The role of EGF and TGF-β signaling in specifying the polarity of the *Drosophila* egg and embryo

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1. INTRODUCTION

Exchange of extracellular signals between different cell populations is a key event in many developmental contexts.

In the last years, work from many labs has shown how an extracellular signal is received by a cell, how it is transduced into it and how it can finally promote gene activation or repression. It is now important to move further in our understanding and to consider single pathways as part of a network. This will provide us with a more authentic picture of cell signaling in which a single pathway is no longer a separate linear entity but part of a large web, exercising and enduring stimulatory and inhibitory inputs.

This new way to look at cell signaling has influenced this research project whose aim was to elucidate the role of the TGF-β (Transforming Growth Factor) signaling pathway during *Drosophila* oogenesis, focusing in particular on its interaction with the EGF (Epidermal Growth Factor) signaling pathway. The result of this research provides evidence of coupling between the two signaling pathways in setting up the developmental coordinates of the *Drosophila melanogaster* egg and embryo.

1.1 Inductive interactions between cells: the developmental concepts of induction and competence and their conservation in the animal kingdom

The development of an organism from an egg is undoubtedly a marvel of nature. The more we learn about the mechanism, in terms of cells and molecules, the more we wonder at it. To make a fully formed organism, single cells, groups of cells and tissues, communicate and instruct each other.

The first experimental attempts conducted in order to understand how cells interact during embryonic development mainly revolved around elegant manipulations that allowed the potential of specific regions to be assayed. They usually involved the ablation of cells or the transplantation of specific embryonic regions to ectopic sites, and the effects of these changes were then followed. Most of these technically demanding experiments were done in vertebrate species whose embryos were accessible and large enough to survive the surgery, such as amphibia or birds. In 1924 Hans Spemann and Hilde Mangold showed the importance of communication between cells during the embryonic development of an

amphibia embryo identifying the "organiser". This dorsal region of a gastrulating amphibian embryo causes striking axis duplication when transplanted to the ventral side of an equivalent stage host embryo. Thus, Spemann and Mangold provided direct evidence for the existence of the phenomenon of induction during embryonic development by showing that cells taken from the organiser could induce a second partial embryo when transplanted into a new site in another embryo.

The existence of cells with inducing capacity implies the presence of other cells possessing the competence to "understand" the inducing signal. An inducing signal can alter how the receiving cells develop only if these are competent to respond to that signal, for instance, they should present the appropriate receptors and possess the complete signal transducing machinery.

Several experiments conducted in many different laboratories demonstrated that equivalent regions in other vertebrates, the chick "Henson´s node", the anterior region of the mammalian primitive streak and the teleost embryonic shield, have "organising" activity (Waddington, 1932; Beddington, 1994; Oppenheimer, 1936; Ho, 1992). This functional conservation is surprising, as the early development of these vertebrate groups appears quite different in terms of morphology and timing.

Many proteins that can mimic the organiser functions have been biochemically purified and the genes encoding these factors have been cloned. For example, the organiser activity of the amphibian blastopore lip can be replaced by factors such as *noggin* (Smith and Harland, 1992; Smith, et al., 1993) and *chordin* (Sasai, et al., 1994). The discovery of proteins with such a function often leads to the isolation of many proteins of similar sequence or structure, which often have closely related activities. This results in genes classified into large functional groups, such as for example the Transforming Growth Factor-beta (TGF-β superfamily; Kingsley, 1994). Work on the fruit fly *Drosophila melanogaster* has led to the identification of a secreted morphogen, *decapentaplegic* (*dpp*), related to the mouse BMP-2 and BMP-4 gene and thus itself part of the TGF-β superfamily (St Johnston, 1987). Mutations at the *short gastrulation* (*sog*) locus can rescue the hapolethality of strong *dpp* alleles and a double dose of Dpp increases the degree of dorsalisation of *sog* mutants, indicating that *sog* is a genetic antagonist of *dpp* (Ferguson and Anderson, 1992). The cloning of *sog* (Fancois, et al., 1994) has shown that the protein is similar to the *Xenopus chordin* (Sasai, et al., 1994). In the amphibian embryo *chordin* exerts

its dorsalising effect by inhibiting BMP-4 activity (Fancois and Bier, 1995), and in the fly embryo *sog* exerts its ventralising activity by inhibiting *dpp*. It has been shown that *sog* expressed in Xenopus behaves just like *chordin* causing a dorsalisation of the embryo (Holley, et al., 1995).

This remarkable mechanistic conservation in the way different organisms use similar signaling molecules and pathways is one of the most important themes in modern developmental biology. Therefore, it makes sense for scientists working on different model systems and on various developmental contests to share knowledge and to exchange findings.

1.2 Juxtacrine and paracrine factors

A signal may pass between cells in different ways. Cells may interact directly with each other through molecules located on their membranes; these events are called juxtacrine interactions. This type of cell-cell communication plays a role in triggering equivalent precursors into distinct cell fates and this is achieved by a process called lateral inhibition, in which one cell, acquiring one fate, suppresses the neighboring cells from taking on the same fate. Lateral inhibition is achieved via the Notch signaling pathway (reviewed in Lendahl, 1998; Fleming et al., 1997). The signal may also be transferred through the extracellular space, as secreted molecule; these events are referred to as paracrine interactions and the corresponding molecules are known as paracrine factors or growth and differentiation factors (GDFs). During the past decades, classes of secreted peptides have been identified in both vertebrates and invertebrates including the TGF-β superfamily, the Fibroblast Growth Factor (FGF) family, the Epidermal Growth Factor (EGF) family and others. These molecules have attracted much interest being a relatively small group employed all over the animal kingdom and in many different developmental processes.

1.3 Signal transduction pathways

The process of signal reception by the responding tissue usually requires a membrane receptor and a cascade of interacting proteins that transmits the signal to the nucleus. These are collectively called signal transduction pathway; they are specific to the different classes of inducing factors but they share rather simple and elegant features. In general, the receptor spans the cell membrane having an extracellular region, a

transmembrane region and a cytoplasmic portion. The binding of the ligand to its receptor induces a conformational change, which often confers to the receptor an enzymatic activity (frequently they are kinases that can use ATP to phosphorylate proteins, including the receptor molecule itself). The activated receptor can now phosphorylate other proteins stimulating their latent activities. Eventually the cascade ends with the activation of a transcription factor capable to activate or to repress particular sets of genes.

This linear scheme although quite simplified is common to many well-known and conserved pathways, for instance the EGF and the TGF-β signaling cascades.

1.4 The TGF-β signaling pathway

Members of TGF-β superfamily take part in cell growth, cell cycle regulation, extracellular matrix secretion, adhesion and cell fate determination (reviewed in Wall and Hogan, 1994; Moses and Serra, 1996). They act through a signaling pathway conserved thought out the animal kingdom.

The superfamily includes the prototypic TGF-β, the Activins, the Bone Morphogenic Proteins (BMPs) and others. In *Drosophila* five TGF-β like ligands have been identified: a Müllerian inhibiting substance-like protein, a dActivin and three BMP-related proteins: Decapentaplegic (Dpp), Screw (Scw) and Glass Bottom Boat (Gbb/60A) (reviewed in Raftery and Sutherland, 1999).

It has been shown that Activins and BMPs are able to induce different developmental responses by acting in a concentration depending manner. For example, animal cap cells from amphibian embryos can respond to Activins by expressing specific genes. John Gurdon and his colleagues placed Activin-releasing beads or control beads in contact with Xenopus animal cap cells. They found that cells exposed to little or no Activins do not express any known mesodermal marker gene and therefore differentiate into ectoderm. Higher concentrations of Activins can turn on *Brachyury*, a gene responsible for directing cells to become mesoderm. Even higher concentrations of Activins induce cells to express the gene *goosecoid*, a marker gene associated with the notochord, the most dorsal mesodermal structure (Dyson and Gurdon, 1998; Shimizu and Gurdon 1999)

Soluble signaling molecules may specify more than one cell type within a tissue by forming a concentration gradient. These molecules are known as morphogens. The morphogen diffuses from its site of synthesis (source) to its site of degradation (sink).

Morphogen gradients can form by passive diffusion of the molecule from its source through the extracellular space, as demonstrated for Activins (McDowell et al., 1997). In addition, recent studies have described other mechanisms that are able to shape TGF-β gradients, such as the presence or absence of extracellular inhibitors able to inactivate the ligand, the balance between other competing or synergistic TGF-β ligands and the intracellular modulation of signal propagation. Each level of regulation applies various options depending on the developmental context, so finally the spectrum of mechanisms involved in establishing the gradient is very broad. In *Drosophila*, Dpp, the fly homologue of the vertebrate BMP-4, has been shown to act as a morphogen providing positional information in two well investigated examples, namely in the blastoderm embryo and in imaginal disc patterning.

In the embryo Dpp acts as a morphogen in the region of its own expression domain, whereas in imaginal discs Dpp emanates from its central source towards more distant regions of the tissue. Gradient formation in these two developmental contexts is controlled by two different mechanisms.

In the embryo, Dpp activity is counteracted by the secreted inhibitor Short gastrulation (Sog), which diffuses into the Dpp domain from an adjacent expression region (Francois et al., 1994; Biehs et al., 1996). In the imaginal discs *dpp* mRNA is expressed in a central stripe of cells, thus the protein concentration is high at the AP compartment boundary decreasing progressively towards the edges of the disc (Nellen et al., 1996; Lecuit et al., 1996). No diffusible inhibitors have been identified, however, there is evidence that the level of the Dpp receptor *thick veins* (*tkv)* influences the effective range of the Dpp gradient (Lecuit and Cohen, 1998; Haerry et al., 1998).

The basic molecular mechanism of TGF−β signaling pathway has been unravelled (reviewed in Heldin et al., 1997; Whitman, 1998; Attisano and Wrana, 1998). TGF-β family members promote signaling by binding to a complex of structurally similar type I and type II transmembrane serine-threonine kinase receptors. Following ligand binding, the type II receptor transphosphorylates the type I receptor, activating in turn its kinase activity. The active type I receptor transmits the signal phosphorylating intracellular components of the pathway, namely the Smads. First, Receptor activated Smads (R-Smads) are phosphorylated acquiring the capability to recognize and bind to the Co-activated Smads (Co-Smads). Then, the heteromeric Smad-complex translocates into the nucleus to directly bind to DNA recognition sites in target gene promoters.

In *Drosophila,* one type II receptor, *punt* (*pnt*) and two type I receptors, *tkv* and *saxophone* (*sax*), have been described (Letsou, 1995; Ruberte, 1995 and Brummel, 1994). In vertebrate several different type I and II receptors have been identified which show different ligand affinities and the ability to discriminate between BMPs or Activins. Furthermore, R-Smads, substrates of the type I receptor, show a pathway-specificity since vertebrate Smad1 and Smad5 are specific for BMPs signaling, whereas, Smad2 and Smad3 transduce TGF-βs and Activins. The discrimination between R-Smads provides a first level of target gene selection. *Mothers against dpp* (*Mad*) is the only Dpp/BMP-specific R-Smad that has been found in *Drosophila* (Sekelsky et al., 1995). In addition, one Activin-specific Smad, *dSmad2*, has been identified (Raftery et al., 1995; Sekelsky et al., 1995; Raftery and Sutherland, 1999). R-Smads associate with Co-Smads, which are the common mediators. They are not direct targets of receptor kinase activity, but they are able to participate in signaling by association with all pathway-restricted R-Smads. Only one member of this subfamily has been identified in each model system, namely Smad4 (also called DPC4) in vertebrates (Hahn et al., 1996; Lagna et al.,1996), *Medea* in *Drosophila* (Raftery et al., 1995; Hudson et al., 1998; Das et al., 1998; Wisotzkey et al., 1998) and *sma-4* in C. elegans (Savage et al., 1996). Co-Smads are therefore shared by all R-Smads and they are not required for nuclear translocation but for the formation of functional transcriptional complexes (Liu et al., 1997).

Once in the nucleus, the Smad complex can modulate transcription of TGF-β target genes. Both R-Smads and Co-Smads can bind to DNA via the MH1 domain (Kim et al., 1997; Shi et al., 1998). Optimal binding is achieved with a 5bp sequence CAGAC, known as Smads Binding Element (SBE) (Shi et al., 1998; Zawel al., 1998). In *Drosophila* Mad or Medea binding sites have been identified in promoters of several Dpp target genes, e.g., *vestigial* (*vg*) and *tinman* (*tin)* (Kim et al., 1997, Xu et al., 1998). The SBE sequence is calculated to be present on average once every 1024 bp in the genome or about once in the regulatory region of any average size gene. Thus, this site alone has no selectivity. However, by associating with DNA-binding partners, forming complexes of specific composition and geometry, the Smads can achieve high-affinity and selective interaction with cognate DNA. *Xenopus* FAST-1 protein was the first described partner of the Smad complex in providing the supplementary DNA binding activity necessary for a specific target gene transcription (Chen et al., 1997). In addition, transcripional activation by R-Smads has been shown to occur, in part at least, by their ability to recruit the general coactivators p300 and CBP (Luo

et al., 1999). Smads proteins have also been proposed to activate transcription by relieving the action of transcriptional repressors. In Drosophila, Brinker protein is likely to be a transcriptional repressor, the action of which is prevented by Smads signaling (Campbell and Tomlinson, 1999; Jazwinska et al., 1999: Minami et al., 1999). A Smad complex bound to DNA has the option to recruit not only coactivators but also corepressors. Recently, C-Ski and Sno-N, originally discovered as products of a retroviral oncogene (v-ski), have been identified as Smad3 and Smad4 interacting proteins, and both have been shown to act as corepressors (Akiyoshi, 1999; Luo, 1999 and Sun, 1999)

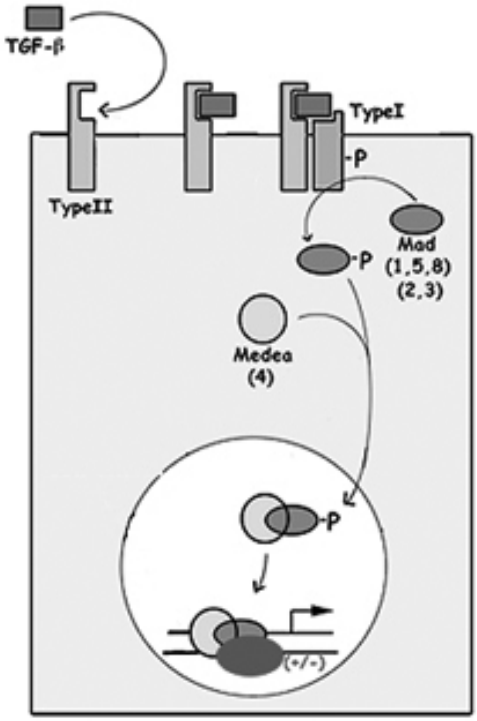

Figure 1.
Schematic view of the TGF−β ***signaling pathway***
TGF−β family members promote signaling by binding to a complex formed by the type I and type II transmembrane serine-threonine kinase receptors. Receptor activated Smads (Mad, Smads 1, 5, 8, 2 and 3) are the substrates of the type I receptor kinase, once phosphorylated they recognize and bind to the Co-activated Smads (Medea, Smad 4). The heteromeric Smad-complex translocates into the nucleus to directly bind to DNA recognition sites in target gene promoters.

1.5 The EGF signaling pathway

One large family of cell surface receptors exerts its function via an protein tyrosine kinase activity. These Receptor Tyrosine Kinases (RTKs) catalyse transfer of the γ phosphate of ATP to hydroxyl groups of tyrosines on target proteins (Hunter, 1998). RTKs play an important role in the control of most fundamental cellular processes including cell cycle, cell migration, cell metabolism, and survival, as well as cell proliferation and differentiation. The RTKs are monomers in the cell membrane. Ligand binding induces dimerisation of the receptor resulting in crossphosphorylation of their cytoplasmic domains (Schlessing, 1988; Lemmon and Schlessingen, 1994; Jiang and Hunter, 1999).

The Drosophila gene *torpedo* encodes an RTK receptor, homologue of the vertebrate EGF Receptor (DER; Livneh et al., 1985; Wadsworth et al., 1985; Price et al., 1989). This receptor fulfills multiple roles during development and in recent years its activation has been shown to play a role in cell fate specification during oogenesis, embryogenesis and immaginal discs development. During these processes the receptor activation is tightly regulated by the precise localisation or activation of its ligands together with positive and negative feedback loops generated by activating and inhibitory factors. The vertebrate EGFR has several ligands: EGF, TGF-α, HB-EGF and Neuregulins (NRG). In Drosophila only three ligands, Spitz, Gurken and Vein, have been identified. *gurken* encodes a TGF-α like molecule with a single EGF domain, a signal peptide and a transmembrane domain. It is expressed and used exclusively during oogenesis in the female fly where it sets up both the anterior-posterior and dorsal-ventral axis of the egg and the future embryo. Spitz, similar in structure to Gurken and TGF-α molecules in general, is used more widely (Rutledge, 1992; Neuman-Silberberg, 1993). It is expressed as a transmemebrane inactive precursor and only the secreted cleaved form is active as an EGFR ligand (Schweitzer et al. 1995; Freeman 1994). Finally, Vein is a secreted molecule with a single EGF repeat and an immunoglobin domain (similar in structure to the vertebrate Neuregulin). Vein is constitutively active, it is not cleaved and it has an intrinsic capacity activate the receptor (Golembo, 1999). It is believed that more EGFR ligands exist in the Drosophila genome since some functions fulfilled by the EGF receptor (e. g. cell proliferation in the eye discs) cannot be explained by the phenotypes resulting from mutations in the known ligands.

A set of mutations, named the spitz group has been connected with the EGF signaling pathway (Mayer, 1988). Their study has greatly advanced our understanding of EGF pathway regulation. Among the spitz group are genes like *rhomboid* and *star* (Bier,

1990; Rouhola-Baker, 1993; Sturtervant, 1993; Freeman, 1994). They encode transmembrane molecules involved in the processing of the Spitz ligand. During development, *rhomboid* expression is always tightly regulated and its ectopic expression gives rise to phenotypes similar to ectopic EGF receptor activation. Therefore, it is the restricted expression of *rhomboid*, preceding Spitz processing which controls the precise EGFR activation pattern. In the Drosophila embryo Spitz precursor is ubiquitously expressed after gastrulation but its proccesing is restricted to the midline cells since Rhomboid and Star are only present in those cells. Their activity gives rise to a localised source of secreted Spitz. At the midline, high levels of EGFR activation induce the ventral-most ectodermal cell fate. The adjacent ventrolateral fates dependent also on the EGFR activation since Spitz is functioning as a morphogen inducing ventralmost and ventrolateral fates in a concentration dependent manner. In addition to the Spitz diffusion, it has been shown that the activation of the EGFR leads to the production of the second ligand Vein that is able to diffuse laterally. Vein guarantees the specification of lateral cell fates even when normal levels of secreted Spitz are compromised (Golembo, 1999). Ultimately, the expansion of ventrolateral cell fates is impaired by the negative activity of Argos, a secreted diffusible protein with a single EGF domain (Freeman, 1992). Argos is capable of antagonizing the activity of the EGF pathway and its expression is controlled by EGF pathway activity. Thus, *argos* activation finally carries out an inhibitory feedback loop on the receptor activation (Golembo, 1999).

Activation of the EGFR leads to the induction of its tyrosine-kinase activity starting a downstream kinase cascade (Schlessinger, 1994; Pawson, 1995; Bar-Sagi and Hall, 2000). The adapter protein Grb binds to the phosphorylated receptor as well as to the guanine nucleotide exchange factor Son-of-Sevenless (Sos). Upon binding with Grb, Sos translocates to the plasma membrane where it can stimulate the GTP/GDP exchange on the G protein Ras (Margilis, 1999). GTP-activated Ras can interact with the effector protein Raf which in turn phosphorylates MAP-kinase-kinase (MAPKK, MEK and in *Drosophila* also known as D-sor). In turn MAPKK activates via phosphorylation MAPK (ERK, known as Rolled in *Drosophila*). At this point the pathway branches since MAPK can phosphorylate several cytoplasmic and membrane linked molecules. This signaling cascade, also known as the Ras pathway, is very conserved through evolution and exists bothin invertebrates and vertebrates.

Many of the EGF signaling pathway components have been identified in genetic screens in *Drosophila melanogaster* and *Caenorhabditis elegans*. In both organisms EGF signaling controls a large variety of distinct developmental decisions and these seem to have no common theme. Thus, processes as diverse as vulva induction in *C. elegans* and formation of retina photoreceptors in *D. melanogaster* depend on EGF signaling. Moreover, in Drosophila the EGF signaling pathway controls the establishment of both embryonic axes. Its role in this process will be illustrated in detail in the following paragraphs.

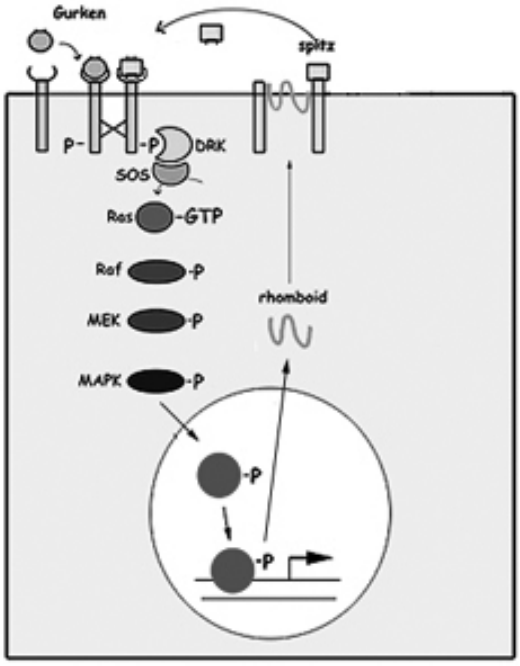

Figure 2.
The EGFsignaling transduction pathway
Signaling by Gurken or Spitz induces Grb binds to the phosphorylated receptor and to Son-of-Sevenless (Sos). Upon binding Sos stimulates GTP/GDP exchange on the G protein Ras. Activated Ras interacts with the effector protein Raf which in turn phosphorilates MAP-kinase-kinase (MAPKK, MEK and in Drosophila also known as D-sor). As a consequence, MAPKK activates MAPK (ERK, known as Rolled in Drosophila). MAPK can phosphorylate several other molecules In addition, rhomboid expression depends on EGF activation. Rho can process Spitz leading to a positive feed back loop on the pathway activation.

