Embryonic ecdysone-induced gene expression and progression of organ morphogenesis

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"You are here to enable the divine purpose of the universe to unfold.
That is how important you are."
Eckhart Tolle

To my precious Keyvan & Kiana,

To my beautiful maman & baba &

To Anne Uv

ABSTRACT

The formation of an epithelial organ requires a set of organ-specific gene programs that instruct parallel and successive developmental events. Still, it is unclear what are the core regulatory programs and how such programs are timely coordinated within the organ. We use mainly the *Drosophila* trachea (respiratory system) as a model to understand epithelial organ development. The trachea is a network of epithelial tubes, and its morphology is sensitive to mutations in genes whose products participate in consecutive steps of branching morphogenesis and tube size maturation. In paper I, we identified two gene functions required for tracheal tube elongation. We show that tracheal cells, at a specific time in development, acquire an ability to elongate that is mediated by a protein involved in actin organization. A luminal matrix holds back this elongation, and temporal expression of an anion channel appears required to modify the luminal matrix and thereby permit a controlled extent of elongation. In paper II, we show that a mucin-like protein is temporally expressed in the trachea and is required for tube elongation. The protein also drives diameter expansion of the hindgut, where it fills the growing lumen and appears to act as an expanding mucin to mechanically dilate the tube. The work demonstrates that regulated expression of a single protein can model epithelial tube diameter. In papers III and IV, we focused on the temporal regulation of tracheal gene expression, and uncovered an important function for the mid-embryonic ecdysone hormone pulse in progression of organ development. In paper III, we analysed the mechanism of embryonic ecdysone signalling and found that the hormone causes pan-embryonic activation of Ecdysone Receptor (EcR). EcR acts tissue-autonomously together with Ultraspiracle to promote concurrent progression of organ development. In paper IV, we show that ecdysone, via EcR and a downstream cascade of gene regulators is needed to advance parallel tracheal-specific gene programs. Together, the results reveal novel gene functions during epithelial tube formation, and show that correct temporal unfolding of the tracheal gene network relies on gene-regulatory input from an external cue in form of a hormone pulse.

Key words: *Drosophila*, trachea, hindgut**,** tubulogenesis, luminal matrix, ecdysteroid, EcR:USP.

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PAPERS AND MANUSCRIPTS

This thesis is based on the following papers, which will be referred to in the text by their roman numbers (I-IV):

I **Tång E.*, Byri S.*, Chavoshi T.M., Norum M. and Uv A.**

A gene program that regulates tube length in the *Drosophila* trachea

Manuscript

II **Zulfaqhar A. S., Bougé A-L*, Chavoshi T.M.*, Byri S., Tång E., Bouhin H., Härd I., Uv A.**

The luminal mucin-like protein promotes diamater expansion of the *Drosophila* hindgut.

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III **Chavoshi T.M., Moussian B., Uv A.**

Tissue-autonomous EcR functions are required for concurrent organ morphogenesis in the *Drosophila* embryo

Mechanisms of Development 127 (2010) 308-319

IV **Chavoshi T.M.** and **Uv A.**

Embryonic ecdysone is required for progression of tracheal gene programs in *Drosophila*

Manuscript

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ABBREVIATIONS

20E	20-hydroxyecdysone
AEL	after egg lay
AP	Anterior-Posterior
BDGP	Berkley Drosophila genome project
Bnl	Branchless
Btl	Breathless
CBP	Chitin binding protein
Crb	Crumbs
CS-1	Chitin synthase 1
Dib	*disembodied*
DBD	DNA-binding domain
DNEcR	dominant negative EcR
DT	dorsal trunk
DV	Dorsal-Ventral
EcR	Ecdysone receptor
EcRElacZ	EcR reporter gene
Fas II	Fasciclin II
FasIII	Fasciclin III
fg	foregut
FGF	Fibroblast growth factor
GB	Ganglionic branch
GlcNac	N-acetylglucoseamine
hg	hindgut
kkv	*krotzkopf verkert*
Knk	Knickkopf
LBD	Ligand-binding domain
LT	Lateral trunk
mg	midgut
mt	Malpighian tubules
PKD	Polycystic kidney disease
Phm	*phantom*
PC-1	Polycystin-1
PC-2	Polycystin-2
Pv	proventriculus
RA	Retinoic acid
RAR	nuclear RA receptors

Serp	Serpentine
SRF	serum response factor
sad	*shadow*
shd	*shade*
TC	Transverse connective
tr	Trachea
Trh	Trachealess
VB	Visceral branch
Verm	Vermiform
vm	visceral mesoderm
vnc	Ventral nerve
Vvl	Ventral veins lacking
wt	wild-type
Usp	Ultraspricle

INTRODUCTION

Organogenesis includes distinct phases of cell determination, differentiation, shape changes and specialization. It commences when the body plan is set and continues until the end of embryogenesis, accordingly occupying a major period of development. Specific gene programs (temporal and spatial) regulate the different developmental steps. Development of multi-cellular organisms has been exponentially explored at the molecular level over the last 30 years. *Drosophila Melanogaster* was recognized as a model organism in genetic studies and developmental biology in 1910, owing to Thomas Hunt Morgan and his colleagues. Due to the possibility of performing large-scale genetic screens in the fly, accumulating advanced methods and a large pool of useful tools for investigating developmental processes, *Drosophila* has today become an important model organism for analyzing different molecular and cellular mechanisms in biological events, such as organogenesis, cellular signaling and immunological and behavioral responses. The developing respiratory organ of *Drosophila* (trachea) is a well-studied model system, and was used here to gain further understanding of gene regulation that steer organ development. The trachea is a network of plain epithelial tubes that extend throughout the organism, and leads oxygen from the exterior through gradually narrower tubes to fine branches, where gas-exchange with target tissues occurs. Formation of the tracheal network proceeds through several distinct phases that are relatively simple to study, as they are highly stereotype and do not involve cell proliferation, and has been applied to dissect mechanisms of branching morphogenesis and formation and growth of epithelial tubes.

