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From Cell Shape to Body Shape: Epithelial Morphogenesis in *Drosophila melanogaster*

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2008

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About the cover: Segmental grooves in the *Drosophila* embryo.

Longitudinal cross-section through the epidermis of a *Drosophila* embryo at the time of segmental groove morphogenesis. Formation of segmental grooves is associated with the apical constriction and inward movement of a single row of cells, termed groove founder cells, in each segment. In the image, groove founder cells have been highlighted in green. The process of segmental groove morphogenesis is the main focus of this thesis. The cover shows a digitally modified version of a laser-scanning confocal micrograph.

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To Felicia, Leo, Paula, Riqui, and Runo

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Abbreviations

AMG	Anterior Midgut
AJ	Adherens Junction
A/P	Anterior-Posterior
Arp	Actin related protein
CR	Conserved Region
C-terminal	Carboxy-terminal
DAD	Diaphanous Auto-inhibitor Domain
DAG	Diacylglycerol
DH	Dbl-Homology
DRF	Diaphanous Related Formin
D/V	Dorsal-Ventral
EGFR	Epidermal Growth Factor Receptor
EGFP	Enhanced Green Fluorescent Protein
FH	Formin-Homology
GAP	GTPase Activating Protein
GDB	GTPase Binding Domain
GDI	Guanine nucleotide Dissociation Inhibitor
GDP	Guanosine Diphosphate
GEF	Guanine nucleotide Exchange Factor
GPCR	G Protein-Coupled Receptor
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
Hox	Homeotic selector
MHC	Myosin Heavy Chain
MLCP	Myosin Light-Chain Phosphatase
MRLC	Myosin Regulatory Light-Chain
MT	Microtubule
N-terminal	Amino-terminal
PDZ	PSD-95/Dlg/ZO-1
PH	Pleckstrin Homology
PMG	Posterior Midgut
PS	Parasegment
RGS	Regulator of G protein Signaling
ROCK/Rok	Rho kinase
UAS	Upstream Activating Sequence
VF	Ventral Furrow
WASP	Wiskott-Aldrich Syndrome Protein
WAVE	Wasp-family Verprolin-homologous protein

List of Papers

The thesis is based on the following papers, which are referred to in the text by their Roman numerals:

Mulinari, S., Padash Barmchi., M. and Häcker, U. *DRhoGEF2* and *Diaphanous* regulate contractile force during segmental groove morphogenesis in the *Drosophila* embryo. *Molecular Biology of the Cell*, 19, 1880–1889 (2008).

Mulinari, S., and Häcker, U. Functional dissection of the *Drosophila* Rho Guanine Nucleotide Exchange Factor DRhoGEF2. Manuscript.

Mulinari, S., and Häcker, U. Role of Hedgehog during segmental groove formation in the *Drosophila* embryo. Manuscript.

1 Introduction

Multicellular organisms exist in an amazing variety of shapes and sizes. The species-specific body plan of an organism is created by the precisely orchestrated arrangement of tissues and organs during embryonic development. This arrangement, in turn, is powered by dynamic and coordinated changes in the shape of individual cells. During the course of development, cells can take on a broad spectrum of morphologies, ranging from the complex branched shape of neurons, to the columnar architecture of epithelial cells, which arrange themselves into polarized sheets that line the inner and outer surfaces of the body. A fundamental question in the field of developmental biology is how such morphological order is created in a highly reproducible fashion – both at a cellular and organism level? In other words, what determines the shape and positions of various cell types, tissues, and organs in the body plan of different species? To answer this question one must turn to the study of morphogenesis, which is concerned with the processes that shape the species-specific body plan during development. In the present thesis, we have used the fruit fly *Drosophila melanogaster* as a model system to study some of the molecular mechanisms that control tissue morphogenesis during development.

From a historical perspective, research in the field of development biology has been particularly successful in identifying transcription factors and signaling molecules that subdivide the developing organisms into regions of specific gene activities. One of the great achievements of developmental biology was the discovery of a set of evolutionary conserved signaling pathways that spatially and temporally control cell fate during development. By contrast, the connection between these signaling pathways and the effectors that execute developmental programs during morphogenesis have been difficult to uncover. Instead, insight into the molecular mechanisms controlling cell morphology first emerged from pioneering work in the field of cell biology. Early studies in the 1940–50's discovered a system of interconnected fibers, the cytoskeleton, that could be observed inside cells in the electron microscope, as crucial for the establishment of cellular shape (Frixione, 2000). During subsequent years, cell biologists and biochemists successfully characterized the function of many constituents and regulators of the cytoskeleton including two conserved proteins, Actin and Myosin. However, this did not resolve the question how the cytoskeleton was regulated during morphogenetic processes in the embryo (Schöck and Perrimon, 2002). Only more recently has the availability of advanced imaging tools and genetic techniques enabled cell and developmental biologists to interface and advance our understanding of the molecular basis of morphogenesis. Amongst the different model organisms

available to cell and developmental biology, the fruit fly *Drosophila* offers a particularly attractive system to study regulation of the cytoskeleton. Flies have a rapid life cycle, they can be handled and bred with relative ease in a cost effective fashion, and limit the use of higher animals in experimental research. In addition, a broad range of tools for gene manipulation are available in the fly system and genetic redundancy that is often a problem in higher animals is negligible. Consequently, this genetically tractable model organism has become the system of choice for many researchers interested in the molecular mechanisms of morphogenesis.

The *Drosophila* model system can look back on a century long history of successful research (Ashburner, 1993; Rubin and Lewis, 2000). It was in *Drosophila* that Thomas H. Morgan and his co-workers discovered that genes carried on chromosomes are the physical basis of heredity, a true milestone in the history of biology. In 1933, Thomas H. Morgan was honored with the Nobel Prize in Physiology or Medicine for this groundbreaking discovery. During the early days of *Drosophila* genetics research, many genes were identified on the basis of adult phenotypes that resulted from spontaneous mutations in a locus. However, thanks to the seminal work of another Noble Prize laureate, Hermann J. Muller, a former student in the Morgan lab, it soon became evident that the mutation rate could be increased by means of X-ray irradiation (Muller, 1927; Rubin and Lewis, 2000). In what might appear today as a somewhat ironic turn of events, these early revolutionizing discoveries in *Drosophila* created a division of genetics and embryology into two distinct fields of research (despite the fact that many early geneticists, including Morgan, were embryologists by training). Genetics, on the one hand, became concerned with the basis of inheritable differences between individuals, observed by easily detectable adult traits. Embryology, however, was focused on the reproducibility of development within species. The different approaches are exemplified by the words of embryologist Ernest E. Just in 1937, quoted by Scott F. Gilbert (Gilbert, 2003): "embryologists were interested in how a fly forms a back, not in the number of bristles on its back". The separation of the fields of genetics and embryology was further reflected in the respective experimental system of choice. For a long time, *Drosophila* remained the favorable system of geneticists. Embryologists tended to study amphibians, sea urchins, and chicken, all animals with large eggs whose cells could be transplanted (Gilbert, 2003).

Despite some early and important attempts, particularly by *Drosophila* researcher Don Poulson, it was not until the late 1960's that genetics and embryology came together, catalyzed by new techniques and findings in molecular biology, to create the field of modern developmental biology (Ashburner, 1993). This new synthesis, as described by Michael Ashburner, culminated in three historical events that revolutionized the world of *Drosophila* research.

The first was the successful attempt by Christiane Nüsslein-Volhard and Erich Wieschaus to systematically identify and categorize all genes involved in embryonic segmentation through large-scale, genome saturating mutagenesis screens (Nusslein-Volhard and Wieschaus, 1980). In recognition of their discoveries regarding the genetic control of early embryonic development, Nüsslein-Volhard and Wieschaus shared the Nobel Prize in Physiology or Medicine in 1995. The second breakthrough came with the introduction of techniques that enabled the making of stable transgenic flies by use of transposable element vectors, a method developed by Allan C. Spradling and Gerald M. Rubin (Rubin and Spradling, 1982; Spradling and Rubin, 1982). The third seminal event was the work of Michael E. Akam and John R. Carlson on the development of an *in situ* hybridization method that allowed the direct spatial and temporal visualization of RNA distributions in tissues (Akam, 1983; Akam and Carlson, 1985).

More recently, at the turn of the millennium, another great achievement was presented, the completion and annotation of the *Drosophila melanogaster* genome sequence (Adams *et al.*, 2000). The availability of the genome sequence has greatly facilitated research in the field. The deciphering of the *Drosophila melanogaster* genome has been followed by the completion of several other eukaryotic genomes, most notably the human genome in 2001 (McPherson *et al.*, 2001). An important realization accompanying these sequencing efforts has been that a majority of essential *Drosophila* genes have orthologs in higher animals, such as humans. Conversely, many disease genes in humans have apparent orthologs in the fly genome. Significantly, experimental evidence suggests that this conservation on the genetic level is often paralleled by a functional conservation on the protein level. In many instances, conserved proteins are organized into universally redeployed signaling networks that collectively regulate a set of fundamental biological and developmental processes such as cell division, cell differentiation, growth, and morphogenesis that have been retained throughout evolution.

The focus of this thesis is on one such conserved signaling network that involves members of the Rho-family of GTPases. Signaling through Rho-family GTPases drives reorganization of the Actin-based cytoskeleton in eukaryotic cells, thus providing the driving force for morphogenesis. This thesis is specifically concerned with the question how signaling through Rho-family GTPases drives morphogenesis of epithelial tissues during *Drosophila* embryogenesis. Importantly, the mechanisms of cytoskeletal regulation investigated here are conserved throughout the animal kingdom and play an essential role in the development of all animals. Our findings thus contribute to a better understanding of how the shape of individual cells is regulated and how changes in cell morphology shape the body plan of the organism.

This thesis can be subdivided into six parts. The first part: chapters 2–4 provide an overview and background to the development of the *Drosophila* embryo from both a morphological and molecular perspective. The second part: chapter 5 reviews the regulation of the Actin-based cytoskeleton by Rho-family GTPase signaling pathways, from a general viewpoint. The third part: chapters 6 and 7 address the specific roles of these signaling pathways during epithelial morphogenesis in the *Drosophila* embryo. Chapter 7 specifically focuses on the role of one particular factor, DRhoGEF2, whose function has been analyzed in detail in this thesis. The following chapters, 8 and 9, briefly describe important genetic techniques applied in this work and provide a summary of the aims and results of the individual papers on which the thesis is based. The fifth part, which is comprised of chapter 10, contains some concluding remarks to the present study. Finally, in the closing chapter of the thesis, chapter 11, a popular scientific summary of the work is presented in Swedish.

2 The *Drosophila melanogaster* Life Cycle

The fruit fly *Drosophila melanogaster* is a holometabolous insect that belongs to the Diptera order of Arthropods. The *Drosophila* life cycle is summarized in Fig. 1. Development from egg to adult takes approximately 10 days at 25° C and includes four distinct developmental phases: egg, larva, pupa, and adult (imago). Embryonic development lasts for about 22–24 hours, after which a feeding larva hatches from the egg. After hatching, larvae undergo three successive stages – referred to as larval instars – L1, L2 and, L3, which last for about 24 hr, 24 hr, and 48 hr, respectively. At the end of the third instar, larvae cease to feed and enter a wandering stage in search of a suitable site for pupation. Over the next four days, the larval tissues are histolysed and the adult body plan is established from imaginal discs in a process known as metamorphosis. On the fifth day, flies eclose from their pupal cases. Newly emerged flies become fertile within about 4 hours.

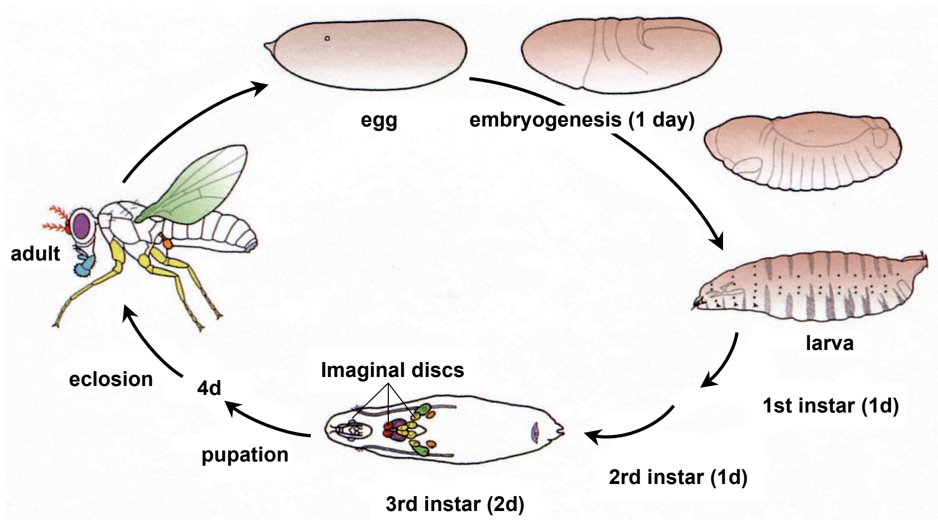

Figure 1. The *Drosophila* melanogaster life cycle. Development from fertilized egg to adult takes approximately 10 days. Morphogenesis and differentiation of imaginal discs to form adult tissues occurs during the pupal stage, before eclosion. Modified from (Carroll *et al.*, 2001).

3 Development of the *Drosophila* Embryo

The mature egg of *Drosophila* has an average length of 500 µm and a diameter of 180 µm and is enclosed by two envelopes, an inner impermeable vitelline membrane and an outer opaque chorion that protects the egg from mechanical damage. As the egg passes down the oviduct, the sperm enters the egg through a hole in the anterior of the chorion, called the micropyle. Following fertilization, the egg initiates embryonic development and is deposited by the female on a substrate. Within about a day, the fertilized egg – or zygote – is transformed into a viable larva with defined body axes and functional organs. The transformation from zygote to larvae relies on a remarkable spatial and temporal coordination of cell proliferation and tissue morphogenesis, coupled to the progressive sub-divisions of the embryo into successively smaller units that can be patterned separately. The following section provides a general overview of major events during *Drosophila* embryogenesis, with emphasis on the morphogenesis of epithelial tissues. For a timeline of *Drosophila* embryogenesis as well as embryonic stages according to (Campos-Ortega and Hartenstein, 1997), see Fig. 2.

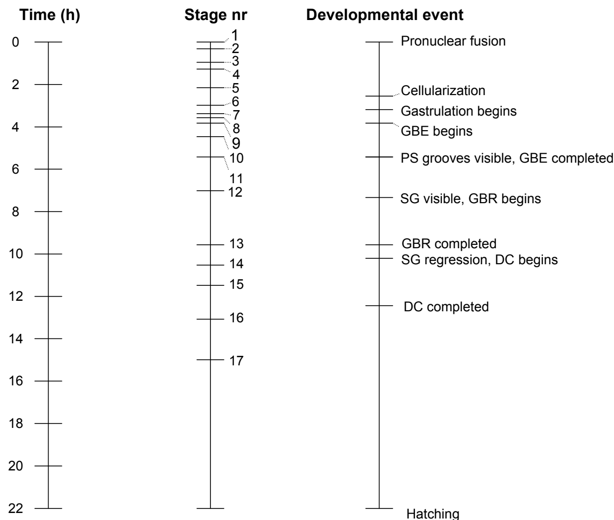

Figure 2. Timeline of *Drosophila* embryogenesis. Development of the *Drosophila* embryo takes approximately 22 hours, and is subdivided into 17 stages, each characterized by specific morphological features (Campos-Ortega and Hartenstein, 1997). Some important developmental events that are referred to in the text are indicated to the right. (GBE) germ band extension; (PS grooves) parasegmental grooves; (GBR) germ band retraction; (SG) segmental grooves; (DC) dorsal closure.

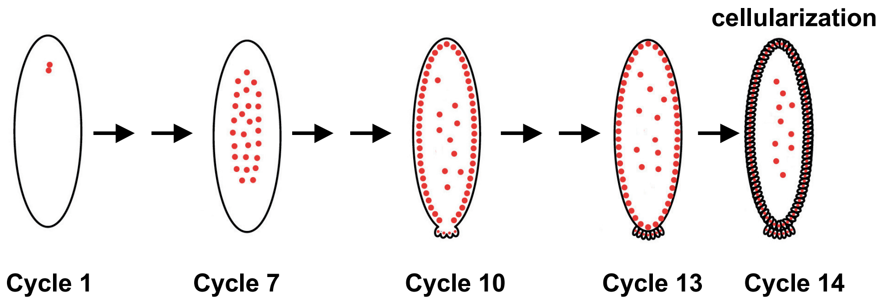

Figure 3. Syncytial divisions and cellularization. The first seven syncytial division cycles take place in the interior of the embryo. At cycle 10, most nuclei have reached the margin of the embryo, leaving behind a small number of yolk cell nuclei in the central region. At the posterior pole of the embryo, pole cells are formed. Subsequently, peripheral nuclei undergo four additional rounds of divisions after which they become entirely enclosed by cell membranes during cellularization. Anterior is up.

3.1 Early Development

3.1.1 Syncytial divisions and pole cell formation

After fertilization, the zygote undergoes 13 synchronous nuclear divisions without cytokinesis (Fig. 3) (Foe and Alberts, 1983; Campos-Ortega and Hartenstein, 1997). The first seven divisions are rapid and take place in the central portion of the embryo. During the course of the next three divisions, most nuclei migrate to the periphery, where mitoses continue. After the eighth division cycle, 3–4 nuclei reach the surface at the posterior pole of the embryo. These nuclei undergo two successive divisions, after which they become enclosed by cell membranes and form the pole cells that give rise to the gametes of the adult. Most of the other nuclei reach the periphery of the embryo at cycle 10. They undergo four more divisions, albeit at progressively slower rates, to form the syncytial blastoderm embryo. The latter consists of approximately 5 000 nuclei aligned in a monolayer at the periphery, all contained within a common cytoplasm. The zygotic genome remains transcriptionally silent throughout most of this early phase of development. However, as the nuclei undergo the last four rounds of syncytial divisions, zygotic transcription is initiated, increasing in level with each successive division cycle.

3.1.2 Cellularization

In the interphase of nuclear cycle 14, the plasma membrane of the embryo grows in radially between individual nuclei to enclose each of them into a single columnar cell (Fig. 3) (Mazumdar and Mazumdar, 2002). This creates a single-layered, cellular blastoderm embryo in a process known as cellularization. During cellularization, blastoderm cells acquire an epithelial character,

with a typical apical-basal polarity and a subdivision of the plasma membrane into discrete domains (Tepass *et al.*, 2001). The first phase of cellularization is characterized by slow ingression of cell membranes in between the nuclei to form a furrow canal. After the furrow canal has passed the level of the nuclei, the second phase of cellularization commences. During this second phase, the rate of membrane invagination doubles as the furrow canal grows further inward and expands to close the newly created individual cells basally. Towards the end of cellularization, the embryo consists of a uniform symmetric epithelium without clear regional differences in cell shapes or cell seizes, with the exception of a group of about 30 pole cells positioned at the posterior end of the embryo (Fig. 3). Gastrulation, the next phase of embryonic development, reorganizes this single-layered, columnar epithelium into a three-dimensional body plan.

3.1.3 Gastrulation

Gastrulation defines the morphogenetic events that position the three germ layers – endoderm, mesoderm, and ectoderm – with respect to the body plan of the embryo. In *Drosophila*, the endoderm, which is the most internal germ layer, forms the lining of the midgut. The ectoderm, the most exterior germ layer, forms several tissues, including epidermis, nervous system, and the most anterior and posterior sections of the gut. The mesoderm, the middle germ layer, develops into cell types such as muscle and fat body (Campos-Ortega and Hartenstein, 1997).

Gastrulation begins approximately three hours after egg laying. It is characterized by a reproducible series of cell shape changes that ultimately lead to complete invagination of the mesodermal and endodermal primordia into the interior of the embryo (Fig. 4) (Kam *et al.*, 1991; Sweeton *et al.*, 1991; Leptin, 1995). The prospective mesoderm internalizes from the ventral side of the embryo, forming a transient structure known as the ventral furrow (VF). The VF encompasses a band of approximately 20 cells along the anterior-posterior (A/P) axis of the embryo, not including the terminal areas. To the anterior, the VF is delimited by the cephalic furrow that separates the thorax and head regions. After internalization through the VF, mesodermal cells undergo an epithelial to mesenchymal transition and migrate dorsally to form a monolayer under the overlying ectoderm (Leptin, 1999).