1.6 Drosophila oogenesis

1.6.1 The egg chamber and the mature egg

The making of the fly does not start with the fertilisation but much earlier during oogenesis when the mature egg forms. Oogenesis takes place in the ovaries of the female fly (for review see King, 1970 and Spradling, 1993). The *Drosophila* egg develops from a distinct structure called egg chamber. This forms at the anterior tip of the ovary and moves posteriorly as it develops. In the ovaries there are strings of developing egg chambers in a linear array of developmental stages. These are called ovarioles. The whole oogenesis process takes around three days (79 hours at 25C) and it is conventionally divided into 14 stages, each being defined by changes in the morphological structure of the egg chamber. Their approximate duration was inferred from their relative abundance in ovaries of flies with high vitality and in an optimum physiological state, where all ovarioles are functional and without any retention of mature eggs (Lin and Spradling, 1993).

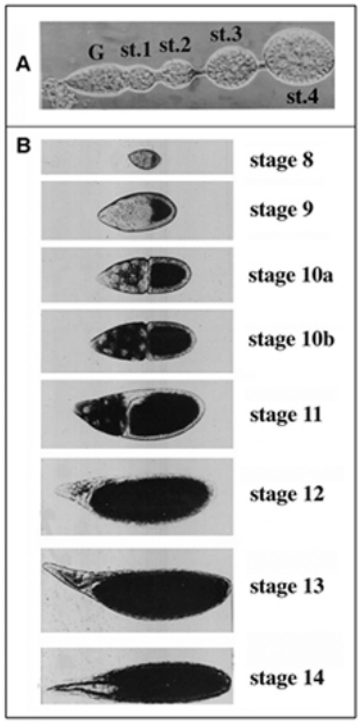

Figure 3
Stages of Drosophila oogenesis
(A) The anterior tip of a dissected ovariole showing the germarium (G) with young egg chambers (st. 1-4). (B) Dissected egg chambers from stage 8 to 14. Anterior in all figures is the left and dorsal to the top

The egg chamber is formed by the germ cells, the oocyte and the 15 nurse cells, and by a monolayer epithelium of somatic follicle cells derived from the gonadal mesoderm. The germline stem cells are located at the distal tip of the germarium which is the anterior most part of each ovariole.

In the region 1 of the germarium each stem cell divides asymmetrically to produce a new stem cell and a cystoblast; the latter undergoes four incomplete cell divisions to form a syncytial cyst. Cytoplasmic bridges, known as ring canals, interconnect the 16 cells forming the cyst. While the cyst moves posteriorly within the germarium, one of the 16 germ cells is selected as the oocyte while the other 15 cells become the polyploid nurse cells. At the present, it is not known how the oocyte is chosen. However, the fusome, a vascular organelle rich in membrane cytoskeletal proteins and Dynein, seems to play an essential role. It has been proposed that the fusome during the initial four incomplete cell divisions grows in a polar manner with one end always associated with the mitotic spindle of one single cell, namely the future oocyte. The growing ends of the fusome enter the other cells of the cyst as they are generated by further cell cleavages (de Cuevas and Spradling, 1998)

Follicle stem cells reside in region 2a of the germarium (Mangolis and Spradling, 1995). In the region 2b of the germarium the follicle cells encase the cyst forming a monolayer epithelium.

As the newly formed egg chamber moves from region 2b to region 3 of the germarium the oocyte acquires an asymmetric localisation within the cyst and its localisation specifies the polarity of the anterior-posterior axis. The DE-cadherin adhesion complex is involved in this process being transiently upregulated in the oocyte, as well as in a corresponding region within the follicle epithelium. This leads to the recruitment of the oocyte at the future posterior side of the egg chamber (Gonzalez-Reyes, 1998 and Godt, 1998).

The so formed egg chamber now buds off from the germarium and grows in size.

Transfer of cytoplasm from synthetically active nurse cells to the transcriptionaly silent oocyte occurs via the ring canals. By the end of oogenesis the nurse cells rapidly transfer all their content to the oocyte in a process known as “dumping”. As a consequence, at the end of the process the nurse cells are reduced to a cluster of apoptotic nuclei anterior to the big oocyte.

Follicle cell proliferation occurs until stage 6 when the follicle cells reach their final number (around 1000) and stop dividing. After this point the epithelium undergoes stereotypic migrations and a general movement of follicle cells towards the posterior of the egg chamber is visible. This generates a population of stretched follicle cells lying over the

nurse cell cluster at the anterior of the egg chamber and a population of columnar shaped cells, or main-body follicle cells, abutting the growing oocyte at the posterior.

At the same time, a small group of cells, the border cells, 6-7 in total, migrates trough the nurse cell cluster from the anterior tip of the egg chamber towards the anterior margin of the growing oocyte. At the end of oogenesis these cells will shape the pore channel of the micropyle allowing sperm entry. The micropyle is an anterior protrusion of the mature egg and it is formed by centripetal follicle cells, which during stage 10, at the end of the general posterior migration, position themselves between the nurse cell cluster and the oocyte.

Two large groups of follicle cells lay down the dorsal appendages, two dorsal filaments with respiratory function positioned at the dorsal anterior site of the egg. Each dorsal filament is formed by a group of follicle cells (around 55-65; Roth, 1999) that migrate over the anterior part of the oocyte at stage 11.

During stage 7-10 the follicle cells undergo endoreduplication and become polyploid and it is after this time point that the active transcription of chorion specific genes starts.

The eggshell is composed of the inner layers (the vitelline membrane, the waxy layer, and the inner chorion layer) and the outer layers (the endochorion and the exochorion). Follicle cells deposit the proteins that comprise each layer with precise temporal control. The follicle cells leave imprints on the eggshell and their final organisation is therefore visible. The hard outer covering contains several prominent features, which allow it to be oriented. The dorsal appendages define the anterior-dorsal side of the egg. The operculum structure, through which the larva hatches, is at the anterior and it is a flat plate ringed ventrally by a prominent collar. In the center of the operculum there is the micropyle. A second respiratory structure, the aeropyle, is present at the posterior pole.

Figure 4.
The Drosophila egg.
A dark field image of a wildtype Drosophila egg. The anteriorly located operculum and micropyle can clearly be seen as can be the aeropyle, a posterior structure. The dorsal side is marked by the anteriorly located dorsal appendages.

1.6.2 Establishment of the anteroposterior and dorsoventral polarity during oogenesis

In recent years much progress has been made in understanding the establishment of anteroposterior (AP) and dorsoventral (DV) polarity of the embryo of *Drosophila*. It is now clear that communication between the oocyte and the overlying somatic follicle cells in the egg chamber is necessary for the asymmetric localisation of instructive molecules and leads to the specification of two spatially distinct cell populations within the follicular epithelium (Gonzalez-Reyes, 1995 and Roth, 1995)

To date, three genes have been identified which are directly involved in the communication between germline and soma. The study of the gene *gurken* and *torpedo* (DER) has revealed the role of EGF signaling in setting up polarity during oogenesis. *gurken* homozygous females are sterile and produce apolar eggs lacking both AP and DV axes (Neuman-Silberberg and Schüpbach, 1993). Female flies lacking DER function in the follicular epithelium show the same phenotype. Analysis of mosaic females demonstrates that *gurken* gene expression is required in the germline while DER expression is required in the soma. As previously mentioned, *torpedo* encodes the *Drosophila* homologue of the EGF receptor, while Gurken has homology with the transforming growth factor (TGF)-α family of vertebrate growth factors and therefore it is a potential ligand for the EGF receptor (Neuman-Silberberg and Schüpbach, 1993). *cornichon* gene has been found to be essential in the generation of Gurken signaling. Cornichon is a small (144aa) hydrophobic protein expressed in the germline (Roth et al., 1995) and is the founding member of a conserved protein family that includes Erv14p, an integral component of the COPII coated vesicles mediating cargo export from the endoplasmatic reticulum (Bockel in preparation). Cornichon is required for the transport of Gurken to the oocyte surface. It has been proposed that an early signal from the oocyte, generated by the interaction between Gurken and DER, induces the abutting terminal follicle cells to acquire a posterior fate.

This group of cells, which go on to produce posterior structures such as the aeropyle in the mature egg, in turn signal back to the oocyte and promote the reorganisation of the cytoskeleton network in that cell (Theurkauf, 1993; Theurkauf, 1994). The nature of this “back signaling" is not yet known but it appears to require the function of some known genes. Mago nashi (Mago) is likely to be part of the machinery able to receive the signal in the oocyte but its exact role in the process is unknown (Micklem, 1997; Newmark, 1997).

Recently, Merlin, a homologue of the human tumor suppressor neurofibromatosis-2 (NF2) has also been shown to be required in the posterior follicle cells. Merlin is thought to link actin to transmembrane proteins and to be required for mRNA localisation. The precise role of Merlin is unknown and the molecule has been suggested to target to the posterior the unknown polarizing signal (MacDougall, 2001).

The signal from the posterior follicle cells is required for the correct repolarization of the oocyte cytoskeleton during midoogenesis (Gonzalez-Rejes, 1994). Before back sinaling occurs the MicroTuble Organising Center (MTOC) is located at the posterior pole of the oocyte and the plus ends of the microtubles extend anteriorly into the nurse cells. After back signaling the posterior MTOC is inactivated and the microtubles are then organized from a more diffuse anterior MTOC. This results in the reversal polarity of the microtuble network, which finally is required for the correct localisation of the anteroposterior determinants, *bicoid* and *oskar*. *bicoid* is localised at the anterior and *oskar* RNA is localised at the posterior. In *gurken* and *cornichon* mutants, the defect in posterior follicle cell fate determination results in improper cytoskeletal reorganization. The posterior MTOC persists, resulting in a "bipolar" microtuble organisation, which leads the mislocalisation of *oskar* RNA to the middle of the oocyte and *bicoid* RNA to both ends (Gonzalez-Reyes, 1995 and Roth, 1995)

The reorganization of the microtuble network is also associated with the movement of the oocyte nucleus from a posterior position to a new asymmetric anterior cortical localisation.

Thus, the movement of the nucleus is also affected in *gurken* and *cornichon* mutants. It is found posteriorly in 70% of the egg chambers, in a cortical anterior position in 26% and in a cortical central position in 4% of the cases. Since *gurken* mRNA is present and co-localises with the nucleus in *cornichon* mutants, it can be found at different positions in the egg chamber depending on the localisation of the nucleus (Roth et al., 1995).

The new anterior cortical localisation of Gurken and the oocyte nucleus marks the future dorsal side of the egg and embryo. This new position along the anterior circumference is not predetermined since in *spaghetti-squash* mutant egg chambers two oocyte nuclei are present and they move to random positions with respect to each other (Roth et al., 1999). During midoogenesis Gurken interacts again with the EGF receptor and this signaling event specifies the dorsal fate in a cell population overlying the new site of Gurken localisation. The localisation of *gurken* to the anterior dorsal region surrounding the oocyte nucleus

requires the function of several genes. One of them is *fs (1)K10 (K10),* a gene encoding a helix-loop-helix DNA binding protein (Wieschaus et al., 1978). In *fs(1)K10* mutant egg chambers, *gurken* mRNA is localised in an anterior cortical ring in the oocyte and, as a consequence, signaling induces an anterior ring of follicle cells to become dorsalised. *orb*, *Bic-D* and *squid* also appear to be required in *gurken* mRNA localisation (Lantz et al., 1992, 1994; Kelly, 1993; Christerson and McKearin, 1994; Ran et al., 1994; Swan and Suter, 1996).

Furthermore, mutations in the *spindle*-like genes – *spindle A-E*, *okra, aubergine*, *squash*, *zucchini*, and *deadlock* – impair both Gurken signals from the oocyte, this phenotype is most probably caused by a reduction in gurken mRNA translation (Gonzalez-Reyes et al., 1997 and Ghabrial and Schüpbach, 1998). *okra* and *spindle-B* encode proteins similar to yeast Rad54 and DMC1, respectively, and are part of the double strand repair pathway (Ghabrial and Schüpbach, 1998). A reduced recombination rate and an enhanced chromosome non-disjunction are observed in these mutants.

The failure to repair DNA breaks generated during recombination is supposed to be the cause of the activation of a checkpoint that inhibits Gurken translation. In addition, *vasa* and *spindleE* encode RNA helicases most likely directly involved in the translational control of Gurken (Gillespie and Berg, 1995; Styhler et al., 1998: Tomancak et at., 1998).

The second Gurken signaling event during midoogenesis establishes the DV pattern of the follicular epithelium, which both defines eggshell polarity and provides spatial information that will later define the DV axis of the future embryo. Gurken signaling induces the dorsal fate in the adjacent follicle cells during midoogenesis and thus concomitantly a ventral region within the follicular epithelium is delimited which will provide spatial cues governing the establishment of the future embryonic dorsoventral axis.

Figure 5.
Model for the induction of follicle cell fates by Gurken.
Schematic rappresentation of egg chambers. (top left) Previous to Gurken signaling the follicle cells at both termini of the egg chamber have an anterior fate (shown in green). The Gurken signal (purple arrows) instructs the terminal follicle cells abutting the oocyte to adopt a posterior fate (red). (center) The newly induced posterior follicle cells induce the migration of the oocyte nucleus (yellow). (low panel) Gurken signals to the overlying follicle cells (purple arrows) from its new anterior cortical location. The induced follicle cells acquire a dorsal fate (blue).

1.6.3 Patterning of the follicular epithelium

As Gurken signals twice to the follicular epithelium using the same transduction machinery, how is the outcome different in each case? It has been shown that the competence to adopt a posterior fate is restricted to the terminal follicle cells at both poles of the egg chamber. This has been nicely demonstrated by Gonzalez-Reyes and St Johnston analysing *dicephalic* mutant egg chambers in which the oocyte lies ectopically at the anterior of the whole structure without Gurken signaling being affected (Lohs-Schardin, 1982; Gonzalez-Reyes et al., 1997). As a consequence, posterior markers are expressed in the anterior follicle cells of the egg chamber which can develop normally although with reversed polarity with respect to the anteroposterior axis of the whole ovariole (Gonzalez-Reyes and St Johnston, 1998). In addition, clonal analysis using the putative Gurken receptor, *top/EGFR*, suggests that the terminal cell population is composed of 200 cells and extends 10-11 cell diameter from the pole. In the absence of Gurken signaling these cells express anterior marker genes rather than posterior markers. Cell clones lacking *top/EGFR* can be seen to cross the boundary between the posterior terminal cells and the main-body follicle cells indicating that the progeny of a single cell can contribute to both populations. Notch-Delta signaling has been proposed to be required in the patterning of the terminal follicle cells. Notch signaling seems to play a role in the determination of the terminal cell population since temperature sensitive Notch mutant egg chambers show a reduction in the size of the terminal cell group (Gonzalez-Reyes and St Johnston, 1998).

However, a recent publication has finally clarified the role of Notch signaling during oogenesis. Using clonal analysis, Lopez-Schier and St Johnston (2001) have shown that Notch signaling is not involved in the patterning of the follicular epithelium but controls cell proliferation and differentiation in the whole epithelium. Notch mutant follicle cells fail to differentiate and remain in a precursor state.

The timing of follicle cells differentiation is controlled by a germline signal produced by the Notch ligand Delta. Undifferentiated follicle cells do not participate in normal axes specification and thus AP defects are observed in Notch mutant egg chambers. Finally, the authors have proposed that Delta signals twice from the germline. As previously described, early during oogenesis, Delta signaling controls the differentiation of the polar

and stalk follicle cells, later it instructs the follicle cells to exit the mitotic cell cycle and to enter endocycles becoming polyploid and finaly differentiating.

The ectopic expression of an activated *top/EGFR* form (λtop), obtained by fusion with a heterologous dimerization domain, suggested that the main-body follicle cells are patterned along the anteriposterior axis previous to the Gurken signaling. The miss-expression of λtop in the whole follicular epithelium induces the expression of certain genes known to respond to the receptor activation, such as *kekkon1* (Ghiglione et al., 1999). However, under the same experimental conditions other targets, as *rhomboid*, are expressed only in the anterior follicle cells (Queenan et al., 1997). This indicates that the main-body follicle cells respond to *top/EGFR* activation in different ways. The authors suggest that the TGF-β like protein Dpp, expressed in the centripetal follicle cells, may play a role in the anteroposterior patterning of the main-body follicle cells (Queenan et al., 1997).

Previous work has shown that *dpp* function is required during oogenesis. Its expression is restricted to a subset of migrating anterior follicle cells, the centripetal follicle cells, and its functions is to maintain egg chamber integrity and generate anterior eggshell structures (Twombly et al., 1996). Increase or decrease of Dpp levels correlate with anterodorsal eggshell defects. When Dpp is overexpressed in all follicle cells eggs display enlarged operculum and absence of respiratory appendages (Twombly et al., 1996).

Recent work has shown how EGF signaling specifies and positions the respiratory appendages at each side of the midline and the reduction in EGF signaling leads to the fusion of the two appendages at the midline (Wasserman and Freeman, 1998).

It has been proposed that the interplay between Gurken, expressed in the oocyte, and Spitz, present in the follicular epithelium, promotes an autoregulatory circuit able to pattern the dorsal appendage anlage. During midoogenesis the paracrine activation of the EGF receptor by Gurken leads to *rhomboid* expression. Freeman and colleagues (Wasserman and Freeman, 1998) proposed that Rhomboid functions in oogenesis to trigger the autocrine activation of EGF receptor by Spitz, which amplifies EGF receptor activation profile. This allows the overall signal to increase in width and amplitude. Finally, the Rhomboid/Spitz amplification induces *argos* expression in the dorsal midline cells leading to the local inhibition of signaling at the midline. The resulting signaling profile, two peaks, specifies the position of the two dorsal appendages one at each side of the midline. Egg chambers lacking *rhomboid* and *argos* functions in the follicular epithelium give rise to eggs with a single dorsal appendage located at the midline (Wasseman and Freeman, 1998).

Early EGFR signaling, required for the posterior fate determination, does not appear to call for Spitz function, as no anterior-posterior phenotypes are observed in *rhomboid* or *spitz* clonal egg chambers. Moreover, the *rhomboid* gene is not expressed in posterior follicle cells and thus the positive feedback-loop on the receptor activation is restricted to the anterior-dorsal side of the egg chamber. This raises the question of how the same ligand/receptor interaction at the level of the follicular epithelium leads to differential gene expression and thus different patterning mechanisms.

1.6.4 Establishment of the embryonic dorsoventral polarity

Dorsal, a member of the Rel/NfK-b family of transcription factors, acts in a concentration dependent manner to specify different cell fates along the DV axis in the Drosophila embryo (Roth, 1989). It enters the ventral nuclei but it remains in the cytoplasm in dorsal regions of the blastoderm embryo. The graded distribution of the Dorsal protein is established in response to the activation of the transmembrane receptor Toll by its extracellular ligand, Spätzle. Molecules have been identified that are required for the proteolytic processing of Spätzle. These are the products of the genes *easter* (*ea*) (Chasan and Anderson, 1989), *snake* (*snk*) (DeLotto and Spierer, 1986) and *gastrulation defective* (*dg*) (Konrad, 1998). These molecules diffuse freely in the perivitelline space of the embryo however the activity of the proteolytic cascade is somehow limited to the ventral side. In addition, three other genes, *nudel* (*ndl*), *pipe* (*pip*) and *windbeutel* (*wind*) are required for Spätzle processing and are expressed in the follicular epithelium thus, they couple follicular and the embryonic DV patterning (Schüpbach, 1991; Stein, 1991). Nilson and Schüpbach (1998) have shown with genetic mosaic experiments that Pipe and Windbeutel activities are required specifically in the ventral follicle cells suggesting that these two genes are the best candidates for triggering and spatially restricting the ventral proteolytic cascade. However, *windbeutel* and *nudel* are expressed in all follicle cells. In contrast, in a landmark paper, Stein and colleagues (1998) have shown that *pipe* RNA is specifically localised to the ventral follicle cells (Sen J., 1998). This localised expression depends on Gurken signaling since *pipe* is expressed in all follicle cells in mutant egg chambers lacking EGFR signaling (Sen, 1998). In addition, miss-expression of *pipe* RNA in dorsal follicle cells reveres the polarity of the future embryo (Sen J., 1998). Thus, Pipe activity is sufficient to define the embryonic DV axis.

The *pipe* gene encodes a heparan sulfate 2-O sulfotransferase that modifies the glycosaminoglycan (GAG) side chain of proteoglycans. A Pipe-GFP fusion protein has been shown to localize in the Golgi and this subcellular localisation depends on the activity of Windbeutel (Sen, 2000). In the absence of Windbeutel, Pipe-GFP seems to be inactive and localised in the ER.

The earliest acting protease in the Toll signaling pathway is Nudel. The *nudel* gene encodes a large protein with sequence similarity to extracellular matrix proteins (Hong and Hashimoto, 1995). However, Nudel has a centrally located domain with homology to the catalytic domains of trypsin-like serine proteases and site-directed mutagenesis of this domain shows that Nudel is a protease generated by apparent autoproteolytic zymogen cleavage and acting during early embryogenesis (LeMosy, 1998).

However, Nudel is probably not the Pipe substrate as Nudel protease activation occurs independently of Pipe activity and uniformly around the embryo circumference (LeMosy, 1998). In addition, Nudel has a role in the biogenesis of the vitelline membrane surrounding the developing embryo (LeMosy and Hashimoto, 2000). *nudel* mutant eggs show defects in their structural integrity indicating that Nudel function maybe also be to provide the embryo with a matrix structure necessary for the activity of the proteolytic cascade (LeMosy and Hashimoto, 2000).

