Signaling networks involved in patterning dorsal chorion structures in *Drosophila*

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Bhupendra Shravage

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Bericherstatter:

Prof. Dr. Siegfried Roth

PD Dr. Thomas Klein

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Bhupendra Shravage

आई, दादांस

(*To my parents*)

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Abbreviations

TGF-β	Transforming Growth Factor β
aa	Amino acid
Alp	Activin like Protein
Anti β gal	Anti β galactosidase
Aos	Argos
AP	Anterior-Posterior
Babo	Baboon
BMP	Bone Morphogenic Protein
bp	base pairs
BR-C	Broad-Complex
Brk	Brinker
cAMP	cyclic Adenosine mono-phosphate
CBP	cAMP binding protein
CMFCs	Centripetally Migrating Follicle Cells
Cni	Cornichon
Co-Smad	Common Smad
CREB	cAMP response element binding protein
Cys	Cysteine
Dac	Dachshund
Dad	Daughters against Dpp
dl	Dorsal
Dpp	Decapentaplegic
Drk	Downstream of Receptor Kinase
DV	Dorsal-Ventral
E2F	Elongation Factor 2
E2F-4	E2F transcription factor 4
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ETS	E-twenty six
Fas III	Fasciclin III
FGF	Fibroblast Growth Factor
Gbb	Glass bottom boat
GDP	Guanine Di-Phosphate
GFP	Green Fluorescent Protein
Grk	Gurken
GS	Glycins and Serine rich
GTP	Guanine Tri-Phosphate
HDAC	Histone deacetylase complex
His	Histidine
hop	hopscotch
Iro-C	Iroquious-C
I-Smads	Inhibitory Smads
JAK	Janus activated kinase
kb	kilo bases
kDa	Kilo Dalton
Kek	Kekkon

Krn	Keren
LRR	Leucine Rich Repeat
Mad	Mothers against Dpp
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
Mav	Maverick
Med	Medea
MH1	Mad homology domain 1
MH2	Mad homology domain 2
Mirr	Mirror
Myo	Myoglianin
ng	Nanogram
Omb	Optomotor Blind
ORF	Open Readinfg Frame
pMad	Phophorylated Mad
Pnt	Pointed
Put	Punt
Rho	Rhomboid
R-Smad	Receptor regulated Smad
Sal	Spalt
Sara	Smad anchor for receptor activation
Sax	Saxophone
SBE	Smad Binding Element
Scw	Screw
SEM	Scanning Electron Microscopy
Ski	Sloan Kettering virus induced protein
Sl	semi-lethal
Sno	Ski related novel
Sog	Short Gastrulation
Sos	Son of sevenless
Spi	Spitz
Sty	Sprouty
TGF-α	Transforming Growth Factor α
TIE	TGF-β inhibitory element
Tkv	Thickveins
Tld	Tolliod
Tsg	Twisted Gastrulation
UAS	Upstream Activator Sequence
Upd	Unpaired
Vg	Vestigial
Vn	Vein
Wg	Wingless
Wit	Wishful thinking
wt	Wild type
λtop	Lambda-torpedo

1. Introduction

Signaling between cells is a widely used mechanism by which cell fate and patterning is determined in development. It has emerged in the recent years that only a relatively small number of genetic networks are essential for designing the body plan during development. Signaling molecules are key to such genetic networks.

The work presented in this thesis was inspired by the question "How Transforming Growth Factor–β (Decapentaplegic) signaling pathway and Epidermal Growth Factor (Gurken) signaling pathway collaborate to pattern the follicular epithelium during *Drosophila* oogenesis? ". Emphasis was laid on elucidating the role of Dpp signaling and its regulation by SnoN, a transcriptional repressor of TGF–β pathway.

1.1 Patterning of a developmental field

Patterning of a developmental field is primarily achieved by two distinct mechanisms involving cell-cell communication: short range signaling and long range signaling. Short range signaling functions across a few cell diameters and specifies distinct cell fates in a developmentally equivalent group of cells. A well known example is the phenomenon of lateral inhibition governed by Notch signaling during neurogenesis (reviewed in Beatus and Lendahl, 1998). Patterning by long range signaling can occur over several cell diameters and is achieved by two different mechanisms: Relay signaling and Morphogen signaling. In relay signaling, different cell identities are designated by a series of sequential inductive signaling events that are relayed between adjacent cells in the developmental field. One of the best studied examples of relay signaling is that of Epidermal Growth Factor Receptor (EGFR) controlled vulval development in *Caenorhabditis elegans* (Dutt et al., 2004). LIN-3, one of the EGF-like ligands in *C. elegans*, is expressed in the anchor cell of the developing gonad. It induces primary vulval cell fates in adjacent precursor cells by activating the EGFR cascade, which leads to the induction of ROM-1. ROM-1

(the Rhomboid homolog in *C. elegans)* in turn positively amplifies the signal in these cells by activating LIN-3, thus relaying the signal further to additional precursor cells. In contrast, morphogen signaling is executed by secreted ligands (peptides) which have an ability to diffuse away from the source tissue. Secreted signaling molecules belonging to the TGF−β superfamily, Wingless/Wnt, Hedgehog and Fibroblast Growth Factor (FGF) family have been identified and implicated as organizers of pattern and growth in many developmental contexts both, in vertebrates and in invertebrates (reviewed in Tabata, 2001). These molecules are termed as morphogens (literally "form giving") because of their ability to specify cell fates (activate genes) in a concentration dependent manner. Thus, by the process of long range signaling distinct cell fates can be induced over several cell diameters in a developmentally competent field.

1.2 Competence of cells

Patterning can be induced only when two different components of the system are present. First component is the inducing cells (tissue) that produce a signal(s) and the second component is the responding cells (tissue) which have an ability to react to the inductive signal(s). Competence is the ability to respond to specific inductive signal (Waddington, 1940; Stern, 2000- in this article Waddington's contributions to developmental biology are described). It is an essential pre-requisite of the responding cells. Competence is not a passive state but an actively acquired condition. It may depend on, for example, the presence of appropriate receptor and transducing mechanism or on the presence of particular transcription factor(s) needed for gene activation. For example, in the developing mammalian eye, Pax6 protein appears to be important in making the ectoderm competent to respond to the inductive signal from the optic vesicle. Pax6 is expressed only in the head ectoderm which can respond to signals from the optic vesicle by inducing the lens (Li et al., 1994). The final proof for Pax6 acting as a competence factor was demonstrated by recombination experiments using embryonic rat eye tissue. When the head

ectoderm from Pax-6 mutant rat embryos was combined with wild type optic vesicle no lenses were formed, however lens tissue was formed when the head ectoderm was derived from wild type embryos even though optic vesicles were obtained from Pax6 mutant embryos (Fujiwara et al., 1994).

1.3 Drosophila oogenesis

Drosophila oogenesis involves co-coordinated development of the germ cells and the overlying follicular epithelium. The follicle cells provide a genetically tractable system to investigate the process of patterning and morphogenesis. Both long range and short range cell-cell communications pattern the domains of follicle cells that will create specific eggshell structures. Hence, the *Drosophila* ovary provides an excellent system for the study of epithelial patterning.

1.3.1 Egg chamber formation

The mature *Drosophila* egg is an amazingly complex cell. It is over 0.5 mm long, contains patterning information to establish the anterior-posterior (AP) and dorsal-ventral (DV) axes of the embryo and bears a highly complex eggshell that facilitates embryonic development in harsh external environment. This highly sophisticated egg develops from a discrete structure called the "egg chamber" (King, 1970; Spradling, 1993). Egg chambers are formed at the anterior tip of the ovary and move posteriorly as they develop. They are connected via stalk cells to form a linear array called the "ovariole". Each ovary contains on an average 16-17 ovarioles. The ovariole is the functional unit of the ovary. The whole process of oogenesis takes about 79 hours at 25^0C and is divided into various stages based on morphological details (Figure 1A, B).

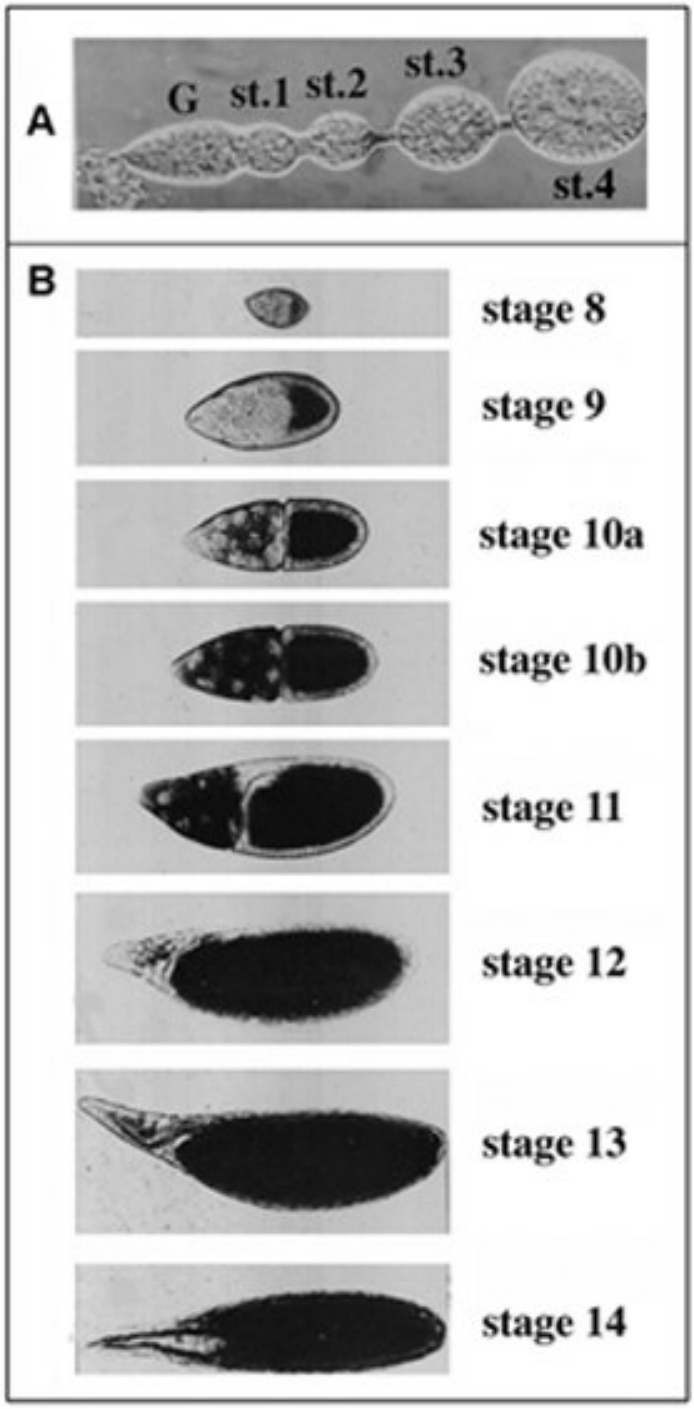

Figure 1

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 except in stage 14 where it is facing the viewer. The developing oocyte can be seen here as an opaque structure (the posterior half) at stage 9 onwards. Follicular epithelium surrounds the ooctye and can be seen as a transperant layer around the oocyte. Adapted from King (1970).

The egg chamber forms from two distinct cell types: germ cells and somatic cells. The germ cells form the developing oocyte and 15 nurse cells while the somatic cells form a monolayer epithelium surrounding the germ cells. The germarium, present at the anterior tip of the ovariole, is an assembly line in which new egg chambers are produced from progeny of germline and somatic stem cells. Based on morphological differentiation, the germarium is subdivided into three regions: region 1, 2 and 3 (Spradling, 1993). The process of formation of the egg chamber starts in region 1 of the germarium where each stem cell divides to form a new stem cell and a cystoblast. The cystoblast undergoes four incomplete divisions to form a syncytial cyst containing 16 cells. These cells are interconnected by cytoplasmic bridges called the "ring canals". As the cyst moves posteriorly in the germarium, one of the cells is selected as an oocyte while the remaining 15 cells become nurse cells. The follicle stem cells reside in region 2a of the germarium and encase the cyst in region 2b to form a monolayer epithelium (Margolis and Spradling, 1995).

As the newly formed egg chamber moves from region 2b to region 3 of the germarium, the developing oocyte acquires an asymmetric localization within the cyst and its localization provides first co-ordinates for the anterior-posterior axis. Thus, the oocyte acquires posterior position in the newly formed egg chamber (Spradling, 1993; Huynh and St Johnston, 2004). The synthetically active nurse cells continuously transfer their contents via the ring canals to the transcriptionally silent oocyte during oogenesis. At the end of oogenesis, the nurse cells rapidly transfer their contents into the oocyte in a process known as "nurse cell dumping". As a result, the nurse cells are reduced to a small group of apoptotic nuclei at the anterior end of a large oocyte.

The follicle cells proliferate untill stage 6 of oogenesis to a number of around 1000 and stop dividing. At this stage of oogenesis, the follicle cells start migrating towards the posterior of the egg chamber. This leaves the anterior end of the egg chamber with very few follicle cells which become stretched to cover the nurse cells, while the follicle cells covering the oocyte, the "main body follicle cells", become columnar in shape. At the same time a small group of follicle cells, called the "border cells", migrate from the anterior tip of the egg

chamber to the anterior tip of the growing oocyte. At the end of oogenesis these cells form the channel for sperm entry called the “micropyle”. Finally, at stage 10 of oogenesis a group of follicle cells migrate anteriorly in between the oocyte and the nurse cells called the “centripetally migrating follicle cells” (CMFCs).

During stage 7 till 10, the follicle cells undergo endoreduplication and become polyploid and it is at this time transcription of genes for forming the chorion starts. Two large groups of follicle cells lay down respiratory appendages, called “the dorsal appendages”, which are positioned at the dorsal anterior of the egg. Each dorsal filament is formed by a group of follicle cells (50-55 cells; Roth, 1999; James and Berg, 2003) that migrate over the anterior part of the oocyte at stage 11. Imprints of the follicle cells are visible on the mature egg. Several structures on the chorion allow orientation of the axes of the egg. The anterior end bears the micropyle and operculum while the posterior end is marked by an aeropyle. Figure 2 shows wild type (wt) *Drosophila* egg.

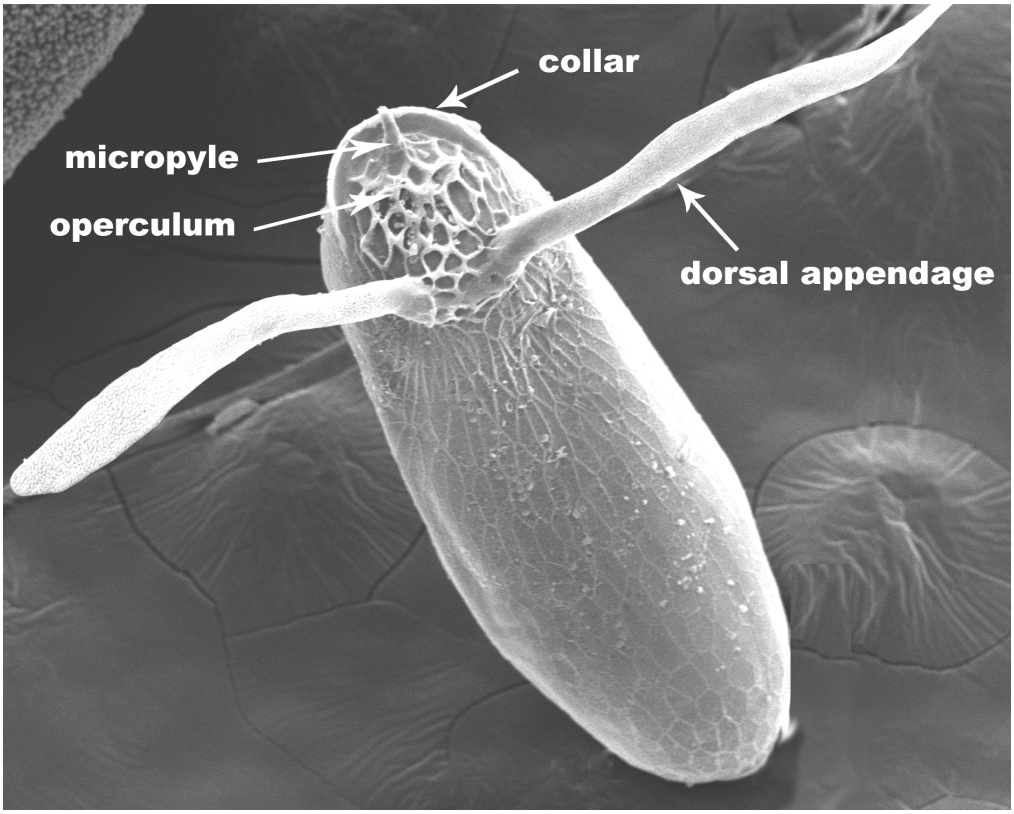

Figure 2

The *Drosophila* egg

A Scanning Electron Microscope (SEM) image of a wildtype *Drosophila* egg (dorsal view). The anteriorly located micropyle, operculum and dorsal appendages can clearly be seen. The dorsal boundary of the operculum is limited by a collar.

1.4 Patterning of the follicular epithelium

In Drosophila, patterning of the follicular epithelium covering the developing oocyte is achieved by inductive signaling. Two major signal pathways converge to induce a subpopulation of dorsal anterior follicle cells to adopt cell fates which give rise to the dorsal appendages and the operculum (Dobens and Raftery, 2000; Peri and Roth, 2000; Roth, 2003; Berg, 2005; Horne-Badovinac and Bilder, 2005). One of the signals is initiated by the EGF signaling pathway. The EGF/TGF-α (Transforming Growth Factor-α) like ligand Gurken signals twice from the developing oocyte to the overlying follicle cells at stage 5-6 in order to specify first, posterior cell fates and subsequently dorsal cell fates at stage 9-10 (Gonzalez-Reyes et al., 1995; Roth et al., 1995; Nilson and

Schupbach, 1999; Roth, 2003). Following the first Grk signal, a cytoskeletal rearrangement triggers the migration of the oocyte nucleus towards the anterior of the oocyte, where it lies at an asymmetric position (Koch and Spitzer, 1983; Peri and Roth, 2000). Grk, which is tightly associated with the oocyte nucleus, signals to the adjacent follicle cells inducing them to take on dorsal fates (Schupbach, 1987). The doralizing Grk signal has two effects on the follicle cells. First, it restricts *pipe* to a ventral stripe in the follicular epithelium (Sen et al., 1998; Peri et al., 2002). This event is essential for DV patterning of the embryo (Nilson and Schupbach, 1999; van Eeden and St Johnston, 1999; Peri et al., 2002; Roth, 2003). Second, it induces dorsal anterior follicle cells to produce operculum and dorsal appendages. Dorsal appendages are derived from two dorsal patches of *Broad-Complex* (*BR-C*) expressing cells (Deng and Bownes, 1997). The characteristic pattern and placement of dorsal appendages along the DV axis is suggested to be established by an intricate mechanism involving signal amplification by two genes: *rhomboid* (*rho*) and *spitz* (*spi*) and feedback inhibition by *argos* (*aos*) (Wasserman and Freeman, 1998; Shvartsman et al., 2002). This aspect is discussed in detail in later sections.

The restriction of *pipe* to the ventral follicle cells occurs along the entire AP axis. However, the operculum and dorsal appendages form only at the anterior. In addition, Queenan et al. (1997) also found that the expression of the dorsal genes: *rho* and *aos* is still confined to the anterior half when an activated form of the EGFR (λ*top*) was misexpressed in the whole follicular epithelium. How is the response to the second Grk signal localized? It is suggested that formation of the operculum and dorsal appendages require a combination of both dorsalizing Grk and a second anterior signal provided by Dpp, one of the TGF-β family member in *Drosophila* (Deng and Bownes, 1997; Queenan et al., 1997; Peri and Roth, 2000). *dpp* expression can be first detected in the follicle cells present at the anteriormost tip of the egg chamber at stage 8 of oogenesis (Twombly et al., 1996; Dobens and Raftery, 2000; Peri and Roth, 2000). These cells start migration at stage 9 of oogenesis towards the posterior to cover the developing oocyte. In late oogenesis, *dpp* is associated with the stretched follicle cells and the CMFCs. Partial loss of function alleles of *dpp* and/or its components lead to production of eggs which exhibit extremely reduced

opercula and dorsal appendages (Twombly et al., 1996). Conversely, eggs produced by females ubiquitously expressing *dpp* display expanded anterior eggshell structures (Twombly et al., 1996; Dobens et al., 1997; Dobens and Raftery, 2000; Gupta and Schupbach, 2003). Based on these findings, Dpp is suggested to function in two different ways. First, it is suggested to form a morphogen gradient in the follicular epithelium along the AP axis (Twombly et al., 1996; Peri and Roth, 2000). Second, Dpp is suggested to induce competence in the anterior subpopulation of main body follicle cells so that they are able to respond to the dorsalizing Grk signal (Peri and Roth, 2000). In addtition, Dpp is suggested to define the boundary between operculum and dorsal appendages (Twombly et al., 1996; Dobens et al., 1997). We would like to address these questions and define the molecular role of Dpp in the follicular epithelium.

Peri and Roth (2000) showed that the combined activation of EGFR and Dpp signaling cascades in a posterior subpopulation of main body follicle cells leads to induction of dorsal appendages at the posterior of the egg. Moreover, induction of *rho* and *BR-C* was observed at the posterior in such egg chambers. This proved that in addition to Grk, *rho* needs Dpp for its expression (Peri and Roth, 2000). Thus, how the Dpp and EGFR activities specify domains (distinct cell populations) along the AP and DV axis in the follicular epithelium needs to be clarified.

1.5 Genes affecting patterning of the follicular epithelium

The specification of dorsal appendages and their subsequent morphogenesis requires induction and interaction of several different signaling pathways within the follicular epithelium. Thus, several genes control the specification of DV pattern in the follicle cells (Spradling, 1993). Many genetic screens have identified mutants which affect the patterning process. Indeed, over five-dozen genes which affect the dorsal appendage patterning have been identified so far (Table 1; reviewed in Berg, 2005).

Dorsoventral patterning mutations are essentially of three general types – ventralizing (e.g. *grk*), dorsalizing (e.g. *K10*) and midline-minus (e.g. *pointed*). All genes in these three classes are required to establish correct cell fate and/or placement of the dorsal appendages. Loss of Grk signal ventralizes the egg, producing elongated eggs lacking dorsal structures. Null alleles of *grk* produce eggs which have a micropyle at both the termini indicating that Grk signaling is needed for establishing posterior cell fates (Gonzalez-Reyes et al., 1995; Roth et al., 1995). In fact, the correct dosage of Grk is needed to induce two dorsal appendages. Hypomorphic alleles of *grk* lead to production of eggs with single thin dorsal appendage (Schupbach and Wieschaus, 1991; Neuman-Silberberg and Schupbach, 1994). The dorsalized egg phenotype can result from mutations in genes affecting several aspects of Grk signaling i.e. mislocalization of *grk* mRNA during oogenesis or loss of inhibitory control on the signaling cascade. *K10* encodes for DNA binding protein and has been shown to restrict localization of *grk* to the dorsal anterior corner of the oocyte, close to the oocyte nucleus (Prost et al., 1988). Midline minus mutation(s) produces eggs with single broad dorsal appendage. This phenotype results from defects in genes required for modulating Grk signaling such as *spitz* or *pointed* (Golembo et al., 1996b; Wasserman and Freeman, 1998).

The genes listed in Table 1 belong to and/or are regulated by several signaling cascades including TGF-β, EGF and Notch signaling pathways. The exact mechanisms that achieve the patterning, the identity of all molecules and the role that each gene has in patterning remain unclear. Knowledge of their molecular role and their interactions with each other would not only help us understand the patterning of follicular epithelium but also on larger scale aid us in understanding their roles in relation to one another in development of several model organisms.

A detailed description and illustration of Dpp and Grk pathways in *Drosophila* is described in later sections.

Table 1

Genes involved in dorsal appendage patterning

Genes marked with an asterick (*) were used for mutant studies and/or for expression analysis in this work. Table Adapted and modified from Berg, CA (2005).

VENTRALIZING	DORSALIZING
aubergine	*capicua*
beadex	*cappuccino*
blistered	*Cbl*
cactus	*Cf2*
cap-n-collar	***Fs(1)K10*** *
COP9 complex homolog subunit 5 (CSN5)	*GTPase-activating protein 1*
cornichon *	*Heterogenous nuclear ribonucleoprotein at 27C*
corkscrew	***kekkon-1*** *
dodo	*Lamin*
DP transcription factor	*ovarian tumor*
Downstream of Raf 1 (MEK1)	*pipsqueak*
encore	*half pint*
Epidermal growth factor receptor (Egfr)	*rhino*
gurken *	***short gastrulation*** *
gustavus	*spire*
Kinesin heavy chain	*sprouty*
licorne	*squid*
Lissencephaly-1	
maelstrom	**MIDLINE MINUS**
magonashi	***argos*** *
Multiple ankyrin repeats single KH domain	*brainiac*
Merlin	*egghead*
Misexpression suppressor of KSR2	*fringe*
mirror	*Notch*
modifier of mdg4	*pointed*
spindle-c	*spitz*
okra	*toucan*
orb	*vein*
pka-C1	
pole hole	**ANTERIOR**
Ras oncogene at 85D	*bunched*
rhomboid *	*CTP-phophocoline cytidylyltranferase 1*
Sec61B	***decapentaplegic*** *
SHC-adaptor protein	*Myocyte enhancing factor 2*
spindle-A	*Mothers against Dpp*
spindle-B	*saxophone*
spindle-D	*Stat92E*
spindle-E	***thick vein*** *
Star	*ultraspiracle*
tsunagi	***Medea*** *
vasa	

1.6 EGF signaling cascade

The evolutionary conserved receptor tyrosine kinase (RTK) pathway is extensively used intercellular signal transduction mechanism that regulates cell fate specification, proliferation, migration and survival (Moghal and Sternberg, 1999). RTKs comprise a large family of integral membrane proteins with highly divergent extracellular domains coupled to a conserved intracellular protein tyrosine kinase motif (Freeman, 1998; Schlessinger, 2000). RTKs can be activated by several classes of ligands one of them being EGF. Ligand binding induces dimerization of RTKs which results in both auto and trans phosphorylation of specific tyrosine residues on the C-terminal cytoplasmic tail of the receptor (Yarden and Ullrich, 1988; Schlessinger, 2000). This modification generates docking site for phosphotyrosine binding adaptor proteins (PTB) known as (DRK) Downstream of Receptor Kinase (vertebrate homologue is designated as Grb; Lowenstein et al., 1992; Olivier et al., 1993; Simon et al., 1993). DRK recruits the guanidine nucleotide exchange factor which activates the GTPase-Ras by catalyzing the exchange of GDP for GTP. Once Ras is active, it recruits and activates the serine/theronine kinase Raf and many other cytoplasmic components (Kolch, 2000; Avruch et al., 2001). Activated Raf phosphorylates and activates MEK, a dual specificity kinase which phosphorylates tyrosine and serine/threonine residues (also called Mitogen Activated Protein Kinase Kinase-MAPKK). Activated MAPKK in turn phosphorylates and activates MAPK which translocates to the nucleus where it phosphorylates and modulates the activity of specific transcription factors (Marshall, 1994; Kolch, 2000).

The *Drosophila* EGF signaling cascade is depicted in Figure 3. In *Drosophila*, only a single gene has been found to encode a homologue of the vertebrate EGF receptor called “EGFR” (Wadsworth et al., 1985; Schejter et al., 1986). EGFR is used several times during *Drosophila* development and has separate roles in oogenesis, embryogenesis and imaginal disc development (Schweitzer and Shilo, 1997). *Drosophila* EGFR can be activated by four different ligands Spi, Keren (Krn), Grk and Vein (Vn) (Shilo, 2003). Three of the ligands namely Spi, Krn and Grk, belonging to EGF/TGF-α family, are produced as transmembrane

precursors. The primary activating ligand Spi is responsible for EGFR activation in most tissues (Rutledge et al., 1992). A ligand structurally related to Spi has been recently identified and termed as Krn (Reich and Shilo, 2002; Urban et al., 2002). Krn can substitute Spi in most tissues during development. Grk, a third TGF-α homologue, is restricted to the activation of EGFR in the follicle cells of the ovary (Neuman-Silberberg and Schupbach, 1993; Gonzalez-Reyes et al., 1995; Roth et al., 1995). Vn, a secreted ligand similar to neuregulins, is used in tissues where low activation of EGF pathway is needed (Schnepp et al., 1996). It functions as a main ligand in some tissues. For example, Vn induces several distal cell fates in the leg imaginal discs (Schnepp et al., 1998). Spi, Krn and Grk have to be processed to an active form by proteolysis. A clue to the mechanism regulating Spi processing emerged from the identification of mutations in two genes, *Star* and *rho*, that gave rise to phenotypes surprisingly similar to *spi* (Mayer and Nusslein-Volhard, 1988). Star, a novel transmembrane protein was shown to act as a cargo receptor in trafficking Spi from the endoplasmic reticulum to Golgi (Lee et al., 2001). Once reaching the Golgi, Spi is proteolytically cleaved by Rho. This releases Spi from the membrane and thus is able to activate the EGFR once it reaches the plasma membrane. Spi and Star are broadly expressed while the expression of Rho is tightly regulated and is dynamic. It thus appears that expression of Rho is the limiting step in EGFR activation. This was demonstrated experimentally. Ectopically expressing *rho* in diverse tissues and contexts is sufficient to increase the level of EGFR activation (Golembo et al., 1996a). Similarly, increased levels of Grk lead to increase in the level of EGFR activation (Ghiglione et al., 2002).

EGFR signaling can occur over several cell diameters due to secretion of diffusible ligands. This has been very well demonstrated in case of patterning of the embryonic ventral ectoderm by Spi and induction of the leg segments by Vn (Schweitzer et al., 1995b; Golembo et al., 1996a; Campbell, 2002). In both these cases the ligands are believed to function as morphogens inducing more than one cell fates depending upon the concentration of the ligand.

EGFR activation is regulated at the extracellular, the receptor and the intracellular level by various inhibitors and repressors which constitute negative feedback loops (for a recent review see Shilo, 2005). At the extracellular level, Aos is the primary inhibitor and is expressed at highest levels of EGF signaling. Aos was believed to function by competing with the activating ligands to bind to the EGF receptor (Freeman et al., 1992; Schweitzer et al., 1995a; Jin et al., 2000). However, Klein and colleagues (2004) proved that Aos inhibits EGFR signaling without interacting with the receptor directly but instead by sequestering the EGFR activating ligand Spi. Aos binds tightly to the EGF motif of Spi and forms (1:1) Spi: Aos complex that does not bind to the EGFR in vitro or at the cell surface (Klein et al., 2004). Aos is a secreted protein and hence can inhibit EGFR signaling several diameters away from its site of synthesis. Kekkon (Kek) is a transmembrane protein that binds the EGFR extracellular domain and attenuates receptor dimerizaton (Ghiglione et al., 1999). Another inhibitor Sprouty (Sty), an intracellular protein, acts by intercepting essential elements of the Ras/MAPK cascade through diverse mechanisms (Casci et al., 1999; Kim and Bar-Sagi, 2004). Both Kek and Sty act cell autonomously and in a context dependent way. At the transcriptional level, the EGFR target genes can be repressed in the nucleus by Pointed (Pnt), an ETS-domain transcriptional repressor (Gabay et al., 1996; Morimoto et al., 1996). Expression of D-Cbl, an E3 ubiquitin ligase, was shown to be critical for attenuating EGF signaling in the follicle cells. D-Cbl ubiquitinates the activated EGFR and targets it to the proteosome for subsequent degradation (Pai et al., 2000).

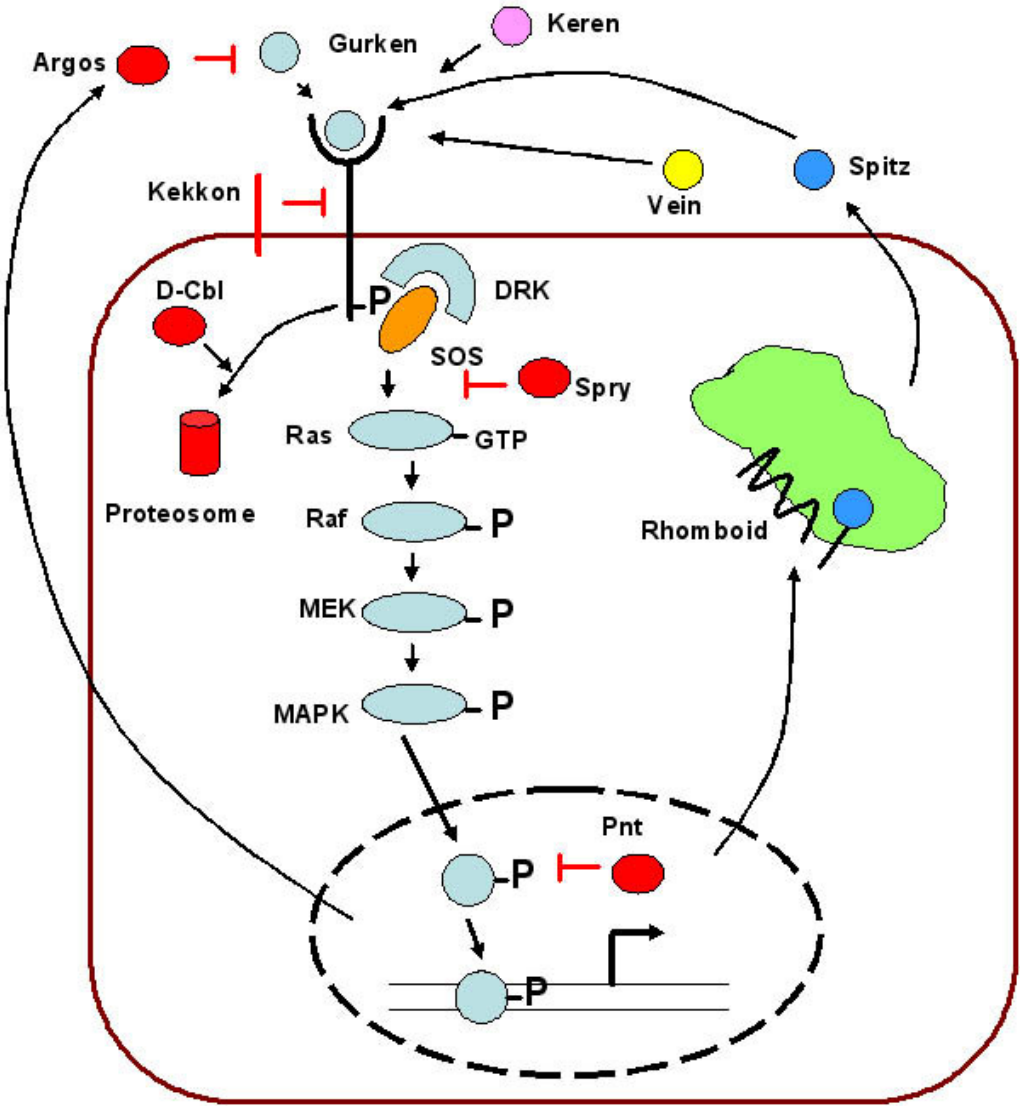

Figure 3

The EGF signaling pathway in *Drosophila*

Binding of the any of the four ligands- Spi, Grk, Vn, Krn to the EGFR induces Drk binding to the phosphorylated receptor and to Sos (Son of Sevenless). Sos stimulates GTP/GDP exchange on the G protein Ras. Activated Ras interacts with the effector protein Raf which phosphorylates MAP-kinase-kinase (MAPKK, MEK; D-sor in *Drosophila*). As a result, MAPKK activates MAPK (ERK, rolled in *Drosophila*); MAPK can phosphorylate several other molecules in the nucleus which control gene activation or repression. Rho is expressed in response to EGFR activation. Rho processes Spi in the Golgi (shown in green) leading to positive feedback loop on pathway activation. Induction of Aos in turn downregulates the signal thus forming a negative feedback loop. D-Cbl targets the phosphorylated EGFR to the proteosome. In addition, Sty and Pnt (in the nucleus) can inhibit EGFR signaling. Positive inputs are represented with arrows while inhibitory inputs are depicted by a bar. Nucleus is depicted as a oval shaped structure (dotted black oval). P denotes phosphorylation. Black arrow in the nucleus represents the direction of transcription.

1.7 Model for patterning of dorsal appendages

Based on the sequence of EGFR mediated signaling events an elegant model for patterning of the dorsal appendages was proposed by Wasserman and Freeman in 1998 (Figure 4). The model is described as following. At stage 8/9 Grk, localized close to the oocyte nucleus, initiates signaling to the overlying follicle cells. Grk signal induces expression of *rho* in a dorsoanterior patch of follicle cells where it activates Spi by proteolytic processing (Urban et al., 2001). Cleavage of Spi leads to an increase in EGFR signaling visualized by increase in MAP kinase activation in width and amplitude. At stage 10, the vitelline membrane is secreted around the developing oocyte and therefore may prevent further signaling from the oocyte to the follicle cells. Vn also contributes to this autocrine amplification of EGFR signaling. The Rho/Spi/Vn amplification induces Aos expression in dorsal midline cells at stage 10, leading to local inhibition of signaling at the dorsal midline. The resulting signaling profile has twin peaks that eventually specify the position of the dorsal appendages.