A few minutes after the VF has begun to invaginate, a similar sequence of events occurs in the posterior midgut (PMG) primordium, which is located at the posterior pole of the embryo. These posterior endodermal cells will later fuse with cells derived from the anterior midgut (AMG) to form the midgut epithelium. As the PMG invaginates, the posterior end of the embryo is pushed dorsally and anteriorly on the dorsal side by the force generated by germ band extension (Leptin, 1995), a process driven by cell intercalations

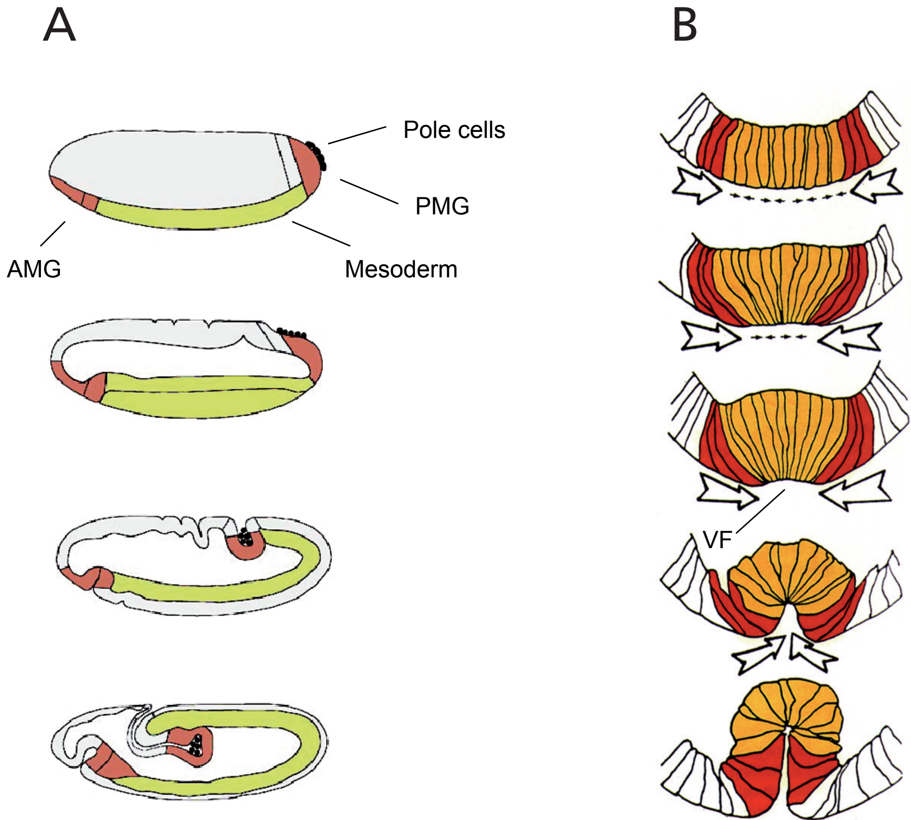

Figure 4. Gastrulation in *Drosophila*. (A) Diagrams of whole embryos indicating location of the mesodermal, endodermal, and ectodermal primordia. Prior to gastrulation the primordia lie in one plane adjacent to each other at the surface of the embryo (top). Gastrulation is initiated when the prospective mesoderm begins to internalize from the ventral surface of the embryo (second embryo). This is followed by invagination of the endodermal posterior midgut (PMG) primordium, which is simultaneously pushed in an anterior and dorsal direction by the extending germ band (third embryo). Gastrulation is completed when the anterior part of the endoderm, the anterior midgut (AMG) primordium, has invaginated (bottom embryo). (B) Cartoon of transverse sections through the ventral epithelium at different time points during mesoderm invagination. Invagination of the mesoderm is driven by apical constriction of mesodermal cells (arrows) that results in a bending of the ventral epithelium, creating a deep groove known as the ventral furrow (VF). Modified from (Leptin, 1999).

(Irvine and Wieschaus, 1994; Bertet *et al.*, 2004) and oriented cell divisions (da Silva and Vincent, 2007) in distinct areas of the ectoderm.

Gastrulation is completed when the germ layers have become separated from each other. The embryo is then ready to enter the next phase of development. Essentially, all later morphogenetic events operate to refine the original body plan set up during gastrulation. This fundamental aspect of animal development (and maybe even more so of students of animal development) is

epitomized in the well-cited words of developmental biologist Lewis Wolpert: "It is not birth, marriage, or death, but gastrulation, which is truly the most important time in your life."

3.2 Late Development

3.2.1 Invagination of the salivary gland primordia and formation of the tracheal pits

The germ band (the visibly metameric region of the embryo) reaches a fully elongated state approximately six hours into development when the posterior tip of the germ band reaches approximately 75 percent egg-length, where zero percent egg-length is defined as the posterior pole of the embryo. At this stage, two bilateral populations of ventral cells located in the anterior ectoderm initiate invagination to eventually give rise to the larval salivary glands (Campos-Ortega and Hartenstein, 1997). Around the same time, ten tracheal pits become visible in the germ band, which will later fuse to create the respiratory system of the larva. Both processes are driven by intricate changes in epithelial cell shape, including apical cell constriction. Following invagination of the salivary gland primordia and tracheal pits, the surface ectoderm differentiates into epidermis.

3.2.2 Parasegmental and segmental grooves

The body plan of insects and that of higher animals is segmented to allow controlled patterning and growth in well-defined compartments (Vincent, 1998). The segmental organization of Arthropods is also important for locomotion that is enabled by the bending of adjacent segments (Deutsch, 2004). In *Drosophila*, which belongs to the long germ band insects, all segments form nearly simultaneously (Gilbert, 2003). The subdivision of the body plan into metameric units at the molecular level occurs in two steps. First, towards the end of germ band extension, parasegmental grooves become apparent as transient furrows in the ectoderm (Campos-Ortega and Hartenstein, 1997). The interval between two parsegmental grooves defines a parasegment (PS). PS are out of register with the definitive segments that form slightly later, following the onset of germ band retraction (see below). These definitive segments can be morphologically distinguishable as periodic grooves – so-called segmental grooves – that form in a position slightly posterior to parasegmental grooves (Fig. 5). Formation of segmental grooves occurs first in thoracic segments and gradually proceeds towards more posterior segments in the course of germ band retraction. Furthermore, segmental grooves form first in the lateral epidermis with grooves appearing in the ventral epidermis only after the germ band is fully retracted. Detailed analysis of cellular behavior

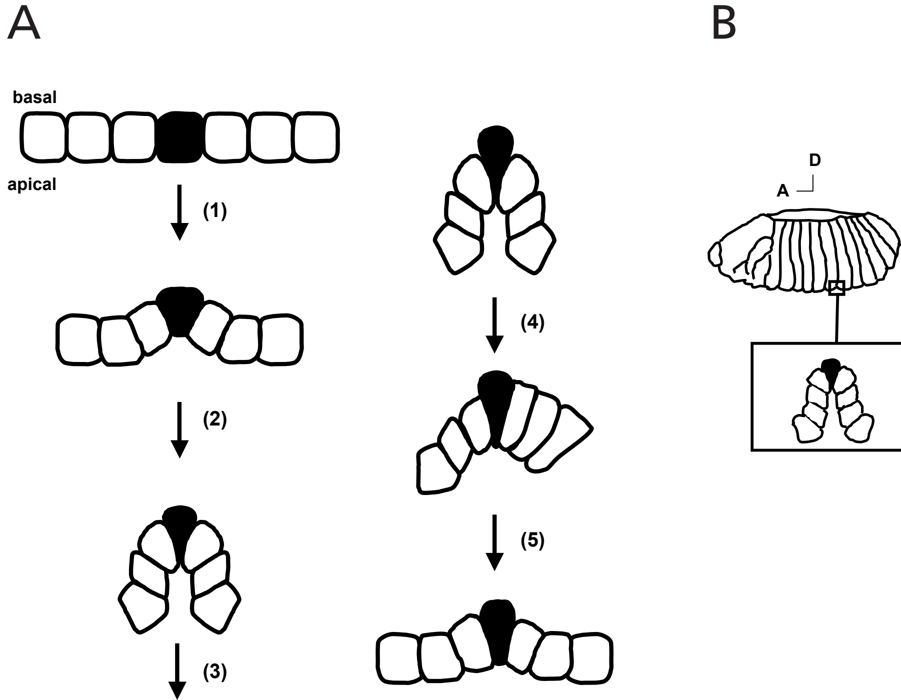

Figure 5. Morphogenesis of segmental grooves. (A) Schematic representation of morphological changes during segmental groove morphogenesis (lateral view). (1) Invagination begins during stage 12, when one row of cells in each segment – the groove founder cells – exemplified by the black cell, undergo apical constriction; (2) Continued apical constriction of groove founder cells causes grooves to deepen; (3) This is followed by apical-basal elongation of groove founder cells; (4) Subsequently, during stage 13, segmental grooves start to regress. Regression is associated with apical constriction of cells located posteriorly adjacent to the groove. In parallel to constricting their apices, cells elongate their apical-basal axis; (5) As regression is completed, only shallow indentations remain in the epidermis. (B) Embryo at stage 13, showing the morphology and location of segmental grooves in the epidermis. D: Dorsal, A: Anterior.

has revealed that segmental groove morphogenesis is associated with distinct morphologic changes in groove founder cells, including apical constriction followed by apical-basal elongation (Fig. 5) (Larsen *et al.*, 2003: Mulinari *et al.*, 2008). Segmental grooves are dynamic structures; shortly after grooves reach their maximum depth, they begin to regress, first on the ventral side followed by more lateral regions. Ventral grooves regress completely whereas shallow lateral segmental grooves will remain throughout embryogenesis. During groove regression, cells located immediately posterior to segmental grooves undergo stereotyped changes in cell shapes that may contribute to the outward movement of groove founder cells (Fig. 5) (Mulinari *et al.*, 2008).

3.2.3 Germ band retraction, dorsal closure, and head involution

When the germ band reaches its fully extended state, cells that are destined to form posterior-most structures are located just behind the future head region. The process of germ band retraction restores the normal A/P sequence of tissues in the larva and brings the posterior tip of the germ band to the posterior tip of the embryo (Campos-Ortega and Hartenstein, 1997). Following germ band retraction, a hole is left on the dorsal side of the embryo, which is occupied by the large, flat cells of the amnioserosa. Dorsal closure of the embryo occurs by dorsally directed extension of the lateral epidermis on both sides of the embryo. The two extending sheets of the epidermis move over the constricting amnioserosa and meet along the dorsal midline, completely sealing the hole off (Jacinto *et al.*, 2000). Simultaneously, involution of head structures takes place at the anterior of the embryo. As these two processes are completed, the period of extensive morphologic rearrangements in the embryo is brought to an end.

3.2.4 Secretion of the larval cuticle

At the end of embryogenesis, epidermal cells secrete a chitinuos cuticular sheet that forms the exoskeleton of the larva. Rows of epidermal cells secrete different types of cuticle depending on their respective position in the embryo. Some cells secrete a smooth cuticle, whereas others secrete hairs or denticles of various shapes and sizes. On the ventral side of the abdominal epidermis, six denticle-secreting cell rows in the anterior of each segment are followed by six smooth cuticle-secreting rows (see Fig. 8). This creates an alternating, segmentally repeated pattern of cuticle deposits in the larval epidermis. To make a proper cuticle, the epidermis must retain its integrity and morphogenetic movements such as ventral furrow formation, germ band retraction, dorsal closure, and head involution must occur properly. Because of its sensitivity to alterations in the underlying epidermis, the *Drosophila* larval cuticle has served as an excellent readout system in numerous genetic screens for genes involved in embryonic patterning and morphogenesis (e.g. Nusslein-Volhard and Wieschaus, 1980; Jürgens *et al.*, 1984; Perrimon *et al.*, 1989; Perrimon *et al.*, 1996).

4 Patterning of the *Drosophila* Embryo

Cells in a developing multicellular organism acquire different developmental fates depending on their relative positions with respect to the body axes. Thus, in the *Drosophila* embryo, cell populations located in the anterior of the embryo develop into head structures whereas cell populations located in the posterior develop into tail structures. Acquisition of different cell fates during embryonic development relies on the activities of specific genes in discrete regions of the embryo, for example, expression of certain genes in the anterior but not posterior of the embryo. In the case of *Drosophila*, the genetic program that subdivides the embryo into discrete regions of gene expression is well characterized. Along the A/P axis, patterning relies on a hierarchy of gene activities that, in a step-wise manner, act to partition the embryo into repeated units, the PS, with only the most terminal areas of the embryo remaining unsegmented (Fig. 6). The boundary between each PS constitutes a signaling center – an organizer – that specifies cell fates across the PS. Finally, the superimposed action of homeotic selector (Hox) genes determines the identity of each PS. Patterning along the dorsal-ventral (D/V) axis similarly involves a subdivision of the body axis into regions of specific gene activities. As a result of the activity of patterning genes along the D/V and A/P axes, a coordinate system is created over the entire embryo and within each segment. Cells interpret this coordinate system and respond by adopting particular developmental fates and behaviors depending on their respective positions in the embryo.

4.1 The Anterior-Posterior Axis

4.1.1 The segmentation cascade

The A/P polarity of the *Drosophila* embryo is determined during oogenesis. The ovarian nurse cells deposit mRNA and protein into the oocyte, thus providing the future embryo with the information required to initiate development and axial patterning (Nasiadka *et al.*, 2002). Genes that are expressed during oogenesis and whose products function in the development of the embryo are known as maternal effect genes (Fig. 6). Two important maternal effect genes are *bicoid* (*bcd*) and *nanos* (*nos*). During oogenesis, *bcd* and *nos* mRNAs become anchored to the anterior and posterior pole of the egg, respectively (Berleth *et al.*, 1988; Ephrussi *et al.*, 1991; Kim-Ha *et al.*, 1991). Fertilization triggers translation of Bcd and Nos gene products, which are free to diffuse away from the respective poles to form two opposing exponential protein

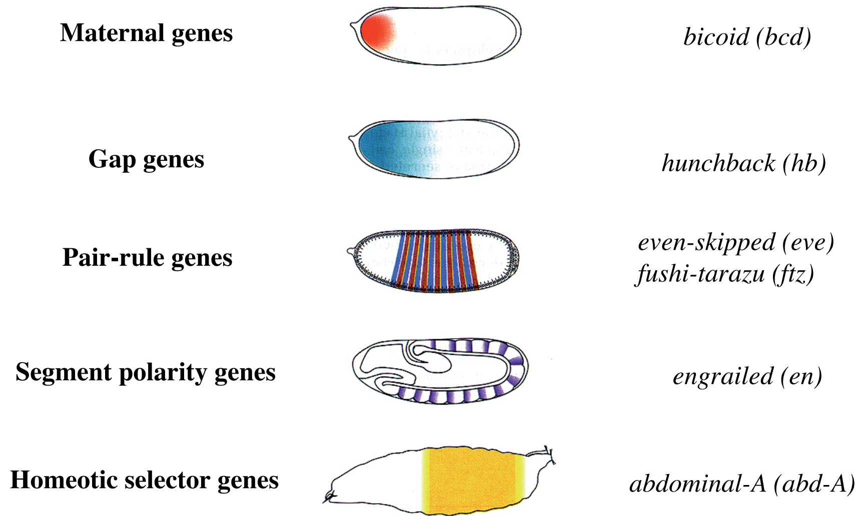

Figure 6. Segmentation of the *Drosophila* embryo. The anterior-posterior axis of the embryo is subdivided into distinct metameric units by the activity of five classes of genes. Maternally encoded factors such as Bcd form protein gradients in the early embryo and regulate expression of gap genes such as *hb* . Proteins encoded by maternal and gap genes act in a combinatorial fashion to switch on pair-rule genes in seven-striped patterns. In turn, pair-rule gene products establish the expression of segment polarity genes that subdivide the embryo into parasegments at the time of germ band extension. Together, these genes enable the expression of homeotic selector (Hox) genes that define the identity of each segment. Modified from (Wolpert, 2002).

gradients in the early syncytial embryo (Driever and Nusslein-Volhard, 1988; Wang and Lehmann, 1991). Bcd and Nos act as translational repressors for the ubiquitously distributed mRNAs of two other maternal effect genes, *caudal* (*cad*) and *hunchback* (*hb*). Bcd represses translation of *cad* (Dubnau and Struhl, 1996; Rivera-Pomar *et al.*, 1996) whereas Nos represses *hb* (Murata and Wharton, 1995). As a result, two new protein gradients are set up in the early embryo: a gradient of Hb in an anterior-to-posterior direction, and a gradient of Cad in a posterior-to-anterior direction. Morphogen gradients of maternal effect gene products activate the transcription of gap genes, which are the first zygotic genes to be expressed in discrete regions along the A/P axis of the embryo (Fig. 6) (Nasiadka *et al.*, 2002). All gap genes encode transcription factors, and loss of gap gene function causes deletions of entire body regions – hence the name gap genes. The promoters of gap genes respond to different concentrations and combinations of maternal transcription factors. As a consequence, different gap genes are expressed in distinct regions of the embryo, depending on the levels of Bcd, Hb and Cad. Further spatial

and temporal refinement of gap gene expression domains is controlled by regulatory interactions among the gap genes themselves.

The products of gap genes and maternal effect genes cooperate in the activation of a third set of segmentation genes, the pair-rule genes. Most pair-rule genes encode transcriptional repressors or activators, and they are all expressed in seven transverse stripes in the syncytial embryo with a double segment periodicity (Fig. 6). Loss of pair-rule gene function results in characteristic cuticular deletions or duplications of discrete domains within alternating segments along the A/P axis of the embryo, each pair-rule gene phenotype with a different phasing (Nusslein-Volhard and Wieschaus, 1980). The translation of the non-periodic maternal and gap protein expression pattern into the periodic pair-rule pattern has been an area of intense research. Pair-rule genes have complex regulatory regions containing several enhancers that include overlapping binding sites for activators and repressors. The *even-skipped* (*eve*) gene has been subject to particularly detailed analysis in this respect. The second stripe of *eve* is controlled by a discrete enhancer element that integrates positive inputs from Bcd and Hb, as well as negative inputs from gap gene products Giant (Gt) and Krüppel (Kr) (Small *et al.*, 1991; Small *et al.*, 1992; Fujioka *et al.*, 1999). Strikingly, the expression pattern of several pair-rule genes, including *eve*, *odd-skipped* (*odd*), and *paired* (*prd*), resolves into fourteen stripes during later development. Neither the mechanism nor the function of this transition is completely understood (Nasiadka *et al.*, 2002).