EGF receptor activation represses the transcription of *pipe* at the dorsal side (Sen, 1998). In turn, Pipe protein at the ventral side is likely to modify an unknown protein triggering and spatially restricting the proteolytic cascade required to set up the Dorsal nuclear gradient. However, it is unclear if Pipe repression is direct or it involves the activation of another signaling pathway downstream of the EGF receptor activation.

The Dorsal nuclear gradient along the dorsoventral axis subdivides the blastoderm embryo into 4 major regions visualized by specific gene expression domains. At the dorsal midline, the extra-embryonic amnioserosa is defined by *zerknüllt* gene expression. The dorsal ectoderm, at more dorsolateral positions, is defined by *tolloid* and *decapentaplegic* expression. Moreover, the ventrolateral neurogenic ectoderm is delimited by *rhomboid* expression and finally the mesoderm at the ventral most side is outlined by the expression of *twist* and *snail*.

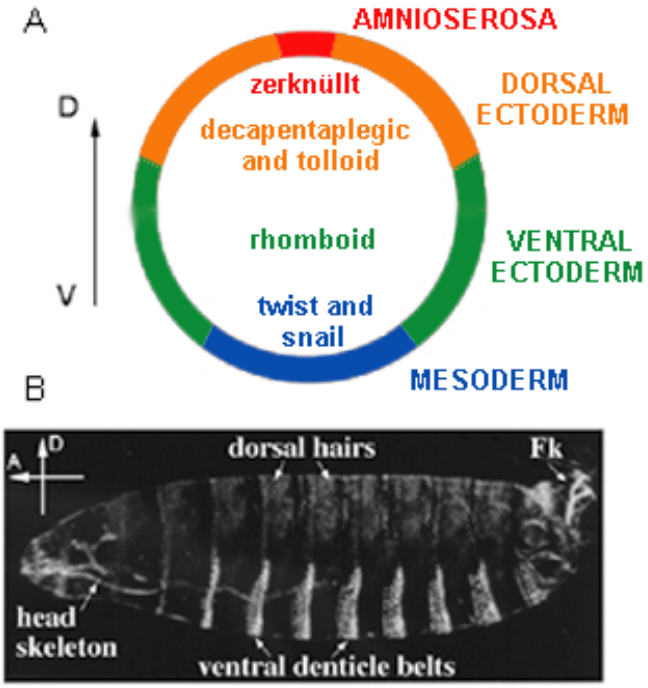

Figure 6.
Fate map of the DV axis.
(A) Schematic representation of the cell fates, defined by specific gene expression, in the cellular blastoderm embryo. The dorsalmost region gives rise to the amnioserosa (red). This is defined by the expression of *zerknüllt*. Dorsolateral cells develop as dorsal ectoderm (orange) forming the dorsal epidermis, the peripheral nervous system, and the tracheal system. The genes *tolloid* and *decapentaplegic* are expressed in this region. Ventrolateral cells give rise to ventral ectoderm (green), which will form the ventral epidermis and the central nervous system. This rigion is defined by the expression of *rhomboid*. The ventralmost region will become the mesoderm (blue). *twist* and *snail* are expressed in this portion of the embryo. (B) Cuticle preparation of a first instar larva shows the ventral denticle belts and the finer hairs of the dorsal epidermis. The head skeleton and the Filzkörper (Fk) derive from a defined dorsolateral position within the dorsal ectoderm.

2. RESULTS

2.1 Modulation of Gurken signaling during oogenesis: a way to understand the patterning of the follicular epithelium.

The AP and DV polarity of the fly egg and embryo are set up during oogenesis by the induction of two spatially distinct cell populations within the follicular epithelium, namely the posterior and dorsal populations. The determination these two populations depends on the activation of the EGF receptor by its ligand Gurken. Therefore, during oogenesis a single signaling molecule is able to induce two different cell fates within the same epithelium. How is this achieved? How is the same extracellular signal able to determine different cell fates?

It has been proposed that the follicular epithelium is divided into two spatially disinct cell groups with different competence for responding to Gurken signaling. These are the terminal group, which upon receiving the signal switches the previous anterior fate to a posterior one, and a lateral group which adopts a dorsal fate in the presence of Gurken activity (Gonzalez-Reyes and St Johnston, 1998). However, it is also possible that the follicular epithelium is a homogeneous cell population with no spatial distinctions and the repsonse upon Gurken signaling is rather a feature of when cells receive this signal. This model suggests that young cells, when induced, assume posterior fate wherease older cells can only give dorsal fate. If this is indeed the case then we would expect a late signaling event to give rise to dorsal follicular cells exclusively.

In order to discriminate between these two scenarios we have investigated follicle cell patterning and its competence in responding to Gurken signaling by supplying Gurken ectopically and at different time points during oogenesis.

2.1.1 Egg phenotypes produced by activating Gurken at different stages of oogenesis

In order to investigate the spatio-temporal competence of the follicular epithelium, the *cornichon* cDNA (0.9 kb) was cloned into the Hsp70-pCaSpeR vector (Pirrotta, 1988) and thus placed under the control of a heat-shock-inducible promoter (see Material and Methods). Four independent lines were obtained from the injection of the P (w; hs cDNA*cni*) vector. All lines where kept at 18C to prevent residual heat-shock promotor activity. Consequently, transgenic lines carrying the construct were crossed into a cornichon minus background (Roth et al., 1995). For brevity we will refer to homozygous *cni* mutant flies carrying one copy of the heat shock cni construct as *hs-cni* flies.

Cornichon protein is required for normal Gurken signaling by controlling the transport of Gurken protein to the plasma membrane of the oocyte (Bökel et al., unpublished data). In the absence of Cornichon a functional Gurken protein is present in the oocyte but it is not correctly exported towards the oocyte plasma membrane. Thus, in the absence of heat shock this construct does not rescue the *cornichon* egg phenotype laid by the *hs-cni* flies, suggesting that under normal conditions the *cni* cDNA is not expressed. As described in the introduction, loss of Cornichon activity results in eggs with anterior structures, such as the operculum and the micropyle, at both ends (Fig. 7). In addition, these eggs do not have any DV polarity and lack structures such as the dorsal appendages (Fig. 7).

Figure. 7
The cornichon egg
The egg has anterior structures, namely the operculum and the micropyle, at both ends. It also lacks DV polarity and as result has no dorsal appendages.

hs-cni flies were heat shocked in order to induce the expression of *cni* and thus promote Gurken signaling. It was found that 10 min. at 39C was the minimum heat shock pulse required. Heat shocked females were then placed in laying blocks at room temperature, and the eggs from several independent heat shock experiments were collected at different

time intervals for several days. *hs-cni* females, which had previously produced apolar eggs, deposited new egg phenotypes that differed in a temporal sequence. Such variation can be explained when one examines the way this heat shock experiment works. An adult *hs-cni* female, such as the one selected for the experiment, has in her ovarioles a sequence of maturating mutant egg chambers. When this female is exposed to the heat shock pulse the Gurken signaling pathway is activated in every egg chamber and thus in egg chambers of different developmental ages. The temporal sequence of egg phenotypes was reproducible and indeed caused by the activation of Gurken signaling at different stages of oogenesis. This idea is confirmed by the observation that often eggs laid at the same time point fall into the same phenotypic class (discussed below). It is possible to have a more precise estimation of the oogenesis stage in which a certain egg phenotype received the heat shock pulse by correlating the time of egg deposition with the oogenesis time scale for females kept at room temperature (Lin and Spradling, 1993).

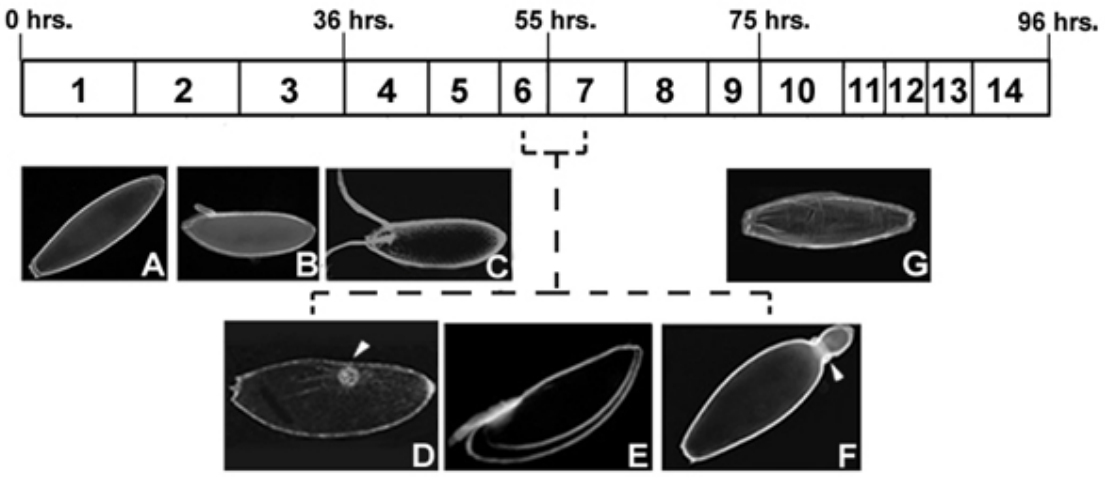

Figure 8.
Activation of Gurken signaling at different stages of oogenesis leads to distinct egg phenotypes.
The upper panel shows a timetable of oogenesis according to Lin and Spradling, 1993. The approximate time is indicated in hours. (A-G) Darkfield micrographs of eggs produced by P[hs-cni, w^{+}], cni^{AR55}/+, cni^{AR55} females at different intervals after heat shock (see Materials and Methods). Anterior is to the left. The micrographs in D- F have slightly higher magnification than those in A-G. (A, B) Heat shock cni during early oogenesis (stage 1 to 3) leads to ventralised eggshells. The egg shown in (A) has AP, but no DV, polarity while that shown in (B) has in addition a weak rescue of the DV axis. (C) Signaling between stage 3 and stage 6 leads to a complete rescue of the cni phenotype (D, E, F) Eggs produced by a partial rescue of the cni phenotype at midoogenesis. (D) Egg with normal AP polarity and one patch of DA material in an intermediate position between the poles of the egg (arrowhead). (E) Egg with a rescued dorsal-ventral polarity exhibiting two DAs, but lacking AP polarity as a micropyle is present at both poles. (F) Egg with a ring of DA material at the posterior pole (arrowhead). (G) Egg lacking both AP and DV polarity. The estimation of the developmental stages given in Fig. 1 is only approximate since abnormal eggs are often retained in the ovaries and laid less efficiently than wildtype eggs.

Thus, the apolar eggs laid on the first day after heat shock show that Cni activity provided at late stages of oogenesis (stage 8-14) is unable to revert the *cni* mutant phenotype (Fig. 8 G). However Cni provided during mid-oogenesis (between stage 4 and 6) rescues the mutant phenotype completly and leads to wild-type eggs that are able to undergo normal embryonic development (Fig. 8C). The presence of wild-type eggs proves that there is a time window during which the Cornichon protein promotes sufficient Gurken activity to completely rescue the mutant phenotype. Activation during earlier stages results in eggs with different degrees of ventralisation defined by the reduction of the dorsal appendage length. This phenotype is likely due to progressive reduction of the dorsalising Gurken activity caused by decreasing amounts of Cornichon protein (Fig 8B). In this case the Cornichon protein activates the Gurken signaling pathway to promote the establishment of the AP polarity during early oogenesis but it is unable to promote the second dorsalising activity of Gurken later in oogenesis. Such ventralised phenotypes are also characteristic of eggs laid by females homozygous for weak *cornichon* alleles. Work with different cni allelic combinations has shown that low levels of Gurken protein are required to induce the posterior fate, since the laid eggs are ventralised but have normal AP polarity (Neuman-Silberberg and Schüpbach, 1993). Females lay severely ventralised eggs with normal anterior-posterior polarity even 10-12 days after heat shock pulse supporting the idea that lower levels of Gurken signal are required for posterior determination than dorsal follicle cell determination (Fig. 8A). These eggs received the heat shock pulse when they where in the germarium, that is, several days before posterior fate induction and yet the Cornichon product present in the oocyte is sufficient to promote the AP polarity. After 12-13 days the heat shock flies lay again eggs with a strong *cornichon* phenotype (Fig. 8G).

We were most interested in the eggs resulting from a partial rescue of the mutant phenotype when *cni* is provided at mid-oogenesis (Fig. 8D-F). Three egg phenotypes can be distinguished. The first shows normal AP polarity associated with a patch of dorsal appendage material located at different positions along the AP axis of the egg (Fig. 8D).

The second shows a complete lack of AP polarity having a micropyle at both ends (Fig. 8E and Fig. 9A-C), but shows a rescue of the dorsoventral chorion pattern as these eggs have dorsal appendage material in the correct anterior dorsal position, a novel phenotype.

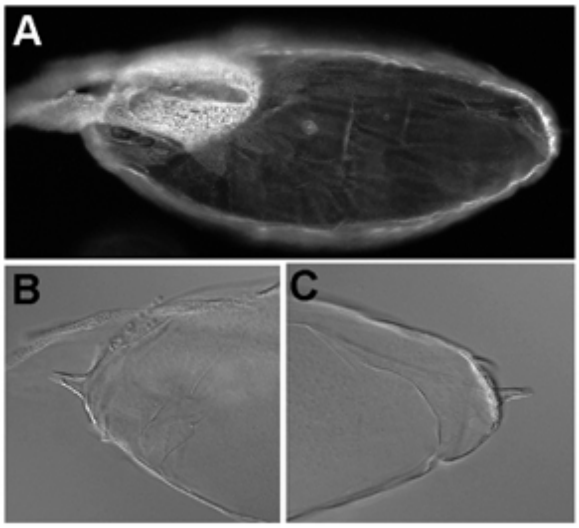

Figure 9
Dorsoventral polarity can be rescue in the absence of an anteroposterior axis.
(A) Darkfield of an egg phenotype resulting from Gurken signaling in a cornichon minus background. This egg has dorsal appendages although it lacks posterior structures. (B and C) Brightfild close up of the anterior end (B) and of the posterior end (C). The anterior micropyle can be seen in both images.

The third phenotype, which is also novel, shows a posterior ring of dorsal appendage material accompanied by a narrowing in the terminal portion of the egg (Fig. 8F). We explain these phenotypes in the following way. The second class is clearly constituted by eggs that have received the heat shock pulse in a *cornichon* mutant background in which the oocyte nucleus has a random anterior cortical localisation. These are the 26% of the all egg chambers present in a *cornichon* mutant female. The first and the third classes are likely to be derived from egg chambers where the oocyte nucleus does not undertake its anterior cortical migration and remains in a posterior position, representing 70% of the all egg chambers present in a *cornichon* mutant female. As Gurken localisation depends on the position of the oocyte nucleus, also the Gurken signal remains confined at the posterior.

We confirmed that the different phenotypes observed in this experiment depend on germline expression of cni as ectopic expression of cni in the follicular epithelium, using a follicle specific GAL4 driver line (see Materials and Methods for details), has no phenotypic consequences.

The *hs-cni* experimental approach was used to further investigate timing and patterning aspects of the oogenesis process.

2.1.2 Gurken signaling promotes nuclear migration as late as stage six.

Although the Gurken protein can be detected in the oocyte when the egg chamber buds off from the germarium (Neuman-Silberberg and Schüpbach, 1993), it is unclear when Gurken first signals to the posterior follicle cells to promote nuclear migration. The nucleus position within the oocyte was monitored in stage nine egg chambers dissected from *hs-cni* flies at different time points after heat shock in order to address the question of how late Gurken can signal to the posterior follicle cells in order to promote nuclear migration. The position of the oocyte nucleus is easily visible in egg chambers at stage nine. These were grouped in three classes depending on whether the nucleus has a wild type anterior localisation, a posterior or an intermediate position (Fig. 10). When the heat shock pulse, and thus the Gurken signal, is provided before or during stage six the oocyte nucleus is able to undergo a wild-type anterior migration and as a consequence all the examined stage nine egg chambers show a normally anteriorly positioned nucleus. Figure 10 shows that for a heat shock pulse occurring at stage six the frequency of stage nine egg chambers with an anteriorly localised oocyte nucleus (green line) is close to 1. Conversely, a heat shock pulse occurring after stage seven can not rescue the posterior *cni* nuclear localisation, in fact 70% of egg chambers show a posteriorly localised nucleus as in the case of null *cornichon* or *gurken* alleles (Roth et al., 1995).

In fact, examining Figure 10 in the interval of time following middle stage seven it is possible to observed that the frequency of stage nine egg chambers showing a posteriorly localised oocyte nucleus (blue line) is around 0,7. A Heat shock pulse between late stage six and middle stage seven leads to a large fraction of egg chambers with nuclei at intermediate positions. Thus, Gurken signaling during this period is ineffective in promoting nuclear migration.

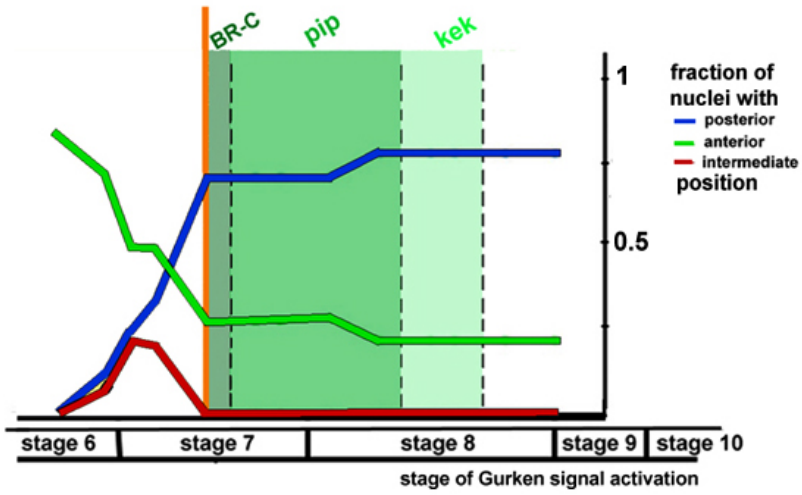

Figure 10.
The effects of delayed Gurken signaling on nuclear migration and follicle cell gene expression.
The position of the oocyte nucleus and the expression of target genes of Grk signaling has been determined in stage 9 (or stage 10) egg chambers at different intervals after heat shock (see Materials and Methods). The frequencies of anterior (green graph), intermediate (red graph) and posterior (blue graph) nuclear positions are shown as a function of developmental time depicted as stages at which the heat shock occurred. Grk signaling at the posterior has to occur before mid-stage 6 in order to efficiently promote the movement of the oocyte nucleus. Activation after mid-stage 7 leads to signaling of the posteriorly localised nucleus to the overlaying follicular epithelium. *BR-C* expression (dark green) can be induced during a small time window at stage 7. *pip* repression (medium green) is achieved by signaling between stages 7 and 8. *kek* expression (light green) is induced by signaling between stages 7 and 9. For each time point an average of 50 egg chambers was analysed.

2.1.3 Gurken signaling promotes egg phenotypes with posterior dorsal appendage material as late as stage seven.

Some eggs laid by hs-cni females show a posterior ring of dorsal appendage material causing a narrowing in this region. This novel phenotype, likely to derive from prolonged Gurken signaling at the posterior of the egg chamber, provides new insights into aspects of follicle cell patterning and in the competence of the epithelium in responding to ectopic and delayed Gurken signaling. Thus, the expression of marker genes involved in follicle cell patterning were monitored in order to define the stage during which this egg phenotype can be produced and the cell fate shifts occurring within the follicular epithelium that lead to this phenotype.

kekkon (*kek*) is a target of Gurken signaling, induced early in posterior and later in dorsal follicle cells, *pipe* (*pip*) is normally repressed by Gurken signaling at the dorsal side and *Broad-Complex* (*BR-C*) is expressed at stage ten of oogenesis in two groups of dorsolaterally located cells that give rise to the dorsal appendages (Fig. 11A,E and I); Ghiglione et al., 1999; Sen et al., 1998; Deng and Bownes, 1997). In the absence of Gurken signaling *kek* is never induced, *pip* is uniformly expressed in all follicle cells and *BR-C* shows a weak expression in anterior terminal follicle cells (Fig. 11B, F and L; Ghiglione et al., 1999; Sen et al., 1998; Deng and Bownes, 1997). The expression of the target genes, as well as the position of the oocyte nucleus has been investigated in stage nine (or stage ten) egg chambers dissected at different time points after heat shock. As previously found Gurken signaling after stage seven is unable to promote nuclear migration. However, Gurken can still signal to the overlying follicle cells. In fact, in stage nine egg chambers, which received the heat shock pulse at stage seven, the posteriorly localised oocyte nucleus is always associated with a small symmetric region of *kek* expression and a broad posterior region of *pip* repression (Fig. 11C and G). As in wild type the relation between activation and repression is maintained and a normal set of DV cell types can be generated along the AP axis (Fig.11 compare A and C with C and G).

When Gurken signals at the posterior of the egg chamber as late as stage seven is unable to promote nuclear migration but can induce *kek* expression and *pip* repression. In addition, in stage ten egg chambers, which have received the pulse at the same time point (stage seven), *BR-C*, marker for the dorsal appendage specification, can be detected in a posterior ring of cells anterior to the nucleus (Fig. 11M). These *BR-C* expressing cells most likely give rise to the ring of dorsal appendage material observed in the deposited eggs (Fig.

8F). However *BR-C* expression is never induced in the posteriormost cells. This observation is in accordance with the phenotype of the deposited eggs, which never showed dorsal appendage material in posteriormost position but always in a ring like manner (Fig. 8F). This is a very important observation since it indicates that the terminal follicle cells can not be induced to adopt dorsal fate even if they receive the Gurken signal late and at a stage when main body follicle cells become dorsalised. When the heat shock pulse occurs towards the end of stage six the oocyte nucleus often undergoes only a partial migration, it does not reach the anterior end of the oocyte and often can be found in the middle of it (Fig. 11D, H and N). However, the nucleus always show a cortical localisation and *kek* expression, *pip* repression and *BR-C* activation are found in intermediate positions depending on the oocyte nucleus localisation (Fig. 11D, H and N). These egg chambers are likely to give rise to the previously described eggs belonging to the first class and thus having a spot of dorsal appendage material at different positions along the AP axis (Fig. 8D).