Although the model integrates both positive and negative feedback loops involved in patterning, there are several drawbacks. First, the clonal analysis of genes involved in patterning namely *rho*, *spi* and *aos* was performed in unmarked clones. Only the resulting eggshell phenotype was observed as readout of the patterning processes (Wasserman and Freeman, 1998). Second, there is a disparity between the results obtained by Wasserman and Freeman (1998) who propose that dorsal appendages are formed at highest levels of MAPK signaling and Peri et al. (1999) who suggest that dorsal appendages are formed at intermediate levels of MAPK signaling. Finally, recent biochemical studies have revealed that Aos associates predominantly with Spi, to form nonfunctional heterodimers (Klein et al., 2004). Therefore, Aos might allow other EGF ligands namely Vn to diffuse readily and allow a lower level of MAPK activation. We have addressed these aspects to better define the role of Rho and Aos mediated feedback loops on patterning aspects

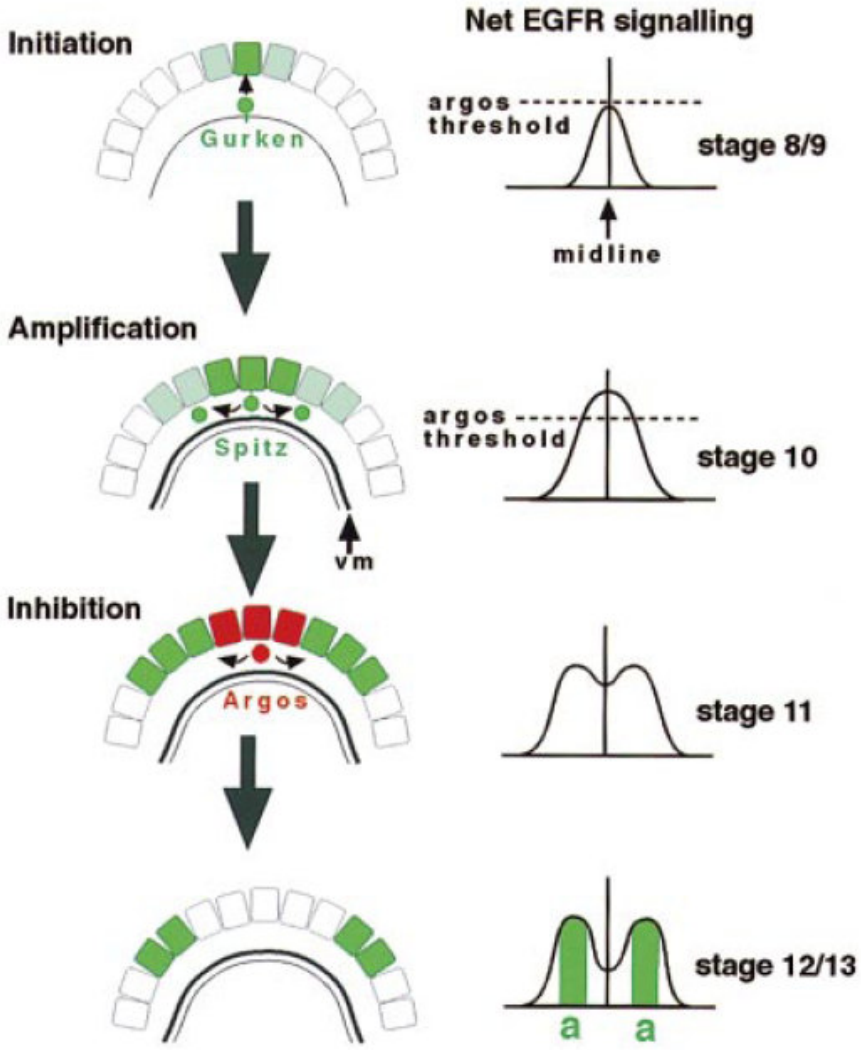

Figure 4

Dorsal patterning of the egg is a three stage process

Left panel shows representative cross section through developing oocyte at different developmental stages. Dorsal side is up. Right panel represents the net EGFR signaling (MAP kinase activity) at the respective stages. See text for more details. Adapted from Wasserman and Freeman (1998).

1.8 TGF–β signaling cascade

The TGF–β signaling cascade regulates numerous cellular responses such as proliferation, differentiation, migration and apoptosis (reviewed in Massague, 1998; Massague et al., 2000). The basic molecular mechanism of TGF–β signaling pathway has been unravelled (reviewed in Attisano and Wrana, 1998; Derynck and Zhang, 2003; Shi and Massague, 2003). TGF–β related ligands bind to a heteromeric complex of type I and type II transmembrane serine threonine kinases. Ligand binding triggers association between the receptors, enabling the constitutively active type II kinase to phosphorylate the type I receptor within a specific domain termed the GS (glycine and serine rich) domain. The activated type I receptor initiates pathway-specific (BMP specific or TGF-–β/Activin specific) signaling by phosphorylating pathway-specific receptor-regulated Smad (R-Smad) family of cytoplasmic signal transduction protein. Activated R-Smads associate with Smad4, a common Smad (Co-Smad) shared by both the pathways. The heteromeric R-Smad/Co-Smad complex translocates to the nucleus and associates with DNA–binding cofactors to directly activate or repress target gene expression. Smad4 is obligately required for most cellular responses; however, in few cases signaling has been reported to occur in absence of Co-Smad (Wisotzkey et al., 1998; Sirard et al., 2000). Both R-Smads and Co-Smads contain highly conserved Mad homology domain (MH1 and MH2) domain separated by a linker region. The MH2 domain of R-Smads has an additional SSXS motif which is substrate for phosphorylation by the type I receptor kinase (Wrana, 2000).

The *Drosophila* TGF–β cascade is depicted in Figure 5. Seven TGF–β related ligands have been identified in *Drosophila* through a combination of molecular genetics and genome annotation techniques (Raftery et al., 1995; Raftery and Sutherland, 1999). Three of the ligands belong to the BMP subfamily while one belongs to the TGF–β/Activin subfamily. The molecular details of ligands Maverick (Mav), Activin like protein (Alp) and Myoglianin (Myo) are still unknown and therefore they are designated as orphan ligands (Raftery and Sutherland, 1999; Parker et al., 2004). Dpp represents the BMP2/4 ortholog in

flies, while Screw (Scw) and Glass bottom boat (Gbb/60A) are related to BMP5/6/7/8 (reviewed in Parker et al., 2004).

Besides its role in specification of embryonic axes and patterning of imaginal discs, Dpp signaling is also essential for *Drosophila* oogenesis (Spencer et al., 1982; Padgett et al., 1987; Twombly et al., 1996). One of the striking properties of Dpp is its ability to function as a morphogen thereby inducing multiple cell fates in a concentration dependent manner (Tabata, 2001; Raftery and Sutherland, 2003). How is a stable gradient of Dpp established and interpreted is one of the key issues in *Drosophila* development? Dpp is suggested to form gradient by at least two different mechanisms in *Drosophila*. One is dependent on long range diffusion of Dpp from its site of synthesis, while the second involves diffusion of extracellular inhibitor, called Short gastrulation (Sog), into the domain of Dpp expression thus resulting in a gradient of signaling activity (Srinivasan et al., 2002; Raftery and Sutherland, 2003). The role of Sog in gradient formation is described in later sections.

Dpp acts as a long range morphogen in the wing imaginal disc (Tabata, 2001). *dpp* is transcribed in a narrow stripe of cells at the AP compartment boundary of the wing disc where it can induce transcription of target genes over 20 cell diameters. The Dpp-responsive genes *vestigial* (*vg*), *optomotor blind* (*omb*) and *spalt* (*sal*) are activated at different signaling thresholds which subsequently subdivide the wing disc into distinct regions (Nellen et al., 1996). Biologically active GFP-tagged Dpp has been visualized to form a gradient in either direction from its site of synthesis in the wing disc (Entchev et al., 2000; Teleman and Cohen, 2000). In the embryo, Dpp is required to induce different cell fates in the dorsal region. High levels of Dpp specify the amnioserosa while lower levels specify the dorsal ectoderm (Raftery and Sutherland, 2003).

In *Drosophila*, only two type II receptors, Punt (Put) and Wishful thinking (Wit) and three type I receptors Thickveins (Tkv), Saxophone (Sax) and Baboon (Babo) have been described (Brummel et al., 1994; Penton et al., 1994; Xie et al., 1994; Letsou et al., 1995; Aberle et al., 2002; Parker et al., 2004). In vertebrates, so far a dozen receptors (I and II combined) and up to 29 ligands

have been identified (for a recent review see Feng and Derynck, 2005). Ligand selectively is determined by the type I receptor in BMP pathway. In contrast, in TGF−β/Activin pathways, ligand selectively is directed primarily by the type II receptor (Parker et al., 2004; Feng and Derynck, 2005). Moreover, R-Smads, the substrates for type I receptor, show pathway specificity. Smads 1, 5 and 8 are specific for BMP signaling while Smad 2 and Smad 3 show specificity towards Activins/TGF−βs mediated signaling (Miyazawa et al., 2002). In *Drosophila*, Mothers against Dpp (Mad) is the only Dpp/BMP-specific R-Smad (Sekelsky et al., 1995) while Smox/dSmad2 (Smad on 'X' chromosome) has been reported to be specific to Activin signaling (Henderson and Andrew, 1998). Only one member of Co-Smad has been identified in each model system, namely Smad4 (also called DPC4) in vertebrates (Lagna et al., 1996), *Medea* in *Drosophila* (Raftery et al., 1995; 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 are required for the formation of functional transcriptional complexes (Parker et al., 2004).

Once translocated to the nucleus, the Smad complex modulates TGF−β target gene expression by binding to Smad binding elements (SBE) via their MH1 domain (Feng and Derynck, 2005). Smad3 MH1 domain binds 5'-GTCT sequence with a higher affinity while Smad1 (Mad) binds to 5'-GCCG (Kim et al., 1997; Shi et al., 1998; Korchynskyi and ten Dijke, 2002). Several target genes of TGF- β have been shown to harbor SBEs in their 5' promoter regions (Jonk et al., 1998; Attisano and Wrana, 2000).

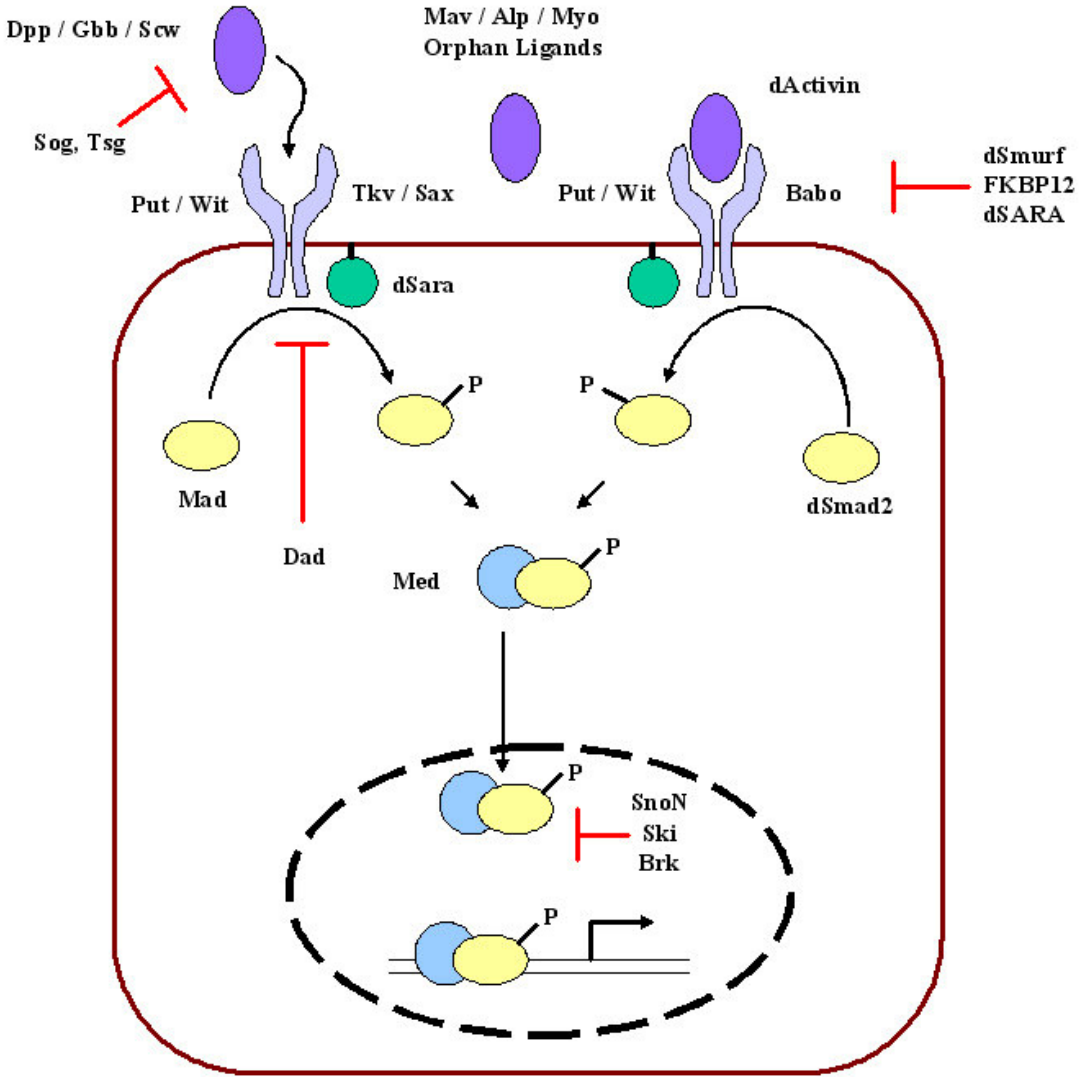

Figure 5

The BMP and Activin signaling pathway in *Drosophila*

The BMP ligands Dpp, Gbb and Scw, act through the type I receptors Tkv and Sax, resulting in phosphorylation of Mad, its association with the co-Smad Med, translocation of the complex into the nucleus and regulation of target gene expression. The type II receptors Put and Wit display dual specificity and function in both BMP and Activin pathways. Activin pathway that involves the type I receptor Babo and dSmad2 has been identified in *Drosophila*. Only one ligand, dActivin, has been shown to signal through Baboon (Babo) in *Drosophila*. It is unknown if Mav, Alp and Myo activate BMP or the Activin pathway. dSmurf, FKBP12, dSara function intracellularly; here they are depicted extracellularly due to space constraints. Positive inputs are represented with arrows while inhibitory inputs are depicted by a red bar. Nucleus is depicted as a oval shaped structure (dotted black oval). P denotes phosphorylation. Black arrow in the nucleus represents the direction of transcription.

1.8.1 Regulation of TGF- β signaling

TGF−β signaling is modulated by several molecules at the extracellular level. These molecules are primarily secreted proteins which result in alteration of ligand-receptor binding, ligand diffusion and/or the local ligand concentration. In *Drosophila,* the function of several proteins has been identified i.e Sog, Tsg (Twisted gastrulation) and Tolloid (tld). Sog, Tsg and Tld function by a unique mechanism to promote Dpp signaling in the dorsal most region of the embryo (Shimell et al., 1991; Francois et al., 1994; Mason et al., 1994). Sog forms a ventral to dorsal gradient in the embryo (Ashe, 2002; Srinivasan et al., 2002). Sog binds Dpp thus inhibiting its function and simultaneously transports it to the dorsalmost region of the embryo alone or together with Tsg (Mason et al., 1994; Srinivasan et al., 2002). Dpp is released in the dorsal region where Tld, a metalloprotease, cleaves Sog from the Dpp-Sog-Tsg complex (Shimell et al., 1991). Thus, the Dpp gradient is formed post-transcriptionally through modulation of ligand distribution. Activins/TGF−βs are proposed to be regulated by follistatins at the extracellular level which function by masking the ability of ligands to associate with the receptor (Keutmann et al., 2004).

sog has a dynamic expression pattern in the follicle cells during oogenesis (Araujo and Bier, 2000). At stage 10 of oogenesis *sog* is expressed in the CMFCs. It is not known if Sog has a function during oogenesis and regulates Dpp activity by a similar mechanism as described above.

At the receptor level, several proteins have been demonstrated to regulate TGF−β signaling. Smad anchor for receptor activation (Sara) also known as dSara in *Drosophila*, is a membrane associated protein that regulates R-Smad recruitment to the receptor (Bennett and Alphey, 2002). A third class of Smads called I-Smads (I for inhibitory) have been shown to inhibit Dpp signaling in the wing imaginal discs (Tsuneizumi et al., 1997). Daughters against Dpp (Dad) is the only known I-Smad in flies and functions by either occupying the Mad binding site at the type I receptor or by competing with Med for oligomerization with R-Smad (Hayashi et al., 1997; Tsuneizumi et al., 1997; Hata et al., 2000). The TGF−β pathway is also regulated by the ubiquitin mediated degradation.

Two E3 ubiquitin ligases Smurf1 and Smurf2 have been shown to target R-Smads, the type I receptor and SnoN, a transcriptional repressor, for ubiquitination and subsequent degradation in the proteosome (Zhu et al., 1999; Bonni et al., 2001; Zhang et al., 2002).

Smads bind to DNA with weak affinity and hence they associate with other DNA–binding proteins for pathway specific gene activation or repression (Massague and Wotton, 2000). Smads can activate target genes by recruiting coactivators of the p300/CBP families to the promoter (Miyazono, 2000). In *Drosophila*, similar activation has been shown between Mad and CBP homolog, Nejire (Takaesu et al., 2002). Smads are also known to associate with several repressors and corepressors. Smad3/4 in combination with E2F-4 and p107 has been demonstrated to bind to a novel sequence element called TGF-β inhibitory element (TIE) in the promoter of *c-myc* gene in vertebrates. This binding represses *c-myc* expression in response to TGF-β (Chen et al., 2002; Frederick et al., 2004). Brinker (Brk) is a nuclear protein that functions as a sequence specific transcriptional repressor of Dpp target genes during embryogenesis and wing imginal disc development (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Jazwinska et al., 1999b). *brk* is expressed during oogenesis in dorsal anterior follicle cells (Peri et al. unpublished). However, its role in regulation Dpp target genes in the follicular epithelium is not known. One of the best characterized corepressors belongs to the Ski/SnoN oncogene family (Liu et al., 2001). A great deal of knowledge has been gathered about the mechanism of Ski/SnoN action from vertebrate model systems. Ski/SnoN homologues are present in *Drosophila* but they have not been characterized either at molecular or functional level.

1.8.2 Ski and Sno oncoproteins

The *v-ski* (viral ski) oncogene was isolated from Sloan-Kettering virus (also known as Avian Leukosis Virus) transformed chicken fibroblasts cells by Ed Stavnezer and named for Sloan Kettering Institute (Stavnezer et al., 1981; Li et al., 1986; Stavnezer et al., 1986). It was the founding member protein of Ski

superfamily of oncoproteins. *c-ski (*cellular ski) proto-oncogene was first isolated from a chicken cDNA library and later found in other vertebrates including Mouse, *Xenopus*, Zebrafish, *Tilapia* (Liu et al., 2001). Recently, a Ski homolog has also been identified in *C. elegans* and termed as Daf-5 (da Graca et al., 2004). The transforming activity of c-Ski proto-oncoprotein was shown to be associated with its nuclear localization (Stavnezer et al., 1989; Sutrave and Hughes, 1989).

A 100 amino acid domain was found conserved in all the Ski proteins identified across different phyla and named as Ski/Sno domain (Figure 6). Recently the crystal structure of the Smad4–binding domain of human c-Ski in complex with the MH2 domain of Smad4 has been solved (Wu et al., 2002). The Smad4–binding fragment of Ski represents a novel class of Cys_2-His_2 type zinc binding module in which these four residues, co-ordinate a bound zinc atom. This structure is highly homologous to the SAND domain named after **S**p100, **A**IRE-1, **N**ucP41/75 and **D**EAF-1 proteins (Bottomley et al., 2001). SAND domain proteins are nuclear proteins that are involved in chromatin dependent transcriptional regulation (Bottomley et al., 2001; Wu et al., 2002).

A related human gene *sno* (for **S**ki related **no**vel) was cloned by low stringency hybridization of the chicken *v-ski* probe to human cDNA libraries (Nomura et al., 1989; Pearson-White, 1993). The *sno* gene, like *ski*, has also been found in vertebrates like chicken, quail, zebrafish and *Xenopus* (Pearson-White, 1993). Sno has also been shown to both transform cells and promote differentiation. However, Sno needed to be expressed at higher levels as compared to Ski (Boyer et al., 1993). Both Sno and Ski share a high homology throughout their entire length of protein sequence. Unlike Ski, Sno is known to form 4 different isoforms by alternative splicing in humans. These isoforms differ by inclusion of different mutually exclusively spliced alternative exon(s) and are named as SnoA (Alu-containing) or SnoN (non Alu-containing) and SnoI (insertion). Functional characterization of SnoA and SnoI has not been performed in vertebrates. Interestingly, Ski is known to form homodimers but preferentially forms heterodimers with SnoN (Cohen et al., 1999). This indicates that they might function together to repress TGF-β target genes.

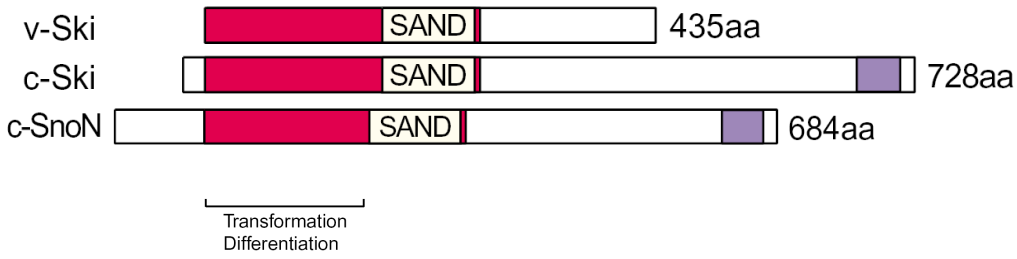

Figure 6

Domain structure of Ski family oncoproteins.

The number of amino acids is indicated to the right. The highly conserved Ski homology region is highlighted in red (including the SAND domain). This region is necessary and sufficient for the transforming and differentiation activities of Ski and SnoN. The C-terminal dimerization domain (highlighted in blue) mediates homo and hetero-dimerization between Ski and SnoN. Adapted from Luo, K (2004)

1.8.3 Mechanism of Ski/SnoN action

Ski and SnoN do not contain any intrinsic catalytic activity and therefore must function through interaction with other cellular proteins (Stavnezer et al., 1989). The mechanism of Ski/Sno action was clarified recently. They have been shown to be important negative regulators of signaling elicited by TGF-βs and BMPs via their interaction with Smad proteins (Luo et al., 1999; Stroschein et al., 1999; Luo, 2004). Ski and SnoN can physically associate with Smad2, Smad3, Smad4 and are recruited to the Smad Binding Element (SBE) present in many TGF–β responsive promoters in a ligand dependent manner (Jonk et al., 1998). Ski uses I-loop in its SAND domain to interact with L3 loop region of Smad4. SAND domain proteins bind DNA through an interaction loop called I-loop (Bottomley et al., 2001). Several amino acid residues in the I-loop of Ski proteins are also conserved in SnoN. Interestingly, the Ski binding surface on Smad4 significantly overlaps with the surface required for interaction with phosphorylated R-Smads (Wu et al., 2002). Thus, by binding to Smad4, Ski and SnoN interfere with formation of active functional R-Smad-Co-Smad complex. Thus, Ski and SnoN function as co-repressors of Smads.

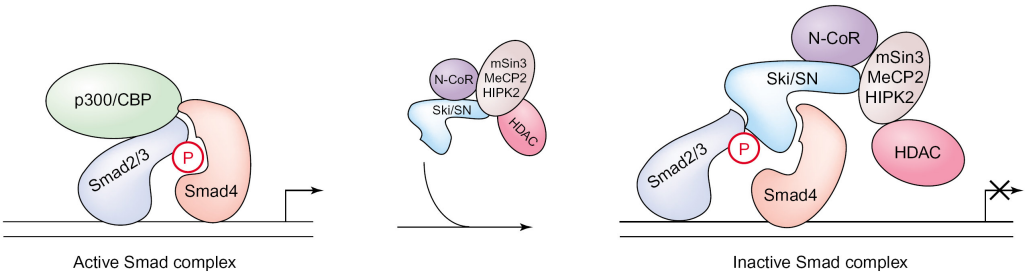

Figure 7

Mechanism of repression of the Smad proteins by Ski and SnoN.

Ski and SnoN can recruit histone deacetylase complex (HDAC) directly or indirectly to DNA via bridging molecules including N-CoR, mSin3 and MeCP2. They induce deacetylation of core histones and thereby repress transcription. Adapted from Luo, K (2004)

Another mechanism by which Ski and SnoN can repress TGF–β signaling is by interacting directly with various transcriptional co-repressors like N-CoR, mSin3A, HIPK2 and methyl-CpG–βinding protein called MeCP2 (Nomura et al., 1999; Tokitou et al., 1999; Kokura et al., 2001; Harada et al., 2003). These co-repressors are found associated with the Histone deactylase complex (HDAC). Thus, by binding to Smads, Ski and SnoN can recruit the HDAC complex to TGF–β target promoters to repress transcription of the target genes (Miyazono, 2000; Luo, 2004). A proposed model for Ski and SnoN action is depicted in Figure 7.

1.8.4 Role of Ski and SnoN in specification and differentiation

The involment of Ski in development was first shown in avian cells, where non-myogenic cells were converted to the myogenic lineage by transfecting chicken fibroblasts with v-*ski* or c-*ski* (Colmenares and Stavnezer, 1989). Following *ski* transfection, induction of muscle specific genes including *myoD* and *myogenin* was observed in the undifferentiated cells. This and other experiments proved that *ski* is a “unique” proto-oncogene which has an ability to increase cell proliferation and simultaneously promoting terminal differentiation (de la

Brousse and Emerson, 1990; Sutrave et al., 1990; Colmenares et al., 1991; Pownall and Emerson, 1992). In, addition, *ski* has an essential role in the development of neuronal and muscle cell lineages. For example, ectopic expression of Ski in *Xenopus* leads to cell-autonomous induction of a secondary neural axis (Amaravadi et al., 1997). In mouse, *ski* is expressed in all normal adult tissues at a low level (Lyons et al., 1994). *ski*-null mice are embryonic lethal (Berk et al., 1997). The embryos show a marked decrease in skeletal muscle mass while transgenic mice overexpressing *ski* display type II skeletal muscle hypertrophy thus confirming its crucial role in proliferation and survival of the muscle and neural crest cell lineages (Sutrave et al., 1990; Berk et al., 1997).

In humans, a genetic syndrome called the "1p36 deletion syndrome" is associated with deletion in the short arm of chromosome 1 at region 1p36 (Shapira et al., 1997; Colmenares et al., 2002). Individuals suffering from this syndrome display craniofacial phenotypes similar to the ones observed in case of *ski*-null mice. The phenotypes include broad forehead, widely separated eyes and ventral midline fusion of the forebrain. Interstingly, *ski* has been mapped to 1p36 region of short arm of chromosome 1 (Colmenares et al., 2002).

SnoN has a very different role inspite of sharing high homology with Ski. *snoN* null mice have been described from two different labs. Shinagawa et al. (2000) showed that replacing *snoN* exon 1 with a neomycin cassette generated a *snoN* null situation. These *snoN*-/- mice die as embryos (Shinagawa et al., 2000). In addition, the authors show that mice heterozygous for *snoN* (+/-) show increased susceptibility to tumors. Another group Pearson-White and McDuffie in 2003 reported two different *snoN* mutant lines of mice. One *snoN*-/- line had a deletion of 5' region of *snoN* which removes *snoN* regulatory sequences. SnoN protein was still detected in this line albeit at a lower level. While the second *snoN*-/- line had a deletion of exon 1 thus generating a molecular null situation. SnoN protein was absent in this *snoN*-/- line. Interestingly, the authors found out to their surprise that *snoN* null line was homozygous viable (Pearson-White and McDuffie, 2003). One of the prominent defects in this knockout line was a defect in T-cell activation (Pearson-White and McDuffie, 2003). Thus, it

is still unclear whether SnoN has an important role in early development in mice. In adult mice, SnoN has been proved to have a vital role in liver regeneration (Macias-Silva et al., 2002). And recently, SnoN has been shown to couple TGF−β signaling to gene expression in the lung epithelial cells (Sarker et al., 2005).

Expression of SnoN, but not Ski is tightly controlled by the TGF−β at the level of both protein stability and transcriptional activation (Stroschein et al., 1999). Immediately after TGF−β stimulation, SnoN is rapidly degraded by the ubiquitin dependent proteosmal pathway (Bonni et al., 2001). Thus, SnoN appears to be specific for TGF-β induced signaling while Ski has been reported to regulate BMP targets in *Xenopus* (Wang et al., 2000).

Ski/Sno domain proteins are largely uncharacterized in *Drosophila*. So far only one gene named *dachshund* (*dac*) has been reported to have Ski/Sno domain, however, it shows poor homology outside the Ski/Sno domain at the amino acid level to c-Ski and c-SnoN (Mardon et al., 1994). Therefore, it is necessary to analyze the *Drosophila* genome for the prescence of additional Ski/Sno genes and characterize their role in development. Interstingly, Dac function is needed in proper cell fate specification in the developing eye and the leg (Miguel-Aliaga et al., 2004). In addition, Dac has been shown to have an important role in axonal guiding (Martini and Davis, 2005).

2. Aim of the research work

The major objectives of this research work were as follows:

I. To elucidate the molecular role of Dpp signaling in patterning of the follicular epithelium.

II. To investigate the interaction between Dpp and Grk signaling in induction of dorsal chorion structures.

III. To decipher the role of Ski family proteins in regulation of Dpp signaling in the follicular epithelium.

IV. To dissect the function and interactions of Dpp inhibitors Sog, Brk, Dad and Ski proteins

V. To understand the role of Rho and Aos in specification of dorsal midline fate.

3. Results

3.1 Dpp forms a gradient along the AP axis in the follicular epithelium

Dpp is known to form a gradient in the wing epithelium and in the embryo. It specifies distinct cell fates in a concentration dependent manner in the embryo and in the developing wing (Wharton et al., 1993; Entchev et al., 2000; Teleman and Cohen, 2000; Wharton et al., 2004). We asked whether Dpp could also form a gradient in the follicular epithelium and therefore function as a patterning molecule. To answer this question it would be necessary to have antibody against Dpp or alternatively to have a tagged version of Dpp transgene (e.g DppGFP) driven by its own promoter. But lack of these tools prevented us from visualizing Dpp gradient directly. Therefore, we decided to follow expression of two components of the Dpp pathway, Med the common signal transducer of the TGF- β cascade in *Drosophila* and *dad* which is a direct target gene of the Dpp cascade (Tsuneizumi et al., 1997; Wisotzkey et al., 1998).

An anti β-galactosidase (anti β-gal) staining was performed on ovaries dissected from females bearing *dad*LacZ transgene shown in Figure 8A-C. Strikingly, *dad*LacZ is expressed in form of a gradient in the follicular epithelium along the AP axis. *dad*LacZ expression was detected first at stage 8 of oogenesis where it is expressed in the follicle cells at the anterior tip of the egg chamber (Figure 8A). The highest intensity of anti β-gal signal was observed in these anterior cells. Towards the posterior the intensity of the staining progressively gets weaker (Figure 8A). At stage 9, *dad*LacZ expression was associated with the posteriorly migrating follicle cells and the stretched follicle cell nuclei (Figure 8B). At stage 10B, *dad*LacZ expression was strongest in the CMFCs and weak expression was observed in two to four follicle cell rows posteriorly (Figure 8C). *dpp*LacZ at these stages is expressed only in a subset of follicle cells expressing *dad*LacZ implying that *dad*LacZ is a sensitive marker of Dpp activity in the follicle cells (Twombly et al., 1996). Thus, *dad*LacZ is expressed even at moderate levels of Dpp signaling (Twombly et al., 1996; Dobens and Raftery, 2000; Casanueva and Ferguson, 2004). Taken

together the above observations suggest that there must be an underlying graded Dpp activity to which *dad*LacZ responds.

Med, the Smad4 homolog in *Drosophila* and is required in patterning of the embryo and the wing imaginal disc (Wisotzkey et al., 1998). Med is localized in the cytoplasm and its localization is not regulated by phosphorylation. For nuclear translocation it requires physical association with phosphorylated Mad (Inoue et al., 1998; Wisotzkey et al., 1998). We asked if translocation of Med in the follicle cell nuclei would be in a graded form (Sutherland et al., 2003). To answer this question we performed anti-Med stainings on wild-type ovaries (Sutherland et al., 2003; Muzzopappa and Wappner, 2005). At stage 8, Med expression was observed to be restricted to the anterior tip of the egg chamber (Figure 8D) similar to expression of *dpp*. At stage 9, Med was detected in the posteriorly migrating follicle cells (Figure 8E and Muzzopappa and Wappner, 2005). At stage 10B, Med was detected in the CMFCs as well as in the stretched follicle cells (Figure 8F and Muzzopappa and Wappner, 2005). In all 3 stages, Med expression was in a broader domain as compared to Dpp expression but narrower than *dad*LacZ expression (Figure 8A-F and Twombly et al., 1996; Dobens and Raftery, 2000).

We decided to analyse more in detail the localization of Med in the follicle cell covering the oocyte (Figure 8G). Med is predominantly nuclear in the CMFCs (Figure 8G') indicating high level of Dpp signaling while in more posterior follicle cells it is mostly cytoplasmic indicating low/no Dpp signaling (Figure 8G'' and Keller Larkin et al., 1999; Guichet et al., 2001; Dobens and Raftery, 2000). In 2001, Guichet et al. reported the staining for pMad (phosphorylated-Mad), one of the core cytoplasmic signal transducers of the Dpp pathway. Anti-pMad stainings at stage 8, stage 9 and 10B show similar distribution as seen for Med (Guichet et al., 2001). In summary, this indicates that both anti-pMad and anti-Med detect high levels of Dpp signaling in the follicle cells while *dad*LacZ is activated even at moderate levels of Dpp signaling.

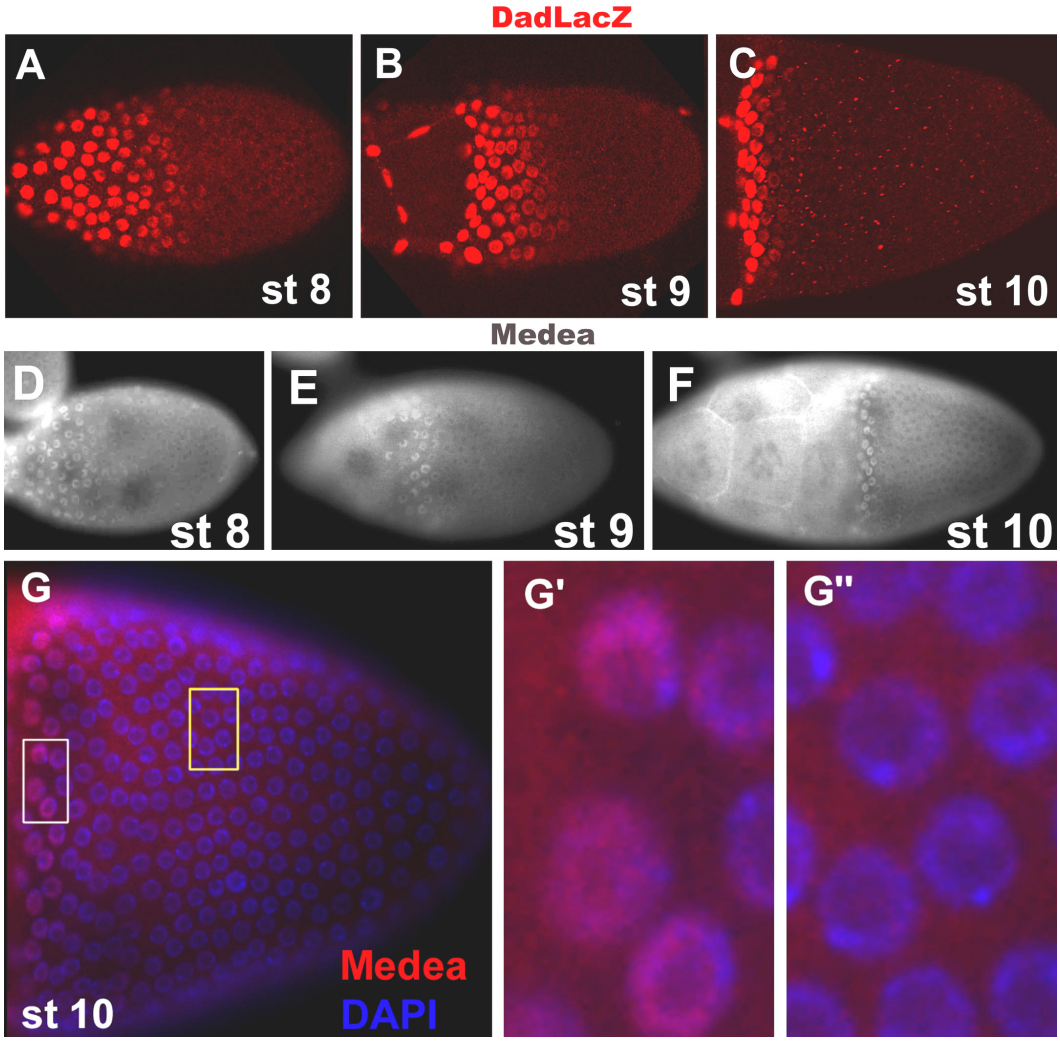

Figure 8

Dpp forms a morphogen gradient along AP axis in the follicular epithelium

Anterior is to left. (A-C) Shows anti β-gal (red) stainings performed on the egg chambers dissected from *dad*LacZ line. Note the AP graded expression of LacZ in the follicular epithelium. (A) At stage 8, high levels of *dad*LacZ can be detected in the anterior most follicle cells. The signal intensity starts to fade in subsequent posterior cells. (B) At stage 9, the gradient of *dad*LacZ expression is prominent in the migrating follicle cells and in the stretched follicle cells (C) At stage 10B, the gradient of *dad*LacZ expression is visualized only in a 3-4 follicle cell rows. The strong signal in the stretched follicle cells still persists. (D-F) Show anti-Med stainings (grey) in wt egg chambers at stage 8, 9, 10B respectively. (D) Stage 8 egg chamber showing the nuclear accumulation of Med in the anteriormost follicle cells. (E) At stage 9, Med can be detected in the posteriorly migrating follicle cells. (F) At stage 10B Med can be dectected in the CMFCs. (G) High magnification of stage 10B egg chamber showing only the posterior half. Anti-Med staining is in red and DNA is labeled in blue by DAPI. G' shows blowup of a section of the CMFCs (white frame) Med accumulates in the nuclei (purple). G" shows blowup of the section of posterior follicle cells (yellow frame) where Med in not localized to the nuclei.