The next round of patterning involves complex interactions between pair-rule genes to set up the expression domains of segment polarity genes in the by now cellularized embryo. Segment polarity genes are typically expressed in fourteen narrow transverse stripes with a single segment periodicity (Fig. 6). Two key segment polarity genes are *engrailed* (*en*) and *wingless* (*wg*), which become activated in adjacent cells flanking the parasegment boundaries (Fig. 7) (Baker, 1987; Lee *et al.*, 1992; Mohler and Vani, 1992). For instance, in the case of *en*, the opposing activities of Fushi-tarazu (Ftz) and Odd control *en* gene expression in even numbered stripes. Ftz activates *en*, whereas Odd, which is expressed out of phase with Ftz, represses *en* to the posterior, thus delimiting the posterior edge of the *en* expression domain (DiNardo and O'Farrell, 1987; Lawrence *et al.*, 1987; Nasiadka *et al.*, 2002). The boundary between *wg* and *en* cells demarcates the PS boundary, which (see above) becomes morphologically distinguishable as a transient groove in the ectoderm. PS boundaries constitute compartment boundaries in the sense that they prevent intermingling of cells on either side of the boundary (Vincent, 1998; Irvine and Rauskolb, 2001). Once activated, Wg and En act to reinforce this parasegmental periodicity through a positive feedback loop (Fig. 7) (DiNardo *et al.*, 1988; Martinez Arias *et al.*, 1988; Heemskerk *et al.*,

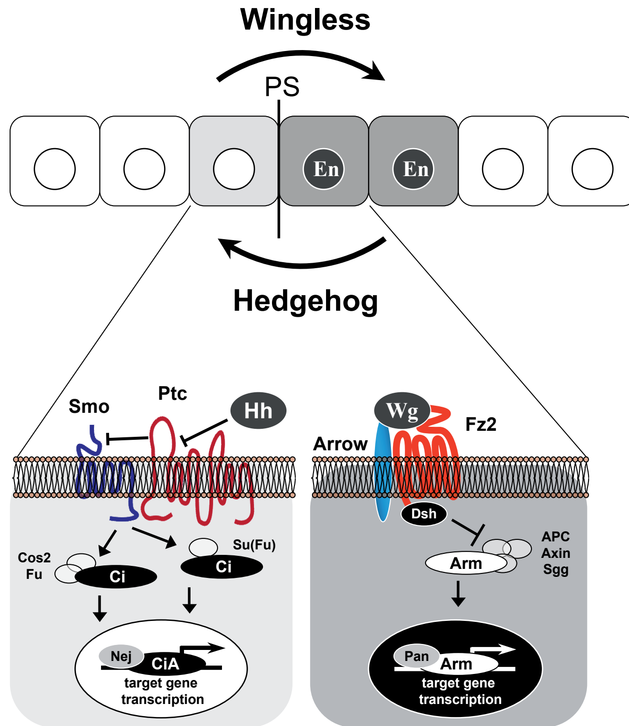

Figure 7. A positive feedback loop maintains expression of Wingless, Engrailed and Hedgehog at the parasegment boundaries. The Wg protein is secreted from cells anterior to the parasegment (PS) boundary and diffuses to surrounding cells. In posterior cells, binding of Wg to its receptor Frizzled 2 (Fz2) elicits the canonical Wnt signal transduction pathway that maintains expression of *en*. Binding of Wg to Fz2 requires interaction with the co-receptor Arrow. Following binding of the Wg ligand to Fz2, a signal is transmitted to Disheveled (Dsh). The role of Dsh is to inhibit a complex including the scaffold protein Axin, the MT binding protein APC (Adenomatous Polyposis Coli), and the kinase Shaggy (Sgg). In resting cells, the transcription factor Arm is phosphorylated and targeted for degradation by this complex. Activation of Dsh inhibits this activity and permits Arm to enter the nucleus where it associates with Pangolin (Pan) to form a transcriptional activator complex that initiates the expression of target genes. At the PS boundary, triggering of Wg signaling promotes expression of *en*. In turn, the En protein activates expression of *hh*. Once synthesized, Hh diffuses away from its source of production and activates a signal transduction pathway in anterior cells to maintain *wg* expression. Hh binds to its receptor Patched (Ptc) to relieve the Ptc-mediated inhibition of Smoothened (Smo) activity. Upon activation, Smo transmits the Hh signal to two complexes. The first complex consists of the kinesin-related protein Costal-2 (Cos2) and the serine/threonine kinase Fused (Fu) together with associated kinases (not shown). In resting cells, this complex phosphorylates and targets the transcription factor Cubitus interruptus (Ci) for proteolytic processing into a repressor form, CiR. The second complex includes the protein Suppressor of Fused, Su(fu), that binds to Ci and retains it in the cytoplasm in the absence of Hh signaling. Smo acts through both complexes, blocking the production of CiR, and permitting full-length Ci (CiA) to enter the nucleus where it interacts with the transcriptional co-activator Nejire (Nej) to activate transcription of Hh target genes.

1991). *en* encodes a homeodomain transcription factor whose function is required to activate another key segment polarity gene, *hedgehog* (*hh*), which codes for a secreted ligand (Forbes *et al.*, 1993; Tabata and Kornberg, 1994). After synthesis, Hh diffuses away form its source of production and activates a signaling cascade in anteriorly adjacent cells to sustain *wg*-expression (Ingham, 1993). Wg, also a secreted ligand, signals back to cells on the other side of the PS boundary to reinforce *en* (and thus *hh*) expression (Fig. 7) (Vincent and O'Farrell, 1992). Integral components in the Wg and Hh signaling cascades such as receptors, transducers, and transcriptional activators, together with factors that mediate the extracellullar diffusion of the respective ligand, are all encoded by segment polarity genes (Perrimon, 1994; Kalderon, 2002; Lum and Beachy, 2004). In their absence, the *wg*/*en* feedback-loop breaks down, resulting in polarity defects within each PS.

As outlined above, segment polarity genes act at the bottom of the segmentation cascade that subdivides the embryonic body into repeated units of PSs. Another group of factors, encoded by the evolutionary and functionally conserved Hox genes, is responsible for conferring specific identities to these units that correspond to their position along the A/P axis (Fig. 6) (Graba *et al.*, 1997). In the genome, these genes are organized into complexes. Two Hox complexes exist in *Drosophila,* the *Anntenapedia* complex and the *Ultrabitorax* complex. Hox genes are expressed in overlapping, non-segmented patterns along the A/P axis of the embryo, and gap and pair-rule genes regulate their nested expression. Strikingly, Hox genes are expressed corresponding to the order in which the genes are encoded in the genome, and the expression boundaries and area of action of individual Hox genes generally respect the parsegmental compartment boundaries (Vincent, 1998; Deutsch, 2004). From a functional perspective, the activities of Hox genes are superimposed on to the segmented body plan and are required for the diversification of segments. Hox genes dictate, for example, whether a segment develops a wing or a haltere.

4.1.2 Patterning the parasegment

After the parasegmental organization is set up in the embryo, cell fates are established within each PS. This is mediated by inductive Wg and Hh signals that emanate from the PS boundary organizer (Hatini and DiNardo, 2001; Sanson, 2001). The role of the organizer is best understood in the ventral epidermis at the level of the abdominal segments (Fig. 8). In an early phase, *wg* and *hh* are involved in a positive feedback loop maintaining each other's expression (Fig. 7). After this initial phase, *wg* and *hh* become independently expressed in adjacent cell rows (Bejsovec and Martinez Arias, 1991; Heemskerk *et al.*, 1991) and interact to make each other's activity asymmetric with respect to the PS boundary; Wg attenuates Hh signaling in the posterior of

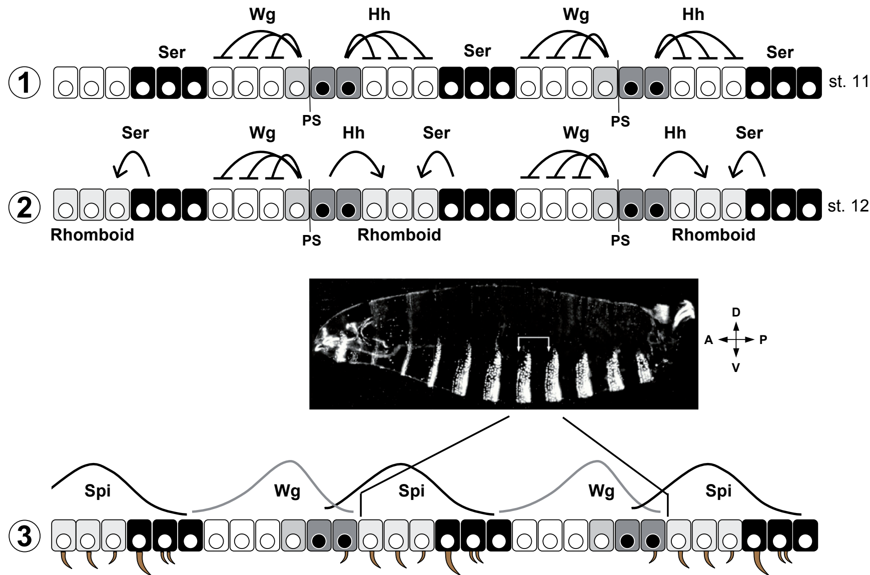

Figure 8. Establishment of segment polarity in the embryonic epidermis. Schematic representation of the ventral abdominal epidermis at different stages. (1) At stage 11, Wg and Hh expression are no longer interdependent. Wg and Hh inhibit *serrate* (*ser*) expression in the posterior and anterior of each PS, respectively. This delimits the Ser domain to the central portion of each PS. (2) Stage 12. Hh activates *rhomboid* in two cell rows posterior to the Hh source; Ser activates *rhomboid* in one cell row anterior to the Ser domain; Wg represses *rhomboid* in the posterior region of each parasegment. As a consequence, Rhomboid expression is confined to a stripe of cells located between Hh and Ser. (3) At the end of embryogenesis, Wg signaling specifies smooth cuticle cell fate in an asymmetric fashion, three to four cell diameters in the anterior direction, but only extending one cell row to the posterior. The denticle cell fate is specified by the EGFR ligand Spitz (Spi) that emanates from the Rhomboid domain in each segment. Corresponding to their respective positions across the segment, cells form denticles of different shapes and sizes. The photograph shows the cuticle of a wild-type larva visualized by dark field microscopy. Note alternating regions of smooth cuticle and denticle belts in the ventral epidermis. Anterior is to the left and apical is down. PS designates the parasegment boundaries.

each PS, whereas Hh restricts Wg action in the anterior (Sanson *et al.*, 1999; Piepenburg *et al.*, 2000; Dubois *et al.*, 2001). This breaks the symmetry across the PS. Finally, Wg and Hh cooperate to subdivide the PS into smaller territories by promoting expression of *rhomboid* and *serrate* (*ser*) in two adjacent but non-overlapping ventral stripes (Fig. 8). *rhomboid* encodes an activator of the Epidermal Growth Factor Receptor (EGFR) ligand Spitz (Spi); *ser* encodes a membrane-bound ligand for the Notch receptor. The expression domains of *ser* and *rhomboid* are established in two steps. First, Hh acts in the posterior direction to repress *ser* expression over three cell diameters while Wg inhibits *ser* to the anterior, thus confining *ser* expression to the center of the PS (Alexandre *et al.*, 1999; Gritzan *et al.*, 1999). In a

second step, Ser collaborates with Hh to set up the *rhomboid* domain. This is counteracted by Wg, which represses *rhomboid* expression in the posterior of each parasegment (Sanson, 2001). As a consequence, *rhomboid* expression becomes confined to a single narrow stripe located between the *hh* and *ser* domains. At the end of embryogenesis, this intra-segmental pattern of gene activities drives the differentiation of epidermal cells into denticle or smooth-cuticle secreting cells, respectively. The posterior row of En cells and the Rhomboid and Ser cells secrete denticles, a fate decision controlled by Spi that emanates from the Rhomboid domain (Fig. 8) (Payre *et al.*, 1999). The action of Spi is counteracted by Wg signaling, which specifies the smooth-cuticle cell fate. Consequently, mutations in genes that disrupt Wg-signaling (either directly or indirectly by affecting the *wg/en* feedback-loop) result in a characteristic "lawn" of denticles on the ventral surface of the larva. As noted in the previous chapter, each denticle secreting cell row produces denticles with a characteristic shape and size. How this diverse pattern is generated is unclear but may involve further rounds of signaling between cells (Hatini and DiNardo, 2001).

The Wg/Hh PS boundary organizer constitutes a paradigm for the study of pattern formation during development. Most research has focused on the role of the organizer in ventral patterning. Accordingly, we have a clear picture of how the ventral epidermis is subdivided into non-overlapping gene expression domains that drive epidermal differentiation. However, the mechanism that controls cell fates decisions dorsally is different from the mechanism employed ventrally. In the dorsal and dorso-lateral epidermis, Hh has been proposed to act in a graded manner to directly pattern several cell types posterior to the Hh domain (Heemskerk and DiNardo, 1994).

4.1.3 From parasegments to segments

Besides reinforcing parasegmental periodicity and controlling the denticle pattern of the larva, segment polarity genes are implicated in the morphological subdivision of the embryonic body into definitive segments. By the time the intra-segmental pattern of gene activities has been established in the epidermis, parasegmental grooves are no longer visible in the epidermis (Sanson, 2001). At this point, definitive segments appear, demarcated by the formation of segmental grooves posterior to the *en/hh* domain. Groove formation is based on a series of controlled shape changes in a single row of specialized cells in each segment termed groove founder cells (see Fig. 5). Previous work has established that formation of segmental grooves requires *en* and *hh* activity (Fig. 9). Thus, embryos mutant for either *en* or *hh* fail to form segmental grooves, even if the activity of the other gene is artificially maintained, and continuous expression of these genes is essential for maintenance of the grooves (Larsen *et al.*, 2003). Anterior to the *en/hh*-expressing

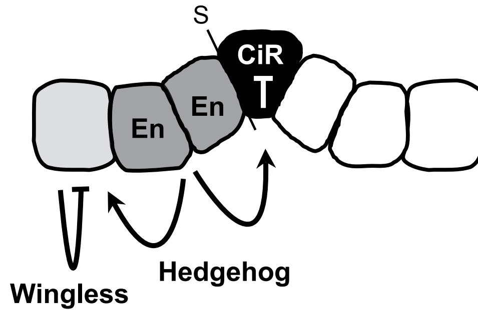

Figure 9. Signaling events during segmental groove formation. Hh emanating from cells immediately anterior to the segment (S) boundary signals to adjacent cells to initiate groove formation. Anteriorly, the inhibiting action of Wg counteracts the Hh-signal. As a consequence, Hh elicits groove morphogenesis only to the posterior where no Wg-signal is present. Hh is thought to act by removing the repressor form of Ci (CiR) from groove founder cells. Groove formation also requires En to be expressed in cells anterior to the segment boundary.

cells, a *wg*-mediated inhibitory signal prevents cells from forming a groove. As a consequence, segmental grooves form only posterior to the *en/hh* cells. In addition, evidence suggests that the morphologic changes accompanying groove formation require elimination of the repressor form of the transcription factor Cubitus Interruptus (Ci) in groove founder cells (Fig. 9) (Larsen *et al.*, 2003). A similar mechanism has recently been demonstrated to drive Hh-dependent cell constrictions in the *Drosophila* eye disc epithelium (Corrigall *et al.*, 2007; Escudero *et al.*, 2007). However, the link between de-repression of a Ci-target and initiation of groove morphogenesis is elusive. In the case of segmental groove morphogenesis, Hh targets such as *rhomboid* or *stripe* are dispensable for invagination (Larsen *et al.*, 2003), suggesting that other, as yet, unidentified Ci targets may control cell fate decisions and cell shape changes during this process. The role of Hh signaling during groove morphogenesis illustrates an important concept: the inductive role of extracellular signals in patterning cellular morphogenesis within a field of cells. Understanding the mechanism that links extracellular signals to changes in cell morphology constitutes a major challenge in the field of developmental biology.

4.2 The Terminal System

The non-segmented, terminal parts of the embryonic A/P axis, the acron and the telson, are specified through the action of a group of proteins encoded by the class of terminal genes (Furriols and Casanova, 2003). Disruption of terminal gene activity results in a characteristic loss of terminal structures

in the embryo. A key terminal gene is *torso*, which encodes a tyrosine kinase receptor. Loss-of-function and gain-of-function studies have demonstrated that Torso-activation induces terminal cell fates in the embryo (Casanova and Struhl, 1989; Sprenger *et al.*, 1989). The Torso receptor is distributed evenly along the surface of the blastoderm but is activated only at the poles, presumably by the Trunk (Trk) protein, which is believed to be the ligand for Torso (Casanova *et al.*, 1995). Similar to Torso, Trk is a ubiquitous factor. However, Trk is thought to exist in an uncleaved, inactive state. Localized activation of Trk requires a third protein, Torso-like (Tsl), that emanates from terminal follicle cells at the poles of the oocyte (Stevens *et al.*, 1990). Tsl-dependent activation of Torso triggers a Ras/Raf/MAPK-signaling cascade that leads to the expression of the terminal gap genes *huckebein* (*hkb*) and *tailless* (*tll*) (Weigel *et al.*, 1990; Furriols and Casanova, 2003). The cascade appears to act by relieving repression of *hkb* and *tll* imposed by a complex that includes the factors Capicua (Cic) and Groucho (Gro) (Paroush *et al.*, 1997; Jimenez *et al.*, 2000). In the posterior region of the embryo, Hkb and Tll cooperate to establish posterior cell fates. However, in the anterior, the combination of Bcd, Hkb and Tll drives cells into anterior fates (Pignoni *et al.*, 1992).

4.3 The Dorsal-Ventral Axis

Similar to the A/P axis, the D/V axis is established during oogenesis. D/V axis determination is initiated by the EGF ligand Gurken (Grk) that is associated with the dorsally located oocyte nucleus. Grk signals to nearby follicle cells to repress ventral cell fate (Amiri and Stein, 2002). The key event is the repression of *pipe* (*pip*) expression, a gene encoding a putative 2-O sulfotransferase, in dorsal follicle cells (Sen *et al.*, 1998). Localized Pip activity in ventral follicle cells activates the protease Nudel (Ndl), which is secreted into the perivitelline space, a fluid filled space between the follicle cells and the oocyte. Ndl initiates a serine protease cascade involving – in order of action – Gastrulation-defective (Gd), Snake (Snk), and Easter (Ea). The last serine protease in the cascade, Ea, cleaves and activates the ligand Spätzle (Spz) in a ventral-to-dorsal gradient (Moussian and Roth, 2005). Binding of Spz to its receptor, Toll, triggers an intracellular signaling pathway that disrupts a cytoplasmic complex of the Cactus (Cact) and Dorsal proteins, thereby allowing Dorsal to enter the nuclei in a ventral-to-dorsal gradient. Dorsal encodes a transcription factor that acts as a morphogen in the syncytial embryo to establish different cell fates along the D/V axis of the embryo (Roth *et al.*, 1989; Rushlow *et al.*, 1989; Steward, 1989). High threshold levels of Dorsal in ventral cells activate expression of the mesodermal cell fate determinants *twist* (*twi*) and *snail* (*sna*), whereas intermediate levels activate expression

of ectodermal genes such as *short gastrulation* (*sog*) in lateral positions. In addition, Dorsal activity represses *decapentaplegic* (*dpp*), thus restricting *dpp* activity to the dorsal ectoderm. *dpp* encodes a secreted factor that forms a dorsal-to-ventral activity gradient through interactions with the Dpp inhibitor Sog that emanates from lateral cells (O'Connor *et al*., 2006). Dpp activity is required for the establishment of dorsal cell fates in the embryo.

5 Regulation of the Actin Cytoskeleton

Cells in a developing organism are in a continuous state of change. Cells modify their shape; they move, divide, grow or die, in a magnificently coordinated interplay with the final objective to reproduce the species-specific body plan. The ability of cells to accommodate this task relies on the function of a dynamic cytoskeleton, an intracellular system of filaments that is instrumental for the shape and function of all cells. Three types of cytoskeletal filaments are found in eukaryotic cells: *Actin filaments* (or microfilaments) determine the shape and motility of cells; *Microtubules* (MT) direct intracellular transport and pull the chromosomes apart during mitosis; and *Intermediate filaments* – although not present in insects (Adams *et al.*, 2000; Tepass *et al.*, 2001) – confer resistance to tensile force. Assembled cytoskeletal filaments also build various cellular structures, including motile MT-based cilia and flagella, and Actin-rich surface protrusions such as the denticles of a *Drosophila* larva or the stereocilia on the surface of hair cells in the inner ear of mammals.

5.1 The Actin Cytoskeleton

Regulation of the dynamic properties of cytoskeletal filaments permits cells to change their shape in response to internal and external cues. More specifically, cell shape changes are driven by dynamic reorganizations of Actin filaments (Jacinto and Baum, 2003). Although dispersed throughout the cell, Actin filaments are highly concentrated at the cortex, just beneath the plasma membrane. This so-called cortical Actin cytoskeleton is organized into bundles and networks of filaments. A large number of conserved proteins have been identified that associate with Actin and that collectively regulate the spatial organization and dynamics of the Actin cytoskeleton (dos Remedios *et al.*, 2003). This includes factors that cap, sever, cross-link, bundle, nucleate, or move Actin filaments. The action of these Actin-regulators needs to be precisely coordinated in time and space to enable the cell to execute a given task. In many instances, this is achieved by a group of proteins that belong to the Rho-family of GTPases (for Guanosine Triphosphatases) (Hall, 1998; Jaffe and Hall, 2005), which act as molecular switches that activate effector pathways regulating cytoskeletal reorganization (Settleman, 2001; Rossman *et al.*, 2005; Bos *et al.*, 2007).

