosin regulatory light chain</td>
<td>EDWARDS and KIEHART 1996</td>
</tr>
<tr>
<td><strong>Ecdysone Inducible Genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Stubble-stubbloid</em></td>
<td>Type II transmembrane serine protease</td>
<td>BEATON <em>et al.</em> 1988</td>
</tr>
<tr>
<td><em>Broad</em></td>
<td>Zinc-finger transcription factors</td>
<td>KISS <em>et al.</em> 1988</td>
</tr>
<tr>
<td><em>E74</em></td>
<td>ETS transcription factors</td>
<td>FLETCHER <em>et al.</em> 1995</td>
</tr>
<tr>
<td><em>crooked legs</em></td>
<td>Zinc-finger transcription factor SAP-90/PSD-95 associated proteins</td>
<td>D'AVINO and THUMMEL 1998</td>
</tr>
<tr>
<td><em>Vulcan</em></td>
<td>Heterogeneous nuclear ribonucleoprotein K</td>
<td>GATES and THUMMEL 2000</td>
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<tr>
<td><em>Bancal</em></td>
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<td>GATES and THUMMEL 2000</td>
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</table>

Genes known to regulate cell shape change required for leg imaginal disc elongation can be distinguished as either components of the Rho pathway or as ecdysone inducible.

a References indicate publications in which the malformed leg phenotype was first described.
The Stubble-stubbloid locus is ecdysone inducible and encodes an extracellular protease that potentially activates Rho1 in imaginal discs.

Most ecdysone inducible genes known to be required for imaginal disc morphogenesis are transcription factors or localized to the nucleus (Table 1). One exception is the Sb-sbd locus, which is a well-characterized, ecdysone-inducible effector of imaginal disc morphogenesis. The Sb-sbd locus encodes an extracellular protease that is required for actin filamentation in bristles and cell shape changes during imaginal disc morphogenesis (CONDIC et al. 1991; APPEL et al. 1993). Mutations in the Sb-sbd locus cause shortened bristles and are associated with malformed legs and wings. With respect to bristle development dominant and recessive alleles have been identified, however all alleles are recessive with respect to imaginal disc morphogenesis (DOBZHANSKY, 1929; APPEL et al. 1993). Sb-sbd mutations interact genetically with components of the intracellular Rho signaling pathway, and genetic evidence suggests that the Sb-sbd protease may act as an activator of the Rho pathway (BAYER et al. 2003).

The Sb-sbd locus encodes a type II transmembrane serine protease (Stubble; Figure 6) (APPEL et al. 1993). Type II transmembrane serine proteases (TTSPs) are a recently characterized group of proteases that are notable for their unique structural organization and extensive associations with human pathologies (HOOPER et al. 2000). All TTSPs contain an extracellular carboxy-terminal (C-terminal) protease domain, an extracellular linker region of variable length, and a short, single-pass transmembrane domain followed by an amino-terminal (N-terminal) intracellular region. The primary structure of the Stubble protein indicates that it is
Figure 6. The structure of the *Stubble-stubbloid* gene protease

A schematic view of the domains in the Stubble-stubbloid gene product, and the mutant *sbd*\textsuperscript{201} gene product. The top line shows protein domains of wild-type Stubble protein (wt): CD (cytoplasmic domain) aa 1–58, TM (transmembrane domain) aa 59–81, knot (disulfide-knotted domain) aa 138–173, S/T-rich (serine and threonine rich region of the stem) aa 260–480, and serine protease from activation cleavage site at aa 542 to the C terminus at aa 786. The position of the zymogen cleavage site is indicated by a solid vertical bar. The catalytic serine at aa 737 is indicated by an open vertical bar. In the diagram of *sbd*\textsuperscript{201} the mutation that results in the interactive nature of this allele, the substitution of histidine to for an arginine residue, is illustrated. Figure and legend are taken in modified form from Hammonds and Fristrom, 2006.
transcribed as a zymogen, and activated by proteolytic cleavage of the extracellular linker region. After activation, the protease domain is thought to remain tethered to the cell surface by a disulfide bridge flanking the cleavage site (APPEL 1993; HAMMOND and FRISTOM 2006). The Stubble extracellular linker region additionally contains a disulfide knotted domain, also referred to as a CLIP domain. CLIP domains are thought to act as protease activator binding sites and appear in several arthropod proteases, including snake and Easter. The snake and Easter proteins are soluble extracellular serine proteases required for establishing dorso-ventral polarity in the _Drosophila_ embryo by modulating a protease cascade that activates the Toll signaling pathway (GAY and KEITH 1992; SMITH and DELOTTO 1992; JAING and KANOST 2000). Mutational analyses indicate that the CLIP domain is essential for snake function (SMITH _et al._ 1994). The Stubble CLIP domain is attached to the transmembrane domain by a serine / threonine rich stem region, which is also seen in the Easter protein and thought to be required for Easter function (SMITH _et al._ 1994). The intracellular domain is 58 amino acids (APPEL _et al._ 1993).

There are 30 identified TTSPs in mammals, and 20 putative TTSPs have been predicted to exist in the _Drosophila_ genome (Ensemble v39, 2006). Stubble was the first _Drosophila_ TTSP identified, and remains the only well characterized invertebrate TTSP (APPEL _et al._ 1993; HAMMONDS and FRISTROM 2006). There is growing evidence that TTSPs play significant roles in transmembrane signaling regulating development and homeostasis of epithelia (reviewed in NETZEL-ARNETT _et al._ 2003). Misexpression of human TTSPs have been shown to be associated with breast, colorectal, ovarian, pancreatic, and prostate cancer, in addition to squamous cell carcinoma of the head and neck (reviewed in NETZEL-ARNETT _et al._ 2003). How TTSPs affect development and homeostasis is not understood on a mechanistic level. One
line of investigation suggests that TTSPs act by cleaving protease activated G-protein coupled-receptors (PARs). For example, in the prostate cancer cell line LNCaP, the TTSP TMPRSS2 has been shown to cleave the protease activated receptor 2 (PAR2), a G-coupled protein receptor that is activated by proteolytic cleavage and known to activate RhoA signaling in humans (WILSON et al., 2005; GREENBERG et al. 2003; VOURET-CRAVIARI et al. 2003). However, it is not known if TMPRSS2 activates RhoA signaling in LNCaP cells. Studies such as these, conducted in cell culture, are sure to provide insight into the function of TTSPs, but a full understanding of how TTSPS act and are regulated during the development of an organism, within specific functioning tissues, can only be garnered by experiments conducted in vivo. How TTSPs, located on the exterior of cells, function to regulated developmental changes occurring within an epithelium, how the activity of TTSPs is regulated during development, and to what effect, are all unanswered questions.

Currently, the von Kalm lab is addressing some of these questions by conducting experiments aimed at elucidating the biological function of the Stubble TTSP in Drosophila development. A series of experiments using transgenic expression of wild-type Stubble protein under the control of a heat-shock inducible promoter have yielded several insights into Stubble function in leg and wing morphogenesis. Wild-type Stubble protein is required for both leg and wing development, however, over-expression of the wild-type Stubble protease at critical times in development causes malformed legs and wings (BAYER et al. 2003; HAMMONDS and FRISTROM, 2006). This period of sensitivity (0-3hrs after the start of pupariation (AP)) corresponds closely with the developmental periods during which the Sb-sbd locus is induced. It is important to note that animals that have been exposed to high levels of exogenous Stubble over-expression do not exhibit any other major developmental abnormalities, indicating that the
effect on leg and wing development is highly specific. Collectively, these data suggest that epithelial cells become competent to respond to Stubble activity in a manner that is temporally coordinated with Stubble expression, possibly by ecdysone, and that the level of Stubble activity is also precisely regulated during normal development.

Capitalizing on the malformed phenotype induced by heat-shock over-expression of Stubble protein, Bayer et al. (2003) provided convincing genetic evidence that Stubble acts upstream of Rho1 signaling during imaginal disc morphogenesis. Based on these data, it was proposed that Stubble protein may act as an ecdysone inducible temporal and spatial regulator of Rho1 signaling during imaginal disc morphogenesis, and Bayer et al. (2003) put forth a model where ecdysone signaling and Rho1 signaling intersect at the cell membrane (Figure 6). In this model, the Stubble protease is induced by ecdysone and stimulates Rho1 activity. Rho1 then induces cytoskeletal rearrangements and cell shape changes through contraction of the sub-apical actin-myosin belt. This model fits well with published data on the ecdysone signaling cascade, provides a reasonable mechanism for the mechanical events that induce leg disc elongation, and explains the preponderance of Stubble alleles that have been identified in genetic screens for enhancers of leg malformation (GOTWALS and FRISTROM 1991; BEATEN et al., 1998; HALSELL et al. 2000; WARD et al. 2003; data discussed below).

Several elements of the Bayer model of imaginal disc epithelial morphogenesis remain to be clarified. What are the specific targets of Stubble proteolysis? How is Stubble protease activity regulated? What signaling components act downstream of Stubble to activate Rho1? Answering these questions is important because they may provide critical insights into how Rho protein activity is regulated to accomplish basic changes in cell shape, a fundamental mechanism
Figure 7. The Bayer model Rho1 signaling in leg imaginal discs elongation
reiterated extensively in the normal development of all animals. As research on TTSPs has advanced, examples of their roles as important regulators of epithelial development have increased (NETZEL-ARNETT et al. 2003). Understanding how this interesting group of proteases function \textit{in vivo} (as opposed to cell cultures isolates) presents the potential to yield insights regarding how key modulators of epithelial morphogenesis (hormones, extracellular matrix components and intracellular regulatory agents) coordinate to accomplish a precisely timed and spatially limited set of cellular responses in a normal developmental context.

Imaginal disc elongation in \textit{Drosophila} provides a model system in which to investigate one example of how global hormone regulated gene expression and intracellular cytoskeletal regulators intersect to transform immature epithelia in to adult organs. In this system the extracellular proteolytic activity of the Stubble protein appears to link the ecdysone and Rho1 pathways at the apical cell membrane, possibly as a hormone induced trigger of cell shape change. In order to further understand this process it will be necessary to identify additional components that act at the cell membrane and potentially interact directly with the Stubble protease. The following work describes a genetic screen conducted to identify a direct interactor with the Stubble protease, with the goal of improving our understanding of the mechanism coordinating hormonal and Rho1 signaling in leg and wing imaginal disc epithelia.
CHAPTER TWO: METHODS

Drosophila Stocks

All Drosophila stocks were maintained on corn meal/yeast/molasses/agar media at a constant temperature of 25°. All deficiency and P-element-insertion stocks used in this study were obtained from the Bloomington Drosophila Stock Center at Indiana University, as were all mutant stocks identified (Bloomington, IN). The mutated line used in the EMS mutagenesis screen, was a w¹¹¹⁸++; + line isogenic on the X, 2nd and 3rd chromosomes. The testor stock was Cy/Pm; red sbd²⁰¹⁰/ TM2, ubx, e. The tester stock was supplied by J.W. Fristrom (University of California at Berkeley). Unless otherwise stated, animals all experiments were maintained in an incubator at a constant temperature of 25°.

EMS mutagenesis and screening

EMS was prepared as described by Ashburner (1989), following precautions outlined by Eherenber and Wachtmeister (1977).

Mutagenesis and brooding

Three hundred cohorts, each consisting of 10 three-to-five-day-old w¹¹¹⁸ males, were isolated, starved for three hours and then allowed to feed on a 25 mM EMS/1% sucrose solution. After 24 hours each cohort was transferred to fresh vials containing 10 Pm/CyO; red, sbd²⁰¹⁰, e /TM6B Tb, Hu, e, virgin females, and allowed to mate for 24 hours. This first brood was then
pseudo-brooded by turning both the males and females into new vials containing fresh cornmeal media and allowed to lay for 24 hours (KISS et al. 1988). After these matings were completed, all flies of the parental generation were placed in new vials, decontaminated and discarded. Progeny were maintained at room temperature inside a fume hood for three days and then transferred to a 25° incubator.

**G1 Flies**

Animals of the G1 generation were scored on a daily basis for 9 days. Animals exhibiting malformed legs or bristles were isolated, then individually backcrossed to 5 Pm/Cyo; red, sbd201, e/TM6B Tb, Hu, e males or females as appropriate. Crosses of potential interactors to the tester line were turned once, after four days and the progeny were allowed to develop at 25°. All G1 crosses were labeled with the vial and brood of origin.

**G2**

Animals of the G2 generation were scored on a daily basis for 9 days. Animals exhibiting malformed legs or bristles were isolated, then individually backcrossed to 5 Pm/Cyo; red, sbd201, e/TM6B Tb, Hu, e males or females as appropriate. Crosses of potential interactors to the tester line were turned once, after four days and the progeny were allowed to develop at 25°. All G2 crosses were labeled so as to appropriately represent the origin of potential mutant alleles.

**G3**

Animals of the G3 generation were scored on a vial by vial basis. For each vial, 40 animals were initially scored. If several malformed animals were apparent within a vial, animals were separated and either out crossed or sib-mated in order to isolate chromosome carrying the candidate enhancer mutation (see Figure 9). If less than 2 malformed animals were observed from the first 40 animals scored, the vials were returned to the incubator and typically scored
once more after two to three days. Vials were discarded only if no malformed animals were seen in after it this second round of scoring.

**Isolation of candidate enhancer of *sbd* chromosomes**

Chromosomes carrying potential enhancer of sbd mutations were isolated using the protocol described in below (Figure 8).
Figure 8. Placing candidate enhancers of *sbd* into a wild-type genetic background.

Using this strategy the third chromosome was double balanced, and then outcrossed to wild type chromosomes. This ensured that the only chromosome in the stock containing EMS-induced mutations was the enhancer chromosome.
**Phenotypic characterization**

Tests for second site non-complementation interactions were generally performed by mating five virgin females of the balanced enhancer of sbd201 line to five to seven mutation-bearing heterozygous males in vials containing standard Drosophila media. After 3 days the adults were transferred to fresh vials and, subsequently, into a third vial after two additional days. Newly eclosing F1 flies were separated by genotype and examined for malformed legs each day for a total of 10 days. Animals were considered mildly malformed if they contained one leg with structural aberrations but no bends or twists that contorted the normally straight femur or tibia beyond an angle of 45 degrees and the leg was at least half the length of its wild-type counterpart. Animals were considered severely malformed if they exhibited two mildly malformed legs, or if they exhibited one severely malformed leg that was either less than half the length of a wild-type leg or contained regions that twisted more than 45 degrees.

**Complementation testing**

Five virgin female Enhancers of sbd201 were crossed to five males carrying candidate mutations or deficiencies, and turned three times. As we were interested in identifying non-complementers, F1 progeny were scored until 10 progeny carrying one copy of the Enhancer of sbd201 and one copy of the candidate mutation or deletion were identified. If all 10 of these animals were phenotypically wild-type, then the alleles were considered to complement and the cross was discarded. In cases where malformation was observed or less than 10 flies of the relevant class emerged from all three vials, the mutant alleles were tested again for lethality and malformation.
Lethal phase analysis

Embryonic lethality was determined by collecting 0- to 1-hr embryos from $Np^{125A}/CyO$, $P^{w^+, Dfd: EYFP}$ stocks. The embryos were aged at 25° for 15-18 hr. Homozygous $Np^{125A}$ embryos were identified on the basis of absence of green fluorescent protein (GFP) expression using a Lieca fluorescent dissecting microscope, with a GFP filter. Viable GFP+ larvae were counted and transferred to cornmeal plates and allowed to develop as a control group at 25°. The $Np^{125A}$ homozygous embryos were counted, left on grape plates and allowed to age for a further 24 hr at 25°. Dead embryos were counted and subsequently preserved in an ethanol/glycerol solution. Whole embryos and cuticle preparations were examined for terminal phenotypes.

