Introduction

1. Epithelium importance

The Epithelium is crucial for organogenesis and morphogenesis. It allows for tissue and organ shape throughout development, allowing the organization of different physiological compartments. During epithelium morphogenesis this tissue is under stresses, and has to maintain integrity. Regulators that maintain epithelial integrity are known to play conserved roles along different tissues. However, for example in multiple diffuse gastric carcinoma a specific mutation in E-cadherin, a protein essential in all cell-cell contacts, is only associate to defects in the gastric epithelium (Bamba, Sugihara et al. 2008). This suggests that although epithelial tissues have similar architecture they may have different requirements to maintain tissue integrity.

2. Epithelium remodeling and cell shape

To form complex epithelial structures, the cells undergo a series of morphogenetic events during embryonic development: cell intercalation, cell shape change and cell-cell and cell-extracelular matrix contact remodeling. This events are essential for proper organism development (Pilot and Lecuit 2005). In the particular case of *Drosophila*, some of these processes will be here addressed in more detail (see below):

2.1. Apical constriction

One important morphogenetic process that occurs during gastrulation is the invagination of epithelial tissues. *Drosophila* ventral furrow invagination is an example of this event (Leptin and Grunewald 1990; Sweeton, Parks et al. 1991) (Fig.1). Essential to this mechanism is the change of cell shape from cuboidal to bottle shape. This was first observed in *Xenopus leavis* and denominated apical constriction (Keller 1981). Recruitment of non-muscle myosin-II (Myo II) to the apical cell cortex together with F-actin has been shown to be important to reduce the apical cell perimeter. The contraction of the cell apex was suggested to be a pulsed process, where the acto–myosin network contractility is regulated by the transcription factor Twist, followed

then by pauses where the constricted state is maintained through *Snail* regulation (Martin, Kaschube et al. 2009).

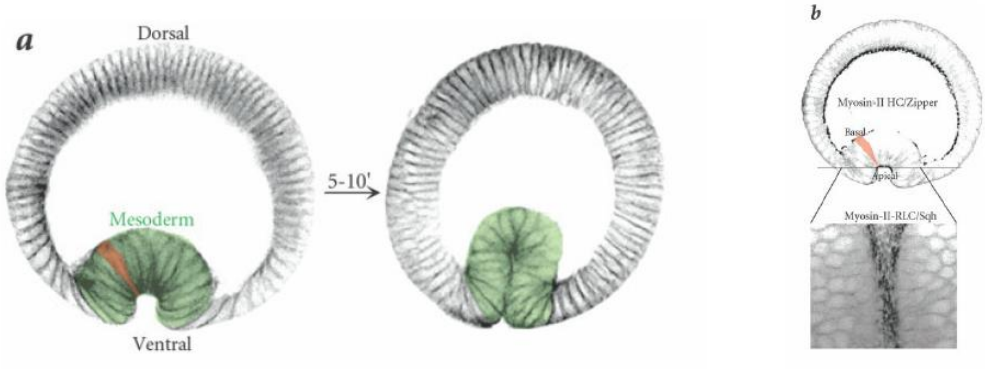

Fig.1- Apical constriction during ventral furrow formation. a: Transverse section of a gastrulating embryo showing the ventral invaginating mesoderm (green). **b:** Enfolding cells (red) undergo apical constriction, which correlates with the apical recruitment of Myosin II (black). Inset: ventral view of the apical surface of the invaginating cells showing the localization of a Myosin-II Light Chain green fluorescent protein fusion protein (black). HC, Heavy Chain; RLC, Regulatory Light Chain. Adapted from (Pilot and Lecuit 2005).

2.2. Cell Intercalation and planar cell polarity

Cell intercalation is another important morphogenetic mechanism that promotes epithelial elongation in one axis, coupled with perpendicular retraction of the tissue. This event, together with apical constriction, forces the epithelium to fold in a 3D complex remodeling its shape. These events occur during *Drosophila* posterior midgut invagination and germ-band elongation, in which the embryo increases 2,5 times in length (Irvine and Wieschaus 1994).

Recent studies report that cell intercalation in structures with more than 5 cells implies the organization of the cells into two columns, that intercalate to form a rosette, meeting at a single point in clusters. During this process F-actin and Myo II show planar polarization in the anterior-posterior (AP) interfaces between cells. After the formation of the rosette, the cluster resolves and allows the AP elongation of the tissue. F-actin and DE-cadherin accumulate at the new cell contacts, after which *bazooka* is also

enriched at these interfaces (Fig.2) (Bertet, Sulak et al. 2004; Blankenship, Backovic et al. 2006).

