Grainy head target genes in epithelial morphogenesis and wound healing

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The cover shows the increase of Stitcher protein levels (in magenta) and phosphotyrosine concentration (green) in cells around the wound of a *Drosophila* embryo.
To my beloved family…
Abstract

grainy head (grh) genes encode a family of transcription factors conserved from fly to human. Drosophila grh is the founding member of this gene family and has multiple functions, including tracheal tube size control, epidermal barrier formation and reconstruction after wounding. To understand the underlying molecular mechanism of grh functions, we tried to isolate its direct targets and analyze their function. We identified ten grh targets by combining bioinformatics and genetics. Grh directly controls the expression of stitcher (stit), which encodes a Ret family receptor tyrosine kinase (RTK), during both development and wound healing. Stit promotes actin cable assembly and induces extracellular signal-regulated kinase (ERK) phosphorylation around the wound edges upon injury. Stit also activates barrier repair genes and its own expression at the wound sites in a Grh-dependent manner. This positive feedback loop ensures efficient epidermal wound repair. In addition, Grh regulates the expression of multiple genes involved in chitin biosynthesis or modification. Most of the genes are required for tracheal tube size control. Two of them, vermiform (verm) and serpentine (serp), encode related putative luminal chitin deacetylases. The functional analysis of verm and serp identifies an important role of luminal chitin matrix modification in limiting tracheal tube elongation. Therefore, it is likely that Grh controls tracheal tube size through regulating multiple targets involved in the assembly or modification of luminal chitin matrix. Grh also directly activates the epidermal expression of Peptidoglycan Recognition Protein LC (PGRP-LC) gene that is required for the induction of antimicrobial peptides (AMPs) upon infection. Furthermore, ectopical expression of Grh is sufficient to induce the AMP Cecropin A lacZ reporter (CecA-LacZ) in the embryonic epidermis. These results suggest a new function of Grh in the local immune responses in Drosophila barrier epithelia.
List of papers

I  Grainy head regulates genes involved in cuticle maturation and immune response in Drosophila. (Manuscript)
   Wang S, Dai Q, Lai E, Samakovlis C.

II Septate-junction-dependent luminal deposition of chitin deacetylases restricts tube elongation in the Drosophila trachea.

III The tyrosine kinase Stitcher activates Grainy head and epidermal wound healing in Drosophila.
   Wang S, Tsarouhas V, Xylourgidis N, Sabri N, Tiklová K, Nautiyal N,
### Commonly used abbreviations

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<td>AMPs</td>
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# Table of contents

## Introduction 10

### The grh gene family 10

### The expression and biological function of grhl genes 11

### Grh in Drosophila 12

#### I. Drosophila as an experimental system 12

#### II. Biological function of Grh in Drosophila 13

##### a. Epidermal barrier maturation and repair after wounding in Drosophila 14

*Formation of the protective barrier* 15

*Wound healing in Drosophila* 16

1) *The purse-string mechanism of wound closure* 18

2) *Dramatic cell shape changes in Drosophila embryonic wound healing* 20

3) *Wound inflammation in Drosophila embryos* 21

4) *Signaling pathways in Drosophila wound healing* 21

*Grh in the maturation and healing of the epidermal barrier in Drosophila* 23

##### b. Tracheal development 25

*Control of tracheal tube size* 26

*grh in tracheal tube size control* 31

##### c. Other Grh functions 32

#### III. Molecular mechanisms of Grh function 33

1) *Upstream regulation by signaling* 33

2) *The partners of Grh* 34

#### IV. Grh downstream genes 35

#### V. Grh binding sites 37

### Aims of this thesis 39

### Results and Discussion 40

### Identification of potential Grh target genes (Paper I) 40

1) *Bioinformatics screening for genes containing Grh binding sites* 40

2) *Previously identified mutations with similar phenotypes to grh* 41

3) *Search for tracheal genes causing tracheal tube overgrowth phenotype* 41
4) Grh controls tracheal tube size through multiple direct targets involved in luminal chitin assembly and modification

Luminal matrix modifications in tube size control (Paper II)
1) Verm and serp are required for tube length restriction
2) verm and serp mutants affect the assembly and structure of the luminal chitin matrix
3) The apical secretion of Verm is dependent on SJs
4) The deposition and modification of the luminal chitin matrix play a central role in tracheal tube size control

A two-tier mechanism ensures efficient epidermal wound repair (Paper III)
1) Stit is a direct target gene of Grh both during development and wound healing
2) Stit is not required for embryonic epidermal and tracheal development but is essential for metamorphosis
3) Stit coordinates the formation of the actin cable during wound re-epithelialization
4) stit encodes a putative RTK that can activate ERK
5) Stit amplifies the wound response through a Grh mediated positive feedback loop
6) How is Stit activated upon wounding?

Conclusions and Perspectives
Acknowledgements
References
Introduction

The *grh* gene family

*grh* genes are conserved in metazoans. They encode a family of transcription factors with a unique, unusually large DNA-binding and dimerization domain, and an isoleucine-rich activation domain (Attardi and Tjian, 1993; Gustavsson et al., 2008; Moussian and Uv, 2005; Ting et al., 2003b; Uv et al., 1994; Venkatesan et al., 2003; Wilanowski et al., 2002). Grh factors were first identified in *Drosophila* (Bray et al., 1989; Bray and Kafatos, 1991; Dynlacht et al., 1989; Johnson et al., 1989) and since then, they were also found in animals as diverse as nematodes and humans. Grh proteins have not yet been detected in unicellular organisms. Phylogenetic analysis subdivides this gene family into two main classes, the Grh-like sub-family and the CP2 sub-family, depending whether the family members are more related to the *Drosophila grh*, or to another *Drosophila* gene, *dCP2* (Ting et al., 2003b; Venkatesan et al., 2003; Wilanowski et al., 2002). The fly and worm genomes each contain a single *grh* gene. Mammals, both mice and humans, have evolved three Grh homologues: Grh-like-1 (*Grhl-1*, or Mammalian Grainyhead (MGR)/TFCP2L2), Grhl-2 (Brother-of-MGR (BOM)/TFCP2L3) and Grhl-3 (Sister-of-MGR (SOM)/TFCP2L4). This group of genes encodes proteins with highly homologous DNA-binding and dimerization domains. They show restricted expression pattern during embryogenesis and play important roles in organogenesis and epidermal development and regeneration after wounding (Almeida and Bray, 2005; Bray and Kafatos, 1991; Hemphala et al., 2003; Lee and Adler, 2004; Mace et al., 2005; Moussian and Uv, 2005; Ting et al., 2005a; Ting et al., 2003b).

The second group of genes in the *grh* family encodes proteins with higher similarity to *dCP2*. It includes *CP2*, *LBP-1a* and *LBP-9* in mammals (Wilanowski et al., 2002). In contrast to the Grh-like subfamily, the CP2-group genes are widely expressed and involved in diverse cellular processes, such as cell cycle control, growth and differentiation (Jane et al., 1995; Sueyoshi et al., 1995; Volker et al., 1997; Zhou et al., 2000). An additional distinction between the groups is the ability of the Grh-like factors...
Grhl-1, Grhl-2 and Grh to interact with each other, but not with members of CP2-like group (Uv et al., 1994; Wilanowski et al., 2002).

Figure 1. Schematic illustration shows the protein domains organization of *Drosophila* Grh. Red, green and blue boxes indicate activation domain, DNA-binding motif and dimerization domain, respectively. The numbers show the protein length in amino acids (aa). This protein domain organization is shared by Grh proteins.

**The expression and biological function of *grhl* genes**

The mouse *grh* like (*grhl1*-3) genes are expressed in the surface ectoderm as well as other epithelial tissues, including the oral cavity, urogenital bladder and gastrointestinal tract (Auden et al., 2006). They each exhibit differential spatio-temporal expression patterns during development. For example, *grhl*-1 expression in the surface ectoderm is dramatically increased from E10.5 to E17.5 while *grhl*-2 and -3 expression is progressively decreased during the same period. In addition, *grhl*-2 is widely expressed throughout the surface ectoderm at E8.5, whereas *grhl*-3 is only expressed in a small population of non-neural ectoderm cells during this period. The non-overlapping expression patterns of *grhl* genes are also detected in non-ectodermal tissues, such as the heart, the lung and the kidney. The different spatial and temporal expression patterns suggest that the *grhl* genes have non-redundant roles despite their extensive sequence identity (Auden et al., 2006).

Gene-targeting in mice demonstrated that *grhl*-1 and *grhl*-3 have related but remarkably different functions during mouse embryogenesis. Mice lacking *grhl*-1 exhibit abnormal hair coat, defective hair anchoring and palmoplantar keratoderma (Wilanowski et al., 2008). In contrast, *grhl*-3 null mice display severe defects in the epidermal barrier function, which is associated with impaired differentiation of the epidermis (Ting et al., 2005a; Ting et al., 2005b; Yu et al., 2008; Yu et al., 2006; Yu et al., 2009). *grhl*-3 is also
required for efficient wound healing, neural tube closure and eyelid closure (Gustavsson et al., 2008; Hislop et al., 2008; Ting et al., 2005a; Ting et al., 2003a; Yu et al., 2008). The analysis of grhl-2 null mice has not been reported so far. However, a mutation in the human grhl-2 gene has been associated with age-related hearing loss (Peters et al., 2002; Van Laer et al., 2008). Human grhl-2 is also up regulated in human oral squamous cell carcinoma cells and is likely to play a crucial role in telomerase activation during cellular immortalization (Kang et al., 2009).

Although the different mammalian grhl genes play diverse roles, the function of grhl-3 in epithelial barrier formation and wound healing is highly conserved during evolution. grh mutants in flies also show multiple cuticle defects and are compromised in epidermal barrier repair (Bray and Kafatos, 1991; Mace et al., 2005). Similarly, knockdown of the nematode (Caenorhabditis elegans) grh function by RNA interference causes fragile cuticles (Venkatesan et al., 2003). Disruption of grhl-1 activity in the clawed frog (Xenopus laevis) also results in a severe defect in terminal epidermal differentiation (Tao et al., 2005). Thus Grhl transcription factors emerge as phylogenetically conserved regulators of epidermal barrier formation and repair.

**Grh in Drosophila**

**I. Drosophila as an experimental system**

Drosophila research relies on an extensive collection of molecular methods applicable to most animals. In addition, a large number of specialized experimental techniques have been developed to allow the precise dissection of gene function in vivo. Large-scale screens using transposon- or chemical-induced mutagenesis can be used to readily isolate mutant strains. The functional study of genes involved in early embryogenesis during adult life is also readily accessible by the generation of mosaic animals carrying patches of mutant tissue in a wild type background (Xu and Rubin, 1993). Stably integrated transgenes can be temporally or spatially controlled by binary systems like the UAS/GAL4 system. In this strategy a transgenic strain carrying any cDNA under the control of the GAL4-responsive UAS elements (upstream activating sequence) can be crossed to strains expressing the yeast transactivator GAL4 in different
tissues and at different stages. Thereby the expression of any gene of interest can be activated and studied in different tissues at distinct developmental stages (Brand and Perrimon, 1993). Additionally, *Drosophila* embryos are ideally suited for live imaging analysis of many developmental events at high cellular resolution because they are relatively transparent and develop rapidly. The *Drosophila* life cycle includes an embryonic stage (0-24 hours after egg laying (AEL), three different larval (or instars) stages: the first instar (24-48 hours AEL), the second instar (48-72 hours AEL) and the third instar (72-120 hours AEL) followed by the pupa (120-220 hours AEL), and adult flies.