Hs-Stubble suppression

Thirty $Np^{125A}/CyO$ $P^{GMR:Hepsin}$ or 30 $Np^{125A}/CyO$, $P^{sevRas1.V12}FK1$ females were crossed to 20 $w^{118}$, $hs-Stubble / hs-Stubble$ males in bottles at 25° and turned into fresh bottles every 3 days. The progeny were selected as 0-hr white prepupae and transferred to food vials. They were then aged for three hours at 25° before the Stubble tranegene was induced by sumerging vials in a 37° water bath for one hour. Animals were returned to the incubator immediately after heat treatment and allowed to continue development at 25°. Eclosed animals were sorted into two progeny classes, $Cy/hs-Stubble$ or $Np^{125A}/hs-Stubble$, and scored for leg malformation. The second and third pairs of legs of each animal were scored separately, and each leg was individually scored and recorded as either wild-type, mildly malformed, or severely malformed. Legs were considered mildly malformed if they contained structural aberrations but no bends or twists that contorted the normally straight femur or tibia beyond an angle of 45 degrees and the leg was at least half the length of its wild-type counter part. Legs were
considered severely malformed if they were either less than half the length of a wild type leg or containing regions that twisted more than 45 degrees.

### Third-site suppressor analysis

Genetic crosses were set up according to the strategies described below (see Results). Animals were crossed, allowed to lay for three days, turned into new vials, and allowed to lay for two days. Crosses were turned a third time and allowed to lay for two days before the parents were discarded. F1 progeny were sorted into appropriate classes based on genotype and scored on the basis of leg malformation. In experiments investigating the suppression of strong genetic interactions (e.g. combinations of *Stubble* and *Rho* mutations) whole animals were scored as either being mildly or severely malformed based on the criteria described above. In experiments investigating the suppression of mild genetic interactions the second and third pairs of legs of each animal were scored separately, and each leg was individually scored and recorded as either wild-type, mildly malformed, or severely malformed, as described above.

### P-element mediated recombination mapping

The method described by Zhai *et al.* (2003) was used, with modifications, to predict a molecular chromosomal position of the mutation in line 125A. In our hands the *hs-Hid* balancer chromosome used by Zhai *et al.* was difficult to maintain and work with at a large scale. We substituted a balancer regularly used in our lab CyO, P{w+, GMR-Hepsin} (CyH) which is easily scored based on a severely-glazed, dark red, reduced eye phenotype. Using the CyH chromosome provided an additional benefit compared to the *HS-hid* used by Zhai *et al.* in that the viable balancer class, which does not contribute to final recombination and mapping
calculations, could be used as an internal control to ensure that large-scale sorting of chromosome is occurring at expected frequencies.

Initial, rough-scale mapping experiments were conducted in vials using 25 or 50 vials, turned once, after four days. Each vial contained 10 virgin females and 5 males, and the total progeny scored from these crosses was between 2,000 and 6,000 animals of the classes of interest. Larger, fine-scale mapping experiments were conducted in 10 bottles, each containing 30 virgin females and 30 males. These were turned four times for a total of 40 bottles, which resulted in 8,000-10,000 progeny of the relevant classes scored.
CHAPTER THREE: RESULTS

EMS mutagenesis screen for enhancers of \textit{sbd}^{201}

In an effort to identify gene products that interact directly with the Stubble protease, we conducted an F1 EMS mutagenesis screen for enhancers of the recessive \textit{Sb-sbd} mutant \textit{sbd}^{201}. The \textit{sbd}^{201} allele was selected to sensitize the genetic background in this screen because it is associated with a missense mutation in the substrate-binding domain which might disrupt interactions between Stubble and direct targets of Stubble protease activity (FRISTROM and HAMMONDS, 2006). In heterozygous condition \textit{sbd}^{201} mutants exhibit very low but detectable levels of leg and wing malformation, indicating that these heterozygous animals are genetically sensitized with respect to defects in cell shape changes during leg morphogenesis. We reasoned that a mutation in one copy of a gene encoding a product that interacts directly with the Stubble protease would significantly increase (enhance) the frequency of leg and wing malformations in double heterozygotes.
G1) Cross mutagenized males to females from the tester stock

\[
\begin{array}{cccc}
\text{w}^{1118} & + & + & X \\
\text{y} & + & + & \text{Pm} \\
\text{Cy} & + & \text{red, sbd}^{201}, \text{e} & \text{TM6B, Hu, e}
\end{array}
\]

G2) Score all G2 progeny for leg and bristle defects

- Isolate all malformed animals, and cross each individually to tester stock to test if malformations are heritable.

\[
\begin{array}{cccc}
Pm & \text{red, sbd}^{201}, \text{e} \\
\text{Cy} & + & \text{TM6B, Hu, e}
\end{array}
\]

G3) Score G3 progeny to identify line that breed true for mutant phenotypes

- Isolate single chromosomes carrying enhancer mutations by crossing to balancer lines

A) Cross malformed animals to second and third chromosome balancer lines

  i) on the second chromosome

  \[
  \begin{array}{cccc}
  ? & ? & ? & \text{CR2} \\
  \text{Y} & \text{Cy} & \text{red, sbd}^{201}, \text{e} & \text{sco} \\
  \text{X} & + & + & \text{+}
  \end{array}
  \]

  ii) on the third chromosome

  \[
  \begin{array}{cccc}
  + & + & + & \text{Sb}^{1} \\
  \text{X} & + & + & \text{Tb}
  \end{array}
  \]

B) Cross virgin females to males carrying first chromosome balancers

\[
\begin{array}{cccc}
? & ? & ? & \text{M5} \\
? & \text{Cy} & \text{red, sbd}^{201}, \text{e} & \text{y}
\end{array}
\]

C) Mate sibling malformed males and females

G4) Generate stock by crossing siblings carrying the isolated enhancer over a balancer chromosome

Figure 9. A schematic overview of the enhancer screen strategy
The strategy and results of this screen are illustrated in Figure 9 and Table 1. XX white-eye males were mutagenized with EMS (see Methods) and mated to \( sbd^{201} \) heterozygote virgin females (G1 in figure 9). 50,437 offspring were screened for leg, wing, and bristle abnormalities. As bona fide wing defects are typically only seen in cases where leg defects are already evident, animals were primarily scored on the basis of leg malformation and short bristle phenotypes. The bristle phenotype was scored because mutations in the \( Sb-sbd \) locus also affect bristle development (Dobzhansky, 1929). 138 G2 animals with malformed legs or short bristles were isolated as candidate enhancers of \( sbd^{201} \). To determine if these aberrations were heritable each candidate animal was crossed back to the tester stock. 14 potential enhancers of \( sbd^{201} \), hereafter referred to as \( E(sbd) \) alleles, bred true and were recovered in the G3. Of these 14, six exhibited leg and wing malformations, and 8 were associated with a reduced bristle phenotype. Six lines of the latter class (short bristles) were subsequently determined to carry alleles of \( Sb-sbd \), and one, line 125A, was found to be a second chromosome enhancer. G3 \( E(sbd) \) males were mated sequentially to females carrying balanced second and third chromosomes (G3 part A), whereas \( E(sbd) \) females were mated to M5 males (G3 part B). This permitted mapping of the \( E(sbd) \) alleles and also enabled the isolation of \( E(sbd) \) alleles in balanced condition in the G4 generation. Balanced \( E(sbd) \) animals were then used to generate permanent stocks of each allele. As a precaution, G3 sibling males and females carrying identical \( E(sbd) \) mutations were mated and maintained until balanced lines were recovered.

After \( E(sbd) \) alleles were mapped to a chromosome and balanced, stocks were out-crossed to exchange chromosomes that may have originated from parental lines exposed to EMS, and therefore remove any extraneous third-site mutations from the \( E(sbd) \) genetic background.
Table 2. Summary of candidate enhancer of \(sbd^{201}\) lines identified in this screen

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<tr>
<th>Lines</th>
<th>Chromosome</th>
<th>Description</th>
<th>Lethality</th>
</tr>
</thead>
<tbody>
<tr>
<td>E(sbd) 126A</td>
<td>2</td>
<td>frequent LMF with (sbd^{201}) and (Sb^1)</td>
<td>homozygous lethal</td>
</tr>
<tr>
<td>E(sbd) 161A</td>
<td>2</td>
<td>frequent LMF with (sbd^{201}) and (Sb^1)</td>
<td>homozygous lethal</td>
</tr>
<tr>
<td>E(sbd) 195B</td>
<td>2</td>
<td>frequent LMF with (sbd^{201}) and (Sb^1)</td>
<td>homozygous lethal</td>
</tr>
<tr>
<td>E(sbd) 107A</td>
<td>2</td>
<td>short, fat legs and wings</td>
<td>homozygous lethal</td>
</tr>
<tr>
<td>E(sbd) 162B</td>
<td>2</td>
<td>short, fat legs and wings</td>
<td>homozygous lethal</td>
</tr>
<tr>
<td>E(sbd) 125A</td>
<td>2</td>
<td>variable short bristle phenotype,</td>
<td>homozygous lethal</td>
</tr>
<tr>
<td>E(sbd) 278A</td>
<td>2</td>
<td>bent or kinked femur</td>
<td>homozygous lethal</td>
</tr>
<tr>
<td>sbd 46A</td>
<td>3</td>
<td>short bristles, &lt;1/2 normal length</td>
<td>rare escapers\textsuperscript{a}</td>
</tr>
<tr>
<td>sbd 173A</td>
<td>3</td>
<td>short bristles, &lt;1/2 normal length</td>
<td>homozygous viable</td>
</tr>
<tr>
<td>sbd 177A</td>
<td>3</td>
<td>short bristles, &lt;1/2 normal length</td>
<td>homozygous lethal</td>
</tr>
<tr>
<td>sbd 241A</td>
<td>3</td>
<td>short bristles, &lt;1/2 normal length</td>
<td>homozygous lethal</td>
</tr>
<tr>
<td>sbd 258A</td>
<td>3</td>
<td>short bristles, &lt;1/2 normal length</td>
<td>rare escapers\textsuperscript{a}</td>
</tr>
<tr>
<td>sbd 266A</td>
<td>3</td>
<td>short bristles, &lt;1/2 normal length</td>
<td>rare escapers\textsuperscript{a}</td>
</tr>
<tr>
<td>sbd 277A</td>
<td>3</td>
<td>short bristles, &lt;1/2 normal length</td>
<td>homozygous lethal</td>
</tr>
</tbody>
</table>

50,437 G2 flies were scored for leg or bristle malformation, 138 of which exhibited some form of malformation and were considered potential interactors and crossed back to the tester line. 106 of the G3 crosses were viable and 14 exhibited malformed offspring. Six lines exhibited leg malformations and seven lines showed bristle malformations. Phenotypes described form lines Phenotype exhibited by 107A, 162B, 125A, and 278A were determined to not be dependent on the presence of \(sbd^{201}\), though lines 125A and 278 were found to interact with mutants in the \(Sb\)-\(sbd\) locus. LMF = Leg Malformation

\textsuperscript{a} Homozygotes appear in stocks at an estimated frequency of less than 1%
(see Methods, Figure 8). It should be noted that this process does not allow free recombination, and would not remove EMS induced mutations on an enhancer chromosome itself. After out-crossing, potential enhancer lines were crossed to $sbd^{201}$ ($sbd^{201}, e / TM6B, Tb, Hu, e$) and again to the original tester stock ($Pm / CyO; red, sbd^{201}, e / TM2, ubx, e$) to determine if mutations on the balancer chromosomes had any effect on the interaction between the newly isolated enhancer and $sbd^{201}$. In four cases the rate of leg malformation increased in the presence of the $Pm$ chromosome (see Table 3). In a fifth case of $E(sbd)$ 162B, an increase in the frequency of a dachsous-like leg phenotype was observed in the presence of the $Pm$ chromosome. The $Pm$ marker chromosome carries two mutations, an allele of $dumpy$ and an allele of $dachsous$, in addition to the inversion that produces the $Pm$ eye phenotype. It appears that the mutations on the $Pm$ chromosome, either individually or together, act as third-site enhancers in regards to some of the interactions between $sbd^{201}$ and $E(sbd)$ alleles identified in this screen.
Table 3 The *Pm* chromosome acts as a third-site enhancer of second chromosome enhancers

<table>
<thead>
<tr>
<th>Candidate <em>E(sbd)</em> lines</th>
<th>126A</th>
<th>161A</th>
<th>195B</th>
<th>107A</th>
<th>162B</th>
<th>125A</th>
<th>278A</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>sbd</em>&lt;sup&gt;201&lt;/sup&gt;, <em>e</em></td>
<td>20%</td>
<td>11%</td>
<td>85%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>(46)</td>
<td>(72)</td>
<td>(101)</td>
<td>(NA)</td>
<td>(57)</td>
<td>(90)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(11)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Pm; red, sbd</em>&lt;sup&gt;201&lt;/sup&gt;, <em>e</em></td>
<td>52%</td>
<td>67%</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>20%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>(73)</td>
<td>(92)</td>
<td>(139)</td>
<td>(NA)</td>
<td>(45)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(40)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(69)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Second chromosome *E(sbd)* lines were tested for interactions with *sbd*<sup>201</sup> in the presence and absence of the *Pm* chromosome. The *Pm* chromosome acts to enhance the rate of leg malformation in *sbd*<sup>201</sup> combinations with *E(sbd)* lines 126A, 161A, 195B, and 125A. The numbers shown indicate the percentage of animals with malformed legs with the total number of animals of the indicated genotype scored shown in parentheses below. **NA**=not available.

a) 100% of animals in the *Pm* class exhibit, short fat legs  
b) high frequencies of short thoracic bristles observed  
c) high frequencies of thoracic dents and kinked femurs observed
Characterization of the Enhancers of sbd$^{201}$

Considering the data in Tables 2 and 3, the \( E(sbd) \) lines identified in this screen can be divided into three general categories: 1) putative stubbloid alleles based on chromosome location and bristle phenotype; 2) interactors exhibiting traditional leg malformations in combination with \( sbd^{201} \); and 3) interactors that do not exhibit traditional leg malformations in heterozygous combinations with \( sbd^{201} \), but instead exhibit interesting, unanticipated phenotypes. I will discuss each of these groups in detail below, with a special focus on line 125A, which was found to be of particular interest.