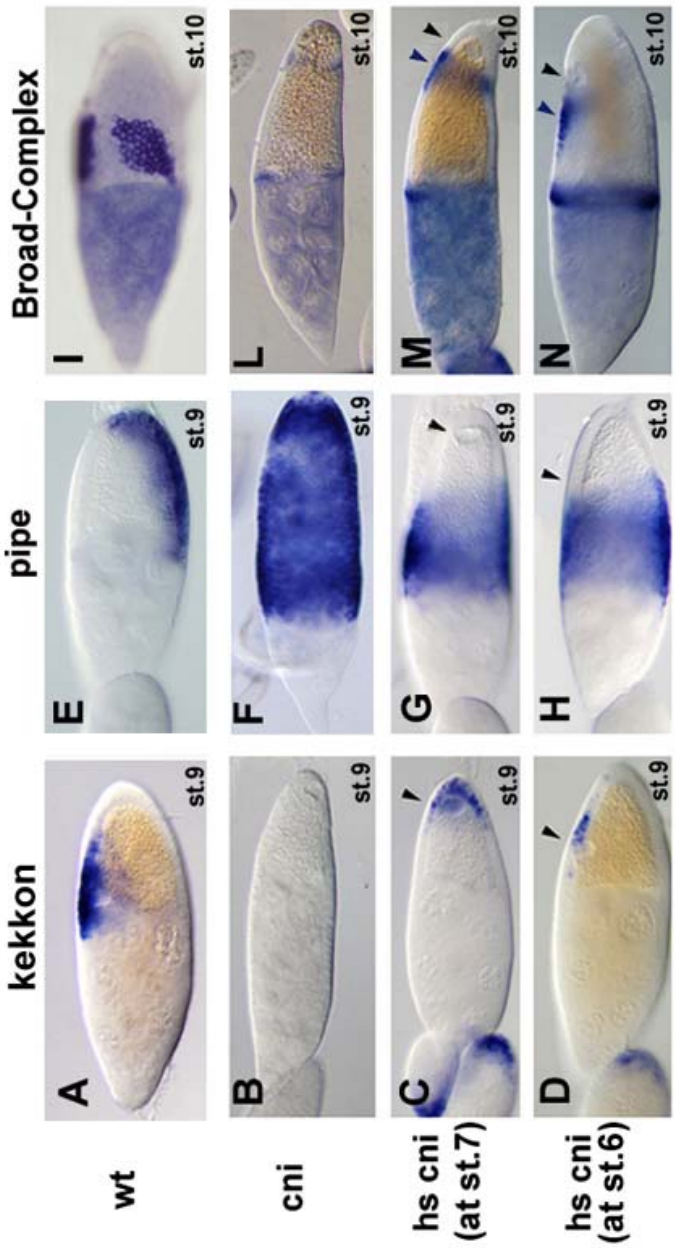

Figure 11.
Follicle cell patterning in egg chambers with mislocalised oocyte nucleus.
(A, E, I) Egg chambers from wildtype females. (B, F, L) Egg chambers from cni^{AR55}/cni^{AR55} mutant females. (C, G, M) Egg chambers from by *P[hs-cni, w^{+}]*, $cni^{AR55}/+$, cni^{AR55} females heat shocked at stage 7. (D, H, N) Egg chambers from by *P[hs-cni, w^{+}]*, $cni^{AR55}/+$, cni^{AR55} females heat shocked at stage 6. (A-D) *kekkon (kek)* mRNA distribution in stage 9 egg chambers. (E-H) *pipe (pip)* mRNA distribution in stage 9 egg chambers. (I-N) *Broad-Complex (BR-C)* mRNA distribution in stage 10 egg chambers. The black arrowheads mark the position of the oocyte nucleus. The blue arrowheads mark the ectopic BR-C expression.

2.1.4 The behavior the terminal follicle cells upon Gurken signaling

Upon closer inspection, the eggs with a posterior ring of dorsal appendage material can in fact be split into two groups based on the type of structures induced at the posterior terminus. Egg of the first group show at the posterior most tip a structures that can be considered genuinely posterior resembling an aeropyle (Fig. 12 A-C). Eggs of the second group have a posterior tip with anterior structures such a micropyle (Fig. 12 D-F). However, not significant difference can be observed in the ring of posteriorly located dorsal appendage material observed in these eggs (Fig. 12 A-F).

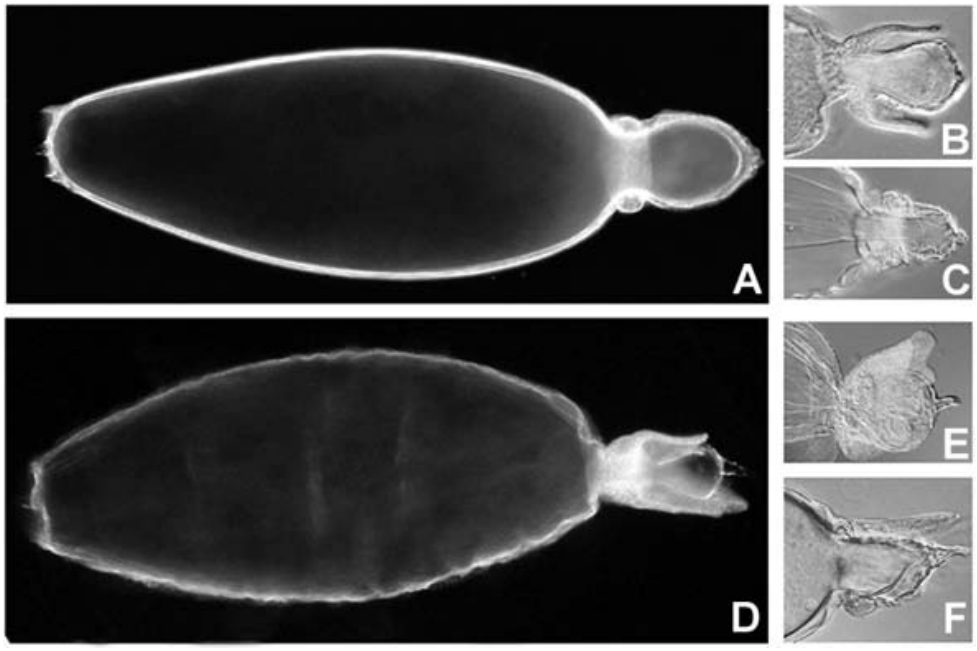

Figure 12.
Gurken signaling from posteriorly localised nuclei leads to eggs with posterior dorsal appendages.
(A, D) Dark field micrographs of eggs with dorsal appendage (DA) material at the posterior. (B, C; E, F) Phase contrast micrographs showing details of the posterior tip of eggs displaying posterior rings of DA material. (A-C) Posterior DA material is associated with an aeropyle. (D-F) Posterior DA material is associated with a micropyle.

This indicated that Gurken siganling at stage seven can induce dorsal fate in main-body follicle cells and simultaneously can promote posterior fates in terminal follicle cells. *dpp* during oogenesis is expressed in anterior follicle and it is known to be involved in the specification of an anterior chorion structure of the mature egg, the operculum (Fig. 13 A

and Twombly et al., 1996) . In egg chambers lacking Gurken siganling *dpp* is expressed at both ends since the terminal follicle cells at the posterior pole acquire anterior fates by default (Fig. 13 B; Twombly et al., 1996). We examined the expression of *dpp* in stage ten egg chambers giving rise to eggs with posteriorly localised dorsal appendages. In all egg chambers, with a posterior ring of *BR-C* expression, *dpp* could be seen weakly expressed in the posterior most follicle cells. (Fig. 13 D). Therefore, we assumed that deposited eggs with a posterior ring of dorsal appendage material combined with an aeropyle are also derived from egg chambers expressing *dpp* at the posterior end. Apparently, the expression of *dpp* is not always linked to the formation of anterior chorion structures, like the micropyle or the operculum. In agreement with this assumption *dpp* expression can be found at the posterior of egg chambers from strong hypomorphic *grk* and *cni* allele combinations even though the eggs display a posterior aeropyle. For example, cniAR55/cniAA12 females lay ventralised eggs with apparently normal AP polarity, However, all egg chambers show posterior dpp expression (Fig. 13 C). Thus, in this case also, weak *dpp* expression at the posterior is not linked to the formation of anterior chorion structures in the mature egg. In variance with *hs-cni* egg chambers, however, the oocyte nucleus, is localised normally in egg chamber from cniAR55/cniAA12 females, indicating that back signaling followed by cytoskeletal repolarisation has occurred. The difference between the two phenotypes might result from the fact that late Gurken activation in the hs-cni experiment does not allow enough time for the back signaling to induce the cytoskeletal rearrangements necessary for the oocyte nucleus movement.

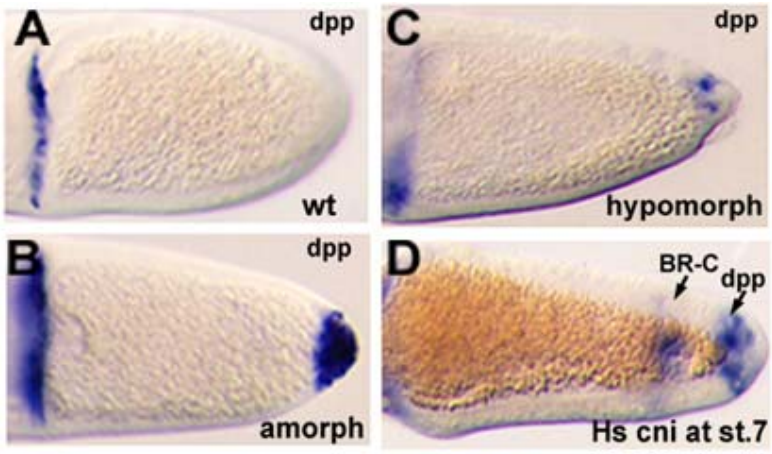

Figure 13
Posterior expression of Dpp in egg chambers with partially posteriorised terminal follicle cells.
(A-C) *dpp* mRNA distribution in stage 10 egg chambers. (A) Wildtype. (B) Complete loss-of-function: $cni^{AR55}/Df(2L)H60$. (C) Partial loss-of-function: cni^{AR55}/cni^{AA12}. (D) *dpp* mRNA and *BR-C* mRNA distribution in stage 10 egg chambers from by *P[hs-cni, w*$^{+}$*]*, $cni^{AR55}/+, cni^{AR55}$ females heat shocked at stage 7. Arrows indicate the *BR-C* and *dpp* domains. The level of *dpp* expression in (D) is lower than that in (B) and similar to that seen in the *cni* hypomorph (C).

2.2 Both Gurken and Decapentaplegic signaling are required for dorsal appendage formation.

Gurken signaling at stage seven directed towards the terminal portion of the cni mutant egg chamber induces dorsal fate in a ring of main-body follicle cells resulting in the production of dorsal appendage material at the posterior of the mature egg (Fig. 8 F and 12 A and D). We have shown that during stage-7 Grk signaling towards the terminal portion of cni mutant egg chambers induces dorsal fates in a ring of main-body follicle cells resulting in the production of dorsal appendage material at the posterior of the mature egg . However, mutants have been described in which late Grk signaling at the posterior does not lead to dorsal appendage formation. In *mago nashi* (*mago*) mutant egg chambers a delay in the repolarisation of the microtubule network prevents oocyte nucleus movement and leads to Grk signaling to posterior cells until stage 9 (Micklem et al., 1997; Newmark et al., 1997). Nevertheless, *mago* mutant eggs do not form dorsal appendages at the posterior.

Posterior localisation of the oocyte nucleus during late stages of oogenesis can also be obtained by feeding flies with colchicine, a drug that disrupts the microtubule network of the oocyte (Koch and Spitzer, 1983; Saunders and Cohen, 1999). Colchicine-treated females lay eggs with a mago-like phenotype (Fig. 15 A). Both in *mago* mutants and after colchicine treatment, signaling to posterior follicle cells starts at early stages and therefore most likely

leads to a complete suppression of anterior fates. Thus, the anterior characteristics of the terminal follicle cells observed in *hs cni* egg chambers with a posterior nucleus, seem to distinguish the heat-shock experiment from the loss of *mago* or from the colchicine treatment.

We tried to identify the anterior molecular components that, together with Grk signaling, promote the formation of dorsal appendages. In addition to *dpp*, *rho* is also expressed in anterior follicle cells independently from Grk signaling (Ruohola-Baker et al., 1993; Sapir et al., 1998; Wasserman and Freeman, 1998). In order to investigate a possible function of these two genes in specifying the DA anlagen, UAS-rho and UAS-dpp flies were independently crossed with the E4-Gal4 line that drives expression at the posterior of the follicular epithelium (Brand and Perrimon, 1993; Queenan et al., 1997). In a wild-type background the ectopic posterior expression of these genes has no visible effect on the chorion structure (data not shown). Rho dependent EGF receptor activation leads, however, to a posterior repression of *pip* (Fig. 14C) and consequently dorsalises the embryo at the posterior end (Fig. 14 D).

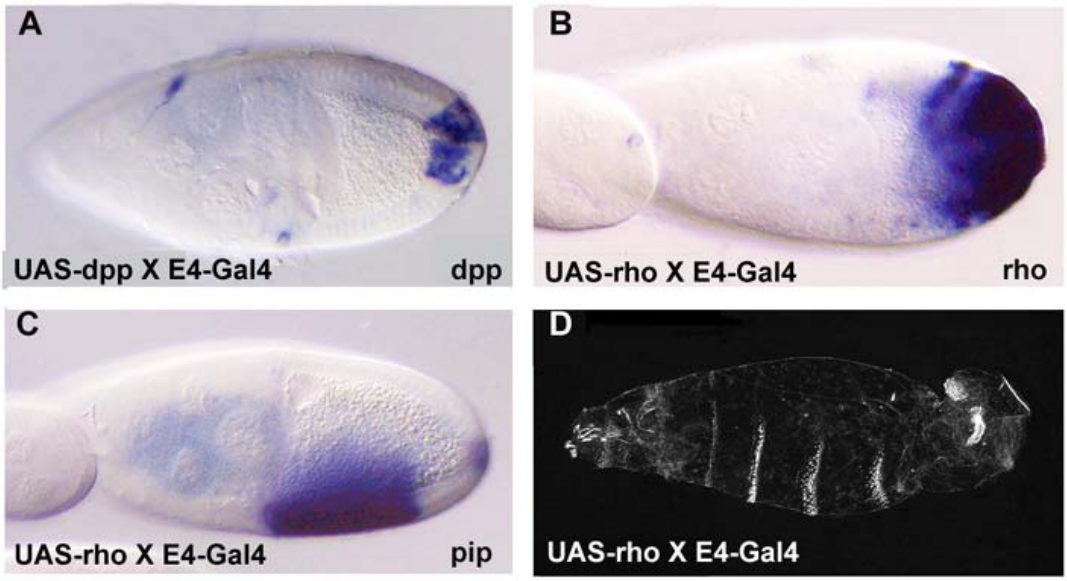

Figure 14.
Ectopic expression of dpp and rhomboid at the posterior of wildtype egg chambers.
(A) *dpp* mRNA distribution in egg chambers from *UAS-dpp*; *E4-Gal4* females. *dpp* is expressed ectopically at the posterior of the egg chamber. The staining procedure had to be stopped before endogenous *dpp* transcripts in centripetal follicle cells (see Fig. 6A) were fully visible. (B-D) Egg chambers or embryo from *UAS-rho*/*E4-Gal4* females. (B) Ectopic *rho* mRNA at the posterior of a stage 9 egg chamber. The staining procedure had to be stopped before endogenous *rho* transcripts in centripetal follicle cells could be detected. Despite using the same Gal4 driver *dpp* was always expressed in a narrower domain than *rho*. Probably, *rho* induces its own expression as a result of Spi mediated DER activation. (C) *pip* expression in stage 10 egg chambers. *pip* is repressed at a narrow posterior domain corresponding to the region of ectopic *rho* expression. (D) Dark field micrograph of a posteriorly dorsalised embryo derived from an egg chamber expressing high levels of *rho* at the posterior.

In order to combine the expression of these two genes at the posterior with Grk signaling we fed colchicine to UAS-rho/E4-Gal4 and UAS-dpp; E4-Gal4 flies. Under these circumstances UAS-rho/E4-Gal4 females produced eggs with the above-described mago-like phenotype, indicating that even in combination with Grk signaling rho cannot induce dorsal anterior fates (data not shown). Interestingly, colchicine-treated UAS-dpp; E4-Gal4 females produce eggs with dorsal appendage material posteriorly (Fig. 15 D). Egg chambers from such females express BR-C in a posterior ring (Fig. 15 F).

Thus, ectopic posterior expression of *dpp* in combination with DER activation by Grk induces dorsal anterior fates in main-body follicle cells. This indicates that a positive interaction between the EGF and the TGF-β pathways is required to specify these cell fates. To confirm the requirement of Dpp signaling for BR-C expression we induced follicle cell

clones mutant for Mad, an essential cytoplasmic signal transducer of the Dpp pathway (Sekelsky et al., 1995).

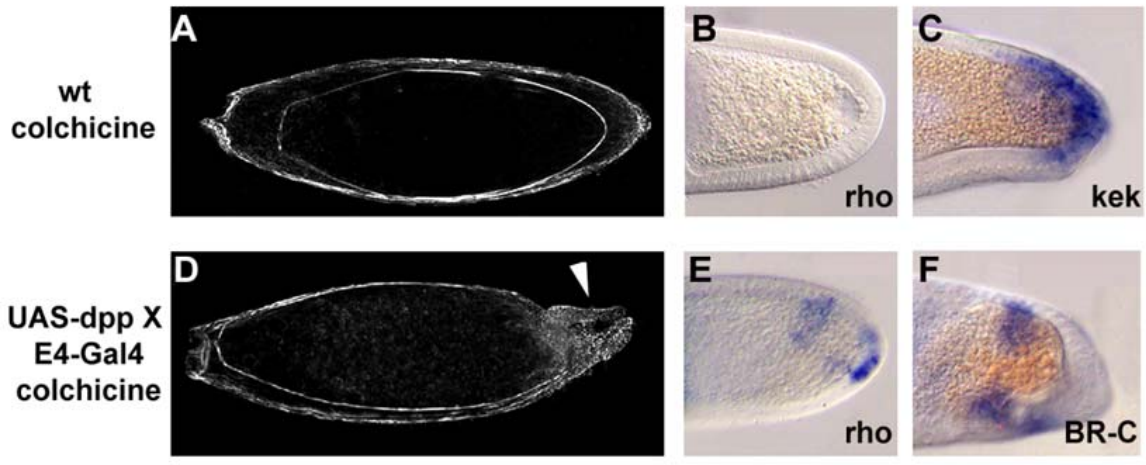

Figure 15.
TGFβ and EGF pathways collaborate to specify and pattern the dorsal appendages of the egg.
(A-C) Egg and egg chambers derived from wildtype females fed with colchicine. (D-F) Egg and egg chambers derived from *UAS-dpp*; *E4-Gal4* females fed with colchicine. (A, D) Dark field micrographs of eggs. The eggs lack normal dorsal-ventral polarity due to a failure of oocyte nucleus movement. Expression of *dpp* at the posterior (D) leads to the formation of a ring of dorsal appendages (arrowhead). (B, C, E, F) Micrograph of posterior region of stage 10 egg chambers.
(B, E) *rho* mRNA distribution. (C) *kek* mRNA distribution. (F) *BR-C* mRNA distribution.
In wild type egg chambers treated with colchicine the presence of the oocyte nucleus at the posterior leads to *kek* (C) but not to *rho* expression (B). However, in egg chambers from *UAS-dpp*; *E4-Gal4* females treated with colchicine *rho* (E) and *BR-C* (F) expressions can be detected at the posterior.

In Mad mutant clones located in anterior-dorsal regions of wild-type egg chambers BR-C expression is abolished (Fig. 16, compare the BR-C wild-type expression patterns in G and I with the expression patterns in H and L).

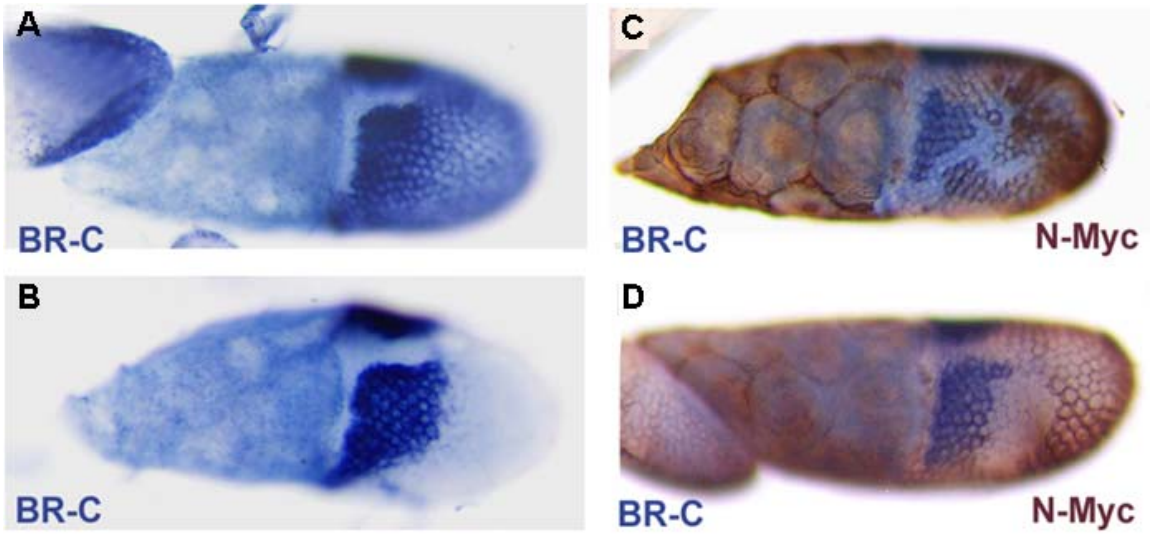

Figure 16
Mad mutant clones in the follicular epithelium.
(A-D) *BR-C* mRNA distribution in stage 10A (A, C) and 10B (B, D) egg chambers. *BR-C* expression gradually becomes confined to two lateral patches. (A, B) wild-type. (C, D) Egg chambers carrying *Mad* mutant clones which are marked by the absence of N-Myc (brown). *BR-C* expression is abolished within the clones of both stage 10A and stage 10B egg chambers.