II. Biological function of grh in *Drosophila*

The *Drosophila melanogaster* genome contains a single *grh* gene, which provides a simple and tractable model system for understanding some of the functions of the *grhl* gene family at the molecular level. *Drosophila grh* (also known as NTF-1 or Elf-1) is the founding member of *grh* gene family. This factor was originally identified through its ability to bind a cis-element of the *Dopa decarboxlase* (*Ddc*) gene and to regulate its late epidermal expression (Bray and Kafatos, 1991; Johnson et al., 1989). The name *grh* reflects the phenotypes of mutants, which develop flimsy cuticles, and a grainy and discontinuous head skeleton (Bray and Kafatos, 1991; Nusslein-Volhard, 1984). *grh* is predominantly expressed in ectodermally derived tissues, such as embryonic epidermis, trachea, foregut and hindgut (Bray and Kafatos, 1991; Hemphala et al., 2003). Grh is also present in these tissues during larval development (Uv et al., 1997). In addition, Grh is detected in the embryonic central nervous system (CNS), larval neuroblasts and optic lobes, as well as in imaginal discs (Uv et al., 1997). Interestingly, alternative splicing is responsible for generating the neuroblast-specific isoform of the Grh protein (Uv et al., 1997).

Grh function has been implicated in many aspects of *Drosophila* development, including epidermal barrier formation and reconstruction after wounding (Bray and Kafatos, 1991; Mace et al., 2005), tracheal tube size control (Hemphala et al., 2003), in establishing normal wing hair polarity (Lee and Adler, 2004) and development of the CNS (Almeida and Bray, 2005; Baumgardt et al., 2009; Cenci and Gould, 2005;
Maurange et al., 2008). The first two functions of grh will be discussed in some details below, as they are relevant to this work.

**a. Epidermal barrier maturation and repair after wounding in *Drosophila***

In *Drosophila*, the epidermal barrier consists of a single epithelial cell layer that is covered by a rigid exoskeleton composed of cross-linked chitin, proteins and quinones (Moussian et al., 2006a). During embryogenesis, a series of morphogenetic events including epithelial stretching, and fusion form the continuous embryonic epidermis. Following germ band extension and its subsequent retraction, a large hole is left behind on the dorsal surface of the embryo. This hole is transiently covered by the amnioserosa, a sheet of large flat cells. Dorsal closure describes the process whereby the lateral epidermal cells migrate over the amnioserosa to close the hole and to seam the embryo along the dorsal midline. Dorsal closure is the last major morphogenetic epidermal movement during *Drosophila* embryogenesis. The process initiates at around stage 12 and completes at the end of stage 15. It takes about two hours and can be divided into four phases: 1) Initiation 2) Epithelial sweeping 3) Zippering and 4) Termination (Harden, 2002; Jacinto et al., 2002b; Martin and Parkhurst, 2004). In the first phase, the two opposing epithelial sheets move slowly towards each other as the consequence of the amnioserosal cell contraction. During the second phase, the dorsal-most epidermal cells termed leading edge cells, which are connecting to the amnioserosa, accumulate actin and myosin their dorsal sides. The accumulation of F-actin forms a contractile cable, which will pull the leading edge of the epithelial sheets together. The cells located more laterally gradually lose their polygonal shape and elongate, thereby shifting the whole epidermis dorsally. As the epithelial sheets meet each other at the midline, the third phase starts. Lamellipodial and filopodial protrusions from the cells on opposing epithelia meet and interact with each other, zipper the two epithelia sheets together. During the final phase, filopodia regress and their transient adhesion become stable and produce the seamless midline (Harden, 2002; Jacinto et al., 2002b; Martin and Parkhurst, 2004). The cellular processes underlying embryonic dorsal closure strongly resemble the characteristic behavior of cells during epithelial wound closure. Additionally many of the molecules required for dorsal closure have been shown to play important roles in
epithelial wound healing (Grose and Martin, 1999; Martin and Parkhurst, 2004; Wood et al., 2002).

**Formation of the protective barrier**

The epidermis of many animals is covered with a protective extracellular matrix (ECM) layer. In insects, this is known as the exoskeleton or cuticle. The cuticle is comprised of fibrils of chitin, a β1-4 linked polymer of N-acetyl-D-glucosamine, and also contains proteins and lipids (Cohen, 2001). Chitin is a long linear sugar formed by transmembrane enzymes, called chitin synthases, that link cytosolic UDP-N-acetyl-D-glucosamine (UDP-GlcNAc) into chains of repeating GlcNAc residues that extrude from the apical cell surface (Merzendorfer, 2006). Chitin can be degraded by chitinases, which are secreted enzymes fragmenting long chitin polysaccharides into shorter chains (Merzendorfer and Zimoch, 2003). Chitin can also be converted to chitosan by deacetylation. This process, which is mediated by chitin deacetylases, removes acetyl groups (CH3-CO) converting chitin into chitosan (Arakane et al., 2009; Christodoulidou et al., 1999).

Mature cuticle consists of three functional layers, the waterproof envelope, the proteinaceous epicuticle and the chitinaceous procuticle that are produced by the underlying epidermal cells. Procuticle is the stratified cuticle, and chitin is found as a lamellar structure in this layer abutting the epidermal cells. The lamellae are built from sheets of chitin microfibrils and are tightly packed to confer stability and elasticity to the exoskeleton (Merzendorfer, 2006; Moussian et al., 2005). The embryonic cuticle of *Drosophila* is deposited by the epidermal epithelium during stage16 of development after dorsal closure and is essential for maintaining the structural integrity of the larval body. Cuticle differentiation in the *Drosophila* embryo occurs in three phases. In the first phase, the three layers are established and occur partially simultaneously rather than in a strict sequential manner. In the second phase the cuticle thickens. Finally, during the third phase, when the cuticle material deposition has ceased, the chitin lamellae acquire their typical orientation, and the epicuticle of the denticles and the head skeleton darken (Moussian et al., 2006a).

Protein and lipids are cross-linked in the cuticle. *Ddc* and *pale* (or *ple*), which encode Dopa Decarboxylase and Tyrosine Hydroxylase respectively, are crucial for this
cross-linking (Wright, 1987b). Both enzymes are components of an enzymatic pathway that convert tyrosine to N-acetyldopamine and N-β-alanyldopamine, both of which are subsequently oxidized to quinones by phenol oxidases. The quinones form covalent bonds with histidine residues in the cuticular protein, which results in cuticle hardening (Schaefer et al., 1987).

**Wound healing in Drosophila**

The capacity to heal wounds is essential for survival upon injury. Any damage of the animal’s outer protective layer, must quickly repair to prevent blood and tissue loss. This is even more important for insects because of their open circulatory system. Epidermal wound healing in the adult skin is complicated and contains various overlapping phases: the immediate response, the inflammatory response, proliferation, migration and contraction, and the resolution phase (Martin, 1997; Shaw and Martin, 2009; Singer and Clark, 1999). In mammals, injury first induces the rapid formation of a fibrin clot at wound. Soon inflammatory cells are recruited to the wound sites. These cells clear invading microbes and cell debris. They also provide growth factors and cytokines that may promote epithelial healing. After a period of several hours the proliferation and spreading of the surrounding keratinocyte sheets across the wound gap restores tissue integrity. This process is aided by activated fibroblasts that migrate to form a granular plug infiltrated by newly formed endothelial capillaries. Finally, resolution phase starts and restores the full functionality of the wounded epithelia. The duration of the healing process *in vivo* depends on the affected tissue, the nature of the injury and the developmental stage. For example, a common remnant of the wound healing process in adult humans is a non-functional fibrotic tissue known as the scar. By contrast, embryonic tissues fully regenerate without fibrosis (Martin, 1997; Redd et al., 2004; Singer and Clark, 1999). The complexity of the wound healing process *in vivo* has led researchers to establish simpler, *in vitro* wound healing assays to investigate the molecular mechanisms of wound regeneration. *In vitro* epithelial scratch-wound closure assays are now widely used in cell biology and can partly reproduce the migratory behavior of cells at wounds. They also offer the possibility to genetically manipulate the process by siRNA and have been successfully used to identify regulators and components of the actin cytoskeleton required for cell migration (Simpson et al., 2008). However, the
in vitro assays are limited because they can neither address the interplay of different cell types during wound responses nor can they reproduce the regeneration of a functional epithelium. A simple, quantitative and genetically tractable system to study wound healing would allow systematic dissection of the process in vivo and strongly complement the studies in vertebrate model systems and clinical investigations.

Drosophila has become a key model system to explore the molecular mechanism of epithelial repair although it is introduced for wound healing research only in recent years. Wound healing in Drosophila has been documented at different developmental stages, including embryos, larvae, and adults (Babcock et al., 2008; Baek et al., 2010; Campos et al., 2010; Galko and Krasnow, 2004; Kwon et al., 2010; Mace et al., 2005; Ramet et al., 2002; Stramer et al., 2005; Wood et al., 2002; Wu et al., 2009). Although there are distinct differences, the wound healing process in all the developmental stages also share some common features. It has been shown that an actin cable operates as a purse-string to draw the wound close whereas dynamic filopodia protrusions are essential for the final knitting together of epithelial cells during Drosophila embryonic epidermal wound healing (Wood et al., 2002). The actin cable-like structure was not very obvious during epidermal wound re-epithelialization in Drosophila larvae. Galko et al reported that upon wounding the larval epidermal cells extend actin-based protrusions (Galko and Krasnow, 2004; Wu et al., 2009). A clot is formed to first cover the hole and after clotting, epidermal cells spread along and through the plug until they meet and reestablish a continuous epithelial sheet (Galko and Krasnow, 2004). However, Kwon et al recently reported that 6 hours after wounding the larval epidermis, F-actin and nonmuscle myosin GFP (Sqh-GFP) accumulate at the wound leading edges and form an actomyosin cable-like structure (Kwon et al., 2010). The growing epithelia of the imaginal discs provide an additional system for the study of epithelial wound repair and regeneration (Bergantinos et al.; Bosch et al., 2005; Mattila et al., 2005; Smith-Bolton et al., 2009). There, the rapid reorganization of actin at the adherens junctions of the edge cells forms a cable-like structure. A few minutes after injury of larval wing discs the cells around the wounds elongate and the leading edge cells extend actin protrusions similar to the filopodia seen in embryonic wound healing (Bosch et al., 2005; Mattila et al., 2005). In adult flies epidermal wounding also induces cytoplasmic extensions in the cells around the wound
but the potential formation of an actin cable has not been studied. In the adult, the filopodia-like extensions allow cells to migrate towards each other under the melanin clot and form the new epidermal sheet (Ramet et al., 2002). Thus, the rearrangement of actin cytoskeleton upon wounding is common and crucial for efficient wound closure regardless of the damaged tissues and developmental stages.

I will discuss the embryonic wound healing process in more detail below, as this work has focused on embryonic stage.

1) The purse-string mechanism of wound closure

It is well known that the embryonic epithelial tissues have a remarkable capacity to seal small wounds very rapidly and efficiently by a ‘purse-string’ mechanism. In this mechanism, the injured epidermal cells close their wounds via an intracellular actomyosin cable. Martin and Lewis initially described this mechanism in chicken embryos. Using a fluorescently tagged phalloidin, which can visualize filamentous actin, they detected a thick actin cable at the leading edge of the marginal cells surrounding the wound (Martin and Lewis, 1992). Subsequently, it was reported that blocking the assembly of the filamentous actin by the addition of cytochalasin D results in complete failure of re-epithelialization in embryonic mouse wounds (McCluskey et al., 1993; McCluskey and Martin, 1995). A few years later, a cable of the motor protein Myosin II was also reported assembled in a similar manner to the actin cable in wounded chicken embryos (Brock et al., 1996). Interestingly, Xenopus oocytes seem to heal wounds using a similar mechanism as well (Bement et al., 1999; Davidson et al., 2002).