5.2 Rho-family GTPases as Regulators of the Actin Cytoskeleton

Rho-family GTPases (also known as Rho-GTPases) constitute a branch of the large superfamily of Ras-related small GTPases. So far, twenty-three Rho-family GTPases have been identified in mammals and numerous members of this family have been identified in other organisms, including eleven in *Arabidopsis thaliana*, nine in *Drosophila*, nine in *Caenorhabditis elegans*, and seven in *Saccharomyces cerevisiae* (Bustelo *et al*., 2007). Similar to other members of the Ras superfamily, Rho-family GTPases cycle between an inactive GDP (guanine diphosphate)-bound and an active GTP (guanine triphosphate)-bound form (Schmidt and Hall, 2002; Rossman *et al*., 2005; Bos *et al*., 2007). Their activation state is highly regulated by three classes of proteins (Fig. 10); (1) Rho-guanine nucleotide exchange factors (RhoGEFs) that catalyze exchange of GDP for GTP, thereby activating the GTPase during signal transduction; (2) GTPase activating proteins (RhoGAPs) that stimu-

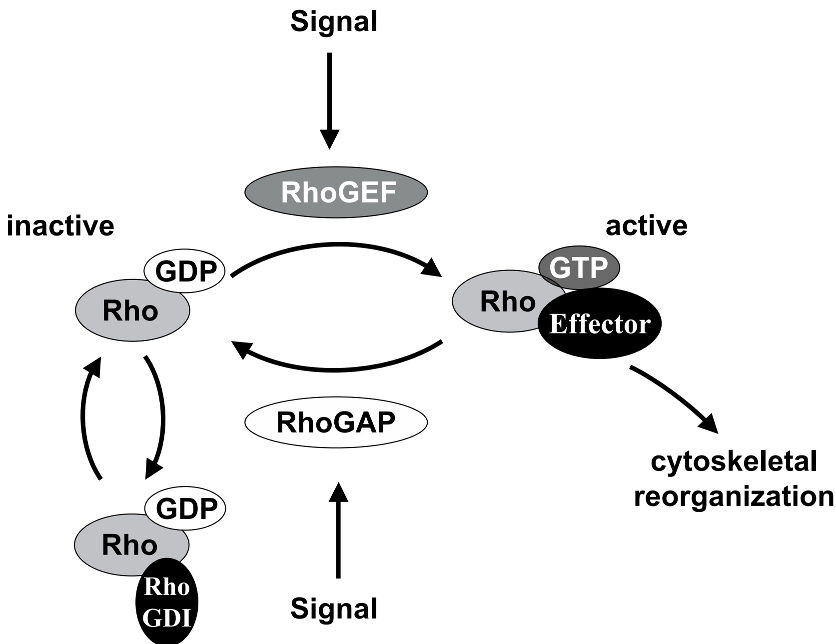

Figure 10. The function of Rho-family GTPases. Rho-family GTPases exist in an inactive GDP-bound and an active GTP-bound state. RhoGDIs bind to the inactive form of the protein and sequester it to the cytosol. During signal transduction, RhoGEFs promote the exchange of GDP for GTP on the GTPase, thus transferring the protein into an active form. The activated GTPase can bind to an array of downstream effectors to promote a spectrum of cellular responses, usually by stimulating cytoskeletal reorganizations. RhoGAPs catalyze conversion of the GTPase back to an inactive state by increasing the intrinsic hydrolytic activity of Rho-family GTPases.

late the low intrinsic GTPase activity of Rho-family GTPases, thus allowing transfer of the GTPase back to the inactive state; and (3) Rho-guanine nucleotide dissociation inhibitors (RhoGDIs), whose function is less clear, but which appear to block spontaneous activation by stabilizing the inactive GDP-bound form of the GTPase.

In their active GTP-bound state, Rho-family GTPases relay extrinsic signals to a wide array of downstream effectors via direct molecular interaction. To date, over 70 potential effector proteins have been identified, and many of them have been tested *in vivo* (Bishop and Hall, 2000; Bustelo *et al.*, 2007). Typically, in the absence of GTPase signaling, downstream effectors are kept in a closed auto-inhibitory state. However, upon GTPase activation by upstream signals, the active GTPase binds to its cognate effector to trigger a conformational change that converts the effector protein into its activated state.

Rho, the founding member of the Rho-family of GTPases, was identified in 1985 but it was not until the mid 1990's that the cellular function of Rho-family molecules began to be revealed. In pioneering experiments carried out in fibroblast cell culture (Ridley and Hall, 1992; Ridley *et al.*, 1992; Kozma *et al.*, 1995; Nobes and Hall, 1995), constitutively activated mutants of the prototypical Rho-family proteins Rho, Rac, and Cdc42 were shown to induce assembly of contractile Actin-based filaments (stress fibers), Actin-rich surface protrusions (lamellipodia), or Actin-rich finger-like membrane extensions (filopdia), respectively. A major conclusion from these early studies was that Rac, Rho, and Cdc42 regulate the assembly of distinct filamentous Actin structures. Since then, a large body of evidence has accumulated on the role of Rho-family GTPases in regulating various aspects of the Actin cytoskeleton in a wide spectrum of cell types, model organisms, and developmental contexts. Consequently, the canonical family members, Rho, Rac, and Cdc42, have been implicated in a variety of cellular processes associated with cytoskeletal rearrangements, including cell division, migration, polarity, shape, and adhesion (Etienne-Manneville and Hall, 2002; Van Aelst and Symons, 2002; Jaffe and Hall, 2005). Current models depict a set of parallel and evolutionary conserved effector pathways controlled by each Rho-family GTPase. In addition to the pivotal role of these GTPases to regulate the Actin cytoskeleton, more recent work has implicated Rho-family GTPases in several other processes, including MT dynamics, gene expression, control of cell cycle progression, and growth (Jaffe and Hall, 2005).

5.2.1 Regulation of Actin polymerization

A major function of Rho-family GTPases is to control the rate and site of Actin polymerization by regulating the head-to-tail assembly of monomeric, globular G-Actin subunits into long, polar filamentous polymers, known as F-Actin. The assembly of Actin filaments is a multi-step process, with a

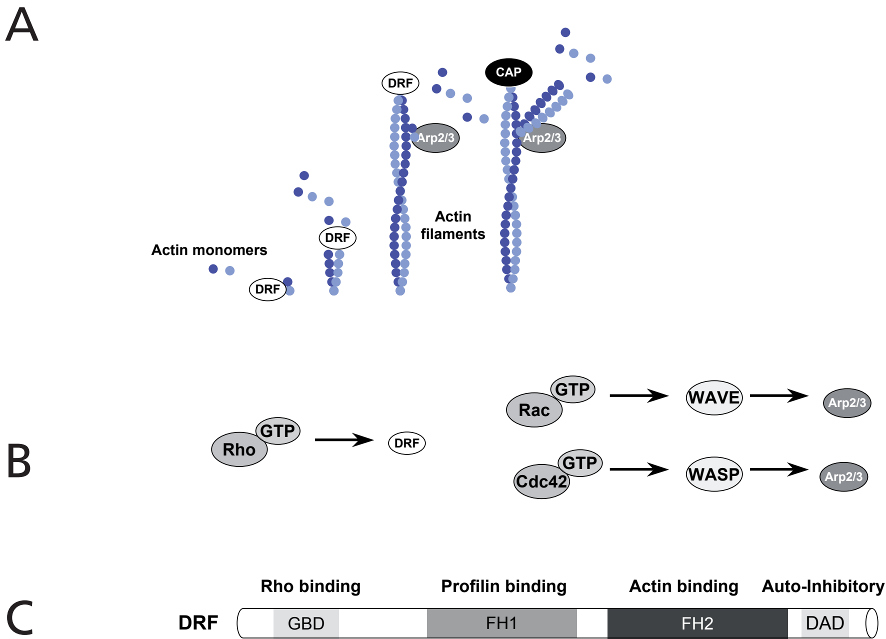

Figure 11. Rho-family GTPases promote two modes of Actin polymerization. (A) DRFs nucleate Actin polymerization and promote linear elongation of filaments. During the elongation phase, DRFs remain bound to the growing filament end to add new Actin monomers and antagonize the activity of Capping Proteins (CAP). Arp2/3 initiates a branched filament network by binding to the sides of pre-existing filaments. (B) Rho activates DRFs through direct binding while Rac and Cdc42 activate Arp2/3 via WAVE and WASP, respectively. (C) Schematic representation of DRFs. Four protein domains are frequently found in DRFs. Binding of Rho to the GBD domain disrupts the auto-inhibitory interaction of the GDB and DAD domains. This disruption exposes the catalytic FH2 domain that drives the polymerization reaction together with the adjacent FH1 domain, which is responsible for recruiting new Actin monomers from Profilin-G-Actin complexes.

slow and rate limiting nucleation phase followed by a rapid elongation phase (Pollard and Borisy, 2003). Rho-family GTPases control F-Actin assembly via two main effectors, the DRFs (Diaphanous Related Formins) and the multi-subunit Arp2/3 complex, which promote the formation of linear and branched Actin filaments, respectively (Fig. 11A). Both factors increase the rate of Actin polymerization by enhancing the rate-limiting nucleation step. In the case of Arp2/3, the mode of action involves binding to the sides of pre-existing filaments to initiate growth of a new filament at a distinctive 70-degree angle from the old filament (Pollard and Borisy, 2003). Nucleation by this heptameric protein complex is mediated by two of its subunits, the Actin-Related Proteins ARP2 and ARP3 that closely resemble the structure of monomeric Actin and serve as initiation sites for new branch points. Both

Cdc42 and Rac control Arp2/3 activation; however, while Cdc42 activates Arp2/3 via members of the WASP (Wiskott-Aldrich Syndrome Protein) family, Rac-dependent activation relies on the structurally related SCAR/WAVE (WASP family Verprolin-homologous protein) family (Fig. 11B) (Pollard and Borisy, 2003; Bompard and Caron, 2004). In the former case, biochemical data suggest a two-tiered regulation of WASP by Cdc42 involving either direct binding of Cdc42 to WASP to relieve an intra-molecular, auto-inhibitory interaction, or, alternatively, Cdc42-mediated activation of the factor Toca-1 that, in turn, relieves WASP from a trans-inhibitory constrain imposed by the protein WIP (Martinez-Quiles *et al.*, 2001; Ho *et al.*, 2004). The mechanism of Rac-dependent activation of SCAR/WAVE appears to involve formation of a complex that includes SCAR/WAVE and four other proteins, PIR121, Nap125, Abi, and HSPC300 (Eden *et al.*, 2002; Gautreau *et al.*, 2004). Consistent with this, work in *Drosophila* cell culture has shown that fly orthologs of PIR121, Nap125, and Abi protect SCAR/WAVE from proteasome-mediated degradation and are critical for its localization and for the generation of Arp2/3-dependent protrusions (Kunda *et al.*, 2003; Rogers *et al.*, 2003).

In contrast to Rac and Cdc42, Rho stimulates Actin polymerization mainly through the second type of nucleators, the DRFs (Fig. 11B) (Wallar and Alberts, 2003; Faix and Grosse, 2006). Many DRF members, including the founding member encoded by *Drosophila diaphanous* (*dia*) (Castrillon and Wasserman, 1994; Afshar *et al.*, 2000), as well as the murine mDia1 (Watanabe *et al.*, 1997) act as Rho-specific effectors. However, individual DRFs can be subjected to regulation by multiple Rho-family GTPases, as shown for mDia2 and mDia3, which can act as effectors for RhoA as well as for Cdc42 and/or Rac1 (Peng *et al.*, 2003; Yasuda *et al.*, 2004; Ji *et al.*, 2008). DRFs constitute a subfamily within the Formin-family, which in contrast to the Arp2/3 complex, promote formation of linear filaments. Besides the Formin-family characteristic FH1 and FH2 (for Formin Homology) domains, two other functional domains distinguish DRFs: the GTPase Binding Domain (GDB) that interacts with active Rho-GTPases, and the Diaphanous Auto-inhibitory Domain (DAD) (Fig. 11C). In the absence of Rho-signaling, the DAD domain binds to the GDB domain, thus keeping the protein in an inactive, dormant state. Binding of active Rho-family GTPase to the GDB domain is believed to relieve this auto-inhibitory interaction and expose the catalytic FH2 domain, which is then free to initiate *de novo* nucleation of Actin filaments (Fig. 11A) (Goode and Eck, 2007), most likely through stabilizing a G-Actin dimer (Pring *et al.*, 2003). The adjacent FH1 domain participates in the polymerization reaction by recruiting and delivering new G-Actin subunits from Profilin-G-Actin-complexes to the FH2 domain for incorporation into growing filaments (Chang *et al.*, 1997; Sagot *et al.*, 2002). After nucleation, DRFs remain stably associated with the growing end of

Actin filaments (Fig. 11A) (Pruyne *et al.*, 2002). From this position, DRFs continue to insert new Actin monomers to the growing filament end and, in some cases, antagonize the inhibitory activities of filament capping proteins (Faix and Grosse, 2006). In this way, DRFs promote formation of various Actin-rich structures, including the contractile ring during cytokinesis (Castrillon and Wasserman, 1994; Chang *et al.*, 1997; Afshar *et al.*, 2000) and filopodia that extend from the leading edge of migrating cells (Schirenbeck *et al.*, 2005; Williams *et al.*, 2007).

Cell culture studies have identified several additional roles for DRFs. For example, mDia1 has been implicated in promoting AJ (Adherens Junction) stability and organizing MT networks in cultured epithelial cells downstream of RhoA (Sahai and Marshall, 2002; Carramusa *et al.*, 2007). While the effect of mDia1 on AJ stability is dependent on its ability to induce Actin polymerization (Sahai and Marshall, 2002), the effect on the MT network was shown to be independent of this function (Ishizaki *et al.*, 2001). Instead, mDia1 has been suggested to stabilize MTs by forming a complex with the MT plus-end binding protein EB1 and the tumor suppressor gene product APC (Adenomatous Polyposis Coli) at the tips of MTs (Wen *et al.*, 2004). DRFs can also regulate non-muscle Myosin II (hereafter referred to as Myosin II) stability. In cultured *Drosophila* cells, Dia-dependent F-Actin retains Myosin II at the cleavage furrow during cytokinesis (Dean *et al.*, 2005). Finally, DRFs have been implicated in regulatory loops during signal transduction. Thus, mDia as well as *Drosophila* Dia provide a direct link between Rho-family GTPases and the transcriptional activation of many cytoskeletal genes, including *actin*, via the transcription factor SRF (Serum Response Factor) and its binding partner MAL (Treisman, 1987; Sotiropoulos *et al.*, 1999; Copeland and Treisman, 2002; Miralles *et al.*, 2003; Somogyi and Rørth, 2004). In resting cells, MAL is associated with a pool of monomeric G-Actin. Upon G-Actin depletion caused for example by DRF-induced Actin polymerization, MAL becomes translocated into the nucleus and forms a transcriptional activator complex with SRF. In the nucleus, the MAL/SRF complex binds to the *SRE* (*Serum Response Element*) of various cytoskeletal target genes to initiate their expression. This is likely to provide an efficient feedback mechanism that refills the pool of G-Actin and of Actin-regulatory proteins, which is essential for the cell to perform a range of cellular functions including motility, adhesion, and shape changes. An additional feedback mechanism that involves a DRF was uncovered recently when mDia1 was shown to participate in a positive feedback mechanism towards RhoA by direct stimulation of the Rho-specific GEF LARG (Leukemia-associated RhoGEF) (Kitzing *et al.*, 2007).

5.2.2 Regulation of Actin filament dynamics

In addition to regulating Actin filament elongation and branching, Rho-family GTPases have been implicated in the spatial and temporal arrangement of Actin filaments; they regulate the formation of filament bundles; promote tethering of the Actin network to the plasma membrane; and trigger Myosin II-dependent sliding of Actin filaments past each other to create the contractile force that drives cellular motility and cytokinesis. In all cases, Rho-specific effectors of the Rho-kinase (ROCK) family of serine/threonine kinases play an essential role (Leung *et al.*, 1996; Riento and Ridley, 2003). Various conserved ROCK substrates have been identified (Fig. 12), including Myosin II Regulatory Light-Chain (MRLC) (Amano *et al.*, 1996), the Myosin II Binding Subunit (MBS) of Myosin II Light-Chain Phosphatase (MLCP) (Kimura *et al.*, 1996; Kawano *et al.*, 1999), LIM-kinase (LIMK) (Ohashi *et al.*, 2000), Adducin (Fukata *et al.*, 1999), and the ERM proteins (Ezrin, Radixin, Moesin) (Matsui *et al.*, 1998). By acting through ROCK, Rho promotes assembly of

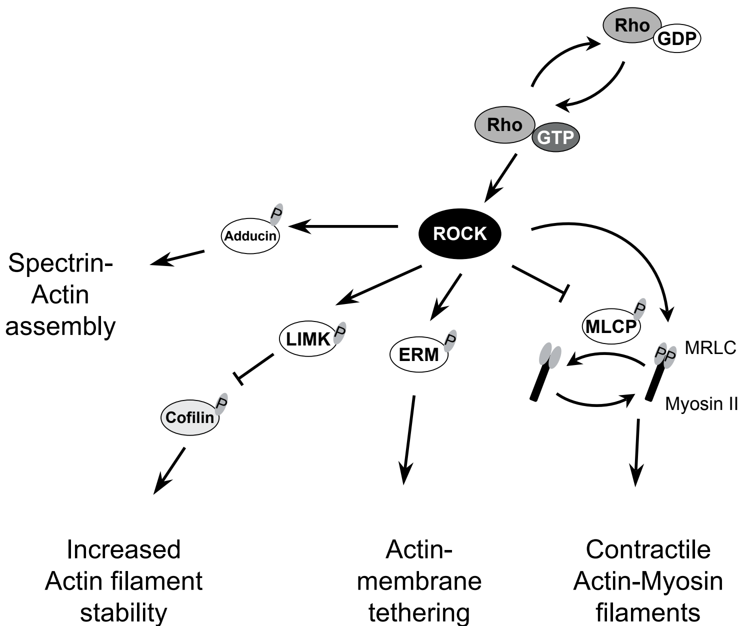

Figure 12. Model illustrating the functions of ROCK. Upon stimulation by Rho, ROCK can trigger reorganization of the Actin-cytoskeleton through phosphorylation of several downstream effectors. ROCK triggers contraction of Actin-Myosin filaments either by directly activating MRLC or by inhibiting the MBS of MLCP. Through ERM family proteins, ROCK promotes the tethering of Actin filaments to the plasma membrane. ROCK-mediated activation of LIMK inhibits Cofilin-mediated Actin de-polymerization resulting in increased Actin-filament stability. Finally, Adducin activation promotes the interaction of Spectrin with Actin.

contractile Actin-Myosin filaments in non-muscle cells (Tan *et al.*, 1992; Somlyo and Somlyo, 2000). It is believed that phosphorylation of MRLC on two conserved residues activates the motor properties of Myosin II and promotes interaction of Myosin II with F-Actin. ROCK increases the phosphorylation state of MRLC either directly, or indirectly by phoshorylating MBS which in turn inactivates MLCP. In addition to regulating MRLC, ROCK influences Actin-filament dynamics by phosphorylating and activating LIMK. Following activation, LIMK inactivates the Actin severing factor Cofilin, leading to reduced turnover and increased Actin filament stability (Maekawa *et al.*, 1999; Bernard, 2007). In addition to its role in the Rho-ROCK pathway, LIMK can act downstream of Rac or Cdc42 via the effector p21-associated kinase (PAK) to inhibit Actin de-polymerization (Edwards *et al.*, 1999). Two other targets of ROCK are Adducin and the ERM proteins, which promote Spectrin-Actin network assembly (Matsuoka *et al.*, 2000) and tether Actin filaments to the plasma membrane (Hughes and Fehon, 2007), respectively.

5.2.3 Coordination of Rho-family GTPase effector pathways

Complex cellular behaviors such as the changes in cellular morphology that accommodate embryonic development require the coordinated spatio-temporal activation of multiple effector pathways acting downstream of multiple Rho-family GTPases. Additionally, specific cell responses require an appropriate balance between parallel effector pathways downstream of individual Rho-family GTPases. For instance, in the case of RhoA activation, the thickness and density of RhoA-induced stress fibers depends on the balance of activity between the mDia1 and ROCK pathways (Watanabe *et al.*, 1999). A delicate balance between ROCK and mDia1 is crucial also in epithelial cells in which RhoA-mediated mDia1-activation results in polymerization of Actin that stabilizes AJs, whereas activation of ROCK promotes contractile force that disrupts AJs (Fig. 13) (Sahai and Marshall, 2002). Moreover, the cellular outcome of mDia1 activation depends on the state of Myosin II activity, which is RhoA/ROCK-dependent. Thus, inhibition of Myosin II activity abolishes mDia1-mediated reinforcement of cell-cell junctions and instead induces formation of numerous mDia1-dependent filopodia-like protrusions (Carramusa *et al.*, 2007).