In order to characterize the genetic interactions of candidate \( E(sbd) \) lines, individuals from each \( E(sbd) \) line were crossed to mutant alleles of genes previously shown to be required for leg imaginal disc morphogenesis. Each \( E(sbd) \) line was tested in doubly heterozygous combination mutants with the highly interactive \( Sb-sbd \) alleles \( Sb^{63b} \) and \( Sb^{70} \), and second chromosome \( E(sbd) \) lines were also tested for dominant interactions with a transheterozygous combination of \( sbd \) alleles (\( sbd^{201}/sbd^{d} \)). \( Sb^{63b} \) and \( Sb^{70} \) are alleles that exhibit dominant bristle phenotypes, and both are associated with the insertion of transposable elements that disrupt the Stubble proteolytic domain, and interestingly cause the mutant transcript to be overexpressed (HAMMONDS and FRISTROM, 2006). Mutations in the ecdysone responsive \textit{broad} \ locus which plays a major role in leg morphogenesis, were also tested. Because the \( br^{d} \) allele is temperature sensitive, showing higher rates of leg malformation at 18° than 25°; \( E(sbd) \) lines were tested for dominant interactions with \( br^{d} \) at both of these temperatures. Additionally, \( E(sbd) \) lines were tested in doubly heterozygous combinations with mutant alleles of Rho pathway genes (\( Rho1^{3.8} \), \( Rho1^{E3.10} \), \( zip^{Ebr} \), \( GEF2^{11-3} \), \( drok^{2} \)) that have previously been shown to interact in regards to leg malformation (BAYER \textit{et al.} 2003 and references there in).
**Stubbloid alleles**

Eight lines that exhibited short bristles in combination with $sbd^{201}$ were isolated. One of these, as noted above, was found to be a second chromosome enhancer of the bristle phenotype and analyzed separately. Another line, $sbd^{l77A}$, showed poor viability even as a heterozygote and was lost before it could be thoroughly characterized. The remaining six of these mutants were confirmed as *stubbloid* alleles by complementation tests to *Stubble* and *stubbloid* mutations. In all cases non-complementing animals showed shortened and tapered bristles typical of *sbd* combinations, variable rates of leg and wing malformation, and reduced viability. All of the $sbd^{E(sbd)}$ alleles show similar patterns of interactions with *Sb-sbd*, *Rho1*, and *broa d* mutations (Table 4). No interactions we observed with the $Rho1^{l3.8}$ allele in double heterozygous combinations (i.e. $Rho1^{l3.8}/+$; $sbd^{E(sbd)}/+$), or in dominant interactions with $br^I$ hemizygotes at 25°C. In every $sbd^{E(sbd)}$ line tested, 100% of animals had malformed legs and wings and short bristles in transheterozygous combination with the $Sb^{62b}$ allele. Significant leg and wing malformations ranging from 38% to 100% of animals affected were observed in transheterozygous combination with the $sbd^{201}$ allele. To determine if the $sbd^{E(sbd)}$ transheterozygous combinations can be enhanced by *Rho1* mutations, $sbd^{E(sbd)}$ lines were crossed to a stock containing both the null *Rho1* allele, $Rho1^{720}$ and the weak *sbd* allele, $sbd^I$ ($Rho720/CR2$; $sbd^I/TM2, ubx, e$). The resulting $sbd^{E(sbd)}/sbd^I$ transheterozygotes exhibited rates of malformation ranging from 38% to 62%, which increased significantly (to between 64% and 92%) in $Rho1, sbd^I; sbd^{E(sbd)}$ triple mutants.
Table 4. \( sbd^{E(sbd)} \) allele interactions

<table>
<thead>
<tr>
<th>sbd alleles</th>
<th>46A</th>
<th>173A</th>
<th>241A</th>
<th>258A</th>
<th>266A</th>
<th>277A</th>
</tr>
</thead>
<tbody>
<tr>
<td>( br^1/Y; sbd^{E(sbd)}/+ ) (25°)</td>
<td>1%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>(101)</td>
<td>(121)</td>
<td>(75)</td>
<td>(105)</td>
<td>(62)</td>
<td>(42)</td>
</tr>
<tr>
<td>( Rho1^{J3.8}/+; sbd^{E(sbd)}/+ )</td>
<td>2%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>(94)</td>
<td>(118)</td>
<td>(111)</td>
<td>(124)</td>
<td>(126)</td>
<td>(144)</td>
</tr>
<tr>
<td>( Sb^{63B}/sbd^{E(sbd)} )</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>(86)</td>
<td>(61)</td>
<td>(72)</td>
<td>(46)</td>
<td>(43)</td>
<td>(181)</td>
</tr>
<tr>
<td>( sbd^{201}/sbd^{E(sbd)} )</td>
<td>38%</td>
<td>85%</td>
<td>44%</td>
<td>97%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>(145)</td>
<td>(40)</td>
<td>(136)</td>
<td>(92)</td>
<td>(41)</td>
<td>(107)</td>
</tr>
<tr>
<td>( sbd^1/sbd^{E(sbd)} )</td>
<td>36%</td>
<td>32%</td>
<td>39%</td>
<td>52%</td>
<td>61%</td>
<td>30%</td>
</tr>
<tr>
<td></td>
<td>(196)</td>
<td>(130)</td>
<td>(150)</td>
<td>(81)</td>
<td>(177)</td>
<td>(179)</td>
</tr>
<tr>
<td>( Rho1^{720}/+; sbd^1/sbd^{E(sbd)} )</td>
<td>75%</td>
<td>85%</td>
<td>73%</td>
<td>87%</td>
<td>92%</td>
<td>64%</td>
</tr>
<tr>
<td></td>
<td>(170)</td>
<td>(112)</td>
<td>(159)</td>
<td>(79)</td>
<td>(188)</td>
<td>(160)</td>
</tr>
</tbody>
</table>

The numbers shown indicate the percentage of animals with malformed legs with the total number of animals of the indicated genotype scored shown in parentheses. Progeny from crosses to \( Sb^{63b} \) and \( sbd^{201} \) alleles were scored in transheterozygous condition (i.e. \( E(sbd)/Sb^{63b} \) or \( E(sbd)/sbd^{201} \)). Crosses to \( Rho1 \) were scored in doubly heterozygous condition (i.e. \( Rho1^{J3.8}/+; E(sbd)/+ \)), and crosses to \( br^1 \) were scored as dominant interactions (i.e. \( br^1/Y; E(sbd)/+ \)) at 25°C. Two classes resulting from crosses to \( Rho1^{720}/CR2; sbd^1/TM2,ubx,e \) were scored, one resulting in the transheterozygous condition (i.e. \( E(sbd)/sbd^1 \)) and its sibling class exhibiting third-site enhancement interactions (\( Rho1^{J3.8}/+; E(sbd)/sbd^1 \)).
$E(sbd)126A$ is an allele of $Rho1$

Complementation tests demonstrated that $E(sbd)\, 126A$ is an allele of $Rho1$, which will henceforth be referred to as $Rho1^{126A}$. Identifying a $Rho1$ allele that interacts with $sbd^{201}$ in regards to leg malformations confirms our understanding of the genetic components required for imaginal disc morphogenesis. $Rho1^{126A}$ showed high rates of leg malformation when combined with mutants in both ecdysone inducible and Rho pathway genes (Table 5). Doubly heterozygous combinations of $Sb^{63b}$ and $Sb^{70}$ with $Rho1^{126A}$ showed high rates of malformation (79% and 97%, respectively). Dominant interactions between $Rho1^{126A}$ and $br^I$ also showed very high rates of malformation at both 25°C and 18°C (71% and 100%, respectively). All animals doubly heterozygous for $Rho1^{126A}$ and $zip^{Ebr}$ or $GEF2^{11-3}$ exhibited a high frequency of leg malformations. In contrast to the otherwise strong interactions seen in combinations of $Rho1^{126A}$ and mutations in both ecdysone inducible and Rho pathway genes, $Rho1^{126A}$ did not show significant interactions with the $Rho$ kinase mutant $drok^2$. 
Table 5. The novel allele, *Rho1*<sup>126A</sup>, shows strong genetic interactions.

<table>
<thead>
<tr>
<th></th>
<th>Ecdysone inducible mutants</th>
<th></th>
<th>Rho pathway mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Sb</em>&lt;sup&gt;63b&lt;/sup&gt;</td>
<td><em>Sb</em>&lt;sup&gt;70&lt;/sup&gt;</td>
<td><em>sbd</em>&lt;sup&gt;201&lt;/sup&gt;/</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>sbd</em>&lt;sup&gt;l&lt;/sup&gt; (25°)</td>
</tr>
<tr>
<td><em>Rho1</em>&lt;sup&gt;126A&lt;/sup&gt;</td>
<td>79% (99)</td>
<td>97% (85)</td>
<td>20% (46)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Df(2R)Jp8</em></td>
<td>85% (198)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86% (180)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>89% (230)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|                      |                      |                      |                      |                      |
|                      | *RhoA*<sup>13.8</sup> | *RhoA*<sup>E3.10</sup> | *zip*<sup>Ebr</sup> | *GEF2*<sup>11-3</sup> |
|                      |                      |                      |                      |                      |
| *Rho1*<sup>126A</sup> | Lethal               | Lethal               | 100% (57)           | 100% (39) |
| *Df(2R)Jp8*         | Lethal<sup>b</sup>   | Lethal<sup>b</sup>   | 98% (86)<sup>b</sup> | 94% (150)<sup>a</sup> |
|                      |                      |                      |                      | NT |

The numbers shown indicate the percentage of animals with malformed legs with the total number of animals of the indicated genotype scored shown in parentheses. Progeny from crosses to *Sb* alleles and Rho pathway mutants were scored in the doubly heterozygous condition (e.g. *E(sbd)/+; Sb*<sup>63b</sup>/+), and in dominant interactions with *sbd* alleles (*sbd*<sup>201</sup>/ *sbd*<sup>l</sup>). Interactions with Rho1 mutants were scored as transheterozygotes (e.g. *Rho1*<sup>13.8</sup>/ *Rho1*<sup>126A</sup>). Crosses to *br*<sup>l</sup> were scored as dominant interactions (e.g. *br*<sup>l</sup>/Y; *E(sbd)/+*) at 25°C or 18°C. Genetic interactions exhibited by *Df(2R)Jp8*, a deficiency that uncovers the *Rho1* locus, have been included for comparison. NT = not tested.

**E(sbd)161A and E(sbd)195B are alleles of zipper**

In initial crosses to Rho pathway genes, both lines 161A and 195B failed to complement \( zip^{Ebr} \). Subsequent testing confirmed that these mutants fail to complement the amorphic \( zip^1 \) allele and each other. For these reasons it was concluded that lines 161A and 1965B are both alleles of zipper (zip), which non-muscle myosin II heavy chain. These two alleles show distinct patterns of genetic interactions. The \( zip^{161A} \) allele exhibited low rates of malformation with mutants in the \( Sb-sbd \) locus (9%–17%), strong interaction with \( br^j \) at both 25°C and 18°C (55% and 100%, respectively), and highly variable interactions with Rho pathway mutants. Interestingly, \( zip^{161A} \) showed very distinct rates of malformation in heterozygous combination with \( Rho1 \): in combination with \( Rho1^{31.8} \) low rates of malformation were observed (11%), while in combination with \( Rho1^{E3.10} \) alleles relatively high rates were observed (67%) (Table 6). In contrast, the \( zip^{195B} \) allele shows extremely strong interactions with \( Sb \) and mutants, \( br^j \), and mutations in the Rho signaling pathway. As a heterozygote \( zip^{195B} \) exhibits moderately high levels of leg malformation (35%) and is therefore a dominant allele.

As with \( Rho1 \), the role of \( zip \) in imaginal disc morphogenesis has been well established in previous studies (BAYER et al. 2003; HALSELL et al. 2002; WARD et al. 2003). The identification of these two new \( zip \) alleles strongly supports the current model that Stubble activity and actin myosin contraction are linked during imaginal disc development.
Table 6. *zip*\textsuperscript{161A} and *zip*\textsuperscript{195B} exhibit distinct patterns of genetic interaction

<table>
<thead>
<tr>
<th>Ecdysone inducible mutants</th>
<th>Sb\textsuperscript{63b}</th>
<th>Sb\textsuperscript{70}</th>
<th>sbd\textsuperscript{201}/sbd\textsuperscript{1}</th>
<th>br\textsuperscript{1} (25(^\circ))</th>
<th>br\textsuperscript{1} (18(^\circ))</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>zip</em>\textsuperscript{161A}</td>
<td>9% (139)</td>
<td>17% (217)</td>
<td>14% (149)</td>
<td>55% (117)</td>
<td>100% (69)</td>
</tr>
<tr>
<td><em>zip</em>\textsuperscript{195B}</td>
<td>96% (128)</td>
<td>74% (160)</td>
<td>72% (184)</td>
<td>100% (67)</td>
<td>100% (59)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rho pathway mutants</th>
<th>Rho\textsuperscript{A13.8}</th>
<th>Rho\textsuperscript{A E3.10}</th>
<th><em>zip</em>\textsuperscript{Ebr}</th>
<th>GEF\textsuperscript{211-3}</th>
<th>drok\textsuperscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>zip</em>\textsuperscript{161A}</td>
<td>11% (72)</td>
<td>67% (92)</td>
<td>Lethal</td>
<td>50% (127)</td>
<td>22% (36)</td>
</tr>
<tr>
<td><em>zip</em>\textsuperscript{195B}</td>
<td>85% (101)</td>
<td>100% (139)</td>
<td>Lethal</td>
<td>100% (87)</td>
<td>94% (35)</td>
</tr>
</tbody>
</table>

The numbers shown indicate the percentage of animals with malformed legs with the total number of animals of the indicated genotype scored shown in parentheses. Progeny from crosses to *Sb* alleles and Rho pathway mutants were scored in the doubly heterozygous condition (e.g. *E(sbd)/+; Sb\textsuperscript{63b}/+*), and in dominant interactions with *sbd* alleles (*sbd\textsuperscript{201}/sbd\textsuperscript{1}*). Interactions with *zip* mutants were scored for transheterozygotes (e.g. *zip*\textsuperscript{161A}/*zip*\textsuperscript{Ebr A}). Crosses to *br*\textsuperscript{1} were scored as dominant interactions (i.e. *br*\textsuperscript{1}/*Y; E(sbd)/+) at 25\(^\circ\)C or 18\(^\circ\)C.
**Line 107A carries a mutation in the *dumpy* locus**

Animals in the 107A line exhibited high rates of an unexpected malformation where all three sets of legs were short and fat but not twisted or otherwise irregular. This phenotype was qualitatively distinct from the traditional leg malformation associated with mutations in the *br*, *Sb-sbd*, *Rho*, or *zip* loci. 107A animals also exhibited wide wings with flattened posterior regions. Additionally, animals carrying a 107A chromosome over a *Pm* chromosome exhibited occasional thoracic eruptions, a cuticle malformation characteristic of a number of *dumpy* transheterozygous combinations (WILKIN *et al.*, 2000 and references therein). The progenitor of the 107A line was isolated over a *Pm* chromosome, and after observing this line for several generations it was determined that the fat-leg and thoracic eruption phenotypes were found to be dependent on the presence of the *Pm* chromosome and not affected by the presence of *sbd*^{201}. As both of the fat leg and thoracic eruption phenotypes are common in transheterozygous combinations of *dumpy* alleles, it was speculated that 107A was a mutant in the *dumpy* locus. The *dumpy* gene spans 200 kb, and encodes a transmembrane protein over 23,000 amino acids long (WILKIN *et al.*, 2000). Due to its size, the *dumpy* locus is often mutated in EMS screens. Subsequent complementation tests showed that line 107A was indeed an allele of *dumpy*. Thus, it appears that the interactions between 107A and the *Pm* marker chromosome results from the failure of 107A to complement a *dumpy* allele, *dp*^{6}, found on the *Pm* chromosome. As 107A showed no interaction with *Sb-sbd* alleles, *br*^{1}, or mutants in the Rho signaling pathway, this line was discarded.
E(sbd)162A is an allele of the daschous locus