Drosophila as a model system for embryonic organ development

The *Drosophila* life cycle includes two periods of organ development, embryogenesis and metamorphosis. During embryogenesis, the fertilized egg develops into a larva and, after two rounds of molting, the larva pupariates and undergoes metamorphosis, when most larval tissues brake down and the adult fly forms. The fertilized *Drosophila* egg requires less than 24 hours to build a larva. Despite its apparently simple external shape, the hatching larva has multiple internal organs that have developed in a reproducible and highly genetically regulated manner to form the functional organism. Here, I will highlight the main organs in the larva to underline the complexity of *Drosophila* embryonic development.

Tubular organs

Biological tubes most commonly consist of a single-cell-layered epithelium with the apical surface facing the tubular lumen. They are important to carry out numerous essential physiological functions in molecular absorption and secretion and in transport of gases and liquids. Malformation of each one of these tubular structures can result in failure of organ functions that are essential for the animal. Their morphogenesis is dynamic and is set by cell division, cell growth, cell shape changes and migration, as well as apoptosis. Any error when plumbing tubular compartments can lead to serious pathological abnormalities, such as polycystic kidney disease (PKD) and stenotic tubes, causing a block in tubular organs (Lurie et al., 1995; Boletta and Germino, 2003; Lubarsky and Krasnow, 2003). Characterization of the mechanisms involved in tubular organ development is therefore of significant importance to

understand the pathogenesis of diseases. Tubular organ development, which sets exceptionally high demands on cell behavior to form the precise organ shape, is also valuable in studies of basic mechanisms that regulate epithelial morphogenesis.

Salivary gland

The *Drosophila* salivary gland (sg) consists of two unbranched elongated secretory tubes that are attached to each other via a "Y"-shaped salivary duct (Demerec, 1950). The salivary gland is connected to the larval mouth, and the columnar cells of the secretory tubes synthesize high levels of proteins to produce secretions (saliva) that are mixed with the food during feeding and ingested along with the food.

Alimentary tract

This comprises three regions; *foregut, midgut and hindgut*. The midgut (mg) originates from the endoderm, while the foregut (fg) and hindgut (hg) derive from the ectoderm (Campos-Ortega, 1997). Unlike the salivary glands, the epithelial tubes of the alimentary tract are covered by mesodermal cells at their basal surface. The hindgut is divided into three compartments; the small intestine, the large intestine and the rectum (Lengyel and Iwaki, 2002). The foregut consists of four parts; atrium, pharynx, esophagus and the proventriculus (pv) at the most posterior part of foregut, which connects with the anterior part of the midgut. The proventriculus is a multilayered organ that controls entrance of food into the midgut (Demerec, 1950). The malpighian tubules (mt) are long two-cell-wide tubes located at the junction between the midgut and the hindgut.

The tubules are proposed to have similar properties to vertebrate kidney (Denholm et al., 2003).

Trachea

The trachea (tr), described in details later, is a branched tubular organ. It consists of elongated epithelial tubes with different cellular architecture, and carries air through the entire embryo.

Dorsal vessel

In *Drosophila*, a heart-like organ, called the dorsal vessel, is an open tube. The vessel is composed of two major cell types: cardioblasts that form the simple contractile tube of the heart, and pericardial cells that lie around the cardioblasts (Perrin et al., 2004).

Epidermis

The epidermis is a sheet of epithelial cells that line the body. This organ produces an apical culticular lining that gives stability to the organism and also protects the animal from dehydration, infection and mechanical damage. The epidermis has a complex task in generating different types of cuticular structures at specific anatomical positions.

The nervous system

The Nervous System derives from the neuroectoderm and controls the body. The central nervous system (CNS) includes the brain and the ventral nerve cord (vnc), the peripheral nervous system (PNS) that connects the

CNS to organs, and the stomatogastric nervous system (SNS) that controls gut movements (Marder and Bucher, 2001). Sensory axons in the PNS reach their targets in the CNS and motor axons connect with the musculature before hatching, enabling late embryonic movement within the eggshell.

Musculature and fat body

The somatic and visceral musculature and the fatbody derive from the mesoderm. The somatic musculature makes up the body wall muscles and muscles in the cephalic region. These muscles enable the larvae to move and retract the head skeleton. At the end of embryogenesis the muscle pattern is fully developed (Bate, 1990). The visceral musculature that lines the basal surface of the alimentary tract is responsible for the peristaltic movements. Some segments of the foregut (which form the inner part of the proventriculus) and hindgut (where malpighian tubules attach) lack visceral mesoderm attachments (Hartenstein et al., 1992). The insect fat body plays an essential role in energy storage and ingestion. It is the central storage place for extra nutrients. In addition, it is an organ of great biosynthetic and metabolic activity (Law and Wells, 1989). The fat body is an elongated sheet of cells that becomes inserted between the developing visceral musculature and the body wall. A group of fat body cells form horizontal plates of fat body under the foregut and hindgut (Demerec, 1950).