However, although the ‘purse-string’ mechanism of wound healing was initially described in vertebrates, it was most extensively studied during Drosophila embryonic wound re-epithelialization by a combination of live imaging and genetics (Wood et al., 2002). Importantly, the dynamic actin based cell protrusions such as filopodia and lamellipodia were also observed by using real time imaging. A green fluorescent protein (GFP)–actin fusion protein was used to visualize the actin cytoskeleton dynamics after laser-induced wounding. Within minutes of injury, an actin cable and dynamic filopodia that extend up to 5 μm in length were formed from epidermal cells around the wound edge. While the wound edge contracted and the opposite sides approached one another, more filopodia formed, which eventually stitched the two epithelia sheets together. The
filopodia were very dynamic, extending and retracting at up to 1 μm/min, as well as sweeping from side to side as if sampling the substratum ahead of them.

![Diagram of actin dynamics](image)

**Figure 2.** Model of actin dynamics during epidermal wound healing in *Drosophila* embryos.

An actin cable is formed around the wound edge shortly after injury and persists during wound healing. The epidermal cells at the wound edges also extend actin protrusions, filopodia/lamellipodia, during wound closure. Modified from (Noselli, 2002).

To analyze the function of actin cable and filopodia in living embryos, mutants for the small GTPases were injured and the actin dynamics were monitored. Small GTPases of the Rho family, including Rho, Cdc42 and Rac, are key signaling molecules implicated in rapid re-organization of actin cytoskeletal structures (Tapon and Hall, 1997). More specifically, Rho1 regulates the formation of cable-like actin stress fibers in serum-starved tissue-culture fibroblasts and Cdc42 mediates the rapid extension of filopodia, Rac is required for proper formation of lamellipodia (Nobes and Hall, 1995; Ridley and Hall, 1992; Ridley et al., 1992). Interestingly, mutants of *Drosophila* small GTPase Rho (*Drosophila* gene *Rho1*), Cdc42 (*Drosophila* gene *Cdc42*) or Rac (*Drosophila* genes *Drac1, Drac2* and *mtl*) are compromised in dorsal closure (Hakeda-Suzuki et al., 2002; Jacinto et al., 2000; Jacinto et al., 2002a; Magie et al., 1999). *Rho1* mutants fail to form a uniform actin cable around the wound and the edges do not contract as wild-type lesions. Nevertheless, *Rho1* mutants close their wounds but require twice as long to complete the process. Once the wound contraction initiates, *Rho1* wounds are closing with the same kinetics as wild type. Notably, *Rho1* cells compensate for the absence of the actin cable by forming numerous, longer filopodia and zippering several of them to form lamellipodia. In contrast, the leading edge cells surrounding
small wounds induced on embryos expressing dominant-negative Cdc42 in epidermal
stripes failed to extend the filopodial or lamellipodial protrusions although they were able
to assemble an actin cable. As a consequence, those wounds remained open. Surprisingly,
the wounds made in triple mutants of Drac1, Drac2 and mtl were able to close like wild
type despite the clearly disrupted dorsal closure in these mutants. However, this is
consisted with the observation that in chicken embryos inactivating Rac activity by
inhibitors does not affect wound closure (Brock et al., 1996). The different requirement
of Drac1, Drac2 and mtl in dorsal closure and wound closure suggests that there are
some differences in the mechanisms controlling these striking similar processes. In
summary, the proper formation of an actin cable is required for efficient wound healing
while the assembly of filopodia is essential for the final knitting together of the wound
edges.

2) Dramatic cell shape changes in Drosophila embryonic wound healing

Along with the dynamic reorganization of the actin cytoskeleton, the epidermal
cell shapes also change dramatically during wound healing. To study cell shape changes
during wound repair, live confocal time-lapse analysis was performed with laser-
wounded embryos expressing a GFP–α-catenin fusion protein in the epidermis (Wood et
al., 2002). No activation of cell division was observed at the wound margin, which
suggests that wound closure relies entirely on cell shape changes and rearrangements.
The cells around the wound margin undergo marked alterations in shape and move
relatively to each other, exchanging their neighbors as the wound closes. The front-row
cells constrict their leading edges towards the wound center and elongate remarkably as
the wound closes. Occasionally, some leading-edge cells leave the front row and move
further back so that the number of cells at the edge decreases during the wound closure.

Interestingly, epidermal cells at the edge of the wound accumulate α-catenin and
E-cadherin upon wounding (Garcia-Fernandez et al., 2009; Wood et al., 2002). This
accumulation likely contributes to the formation of new adherens junctions between cells.
It has been proposed that the filopodial and lamellipodial contacts contribute in the
formation of stable adherens structures (Raich et al., 1999; Vasioukhin et al., 2000) but
the mechanism of junctional protein recruitment and junction maturation remains elusive.
3) Wound inflammation in Drosophila embryos

It is well known that tissue damage triggers an acute and robust inflammatory response. Upon injury, blood cells such as neutrophils and macrophages rapidly migrate to the wound site, where they combat the invading pathogens and are involved in tissue degradation and regeneration (Martin, 1997).

In Drosophila, hemocytes are functionally equivalent to neutrophils and macrophages (Tepass et al., 1994). Laser wounding triggers hemocytes immediately adjacent to the wound to polarize and migrate directionally toward the wound zone within minutes in embryos (Stramer et al., 2005). These hemocytes engulf cell debris as well as pathogens once they are at the wound site. The Rho-family small GTPases are required for efficient migration of the hemocytes towards the wounds during embryonic wound inflammation (Stramer et al., 2005). Loss of Rac activity (Drac1, Drac2, mtl triple mutants) resulted in recruitment of very few hemocytes, likely due to the significantly reduced lamellar protrusions of the mutant hemocytes and a motility decrease. Wounding Rho1 null embryos caused an even lower recruitment of hemocytes. The hemocytes in the wound vicinity attempted to migrate towards but failed to retract their tails, suggesting that Rho signaling is necessary for cells to remodel their matrix- and cell-cell contacts (Stramer et al., 2005). Cdc42 is required for the hemocytes to polarize towards the wounds upon injury. However, the hemocyte recruitment to wounds was normal probably because these cells migrated at approximately twice the normal speed (Stramer et al., 2005).

Once at the wound sites, hemocytes may release signals that regulate the wound healing process. However, wounds made in serpent mutant embryos that lack all hemocytes closed their wounds just like in wild type (Stramer et al., 2005). This result suggested that wound healing (or at least the re-epithelialization) in embryos doesn’t require signals from invading hemocytes and the debris left behind in the absence of an inflammatory response doesn’t influence re-epithelialization.

4) Signaling pathways in Drosophila wound healing

Numerous studies in vertebrates and invertebrates have examined the signaling pathways regulating wound healing and highlighted the genes required for epithelial sheet repair (Garcia-Fernandez et al., 2009; Jacinto et al., 2001; Martin, 1997; Schafer
and Werner, 2007; Shaw and Martin, 2009). Except for the Rho-family GTPases mentioned above, the JUN N-terminal kinase (JNK) signaling pathway also plays a vital role during wound healing in *Drosophila*. The *Drosophila* JNK (DJNK) signaling pathway includes the MAPK kinase kinase (MAPKKK) *misshapen* (*msn*), the MAPKK *hemipterous* (*hep*), the MAPK *basket* (*bsk*), the AP-1 transcription activator consisting of the *Jun-related antigen* (*Jra* or *Djun*) and *kayak* (*kay* or *Dfos*), and the dual specificity phosphatase *puckered* (*puc*) (Xia and Karin, 2004). *puc* is a classical JNK pathway downstream target, which negatively regulates JNK signaling (Martin-Blanco et al., 1998). Interestingly, mutants in the components of JNK pathway are all compromised in dorsal closure (Harden, 2002; Jacinto et al., 2002b; Xia and Karin, 2004). JNK phosphorylation is increased in epidermal cells of wounded flies compared to untreated controls and the expression of *puc, msn* and *kay* is activated at the wounds of adults or larvae (Galko and Krasnow, 2004; Ramet et al., 2002). These observations indicate that the JNK pathway is activated at wounds and that it maybe necessary for efficient wound healing. Consistent with this, mutant flies of *kay* failed to close their wounds and did not induce *puc* expression at the wound edges (Ramet et al., 2002). *kay* is also crucial for epithelial re-epithelialization in *Drosophila* embryos and larvae (Bosch et al., 2005; Campos et al., 2010). *hep* and *bsk*, activities are also required for wound repair in larval epidermis and/or wing imaginal discs (Bosch et al., 2005; Galko and Krasnow, 2004; Mattila et al., 2005).

The JNK pathway has also been implicated in epidermal wound closure in mammals. *c-fos* is induced in epidermal cells at the leading edge in wounded rat embryos (Martin and Nobes, 1992). In line with this, conditional inactivation of c-Jun in the epidermis of mice resulted in defective epidermal wound healing (Li et al., 2003). Interestingly, the mutant mice also showed defective eyelid closure (Li et al., 2003; Zenz et al., 2003), which is similar to wound healing in many aspects of cellular behavior (Martin and Parkhurst, 2004). JNK1 and JNK2 were also rapidly activated in cultured Madin-Darby Canine Kidney (MDCK) cells upon scratch wounding and were required for the formation of lamellipodial protrusions at the edges of the scratch (Altan and Fenteany, 2004). Although the JNK signaling pathway is pivotal for wound healing in both invertebrates and vertebrates, the upstream signals activating the JNK signaling
cascade remain elusive. In flies, mutants in the necrotic gene encoding a putative serine protease inhibitor, develop random necrosis in the epidermis. puc expression is induced in the tissues around these necrotic regions, suggesting there is link between the necrotic signaling and the activation of JNK pathway (Ramet et al., 2002). In cultured mouse fibroblast, the JNK pathway is activated in the leading edge cells after platelet-derived growth factor (PDGF) and Epidermal growth factor (EGF) stimulation (Lallemand et al., 1998). PDGF and EGF signaling therefore may activate JNK pathway in mouse epidermal wound healing.

pvr encodes a RTK related to PDGF and vascular endothelial growth factor (VEGF) receptors (Duchek et al., 2001). Pvr signaling has been shown upstream of JNK signaling during Drosophila thorax closure (Ishimaru et al., 2004). Pvr and one of its ligands, PDGF/VEGF factor 1 (Pvf1), are also required for epidermal wound closure in Drosophila larvae (Wu et al., 2009). However, the activation of JNK signaling is not affected in the pvr or pvf1 mutant upon wounding. In addition, Pvr signaling promotes wound-margin epidermal cells to extend actin-based cell processes into the wound gap while JNK mediates transient dedifferentiation of cells at the wound margin. Therefore, JNK and Pvr signaling pathways act in parallel during wound healing.

In addition, mutations of the scab gene, which encodes Drosophila αPS3 integrin, results in defective embryonic epidermal wound healing. This suggests that integrin signaling is involved in epithelia wound repair (Campos et al., 2010). Integrins are transmembrane glycoproteins that attach a cell to its neighbouring cells or ECM. They bind cell surface and ECM components such as fibronectin, vitronectin, collagen, and laminin and mediate cell-cell and cell-matrix interaction and communication.