Line 162A also exhibited a fat leg phenotype that affected all three sets of legs. Line 162A showed no interactions with \( sbd^{201} \), but it did show a short, fat leg phenotype with the \( Pm \) balancer chromosome. Line 162A was complementation tested against \( dumpy \) and \( dachsous \) alleles, because mutations in both of these loci are found on the \( Pm \) chromosome, and both are known to induce short, fat leg phenotypes. Line 162A complemented \( dumpy \) alleles, but failed to complement \( ds \) alleles and will henceforth be referred to as \( ds^{162A} \). The \( dachsous \) gene encodes a large cadherin protein that is known to be involved in cell adhesion, as well as intercellular signaling (CLARK et al. 1995). Mutants in the \( daschous \) gene exhibit defects in the regulation of cell growth, planar polarity, and exhibit short fat legs reminiscent of a daschound. The \( ds^{162A} \) allele was tested for interactions with \( Sb-sbd, bv^{l}, Rho1 \) and \( zip \) mutations (Table 7). The \( ds^{162A} \) line showed no interactions with mutations in Rho pathway genes, weak interactions with \( Sb \) alleles (11-17%), but significant enhancement (43%) of leg malformation in \( sbd^{l}/sbd^{201} \) transheterozygotes. Several additional \( ds \) alleles were tested including \( ds^{33K}, ds^{38K} \), and \( ds^{w} \). The \( ds^{33K} \) allele, which is found on the \( Pm \) chromosome, exhibited leg malformation in combination with \( sbd^{l}/sbd^{201} \), though the rate of malformation observed in this interaction was low (13%). Other \( ds \) alleles tested did not interact with \( Sb-sbd \) alleles or \( Rho1 \) alleles with the exception of \( ds^{w} \) which showed significant interactions with \( zip^{Ebr} \) (31%). Why the \( ds \) alleles that were tested showed such variability in their genetic interactions is not clear, however, it appears the some \( ds \) mutations can contribute to the malformed leg phenotype.
Table 7. The $ds^{162A}$ allele enhances mutations in the $Sb-sbd$ locus

<table>
<thead>
<tr>
<th>Ecdysone inducible mutants</th>
<th>Rho pathway mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Sb^{63b}$</td>
<td>$Sb^{70}$</td>
</tr>
<tr>
<td>$ds^{162A}$</td>
<td>17% (115)</td>
</tr>
<tr>
<td>$ds^{33K}$</td>
<td>0% (100)</td>
</tr>
<tr>
<td>$ds^{38K}$</td>
<td>0% (76)</td>
</tr>
<tr>
<td>$ds^{w}$</td>
<td>3% (162)</td>
</tr>
</tbody>
</table>

The numbers shown indicate the percentage of animals with malformed legs with the total number of animals of the indicated genotype scored shown in parentheses. Progeny from crosses to $Sb$ alleles and Rho pathway mutants were scored in the doubly heterozygous condition (e.g. $E(sbd)/+; Sb^{63b}/+$) and in dominant interactions with $sbd$ alleles ($sbd^{201}/sbd^{l}$).
\textit{E(sbd)278A is an allele of muscle-myosin heavy chain}

The 278A line was highly interesting because it exhibited two apparently dominant phenotypes. One phenotype was a mild but distinct bending or “kink” in the femur of second and third legs, and the other a depressed or dented thorax. These phenotypes are manifest at highly variable rates of expressivity, and may be influenced by genetic background. The kinked femur phenotype appeared consistently, even if at variable levels.

To investigate the identity of the mutant locus responsible for the 278A phenotypes, this mutation was complemented against 10 second chromosome deficiencies previously isolated in the von Kalm lab as second site non-complementers of \textit{Sb} alleles. One of these deficiencies, \textit{Df(2L)cact-255rv64} failed to complement 278A. This deficiency uncovers the genomic region spanning cytological bands 35F-36A to 36D. After reviewing the genes found in this interval in search of mutants to complement against 278A, it was noted that this region included the \textit{muscle myosin heavy chain} gene (\textit{Mhc}) which is found within the breakpoints of \textit{Df(2L)cact-255rv64}. A brief literature review revealed that several \textit{Mhc} alleles exhibit phenotypes highly similar in description to 278A, including ones originally called \textit{Bashed} and \textit{Shrunken thorax}. A hypomorphic allele, \textit{Mhc^{k10423}}, associated with a P-element insertion, was acquired from the Bloomington Stock Center and complemented to 278A animals. These alleles failed to complement and it was concluded that Line 278A carries a mutant allele of \textit{Mhc}. This allele is now referred to as \textit{Mhc^{278A}}.

The \textit{Mhc^{278A}} line was tested for interactions against \textit{Sb-sbd} alleles, \textit{br^I}, and Rho pathway mutants (Table 8). It was found that \textit{Mhc^{278A}} showed moderate to high rates of leg malformation in double heterozygote combinations with \textit{Sb} mutants, and in combination with \textit{sbd} transheterozygotes. In contrast, \textit{Mhc^{278A}} shows very weak interactions with \textit{br^I} and three alleles.
of Rho pathway genes. In contrast to $Mhc^{278A}$, $Mhc^{K10423}$ does not show significant rates of leg malformation, except in combination with $sbd$ transheterozygotes (Table 8).
Table 8. \(Mhc^{278A}\) interacts genetically with mutations in the \(Sb-sbd\) locus

<table>
<thead>
<tr>
<th>Ec dysone inducible mutants</th>
<th>(Sb^{63b})</th>
<th>(Sb^{70})</th>
<th>(sbd^{201}/)</th>
<th>(br^{1}) (25(^\circ))</th>
<th>(br^{1}) (18(^\circ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Mhc^{278})</td>
<td>71% (62)</td>
<td>44% (87)</td>
<td>46% (187)</td>
<td>0% (77)</td>
<td>11% (43)</td>
</tr>
<tr>
<td>(Mhc^{K10423})</td>
<td>0% (71)</td>
<td>1% (75)</td>
<td>17% (84)</td>
<td>0% (85)</td>
<td>5% (45)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rho pathway mutants</th>
<th>(\text{Rho}^{J3,8})</th>
<th>(\text{Rho}^{E3,10})</th>
<th>(\text{zip}^{Ebr})</th>
<th>(\text{GEF}^{11-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Mhc^{278})</td>
<td>12%</td>
<td>2%</td>
<td>11%</td>
<td>0%</td>
</tr>
<tr>
<td>(Mhc^{K10423})</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>3%</td>
</tr>
</tbody>
</table>

The numbers shown indicate the percentage of animals with malformed legs with the total number of animals of the indicated genotype scored shown in parentheses. Progeny from crosses to \(Sb\) alleles and Rho pathway mutants were scored in the doubly heterozygous condition (e.g. \(E(sbd)/+; Sb^{63b}/+\)) and in dominant interactions with \(sbd\) alleles (\(sbd^{201}/ sbd^{1}\)). Crosses to \(br^{1}\) were scored as dominant interactions (i.e. \(br^{1}/Y; E(sbd)/+\)) at 25\(^\circ\)C or 18\(^\circ\)C.
**E(sbd)125A is an allele of Notopleural**

The 125A line enhances the $sbd^{201}$ bristle phenotype and exhibits a weakly penetrant dominant thoracic bristle phenotype in which the bristles are shortened and uneven in length. These qualities make the 125A mutation a uniquely valuable discovery because the nature of how Stubble activity regulates actin accumulation during bristle development is currently not understood, and no second site enhancers of the $sbd$ bristle phenotypes have been previously identified.

To investigate the identity of the mutant locus responsible for the 125A phenotype, this line was complemented against a series of 10 second chromosomal deficiencies previously isolated in the von Kalm lab as second site non-complementers of $Sb$ alleles. One of these deficiencies that uncovers a region containing the *Notopleural* ($Np$) locus, $Df(2)Np4$, failed to complement 125A. In subsequent analyses all deficiencies tested that delete the $Np$ locus ($Df(2)Np1$, $Df(2)Np2$, $Df(2)Np3$, $Df(2)Np4$ and $Df(2)Np5$) failed to complement 125A. Additionally, all of these deficiencies, $Np$ mutants, and 125A exhibit a similar dominant bristle defect, suggesting that 125A might be an $Np$ allele. When 125A was tested against an EMS induced $Np$ allele, $Np2$, these alleles failed to complement. Therefore, 125A is an allele of *Notopleural*, and it will henceforth be referred to it as $Np^{125A}$.

*Notopleural* was first described by Calvin Bridges in a 1936 paper that mapped the bristle phenotype and associated embryonic lethality to a chromosomal deletion spanning 44E1 to 45E1 (BRIDGES et al. 1936). The $Np^{125A}$ adult phenotype closely matches the description originally provided by Bridges, which states that *Notopleural* “is characterized by numerous departures from the wild type, especially by shortened *Notopleural*, humeral and pretarsal bristles, by branched straggly microcheatea, by blunter wings with somewhat thickened and branched
venation.” In the past 70 years, the biology and molecular position of the \( Np \) locus have not been elucidated. Several large deficiencies uncover the \( Np \) locus and result in dominant phenotypes, indicating that all null \( Np \) alleles are dominant. In the remaining sections I will discuss work carried out with the goal of genetically characterizing the role of \( Np^{125A} \) in leg and bristle development, and defining the precise molecular position of the \( Np \) locus.

**Genetic and phenotypic characterization of the \( Np^{125A} \) allele**

In addition to acting as a second-site enhancer of the \( sbd \) bristle phenotype, the \( Np^{125A} \) allele shows moderate rates of leg malformation (20%-44%) when combined with mutants in the \( Sb-sbd \) locus, and strong interactions (63%) with \( br^I \) alleles at 18\(^\circ\)C (Table 9). \( Np^{125A} \) shows relatively low rates of malformation with Rho Pathway mutants (10%-13%), though it should be noted that \( Np^{125A} \) shows stronger interactions (20%) with the Rho kinase mutant \( drok^2 \) than does \( Rho1^{126A} \) (4%, Table 9). \( Np^2 \) showed similar interactions to \( Np^{125A} \). Collectively, these data clearly indicate that the \( Np \) locus is involved prepupal leg imaginal disc development, a role not previously described for this locus.

In order to further investigate the behavior of \( Np^{125A} \), and the \( Np \) locus in general, several deficiencies that uncover the \( Np \) gene region were tested for genetic interactions with mutants in \( Sb-sbd \) and mutations in components of the Rho signaling pathway. \( Np \) deficiencies exhibited a similar trend as seen with the EMS generated mutants \( Np^{125A} \) and \( Np^2 \), in that they showed higher rates of leg malformation in combination with \( Sb-sbd \) mutants than with with \( Rho1 \) pathway mutants (Table 10). Except for \( Df(2R)Np4 \), the \( Np \) deficiencies tested showed stronger interactions with \( Sb-sbd \) and \( Rho1 \) mutants than did the \( Np^{125A} \) and \( Np^2 \) alleles.
Table 9. *Np* allele genetic interactions

<table>
<thead>
<tr>
<th></th>
<th><em>Sb</em>&lt;sup&gt;63b&lt;/sup&gt;</th>
<th><em>Sb</em>&lt;sup&gt;70&lt;/sup&gt;</th>
<th><em>sbd&lt;sup&gt;201&lt;/sup&gt;</em>/&lt;sup&gt;sbd&lt;/sup&gt;&lt;sup&gt;1&lt;/sup&gt;</th>
<th><em>br&lt;sup&gt;1&lt;/sup&gt;</em>/&lt;sup&gt;(25°C)&lt;/sup&gt;</th>
<th><em>br&lt;sup&gt;1&lt;/sup&gt;</em>/&lt;sup&gt;(18°C)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Np&lt;sup&gt;125A&lt;/sup&gt;</em></td>
<td>36% (117)</td>
<td>44% (94)</td>
<td>20% (152)</td>
<td>0% (122)</td>
<td>63% (80)</td>
</tr>
<tr>
<td><em>Np&lt;sup&gt;2&lt;/sup&gt;</em></td>
<td>22% (78)</td>
<td>43% (99)</td>
<td>29% (93)</td>
<td>0% (75)</td>
<td>52% (49)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th><em>RhoA&lt;sup&gt;13.8&lt;/sup&gt;</em></th>
<th><em>RhoA&lt;sup&gt;E3.10&lt;/sup&gt;</em></th>
<th><em>zip&lt;sup&gt;Ebr&lt;/sup&gt;</em></th>
<th><em>GEF2&lt;sup&gt;11-3&lt;/sup&gt;</em></th>
<th><em>drok&lt;sup&gt;2&lt;/sup&gt;</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Np&lt;sup&gt;125A&lt;/sup&gt;</em></td>
<td>10% (80)</td>
<td>13% (52)</td>
<td>18% (153)</td>
<td>0% (106)</td>
<td>20%</td>
</tr>
<tr>
<td><em>Np&lt;sup&gt;2&lt;/sup&gt;</em></td>
<td>14% (70)</td>
<td>0% (46)</td>
<td>18% (88)</td>
<td>2% (103)</td>
<td>NT</td>
</tr>
</tbody>
</table>

The numbers shown indicate the percentage of animals with malformed legs with the total number of animals of the indicated genotype scored shown in parentheses. Progeny from crosses to *Sb* alleles and Rho pathway mutants were scored in the doubly heterozygous condition (e.g. *E(sbd)/+; Sb<sup>63b</sup>/+*) and in dominant interactions with *sbd* alleles (*sbd<sup>201</sup>/sbd<sup>1</sup>*). Crosses to *br<sup>1</sup>* were scored as dominant interactions (i.e. *br<sup>1</sup>/Y; E(sbd)/+*) at 25°C or 18°C.
Table 10. *Notopleural* deficiencies and EMS mutants show similar genetic interactions

<table>
<thead>
<tr>
<th>Np</th>
<th>Sb&lt;sup&gt;63b&lt;/sup&gt;</th>
<th>Sb&lt;sup&gt;70&lt;/sup&gt;</th>
<th>sbd&lt;sup&gt;201&lt;/sup&gt;/sbd&lt;sup&gt;l&lt;/sup&gt;</th>
<th>RhoA&lt;sup&gt;13.8&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Np&lt;sup&gt;125A&lt;/sup&gt;</td>
<td>36% (117)</td>
<td>44% (94)</td>
<td>20% (152)</td>
<td>10% (80)</td>
</tr>
<tr>
<td>Np&lt;sup&gt;2&lt;/sup&gt;</td>
<td>22% (78)</td>
<td>43% (99)</td>
<td>29% (93)</td>
<td>14% (46)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Np Deficiencies</th>
<th>Sb&lt;sup&gt;63b&lt;/sup&gt;</th>
<th>Sb&lt;sup&gt;70&lt;/sup&gt;</th>
<th>sbd&lt;sup&gt;201&lt;/sup&gt;/sbd&lt;sup&gt;l&lt;/sup&gt;</th>
<th>RhoA&lt;sup&gt;13.8&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(2R)Np1</td>
<td>51%</td>
<td>64%</td>
<td>75%</td>
<td>30%</td>
</tr>
<tr>
<td>Df(2R)Np3</td>
<td>97%</td>
<td>47%</td>
<td>38%</td>
<td>18%</td>
</tr>
<tr>
<td>Df(2R)Np4</td>
<td>21%</td>
<td>41%</td>
<td>46%</td>
<td>8%</td>
</tr>
<tr>
<td>Df(2R)Np5</td>
<td>56%</td>
<td>55%</td>
<td>57%</td>
<td>16%</td>
</tr>
</tbody>
</table>

The numbers shown indicate the percentage of animals with malformed legs with the total number of animals of the indicated genotype scored shown in parentheses. Progeny from crosses to *Sb* alleles and Rho pathway mutants were scored in the doubly heterozygous condition (e.g. *E(sbd)/+; Sb<sup>63b</sup>/+*) and in dominant interactions with *sbd* alleles (*sbd<sup>201</sup>/sbd<sup>l</sup>*). Crosses to *br<sup>j</sup>* were scored as dominant interactions (i.e. *br<sup>j</sup>/Y; E(sbd)/+*) at 25°C or 18°C.
Phenotypic analysis of Np^{125A}

A primary phenotypic analysis of Np^{125A} was conducted. The question of whether Np^{125A} is homozygous lethal was addressed by observing large scale self-crosses of Np^{125A} /CR2 conducted in bottles. Over 5000 progeny were screened for evidence of Np^{125A} homozygotes, and none were found. To eliminate the possibility that lethality may be associated with a second-site lethal on the Np^{125A} chromosome, the Np^{125A}/Np^{2} combination was also studied. Over 2000 progeny were screened for evidence of surviving Np transheterozygotes and none were found. The results of these experiments are confirmed by data from mapping experiments discussed below, in which over 20,000 animals were scored and at no point was the appearance of a homozygote observed.

