PATTERNING OF CHORION PROTEINS IN THE *DROSOPHILA* EGGSHELL

by

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A thesis submitted to the

Graduate School-Camden

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Biology

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May, 2011

ABSTRACT OF THE THESIS

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Oogenesis in *Drosophila melanogaster* is an established system to study how changes in tissue patterning affect eggshell morphology. During oogenesis, the mono-layer of epithelial cells surrounding the developing oocyte, called the follicle cells (FCs), is patterned and instructed to fold into the three dimensional (3D) structures of the eggshell. Eggshells display great morphological diversity among *Drosophila* species. Specifically, a structure reports unique to the eggshell of Hawaiian *Drosophila* species, the dorsal ridge, was found in the eggshells of numerous species from the subgenus *Sophophora*. The eggshell of *D. melanogaster* consists of two major protein families; the vitelline membrane (Vm) and Chorion proteins (Cp). In my work, I focused on the Cp family and three *Drosophila* species; one without a dorsal ridge (*D. melanogaster*) and two with a dorsal ridge (*D. willistoni* and *D. nebulosa*). The Cp genes are expressed in the FCs of *D. melanogaster*, so I reasoned that the spatial changes in Cp genes' patterning should reflect the presence or absence of a dorsal ridge. I performed a screen for the full dynamics of nine members of the Cp genes. I found three major categories;

the first are expressed in the dorsal ridge, the second are absent from the dorsal ridge, and the last are uniformly expressed. The formation of dorsal structures on the eggshell of *D. melanogaster* is governed by the epidermal growth factor receptor (EGFR). The ligand for EGFR is the transforming growth factor (TGFα) like Gurken (GRK), which is localized to the oocyte's nucleus, and activates EGFR in the FCs. Using colchicine, I mislocalized the nucleus, which in turn, abolished the patterns of Cp genes and the dorsal ridge. Together with genetic perturbation of EGFR signaling in *D. melanogaster*, I conclude that expression of Cp genes is regulated by EGFR signaling, and the patterning of these genes reflects the interspecies diversity of the dorsal ridge.

ACKNOWLEDGEMENT

I would like to first thank Nir Yakoby for providing me with the opportunity to work in his lab. His work ethic and leadership through example will always be a standard I will try to hold myself to. The difficult yet rewarding nature of working in a fly lab has taught me an invaluable lesson in working towards my goals.

I would also like to take this opportunity to thank the many members of the Yakoby lab (past and present), Cui Tiange, Matthew Niepielko, Robert Marmion, David Lemon, David Luor, Chelsea Ray, Miranda Converse, Andrew Roman, Joanli Tineo, Justin Torres, and Khai Tran. They all helped me tremendously with my project, and I am grateful that I had the opportunity to be fellow lab mates with them.

I would like to thank my loving girlfriend Jennifer as well. She was always there to support my goals, and give me motivation to better myself by believing in me.

Finally, I would like to thank my mother and father for supporting me through all of these years and continuously encouraging me to complete my higher education. I will always remember how valuable an opportunity can be when providing them to my own family one day.

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Introduction

Oogenesis in *Drosophila melanogaster* is a model system to study tissue patterning and morphogenesis (Horn-Badovinac and Bilder, 2005; Berg, 2005). Oogenesis consists of 14 developmental stages (Fig. 1) that are based on distinct morphologies of the egg chambers(King et al., 1970; Spradling et al., 1993). The ovary of each female has 14-16 ovarioles, all of which have multiple stages of developing egg chambers (Cavaliere et al., 2008). When the egg chamber reaches the final stage of oogenesis, stage 14, it is ready to be fertilized and laid.

The ovariole can be divided into two parts: the vitellarium and the germarium. In the germarium, each germline stem cell divides, producing a cystoblast that experiences four mitotic divisions; the end result comprises 16 cystocytes. One of the cystocytes eventually develops into the oocyte, whereas the rest develop into nurse cells (Cavaliere, et al., 2008). Nearby, the somatic stem cells eventually develop into a part of the egg chamber known as the follicle cells (FCs), a mono layer of epithelial cells engulfing the cystocytes. The FCs undergo intensive gene patterning before folding into the three-dimensional structure of the eggshell(Hinton, 1993).

Numerous signaling pathways govern FCs patterning including Notch, bone morphogenic protein (BMP), epidermal growth factor receptor (EGFR), and transforming growth factor (Tgf-β) (Assa-Kunik et al., 2007; Wu et al., 2008; Peri et al., 2000; Ward et al., 2006). One of the main signaling pathways

involved in FC patterning is the epidermal growth factor receptor (EGFR) (Nilson and Schupbach, 1999). Recently, a correlation between the EGFR activation and the formation of the dorsal ridge domain was found (Niepielko, Yakoby, unpublished). EGFR activation is regulated by a TFG-α like ligand Gurken (grk) (Neuman-Silberberg and Shupbach, 1996). The grk RNA is localized to the oocyte's nucleus. Under normal circumstances, the nucleus migrates from the posterior end of the oocyte to the dorsal anterior end during oogenesis (Fig. 2). Nuclear migration determines sequencially the anterior-posterior and dorsal-ventral polarity of the egg (Neuman-Silberberg and Schupbach, 1996; Berg et al., 2005).

EGFR activation is at the beginning of a signaling pathway, where downstream target genes are activated as well, resulting in the regulation of additional target genes that create the patterning to construct the dorsal ridge. In the case of the EGFR signaling, a dorsal-ventral polarity is observed (Yakoby et al., 2008, peri et al., 2000). Another important signaling pathway is called the Bone Morphogenic Protein (BMP) pathway. The TGF-β family member, Decapentaplegic (Dpp) is a morphogen that is required for BMP activation. It is expressed in an anterior-posterior gradient in the centripetal cells and nurse cell associated follicle cells at stage 10(Wu, 1997). Gene expression is governed by multiple overlapping pathways and results in complex patterns that are reflected by the morphologies.

A multi-layer structure of the eggshell develops during the last 5-6 hours of *D. melanogaster* oogenesis. This outer layer of the eggshell, formed by the

inward folding of the FCs, is called the chorion. Structurally, the chorion consists of four layers; the Exochorion (Ex), the Endochorion (En), the Inner Chorion Layer (ICL), and the Vitelline membrane (VM) (Cavaliere et al., 2008) (Fig 3). This structure is responsible for protecting the developing embryo (Cavaliere et al., 2008). The FCs secrete Cps between stages 9 and 14 of oogenesis (Table. 1) (Cavaliere et al., 2008). The Cp genes having a variety of expression patterns is an example of tissue patterning, which is the result of signaling pathways. Although preliminary experiments have been performed indicating when exactly some of the Cps are expressed (Parks, 1987), the images of the results are unclear. There are two families of gene expression that contribute to the chorion formation; the Vitelline membrane genes, and the chorion protein genes (Table. 1). Here, I take a second look at the entire family (9 genes) of annotated Cps. Since Cp proteins participate in eggshell formation, I hypothesized that the pattern of Cp genes would be reflect the absence or presence of a dorsal ridge.

Morphological diversity can be seen in many structures spread across animals. In the example of the finches that were studied by Darwin, a wide variety in the morphology of the beaks amongst the many different species of finches is found. The variation reflects the result of adaptation to different ecological niches; the signaling of the Bmp4 morphogen differs for different variants of the beaks (Abzhanov et al., 2004). In the same way, there is morphological diversity in the eggshells of *Drosophila* (Fig. 4). In a recent screen for new structures on the *Drosophila* eggshell, we found a lumen-like

structure, the dorsal ridge in numerous fly species. This structure was reported to be unique to the eggshells of the Hawaiian *Drosophila* species (Figs. 5); however, it was only studied at the morphological level (Margaritis et al., 1983).

Because this experiment produced a large number of results, I used a system of primitives for labeling the expression patterns (Fig. 6 A). The patterns could be divided into three categories: Expressed in the dorsal ridge domain, excluded from the dorsal ridge domain, and expressed uniformly. However, due to the complexity of the nature of patterning, additional Boolean operators were implemented to accurately describe patterns that had mixed primitives (Fig. 6B).

I found that the Cp genes are expressed dynamically during egg development. Several of the genes reflect the presence of a dorsal ridge by being expressed or repressed in the future ridge domain. The disruptions of nuclear localization have successfully disrupted the dorsal ridge formation and Cp patterning. This was confirmed in *D. melanogaster* by genetic perturbations. I would like to emphasize that while I discuss the dorsal ridge structure, the main focus of my thesis is the patterns of Cp in different species, and I use the dorsal ridge as a structure to learn if modifications in gene patterning are unique to the future dorsal ridge domain.

Materials and Methods

Fly species: The following *Drosophila* species were used in this study: *D. melanogaster (wild-type OreR), D nebulosa, D willistoni* (The San Diego Stock Center). Additional flies were CY2-Gal4, E4-Gal4, UAS- λtop (Queenan et al., 1997), and UAS-dnEGFR (Peri at al 2000).

Cp cloning: A partial region of each of the Cp genes were amplified from the cDNAs of *D. melanogaster* and *D. willistoni*. The primers (Table. 2) were designed using a Primer 3(http://frodo.wi.mit.edu/primer3/) based on the sequences of Cp genes which were available at flybase (http://www.flybase.org/). Polymerase Chain Reaction (PCR) was performed using the MJ Mini (BioRad) thermocycler using the StrataClone Cloning kit (Stratagene). Afterwards, plasmids were extracted with a QIAprep spin Miniprep Kit (Qiagen). Finally, each gene was sent to sequencing (GeneWiz). Sequences were blasted against the known sequence in the corresponding species on FlyBase.

***In situ* hybridization**: The *in situ* hybridization was performed as previously described (Wang et al., 2006, Yakoby et al., 2008) with the exclusion of the RNase digestion step. *Drosophila* ovaries were dissected in Grace's media and dehydrated using 33%, 66%, and 100% concentrations of methanol. The ovaries then were washed with a 0.2% Tween-20 solution in PBS. This was followed by three RIPA washes to permeabilize the ovaries. They were then

post-fixed by a wash of 0.2% glutaraldehyde and 4% formaldehyde. The ovaries were then prehybridized for three hours in a pre-hybridization solution and immediately put into hybridization solution for an overnight hybridization at 65 degrees Celsius with probe dilutions that varied from 1:5 to 1:10 in hybridization buffer. Ovaries were then blocked for 1 hour using 1% BSA solution. The ovaries were developed with 1ml of alkaline phosphate buffer with 6.6 µl NBT and 3.3 µl BCIP. Since *D. nebulosa* is not sequenced, we used the genes cloned from a closely related species, *D. willistoni*.