Hormones are temporal signals in animal development

Although many processes of organ development rely on intrinsic feed-

forward mechanisms of gene expression, temporal signals arising from hormones, have the potential to induce global changes in gene expression to affect organ development. One example is Retinoic acid (RA), a signaling molecule synthesized from vitamin A that controls gene expression at the transcriptional level by functioning as a ligand for nuclear RA receptors (RAR). The RA signal itself is a prerequisite for morphogenesis past day 9 of gestation and is transduced by functionally overlapping isotypes and isoforms of RXR/RAR heterodimers, whose single or combinatorial loss demonstrates their requirement in many organs at different stages. These include segmentation and closure of the hindbrain, development of pharyngeal arches and forelimb buds, closure of the primitive gut, histogenesis of the retina, epithelial-mesenchymal interactions in the kidney, lung branching morphogenesis and lung alveoli septation (Mark et al., 2006).

Hormones are also known to act as inducers of major developmental transitions from one stage to another. One example from humans is the hormonally triggered changes that occur during puberty and adolescence to promote maturation of a non-reproductive juvenile to a mature adult form. In the amphibian larva, a complex interaction of hormones precipitates metamorphosis, where two major classes of hormones act together: the thyroid hormones (made by the thyroid gland) and prolactin (made by the pituitary gland) (Brown and Cai, 2007). In insects, including *Drosophila melanogaster,* the steroid hormone 20-hydroxyecdysone (hereafter called ecdysone) functions as a molting hormone and to trigger metamorphosis, transforming the larvae into the adult fly (Baehrecke, 1996).

Ecdysone is also essential during embryogenesis (Figure 2). The level of the hormone rises when gastrulation is completed and organ morphogenesis has just commenced (eight hours after egg laying AEL). This corresponds to stage 12 of embryogenesis, also referred to as the mid-embryonic stage. Mutants that lack enzymes required for ecdysone biosynthesis fail to complete major developmental processes, such as head involution, dorsal closure, midgut constriction, nervous system formation, and late cuticle production (Chavez et al., 2000; Giesen et al., 2003; Kozlova and Thummel, 2003). The rise in ecdysone-levels is therefore essential for organ developmental past mid-embryogenesis.

Ecdysone biosynthesis

Ecdysteroids are molting hormones in insects, and 20-hydroxyecdysone is the main active form of the hormone in these animals. Thus, insects utilize the same active ecdysteroid, synthesized from cholesterol through the same series of sterol modifications (Rewitz et al., 2006). Four P450 enzymes have been found to be required for ecdysone biosynthesis in *Drosophila*: *phantom* (*phm*), *disembodied* (*dib*), *shadow* (*sad*) and *shade* (*shd*). *phm, dib*, *sad* and *shd* belong to a group of mutant called the Halloween mutants, due to their faint and ghost-like cuticle. Recently, it was shown that *shroud*, a fifth member of the Halloween gene family, encodes an enzyme required for the conversion of 7-dehydrocholesterol to 5 β -ketodiol (Niwa et al., 2010). Mutants for any of the five genes are embryonic lethal.

Ecdysone signaling via nuclear receptors

Ecdysone mainly exerts its effects by binding to a nuclear receptor heterodimer, consisting of the Ecdysone receptor (EcR) and the RXR homologue Ultraspiracle (Usp). Nuclear receptors consist of a DNA-binding domain (DBD) and a ligand-binding domain (LBD).

EcR and Usp associate with each other and localize to the nucleus, even in the absence of ligand. A model for the transcriptional activating and repressing functions of EcR:Usp is illustrated in Figure 4. In the absence of ligand, the receptor complex can act as repressors, while ligand-binding to EcR:Usp causes changes in protein interactions, leading to transcriptional activation. For Usp, the repressor function, but not the activating function, is shown to require its DNA-binding domain (Ghbeish et al., 2001).

During larval molting and metamorphosis, ligand-bound EcR:Usp induces a series of ordered gene activities that lasts long after the actual