3.2 Misexpression of *dpp* in the follicle cells expands dorsal cell fates along the AP axis.

Dpp the fly BMP2/4 homologue has dynamic role in the fly development. During oogenesis, it is absolutely essential in the germ stem cells and their maintenance (St Johnston and Gelbart, 1987; McKearin and Spradling, 1990; Twombly et al., 1996). As mentioned in the previous section, *dad*LacZ stainings suggests that Dpp forms a gradient in the follicular epithelium. Therefore, it is possible that Dpp specifies different cell fates along the AP axis in a concentration dependent manner. We tested this possibility by modifying the slope of Dpp gradient along the AP axis. Thus, by increasing the range of the gradient the operculum and dorsal appendage fate should shift posteriorly along the AP axis.

We followed expression of three genes to test our hypothesis – Fasciclin III (Fas III), *BR-C* and *pipe*. Fas III is first detected in polar cells and later in all columnar follicle cells at the apico-lateral junctions. At stage 10B of oogenesis, Fas III is upregulated in CMFCs and in the dorsal midline follicle cells which contribute to operculum formation (Figure 9E and Kose et al., 1997; Ward and Berg, 2005). At stage 10B, the intersection of Dpp and EGFR pathways upregulates *BR-C* expression in two anterior patches (Figure 9I and Deng and Bownes, 1997; Peri et al., 1999; Peri and Roth, 2000). These cells later on form the dorsal appendages. *pipe*, a putative heparin sulfate 2-sulfotransferase is restricted ventrally by the dorsalizing Grk signal (Figure 9M and Sen et al., 1998; Peri et al., 2002). We analyzed *pipe* expression because of two reasons – first, to understand the effect of *dpp* misexpression in ventral follicle cells and second to investigate its effect on *pipe* expression itself.

We decided to provide excess amounts of Dpp in the follicle cells using the UAS/Gal4 system in a controlled manner. Two different Gal4 driver lines were utilized for this purpose: GR1Gal4 and CY2Gal4 which express in all main body follicle cells to drive a UAS*dpp* transgene in females. Both the Gal4 driver lines vary in their strength of expression in space and time. To visualize the strength of individual Gal4 driver line *dpp* RNA insitu hybridizations were

performed on ovaries dissected from females beraing GR1Gal4/UAS*dpp* and CY2Gal4/UAS*dpp*. GR1Gal4 is turned on moderately at stage 5 in oogenesis and has ability to express until stage 9 while CY2Gal4 is turned on strongly at stage 5 and it persits till late stages of oogenesis (Queenan et al., 1997).

Females misexpressing moderate levels of *dpp* (UAS*dpp* X GR1Gal4) laid two classes of eggs: First, eggs which displayed larger operculum and thicker dorsal appendages. Both the operculum and the dorsal appendages were pushed slightly towards the posterior along the AP axis (compare Figure 9A and B; Twombly et al., 1996; Muzzopappa and Wappner, 2005). Correspondingly, Fas III and *BR-C* expression was expanded along the AP axis indicating an increased number of follicle cells were committed to operculum and dorsal appendage fates respectively (compare Figure 9E, F and I, J). The above observations suggest that graded Dpp controls both the position and specification of operculum and dorsal appendage cell fates along the AP axis.

Second class of eggs showed prescence of multiple dorsal appendages (compare Figure 9A and C; Twombly et al., 1996; Muzzopappa and Wappner, 2005). Fas III and *BR-C* expression was expanded along the AP axis in this case too (compare Figure 9E and G; I and K). Moreover, we could clearly observe few "isolated" groups of cells expressing Fas III and *BR-C* ectopically (Figure 9 G, K). These cells would give rise to ectopic operculum and dorsal appendages (cells). In addition, upon careful analysis of *BR-C* expression we found that the *BR-C* domain was fragmented (compare Figure 10A and B). Similar observations were made by James and Berg (2003) and by Nakamura and Matsuno (2003) in case of *Drosophila virilis* where eggs bear four dorsal appendages instead of two. Interestingly, *grk* mRNA levels in *D. virilis* stage 10 egg chambers are comparable to those of *D. melanogaster* at a similar stage (Peri et al., 1999). This clearly suggests that difference in Dpp signaling between species must be responsible for specification and observed variation of dorsal appendage number among Drosophilids.

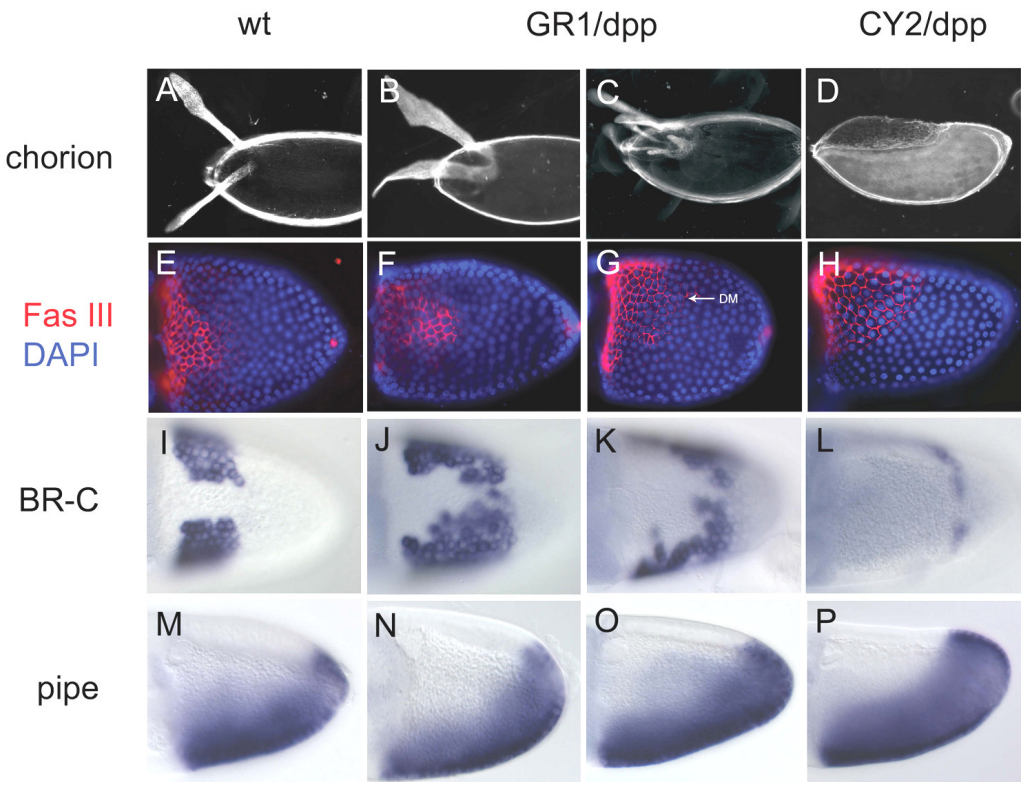

Figure 9

Misexpression of *dpp* in the follicular epithelium dorsalizes the eggshell along the AP axis.

Anterior is to the left. (A-L) Stage 10B egg chambers (M-P) Stage 9 egg chambers (A-D) Dorsolateral views of chorion. (E-G and I-L) Dorsal views. (H and M-P) Lateral views. (A, E, I, M) Egg shell phenotype, anti-FAS III stainings (red) and RNA in situ hybridization patterns for *BR-C* and *pipe* in wt egg chambers. (B,F, J, N) and (C,G,K,O) Egg shell phenotypes, anti-FAS III stainings (red) and RNA in situ hybridization patterns for *BR-C* and *pipe* in moderate misexpression of *dpp* (UAS*dpp* X GR1-Gal4). (D, H, L, P) Egg shell phenotypes, anti-FAS III stainings (red) and RNA in situ hybridization patterns for *BR-C* and *pipe* in strong misexpression of *dpp* (UAS*dpp* X CY2-Gal4). DNA is stained in blue (DAPI). Arrow in G marks dorsal midline.

Eggs with enlarged opercula but with no dorsal appendages were laid when *dpp* was strongly misexpressed (UAS*dpp* X CY2Gal4) in the whole follicular epithelium (Figure 9D and Twombly et al., 1996; Muzzopappa and Wappner, 2005). Fas III expression was expanded along both axes in such egg chambers (compare Figure 9E and H). However, the asymmetry of its expression was still maintained. Consistent with the loss of dorsal appendages, *BR-C* was dramatically reduced to few cells and was detected as a dorsolateral stripe in the

follicular epithelium (Figure 9I, L). This indicates that Dpp levels obtained with CY2-Gal4 cross a certain threshold beyond which specification of dorsal appendage fate becomes impossible. In addition, it consolidates the point that at highest levels of Dpp operculum cell fate is specified.

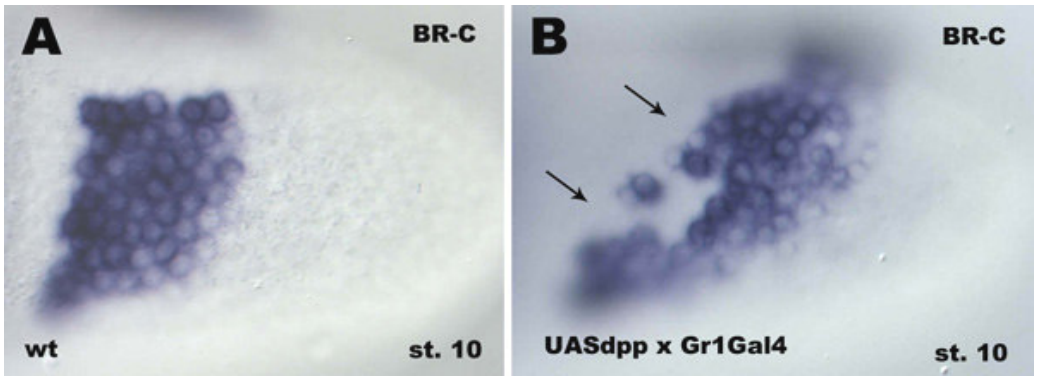

Figure 10

Multiple domains of BR-C expression correspond to multiple dorsal appendages.

(A, B) Lateral view of stage 10B egg chambers stained for *BR-C*. (A) BR-C staining is seen as single domain in wt egg chambers. (B) In moderate expression of *dpp* in follicular epithelium *BR-C* domain was fragmented. Black arrows point two fragments of *BR-C* expression.

In the above *dpp* misexpression situations, *pipe* mRNA distribution was similar to that in wt except in the posterior follicle cells where it expanded dorsally (compare Figure 9M and N, O, P) Thus, excess amounts of Dpp do not affect *pipe* expression in the main body follicle cells but ectopically induce its expression in the dorsal posterior cells.

From the above observations it could be concluded that highest levels of Dpp signaling in the follicle cells promote operculum and suppress dorsal appendage fate, while moderate levels of signaling promotes the latter cell fate (Twombly et al., 1996; Dobens and Raftery, 2000). These observations support our hypothesis that Dpp is indeed acting as a morphogen and patterning molecule in the follicle cells and different thresholds of Dpp signaling specify different cell fates along the AP axis. We also conclude that Dpp does not provide any

dorsalizing cue. Two observations support our conclusion. First, higher levels of Dpp expand of all dorsal cell fates only along the AP axis. And second, the ventral follicle cells still express *pipe* in *dpp* misexpression situations confirming absence of dorsalization at molecular level.

3.3 Loss of Dpp signaling in the follicular epithelium renders them unresponsive to Grk signaling

We asked if Dpp signaling is required positively for specification of the operculum and dorsal appendage fate. To address this question we analysed the expression of BR-C and Fas III in *tkv* and *Med* mutant follicle cell clones. Tkv and Med are core components of the Dpp signaling cascade in *Drosophila* (Raftery and Sutherland, 1999). Follicle cell clones in adult females were induced using the FRT-FLP system (Xu and Rubin, 1993).

Homozygous *Med* mutant clones were detected by absence of GFP. Clones induced in the dorsal follicle cells show downregulation of both FAS III and BR-C in a cell autonomous manner (compare Figure 11A-D and E-J). No change in the basal level of expression of BR-C and Fas III was observed in clones located in posterior or ventral follicle cells (Figure 11E-J). Fas III expression can still be detected in the polar follicle cells (two red dots in Figure 11I at the anterior and posterior which mark the polar follicle cells) as its expression is independent of Dpp or Grk signaling and therefore served as an important internal control. This demonstrates that Dpp signaling is absolutely essential for the upregulation of both BR-C and Fas III in the dorsal anterior follicle cells.

Activins/TGF-βs and BMPs signal through the Co-Smad Med (Wisotzkey et al., 1998; Parker et al., 2004). To ensure that the phenotype obtained with *Med* mutant clones is specific to Dpp signaling we induced homozygous mutant clones for *tkv*, the type I receptor specific for Dpp. We obtained exactly the same results (Figure 12A-F) confirming the positive requirement of Dpp in

formation of the dorsal appendages and operculum (Twombly et al., 1996; Peri and Roth, 2000).

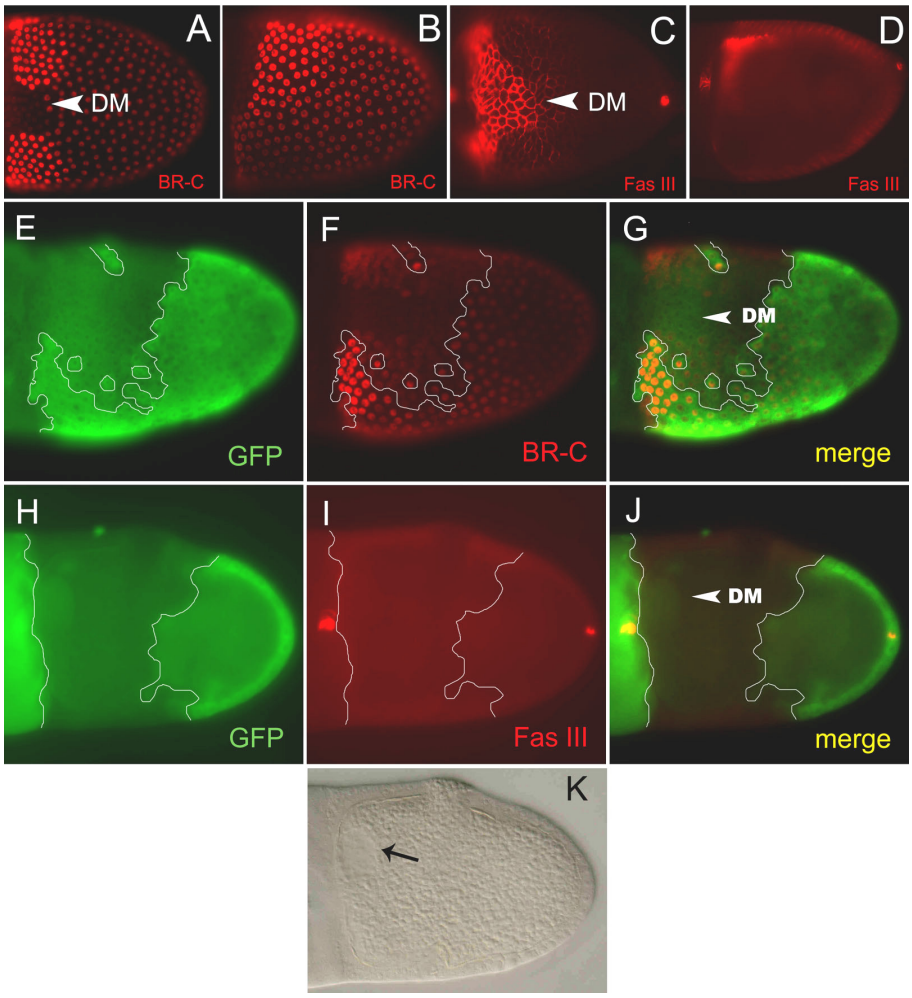

Figure 11

Med loss of function clones affect BR-C and Fas III expression in cell autonomous manner.

Anterior is to the left (A, C and E-G) Dorsal views and (B, D and H-J) Lateral views of stage 10B egg chambers. (A, B) wt egg chamber stained for anti-BR-C (red) which marks two patches of follicle cells one on either side of the dorsal midline (white arrow-DM). (B) Lateral view of wt egg chamber stained for anti-BR-C (red). (C, D) Wt egg chamber stained with anti-FAS III (red) which marks the dorsal midline cells (D) Lateral view of wt egg chamber stained with anti-FAS III (red). (E and H) Clones of cells lacking Med marked by absence of GFP (green) expression. (Fand I) BR-C (red) and Fas III (red) expression in *Med* mutant follicle cell clones. (G and J) Merge. BR-C and Fas III expression is not upregulated in *Med* mutant follicle cell clone. The white arrow marks the dorsal midline (DM). (K) Nomarsky image of egg chamber shown in (H). The oocyte nucleus is pointed by black arrow. White lines mark the clone boundary.

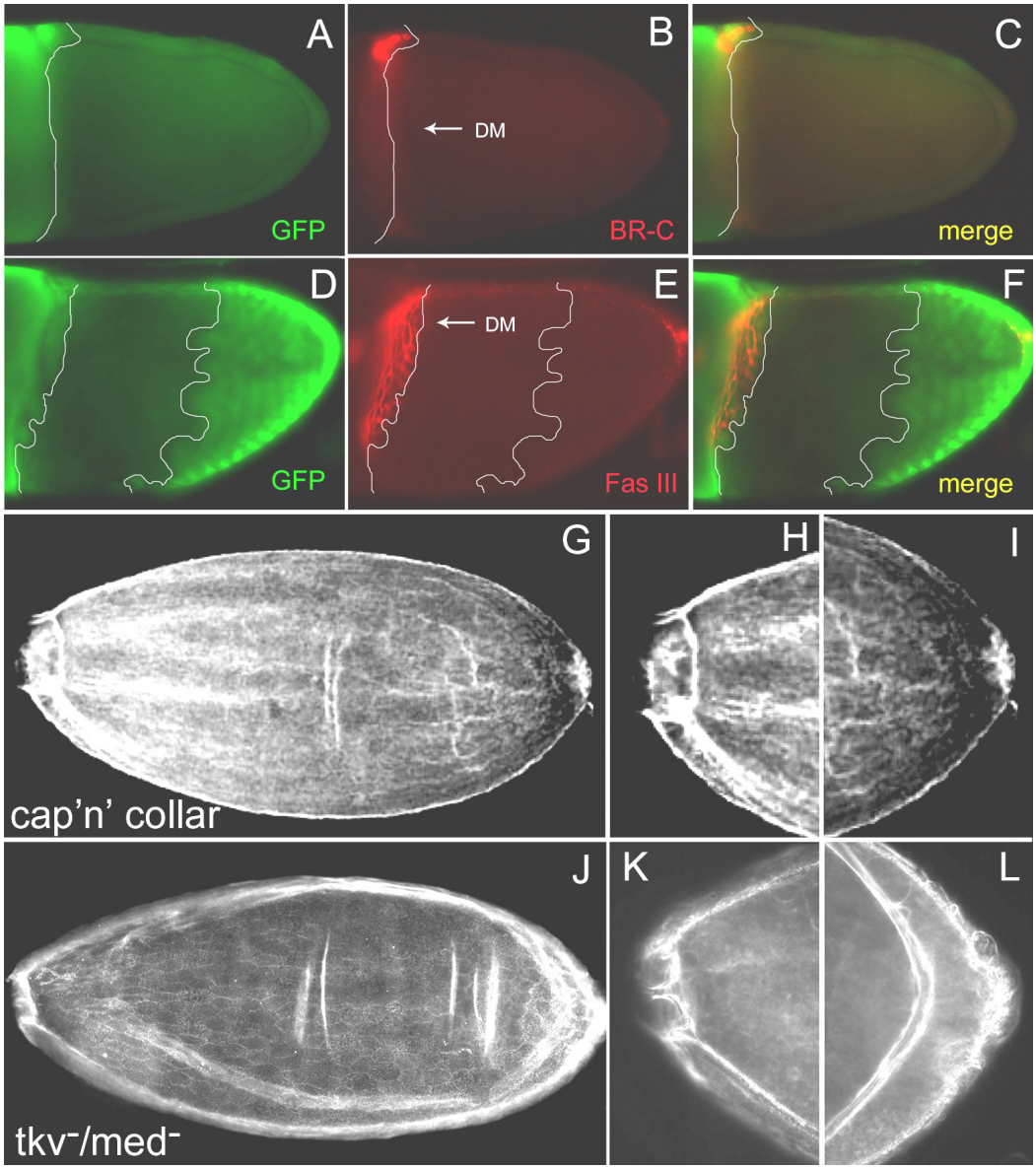

Figure 12

Tkv loss of function clones affect BR-C and Fas III expression in cell autonomous manner

Anterior is to the left (A-C) Dorsal views and (D-F) Lateral views of stage 10B egg chambers. (A and D) Clones of cells lacking Tkv marked by absence of GFP (green) expression. (B and E) BR-C (red) and Fas III (red) expression in *tkv* mutant follicle cell clones. (C and F) Merge. BR-C and Fas III expression is not upregulated in *tkv* mutant clones. The white arrow marks the dorsal midline (DM). White lines mark the clone boundary. (G) Egg(s) laid by females lacking Cap'n'Collar function in oocyte showing complete lack of DV polarity. Adapted from Guichet et al. 2001 (H) Higher magnification of anterior end of egg in G showing micropyle and collar. (I) Higher magnification of posterior end of egg in G. (J) Egg(s) laid by females lacking Med or Tkv function in follicle cells showing complete lack of DV polarity. (H) Higher magnification of anterior end of egg in J showing micropyle and collar. (I) Higher magnification of posterior end of egg in J.

These results indicate that both *Med* and *tkv* mutant follicle cells behave as if they haven't received the dorsalizing Grk signal. Indeed, eggs laid by females in which *Med* and *tkv* mutant follicle cells were induced (henceforth termed as tkv^{-} /Med^{-} eggs) lack DV polarity and resemble mutants in which dorsalizing Grk signal is compromised (Figure 12G-L). These results suggest that in absence of Dpp signaling, Grk cannot induce dorsal cell fates in the follicular epithelium.

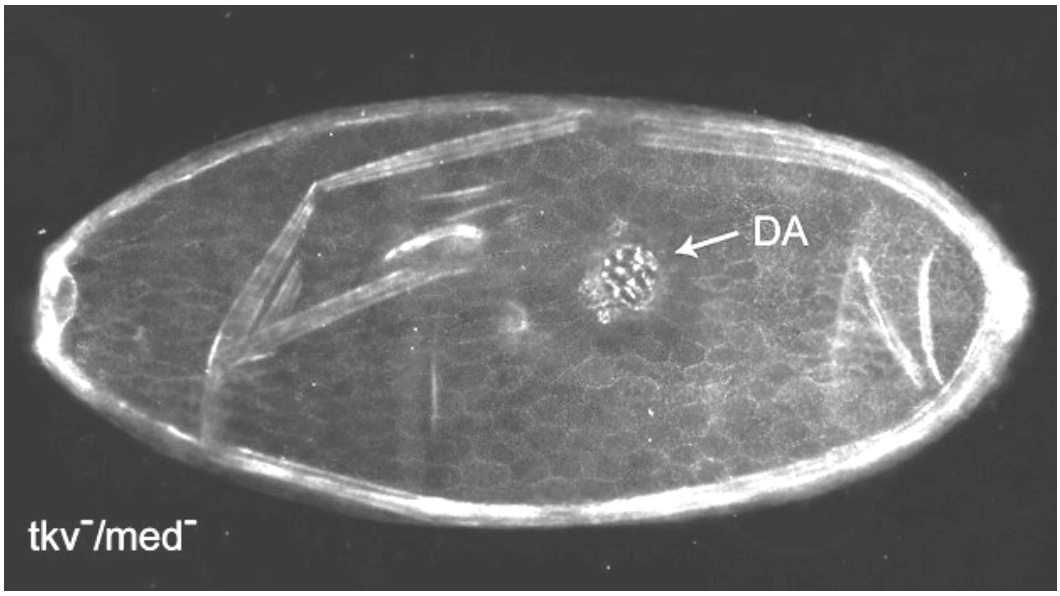

Figure 13

Grk signaling is not affected in tkv^{-}/med^{-} eggs

Anterior is to the left. tkv^{-}/Med^{-} egg showing residual dorsal appendage material (DA shown by white arrow).

We believe that Grk signaling occurs normally in these egg chambers as both the AP polarity specification and induction of oocyte nuclear migration occur normally (Figure 11K; Koch and Spitzer, 1983; Roth et al., 1995). Indeed, a certain percentage of tkv^{-}/Med^{-} eggs show reminiscent dorsal appendage material confirming that Grk signaling does occur (Figure 13). This can be explained in the following way. Such eggs would be derived from egg chambers in which *tkv* or *Med* mutant clone would encompass only a part of the anterior half of the follicular epithelium. Thus, in absence of in Tkv/Med in the anterior

follicle cell clones, the effective number of Dpp molecules would increase. This would allow Dpp to diffuse further in the posterior follicle cells. In such posterior follicle cells, Dpp in concert with Grk can induce dorsal appendage fate. Thus, from above results we conclude that Dpp not only acts as a patterning molecule but indeed makes cells competent to respond to Grk signal.

We asked if the tkv^{-}/Med^{-} egg phenotype would be similar to that of *grk*$^{-}$ at the molecular level. We decided to use *chornichon* (*cni*) mutant(s) for our analysis which resemble(s) a loss of *grk* phenotype (Roth et al., 1995). We used two mutant alleles of *cni* in our study: - *cni* ^{aa12} (hypomorphic allele) and *cni* ^{ar55}(null allele). Ovaries dissected from *cni* ^{aa12}/*cni* ^{ar55} and *cni* ^{ar55}/Df(*cni*) mothers were stained for Fas III. Eggs laid by *cni* ^{aa12}/*cni* ^{ar55} mothers have residual dorsal appendage material indicating that small amount of Grk signaling occurs during oogenesis (Figure 14A). Fas III expression was weak in the dorsal midline cells but was normal in the polar cells and CMFCs in stage 10 egg chambers (Figure 14E). Additionally, Fas III was detected in the terminal and dorsal anterior follicle cells at stage 14 (Figure 14C). *cni* ^{ar55}/Df(*cni*) combination generates a true *cni* null situation resembling *grk*$^{-}$. Eggs derived from *cni* ^{ar55}/Df(*cni*) females lack both the AP and DV polarity (Figure 14B and Roth et al., 1995). Fas III was detected at both the termini in late stages and in the CMFCs at stage 10 in *cni* ^{ar55}/Df(*cni*) egg chambers (Figure 14D, F). Fas III was not detected in the dorsal midline cells. These observations suggest that the *cni*$^{-}$ and tkv^{-}/Med^{-} eggs are different from each other atleast with respect to Fas III expression.

We asked if the presence of absence of Fas III would result in difference in the morphology of anterior follicle cells in *grk*$^{-}$ and tkv^{-}/Med^{-} eggs as Fas III is suggested to have a role in maintaining integrity of cells (Snow et al., 1989; Ward and Berg, 2005). Additionally, it is known that the operculum cells can be further subdivided into three different sub types- type I, II and III, based on their morphology (Figure 15A-C and Margaritis et al., 1980; Dobens and Raftery, 2000). We performed Scanning Electron Microscopy (SEM) on wt, *grk*$^{-}$ and tkv^{-}/Med^{-} eggs (Figure 15D-I). Detailed observation revealed that in *grk*$^{-}$ eggs but not in tkv^{-}/Med^{-} eggs type I operculum cells are specified (Figure 15H, I-

yellow arrow). Thus, in tkv^{-}/Med^{-} eggs, cells adjacent to collar expand around the micropyle.

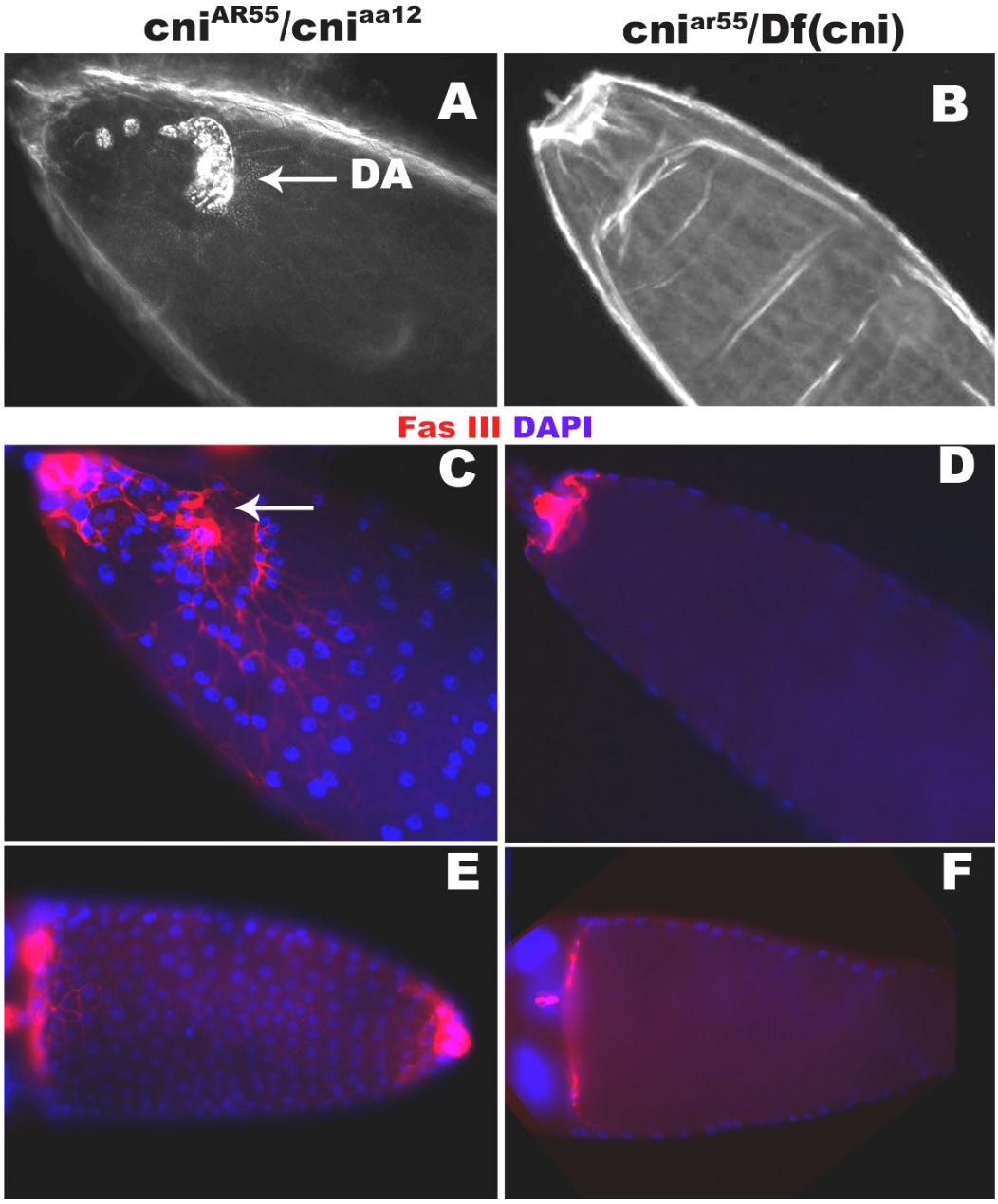

Figure 14

Fas III is still expressed in CMFCs in *cni*$^{-}$ (*grk*$^{-}$) egg chambers.

(A-D) Dorsal view of stage 14 egg chambers (E-F) Dorsal view of stage 10B egg chambers (A and C) Chorion phenotype and Fas III staining (red) of stage 14 egg chamber derived from *cni* ^{aa12} /*cni* ^{ar55} mothers. (E) Fas III staining (red) of stage 10B egg chamber derived from *cni* ^{aa12} /*cni* ^{ar55} mothers (B and D) Chorion phenotype and Fas III stainings (red) in stage 14 egg chambers derived from *cni* ^{ar55}/Df(cni) mothers. (F) Fas III stainings (red) in stage 10B egg chambers derived from *cni* ^{ar55}/Df(cni) mothers. DNA is stained in blue by DAPI. White arrow in A marks the residual dorsal appendage material and in C white arrow shows associated with the DA cells.

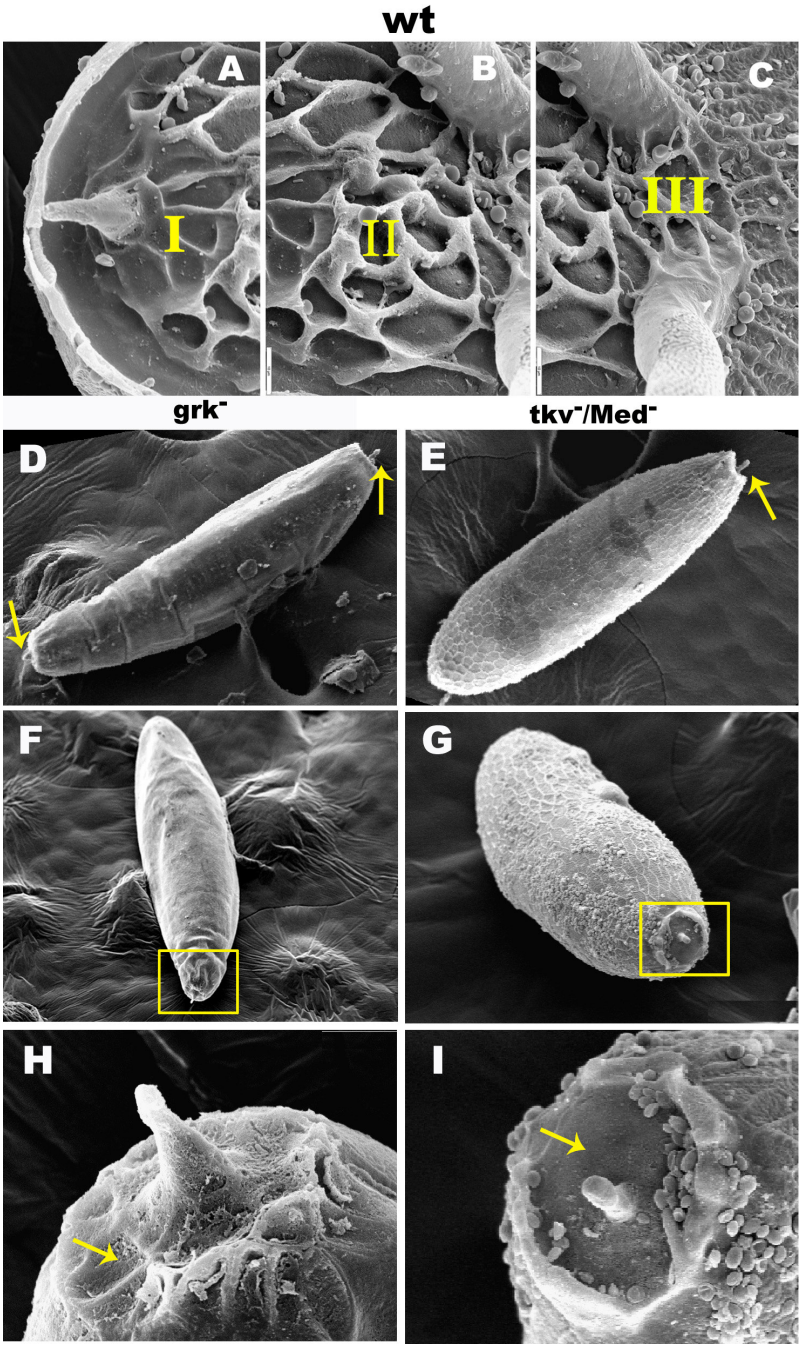

Figure 15

Specialized features of *Drosophila* operculum

SEM images of *Drosophila* eggshell in wt, *grk*$^{-}$ and tkv^{-}/Med^{-} eggs. (A-C) Close up view of operculum showing three different cell types classified based on their morphology. (D) Chorion phenotype of *grk*$^{-}$ eggs. Yellow arrows mark micropyle at the both the termini. (E) Chorion phenotype of tkv^{-}/Med^{-} eggs. Yellow arrow marks micropyle at the anterior end. (F and G) Another example of *grk*$^{-}$ egg and tkv^{-}/Med^{-} egg. (H and I) Blow up sections of anterior end of *grk* and tkv^{-}/Med^{-} egg shown in F and G (yellow frames) respectively. Type I (yellow arrow in H) but not Type II and III operculum cells are specified in *grk*$^{-}$ egg. None of the operculum cell types (yellow arrow in I) are specified in tkv^{-}/Med^{-} egg.