Np^{125A}, like all previously described EMS-induced Np mutants, exhibits embryonic lethality as homozygotes (DOCKENDORF et al. 2000; MOHR and BOSWELL, 2002). A stock of Np^{125A} balanced over a Cy, Dfd;EYFP chromosome was constructed in order to investigate the embryonic viability of 125A. In six trials, over 600 embryos were screened, 200 of which lacked EYFP expression. In no cases were any larva lacking EYFP expression observed, and it was concluded that Np^{125A} exhibits 100% embryonic lethality in the homozygous condition. Though extensive characterization of embryonic phenotypes associated with Np^{125A} homozygote lethality were not conducted, it was observed that many (over 10%) of the dead Np^{125A} embryos had advanced sufficiently far to develop clearly recognizable mouth-hooks that were apparent through the embryonic cuticle.
Np alleles act as second site enhancers of the sbd\textsuperscript{1} bristle phenotype

Np\textsuperscript{125A} and Np\textsuperscript{2} both enhance leg malformations associated with Sb alleles and, to a much lesser degree, Rho pathway mutants. Np\textsuperscript{125A} was initially identified as an enhancer of the sbd\textsuperscript{201} bristle phenotype, and thus appears to be closely linked to Stubble protease activity in both bristle and appendage development. Though Rho1 activity is known to modulate actin dynamics and the Stubble protease is required for actin accumulation in developing bristles, Rho1 activity has never been linked to bristle development. In order to investigate the possibility that Rho1 plays a role in actin cytoskeletal dynamics of developing bristles, a third site enhancer assay was constructed in order to quantify the frequency of bristle abnormalities seen in Np, sbd double heterozygotes, and to determine whether this interaction is enhanced by the presence of a mutant Rho1 allele. In these experiments the weak hypomorphic sbd\textsuperscript{1} allele was used along with the null Rho1 allele, Rho1\textsuperscript{720}.

Males carrying Rho1\textsuperscript{720} and sbd\textsuperscript{1} were crossed to virgin Np\textsuperscript{125A}/CyH flies and the resulting progeny were scored on the basis of bristle length. Np\textsuperscript{125A} associated reductions in bristle length are most penetrant and recognizable in the postscutellar bristles, so progeny were scored as being in one of three classes: those exhibiting two wild-type post-scute, those exhibiting at least one postscutellar that was less than ¾ wild-type length, and those exhibiting two post scutellars bristle less than ½ wild-type length. Each of these classes was exclusive, i.e. animals with two ½ wild-type length bristles were not included in the ¾ wild-type length class. No animals heterozygous for Rho1\textsuperscript{720} or sbd\textsuperscript{1}, or doubly heterozygous for Rho1\textsuperscript{720} and sbd\textsuperscript{1} showed any reduction in bristle length (Table 11). Less than 10% of the animals heterozygous for Np\textsuperscript{125A} and doubly heterozygous for Np\textsuperscript{125A} and Rho1\textsuperscript{720} showed a reduction in bristle length, and none of these were in the more severe class (data not shown). 100% of animals doubly
heterozygous for \(Np^{125A}\) and \(sbd^l\) exhibited scutellar bristles that were clearly reduced in length. 45% of these animals exhibited two postscutellar bristles that were less than one half the length of wild type bristles, and the remaining 55% exhibited at least one bristle that was less than three-fourths of wild-type length. In triple heterozygotes carrying one copy of \(Np^{125A}\), one copy of \(Rho1^{720}\), and one copy of \(sbd^l\), 76% of the animals exhibited two postscutellar bristles that were less than one half the length of wild type bristles, and the remaining 24% exhibited at least one bristle that was less than three-fourths of wild-type length.

Similar, though weaker interactions were observed when \(Np^2\) was tested in the same manner. 37% of \(Np^2\), \(sbd^l\) double heterozygotes exhibited wild-type bristles, 10% exhibited two postscutellar bristles that were less than \(\frac{1}{2}\) wild-type length, and the remaining 53% exhibited at least one postscutellar bristle that was less than \(\frac{3}{4}\) wild-type length. In \(Np^2\), \(Rho1^{720}\), \(sbd^l\) triple heterozygous animals, 6% exhibited wild-type length bristles, 36% exhibited two postscutellar bristles that were less than one half the length of wild type bristles, and the remaining 56% exhibited at least one bristle that was less than three-fourths of wild-type length. These data confirm that \(sbd\) alleles enhance the weak but dominant \(Np^{125A}\) bristle phenotype, and suggest that the severity of this phenotype can be further enhanced by reducing the gene dosage of \(Rho1\).
Table 11. *Np* alleles interact with and *Rho1720* in regards to bristle elongation

<table>
<thead>
<tr>
<th>Bristle length</th>
<th>WT</th>
<th>&gt;3/4 WT</th>
<th>&gt;1/2 WT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Np</em>&lt;sup&gt;125&lt;/sup&gt;/CR2; sbd1/+</td>
<td>0%</td>
<td>55%</td>
<td>45%</td>
<td>164</td>
</tr>
<tr>
<td><em>Np</em>&lt;sup&gt;125&lt;/sup&gt;/ <em>Rho1720</em>; sbd1/+</td>
<td>0%</td>
<td>24%</td>
<td>76%</td>
<td>203</td>
</tr>
<tr>
<td><em>Rho1720</em>/CR2; sbd1/+</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>178</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bristle length</th>
<th>WT</th>
<th>&gt;3/4 WT</th>
<th>&gt;1/2 WT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Np</em>&lt;sup&gt;2&lt;/sup&gt;/CR2; sbd1/+</td>
<td>37%</td>
<td>53%</td>
<td>10%</td>
<td>123</td>
</tr>
<tr>
<td><em>Rho1720</em>/ <em>Np</em>&lt;sup&gt;2&lt;/sup&gt;; sbd1/+</td>
<td>6%</td>
<td>58%</td>
<td>36%</td>
<td>160</td>
</tr>
<tr>
<td><em>Rho1720</em>/CyH; sbd1/+</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>198</td>
</tr>
</tbody>
</table>

The table above provides the percent of animals in each class with wild-type bristles (WT), with at least one post-scutellar bristle less than ¾ wild-type length (>3/4 WT), or with two bristles that were less than ½ wild-type length (>1/2 WT). These phenotypic classes are exclusive, i.e. animals in the >1/2 WT class were not included in the >3/4 WT class. The total number of animals scored in each class in the last column on the right under Totals.
**Np^{125A} suppresses leg malformations resulting from hs-Stubble over-expression**

In order to further investigate the link between \( Np^{125A} \) and Stubble protease activity, it was tested whether \( Np^{125A} \) could suppress leg malformation caused by early prepupal over-expression of wild-type Stubble protein. These experiments were based on work carried out by Bayer et al. (2003), in which animals carrying a heat-shock inducible full length wild-type Stubble cDNA transgene (\( hs-Stubble \)) were exposed to a 1hr heat-shock, 3 hours after pupariating (3hr AP). Bayer et al. reported that this treatment resulted in high levels of second and third leg malformations (69% and 84%, respectively) in animals carrying one copy of the \( hs-Stubble \) transgene, but not in control animals (0.1%). In the same study, it was found that the presence of a Rho1 loss of function mutation reduced the rate of leg malformation resulting from Stubble over-expression by between 2 and 3 fold (BAYER et al. 2003). Based on this data Bayer et al. suggested that Stubble proteolysis acts upstream of Rho1 signaling during leg imaginal disc morphogenesis.

In order to determine if \( Np^{125A} \) was capable of suppressing leg malformations induced by over-expression of Stubble protein, \( Np^{125A}/CR2 \) animals were crossed to \( hs-Stubble \) homozygotes, which yielded CR2/\( hs-Stubble \) and \( Np^{125A}/hs-Stubble \) offspring. Animals were collected as white-prepupae (0 hrs-AP) and exposed to a one hour heat-shock at 37°C at 3hr AP. In these experiments the CR2/\( hs-Stubble \) class served as a control making it possible to compare the effects of Stubble over-expression in the presence and absence of \( Np^{125A} \). In second legs the rate of malformations was reduced by 2.3 fold, from 48% to 21% in animals carrying \( Np^{125A} \) (Table 12). Similar results were observed for third legs, with the rate of malformation decreasing 2-fold from 84% to 42%. A simple interpretation of these data is that Np gene product acts downstream of Stubble during prepupal leg morphogenesis.
Table 12. $Np^{1254}$ suppresses leg malformation associated with Stubble over-expression

<table>
<thead>
<tr>
<th></th>
<th>HS 0 hr AP</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/+ (%)</td>
<td>$Np^{1254}$/+ (%)</td>
<td>Fold reduction</td>
<td></td>
</tr>
<tr>
<td>Second legs</td>
<td>48% (132)</td>
<td>21% (200)</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Third legs</td>
<td>84% (132)</td>
<td>42% (200)</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

All animals carry one copy of the third-chromosome $hs$-$Stubble$ transgene. The transgene was induced by heat-shocking 3-hr prepupae for 1 hr at $37^\circ$. The numbers shown indicate the percentage of malformed legs of the given class with the total no. of legs of the indicated genotype scored shown in parentheses. +/+ indicates that animals carry two wild-type $Np$ alleles.
Third-site suppression analysis of Np^{125A}

In order to further investigate the role of Np in the Stubble-dependent genetic pathway required for leg disc morphogenesis, third-site suppression analyses were conducted. When doubly heterozygous animals exhibit a phenotype that neither allele manifests independently, this is called second site non-complementation (SSNC). SSNC results in developmental defects when a reduction in the wild-type gene dosage of two components contributing to the same process causes that process to fail. The addition of a third mutant allele into a genetic background that that exhibits SSNC will typically further impede normal development; i.e. the severity of the phenotype will be enhanced. The increased bristle malformation interactions seen in Np^{125A}, Rho1^{720}, sbd^I triple heterozygotes, discussed above, is an example of this phenomena, which is referred to as third-site enhancement. If the rate or severity of expression of a SSNC induced phenotype is reduced by the presence of a third mutation, a third-site suppressor, this is referred to as third site suppression. Such interactions, where the addition of a third mutation drives development towards the wild-type condition, are rare and thought to be highly specific to genes acting in the same biochemical pathway regulating a developmental process. Third site suppression can occur if an SSNC interaction caused by the disruption of two key components in a pathway is suppressed by the presence of a loss of function allele of a negative regulator of that same pathway.

In work not described in this thesis I carried out third-site suppression analyses to place Stubble activity at or above the level of RHOK activity by showing that loss of function mutations in negative regulators of two pathways downstream of RHOK suppress the malformed leg phenotype caused by SSNC interactions between Sb and Rho1 mutants (Figure 7). The first regulator that was tested was myosin phosphatase, which inhibits the activation of the myosin
light chain and the actin-myosin contraction pathway required for cell shape changes. For these experiment the mutant \( mbs^3 \), which is a hypomorphoic allele of the myosin binding subunit of myosin phosphatase was tested (Kimura et al. 1996). The second regulator tested was cofilin phosphatase, which inhibits the F-actin polymerization dependent pathway downstream of RHOK (Figure 7). Cofilin phosphatase, also called slingshot (ssh), activates cofilin, which in turn inhibits F-actin polymerization and changes in cell shape. To test if mutations in negative regulators of the F-actin polymerization-dependent pathway can suppress \( Sb, Rho1 \) interactions the hypomorphic allele \( ssh^{01207} \) was used (SOMOGYI and RORTH, 2004). It was found that the presence of \( mbs^3 \) or \( ssh^{01207} \) suppressed the rate of malformation seen in animals doubly heterozygous for \( Sb^{63b} \) and \( Rho^{720} \) by nearly 2 fold. These observations indicate that Stubble and Rho act together to induce both myosin contraction and actin polymerization. As both of these processes are regulated in two distinct pathways downstream of RHOK, it can be concluded that Stubble acts at or above the level of RHOK.