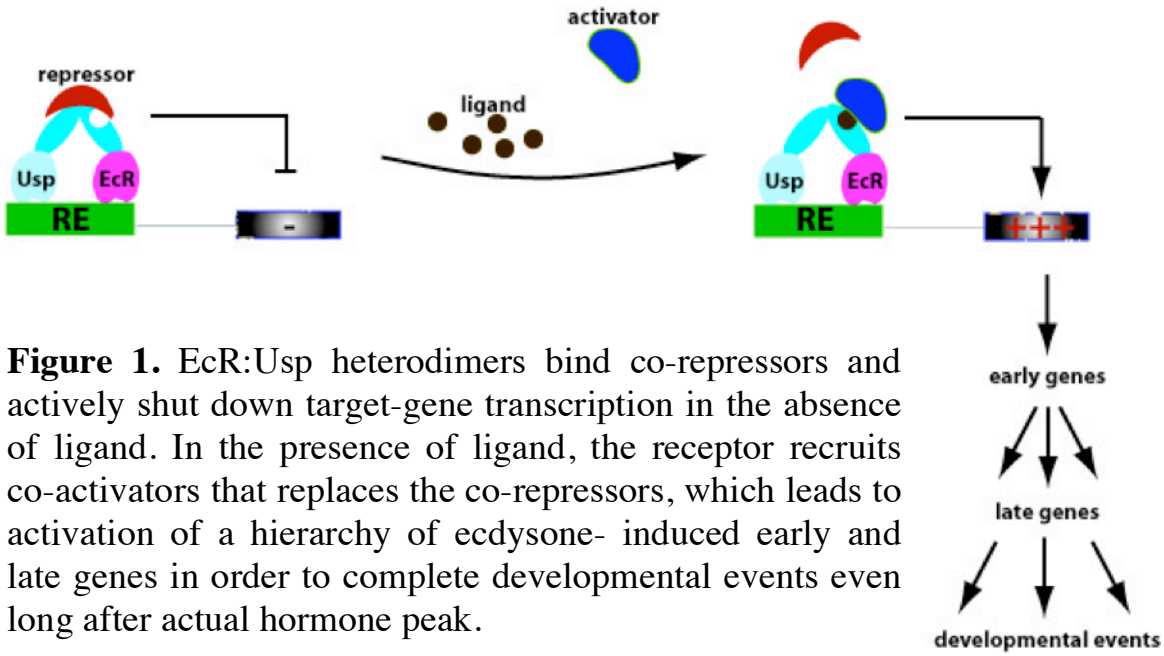

Figure 1. EcR:Usp heterodimers bind co-repressors and actively shut down target-gene transcription in the absence of ligand. In the presence of ligand, the receptor recruits co-activators that replaces the co-repressors, which leads to activation of a hierarchy of ecdysone- induced early and late genes in order to complete developmental events even long after actual hormone peak.

hormone pulse. Among the ecdysone-induced genes are activators and repressors, which interact to cause a temporal profile of gene activities. It is thus believed that ecdysone sets off a cascade of global events that promotes molting and the transition from larvae to flies (King-Jones and Thummel, 2005) (Figure 1).

Ecdysone-function during larval molts and metamorphosis

Pulses of ecdysone organize many features of *Drosophila* development. These occur at major post-embryonic transitions such as molting, larval-prepupal and pupal transitions. Ecdysone is the direct initiator of molting, the periodical shedding of the cuticle that occurs twice during larval life (Riddiford, 1993). At the end of the third larval instar, comprehensive changes appear across the whole insect and promote the transition to prepupal development. The high titer of ecdysone is involved in initiation of glue molecule secretion by larval salivary glands for attachment of the larva to a hard surface, body length shortening and darkening and solidification of the larval cuticle to form a protective pupa. Ten to twelve hours later, a second ecdysone pulse drives the prepupal to pupal transition. During metamorphosis, ecdysone induces programmed cell death to destruct the "old" larval tissues and the differentiation and metamorphosis of new adult structures (Baehrecke, 1996; Truman et al., 1996).

Ecdysone-response genes

Binding to EcR leads to activation of characteristic sets of target genes, such as *Broad-Complex* (*BR-C*), *E74 (Eip74EF), E75B (Eip75B),*

DHR4, DHR39, E78 (Eip78C) and *Hr46* during larval phases and metamorphosis. These genes encode transcription factors that, in turn, activate or repress the expression of later response genes, such as *β-ftz-f1*, and transduce the hormone signal into developmental responses (Woodard et al., 1994; Thummel, 1996).

Ecdysone activity during embryogenesis

In contrast to the extensive studies of ecdysone functions during larval molts and metamorphosis of *Drosophila*, the cellular and genetic pathways regulated by embryonic ecdysone have not been well characterized. A main reason for this is that the requirements for EcR and Usp during embryogenesis have been difficult to assess. Both EcR and Usp gene products are maternally deposited in the egg, and zygotic mutants of either EcR or Usp develop past stage 14 and fail to reproduce the phenotypes seen upon loss of ecdysone. Removal of maternal EcR to generate embryos that completely lack EcR function causes arrest in oocyte development, and no eggs are produced (Buszczak et al., 1999). Germ line clones mutant for *usp* alleles with mutations within the DBD, have been generated, but produce embryos with little morphological defects, showing that at least the Usp DBD activity is not necessary for ecdysone-dependent embryonic morphogenesis (Ghbeish et al., 2001). Thus, it is speculated that EcR might have partner other than Usp during embryogenesis.