3.4 Misexpression of Grk from the germline dorsalizes the eggshell

The *Drosophila* EGF receptor mediates two inductive events that establish both the body axes during oogenesis (Gonzalez-Reyes et al., 1995; Roth et al., 1995). This is achieved by inducing two different follicle cell populations, first the posterior and then dorsal populations, and is mediated through activation of the EGFR cascade in the follicular epithelium by Grk secreted from the developing oocyte (Schupbach and Roth, 1994; Roth et al., 1995; Nilson and Schupbach, 1999). The mechanism by which Grk is able to specify two different cell fates is attributed to a difference in the competence of the cells (Queenan et al., 1997; Peri and Roth, 2000).

Misexpression of the activated form of EGFR in the whole follicular epithelium dorsalizes the eggshell and embryo to different degrees based on the strength of the follicle cell specific Gal4 driver line used (Queenan et. al., 1997). These experiments suggested that moderate levels of EGFR signaling specify the dorsal appendage fate while higher levels specify the operculum fate. We asked whether providing excess Grk from the germline could affect cell fate determination in the follicular epithelium in a similar way. To achieve *grk* misexpression during oogenesis, UASp*grk* transgene was driven by tubGal4VP16 driver line (Brand and Perrimon, 1993). This driver line is expressed exclusively in the germ cells (nurse cells and the developing oocyte) as early as stage 4 of oogenesis (Sajith Dass, PhD thesis). We investigated the expression of the cell fate specific marker genes FasIII, BR-C and *pipe* in a Grk misexpression background.

Misexpression of *grk* in the germline generated a spectrum of dorsalized egg phenotypes (Figure 16A-F). From our analysis of the egg phenotypes we could distinguish two levels of Grk which give rise to two distinct egg phenotypes.

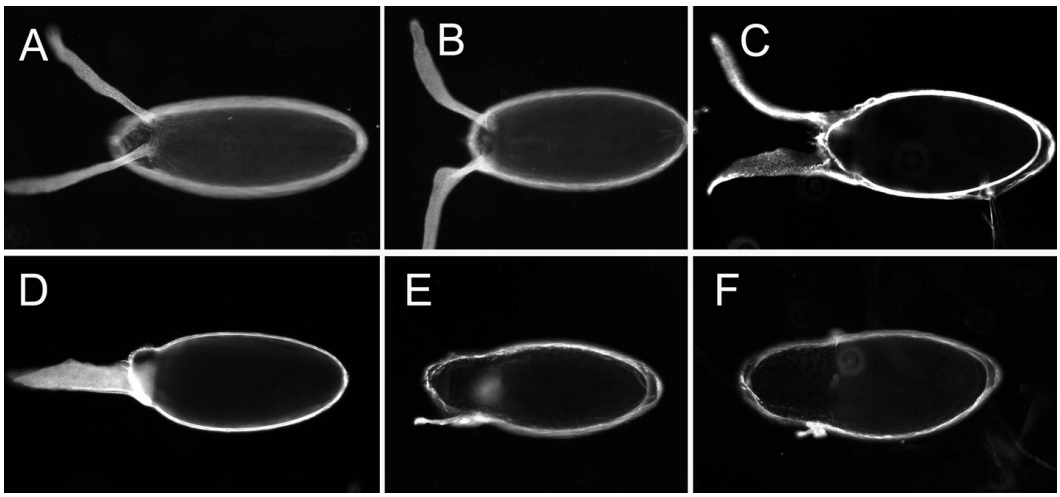

Figure 16

Misexpression of *grk* from the germline results in a series of dorsalized egg phenotypes.

(A-F) Series of increasingly dorsalized chorion phenotypes obtained by misexpressing *grk* in germline. Anterior is to the left. (A-C) Dorsal views and (D-F)) Lateral views. (A) Wt chorion showing the characteristic placement of dorsal appendages. (B) Mild dorsalization of the chorion leading to increase in distance between the dorsal appendage. (C) A slightly stronger doralization leads to lateral placement of dorsal appendages. (D) Moderate dorsalization leads to formation of dorsal appendage in ventral region of the egg. (E) A stronger dorsalization leads to expansion of operculum to the ventral region of the egg with a strong suppression the dorsal appendages. (F) Strongest dorsalization leads to production of egg with symmetrical operculum with no dorsal appendages.

First, moderate misexpression of *grk* promotes dorsal appendage fate confirmed by presence of the dorsal appendages in ventral anterior regions of the eggs (Figure 17A and Queenan et al., 1997). Fas III expression was found to be expanded (Figure 17C) laterally and *BR-C* was induced ventrally (Figure 17E) which was in congruence with the observed egg phenotype (Prost et al., 1988; Ward and Berg, 2005). Thus we could conclude that high level of Grk signaling at the dorsalmost regions induces operculum fate while moderate levels which spread ventrally induce dorsal appendage fate.

Second, strong misexpression of *grk* abolished the dorsal appendage fate and the eggshell showed an expansion of the operculum along the DV axis (Figure 17B and Queenan et al., 1997). Fas III expression as expected was highly expanded along the DV axis (Figure 17D). *BR-C* was completely lost in these

egg chambers, thus, supporting the observation of absence of the dorsal appendages (Figure 17F). Upon moderate and strong misexpression of *grk*, *pipe* was repressed completely in the ventral main body follicle cells and its expression was restricted to a few posterior follicle cells (Figure 17G, H and Sen et al., 1998; Peri et al., 2002).

The expansion of dorsal cell fates was observed only along the DV axis in the anterior half of the follicular epithelium implying the restrictive nature of competence in the anterior follicle cells conferred by Dpp.

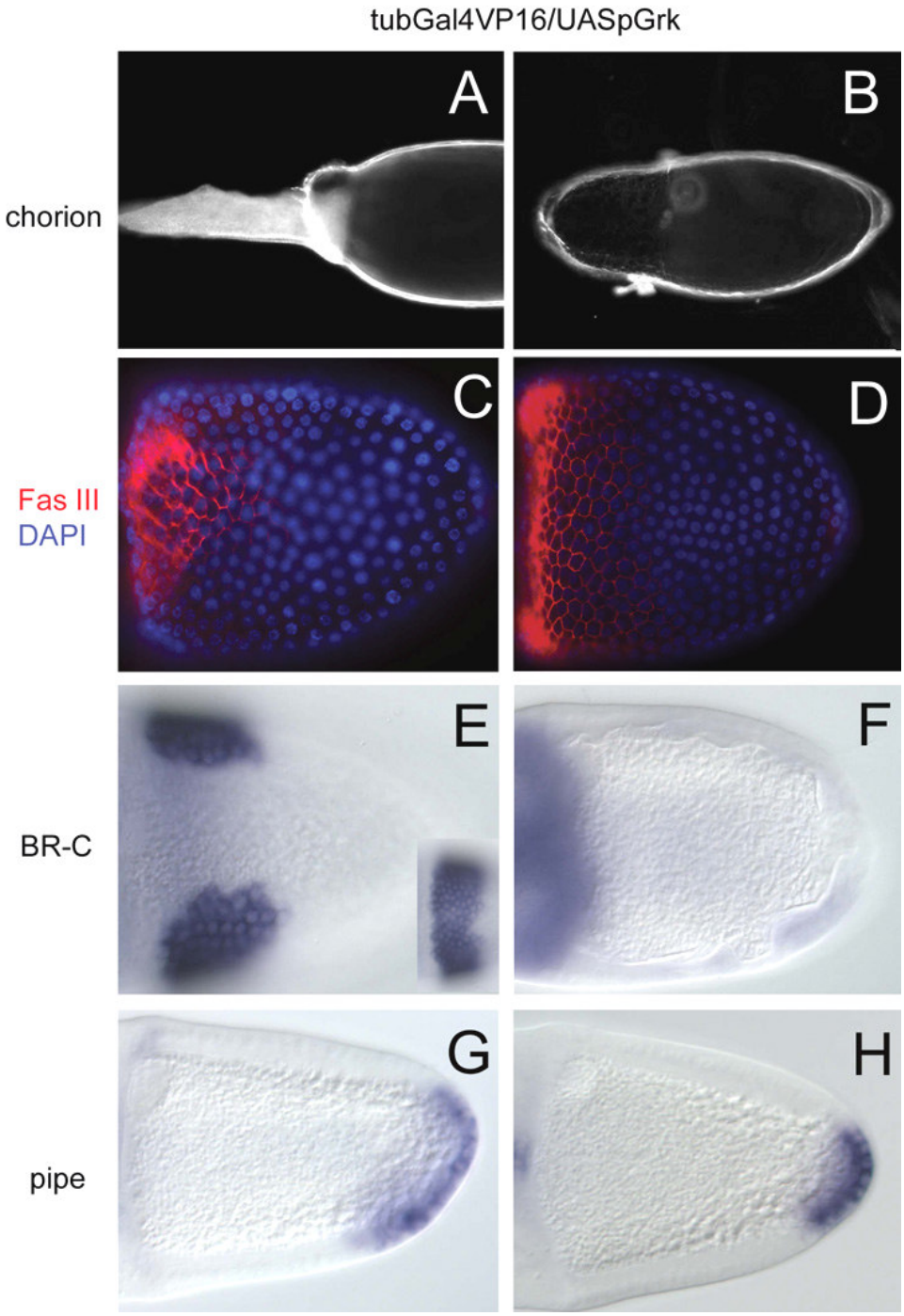

Figure 17

Misexpression of *grk* in the germline dorsalizes the eggshell

Anterior is to the left. (A-B) Lateral views of chorion. (C-H) Only posterior half of the egg chambers at stage 10B are shown here (C-F) Dorsal views stage 10B egg chambers. (G-H) Lateral view of stage 9 egg chambers. (A, C, E, G) Egg shell phenotype, anti-Fas III stainings (red) and RNA in situ hybridization patterns for *BR-C* and *pipe* in moderate misexpression of *grk*. (B, D, F, H) Egg shell phenotype, anti-Fas III stainings (red) and RNA in situ hybridization patterns for *BR-C* and *pipe* in strong misexpression of *grk*. Inset in E shows the expansion of *BR-C* to the ventral follicle cell at a lower magnification.DNA is stained in blue by DAPI

3.5 Combined misexpression of Grk and Dpp leads to novel eggshell phenotypes

To get a deeper insight in understanding the role of Grk and Dpp together in specification of the dorsal chorion structures we misexpressed *grk* from germline and *dpp* in the follicle cells simultaneously. The necessity of designing such an experiment was felt due to the following reasons. First, Dpp is expressed in the posteriorly migrating follicle cells at stage 9 and at the same time these cells receive second Grk signal from the developing oocyte which induces dorsal fates and consequently patterns the follicular epithelium. Second, several groups including Peri et al. in 2001 have suggested that both Grk and Dpp collaborate to specify dorsal chorion structures of the egg. Finally, from our single misexpression data we observed that Grk provides the dorsalizing cue primarily across the DV axis while Dpp modulates the competence of follicle cells thereby restricting the extent of Grk effect in the follicle cells along the AP axis.

To achieve combined misexpression, UAS*dpp*/tubGal4VP16 and UASp*grk*/GR1Gal4 (or UASp*grk*/CY2Gal4) flies were generated and reared individually. These transgenic flies were then crossed to generate two different genotypes UASp*grk*/ tubGal4VP16; UAS*dpp*/GR1Gal4 and UASp*grk*/ tubGal4VP16; UAS*dpp*/CY2Gal4.

Eggs laid by females having either of the two combinations were novel with respect to their morphology (Figure 18A, B). Females possessing the combination UASp*grk*/ tubGal4VP16 and UAS*dpp*/GR1-Gal4 laid eggs which had a large operculum covering up to 50% of the egg length and a ring of dorsal appendage material at the middle of the egg (Figure 18A). Fas III expression expanded along both the axes and was detected in all the main body follicle cells with a exception of few (Figure 18C). *BR-C* expression was symmetrical and its expression was detected in posterior half of the main body follicle cells an indication of high levels of Grk and Dpp in the anterior repressing it (Figure 18E). *pipe* was repressed in such egg chambers except in the posterior follicle cells further confirming the extent of dorsalization at the molecular level

(Figure 18G). This observation was consistent with the expansion of *pipe* domain in posterior follicle cells observed in the moderate misexpression of *dpp* (Figure 9N, 0).

Females possessing the combination UASp*grk*/tubGal4VP16 and UAS*dpp*/CY2Gal4 laid eggs with huge symmetrical operculum covering up to 75% of the egg length and had no dorsal appendage material (Figure 18B). Fas III expression was upregulated in all the follicle cells except in the most posterior follicle cells (Figure 18D). *BR-C* was severely reduced and was detected as a symmetrical ring of few cells wide close to the boundary of main body and posterior follicle cells (Figure 18F). *pipe* was upregulated only in the posterior follicle cells indicating that Dpp has a positive input on *pipe* expression only in the posterior follicle cells (Figure 18H and Figure 9P). This also suggests that Grk can no longer repress *pipe* in the posterior follicle cells in prescence of excess Dpp.

By co-activating two cascades simultaneously, we could show that combination of high levels of Grk and high Dpp co-operate to specify operculum fate whereas combination of moderate levels of Grk and moderate levels of Dpp specify dorsal appendage fate. In addition, we could also make the chorion pattern symmetrical.

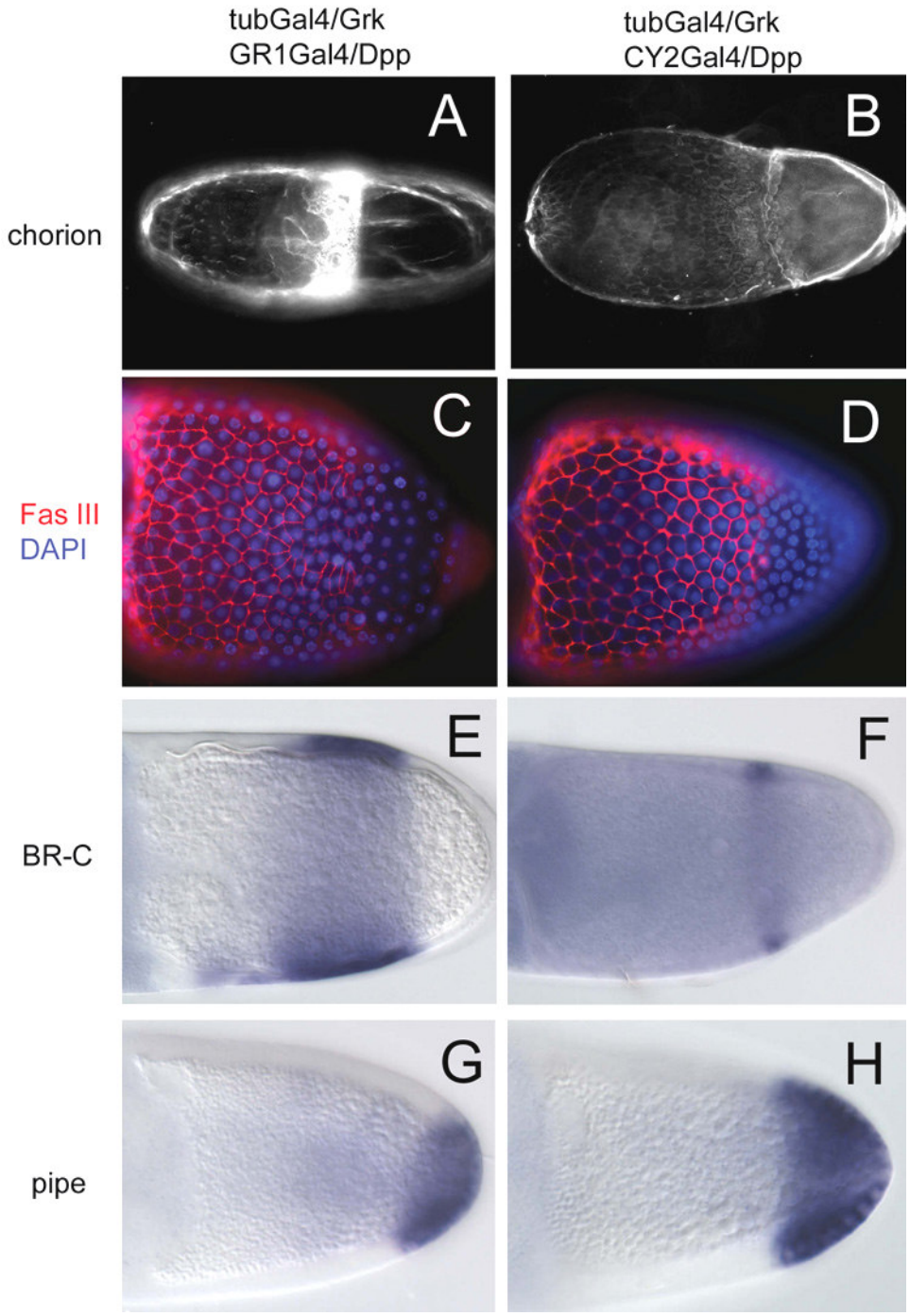

Figure 18

Combined misexpression of *dpp* in the follicle cells and *grk* in the germline generates novel chorion phenotypes.

Anterior is to the left. (A-B) Lateral views of chorion. (C-H) Only posterior half of the egg chambers at stage 10B are shown here (C-F) Dorsal views stage 10B eggchambers. (G-H) are lateral of stage 9 egg chambers. (A, C, E, G) Egg shell phenotype, anti-Fas III stainings (red) and RNA in situ hybridization patterns for *BR-C* and *pipe* in combined moderate misexpression of *grk* and *dpp* (B, D, F, H) Egg shell phenotype, anti-Fas III stainings (red) and RNA in situ hybridization patterns for *BR-C* and *pipe* combined strong misexpression of *grk* and *dpp*.

3.6 Cloning of SnoN and its expression of during oogenesis

We were interested in deciphering the role of genes and the mechanisms by which they regulate Dpp signaling in the follicular epithelium. Our efforts were concentrated on studying the inhibitors and repressors of Dpp signaling. We investigated the role of three known proteins Brk, Sog and Dad (discussed in later sections) and characterized Ski proteins, the function of which had not been analyzed previously in *Drosophila*.

Sequence information from Berkeley *Drosophila* Genome project (BDGP) (Adams et al., 2000) revealed that homologues of Ski family of proteins are encoded by genes CG7233 and CG11093, located on chromosome 2, left arm: cytological location 28D3 and chromosome 4, right arm: cytological location 102F4 respectively. cDNAs for both genes were isolated from ovarian cDNA library (see Material and Methods), which are predicted to encode proteins of 338 and 333 amino acids respectively. Comparison of the genomic and cDNA sequences indicate that CG7233 encodes only single transcript while CG11093 may encode more than one mRNA products as its coding sequence is distributed among 5 exons (Figure 19).

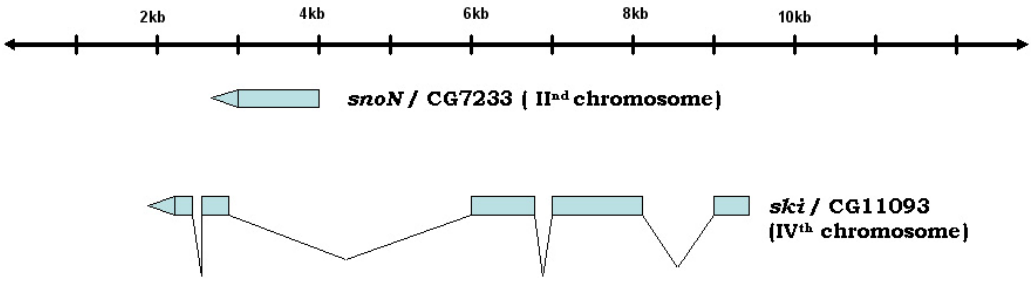

Figure 19

Gene structure of *Drosophila snoN* and *ski*

Arrangement of *snoN* and *ski* on chromosome II and IV respectively. The annotation of these genes is based on the latest version 4.2.1 of *Drosophila* melanogaster genome annotation release (Flybase September 2005). *snoN* is encoded by a single Open Reading Frame (ORF) of about 1017 base pairs. *ski* is encoded by five different exons and the whole coding sequence spreads over 8 kilo bases. The second exon is the largest and also codes for the Ski/Sno domain. The direction of the arrowhead depicts the direction of mRNA synthesis (Flybase V4.2.1, Sept 2005).Both *snoN* and *ski* are transcribed from the minus strand.

Sequence comparison shows that CG7233 and CG11093 proteins share a high degree of similarity within their Sno/Ski domain (98.2 % and 89.5 %; shown as bold purple line in aligment Figure 20) as well as short sequence stretches at the N and C-terminal region (Figure 20). The Ski superfamily of oncoproteins shares a conserved CLPQ motif in the Ski/Sno domain (Figure 20-shown as red bar). *Drosophila* CG7233 has this motif, however, CG11093 has a substitution of P to A in the CLPQ motif (see Figure 20). Finally, both genes share a conserved SAND domain (Cys_2His_2) between amino acids 255 and 285 (Figure 20-highlighted by green bar). Additional sequence comparisons with the Ski family proteins from vertebrates and phylogenetic analysis show that *Drosophila* CG7233 is closer to vertebrate SnoN homologs as compared to *Drosophila* CG11093 which does not cluster with either Ski or SnoN proteins (Figure 21). We therefore decided to name CG7233 as *Drosophila snoN* and CG11093 as *Drosophila ski*.

Interestingly, another gene in *Drosophila* named *dac* also shows prescence of the Ski/Sno domain and functions as a transcriptional co-repressor (Mardon et al., 1994; Keisman and Baker, 2001). Dac function has been very well characterized in patterning of the leg and eye imaginal disc and interestingly in mushroom body development, the higher learning center in *Drosophila* (Mardon et al., 1994; Miguel-Aliaga et al., 2004). Several Dac isoforms have been predicted to be synthesized, the largest of them being 1072 aa in length. However, Dac bears significant homology only in its Ski/Sno domain with *Drosophila* Ski and SnoN. Moreover, Dac protein lacks the characteristic SAND domain of Ski family proteins. Hence, we omitted Dac from our clustal, phylogenetic and functional analysis of Ski/Sno proteins.

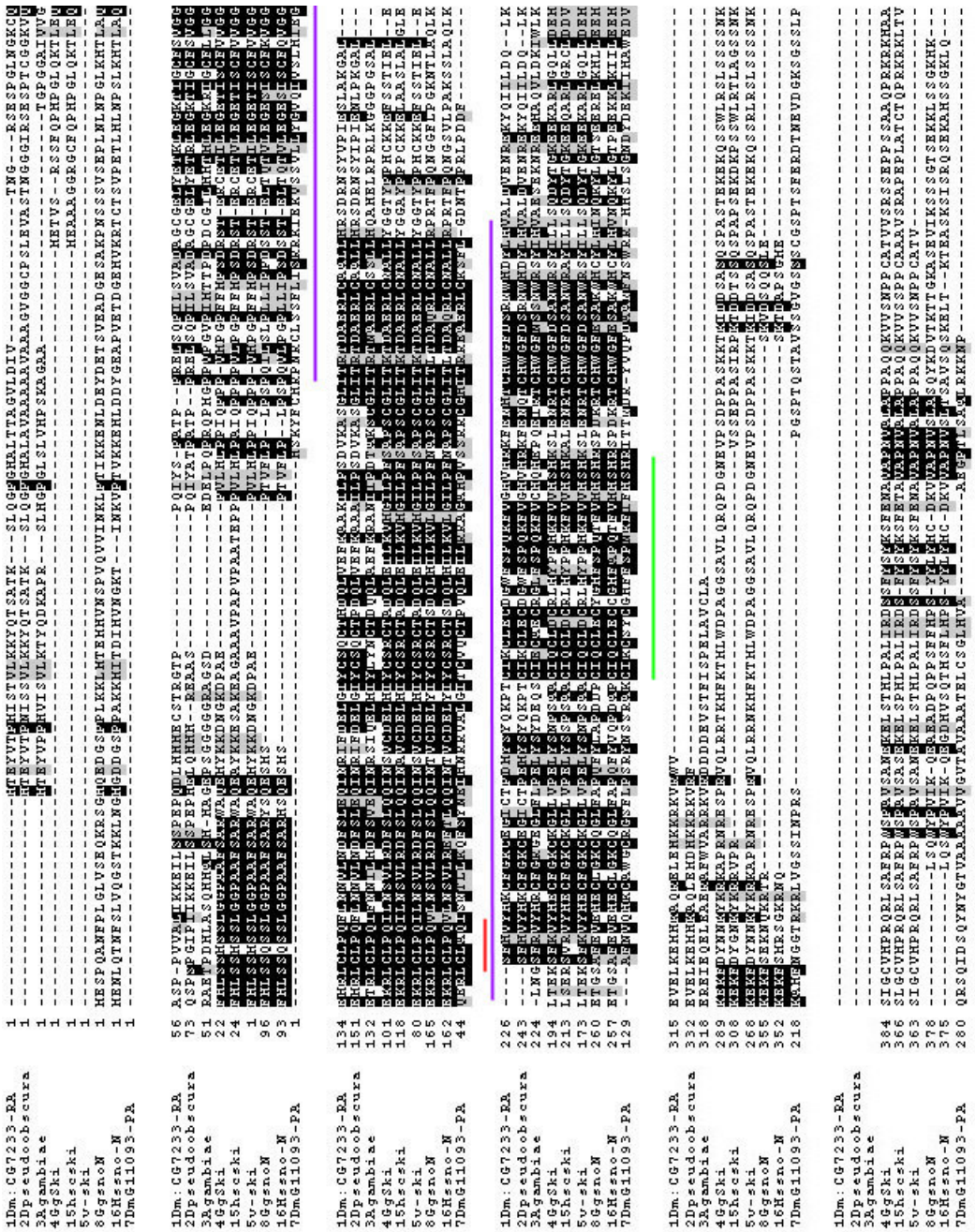

Figure 20

Clustal X generated alignment of SnoN and Ski proteins.

Multiple aligment of Ski and SnoN proteins from various species generated using the Clustal X method. Conserved amino acid residues are marked with black boxes. Similar amino acid residues are marked with grey boxes. All the proteins share a highly conserved Ski/Sno domain (purple line) which is located after the first 100 N-terminal aa. Only an aligment of first 400 aa is shown here as Drosophila *snoN* (CG7233) and Ski (CG11093) are 338 and 333 aa long. Vertebrate proteins are larger and bear additional domains. Red line indicates the conserved CLPQ motif. Green line indicates the SAND domain.

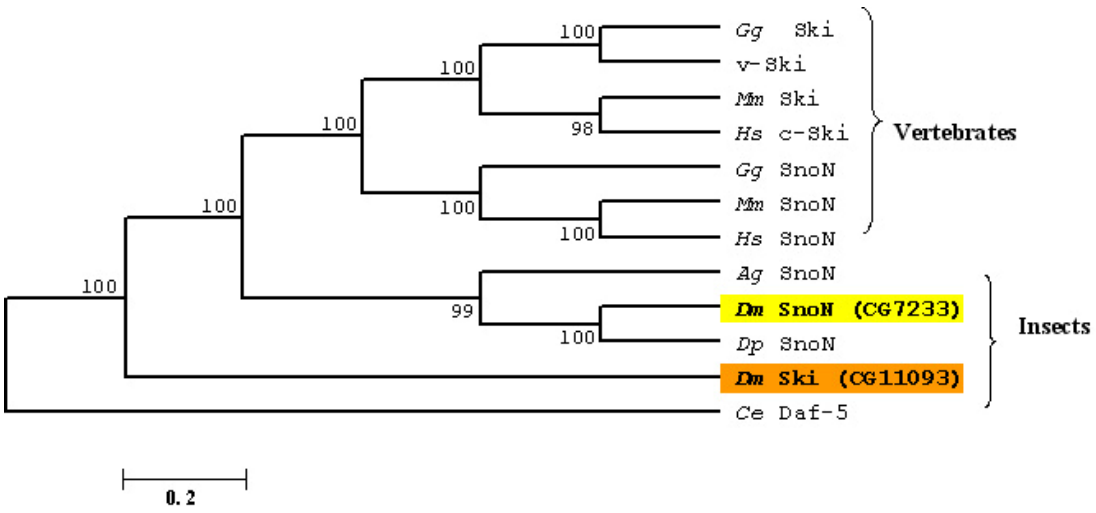

Figure 21

Phylogeny of SnoN and Ski proteins.

Phylogenetic Neighbour-Joining rooted tree derived from Clustal X analysis, based on alignment shown in Figure 20 and using 1000 bootstrap trials (bootstrap values at tree node represent confidence values; branches with values below 70 are considered less reliable and below 50 unreliable. Bar represent amino acid exchanges as a fraction of 1). *Caenorhabditis elegans* (*Ce*) Daf-5 was included as an outgroup member. (*Gg – Gallus gallus*; *Mm – Mus musculus*; *Hs – Homo sapiens*; *Ag – Anopheles gambiae*; *Dm – Drosophila melanogaster*; *Dp – Drosophila pseudobscura*).

3.7 *snoN* is expressed as a dorsolateral stripe in the follicular epithelium during oogenesis.

Both *snoN* and *ski* were cloned from an ovarian cDNA library, a clear indication of a role during oogenesis. Therefore, it was necessary to investigate the spatio-temporal aspects of their expression during oogenesis. To visualize the expression of *snoN* and *ski,* we performed RNA in situ hybridization on ovaries using antisense probes generated from *snoN* and *ski* cDNA.

snoN was detected in the germarium and at high levels in the nurse cells until stage 3 of oogenesis (Figure 22A). During mid oogenesis, its expression was found to be downregulated in the nurse cells. At stage 10B, *snoN* was expressed as a characteristic dorsolateral stripe in the main body follicle cells (Figure 22B, C). The expression in these cells persists until the end of oogenesis. A weak expression of *snoN* was still visible in the nurse cells in late stages of oogenesis

(Figure 22B, C). At stage 14, *snoN* was found associated with developing dorsal appendages (Figure 22D). An enhancer trap line, l(2)Sh1402 (referred to *snoN*LacZ henceforth) mimicks the *snoN* expression pattern during oogenesis (Figure 22E-H and Oh et al., 2003).

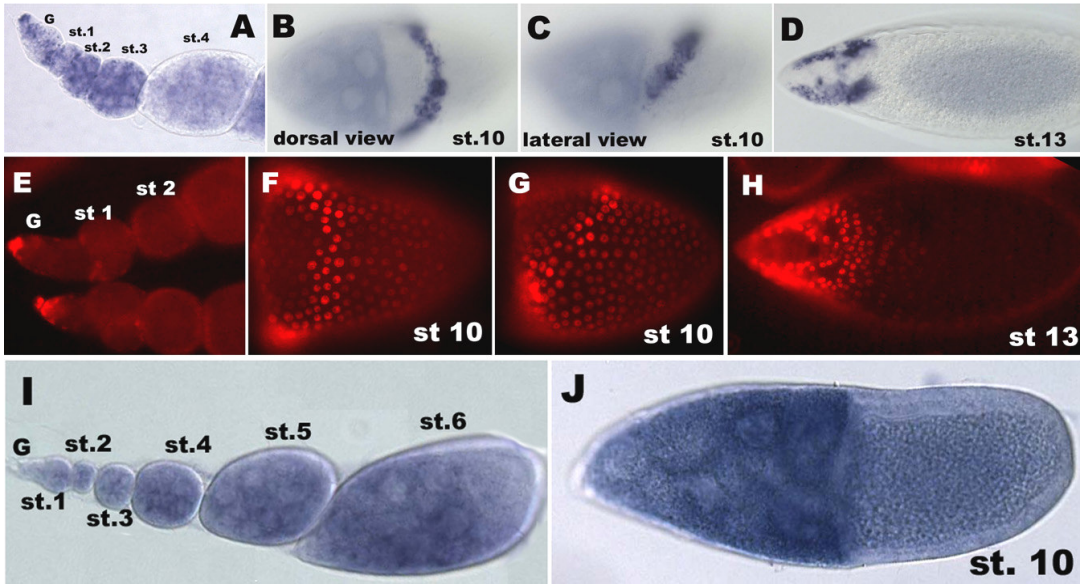

Figure 22

Expression pattern of *snoN* and *ski* in wild type ovaries.

Anterior is to the left. (A-D) *snoN* expression detected by whole mount RNA in situ hybridization. (A) *snoN* is first expressed in germarium (G) *snoN* transcript can be detected in early egg chambers stages 1-4. (B) At stage 10B, *snoN* is expressed as a dorsolateral stripe. Shown here is the dorsal expression domain of *snoN* (C) Depicts the lateral view of egg chamber in B. (D) In late stages, *snoN* expression is associated with dorsal appendages. (E-H) Anti β-gal stainings showing *snoN*LacZ enhancer trap expression which mimics *snoN* RNA in situ expression pattern except in (E) where *snoN*LacZ is expressed in the germarium (G) but not in early stages.(I, J) *ski* expression detected by whole mount RNA in situ hybridization. (I) *ski* message was detected in nurse cells and follicle cells during all stages of oogenesis. Shown here stage 1-6 (J) *ski* expression at stage 10B (lateral view). *ski* in situs were developed for a longer time as compared to *snoN* in situs.

ski, too is expressed during oogenesis albeit at a very low level in the germarium and in the nurse cells at early stages (Figure 22I; see Figure legend). No characteristic pattern was detectable in any of the cell types during mid oogenesis. In stage 10B egg chambers, *ski* expression was dectable at uniform levels in the nurse cells and in the follicle cell layer (Figure 22J).

To get an idea of relative expression of BR-C and *snoN* with respect to each other we monitored their expression simultaneously in the follicular epithelium. We detected β-gal and BR-C protein in ovaries dissected from *snoN*LacZ line. It was found that *snoN* is expressed at both the posterior and lateral boundaries of BR-C expression (Figure 23A-F).

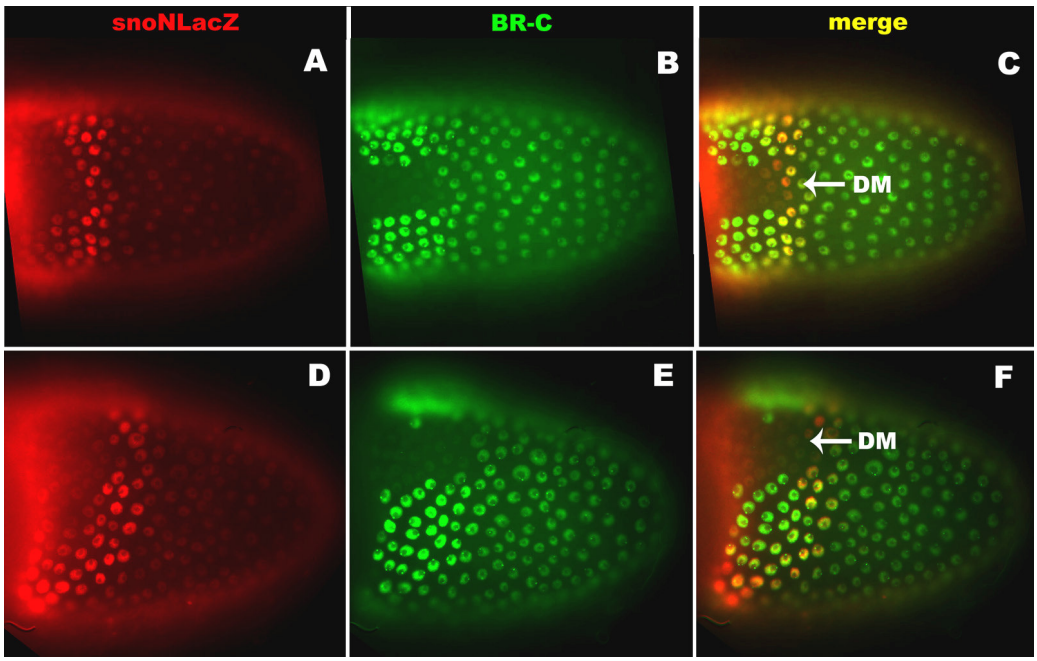

Figure 23

***snoN* is expressed at the posterior and lateral boundaries of BR-C expression domain in wt**

Anterior is to the left. Only posterior half of the egg chambers at stage 10B are shown here (A-C) Dorsal view of stage 10B egg chambers. (D-F) Dorsolateral view of stage 10B egg chambers. Anti β-gal (red) and anti-BR-C (green) show overlap (yellow) at the posterior and lateral boundaries of BR-C expression. (A, D) *snoN* expression detected by anti β-gal stainings performed on *snoN*LacZ line. (B, E) BR-C protein detected by anti BR-C antibody. (C, F) Merge showing the expression domains with respect to each other. White arrows mark the dorsal midline (DM).