Colchicine treatment: The colchicine treatment was performed as previously described (Koch et al, 1983), with the exception of exposing the flies to the colchicine for two days at a time. In addition, the flies were fed colchicine treated food (yeast mixed with 10 mg/mL concentration of colchicine) on top of agar media. The success of the treatment was initially evaluated by deformation of eggshell structures.

Genetic Tools: The EGFR signaling was disrupted using the GAL4/UAS system to express the CP in specific domains (Duffy, 2001) (Fig. 7). Virgin females from a GAL4 driver stock, and the males from stocks that have specific genes of interest that were crossed. The resulting changes in Cp patterning was monitored under light microscopy. The UAS-dnEGFR driven by the uniform driver CY2-GAL4 repressed EGFR activation in all the oocyte associated FCs.

Results

Cp expression shows diversity across the different species

We screened three species of *Drosophila* for 9 different Cp genes in the FCs. The specific stages of oogenesis that were looked at were stage 9 through 13. Based on the resulting patterns, there were three patterning groups established. The groups represented expression in the dorsal ridge domain, expression that was excluded from the dorsal ridge domain, and uniform expression (Fig. 6).

Expression patterns in the dorsal ridge (Cp 7fa, Cp 16, Cp 19)

Cp 7fa was expressed uniformly excluding the dorsal cells as well as floor cell expression in *D. melanogaster* during stages 11 and 12. *D. nebulosa* showed midline expression beginning at stage 12. *D. willistoni* also showed midline expression at stage 12 (Fig. 8A).

Cp 16 was expressed in an anterior band in *D. melanogaster* starting at stage 10B and continued until stage 12. *D. nebulosa* on the other hand, showed anterior expression as well dorsal expression without roof starting at stage 10B that extended completely to the posterior end by stage 12. *D. willistoni* showed midline and posterior expression starting at stage 11 and continued to stage 12(Fig 8B).

Cp 19 was expressed in the anterior and roof cells as early as stage 10B in *D. melanogaster*. However, by stage 11, uniform expression could be seen that continued to stage 12. In *D. nebulosa*, there was dorsal and anterior

expression starting at stage 10B. At stage 11, there was uniform expression with anterior expression as well as dorsal expression without roof and posterior cell expression. This continued to stage 12. *D. willistoni* also showed dorsal and anterior expression starting at stage 10B. At stage 11, there was uniform expression with anterior expression as well as dorsal expression without roof and posterior cell expression. This continued to stage 12, however the midline did not extend as far as the one in *D. nebulosa*(Fig 8C).

Expression patterns excluded from the dorsal ridge domain (Cp 7fc, Cp 36, Cp 38)

Cp 7fc was expressed in the roof cells in *D. melanogaster* starting at stage 10B. This expression spread to the anterior and floor cells by stage 11 and continued to stage 12. In *D. nebulosa*, there was uniform expression without midline and roof cell expression starting at stage 11 and continued to stage 12. In *D. willistoni*, there was uniform expression without midline and roof cell expression starting from stage 10b that continued to stage 12 (Fig 9A).

Cp 36 was expressed uniformly with no midline expression in *D. melanogaster* beginning at stage 10B and the expression continued to stage 12 when it changed into uniform expression. In contrast, *D. nebulosa* showed a uniform expression starting at stage 10A, and added roof cell expression at stage 10B. This returned to uniform expression up to stage 12. *D willistoni* showed roof cell expression at stage 10B, and became uniformly expressed

with no midline at stage 11. By stage 12, it became uniform expression (Fig. 9B).

Cp 38 was expressed in the anterior and roof cells in *D. melanogaster* starting at stage 10B. At stage 11, posterior expression was added. By stage 12, it became uniform expression except midline. *D. nebulosa* showed roof cell expression starting at stage 10B, but by stage 11 displays dorsal expression with no floor and anterior expression. By stage 12, uniform expression was seen. *D. willistoni* shows roof cell expression at stage 10B and becane uniformly expressed with no midline at stage 11. At stage 12, it became uniformly expressed (Fig. 9C).

Expression patterns that were uniformly expressed (Cp 7fb, Cp 15, Cp 18)

Cp 7fb was expressed uniformly in *D. melanogaster* starting at stage 10A and became uniform with roof and floor cell expression starting at stage 10B. This continued to stage 12. In *D. nebulosa*, uniform expression was seen beginning at stage 10B and continued until stage 12. In *D. willistoni* uniform expression was seen at stage 10B and continued until stage 12 (Fig. 10A).

Cp 15 showed uniform expression in *D. melanogaster* starting at stage 10B and became uniform with anterior expression at stage 11. This continued to stage 12. *D nebulosa* showed uniform expression beginning at stage 11, and continued until stage 12. *D. willistoni* showed uniform expression beginning at stage 12 (Fig. 10B).

Cp 18 showed uniform expression in *D. melanogaster* starting in stage 9 and continued until stage 12. *D nebulosa* showed uniform expression beginning at stage 9 and continued until stage 12. *D willistoni* showed uniform expression beginning at stage 9 and continued until stage 12 (Fig. 10C).

Cp expression patterns are lost upon colchicine treatment

Colchicine is known to depolymerize the microtubules which are responsible for the position of the nucleus in the oocyte (Koch, 2008). As a result of this disruption, the morphogen GRK is also mislocalized, that in turn mislocalizes EGFR activation and disrupts normal Cp expression.

When comparing the wild-type egg chambers to the colchicine treated egg chambers, a trend is observed. Wild-type anterior expression typically leads to a strong expression in the form of a posterior cap for the most disrupted eggshells. Dorsal ridge formation does not occur in the disrupted eggshells, and thus is reflected in the Cp expression patterns.

Cp 7fa showed a posterior cap forming in *D. melanogaster* (Fig. 11A, B) and *D. nebulosa* (Fig. 11C, D). For *D. willistoni,* the anterior pattern was preserved but the midline expression shifted into a band that migrated towards the posterior (Fig. 11E, F).

Cp 16 showed the anterior band being preserved for *D. melanogaster* (Fig. 12A, B), *D. nebulosa* (Fig. 12C, D), and *D. willistoni* (Fig. 12E, F). *D. nebulosa* and *D. willistoni* had their midlines disrupted but a posterior cap formed for *D. nebulosa*.

Cp19 showed a posterior cap forming for *D. melanogaster*. The uniform, anterior, and roof cell expression was perturbed (Fig. 13A, B). *D. nebulosa* retained anterior expression but also formed a posterior cap (Fig. 13C, D). *D. willistoni* lost its anterior expression but formed a posterior cap (Fig. 13E, F).

Cp 7fc lost its anterior, roof and floor cell expression and formed a posterior cap for *D. melanogaster* (Fig. 14A, B). *D. Nebulosa* lost its uniform and roof cell expression but showed uniform expression afterwards (Fig. 14C, D). *D. willistoni* showed a shift towards posterior expression, with no anterior pattern preserved (Fig. 14E, F).

Cp 36 lost its uniform expression and formed a posterior cap for *D. melanogaster* (Fig. 15A, B). *D. nebulosa* (Fig. 15C, D) and *D. willistoni* (Fig. 15E, F) showed disrupted patterns but showed no expression and uniform expression respectively.

Cp 38 showed uniform expression, and lost anterior and roof expression for *D. melanogaster* (Fig. 16A, B). *D. nebulosa* lost its dorsal and anterior expression and formed a posterior cap (Fig. 16C, D). *D. willistoni* showed a unique disruption where roof and floor cell expression became an anterior band (Fig. 16E, F).

Cp 7fb showed a posterior cap for *D. melanogaster* (Fig. 17A, B). *D. nebulosa* had no observable change. (Fig. 17C, D) *D. willistoni* showed a retention of anterior expression as well as a posterior cap (Fig. 17E, F).

Genetic perturbation using the GAL4/UAS system leads to disrupted Cp patterning

The UAS-dnEGFR driven by the uniform driver CY2-GAL4 showed considerably disrupted expression compared to the wild type. Cp 7fa lost its floor cell expression and showed only anterior expression (Fig. 18 A, B). Cp 16 retained its anterior band (Fig. 18C, D). Cp 19 lost its anterior and roof cell expression and only showed slight anterior expression (Fig. 18E, F). Cp 7fc lost its roof and floor cell expression and showed a strong anterior pattern (Fig. 18G, H). Cp 36 lost its uniform expression and became anterior and posterior expression (Fig. 18I, J). Cp 38 lost its roof and posterior expression and only retained anterior expression (Fig. 18K, L). Cp 7fb lost its roof and floor cell expression and had slight anterior expression (Figs. 18M, N).

Discussion

The pattern of Cp genes is dynamic

The results of the in-situ hybridization of all species were examined and annotated according to the known primitives and Boolean operators. The expression patterns of the Cp genes are dynamic, and several trends can be seen in the results. Most of the patterns begin uniformly expressed and at stage 10B begin their unique pattern.

When compared to the published literature (Yakoby et al, 2008), certain differences can be seen. For Cp 38, I discovered more anterior expression in stage 10B in addition to the roof cell expression which was published. In stage 12, I show that there is a midline clearing in addition to the uniform expression which was published.

The expression in Cp 7fc is in agreement with the published results, except for stage 11, where I show that there is also anterior expression. The expression in Cp 15 and Cp 7fb is in agreement with the published results (Table. 1) The results also are in line with the timing of the expression that was shown in a previous study, with the exception of Cp 15 and Cp 18. In my results, Cp 15 shows expression as early as stage 10B, but in the table was said to only be expressed at stage 13. Cp 18 shows expression as early as stage 9, whereas in the table it was listed as being expressed beginning at stage 11.