Third-site suppression analyses were conducted to determine if loss of function mutations in negative regulators of the myosin-dependent or the F-actin polymerization dependent pathways downstream of RHOK could suppress leg malformations seen in \( Np^{125A}, Sb^{70} \) SSNC interactions. In these experiments animals carrying \( Np^{125A} \) and one copy of a negative regulator (\( Mbs^3 \) or \( ssh^{01207} \)) were crossed to animals carrying the \( Sb^{70} \) allele. The progeny classes resulting from these crosses contained animals that were doubly heterozygous for \( Np^{125A} \) and \( Sb^{70} \), and triple heterozygotes that carried one copy of \( Np^{125A}, Sb^{70} \) and one copy of either \( Mbs^3 \) or \( ssh^{01207} \). In order to make these assays more sensitive, each leg was individually was scored and the rates of malformation in second and third legs were tabulated separately. \( Np^{125A}- Sb^{70} \) interactions were significantly suppressed by the presence of \( Mbs^3 \). These effects ranged from 3.5 fold
suppression in second legs to 2.9 fold suppression in third legs (Table 13). However, $Np^{125A}$-$Sb^{70}$ interactions were not significantly suppressed by $ssh^{01207}$ in second or third legs. These data are consistent with a model where Stubble protein interacts with the Np gene product to promote $Rho1$- dependent actin-myosin contraction, but not F-actin polymerization, during the process of imaginal disc morphogenesis. Theses results are surprising in light of the fact that $Np^{125A}$ interacts with $sbd$ alleles in regards to bristle morphogenesis, a process that is F-actin polymerization dependent, but is not thought to require myosin activity.
Table 13. Third-site suppression analyses of $Np^{125A}$–$Sb^{70}$ interactions in leg development

<table>
<thead>
<tr>
<th>Myosin-contraction</th>
<th>2nd Leg</th>
<th>3rd Leg</th>
<th>Total Animals Scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Np^{125A} /+; Sb^{70}$/TB6B</td>
<td>22%</td>
<td>33%</td>
<td>234</td>
</tr>
<tr>
<td>$Np^{125A} /+; Sb^{70}/Mbs^3$</td>
<td>6%</td>
<td>11%</td>
<td>316</td>
</tr>
<tr>
<td>Fold Suppression</td>
<td>3.5</td>
<td>2.9</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>F-actin polymerization</th>
<th>2nd Leg</th>
<th>3rd Leg</th>
<th>Total Animals Scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Np^{125A} /+; Sb^{70}$/TB6b</td>
<td>23%</td>
<td>34%</td>
<td>204</td>
</tr>
<tr>
<td>$Np^{125A} /+; Sb^{70}/ssh01207$</td>
<td>22%</td>
<td>32%</td>
<td>166</td>
</tr>
<tr>
<td>Fold Suppression</td>
<td>1.0</td>
<td>1.1</td>
<td></td>
</tr>
</tbody>
</table>

Third-site suppression analyses indicate that $Np^{125A}$ is linked to Stubble function in stimulating actin-myosin contraction, but not F-actin polymerization during leg development. To test if the Np gene product is required along with Stubble to induce actin-myosin contraction, the rate of leg malformation seen in animals double heterozygous for $Np^{125A}$ and $Sb^{70}$ was compared to the rate of leg malformation seen in sibling animals that were triple heterozygotes for $Np^{125}, Sb^{70}$, and $Mbs^3$. To test if the Np gene product is required along with Stubble to induce F-actin-polymerization, the rate of leg malformation seen in animals double heterozygous for $Np^{125A}$ and $Sb^{70}$ was compared to the rate of leg malformation seen in sibling animals that were triple heterozygotes for $Np^{125}, Sb^{70}$, and $ssh01207$. Second and third legs were scored separately. The total number of animals scored is given in the last column on the right.
Mapping the Np locus

To fully understand the biological function of the Np locus it will be essential to identify and clone this gene. The first step in this process is generating a clear prediction of the position of the locus. By complementing \( Np^{125A} \) to chromosomal deficiencies it was possible to reduce the candidate \( Np^{125A} \) gene region to an interval spanning from 44F12 to 45A7 (Table 14). This interval is roughly 160 kb in length and contains 30 molecularly defined coding regions, 21 of which have not been investigated (Flybase, 2006). This region contains only a limited number (13) of mutations and transgene insertions. All of these were tested, and all complemented \( Np^{125A} \) (see Appendix A).
Table 14. Deficiency data indicate $Np^{125A}$ is located between 44F12 and 45A7

<table>
<thead>
<tr>
<th>Deficiency</th>
<th>Region uncovered</th>
<th>Results of cross to 125A</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Df(2R)Np1$</td>
<td>44F2--45C6</td>
<td>fails to complement</td>
</tr>
<tr>
<td>$Df(2R)Np3$</td>
<td>44D2--45C1</td>
<td>fails to complement</td>
</tr>
<tr>
<td>$Df(2R)Np4$</td>
<td>44F11--45C1</td>
<td>fails to complement</td>
</tr>
<tr>
<td>$Df(2R)Np5$</td>
<td>44F11--45E1</td>
<td>fails to complement</td>
</tr>
<tr>
<td>$Df(2R)H3E1$</td>
<td>44D1-4--44F12</td>
<td>complements</td>
</tr>
<tr>
<td>$Df(2R)G75$</td>
<td>44F4-5--44F9-11</td>
<td>complements</td>
</tr>
<tr>
<td>$Df(2R)G50-31$</td>
<td>45A6/7--45B8/C1</td>
<td>Previously shown to complement $Np$ alleles</td>
</tr>
</tbody>
</table>
In order to identify the precise molecular position of \(Np^{125A}\) meiotic recombination mapping using molecularly defined P-elements was conducted as described by Zhai et al. (2003). In this method, the rate of recombination between an unmapped lethal mutation (in this case \(Np^{125A}\)) and two molecularly defined P-elements (one to the left and one to the right of the lethal mutation) are used to generate a predicted molecular position (PMP) of the lethal mutation (Figure 10). Briefly, the rate of recombination between \(Np^{125A}\) and each molecularly defined P-element was determined. Next, using the molecular distance between the two P-elements and their rates of recombination with \(Np^{125A}\), the predicted molecular distance between each P-element and \(Np^{125A}\) was calculated. These data were then used to determine the PMP of \(Np^{125A}\) within the genome.
Recombination rates between molecularly defined P-elements (P1 and P2) and the mutation \( Np^{125A} \) were used to determine the predicted molecular position (PMP) of \( Np^{125A} \).

MD = molecular distance in base pairs; RD = recombination distance in cM.

Figure 10. Calculations used for predicting the molecular position of \( Np^{125A} \)

\[
PMD_B = \frac{MD_A}{RD_A + RD_B} \times RD_B
\]

\[
PMP(Np^{125A}) = P1 + PMD_B
\]
Two rounds of P-element recombination mapping were carried out to identify a predicted molecular position for \(Np^{125A}\). The first round was conducted using P-elements several hundred kb away from the \(Np\) candidate interval defined by deficiency mapping, 44F12-45A7, to obtain an approximate map position for \(Np^{125A}\) within the 44F12-45A7 region. The left flanking P-element was \(P\{EPgy2\}CG8712^{EY07021}\), which occurs at position 3,610,261 on the genome sequence and is 850 Kb away from the left-most position of cytological band 44F12. The right flanking P-element was \(P\{EPgy2\}CG1888^{EY02539}\), which occurs at position 5,058,638 and is 440 Kb away from the right-most position of cytological band 45A7. Recombination mapping using these P-elements will be referred to as rough mapping due to the distance from the candidate region. Results from these experiments are shown in Table 15. The rate of recombination between the left flanking P-element and \(Np^{125A}\) was 1.13159cM, while the rate of recombination between the right flanking P-element was 0.98289cM. These recombination rates were relatively similar even though the left flanking P-element was almost twice as far from the \(Np\) candidate region. Calculations based on the results from these rough mapping experiments gave a predicted molecular position of 4,385,379, which would place the \(Np\) locus at in the cytological band 44F2, approximately 79kb to the left of the region predicted to contain the \(Np\) gene by deficiency mapping.
Table 15. Rough mapping of \( Np^{125A} \) using molecularly defined P-elements

<table>
<thead>
<tr>
<th>P-element</th>
<th>Molecular Position</th>
<th>Animal scored Non recomb.</th>
<th>Recomb.</th>
<th>RD(cM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1: ( P{EPgy2}CG8712^{EY07021} )</td>
<td>44A4</td>
<td>3,610,261</td>
<td>5,479</td>
<td>62</td>
</tr>
<tr>
<td>P2: ( P{EPgy2}CG1888^{EY02539} )</td>
<td>45F1</td>
<td>5,058,638</td>
<td>5,494</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA(P1,.P2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMD(( Np^{125A} ))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P1 = left flanking P-element; P2 = right flanking P-element
Cyto= Cytological position; Non-Recomb. = Nonrecombinant animals scored; Recomb = Recombinant animals scored
Molecular position, MDA and PMD are given in base pairs
The preliminary round of rough mapping yielded two useful pieces of data: 1) the rough mapping confirmed that the position of \( Np^{125A} \) is close to the region defined by deficiency mapping; and 2) the rate of recombination in the areas surrounding the \( Np^{125A} \) candidate gene region are high enough that P-elements positioned very close to this interval can be expected to recombine at a rate that will be useful for fine scale mapping. In order to conduct a second round of recombination mapping molecularly defined P-elements positioned very close to the \( Np \) candidate region were selected. The left flanking P-element was \( P\{EPgy2\}Pgi^{EY09730} \), which occurs at position 4,425,835 and is 71 Kb away from the left most position of cytological band 44F12. The right flanking P-element was \( P\{XP\}CG30345^{d04911} \), which occurs at position 4,660,422 and is 40 kb away from the left most position of cytological band 45A7. The rate of recombination between the left flanking P-element and \( Np^{125A} \) was 0.03359 cM, while the rate of recombination between the right flanking P-element was 0.16743 cM (Table 14). Calculations based on the results from these mapping experiments gave a predicted molecular position of 4,465,036, placing \( Np \) within the candidate interval previously defined by deficiency mapping. This molecular position occurs in the gene \( baboon \), a previously described locus located in cytological band 44F12 that encodes a G-coupled protein receptor. Previous reports have determined that \( baboon \) (\( babo \)) and \( Np \) are inseparable by recombination, so it is not unanticipated that the predicted molecular position of \( Np \) would be close to the \( baboon \) locus. However, two important data indicate that \( Np^{125A} \) is not an allele of baboon: 1) \( Np^{125A} \) fully complements two lethal alleles of \( baboon \), and 2) while \( baboon \) fails to complement the deficiency \( Df(2R)H3E1 \), both \( Np^{125A} \) and \( Np^{2} \) fully complement this deficiency (MOHR and BOSWELL, 2002; ZHENG et al., 2003).
Table 16. Fine-scale mapping of \( Np^{1254} \) using molecularly defined P-elements

<table>
<thead>
<tr>
<th>P-element</th>
<th>Cyto</th>
<th>Molecular Position</th>
<th>Animal scored</th>
<th>RD(cM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1: ( P{EPgy2}Pg^{EY09730} )</td>
<td>44F6</td>
<td>4,425,835</td>
<td>8,931</td>
<td>3</td>
</tr>
<tr>
<td>P2: ( P{XP}CG30345^{404911} )</td>
<td>45A13</td>
<td>4,660,422</td>
<td>10,751</td>
<td>18</td>
</tr>
</tbody>
</table>

MDA(P1,P2) 234,587

PMD(\( Np^{1254} \)) 4,465,036

P1 = left flanking P-element; P2 = right flanking P-element
Cyto= Cytological position Non-Recomb. = Nonrecombinant animals scored; Recomb = Recombinant animals scored
Molecular position, MDA and PMD are given in base pairs
Zhai et al. (2003) found that P-element recombination mapping commonly yielded PMPs ranging from 50kb from their target gene to within the gene itself. Regions to the left of the \textit{bab}o locus can be ruled out of the \textit{Np} candidate gene region, as they are known to be uncovered by \textit{Df(2R)H3E1}. Because the precise limits of this deficiency are not known, the two genes found to the right of \textit{bab}o, \textit{CG8216} and \textit{CG8213} (fig 3), are still considered potential candidates for the \textit{Np} locus. This leaves us with a predicted gene region for \textit{Np} which would span the last 25kb of the 44F12 region and the first 25 kb of the adjacent 44A1 region. To be exhaustive, all genes within 100kb of the PMP were investigated, Table 17 lists the genes found within this interval.

Inspection of the genes in and around the \textit{Np} candidate region revealed the presence of a surprisingly high concentration of serine proteases (Figure 11). 16kb downstream of the \textit{Np}^{125A} PMP is \textit{CG8123}, a large (over 1600 amino acid long) TTSP. The next gene, \textit{CG11824}, is located 40kb downstream of the \textit{Np}^{1245} PMP and encodes a short S1 protease, which is followed 1.41kb downstream by \textit{CG8181}, which is also been labeled a protease \citep{Flybase, 2006}. Based on a visual inspection of the cDNA translations for these two genes, \textit{CG11824} encodes only a C-terminal protease domain and \textit{CG8181} encodes an N-terminal CLIP domain and a serine-threonine rich domain. For this reason I suggest that there has been an annotation error and \textit{CG8181} and \textit{CG11824} are not two separate loci, but rather one gene encoding single secreted protease. The next gene, \textit{CG8172}, is roughly 54 kb from the \textit{Np}^{125A} PMP and encodes a TTSP. The following gene, \textit{CG13744}, appears 61kb downstream of the \textit{Np}^{125A} PMP and encodes another TTSP. The following gene, 65 kb downstream from the \textit{Np}^{125A} PMP, \textit{CG1870}, encodes a serine protease. To put this information in a genomic context, the Ensembl data base lists 21 loci putatively encoding TTSPs in the \textit{Drosophila} genome and four of these are found within 70kb of the \textit{Np}^{125A} PMP, along with two additional serine protease encoding genes. The catalytic domains
of CG8213, CG11824, CG8172, CG13744, and CG8170 are readily alignable with each other and the Stubble protease domain (Appendix). It should be noted that based on protease domain alignments, \textit{CG11824} is thought to encode the most closely related protease to Stubble, while based on CLIP domain similarities, \textit{CG8213} appears to have the highest homology to Stubble (Ross \textit{et al.} 2003).
Table 17. Genes located near the predicted molecular position of $Np^{125A}$

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cyto</th>
<th>Molecular position</th>
<th>Distance from $Np^{125}$ PMP</th>
<th>Available Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>babo</td>
<td>44F12</td>
<td>4,464,156.</td>
<td>X</td>
<td>G-protein coupled receptor kinase; activin receptor activity, type I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4,473,449.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG8216</td>
<td>44F12</td>
<td>4,474,977.</td>
<td>4,473,449.</td>
<td>DNA binding; transposase activity</td>
</tr>
<tr>
<td>CG8213</td>
<td>44F12</td>
<td>4,481,149.</td>
<td>9,941</td>
<td>DNA binding; transposase activity</td>
</tr>
<tr>
<td>CG11824$^a$</td>
<td>45A1</td>
<td>4,505,355.</td>
<td>4,506,288</td>
<td>serine protease</td>
</tr>
<tr>
<td>CG8181$^a$</td>
<td>45A1</td>
<td>4,506,810.</td>
<td>4,509,673</td>
<td>serine protease</td>
</tr>
<tr>
<td>CG8172</td>
<td>45A1</td>
<td>4,519,384.</td>
<td>4,520,321</td>
<td>type II transmembrane serine protease</td>
</tr>
<tr>
<td>G13744</td>
<td>45A1</td>
<td>4,526,319.</td>
<td>4,528,492</td>
<td>type II transmembrane serine protease</td>
</tr>
<tr>
<td>CG13747</td>
<td>45A1</td>
<td>4,529,668.</td>
<td>4,530,687</td>
<td>NA</td>
</tr>
<tr>
<td>CG8170</td>
<td>45A1</td>
<td>4,530,838.</td>
<td>4,542,854</td>
<td>type II transmembrane serine protease</td>
</tr>
<tr>
<td>Ance-4</td>
<td>45A1</td>
<td>4,551,533.</td>
<td>4,553,955</td>
<td>Metalo-protease, transmembrane</td>
</tr>
<tr>
<td>CG8193</td>
<td>45A1</td>
<td>4,554,073.</td>
<td>4,556,521</td>
<td>monophenol monooxygenase transporter</td>
</tr>
<tr>
<td>CG13743</td>
<td>45A1</td>
<td>4,556,663.</td>
<td>4,571,719</td>
<td>amino acid-polyamine transporter</td>
</tr>
</tbody>
</table>

Molecular position and the distance of each gene from the $Np^{125A}$ projected molecular position are given in base pairs. Gene listed in blue fall in the 44F12 region. Genes listed in yellow fall occur within cytological band 45A1 and are within 55 kb of the $Np^{125A}$ PMP. Genes listed in green are located between 55 and 100 Kb from the $Np^{125A}$ PMP.

a) Personal inspection of these two sequences revealed they are probably one gene.
Figure 11. The $Np^{125A}$ candidate gene region

The genome region represented above starts on the left at the predicted molecular position of $Np^{125A}$, position 4,465,036 bp on the right arm of the second chromosome, and continues for 100kb. The $babo$ locus, in which the $Np^{125A}$ PMP occurs, has been ruled out as a candidate for the $Np$ locus based on complementation tests and deficiency mapping. The red bar indicates 50kb range from the $Np^{125A}$ PMP. Red stars indicate the position of genes encoding type II serine proteases.
CHAPTER FOUR: DISCUSSION

The goal of this project was to identify a direct interactor with the Stubble protease with the aim of improving our understanding of the mechanism coordinating hormonal and Rho1 signaling during leg imaginal disc epithelial morphogenesis. In pursuit of this goal, a forward genetic screen was conducted that identified six second-chromosome enhancers of \(sbd^{201}\) and seven new \(sbd\) alleles. Five of the second-chromosome enhancers of \(sbd^{201}\) were found to be alleles of previously characterized genes, and one (line 125A) was found to be a mutant allele of the \(\text{Notopleural}\) locus. \(\text{Notopleural}\), first identified by Calvin Bridges 70 years ago, remains relatively uncharacterized and has yet to be cloned.