During embryogeneis, initial weak ubiquitous expression of *snoN* and *ski* was observed at the cellular blastoderm stage of the embryo (Figure 24A, C). In later stages of embryogenesis, both *snoN* and *ski* have different expression patterns. *snoN* was upregulated in the central nervous system: the nerve cord and the developing larval brain (Figure 24B). Interestingly, *ski* expression was detected

as punctate staining in three groups of cells (probably a subset of neurons) one on either side of ventral midline in every parasegment (Figure 24D). In addition, *ski* is also expressed in specific neurons in the larval brain.

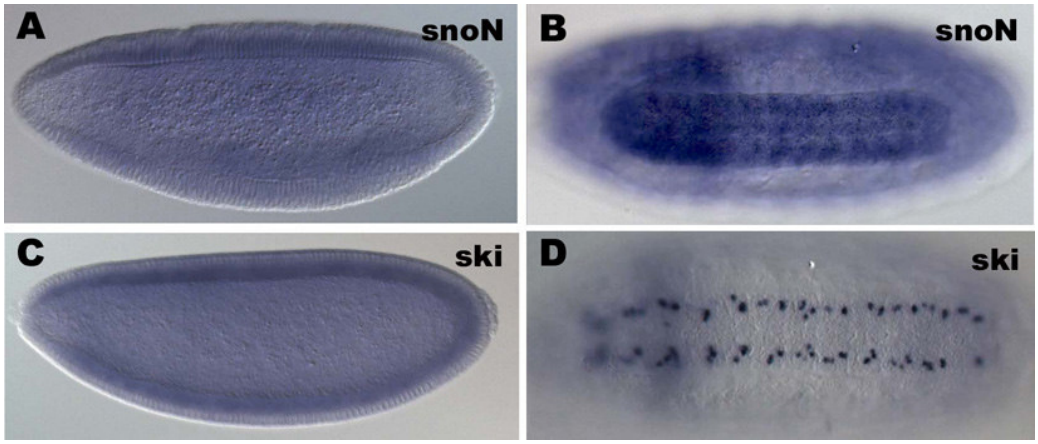

Figure 24

Expression pattern of *snoN* and *ski* in wild type embryos.

Anterior is to the left. (A, C) Dorsal is facing up. (B, D) Ventral views of stage 14 embryos. (A-D) *snoN* and *ski* expression detected by whole mount RNA in situ hybridization in embryos (A) *snoN* is first expressed at low levels in the blastoderm embryos. (B) Later in embryogenesis *snoN* expression is detected in the developing nerve cord and the head region. (C) *ski* is expressed at uniform levels in the embryo at blastoderm stage. (D) At stage 14 *ski* is expressed in groups of cells on either side of ventral midline.

Lack of mutants, P element insertions and deficiencies available for the study of *ski* (4^{th} chromosome) prevented us from investigating its function during *Drosophila* development. Efforts were concentrated on elucidating the role of *snoN* in patterning of follicular epithelium.

To sudy the SnoN protein expression profile and its subcellular localization in wt egg chambers, we generated polyclonal antibodies against SnoN by injecting SnoN-myc-his tagged fusion protein expressed in *Escherichia coli* (see Materials and Methods). To test the quality and titre of antibodies generated from rats we performed western blotting using partially purifired SnoN-myc-his tagged fusion protein. Upon developing the western blot it was found that a high titre of anti-SnoN antibodies were present in the rat serum (Figure 25A- see

Figure legend). SnoN fusion protein was detected in an independent experiment using anti-myc antibodies which served as an important control (Figure 25B).

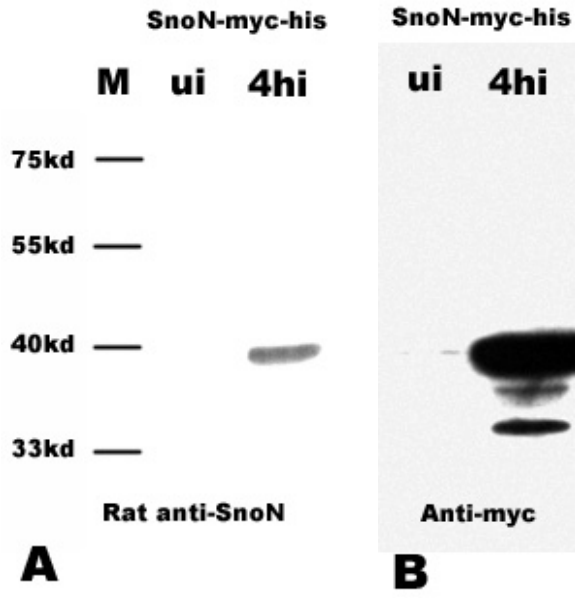

Figure 25

Detection of SnoN fusion protein using anti-SnoN antibodies.

(A, B) Detection of purified SnoN-myc-his fusion protein (~40kDa) using immunoblotting technique (A) Using Rat anti-SnoN antibodies (dilution used 1:100,000; 3 sec exposure) (B) Using Anti-myc antibody (control; dilution used 1:5000, 10 sec exposure). M –10-180 kDa Standard Protein Marker (MBI Fermentas), ui-protein extracted from uninduced bacterial culture and 4hi- protein extracted from bacterial cells 4h postinduction. Approximately 500 ng protein was loaded in each lane.

We were not able to detect endogenous SnoN protein using the anti-SnoN antibodies in whole mount immunostainings performed on wt ovaries. There could be two possible reasons. We were unable to obtain 100% pure SnoN fusion protein (antigen) from crude bacterial lysates. Therefore, we performed preparative SDS gel electrophoresis to further purify it. The ~ 40kDa band from the preparative SDS gel was cut and delivered for immunizing rats. It is possible that SnoN fusion protein (antigen) was still in its denatured form and the anti-SnoN antibodies generated against it are able to detect only the denatured form of SnoN. Second, the endogenous SnoN protein levels could be very low and hence undetectable by the anti-SnoN antibodies.

3.8 *snoN* expression in the follicular epithelium depends on Grk and Dpp

The gradient of Dpp is established along the AP axis of the egg chambers. Therefore, based on the expression pattern of *snoN* it is conceivable that high as well as moderate levels of Dpp signaling activate *snoN*. To test the input from Dpp pathway, *snoN* expression was monitored in two different genetic backgrounds. First in which Dpp signaling is hyperactivated in the follicle cells (UAS*dpp* X CY2Gal4) and a second in which Dpp signaling activity is compromised in the follicle cells (*Med* mutant follicle cell clones). We have followed *snoN* expression by tracking expression of *snoN*LacZ in *Med* mutant clones.

snoN was observed to be ectopically expressed in stage 10 egg chambers derived from females misexpressing *dpp* in the whole follicular epithelium under the control of CY2Gal4 (Figure 26A, B). Interestingly, *snoN* was also detected in the dorsal anterior follicle cells (Figure 26B). Thus, Dpp can ectopically induce *snoN* expression even in dorsal anterior region where it is normally repressed. *Med* mutant clones were generated in follicle cell using the FRT/FLP system described by Xu and Rubin (1993). Homozygous *Med* mutant clones marked by absence of GFP showed a cell autonomous abscence of *snoN*LacZ expression (Figure 26C, D). This result proved that in absence of Dpp signaling *snoN* is not activated. Thus, we can conclude that Dpp is absolutely necessary for inducing *snoN* expression in the follicular epithelium.

We performed yeast two hybrid experiments in order to investigate whether SnoN interacts with BMP activated R-Smad, Mad or Activin/TGF-β specific R-Smad, dSmad2. We found that snoN interacts with neither of them nor with the common Smad, Med. This led us to conclude that SnoN might need an additional protein in order to associate with the Smad proteins or SnoN might need a specific post-translational modification(s) to interact with Smads.

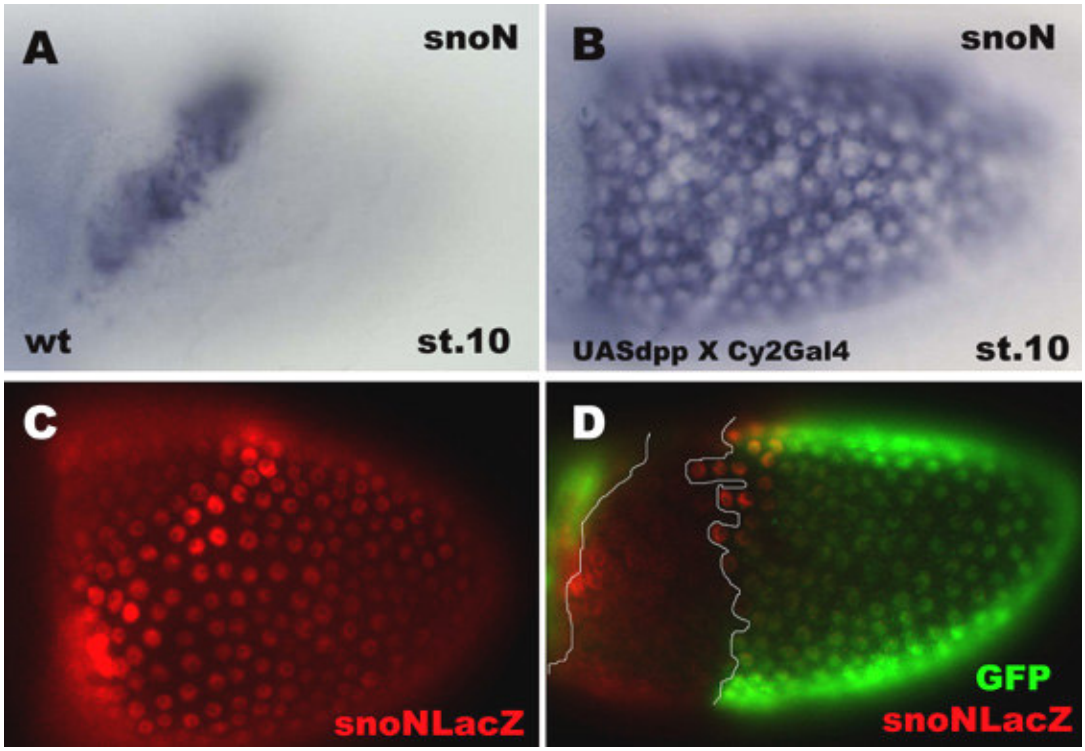

Figure 26

***snoN* expression is regulated by Dpp signaling in the follicular epithelium.**

Anterior is to the left. Only posterior half of the egg chambers at stage 10B are shown here (A-D) Dorsal is facing up. (A-B) *snoN* expression detected by whole mount RNA in situ hybridization in ovaries (A) wt *snoN* expression pattern in stage 10B egg chamber. (B) *snoN* expression in egg chambers obtained from females misexpressing *dpp* in the follicular epithelium. (C) *snoN* expression visualized by using *snoN*LacZ (red) line in wt.(D) Loss of GFP (green) marks follicle cells lacking Med. *snoN*LacZ (red) is not expressed in *Med* mutant follicle cell clones.

The asymmetry of *snoN* expression across the DV axis in the follicle cells lead us to test if *snoN* expression is regulated by Grk signaling pathway. To test the input from Grk pathway, *snoN* expression was monitored in two different genetic backgrounds; first when the Grk signaling is hyperactivated in the follicle cells and second when Grk activity is diminshed in the follicle cells.

To hyperactivate the Grk/EGFR pathway we generated the same genetic situation as used for testing the role of Grk signaling in patterning of eggshell structures (see Section 3.4 for details). In stage 10B egg chambers dissected from UASp*grk*/tubGal4VP16 females, expression of *snoN* was observed to be

altered from a normal asymmetric dorsolateral expression to a completely symmetric pattern (Figure 27A, B). In addition, *snoN* domain appeared to be shifted posteriorly along the AP axis. Females homozygous for *cni* mutation were generated and *snoN* expression was examined in the ovaries of these females. *snoN* expression was neither detected at early stages nor at stage 10B or at later stages of oogenesis (Figure 27A, C). This confirmed that Grk signaling is not only repressing *snoN* at highest levels but also that it is essential for the activation of *snoN* expression.

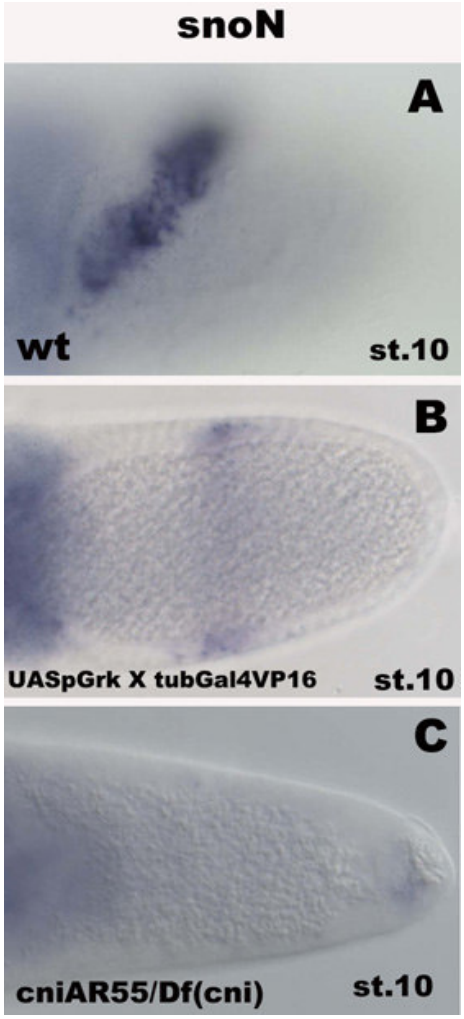

Figure 27

***snoN* expression is regulated by Grk signaling in the follicular epithelium.**

Anterior is to the left. Only posterior half of the egg chambers at stage 10B are shown here (A, B) Dorsal is facing up. (A-C) *snoN* expression detected by whole mount RNA in situ hybridization in ovaries (A) Wt *snoN* expression pattern in stage 10B egg chamber. (B) *snoN* expression in egg chambers misexpressing *grk* in the germline cells. (C) *snoN* expression is absent in *cni*$^{-}$ egg chambers.

3.9 Generation of a *snoN* mutant

Mutations in *snoN* were generated by imprecise excisions of the P element insertion, designated as l(2)Sh1402 (Figure 28; Oh et. al 2003). The breakpoints of the deletions were mapped by PCR revealing two classes of mutation. First, deletions extending 3' with respect to the P element orientation which remove a part of *snoN* coding sequence and second, deletions which remove the *snoN* coding region completely. Both classes of the deletions remove predicted *snoN* promoter sequences (Flybase v4.2.1). We uncovered a total of 17 different *Drosophila* lines which fall in either of the above mentioned classes. The second class of deletions is comparatively large and in one of the mutant line $snoN^{174}$, the deletion extends upto 9kb including the *snoN* coding region (Figure 28-blue dotted line). This excision $snoN^{174}$ is denoted as *snoN*-/- henceforth. Suprisingly, lines representing both the deletion classes are homozygous viable.

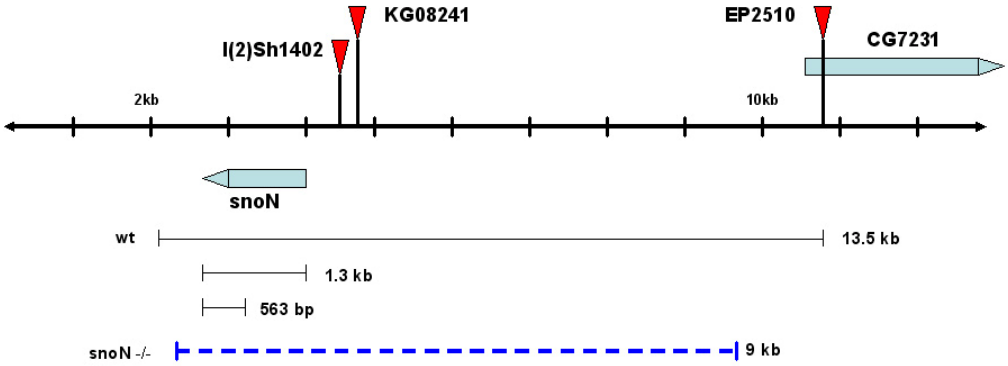

Figure 28

Map of *snoN* genomic region.

Genomic region in the vicinity of *snoN*. Representative illustration of band 28D3 (cytological location) of the left arm of second chromosome of *Drosophila* melanogaster. Approximate length of the genomic region shown here is ~17 kb. Light blue bars depict the predicted Open reading frames (ORFs). Red arowheads depict the P element insertions in this region. Deletions of *snoN* were generated by mobilizing the P element l(2)Sh1402, which is inserted 719 bp upstream of *snoN*. PCR amplification was performed on the potential deletions obtained. Dotted blue line depicts the genomic region missing (9kb deletion) in *snoN* mutant. The mapping of deletion was based on three different amplicons with different sizes 563bp, 1.3 kb, and 13.5 kb. The direction of arrowhead shows the direction of transcription. The P element l(2)Sh1402 is oriented in same direction as the direction of transcription of *snoN*.

3.9.1 *sno N*-/- is a molecular null allele.

In order to confirm *snoN*-/- is a molecular null allele we performed *snoN* in situ hybridization on ovaries dissected from homozygous *snoN*-/- mothers. The characteristic *snoN* expression as a dorsolateral stripe in the follicular epithelium was absent in *snoN* mutants (Figure 29 A, B). This confirms our earlier observation of the deletion of *snoN* coding sequence. Thus, *snoN*-/- flies are unable to synthesize *snoN* mRNA and hence no SnoN protein would be formed confirming that the *snoN-/-* is a null allele of *snoN*.

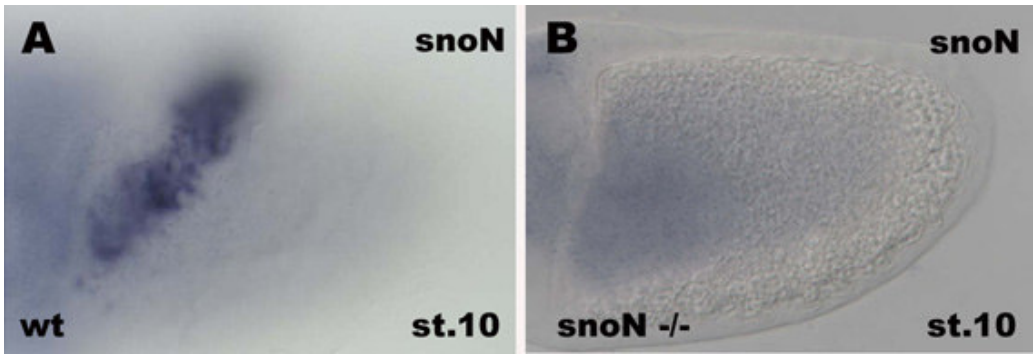

Figure 29

***snoN*-/- is a molecular null allele**

Anterior is to the left. Dorsal is to the top. Only posterior half of the egg chambers at stage 10B are shown here (A) *snoN* mRNA distribution in wt stage 10B egg chamber. (B) *snoN* mRNA is absent in *snoN*-/- mutant egg chambers.

snoN-/- flies are homozygous viable and produce viable progeny at laborotary conditions. However, a detailed analysis revealed that as compared to wt flies, *snoN*-/- flies have reduced viability at every stage in the fly life cycle (Figure 30). Out of 455 hatched larvae we obtained 341 adults in case of wt while only 155 adults were obtained out of 285 *snoN* mutant larvae. Thus, there was up to 50% reduction in viable progeny from larval stages till adult flies in case of *snoN* mutant. This effect was more pronounced when the flies were reared at 29^{o} C.

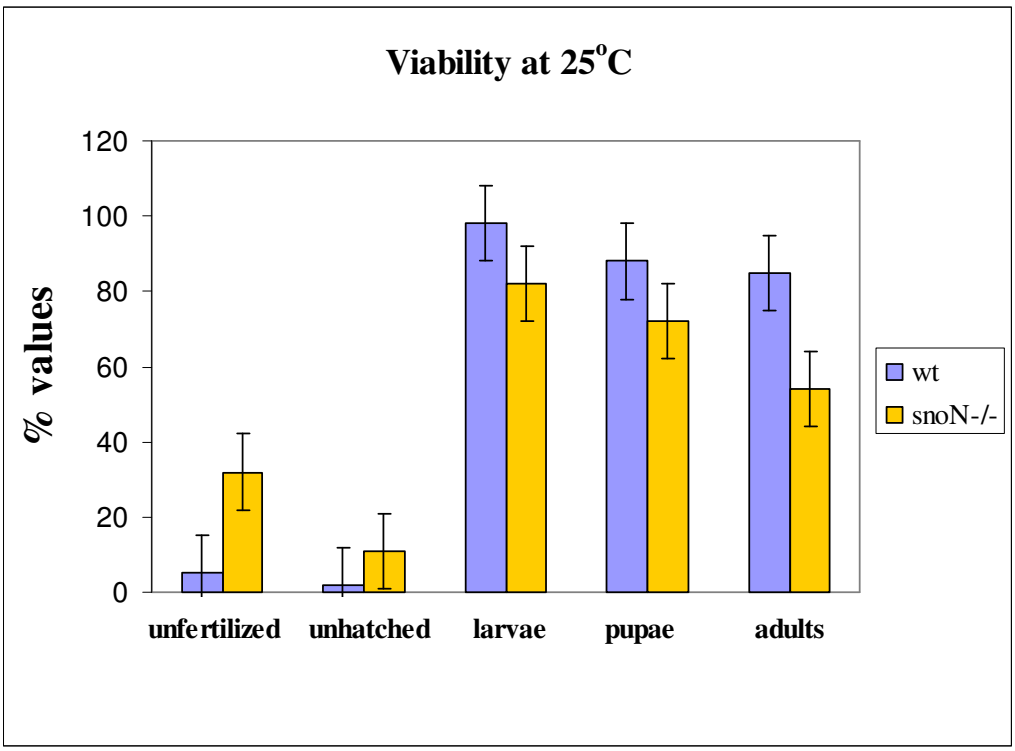

Figure 30

***snoN* mutant flies have reduced viability.**

Histogram illustrating the comparative fertilization and viability analysis of wt vs *snoN* mutant flies on standard cornmeal agar medium at 25°C. Y axis coordinates depict percent values at each stage during *Drosophila* life cycle. X axis coordinates depict the various stages in *Drosophila* life cycle: unfertilized eggs, fertilized but unhatched eggs, 1st 2nd and 3rd instar larval stages, pupae and adults. Light blue bars show data for wt while yellow bars show data for *snoN* mutant. Standard deviation values are shown as vertical segments in each bar.

3.9.2 SnoN is required for formation of operculum and dorsal appendages

Although, flies mutant for *snoN* have reduced viability, a sufficient number of eggs are laid by females during their lifespan. We examined if the eggshell pattern was disrupted in *snoN* mutants. One of the eggshell defects was an increase in average length of the operculum (Figure 31A-D). The increase in the operculum length was quantified by measuring the angle between a line drawn horizontally through each egg and a line drawn from the anterior most tip to the posterior most end of the operculum (Gupta and Schupbach, 2003). Angles for all the eggs were then averaged. Smaller angles indicate larger opercula. Eggs from wt female had an average angle of 29.58° (Gupta and Schupbach, 2003).

By contrast, eggs from females homozygous for *snoN* mutation had an average angle of 18.70^{o} (Figure 31A, B). To further confirm the expansion of operculum in *snoN* mutants we performed Fas III stainings on *snoN* mutant ovaries. Fas III was observed to be expanded along the AP axis in *snoN* mutants (compare Figure 31E and F). Thus, elimination of *snoN* in follicle cells results in significant increase of the operculum.

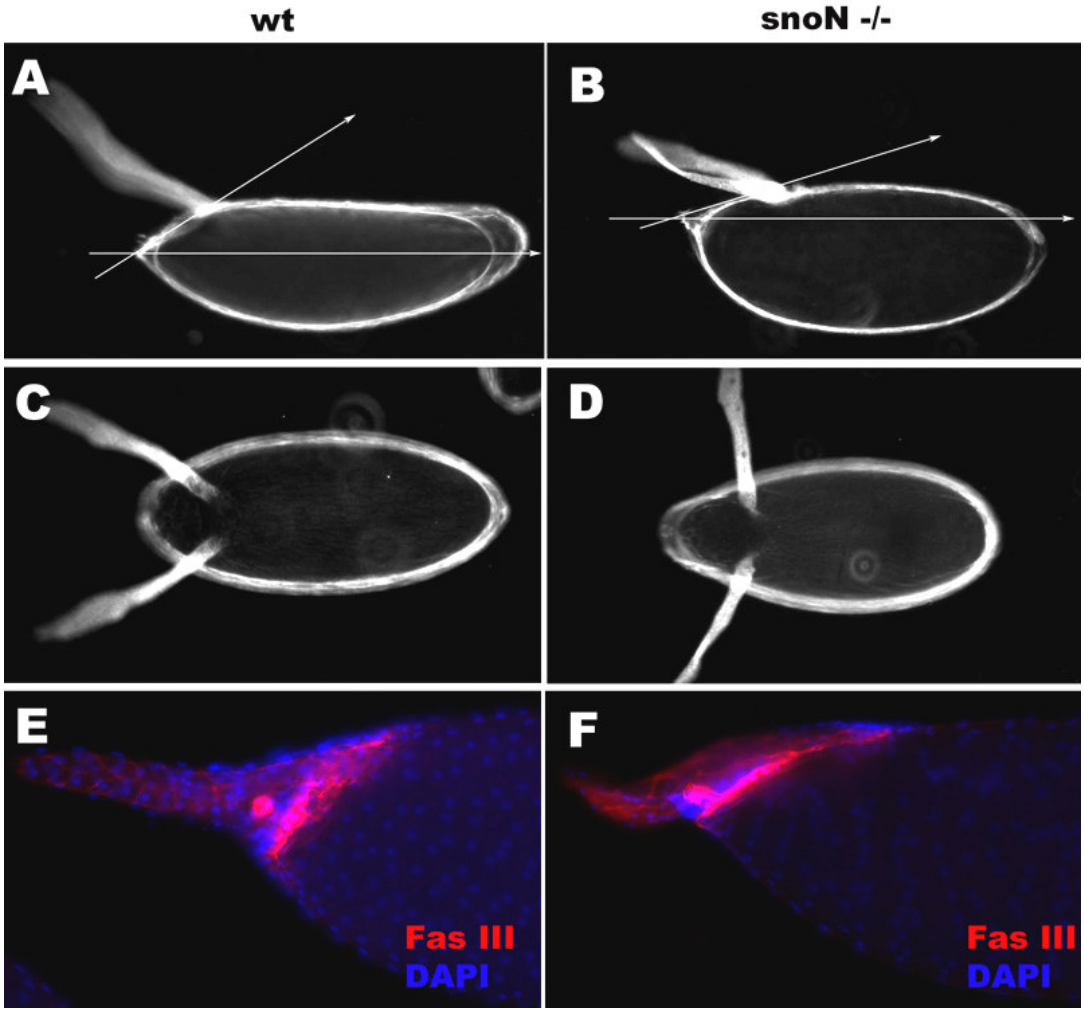

Figure 31

Operculum size is increased in *snoN* mutant eggs

Anterior is to the left. (A, C, E) Wt eggs and stage 14 egg chamber. (B, D, F) *snoN* mutant eggs and stage 14 egg chamber. (A) Lateral view of wt egg (B) Lateral view of egg laid by *snoN* mutant mothers. (C) Dorsal view of wt egg. (D) Dorsal view of egg laid by *snoN* mutant mothers. (E) Stage 14 egg chambers stained with anti-Fas III (red) and DNA (blue = DAPI) (F) Stage 14 egg chamber from *snoN* mutant ovary stained with anti-Fas III (red) and DNA (blue = DAPI). White lines in A, B represent the angle measured as a function of operculum length

During our examination of *snoN* mutant eggs we discovered that the average length of dorsal appendages was also significantly reduced (Figure 32 compare A and B). *BR-C* expression was monitored in *snoN* mutant egg chambers. In contrast to wild type egg chambers where 50-55 (n=10) cells express *BR-C* in *snoN* mutant egg chambers only 35-40 (n=13) cells express *BR-C*. Interestingly, the two BR-C domains in *snoN* mutant shift laterally as compared to wt. Thus, there is upto 20% reduction in *BR-C* expressing cells and hence fewer cells participated in making dorsal appendages resulting in smaller dorsal appendage.

pipe is predicted to be regulated by the Dpp signaling pathway (F. Peri thesis). A careful analysis of the *pipe* promoter reveals characteristic *snoN* binding sites (5' GTCTAGAC). We therefore analysed *pipe* mRNA distribution in *snoN* mutant egg chambers. *pipe* was found to be derepressed laterally and in the posterior dorsal follicle cells in *snoN* mutants (Figure 32C, D). However, the derepression of pipe was very subtle.

We investigated if expression of *rho* and *aos* which are also regulated by Dpp had an altered expression pattern in *snoN* mutants (Dobens and Raftery, 2000; Peri and Roth, 2000). *rho* expression in the follicle cells begins as a dorsal anterior patch at stage 9 and then resolves into two dorsolateral stripes at stage 10B of oogenesis (Neuman-Silberberg and Schupbach, 1994; Peri et al., 1999). *aos* is expressed in dorsal centripetal follicle cells and as a faint dorsal patch at stage 10A which refines into a T shaped pattern at stage 12 of oogenesis (Zhao and Bownes, 1999). *rho* and *aos* expression in *snoN* mutants is upregulated and spreads more laterally (compare Figure 32E, G and F, H). Thus, expression of all three genes *rho*, *aos* and *pipe* appears to be derepressed in *snoN* mutant.

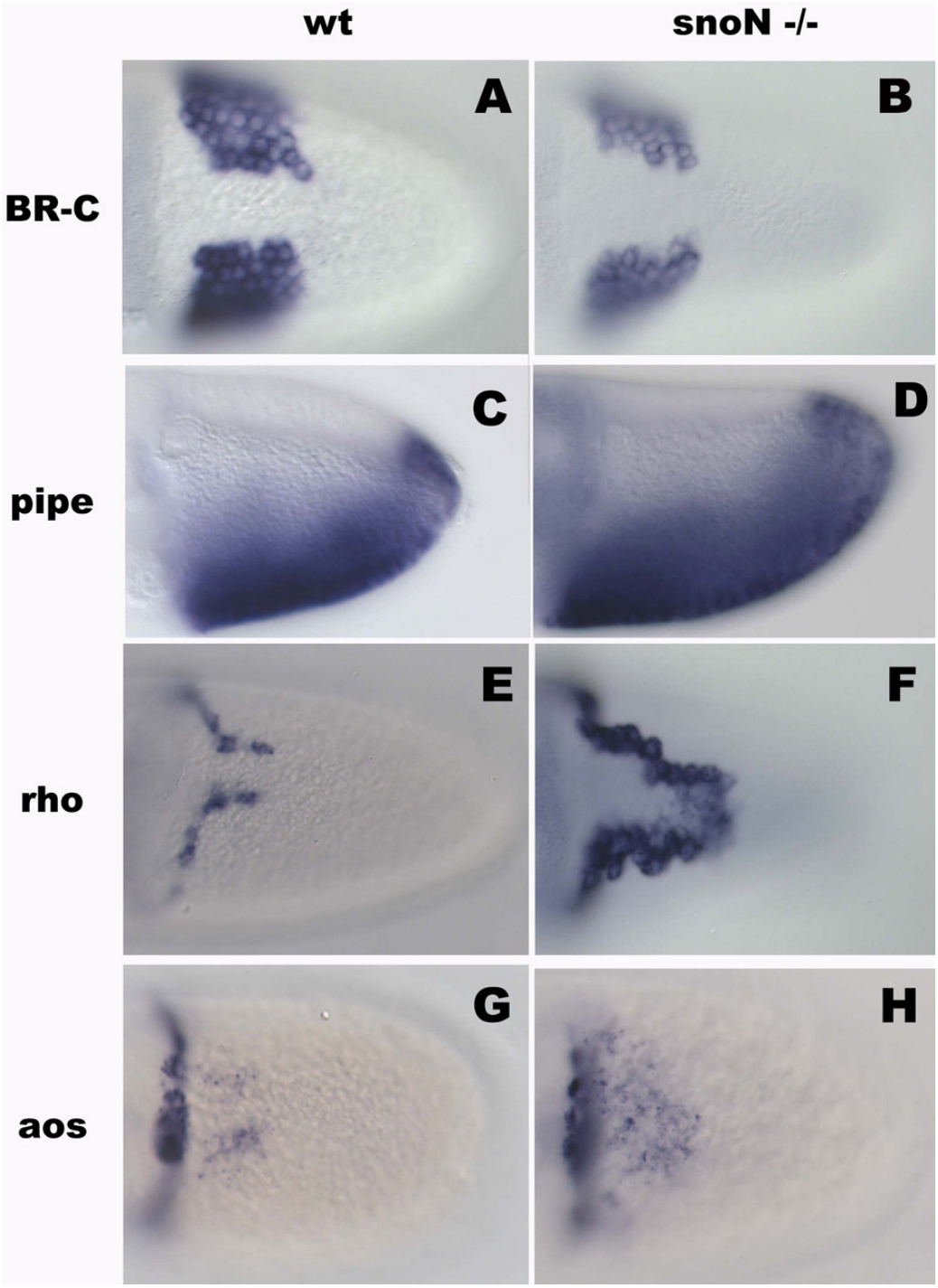

Figure 32

***snoN* mutant exhibit defects in patterning of follicular epithelium.**

Anterior is to the left. Only posterior half of the egg chambers are shown here (A-B) and (E-H) Dorsal views of stage 10B egg chambers. (C, D) Lateral views of stage 9 egg chambers. (A, B) *BR-C* mRNA distribution in wt and *snoN* mutant egg chamber. (C, D) *pipe* mRNA distribution in wt and *snoN* mutant egg chamber at stage 10A. (E, F) *rho* mRNA distribution in wt and *snoN* mutant egg chamber and (G, H) *aos* mRNA distribution in wt and *snoN* mutant egg chamber at stage 10B respectively.

3.9.3 SnoN inhibits Dpp signaling in ovary and in wing

SnoN is a well characterized transcriptional repressor of TGF-β signaling pathway in vertebrates (Stroschein et al., 1999; Miyazono, 2000; Luo, 2004). We have shown that loss of function of *snoN* leads to increase in the operculum size as well as decrease in the dorsal appendage anlagen. We asked if misexpressing *snoN* in the whole follicular epithelium during oogenesis would have an opposite effect. Indeed, females misexpressing *snoN* in whole follicular epithelium (UAS*snoN* X CY2Gal4) laid eggs with decreased operculum (Figure 33A-D). The average operculum length was decreased from 29.58^{o} to 35.1^{o} (compare Figure 33A, B).

The *Drosophila* wing tissue is sensitive to Dpp levels and a decrease in Dpp signaling can lead to dramatic decrease in the size of wing. Such a decrease in wing size was observed when *sog* and *brk* were misexpressed in the wing epithelium (Jazwinska et al., 1999a; Jazwinska et al., 1999b; Yu et al., 2004). We misexpressed UAS*snoN* in the *optomotor blind* (*omb*) domain (using ombGal4 driver line) in the wing imaginal disc. The *omb*Gal4 driven UAS-transgenes express in the central region of the wing disc (Grimm and Pflugfelder, 1996). Flies misexpressing *snoN* in the wing had a characteristic "held out" wing phenotype, typical of Dpp pathway mutants (Spencer et al., 1982; Tabata, 2001). Moreover, the overall wing size was dramatically reduced. In addtition, the wings showed pronounced venation defects as compared to wt wing indicating a strong repression of Dpp activity in the wing (Figure 33E, F). In brief, SnoN acts as a repressor of Dpp activity in patterning of follicular epithelium and wing imaginal disc.

To get a deeper insight into the mechanism of SnoN action on the Dpp pathway we generated UAS*snoN* constructs fused to either engrailed repressor domain or VP16 acitivation domain separately (Triezenberg et al., 1988; Smith and Jaynes, 1996). Fusion of SnoN to engrailed repressor domain would convert it into a more potent repressor while fusion to VP16 activation domain would turn it into an activator. However, when msiexpressed neither of the fusion

constructs showed a stronger or a novel egg shell or wing phenotype as compared to *snoN* misexpression.

