Methods

1. Mutants and alleles used in this work

The two novel aPKC alleles described in this work (*aPKC*^{TS} and *aPKC*^{PB1}) were generated in an EMS-induced mutagenesis maternal mutant collection for the 2R chromosome. The screen was designed to select lethal or sterile mutants with defects in blastoderm or embryonic cuticle formation, but with normal germ cell differentiation and localization. The mutagenesis was induced in OregonR (OR), with the following p-element insertion: P[FRT 42B; mini-w+, FRT 42B]. Lethal or sterile mutants were balanced with Curly of Oyster (CyO) balancer chromosome, and complementation analyses was performed between all established lines.

For several analyses were used embryos and adult flies that were aPKC^{TS}/DF6482 with the positive control DF6482/+. To obtain females aPKC^{TS}/DF6482, females of aPKC^{TS}/Cyo were crossed with males DF6482/Cyo, and F1 Females were selected for absence of Cyo balancer. Concerning to DF6482/+, females DF6482/Cyo were crossed with OR males and females were selected as previously case.

2. Germ Line Clones (GLC) generation

Since one of the novel aPKC alleles is maternally lethal, we used the FRT/ovo^{D} system to generate homozygous mutant embryos. This is referred to as germ line clones (GLC) (Chou e Perrimon, 1996)

Briefly, the generation of GLC implies the actin of the flipase (FLP, which induces mitotic recombination) on specific target sequences (FRT, flipase recombination targets). In this case, we used and heat-shock inducible flipase (HS-FLP) to control the timing of recombination. The ovo^{D} dominant mutation leads to defects during oogenesis, and to the degeneration of the ovarioles at around stage 7. Only the egg chambers that effectively underwent the process to mitotic recombination will lose the ovo^{D} mutation and will develop into embryos (Fig.9). This strategy allows for the selection of the maternal mutant egg chambers/embryos.

To generate aPKC GLC, we crossed FRT42B OVO^{D}/CyO males with HS-FLP22; If/CyO hshid females to obtain males bearing both the FLP and the FRT site: HS-FLP22;FRT42B OVO^{D}/CyO hshid. These males were then crossed with aPKC mutant virgins: w/w; FRT42B aPKC/CyO x HS-FLP22/;FRT42B OVO^{D}/CyO hshid. The F1 descendants from this cross were heat-shocked 1 hour at 37ºC induce the mitotic recombination for 1 hour, during late 2nd-3rd instar larvae. From this cross were selected the females that lost the CyO balancer: HS-FLP22/w; FRt42B OVO^{D}/ FRT42B aPKC and crossed with OR males. aPKC mutant embryos were then collected and processed accoding to the desired protocol.

All crosses were done in standard corn meal fly food and kept at 25ºC (except when otherwise indicated).

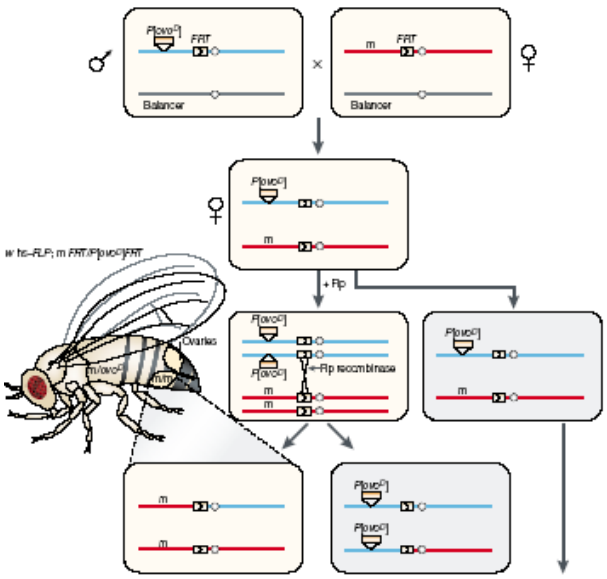

Fig.9 - FRT/ovo^{D} system – The ovo^{D} mutation is brought for the female (blue) and the desired mutation for the male (red). F1 females that are absent of balancer, will receive both ovo^{D} and mutation. Induced mitotic recombination by FLP22 at 37ºC, gives rise to homozygous mutant egg chambers that will develop into embryos. Whereas the ones that are homozygous for ovo^{D} or that does not recombined, does not develop due to the presence of dominant mutation ovo^{D}.