**Grh in the maturation and healing of the epidermal barrier in Drosophila**

grh mutant embryos show a granular head skeleton. The mouth hooks are much smaller than usual in the mutants and the denticles are frequently disordered, with cases of reversed polarity. The ventrolateral cuticle of grh mutant embryos is also bulging and distended compared to that of wild type (Bray and Kafatos, 1991). The cuticle phenotypes of grh mutants suggest that genes regulated by grh are involved in many aspects of exoskeleton specialization. Interestingly, when grh mutant embryos are removed from the vitelline membrane their cuticle stretches to a much greater extent than
the wild type cuticle. This results in grossly inflated embryonic cuticle preparations compared to wild type. This phenotype has been referred to as the “blimp” cuticle and suggests poor structural integrity of the cuticular layer (Bray and Kafatos, 1991; Ostrowski et al., 2002).

grh mutants show no defects in embryonic dorsal closure. However, Grh overexpression in embryos causes a failure in dorsal closure (Attardi et al., 1993; Narasimha et al., 2008). This is likely due to ectopic activation of genes encoding septate junction (SJ) proteins in the amnioserosa or due to their overexpression in the epidermis (Narasimha et al., 2008). The grh overexpression experiments suggest that it has a redundant role in embryonic dorsal closure.

Grh is also crucial for the repair of the protective cuticular layer of the epidermis in wounded Drosophila embryos. Upon injury, it activates the expression of cuticle repair genes like Ddc and ple, which encode cuticle cross-linking enzymes, in cells surrounding epidermal wounds (Mace et al., 2005; Pearson et al., 2009). Analysis of the regulatory elements required for the expression of both genes upon wounding demonstrates that their activation is dependent on a Grh binding site in these elements. ERK activation is also detected around the wounds and required for the efficient induction of Ddc wound response reporter (Mace et al., 2005). The induction of Ddc seems crucial for cuticle repair upon wounding since grh mutants, which fail to activate Ddc around wounds, are defective in cuticle repair, as well as in displacement of the melanin plug that forms immediately after wounding when compared with wild type embryos (Mace et al., 2005).

The structure of the mammalian skin is very different from the insect epidermis. In insects, a single layer of epidermal cells and the extracellular cuticle forms the surface barrier. In contrast, the mammalian epidermis is composed of several cell layers. The outermost layer is the stratum corneum, which contains dead granulocytes, encaged in a cornified envelope of cross-linked proteins, keratin cables and lipids (Jane et al., 2005). In mice, transglutaminase 1 (TGase 1) encodes the enzyme that cross-links components of stratum corneum and renders it impermeable to water and solutes. Not surprisingly, mice lacking TGase 1 show defects in the development and maturation of the surface epidermal barriers (Matsuki et al., 1998). The degradation of nuclei and the keratohyalin F-granules in the stratum corneum was incomplete and consequently the mutants died 4-5
hours after birth. Homology searches and genetic screens for cuticle phenotype genes in *Drosophila* had failed to identify homologs of the genes encoding keratin and other structural components of the mammalian barrier. The two enzymes, Ddc and Ple, which cross-link and harden the *Drosophila* cuticle, have completely different chemical functions than TGase1 but perform similar biological functions. Mouse TGase 1 is a direct target of *grhl3* (Ting et al., 2005a) while in flies *grh* directly regulates the epidermal expression of *Ddc* during development (Bray and Kafatos, 1991). In addition, *grh* activates *Ddc* and *ple* expression at the wound sites in flies (Mace et al., 2005). And both *grhl3* and TGase 1 mutant mice are defective in formation of an impermeable epidermal barrier and efficient wound healing (Inada et al., 2000; Matsuki et al., 1998; Ting et al., 2005a), in keeping with the reduced expression of TGase 1 in *grhl3* mutant mice (Ting et al., 2005a). Thus, despite the structural differences of the surface barriers of flies and mice, the molecular mechanism controlling their formation and repairing after wounding appears evolutionarily conserved.

**b. Tracheal development**

Oxygen is vital to all organisms that rely on the respiratory chain of oxidative phosphorylation, and animals have evolved elaborate organs for its transport and delivery to different tissues. In insects, gas exchange is ensured by a network of epithelial tubes known as the tracheal system, which extends branches to the whole organism. The *Drosophila* tracheal system receives air directly from the environment through specialized valve openings called spiracles and delivers it to all tissues through capillaries that penetrate the target organs. The tracheal network is strictly patterned during embryogenesis. The tracheal branches arise by ectodermal invaginations of 20 independent clusters, called the tracheal placodes. The clusters are symmetrically located on each side of the embryo. Tracheal cell invagination, cell shape changes and rearrangements result in a distinct branch sprouting pattern, directed branch outgrowth and branch fusion. Embryonic tracheal branching and morphogenesis rely entirely on epithelial cell movements and rearrangements. The embryonic tracheal cells divide for the last time during their invagination from the ectoderm and they very rarely undergo apoptosis (Samakovlis et al., 1996). At the end of embryogenesis just before hatching,
luminal material is rapidly cleared from the tracheal tubes, and shortly thereafter liquid is removed. Then the trachea system fills with air and becomes functional (Tsarouhas et al., 2007).

**Control of tracheal tube size**

An important late step in the morphogenesis of tubular organs is the final acquisition of distinct and uniform sizes of their branches. The fixed length and diameter of tubes dictate the flow rates of the transported gas or liquid and are therefore major determinants of optimal organ function. The pathology of patients with Autosomal Dominant Polycystic Kidney Disease for example, is intimately linked with aberrant tube sizes and cysts in the kidney tubules and collecting duct (Harris and Torres, 2009). Despite difference in tube structure and construction mechanisms in various organs and animals, tube size regulation commonly involves the apical cell membrane growth, cell shape changes and cytoskeletal reorganization.

The major tracheal airways, the dorsal trunks are made by a single epithelial cell layer. At the end of embryogenesis they are surrounded by a basal lamina and an apical specialized cuticular lining forming the characteristic taenidia (Uv et al., 2003). The major component of the taenidial lining is chitin, an assembly of cross-linked glucan polymers. This apical ECM is thought to provide elasticity to the tubes and a tough, protective barrier against dehydration and pathogens. The length and diameter of the different tracheal branches are controlled independently, with the length of branches increasing continuously through development, and the expansion of tube diameter occurring step-wise. Furthermore, neither cell size or cell number is a primary determinant of tube length and diameters in the embryonic trachea (Beitel and Krasnow, 2000). Analysis of mutants with selective size defects indicates that genes encoding proteins with very diverse molecular functions, control tracheal tube size in *Drosophila* embryos.

The first group of genes encodes proteins involved in chitin biogenesis or assembly. These genes include cystic/mummy (*mmy*), krotzkopf verkehrt (*kkv*), gnarled/*knickkopf* (*knk*) and retroactive (*rtv*). *mmy* encodes the *Drosophila* homolog of UDP-N-acetylglucosamine diphosphorylase. This enzyme is required for the production of UDP-N-acetylglucosamine, a substrate for chitin synthesis (Araujo et al., 2005; Devine et al.,
2005; Tonning et al., 2006). *kkv* encodes a chitin synthase (Ostrowski et al., 2002; Tonning et al., 2005). *knk* and *rtv* encode novel proteins implicated in chitin filament organization (Moussian et al., 2006b; Ostrowski et al., 2002). In *Drosophila* trachea, a luminal chitin cable is detected at stage 13, just before the expansion of the tracheal tubes. The luminal chitin matrix is a tightly packed cylindrical structure that grows during tube expansion at stages 14 and 15 and disappears during late stage 16 (Devine et al., 2005; Tonning et al., 2005). Mutations in *mmy, kkv, knk* or *rtv* result in severe defects in the formation and assembly of the luminal chitin matrix (Araujo et al., 2005; Devine et al., 2005; Moussian et al., 2006b; Tonning et al., 2006; Tonning et al., 2005). These defects are accompanied by irregular diametric expansion of the tracheal tubes that show local constrictions and dilations. In addition, these mutants develop overelongated and tortuous tracheal branches. The overall apical basal polarity, the organization of the Septate Junctions (SJs) and the paracellular barrier function of the tracheal epithelium are not affected by mutations interfering with chitin biosynthesis and assembly (see more discussion about SJs below). This suggested that the transient apical matrix provides a signal or a template for the uniform expansion of the tracheal tubes. Interestingly, *kkv* mutants show an irregular subapical cytoskeleton (Tonning et al., 2005), suggesting that the chitin cable provides cues for the organization of the apical cell surface and cytoskeleton. The apical ECM defects of mutants in the “chitin” group are not confined to the trachea but they are also evident in the epidermis and other ectodermally derived epithelial tissues. For example, *kkv, knk* and *rtv* mutants also show the characteristic “blimp” cuticle phenotype, which is similar to the cuticle defect of *grh* mutant embryos (Ostrowski et al., 2002). Collectively, the analysis of the mutant phenotypes in the “chitin” group of genes suggests that the apical chitin matrix provides either a physical scaffold that defines the shape of the underlying epithelial cells or it signals to the epithelial cells to adjust their shape in a coordinate manner.
Figure 3. Model of luminal chitin matrix function in tracheal tube size control (modified from Devine et al., 2005).

In wide type (wt) embryos, an expanding cylinder structure of chitin fibers in tracheal lumen coordinates the behavior of surrounding tracheal cells and controlling the tube size. In contrast, this luminal chitin matrix is absent in chitin synthesis mutants. Consequently, the tracheal tube expands in an uncoordinated manner and becomes cystic and overelongated.

The second group of tube size regulators consist of genes encoding proteins associated with the paracellular SJs. SJs are located just basally to the adherens junctions (AJs) in ectodermally derived epithelial tissues in insects. SJs function as a barrier to prevent the diffusion of water and solutes between epithelial cells. This paracellular barrier function is analogous to the function of tight junctions (TJs) in vertebrates. Notably SJs and TJs also share a subset of their constituent proteins like claudins (Jane et al., 2005; Wu and Beitel, 2004). The first evidence indicating that SJ-associated proteins are crucial for tube size control came from the analysis of a mutation in the Na-K ATPase α subunit gene (ATPα) (Paul et al., 2003). ATPα mutants show overelongated and convoluted tracheal tubes along with defective paracellular barrier function. The number of paracellular septa was greatly reduced in the mutants and several SJs components like Neurexin IV (Nrx IV) and Coracle (Cora) were mislocalized. Other genes encoding SJ-associated proteins were subsequently found to be vital for tracheal tube size control. These genes include Nrx IV, megatrachea (mega), sinuous (sinu), bulbous/lachesin (lac), nervana 2 (nrv2), Neuroglian (nrg), fasciclin II (fasII), scribble (scrib), lethal giant larvae (lgl), discs large (dlg), cora and yurt (yrt) (Behr et al., 2003; Beitel and Krasnow, 2000; Genova and Fehon, 2003; Hemphala et al., 2003; Lamb et al., 1998; Laprise et al., 2010; Llimargas et al., 2004; Paul et al., 2007; Paul et al., 2003; Wu and Beitel, 2004;
Wu et al., 2004; Wu et al., 2007). Mutants of these genes show overelongated and convoluted tracheal tubes, and many of them are also compromised in the paracellular barrier function.