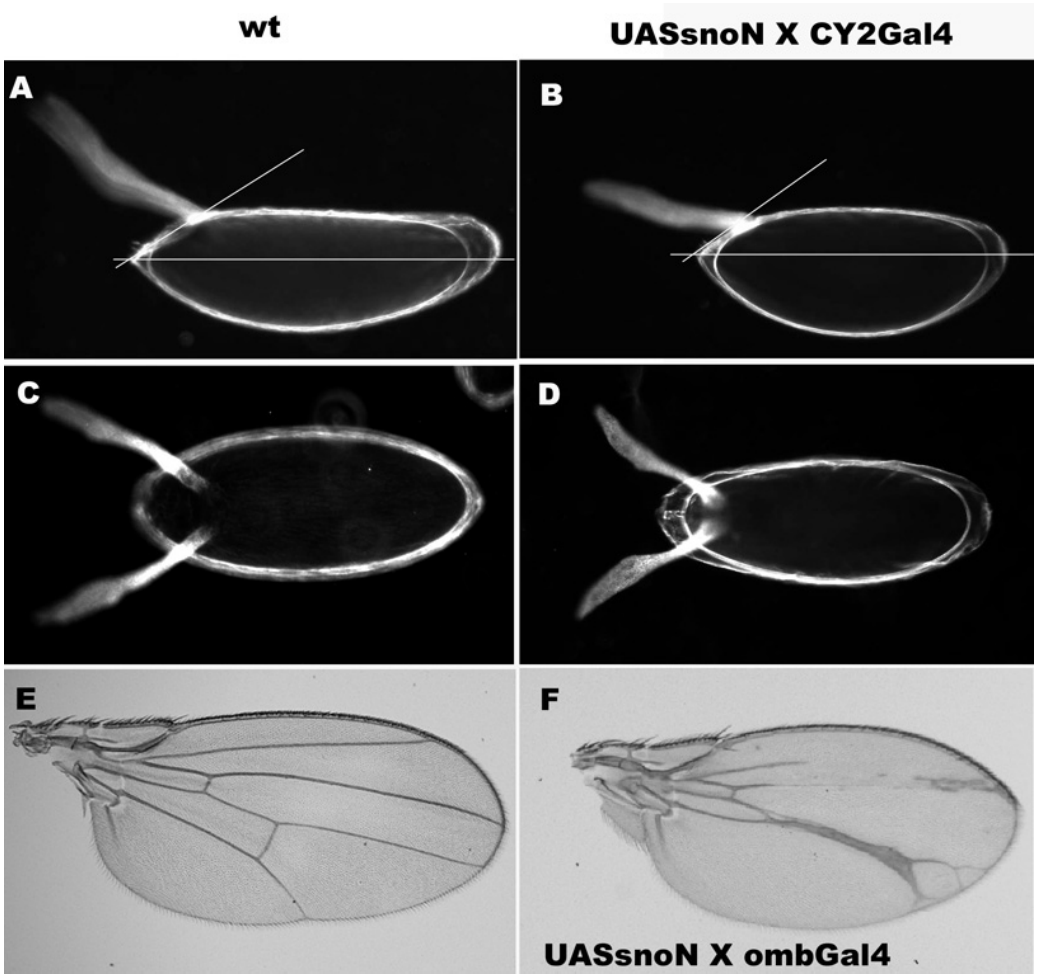

Figure 33

***snoN* acts as a repressor of Dpp signaling.**

In (A-D) Anterior is to the left. (E, F) Whole mount of adult wings.(A) Lateral view of wt egg (B) Lateral view of egg laid by mothers misexpressing UAS*snoN* in the whole follicular epithelium. (C) Dorsal view of wt egg. (D) Dorsal view of egg laid by mothers misexpressing UAS*snoN* in the whole follicular epithelium. (E) Wt adult wing (F) Adult wing produced upon misexpressing UAS*snoN* in the *optomotor blind* (*omb*) domain. White lines in A, B represent the angle measured as a function of operculum length.

3.10 *brk* and *sog* are expressed in distinct domains in the follicular epithelium

Brk and Sog are known to negatively regulate Dpp signaling and act via two distinct mechanisms in the embryo. Brk acts as a differential repressor of Dpp target genes during embryogenesis and wing development (Jazwinska et al., 1999a; Jazwinska et al., 1999b; Kirkpatrick et al., 2001). Sog affects BMP signaling by two modes of action in the embryo–short range inhibition (in lateral and ventral region) and long range enhancement (in the dorsal most region) (Ashe and Levine, 1999; Ashe, 2002; Marques et al., 2002; Srinivasan et al., 2002). Whether Sog and Brk have a similar function or act via completely different mechanisms during oogenesis is unknown. To understand this in detail we started by analyzing *sog* and *brk* expression during oogenesis.

We examined *sog* expression during oogenesis by performing RNA in situ hybridization on wt ovaries. At stage 10B, *sog* was detected in all the CMFCs exactly where *dpp* is also expressed at this stage of oogenesis (Figure 34A, B; Araujo and Bier, 2000). However, *sog* was aymmetrically expressed in these cells. Dorsal CMFCs showed a stronger signal compared to ventral cells (Figure 34B). *brk* was found to be expressed asymmetrically in the follicular epithelium (Figure 34C). Its expression has two features. First, it is excluded from the very anteriormost region of the follicle cell layer (Figure 34C, D). Second, its expression has a DV asymmetry – strong expression was observed in the dorsal follicle cells as compared to ventral follicle cells (Figure 34C, D).

From the expression patterns of *dpp*, *sog* and *brk* it can be concluded that *sog* is expressed at highest levels of Dpp and Grk signaling while *brk* is expressed at relatively lower levels of Dpp and high levels of Grk signaling. Due to the characteristic restriction of *sog* and *brk* to two distinct domains we asked if their expression would be regulated by Dpp and Grk signaling. We addressed this question by analyzing *sog* and *brk* in *dpp* and *grk* misexpression separately.

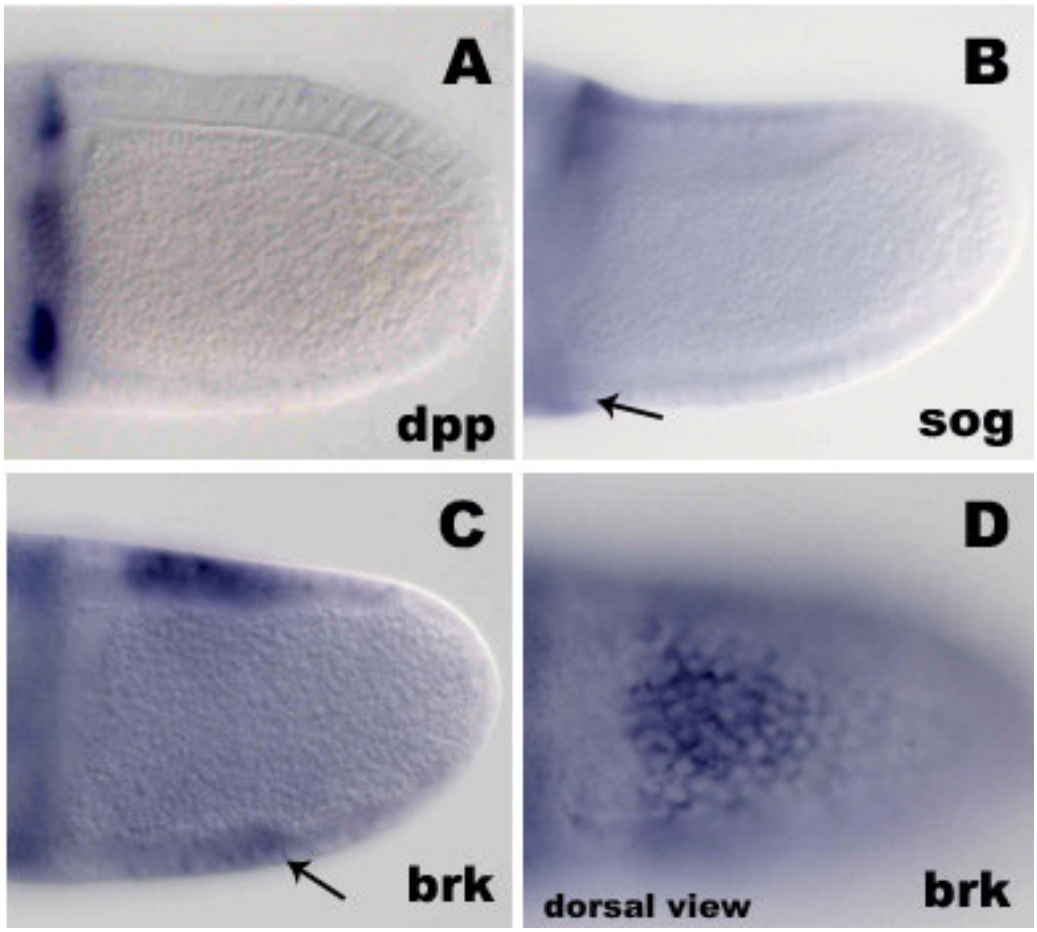

Figure 34

Expression of *dpp*, *brk* and *sog* during oogenesis

Anterior is to the left. Only posterior half of the egg chambers at stage 10B are shown here (A-C) Lateral view and (D) Dorsal view of stage 10B egg chamber. RNA in situ hybridization pattern for (A) *dpp* (B) *sog* and (C, D) *brk*. Black arrows in B and C point the low level of expression in ventral follicle cells.

sog expression was upregulated asymmetrically in the dorsal anterior follicle cells in both moderate and strong *dpp* misexpression in follicular epithelium (Figure 35A, B). This was a suprising result and suggests that there is a hidden dual input on *sog* expression most likely by Grk signaling. In *grk* misexpression, *sog* expands along the AP axis. High levels of *sog* were still detected in the CMFCs but in addition, low levels of *sog* were detected in anterior ring of follicle cells (Figure 35C). In combined misexpression situation, *sog* expression expands along both axis in almost all main body follicle cells (Figure 35D, E). These data suggest that *sog* is regulated by both Dpp and Grk which is consistent with its wild type expression pattern.

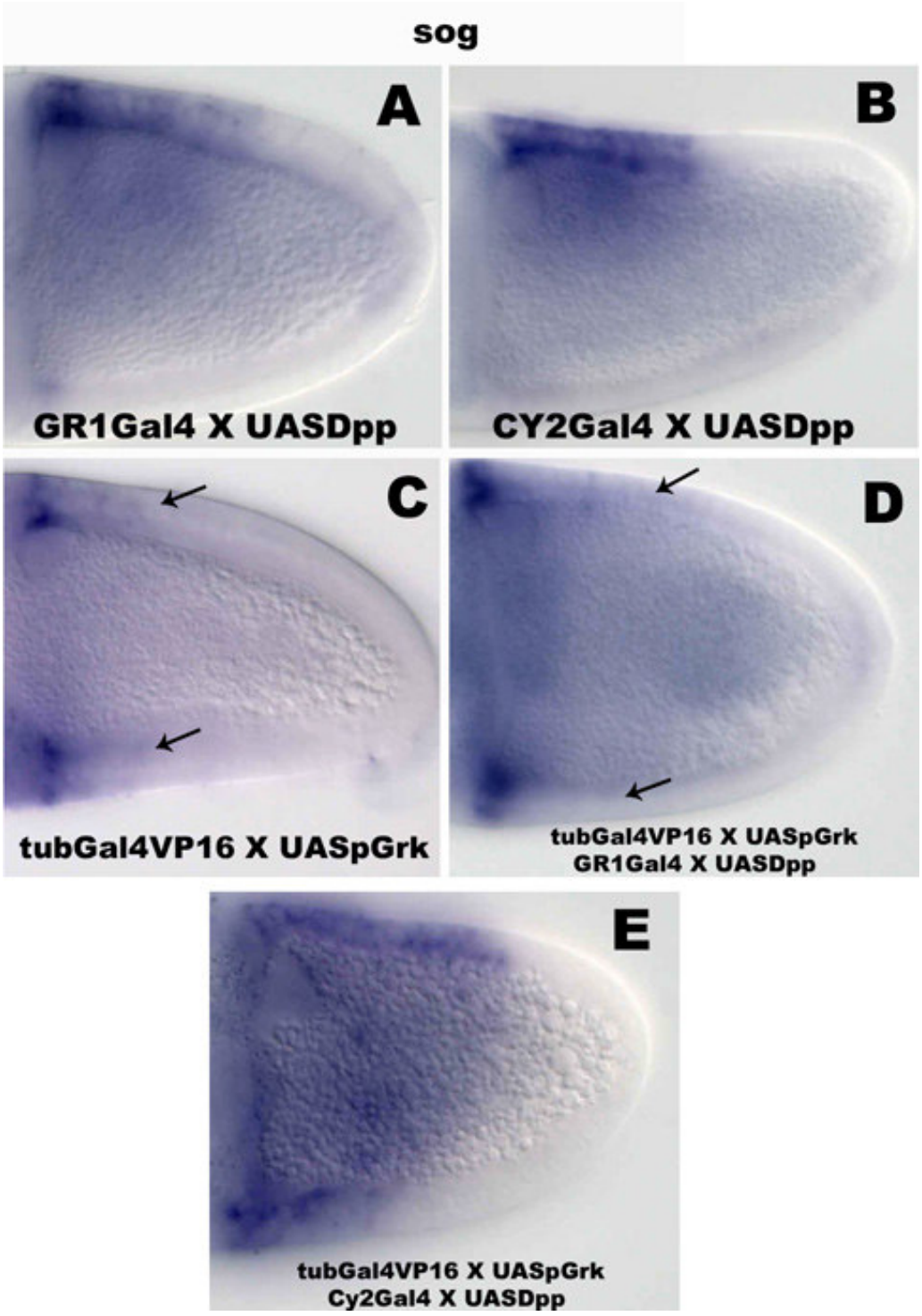

Figure 35

Expression of *sog* in *dpp* and *grk* misexpression

Anterior is to the left. Only posterior half of the egg chambers at stage 10B are shown here (A-E) Lateral views of stage 10B egg chambers. RNA in situ hybridization for *sog* in various misexpression conditions (A) In moderate and (B) In strong *dpp* misexpression (C) In *grk* misexpression and (D) In combined misexpression- moderate *dpp* and strong *grk*. (E) in combined misexpression- strong *dpp* and strong *grk*. Black arrows in C and D point lower levels of *sog* expression.

Consistent with its wt expression pattern, *brk* expression was downregulated by Dpp signaling. Moderate levels of Dpp (generated by GR1Gal4 X UAS*dpp*) shift the *brk* expression domain more posteriorly (Figure 36A) while high levels of Dpp (generated by CY2Gal4 X UAS*dpp*) completely suppress it (Figure 36B). Two different levels of Grk signaling result in two distinct chorion phenotypes. Correspondingly, *brk* was detected exclusively in ventral region of the egg chambers in which *grk* was moderately misexpressed (Figure 36C) while expression of *brk* is completely downregulated in stage 10B egg chambers where *grk* was strongly misexpressed (Figure 36D). In high *grk* and moderate *dpp* combined misexpression situation, *brk* expression was found in posterior half of main body follicle cells (Figure 36E). *brk* was totally repressed in prescence of high Dpp and high Grk signaling indicating a negative input on *brk* expression (Figure 36F). Taken together, from the above observations we can correlate prescence of dorsal appendages with expression of *brk*. This suggests that Brk might have a role in specification of dorsal appendage fate.

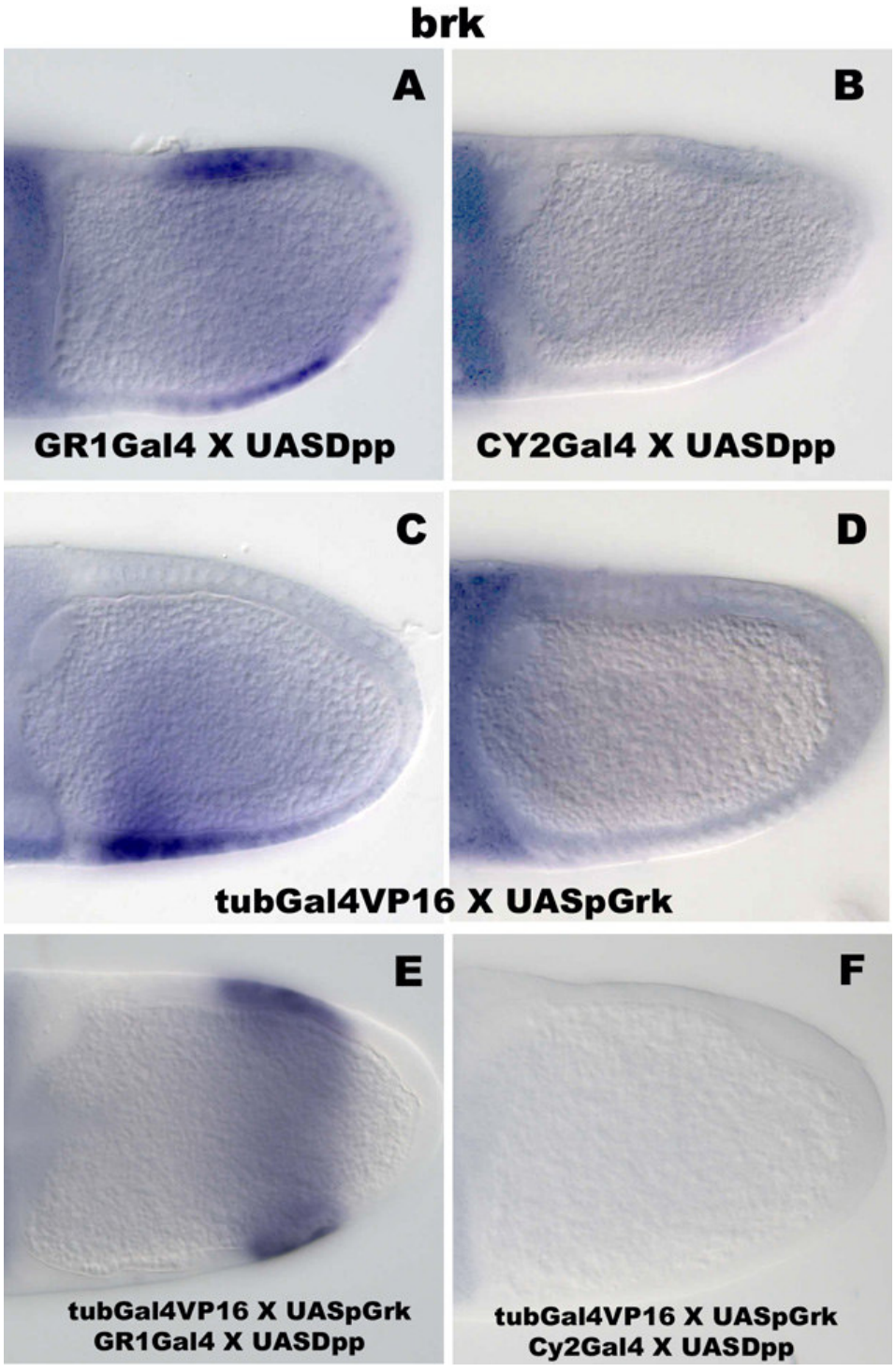

Figure 36

Expression of *brk* in *dpp* and *grk* misexpression

Anterior is to the left. Only posterior half of the egg chambers at stage 10B are shown here (A-F) Lateral views of stage 10B egg chamber. RNA in situ hybridization for *brk* in different misexpression situations (A) In moderate *dpp* misexpression (B) In strong *dpp* misexpression (C) In moderate *grk* misexpression (D) In strong *grk* misexpression (E) In combined misexpression moderate *dpp* and strong *grk*. (F) In combined misexpression strong *dpp* and strong *grk*.

3.10.1 Loss of Sog in follicle cells leads to induction of ectopic dorsal appendage material

We have seen that *sog* is regulated by both Dpp and Grk in the follicular epithelium. We asked if *sog* has a role in patterning of the follicular epithelium. We addressed this question by inducing follicle cells clones mutant for *sog*. To aid in our analysis we examined BR-C expression which marks the dorsal appendage fate

Eggs laid by females in which *sog* mutant follicle cell clones were induced had thinner and shorter dorsal appendages (Figure 37A, B). In addition, these eggs had ectopic dorsal appendage material restricted to the dorsal side of egg (Figure 37A, B). In a few eggs, dorsal appendage material expanded anteriorly (Figure 37B- marked by white arrow) and correspondingly there was decrease in operculum size.

In *sog* mutant follicle cell clones that included the CMFCs we observed that BR-C domain expanded anteriorly as well as posteriorly (Figure 37C-E; In D marked by white arrow). A clear example shown here where the number of BR-C expressing cells in *sog* mutant follicle cell clone is ~ 65 where as in wt is around 50-55 cells (Figure 37D, E; Roth et al., 1999). However, the BR-C expression domain remained unchanged in *sog* clones which did not include the CMFCs (Figure 37C-E). Loss of *sog* in follicle cells other than the CFMCs would not affect BR-C as *sog* mRNA is synthesized only in the CMFCs. However, as Sog is a diffusuble protein, loss of Sog function only in the CMFCs should lead to cell non-autonomous effects in the whole follicular epithelium. *BR-C* encodes for a Zinc finger transcription factor and it localized strictly to the nucleus in wt egg chambers (Figure 37F). In *sog* mutant clones, we observed that BR-C was no longer restricted to the nuclei (Figure 37G). Thus, loss of Sog in the CMFCs leads to induction of ectopic dorsal appendage material.

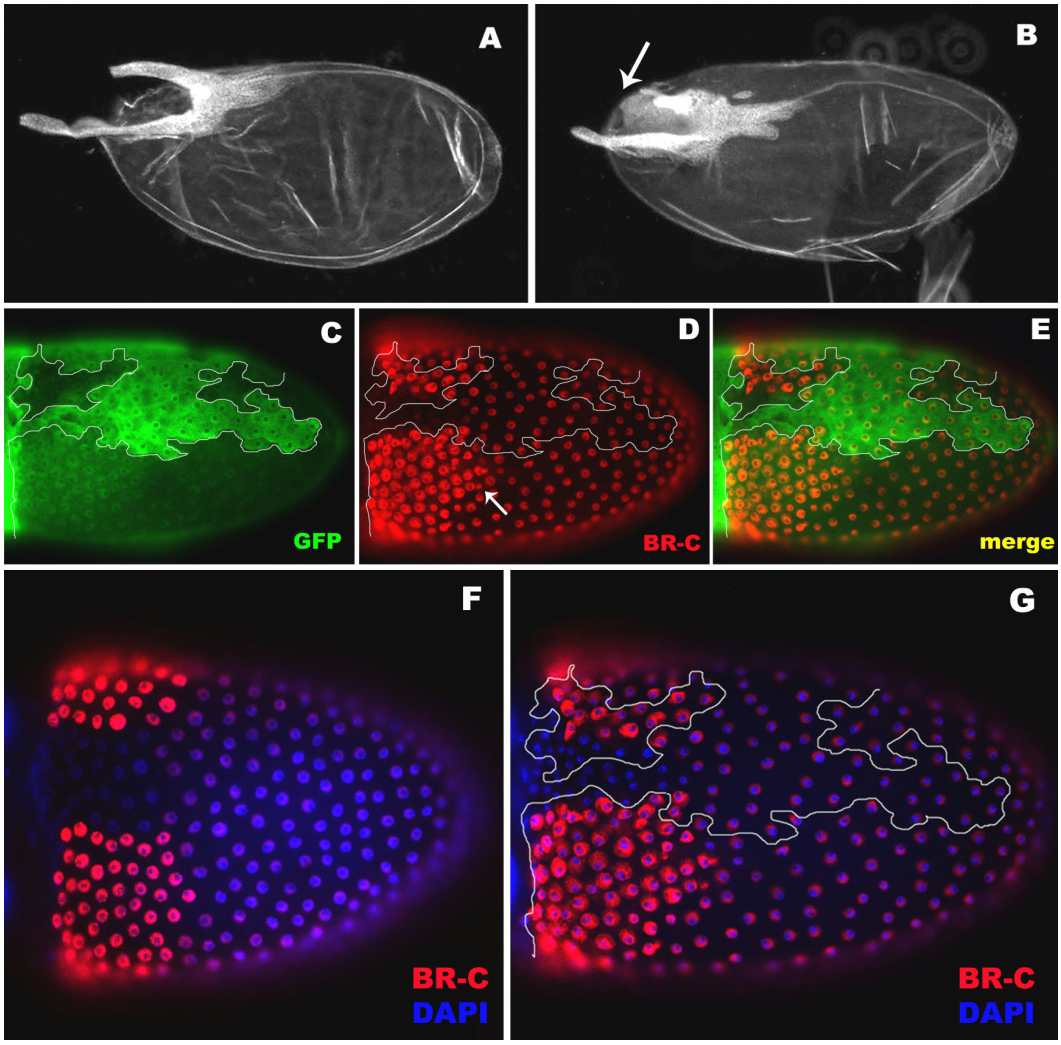

Figure 37

Sog loss of function clones lead to ectopic BR-C expression

Anterior is to the left. (A, B) Dorsolateral views of eggs derived from mothers lacking Sog function in follicle cell clones. (C-G) Only posterior half of the egg chambers at stage 10B are shown here (C) Anterior dorsolateral clone of mutant cells lacking *sog* marked by absence of GFP (green) expression. (D) BR-C (red) expression in *sog* mutant follicle cell clones. White arrow marks the cells expressing ectopic BR-C and (E) merge showing expansion of BR-C in *sog* mutant follicle cell clone. (F) BR-C protein is localized to the nucleus (blue = stained with DAPI) in wt egg chambers. (G) BR-C (red) in no longer restricted to the nucleus (blue) in *sog* mutant follicle cell clones. Thin grey line marks the clone boundary. Arrow in B shows dorsal appendage material at the anterior where it is normally absent.

3.10.2 Loss of Brk in follicle cells leads to expansion of operculum fate

Brk acts as a sequence-specific transcriptional repressor by binding to TGGCGc/tc/t sequence motif of target genes of Dpp during embryogenesis and wing development (Jazwinska et al., 1999a; Zhang et al., 2001; Winter and Campbell, 2004). Based on the expression pattern of *brk* in the follicular epithelium we hypothesized that Brk functions by repressing targets genes of Dpp that are essential for operculum fate. We tested the hypothesis by inducing follicle cells clones mutant for *brk*. To aid in our analysis we examined the expression of BR-C and Fas III which mark the dorsal appendage and operculum fate respectively.

Eggs laid by females in which *brk* mutant follicle cell clones were induced lack one or both of the dorsal appendages (Figure 38A, B). In addition, these eggs show expansion of the operculum. The expansion of the operculum occurs only at the very anterior of the egg (Figure 38B). BR-C expression in *brk* mutant follicle cell clone was downregulated cell autonomously in the anterior (Figure 38C, D). In addition, BR-C protein appeared to be upregulated in posterior cells in the clone, however the intensity of staining never reached the same level as seen in wt cells (Figure 38E). Fas III was upregulated in *brk* mutant follicle cell clone only in the anterior while no change was observed in posterior clones (Figure 38E-G). Thus, this clonal analysis proves our hypothesis that *brk* expression in the dorsal anterior follicle cells is needed for repressing genes (Fas III) needed for operculum fate.

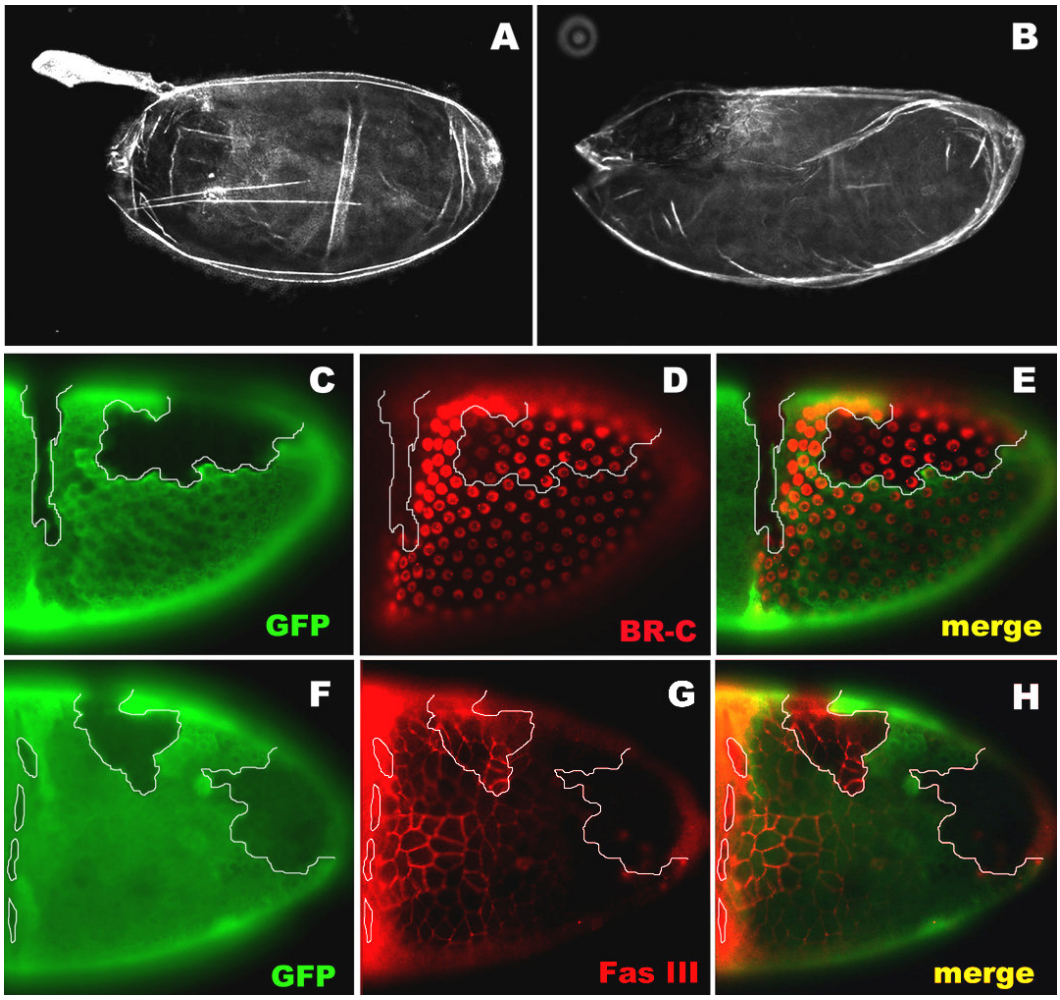

Figure 38

Brk loss of function clones lead to downregulation of BR-C and upregulation of Fas III expression

Anterior is to the left. (A, B) Lateral views of eggs derived from mothers lacking Brk function in follicle cell clones. (C-H) Only posterior half of the egg chambers at stage 10B are shown here (C-E) Lateral view of stage 10B egg chamber. (F-H) Dorsal view of stage 10B egg chamber. (C, F) *brk* mutant follicle cell clone marked by absence of GFP (green) expression. (D and G) BR-C (red) and Fas III (red) expression in *brk* mutant follicle clones (E) Merge showing loss of BR-C in *brk* mutant follicle cell clone. (E) Merge showing upregulation of Fas III in *brk* mutant follicle cell clone laterally but not posteriorly. Thin grey line marks the clone boundary.

3.11 Dpp inihibitors function redundantly in the follicular epithelium to specify operculum and dorsal appendages

In contrast to *snoN* mutants, mutations in *brk*, *sog* and *dad* are homozygous lethal (Francois et al., 1994; Tsuneizumi et al., 1997; Jazwinska et al., 1999a). We took advantage of the fact that *snoN*-/- flies are viable and decided to generate double mutant combinations of the Dpp inhibitors. The logic of the experiment was conceptually simple; we tested if *snoN*-/- phenotype would be enhanced by removing one or both copies of other inihibtors namely- *brk* and *dad*.

Dad is similar to vertebrate Smad 7 and is proposed to inhibit Dpp signaling by blocking the transduction of signal further downstream from the receptor (Tsuneizumi et al., 1997). To generate *snoN/dad* double mutants we took advantage of the existence of a semilethal P insertion (called P1883) in the 5' promoter region of *dad* gene denoted here as *dad(sl)* (Tsuneizumi et al., 1997). Females homozygous for this P insertion are viable and lay eggs which resemble wt eggs and show normal *BR-C* expression (compare Figure 39A, B and E, F). Eggs laid by double homozygous *snoN/ dad(sl)* mutant females had a striking chorion phenotype which resembled eggs laid by females weakly misexpressing *dpp* in the follicular epithelium (Figure 39G). These eggs have thicker dorsal appendages with highly irregular ends in addition to slightly enlarged operculum (Figure 39G). In few eggs the chorion showed ectopic dorsal appendages. In accordance, *BR-C* expression was irregular and pushed laterally in stage10B egg chambers (Figure 39H).

Lack of availability of a hypomorphic and/or a semilethal allele of *brk* prevented us from generating *brk/snoN* double homozygous mutants. However, we generated a mutant line, which was homozygous for *snoN* mutation and lacked one copy of functional *brk* (termed *brk:snoN* double mutant henceforth). Eggs laid by such females had a very distinct phenotype than the *snoN:dad(sl)* double mutant combination. On an average these eggs were smaller in length and had a significantly larger operculum as compared to *snoN* and or *brk* single mutants (Figure 39C, I and K). Furthermore, the dorsal appendages were stout

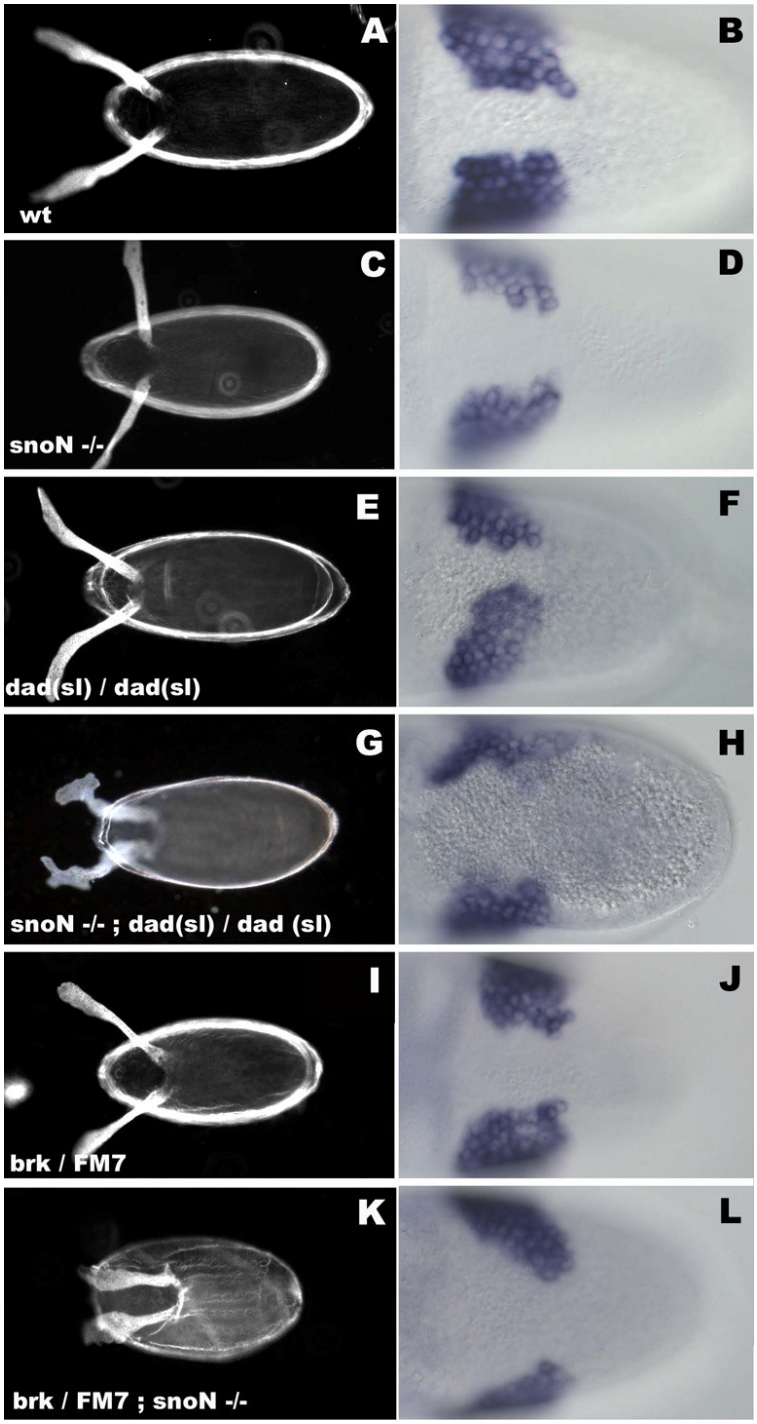

Figure 39

***snoN*, *dad*, *brk* function redundantly in the follicular epithelium to inihibit Dpp signaling.**

Anterior is to the left. (A-L) Dorsal views of chorion and stage 10B egg chambers. (A, B) wt chorion and *BR-C* expression (C, D) *snoN-/-* chorion and *BR-C* expression (E, F) Egg laid by females homozygous for *dad(sl)* mutation and corresponding *BR-C* expression in *dad(sl)* egg chamber (G,H) Egg laid by *snoN/dad(sl)* double homozygous females and corresponding *BR-C* expression. (I, J) Egg laid by female heterozygous for *brk* mutation and *BR-C* expression in such an egg chamber (K, L) Egg laid by *brk :snoN* mutant female and corresponding *BR-C* expression.

and massively thick at their ends. *BR-C* expression was pushed more posteriorly and laterally (compare Figure 39D, J and L). The enlarged operculum *in brk:snoN* double mutants emphasized the role of *brk* in specification of dorsal appendages by repressing genes needed for operculum formation.