However, despite the need to balance effector pathways during cellular morphogenesis it is currently unclear how this is achieved. An emerging theme is that signaling events upstream of Rho-family GTPases which involve RhoGEFs and RhoGAPs may specify signaling downstream of Rho-family GTPases (Settleman, 2001; Buchsbaum, 2007). Although incompletely understood, several RhoGEFs participate in multi-protein complexes that include specific GTPase effector proteins, which could provide a mechanism for selective activation of effector pathways. For instance, the Cdc42/RacGEF

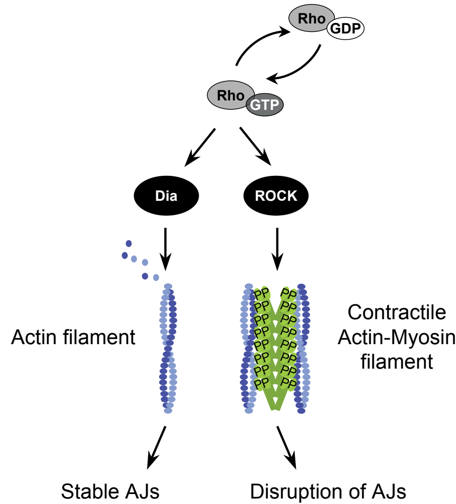

Figure 13. Rho effector pathways regulating adherens junctions in cultured epithelial cells. Rho-mediated activation of Dia induces Actin polymerization that maintains stable adherens junctions (AJs), whereas activation of ROCK can destabilize AJs by generating contractile force.

COOL-2/αPIX binds PAK (Bagrodia *et al.*, 1998; Baird *et al.*, 2005) and the Cdc42-GEF ITSN binds WASP (Hussain *et al.*, 2001), whereas the RacGEF Tiam1 is found in complexes that include Par3 and PKC (Mertens *et al.*, 2005; Pegtel *et al.*, 2007) or WAVE (Connolly *et al.*, 2005), respectively. Another possibility is that specific RhoGEFs and RhoGAPs cooperate to achieve a distinct level, duration or subcellularly localized activation of Rho-family GTPases, which may allow stimulation of specific downstream effector pathways. In either case, understanding GTPase regulation by RhoGEFs and RhoGAPs is imperative to understand Rho-family GTPase function *in vivo*.

5.3 The Role of RhoGEFs and RhoGAPs in the Regulation of Rho-family GTPases

As outlined above, Rho-family GTPases are regulated by a balance between RhoGAP and RhoGEF activities. The importance of this regulation is reflected by the involvement of RhoGAPs and RhoGEFs in development and disease (Settleman, 2001; Schmidt and Hall, 2002; Bos *et al.*, 2007; Tcherkezian and Lamarche-Vane, 2007). During the last decade, much work has been devoted to understanding the function of these two protein classes in cytoskeletal regulation. The emerging picture is that both RhoGAPs and RhoGEFs operate as components in signaling pathways where they convey extrinsic stimuli mediated through various cell surface-receptors, including the cytokine,

growth factor, and adhesion receptors, to Rho-family GTPases (Schmidt and Hall, 2002; Rossman *et al*., 2005; Tcherkezian and Lamarche-Vane, 2007). In addition, particular RhoGEFs can act downstream of G-Protein Coupled Receptors (GPCRs).

Another important realization, which followed the completion of the genome sequence of several organisms, was that the number of RhoGEFs and RhoGAPs greatly exceeds the number of Rho-family GTPases, a relation conserved across phylogeny. The *Drosophila* genome, for instance, encodes over 20 RhoGEFs and 20 RhoGAPs but only nine Rho-family GTPases (Adams *et al*., 2000; Settleman, 2001; Bustelo *et al*., 2007). In the human genome, RhoGAPs and RhoGEFs constitute large gene families with 60 RhoGAP- and 53 RhoGEF-related genes, respectively (Bernards, 2003). This has led to the hypothesis that different RhoGEFs and RhoGAPs may regulate specific aspects of Rho-family GTPase function in cells, a hypothesis also supported by *in vivo* studies in several model organisms. Accordingly, the conserved *Drosophila* RhoGEF Pebble (Pbl) regulates Rho1-dependent events during cytokinesis in post-blastoderm embryos but is dispensable for other Rho1-dependent processes such as cellularization and cell constriction during mesoderm invagination (Hime and Saint, 1992; Lehner, 1992; Prokopenko *et al*., 1999). The opposite applies to another conserved RhoGEF, DRhoGEF2, which has no essential function during cytokinesis but activates Rho1 in both cellularization and mesoderm invagination (Barrett *et al*., 1997; Häcker and Perrimon, 1998; Grosshans *et al*., 2005; Padash Barmchi *et al*., 2005).

5.3.1 The RhoGEFs

The first RhoGEF gene to be identified was the *DBL* (Diffuse B-cell Lymphoma) oncogene (Eva and Aaronson, 1985). In subsequent studies, DBL was shown to induce nucleotide exchange on Cdc42 (Hart *et al*., 1991) by means of a catalytic domain that encompasses approximately 180 amino acids (Hart *et al*., 1994). This domain, which is now known as the DH domain (for DBL homology), is necessary for GEF activity and is conserved in all DBL-related RhoGEFs subsequently identified, which constitutes the largest group of RhoGEFs (Rossman *et al*., 2005). In the course of the exchange reaction, the DH domain drives the displacement of GDP from the inactive GTPase. The subsequent step, the addition of GTP to the GTPase, is promoted by the high intracellular ratio of GTP over GDP. With the exception of three conserved regions (CR1, CR2, CR3), each 10–30 amino acid long, DH domains share little identity with each other. Structure-function analyses have revealed that CR1, CR2, and CR3 form helical structures that in the case of CR1 and CR3 are exposed to the surface of the RhoGEF and participate in formation of the Rho-family GTPase binding pocket (Rossman *et al*., 2005; Bos *et al*., 2007). With respect to specificity, analyses of the ability of RhoGEFs to activate

different Rho-family GTPases has revealed that several RhoGEFs act exclusively on a single GTPase while others, such as COOL-2/αPIX mentioned above (Baird *et al.*, 2005), may act more promiscuously to activate several downstream Rho-family GTPases (Rossman *et al.*, 2005; Bos *et al.*, 2007).

RhoGEFs possess a pleckstrin homology (PH) domain carboxy (C)-terminally adjacent to the catalytic DH domain. Together, this DH/PH module is the minimal structural unit that can promote nucleotide exchange *in vivo* (Rossman et al., 2005). Evidence suggests that PH domains can regulate GEF activity by direct modulation of DH domain function as well as by targeting of the RhoGEF to its proper intracellular location, likely via binding to membrane phospholipids (Schmidt and Hall, 2002). However, the PH domains of several RhoGEFs have been shown to be dispensable for membrane localization, implicating other domains or motifs in proper protein targeting (Snyder *et al.*, 2001; Rossman et al., 2005). Outside the DH/PH module, domain composition differs significantly between RhoGEF family members, and often additional domains mediate protein-protein and protein-lipid interactions, which couple GEF activity to upstream regulators and downstream effectors.

A second subfamily of RhoGEFs that is not related to the DH domain GEFs has been recently described (Rossman et al., 2005). Members of this so-called Dock-180-GEF subfamily share the presence of two highly conserved domains, designated Dock-homology region-1 and -2 (DHR1 and DHR2), of which the DHR2 domain has been implicated in regulating nucleotide exchange on Rho-family GTPases. The prototypical member of this family is the evolutionary conserved GEF Dock180/Myoblast City, which has been shown to function upstream of Rac in several contexts (Cote and Vuori, 2007), including dorsal closure of the *Drosophila* embryo (Rushton et al., 1995; Erickson et al., 1997).

5.3.2 The RhoGAPs

Despite their name, Rho-family GTPases are inefficient in hydrolyzing GTP to GDP. RhoGAPs accelerate this reaction several-fold (Bos *et al.*, 2007). Typically, members of this diverse protein family possess a conserved catalytic RhoGAP domain. Structural analysis of the GAP domain has revealed a core of four bundled helices that include the most conserved residues amongst RhoGAPs (Bernards, 2003). Although originally considered as signaling terminators, RhoGAPs are now acknowledged as equally important to RhoGEFs when it comes to regulating Rho-family GTPase activity in response to upstream signaling. Consequently, much like RhoGEFs, the majority of RhoGAPs contain protein domains that connect them to various upstream cues and downstream effectors during signal transduction (Tcherkezian and Lamarche-Vane, 2007).

6 Rho-Family GTPase Signaling During Ephithelial Morphogenesis in the *Drosophila* Embryo

Embryonic morphogenesis is driven by dynamic changes in cell shapes that collectively act to sculpture tissues, and in extension, the whole organism. As outlined in the previous chapter, cell shape changes are highly dependent on reorganization of the Actin-based cytoskeleton. It is therefore no surprise that Rho-family GTPases, the principal regulators of Actin dynamics in cells, contribute to virtually every aspect of embryonic morphogenesis (Settleman, 2001; Van Aelst and Symons, 2002). The function of Rho-family GTPases has been extensively investigated in cell culture systems. However, in the context of a developing organism, cytoskeletal regulation by Rho-family GTPases is more complex than in cultured cells. In the four-dimensional entity that is the embryo, the activities of Rho-family GTPases have to be coordinately regulated in such a way that groups of cells throughout the embryo at any given time-point in development adopt appropriate morphological configurations.

Within this larger framework, the present chapter focuses on the role of Rho-family GTPases and their regulators during epithelial morphogenesis in the *Drosophila* embryo. Epithelial cells are organized into laterally coherent sheets that line cavities and surfaces of the body (Tepass *et al.*, 2001; Schöck and Perrimon, 2002). During the course of development, epithelial sheets play important roles in the sculpturing and compartmentalization of the embryo. Groups of epithelial cells can give rise to various three-dimensional structures, including shallow grooves, deep invaginations, plate-like placodes, small pits, or hollow tubes by undergoing intricate changes in cellular shapes (Pilot and Lecuit, 2005; Lecuit and Lenne, 2007). Typically, initiation of epithelial morphogenesis is associated with three distinct steps (Schöck and Perrimon, 2002). First, diverse cell fates are established throughout a tissue, often demarcated by the expression of transcription factors in specific areas of the tissue. In a second step, signaling molecules are produced locally to trigger the morphogenetic event. Finally, in response to these signals, groups of cells reorganize their cytoskeleton or modulate their adhesive properties to undergo cell shape changes.

6.1 Mechanisms of Epithelial Morphogenesis

Epithelial cells are characterized by a polarized architecture and by regionalization of the plasma membrane into distinct apical and basolateral domains

(Tepass *et al.*, 2001; Nelson, 2003). The apical membrane is organized into a domain that faces the external or internal milieu and a more lateral domain that faces a neighboring cell, known in *Drosophila* as the marginal zone (Fig. 14). Similarly, the basolateral domain is divided into a basal domain that mediates cell-matrix adhesion and a lateral domain where cells adhere to each other. Polarization of epithelial cells depends on the asymmetric distribution of several protein complexes to these different membrane domains. For instance, a complex consisting of the transmembrane protein Crumbs (Crb), Pals1/Stardust (Std), and Patj is concentrated at the marginal zone. Basal to the marginal zone, a circumferential belt of AJs (the Zonula Adherens) is formed which provides a strong mechanical link between adjacent cells. AJs consist of a conserved core cadherin-catenin complex (Halbleib and Nelson, 2006). The complex is organized around membrane-spanning cadherins that mediate intercellular adhesion by means of homophilic interactions and that use their cytoplasmatic tails to assemble an intracellular catenin complex that includes β-catenin (β-cat) bound to α-catenin (α-cat). The assembly of cell-cell adhesion complexes is concomitant with the establishment of cell polarity (Tepass *et al.*, 2001), and in the absence of AJs there is a failure to maintain the epithelial organization of tissues (Muller and Wieschaus, 1996; Harris and Peifer, 2004). Importantly, the requirement of epithelial cells to maintain an apical-basal polarity and to remain in intimate contact throughout morphogenesis constrains their morphogenetic potential.

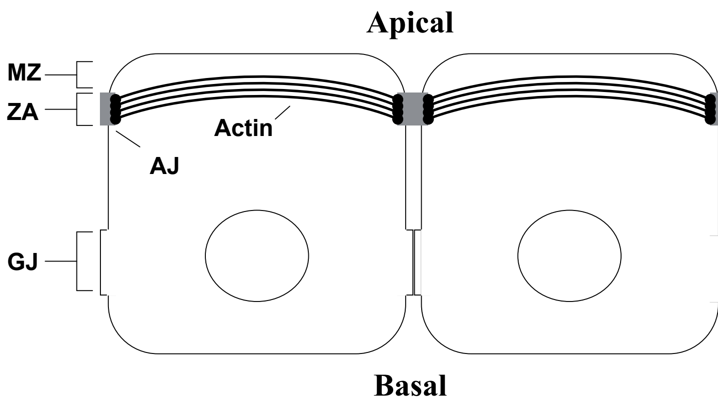

Figure 14. Schematic representation of epithelial cell structure during early and mid embryogenesis in *Drosophila*. Epithelial cells are arranged into laterally coherent sheets. Cells are linked to each other through adherens junctions (AJs) that form a circumferential belt known as the Zonula Adherens (ZA) surrounding the apical pole of each cell. Under the plasma membrane, at the level of AJs, Actin filaments are organized into a ring-like structure. The marginal zone (MZ) is located apical to AJs. Epithelial cells also contain gap junctions (GJ) that permit small molecules and ions to pass freely between cells.

A small number of basic mechanisms drive changes in epithelial tissue architecture during development. Common mechanisms include: (1) local invagination of a tissue to form an infolding; (2) delamination of a cell sheet that splits one sheet into two; (3) involution of cells by coordinated inward migration of a group of cells; (4) ingression of individual cells through migration; (5) cell intercalations in the plane of the tissue to drive tissue elongation; and (6) epiboly whereby one cell sheet spreads as a unit to enclose underlying layers. Each of these types of tissue rearrangements depends on the interplay between AJs and the Actin cytoskeleton and its associated proteins. For example, Myosin II regulates AJ remodeling during cell intercalations that drive germ band extension in *Drosophila* (Bertet *et al.*, 2004; Lecuit, 2005). Coordination of cell adhesion and Actin contraction is also essential for apical constriction of epithelial cells during tissue invagination, which provides the driving force for gastrulation movements or neurulation in many invertebrates and vertebrates (Lecuit and Lenne, 2007). Apical constriction requires assembly of AJs and a contractile network at the apical cell cortex (Cox *et al.*, 1996; Dawes-Hoang *et al.*, 2005). This network is made up of Actin-Myosin filaments that are tethered to AJs. The filaments consist of overlapping, antiparallel F-Actin arrays, with Myosin II located in between Actin filaments. In a simple model, Myosin II-dependent sliding of Actin filaments past one another induces contraction of the microfilament network. As a consequence, the apical cell perimeter is reduced and cells constrict. This creates tension in the tissue, which is transmitted from cell to cell through AJs and causes the whole tissue to bend. This model assumes the existence of a stable link between Actin and AJs, and the role of this Actin-AJ cross-linker was long-thought to be played by α-cat. This idea has recently been called into question by the finding that α-cat cannot bind to both Actin and β-cat simultaneously (Drees *et al.*, 2005; Yamada *et al.*, 2005a). How, then, contractile Actin filaments are linked to AJs is currently unknown (Gates and Peifer, 2005). Nevertheless, the tethering of the cortical Actin-Myosin network to AJs is illustrated by an elegant experiment demonstrating that in the absence of AJs, the Actin-Myosin network can contract while at the same time the cell membrane remains unconstricted (Dawes-Hoang *et al.*, 2005).

6.2 Rho-family GTPases During Epithelial Morphogenesis in the *Drosophila* Embryo

The profound effects of Rho-family GTPases on the behavior of cultured cells has led many investigators to explore the roles of these GTPases in developing embryos. The *Drosophila* embryo is a particularly useful system to investigate how epithelia are structured and how tissue movement is regulated during

development. In addition, the power of *Drosophila* genetics has provided a tool to investigate the role of Rho-family GTPases during epithelial sculpturing, as well as to unveil the signaling networks within which these factors are embedded (Settleman, 2001; Van Aelst and Symons, 2002). In *Drosophila*, genes encoding Rho1, Rac1, Rac2, and Cdc42 were originally identified by sequence similarity to Rho-family homologs in other organisms, and their gene products are 70–90 percent identical to their mammalian counterparts (Luo *et al.*, 1994; Harden *et al.*, 1995; Hariharan *et al.*, 1995). Subsequent work led to the characterization of three additional Rho-family GTPase homologs, Mtl, RhoL, and RhoBTB (Murphy and Montell, 1996; Newsome *et al.*, 2000). Similar to studies in cell culture systems, initial efforts to investigate Rho, Rac, and Cdc42 function during *Drosophila* embryogenesis relied on the use of constitutively activated or dominant-negative forms of the GTPases. Expression of dominant-negative forms disrupted epithelial morphogenesis due to defects in epidermal cell shape (Harden *et al.*, 1995; Barrett *et al.*, 1997; Häcker and Perrimon, 1998; Harden *et al.*, 1999). Important conclusions from these studies were that different Rho-family GTPases had distinct roles in morphogenesis and that they largely act in parallel during development.

A major breakthrough came with the identification of loss-of-function mutations in genes that encoded the respective Rho-family GTPases (Fehon *et al.*, 1997; Magie *et al.*, 1999; Hakeda-Suzuki *et al.*, 2002; Ng *et al.*, 2002). From analysis of zygotic *Rho1* mutants it became evident that *Rho1* was required for dorsal closure and head involution (Magie *et al.*, 1999). This phenotype was distinct from that of *Cdc42* mutants, which exhibited a breakdown of the ventral epidermis, in addition to defects in germ band retraction and dorsal closure (Genova *et al.*, 2000). Lastly, *Rac1*, *Rac2*, and *Mtl* were shown to have overlapping functions during dorsal closure (Hakeda-Suzuki *et al.*, 2002). Since then, a more detailed analysis of cellular behavior in Rho-family GTPase mutant backgrounds has revealed that the *in vivo* function of these GTPases mirror, to a large extent, the findings of classical cell culture experiments with *Rho1* being required for actin cable assembly (Jacinto *et al.*, 2002b), *Cdc42* for the formation of filopodia (Jacinto *et al.*, 2000), and *Rac1*, *Rac2,* and *Mtl* for actin organization and protrusiveness (Woolner *et al.*, 2005). In addition, Rho1 regulates AJ dynamics in the epidermis (Magie *et al.*, 2002; Fox *et al.*, 2005); Rac1, Rac2 and Mtl act upstream of the Jun N-terminal kinase (JNK) pathway to regulate gene expression and shape changes in leading edge cells during dorsal closure (Woolner *et al.*, 2005); and Cdc42 is required to set up apical-basal polarity in epithelial cells (Hutterer *et al.*, 2004).

During the past decade, great effort has been invested to delineate effector pathways downstream of Rho-family GTPases. Consequently, many of the GTPase effectors and downstream factors described in other systems, such as Dia (Castrillon and Wasserman, 1994; Afshar *et al.*, 2000; Grosshans *et*

al., 2005; Homem and Peifer, 2008; Mulinari *et al.*, 2008), Rho-kinase (Rok) (Mizuno *et al.*, 1999; Bertet *et al.*, 2004; Dawes-Hoang *et al.*, 2005), SCAR/WAVE, WASP and Arp2/3 (Zallen *et al.*, 2002), MRLC/Spaghetti-Squash (Sqh) (Karess *et al.*, 1991; Bertet *et al.*, 2004), non muscle-Myosin Heavy Chain (nm-MHC)/Zipper (Zip) (Young *et al.*, 1993; Jacinto *et al.*, 2002a; Franke *et al.*, 2005), MBS (Mizuno *et al.*, 2002; Tan *et al.*, 2003), Protein kinase N (dPKN) (Lu and Settleman, 1999), and dPAK (Harden *et al.*, 1996; Conder *et al.*, 2004), have all been analyzed during epithelial morphogenesis in the *Drosophila* embryo. Similarly, much research has been devoted to deciphering the events upstream of Rho-family GTPases during epithelial morphogenesis, in particular the RhoGEFs and RhoGAPs that regulate Rho-family GTPase activity. These studies have outlined the importance of RhoGEFs and RhoGAPs for a spectrum of cell functions and have started to elucidate the regulatory network within which these factor act.