One possible mechanism of SJ-mediated tube size control is through the regulation of apical polarity proteins such as Crumbs (Crb) and atypical protein kinase C (aPKC) (Wu and Beitel, 2004). scrib, lgl, dlg, cora and yrt encode SJ-associated proteins that are also required for the establishment of epithelial apico-basolateral polarity (Laprise et al., 2010; Wu and Beitel, 2004). These SJ-associated epithelial polarity genes normally negatively control apical cell surface specification by antagonizing the activities of the apical domain determinants Crb and aPKC (Bilder et al., 2003; Tanentzapf and Tepass, 2003). Zygotic inactivation of genes encoding SJ-associated polarity proteins results in excessively elongated and convoluted tracheal tubes while complete loss of both the maternal and zygotic products cause grossly disrupted epithelial polarization and morphogenesis (Laprise et al., 2006; Laprise et al., 2009; Laprise et al., 2010; Tanentzapf and Tepass, 2003). In addition, the surviving mutant cells form cysts with expanded apical surfaces. Therefore, it is likely that the SJ-associated epithelial polarity genes also control tracheal tube size by antagonizing the activities of apical domain determinants. Indeed, the reduction in the levels of the apical polarity protein Crb suppresses the tube length defects in cora and yrt mutants (Laprise et al., 2010). However, removing one copy of crb does not suppress the overgrowth tracheal tube phenotype in scrib or other SJ-associated mutants like sinu (Laprise et al., 2010). Thus, additional mechanism(s) are involved in the tube size control for other SJ-associated proteins.

A possible mechanism for the additional functions of the SJ-associated proteins in tube size control is their potential involvement in the assembly of the apical and luminal ECM (Wu and Beitel, 2004). This model is based on observations suggesting that the luminal and apical chitin matrix is compromised in SJ mutants. 1) Mutations in mega, ATPα or fasII leads to more diffuse luminal chitin matrix compared to wild type (Tonning et al., 2005). 2) Removal of one copy of kkv in mega, fasII, ATPα, lac, nrxIV, cora or sinu mutant results in a remarkable reduction in the levels of the luminal antigen 2A12 compared to the single mutants (Tonning et al., 2005). 3) In sinu mutants the
taenidia are disorganized compared to wild type (Wu et al., 2004). 4) Nrx is required for the normal formation of the cuticle in epidermal cells (Baumgartner et al., 1996).

Thus, there are at least two possible ways that SJs control tube size: A) By antagonizing the activation of Crb at the apical surface and B) By controlling the deposition of the luminal or apical ECM.

Figure 4. Model of the possible mechanisms involved in tube size regulation by SJ proteins (Modified from (Laprise et al., 2010)).

(I) Some SJ proteins, such as Yrt and Cora, control tube length by antagonizing the activation of the apical domain determinant Crb. Loss of either Yrt or Cora results in excessive apical surface specification, leading to tracheal tube overelongation. (II) Other SJ proteins like Sinu may control tube size by regulating the formation of the luminal or apical ECM. This could be achieved by
their contributions to the secretion of luminal proteins that play a role in the assembly and/or modification of luminal or apical ECM.

**grh in tracheal tube size control**

Grh is so far the only transcription factor among the genes implicated in tracheal tube size control. Grh regulates tube elongation selectively because *grh* mutants show overelongated and convoluted tubes without any apparent cysts or dilations (Hemphala et al., 2003). The SJs and the localization of apical basal polarity markers were not affected in *grh* embryos. Labeling *grh* mutant embryos for the adherens junction protein DE-Cadherin (DECad) shows that the apical surface of *grh* tracheal cells is highly irregular and elongated compared to the cobblestone-shaped cells in wild type embryos. The cellular defects have been further analyzed by transmission electron microscopy (TEM). Grh mutant embryos show normal morphology along the basal and lateral membrane of the tracheal cells. However, the apical cell domain is excessively overgrown and distorted. The apical cell membrane is so much expanded that it grows into the tracheal lumen. Cuticle deposition is also highly abnormal with strong defects in the procuticle layer and the taenidial organization. Furthermore, the transient luminal chitin matrix in the tracheal lumen is also reduced in *grh* mutants (Luschnig et al., 2006). On the other hand, overexpression of Grh in all tracheal cells throughout embryonic tracheal development is sufficient to prevent luminal extension and results in a partial failure in branch outgrowth (Hemphala et al., 2003). This phenotype suggests that Grh may also have a redundant function during early development of trachea system. *grh* targets in tracheal tube size control are not identified and the molecular mechanism by which *grh* controls tracheal tube size remains unknown.

Although both Grh and SJ-associated proteins control tracheal tube elongation, it is likely that Grh regulates tube elongation independently of SJs protein function since: 1) *grh* mutants show normal levels and localization of the typical SJs proteins, Nrx and Cora, while mutations in any of the bona fide SJs components disrupt the localization of the other components and SJs assembly 2) *grh* mutants show overelongated tubes and overgrowth of the apical cell membrane into the lumen, but SJ mutants don't 3) Double mutants for *grh* and either of the two SJs proteins, *fasII* and *ATPα*, show enhancement of
the overelongated and convoluted tracheal tube phenotype seen in the single mutants (Hemphala et al., 2003).

It is also unlikely that Grh regulates tracheal tube size by antagonizing the expression or the activity of the apical domain determinant Crb, as the levels and localization of Crb and its negative regulator Cor are intact in grh mutants (Hemphala et al., 2003). Instead, grh mutant embryos show compromised tracheal taenidial structure and a reduction of the luminal chitin matrix (Luschnig et al., 2006). Additionally they share the characteristic “blimp” cuticle phenotype with two mutants, kkv and knk, involved in chitin biosynthesis. These observations suggest that grh controls tracheal tube size at least partially by regulating the expression of genes involved in chitin biosynthesis, secretion or maturation.

It will be of interest to test whether the grhl genes in mice also play roles during tube morphogenesis since the expression of these genes is detected in a wide range of tissues undergoing branching morphogenesis, such as the lung and the kidney (Auden et al., 2006).

c. Other Grh functions

Apart from its function in embryonic epithelial maturation and tracheal tube size control, Grh is also required for several processes during the differentiation of the adult epidermis in Drosophila (Lee and Adler, 2004). For example, grh mutant clones in the wing show a delay in the formation of wing hairs and often form multiple wing hairs with planar cell polarity defects. grh mutant clones are also defective in pigmentation as well as wing vein specification. Occasionally, grh mutant cells are larger than normal in the pupal wing.

One of the grh splicing isoforms, the O isoform (Grh-O), is specifically expressed in the neuroblasts in the CNS after stage 14 and is a terminal the embryonic CNS lineage marker (Almeida and Bray, 2005; Cenci and Gould, 2005; Uv et al., 1997). Loss of the activity of Grh-O in thoracic neuroblasts leads to delay in cell cycle, reduction in cell size and neuroblast disappearance during larval stages (Almeida and Bray, 2005; Cenci and Gould, 2005).
III. Molecular mechanisms of Grh function

1) Upstream regulation by signaling

The spatial and temporal expression of *GBE-LacZ*, an artificial *lacZ* reporter containing Grh be-1 (see below), in the trachea has provided the first indication that Grh activity may be regulated *in vivo* (Hemphala et al., 2003; Uv et al., 1997). *GBE-LacZ* is selectively up regulated in fusion and terminal cells at the tips of the tracheal branches at stage 14. Further analysis in the trachea has shown that Fibroblast growth factor (FGF) signaling regulates Grh activity. When *Branchless* (*Bnl*, a *Drosophila* FGF) or the activated form of Breathless (*Btl*, a *Drosophila* FGFR) is ectopically expressed in tracheal cells, *GBE-LacZ* expression becomes significantly up regulated. The levels of Grh protein remain unchanged under these conditions. The activation of the *GBE-LacZ* reporter is dependent on Grh since its expression disappears in *grh* mutants. Bnl signaling is also required for the transcriptional activity of Grh in the trachea since the expression of *GBE-LacZ* is both reduced and uniform in *bnl* and *btl* mutant embryos. Importantly, the level of *GBE-LacZ* expression was not altered by mutations in the *bnl* signaling nuclear effector *pointed*. Because mitogen-activated protein kinase (MAPK, also called ERK, encoded by the *rolled* gene in *Drosophila*) can phosphorylate Grh *in vitro* (Liaw et al., 1995), it is likely that Grh activity is directly modulated by Bnl-induced phosphorylation during tracheal development.

MAPK activity is also rapidly elevated in epidermal cells upon wounding and it contributes to the induction of the *Ddc* wound reporter, which also carries essential Grh binding sites (Mace et al., 2005). Thus, it is reasonable to speculate that wounding may trigger a signaling cascade that activates MAP kinase, which in turn phosphorylates Grh protein and activates Grh-mediated gene expression. However, Bnl signaling is unlikely to account for this, since the *btl* receptor is not present in the epidermis. A candidate signaling receptor for the regulation of the epidermal Grh activity should fulfill the following criteria: it should be present in the epidermis where it may be inactive before wounding, it should become activated around wound edges, and it should be able to induce MAPK activation.
Other RTK and non-canonical Wingless signaling may also regulate Grh activity in flies. Grh binds to the torso response elements (tor-RE) of the tailless (tll) promoter and tll expression during early embryogenesis is expanded in embryos lacking maternal Grh (Liaw et al., 1995). Additionally, clones of grh mutant cells in the wing display planar cell polarity defects. In this context grh shows dosage sensitive genetic interactions with several mutants in genes involved in planar epithelial polarity (Lee and Adler, 2004).

Interestingly, the expression of Xenopus grhl-1 gene has been reported to be under the control of BMP4 signaling (Tao et al., 2005). Also the rat grhl-3 gene has been identified as a NGF-dependent transcriptional target of p53 in the ectoderm-derived PC12 pheochromocytoma cells (Brynczka et al., 2007). In addition, Tumor Necrosis Factor alpha (TNFα) can induce the expression of human grhl-3 in the mammary carcinoma cell line MCF7 (Guardiola-Serrano et al., 2008). Collectively, these data suggest that multiple signaling pathways control the expression and/or the activity of Grhl factors during development and pathogenesis.

2) The partners of Grh

Grh activity may also rely on its association with other factors. An artificial lacZ reporter containing both Grh be-1 binding site combined with a Suppressor of hairless binding site (termed Gbe+Su(H)_m8 is expressed only in tracheal fusion cells. This localized expression reflects the activation and requirement of Notch signaling in the trachea (Furriols and Bray, 2001) and contrasts the wide expression of Gbe-LacZ in the epidermis and the entire trachea. Removing Su(H) converts the expression of Gbe+Su(H)_m8 to the same tracheal expression pattern of Gbe-LacZ, which indicates that Su(H) suppresses the Grh dependent expression of Gbe-LacZ when its binding site is combined with Grh binding sites. Furthermore, ectopic expression of the Notch intracellular domain, which is known to convert Su(H) from a repressor to an activator, dramatically induces the expression of Gbe+Su(H)_m8 (Furriols and Bray, 2001). Although the data do not show that Notch signaling regulates Grh activity on endogenous promoters, they suggest that Grh co-operates with other factors and that its transcriptional activity is modulated by the input of the associated factors.

In line with this hypothesis, it has been reported that Grh forms complexes with
the polycomb group protein Pleiohomeotic \textit{in vitro}. This association facilitates the binding of both proteins to their respective targets. Consistently, Grh also interacts genetically with Pleiohomeotic (Blastyak et al., 2006). Furthermore, Grh has also been shown to interact with another polycomb protein, Ding \textit{in vitro} and \textit{in vivo} (Tuckfield et al., 2002). In mice, Grhl-3 interacts with the LIM-only protein LI MO4, which is also expressed in the developing mammalian epidermis (Hislop et al., 2008; Yu et al., 2006).