In previous studies on embryonic ecdysone, it has been suggested that the source of active ecdysteroids resides in a tissue called the amnioserosa (Kozlova and Thummel, 2003). The amnioserosa is an extra-embryonic,

epithelial tissue that covers the dorsal side of the *Drosophila* embryo. This conclusion is based on two observations: First, EcR-activities were selectively detected in the amnioserosa from stage 12, using either a ligand sensor system or an EcR reporter gene (*EcRElacZ*) containing 7 multimerized EcR binding sites upstream of the *lacZ* coding region (Kozlova and Thummel, 2003; Palanker et al., 2006). Second, incubation of whole embryo in 20E caused broad *EcRElacZ* expression. Consequently, it was suggested that ecdysone is confined to the amnioserosa at the time of its peak, a situation that parallels the production of mammalian placental hormones during pregnancy (Kozlova and Thummel, 2003). In addition, it was shown that inhibition of EcR activities in the amnioserosa, by expression of a dominant negative EcR (DNEcR) specifically in the amnioserosa cells, caused defects in germ band retraction and head involution (Kozlova and Thummel, 2003). As mutants lacking zygotic ecdysone production do not show defect in germ band retraction, the early role of EcR in this process was believed to depend on maternal ecdysone. A later study (Palanker et al., 2006), however, dismissed this conclusion after observing the fact that mutants for *disembodied* (i.e. lacking zygotic ecdysone) show no EcR activity in the amnioserosa, but still carry out germ band retraction successfully. The way that ecdysone signals to effect embryonic morphogenetic movements has thereby been unclear.

The respiratory organ of *Drosophila* as a model system for epithelial organ development

To explore the importance of ecdysone for embryonic organ formation, we began by analyzing its role in the developing respiratory organ (trachea). The trachea is formed when tracheal precursor cells undergo genetic specification from the ectoderm. The cells then invaginate to form 20 pockets, and prior to or during invagination they become patterned within each pocket. Tracheal branching morphogenesis is a highly stereotyped process and is invariable from embryo to embryo (Samakovlis et al., 1996a; Uv et al., 2003). During branching morphogenesis, each metamere connects with its neighboring metameres, both on the same side and on the contra-lateral side of the embryo, to form a continuous tracheal network, similar to the formation of vertebrate capillary anastomoses (Samakovlis et al., 1996b; Gerhardt et al., 2003). The tubes continue to sprout and mature in size and length. Cuticle differentiation and air filling are terminal events of tracheal developmental. These developmental steps are described below.

Tracheal formation and branching morphogenesis

The tracheal precursor cells (1600 cells) are genetically specified from the ectodermal tissue. Specification occurs at stage 10 at twenty sites, ten on each side of the embryo, and is mediated by transcription factors, mainly Trachealess (Trh) and Ventral veinless (Vvl) (Anderson et al., 1995; Wilk et al., 1996) (Zelzer and Shilo, 2000). Soon after, these clusters of cells invaginate to form 20 pockets. During invagination, the tracheal

cells undergo their last cell division, which result in 80 cells per pocket (Samakovlis et al., 1996a). Once the tracheal cells are specified they become patterned. Such patterning is thought to confer branch-specific properties to the tracheal cells, so that they later form correct size and migrate in the correct orientation. This is mediated by Wingless (Wnl), Decapentapledic (Dpp), Epidermal growth factor (EGF) and Hedgehog (Hgg) (Chihara and Hayashi, 2000; Llimargas, 2000). Thus the invagination process involves different domains with different gene expression. After invagination, the cells in each pocket begin to form six primary branches with different types of tube architecture), called the Dorsal trunk anterior (DTa), dorsal trunk posterior (DTp) and transverse connectives (TC), lateral trunk anterior (LTa), lateral trunk posterior (LTp), dorsal branch and ganglionic branch (GB).

Initially, all these branches are type-I tubes that contain several wedge-shaped cells around the lumen. Later, the dorsal branch, lateral trunk anterior, lateral trunk posterior and ganglionic branch form type-II tubes, where the lumen edge is surrounded by a single cell that folds over its' own axis and is sealed by autocellular junctions, thereby forming a small lumen. Branchless (Bnl, an FGF homologue) is expressed in tissues surrounding the tracheal cells and acts as a chemo-attractant for branch migration. Bnl binds its receptor Breathless (Btl), which is expressed in tracheal cells (Sutherland et al., 1996). Branch migration continues until the branch arrives at the signal sources. At stage 14, secondary branching commences. Bnl and Btl are involved also in this process, but here, Bnl-signaling causes differentiation of the tip cells by inducing gene expression (Samakovlis et al., 1996a). One such gene is *pointed* (*pnt*), which encodes

an ETS transcription factor. Pnt plays at least two roles in the tip cells. First, it induces a gene, *pruned,* which encodes *Drosophila* Serum Response Factor (SRF) and second, it inhibits the expression of a fusion gene, *escargot*, found in the terminal cells, which induces expression of later fusion genes and suppresses terminal branching in the fusion cells (Affolter et al., 1994; Samakovlis et al., 1996b).

Bnl also induces expression of *sprouty*, which is inhibits *bnl* signaling and is needed to limit cell tip identity in tip cell differentiation (Hacohen et al., 1998). The two tip cells in the dorsal branch specialize to one fusion cell and one terminal cells via lateral inhibition. To form fusion anastomoses, two cells positioned at the tip of the two fusing branches connect and form unicellular doughnut shaped seamless cells (type-III). DT branch fusion occurs during stage 14, LT branch fusion at stage 15 and DB fusion at stage 16. Finally, the terminal cells form hollow intracellular extensions to build the type-IV seamless capillaries to supply the surrounding tissues with oxygen (Uv et al., 2003).