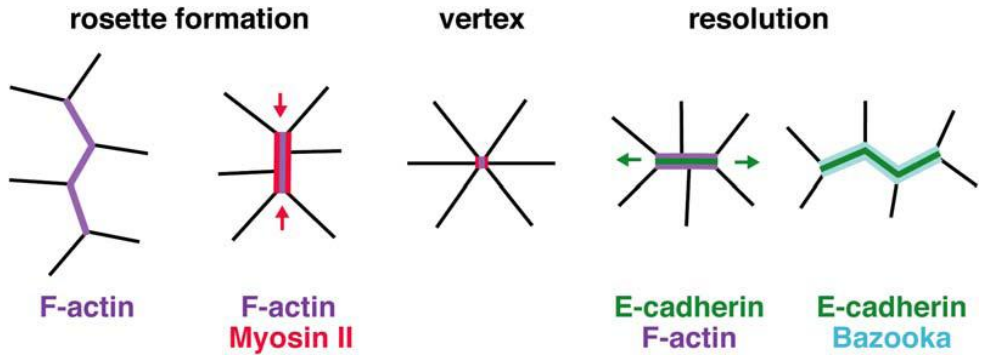

Fig. 2- Rosette formation and resolution. F-actin enrichment (purple) at anterior-posterior (AP) contacts are followed by Myosin II (pink) and subsequent accumulation at AP interfaces to coordinate the contraction and rosette formation. F-actin and Myosin II colocalize at the vertex where multiple cells meet (center). Rosettes resolve in a directional fashion to create contacts between cells that were previously separated along the dorso-ventral (DV) axis. DE-cadherin accumulates (green) at new contacts together with F-actin. Bazooka (blue) is recruited to new interfaces after a delay, which may create local differences in adhesion that are important for rosette resolution. Adapted from (Blankenship, Backovic et al. 2006).

Throughout all of these events, the epithelial cells depend highly on their apico-basal polarity. This is essential to allow the remodeling of the cell-cell contacts without the collapse of the tissue.

3. Cell Adhesion

Adherens junctions (AJs) are conserved structures critical to cellular adhesion, both in vertebrates and insects. Besides AJs, vertebrate cells possess tight junctions and desmosomes. On the other hand, in insects there are other adhesion complexes like the septed junctions (SJ) and the sub-apical region (SAR) (Fig.3).

The AJs contacts result from the homophilic Ca^{2+}-dependent interactions between cadherin proteins on adjacent cells (Nelson 2008). Actin and AJs are brought together by the transient set of dynamic interactions between DE-Cad, β-catenin and α-catenin.

α-catenin binds to actin. α-catenin binds also to Armadillo (Arm), the *Drosophila* homologue of β-catenin, which in its turn binds DE-Cad. This brings α-catenin (and actin) and DE-Cad (AJs) in close proximity, and allows their intercalation (Yamada, Pokutta et al. 2005). Other important component is p120, which is responsible for the regulation of E-Cadherin (Davis, Ireton et al. 2003).

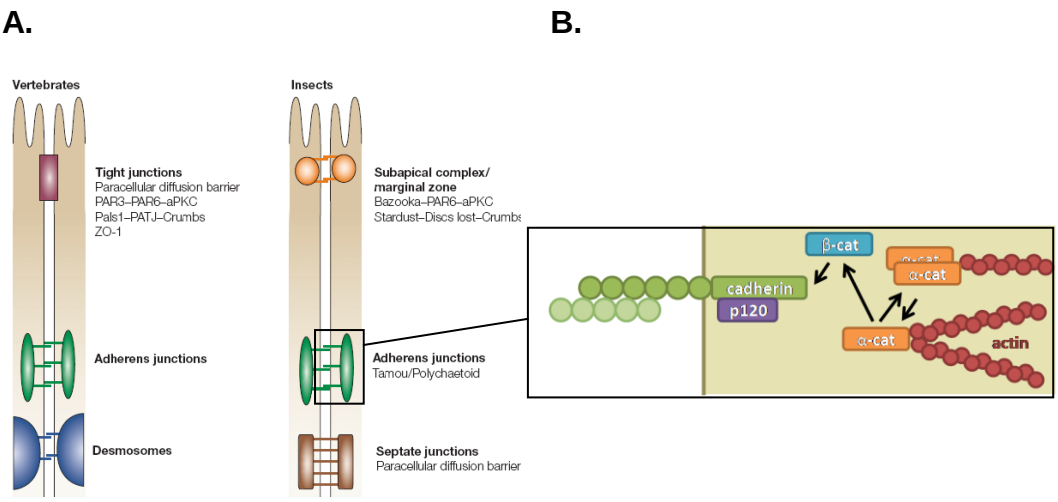

Fig.3- Cell-cell junctions in Vertebrates and Insects. (A) Both types of organisms have AJs in the apical domain (green in A). Vertebrate cell-cell contacts are composed by Tight Junctions (purple in A) and Desmosomes (blue in A). Insects contain a baso-lateral. Septed Junction (brown in A) and a Subapical Complex (orange in A). AJs are composed by Cadherin (green in B), p120 (purple in B), β-catenin (blue in B), α-catenin (orange in B) and actin filaments (red in B). Adapted from (Matter and Balda 2003).