Kekkon-1 is a Leucine Rich Repeat (LRR) domain transmembrane protein and inihibits EGF signaling by interfereing with ligand binding, thus, masking the ligand binding site on the EGFR (Ghiglione et al 1999; Ghiglione et al 2003). *kek* mutatns are homozygous viable. Eggs laid by *kek* mutant homozygous females have widely spaced dorsal appendages (Ghiglione et al., 1999). We generated homozygous double mutants for *kek* and *snoN* expecting to obtain chorion phenotype which resembles weak misexpression of both Grk and Dpp. However, this was not the case.

In brief, Dpp inhibitors function in concert to specify different cell fates in the follicular epithelium.

3.12 EGF targets *rho*, *aos* and *kek* are regulated by Dpp signaling

How does the second Gurken signal specifies two different cell fates in the dorsal anterior follicular epithelium is still not completely understood. Although, an elaborate model for patterning of the follicular epithelium by Grk signaling has been proposed, it has several drawbacks (see Introduction 1.7; Wasserman and Freeman, 1998). It is argued that Grk specifies operculum and dorsal appendage fate based on an activity gradient which is induced upon EGFR activation (Peri et al., 2002; Roth, 2003). However, the situation becomes more complex as *rho* and *aos* are induced in the dorsal follicle cells in response to Grk signaling (Sapir et al., 1998; Wasserman and Freeman, 1998). Rho and Aos function by forming regulatory loops (positive and negative respectively) and modulate MAPK activity. We asked if the expression of *rho*, *aos* and *kek* is dependent on the Grk gradient and if Dpp too modulates their expression. We tested this by analyzing *rho*, *aos* and *kek* expression in Grk, Dpp and in combined misexpression situations. In addition, we performed clonal analysis for *aos* and *rho* to pinpoint their role in patterning.

In wt stage 9 egg chambers, *kek* is expressed in a broad dorsal anterior patch of follicle cells in response both high and moderate levels of Grk signaling (Figure 40C and Musacchio and Perrimon, 1996; Ghiglione et al., 1999). In both moderate and strong *dpp* misexpression; *rho*, *aos* and *kek* expression domains expanded along both the AP and DV axes (Figure 40A-I). The distance between the *rho* stripes increased with increase in dosage of Dpp until it was detected in form of dorsolateral stripe in the follicular epithelium (Figure 40A, D, G and J). This observation is consistent with earlier findings from Peri and Roth (2000) where Dpp was shown to have a positive input on *rho* expression. *aos* expression too was influenced by Dpp in a dose dependent manner. *aos* expression was detected in a broader domain in moderate misexpression of Dpp (Figure 40B, E and H) while it was detected in the whole dorsal anterior follicle cells when Dpp levels were high (Figure 40K). *kek* is a primary target of Grk signaling and a direct read out of high and moderate EGFR activation (Musacchio and Perrimon, 1996; Ghiglione et al., 1999). Suprisingly, *kek* expression expanded both posteriorly and in lateral follicle cells in both cases of

moderate and strong misexpression of *dpp* (Figure 40C, F, I and L). These results proved that Dpp has a positive influence on the modulators of Grk signaling and that Dpp affects patterning of the dorsal chorion structures (Peri and Roth, 2000). Thus, Dpp itself can change the pattern of EGFR activation (MAPK activation) by modulating the activity of *rho*, *aos* and *kek* in the follicular epithelium.

In moderate misexpression of *grk*; *rho,* aos and *kek* expanded laterally along the DV axis (Figure 40M-O). The distance between the *rho* stripes increases in response to higher Grk signaling (Figure 40M). In addition, *rho* was detected even in ventral anterior follicle cells. *aos* too was expressed in a broader domain (Figure 40N). *kek* mRNA spread more laterally indicating the range of EGFR activation (Figure 40O). In strong Grk misexrpression; *rho*, *aos* and *kek* expression was massively increased and their expression was detected as symmetrical broad ring in the anterior half of main body follicle cells (Figure 40P-R). Similar expression patterns for *rho, aos* and *kek* were obtained when λtop was misexpressed with CY2-Gal4 (Queenan et al., 1997). Thus, the lateral expansion of *rho, aos* and *kek* in response to increasing Grk signaling from the germline suggests an underlying gradient of EGFR activation.

In combined misexpression of *dpp* and *grk* we obtained unique expression patterns for *rho*, *aos* and *kek*. In moderate *dpp* misexpression combined with strong misexpression of *grk*, *rho* expression was symmetrical in form of a broad ring but was excluded from the anterior half of main body follicle cells (Figure 40S). *aos* expression was restricted to the anterior half of main body follicle cells in form of a symmetrical ring (Figure 40T). In strong misexpression of both *dpp* and *grk*, *rho* expression was detected as a thin symmetrical ring at the posterior most limits of the main body follicle cells (Figure 40V). Suprisingly, *aos* expression was absent in such egg chambers (Figure 40W). In both the combined misexpression studies, *kek* was detected in all the main body follicle cells indicating the presence of high level of Grk activity in these cells and that its expression is also regulated by Dpp (Figure 40U, X). The above observations suggest that both Dpp and Grk inputs are needed to establish the spatiotemporal domains of *rho*, *aos* and *kek* during oogenesis.

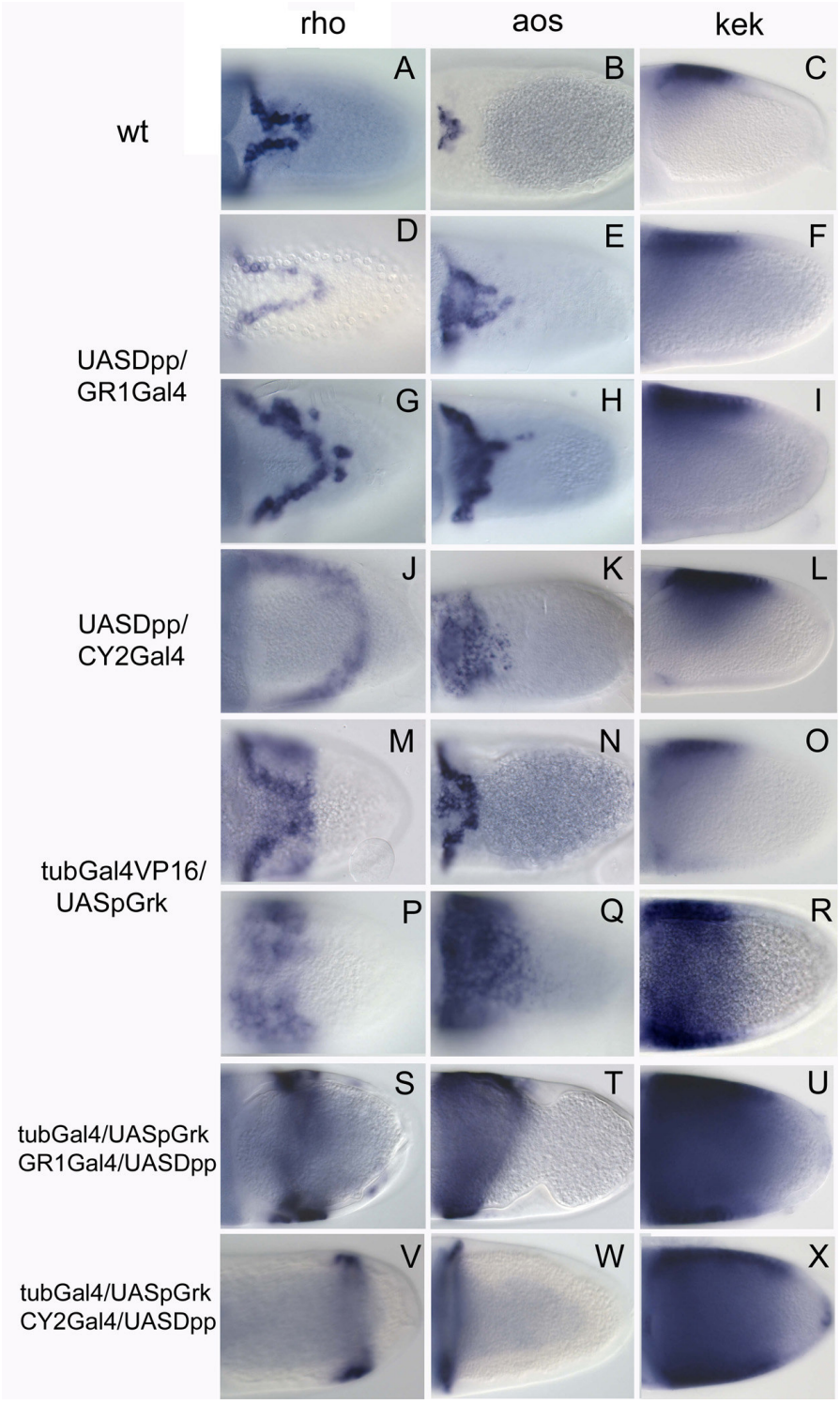

Figure 40

Expression of *rho*, aos and kek is regulated by both Dpp and Grk

Anterior is to the left. (A, B, D, E, G, H, J, K, M, N, P, Q, S, T, V, W) Dorsal views of stage 10B egg chambers. (C, F, I, L, O, R, U, X) Lateral views of stage 10A egg chambers. *rho*, *aos* and *kek* mRNA distribution in (A-C) Wt (D-I) Moderate *dpp* misexpression. (J-L) Strong *dpp* misexpression. (M-O) Moderate *grk* misexpression. (P-R) Strong *grk* misexpression. (S-U) Combination of moderate *dpp* and strong *grk* misexpression. (V-W) Combination of strong *dpp* and strong *grk* misexpression.

3.13 Rho and Aos function are not essential for specification of dorsal midline

In order to the test the model for patterning of dorsal chorion structures proposed by Wasserman and Freeman (1998) we performed clonal analysis for *rho* and *aos* in the follicular epithelium. We followed the expression of BR-C and Fas III, the markers for dorsal appendage anlagen and operculum respectively.

Rho loss of function clones were induced in follicle cells using the FRT/FLP system (Xu and Rubin, 1993). Homozygous *rho* mutant follicle cell clones, detected by absence of GFP expression showed no effect on BR-C expression domain *per se*. However, BR-C localization was no longer restricted to the nucleus. Suprisingly, the specification of dorsal midline occurs in absence of *rho* which indicates that the proposed splitting of MAPK activity into two peaks (dorsal appendage anlagen) is independent of Rho (Wasserman and Freeman, 1998). Thus, it appears that dorsal midline fate is specified before Rho initiates positive amplification loop through cleavage of Spi. These data suggest that a gradient of EGFR activation initiated by Grk from the developing oocyte is sufficient to induce two dorsal fates.

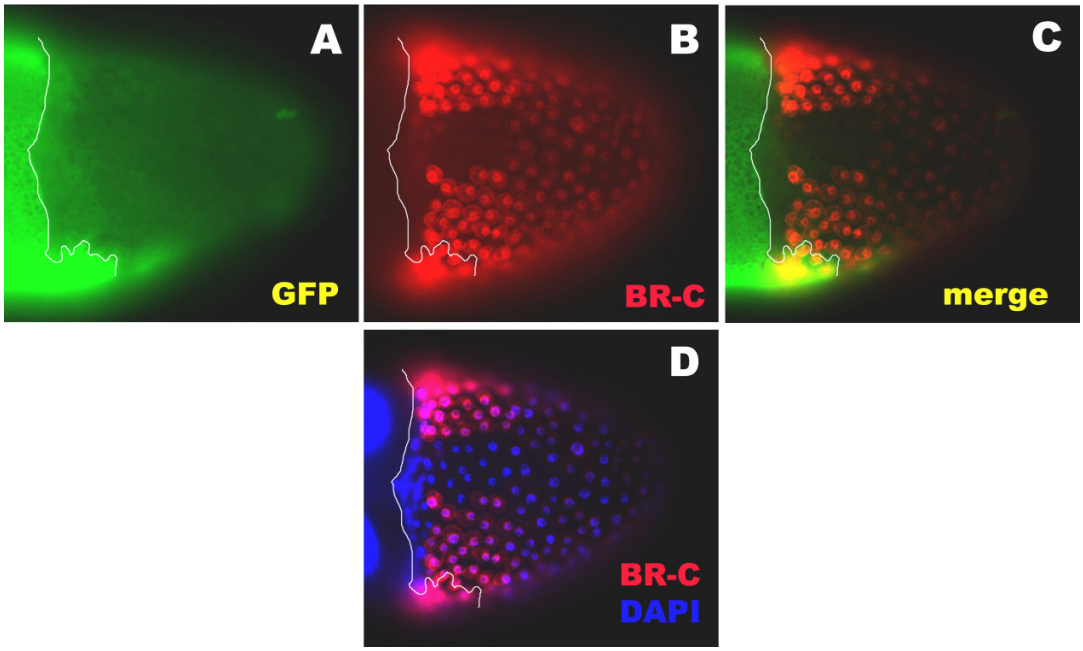

Figure 41

Rho loss of function clones affect do not affect BR-C expression

(A-D) Dorsal views of stage 10B egg chambers. Anterior is towards left. (A-C) BR-C expression largely remains unchanged in follicle cells lacking Rho. (A) Follicle cell clone of mutant cells lacking Rho marked by absence of GFP (green) expression. (B) BR-C (red) expression in *rho* mutant follicle cells. (C) Merge showing BR-C pattern in the *rho* mutant clone.(D) BR-C protein (red) is no longer restricted to the nucleus (DAPI-blue) *rho* mutant follicle cell clone. White lines mark the clone boundary.

Aos was recently shown to act as a competitive inhibitor by binding to secreted EGF ligand Spi in turn preventing its binding to EGFR (Klein et al., 2004). This action of Aos prevents high level EGFR activation thus enabling other EGF ligands to activate EGFR at moderate levels. We hypothesized if Aos functions in a similar way in the dorsal anterior follicular epithelium. Thus, by restricting the spread of Spi, Aos would allow Vn to activate EGF cascade at moderate levels. This in turn would lead allow upregulation of BR-C in the follicle cells. On the contrary, loss of Aos function would allow Spi to diffuse more laterally allowing it activate EGF cascade at high levels which in turn would result in downregulation of BR-C. Thus, our hypothesis is in contrast to Freeman model which implies that BR-C is induced at high levels of EGFR activation. To test this hypothesis we induced loss of function *aos* clones in the follicular epithelium and analysed expression of BR-C and Fas III.

We recombined *aos*$^{1\delta7}$ (a null allele of *aos*) chromosome on FRT79D(w+) and induced homozygous loss of function *aos* clones in the follicular epithelium. Rather surprisingly and in agreement with our proposed hypothesis, BR-C expression was downregulated in *aos* mutant follicle cell clones (Figure 42A-C). Fas III expression however remained unchanged in *aos* clones (Figure 42D-F).

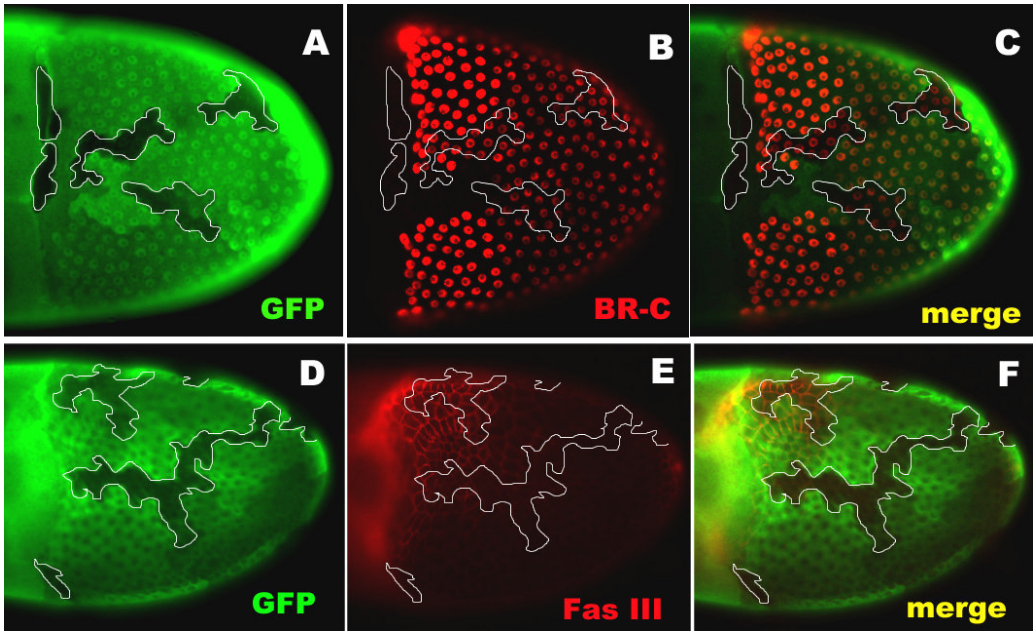

Figure 42

Aos loss of function clones show cell autonomous downregulation of BR-C

Anterior is towards left (A-C) are dorsal views views (D-F) are lateral views of stage 10B egg chambers.. (A-C) BR-C expression is downregulated in cell autonomous manner in follicle cells lacking Aos function. (A and D) *aos* mutant follicle cell clone marked by absence of GFP (green) expression. (B and E) BR-C (red) and FAS III (red) expression in *aos* mutant follicle cells. (G) merge showing downregulation of BR-C in *aos* mutant follicle cell clone. (J) Merge showing FAS III in *aos* mutant follicle cell clone. White lines mark the clone boundary.

4. Discussion

The results are discussed in three sections. First, we discuss the distinct functions of Dpp and Grk in the patterning of the dorsal chorion structures. In the second section we describe the regulation of Dpp activity by its inhibitors. Finally, we discuss the role of feedback loops in regulation of Grk activity in the follicular epithelium.

4.1 The Dpp and Grk function in follicular epithelium

Here we describe a central role of Dpp signaling in the anterior-posterior patterning of the follicular epithelium. We demonstrate that the Dpp pathway is the molecular foundation for differential AP pattern in main body follicle cells. In addition, we confirm that Grk is the instructive signal and needs Dpp which acts as a competence factor for Grk signaling in the follicular epithelium. Finally, we provide evidence for the existence of a combinatorial code of Dpp and Grk in specifying distinct dorsal cell fates.

4.1.1 The Dpp gradient and prepatterning of follicular epithelium.

Many secreted proteins accumulate in a gradient as they diffuse from their cellular sources. Such a gradient of Dpp has been seen in the developing wing imaginal disc. *dpp* mRNA is expressed along the AP compartment boundary of the wing imaginal disc, however, Dpp protein has an ability to diffuse and was shown to form a gradient across the AP compartment (Entchev et al., 2000; Teleman and Cohen, 2000). Dpp patterns the wing by activating target genes such as *spalt* (*sal*) and *optomotor blind* (*omb*) in a concentration dependent manner (Nellen et al., 1996). Dpp has been shown to have a function in patterning of the follicular epithelium during oogenesis however its precise role was not clear (Twombly et al., 1996). We have uncovered two novel aspects of Dpp function in patterning of follicular epithelium. First, Dpp forms a gradient

in the follicular epithelium along AP axis and subsequently prepatterns it which is consistent with Dpp loss of function and Dpp misexpression egg phenotypes (Twombly et al., 1996; Muzopappa and Wappner, 2005). Second, Dpp acts as a competence factor in follicular epithelium for Grk signaling. We have also ruled out the possibility of Dpp providing a dorsalizing cue in the follicular epithelium.

Although we did not detect Dpp protein directly in the ovary, several lines of evidence indicate that the Dpp pathway is activated in a graded fashion in the follicular epithelium along the AP axis, with highest levels in the CMFCs present at the nurse cell oocyte boundary. First, *dad* a direct primary target gene of Dpp is expressed in a graded fashion in response to Dpp signaling indicating indirectly a prescence of graded Dpp activity (Figure 8A-C). Second, ectopic Dpp expands and shifts the expression of Fas III and *BR-C* which coincides with the observed chorion phenotypes (Figure 9F, J). This can be best explained by an existence of a transcriptional control on the promoters of these genes by Dpp. Additionally, support for graded activity of Dpp comes from chorion phenotypes seen in weak *dpp* allele combinations (Twombly et al., 1996). In such eggs there is a significant reduction in operclum size and a considerable shift of dorsal appendages to the anterior of the egg (Twombly et al., 1996).

The scheme of shifted fates observed in this study is similar to that observed in other systems with well established graded activities. For example, reduction of the Drosophila JAK signaling, which is graded with highest levels at both the termini, as seen in weak *hopscotch* (*hop*) mutant animals leads to the loss of the anteriormost fates and dramatic reduction of the posteriormost cell fates (Xi et al., 2003). Simultaneously, the central fate, main body follicle cells, expands while all the other cell fates are shifted to the termini (Xi et al., 2003). Similarly, reduction of *Drosophila* transcription factor and graded determinant Dorsal (*dl*) causes the loss of cell fates along the dorsoventral axis in the embryo corresponding to the strength of alleles. Dorsal shows graded nuclear accumulation in response to proposed graded activation of Toll pathway (Roth et al., 1989). Peak levels of nuclear Dorsal are present in the ventral cells while in dorsal ectodermal cells it is mostly cytoplasmic. In *dl* minus, embryos lack all

dorsoventral polarity (reviewed in Nusslein-Volhard, 1979; Morisato and Anderson, 1995). Correspondingly, the embryos show uniform expression of *dpp* which is normally restricted to a dorsal stripe in wt embryos (Stathopoulos and Levine, 2002). The loss of one copy of *dl* (at 29^{o}C) leads to reduction in size or complete loss of ventralmost region, the mesoderm (Nusslein-Volhard, 1979). Correspondingly, neuroectoderm expands to the ventral side confirmed by presence of *sog* which is normally restricted to ventrolateral region in the wt embryo. Thus, there is a coordinated fate shift in response to reduction in Dorsal protein levels. The similarity in behaviour of mutants from graded morphogen systems to that of Dpp mutants suggests a similar mechansim of graded Dpp activity in the follicular epithelium.

How the Dpp gradient is established in the follicular epithelium is still an open question. Several models have been proposed to explain the formation of morphogen gradient(s) which include transport of a morphogen by passive diffusion, or via endocytic trafficking (planar transcytosis), or by argosomes (membranous structures) and finally via actin based extensions called "cytonemes" (Ramirez-Weber and Kornberg, 1999; Greco et al., 2001; Entchev and Gonzalez-Gaitan, 2002; reviewed in Tabata, 2004). We propose that follicular epithelium would be an additional good model system to study morphogen trafficking as it offers several advantages.

4.1.2 Dpp acts as a competence factor in the follicular epithelium

During oogenesis, Grk signaling induces first posterior and subsequently dorsal cell fates in the follicular epithelium (Roth et al., 1995; Nilson and Schupbach, 1999). Differential competence of follicle cells is suggested to account for distinct response of the follicle cells to Grk signaling. Both early (stage 6) and late (stage 9) Gurken signaling is directed to distinct regions of the follicular epithelium (Roth et al., 1995). Thus, it could be deduced that two groups of cells are differentially prepatterned and are able to interpret Grk signaling differently by adopting different cell fates. Prior to Grk signaling the follicle cells are prepatterned in two major subpopulations- the "terminal" follicle cells

and the "main body" follicle cells present between the two terminal subpopulations (Ray and Schupbach, 1996; van Eeden and St Johnston, 1999). Several elegant experiments have shown that underlying prepattern of the follicular epithelium displays mirror image symmetry at the termini in the AP axis (Roth et al., 1995; Gonzalez-Reyes and St Johnston, 1998; Keller Larkin et al., 1999; Xi et al., 2003). This indicates that the terminal follicle cells are equally competent to respond to Grk signaling.

We have provided conclusive evidence that competence of main body follicle cells is controlled by Dpp. Two observations confirm our findings. First, follicle cells which cover the developing oocyte are exposed to Dpp much earlier (at stage 8) than they are exposed to Grk. This has been well supported by expression profiles of *dpp* and pMad earlier and by *dad*LacZ and Med in this study (Twombly et al., 1996; Guichet et al., 2001). Second, elimination of Dpp signaling achieved by generating *tkv* or *Med* mutant follicle cell clones renders them unresponsive to the dorsalizing cue generated by Grk. However, this is not true in case of *pipe* expression. Preliminary data suggests that in *tkv* mutant follicle cell clones, *pipe* is still expressed and restricted to ventral follicle cells like in wt egg chambers (F. Peri unpublished). On the contrary, misexpression of *dpp* alone or in combination with *grk* induced ectopic competence in all main body follicle cells as seen by expansion of Fas III and BR-C expression.

Interestingly, when activated forms of EGFR or UAS*rho* are misexpressed in the whole follicular epithelium in homozygous *grk* mutant females, ectopic dorsal appendage material can be detected randomly distributed in the whole chorion (Queenan et al., 1997; Sapir et al., 1998). In *grk* mutants the terminal follicle cells remain in their default anterior state and continue expressing Dpp (Peri and Roth, 2000). This suggests that Dpp emanating from both poles of the egg chamber can induce competence in the main body follicle cells along the whole AP axis. These observations and the temporal and spatial expression of Dpp are in complete agreement with proposed competence generating activity. This mode of action of Dpp is similar to that of JAK signaling which induces competence in terminal follicle cells. Unpaired (Upd), the ligand of JAK signaling, emanating from polar follicle cells present at both the termini

specifies different cell fates in a concentration dependent manner (Xi et al., 2003). Elimination of JAK signaling in these terminal cells disrupts their ability to respond to *grk* signaling suggesting that JAK acts as a competence factor for Grk signaling in posterior follicle cells. Taken together, it can be concluded that Dpp acts as a competence factor for Grk signaling in the main body follicle cells.

It is interesting to note that both Dpp and Grk signaling interact differently with each other in order to specify posterior and dorsal cell fates. Grk signaling controls Dpp expression in posterior follicle cells (Twombly et al., 1996). Ectopic expression of *dpp* occurs at the posterior pole in *grk* mutant egg chambers as the posterior follicle cells remain in their default anterior state in absence of Grk signal (Roth et al., 1995). However, it is unclear if Grk regulates *dpp* expression in posterior cells directly or indirectly (Twombly et al., 1996). We have shown that Dpp acts as an essential factor for Grk signaling in specifying dorsal cell fates. In addition, Dpp also controls activation of Grk targets like *rho*, *aos* and *kek*. This suggests that both Dpp and Grk cascades communicate with each other differently in distinct contexts.

Earlier reports suggest that loss of function Mirror (Mirr) clones in the follicular epithelium generate eggs which resemble eggs laid by females where Dpp function is compromised (tkv or Med clones; Jordan et al., 2000). The *mirr* locus encodes a homeodomain-containing protein belonging to Iroquious complex (Iro-C). Mirr is expressed in the dorsal anterior follicle cells and is activated by Grk signaling (Jordan et al., 2005). It is not known if Dpp is also needed for *mirr* expression and function in the dorsal follicle cells. On the other hand, it would be interesting to know if Dpp functions via Mirr to induce competence in main body follicle cells.

Competence appears to be an essential pre-requisite for EGF/Ras/MAPK signaling to induce different cell fates. As described earlier JAK signaling induces competence in terminal follicle cells to respond to Grk signaling (Xi et al., 2003). Compelling evidence comes from the work of Carmena et al (1998) where they show that specification of cell fates by EGF/Ras/MAPK signaling in *Drosophila* embryonic mesoderm is dependent on Wg and Dpp. In the trunk

region of *Drosophila* embryo several tissues develop from the mesoderm in a segmentally repeated fashion. In this work, the authors have shown that Wg and Dpp create a competent state in the dorsal mesoderm such that all cells in the region have an ability to respond to signal from receptor tyrosine kinase. This leads to specification pericardial and somatic muscle progenitor cells (Carmena et al., 1998). Taken together these facts suggest that in certain contexts EGF signaling in *Drosophila* can only act if the responding tissue is competent.

Although the concept of competence was first introduced in the early 40's its molecular nature still remains elusive (Waddington, 1940; Stern, 2000). Several propositions have been made till to date. In simplest terms competence is an active state that defines the way in which cells respond to an inductive signal (Waddington, 1940). Growing evidence suggests that induction of competence involves production of at least one or more proteins which could be specific transcription factor(s) or a more general chromatin modulator protein (Fujiwara et al., 1994; Steinbach et al., 1997). Thus, it is possible that cell type specific transcriptional activators or repressors induced by Dpp signaling would assist the core transcriptional assembly of the cell to react to Grk signaling in the follicular epithelium. At DNA sequence level it would mean that the transcription modulatory elements of the dorsal set of genes have binding sites for transcription factor(s) induced by both Dpp and Grk. In absence of Dpp signal, transcription factor(s) induced by Grk would not be able to bind the promoter elements of target genes or they can still bind the promoter sequences but would not be able to recruit the transcription complex to induce gene expression. On a global level Dpp would activate histone modifying enzymes which modulate chromatin topography so that promoter elements are accessible to transcription factors induced by Grk. Indeed, activation of Dpp targets genes in the embryo is shown to be dependent on CBP (CREB-binding protein; CREB - cAMP response element binding protein) histone acetyltransferase (Ashe et al., 2000)

4.1.3 Dpp promotes at least two different cell fates in the main body follicle cells

We have shown that Dpp forms a morphogen gradient along the AP axis. Based on Dpp misexpression experiments performed by us and several other groups it can be suggested that two different concentrations of Dpp promote two different cells fates (Twombly et al., 1996; Muzopappa and Wappner, 2005). Highest levels of Dpp are present in the CMFCs and three to four rows of cells close to the CMFCs which contribute to operculum formation while in more posterior cells moderate levels of Dpp promote dorsal appendage fate. Similarly, Dpp has been shown to specify different cell fates along the embryonic DV axis in a concentration dependent manner (Ferguson and Anderson, 1992; Ashe et al., 2000). Highest levels of Dpp in the dorsal ectoderm are essential for specification of amnioserosa while intermediate levels of Dpp specify dorsal ectoderm in the embryo (Ferguson and Anderson, 1992).

Upon alteration of Dpp levels we modify the prepattern and competence of the follicle cells which is evident from the egg phenotypes obtained. Although, the prepattern and competence generated by Dpp is symmetrical in nature the induction of the operculum and the dorsal appendages is only in the dorsal follicle cell which is due to the restricted asymmetric activation of Grk cascade and its effectors across the DV axis. Thus, by inducing ectopic competence in follicle cells we could induce more number of main body follicle cells to participate in formation of the operculum and the dorsal appendages.

Interestingly, the expression of *rho*, *aos* and *kek* which are primary targets of Grk signaling also respond to Dpp in a concentration dependent manner. Our data indicate that high levels of Dpp repress *rho* expression and activate aos and *kek* expression. Peri et al. (2000) have reported that Dpp signaling is essential for *rho* expression. Strikingly, misexpression of Dpp shifts expression of *rho*, *aos* and *kek* not only along the AP axis but also across DV axis. This clearly suggests that Dpp also influences EGF target genes in a positive way probably via MAPK. Similar findings have been made in vertebrate systems where TGF-β signaling has been shown to influence MAPK activation (reviewed in

Javelaud and Mauviel, 2005). In addition, it has been found that TGF-β induced MAPK activation, is both cell type specific and cell-type dependent (reviewed in Derynck and Zhang, 2003). This would be one possible explanation of the observed shift in *rho*, *aos* and *kek* domains in *dpp* misexpression in this study. Thus, monitoring of MAPK activation in different misexpression situations might provide valuable insights into the patterning process. However, it is also possible that Dpp influences *rho*, *aos* and *kek* expression via another transcription factor(s).

Surprisingly, misexpression of *dpp* in the follicle cells does not affect *pipe* transcription in main body follicle cells indicating that higher levels of Dpp cannot counteract repression of *pipe* by Grk. However, Dpp upregulates pipe expression in the posterior follicle cells (Morimoto et al., 1996; Zhao and Bownes, 1999). Thus, it is possible that *pipe* promoter has two different enhancers which regulate its expression in main body follicle and posterior follicle cells. This would suggest that Dpp is able to act directly or indirectly (via another transcription factor) on the posterior follicle cell specific *pipe* enhancer to upregulate its transcription.

4.1.4 Distinct levels of Grk signaling specify operculum and dorsal appendage cell fates

We have confirmed that two distinct levels of Grk specify two different cell fates in the dorsal follicle cells similar to the results obtained by other groups (Queenan et al., 1997; Nilson and Schupbach 1999). Follicle cells receiving highest levels of Grk are induced to form operculum fate while lower levels of Grk specify dorsal appendage fate. Grk is suggested to form a activity gradient i.e activate *kek* in dorsal follicle cells at highest activity while repress *pipe* at a distance and restrict its expression in ventral follicle cells (reviewed in Roth, 2003). Evidence from several genetic mosaic experiments supports this gradient hypothesis (Pai et al., 2000; James et al., 2002; Peri et al., 2002). We have shown that strong ectopic activation of EGFR in follicle cells (achieved by misexpressing *grk* from the germline) activates *kek* in whole anterior follicle

cell subset and represses *pipe* in all the main body follicle cells leaving only a few cells in the posterior expressing *pipe*. This shows the long range inhibition of *pipe* by Grk strongly supporting the gradient hypothesis. In addition, the lateral expansion of *rho* and *aos* in response to excess Grk (obtained by misexpression of *grk*) also supports gradient hypothesis (Figure 40M,N).

The expansion of dorsal cell fates in Grk misexpression occurs only in the anterior follicle subset which was also observed by Queenan et al (1997). We believe that this is due to restricted competence of follicle cells controlled by Dpp. Competence of follicle cells is not necessary for repression of pipe suggesting that repression is achieved via a different unknown mechanism. The exact molecular mechanism by which Grk achieves *pipe* repression still remains to be discovered.

4.1.5 Combinatorial signaling by Dpp and Grk specify operculum and dorsal appendage cell fates

Ectopic posterior expression of *dpp* in combination with EGFR activation by *grk* induces dorsal appendages at the posterior end of the egg (Peri and Roth, 2000; Roth, 2003). Based on this observation the authors suggested that both Dpp and Grk specify dorsal chorion structures. We confirmed this intersection of Dpp and Grk signaling by modulating both pathways simultaneously. Eggshell phenotypes obtained in our combined misexpression experiments clearly suggest that moderate levels of Dpp and Grk together specify the dorsal appendage fate while a combination of high Grk and high Dpp induces the operculum fate (Figure 18A, B). Thus, we could convert all main body follicle cells to the operculum and dorsal appendage fates by inducing competence achieved by misexpressing Dpp in the whole follicular epithelium and at the same time providing excess amounts of Grk from the germline.

We have provided evidence of existence of cross-talk between Dpp and Grk signaling pathway. Although, we didn't study the cross regulatory aspects of core cytoplasmic components like MAPK (for EGF pathway) and pMad (for

Dpp pathway), we have studied the cross regulation at transcription level of pathway specific genes. From our combined misexpression experiments we can conclude that all genes tested in this study – *rho*, *aos*, *kek*, *BR-C*, *fas III*, *brk* and *sog* are dual targets of both Dpp and Grk signaling. An interesting feature worth noting is that *rho*, *aos*, *kek*, *BR-C*, *fas III*, *brk* and *sog* have an asymmetric expression pattern in the follicular epithelium.

A limited number of signaling cascades exist in animal kingdom and hence are used repeatedly during development in various combinations and permutations. Our study reiterates this point. Several other studies have also support this fact. For example, in *Drosophila,* induction of leg imaginal disc precursors from ectodermal cells needs cross-talk between EGF and Dpp. EGFR antagonizes wing promotion function of Dpp and allows recruitment of leg precursors cells from uncommitted ectodermal cells (Kubota et al., 2000). Thus, it appears that in many developmental settings, signaling from EGFR and Dpp is integrated with signaling from other pathways.

4.1.5.1 Model for patterning of dorsal chorion structures

Patterning of the follicular epithelium requires coordination of several signaling pathways. In AP axis prominent roles of EGFR, Notch and JAK pathway in determining terminal cell fates have been established (Gonzalez-Reyes et al., 1995; Roth et al., 1995; Keller Larkin et al., 1999). We have shown that Dpp gradient primarily provides postional information in the follicular epithelium along the AP axis. We propose a model for patterning of dorsal chorion structures that integrates the network formed by Dpp and Grk (Figure 43). The model is described as following. Prior to second Grk signal (stage 9), the Dpp gradient specifies two domains in the follicular epithelium by inducing differential competence. Anterior half of main body follicle cells closer to the CMFCs (Dpp source) receive high to moderate levels of Dpp and become competent to form dorsal chorion structures. The posterior half of main body follicle cells which are exposed to low or no of Dpp form the non-competent zone and hence, do not participate in forming dorsal chorion structures. The

boundary between the competent and non competent zones is primarily controlled by the Dpp gradient itself. Grk can then function in such prepatterned competent follicle cell zone by inducing genes conferring operculum fate (Fas III) and dorsal appendage fate (BR-C). It should be noted that the induction of operculum and dorsal appendages is dependent on a "combinatorial code" designated by the gradients of both Dpp and Grk. Based on proposed roles of Dpp and Grk a similar model was recently published by Berg, CA (2005).