Anterior patterns for *D. melanogaster* for Cp genes have a correlation with midline expression in *D. nebulosa* and *D. willistoni.*

For the three genes, Cp 7fa, Cp 16, and Cp 19, a trend can be observed between the species with a dorsal ridge and the species without. *D. melanogaster* displays no midline expression for any of the Cp genes, however for the stages that have midline expression in *D. nebulosa* and *D. willistoni*, it has a strong anterior pattern (floor cell expression in Cp 7fa). For Cp 19, not only do the species with dorsal ridges express a midline, they also show floor and roof cell expression which is also expressed in *D. melanogaster*. This suggests a potential trend that can be used to predict the difference in expression of genes between these species if present in *D. melanogaster* in the anterior end.

The pattern of Cp genes is diverse between species

The eggshells of *D. melanogaster*, *D. nebulosa* and *D. willistoni* can be separated into three different categories. *D. melanogaster* has no dorsal ridge present in its eggshell. *D. nebulosa* has a long dorsal ridge that goes to the posterior end. And *D. willistoni* has a shorter dorsal ridge that only extends partially to the posterior end. When comparing the Cp expression between *D. melanogaster* and *D. nebulosa* and *D. willistoni*, trends are visible that indicate a correlation with the presence (and lack of) a dorsal ridge. More specifically, Cp 7fa, Cp 16, and Cp 19 are all expressed in the dorsal ridge domain beginning as early as stage 10B in *D. nebulosa* and *D. willistoni*. When compared to *D. melanogaster*, none of the three Cp genes show midline expression, but instead either floor cell expression or an anterior band. This suggests that changes in Cp expression change causes altercations in the

composition of the chorion. One interesting point, is that when a Cp gene is expressed strongly in the anterior for *D. melanogaster*, the species with dorsal ridges display expression in the midline.

The pattern of Cp genes is diverse between species with a dorsal ridge

Numerous Cp genes that are expressed in the future dorsal ridge domain show diversity between species, and these reflect the final dorsal ridge structure. For example, Cp 7fa in *D. nebulosa* has midline expression that extends all the way to the posterior end, whereas *D. willistoni* has midline expression that stops midway through. A similar pattern is found in Cp 16 in *D. nebulosa.* It shows a midline extending all the way again, whereas *D. willistoni* only has a slight midline extension. In *Cp 19,* the same trend continues where *D. nebulosa* has a completely extending dorsal ridge, and *D. willistoni* shows a partially extending dorsal ridge. These patterns suggest that they reflect the morphology of the final ridge.

Disruption of Grk localization modifies Cp gene expression

The ligand GRK is localized to the oocyte nucleus. It is responsible for the formation of the gradient of EGFR activation in the FCs beginning at stage 8 of oogenesis (Wu et al., 1997). At stage 8, the nucleus migrates from the posterior end of the egg chamber to the anterior end. This migration is facilitated by the actions of microtubules (Koch et al., 2008). When the nuclear localization as well as the GRK localization was disrupted via colchicine, a change could be seen in all three species when compared to the wild type. These changes have two categories: patterns turning into a

posterior cap, and patterns retaining an anterior expression while also generating a posterior cap. The use of colchicine leaves the nucleus in a posterior position, thus creating a posterior cap like pattern. This suggests that these genes are regulated by EGFR activation. When the existing pattern becomes a posterior cap, it suggests that the GRK only activated EGFR around where the nucleus remained and because the pattern was dependent on EGFR, became disrupted in that manner.

More than one signaling pathway controls Cp gene patterning

The patterns that retained an anterior band as well as a posterior cap imply that EGFR was not the only factor regulates that expression. The perturbations using colchicine disrupted the ligand from properly activating EGFR, and the genetic perturbations suppressed the EGFR receptors in the follicle cells. Any remaining patterns, especially in the anterior end, can be attributed to the presence of another signaling pathway. It is possible that BMP activation was responsible, due to its anterior/posterior gradient. When the patterns were disrupted, the only polarity that remained was in the anterior/posterior axis which further supports this theory.

Genetic perturbations that disrupt EGFR signaling disrupt patterning.

In the egg chambers of the genetically perturbed flies (CY2>dnEGFR), EGFR activation is uniformly repressed. By looking at the resulting patterns, it can be seen that all patterns except for Cp 16 have been disrupted completely. The patterns for Cp 16 and Cp 7fc retained their anterior expression, whereas the rest of the Cp genes showed no expression. This

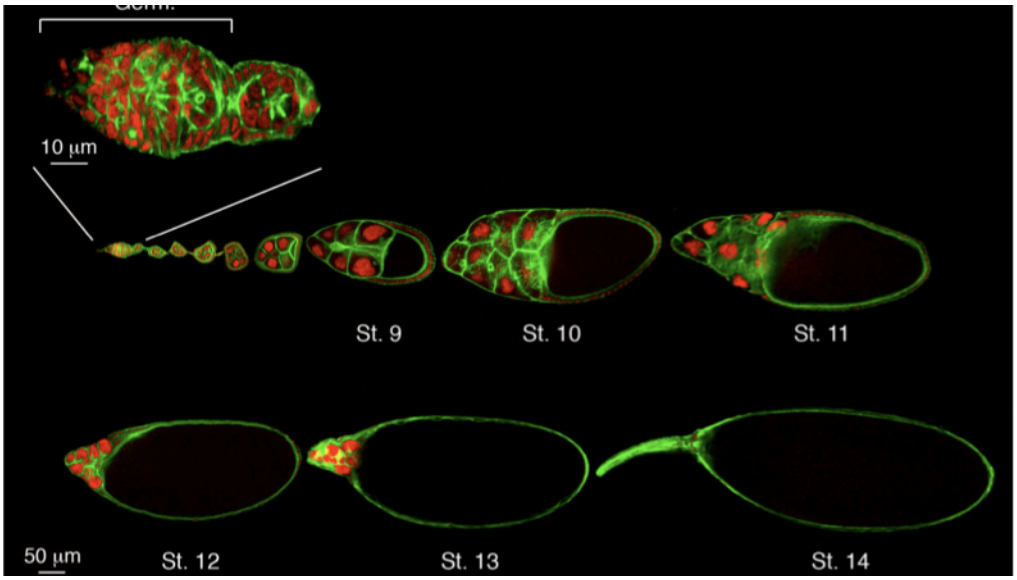
further emphasizes the hypothesis that the Cps are regulated by EGFR activation. The two genes that were not disrupted fully show that it is possible the BMP pathway also regulates part of the expression.

Summary

I explored the dynamic expression of a family of Cp genes and the involvement of EGFR signaling in their regulation. These patterns are complex, and analysis of the patterns suggests that other signaling pathways are involved in their regulation. For example, anterior expression patterns are considered to be targets of BMP signaling, thus it will be interesting to look at the effect of perturbations in BMP signaling on Cp genes patterning. An important aspect of this work is the discovery of genes that are expressed or repressed in the future dorsal ridge domain. To determine the action of these genes in chorion formation, I propose to disrupt them in all species, and monitor the changes in eggshell formation.

Figures

Figure 1



(Cavaliere et al. 2008)

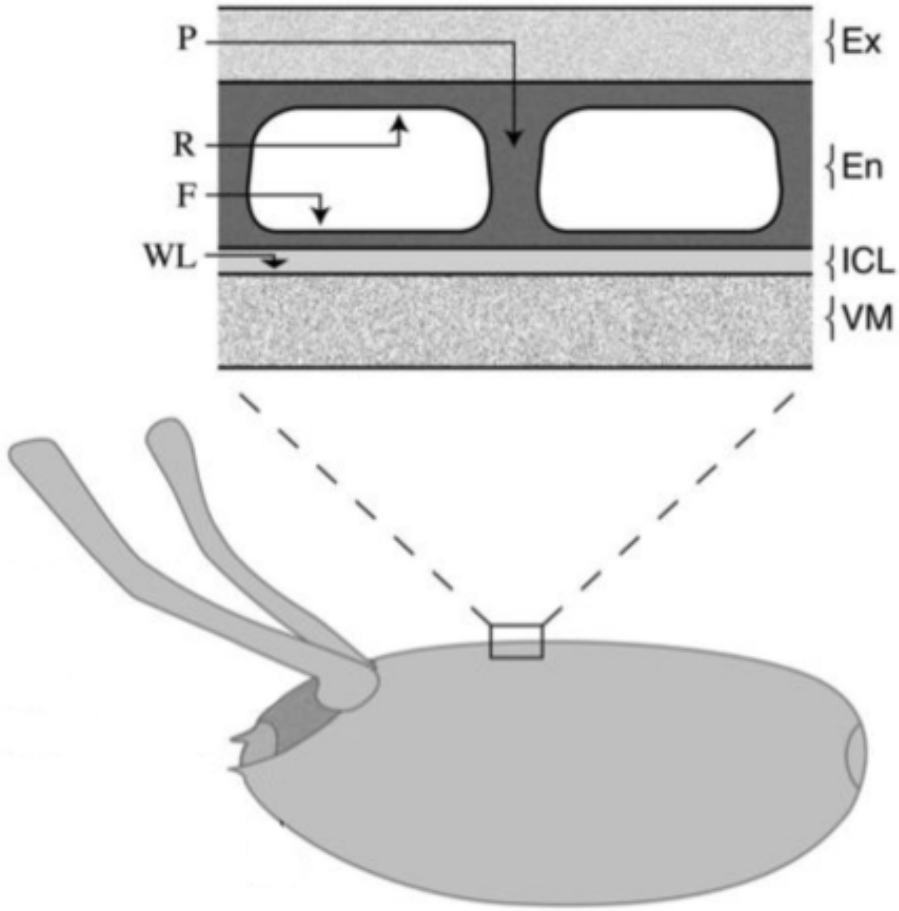
Figure 1: Oogenesis in *D.* melanogaster. Oogenesis consists of 14 distinct morphologies of the egg chamber. Stages 9 to 14 are marked. Anterior to the left.

Figure 2

(Van Buskirk and Schupbach, 1999)

Figure 2: A diagram showing the position of the oocyte nucleus during *D. melanogaster* oogenesis. The colored follicle cells (FCs) represent the EGFR activation.