3.1. Vesicle trafficking and Actin regulation

To allow for the plasticity and rapid remodeling of cell contacts during morphogenesis, the cell must regulate the delivery of cadherin-catenin complexes to the AJs. Membrane trafficking and recycling are crucial for this event (Delva and Kowalczyk 2009). Rab proteins play an essential role in targeting and fusing transport vesicles with their appropriate acceptor membranes. The membrane proteins are internalized into early endosomes that are positive for Rab5. Rab5 positive endosomes are then recycled via the Rab11-recycling endosome, which is important for E-cadherin targeting to the

basolateral membrane (Lock and Stow 2005). Alternatively, RAB 5 endosomes can mature into Rab7/late endosomes and be targeted to destruction.

In epithelial tissues the actin cytoskeleton is important to maintain cellular shape and consequently its integrity. It has also a pivotal role in cell motility, cytokinesis, phagocytosis and intracellular transport processes. Rho, Rac and Cdc42 are small GTPases that belong to the Ras-related super family of proteins, known to be involved in actin regulation and dynamics (Nobes and Hall 1995).

RhoGTP is frequently associated with the formation of contractile actin structures. RhoGTP activates Rho associated kinase (Rok) allowing it to phosphorylate myosin regulatory light chain (MRLC) (Amano, Ito et al. 1996; Matsui, Amano et al. 1996) and therefore promote contractility. Consistent with this, RhoGEF2 (a Rho effector), was shown to be important to regulate actomyosin contraction events throughout *Drosophila* development (Barrett, Leptin et al. 1997; Padash Barmchi, Rogers et al. 2005). Additionally, Diaphanous (Dia), which is involved in actin polymerization, is known to be downregulated by RhoGTP (Castrillon and Wasserman 1994; Watanabe, Madaule et al. 1997).

Cdc42 is known to regulate the Arp2/3 actin nucleator. Cdc42 activates the Wiskott-Aldrich Syndrome protein (WASp) which in turn is an activator of the Arp2/3 complex, which promotes actin polymerization (Pollard, Blanchoin et al. 2000; Rohatgi, Ho et al. 2000). It was reported that aPKC-PAR6-Cdc42 complex regulates WASp, Cdc42-interacting protein 4 (Cip4) and Arp2/3. This regulation was shown to be important to AJ remodeling by endocytosis (Georgiou, Marinari et al. 2008; Leibfried, Fricke et al. 2008).

4. Apico-Basal Polarity

An epithelial cell is a polarized structure with two distinct domains: an apical domain, usually in contact with the lumen of the biological compartment/organ, and a basal domain, that faces the interstitial region or extracellular matrix. Apico-basal polarization is controlled by several protein complexes, which include very different

types of proteins, from scaffold to kinase proteins. All these complexes need to integrated and crosstalk to allow the establishment and modulation of cell polarization.

4.1. Atypical Protein Kinase-C (aPKC)

The isolation of the first Protein kinase C (PKC) protein was reported by Nishizuka and coworkers and described, as a histone protein kinase (Inoue, Kishimoto et al. 1977). The PKC superfamily of serine-threonine kinases includes the subgroup, of the atypical protein kinase C (aPKC) (Fig. 4). aPKCs do not require Ca^{2+} or diacylglycerol for activation (Hug and Sarre 1993) because the N-terminal C1 domain (the diacylglycerol sensor in PKCs) is incomplete in the aPKC (Fig. 4, orange). Like the other PKCs they have three phosphorylation sites, located within the activation loop, the turn and the hydrophobic motifs (Fig. 4, pink) of kinase domain (Fig. 4, blue) (Nelson 2003). Two of them may be activated by auto-phosphorylation (Keranen, Dutil et al. 1995). Atypical PKCs have the particularity of having a N-terminal Protein Binding 1 (PB1) domain, that allows the interaction with other PB1 containing proteins (Fig.4, yellow) (Hirano, Yoshinaga et al. 2004).