We conclude that dpp not only is essential for the formation of the operculum (Twombly et al., 1996), but also for that of the dorsal appendages.

2.2.1 Dpp signaling controls the positive feed back loop of EGF receptor activation

One of the distinctive features of Grk signaling to terminal follicle cells as compared to main-body follicle cells is that Grk induces rho expression only in the latter case and thereby initiates a positive feed-back loop of DER activation (Wasserman and Freeman, 1998). Since this mechanism is required to pattern the dorsal appendage anlagen, which we have shown to be specified by an interaction of Dpp and Grk signaling, we wondered whether it itself depends on *dpp*. In wild-type egg chambers, treated with colchicine, the posteriorly localized oocyte nucleus leads to DER activation in the overlying follicle cells, as can be seen from the posterior expression of kek. However, the transcription of *rho* is not induced, even though rho has been described previously as a target gene in the follicular epithelium (Ruohola-Baker et al., 1993; Sapir et al., 1998; Wasserman and Freeman, 1998).

Surprisingly, *rho* expression is observed at the posterior of UAS-dpp; E4-Gal4 flies treated with colchicine (Fig. 15 E). We conclude that rho expression in main-body follicle cells occurs only where Grk and Dpp signaling coincide, i.e. at the dorsal anterior corner of wild-type egg chambers. Thus, the interaction between the EGF and TGF-β pathways controls the specification of the dorsal cell fate leading to dorsal appendage production, their positioning along the AP axis and the mechanism that patterns them along the DV axis. Since Spitz, the DER ligand activated by rho is ubiquitously expressed in all follicle cells (Wasserman and Freeman; 1998), anterior restriction of *rho* expression in wild type might be essential to prevent the spreading of EGF receptor activation throughout the main-body follicle cells.

2.3 Modulation of Dpp signaling: is Dpp providing a graded information during oogenesis?

During oogenesis *dpp* expression in the centripetal follicle cells specifies the operculum fate, the anteriormost structure of the egg (Twombly et al., 1996). In addition, Dpp signaling, emanating from the centripetal follicle cells, positions the dorsal appendages along the AP axis of the egg.

It has been shown that strong *dpp* overepression in all follicle cells, using a potent Gal4 driver line, results in eggs with an enlarged operculum (Fig 17; Twombly et al., 1996). Futhermore, these eggs do not have dorsal appendages. However, when *dpp* is overexpressed in all follicle cells using a much weaker Gal4 driver line, as the one described in Queenan et al. 1997, the deposited eggs have enlarged dorsal appendages (Fig. 17).

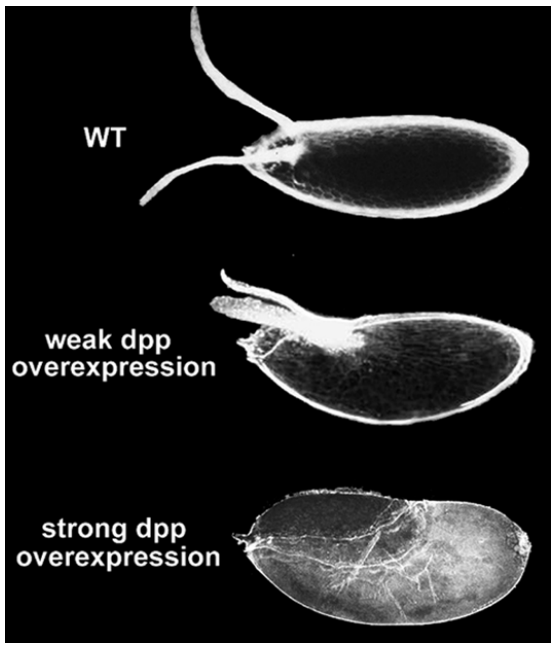

Figure 17
dpp over-expression in the follicular epithelium.
Darkfield micrographs of eggs.
The egg in the upper panel is wildtype and it has a pair of dorsally located respiratory appendages.
The egg phenotype in the midle pannel is produced by weak *dpp* over-expression in the whole follicular epithelium. The dorsal appendages are thicker than in wild-type.
The egg phenotype in the lower panel is obtained by strong *dpp* over-expression in the whole follicular epithelium. The operculum is enlarged.

In these egg chambers where *dpp* is overexpressed using the weak Gal4 driver, *BR-C* expression expands (Fig. 18).

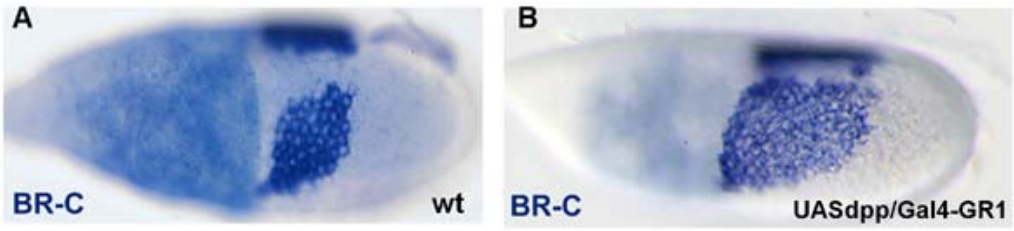

Figure 18.
Weak dpp over-expression in the whole follicular epithelium leads to the expansion of the BR-C expression domain.
(A and B) *BR-C* mRNA distribution in stage 10 B egg chambers. (A) wildtype egg chamber. (B) egg chamber from a *UAS-dpp/GR1-Gal4* female. BR-C expression domain expands posteriorly when dpp is weakly over-expressed in the whole follicular epithelium.

Thus, altering Dpp signaling strength indicates that high levels of signaling, in the centripetal follicle cells, specify the anterior most fate, namely the operculum, while, lower signaling levels, emanating from the centripetal follicle cells, induce a more posterior fate which leads to dorsal appendages formation. This suggestes that during oogenesis Dpp is indeed behaving as a morphogen able to specify different cell fates in a concentration depending maner

However, during oogenesis EGF and TGF-β signaling collaborate to specify the operculum and the dorsal appendages. This collaboration between the two pathways makes it difficult to investigate the precise nature of the Dpp gradient during oogenesis.

2.4 EGF and TGF-β signaling specify the polarity of the *Drosophila* embryo.

The DV polarity of the embryo is defined by a signal encoded by the *spätzle* locus. Spätzle activates the Toll receptor on the ventral half of the embryo at the blastoderm stage. Three genes, *nudel*, *windbeutel* and *pipe,* are expressed in the follicular epithelium and act

upstream of Späzle activation. *windbeutel* and *pipe* functions are required in ventral follicle cells but only *pipe* expression is ventrally restricted. It has been shown that the EGF receptor, activated by its ligand Gurken, restricts the pipe expression domain at the ventral side of the egg chamber. Gurken has an anterior cortical localisation and, as a consequence, its target genes, *kekkon* and *mirror*, are activated only in anterodorsal follicle cells. However, *pipe* is repressed in all dorsal follicle cells along the AP axis. Thus, Gurken seems to act as a short range signaling molecule in activating target genes but as a long range signaling molecule in repressing *pipe* (Fig. 11 compare A and E).

This rises the question whether all cell fates, along the DV axis, are indeed specified by Gurken signaling. Is Gurken a long range morphogen or is it only activating secondary signals? Futhermore, pre-patterning of the follicular epithelium by TGF-β signaling may confer the somatic cells with different competences and thus the ability to differentialy read the EGF signaling.

2.4.1 pipe shows a dynamic expression pattern

We have re-examined pipe mRNA expression during oogenesis and found that in wild type stage nine egg chambers, when Gurken signals to the dorsal follicle cells, *pipe* is expressed in two distinct domains, namely a strong posterior-ventral domain and a broad weak anterior domain (Fig. 19 A). As the anterior to posterior migration of the follicular epithelium takes place, the two domains approach each other (Fig. 19 B and C; Sen et al.; 2000) and finally they fuse forming a stripe at stage 10 of oogenesis (Fig. 19 D). Ultimately, the *pipe* transcript disappears from the posterior follicular cells and by the end of stage 10 *pipe* mRNA is no longer detected in the follicular epithelium (data not shown).

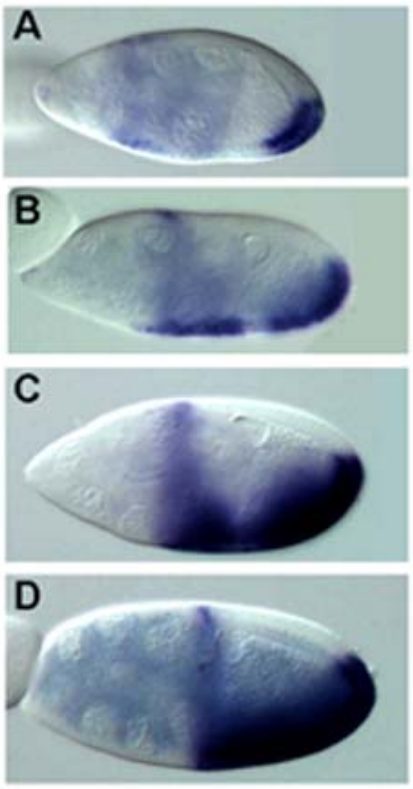

Figure. 19
pipe mRNA expression during Drosophila oogenesis
(A-D) pipe mRNA distribution in wildtype egg chambers. (A) During early stage 9 pipe is expressed in two domanis, a broad and weak anterior domain and a strong posterior-ventral domain. (B and C) The two domains of expression approach each other and (D) they finaly fuse forming a stripe at stage 10 of oogenesis.

These dynamic and temporal changes in *pipe* expression should be considered in attempting to understand how the *pipe* gene is regulated.

2.4.2 Raf is required cell autonomously in the follicular epithelium for pipe repression

EGF signaling in the somatic follicle cells is transduced through the canonical Ras pathway in which the GTP-activated Ras interacts with the effector protein Raf able in turn to phosphorylate the downstream component MAPKK-D-sor (for review Rubin, 1997).

To investigate the role of EGF signaling on *pipe* expression we have analyzed the effect of Raf mutant clones. For simplicity, we have looked at *pipe* expression by using a construct in which a lac-Z reporter gene was cloned under the control of an 8 kb fragment known to contain the promoter and the small first exon of *pipe*. For brevity we will refer to this construct as pipe-LacZ. The line was generated in the laboratory of Dave Stein who showed that beta-Gal expression during stage 10 of oogenesis mimics *pipe* expression (Fig.

20B and C). In addition, the construct reproduces early features of *pipe* expression (Fig. 20A).

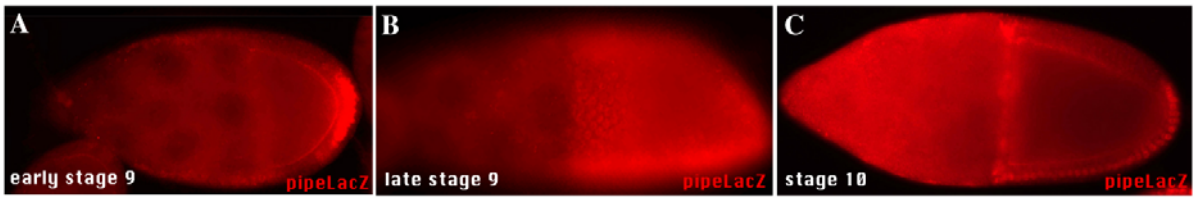

Figure. 20
Pipe-lacZ expression
(A-C) pipe-LacZ distribution in wildtype egg chambers. (A) During early stage 9 pipe is expressed in two domanis, a broad and weak anterior domain and a strong posterior-ventral domain. (B) The two domains of expression approach each other and (C) they finaly fuse forming a stripe at stage 10 of oogenesis.

To visualize the Raf mutant clones, we placed the Raf FRT101 chromosome in trans to a GFP FRT101 chromosome. Homozygous mutant clones were detected by the absence of GFP expression. Clones induced at the dorsal side show a strong cell-autonomous de-repression of *pipe* (Fig. 21 A-F). This result shows that Raf mutant cells behave as if they did not receive EGF and this outcome is consistent with its molecular nature of Raf, an intracellular component of the EGF pathway. In addition, no difference could be observed between clones differentially positioned along the AP axis of the egg chamber (Fig. 21 compareA-C with D-F).

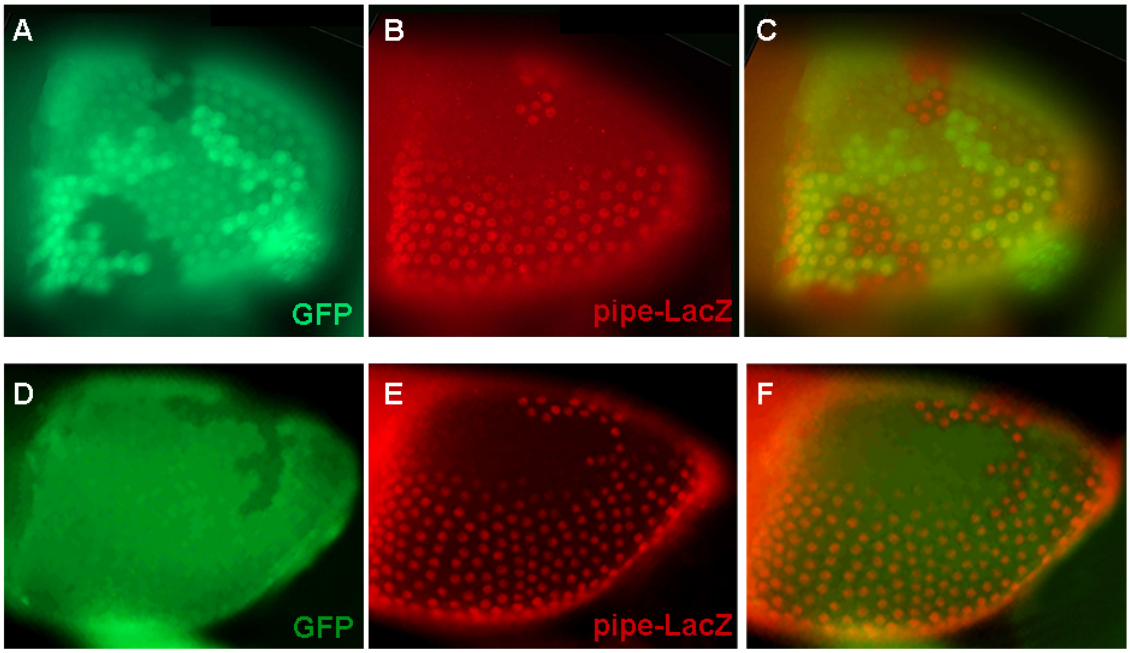

Figure. 21
Raf mutant clones show cell-autonomous de-repression of pipe
(A-F) Stage 10 egg chambers (anterior to the left and dorsal to the top). (A and D) Clones of mutant cells missing Raf are marked by the absence of GFP expression. (B and E) pipe expression is visualized using the pipe-LacZ construct. (C and F) merge.
pipe expression (red) is ectopically activated in Raf mutant clones (lack of green) located at the dorsal side. This induction is cell autonomous and it is not influenced by the position of the clone along the AP axis.

These observations suggest that EGF signaling pathway must be actived along the whole AP axis to limit *pipe* expression to the ventral side of the egg chamber. Moreover, the observed cell-autonomy suggests that EGF signaling acts directly on *pipe* and does not just initiate a second signaling pathway.

2.4.3 Dof activity is not required in the follicular epithelium for pipe repression

The Ras signaling cassette acts downstream of many Receptor Tyrosine Kinases (RTKs). Thus, homozygous mutant Raf clones could impair signaling initiated by other RTKs, such as the Fibroblast Growth Factor receptor (FGF), as well as EGF signaling. In Drosophila two FGF receptors have been identified so far, namely Heartless, which controls the spreading of the mesodermal cells, and Breathless, which organizes the migration of the tracheal cells (Sutherland, 1996 and Shishido, 1997). Dof was identified as a specific component of the FGF pathway and it has been shown to be essential in the signal transduction cascade downstream of both receptors (Vincent, 1998 and Michelson, 1998). We have used Dof in order to investigate a possible role for FGF signaling in shaping the *pipe* expression domain. To visualize Dof mutant clones, the Dof FRT82 chromosome was placed in trans to a GFP FRT82 chromosome. Homozygous Dof mutant clones, marked by the absence of GFP expression, do not show any effect on *pipe* expression suggesting that signaling initiated by FGF receptor activation does not play any role in *pipe* expression regulation (Fig 22).

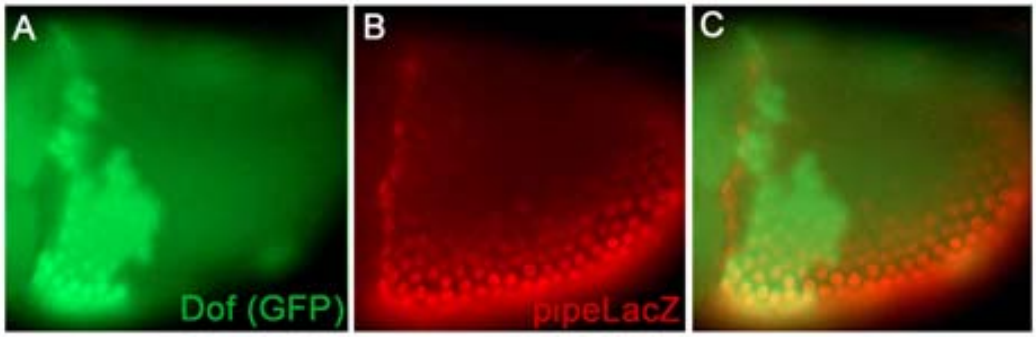

Figure. 22
pipe expression is not effected in Dof mutant clones
(A-C) Stage 10 egg chamber (anterior to the left and dorsal to the top). (A) A big clone of mutant cells missing Dof is marked by the absence of GFP expression. (B) *pipe* expression is visualized using the pipe-LacZ construct. (C) merge. *pipe* expression (red) is not effected by loss of Dof activity (lack of green).

Thus, the effect of Raf clones on *pipe* expression it is likely to reflect lack of EGF signaling.

2.4.4 Mirror is required cell autonomously in the follicular epithelium for pipe repression

The *mirro*r (*mirr*) locus encodes an homeodomain-containing protein most closely related to two other homeodomain proteins of the Iroquois complex, Araucan (Ara) and Caupolican (Caup; Cavodessi, 1999). *mirror* has been cloned and described by McNeill and colleagues (1997) as involved in setting up the equator, a boundary where the dorsal and ventral cells meet, in the eye immaginal disc (Yan, 1999 and McNeill, 1997). Jordan and colleagues (2000) have shown that in Drosophila oogenesis *mirror* is a target of EGF activation and it is expressed in dorsal-anterior follicle cells. Consequently, they have proposed that Mirror initiates an unknown long-range signal able to pattern the follicular epithelium and thus to shape pipe expression. In their model Mirrow limits the expression of *fringe*, which in turn restricts Notch pathway activation. They have suggested that the dorsal activation of Notch then induces the expression of an unidentified molecule that is able to diffuse and to repress *pipe*.

In order to test their model we have place the *mirr* FRT80 chromosome in trans to a GFP FRT80 chromosome. Homozygous *mirr* mutant clones, detected by the absence of GFP

expression and located at the dorsal side show a strong cell-autonomous de-repression of *pipe* (Fig. 23).

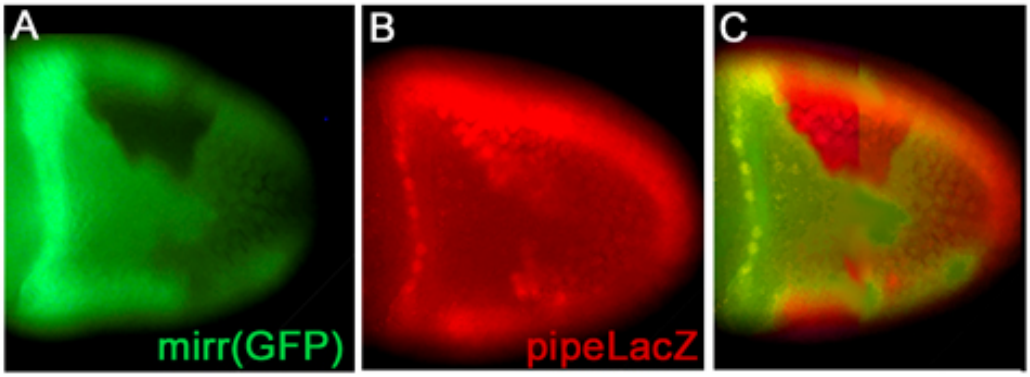

Figure. 23
Mirror mutant clones show cell-autonomous de-repression of pipe
(A-C) Stage 10 egg chambers (dorsal view and anterior is to the left). (A) Clones of mutant cells missing *mirr* are marked by the absence of GFP expression. (B) pipe expression is visualized using the pipe-LacZ construct. (C) merge.
pipe expression (red) is ectopicaly activated in Mirr mutant clones (lack of green) located at the dorsal side. This induction is cell autonomous.

The observed cell autonomy is consistent with the molecular nature of Mirr which is a trascription factor and thus an intracellular component of the pathway. Moreover, the observed cell-autonomy contrasts with the proposed model and it suggests that Mirr acts directly on *pipe* regulation without initiating a downstream signaling pathway or the production of a diffusible molecule.