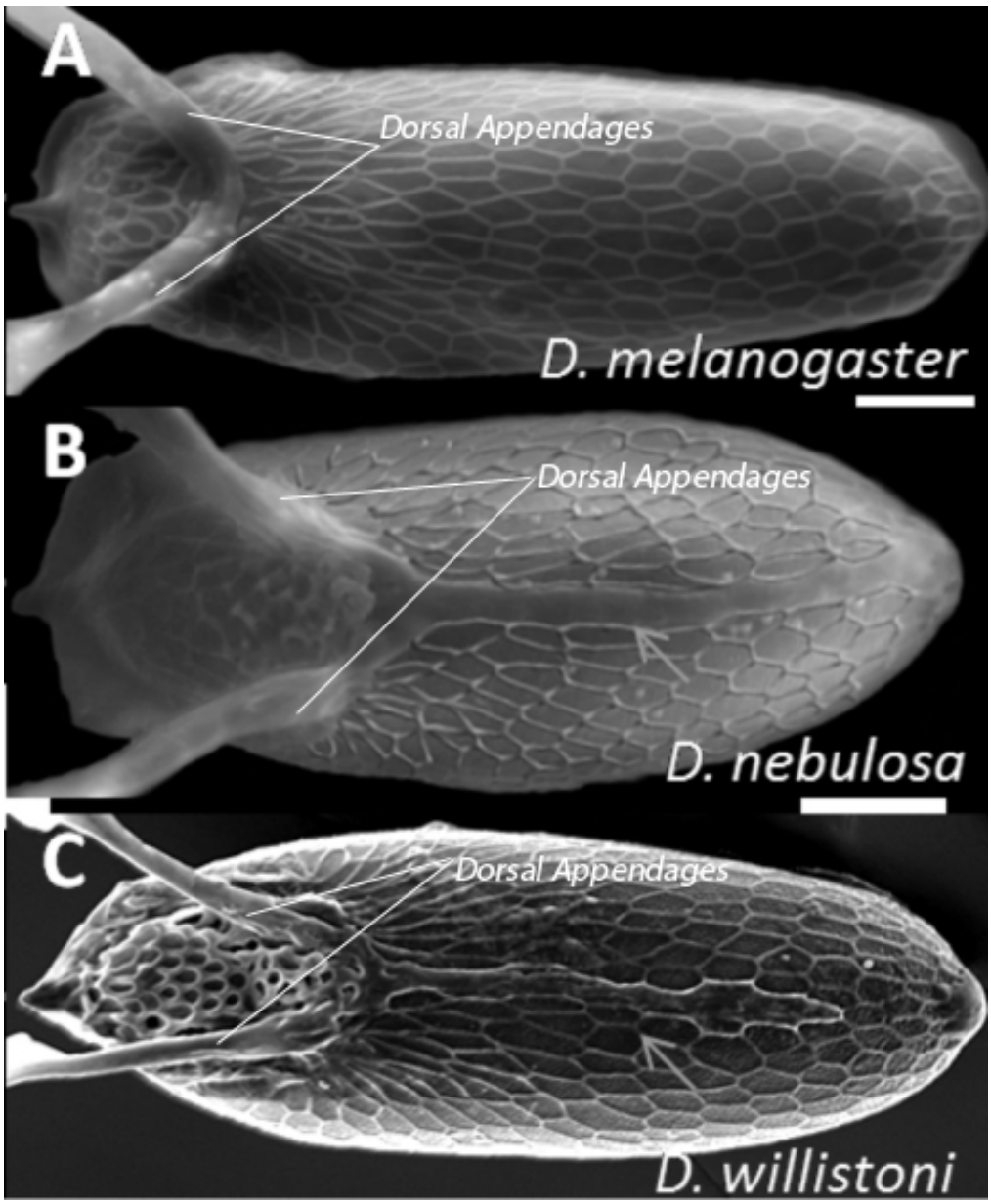
Figure 3



(Cavaliere et al., 2008)

Figure 3: Schematics of the chorion structure in a *Drosophila* eggshell. (Ex) Exochorion. (En) Endochorion. (ICL) Inner chorion layer. (VM) Vitelline membrane. Additional structures: (P) Vertical pillar. (R) Outer roof. (F) Thin fenestrated floor. (WL) Wax layer.

Figure 4



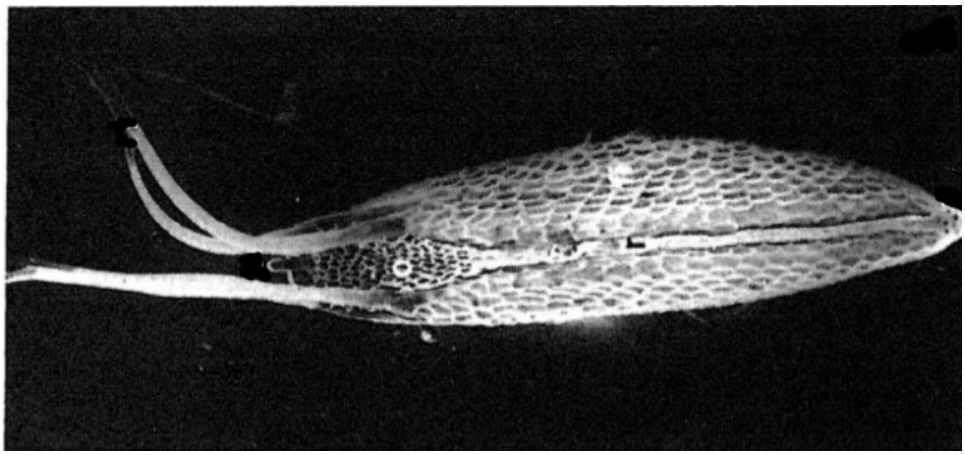
(M. Niepielko, unpublished)

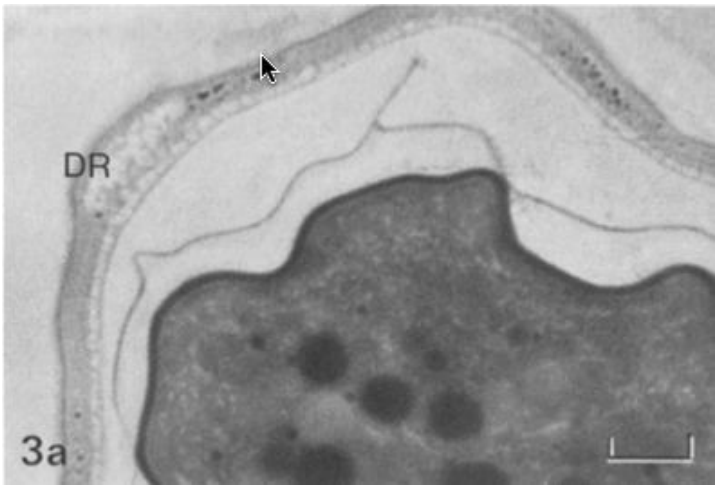
Figure 4: Micrograph of *Drosophila* eggshells. (A) *D.* melanogaster. (B) *D.* nebulosa with the dorsal ridge (arrow) extending completely to the posterior end. (C) *D. willistoni* with the dorsal ridge (arrow) shown to extend only

partially to the posterior end. All images are anterior to the left and a dorsal view.

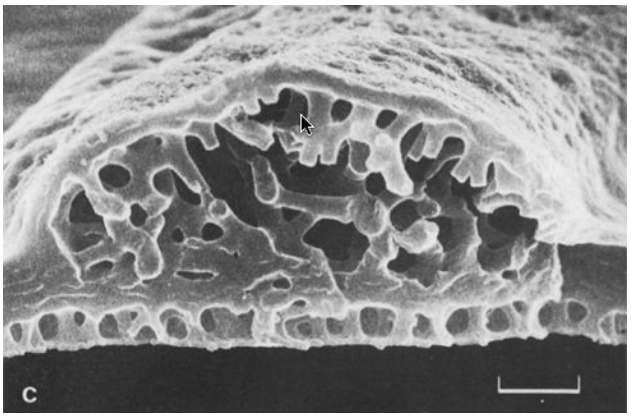
Figure 5

A





B



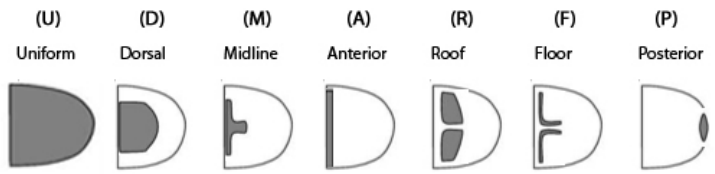
C

Figure 5: The dorsal ridge and its cross sections in *D.* grimshawi. (A) A Dorsal view of the mature eggshell of *D.* grimshawi. "o": operculum, "r":

dorsal ridge (B) A cross section (light microscope) of the eggshell of *D.* grimshawi. The dorsal ridge (DR) is porous. (C) A cross section (SEM) of the dorsal ridge in *D.* grimshawi. (Margaritis et al, 1983)

Figure 6

A:



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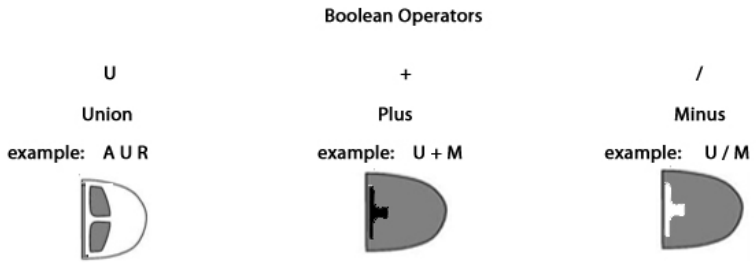
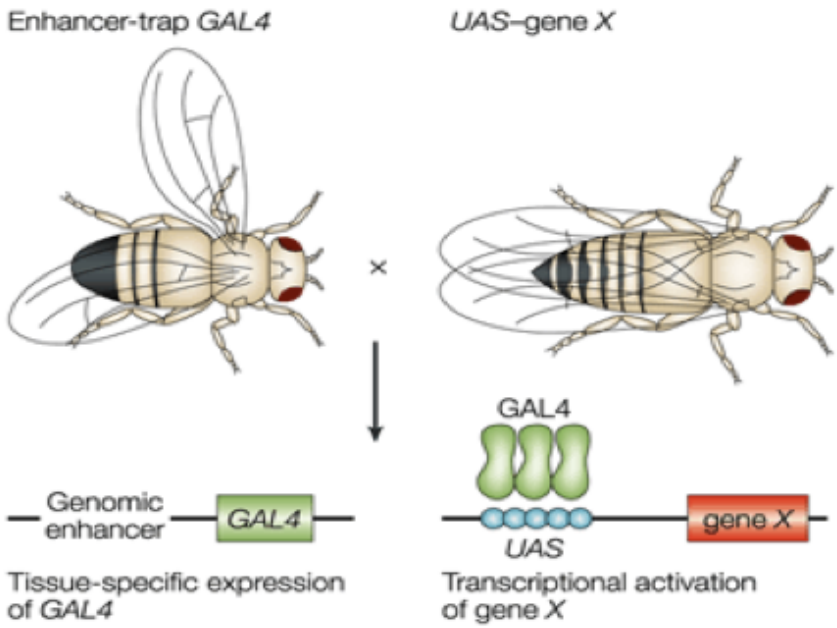


Figure 6: Diagram of terminology for domains of expression in the follicle cells of *Drosophila*. (A) Table of primitives with label scheme. (B) Boolean operators and examples of combinations of primitives.