The first evidence of the role of aPKC in polarity was shown in *Caenorhabditis elegans* (*C. elegans*) where the homologous PKC-3 revealed to be essential for proper asymmetric cell divisions and to colocalizes with Partitoning defective 3 (PAR3) (Tabuse, Izumi et al. 1998) the homologue of Baz. This interaction and colocalization were confirmed in mammalian cells for ASIP, the mammalian homologue of PAR3 (Izumi, Hirose et al. 1998).

Similarly, *Drosophila* aPKC loss-of-function mutants show loss of apico-basal polarity, multilayering of the epithelia and abnormal spindle orientation in neuroblasts (Wodarz, Ramrath et al. 2000; Harris and Peifer 2007).

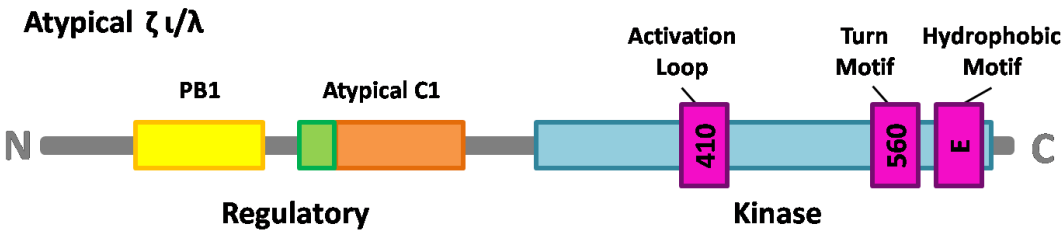

Fig.4- Domain composition of Human atypical Protein Kinase C. Regulatory region containing the PB1 domain (yellow), the atypical C1 domain (orange) and the autoinhibitory pseudo-substrate sequence (green). The kinase domain (blue) has three phosphorylation sites (pink). In aPKCs one of phosphorylation sites is substituted by a glutamate. Adapted from (Nelson 2003).

4.2. Apical aPKC-PAR complex

Before the report of the role of aPKC, another group of proteins was shown to be important to establish early asymmetries in *C. elegans*. These proteins were denominated by PAR proteins (from Partitioning defective), and are composed by PAR-1, PAR-2, PAR-3, PAR-4, PAR-5 and PAR-6 (Kemphues, Priess et al. 1988; Bowerman, Ingram et al. 1997; Crittenden, Rudel et al. 1997). Two of them, PAR3 and PAR6, are known to form a complex with aPKC.

Bazooka, the *Drosophila* homologue of C. elegans PAR-3, encodes a protein with three PDZ domains (Post synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (DlgA), and Zonula occludens-1 protein (zo-1)) important for protein-protein interactions. It is localized to the apical cortex of the cells (Kuchinke, Grawe et al. 1998) and in Madin-Darby Canine Kidney cells (MDCK) it localizes to tight junctions. It is stabilized by aPKC phosphorylation (Fig. 5) at the S827 residue (Nagai-Tamai, Mizuno et al. 2002).

The *Drosophila* PAR-6 homologue (DmPAR6) binds to the aPKC N-terminal PB1 domain. aPKC also interacts with Bazooka (*in vitro*) via its PDZ domain (Joberty, Petersen et al. 2000; Petronczki and Knoblich 2001). The Cdc42- and Rac-interactive binding domain (CRIB) in PAR6 is responsible for its binding to Cdc42/Rac1 GTPases. This interaction was shown to be important for the activation of aPKC, after which

aPKC phosphorylates Bazooka (Yamanaka, Horikoshi et al. 2001) (Fig. 5) and stabilizes it.

In *Drosophila* early development, epithelial polarity is established during cellularization, when Bazooka accumulates apically in the cells. This apical localization is independent of PAR6 and aPKC, and occurs in the absence of proper AJs (Harris and Peifer 2004; Harris and Peifer 2005).

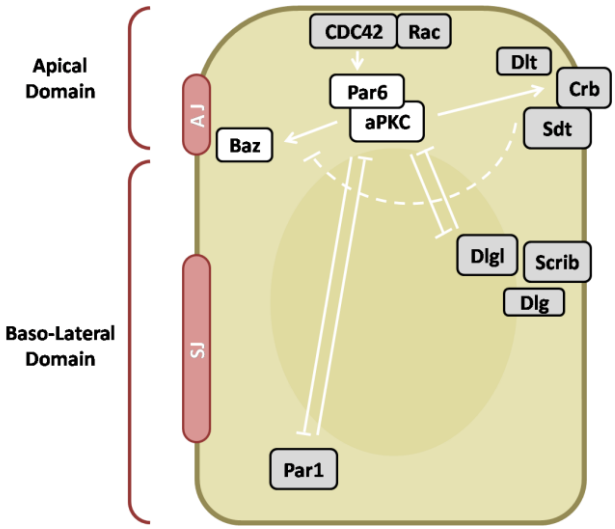

Fig.5- Apico-Basal regulation in *Drosophila* epithelial cells. Adherent Junctions (AJ, red) are located in apical domain, whereas Septate Junctions (SJ, red) are located in baso-lateral domain. Apically, Cdc42 activates aPKC through PAR6, this complex regulates AJs through phosphorylation of Baz, and also maintain Crb apically by phosphorylation. Crb is thought to dissociate aPKC/Par6 complex from Baz/AJs to the apical most region (dashed line) during gastrulation. The aPKC/PAR6 complex excludes Scrible (Scrib) complex to the baso-lateral domain as well as PAR1.