2.4.5 Rhomboid function is not required for pipe repression

Freeman and colleagues (Wasserman and Freeman, 1999) have proposed that the function of Rhomboid during oogenesis is to trigger the autocrine Spitz activation of the EGF receptor, which amplifies pathway activation. This allows the overal signal to increase in width and amplitude. The generated positive feed back loop on receptor activation positions the dorsal appendages lateral to the midline. Nevertheless, as shown by the authors, the establishment of the embryonic DV axis seems not to require Spitz function, as no dorsoventral defects were observed in cuticles prepared from embryos laid by mothers with rhomboid or spitz clonal egg chambers.

We have tested this observation by looking directly at the pipe expression pattern in *rhomboid* clonal egg chambers. Large homozygous rho mutant clones detected by the absence of GFP expression and covering the dorsal anterior half of the egg chamber do not effect pipe expression (Fig. 24 A-C).

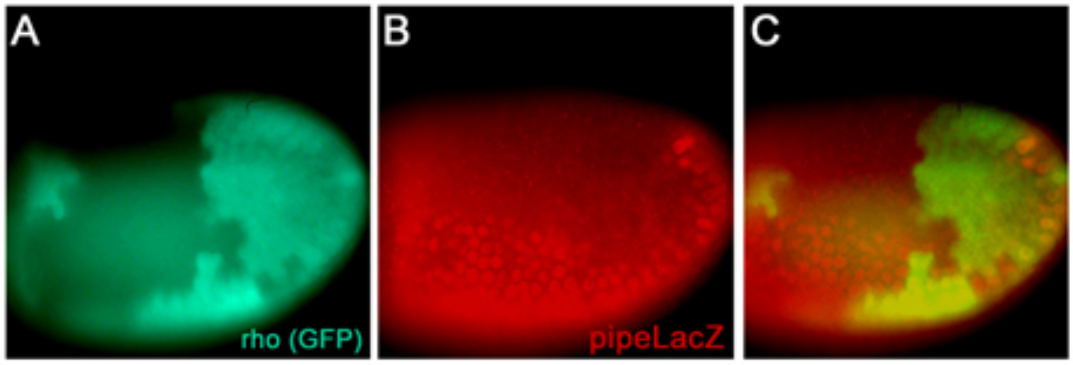

Figure.24
pipe expression is not effected in rhomboid mutant clones
(A-C) Stage 10 egg chamber (anterior to the left and dorsal to the top). (A) A big Clone of mutant cells missing Rho is marked by the absence of GFP expression. (B) pipe expression is visualized using the pipe-LacZ construct. (C) merge.
pipe expression (red) is not effected by loss of Rho activity (lack of green).

Embryos develop form these clonal egg chambers are likely to have a normal DV polarity (Wasserman and Freeman, 1998). Moreover, this observation supports the previous findings indicating that Gurken signaling is sufficient to directly repress *pipe* expression at the dorsal side of the egg chamber.

2.4.6 Ectopic EGF signaling can repress pipe expression

Clonal analysis with members of the EGF signalling pathway has suggested that activation of the receptor by Gurken directly represses *pipe* expression.

We have further tested this hypothesis by looking at the effect of clones in which the EGF signalling transduction pathway is constitutively activated by expressing a ligand-independent form of the EGF receptor (Queenan et al., 1997). Queenan and colleagues generated this construct by fusion of the receptor with the lambda repressor dimerizaton domain, the construct was named λtop and cloned under the control of a UAS sequence. Clones of genetically marked cells expressing λtop were generated using the combined GAL4, flip-out system.

Ventrally located λtop expressing cells, marked by GFP expression, act autonomously to repress pipe (Fig. 25 A-D).

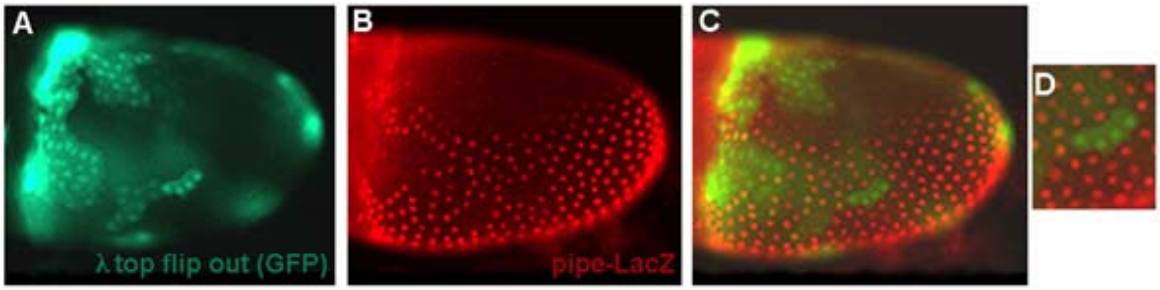

Figure. 25.
λtop flip out clones promote cell-autonomous pipe repression
(A-C) Egg chamber at stage 10 of oogenesis (anterior to the left and dorsal to the top). (A) ectopic EGF signaling is marked by GFP expression. (B) *pipe* expression is visualized using the pipe-LacZ construct. (C) merge. (D) close up of the clone
Ectopic EGF activation (green) leads to cell-autonomous repression of pipe (red).

This result is consistent with the observation of Queenan and colleagues that, expression of **λtop** in the follicle cells of the ovary is effective and can dorsalise the embryo (Queenan et al., 1997). By looking directly at *pipe* we observed that this is achieved in a cell autonomous manner.

However, clones expressing *rhomboid* and marked by GFP expression act non-autonomously and *pipe* is repressed in the cells surrounding the clone (Fig. 26 A-D).

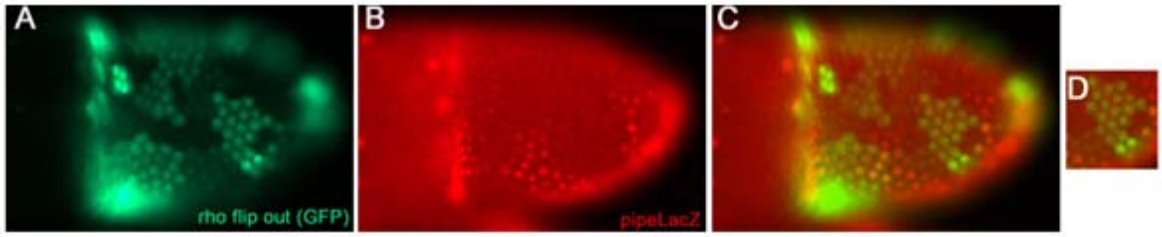

Figure. 26
Rho flip out clones promote a cell-non-autonomous repression of pipe
(A-C) Egg chamber at stage 10 of oogenesis (anterior to the left and dorsal to the top). (A) ectopic Rho activity is marked by GFP expression. (B) *pipe* expression is visualized using the pipe-LacZ construct. (C) merge. (D) close up of the clone Ectopic Rho activity (green) leads to cell-non-autonomous repression of pipe (red).

The molecular nature of *rhomboid* explains this observation. In fact, Rhomboid activates Spitz, the diffusible ligand of the EGF receptor, leading to the expansion in with of the domain of the pathway activation. Nevertheless, mitotic *rhomboid* clones do not effect *pipe* expression. Thus, Rhomboid and Spitz do not take part in *pipe* repression.

2.4.7 EGF and TGF-β *signaling differentially influences pipe expression*

We have previously shown that EGF and TGF-β signaling contribute in the patterning of the dorsal half of the egg chamber. We have investigated whether Dpp signaling is also required together with the EGF pathway to shape the *pipe* expression domain. The repressing capacity of the EGF pathway on *pipe* expression decreases and it is often abolished by dpp, something we observed in clones that co-express λtop and *dpp*. In these clones, marked by GFP, *pipe* is still expressed (Fig. 27 A-D and E-H).

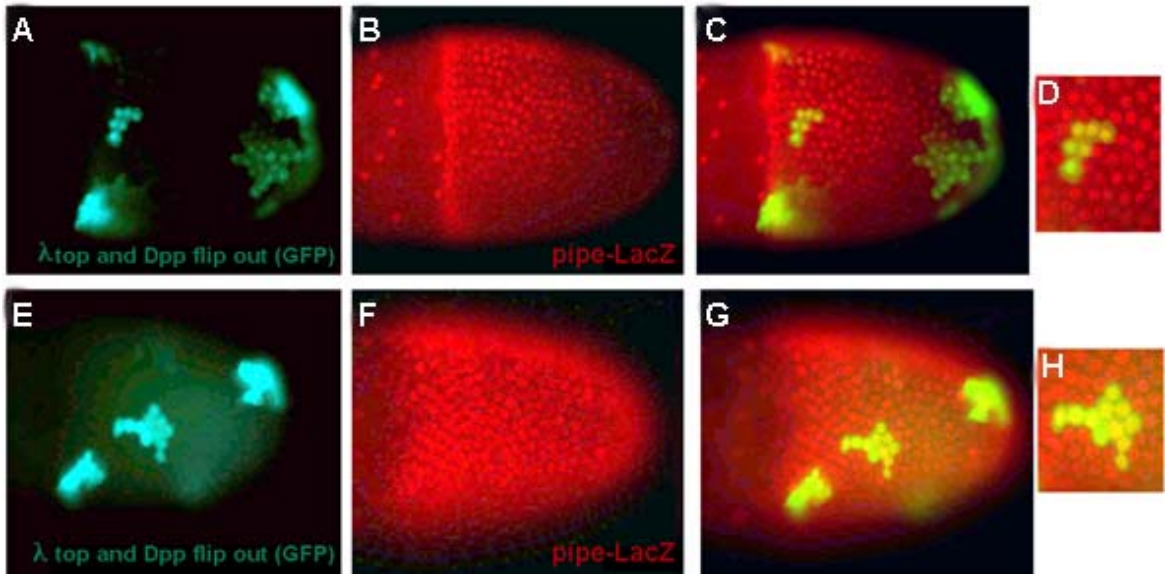

Figure. 27
Simultaneous activation of the TGFβ ***and EGF pathway***
(A-C and E-G) Egg chambers at stage 10 of oogenesis (ventral view and anterior is to the left). (A-E) ectopic TGFβ and EGF signaling is marked by GFP expression. (B and F) *pipe* expression is visualized using the pipe-LacZ construct. (C and G) merge. (D and H) close up of the clones.
Not all the cells within the double flip out clones are able to repress pipe expression (yellow cells).

This result seems to indicate that TGF-β signalling counteracts the repressing nature of EGF signaling on *pipe*. Thus, TGF-β may be able to turn on *pipe* expression or to prevent cells from responding to EGF signaling.

We have also looked at *pipe* mRNA expression in *UAS-dpp; E4-Gal4* egg chambers where *dpp* is mis-expressed at the posterior. Interestingly, *pipe* expression pattern changes and the gene is strongly expressed in posterior follicle cells where TGF-β signaling is ectopicaly activated (Fig. 28 A). Consequently, posteriorly ventralised embryos are produced

(Fig. 28 B). This result seems to support the hypothesis that TGF-β signaling turns on pipe transcription.

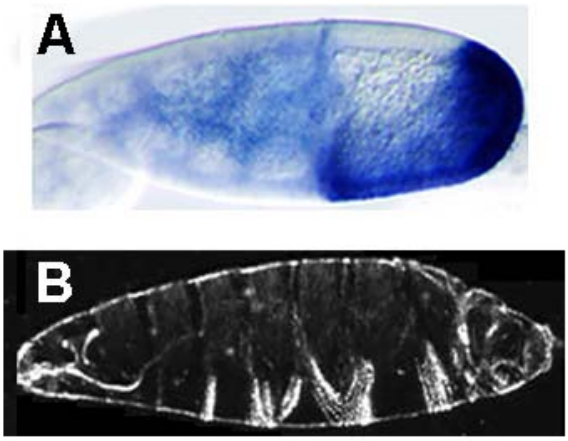

Figure. 28
Ectopic dpp expression at the posterior leads to ventralisation
(A) *pipe* mRNA expression in an stage 10 egg chamber derived from a UAS-dpp/E4-Gal4 mother. *pipe* expression is strong at the posterior. (B) Dark field micrograph of a posteriorly ventralised embryo derived form an egg chamber expressing dpp at the posterior.

We confirmed this finding by generating random clones of cells co-expressing dpp and GFP and by looking at the *pipe* transcript. Clones marked by GFP (brown) and expressing *dpp* show a strong *pipe* (blue) expression, not detected in neighbouring cells, where the TGF-β pathway has not been activated (Fig. 29 A). Control clones that express only GFP (brown) do not show higher levels of *pipe* expression when compared with the neighbouring cells (Fig. 29C).

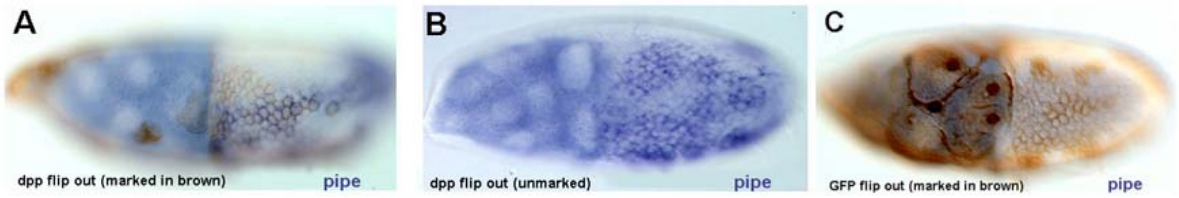

Figure. 29
dpp flip-out clones in the follicular epithelium lead to ectopic pipe expression
(A-C) Egg chambers at late stage 10 of oogenesis. (A) *pipe* mRNA expression (blue) is induced by ectopic Dpp signaling marked by GFP expression (brown). (B) *pipe* mRNA expression (blue) in an egg chamber with unmarked dpp flip out clones. At late stage 10 in a wildtype egg chamber *pipe* mRNA is no longer detected, thus, ectopic Dpp signaling leads to *pipe* expression. (C) *pipe* mRNA expression (blue) is not influenced by GFP ectopic expression (brown).

2.4.8 Lack of TGF-β signaling in somatic follicle cells leads to embryonic DV defects

We further investigated the role of the TGF-β signaling pathway in the establishment of the embryonic DV axis by performing clonal analysis with members of the pathway. Mutant follicle cell clones for *Mothers against dpp* and for *Medea* were generated using a Gal4/UAS-flip line. The Gal4/UAS system is under the control of a specific follicle cell promoter active early during oogenesis; this guarantees that mitotic clones are generated only in the somatic follicle cells and not in the germline and these eggs were allowed to futher develop. A fraction of the eggs (10%) did not hatch. Among these, there were eggs showing anterior chorion defects, a result that is expected when we consider the role of the TGF-β signalling pathway in patterning the anteriodorsal egg shell, as defects in anterior chorionic structures can impair fertilisation. However, a fraction of the unhatched eggs were in fact fertilized. Cuticles prepared from these eggs show different degrees of dorsalisation along the AP axis (Fig. 30).

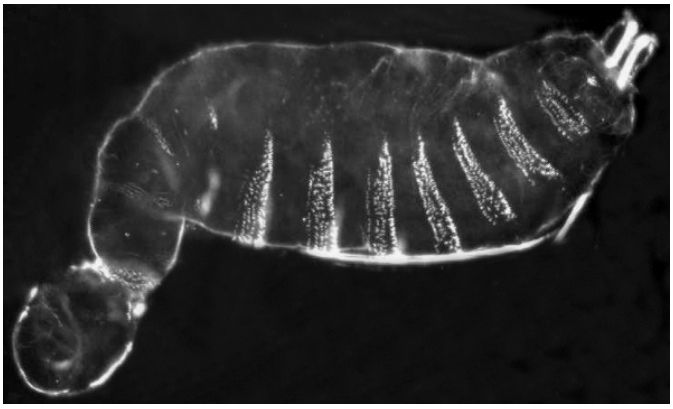

Figure. 30
Follicle cell clones lacking Mad function cause dorsalisation along the embryonic AP axis.
Dark field micrograph of an anteriorly dorsalised embryo derived from a mother in which mutant cell clones lacking Mad function have been induced in the follicular epithelium

In addition, blastoderm stage embryos collected from *mad* clonal mothers were stained with DV embryonic markers *twist* and *rhomboid.* This analysis revealed the presence of embryos lacking *twist* expression along the AP axis (Fig. 31B).

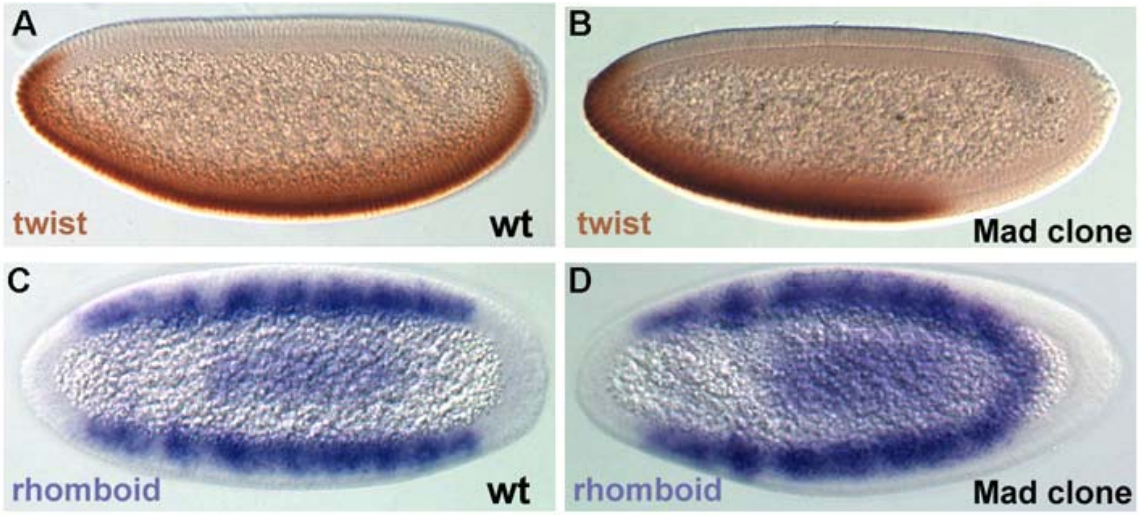

Figure. 31
Mad mutant clones in the follicular epithelium cause dorsalisation along the AP axis in the blastoderm embryo.
(A-D) Embryos at blastoderm stage. (A and C) Wild type. (B and D) Embryos laid by a female fly with Mad mutant clones in the follicular epithelium. (A and B) twist antibody staining. Lack of TGF-β signaling during oogenesis leads to loss of twist expression along the AP axis. (C and D) *rho* mRNA expression. Lack of TGF-β signaling during oogenesis leads to loss of rho expression along the AP axis. In this embryo, the two lateral stripes of *rho* expression fuse at the micell fate□□at the posterior of the egg chamber.

Moreover, loss of the ventralmost fate, the mesoderm, along the AP axis causes a shift of the ventrolateral fate, the ventral ectoderm, towards the midline of the embryo. This can be observed in embryos collected from mad clonal mothers and stained for the venrolateral marker rhomboid. We have identified a clear case in which the two lateral stripes of *rho* expression fuse posteriorly at the midline (Fig. 31 D).

2.4.9 Lack of TGF-β signaling in somatic follicle cells surprisingly leads to an up-regulation of pipe and nudel expression.

Mitotic clonal analysis with members of the TGF-β signalling cascade suggests a role for the pathway in the establisment of the embryonic DV axis. In addition, as previously described, when *dpp* is ectopically expressed at the posterior of the egg chamber, the *pipe* expression profile is affected and the gene is up–regulated in response to TGF-β signaling. Taken together, these data seem to suggest that TGF-β signaling partecipates in the establishment of the embryonic DV axis by turning on *pipe* at the ventral side of the egg chamber.

We have tested this hypothesis by analysing follicle cell clones mutant for *Mad* and for *Medea* and by looking directly at how a lack of TGF-β signaling affects *pipe* or *nudel* expression. Surprisingly, ventrally located *Mad* clones, induced exclusively in the follicular epithelium and detected by the absence of GFP, do not lack *pipe* nor *nudel* expression, as expected. On the contrary, the expression of the two genes is stronger within the clone if compared to neighbouring cells (Fig. 32A-C and D-F).

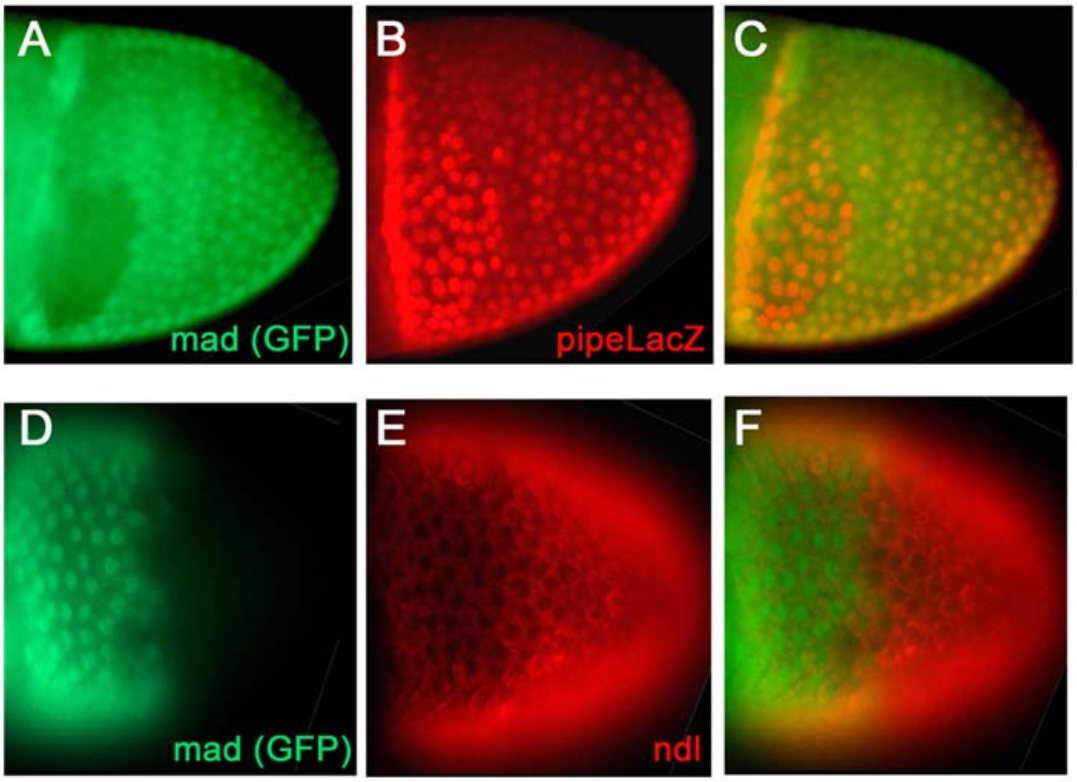

Figure. 32
Mad mutant clones show cell-autonomous up-regulation of pipe and Nudle expression.
(A-F) Stage 10 egg chambers (ventral view and anterior is to the left). (A and D) Clones of mutant cells missing Mad function and marked by the absence of GFP expression. (B-E) *pipe* expression (B) and *nudle* expression (E). (C and F) merge.
pipe and Nudle expression (red) is stronger within the clone (lack of green).