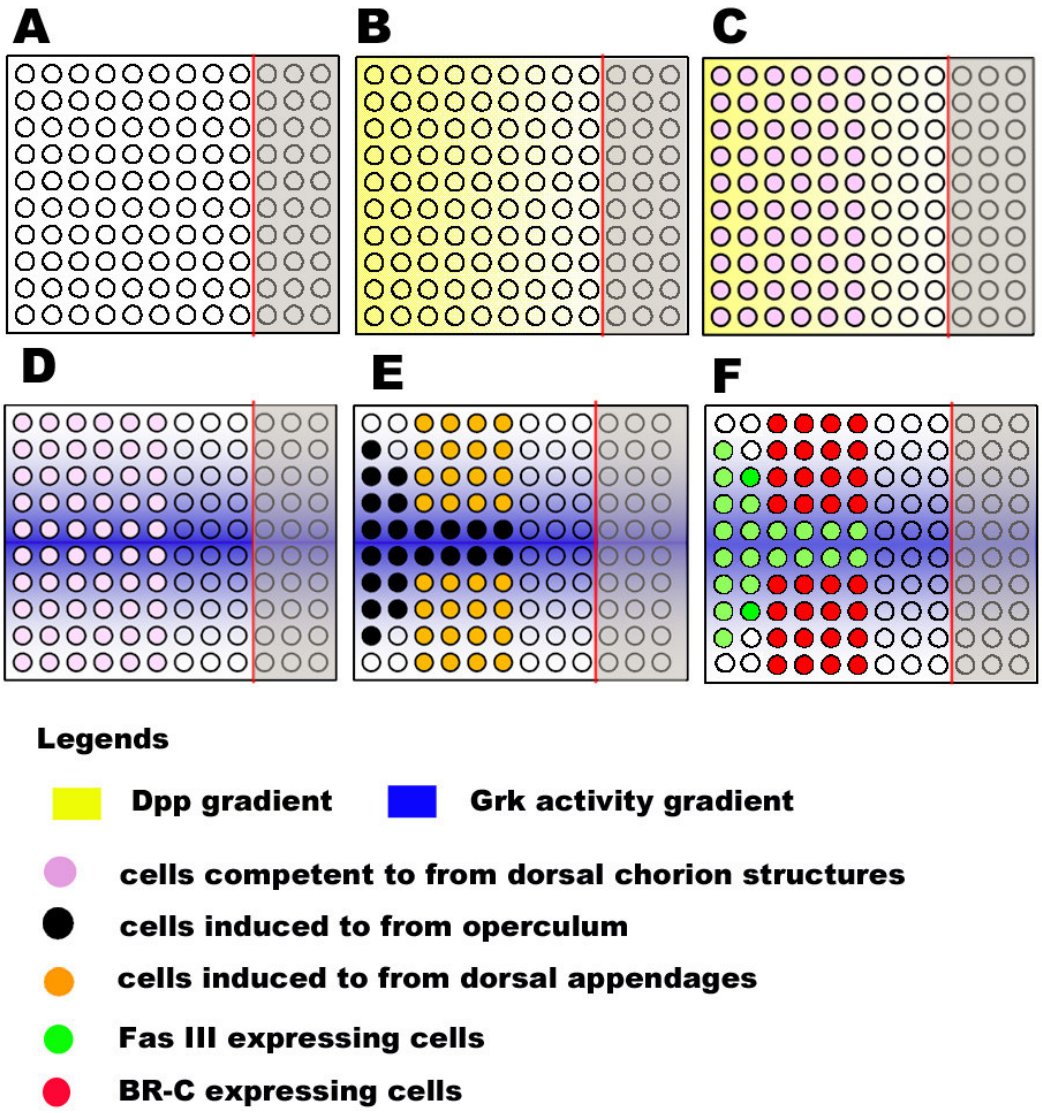

Figure 43

Model for patterning of main body follicle cells by Dpp and Grk

Anterior is to the left and dorsal is facing the viewer. (A-F) Illustration showing dorsal follicle cells (Naïve main body follicle cells as open circles and posterior follicle cells as grey area). (A) Follicle cells at stage 7 of oogenesis are divided into two main sub populations: the main body follicle cells and terminal follicle cells. Red line demarcates the boundary between the two cell types (B) At stage 8, the main body follicle cells receive graded Dpp signal:-shown as a yellow gradient. (C) Dpp signal prepatterns the follicle into atleast two groups – high to moderate levels of Dpp are competent to dorsal chorion structures (faint purple circles) and second, cells which receive low to no Dpp form the non-competent group (open circles). (D) At stage 9, a dorsal to ventral activity gradient of Grk (blue) can now induce different cell fates in concentration dependent maner in the prepatterned competent follicular epithelium. (E) Grk signaling induces two different cell fates i.e operculum fate (black circles) and dorsal appendage fate (amber circles) in combination with Dpp. (F) This leads to induction of Fas III and BR-C in operculum and dorsal appendage forming cells respectively.

4.2 Regulation of Dpp gradient activity

Morphogens are potent signaling molecules. They are particularly significant in developmental patterning because a single event causing release of a morphogen can generate several different cell types spatially related to the source of the signal. If the amount or duration of morphogen signaling is too great, development is diverted from its normal course. Clearly, these kind of signaling needs to be quantitatively regulated precisely to ensure that correct cell fate decisions are taken. In this context, we have addressed the regulation of Dpp morphogen signaling by its inhibitors Sog, Brk, Dad and Ski (SnoN) family proteins. SnoN had not been studied in *Drosophila* before. In addition, we demonstrate that the readout of Dpp gradient is regulated redundantly by inhibitors such as SnoN, Brk and Dad. Furthermore, we deciphered the role of Brk and Sog individually by clonal analysis.

4.2.1 SnoN acts as a repressor of Dpp target genes.

We have cloned two genes belonging to Ski superfamily of oncoproteins and characterized the expression and function of *Drosophila snoN* during oogenesis. The study described here is one of the first attempts to characterize Ski family proteins from *Drosophila*. A prominent feature of *snoN* (expression in follicle cells and embryo) and *ski* expression (in embryo) is that they are both expressed in areas that receive low level input from Dpp. In addition, we have shown that stable induction of *snoN* expression as a dorsolateral stripe in follicle cells requires inputs from both Grk and Dpp signaling.

snoN mutant flies are homozygous viable but exhibit reduced viability at every developmental stage during the fly life cycle. Surprisingly, *snoN* mutant flies do not display any tumorous growth of any tissue as observed in case of *snoN* mutants in vertebrates (Shinagawa et al., 2000). Viability of snoN mutant flies could be explained in two possible ways. First, *snoN* might function redundantly with *ski* during oogenesis. Thus, obtaining mutants for *ski* would be crucial to answer this question. Redundancy is one of the common features in

development where two or more genes control same process, for example, as observed in case of three *dorsocross* genes which control amnioserosa development in *Drosophila* (Reim et al., 2003). Second, in *Drosophila* the BMP pathway has been adapted to control several aspects of early development as against Activin/TGF-β pathway in vertebrates (Arendt and Nubler-Jung, 1994; Gerhart, 2000; DeRobertis and Kuroda, 2004). *snoN* is known to be preferentially activated by Activins/TGF-β in vertebrates (Luo, 2004). In addition, it was observed that only one out of three *snoN* knockout mice lines are embryonic lethal (Shinagawa et al., 2000; Pearson-White and McDuffie, 2003). Thus we can conclude that *snoN* is dispensible during development in flies and probably in mice.

The *snoN* mutant and ubiquitous misexpression of *snoN* in the follicular epithelium have opposite effects on the size of operculum. From studies on vertebrates, it is known that SnoN can interact with Smads and subsequently get recruited to the Smad binding elements that are present in TGF-β responsive promoters (Stroschein et al., 1999; Luo, 2004). Thus, it acts as a transcriptional (co) repressor of TGF-β target genes by negatively regulating them.

We would like to propose that SnoN in *Drosophila*, acts as a transcriptional repressor of Dpp targets in the follicular epithelium. Several lines of evidence support our argument. First, in *snoN* mutants operculum fate is expanded at the expense of dorsal appendage fate which suggests that SnoN acts as a transcriptional repressor of operculum fate genes. Second, *rho* and *aos* expression was upregulated in a broader domain in *snoN* mutant. We have shown that both *rho* and *aos* are targets of Dpp signaling. Thus, *snoN* might be repressing *rho* and *aos* indirectly by repressing known or unknown Dpp targets which in turn repress *rho* and *aos* transcription. Indeed, a careful analysis of *BR-C* expression pattern in *snoN* mutants reveals an increase in distance between the two *BR-C* domains (Figure 23A-F). Thus, SnoN indirectly controls the lateral limits of BR-C expression which is consistent with BR-C and SnoN expression domain overlap in wt egg chambers. Finally, one of the mechanisms by which SnoN negatively regulates TGF-β target gene expression is by recruiting histone deacetylase I to remodel chromatin topography (Luo, 2004).

Histone deacetylases remove acetyl group from histones, which allows them to bind DNA and inhibit gene transcription. Thus, probably due to altered chromatin topography in *snoN* mutant we observe an upregulation of Dpp target genes in the follicular epithelium.

4.2.2 Brk acts as a transcriptional repressor of operculum fate genes

Brk acts as a differential repressor of Dpp target genes in the embryo (Jazwinska et al., 1999b). Upon comparison of *BR-C* and *brk* expression in dorsal follicle cells we found that *brk* is expressed along the dorsal midline as well as in follicle cells which give rise to dorsal appendage anlagen. This suggests that Brk function is essential in these cells to represses genes which are involved in specification of operculum. Our argument is strongly supported by the fact that Fas III is upregulated cell autonomously in follicle cell clones mutant for *brk*. Further, eggs laid by such females show expansion of operculum at the expense of dorsal appendage fate. This suggests that Brk does not regulate competence or the prepattern in the follicle cells established by Dpp.

A recent study identified Brk binding sequences (TGGCGc/tc/t) in Dpp target genes in the wing epithelium (Winter and Campbell, 2004). We searched ~ 10kb region upstream and downstream of FasIII coding sequence for Brk binding sites. The analysis revealed several putative *brk* binding sites, two of them clustered together, separated by just 84 bp approximately and present 7 kb upstream of transcription initiation site. Another cluster was found in the first intron of the gene seperated by a mere 205 bp. This analysis further supports our argument. Interestingly, *brk* is also needed for BR-C expression. In *brk* mutant follicle cell clones which spanned most of the dorsal main body follicle cells, BR-C is cell autonomously downregulated. This indicates that Brk acts as a cell type specific repressor and therefore could function by repressing another repressor which regulates BR-C expression. Thus, we propose that Brk acts as a differential transcriptional repressor of operculum fate genes.

Interstingly, FasIII is upregulated in the dorsal midline even in prescence of Brk (as *brk* levels are highest in these cells) in wt egg chambers. This indicates that the FasIII expression in dorsal midline is independent of Brk. Alternatively, it is also possible that Brk activity is counteracted by an unknown mechanism.

4.2.3 Sog controls diffusion of Dpp in the follicular epithelium

Sog functions in specification of embryonic DV polarity. Its main function is to sequester Dpp in the ventral and lateral regions of the embryo and transport it to the dorsal most regions of the embryo (Ashe and Levine, 1999; Srinivasan et al., 2002). In fact, Sog forms a ventral to dorsal gradient exactly opposite to that of Dpp in the embryo (Srinivasan et al., 2002). In pupal wing, *sog* and *dpp* are expressed in complementary domains. *sog* is expressed in the intervein region of the wing while *dpp* is expressed in the vein primordia (Yu et al., 1996). Recently, it has been shown that Sog function is needed in transporting Dpp from the longitudinal veins to crossvein regions where it induces crossvein formation (Shimmi et al., 2005). However, this appears not to be the case in the follicle cells. First, *sog* is expressed in the CMFCs at stage 10 of oogenesis when Dpp levels are highest in these cells. Therefore, a gradient of Sog protein would form along the AP axis similar to that of Dpp. Thus, highest levels of Sog would be present close to source of Sog expressing cells, the CMFCs. This suggests that in turn Sog-Dpp complexes would be formed in form of a gradient along the AP axis with highest number of such complexes being present close to CMFCs. Thus, it is possible that Sog functions by another mechanism or has a different function in the follicular epithelium as compared to that in embryo and wing.

We propose that Sog controls the diffusibility of Dpp along the AP axis. Sog sequesters free Dpp molecules close to the CMFCs thereby preventing its diffusion further in posterior follicle cells. Thus, it can be predicted that in absence of Sog in the follicular epithelium, Dpp should be able to diffuse further in posterior follicle cells. This is very well supported by observations obtained from Sog mutant follicle cells clones. Eggs obtained from mothers

where *sog* clones were induced, show expansion of dorsal appendage material in both anterior and posterior direction. Corresspondingly, we observed expansion of BR-C expression along the AP axis. This is exactly opposite to the proposed mechanism of Sog in the embryo and in the pupal wing where binding of Sog to Dpp is essential for its diffusion to the dorsalmost and crossevein regions respectively (Ashe and Levine, 1999; Srinivasan et al., 2002; Shimmi et al., 2005). Due to enhanced diffusibility of Dpp, the gradient of Dpp would flatten throughout the follicular epithelium. The flattening of the Dpp gradient would result in moderate level signaling in the anterior dorsal main body follicle cells which leads to ectopic induction of BR-C. Thus, Sog controls the steepness of Dpp gradient by modulating the diffusibility of Dpp in the follicular epithelium.

Dpp can signal via its receptors only when it is released from Dpp-Sog complex. This is achieved in the embryo by Tld, a putative metalloprotease that liberates Dpp allowing it to signal through to its receptor. It is not known if Tld or another unknown protease is expressed and functions in the follicular epithelium to release Dpp from such a complex. The expression pattern of Tld would provide definitive clues in understainding the role of Sog in follicular epithelium.

4.2.4 Cooperative roles of Dpp inhibitors in patterning of dorsal chorion structures.

We have shown that four Dpp inhibitors i.e *dad*, *snoN*, *sog* and *brk* are expressed in the follicular epithelium during oogenesis. All four inihibitors have distinct expression patterns which suggest distinct functions with respect to patterning of follicular epithelium. However, since they regulate Dpp activity in follicular epithelium it was conceivable that they might have overlapping functions. Indeed, individual mutant anaylsis of *brk*, *sog* and *snoN* provides hints on their cooperative nature in specifying operculum and dorsal appendage fate. Sog functions at the extracellular level by modulating the diffusion of Dpp

while Brk and SnoN act as transcriptional repressors Dpp targets involved in operculum fate induction.

Experiment performed by reducing Brk function by 50% in *snoN* mutant homozygous background suggests that both Brk and SnoN function together in the same pathway involved in repressing operculum fate. The egg phenotypes obtained from females homozygous for both *snoN* and a semilethal allele of *dad* reveals their collaborative action in specification of operculum and dorsal appendages.

Vertebrate homologue of *dad*, called Smad7, is believed to stably interact with the receptor and negatively modulate TGF-β signaling (Matsuzaki et al., 2000). The expression pattern of *dad* and egg phenotypes from *snoN*::*dad* double mutants suggest an existence of Dad mediated negative feedback circuit in the follicular epithelium that might sharpen the Dpp gradient (Tsuneizumi et al., 1997). Thus, by dampening the Dpp activity in a graded manner, Dad would be providing positional information to the follicle cells with respect to each other. Inducing loss of function follicle cell clones of lethal allele of *dad* would definately provide insights into its role in patterning. In summary, Dad is a general modulator of Dpp function in the follicular epithelium implying that it directly controls the prepattern and competence induced by Dpp.

One of interesting questions in patterning of a developmental field is how boundaries between different cell fates are determined and maintained? The formation of such boundaries is suggested to involve differential local expression of inhibitors and activators. We propose that Dpp inhibitors in addition also maintain boundaries between the operculum, dorsal appendage and dorsal midline cell fates in the follicular epithelium. Indeed, wt expression patterns of the inhibitors and mutant analyses support our proposition. Thus, Sog indirectly controls the boundary between the operculum and dorsal appendage fate. Brk controls the posterior limit of the operculum fate. SnoN controls the posterior and lateral boundaries of dorsal appendage fate. Finally, Dad strengthens the boundaries between the operculum and dorsal appendage fates.

4.3 Regulation of Grk activity

Our data supports the existence of a Grk activity gradient in the follicular epithelium across the DV axis. The induction of EGFR by Grk leads to expression of several modulators like Kek, Rho and Aos. Rho and Aos form feedback loops on EGFR activation (see Introduction). Wasserman and Freeman integrated these feedback loops and proposed a model for patterning of dorsal appendages. However, due to several inherent drawbacks (see Introduction) in their experimental setup we decided to validate the model by analyzing the patterning process in Grk misexpression and in Rho and Aos loss of function clones.

4.3.1 Rho and Aos function is essential for maintaining dorsal midline fate

We performed clonal analyses for Rho and Aos in marked clones. Loss of function clones were marked by absence of GFP in our experimental setup. To get a better understanding of the patterning process we followed the expression of Fas III and BR-C in marked clones. This was not the case in the experiments designed by Wasserman and Freeman. Only the resultant eggs were analyzed and the patterning events interpreted based on that.

We found that BR-C was still expressed in its characteristic two dorsolateral domains in loss of function Rho follicle cell clones. However, BR-C protein was no longer restricted to the nucleus. This suggests that in absence of feedback amplification loop initiated by Rho, Grk alone is able to specify dorsal midline as well as dorsal appendage fate.

A recent study from Ward and Berg (2005) suggests that Rho and BR-C are involved in dorsal appendage morphogenesis. The authors suggest that morphogenesis of dorsal appendages needs coordination between the floor cells (Rho expressing) and the roof cells (BR-C expressing). They propose that a boundary exists between the floor cells and roof cells. Loss of Rho would therefore allow mingling of roof cells into one another and would lead to production of eggs with a single dorsal appendage. However, this would occur

only at later stages of oogenesis during dorsal appendage morphogenesis when there are pronounced rearrangements between the floor and roof cells. This could be one of the reasons why Wasserman and Freeman obtained eggs with a single dorsal appendage. Analyzing *rho* mutant clones in late egg chambers would be provide a definitive answer.

Wasserman and Freeman imply that Aos is induced in dorsal midline response to amplification of EGF signal by Rho (Wasserman and Freeman, 1998). However, since we find that dorsal midline is specified in absence of the amplification step, it is possible that Aos is expressed in the midline. We therefore suggest that analyzing *aos* expression in *rho* mutant clones would be important experiment for validating the Freeman model.

In Aos loss of function clones located close to the dorsal midline we observed cell autonomous downregulation of BR-C but not of Fas III. This was a striking result as it is in disagreement with the Freeman model. However, it totally supports the recently discovered mechanism by which Aos mediates inihibition of EGF signaling (Klein et al., 2004). Argos associates predominantly with Spitz, to form nonfunctional heterodimers (Klein et al., 2004). Thus, Aos serves as a molecular sieve by restricting the range of Spi action and allowing only a few molecules of Spi to reach and activate EGFR in more distant cells. As Aos specifically sequesters Spi, it is also likely that other low activating EGF ligands like Vein can readily diffuse and elicit a lower level of EGFR activation. In addition, recent computational analysis has demonstrated that short range activity of Aos is sufficient to restrict the range of Spi diffusion and buffer fluctuations in levels of Spi (Reeves et al., 2005; Shilo, 2005). Thus, loss of Aos in the dorsal anterior follicle cells would result in diffusion of Spi leading to higher level of MAPK which in turn would lead to downregulation of BR-C.

We propose that induction of dorsal midline fate occurs at high level of MAPK signaling while assumption of dorsal appendage fate occurs at lower levels of MAPK activation. In fact, MAPK activation profile in follicle cells at stage 10 of oogenesis as reported by Peri et al. (1999) suggests that dorsal midline fate is specified at highest levels of MAPK while dorsal appendages would be formed at lower levels of MAPK. Our misexpression data supports

this proposition since moderate misexpression of *grk* in the germline leads to induction of dorsal appendage fates at ventral region while higher levels totally suppress it. Thus, tracking MAPK activation in loss of function Aos and Rho clones would be an important experiment in this context and would provide a definitive answer.

5. Summary

In *Drosophila* oogenesis, patterning of the follicle cells covering the developing oocyte is achieved by inductive signaling. Two major signaling pathways converge to induce a subpopulation of dorsal anterior follicle cells to adopt cell fates which give rise to operculum and dorsal appendages. One of the signals is initiated by the TGF-β/BMP signaling pathway. Decapentaplegic (Dpp), one of the BMP like ligands in *Drosophila*, forms a morphogenetic gradient along the AP axis in the follicular epithelium and promotes operculum fate at highest level while moderate levels promote dorsal appendage fate. The second signal is provided by EGF/TGF-α like ligand Gurken (Grk) which is locally secreted by from the developing oocyte and forms a gradient along the dorsoventral axis. High concentrations of Grk induce the operculum fate, moderate concentrations the dorsal appendage fate.

Clonal analysis shows that in absence of Dpp activity Grk cannot induce any of the dorsal cell fates in the follicular epithelium indicating that Dpp acts as a competence factor for Grk signaling. Moreover, Dpp also restricts the range of Grk signaling. The combined misexpression of Grk and Dpp leads to an expansion of dorsal fates along both the axes. A phenotype could be generated in which all main body follicle cells except those at the termini of the egg chamber were tansformed into operculum fate.

The Dpp gradient and its action on target genes is modulated by several inhibitors which themselves are targets of the Dpp and EGF pathways. This results in a complex network of feedback control. Based on its intriguing expression pattern within the follicular epithelium I investigated the function of *Drosophila snoN*, a member of the Ski family proteins which are known as transcriptional co-factors of TGF-ß signaling in vertebrates. *snoN* mutant females lay eggs with enlarged operculum while misexpression of *snoN* in the whole follicular epithelium reduces the operculum size. Thus, SnoN acts as a transcriptional repressor of operculum fate genes. The intracellular Dpp inhibitors, *brinker* (*brk*) and *daughters against dpp* (*dad*) act together with *snoN* to regulate the Dpp readout in the follicular epithelium. Loss of function clones

for *brk,* show that *brk* acts as a transcriptional repressor of operculum fate genes. Interestingly, loss of function clones of the extracellular Dpp inhibitor *short gastrulation* (*sog*) lead to a posterior expansion of the dorsal appendage fate indicating that in contrast to its role in the embryo Sog limits the diffusion of Dpp within the follicular epithelium.

Like the Dpp pathway, the EGF pathway induced by Grk is modulated by several genes which themselves are targets of both pathways. In particular, the activation of *rhomboid* (*rho*) leads to a secondary amplification of the EGF signal which is thought to play an important role in follicle cell patterning. By performing clonal analysis for *rho*, we disprove this hypothesis and show that that a graded activity of Grk itself is sufficient to induce different dorsal fates and that the amplification is not essential for defining the midline fate.

6. Zusammenfassung

In *Drosophila* erfolgt die Musterbildung der Follikelzellen, welche die sich entwickelnde Oozyte umgeben, durch induktive Signale. Zwei zentrale Signalwege wirken zusammen, um in dem dorsal-anterioren Follikelepithel Zellen zu spezifizieren, die das Operculum und die dorsalen Anhänge bilden. Eines der Signale wird durch den TGF-ß/BMP ähnlichen sezernierten Liganden Decapentaplegic (Dpp) bereitgestellt. Dpp bildet einen Morphogengradienten entlang der anteriorposterioren Achse des Follikelepithels aus. Hohe Dpp-Konzentrationen werden für die Bildung des Operculums, mittlere für die Bildung der dorsalen Anhänge benötigt. Das zweite Signal wird durch den EGF/TGF-α ähnlichen Liganden Gurken (Grk) bereitgestellt, der von der Oozyte lokal sezerniert wird und einen Konzentrationsgradienten entlang der dorsoventralen Achse ausbildet. Hohe Grk Konzentrationen induzieren Operculumzellen in dorsalen Positionen, mittlere Konzentrationen induzieren dorsale Anhänge in mehr lateralen Positionen.

Mit Hilfe klonaler Analysen wurde gezeigt, dass Grk in Abwesenheit von Dpp keine der dorsalen Zellschicksale im Follikelepithel induzieren kann. Dpp wirkt also als Kompetenzfaktor für das Grk-Signal. In dieser Funktion begrenzt Dpp die anteriorposteriore Reichweite des dorsalisierenden Grk-Signals. Die ektopische Expression beider Liganden führt zu einer Expansion des dorsalen Follikelzellschicksals entlang beider Achsen. Im Extremfall ist es möglich, das gesamte Follikelepithel über der Ooyzte mit Ausnahme der posterioren Follikelzellen in Operculum zu transformieren.

Der Dpp-Gradient und seine Wirkung auf die Expression von Zielgenen im Follikelepithel werden durch mehrere Inhibitoren reguliert, die selbst Zielgene des Dpp-Signalwegs und meist auch des EGF-Signalwegs sind. Hieraus ergibt sich ein komplexes Rückkopplungs-netzwerk. Als neue Inhibitoren in *Drosophila* wurden Homologe der Ski-Familie untersucht, die in Vertebraten als transkriptionelle Kofaktoren der TGF-ß Kaskade wirken. *snoN* mutante Weibchen legen Eier mit einem vergrößerten Operculum während die Missexpression von *snoN* im gesamten Follikelepithel die Größe des

Operculums reduziert. *snoN* wirkt also als ein Repressor des Operculumzellschicksals. Die intrazellulären Dpp Inhibitoren *brinker* (*brk*) und *daughters against dpp* wirken mit *snoN* zusammen, um die Zielgenregulation durch den Dpp-Gradienten zu modulieren. In *brk* mutanten Follikelzellklonen kommt es zu einem Verlust des Zellschicksals der dorsalen Anhänge und zu einer Ausdehnung des Operculumschicksals. Interessanterweise hat auch der extrazelluläre Dpp-Inhibitor *short gastrulation* (*sog*) einen Einfluss auf die Musterbildung der dorsalen Follikelzellen. *sog* mutante Zellklone führen zu einer posterioren Expansion der dorsalen Anhänge. Vermutlich schränkt *sog* anders als im Embryo die Diffusion von Dpp im Follikelepithel ein.

Ähnlich wie der Dpp-Signalweg wird auch der durch Grk aktivierte EGF-Signalweg durch zahlreiche Faktoren moduliert, die von Zielgenen beider Signalwege kodiert werden. Insbesondere führt die Aktivierung von *rhomboid* zu einer sekundären Amplifikation des EGF-Signals. Durch klonale Analyse konnte aber gezeigt werde, dass im Unterschied zu früheren Modellvorstellungen dieser Amplifikationsprozess keinen entscheidenden Einfluss auf die Musterbildung im Follikelepithel hat. Im Bereich der durch Dpp festgelegten Kompetenzzone ist der Grk-Gradient alleine in der Lage die unterschiedlichen dorsoventralen Zellschicksale im Follikelepithel festzulegen.

7. MATERIALS AND METHODS

7.1 Fly stocks and genetics

To analyze the consequences of misexpression of different genes on patterning in the follicular epithelium the following lines were used:

UAS-transgenic lines used:

UAS-dpp: UAS-Dpp/CyO (was a gift from FM Hoffmann)

UASp-grk: UASp-Grk/w (on X chromosome- generated in our lab). This line has the ability to express in germ cells

UAS-snoN: UAS-SnoN/CyO (on second chromosome-generated in our lab)

Gal4 drivers lines used:

Tub-Gal4VP16: Tub-Gal4VP16/w (on the X chromosome; St Johnston Lab)

GR1-Gal4: GR1-Gal4/TM3 (on the third chromosome; Queenan et. al., 1997)

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

omb-Gal4: omb-Gal4/FM7 (Bloomington Stock Center)

Lines generated for **double misexpression** studies

UASp-Grk: GR1-Gal4

UASp-Grk: Cy2-Gal4

Tub-Gal4VP16: UASp-Grk

Mutants used in this study:

dad-LacZ(L): dad-LacZ/TM6 (on the third chromosome; described as l(3)1E4 in Tabata et. al., 1997)

dad-LacZ(SL): dad-LacZ/TM6 (on the third chromosome; described as P1883 in Tabata et. al., 1997)

cni^{AR55}: b cniAR55 pr cn/CyO (cniAR55 is a null allele; Roth et. al., 1995)

cni^{AA12}: b cniAR55 pr cn/CyO (cniAR55 is a null allele; Roth et. al., 1995)

Df(2L)H60: w; sco Df(2L)H60/CyO This deficiency removes chornichon (Roth et. al., 1995)

Df(2L)Mz-Sz/CyO: Df(2L)Mz-Sz/CyO. This deficiency removes *snoN* (Bloomington Stock Center)

snoN-LacZ: sno-LacZ/CyO (on the second chromosome; described as l(2) Sh1402 Oh et al., 2003)

brk: yw brk^{M68}/FM7cftz-LacZ (Jaswinska et al 1999a)

sog: sog^{ys06}/ FM7cftz-LacZ (Jaswinska et al 1999a)

snoN-/- : snoN^{174} (This study)

Df (3L) st4: Df (3L) st4 This deficiency removes *aos* gene (Bloomington Stock Center)

Aos1δ7: aos$^{1\delta7}$. Null allele of *aos* (Freeman et al., 1992)

Mutant Follicle cells clones were generated using the following lines;

Brk clones: yw brk^{M68} FRT18A/FM7cftz-LacZ

Sog clones: yw sog^{ys06} FRT18A/FM7cftz-LacZ

Tkv clones: tkv ^{a12} FRT 40A (neo)/CyO

Medea clones: w; eMed ^{13} FRT (w+)/TM3 sb

Rho mutant clones: w; sco/cyo;rho ^{7M3} FRT80b(neo)/TM6 tb

Aos mutant clones: w; If/CyO; aos ^{1d7} 79DFRT(w+)/TM6 tb

FRT chromosomes were used for marking clones in the follicular epithelium.

FRT18A GFP; MKRS hs-flp/+

ywhsflp: 40AGFP FRT

ywhsflp: If/CyO;79DGFP FRT/TM6 tb

ywhsflp: If/CyO; 80BGFP FRT/TM6 tb

ywhsflp: If/CyO; 82BGFP FRT/TM6 tb

7.1.1 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 18°C. To ensure genetic purity for the progeny of crosses, only non-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 16h at 18°C before collecting the virgins. Oregon R, wy1 served as the wildtype strain.

7.2 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 with water and mounted in the Hoyer's medium. The mounted samples were incubated at 60°C for at least 24 hours before they were photographed Zeiss Axiovert.

7.3 Immunohistochemistry and in situ hybridization

7.3.1 Fixation of ovaries for immunostainings

The ovaries were disected and transferred to heptan fix (200µl4% paraformaldehyde + 20µl DMSO + 600µl heptan) for 20 min.

7.3.2 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. Preabsorbed secondary antibody was added for 1,5 h incubation. The antibody was removed and the ovaries were rinsed and washed twice over 45 min. Secondary antibody either coupled with fluorochrome Alexa 488 or Alexa555 were used and finally the ovaries were mounted in Vectashield (Linaris).

7.3.3 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 –20^{o}C.

7.3.4 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 minand incubated for 10 min in 50 µ/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 MgCl2, 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

7.3.5 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. Images were obtained using a Zeiss microscope.

7.4 Molecular Cloning

All DNA methods were performed according to Sambrook et. al. (1989) and or according to manufacturers instructions in situations where kits were utilized.

7.4.1 Cloning of snoN/ski

Chicken snoN (c-*snoN*) cDNA sequence was Blast searched against the whole Drosophila genome. Two genes similar to c-*snoN* were identified. One could be located on the second chromosome, CG7233 and the other on fourth chromosome, CG11093. Full length CG7233 cDNA was amplified from Drosophila ovarian cDNA library (Tolias et. al.) using the following primers: SnoNcoIR (5´ CATGCCATGGCCAACGCACCTTTCTTTTCT) and SnoNcoIF (5' CATGCCATGGCCGAATACGTGACGCCAATG). The 1044bp amplified fragment was then cloned in pCR2.1-TOPO vector (Invitrogen) and the E. coli competent cells were transformed with the recombianant plasmid pCR-TOPO-SnoN according to manufacturer's instructions. CG11093 message could be amplified from Drosophila ovarian cDNA library (Tolias et. al.) using primers ski5x2 (5'GCTGTACGGAGTGCAGATTGTATC) and ski3x2 (5'GTAGTTGTACTGGCTGTCGATCTG) specific for the second exon. The 852bp fragment was then cloned in pCR2.1-TOPO vector (Invitrogen) and the *E. coli* competent cells were transformed with the recombianant plasmid pCR-TOPO-II exSki according to manufacturer's instructions. Both the inserts were sequenced using BigDye Sequencing Kit (Perkin Elmer, USA) and further used for molecular biology applications.

7.4.2 DNA work and germline transformation

The *snoN* cDNA (1.325kb) was cloned into the pCRTOPO II vector according to the manufacturer's instructions. The *snoN* cDNA was restricted from pCR-

TOPO-SnoN and ligated into pUAST vector using appropriate restriction enzymes. 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 1nl of the solution was injected into the posterior region of the 20- to 30 min old w8 flies preblastoderm embryos. Embryos were covered with Voltalef hydrocarbon 5S and allowed to hatch at 18°C. The second instar larvae 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.

7.4.3 Production of antibody against SnoN

Full length (1026bp) *snoN* was amplified from pCR-TOPO-SnoN using appropriate primers and then restricted digested with NcoI and SacI to give a 960bp fragment. This fragment was then ligated into pBADmychisB (Invitrogen) plasmid so as to obtain recombinant plasmid. *E.coli* JM109 was transformed with the pBADsnoNmychis recombinant plasmid. *snoNmychis* was expressed under the control of T7 promoter. JM109 harbours plasmid which expresses T7 polymerase under the control of *arabinose* promoter (Kosiba and Schleif, 1982). 100mg/ml of arabinose was used to induce the SnoN-myc-his protein. The bacteria were harvested after 4h postinduction and pellets stored at -70^{o}C for downstream processing. The recombinant protein was partially purified using Ni-NTA agarose (Qiagen) as described in the protocol handbook. A 100% efficient purification of SnoN fusion protein from bacterial extracts was not possible. Therefore, large amounts of partially purified SnoN fusion protein was loaded on 10% SDS gels. After electrophoresis the gel was stained by reversible protein stain Ponceau-S so as to localize the SnoN-myc-his protein band (~ 40kd). The gel slice containing SnoN fusion protein was cut and washed overnight with distilled water to remove traces of SDS. Next day the gel slices were sent to Eurogentec Corporation, Belgium for immunization of rats.

Three bleeds were obtained from them. The rat serum was tested for prescence of anti-SnoN antibodies by standard procedures.

7.5 Induction of Mitotic clones

FLP/FRT system of mitotic recombination was used to generate marked follicle cell clones. All follicle cell clones generated in this study were marked by the loss of GFP. Clones were induced by either heat shocking 1st, 2nd and 3rd instar larvae for 4 days 2 h/day or by heat shocking adult females for 1h at 37^{o}C. The heat shocked adult females were transferred to a fresh yeasted vial with wy males and were placed at 25^{o}C for max 3-4 days after which the ovries were dissected out and stained for GFP and appropriate markers.

7.6 Western blotting

Electrophoresis of protein extracts was carried out on 10% SDS gels according to Sambrook et al (1989). Western blotting techinique was carried out according to manufacturers' instructions (Trans-Blot Semi-Dry apparatus, Bio-Rad Labs, USA). Primary and secondary antibodies were diluted to desired concentration and applied to the blot. Detection of protein was done ECL chemiluminescent detection kit from (Amersham Biosciences, USA).

7.7 Scanning Electron microscopy (SEM)

Fixation of eggs was followed according to protocol described in (Margaritis et al., 1980). Scanning of gold sputtered samples was performed using LEO 430 scanning electron microscope (Oxford Instruments, England)

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Bhupendra V. Shravage

LEBENSLAUF

Bhupendra V. Shravage

Staatsangehoerigkeit: Indien

18.10.1977	geboren in Pune- MS, Indien Eltern:Vinayak H. Shravage und Sarojini V. Shravage
1983-1993	Grundschule und Hauptschule, S.D Katariya High School, Pune, Indien
1993-1995	Gymnasium in Abasaheb Garware College, Pune, Indien Abitur im Juni 1995
1995-1998	Bachelor of.Science (B.Sc.) in Microbiology-University of Pune, Pune, Indien
1998-2000	Masters of Science (M.Sc.) in Marine Biotechnology-Goa University, Goa, Indien
	M.Sc Thesis (Diplomarbeit) bei Dr. Sanjeev Ghadi,Goa University, Goa, Indien **Titel**: DNA extraction from Seaweeds for RAPD analysis
Aug 2000-Sept 2000	Wissenschaftlicher Mitarbeiter bei Dr. Rohit Mittal an der Tata Institute for Fundamental Research (TIFR), Mumbai, Indien
Oct 2000-Sept 2001	Wissenschaftlicher Mitarbeiter bei Dr. Yogesh Shouche an der National Center for Cell Science (NCCS), Pune, Indien
Seit 2001	Doktorarbeit bei Prof. Siegfried Roth am Institut für Entwicklungsbiologie, Der Universität zu Köln
	Titel: Signaling networks involved in patterning dorsal chorion structures in Drosophila