Figure 7

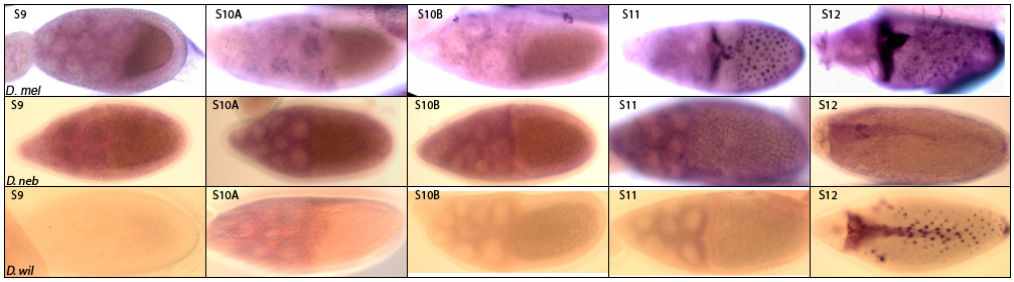


(Johnston, 2002)

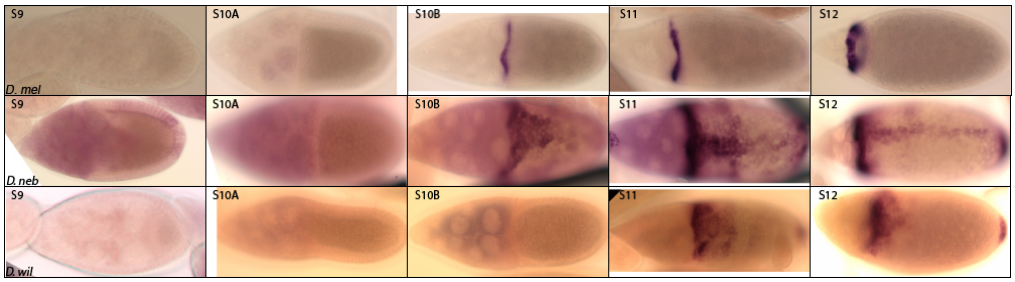
Figure 7: Diagram of the GAL4/UAS system. The GAL4 transcription factor is linked with a genomic enhancer that causes a tissue specific expression. The upstream activation sequence (UAS) controls the gene of interest (for this experiment, dnEGFR was used). When an enhancer-trap GAL4 possessing fly is crossed with a UAS fly that has the gene of interest, the F1 generation will express that gene of interest in a specific domain.

Figure 8

A. Cp 7fa



B Cp 16



C Cp 19

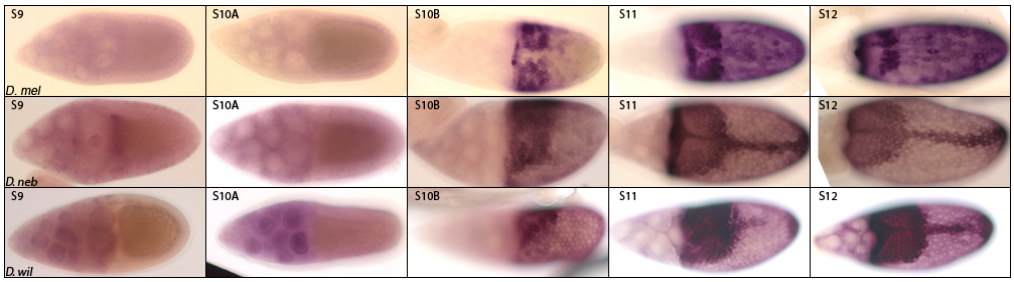
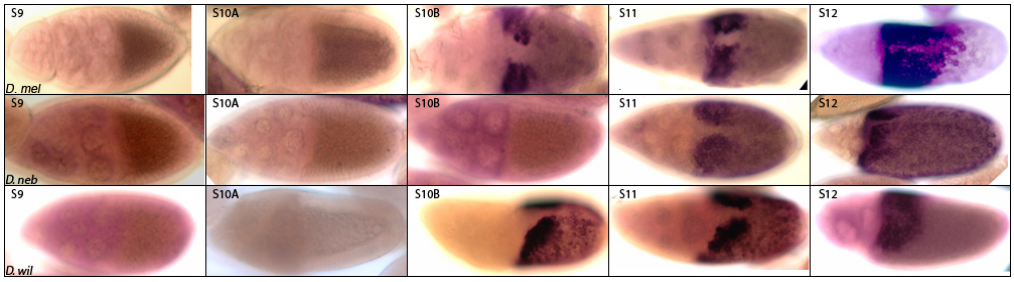


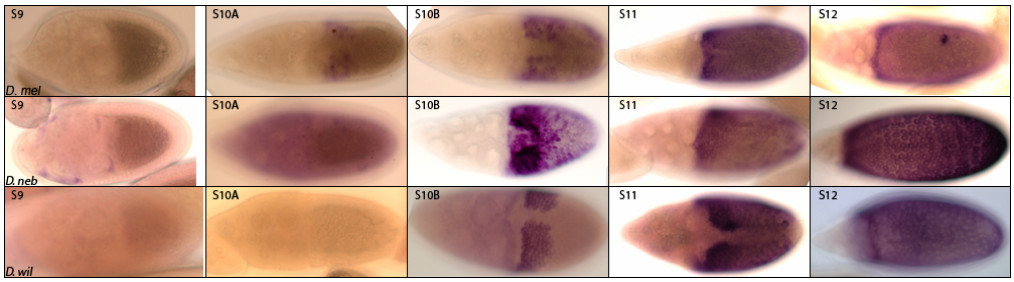
Figure 8: In-situ hybridization of the first group with the midline expression for their respective Cp gene. Order from top to bottom: *D. melanogaster* (*D. mel*), *D. nebulosa* (*D. neb*), and *D. willistoni* (*D. wil*): (A) Chorion protein 7fa (Cp 7fa). (B) Chorion protein 16 (Cp 16). (C) Chorion protein 19 (Cp 19)

Figure 9

A Cp 7fc



B Cp 36



C Cp 38

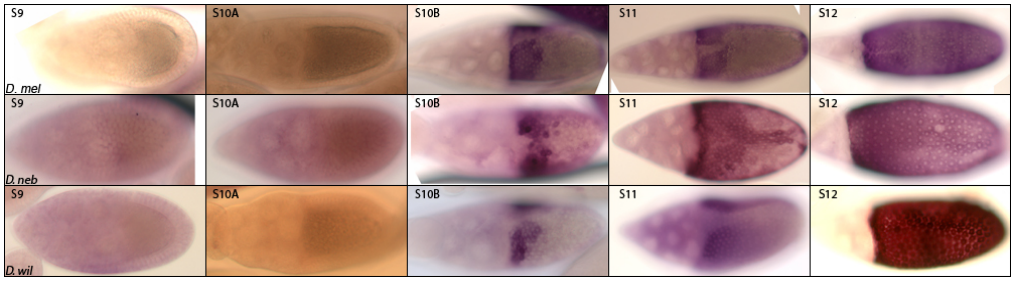
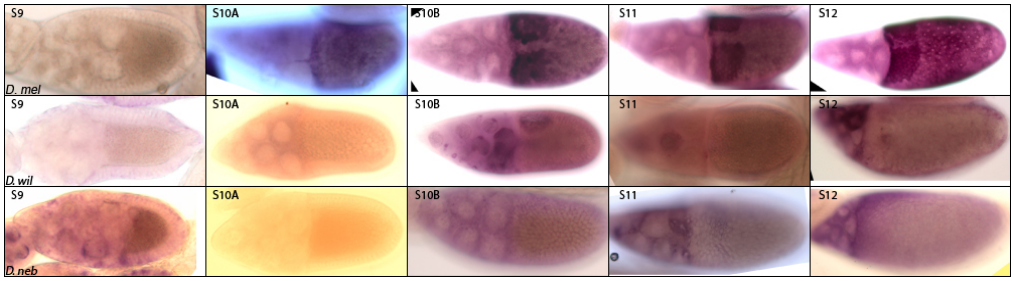


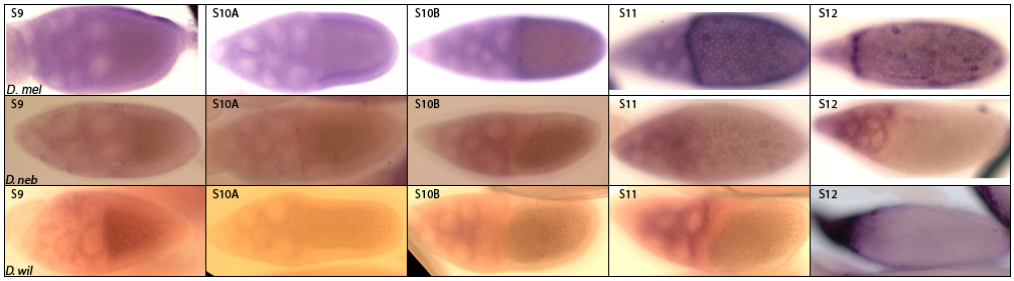
Figure 9: Expression pattern of Cp genes belonging to the group that showed exclusion from the dorsal ridge. Order from top to bottom: *D. melanogaster* (*D. mel*), *D. nebulosa* (*D. neb*), and *D. willistoni* (*D. wil*): (A) Chorion protein 7fc (Cp 7fc). (B) Chorion protein 36 (Cp 36). (C) Chorion protein 38 (Cp 38)