In the context of wound responses, both Grh and AP-1 binding sites are present in the wound responsive enhancers isolated from \textit{Ddc}, \textit{ple}, \textit{msn} and \textit{kkv}. These results strongly suggest that Grh and AP-1 factors cooperatively regulate common downstream genes at least during wound healing. This hypothesis is further supported by the fact that both Grh and AP-1 binding sites are essential for the activation of the \textit{Ddc}, \textit{ple} and \textit{msn} wound reporters (Mace et al., 2005; Pearson et al., 2009).

\section*{IV. Grh downstream genes}

Loss of Grh function in \textit{Drosophila} results in diverse phenotypes, which include a “blimp” cuticle, inefficient cuticle repair, overelongation of the tracheal tubes, disordered planar cell polarity and defective neuroblast development. This suggests that Grh may regulate a wide variety of target genes in diverse tissues during development and wound repair. The expression of Grh in epithelial tissues producing cuticular structures and the phenotypes in \textit{grh} mutants suggest that Grh activates genes involved in cuticle formation. One such target gene of Grh is \textit{Ddc} (Bray and Kafatos, 1991). \textit{Ddc} is expressed in epidermal cells during cuticle secretion and is essential for hardening and coloration of the cuticle. Furthermore, \textit{Ddc} and \textit{ple} double mutant larvae develop an almost colorless head skeleton (Mace et al., 2005). This phenotype suggests a reduction in the melanization and sclerotization of the head skeleton. Loss of \textit{grh} results in the decreased expression of \textit{Ddc} in the epidermis, which may be partly responsible for the weakening of the cuticle and the “blimp” phenotype of \textit{grh} mutants. Failure in \textit{Ddc} expression may also partially explain the lowered pigmentation of clones containing \textit{grh} mutant cells (Lee and Adler, 2004). It is however clear that the reduction of \textit{Ddc} is not the only cause for all the cuticle-related phenotypes of \textit{grh} mutants. For example, null \textit{Ddc} mutants have underpigmented mouth hooks and denticle belts, but no other obvious structural defects
in their cuticle. Ddc but not grh mutants can survive into larval instars if manually removed from the vitelline membrane (Wright, 1987a). In addition, Ddc functions cell non-autonomously and clones of Ddc mutant cells display a less severe pigmentation phenotype than grh clones (Lee and Adler, 2004). Therefore Grh is expected to regulate more genes than Ddc in the epidermis.

Other candidate grh targets during epithelial development are the genes encoding the SJ proteins Fasciclin III (FasIII), Cora and Sinu (Narasimha et al., 2008). Grh-binding sites have been noticed in their regulatory regions, and Grh overexpression in the embryonic amnioserosa robustly induces their expression. Furthermore, the protein levels of FasIII and Cora are reduced in grh mutant clones in the larva wing discs. However, the expression of these genes is not altered in grh mutant during embryonic stages. Another likely direct target of Grh is starry night (stan or flamingo) (Lee and Adler, 2004). stan encodes a protein with multiple transmembrane domains, which functions in planar cell polarity. Stan protein levels are directly related to the levels of Grh both during larval and pupa wing development. In addition Grh does not affect Stan protein stability, suggesting that grh regulates stan transcription. A decrease in stan expression may largely account for the planar cell polarity defect of grh mutant clones. However no putative Grh-binding sites are detected within or around the stan gene. This may reflect a large variability in the Grh-binding sites or that stan regulation by Grh is indirect. Further potential Grh downstream genes include Ultrabithorax (Ubx), fushi tarazu (ftz) and Proliferating Cell Nuclear Antigen (PCNA) (Dynlacht et al., 1989; Hayashi et al., 1999). They all encompass Grh-binding sites and Grh can activate transcription of reporters containing the enhancer elements of these genes in cultured cells. Grh also activates till expression and represses ventral dpp expression during early embryonic development (Huang et al., 1995; Liaw et al., 1995). Additionally it directly regulates DE-Cad expression during larval CNS development (Almeida and Bray, 2005) and the expression of Ddc, msn and ple transcriptional reporters upon wounding (Mace et al., 2005; Pearson et al., 2009).

In vertebrates, TGase 1 is a direct target of Grhl-3 in the mouse epidermis (Ting et al., 2005a). Grhl-3 also directly activates uroplakin II, which encodes an urothelial membrane protein (Yu et al., 2009). In addition, Grhl-3 may also directly or indirectly regulate multiple genes encoding structural proteins, lipid metabolism enzymes and cell
adhesion molecules (Yu et al., 2006). Grhl-1 directly regulates the expression of the desmosomal cadherin, desmoglein1 in mice (Wilanowski et al., 2008). In Xenopus, Grhl-1 activates the expression of the epidermal keratin gene, XK81A1 (Tao et al., 2005).

V. Grh binding sites

Most of the potential grh target genes have been tested for the identification of distinct sites for Grh binding in their sequence. The main conclusion from these experiments is that Grh binds to specific DNA sequences and that Grh-binding sites from different target genes show very high sequence similarity. However, a reliable DNA consensus-binding site of Drosophila Grh has not yet emerged.

Two Grh-binding sites (be-1 and be-2) have been identified within the Ddc gene (Johnson et al., 1989; Uv et al., 1997). The DNA sequences of be-1 and be-2 are ATAACCGGTTTC and TGAACCGGTCCT, and are located 598 and 76 base pairs upstream of the transcription site of Ddc gene, respectively. Both of these elements are sufficient to confer Grh-dependent activation of reporters in vitro. To test whether Grh can activate transcription from these sites in vivo, Grhbe1-lacZ (also named as GBE-LacZ) and Grhbe2-lacZ transgenic lines were generated by inserting trimers of be-1 (5’-CTCGCATAACCGGTTTTCCAAG-3’) or be-2 (5’-GCGATTGAACCGGTTCTGCGG-3’) upstream of the LacZ reporter gene (The be-1 and be-2 Grh-binding sites in these constructs are underlined and the remaining nucleotides are flanking sequence from the endogenous Ddc promoter) (Uv et al., 1997). Both reporters are expressed in Grh-expressing cells during embryonic development. Expression of the be-1 reporter is detected in embryonic epidermis and trachea. By contrast, the be-2 reporter is only expressed in the CNS and is absent from other Grh-expressing tissues. The expression of both reporters is greatly reduced in grh mutant embryos, suggesting that Grh is responsible for most aspects of its expression (Uv et al., 1997). Therefore, these reporters faithfully report Grh transcriptional activity in vivo.

A DNA-binding consensus of Grh-like factors has also been identified for several animal species. For example, the mouse Grhl3 consensus-binding site is WAAACCGGTTWWW (Ting et al., 2005a). The consensus-binding sequence for mouse Grhl1 is AACCGGTT (Wilanowski et al., 2008). The consensus-binding sequence of
C. elegans Grh is WCHGGTT (Almeida and Bray, 2005; Venkatesan et al., 2003).
Aims of this thesis

The aim of this work was to identify Grh target genes and analyze their function during *Drosophila* tracheal development.

The project followed three main lines:

- Identification of potential Grh targets
- Validation of the candidates
- Functional analysis of the confirmed targets genes by generation of loss of function and/or gain of function mutants
Results and Discussion

Identification of potential Grh target genes (Paper I)

We have isolated potential downstream genes by three means:
1) Searching for genes containing putative Grh binding sites in their regulatory regions
2) Directly testing genes whose mutants have been reported in the literature to have similar phenotypes to grh mutants
3) Isolating genes with reported tracheal expression and screening for tracheal tube overgrowth phenotypes.

1) Bioinformatics screening for genes containing Grh binding sites

In this approach we took advantage of the Grh-binding sites from the Ddc promoter (Uv et al., 1997). GBE-LacZ reporter containing a trimerized be-1 site is sufficient to direct the expression of a lacZ reporter gene, in the trachea and epidermis in a grh-dependent manner (Hemphala et al., 2003; Uv et al., 1997). We thus, hypothesized that endogenous targets may contain a combination of Grh-binding sites similar to that used in the GBE-LacZ reporter. We screened the Drosophila genome for clusters containing at least 5 Grh binding sites (be-1 and/or be-2) within 700 bp using the Cis-Analyst algorithm (Berman et al., 2002; Berman et al., 2004). This screen identified 95 clusters located at either upstream, downstream, or in intronic regions of ~120 annotated genes. I generated RNA probes against 20 of these genes and compared their expression in wild type, grh mutant and grh over-expression embryos by in situ hybridization. I found that the expression of 4 of them was altered in grh mutants or embryos over-expressing Grh. These potential Grh targets are: PGRP-LC (encoding Peptidoglycan recognition protein LC); CG10244 (encoding a putative RTK); Cpr11A (encoding a novel larval cuticle protein) and CG32699 (encoding a calcium ion binding protein with acyltransferase activity). We selected CG10244 for further analysis because it was strongly expressed in the ectodermal tissues, like grh, and it encoded an uncharacterized protein with homology to RTKs. We renamed CG10244 stitcher (stit) to reflect its function during wound healing (see below and Paper III).
2) Previously identified mutations with similar phenotypes to grh

grh mutants have been previously classified in the group of the “blimp” cuticle phenotype mutations (Ostrowski et al., 2002). *In situ* hybridization with RNA probes against two of “blimp” genes, *kkv* and *knk*, showed that both were expressed in epithelial tissues including epidermis, trachea, foregut and hindgut. In addition, we found that grh regulates both *kkv* and *knk* expression in embryos. We have also identified several putative Grh binding sites in *kkv* and *knk* gene. Together, this suggested that *kkv* and *knk* are likely to be grh direct target genes. Later on, our laboratory and others showed that *kkv* mutants display cystic and overelongated tracheal tubes (Devine et al., 2005; Tonning et al., 2005). The analysis of *kkv* mutants also led to the discovery of the dynamic chitinous luminal matrix and its role in tube growth. The mechanism by which the luminal chitin matrix conveys its structural integrity to the epithelium remains unknown. Chitin is bound and modified by different enzymes such as chitin deacetylases and chitinases. The *Drosophila* genome contains around 100 genes encoding putative chitin binding proteins. We have identified two such genes and named them *vermiform* (*verm*) and *serpentine* (*serp*) to reflect the tracheal phenotype of the mutants (paper II). Both *verm* and *serp* contain putative Grh binding sites. Grh overexpression in the trachea reduced the expression of both genes, suggesting that they also are potential Grh target genes. Mutations in *mmy* leads to cystic and overelongated tracheal tube similar to that of *kkv* and *knk* mutants (Devine et al., 2005). In addition, overexpressing Chitinase 2 (*Cht2*) in trachea also mimic the phenotype of *kkv* mutants (Tonning et al., 2005). We have also confirmed these two genes as Grh targets.

3) Search for tracheal genes causing tracheal tube overgrowth phenotype

We hypothesized that the Grh target genes should be expressed in trachea and when mutated they would produce phenotypes similar to the tube overelongation defects of grh mutants. By screening the BDGP database I found around 200 genes expressed in trachea (http://insitu.fruitfly.org/cgi-bin/ex/insitu.pl). I obtained transposon insertion mutants for 32 of these 200 genes and checked for their potential tracheal phenotypes. One mutant showed convoluted tracheal tubes similar to the tubes of grh mutant embryos. The disrupted gene encodes a protein with high similarity to mammalian Melanotransferrin (MTf). However, *MTf* expression is not altered in grh mutants or by
Grh overexpression, suggesting that it is unlikely to be a Grh target gene (data not shown).

In summary, we have tested 26 potential Grh target genes and confirmed 10 of them as true targets based on the presence of putative Grh binding sites and changes of their expression in grh mutants and/or embryos overexpressing Grh. Subsequently, we showed that Grh binds to their potential regulatory regions in embryos by chromatin immunoprecipitations suggesting that grh regulates them directly.