4.3. Apical Crumbs complex

Crumbs (Crb) is an apical membrane protein particularly enriched at epithelial membranes of adjacent cells (Tepass, Theres et al. 1990). It has an extracellular domain composed of 29 Epithelial Growth Factors-like (EGF) repeats that together with the cytoplasmatic FERM (protein 4.1, Ezrin, Radixin Moesin) domain is important to recruit beta-heavy-spectrin and Moesin (Medina, Williams et al. 2002; Richard, Muschalik et al. 2009). Crb is known to be involved in photoreceptor morphogenesis and required for extension of the stalk membrane and elongation of the cell, and also for

development of cell polarity. Stardust (Std) the *Drosophila* homologue of PalS1, is known to bind the carboxy-terminal amino acids of the cytoplasmic tail of Crb, and this is important for the proper localization of Std to the sub-apical region (Fig. 5) and for the reorganization of AJs material (Knust, Tepass et al. 1993; Tepass 1996; Bachmann, Schneider et al. 2001).

Another component of this complex is the multi PDZ domain protein Discs lost (Dlt). Dlt was shown to interact with Crb on the apical cortex of both *Drosophila* and mammalian cells (Bhat, Izaddoost et al. 1999; Lemmers, Medina et al. 2002), and is also important to establish and maintain proper cell polarity.

In *Drosophila*, Crb was shown to be a phosphorylation target of aPKC (Fig. 5) (Sotillos, Diaz-Meco et al. 2004). Zygotic mutants of Crb show the formation of aggregates containing AJs components, Baz, PAR6 and aPKC; suggesting that Crb is important to dissociate AJs and Baz from the aPKC/PAR6 complex during gastrulation (Fig. 5, dashed line) (Harris and Peifer 2005). Recently, this complex was shown to have an important role in maintaining cell polarity during morphogenetic movements (Campbell, Knust et al. 2009).

4.4. Baso-lateral Scribble complex

This complex is composed by Scribble (Scrib), Lethal giant larvae (Lgl) and Discs large (Dlg). It localizes to the basolateral region of the cell cortex (Fig. 5) and it is important to maintain cell polarity in *Drosophila* imaginal discs epithelium. Scrib belongs to the LAP family of proteins. It has 16 N-terminal Leucine-rich-repeat (LRR) that allow the protein to be targeted to the Septated Junctions (SJs) in the basolateral cortex of *Drosophila* epithelial cells and interact with Lgl (Fig. 5) (Santoni, Pontarotti et al. 2002; Navarro, Nola et al. 2005; Kallay, McNickle et al. 2006).

Drosophila Dlg is the homolog of mammalian DLG1. It has a L27 domain, which are important for its localization (Bohl, Brimer et al. 2007), and the interaction between Dlg and Scrib is via Guanylate-kinase holder (GUKh) which is an adaptor molecule linking this two proteins (Katoh 2004).

Mammalian and *Drosophila* Lgl proteins were shown to interact with PAR3 during cell polarization. Lgl initially colocalizes with PAR6/aPKC dimmer at apical region, and here is phosphorylated by aPKC (Fig. 5). As a result, Lgl is segregated to the basolateral membrane, where it interacts with Dlt and Scrib (Yamanaka, Horikoshi et al. 2003; Hutterer, Betschinger et al. 2004).

4.5. Basal antagonisms

PAR1 is a Ser/Thr kinase that is posteriorly localized in the zygote of *C.elegans* (Guo and Kemphues 1995). Human PAR1 is phosphorylated at T595 by aPKC and this regulates the activity and localization of the protein (Hurov, Watkins et al. 2004). Once active, PAR1 phosphorylates Bazooka and this inhibits the formation of the Bazooka/PAR6/aPKC complex (Benton and St Johnston 2003). Antagonisms are thus key strategies to regulate the polarized domains in the cell.

5. Epithelial Morphogenesis in *Drosophila melanogaster*

Drosophila oogenesis and early embryogenesis developmental programs involve the formation, maturation and morphogenesis of epithelial tissues. We focused on this processes to understand how epithelial morphogenesis and polarity are established and modulated.