Tracheal tube size regulation

Each branch of the tracheal network initially has a narrow lumen, and grows in lumen size to attain specific dimensions in order to become functional. The dorsal trunks (DT) are the main tracheal tubes, and extend through the body from the anterior to the posterior part of the animal. After fusions of branches, the DT lumen grows in diameter and length to achieve correct size. Tube diameter expansion occurs over a narrow three-hour period resulting in a 3-fold increase in lumen diameter (Beitel and

Krasnow, 2000). Such dilation is associated with elevated levels of apical secretion into the lumen, and mutants with impaired secretion show reduced diameter expansion (Samakovlis et al., 1996a; Jayaram et al., 2008; Forster et al., 2010). Lumen dilation also requires the deposition and organization of chitin filaments, which lie lengthwise inside the tubular lumen interacting with other molecules. The filament is not a requirement for increased lumen volume, but is necessary for uniform diameter expansion upon increase in lumen volume (Devine et al., 2005; Tonning et al., 2005). After diameter expansion, which occurs at stage 15, the lumen continues to grow through tube elongation.

Genes involved in tube dilation and elongation

Many genes are involved in manufacturing tracheal tubes with correct size (diameter and length) and shape. Of these are two transcription factors, Grainy head (Grh) and Ribbon. Mutants for *grh* display excessive growth of apical membrane resulting in convoluted lumens (Hemphala et al., 2003), whereas *rib* mutants present restricted apical membrane growth (Shim et al., 2001). Similar phenotype is attained when overexpressing *grh* in the trachea, as is seen in *rib* mutants, arguing that *grh* is a prerequisite for correct apical membrane expansion. *krotzkopf verkehrt* (*kkv*) is another gene encoding for Chitin synthase-1 (CS-1) that generates chitin chains in the lumen, a requirement for a uniformed lumen expantion (Devine et al., 2005; Tonning et al., 2005). Mutants for *retraactive* (*rtv*) and *knickkopf* (*knk*) have defective chitin filament organization and exhibit uneven tracheal tube diameter, similar to those seen upon loss of *kkv* (Devine et al., 2005; Tonning et al., 2005; Moussian et al., 2006). Two other genes, *serpentine* (*serp*) and *vermiform*, encode proteins with chitin binding

domains that associate with the intraluminal chitin matrix and are required to restrict excess tube elongation (Luschnig et al., 2006; Wang et al., 2006). A member of the Halloween gene family, *mummy* (*mmy*), encodes an intracellular enzyme required to produce UDP-GlcNAc, the substrate for CS-1. Mutants for *mmy* lack the intraluminal chitin matrix and develop severely irregular lumen diameter (Tonning et al., 2006). *ghost* (*gho*) and *haunted* (*hau*), two other members of the Halloween gene family, have recently been shown to play a key role in cell secretion, and mutants for these genes display narrow tracheal tubes, arguing that secretion is fundamental to tube growth (Norum et al., 2010). Genes encoding septate junction (SJ) components are another group of tracheal tube size genes that are required to restrict tube elongation, and include *megatrachea* (*Mega*), *boudin* (*bou*), *Lachesin* (Banerjee and Slack), Na+/K+ ATPase, *sinuous* (*sin*) and *varicose* (*vari*) (Behr et al., 2003; Paul et al., 2003; Llimargas et al., 2004; Wu et al., 2004; Wu et al., 2007; Hijazi et al., 2009). The two polarity genes, *crumbs* and *yurt,* also affect DT length, and the antagonistic mechanisms of the two of them seem to regulate the extent of apical surface expansion and tube elongation (Laprise et al., 2006). Although these findings illuminate our understanding about some of the actors involved in tube size regulation, they do not fully explain the mechanism that control tube size, and it is not clear how their activity is temporally regulated during the different phases of tube growth.

Gas-filling and cuticle differentiation

The developing trachea is liquid-filled and, at the end of embryogenesis, the liquid is cleared and the tube becomes gas-filled

(Manning and Krasnow, 1993). At this time the cuticle, an apical extracellular matrix, has formed to prevent collapse of the tubes. Its rigid form offers structural stability and support to the tubes. The cuticle is composed of three defined layers, an outer envelope containing lipids and waxes, an epicuticlar network of proteins and a procuticle layer, which is closest to the apical side of the epidermis and is composed of sheets of highly ordered protein-chitin network (Moussian et al., 2005). Facing the lumen are ridges that lie perpendicular to tube length, called taenidia. The structure of the taenidia is thought to prevent the collapse of tracheal tubes, while allowing the tubes to expand and contract along their length.

AIMS OF THIS THESIS

The main aims of the presented thesis are to elucidate the impact of ecdysone on embryonic organ development, and to attain further understanding of the genetic and molecular mechanisms that control tube size, using the developing respiratory system (trachea) as a model system.

RESULTS AND DISCUSSION

The work for paper I and II was initiated with the aim of characterizing the onset of tracheal tube elongation after completion of diameter expansion at stage 15. We were interested in the molecular mechanisms that underlie this switch in growth mode. In paper I, we show that tube elongation requires two simultaneous events. First, the tracheal cells acquire an intrinsic ability to elongate, presumably mediated by subapical actin rearrangements. Second, the luminal matrix, which at this stage serves to hold back elongation, undergoes a modification, which appears required to permit a controlled extent of elongation. We conclude that successive morphogenic steps can be regulated through the timely modification of apical matrix properties. In Paper II, we identify another protein that is required for tracheal tube elongation. However, we noted the protein has an even more striking effect on tube size regulation of the hindgut, which became the focus of this article. Papers III and IV describe a role for ecdysone in the temporal progression of tracheal developmental events. In paper III, we characterize the gene regulatory mechanism of embryonic ecdysone receptor. In paper IV, we show that ecdysone is required for the temporal unfolding of tracheal gene programs, including the expression of genes required for tracheal tube elongation. Since ecdysone is required for the progression of parallel tracheal gene programs, the hormone pulse appears to be important to schedule and synchronize organogenic events.