4) Grh controls tracheal tube size through multiple direct targets involved in luminal chitin assembly and modification

Among the 10 identified Grh target genes we found none, whose expression is abolished in grh mutants. This suggested that the reduction in the expression of multiple targets is responsible for the epidermal and tracheal phenotypes of grh mutants. Consistently, we have identified a group of genes that might be collectively responsible for the tracheal phenotype of grh mutants. These genes include mmy, kkv, knk, verm, serp and Cht2. They all function in tracheal tube size control. mmy, kkv and knk are required for chitin synthesis and assembly (Devine et al., 2005; Moussian et al., 2006b; Tonning et al., 2005). Mutations in either gene cause a dramatic reduction or lack of the luminal chitin matrix. The tracheal tubes in these mutants expand in an uncoordinated fashion and result in constrictions, dilations and overelongation. Grh mutants only show overelongated branches suggesting that the reduced levels of kkv and knk are sufficient to support the coordination of radial growth in the tracheal tubes of grh mutants. Verm and Serp are secreted putative chitin deacetylases that are thought to modify the luminal chitin matrix subsequently to its deposition in the lumen. Inactivation of either verm or serp leads to disorganized luminal chitin matrix and tube elongation (see below and (Luschnig et al., 2006)). In contrast to the reduction of kkv and knk in the trachea of grh mutants we did not detect any convincing changes in the tracheal expression of verm or serp in the grh mutant embryos by in situ hybridization. However, tracheal Grh overexpression caused a reduction of verm and serp mRNA in the trachea. The phenotype of Cht2 mutants has not been reported. However, overexpressing Cht2 mimics kkv mutant phenotypes, suggesting that Cht2 has a potential function on trachea tube size control. Given its opposing molecular function to kkv, it is likely that Cht2 antagonizes the
function of \textit{kkv} during tracheal size control. However, \textit{Cht2} expression is also dramatically reduced in \textit{grh} mutants. Therefore, \textit{grh} positively regulates both \textit{kkv} and \textit{Cht2}, and it has opposing effects on the expression of genes involved in luminal chitin synthesis and modification. These results highlight an unexpected complexity of \textit{grh} in the regulation of genes involved in the assembly of luminal chitinous structures. Nevertheless, it is likely that \textit{grh} mutants show a compromised luminal matrix because the expression of all the six genes is regulated by \textit{grh}. Therefore we conclude that a major function of \textit{grh} in tracheal tube size control is to directly regulate the expression of genes related to the luminal chitin matrix assembly or modification. \textit{kkv}, \textit{knk}, \textit{verm} and \textit{serp} mutants also share the characteristic “blimp” cuticle phenotype with \textit{grh} mutants (Luschnig et al., 2006; Ostrowski et al., 2002). Although the phenotypes of mutants of target genes involved in chitin biosynthesis resemble the tube overelongation defects of \textit{grh} mutants, the apical membrane overgrowth detected in \textit{grh} embryos was not found in the mutants of the “chitin” group. This suggests either that additional Grh targets are involved in its function in tube morphogenesis or that the apical membrane defects are only caused by the collective reduction of the products of the “chitin” group genes. It will be interesting to test if the overexpression or reduction in the levels of these Grh targets in \textit{grh} mutants may ameliorate the tube overelongation defect and apical membrane overgrowth.

**Luminal matrix modifications in tube size control (Paper II)**

A transient chitinous luminal matrix is required for uniform tracheal tube growth suggesting that the luminal chitin matrix signals its structural integrity to the epithelium and coordinates the behavior of tracheal cells. Hence, the structural composition of the luminal chitin matrix should be critical for its postulated function. \textit{verm} and \textit{serp} encode two related putative luminal chitin deacetylases with a chitin binding domain. Therefore they presented good candidates for proteins that modify luminal chitin and thereby facilitate its function in tracheal tube size control.

\textit{I) Verm and serp are required for tube length restriction}

\textit{Verm} and \textit{serp} mRNAs were expressed in all trachea cells from stage 12 onwards. Using an antibody against the C-terminus of Verm, we first detected the protein inside all
tracheal cells at stage 12. Verm was then rapidly deposited into the tracheal lumen at stage 14. Verm labeling was predominantly luminal and lining the apical cell surface of the dorsal trunk by stage 15. The protein was then cleared from the lumen at late stage 16 and was only detected along the apical surface of the tracheal cells, presumably associated with the taenidial chitin.

Both verm and serp mutant embryos display overelongation and convolutions of the main tracheal tubes. Df(3L)Exel6135 embryos, lacking 10 genes including verm and serp show more severe tube overextension phenotypes than either single mutant. verm and serp embryos labeled with anti-DE-Cad antibodies revealed overstretched cells in the dorsal trunks compared to wild type. This indicates that both verm and serp are required to halt tube elongation and coordinate apical cell extensions at late stage 15. The aggravated phenotype of embryos deficient for both proteins suggests that they may cooperate in that task.

2) verm and serp mutants affect the assembly and structure of the luminal chitin matrix

We used two chitin makers, a bacterial fluorescent chitin binding protein (ChtB) and a fluorescent chitin binding plant lectin (WGA) to visualize the luminal matrix. We found that both in verm and serp embryos the chitin structure was diffuse and radially expanded compared to the tightly packed cylindrical structure found in the wild type. TEM analysis also showed distorted orientation and assembly of the fibrils in verm mutants. These results indicate that Verm organizes intraluminal chitin packing. In addition, the luminal Verm staining was severely disrupted in kkv mutants, in which the chitinous cable is absent and the tubes dilated and convoluted. Thus, Verm and Serp are primarily required to form or preserve the tight texture of the chitinous luminal cable, and their function and localization require kkv and its product chitin.

3) The apical secretion of Verm is dependent on SJs

Mutations in the components of SJs results in overgrown tracheal tubes similar to verm and serp mutants, which prompted us to investigate the potential mechanistic link between the function of SJs and verm and serp in tube length control. verm and serp mutants do not affect SJs structure or function. However, Verm is retained inside the tracheal cells of many SJs mutants, including Atpα, sinu and lac. Furthermore, Verm
luminal abundance is strongly reduced and becomes gradually undetectable at early stage 16 in those mutants. The defect of Verm intracellular retention and luminal stabilization in SJ mutants is unlikely to reflect a general block of apical secretion, because other apical and luminal markers such as 2A12 and the luminal ZP-protein Piopio are properly deposited in the lumen at stage 15. The results demonstrate a new function for the transepithelial barrier junctions in the apical secretion of Verm and provide molecular evidence that Verm acts downstream of the SJs to regulate tube size. Protein domain deletion analysis indicated that the proper secretion of Verm is depended on its C-terminal putative chitin deacetylase domain. However, whether this domain contains an apical protein sorting signal(s) and how it is related to the SJs mediated apical secretion is still unknown.

4) The deposition and modification of the luminal chitin matrix play a central role in tracheal tube size control

The size of the epithelial tubes is critical for the function of tubular organs. The analysis of the mutants of chitin biosynthesis genes had shed light on how luminal chitin matrix contributes to tube expansion and tube length control. The formation of a transient luminal chitin-based matrix is required for the uniform expansion of the tube size. If lacking this matrix, the epithelial cells expand in an uncoordinated fashion and form dilated and overelongated tubes. The modification of the luminal chitin matrix by the putative chitin deacetylases appears to be specifically required for tube length control. The decreased Verm in SJs mutants suggests that SJs control tracheal tube size indirectly by facilitating Verm secretion and luminal chitin modification. In addition, other apical ECM components, like Piopio and Dumpy, have also been reported to be important to maintain the integrity of the small tracheal branches (Jazwinska et al., 2003). Therefore, the apical matrix is critical at multiple steps in tracheal remodeling.

Is the luminal-matrix based mechanism of tube size control conserved in other systems? Chitin is one of the most abundant biopolymers in nature (Merzendorfer and Zimoch, 2003). Chitin is present in invertebrates ranging from fungi to lobsters. Although chitin is rare in vertebrates, vertebrates do have enzymes that can synthesize short chitin oligosaccharides and express chitinase-like protein in tubular epithelial (Semino and Robbins, 1995) and tissue undergoing remodeling (Bleau et al., 1999). For example, the
mature blood vessels and lung epithelial are lined with an oligosaccharide-based ‘glycocalyx’ (Pries et al., 2000). In addition, many vertebrate tubes, like the notochord contain uncharacterized fibrillar material at their apical surface or in the lumen (Folkman and Haudenschild, 1980; Martins Mde and Bairos, 2002; Pries et al., 2000; Solursh and Morriss, 1977). Some of those molecules, such as chondroitin sulfate and hyaluronic acid, can influence related morphogenic processes such as epithelial invagination (Haddon and Lewis, 1991; Hwang et al., 2003; Lane et al., 1993). Therefore, the apical ECM containing chitin or other components may play important role in vertebrate tube morphogenesis.

A two-tier mechanism ensures efficient epidermal wound repair (Paper III)

Efficient wound healing is crucial for animal to survival from injury, especially when the damage is made to the epidermal protective barrier. The proper reconstruction of the extracellular barrier and damaged tissue repair rely on the precise coordination of epithelial responses triggered by the injury. Grh transcription factors play vital roles in efficient repairing the extracellular barrier in both flies and mice (Mace et al., 2005; Ting et al., 2005a). However, the activation mechanisms and functions of Grh factors in re-epithelialization remain unknown.

1) Stit is a direct target gene of Grh both during development and wound healing

Our bioinformatics scan of the fly genome identified a Grh-binding site cluster located in the stit second intron, and suggested that stit is a potential target of Grh. stit encodes an uncharacterized RTK, which further prompted us to test whether it is a direct target gene of Grh. We found that Grh could bind to the second intron of stit in vivo and in vitro and regulates stit expression during development. This indicated that stit is likely a direct Grh target and the second intron of stit might contain a Grh dependent regulatory element. Indeed, a 2-kb enhancer corresponding to the second intron of stit drives the expression of a GFP transgene in embryonic epidermis in a grh dependent manner. This 2-kb intronic enhancer bears 4 Grh binding sites and mutations altering the 4 Grh binding
sites remarkably reduced the epidermal GFP expression. Hence, Grh directly activates stit during development.

stit expression is also accumulated at the wound sites generated with a glass capillary needle. Consistently, the GFP expression of the Grh dependent stit-GFP reporter was robustly increased around the wounds of wild type embryos. This induction was severely reduced in grh mutants. In addition, wild type embryos carrying the stit-GFP reporter with mutated Grh binding sites barely showed any GFP induction upon wounding. Thus, Grh activates epidermal stit expression during development and wound closure.

It is important to note that stit expression is not abolished in grh mutants, which suggests that grh is not the only gene required for stit expression. In line with this, we have also identified a Grh independent 3-kb enhancer element encompassing the upstream region and the first exon and intron, of the stit gene. Furthermore, the expression of a 5-kb reporter transgene containing both the upstream region, the first exon and intron, and the second intron, is consistently weaker than the expression of the second intron reporters, suggesting that a repressor binding site present in the 5-kb enhancer region. Identifying the additional factors that together with Grh co-regulate stit expression may shed light on the general mechanism by Grh proteins regulate their targets.