Several of the alleles isolated in this screen potentially provide insight into the process of leg imaginal disc epithelial morphogenesis and open novel avenues for investigating how this process is executed. In the following section I briefly review the results of the screen with a special focus on the identity and function of the \(\text{Np}\) locus, and then discuss the future goals and broader impacts of this work.

**Enhancers of \(sbd^{201}\)**

A total of thirteen \(E(sbd)\) alleles were isolated from the genetic screen. Of these, seven were new alleles of the \(\text{Sb-sbd}\) locus identified on the basis of a reduced bristle phenotype in combination with \(sbd^{201}\). A previous screen for enhancers of \(br^{l}\) also resulted in a number of \(sbd\) alleles isolated (Ward et al. 2003), indicating that this is not an unexpected outcome for the current screen. Sequencing of the new \(sbd\) alleles isolated in both screens should prove fruitful
with respect to a structure-function analysis of the Stubble protease. To date, the only mutations characterized are within the catalytic domain, and lesions within the highly conserved CLIP domain have not been reported (Appel et al., 1993; Hammonds and Fristrom, 2006). In addition, the possibility of an important function for the cytoplasmic domain remains to be investigated.

The second chromosome $E(sbd)$ alleles included an allele of $Rho1$, two alleles of $zipper$, and individual alleles of $dachsous (ds)$, $dumpy (dp)$, $muscle-myosin heavy chain (Mhc)$ and $Notopleural (Np)$. The isolation of new $Rho1$ and $zipper$ alleles confirms that the screen successfully identified components of the $Sb-Rho1$ signaling pathway, and gives us confidence that some of the additional genes identified here, and not previously linked to $Sb-Rho1$ signaling, may in fact be components of this pathway.

The $Rho1$ allele identified in this screen, $Rho^{126A}$, showed stronger interactions with the $br^1$ allele than a deficiency, $Df(2R)Jp8$, that uncovers the $Rho1$ locus, suggesting that the $Rho^{126A}$ mutation is weakly antimorphic. However, the $Rho^{126A}$ allele showed markedly weaker interactions than the deficiency when combined with $sbd$ transheterozygotes ($sbd^d/sbd^{201}$) arguing against an antimorphic condition. While the underlying basis of these differences is not understood, these observations reinforce previous conclusions that $Rho1$ interacts with ecdysone inducible gene products required for leg morphogenesis (Bayer et al., 2003; Ward et al., 2003; Chen et al., 2004).

The $zip$ alleles isolated in this screen both enhance $sbd^{201}$, but in several important regards exhibit very distinct genetic interactions with previously identified mutants that affect imaginal disc morphogenesis. The $zip^{161A}$ allele is apparently recessive, and showed relatively low levels of interaction with $Sb^{63b}$, $Sb^{70}$, and $sbd^{201}$, but very strong dominant interactions with $br^1$. While, $zip^{161A}$ interacted at significant rates with $GEF2^{11-3}$ and $dRhok^2$ mutants, it interacted
in a highly allele-dependent manner with Rho1 mutants. \textit{zip}^{161A} showed low rates of malformation with \textit{Rho}^{J2.8} (11%), which carries a C-terminal nonsense mutation, and high rates with \textit{Rho}^{E3.10} (67%), which carries a missense mutation that is thought to disturb post-translational modifications of the Rho1 protein (HALSELL \textit{et al.} 2000).

In contrast to \textit{zip}^{161A}, \textit{zip}^{195A} appears to be an unusual viable, dominant mutation. Sequencing of the \textit{zip}^{Ebr} lesion indicates that it may also be a gain-of-function mutation, albeit a weak antimorph, however this has not been confirmed. In contrast the \textit{zip}^{195B} allele is a strong gain of function mutation that shows high levels of leg malformation as a heterozygote (31%). It will be interesting to determine the molecular basis of the \textit{zip}^{195A} mutation and what this implies about the structure and function of the \textit{zip} gene product.

Several novel interactors were identified in this screen, including alleles of \textit{dumpy}, \textit{ds}^{162}, \textit{Mhc}^{278A} and \textit{Np}^{125A}. The \textit{dp} allele was identified purely on the basis of a short leg with all segments thickened. This phenotype was observed only in combination with the \textit{Pm} chromosome used in the screen which also carries a \textit{dp}^{6} allele. The \textit{dp} allele isolated in this screen did not interact genetically with \textit{Sb-sbd}, \textit{broad}, or \textit{Rho1} pathway mutants, and thus there is no evidence to suggest that \textit{dp} plays a role in \textit{Sb-Rho1} signaling.

The genetic interactions between \textit{ds}^{162} and mutants in the \textit{Sb-sbd} locus will require further investigation and may represent an undescribed function for both the \textit{Sb-sbd} and \textit{ds} loci. The \textit{ds}^{162} allele showed significant interactions with \textit{Sb} alleles and in transheterozygous combinations with \textit{sbd} alleles, but not with components of the Rho1 signaling pathway. It is interesting to note that the \textit{Pm} marker chromosome found in our tester stock also carries a \textit{ds} allele, \textit{ds}^{33K}, and the \textit{Rho1} and \textit{zipper} mutations isolated in this screen, along with \textit{Np}^{125A}, exhibited stronger interactions with \textit{sbd}^{201} when balanced over the \textit{Pm} chromosome. The \textit{Pm}
chromosome is known to carry three mutations: one in the brown locus, \(bw^{VI}\), one in the dumpy locus, \(dp^{6}\), and \(ds^{33K}\). The \(bw^{VI}\) and \(dp^{6}\) mutations found on the \(Pm\) locus are also found on the balancer \(CyO\), which has been used extensively in the von Kalm lab and has never been observed to influence the rate of leg malformation. These data suggest that the \(ds^{33K}\) allele may be responsible for the third-site enhancement caused by the \(Pm\) chromosome. Mutations in the dachsous cadherin have been previously associated with a leg malformation phenotype (Clark et al. 1995) and the genetic interactions described here tend to support a role for the dachsous in leg morphogenesis. It should be noted however that third-site enhancement is not particularly strong evidence that two gene functions are linked in the same genetic pathway. One way to determine if the interaction between \(Sb-sbd\) and \(ds^{1622}\) is specific, and to see if both of these loci contribute to the same pathway, would be to test if \(ds^{1622}\) mutants can suppress leg malformations caused by a heat-shock induced, early prepupal over-expression of wild-type Stubble protein.

Another novel interactor identified in this screen, \(Mhc^{278A}\), was interesting because muscle myosin has never been described as having a role in imaginal disc morphogenesis. As a heterozygote \(Mhc^{278A}\) exhibited dominant thoracic and femur malformations that, based on their similarity to phenotypes exhibited by fully characterized \(Mhc\) alleles, can be assumed to result from muscle defects. Drosophila \(Mhc\) mutations often show dominant phenotypes as heterozygotes, primarily for two reasons. One reason is that mutant \(Mhc\) protein can form nonfunctional complexes and thereby have a dominant negative effect on muscle formation (Cripps et al. 1994). The second reason is that null \(Mhc\) alleles limit the formation of thick filaments, composed of myosin, which in turn leads to an imbalance in the number of thick and thin filaments (composed of actin), resulting in muscle collapse and developmental defects.
(BEALL et al. 1994). Interestingly, it has been shown that reducing the gene dosage of tissue and stage specific actin genes can rescue dominant phenotypes associated with null Mhc alleles.

Preliminary investigations to determine if the presence of Mhc$^{278A}$ can suppress leg malformation caused by over-expression of wild-type Stubble protein, found that Mhc$^{278A}$ enhanced, rather than suppressed the rate of leg malformation. These experiments were conducted on a small scale, with less than 50 animals in each class and were not reported in the results section of this manuscript. It is worth noting that enhancement of the Stubble over-expression phenotype was strong - out of 45 Mhc$^{278A}$/hs-Stubble animals exposed to a one hour heat treatment at 37°C as early prepupa (3hr AP), only one animal with six wild type legs was observed. Overall, these data are open to interpretation and do not strongly support a role for Mhc in Sb-Rho1 signaling. For example, the genetic interactions observed could be indirect and conceivably stem from a partial failure to make proper myo-epidermal attachments during leg morphogenesis coupled with reduced strength of signaling through the Rho1 pathway. The current data do however indicate that the Mhc$^{278A}$ allele does not disrupt signaling or functions downstream of Stubble proteolytic activity.

The Notopleural locus

The Np$^{125A}$ allele is the most interesting mutant isolated in this work because it is linked to Stubble function in both bristle and leg development. In regards to leg development, Np$^{125A}$ showed moderate rates of leg malformation in doubly heterozygous combinations with Sb-sbd mutants, as did another EMS mutant, Np$^{2}$. Several deficiencies that uncover the Np locus showed similar or stronger interactions with mutations in the Sb-sbd locus. The hypothesis that Stubble and Np function are linked in leg imaginal disc morphogenesis is supported by the observation
that the presence of one copy of $Np^{125A}$ reduces by two-fold the rate of leg malformation in animals exposed to early prepupal (3hr AP) over-expression of wild-type Stubble protein. These data are consistent with a model that places Np activity at or below the level of Stubble signaling.

In regards to bristle development, the $Np^{125A}$ allele exhibits a weakly penetrant short bristle phenotype that is expressed at low, but variable rates as a heterozygote. The mild, dominant $Np^{125A}$ bristle phenotype is enhanced by the presence of $sbd$ alleles. Interestingly the $Np^{125A}$, $sbd^1$ bristle phenotype appeared to be enhanced by a loss of function $Rho1$ allele. Subsequent experiments conducted with $Np^2$ showed that this was not an allele specific interaction. To our knowledge, this is the first observation of $Rho1$ activity having any role in the bristle morphogenesis, and may provide a novel avenue of investigating the link between Stubble and $Rho1$ signaling. However, as this interaction is the result of a third site enhancement, further investigations of the role of $Rho1$ signaling in bristle morphogenesis should be conducted with caution. Subsequent studies will need to be fully quantitative in that each scutellar bristle must be measured and statistical analyses will be needed to demonstrate that there is a significant difference between sibling classes of different genotypes.

Precisely how the $Np$ gene product functions in regards to leg imaginal disc morphogenesis remains to be elucidated. The presence of an $Np^{125A}$ allele was found to suppress the rate leg malformation induced by Stubble over-expression by roughly two-fold in both second and third legs. The simplest interpretation of these data is that Np activity is required downstream of Stubble proteolysis. Suppression analyses using $Mbs$ and $ssh$ mutants found that the $Mbs$ loss of function mutant, $Mbs^3$, significantly suppressed the rate of leg malformation (3.5 fold in second legs and 2.9 fold in third legs) in animals doubly heterozygous for $Np^{125A}$ and
$Sb^{70}$. Similar experiments conducted with the $ssh$ loss of function allele $ssh^{01207}$ showed no significant third-site suppression. These data suggest that $Np$ activity is required downstream of Stubble proteolysis in a manner that contributes to myosin-dependent changes in cell shape.

Clearly, cloning the $Np$ locus and identifying the lesions responsible for the phenotypes exhibited by $Np^{125A}$ and $Np^2$ is of critical interest and will provide important insight into the biological function of this locus and its role in epithelial morphogenesis. Recombination mapping using molecularly defined P-elements yielded a predicted molecular position of 4,465,036, which occurs within the baboon ($babo$) locus. As lethal mutants in the babo locus have previously been shown to complement $Np^{125A}$, all genes within 50 kb of the PMP are considered candidate $Np$ loci. Genes within 50 kb of the PMP but located to the left of babo can be ruled out because they, like babo, are uncovered by a deficiency which complements $Np^{125A}$. The candidate region therefore consists of the 50 kb to the right of the $Np^{125A}$ PMP. This region contains one gene that encodes a product containing motifs associated with DNA binding and transposase activity, and two proteases, one of which is a TTSP and both of which contain CLIP domains, as does Stubble. After extending our survey of genes to include the 100 kb to the right of the $Np^{125A}$ PMP it was found that this region contains three additional TTSPs and a matrix metalloprotease. $CG8213$ is the protease-encoding gene located closest to the $Np^{125A}$ PMP. Based on amino acid alignments of all S1 (trypsin-like) proteases in the *Drosophila* genome, the CG8213 CLIP domain is the most similar to Stubble’s CLIP domain, and the CG8213 protease domain is the second most similar to Stubble’s protease domain (ROSS et al. 2003). $CG11824$ is located adjacent to $CG8213$, and encodes the serine protease whose domain shows the highest similarity in the *Drosophila* genome to Stubble’s (67% similarity; ROSS et al. 2003). Based on CLIP and protease domains, the next most similar protease to Stubble is encoded by $CG8172$,
which occurs 55kb to the right of the \( N_p^{125A} \) PMP, adjacent to \( CG11824 \). Phylogenetic analyses conducted by Ross et al. (2003) analyzing the evolutionary relationship of S1 serine proteases found in the \( Drosophila \) genome suggest that the \( CG8213, \ CG11824, \ CG8172 \) and \( Sb-sbd \) loci form a monophyletic group of \( Drosophila \) TTSP paralogs. Paralogs result from gene duplication. Selection will typically remove duplicated genes unless they provide a selective advantage. One way that duplicated genes provide a selective advantage is if the parent gene’s function becomes divided between parent gene and the gene copy, a process referred to as subfunctionalization (Aquileta, et al. 2004). It is tempting to speculate that all the gene products of the \( CG8213, \ CG11824, \ CG8172 \) and \( Sb-sbd \) loci may act together in a proteolytic cascade. This speculation may be supported by the observation that Mohr and Boswell (2002) found that the 31 \( N_p \) alleles they identified in a screen for lethal alleles in the 44D-45F region fell into eight overlapping complementation groups. Mohr and Boswell did not go on to explain this observation, but it would appear to suggest that what they refer to as the \( N_p \) locus is either one gene that encodes several distinct but overlapping genetic functions, or it is a gene complex. One possible explanation of these data is that the \( N_p \) locus is actually a complex of genes, many or all of which encode serine proteases.