Paper III: Tissue-autonomous EcR functions are required for concurrent organ morphogenesis in the *Drosophila* embryo

Here, we investigated the embryonic ecdysone-signaling mechanism. Vi show that both EcR and Usp are essential to mediate the effects of ecdysone on organ morphogenesis, indicating that embryonic ecdysone signals via EcR:Usp. We also uncover that EcR mediates the effects on organ morphogenesis in a tissue-autonomous manner, and that embryonic ecdysone via EcR instructs the temporal and tissue-specific expression of at least four transcription factors that are needed for embryogenesis and are common to the metamorphic ecdysone- response.

Organ morphogenesis is inhibited upon loss of ecdysone

We had previously noted a defective trachea of embryos that lack embryonic ecdysone biosynthesis, and started these studies by reinvestigating the requirement for ecdysone on epithelial organ morphogenesis. Mutants for *shadow* (*sad*) and *shade* (*shd*) lack the last two enzymes essential for embryonic ecdysone biosynthesis, respectively. Both mutants have major defects in dorsal closure, head involution and midgut constrictions. In addition, their tracheal branching morphogenesis stall at stage 14. However, the embryos continue to develop until the end of embryogenesis, since late *sad* and *shd* mutant embryos displayed movements within their eggshell and produced chitin in the epidermis during stages 16 and 17. We analyzed *sad* and *shd* mutants in parallel for all further studies, and

the mutants showed indistinguishable phenotypes.

EcR and Usp are required for embryonic organ development

Although EcR:Usp heterodimers mediate the response to ecdysone during larval moulting and metamorphosis, the embryonic ecdysone signaling mechanism has been unclear. First, we analyzed embryos homozygous for loss of function alleles of EcR (EcR^{M554fs} and EcR^{V559fs}) (Bender et al., 1997). We could show that these embryos had incomplete head involution and abnormal midgut morphogenesis at late stage 16, and a majority of the embryos had incomplete dorsal closure. In addition, their trachea stained only weakly for 2A12 and commonly had a bloated appearance. Thus, EcR is required for morphogenesis of different epithelial organ, and the milder phenotypes of *EcR* mutants compared to those of *sad* and *shd* mutants, is likely to be due to the maternal contribution of *EcR* mRNA and protein (Talbot et al., 1993). Both EcR and Usp are present in the embryo (Sedkov et al., 2003), but it has been unclear whether Usp is required for embryonic organ development; *usp* mutants generated from a *usp* mutant germ line (maternal$^-$, zygotic$^-$ m^-/z^-) die around hatching with few morphological defects (Perrimon et al., 1985; Oro et al., 1992), but the *usp* alleles used in these studies (usp^3 and usp^4) encode stable proteins with impaired DNA-binding domains (Henrich et al., 1994) and are hypomorphic alleles. We instead investigated the requirement for embryonic Usp by using the $usp^{Act\Delta 148}$ allele (Wahlstrom et al., 2006), in which the 5' region of the gene is deleted. Zygotic $usp^{Act\Delta 14}$ mutant animals die at the

molt between 1st and 2nd instar larvae, while embryos derived from homozygous $usp^{ActΔ14}$ germ cells ($usp^{ActΔ14}$ m^{-}/z^{-}) die before hatching. The latter had organ morphogenetic defects, including incomplete head involution and dorsal closure, aberrant midgut morphology and bloated tracheal tubes with reduced 2A12-levels. The phenotypes of $usp^{ActΔ14}$ m^{-}/z^{-} embryos imitate those of zygotic EcR mutants, but are less severe than those seen upon loss of zygotic ecdysone, probably because the $usp^{ActΔ14}$ mutation is not a null allele. Thus, it appears that Usp functions together with EcR to mediate the effects of ecdysone on embryonic organ morphogenesis, like in other stages of the *Drosophila* life cycle.

Embryonic organ formation requires tissue-autonomous EcR activity

Both EcR and Usp have been reported to active in the amnioserosa at the time of the ecdysone pulse (Kozlova and Thummel, 2003). However, when re-examining embryonic EcR:Usp activities using *EcRE-LacZ* (Koelle et al., 1991), which supposedly reflects *in vivo* EcR:Usp activity (White et al., 1999), we found that the reporter gene is expressed in the visceral mesoderm and head-region from stage 12, and later (stage 13/14) in the amnioserosa. No expression was seen in the trachea and other epidermal tissues. We also confirmed that the expression of *EcRE-LacZ* strictly depends on ecdysone. Moreover, when incubating young embryos (0–5 h) for 2 h in ecdysone (20E), we detected premature *EcRE-LacZ* expression, but still, the expression was confined to the visceral mesoderm. We