Figure 10

A Cp 7fb



B Cp 15



C Cp 18

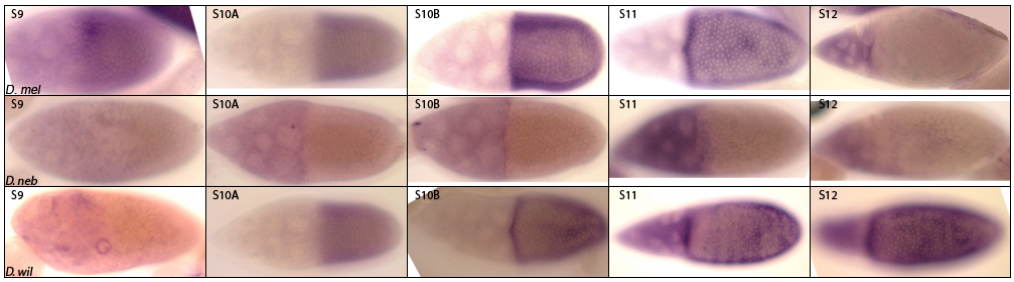


Figure 10: In-situ hybridization of the third group that had their Cp gene uniformly expressed. Order from top to bottom: *D. melanogaster* (*D. mel*), *D. nebulosa* (*D. neb*), and *D. willistoni* (*D. wil*): (A) Chorion protein 7fb (Cp 7fb). (B) Chorion protein 15 (Cp 15). (C) Chorion protein 18 (Cp 18)

Figure 11

WILD TYPE **COLCHICINE**

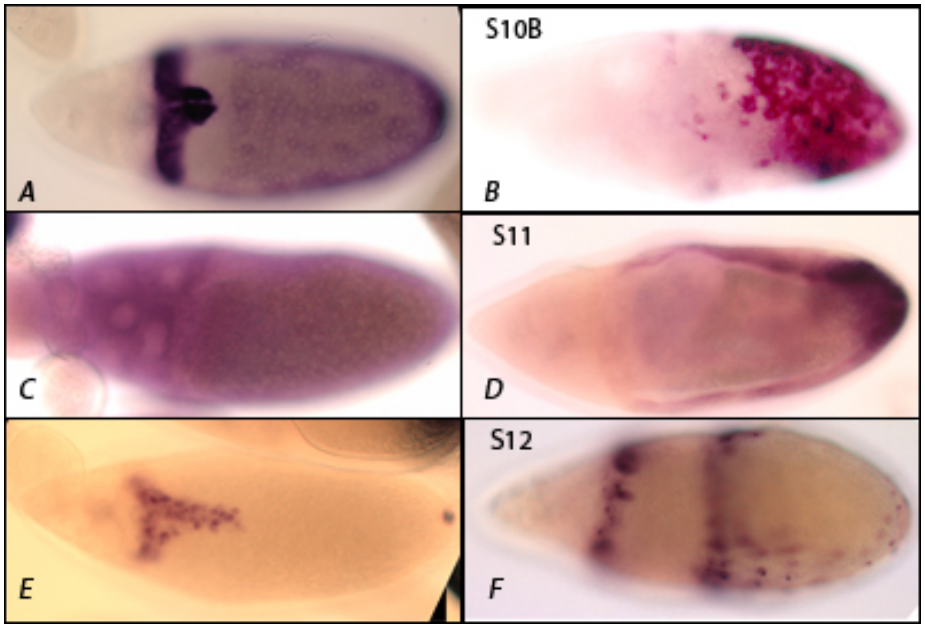


Figure 11: In-situ hybridization results of flies treated with colchicine. Probes were used for Chorion protein 7fa: (A, B) *D. melanogaster* wild type, and colchicine treated respectively. (C, D) *D. nebulosa* wild type, and colchicine treated respectively. (E, F) *D. willistoni* wild type, and colchicine treated respectively.

Figure 12

WILD TYPE **COLCHICINE**

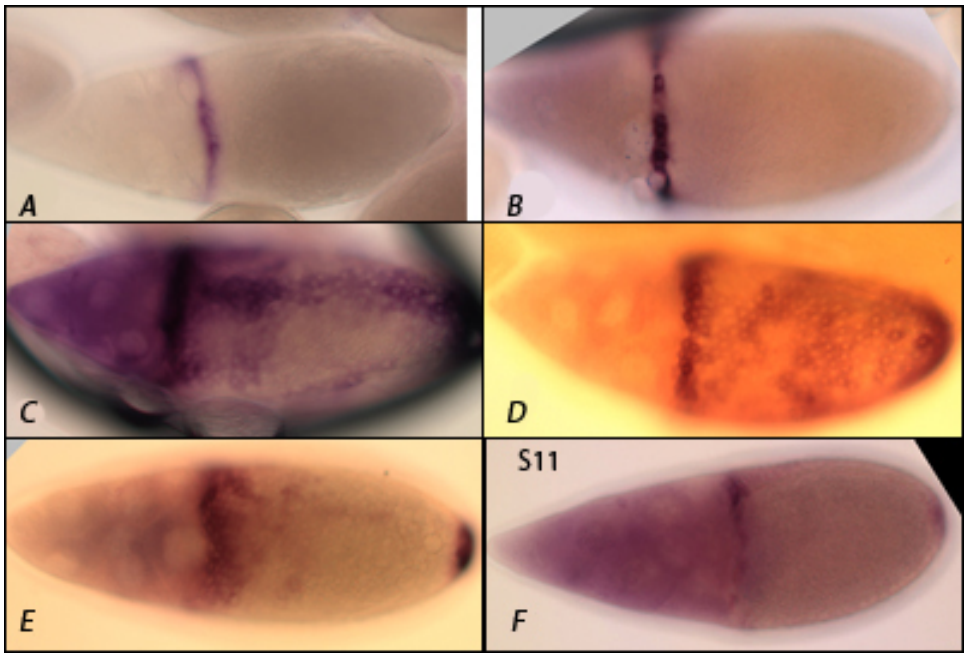


Figure 12: In-situ hybridization results of flies treated with colchicine. Probes were used for Chorion protein 16: (A, B) *D. melanogaster* wild type, and colchicine treated respectively. (C, D) *D. nebulosa* wild type, and colchicine treated respectively. (E, F) *D. willistoni* wild type, and colchicine treated respectively.

Figure 13

WILD TYPE **COLCHICINE**

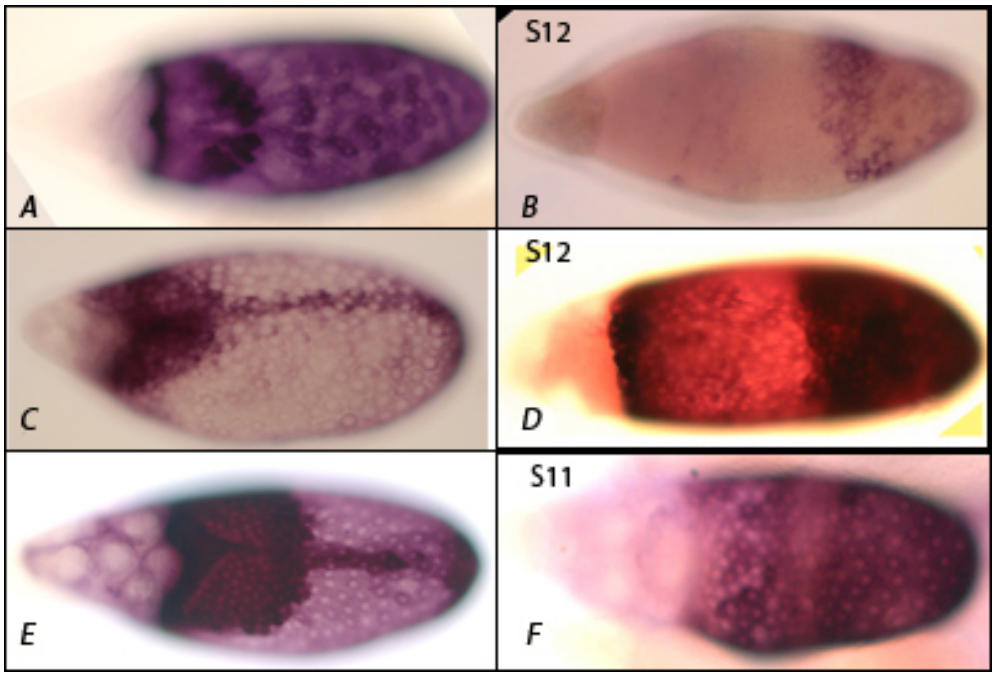


Figure 13: In-situ hybridization results of flies treated with colchicine. Probes were used for Chorion protein 19: (A, B) *D. melanogaster* wild type, and colchicine treated respectively. (C, D) *D. nebulosa* wild type, and colchicine treated respectively. (E, F) *D. willistoni* wild type, and colchicine treated respectively.