Little is know regarding the biology of loci found in the \( N_p \) candidate gene region, though data from yeast two-hybrid data is available for three genes within this interval. The gene located closest to the \( N_p^{125A} \) PMP, does not encode a protease but rather has motifs which suggest it is a component of the nucleus (Flybase, 2006). Yeast two-hybrid assays indicate that \( CG8216 \) interacts with DNA binding, RNA binding and chromatin binding proteins, as well as dystrophin, an actin binding protein required for muscle attachment (Goit et al. 2003). Yeast-two hybrid interactions have also been described for \( CG11824 \), which lists 24 identified interactors.
As stated above, it is the author’s opinion that CG11824 and CG8181 are actually one gene that encodes a secreted serine protease containing a clip domain. The exons labeled as CG11824 encode a 250 amino acid-long protease, consisting of a C-terminal protease domain and three amino acid residues N-terminal to the cleavage site required for zymogen activation. Interactors with CG11824 include several regulators of the cell cycle (cyclin A, Cyclin G. Cdk4, Cdk5, decapo, skpB and skpC), six serine-threonine kinases (Akt, grapes, minibrain, PAK, pan gu. and SAK) a DNA helicase (RecQ4), a transcription coactivator (nejire), a transcription repressor associated with imaginal disc development (E2f2) and RhoGAP (RhoGAP68F) (Stanyon et al. 2004). If the CG11824 gene product is a secreted protease, it would be spatially separated from all of the interactors listed above and therefore not physically interact with them in a biological context.

The remaining *Np* candidate gene with published yeast two hybrid interactors is *CG8170*, which encodes a TTSP. Yeast two hybrid assays identified two CG8170 interactors, one, CG1707, encodes an undescribed protein, and the other *tincar*, encodes a novel transmembrane protein that is required for the development of cardioblasts and ommatidia (HIROTA et al. 2002; HIROTA et al. 2005; Stanyon et al. 2004). The *tincar* gene itself appears to interact in yeast two hybrid assays with 17 gene products, including a rho-type guanine exchange factor, rtGEF, and the Chickadee gene product, profilin (Goit et al. 2003). These data suggest that the *tincar* locus may be worth investigating in future experiments.

The data indicating that Np acts downstream of Stubble are, in principle, consistent with Stubble and Np acting in a membrane associated proteolytic cascade that triggers Rho1 signaling. However, the observation that Np acts through the actin-myosin dependent branch of the Rho1 pathway, and not through the LIM kinase branch leading to SRF activation, is more
difficult to explain in the context of a proteolytic cascade on the cell surface. Third-site suppression analyses indicate Stubble functions at or above the level of RHOK. If the Np gene product were to function in direct conjunction with Stubble, or in any other way upstream of RHOK, then ssh^{01207} would be expected to suppress leg malformations in Np^{125A}, Sb^{70} double heterozygotes, which it does not. Perhaps signaling through each branch of the Rho pathway is independently initiated at the cell membrane, with Np involved in activation of the actin-myosin pathway and Stubble playing a role in the activation of both branches of the pathway. It is also possible that these differences in apparent signaling functions are due to closely linked, but distinct temporal requirements. Studies investigating the process of wound healing (which requires apical actin-myosin contraction) in epithelial monolayers composed of human colonic biopsy specimens used live cell imaging to distinguish between RHOK dependent assembly of an apical actin ring, and subsequent MLCK-dependent actin-myosin contraction (RUSSO et al. 2005). If these two processes are similarly separable in leg imaginal discs, then Stubble activity may be required for stimulating both apical actin polymerization and subsequent actin myosin contraction, while Np activity is required only for inducing actin-myosin contraction, perhaps by functioning to amplify or maintain Stubble signaling. Alternatively, Np may act to promote actin-myosin contraction downstream of RHOK activity, and in parallel to Stubble activity.

The observation that Np and sbd alleles interact in regards to bristle malformation suggests that Np and Stubble activity are closely linked, and is therefore also consistent with the possibility that both are part of a protease cascade. The data indicating that Np does not stimulate the LIMK-dependent actin polymerization pathway, however, is hard to reconcile with this observation, because bristle morphogenesis is an actin polymerization dependent process. It is possible that Np signaling acts to stimulate distinct pathways during leg and bristle development.
Another possibility is that some components of the myosin-dependent pathway have roles in actin regulation that have not been previously characterized. For example, siRNA inhibition of the myosin phosphatase targeting subunit 1 (MYPT1; a human homolog of *Drosophila Mbs*) in HeLa cells, found that reducing the expression of MYPT1 lead to an increase in F-actin concentration in a myosin independent manner (Xia *et al.* 2005). There is no record of *Drosophila* Mbs having a similar effect on actin polymerization, or of this possibility having been investigated. It may, therefore, be useful to test both ssh and Mbs mutants as potential third site suppressors of the *Np*<sup>125A</sup>:*sbd*<sup>l</sup> induced bristle phenotype.

**Future Aims**

This work has the potential to initiate several interesting lines of investigation. One important set of goals will be to sequence the *Rho1* and *zip* mutants identified in this screen. These mutants are the first *Rho1* pathway alleles to show significant interactions with a recessive *sbd* allele, and the identification of the molecular lesions responsible for this quality may present important data regarding how Rho1 and zip activity is linked to Stubble proteolysis. This work may be augmented by also sequencing alleles isolated in a screen for enhancers of *br*<sup>l</sup> conducted by Ward *et al.* (2003), which also identified a *Rho1* allele and several new *sbd* alleles. These new *E(br)* alleles can be sequenced alongside the *E(sbd)* *Rho1* and *sbd* alleles with the aim of potentially linking the mutations found in these alleles to the genetic interactions they exhibit.

Clearly, it is of critical interest to identify and clone the *Np* locus. The *Np*<sup>125A</sup> allele has been mapped using molecularly defined P-element insertions, and work is currently underway to similarly characterize the mutant *Np*<sup>2</sup>. Data generated from these experiments should yield useful insights into the molecular position of the *Np* locus, though, ultimately, the sequence of
molecular lesions that cause these mutations must be identified. Towards this goal, stocks carrying P-element insertions and small deletions in the $Np$ candidate gene region have been ordered from international stock centers. If none of these fail to complement $Np^{125A}$, one strategy is to generate novel deletions using compound transposable elements, as described by Huet et al. (2002). This method uses P-elements containing unpaired insertion sequences from hobo transposable elements. By crossing these hybrid elements to lines containing a heat-shock inducible hobo transposase, the unpaired Hobo sequence can be induced to translocate causing a deletion either to the right or the left of the hybrid element. The presence of selectable mutations located within the P-element, to the left and right of the hobo sequence, make it possible to visually identify animals that carry deletions. Because these markers are placed on either side of the hobo sequence, the absence of a phenotype indicates not only that a deletion occurred, but also the direction in which the deletion occurred relative to the hybrid element. After animals carrying deletions are generated, they can be tested for complementation against $Np$ alleles. The length of deletions can be determined using inverse PCR, and by comparing the lengths of the novel deletions that uncover the $Np$ locus to those that do not, the position of the $Np$ locus can be established.

The final step in demonstrating that a particular mutation causes a mutant phenotype is to rescue the phenotype. This is commonly accomplished using a transgenic source of wild-type protein. Since the number of genes in the $Np$ candidate region is fairly small (three, if only genes within 50kb of the $Np^{125A}$ PMP are included, six, if the next three genes, all of which encode TTSPs are also included) it may be worthwhile to consider using rescue experiments to identify the position of the $Np$ locus. The logic behind this strategy is that making transgenic animals takes several months, and while not a trivial feat, it requires considerably fewer resources and
less labor than the deletion generator strategy outlined above. Additionally, rescue experiments will be required as proof that a lesion in a particular coding region is in fact the source of \(Np\) mutant phenotypes, including embryonic lethality. Thus it will be eventually necessary to generate a heat-shock inducible \(Np\)-cDNA construct and to then rescue the \(Np\) embryonic lethal phenotype using this construct. As cDNA libraries and vectors carrying heat-shock promoters are publicly available, making these clones should be easily accomplished. Generating transgenic animals is a more difficult task, but feasible, given the technical capabilities in the von Kalm lab. After transgenic lines carrying candidate heat-shock \(Np\)-cDNA transgenes have been generated, rescuing animals through embryonic development could be attempted for each in a systematic manner. If this strategy were to work, then the transgenic line that rescued \(Np^{125A}\) homozygotes through to larval life could be used to identify the \(Np\) locus and the mutant \(Np^{125A}\) allele could be sequenced with its relationship to the \(Np\) phenotypes already experimentally verified. The primary concern regarding this strategy is that the \(Np\) phenotype may be difficult to rescue, making this process arduous or impossible. A secondary concern is that several of the closely associated protease genes in the \(Np\) candidate gene region may act together, and that multiple heat-shock cDNA constructs will be capable of rescuing \(Np\) homozygotes through embryonic development equally well. If this is the case, significant data regarding the function of these loci would be garnered, but not much about the molecular position of the \(Np\) gene.

Considering the possible \(Np\) candidates, we are still left with the question of how extracellular proteolysis stimulates intracellular Rho1 signaling. Several genetic screens have been carried out to identify enhancers of leg malformation prior to this work, and though all of these screens identified important loci involved in regulating cell shape change and imaginal disc morphogenesis, none have found an apparent membrane-associated component that provides a
physical link to Rho1 activity (GOTWALS and FRISTROM, 1991; HALSELL et al. 1998; WARD et al. 2003). Mutagenesis is a random event, so it is possible that with further screening a Rho1 activator required for imaginal disc development will be found. Though none of the screens for enhancers of leg malformation have approached saturation, multiple mutations in the Rho1, zipper and Sb-sbd loci have been identified, demonstrating that these investigations have been thorough enough to mutate key regulators of this process multiple times. Provided our current model is correct and there is a target of Stubble proteolysis that acts to stimulate Rho1 signaling, it is worth considering reasons why this target might not be easily identified in enhancer screens. One hypothesis it that Stubble proteolysis may act to degrade a negative regulator of Rho1 signaling. If this is the case then the link between Stubble and Rho1 signaling would not be identifiable using genetic screens for enhancers, as mutating a negative regulator of Rho1 would act to suppress, not enhance, mutations in genes that activate Rho1. Third-site suppression analyses could be used to address this possibility. Experiments conducted using third-site suppression analyses in this work, and for associated projects in von Kalm lab, have shown that negative regulators of Rho1 signaling (e.g. ssh and Mbs) consistently suppress the rate of leg malformation resulting from SSNC interactions between Sb and Rho1 mutants. This suggests that a third-site suppressor strategy could readily be expanded to a large scale screen of either EMS induced mutant or chromosomal deletions. In addition to being a novel means of investigating the relationship between Stubble and Rho1 activity, the benefits of such work may include the characterization of negative regulators of Rho1 signaling and possibly agents that act to regulate them. The findings from a third-site suppression screen could be of particular interest, as mechanisms that control negative regulators of Rho signaling are not well understood.
**Broader impacts**

The results of the work presented here should benefit the understanding of how global signals induce local effectors that coordinate the intracellular activities required for epithelial morphogenesis. Novel alleles of known effectors of epithelial morphogenesis, including $Rho1$, $zip$ and $Sb-sbd$, were identified in this screen and can be sequenced to further our understanding of the relationship between their structure and function. Alleles of well characterized genes, $Mhc$ and $ds$, which were not previously known to interact with mutants in the $Sb-sbd$ locus were also identified. The $Mhc$ and $ds$ mutant interactions suggest that there are factors outside the current model of leg epithelial morphogenesis that rely on Stubble activity, and present the possibility to place the Stubble-$Rho1$ pathway within a greater developmental context. Perhaps the most interesting allele identified in this screen is $Np^{125A}$, which was found to enhance both leg and bristle phenotypes associated with mutants in the $Sb-sbd$ locus. Current data indicate that the $Np$ locus is closely associated with Stubble protease activity, and that the $Np$ locus itself may encode a protease similar to Stubble. If this is the case $Np^{125A}$ will be a useful tool for further investigating how hormonally regulated proteolytic activity functions to influence the development of epithelia.

Type II proteases appear to be important regulators of development and homeostasis, as they are associated with a seemingly disproportionately large number of human pathologies (HOOPER et al. 2001). Understanding Stubble function presents the possibility of generating a coherent model of how hormones, TTSPs and intracellular regulators like $Rho1$ interact *in vivo*, which may be applied to understanding similar interactions in other systems. Such an understanding would be a benefit in terms of disease, as mis-regulation of extracellular protease activity and $Rho1$ signaling are both associated with metastatic cancer progression (FRITZ and
KAINA, 2006; NETZEL-ARNETT et al. 2003). Imaginal discs are composed of cells in tight association, like those composing any epithelium, which then undergo a brief, concerted wave of contraction. Epithelial cancers undergo a similar process as they conduct collective migration, one of the first steps towards invasion and metastasis. These changes in cell shape are absolutely dependent on changes in the actin cytoskeleton and Rho-regulated actin–myosin contraction (YAMAZAKI et al. 2005). Cell migration is also highly associated with the activity of extracellular proteases, which are currently thought to degrade the extracellular matrix to create space for migrating tumor cells and possibly influence signaling into and around tumor cells (AZNAR et al. 2004). Numerous examples exist of concerted changes in the actin cytoskeleton and extracellular protease activity. For example, the extracellular protease thrombin has been shown to rapidly induce the contraction of lung alveolae in a manner that is F-actin and RhoA dependent. As alveolae are composed of single cell thick invaginations, these data suggest there may exist a basic mechanism for coordinating extracellular proteolysis and Rho-dependent actin–myosin constriction that is conserved and utilized in metazoan development. One of the clear benefits of studying such mechanisms in Drosophila is the potential to identify both the effectors that carry out cell shape changes, and the signaling components that determine how and where the changes occur during epithelial morphogenesis. Hopefully, some of the results of this work will contribute to the understanding of these processes.
APPENDIX A: GENES IN THE NOTOPLEURAL REGION
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APPENDIX B: AN AMINO ACID ALIGNMENT OF SERINE PROTEASES IN THE Np CANDIDATE GENE REGION AS DEFINED BY MOLECULARLY DEFINED P-ELEMENT MEDIATED RECOMBINATION MAPPING
LIST OF REFERENCES


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WATANABE, N., P. MADAULE, T. REID, T. ISHIZAKI, G. WATANABE et al., 1997 p140mDia, a mammalian homolog of *Drosophila* diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *EMBO J.* **16**: 3044–3056.