3. Embryo fixation and immunohistochemistry

The aPKC maternal mutants embryos were generated as above mentioned. Zero to seven hours collections were done in standard agar plates, supplemented with apple juice and live baker's yeast.

The embryos were collected and washed in ddH_2O with 0.1% Tween20, bleached during 5 minutes in 50% commercial bleach solution, and then washed 3 times during 5 minutes in ddH_2O.

The embryos were fixed in scintillation flasks with 4ml Heptane: 875µl PBS: 125µl of formaldehyde, during 30 minutes, with rotation (90-110rpm).

After fixation, the vitelinic membrane was removed from the embryos using a Heptane:Methanol solution (1:1). The embryos were vigorously shacked for 60 seconds. These embryos were collected and washed/De-hydrated with 3x 5min MeOH. After the last wash, the embryos were stored at -20ºC or immediately processed for immunostaining. For antibodies that required heat-fix protocol, embryos were submerse in a boiling saline solution (68nM $NaCl_2$ + 0,1% Triton X100) during 1 minute that was immediately cooled by adding the same volume of this solution at 4ºC.

For phaloidin staining, the vitelinic membrane was removed by hand in PBS, and processed immediately for immunostaining.

3.1 Immunostainning

The embryos were re-hydrated (PBST:MeOH, 3:7, 1:1 and 7:3) and washed in PBST, before the blocking ON (over-night) at 4ºC using PBT + 1% Bovine Serum Albumine (BSA) and 1% Donkey serum, with agitation.

1st antibodies were diluted in the same solution of blocking, and incubation was done ON at 4ºC with planar agitation. In table 1 are described the fixation and dilutions used. After incubation with the primary antibodies, the embryos were washed in PBST during 3 times 5 minutes and blocked for 1 hour at RT. The secondary antibodies were diluted (all 1:1000) in the same solution as blocking during and the embryos were incubated for 2 hours at RT or ON at 4ºC.

For DNA staining (using SYTOX®Green nucleic acid stain (Invitrogen S7020) 1:5000) RNAse (5µg/ml) was added to PBST and the incubation was done for 1 hour at RT with planar agitation.

Before mounting (DAKO Fluorescent Mounting Medium) the embryos were washed 3 times 5 minutes and PBS 5 minutes. The slides were stored in the dark, at 4ºC until processed.

Images were acquired using a Leica SP5 Confocal Microscope and processed using Adobe Photoshop CS4 and ImageJ 1.41o.

4. Mosaic ovary analysis

A similar technique to the GLC was used, but instead of using the ovo^D domineant mutation it was used a NLS-GFP signal for selection of clonal tissue: HS-FLP22; FRT42B ubi-nls-GFP/Cyo hshid x w ; FRT42B aPKC/ Cyo. The recombination was induced for 1 hour at 37ºC, 4 days after egg laying (late 2^{nd}- 3^{rd} instar larvae). The cross was maintained at 25ºC. The females were selected by the absence of the Cyo Balancer : HS-FLP22;FRT42B ubi-nls-GFP/ FR42B aPKC. 3 days old females were collected and leaved in a fresh corn meal tube supplemented with fresh yeast during 16 hours at 25ºC for Germarium analysis, or 2 days at 25ºC for egg chambers analysis. After, the ovaries were dissected and fixed for immunostaining (Harrison and Perrimon 1993)

5. Ovaries fixation and staining

The ovaries were dissected in PBS at RT. Fixation was done for 20 minutes at RT and with planar rotation in a solution containing (600µL of heptane, 20µL PBS 10x, 10µL NP-40 10%, 22µL formaldehyde 37% and 149µL ddH_2O). After 3 times 5 minutes washes with PBST2 (PBS + 0,2%Tween20), the ovaries were then separated and cleaned, to allow a more homogeneous incubation with the antibodies. Permeabilization of the ovarioles were done by incubation with PBST2 + 1%Triton-X100) during 1 hour at RT. Blocking was done with PBST2 + 1.0 % BSA for 1 hour at RT. The primary antibodies were diluted in PBST2 (see Table. 1) and incubated ON at 4ºC, with planar rotation. Then after 3 times 20 minutes washing PBST2, the secondary antibodies were diluted in PBST2 + 1% BSA (all 1:1000) and incubated 2 hours at RT or ON at 4ºC. Next, the ovaries were washed again three times during 20 minutes each before DNA staining or direct mounting.

DNA staining and sample mounting was as previously described for the embryos, except that the ovarioles and egg chambers were further separated before placing the couverslip. Image processing was the same as for the embryos.