6.3 *Drosophila* RhoGEFs and RhoGAPs

Several of the approximately 20 RhoGAPs (Bernards, 2003) and 20 RhoGEFs (Settleman, 2001) encoded in the *Drosophila* genome, have been functionally characterized. Collectively, they regulate various cellular processes that depend on Rho-family GTPases, including apical cell constriction (Barrett *et al.*, 1997; Häcker and Perrimon, 1998; Nikolaidou and Barrett, 2004; Denholm *et al.*, 2005; Brodu and Casanova, 2006; Sanny *et al.*, 2006; Simoes *et al.*, 2006; Kolesnikov and Beckendorf, 2007), cell migration (Lundström *et al.*, 2004; Schumacher *et al.*, 2004; Smallhorn *et al.*, 2004), and cytokinesis (Prokopenko *et al.*, 1999; Zavortink *et al.*, 2005). For instance, the conserved RhoGEF Pbl (Tatsumoto *et al.*, 1999; O'Keefe *et al.*, 2001) controls cytokinesis (Prokopenko *et al.*, 1999; Somers and Saint, 2003), but also the lateral migration of mesodermal cells in response to signaling by the Fibroblast Growth Factor Receptor (FGFR) Heartless (Htl) (Schumacher *et al.*, 2004; Smallhorn *et al.*, 2004). Other GEFs, such as the Rho-specific GEF64C (Bashaw *et al.*, 2001) and the Rac-specific Trio (Awasaki *et al.*, 2000; Bateman *et al.*, 2000; Liebl *et al.*, 2000; Newsome *et al.*, 2000), regulate cell shape changes in the developing nervous system. RhoGEF64C has also been implicated in posterior spiracle invagination in conjunction with DRhoGEF2 (see below) (Simoes *et al.*, 2006). In addition, several predicted RhoGEF-encoding genes for which loss-of-function alleles are not yet available exhibit dynamic expression patterns during embryogenesis. Of particular interest are *DRhoGEF3* (Hicks *et al.*, 2001), *DRhoGEF4* (Nahm *et al.*, 2006) and the unnamed RhoGEF gene family member *CG30115*, respectively, which are all expressed in morphogenetically active epithelial tissues. *CG30115*, for instance, is expressed in the

invaginating mesoderm, in the segmental grooves during groove morphogenesis, and at the epidermal leading edge during dorsal closure (Tomancak *et al.*, 2002). Future work will have to address putative roles for these RhoGEFs in *Drosophila* development.

As with the RhoGEFs, several *Drosophila* RhoGAP family members have been implicated in epithelial morphogenesis. This includes RhoGAP88C/Crossveinless-c (CV-C) which links EGFR-signaling to cytoskeletal remodeling during tracheal invagination (Brodu and Casanova, 2006), RhoGAP68F which controls apical cell constriction during VF formation (Sanny *et al.*, 2006), and the Rac/Cdc42GAP Vilse which physically associates with the guidance receptor Robo to regulate GTPase-signaling in response to migratory cues during tracheal development (Lundström *et al.*, 2004). In addition, RhoGAP5A and RhoGAP88C/CV-C are thought to act downstream of a Toll-like receptor protein during salivary gland morphogenesis (Kolesnikov and Beckendorf, 2007).

How the balance between RhoGEF and RhoGAP activities regulates the *in vivo* function of Rho-family GTPases during epithelial morphogenesis is unclear. It is plausible that RhoGEFs and RhoGAPs act in concert to provide spatio-temporal specificity to GTPase activation within cells. Consistent with this, a recent report that used invagination of the posterior spiracles as a system to investigate apical cell constriction, suggested that spatial restriction of activated Rho1 to the apical side of constricting cells is at least partially dependent on the reciprocal intracellular distribution of RhoGAPs and RhoGEFs to distinct compartments of the plasma membrane, with the GEFs localizing apically and GAPs more basally (Simoes *et al.*, 2006). Whether this applies to other morphogenetic processes as well remains to be investigated.

7 The Role of DRhoGEF2 During Epithelial Morphogenesis in the *Drosophila* Embryo

In *Drosophila,* the Rho1-specific GEF DRhoGEF2 has received particular attention for its role in epithelial morphogenesis. Mutations in *DRhoGEF2* were independently isolated in two parallel genetic screens, one attempting to identify novel Rho1 signaling pathway components (Barrett *et al.*, 1997), the other a large P-element based mutagenesis screen designed to characterize the maternal effects of zygotic lethal mutations (Häcker and Perrimon, 1998). DRhoGEF2 belongs to the large DBL-family of RhoGEFs, and is the sole fly member of the Regulator of G-protein Signaling (RGS) domain-containing subfamily, which includes mammalian PDZ-RhoGEF, p115-RhoGEF, and LARG (Hart *et al.*, 1998; Kozasa *et al.*, 1998; Fukuhara *et al.*, 1999; Fukuhara *et al.*, 2000), zebrafish Arhgef11 (Panizzi *et al.*, 2007), and *C. elegans* CeRhoGEF (Yau *et al.*, 2003). A large body of evidence supports the view that this RhoGEF-subfamily promotes nucleotide exchange specifically on the GTPase Rho (Hart *et al.*, 1998; Fukuhara *et al.*, 1999; Fukuhara *et al.*, 2001; Reuther *et al.*, 2001; Suzuki *et al.*, 2003; Derewenda *et al.*, 2004; Kristelly *et al.*, 2004; Oleksy *et al.*, 2006).

7.1 DRhoGEF2 Expression in the Embryo

Transcripts of *DRhoGEF2* are maternally provided to the egg and ubiquitously expressed in the embryo (Barrett *et al.*, 1997; Häcker and Perrimon, 1998). Analysis of DRhoGEF2 protein distribution has revealed that DRhoGEF2 localizes cortically in epithelial cells throughout embryogenesis and is enriched at the apical end of cells (Padash Barmchi *et al.*, 2005). Levels of DRhoGEF2 are specifically elevated in groups of cells that undergo shape changes driven by assembly and contraction of Actin-Myosin-based filaments. Increased DRhoGEF2 levels are, for instance, detected at the membrane front of the invaginating furrow canal that separates blastoderm cells during cellularization, at the apical membrane of constricting ventral furrow cells during gastrulation, and in a polarized fashion at the epidermal leading edge during dorsal closure, where the assembly of a contractile supracellullar Actin-Myosin cable supports closure of the embryo dorsally.

7.2 Structure and Evolutionary Conservation

DRhoGEF2 encodes a large protein of 2 559 amino acids that contains several conserved protein domains (Fig. 15). The C-terminal region of DRhoGEF2 shares a high degree of similarity with conserved DH/PH sequences found in all Dbl-family proteins. In addition to this family characteristic DH/PH module, DRhoGEF2 encompasses an amino (N)-terminal PSD-95/Dlg/ZO-1 (PDZ) domain and an adjacent RGS domain. PDZ domains are known as protein-protein interaction domains that act as scaffolds to concentrate signaling molecules at specific regions in the cell (Harris and Lim, 2001; Garcia-Mata and Burridge, 2007), while RGS domains can interact with α subunits of heterotrimeric G proteins during signal transduction (Watson *et al.*, 1996). In the central part, DRhoGEF2 contains a putative cysteine-rich diacylglycerol (DAG)-binding domain (Barrett *et al.*, 1997). A homologous domain present in Protein kinase C mediates kinase activation in response to DAG, which is generated by phospholipase C-mediated cleavage of membrane lipids (Azzi *et al.*, 1992). There is currently no direct evidence that DAG can activate DRhoGEF2 or any of its orthologs. By contrast, there is strong evidence for direct roles of the PDZ and RGS domains in the regulation of RGS-RhoGEF localization or activity (Sternweis *et al.*, 2007). Rat PDZ-RhoGEF, for instance, has been shown to bind light chain 2 (LC2) of MT-associated protein 1 via its PDZ domain, an interaction that modulates GEF activity and subcellular localization (Longhurst *et al.*, 2006). In addition, the PDZ domains of PDZ-RhoGEF and LARG can associate directly with Plexin-B, a Semaphorin-4D receptor (Aurandt *et al.*, 2002; Swiercz *et al.*, 2002) and/

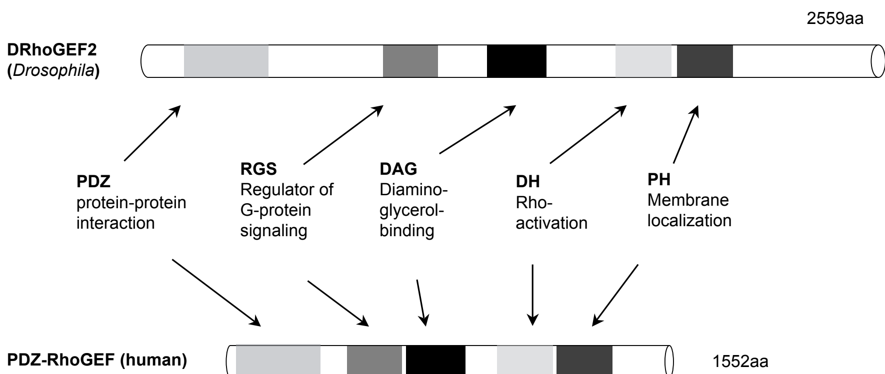

Figure 15. Schematic representation of DRhoGEF2 and one of its mammalian orthologs, PDZ-RhoGEF. The relative position and putative function of conserved protein domains are indicated (see text for details). (aa) amino-acids.

or, in the case of LARG, the insulin-like growth factor 1 (IGF-1) receptor (Taya *et al.*, 2001). Notably, the PDZ-binding motif of Plexin-B is similar to that of the T48 protein, which has recently been shown to bind to the PDZ domain of DRhoGEF2 (Kölsch *et al.*, 2007). The PDZ domains of LARG and PDZ-RhoGEF also interact with the C-terminus of the heterotrimeric GPCRs, $LPAR_1$ and $LPAR_2$ (for Lysophosphatidic Acid Receptors) (Yamada *et al.*, 2005b). In addition, LPAR-activated $G\alpha_{12}$ or $G\alpha_{13}$ subunits mediate signals from these GPCRs to Rho. In this pathway, $G\alpha_{12/13}$ subunits bind to the RGS domains of p115-RhoGEF (Hart *et al.*, 1998; Kozasa *et al.*, 1998), LARG (Suzuki *et al.*, 2003), or PDZ-RhoGEF (Fukuhara *et al.*, 1999), as well as to the DH/PH module of p115RhoGEF (Chen *et al.*, 2003) to promote the intrinsic GEF activity towards RhoA. Significantly, DRhoGEF2 has been implicated to act downstream of the closely related $G\alpha_{12/13}$ fly homolog Concertina (Cta). Thus, it appears likely that a Rho-mediated signaling pathway linked to heterotrimeric $G\alpha$ proteins has been evolutionarily conserved (Fukuhara *et al.*, 2001). This is further supported by the discovery that also zebrafish Arhgef11 (Panizzi *et al.*, 2007) and *C. elegans* CeRhoGEF (Yau *et al.*, 2003) connect GPCR-activated $G\alpha_{12/13}$ subunits to Rho.

7.3 The Role of DRhoGEF2 During *Drosophila* Development

Embryos homozygous mutant for *DRhoGEF2* die during late embryogenesis or early larval stages exhibiting no apparent morphological defects (Barrett *et al.*, 1997; Häcker and Perrimon, 1998). However, since *DRhoGEF2* transcripts are supplied maternally to the egg, this maternal component must be removed in order to reveal the *DRhoGEF2* loss-of-function phenotype during embryogenesis. The most prominent phenotype of embryos lacking maternal *DRhoGEF2* is the failure to invaginate mesodermal and endodermal primordia during gastrulation. Although mesodermal and endodermal cell fates are properly established in maternal *DRhoGEF2* mutants, the coordinated cell shape changes that drive tissue invagination are never initiated. Instead, many cells fail to constrict apically and cell shape becomes erratic. A similar defect can be induced by expression of dominant negative Rho1 but not Rac1 or Cdc42, thus placing DRhoGEF2 upstream of Rho1 in this process. The specificity of DRhoGEF2 for Rho1 has since then been confirmed by both genetic and biochemical data (Rogers *et al.*, 2004; Grosshans *et al.*, 2005; Padash Barmchi *et al.*, 2005).

Evidence identifying factors that connect the DRhoGEF2 to the Actin cytoskeleton emerged first from genetic studies. A screen for second-site non-complementors of a mutation in the *zip* gene, which encodes nm-MHC, identified *DRhoGEF2* and *Rho1* (Halsell *et al.*, 2000), suggesting a link

between DRhoGEF2/Rho1 and Myosin II. Similar to DRhoGEF2, Myosin II concentrates at the apical membrane of invaginating VF cells prior to constriction (Young *et al.*, 1991) and DRhoGEF2 has subsequently been shown to regulate this translocation through Rho1-signaling (Nikolaidou and Barrett, 2004; Dawes-Hoang *et al.*, 2005) (see below).

Although failure in gastrulation is the most striking phenotype of *DRhoGEF2* deficient embryos, detailed analysis of maternal *DRhoGEF2* mutants has established a role for DRhoGEF2 in the maintenance of cell shape throughout early embryogenesis (Häcker and Perrimon, 1998), which fits well with the ubiquitous expression of DRhoGEF2 in embryonic tissues (Padash Barmchi *et al.*, 2005). Further studies have implicated *DRhoGEF2* in other morphogenetic processes such as salivary gland invagination (Nikolaidou and Barrett, 2004), wing epithelial folding (Nikolaidou and Barrett, 2004), leg morphogenesis (Halsell *et al.*, 2000), and posterior spiracle invagination (Simoes *et al.*, 2006). In all cases, DRhoGEF2 appears to regulate the apical constriction of cells, most likely by controlling assembly or contraction of apically localized Actin-Myosin based filaments. Most extensively characterized is, however, the role of DRhoGEF2 in VF formation.

7.3.1 Invagination of the mesoderm

Mesoderm invagination through the VF has served as an excellent system to study how changes in epithelial cell shape are regulated in the context of a developing mutlticellullar organism. The first morphologically distinguishable event in VF formation is the apical flattening of the ventral-most cells of the presumptive mesoderm (see Fig. 4). This is followed by rapid apical constriction, converting the cells from cuboidal to wedge shaped, which induces the epithelium to fold, thereby forcing mesodermal cells to move inside the embryo.

During the last two decades, several factors that are required for VF formation have been identified. These factors can be grouped into three classes: cell fate determinants, signaling molecules, and cytoskeletal regulators. When placed in hierarchical order, they explain development of the mesoderm from the early establishment of cell fate, over the triggering of a signaling cascade in the prospective mesoderm, to the activation of cytoskeletal effector molecules. The latter reorganize the Actin-based cytoskeleton to bring about a series of reproducible changes in cell shape that drive tissue invagination.

Mesodermal fate is established prior to gastrulation. It is marked by the expression of two zygotic transcription factors, Twi and Sna, whose expression is restricted to a band of ventral cells and defines the mesodermal primordium (Kosman *et al.*, 1991; Leptin, 1991, 1995). In *twi* and *sna* double mutants, mesodermal differentiation is blocked and ventral furrow formation does not occur (Leptin, 1991). The differential expression of *twi* and *sna* along the D/V

axis is established by a nuclear gradient of the maternal transcription factor Dorsal which peaks in the most ventral nuclei and decreases steadily towards the more dorsal territories (Roth *et al.*, 1989; Rushlow *et al.*, 1989; Steward, 1989). High nuclear levels of Dorsal are required to trigger expression of *twi* and *sna*, thus ensuring that mesodermal fate is restricted to cells at the ventral surface of the embryo. *twi* and *sna* provide the link between the maternal patterning program and the establishment of cell fate. While Sna generally functions as a repressor of ectodermal genes, Twi acts as a transcriptional activator determining mesodermal cell fate (Leptin, 1991).

Twi positively regulates *sna* (Kosman *et al.*, 1991; Leptin, 1991; Ip *et al.*, 1992), but also triggers expression of two other genes required for efficient gastrulation, *folded gastrulation* (*fog*) (Costa *et al.*, 1994; Morize *et al.*, 1998; Dawes-Hoang *et al.*, 2005) and *T48* (Kölsch *et al.*, 2007), which encode an apically secreted ligand and a cortically localized transmembrane protein, respectively. *fog* is also expressed in the PMG primordium where its expression depends on two other transcription factors, Hkb and Tll (Costa *et al.*, 1994). Thus, *fog* and *T48* expression prefigures the appearance of invaginations in the cellular blastoderm.

T48 and Fog act at the interface between cell fate determinants and the Rho1-signaling cascade that regulates cell constriction (Fig. 16). According to a recent model (Kölsch *et al.*, 2007; Lecuit and Lenne, 2007), Fog and T48 act in parallel to recruit DRhoGEF2 to the apical cell cortex of mesodermal cells. T48 directly binds the PDZ domain of DRhoGEF2 (Kölsch *et al.*, 2007), whereas Fog acts in an autocrine fashion to activate the Gα subunit Cta (Parks and Wieschaus, 1991; Morize *et al.*, 1998), presumably by activating a GPCR that has yet to be identified. By analogy to mammalian DRhoGEF2 orthologs, Cta is thought to interact with the RGS domain of DRhoGEF2 to promote its apical enrichment and activation. Studies in *Drosophila* cell culture have suggested that this could be mediated by Cta-induced dissociation of DRhoGEF2 from MT tips (Rogers *et al.*, 2004).

Once localized to the apical membrane, DRhoGEF2 can catalyze nucleotide exchange on apical Rho1 to induce downstream signaling (Barrett *et al.*, 1997; Häcker and Perrimon, 1998; Grosshans *et al.*, 2005). The identification of a putative negative regulator of Rho1, RhoGAP68F, as a part of the regulatory network that triggers VF formation, further supports the view that regulated Rho1-activivation is important for apical mesodermal cell constriction (Sanny *et al.*, 2006).