2) Stit is not required for embryonic epidermal and tracheal development but is essential for metamorphosis

To analyze the function of stit, we have generated two null mutants and analyzed them for phenotypes both during development and wound repair. To our surprise, stit mutant embryos didn’t show any obvious defects in either epidermis or trachea although Stit protein was clearly detected in these tissues. We have also monitored the dorsal closure of stit embryos by live imaging. stit mutant embryos completed dorsal closure just as wild type. The lack of embryonic phenotypes is unlikely due to maternal contribution since Stit protein was reduced to undetectable levels in the mutants during the stages of phenotypic analysis. These results suggested that stit is not required for embryonic development. However, stit is not fully dispensable for development since
animals lacking stit die at late pupal stages. This lethality could be rescued by re-expressing stit in the mutants.

3) Stit coordinates the formation of the actin cable during wound re-epithelialization

To investigate stit function during wound closure, we inflicted 400-800µm² puncture wounds to wild type and stit mutant embryos expressing a GFP-moesin (GFP-moe) marker that visualizes filamentous actin and imaged them in parallel. stit mutants showed a pronounced delay in epithelial repair. 25% of the mutants show at least a threefold delay, while the rest 75% of stit mutants require double the time needed for wound closure in wild type. Thus, Stit is required for the rapid re-epithelialization of wild type embryos. We analyzed cytoskeletal dynamics in wounded wild type and stit mutants expressing the GFP-moe marker by confocal microscopy. Halfway through wound healing, GFP-moe visualized a pronounced actin cable around the wound edge of wild type embryos. Strikingly, in stit mutants the GFP-moe ring was either discontinuous or showed severely reduced intensity. We calculated the Relative Fluorescence Intensity (RFI) of GFP-moe at the wound edge for each embryo at defined time points of the wound closure period. The rate of cytoskeletal RFI increase during wound closure was much reduced in stit mutants compared to the wild type. Also, the RFI at the wounds of stit mutants was significantly lower during the second half of the wound healing process compared to wild type. Thus, Stit coordinates the formation of the actin cable at the wound perimeter during re-epithelialization.

4) stit encodes a putative RTK that can activate ERK

stit encodes a putative RTK that resembles members of the Ret oncogene RTK family (Runeberg-Roos and Saarma, 2007). RTK activation normally depends on ligand-induced dimerization and autophosphorylation of tyrosine(s) in the intracellular domain (McKay and Morrison, 2007). To test whether Stit could function as a RTK, we expressed a series of wild type and mutated version of Stit in S2 cells, and checked whether they can be autophosphorylated. An artificially dimerized Stit version (λStit) and wild type Stit undergo ligand-independent autophosphorylation, while a mutated StitK504A protein lacking the lysine necessary for the predicted kinase activity doesn’t display phosphorylation on tyrosine. This indicates that Stit itself is a tyrosine kinase.
RTK activation typically leads to phosphorylation of members of the MAPK family. We tested whether Stit can activate ERK in vivo using transgenic strains expressing either stit or the kinase-defective stit\(^{K504A}\) in stripes of the embryonic epidermis. Indeed, Stit but not the mutated version produced ectopic tyrosine and ERK phosphorylation in stripes. Thus, Stit can activate ERK, a common RTK effector. In summary, Stit is a wound-responsive, tyrosine kinase that can induce ERK activation in vivo.

5) Stit amplifies the wound response through a Grh mediated positive feedback loop

ERK activation is rapidly detected around the wounds and required for the efficient induction of the Grh-dependent epithelial barrier repair genes Ddc and ple upon wounding. Is Stit also required for ERK activation at wound sites? We pierced wild type and stit mutants and co-stained for di-phosphorylated ERK (dpERK) and Fas III to visualize surrounding epidermal cells. dpERK staining is not detectable in epidermal cells of stage 16 wild type embryos. Wounding induced a robust dpERK signal extending 2-3 cell diameters around the puncture sites of wild type embryos. By contrast, in stit mutant embryos, this dpERK signal was weaker and restricted to cells immediately adjacent to the edge. Furthermore, we also found that both Ddc and ple induction were much limited in stit mutants, in agreement with the decreased activation of ERK upon wounding. Therefore, Stit regulates ERK phosphorylation and Grh-dependent transcriptional activation of cuticle repair genes.

It has been reported that Grh is activated by RTK signaling during development and can be phosphorylated by ERK in vitro (Hemphala et al., 2003; Liaw et al., 1995). Combined with our results, it suggests that Stit may trigger a signaling cascade that activates ERK, which in turn phosphorylates Grh protein and activates Grh-mediated genes expression. In line with this hypothesis, Ddc mRNA was ectopically induced by stit misexpression in epidermal stripes and this induction was abolished in grh mutants. The genetic analysis places stit both downstream and upstream of grh, suggesting there is a positive feed back loop mechanism in wound repair. Because stit is induced at wound sites, we tested whether the Grh-dependent stit-GFP induction requires endogenous Stit. In contrast to the robust GFP induction in wild type embryos, almost no change of stit-GFP expression was detected in wounded stit mutants. Accordingly, stit-GFP expression
was ectopically induced in embryos overexpressing Stit, and this increase was abolished in grh mutants. Thus, the endogenous Stit is required for the transcriptional up regulation of stit at the wound. Our functional analysis of stit suggests a two-tier mechanism in wound healing. Epidermal injury activates Stit and likely also other receptors to initiate cuticle repair gene expression and cytoskeletal rearrangements. Stit further induces its own expression and a second tier of responses through a Grh-dependent positive feedback loop. The proposed amplification mechanism ensures efficient epidermal wound repair and relies on transcriptional regulation without the need of further injury stimulation.

6) How is Stit activated upon wounding?

Several growth factors, including FGF, EGF, Transforming growth factor alpha (TGF-α), and keratinocyte growth factor (KGF), are released at the wound site (Martin, 1997). It is possible that such growth factors released at wound sites serve as ligand(s) to quickly activates Stit and other RTKs at the wound edges. The rupture of the epithelium may expose epidermal cells to growth factors circulating in the hemolymph. Such a mechanism may allow epithelial RTKs to bind to normally latent ligands, like PVF and become activated. A recent report has proposed this mechanism for the activation of the larval wound healing (Wu et al., 2009). A similar mechanism may provide access to a yet unidentified Stit ligand and facilitate Stit activation during embryonic wound healing. Damage on epidermal cells upon wounding can lead to leakage of small molecules, such as Ca$^{2+}$, which might trigger the activation of Stit in the wound edge cells (Woolley and Martin, 2000). Disruption of an epithelial layer instantaneously also generates endogenous electric fields, and recently, it has been reported that electrical signals control wound healing through phosphatidylinositol-3-OH kinase-γ (PI(3)Kγ) and phosphatase and tensin homolog (PTEN) (Zhao et al., 2006). It is possible that similar electrical signals may activate Stit upon wounding.

At the time of wounding, the epithelium also receives several mechanical cues that may also be part of the mechanism of Stit activation upon wounding. There is good evidence indicating that mechanical forces play an important role during dorsal closure and gastrulation in the fly embryo (Farge, 2003; Hutson et al., 2003; Kiehart et al., 2000). The epidermis is under tension during development, and wounding will result in changes
of tension in all epithelial cells. This change in tension may activate Grh-dependent or independent transcriptional responses during wound healing. In mammalian adult wound healing, mechanical force, combined with growth factors such as TGFβ1, are believed to be the signals that trigger the conversion of fibroblasts into myofibroblasts at a wound site (Grinnell, 1994).

In addition, Stit might be activated by formation of cis homodimers without the need of a ligand(s). Stit contains an extracellular Cadherin domain and therefore may share some properties with cadherin adhesion molecules (Tepass et al., 2000). It has been shown that cadherins can form both cis and trans homodimers. Stit is partially localized on the cell membranes both on the apical surface and adherens junctions. Is it therefore possible that during development, Stit proteins form trans homodimers between neighbouring cells and remains inactive? However, upon wounding, the loss of neighbouring cells may provide a chance for Stit proteins to form the cis homodimers along the membrane of the cells at the wound edges. Since overexpression of wild type Stit alone can induce autophosphorylation and ERK activation, formation of Stit cis homodimers within the same cell might be sufficient to activate signaling.

Alternatively, Stit may be activated by its subcellular re-localization upon wounding. Antibody stainings of wild type embryos localized the Stit protein at the apical surface of all ectodermal epithelial tissues. Observation of double stainings for Stit and the membrane maker CAAX-GFP reveals that a substantial fraction of Stit protein is also found underneath the apical membrane, likely in some vesicles. Wounding may relocate Stit and increase its local levels either at the plasma membrane or in the intracellular vesicles and thereby activate signaling without the need of a specific ligand.
Conclusions and Perspectives

We have identified 10 grh direct targets by combining bioinformatics and genetics. Most of these targets encode proteins involved in chitin assembly or modification, indicating that a major function of Grh during development is to directly regulate the expression of genes involved in cuticle maturation. Furthermore, several genes function in tracheal tube size control by affecting the assembly or modification of luminal chitin matrix, and their expression are altered but not abolished in grh mutants. This suggests that grh controls tracheal tube size by directly modulating the expression of multiple targets involved in chitin biosynthesis or modification in the lumen of tracheal branches.

Two of the chitin-related Grh targets, verm and serp, encode similar putative luminal chitin deacetylases. The functional analysis of verm and serp identifies an important role of luminal matrix modification in limiting tube elongation. Our results, together with the previous discovery that the assembly of a luminal chitin-based matrix is required for the uniform expansion of tube diameter, suggest a new model of tube size control in Drosophila trachea. According to this model, the assembly and growth of the chitin-based luminal matrix is first required to coordinate uniform radial expansion of the tubes. Subsequent modification of luminal chitin by the secreted chitin deacetylases is specifically required to limit the tube elongation. The compromised Verm secretion in SJ mutants suggests a new function of SJ in secreted protein secretion, and plays SJs in a mechanistic pathway for tracheal tube size control. A unifying model for tube growth regulation by changes in the pressure exerted to the epithelium by intraluminal matrices or intraluminal liquid may apply in the growth regulation of different types of tubes in both invertebrates and vertebrates.

Grh also directly controls the expression of stit, which encodes a Ret family RTK, both during development and wound healing. Stit functions in a two-tier mechanism that ensures efficient epidermal wound repair. Through an unknown mechanism Stit activation coordinates the formation of an actin cable around the wound edges during re-epithelialization. Stit also rapidly induces ERK phosphorylation at the wound sites upon injury. Furthermore, Stit activates wound repair genes and its own
expression at the wound site in a Grh-dependent manner. This Grh mediated positive feed back loop ensures efficient epidermal wound healing in Drosophila embryos.

In addition, Grh directly activates the embryonic epidermal expression of PGRP-LC gene. PGRP-LC encodes a receptor for the Imd pathway that is required for inducing AMPs expression both in fat body and epithelial protective barriers upon infection. Furthermore, overexpression of Grh is sufficient to activate the expression AMP reporter CecA-LacZ in the embryonic epidermis. This raises the possibility that grh is not only required for the formation and repair of the barrier epithelia but also for local antimicrobial defense.

Although we identified several grh direct targets, we may miss many. More unbiased methods may help to identify all grh direct targets in Drosophila. We are combining Chip followed by deep sequencing (ChIP-Seq), expression microarrays and bioinformatics to isolate all Grh direct targets. We have got ChIP-Seq results form the mouse monoclonal anti-Grh antibody and are analyzing the data. Meanwhile, we are also trying to investigate Grh function in local immune response in the barrier epithelia and the potential molecular link between wound healing and local immune response.

The elucidation of the mechanism by which Stit is immediately activated upon wounding, and the identification of downstream Stit effectors that coordinate the actin cable around wound edges during re-epithelialization are important future goals.
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