Table1.- Dilution and type of fixation used for listed antibodies

Antibody	Host	Fixation**	Dilution	Origin
aPKC	Rabbit	Chemical fix.	1:2000	Santa Cruz Sc-216
Nrt	Mouse	Heat fix.	1:133	DSHB
Slam	Rabbit	Heat fix.	1:1000	Ruth Lehmann
pTyr	Mouse	Chemical fix.	1:1000	Cell Signaling #9411
Par3	Rabbit	Chemical fix.	1:1000	Andreas Wodarz lab
Par6	Rabbit	Chemical fix.	1:1000	-
Arm	Mouse	Chemical fix.	1:50	DSHB
DE-Cad	Rat	Chemical fix. in embryos + 1mMCa^{2+}	1:20	DSHB
p-Myosin	Rabbit	Chemical fix.	1:50	Cell Signaling #3671
aPKC	Rabbit	Western blot	1:1000	Santa Cruz Sc-216
GFP	mouse	Western blot	1:200	Roche 11814460001
Phaloidin-rhodamin	-	Chemical fix. + hand removal of the vitelinic membrane	1:200	Sigma

*Added to secondary antibody for the last 10 minutes incubation

**Fixation in embryos, ovaries were always fixed as above described.

6. Biochemistry

The crosses to obtain females Par6-GFP*Par6*$^{\Delta 226}$/+; aPKC^{TS}/DF6482 and Par6-GFP*Par6*$^{\Delta 226}$/+; DF6482/+ are described in Annex I.

6.1 Protein extracts preparation

Protein extractions were done from embryo collection with 0 to 7 hours, and collected as described above. The coreon was removed from the embryo as the fixation protocol. The embryos were used immediately or rinsed twice in 10mM TrisCl pH 7,5 solution, and frozen at -80ºC after removing the solution.

To obtain the protein extraction, embryos were crushed in 200-500µl Protein Lyses Buffer (50mM TrisCl pH 7,5 + 150mM NaCl + 2mM EDTA + 1 pill/10ml EDTA-free protease inhibitor + 1mM DTT, 10mM Sodium Floride) and centrifuged at 4ºC (14000 rpm during 5 minutes). The supernatant was transferred to a new eppendorf. This step was repeated twice to maximize pellet and lipid removal.

Protein concentration was measured in spectrophotometer at 595nm, using the BradFord procedure.

6.2 Immuno-Precipitation

0,5mg of protein was diluted in Protein Lyses Buffer to get equal volumes for all the samples. The samples were incubated for 2 hours at 4ºC with 1:1000 anti-GFP Rabbit antibody (Abcam ab6556). Protein A beads (Sigma P-3391) were washed twice with PBS and equilibrated with Protein Lyses Buffer three times. The beads were than incubated with each sample for 2 hours at 4ºC. After this incubation the beads washed seven times with Protein Lyses Buffer, with a centrifuge step between washes (10000rpm at 4ºC during 20 seconds) to pellet the beads.

6.3 Western blotting

To analyze the total amount of protein were used the protein extracts described above. The same volume of Sample Buffer Laemmeli 2x concentrate (Sigma S3431) was added to the samples, protein extract or beads concerning to the type of analysis, and then heated at 95ºC during 5 min. 8% SDS acrylamide/bis-acrylamide gels were prepare. To Phos-tag™ procedure, this component it was added in the referred concentrations (25 and 100µM) plus the same concentration of $MnCl_2$ to gels prepared as indicated above. Electrophoresis was started at 60V (stacking gel) and after it was switched to 100 V (resolving gel). The proteins were transferred to a nitrocellulose membrane (1 hour at 100V) (Biorad wet transfer system). The membranes were then washed 10 min in PBT and blocking was applied ON at 4ºC (PBT + 5% (w/w) of milk powder) with agitation. Primary antibodies were incubated over night at 4ºC in PBT containing 1% powder milk. Dilution for each antibody is on the Table 1. After the membranes were washed three times with PBT during 20 minutes each, before 1 hour blocking step. The membranes were then incubated with the secondary antibodies

(1:1000) in PBT, for 2 hours at room temperature. Finally 3 washes with PBT were made during 5 minutes each, and a last one with PBS. For detection of the samples, ECL solutions 1 and 2 were prepared, and used in 1:1. The membranes were incubated for 1 minute in this solution. The detection was done on Photographic films (Kodak film 853 2665) in the dark room. Revelation was performed in revelation solution, and films fixation in fixation solution. Several exposure times were tested for each sample to maximize analyze.