5.1. Oogenesis in *Drosophila*

During *Drosophila* oogenesis, a stem cell located at the tip of the germarium divides asymmetrically into another stem cell and cystocyte. This differentiated cell (cystocyte) undergoes four consecutive mitotic divisions, with incomplete cytokinesis, and gives rise to sixteen cells that remain connected, forming the cyst (Fig.6-A). Later, one of the two cells contacting with four other cells will differentiate into the oocyte. The other fifteen cells, denominated nurse cells, will polyploidy and produce several mRNAs and proteins that will be transferred into the oocyte (maternal contribution) (Fig.6-A) (Spradling 1993).

Simultaneous to the four mitosis in the germarium, the somatic stem cells (SCCs) divide and give rise to precursor follicle cells (PFC). Sixteen of the PFC stop division and become prepolar cells, which ultimately become polar cells and stalk cells (Laurel A. Raftery, 2008). Mesenquimal PFC divides a few more times and then encapsulate the egg chamber to form the follicular epithelium. This process involves the mesenquimal to epithelial transition of PFC, and the adhesion to germline cells is mediated by DE-cad (Margolis and Spradling 1995; Oda, Uemura et al. 1997).

It was proposed that Crb is required for the formation and maintenance of the follicular epithelium, whereas cadherin-catenin proteins are only required for maintenance. It was also shown that contact with the basement membrane during mesenquimal to epithelial transition was important to provides an initial polarization cue (Fig.6-B) (Tanentzapf, Smith et al. 2000).

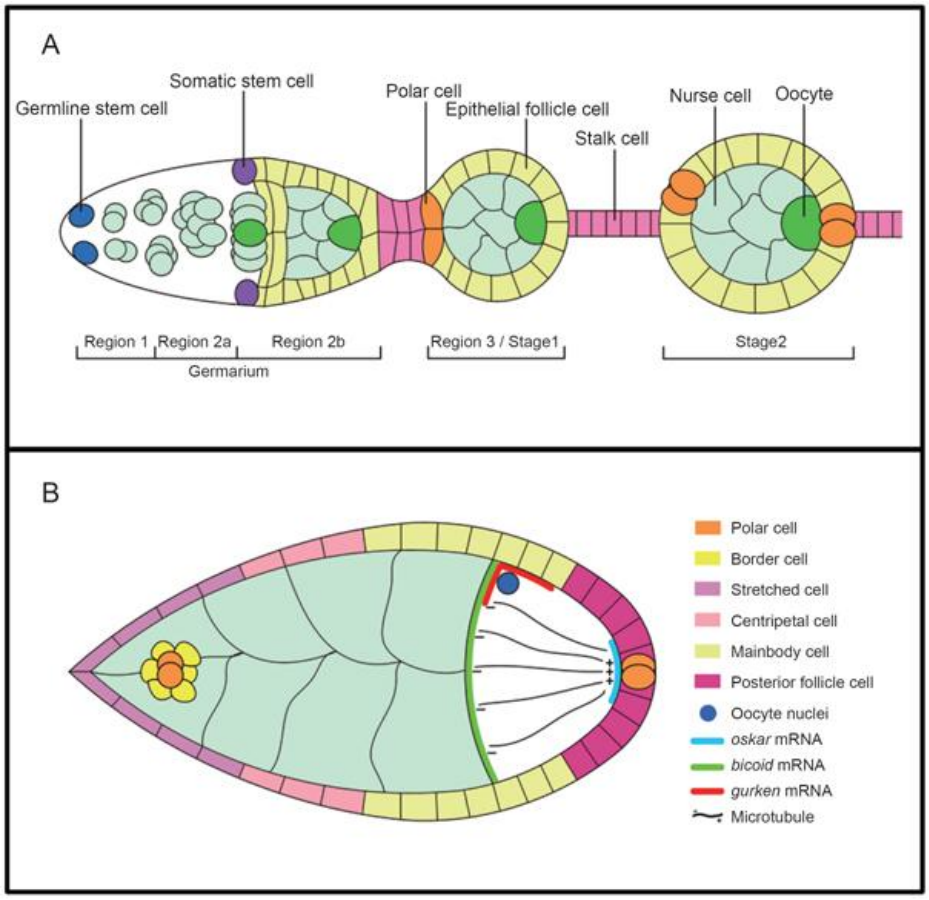

Fig.6 - *Drosophila* oogenesis stages. A: Germline stem cells (dark blue) undergo 4 mitotic divisions in the germarium region 1 and 2a (cystocytes in blue are the future nurse cells). One of the cystocytes becomes the oocyte (green). Somatic stem cells (purple) divide and give rise to epithelial the follicle cells (light green) that surround the nurse cells in region 2b. Some of the precursor follicle cells differentiate into polar cells (orange) and stalk cells (pink). **B:** The oocyte becomes polarized and polar cells migrate through the nurse cells with the border cells (yellow) to surround the Oocyte. Adapted from (Li, Xin et al. 2008).