The same result is observed in M*edea* follicle cell clones when these are located at the ventral side (Fig. 33 A-C). *Medea* clones in the dorsal half of the egg chamber do not affect *pipe* expression (Fig. 33 D-F).

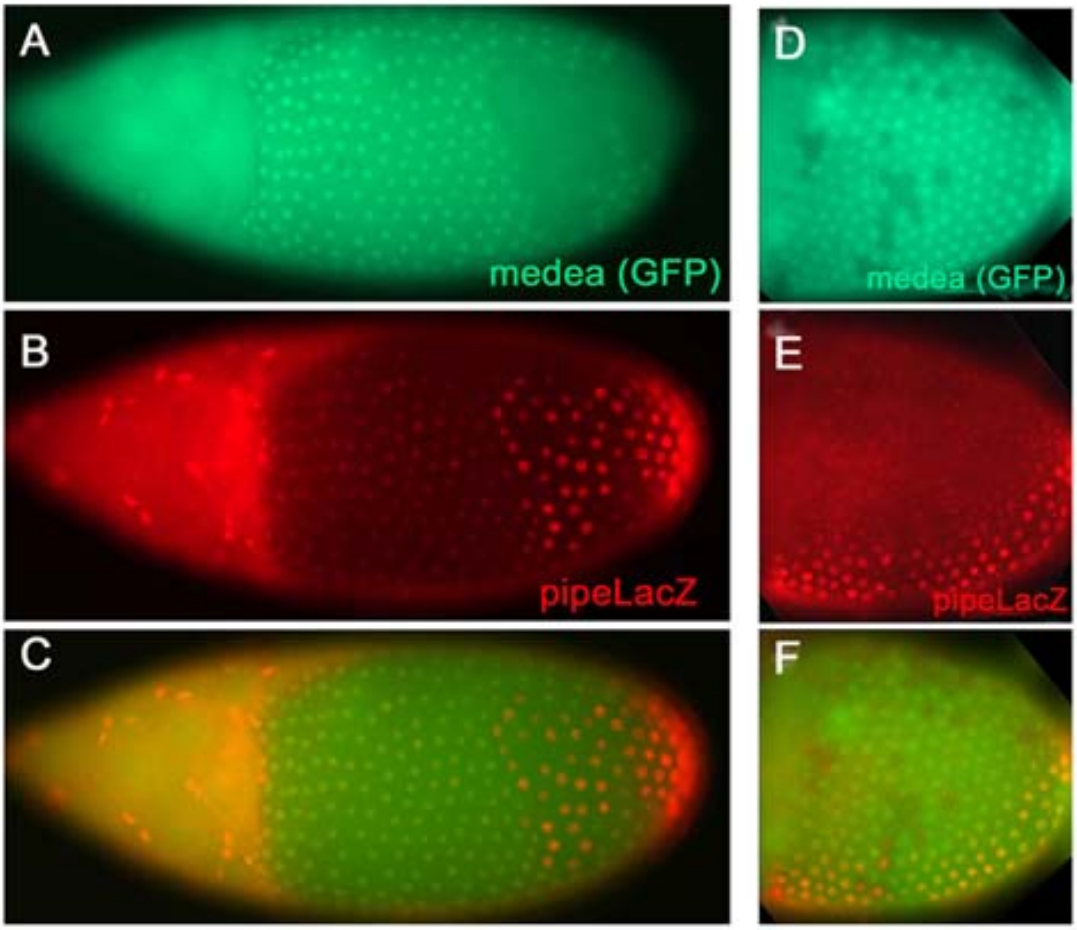

Figure 33
Medea mutant clones show cell-autonomous up-regulation of pipe only ta the ventral side of the egg chamber.
(A-C) Stage 11egg chamber and (D-F) stage 10 egg chamber. (A-C) Ventral view. (D-F) Anterior is to the left and dorsal to the top. (A and C) Clons of medea mutant cells are marked by the absence of GFP expression. (B and E) pipe expression is visuallized by using a pipe-LacZ construct. (C and F) merge.
(A-C) pipe expression (red) is stronger within the clone (lack of green). (D-F) *medea* clones located outside the endogenous *pipe* expression domain do not effect pipe expression.

These results are rather surprising and seem to be in contrast with the previous findings.

3. DISCUSSION

The *Drosophila* egg chamber is an ideal system for studying the crosstalk between signaling pathways. The follicle cells form a monolayer epithelium surrounding the nurse cell cluster and the oocyte. During oogenesis the epithelium is progressively subdivided into multiple cell types that undergo different developmental programs. This is accomplished by signaling events differentially instructing sub-populations of follicle cells.

3.1 The competence of the follicular epithelium in responding to Gurken signaling

The AP and DV axes of the developing *Drosophila* egg are set up by the activation of the EGF receptor through Gurken, a ligand whose localization is spatially restricted within in the oocyte. First, follicle cells abutting the oocyte acquire a posterior fate and signal back to the oocyte. Consequently, this signal reorganizes the oocyte cytoskeleton and the nucleus initiates a movement towards the anterior pole where Gurken signals again inducing the overlying follicle cells to acquire a dorsal fate (González-Reyes et al., 1995; Roth et al., 1995). Thus, Gurken signaling stimulates, sequentially, two subsets of cells within the follicular epithelium to adopt posterior and dorsal fate, respectively.

The fact that early and late Gurken signaling are both directed to a distinct region of the follicular epithelium appears to explain the different responses: the two groups of cell are differentially pre-patterned and thus able to "interpret" the Gurken signaling by adopting different cell fates. Indeed mutant phenotypes, overexpression studies and clonal analysis suggest that the follicular epithelium is subdivided into two major cell populations (Gonzáles-Reyes et al., 1995; Gonzáles-Reyes and St Johnston, 1998; Keller Larkin et al., 1999; Lee and Montell, 1997; Roth et al., 1995). Both ends of the egg chamber are believed to harbor terminal follicle cells, which display default anterior fates unless early Gurken signaling induces them to adopt a posterior fate. Between the two terminal populations are the main-body follicle cells, which can be induced to adopt dorsal rather than ventral fates. However, during embryonic development specification of different cell fates can also result from different levels of signaling and/or be under timing control. In order to test if these two patterning mechanisms apply to axis specification during *Drosophila* oogenesis, we have put Cornichon under heat-shock control and thus we have manipulated the temporal expression

pattern of the endogenous Gurken signal and we have supplied terminal and main-body follicle cells with the same level of Gurken signaling.

3.1.1 Oocyte nucleus movement determines the orthogonal orientation of the axes

Starting from midstage 6 Gurken signaling becomes insufficient to promote nuclear movement although up to stage 7 an influence on nuclear movement can be detected (Fig. 11D, H and N). This transition is probably caused by changes both in the follicular epithelium and in the oocyte. If Gurken activation occurs too late to induce nuclear movement or if the movement is incomplete so that the nucleus resides in an intermediate position, Gurken remains localized with the oocyte nucleus and signals from there to the nearby follicle cells. The resulting pattern of the target gene expression (*BR-C, pipe, and kek*) is dictated by the nuclear position and suggests that there is no intrinsic DV polarity in the egg chamber. This is especially obvious when completely symmetric rings of dorsal appendage material are induced from a posteriorly localized nucleus (Fig. 12). In this situation, a set of marker genes corresponding to the entire DV axis of the follicular epithelium is expressed along the AP axis, indicating that there is, with one exception discussed below, no principal bias to the way these genes are activated or repressed in the main-body follicle cells.

These observations demonstrate that the movement of the nucleus is the sole determinant of the orthogonal orientation of the body axes and that it stochastically determines the position of the dorsal side of the egg.

3.1.2 The responsiveness of terminal and main-body follicle cells

If induction of the oocyte nucleus movement occurs after stage 7 the oocyte nucleus remains posteriorly and Gurken signals to a region that includes both terminal and an abutting ring of main-body follicle cells. Simultaneous Gurken signal to both cells population clearly shows their different developmental responses: the terminal cells form either anterior or posterior structures, while the encircling ring of cells form dorsal cells types characterized at the molecular level by repression of pipe and by the activation of BR-

C. This excludes the possibility that timing controls posterior and dorsal follicle cell-fate specification so that late Gurken signaling always has a dorsalizing effect, irrespective of the cell group receiving the signal. The observation confirms earlier studies, which showed that terminal and main-body follicle cells have different default states in the absence of signaling and respond differentially upon ectopic activation of Ras or DER (Lee and Montell, 1997; Gonzales-Reyes and St Johnston, 1998; Keller Larkin et al., 1999). It also demonstrates that the different responses of terminal and main-body follicle cells to Gurken signaling are not strictly separated in time such that the former have entirely lost their ability to react when the latter are competent. We see posteriorisation occurring simultaneously with dorsal fate induction.

However, whenever dorsal fate, i.e. dorsal appendages were found together with posterior chorion structures a close inspection of these egg chambers revealed that the posteriorisation was incomplete and dpp, normally only found anteriorly, was still present at the posterior pole.

The mixed populations consisting of anterior and posterior follicle are also present in egg chambers from certain hypomorphic cni and grk alleles (Fig. 13C). This is maybe linked to the fact that the terminal cells are not a homogeneous cell group. They seem to be divided into three subgroups by Gurken-independent patterning mechanisms (Leeand Montell, 1997; Gonzalez-Reyes and St Johnston. 1998; Keller Larkin et al., 1999). These subgroups might have differentially sensitive towards Gurken signaling.

3.1.3 EGF and TGF-β *signaling have to coincide to induce anterior-dorsal fates*

The uniform expression in all main-body follicle cells of an activated form of the EGF receptor led to the suggestion of a signal emanating from anterior follicle cells, which modulates the response of the follicle cells upon EGF activation (Sapir et al., 1998; Queenan et al., 1997). In fact, despite uniform activation of primary EGF targets like *kekkon*, other target genes were only activated in proximity of anterior terminal follicle cells. Our results clearly identify Dpp as the actual signaling molecule that pre-patterns the main-body follicle cells. Residual *dpp* expression in posterior terminal cells explains the difference between the *hs-cni* and *mago* phenotypes, and most importantly, ectopic posterior *dpp* expression in egg

chambers with posterior Gurken signaling is sufficient to induce dorsal appendage formation. Currently we can not assess the relative contribution of Dpp signaling to the specification of the dorsal appendages and operculum, respectively. Previous observation (Twombly et al., 1996: Deng and Bownes, 1997) suggest that high levels of Dpp repress dorsal appendages and promote operculum formation. Our analysis of follicle cells clones lacking Mad function, however, demonstrates that Dpp signaling is required for BR-C expression and suggests that low levels of Dpp, insufficient for operculum formation, are likely to specify dorsal appendages. Indeed, when Dpp is overexpressed in the whole follicle epithelium using a weak Gal4 driver the dorsal appendage are enlarged.

The finding that *rho* expression in the follicular epithelium cannot be induced by Gurken alone, but also requires Dpp, shows that both cell-fate specification and cell-fate patterning are controlled by the intersection of the two pathways. Loss-of-function clones have been used to demonstrate that *rho* and *spi* are not required for dorsal appendage formation per se but that they are necessary to separate the two appendages and to position them dorsolaterally (Wasserman and Freeman, 1998). Since this patterning mechanism involves the self-amplification of EGF pathway activation and includes the diffusible ligand Spi, the process must be under tight spatial control to prevent runaway activation in the follicular epithelium. While the localization of Gurken limits the process along the DV axis, we propose that a Dpp gradient emanating from anterior-terminal cells prevents its spreading along the AP axis of the main-body follicle cells.

3.2 Establishment of the dorsoventral embryonic polarity during oogenesis

In has been shown that Gurken signaling at the dorsal side is required to repress *pipe* and thereby it defines the region of the egg from which the embryonic DV axis is initiated (Sen, 1998). The mechanism of *pipe* repression remains elusive as data from different labs support rather opposite working models. On one hand, Gurken has been suggested to simply activate *mirror* which in turn promotes the expression of an unknown diffusible molecule able to repress *pipe* at a distance. *mirror* missexpression appears to support this hypothesis since it leads to long range repression of *pipe*. On the other hand, Gurken may have a graded distribution within the oocyte and be the only signal responsible for *pipe* repression. Indeed, the EGF receptor seems to be activated also in the ventral region of the epithelium where *pipe* is expressed. In fact, the molecule D-cbl has been found to be required in ventral follicle

cells to terminate EGF signaling maybe by targeting the activated EGF complex into the ubiquitination-dependent degradation pathway (Pai, 2000). It has been found that activation of the EGF receptor at the ventral side depends on Gurken signaling indicating that during oogenesis Gurken may work as a long range signaling molecule able to stimulate also ventral follicle cells (Pai, 2000). This finding suggests that *pipe* repression may not involve secondary signaling events downstream of Gurken function and that Gurken signaling is the only cause for *pipe* repression. Our data strongly support this second working hypothesis.

Clonal analysis with Raf, a cytoplasmic component of the EGF signaling pathway, indicates that EGF signaling is required dorsally along the whole AP axis in order to repress *pipe*. The observed cell autonomous de-repression of *pipe* within Raf clone excludes the involvement of a diffusible signaling molecule downsteam of the EGF activation. During *Drosophila* oogenesis two ligands have been shown to activate EGF signaling, these are Gurken and Spitz, respectively. While Gurken is secreted by the oocyte, Spitz is present in all follicle cells but it requires Rhomboid function in order to be active. Wasserman and Freeman (1998) have shown that *spitz* and *rhomboid* clones in the follicular epithelium lead to eggs with an abnormal eggshell but with a normal embryonic DV polarity. This observation suggests that Spitz and Rhomboind are not involved in setting up the embryonic DV axis during *Drosophila* oogenesis. However, *rho* and *spi* clones may still affect *pipe* expression and later molecular events occurring during the embryonic phase of DV axis may compensate for these changes. Thus, we have induced large *rhomboid* clones in the follicular epithelium and have analyzed *pipe* expression directly. We observed that in order to repress *pipe* the EGF receptor needs only to be activated by Gurken. Lack of *rhomboid* in the follicular epithelium does not effect the *pipe* expression pattern, ruling out a requirement for the other ligand Spitz in this process as Spitz activation is dependent upon Rhomboid. If the higher level of the EGF receptor activation induced by Rhomboid and Spitz is not required for *pipe* repression, the ventral *pipe* domain might be defined exclusively by Gurken stimulation. *rhomboid* expression in the follicular epithelium starts at stage 9 and Spitz is likely to be activated at this time point during oogenesis.

However, at stage 9 of oogenesis *pipe* is not yet expressed as an even ventral stripe but rather the domain spreads further dorsally and can be detected in follicle cells over the nurse cell cluster (Fig. 1). By this time point EGF signaling may have already completed its function. Later patterning events required to refine *pipe* expression may be controlled by the

action of other signaling pathways that are not directly initiated or controlled by Gurken signaling. Furthermore, the egg chamber grows during this period and the follicle cells continue to migrate over the oocyte nucleus and thus different egg chamber geometries at the time of pattern induction might also accout for the final *pipe* expression domain.

The homeodomain transcription factor Mirror is a target of Gurken signaling at the dorsal side of the egg chamber and it is likely to be the best candidate to directly bind and repress the *pipe* promoter within this region. Indeed, clonal anlysis with *mirror* leads to cell autonomous derepression of *pipe* indicating that this transcription factor directly controls *pipe* repression at the dorsal side. However, Jordan and colleagues have previously shown that missexpression of *mirror* at the posterior of the egg chamber leads to a long-range repression of *pipe*. This observation is in contrast with the cell autonomous de-repression of *pipe* observed in the lack-of-function *mirror* clones. Nevertheless, as also reported by the authors, missexpression of *mirror* at the posterior of the egg chamber leads to ectopic expression of *rhomboid* in the same region. Indeed, *rhomboid* expression at the posterior leads to an ectopic activation of Spitz which is present in all follicle cells. Spitz is a diffusible ligand and initiates EGF signaling leading to *pipe* repression at a distance. In addition, EGF signaling activates more *rhomboid* expression. This starts a positive feedback loop on pathway activation which finally promotes a "run-away" repression of *pipe*. We have previously reported that posterior expression of *rhomboid* alone leads to repression of *pipe* and to strong dorsalisation at the cuticle level. However, this is an artificial situation, as Spitz signaling is normally not involved on *pipe* repression.

Nevertheless, when ectopicaly activated this ligand stimulates the EGF receptor and thus it promotes *pipe* repression. Moreover, in order to repress *pipe* the EGF receptor has just to be activated and it does not discriminate between the two ligands, Gurken and Spitz. Indeed, we could imagine that stimulation of the receptor by Gurken leads to transcription of a certain set of target genes different from the ones downstream of Spitz binding. However, this is not the case as repression of *pipe* is achieved by the binding of both ligands. Moreover, it is likely that *mirror* is expressed upon low levels of EGF signaling exerted by Gurken signaling only. Such a low level receptor stimulation does not require the amplification step on pathway activation starting at stage 9 and controlled by Rhomboid and Spitz. This may indicate once more that Gurken signaling could repress *pipe* up to stage 9 of

oogenesis. Later processes, independent of Gurken signaling, may then occur to shape the *pipe* expression domain to the final appearance of an even anteroposterior ventral stripe.

3.2.1 EGF and TGF-β *signaling may work together to pattern pipe expression*

After the migration of the oocyte nucleus Gurken and Dpp work together to specify and pattern anterior-dorsal cell-fates. Our data indicate that these two signaling pathways may also collaborate to shape the *pipe* expression domain. However, these data are rather difficult to interpret. On one hand, missexpression or overexpression of Dpp indicates that activation of the TGF-β signaling pathway promotes *pipe* expression and thus counteracts the repressing activity of EGF signaling. However, lack of TGF-β signaling within the follicular epithelium does not lead to lack of *pipe* expression, as expected, but on the contrary, to stronger *pipe* expression. In addition, the same result is observed for Nudel. However, when these clonal egg chambers are allowed to develop further embyos and cuticles show dorsalisation along the AP axis.

Nevertheless, we have observed that both *pipe* mRNA and the Nudel protein expression is downregulated in wild-type follicle cells after stage 10 of oogenesis. Thus, we think that in loss-of-function Mad or Medea clones the strong *pipe* and Nudel expression is due to the persistence of the two products after stage 10 of oogenesis. It is likely that cells within the clones are somehow “retarded” in their molecular program and thus they express *pipe* and Nudel later then normal. Consequently, the two products persist longer in the clone then in the neighboring wildtype follicle cells although without having instructive effects on the axis induction. Thus, after clone induction *pipe* and Nudel expression should be examined in younger egg chambers. Lack-of-function clones are marked by the absence of GFP expression which is unfortunately rather difficult to detect early during oogenesis.

We do not know if our hypothesis is correct and if these mutant cells are indeed retarded, however, we would still like to speculate and to point out that a strong expression of *pipe* and Nudel after stage 10 of oogenesis seems unable to ventralize the future embryo or to fully rescue a previous lack of expression of the two molecules. If this were indeed the case then the function of the two molecules might be fullfilled before stage 10 of oogenesis and thus before *pipe* mRNA is expressed as an even anteroposterior ventral stripe. Indeed,

Pipe may modify its substrate in a broader region of the epithelium than expected. Then later, during the embryonic phase, patterning events and positive and negative feedback loops would finally establish the DV polarity of the embryo.

It is hard to speculate on the exact role of TGF-β signaling on the *pipe* expression pattern. Embryonic defects caused by Mad loss-of-function, are observed along all AP axis. We think unlikely that Dpp emanating from the anterior most cells during midoogenesis, is sufficient to account for such large range defects. However, it is still our opinion that TGF-β during oogenesis plays a decisive role in the patterning the follicular epithelium.