Figure 14

WILD TYPE **COLCHICINE**

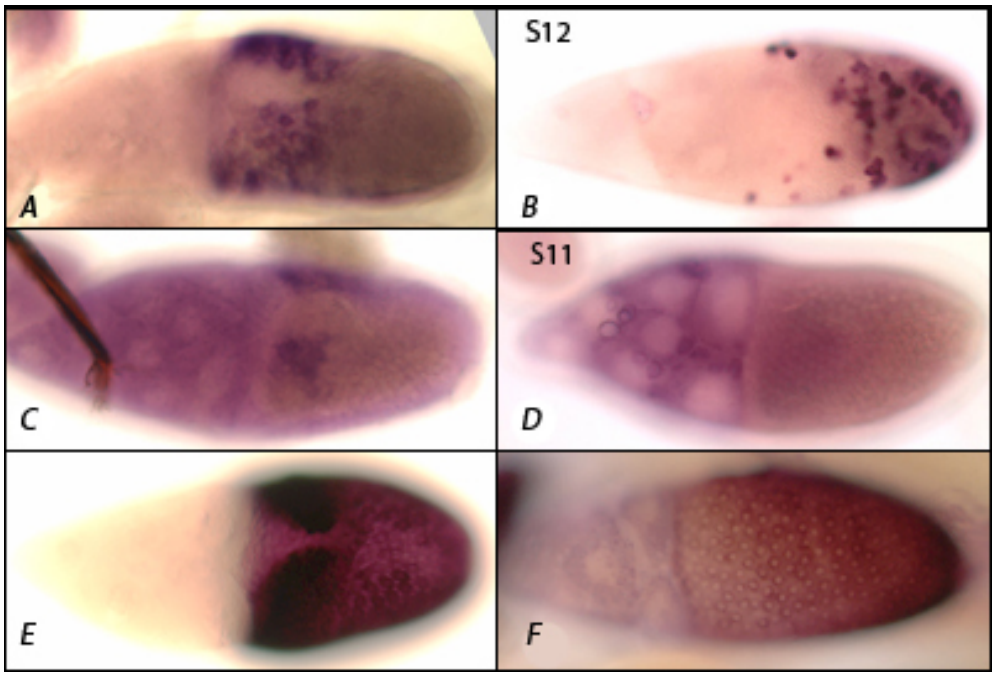


Figure 14: In-situ hybridization results of flies treated with colchicine. Probes were used for Chorion protein 7fc: (A, B) *D. melanogaster* wild type, and colchicine treated respectively. (C, D) *D. nebulosa* wild type, and colchicine treated respectively. (E, F) *D. willistoni* wild type, and colchicine treated respectively.

Figure 15

WILD TYPE **COLCHICINE**

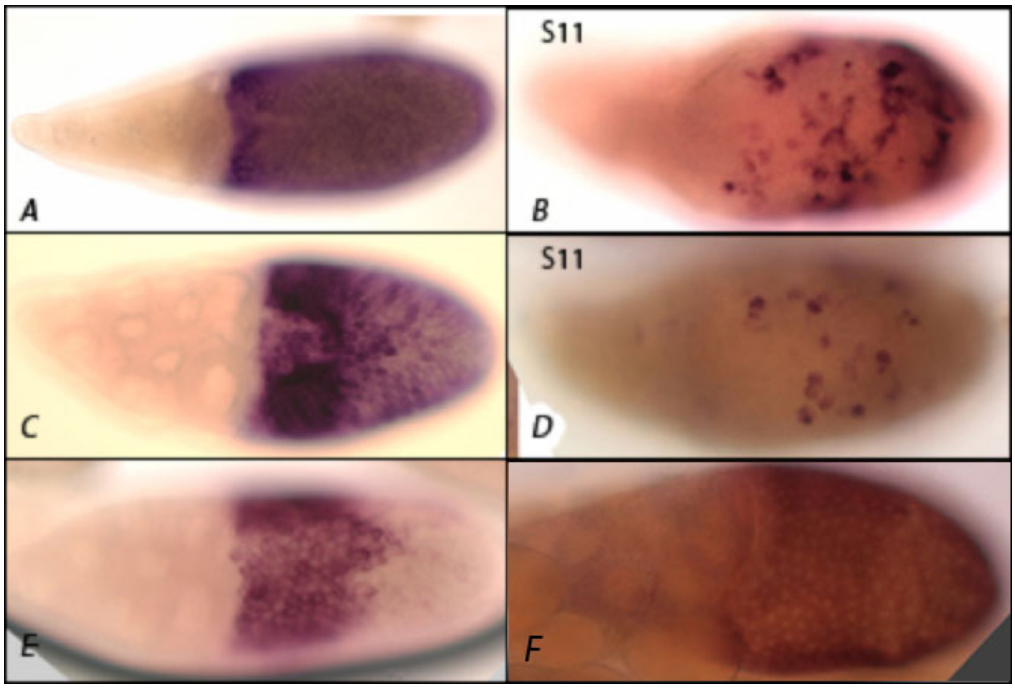


Figure 15: In-situ hybridization results of flies treated with colchicine. Probes were used for Chorion protein 36: (A, B) *D. melanogaster* wild type, and colchicine treated respectively. (C, D) *D. nebulosa* wild type, and colchicine treated respectively. (E, F) *D. willistoni* wild type, and colchicine treated respectively.

Figure 16

WILD TYPE **COLCHICINE**

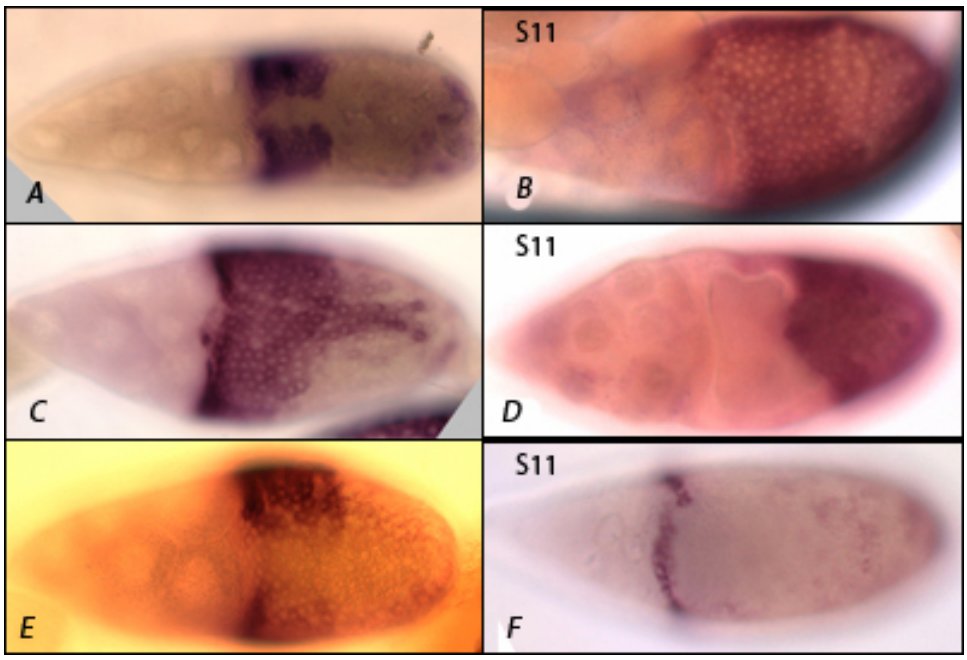


Figure 16: In-situ hybridization results of flies treated with colchicine. Probes were used for Chorion protein 38: (A, B) *D. melanogaster* wild type, and colchicine treated respectively. (C, D) *D. nebulosa* wild type, and colchicine treated respectively. (E, F) *D. willistoni* wild type, and colchicine treated respectively.

Figure 17

WILD TYPE **COLCHICINE**

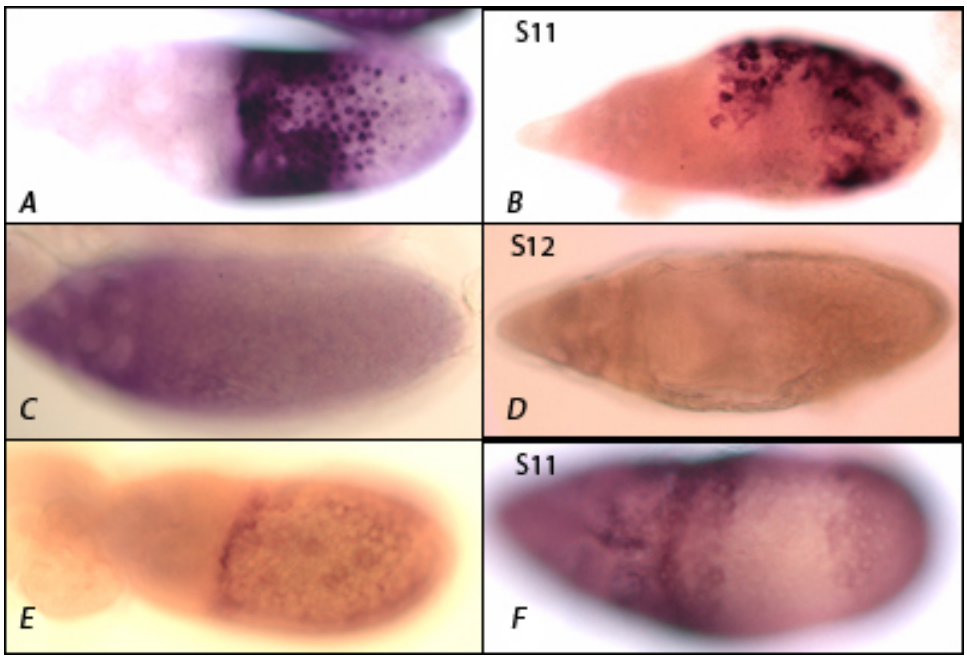


Figure 17: In-situ hybridization results of flies treated with colchicine. Probes were used for Chorion protein 7fb: (A, B) *D. melanogaster* wild type, and colchicine treated respectively. (C, D) *D. nebulosa* wild type, and colchicine treated respectively. (E, F) *D. willistoni* wild type, and colchicine treated respectively.