5.2. Early Embryogenesis

After fertilization of the oocyte, the zygote nucleus undergoes several mitotic divisions without cytokinesis (syncitial divisions). The early divisions are sustained through the maternal contribution (mRNA and proteins produced by the nurse cells during oogenesis) and only from mid-late divisions are zygotic products transcribed actively by the embryo. After the fifth division, the nuclei begin to move to the periphery (stage 2), and at the end of divisions nine-ten (stage 3) the majority of the nuclei are evenly distributed around the embryo surface about, 35µm beneath the membrane. The nuclei

that still remain on the centre of the embryo will give rise to the yolk nuclei. During division nine, about five nuclei reach the posterior pole of the embryo and will be enclosed by membrane. This nuclei will form the pole cells, that later will give rise to the adult gametes (Campos-Ortega, J. A. and Hartenstein, V. 1985).

5.2.1. Cellularization of the blastoderm

At stage 4, the nuclei that reach the surface are surrounded by actin filaments and microtubules (Karr and Alberts 1986). The plasma membrane in the syncytial blastoderm already possesses a polarized organization, which is actin filament-dependent (Mavrakis, Rikhy et al. 2009). During divisions ten to thirteen, the syncytial nuclei at the periphery form the syncytial blastoderm, by a process known as cellularization. Cellularization starts at the interphase of cycle fourteen, with the plasma membrane surrounding the cortical syncytial nuclei (stage 5) (Foe and Alberts 1983). The nuclei elongate and furrow canals form between them, which are highly associated with the actomyosin contractile apparatus (Fig.7). Simultaneously, the actin filaments that are concentrated just below the plasma membrane and the microtubules form an inverted basket over the nuclei, with the centrosomal pair located apically. After this, the actin is delocalized from the apical membrane to the furrow canal, allowing its movement together with the adding of new membrane by vesicle trafficking. When the furrow canal reaches the basal end of the nuclei, the diameter of the actomyosin contractile apparatus starts to decrease and finally pinches off to form the blastoderm cells (Fig.7) (Mazumdar and Mazumdar 2002).

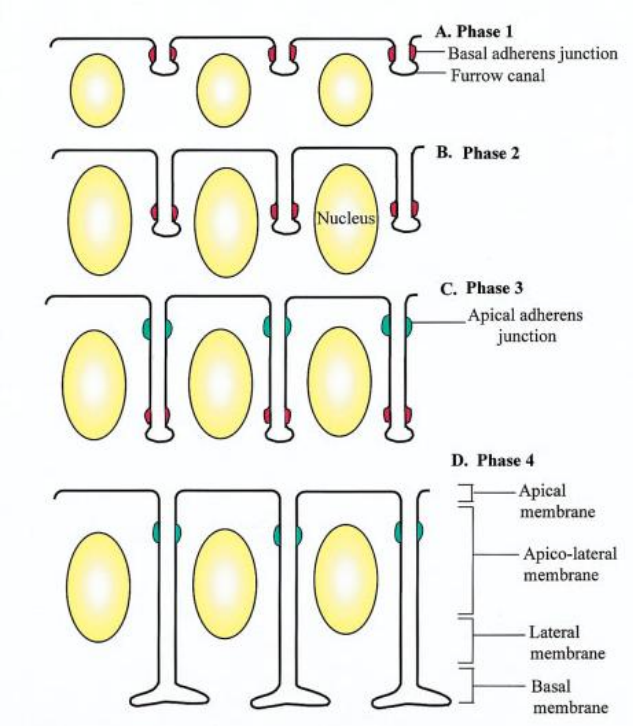

Fig. 7 – Blastoderm Cellularization. CelluIarization can be divided into four different phases. In phase 1 the furrow canal is formed and the basal adherens junctions attach just apical to it. During this phase the nucleus start to elongate. In phase 2 the nuclei complete their elongation. The furrow canals reach the basal end of the nuclei at phase 3. At the end of phase 4, when the maximum invagination of the furrow canals has occurred already four membrane domains are distinguished, as indicated. Adapted from (Mazumdar and Mazumdar 2002).

At the end of stage 5, almost three hours after the egg has been layed, the blastoderm monolayer is formed and polarization markers are properly found in the cells. Pole cells are located at the posterior pole and start to shift dorsally through elongation of germ band. At this stage the embryo is ready to start gastrulation.