Apically activated Rho1 appears to signal to two well-characterized Rho effectors, the kinase Rok (Mizuno *et al.*, 1999; Winter *et al.*, 2001; Dawes-Hoang *et al.*, 2005) and the formin Dia (Afshar *et al.*, 2000; Homem and Peifer, 2008). As described in other systems (Sahai and Marshall, 2002), Rok and Dia may act in parallel to promote assembly and subsequent contraction

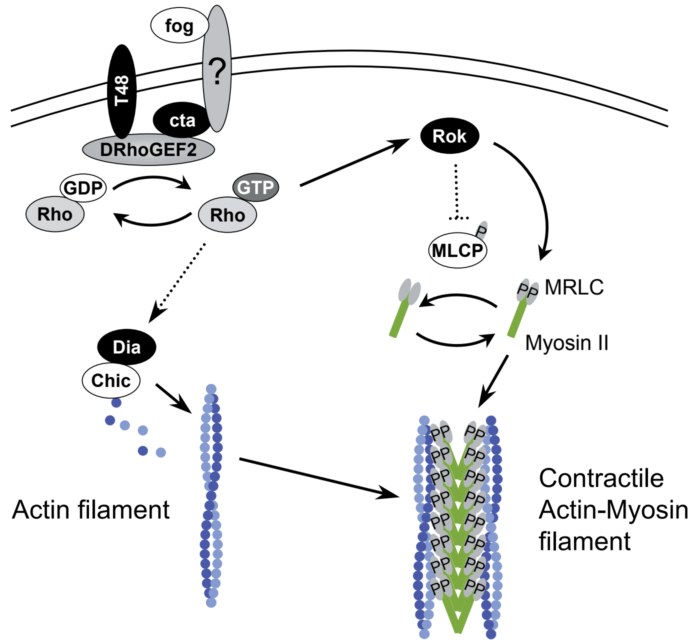

Figure 16. DRhoGEF2-signaling during ventral furrow formation. Constriction of VF cells requires the assembly and contraction of Actin-Myosin filaments at the apical end of cells. The ligand Fog and the transmembrane protein T48 act at the top of the signal transduction pathway that elicits constriction. Fog and T48 function in parallel to recruit DRhoGEF2 to the apical cell cortex. T48 binds to the PDZ domain of DRhoGEF2 while Fog activates the G protein α subunit Cta via an unknown receptor. Apically recruited DRhoGEF2 promotes nucleotide exchange on Rho1 to activate downstream signaling. GTP-bound Rho1 is linked to the Actin cytoskeleton via two parallel effector pathways. Rho1 activates Rok to induce phosphorylation of MRLC, which results in recruitment of Myosin II to the apical cell cortex. MRLC phosphorylation also promotes the association of F-Actin and Myosin II into force-generating filaments. Rho1 may in addition activate Dia to promote production of linear Actin filaments. However, it is unclear whether Rho1 activates Dia in response to DRhoGEF2 signaling (dashed line). Several other factors may be involved in this pathway, such as Profilin/Chickadee (Chic) in Actin monomer delivery, and the MBS of MLCP in MRLC regulation. Dashed lines depict regulatory connections during VF formation that have been postulated but not directly demonstrated.

of the Actin-Myosin network at AJs. In this process, Rok has a conserved function to increase the phosphorylation state of MRLC (Mizuno *et al.*, 1999; Winter *et al.*, 2001), encoded by *sqh* in *Drosophila* (Karess *et al.*, 1991; Jordan and Karess, 1997). Myosin II is a hexameric protein consisting of two heavy chains, two light chains, and two regulatory light chains. In this Myosin II complex, the MHC subunits (*Drosophila* Zip) interact directly with Actin through the region containing the ATPase activity that drives this Actin-based motor (Young *et al.*, 1993). Phosphorylation of MRLC promotes assembly of Myosin II into force-generating filaments, which results in increased motor

activity (Tan *et al.*, 1992; Somlyo and Somlyo, 2000). Phosphorylation also appears to be essential for recruitment of Myosin II to the apex of cells prior to gastrulation. Accordingly, apical Myosin II accumulation is lost and cells fail to constrict in *rok* mutants (Dawes-Hoang *et al.*, 2005) and in embryos treated with the Rok inhibitor Y27632 (Pilot and Lecuit, 2005), a phenotype mimicked by loss of *DRhoGEF2* (Nikolaidou and Barrett, 2004; Dawes-Hoang *et al.*, 2005). In addition, a mutated version of Myosin II that lacks the Actin-binding domain failed to localize to the apical side of VF cells, providing strong evidence that Myosin II localization to VF cells is dependent on Actin binding and/or contractility (Dawes-Hoang *et al.*, 2005). Together, these data suggest that apically recruited DRhoGEF2 activates Rok through Rho1 to trigger phosphorylation of Sqh, which, in turn, is a prerequisite for apical enrichment of Myosin II. In accordance with this view, overexpression of DRhoGEF2-pathway components in Schneider-2 (S2) cells promotes Myosin II accumulation and cell contraction that can be inhibited by Rok inactivation (Rogers *et al.*, 2004).

In contrast to Rok, the role of Dia in the generation of contractile force as well as its mechanistic link to DRhoGEF2 is less clear. Based on the established role of DRFs in nucleation and polymerization of unbranched Actin filaments, it seems likely that Dia participates in the organization or stabilization of the perijunctional Actin ring (Fig. 16). However, recent data has revealed a role for Dia in promoting Myosin II activity and AJ stability in *Drosophila* (Homem and Peifer, 2008; Mulinari *et al.*, 2008) and it will be important to investigate Dia function during apical cell constriction in detail in the future. If Dia, indeed, participates in the regulation of Actin dynamics, it may cooperate with another Actin regulator, the Actin-binding non-receptor tyrosine kinase Abl (Grevengoed *et al.*, 2003). Similar to Dia, Abl regulates apical constriction of VF cells (Fox and Peifer, 2007). The role of Abl in cell constriction is conserved, as double mutants for the two mouse *abl* orthologs – *abl* and *arg* – exhibit disrupted neural tube closure and defects in Actin organization in constricting neuroepithelial cells (Koleske *et al.*, 1998). Interestingly, the *Drosophila abl* mutant phenotype is characterized by the failure of many VF cells to apically constrict despite proper localization of the constriction machinery, as judged by DRhoGEF2 and Myosin II detection (Fox and Peifer, 2007). Instead, Abl appears to regulate ordered apical Actin assembly at the cell cortex in parallel to DRhoGEF2 (Grevengoed *et al.*, 2003; Fox and Peifer, 2007). By contrast to DRhoGEF2, Abl acts specifically by down-regulating the Actin anti-capping protein Enabled (Ena) (Grevengoed *et al.*, 2003; Gates *et al.*, 2007), and embryos mutant for *abl* exhibit ectopic Ena as well as ectopic Actin accumulations in VF cells (Fox and Peifer, 2007).

7.3.2 Cellularization

Drosophila embryogenesis starts with 13 cycles of synchronous nuclear divisions that are not accompanied by cytokinesis to form a syncytial blastoderm embryo. During the 14th division cycle, cell membranes invaginate radially between the nuclei to cellularize the blastoderm. The discovery that DRhoGEF2 localizes to the front of the invaginating membranes during cellularization suggested that DRhoGEF2 might have a role in blastoderm cellularization (Grosshans *et al.*, 2005; Padash Barmchi *et al.*, 2005). Analysis of *DRhoGEF2* mutant embryos revealed that Actin fails to efficiently re-localize to the furrow canal in the absence of DRhoGEF2. Subsequently, interconnected Actin-Myosin hexagons that surround individual nuclei fail to constrict. Despite these defects, DRhoGEF2 mutant embryos complete basal closure of blastoderm cells, most likely due to the contribution of other mechanisms such as membrane insertion or MT-based forces.

Which Rho1-effectors could then connect DRhoGEF2 to contractile Actin-Myosin filaments during cellularization? Rok and Dia are likely candidates since both factors are involved in this process. However, the question whether DRhoGEF2 specifically acts trough Rok, Dia, or both during cellularization is not resolved. Comparison of the *dia*, *rok*, and *DRhoGEF2* mutant phenotypes, respectively, suggests that Dia and Rok may have a broader spectrum of functions than DRhoGEF2 (Afshar *et al.*, 2000; Dawes-Hoang *et al.*, 2005). Specifically, Dia and Rok are both involved in cytokinesis, a process unaffected in *DRhoGEF2* mutant embryos (Nikolaidou and Barrett, 2004; Padash Barmchi *et al.*, 2005). These observations illustrate an important concept: The RhoGEFs activating Rho signaling tend to show greater specificity for individual processes than the effectors downstream of Rho, which are more generally employed.

Nevertheless, phenotypes of *DRhoGEF2* and *rok* mutants during cellularization are strikingly similar suggesting that DRhoGEF2 acts trough Rok (Dawes-Hoang *et al.*, 2005). However, *DRhoGEF2* and *dia* mutants also share phenotypic similarities, which has sparked the hypothesis that also Dia might act as an effector of DRhoGEF2 during cellularization. Thus, in both *dia* and *DRhoGEF2* mutants, morphology of the furrow canal is disrupted and pole cells fail to form properly. Grosshans and co-workers (Grosshans *et al.*, 2005) suggested therefore that DRhoGEF2 might activate Dia downstream of Rho1 to induce assembly of Actin filaments in the furrow canal. Consistent with this view, they reported that Dia localization in the furrow canal was affected in *DRhoGEF2* mutants during the early phase of cellularization. This result is in contrast to Padash Barmchi et al (Padash Barmchi *et al.*, 2005) who found that Dia localization was unaffected in *DRhoGEF2* mutants. Moreover, they reported that the *DRhoGEF2* mutant phenotype was significantly different from that of *dia* mutants (Afshar *et al.*, 2000).

Specifically, *dia* mutants exhibit a failure to form stable contractile rings (Afshar *et al.*, 2000). *DRhoGEF2* mutants, by contrast, form stable Actin rings but these rings fail to constrict. The authors further reported that the temporal and spatial localization of the Septin family protein Peanut, whose recruitment to the cellularization front is disrupted in *dia* mutants (Afshar *et al.*, 2000), was unaffected in *DRhoGEF2* mutants (Padash Barmchi *et al.*, 2005). These observations are consistent with the idea that DRhoGEF2 may regulate Actin-Myosin contractility independent of Dia, and that Dia may specifically regulate the assembly of Actin rings. Importantly, the suggestion that DRhoGEF2 acts trough Rok but not Dia implies that DRhoGEF2 may provide pathway specificity downstream of Rho1.

8 Genetic Tools and Techniques

Drosophila is an attractive model system for the study of various aspects of biology, including genetics, cell biology, development, behavior, and disease (often in a combinatorial fashion). The use of the *Drosophila* system is tightly linked to the availability of sophisticated tools and techniques for gene manipulation that have been introduced and refined during the course of the past three decades. Collectively, the use of this "genetic tool box" has enabled the discovery of gene networks and signaling pathways that orchestrate patterning and morphogenesis throughout development. In the following, two important genetic techniques that were used in this thesis are presented.

8.1 Generation of Germline Clones Using the FLP-DFS Technique

During oogenesis, nurse cells in the female ovary deposit mRNA and protein of a large number of genes in the developing egg. This maternal contribution of gene products provides essential gene function during the early phase of embryogenesis before the onset of zygotic transcription. Importantly, maternally contributed gene products can rescue the loss of zygotic gene function in embryos that are homozygous mutant for the gene in question. In order to permit genetic analysis of such genes, both the maternal and zygotic contribution must be eliminated.

In 1993, Chou and Perrimon developed the "Flipase Recombinase – Dominant Female Sterile" technique (FLP-DFS) that made it possible to efficiently remove the maternal component of a large majority of gene products (Chou *et al.*, 1993; Chou and Perrimon, 1996). This technique, which is outlined in Fig. 17, allows the generation of mosaic females that are homozygous for a lethal mutation in their germline, while heterozygous, and therefore viable, in somatic cells. The technique is based on the placement of a lethal mutation of interest on a chromosome that carries a recognition sequence (known as *FRT*) for the enzyme Flipase Recombinase (the FLP) in a centromere proximal location on the same chromosome arm as the mutation. Female flies carrying such a chromosome are mated to males that carry a chromosome with an *FRT* inserted at the same position but which in addition carries the dominant female sterile allele, ovo^D, distal to the *FRT*. The ovo^D mutation affects female germ cell development at an early stage, and females heterozygous for ovo^D do not lay eggs. In females that are transheterozygous for both the mutation and ovo^D, a FLP recombinase gene under control of a heat shock promoter carried on another chromosome is used to induce site-specific mitotic recombination

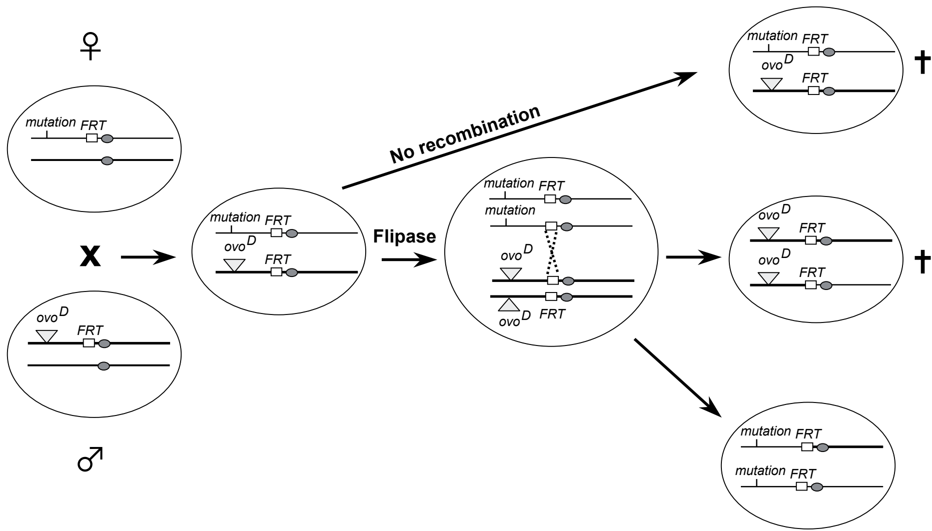

Figure 17. The Flipase Recombinase – Dominant Female Sterile technique. This system allows the generation of germline clones in the female ovary that are homozygous mutant for a gene of interest. (A) Males carrying the dominant female sterile allele *ovo*^{D} and an *FRT* at the base of the chromosome arm are mated to females that carry the same *FRT* and a lethal mutation of interest distal to the *FRT* on the same chromosome arm. (B) In the progeny, FLP expression is induced from a heat-shock transgene. The FLP can induce site-specific mitotic recombination between non-sister chromatids at the *FRT* sites. (C) Following mitosis, daughter cells are generated that are either homozygous for the mutation, homozygous for *ovo*^{D} or, if no mitotic recombination occurred, heterozygous for both the mutation and *ovo*^{D}. In the female germline, only descendents from daughter cells homozygous mutant for the mutation can develop since *ovo*^{D} aborts oogenesis at an early stage. Thus, only eggs that are maternally mutant for the gene of interest will develop.

between non-sister chromatids at the FRT sites. This results in the generation of daughter cells that are either homozygous for the mutation or homozygous for *ovo*^{D}. In the ovary, only descendents from homozygous mutant daughter cells can develop since *ovo*^{D} aborts oogenesis. As the descendent cells undergo subsequent rounds of divisions, a clone of mutant cells is generated in the germline. Female flies that carry germline clones exclusively produce eggs that are maternally mutant for the gene of interest.

8.2 The Gal4/*UAS* System

An important tool for the analysis of gene function is the ability to missexpress or overexpress a gene of interest in a temporally or spatially restricted fashion. In the *Drosophila* system, this can be achieved using the Gal4/*UAS* system (Fig. 18), which was originally developed by Andrea Brand in the Perrimon

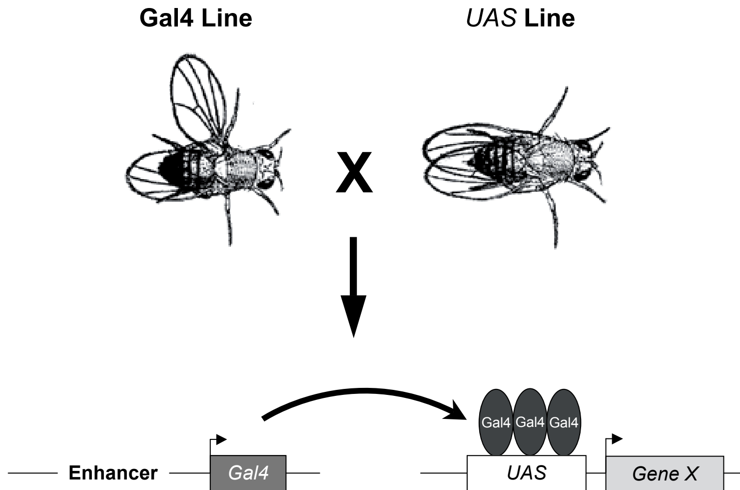

Figure 18. The Gal4/UAS system. This system allows the expression of a gene of interest in a spatio-temporal pattern in a variety of tissues in *Drosophila*. Flies in which the *Gal4*-gene is expressed under control of an endogenous enhancer, which drives its expression in a specific spatio-temporal pattern, are mated to flies that carry the cloned DNA sequence fused to the *UAS*-enhancer. In the progeny, the Gal4 protein binds to the *UAS*-enhancer to activate expression of the target gene.

lab in parallel to the FLP-DFS technique (Brand and Perrimon, 1993). The system makes use of the yeast (*S. cerevisiae*) transcriptional activator, Gal4, to drive the expression of target genes under control of Gal4-specific *Upstream Activating Sequences* (*UAS*) to which it binds. The Gal4/*UAS* system is a bipartite system based on separation of the Gal4 transcriptional activator and the target gene fused to the *UAS*-enhancer in two distinct transgenic fly stocks. In the Gal4-stock, *Gal4* is expressed under control of an endogenous enhancer that drives its expression in a specific spatio-temporal pattern. Importantly, expression of Gal4 in *Drosophila* has no deleterious phenotypic effects since Gal4 has no targets in the fly genome. A large collection of different Gal4-stocks has been generated using an enhancer-trapping approach in which a mobile transposon element that contains the *Gal4* gene is inserted at random genomic sites where it can be expressed under the control of endogenous enhancers (Venken and Bellen, 2007). Most of these stocks are now available to the research community from public stock centers.

The second transgenic stock carries the target gene of interest under control of a *UAS* sequence. Because transcription of the target gene requires Gal4 to bind to the *UAS*-sequence, the gene remains transcriptionally silent in the absence of Gal4. Only when a Gal4-stock is mated to the *UAS*-stock is the target gene switched on in the progeny. The strength of the system arises from

the ability to mate a single *UAS*-stock to a selection of Gal4-stocks, which permits expression of the gene of interest in a variety of spatial and temporal patterns. A further development of the system is the employment of *UAS*-constructs that encode mutated versions of genes producing, for instance, truncated, dominant-negative, or activated proteins. In addition, the system has been adapted to enable mosaic knockdown of gene function by expressing siRNAs that target particular gene products to the RNAi machinery (Duffy, 2002). *UAS*-constructs are introduced into the genome through the use of P-element-based vectors that allow the placement of the gene of interest downstream of a *UAS*-enhancer.

9 Present Investigations

9.1 Paper I: *DRhoGEF2* and *Diaphanous* Regulate Contractile Force During Segmental Groove Morphogenesis in the *Drosophila* Embryo

Embryonic development in all animals is associated with extensive rearrangements of tissues and changes in the shape of individual cells. The driving force for these rearrangements is generated by a dynamic reorganization of the Actin cytoskeleton. In many instances, Rho-family GTPases that are activated by RhoGEFs play an important role in this process. Upon activation, Rho-family GTPases transduce a signal to the Actin cytoskeleton via parallel downstream effector pathways. In the first paper of this thesis, we investigated the roles of the Rho1 activator DRhoGEF2 and the Rho1 effector Dia during epidermal morphogenesis in the *Drosophila* embryo. The study focuses specifically on the morphogenesis of segmental grooves, which are segmentally repeated tissue infoldings that form in the epidermis during mid embryogenesis. Formation of segmental grooves is associated with changes in epidermal cell shape, including apical cell constriction (Larsen *et al.*, 2003).

The aim of this study was two-fold. First, we aimed to provide a detailed spatio-temporal description of the cell shape changes that accompany segmental groove morphogenesis at cellular resolution. Secondly, we sought to use segmental grooves as a system to compare the roles of DRhoGEF2 and Dia during epithelial morphogenesis. The interest to functionally compare DRhoGEF2 to Dia emerged from the hypothesis that individual RhoGEFs such as DRhoGEF2 may confer Rho-family GTPase specificity towards a subset of effector pathways. In this context, it is unclear whether DRhoGEF2, via Rho1, can activate Dia to promote Actin polymerization, or signals specifically through Rok to regulate Actin contractility (Grosshans *et al.*, 2005; Padash Barmchi *et al.*, 2005).

The first part of the paper presents a detailed examination of segmental groove morphogenesis (Fig. 1). We describe five morphologically distinguishable phases during groove morphogenesis that include: (1) apical constriction which results in initial bending of the epithelium during early stage 12; (2) apical-basal elongation of groove founder cells during late stage 12; (3) apical constriction of cells posterior to the groove initiating groove regression during stage 13; (4) apical-basal elongation of cells in the groove; and (5) outward movement of the apical cell surface, followed by subsequent apical-basal shortening and outward movement of the basal end of cells. In

addition, we report the localization of DRhoGEF2, F-Actin, and Myosin II throughout the process. Particularly striking is the accumulation of DRhoGEF2, F-Actin and Myosin II in cells posterior to the groove that undergo apical constriction at the time of groove regression.