Figure 18

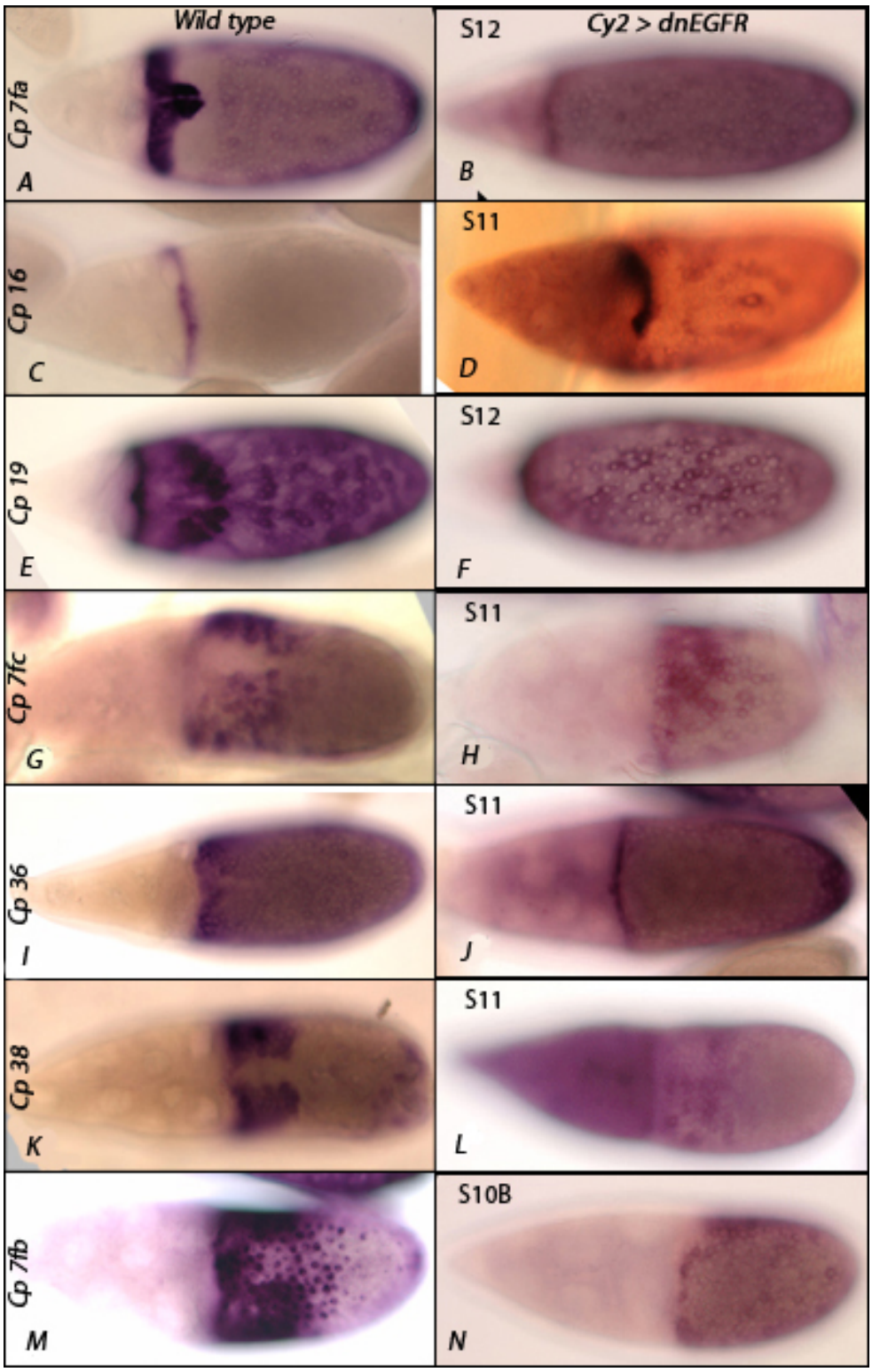

Wild type
Cy2 > dnEGFR
Cp 7fa
A
S12
B
Cp 16
C
S11
D
Cp 19
E
S12
F
Cp 7fc
G
S11
H
Cp 36
I
S11
J
Cp 38
K
S11
L
Cp 7fb
M
S10B
N

Figure 18: In-situ hybridization results of the f1 eggshells from the cross CY2-GAL4 x UAS-dnEGFR (*D. melanogaster*). (A, B) Cp 7fa expression in wild type and genetically perturbed eggshells respectively. (C, D) Cp 16 expression in wild type and genetically perturbed eggshells respectively. (E, F) Cp 19 expression in wild type and genetically perturbed eggshells respectively. (G, H) Cp 7fc expression in wild type and genetically perturbed eggshells respectively. (I, J) Cp 36 expression in wild type and genetically perturbed eggshells respectively. (K, L) Cp 38 expression in wild type and genetically perturbed eggshells respectively. (M, N) Cp 7fb expression in wild type and genetically perturbed eggshells respectively.

Tables

Table 1

TABLE 1. Timing of Major Eggshell Gene Expression During Oogenesis[a]

Gene	Stage 8	Stage 9	Stage 10	Stage 11	Stage 12	Stage 13	Stage 14
VM26A.1	+	+	+++	++			
VM26A.2	+	+	+++	+			
VM34C	+	++	+++				
VM32E			+++				
s36			+	+	+++	+++	+
s38			+	+	+++	+++	+
s16				+	+	+++	+++
s19			+	+	+	+++	+
s15						+++	+++
s18				+	+	+++	+++

[a]The different gene transcripts were described in: Mindrinos et al. (1985), Burke et al. (1987), Popodi et al. (1988), Jin and Petri (1993) (*VM26A.1, VM26A.2,* and *VM34C*), Gigliotti et al. (1989) (*VM32E*), Parks et al. (1986), Parks and Spradling (1987) (*s36* and *s38*), Griffin-Shea et al. (1982), Parks and Spradling (1987) (*s16, s19, s15,* and *s18*). +++, maximum transcript level.

(Cavaliere et al, 2008)

Table 1: A table showing the two main families of of genes that comprise the eggshell (VM and Cp) as well as the corresponding developmental stage of oogenesis that they are expressed at.

Table 2

species	Primer
dmel_cp15_5'	CCCTTTTCGCCTACATCAAC
dmel_cp15_3'	ATTGCGTTCAAGCTGCTTTT
dwil_cp15_5'	CTGTTCGTTTGCATCAGCTT
dwil_cp15_3'	TTGTATCCACCATCGATCTCC
dmel_cp18_5'	TCGCATCGATCAACTAACCA
dmel_cp18_3'	GGCCTCTTGTAGCCCTTCTT
dwil_cp_18_5'	TGCTGTTTCTGCCTATGGTG
dwil_cp_18_3'	TAGCCGGACTTCTTGTAGCC
dmel_cp_19_5'	CAACTGTGCCAAAACCCATA
dmel_cp_19_3'	CACGATCAGGCTGAGATCAA
dwil_cp_19_5'	ATTCATCTGCGCCTATCTGG
dwil_cp_19_3'	TATTTGGGTCCCTCAACACC
dmel_cp_36_5'	ATGCAACTCGGTCTCTGGTT
dmel_cp_36_3'	TGTACAGTGGAGCCTCGTTG
dwil_cp_36_5'	GTCTCTGGTTTGGGCTTTTC
dwil_cp_36_3'	AGTAGTTCTGCTGGCCATAGG
dmel_cp_38_5'	TGCAACTGGGAGACAAGATG
dmel_cp_38_3'	GCCGGATAAGCGAATGACTA
dwil_cp_38_5'	CCTGCCTGATTGCATGTG
dwil_cp_38_3'	AATATGCAGGAGCGCCATAG
dmel_cp_7fa_5'	CTTCTTCTCGCGTTGGTCAT
dmel_cp_7fa_3'	ATTGGAATTGGATTGGATGG
dwil_cp_7fa_5'	GTCAGTGTGCAAGGGCTTCT
dwil_cp_7fa_3'	CAATAACAATTGCCCGCATC
dmel_cp_7fb_5'	GAGGAGCAAGTGCCTCAAAC
dmel_cp_7fb_3'	GTTGCATTCGGTTTGGAGTT
dwil_7fb_5'	TTGCTCGAGCTGTTCGTCTA
dwil_7fb_3'	GATCGACAAGATCGCCAAAT
dmel_7fc_5'	CCCCGATGAGGAATACAATG
dmel_7fc_3'	TCTGGAGACCGCAAGTCTTT

dwil_7fc_5'	CCAATTGGTGGCTATTGTTAC
dwil_7fc_3'	TCCTCGCCTGTAGAGTAAATAGG

Table 2: Table of primers used to clone specific Chorion protein genes from *D. melanogaster* and *D. willistoni.*

Table 3

D. melanogaster	Stage 9	Stage 10A	Stage 10B	Stage 11	Stage 12
Cp 7fa	U	U	U	U/D ∪ F	U/D ∪ F
Cp 7fb	-	U	U + (R ∪ F)	U + (R ∪ F)	U + (R ∪ F)
Cp 7fc	-	-	R	A ∪ R ∪ F	A ∪ R ∪ F
Cp 15	U	U	U	U + A	U + A
Cp 16	-	-	A	A	A
Cp 18	U	U	U	U	U
Cp 19	-	-	A ∪ R	U + (A ∪ R)	U + (A ∪ R)
Cp 36	-	R	U/M	U/M	U
Cp 38	-	-	A ∪ R	A ∪ R ∪ P	U/M
D. nebulosa	Stage 9	Stage 10A	Stage 10B	Stage 11	Stage 12
Cp 7fa	U	U	U	U	U + M
Cp 7fb	-	-	U	U	U
Cp 7fc	-	-	-	U/M + R	U/M + R
Cp 15	U	U	U	U	U
Cp 16	-	-	A ∪ D/R	A ∪ D/R	A ∪ M ∪ P
Cp 18	U	U	U	U	U
Cp 19	U	U	D ∪ A	(U ∪ A) + (D/R) + P	(U ∪ A) + (D/R) + P
Cp 36	-	U	U + R	U	U
Cp 38	-	-	R	D/F + A	U

D. willistoni	**Stage 9**	**Stage 10 A**	**Stage 10B**	**Stage 11**	**Stage 12**
Cp 7fa	**-**	**U**	**U**	**U**	**M P**
Cp 7fb	**-**	**-**	**U**	**U**	**U**
Cp 7fc	**-**	**-**	**U/M + R**	**U/M + R**	**U/M + R**
Cp 15	**-**	**-**	**-**	**-**	**U**
Cp 16	**-**	**-**	**-**	**M U P**	**M U P**
Cp 18	**U**	**U**	**U**	**U**	**U**
Cp 19	**-**	**U**	**U + (D U A)**	**(U U A) + (D/R) + P**	**(U U A) + (D/R) + P**
Cp 36	**-**	**-**	**R**	**U/M**	**U**
Cp 38	**-**	**-**	**R**	**U/M**	**U**

Table 3: Annotations for domains of Cp expression for all three species of *Drosophila*.

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