4. MATERIALS AND METHODS

4.1 Abbreviations

General abbreviations:

AEL	after egg lay
AP	anteroposterior
bp	base pair
BSA	Bovine Serum Albumin
DV	dorsoventral
Fig.	figure
Fk	Filzkörper
g	gram
h	hours
min	minutes
ml	milliliter (10^{-3} litre)
µl	microliter (10^{-6} litre)
PF	paraformaldehyde
PBS	phosphate buffer saline
PBST	PBS + 0,2% Tween
RT	room temperature
WT	wild type

Genetic abbreviations:

b	*black*
BMP	*Bone Morphogenetic Protein*
BR-C	*Broad-Complex*
brk	*brinker*
CyO	*Curley of Oster (balancer for the second chromosome)*
cni	*cornichon*
Df	*deficiency*
dl	*dorsal*
dpp	*decapentaplegic*
FM	*First Multiple (balancer for the first chromosome)*
flp	*flipase*
FRT	*Flipase Recombination Target*
hs	*heat shock promoter*
In	*inversion*
Mad	*Mothers against dpp*
Med	*Medea*
mirr	*mirror*
N	*Notch*
ndl	*nudel*
pip	*pipe*
sna	*snail*
sog	*short gastrulation*

TM *Third Multiple (balancer for the third chromosome)*
Tl *Toll*
tkv *thick veins*
tld *tolloid*
twi *twist*
w *whit*
y *yellow*
zen *zerknüllt*

4.2 Fly stocks

The following Drosophila melanogaster strains were used:

OregonR

cni^{AR55}: b *cni^{AR55}* pr cn / CyO (*cni^{AR55}* is a null alelle; Roth et al., 1995)

cni^{AA12}: b *cni ^{AA12}* pr cn / CyO (*cni ^{AA12}* is an hypomorph allele; Roth et al., 1995)

Df (2L)H60: w; sco Df(2L)H60 / CyO

hs-cni: w; P(*hs-cni*, w+), *cni^{AR55}* / Cyo The experiment was performed by crossing these flies with w; cni^{AR55} / CyO flies and by looking at w; P(*hs-cni*, w+), *cni^{AR55}* / +, *cni^{AR55}* flies

Mad 12: w; *Mad^{12}* FRT40A / CyO (*Mad^{12}* is a null allele; Raftery et al., 1995)

Medea 1: *Medea^{1} e FRT82B* / TM3 (*Medea^{1}* is a null allele; Das et al.,1998)

rho 7M43: *rho^{7M43}* FRT80B / TM6 (*rho^{7M43}* is a null allele; Tearle and Nusslein-Volhard, 1987; Wassrman et al., 2000)

raf LE78: y w *raf ^{LE78}* FRT101 / FM7

mirr e48: w; *mirr ^{e48}* FRT 80B / TM3 (*mirr^{e48}* is a null allele; McNeill et al., 1997)

Dof 1: FRT82B *Dof ^{1}* / TM3 (*Dof^{1}* is a null allele; Vincent et al., 1999)

Crosses and stocks used for genetic mosaic analysis:

Loss-of-function clones of genetically marked cells by the absence GFP expression were generated by Flp-FRT recombination (Xu and Rubin, 1993). Two distinct lines where used to drive flippase expression exclusively in the follicle epithelium:

GR1-Gal4 UAS-flip: GR1-Gal4 UAS-flip / TM3

e22c-Gal4 UAS-flip: e22c-Gal4 UAS-flip / CyO

Flies of the following genotype were analysed:

Mad mutant clones: Mad^{12} FRT40A pipe-LacZ / GFP FRT40A pipe-LacZ ; GR1-Gal4 UAS-flip

Medea mutant clones: w; e22c-Gal4 UAS-flip / pipe-LacZ ; FRT 82B $Medea^{1}$ / FRT 82B GFP

rho mutant clones: w; e22c-Gal4 UAS-flip / pipe-LacZ ; FRT 80B rho^{7m43} / FRT 80B GFP

raf muant clones : raf ^{LE78} FRT 101 / FRT 101 GFP ; e22c-Gal4 UAS-flip / pipe-LacZ

mirr muant clones: w; e22c-Gal4 UAS-flip / pipe-LacZ ; FRT 80B $mirr^{e48}$ / FRT 80B GFP

Dof muant clones: w; e22c-Gal4 UAS-flip / pipe-LacZ ; FRT82B Dof ^{1} / FRT82B GFP

Overexpression and missexpression studies:

To analyze the consequences of overexpression and/or missexpression of differnt genes in the follicular epithelium the following driver lines were used:

Cy2-GAL4: Cy2-Gal4 (on the second chromosome; Queenan et al., 1997)

GR1-Gal4: GR1-Gal4 (on the third chromosome; Queenan et al., 1997)

E4-Gal4: E4-Gal4 (on the third chromosome; Queenan et al., 1997

Hs-flip; actin>CD2>Gal4; UAS-GFP: hs-flip; actin>CD2>Gal4 / CyO ; UAS-GFP / TM3

Flp-out clones of cells genetically marked by GFP expression were generated at the larval stage at 37C for 30 min

The following UAS line were used:

UAS-dpp: UAS-dpp , pipe-Lac-Z / CyO (original line described in Nellen et al., 1996)

UAS-rho: UAS-rho / TM3 (Sapir et al., 1998)

UAS-cni: w; UAS-cni, cni^{AR55} / CyO

The experiment was performed by crossing this line with: w; CY2-Gal4, cni^{AR55} flies and by looking at w; UAS-cni, cni^{AR55} / CY2-Gal4, cni^{AR55} flies
UAS-ltop: UAS-ltop / TM3 (Queenan et al., 1997)

4.2.1 Breeding of Drosophila melanogaster

All *Drosophila* lines were kept as described by Ashburner (1989). Flies were grown in plastic vials on standard cornmeal agar food at 25°C or 17°C. To ensure genetic purity for the progeny of crosses, only none fertilized females were mated with males of the appropriate genotypes. To ensure virginity the vials were emptied and the hatching flies were allowed to grow up to eight hours at 25°C or up to 22h at 17°C before collecting the virgins. Oregon R served as the wildtype strain.

4.3. Preparation of egg shell and embryonic cuticle

For the analysis of the embryonic cuticle, non-hatched larvae were washed in water, dechorionated in 50% NaOCl for 3-5 min, washed rapidly and mounted in a mixture of Hoyer's medium and lactic acid 2:1.

Egg shells were simply washed and mounted in the same medium. The mounted samples were incubated at 60°C for at least 24 hours before they were photographed.

4.4 Application of colchicine

Colchicine was mixed with fresh yeast and used at 25 µg/ml to destabilize microtubles as described in Theuzkauf et al. (1997). 4-day old females of the right genotype were fed with the yeas-colchicine mix. Ovaries dissected 1 day after treatment show a hight frequency of posteriorly localised ooyte nuclei.

4.5. Immunohistochemistry and *in situ* hybridization

4.5.1. Fixation of embryos for immunostainings and in situ hybridization

The dechorionated embryos (in 50% NaOCl) were washed and transferred to heptan fix (5 ml 4% paraformaldehyde in PEMS + 5ml heptan) and shaken for 20 min. The lower phase was removed and replaced by an equal volume of methanol. The strong shaking for 1 min led to the removal of vitelline membrane of most of the embryos, which sank to the bottom of the glass tube. The heptan and most of the methanol was aspirated and the embryos were transferred to an eppendorf tube and stored in methanol at –20ºC.

PEMS buffer pH 6.9 (stored at 4°C):

0,1 M Pipes

2 mM $MgSO_4$

1 mM EGTA

4.5.2. Antibody staining of embryos

Immunostaining of embryos was done as follows:

Fixed embryos were rehydrated by several washes in PBS + 0.2% Tween (PBST) for 5-10 min. To block the non-specific protein binding sites, the embryos were twice incubated in 1% BSA for 30 min. The incubation the first antibody was done over-night at 4°C. On the next day the antibody solution was removed and embryos were rinsed twice with PBST followed by four 30 min washes. Preabserbed secondary antibody was added for 1,5 h incubation. The antibody was removed and the embryos were rinsed and washed twice over 45 min. For biotinylated secondary antibody, 10 µl avidin and 10µl biotinylated horseradish peroxidase solutions from ABC Vector's Kit were mixed in 1 ml PBST and let stand for 30 min before the embryos were resuspended in this mixture for 40 min. After three times 10 min washes with PBST, the embryos were transferred into the solution: 0.75 mg/ml 3,3'diaminobenzidine (DAB) in 0.1 M Tris-HCl, pH 7.3, which after 5 min was replaced by the staining solution containing additionally 0.01% H_2O_2. A brown DAB precipitate has formed within 5-15 min. The reaction was stopped by adding 2 µg/ml of 20% sodium azide and rapid rinsing with PBST.

The embryos were dehydrated and mounted in araldite for the microscopic analysis as described in the section 4.5.8.

4.5.3. Fixation of ovaries for immunostainings

The ovaries were disected and transferred to heptan fix (200µl 4% paraformaldehyde + 20µl DMSO + 600µl heptan) for 20 min.

4.5.4. Antibody staining of ovaries

Immunostaining of ovaries was done as follows:

Fixed ovaries were incubated twice in 1% BSA for 30 min to block the non-specific protein binding sites. The incubation the first antibody was done over-night at 4°C. On the next day the antibody solution was removed and ovaries were rinsed twice with PBST followed by four 30 min washes. Preabserbed secondary antibody was added for 1,5 h incubation. The

antibody was removed and the ovaries were rinsed and washed twice over 45 min. In the case of a secondary antibody coupled with Cy3, the ovaries were simply mounted in *Vectashield* (*Linaris*). For biotinylated secondary antibody, the ovaries were treated as described for embryos in the section 4.5.2.

The ovaries were dehydrated and mounted in araldite for the microscopic analysis as described in the section 4.5.8.

4.5.5. Fixation of ovaries for in situ

The ovaries were disected and transferred to heptan fix (200µl 4% paraformaldehyde + 20µl DMSO + 600µl heptan) for 20 min. The upper phase was removed and the ovaries incubated for 5 additional minutes with 4% paraformaldehyde. The ovaries were then washed several times with methanol and eventually stored at this point at –20C.

4.5.6. In situ hybridisation of ovaries

In situ hybridization was done with digoxigenin-labeled RNA probes synthesized using RNA Labelling Mix (Boehringer Mannheim). Detection of single transcripts was performed as outlined in Tautz and Pfeifle (1989).

The fixed ovaries were rehydrated in PBST, refixed in 4% paraformaldehyd in PBST (PF / PBST) for 20 min, washed four times with PBST over 15 min and incubated for 10 min in 50 µg/ml proteinase K. Proteinase was quickly blocked by adding glycine solution (2 mg/ml in PBST) for 2 min. The ovaries were rapidly rinsed 4 times, and refixed with PF/PBST for 20 min and washed three times with PBST all for 15 min. The ovaries were incubated 10 min in 1:1 hybridization solution / PBST and next 10 min only in hybridization solution (hyb. soln.). Prehybridisation required 1 h incubation of embryos in hyb. soln. + 100 µg/mg salmon sperm DNA (Sigma) at 55°C. 1-2 µl of the probe was added per 50 µl of hyb. soln. and allowed to hybridise over night at 55°C. On the next day the probe was removed and the ovaries were rinsed with the prewarmed hyb. soln. and washed 4 times 30 min each at 55°C in hyb. soln. and in a series of hyb. soln. / PBST mixture in proportions 4:1, 3:2, 2:3 and 1:4 for 10 min each at 55°C except the last wash, which was done at room temperature (RT). The hybridization was detected by the immunoreaction. First the ovaries were incubated in PBST + 1%BSA (PBST / BSA) twice for 20 min each to block non-specific immunoreactivity of proteins. After a short wash in PBST, the preabsorbed anti-Digoxigenin-AP conjugated antibody (Dianova) was added at the final dilution 1:5000 for 1.5 h at RT. The ovaries were washed several times in PBST over 45 min and transferred into alkaline phosphatase staining buffer (APB: 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5, 0.2% Tween).

After three 5 min washes in APB, the antiboby bound to the epitope was visualised by a blue alkaline phosphatase reaction. X-phosphate / NBT staining solution was added (for 1 ml

AP-buffer: 4.4 ml of 75 mg/ml NBT and 3.5 ml of X-phosphate) and the reaction developed in the darkness within 60 min (see Boehringer anti-Dig-AP protocol). The reaction was monitored every 15 min and stopped by washes in PBST. The ovaries were dehydrated and mounted in araldite as described in the section 4.5.8.

4.5.7. Double in situ antibody staining of the ovaries

Ovaries were dissected and fixed as described above. Myc expression was visualized using a rabbit anti-Myc antibody (1:100 Upstate) and antibody stainings were conducted in RNAse-free conditions. Subsequently, *BR-C* expression was detected by in situ hybridization, as described above.

4.5.8. Mounting the stained embryos and ovaries

Embryos and ovaries were dehydrated with a series of ethanol washes (70% and 100%), followed by one wash in dry ethanol and twice in dry acetone, 10 min each wash. The mixture of araldite (Durcupan-ACM from Fluka) / acetone 1:1 was added and the embryos and ovaries were transferred into the depression slides to allow evaporation of acetone for more than 3 hrs in the fume hood. Embryos and ovaries were selected under the dissection microscope and using a wolfram needle transferred individually into a small drop of araldite on a slide and analyzed under the microscope. Ovaries were additionally dissected to separate single egg chambers. Embryos and ovaries mounted in araldite were stored at – 20ºC. Immages were obtained using a Zeiss microscope.

4.6. Heat-shock treatment, ovary dissection and egg collection

3- to 5- day old flies were incubated in a 39C water bath for 10 minutes. They were kept at room temperature (25C) in vials with food and dry yeast ans subsequently used for timed egg collection or for ovary dissections in 1 or 2 hour intervals for a maximum period of 28 hours after heat shock. For each time point, on average, 50 stage 9 egg chambers were scored for the position of the oocyte nucleus and for *pipe* or *kek* expression. Likewise, approximatly 50 stage 10 egg chambers were scored for *BR-C* expression. Staging was according to King (1970) and Spradling (1993). Molecular markers gene expression and/or oocyte nucleus position in egg chambers of known dissection time were correlated with an oogenesis timetable for flies kept at room temperature (Lin and Spradling, 1993)

4.7. DNA work and germline transformation

All DNA methods were performed according to Sambrook et al. (1989).

The *cni* cDNA (0.9kb) was cloned into the Hsp70-pCaSpeR (Pirrotta, 1988) and into the pUAS (Brand and Perrimon, 1993). The constructs for transformation were prepared at the concentration 0.3 μg/ml together with the 0.1 μg/ml Δ2-3 helper DNA (Laski et al., 1986) in the injection buffer (0.1 mM phosphate buffer [pH 7.4], 5 mM KCl) containing 2% phenol red. About 1μl of the solution was injected into the posterior region of the 20- to 30 min old *w; cniAR55 /CyO* preblastoderm embryos. Embryos were covered with Voltalef hydrocarbon 5S and allowed to hatch at 18°C. The second instar larvea were then transferred to fresh food vials, and surviving flies were mated against appropriate *yw* flies. Successful transformation events were identified in the F1 generation by the expression a *mini white* gene [w^+]. Stocks of transformants were established, which carry the P-insertion on the I, II and III chromosome. The tested lines did not vary among each other in the phenotypes when heat-shocked or crossed to the same *Gal4*-containg flies. CY2-Gal4 driver line was used to drive ubiquitous expression of cni in the follicular epithelium, which was confirmed by in situ hybridization. This did not alter the cni mutant phenotype, confirming that any effect observed in *hs-cni* experiments is due to Cni activating Grk in the germline.

5. SUMMARY

During *Drosophila* oogenesis Gurken, associated with the oocyte nucleus, activates the *Drosophila* EGF receptor in the follicular epithelium. Gurken signaling first establishes the anterior-posterior axis by specifying a posterior follicle cell population, which in turn signals back to the oocyte to promote the migration of the oocyte nucleus anteriorly. This leads to a new position of Gurken localization at the anterior of the oocyte and, once here, Gurken signals to dorsal follicle cells and thereby specifies the dorsoventral axis of the egg and embryo. Thus, early and late Gurken signaling are both directed to the folliclular epithelium but lead to different outcomes.

It has been suggested that the follicular epithelium might be subdivided into two major cell populations with different competence in responding to Gurken signaling. The terminal follicle cells at both ends of the egg chamber harbor default anterior fates unless early Gurken signaling induces them to adopt posterior fates. Between the two terminal populations are the main-body follicle cells, which can be induce to adopt dorsal rather than ventral fates. Within this cell population late Gurken signaling activates genes required for dorsal appendages formation and their patterning at the dorsal side and restricts *pipe* expression to a stripe on the ventral side, which provides spatial cues governing the establisment of the future embryonic dorsoventral axis.

In this study, we have further investigated aspects of follicle cell patterning and competence in responding to Gurken signaling by supplying Gurken ectopically and at different time points during oogenesis. We show that even if terminal and main-body follicle cells receive the Gurken signal simutaneously during mid-oogenesis they react differently, one by producing posterior and the other by producing dorsal cell types. In addition, main-body follicle cells are differentially patterned along the anterior-posterior axis.

In fact, the specification of dorsal fates leading to the production of the dorsal appendages in the mature egg is restricted to the anterior half of the egg chamber. By activating Gurken signaling together with *decapentaplegic* at the posterior pole we show that this local restriction derives from TGF-β signaling emanating from the anterior-terminal follicle cells and that both signaling pathways collaborate to induce dorsal appendage formation. Thus, the specification of the dorsal appendages and their correct positioning along the anterior-posterior axis of the egg results from the intersection of EGF signaling

with a presumed anterior-posterior gradient of Decapentaplegic. In addition, we show that the intersection of Gurken and Decapentaplegic signaling is required for *rhomboid* expression and thus controls the fine patterning of the dorsal appendages along the dorsoventral axis.

Although specification of dorsal chorion structures and their patterning is restricted to the anterior region of the egg chamber Gurken signaling acts on all dorsal main-body follicle cells with regard to *pipe* repression. In fact, via clonal analysis with components of the EGF signaling pathway we show that activation of the pathway by Gurken directly represses *pipe* in all dorsal follicle cells. Clonal analysis with the homeodomain transcription factor *mirror* leads to autonomous derepression of *pipe* indicating that Gurken repression of *pipe* is mediated by *mirror*. In addition, preliminary data indicate that modulation of TGF-β signaling during oogenesis can affect *pipe* expression pattern and suggest a general role for the TGF-β pathway in controlling the developmental program of the follicular epithelium.

This study demonstrates the importance of crosstalks between signaling pathways for cell fate specification and patterning and it identifies the *Drosophila* egg chamber as an ideal system to investigate the interaction of signaling pathways in complex developmental contexts.

6. ZUSAMMENFASSUNG

Während der *Drosophila*-Oogenese aktiviert der EGF-artige Ligand Gurken, dessen mRNA mit dem Oozytenkern assoziiert ist, den *Drosophila* EGF Rezeptor im Follikelepithel. Zunächst führt das Gurken-Signal zur Festlegung der anteroposterioren Achse durch die Spezifizierung von posterioren Follikelzellen, welche später zur Oozyte zurücksignalisieren und dadurch die asymmetrische Wanderung des Oozytenkerns zum anterioren Pol der Oozyte auslösen. Am anterioren Pol der Oozyte definiert die asymmetrische Position des Kerns die dorsale Seite der Eikammer. Hier signalisiert Gurken ein zweites Mal und spezifiziert dadurch die dorsoventrale Achse des Eies und des Embryos. Frühes und spätes Gurken-Signal werden also beide vom Follikelepithel empfangen, führen aber zu unterschiedlichen Differenzierungsprozessen.

Um die unterschiedliche Reaktion der Follikelzellen auf das Gurken-Signal zu erklären, wurde angenommen, dass sich das Follikelepithel in zwei Zellgruppen untergliedert. Die terminalen Follikelzellen an beiden Enden der Eikammer prägen ein anteriores Zellschicksal aus, so fern das Gurken-Signal nicht zur Induktion posteriorer Zellen führt. Die zwischen den beiden terminalen Zellpopulationen liegenden Hauptkörperfollikelzellen entwickeln sich im Grundzustand zu ventralen und nach Gurken-Induktion zu dorsalen Zellen. Das Gurken-Signal führt in dieser Zellpopulation zur Aktivierung von Genen, die zur Spezifizierung und Positionierung der dorsalen Anhänge gebraucht werden und es schränkt die *pipe* Expression auf einen ventralen Streifen ein, der die räumliche Information für die dorsoventrale Achse des Embryos liefert.

In der vorliegenden Arbeit wurde die Musterbildung und Kompetenz der Follikelzellen nach ektopischer Expression von Gurken während unterschiedlicher Oogenesestadien untersucht. Selbst wenn das Gurken-Signal gleichzeitig von den terminalen und Hauptkörperfollikelzellen während mittlerer Oogenesestadien empfangen wird, reagieren diese unterschiedlich und differenzieren in posteriore, bzw. dorsale Zelltypen. Die Hauptkörperfollikelzellen reagieren außerdem unterschiedlich entlang der anteroposterioren Achse. Die Spezifizierunng der Anlagen für die dorsalen Anhänge erfolgt normalerweise nur im anterioren Bereich der Eikammer. Dieses räumliche Muster wird von Decapentaplegic, einem TGFß-artigen Wachstums- und Differenzierungsfaktor erzeugt, der normalerweise nur in anterior-terminalen Follikelzellen exprimiert wird. Wenn Dpp und Grk gleichzeitig am posterioren Pol ektopisch exprimiert werden, so führt dies zur Bildung von dorsalen Anhängen am posterioren Pol. Die Spezifizierung der dorsalen Anhänge und ihre korrekte

Positionierung entlang der anteroposterioren Achse erfolgt also dort, wo sowohl Gurken als auch Dpp Signalaktivität vorhanden sind. Beide Signale werden auch dazu benötigt, die *rhomboid* Expression zu aktivieren. Rhomboid kontrolliert die Musterbildung der dorsalen Anhänge.

Obwohl die Bildung dorsaler Chorionstrukturen auf den anterioren Bereich der Eikammer beschränkt ist, scheint Gurken die Expression von *pipe* in allen dorsalen Hauptkörperfollikelzellen zu reprimieren. Dies konnte durch klonale Analyse mit Komponenten der EGF-Signalkette bestätigt werden. Die Gurken abhängige *pipe* Repression wird wahrscheinlich durch den Homeobox-Transkriptionsfaktor Mirror vermittelt. *mirror* mutante Follikelzellklone zeigen autonome Derepression von *pipe*. Einige Daten deuten außerdem darauf hin, dass die *pipe* Expression durch TGFß-Signale moduliert wird. Die TGFß-Signalkette könnte also eine allgemeine Funktion bei der Kontrolle der Differenzierung des Follikelepithels haben.

Die vorliegende Studie zeigt, dass die *Drosophila* Eikammer ein ideales System darstellt, um die Rolle der Kooperation von Signalketten für Differenzierungs- und Musterbildungsprozesse zu untersuchen.

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ERKLÄRUNG

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich der Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe: dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Siegfried Roth betreut worden.

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