In the second part of the paper, we present a functional comparison of DRhoGEF2 and Dia. Mutant analyses showed that *DRhoGEF2* and *dia* are essential for the morphogenesis of segmental grooves, but not for patterning of the epidermis as assayed by the expression of En (Fig. 2 and Fig. 5). In the absence of either *DRhoGEF2* or *dia* gene function, groove founder cells fail to constrict and invaginate. However, the lack of cell constriction could arise either due to lack of F-Actin polymerization or failure to constrict F-Actin. Thus, the loss of function phenotypes of *DRhoGEF2* or *dia* mutants cannot be used to establish epistatic relationships between the two factors. To circumvent this problem, we compared the phenotypic consequences resulting from overexpression of either factor in the epidermis at the time of groove morphogenesis. In the case of Dia, we used an activated form of the molecule, Dia^{CA} (Somogyi and Rørth, 2004), since overexpression of wild type Dia does not activate the pathway. Expression of either factor caused a deepening of segmental grooves (Fig. 3 and Fig. 6). Interestingly, however, morphologic changes elicited by Dia^{CA} at the cellular level were distinct from those observed with DRhoGEF2 (Fig. 3, Fig. 4, and Fig. 6). While DRhoGEF2-overexpression caused cells to contract and take on a rounded shape, thereby reducing cell-cell contact, Dia^{CA} expressing cells remained tightly packed and columnar and produced numerous apical filopodia. In addition, Dia^{CA}-expressing cells showed increased levels of the adherens junction proteins β-cat/Armadillo (Arm) and DE-cad, suggesting a strengthening of cell-cell contacts. The role of Dia in strengthening cell-cell junctions was confirmed in *dia* mutants, which exhibited loss of DE-Cad from the cell cortex in some areas (Fig. 5). Another distinguishing feature between DRhoGEF2 and Dia^{CA} was the accumulation of F-Actin in response to Dia^{CA} expression but not DRhoGEF2 overexpression. However, similar to DRhoGEF2, Dia^{CA} was able to trigger cortical Myosin II accumulations. Taken together, the results presented in Paper I suggest that DRhoGEF2 and Dia regulate different aspects of cytoskeletal reorganization. Our data is consistent with the view that Dia polymerizes Actin filaments whereas DRhoGEF2 regulates F-Actin contraction but not polymerization. We, therefore, hypothesize that DRhoGEF2 and Dia are connected to the Actin cytoskeleton through distinct Rho1 effector pathways. The mechanism for such selective Rho1 effector pathway activation by DRhoGEF2 remains to be elucidated. However, studies of RhoGEFs in other systems have identified RhoGEFs as components of multi-protein complexes that include specific Rho-family GTPase effectors. Further work will have to address whether

DRhoGEF2 is part of multi-protein complexes during signal transduction to provide pathway specificity downstream of Rho1.

9.2 Paper II: Functional Dissection of the *Drosophila* Rho Guanine Nucleotide Exchange Factor DRhoGEF2

DRhoGEF2 encodes a large protein that contains several conserved protein domains. In addition to the DBL-family characteristic catalytic DH/PH module, DRhoGEF2 has an N-terminal PDZ domain adjacent to a RGS domain, and a putative DAG-binding domain in the central region of the protein. Putative functions for these protein domains have emerged mainly from cell culture studies on mammalian DRhoGEF2 homologs (Sternweis *et al.*, 2007). However, the function of PDZ, RGS, and DAG domains for proper DRhoGEF2 localization and activity has remained untested. Previous work has provided evidence that N-terminal domains may be required for protein function. Thus, in the gastrulating embryo, DRhoGEF2 regulates cell shape downstream of the Fog/Cta pathway and the transmembrane protein T48, which are thought to activate or localize DRhoGEF2 via its RGS and PDZ domains, respectively (Barrett *et al.*, 1997; Rogers *et al.*, 2004; Kölsch *et al.*, 2007).

To evaluate the contribution of the various protein domains to DRhoGEF2 function *in vivo*, we constructed several truncated forms of the *DRhoGEF2* open reading frame in which one or several of these protein domains were deleted or inactivated (Fig. 1). Studies carried out in embryos (see also Paper I) as well as in cell culture (Rogers *et al.*, 2004) have shown that overexpression of DRhoGEF2 induces cell-rounding. We therefore used this as an assay to assess the activity of respective construct. Consistent with the idea that cell-rounding is caused by Rho1 activation, a DRhoGEF2-form with a mutated catalytic DH domain – the ΔCR3-EGFP construct – failed to induce cell-rounding (Fig. 2). By contrast, an N-terminally truncated form of DRhoGEF2 that lacked the PDZ, RGS, and DAG domains (referred to as DBL) induced cell-rounding (Fig. 2 and Fig. 4), suggesting that the PDZ, RGS, and DAG domains are not essential for the GEF activity of DRhoGEF2. In addition, because DBL localized cortically in cells, we conclude that the PDZ, RGS, and DAG domains are non-essential for cortical targeting in our assay (Fig. 2).

Since PDZ domains are well-documented protein-protein interaction domains that act as scaffolds to concentrate signaling molecules at specific regions in the cell, we decided to investigate the role of the DRhoGEF2 PDZ domain for protein localization during early embryogenesis (Fig. 3). For this,

we analyzed the localization of a form of DRhoGEF2 lacking the PDZ domain (ΔPDZ-EGFP) as well as that of the DRhoGEF2 PDZ domain alone (PDZ-EGFP). Interestingly, the ΔPDZ-EGFP construct failed to localize to the furrow canal during cellularization suggesting a role for the PDZ domain in DRhoGEF2 localization during this process. However, the PDZ domain alone did also not localize to the furrow canal either, suggesting that recruitment of DRhoGEF2 to the furrow canal may depend on several protein domains besides the PDZ domain.

We also analyzed ΔPDZ-EGFP localization in VF cells. Similarly to endogenous DRhoGEF2, ΔPDZ-EGFP localized to the apical cell cortex in gastrulating embryos, even though the accumulation was less pronounced than with endogenous DRhoGEF2 (Fig. 3). In conclusion, we found that ΔPDZ-EGFP can recapitulate only certain aspects of DRhoGEF2 localization during cellularization and subsequent gastrulation, suggesting that the PDZ domain is necessary for specific aspects of dynamic DRhoGEF2 localization in early embryos.

Next, we investigated whether N-terminal truncation of DRhoGEF2 alters the specificity of DRhoGEF2 towards a subset of Rho1 functions. For this, we compared phenotypes associated with overexpression of DRhoGEF2 or DBL to the phenotypes resulting from expression of activated $Rho1^{V14}$. We found that $Rho1^{V14}$, but not DBL or DRhoGEF2 (see Paper I), induced cortical F-Actin accumulation (Fig. 5). This suggests that N-terminal truncation does not alter the specificity of DRhoGEF2.

We also sought to gain insight into specific DRhoGEF2-dependent and independent functions of Myosin II and, therefore, compared the effect of DRhoGEF2 overexpression to that of activated Myosin Light-Chain Kinase ($MLCK^{CA}$) expression (Fig. 7). Since both DRhoGEF2 and $MLCK^{CA}$ are presumed to increase phosphorylation and subsequent activation of Myosin II, expression of these factors may induce similar phenotypes. Interestingly, $MLCK^{CA}$, unlike DRhoGEF2, triggered accumulation of AJ components and perijunctional F-Actin.

In conclusion, the results presented in Paper II are consistent with a model according to which DRhoGEF2 can act independent of upstream signals to create uniform tension throughout a tissue. During specific developmental processes, inputs through N-terminal domains might be superimposed on this base level activation to promote spatially and temporally restricted increments in DRhoGEF2 activity, leading to stronger contraction of Actin-Myosin filaments. The data also suggest that DRhoGEF2 promotes only a subset of Rho1 and Myosin II functions in epidermal cells, independent of N-terminal domains.

In order to further characterize the function of specific DRhoGEF2 protein motifs, we plan to investigate the ability of individual DRhoGEF2-forms to

rescue the loss-of-function phenotype of *DRhoGEF2* mutants. In addition, the localization of ΔCR3-EGFP during early development will be determined and compared to that of ΔPDZ-EGFP. Finally, a more detailed analysis of the effects on Myosin II localization and on the spatially restricted pattern of MRLC/Sqh phosphorylation induced by expression of various factors, including DRhoGEF2 and $MLCK^{CA}$, will be conducted.

9.3 Paper III: Role of Hedgehog During Segmental Groove Formation in the *Drosophila* Embryo

Segmental grooves form in the epidermis during mid embryogenesis and mark the establishment of definitive segments in the *Drosophila* embryo. The grooves form at segment boundaries, immediately posterior to the segmentally repeated expression of the segment polarity genes *en* and *hh*. It has previously been shown that Hh is required for segmental groove formation (Larsen *et al*., 2003). The action of Hh is counteracted by Wg which emanates from a row of cells anteriorly adjacent to Hh. Consequently, signaling by Wg represses groove formation in the posterior part of each segment. Since both Hh and Wg are secreted ligands that activate signal transduction pathways controlling the expression of target genes, they must act through one or several downstream targets to trigger (for Hh) or block (for Wg) cell shape changes associated with segmental groove morphogenesis.

In this paper, we study how Hh directs shape changes in groove founder cells and organizes the morphogenesis of segmental grooves. We analyzed the spatio-temporal expression of three groove founder cell-markers, the zinc finger transcription factor Odd, the Actin regulator Ena, and the apical polarity determinant Crb (Fig. 2 and Fig. 6). Unlike Ena and Crb, Odd accumulates in groove founder cells prior to the formation of grooves. Initially, Odd is expressed in several cell rows, but its expression condenses into a single cell row prior to groove formation. Moreover, Odd is lost in ventral cells that initiate invagination at a later stage than their lateral counterparts. We present evidence that Hh promotes Odd expression while Wg restricts it (Fig. 4).

We took two approaches to study the role of Hh in the expression of groove cell-markers and the generation of specific cell shape changes associated with groove invagination. First, we overexpressed or ectopically expressed *hh* in the epidermis. Secondly, we analyzed null mutants for the Hh receptor *patched* (*ptc*), which negatively regulates Hh signaling in receiving cells on either side of the Hh source. We found that over-activation of Hh signaling is sufficient to trigger ectopic constriction (Fig. 1 and Fig. 2), apical-basal elongation (Fig. 6), and accumulation of the groove founder cell-markers

Odd, Ena, and Crb (Fig. 3 and Fig. 6) in a subset of epidermal cells, namely in cells located in the lateral epidermis immediately posterior to the segment boundaries. This suggests that high-level Hh-signaling can trigger specific epidermal cells to adopt a groove founder cell-like fate and behavior.

Finally, we investigated the role of *odd* in segmental groove morphogenesis. We find that groove formation is unaffected in the strong allele odd^5 (Fig. 5). Moreover, ectopic expression of Odd in the epidermis did not alter groove morphology indicating that Odd is not sufficient for groove formation. Consistent with this, we find that Odd expression is not sufficient to induce accumulation of Ena or Crb. These results suggest that *odd* may not play an essential role in groove formation. A future task will be to reveal the function of *odd* in the epidermis, as well as to uncover its regulation. In addition, we will need to address putative roles for Ena and Crb in groove morphogenesis.

10 Concluding Remarks

The species-specific body plan of multicellular organisms is established during the course of their development from egg to adult. In each species, the characteristic shape of the organism emerges as cells become organized into tissues and organs. The shape of tissues and the position of individual organs in the developing organism are, in turn, driven by cell migration and by changes in the shape of individual cells in a process known as morphogenesis. Importantly, during morphogenesis, the behavior of individual cells must be highly coordinated in space and time to permit the reproducible generation of a stereotyped body plan.

Morphogenetic processes can be studied in various animal models. This thesis is focused on one particular morphogenetic process, the formation of segmental grooves, which takes place in the *Drosophila* epidermis during mid-embryogenesis. Prior to this work, little was known about the molecular mechanism of this process. We decided to investigate the morphogenesis of segmental grooves because one of the major challenges in the field of developmental biology is to elucidate how conserved gene networks found to determine positional information in the embryo – for example during patterning of a metameric body plan – are linked to cytoskeletal rearrangements that control the shape of individual cells, and in extension, the shape of the entire organism.

One important aim of this thesis was thus to establish segmental grooves as a system for the study of cell shape regulation during epithelial morphogenesis. We began our study with a detailed investigation of the cell shape changes associated with this process (Paper I). This was followed by an analysis of the molecular mechanisms regulating cell shape in the epidermis at the time of groove morphogenesis. We then conducted a molecular dissection of the cytoskeletal regulator DRhoGEF2 that plays a central role in segmental groove formation, in order to gain insight into the upstream processes regulating cell shape (Paper II). Finally, we have begun to investigate how the mechanisms controlling patterning of the segments direct morphological differentiation at segment boundaries (Paper III). We hope that our establishment of segmental grooves as a system to study morphogenesis will stimulate further studies directed towards bridging the knowledge gap that currently exists at the interface between determination of cell fate and the control of cell shape.

As stressed in this thesis, the behavior of cells in a developing organism is extremely complex. Cells divide, they grow, migrate, change shape, and form extensions. All these processes contribute to tissue morphognesis, and require therefore precise temporal and spatial coordination. This is achieved through

a complex system of parallel and interconnected signaling networks that we are only now beginning to understand. The work of this thesis contributes to our understanding of a specific morphogenetic process, the formation of segmental grooves in the *Drosophila* embryo. It identifies some of the molecular players regulating segmental groove formation and elucidates some of the mechanisms that may control specificity in cytoskeletal signaling. It also identifies a few of the components likely to connect the network of patterning genes to cytoskeletal regulators determining cell shape. However, further efforts are necessary if we wish to fully understand the molecular mechanisms that govern cell shape during development and, thus, the body shape of the entire organism.

11 Populärvetenskaplig sammanfattning:

Från cellform till kroppsform – vad bananflugans celler berättar om människokroppen

Hur blir en människa till? Det börjar vid befruktningen, när spermie och ägg smälter samman. Sedan följer flera celldelningar. En cell blir två celler, som blir fyra, och så vidare. En cellklump bildas.

Vid detta tidiga stadium ser en människocellklump inte mycket annorlunda ut än en klump mus- eller råttceller. Men plötsligt börjar cellerna ändra form. Fast bara vissa celler, övriga celler förblir orörliga. Det är alltid samma celler som ändrar form, de gör det alltid på samma sätt och vid exakt samma tillfälle i varje människoembryo.

Men hur vet cellerna i det tidiga människoembryot hur de ska bete sig? Frågan är viktig. Ett felaktigt cellbeteende tidigt i embryoutvecklingen kan leda till att embryot avstannar i sin utveckling. Senare i utvecklingen kan felaktiga cellbeteenden få olika sjukdomar eller missbildningar som följd. Även efter födseln måste celler uppvisa ett kontrollerat beteende, cancertumörer kan annars uppstå genom att några av kroppens celler ohämmat delar på sig.

Att undersöka hur cellers beteende styrs under embryoutvecklingen är dock inte bara viktigt för att förstå hur sjukdomar och missbildningar uppstår. Det är också viktigt inom medicinsk forskning när man vill utveckla nya sjukdomsterapier. Framförallt gäller detta för sjukdomar som Parkinsons och Diabetes. Dessa hoppas man kunna behandla genom att få stamceller som liknar cellerna i det tidiga embryot att återbilda skadade vävnader hos patienterna. En förutsättning för att detta ska bli möjligt är att man först förstår de mekanismer som styr embryocellers beteende.

Ett stort problem är emellertid att embryon är väldigt svåra att studera. Däggdjursembryon måste exempelvis först opereras ut från den gravida honan innan de kan studeras. Många forskare väljer därför att experimentera på enklare organismer som är lättare att manipulera och undersöka. Om man dessutom vill studera generella egenskaper och beteenden hos celler som celldelning, cellform, cellstorlek eller cellrörelse är *Drosophila melanogaster*, eller bananflugan, ett förstahandsval för många. Följaktligen har utvecklingen hos bananflugan, från ägg till vuxen individ, kommit att bli den mest kartlagda av alla arters. En viktig slutsats som man dragit genom de senaste tjugo årens forskning är att likheterna mellan människa och bananfluga bara blir större ju mindre beståndsdelar man studerar. En människa och en fluga

ser ganska olika ut för ögat, även om den generella kroppsbyggnaden hos de två arterna är slående likartad. Tittar man på cellnivå blir likheterna ännu större, och tittar man inne i cellerna blir likheterna än mer framträdande. I grund och botten fungerar bananflugans celler precis som våra, och de senaste uppskattningarna visar att 75 procent av alla kända sjukdomsgener har motsvarigheter i flugans arvsmassa.

Experiment med bananflugor har därför varit och är fortfarande väldigt betydelsefulla inom biologisk och medicinsk forskning. Mycket av det man idag vet om hur celler fungerar i djurembryon har exempelvis vuxit fram genom studier av bananflugsembryots celler. Detta gäller framförallt identifierandet av de generella signaler som celler använder sig av för att kommunicera med varandra.

I denna avhandling har vi använt oss av bananflugsembryon för att studera hur celler ändrar form under embryoutvecklingen, och hur detta, i sin tur, påverkar hela vävnaders form i embryot. En vävnads allmänna utseende bestäms av hur många celler som bygger upp vävnaden, cellernas storlek, deras form och hur de är arrangerade i förhållande till varandra. Genom att enskilda celler ändrar form kan hela vävnaders topografi förändras. Under embryoutvecklingen, när hela vävnader förändrar utseende och byter position, måste cellers form kontrolleras in i minsta detalj. För att cellerna i embryot ska ändra form vid exakt rätt tidpunkt måste cellerna förses med korrekt information som talar om för dem hur de ska bete sig. Exempelvis skickar vissa celler i bananflugembryot ut ett protein, kallat Hedgehog, för att instruera närliggande celler att ändra form och bilda fåror eller gropar i vävnaden. Samma protein skickas ut av vissa celler i människofostret för att instruera närliggande celler att ändra form och bilda det rör som sedan ger upphov till nervsystemet.

I båda fallen tas Hedgehog-proteinet emot på cellytan av de närliggande cellerna. Detta aktiverar en serie av proteiner inne i cellen. Samma proteiner aktiveras i människoceller som i bananflugeceller. Men vad som sedan händer inne i cellerna när de ändrar form är fortfarande relativt okänt. Det man vet är att alla celler har ett inre skelett, ett nätverk av proteintrådar som styr cellformen. Trådarna kan arrangeras på olika sätt inne i cellen, och genom att trådarna dras ihop alstras den kraft som driver fram formförändringar av cellens yta. Om exempelvis en fyrkantig cell ska anta en triangulär form måste en av cellens sidor krympa medan de andra förblir oförändrade. Detta sker genom att proteintrådarna lägst den krympande sidan dras ihop.

Men vad är det då som ser till så att proteintrådarna hamnar på rätt plats i cellerna, och att de kan dra ihop sig vid exakt rätt tidpunkt? Vår forskning har kretsat kring studiet av två bananflugsproteiner som styr detta, DRhoGEF2 och Diaphanous, båda med proteinmotsvarigheter i människoceller. Det som varit oklart är om DRhoGEF2 kan aktivera Diaphanous eller om

proteinerna arbetar helt oberoende av varandra. För att ta reda på detta har vi tittat närmare på bananflugsmutanter som antingen saknar DRhoGEF2 eller Diaphanous, eller som producerar för mycket av proteinerna. Saknas DRhoGEF2 eller Diaphanous kan embryots celler inte längre ändra form på ett organiserat sätt, till exempel bildas då inga fåror eller gropar i vävnaden även om närliggande celler producerar Hedgehog-proteinet. Embryocellerna som producerar för mycket DRhoGEF2 uppvisar ett omvänt beteende, de drar istället ihop sig mer än vanligt och antar en helt rund form. Producerar de för mycket av det andra studerade proteinet, Diaphanous, bildar cellerna långa proteintrådar som sticker ut från cellytan. När vi summerar experimenten tyder våra resultat på att både DRhoGEF2 och Diaphanous är av avgörande vikt när celler ska ändra form, men att de kan arbeta oberoende av varandra. Diaphanous verkar styra produktionen av proteintrådar i cellen medan DRhoGEF2 aktiverar cellers förmåga att dra ihop dessa.

Att ta reda på hur proteiner som DRhoGEF2 och Diaphanous, som svar på yttre signaler som exempelvis Hedgehog-proteinet, samverkar för att förändra cellformen är avgörande om man ska förstå hur den klump celler som utgör det tidiga fostret utvecklas till en fullt fungerande organism. Denna kunskap bidrar inte bara till att besvara frågan om hur en människa blir till. Kunskapen är också grundläggande för att man ska kunna ta fram nya behandlingar och terapier, exempelvis mot cancer och mot sjukdomar som Diabetes och Parkinsons. Denna avhandling kan förhoppningsivs vara ett litet steg mot det målet.

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