therefore concluded that detectable levels of *EcRE-LacZ* expression do not fully reflect the spatial distribution of ecdysone, or the developing organs that depend on EcR-activtiy. To test whether EcR activity in the amnioserosa or the visceral mesoderm could act indirectly to affect epithelial organ development, we expressed Dominant Negative (DN) EcR, EcR-DN (UAS-EcR-W650A, (Cherbas et al., 2003)) in the two tissues using the UAS-GAL4 system. We did not find that amnioserosal expression of DN-EcR caused any defects on embryonic development. EcR-DN expression in the mesoderm did, however, produce defects in midgut morphogenesis. The visceral mesoderm lines the midgut endothelium and is required for midgut closure and constriction (Tepass and Hartenstein, 1994). Strikingly, all embryos showed arrested midgut constrictions, but other ecdysone-dependent processes, such as dorsal closure, tracheal dorsal branch fusions and head involution were not affected. The midgut phenotype could be rescued upon co-expression of EcR, arguing that mesodermal EcR activity is required for midgut development. Based on these results, we speculated that *EcRE-LacZ* expression does not reflect all EcR activities within embryo, and tested whether EcR-DN expression in tracheal cells had any effect on tracheal development. Such embryos developed to hatching larvae, but all had a defective trachea similar to those of seen upon loss of ecdysone. The tracheal phenotypes were rescued by the simultaneous expression of EcR, arguing that they were due to loss of EcR function. We finally asked whether EcR is required tissue-autonomously for head involution, dorsal

closure and cuticle deposition, by expressing EcR-DN in the ectoderm. Most embryos showed stalled head involution and dorsal closure, as well as reduced cuticular structures. The phenotypes were rescued upon co-expression of EcR. Together, the results imply that ecdysone causes pan-embryonic EcR activity, and that EcR is needed in individual tissues for concurrent morphogenetic progression.

Tissue-specific induction of 20E-response genes via EcR

The zygotic functions of two primary ecdysone response genes in larvae, the nuclear receptors Eip75B (E75) and Hr46 (DHR3), are required for embryonic viability (Bilder and Scott, 1995; Carney et al., 1997). We therefore tested if the expression of these genes is induced also by embryonic ecdysone. The mRNA expression of *Eip75B* and *Hr46* is evident in wild type embryos from stage 12, correlating in time with the peak of the ecdysone pulse. The transcripts are first seen in the midgut, and later in the trachea and epidermis. *Eip75B* and *Hr46* mRNA were not detected in *sad* mutants. In addition, when young wild type embryos were incubated with exogenous 20E, *Eip75B* and *Hr46* transcripts were detected prematurely in the midgut, showing that 20E regulates their temporal onset of expression. *Blimp-1*, which encodes a SET-domain protein, is another ecdysone-response gene in larval cells, and *Blimp-1* mutants develop bloated tracheal tubes during embryogenesis (Ng et al., 2006), similar to those of embryos with reduced EcR and Usp functions. *Blimp-1* is expressed from stage 12 in the trachea and epidermis, and we found that this expression depends on ecdysone,

arguing that Blimp-1 is another embryonic ecdysone-response gene. We were also able to show that the expression of *Eip75B*, *Hr46* and *Blimp-1* depends on EcR, since the expression was severely reduced in the trachea of embryos that express EcR-DN in the trachea. A fourth potential embryonic ecdysone response gene that we analyzed was *bftz-f1*. This gene encodes another nuclear receptor that is induced by post-embryonic ecdysone pulses as a late response-gene. In the embryo, expression of *bftz-f1* increases towards the end of embryogenesis and is known to be required for embryonic viability (Yamada et al., 2000). We showed that the late *bftz-f1* expression also depends on ecdysone. Hence, *Eip75B, Hr46* and *Blimp-1* are early ecdysone-response genes, while *bftz-f1* appears to be a late response gene in the embryo, much like they act during larval moulting and metamorphosis. Together, the results imply that the pulse of embryonic ecdysone adds a temporal aspect to the gene regulatory networks that drives organ morphogenesis ahead.

CONCLUSIONS

I. Tracheal tubes undergo successive phases of tube growth that are associated with distinct tracheal gene expression. At the end of diameter expansion, tracheal cells acquire an ability to elongate that depends on an actin-organizing factor. A luminal matrix holds back elongation and a timely anion-dependent matrix modification permits a limited extent of elongation.

II. A third factor required for tracheal tube elongation is a mucin-like protein. The protein also drives hindgut lumen diameter expansion and appears to do so in a dose-dependent manner. This work provides an example where tube diameter is modeled by the regulated expression of a single protein.

III. Ecdysone is essential for epithelial organ morphogenesis past mid-embryogenesis and mediates its effects on organ development by tissue-autonomous EcR functions and Usp. The temporal pan-embryonic activation of EcR and its requirement for continued organ development implies an essential role for ecdysone in concurrent organ development. Embryonic ecdysone, via EcR also activates a gene regulatory hierarchy, similar to that of post-embryonic stages.

IV. Ecdysone advances tracheal development by allowing the temporal progression of tracheal gene programs that control morphogenesis and terminal differentiation. A hierarchy of embryonic ecdysone response genes interferes with late tracheal development, including tube elongation. Ecdysone therefore adds a temporal aspect to the spatially defined tracheal gene network.

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