5.3. Gastrulation and Segmentation

Stage 6 of development is when the major morphogenetic movements, like ventral and cephalic furrow formation, happen. Ventral furrow is an invagination process that gives rise to the mesoderm and to the anterior endodermal midgut primordium. Germ band elongation is another morphogenetic process occurring in the embryo at this time reaching its maximum at the end of stage 10. Germ band retraction returns the hindgut to its dorsal position at stage 12-13 of development. During this process the anterior and

posterior midgut fuse together and the yolk sac is pushed dorsally, with the amnioserosa cells covering it. Epidermal cells start then to spread from a lateral to a more dorsal position, a movement that is initiated by the leading edge cells, until both sides meet mid-way, closing the dorsal hole left in the embryo during the movements of germ band retraction and head involution. This process is called embryonic dorsal closure and occurs during stage 14. A similar process happens during the formation of the adult abdomen (Guerra, Postlethwait et al. 1973). Meanwhile these processes are occurring, patterning and segmentation of the embryonic body are being established, and organogenesis is also. Dorsal closure finishes at stage 15, together with the head involution and segmentation. After full embryonic development, the embryo hatch as first instar larvae (Campos-Ortega, J. A. and Hartenstein, V. 1985).

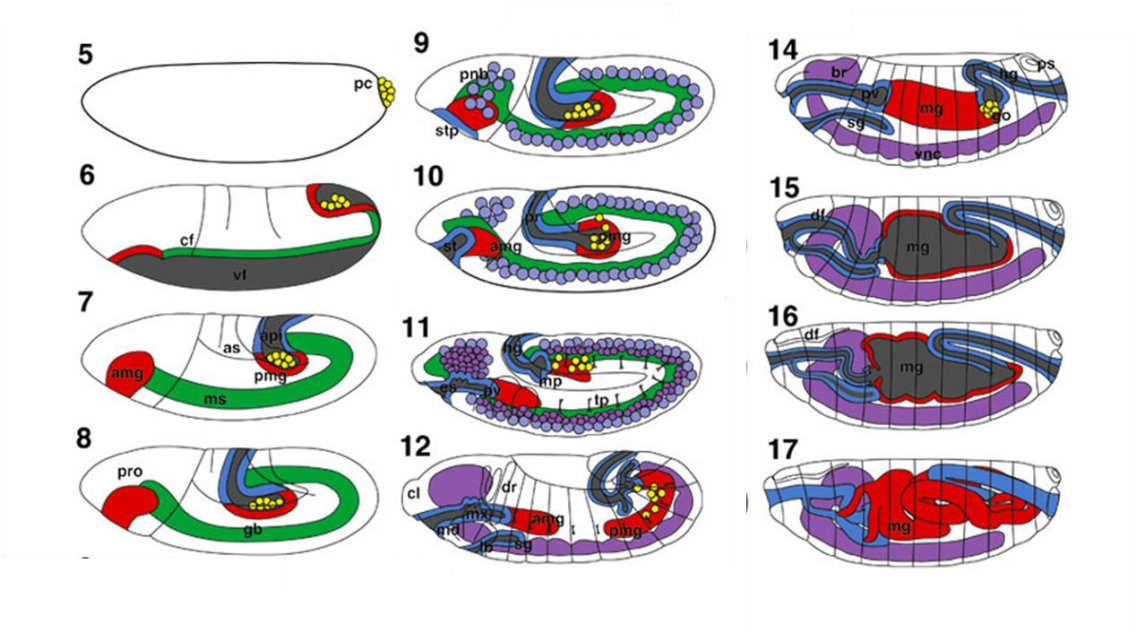

Fig. 8 – Embryogenesis stages – The numbers represent the developmental stages depicted in the corresponding scheme. Cellularization of the blastoderm and posterior pole cell (pc in yellow) localization are represented in stage 5. Gastrulation begins at stage 6 with the formation of the ventral furrow (vf in grey) and cephalic furrow (cf). Simultaneously germ band (gb) extension pushes the anterior part to the dorsal side and helps to form the posterior midgut (pmg in red). The mesoderm (ms in green) and anterior midgut (amg in red) are formed at stage 7. Procephalic neuroblasts (pnb in purple) start to form at stage 9 to give rise to the brain (br in purple) and ventral nerve (vnc in purple). Germ band retraction starts at stage 12, and at stage 14 *amg* and *pmg* are already fused, forming the midgut (mg in red). Dorsal closure is complete at stage 15. Adapted from Campos-Ortega, J. A. and Hartenstein, V. 985.