

SYSTEMATIC ANALYSIS OF BONE MORPHOGENETIC PROTEIN SIGNAL
DIVERSIFICATION ACROSS *DROSOPHILA* SPECIES DURING OOGENESIS

by

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ABSTRACT OF THE THESIS

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Eggshells of *Drosophila* species provide great examples of morphological variation. The eggshell is a three-dimensional structure that protects the developing embryo from the surrounding environment and allows continuous gas exchange via tube-like structures called dorsal appendages (DAs). The number, size, shape, and positions of DAs vary among *Drosophila* species. During animal development, a handful of signaling pathways control tissue differentiation and morphogenesis. In general, mechanisms governing signal diversification that guide morphological variation remain largely unexplored. One conserved signaling pathway involved in guiding tissue development during *Drosophila* oogenesis is the Bone Morphogenetic Protein (BMP) signaling pathway. Representing a variety of eggshell morphologies, 16 *Drosophila* species were screened for diversity in BMP signaling during oogenesis. During early oogenesis, BMP signaling in all species was maintained in similar patterns displaying only anterior-posterior polarity. However, BMP signaling during late oogenesis acquired patterns with distinct dorsal-ventral polarities in all species. Further analyses of late patterns revealed five unique patterning groups. Using genetic tools, we demonstrated that the BMP type I receptor *thickveins* (*tkv*) accounted for BMP signaling diversification. Based on signaling diversity, computational modeling was employed to predict patterns of *tkv* that were

further tested experimentally. For most species, it was concluded that spatial changes to *tkv* guided diversification of late BMP signaling. In species belonging to the *D. virilis-repleta* radiation, *tkv* partially accounted for BMP signaling diversity and, for that radiation, the model proposes the involvement of another receptor in guiding BMP signaling. These results establish *tkv* as a major component in regulating BMP signaling diversification across 45 million years of evolution.

DEDICATION

This research project and thesis is dedicated to those that have truly impacted my life, and continue to influence my life, although they are no longer with me. To my best friend, Michael Frees, for providing me with the motivation necessary to succeed in so many aspects of my life before his passing. I would also like to dedicate this to my high school Biology teacher, Donald Holmquist, for being the first to spark my interest in genetics and specifically *Drosophila* research, and for inspiring me to pass my knowledge onto others. Finally, I would like to dedicate my work to my father, Zygmunt Niepielko: even though you didn't get a chance to witness my growth, you always have and always will influence it.

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Introduction

Drosophila oogenesis is an established model system to study developmental processes including cell signaling, tissue patterning, and morphogenesis (Horn-Badovinac and Bilder, 2005; Berg, 2005). Oogenesis, the process of egg development, comprises of 14 stages divided by distinct morphological characteristics in *Drosophila* (Fig. 1A) (Spradling, 1993). Referred to as an egg chamber, the developing egg has three main compartments; the nurse cells (NCs), the oocyte, and the follicle cells (FCs) (Fig. 1B). The NCs are cells responsible for nourishing the developing oocyte with different RNAs and proteins. The oocyte becomes the developing embryo after fertilization and the FCs surround the oocyte. The FCs includes about 650 cells that collectively form a two-dimensional tissue. This tissue will stretch and move to form the three-dimensional structure of the eggshell that protects and provides gas exchange for the developing embryo (Spradling, 1993; Berg, 2005). In this work, an egg chamber up to stage 10 (~50% NCs, ~50% oocyte) is referred to as “Early”, and stages 11 and up (~25% NCs, ~75% oocyte) are referred as “Late” oogenesis.

At the end of oogenesis, the FCs secrete proteins and form a three-dimensional structure called an eggshell (Fig. 1C). The main structures of the eggshell include the micropyle, operculum, and dorsal appendages (DAs). The micropyle is the structure responsible for directing sperm into the egg for fertilization. The operculum is a weakened structure in the eggshell that acts like a hatch door for the larva to escape once developed. The most prominent structures of the eggshell are the DAs. DAs are tube-like shaped structures providing gas exchange to the developing embryo; especially when the egg is submerged in rotten fruit (Hinton, 1981). The main interest of the project

involves understanding the signaling process guiding the formation of varieties of DAs morphologies across *Drosophila* species. Specifically, we aim to study the elaborate signaling networks evolved to generate diverse inputs that instruct different cellular differentiation strategies (Berg, 2005).

Tissue differentiation is guided by the chemical environment sampled by the tissue. A morphogen is a chemical signal secreted from a localized source that forms a gradient within a tissue; from high to low the further it travels from its source. Remarkably, morphogen gradients transform naïve cells, such as the egg chamber's FCs, into a patterned, non-uniform differentiated tissue (Fig. 2A; Turing, 1952). During development, multiple morphogen gradients may influence the same tissue to form even more complex gene expression patterns (Fig. 2B; Yakoby *et al.*, 2008). Once a tissue is patterned, the cells have received their instructions and the process of morphogenesis begins.

Two main morphogens are involved in patterning FC's: Gurken (GRK) and Decapentaplegic (DPP) (Twombly *et al.*, 1996; Neuman-Silberberg and Schupbach, 1996; Dobens and Raftery, 1998; Berg, 2005). Gurken is a TGF- α -like signaling molecule that activates the Epidermal Growth Factor Receptor (EGFR) signaling pathway. Gurken's RNA (*grk*) is localized to the oocyte's nucleus. Initially, the nucleus is localized at the posterior end of the egg chamber and later migrates asymmetrically to the anterior of the oocyte near the FCs. This process will first determine the anterior-posterior (AP) axis and later the dorsal-ventral (DV) axis of the egg (Neuman-Silberberg and Schupbach, 1996; Berg, 2005). GRK signals by binding to the EGFR and activating,

in the FCs, a series of phosphorylation events via the RAS-RAF-MAPK pathway (Ray and Schupbach, T. 1996).

Decapentaplegic is the homolog to the mammalian Bone Morphogenic Protein (BMP) type 2, 4 and is part of the TGF-Beta super family of signaling molecules (Parker *et al.*, 2004; Massague and Gomis, 2006). In the developing egg chamber, DPP is secreted from cells along the NCs – FCs border, referred to as the anterior region, and creates an anterior-posterior gradient (Fig. 3A; Twombly, *et al.*, 1996). In this pathway, a ligand binds to a receptor and initiates a cascade of phosphorylation. Inside the cells, the type I receptor phosphorylates a mediating protein, SMAD (P-MAD). Two P-MADs then bind to another protein, Medea (MED), and together they translocate to the nucleus to regulate gene expression (Fig. 3B; Wu and Hill, 2009). To monitor BMP signaling, we used an antibody against P-MAD (Yakoby *et al.*, 2008).

During oogenesis, the formation of the dorsal appendages (DAs) has been extensively studied in *D. melanogaster*. The formation of each DA on the eggshell begins from two non-overlapping domains of FCs, the floor cells and roof cells (Berg, 2005) (Fig. 4A). Each population of cells is marked by a non-uniformly expressed gene. The floor (bottom) of the DAs is formed from a domain of cells that are marked with the expression of a protease in the EGFR signaling pathway, *rhomboid (rho)* (Fig. 4B) (Ruohola-Baker *et al.*, 1993). The adjacent cells are monitored by the expression of the zinc-finger transcription factor Broad (BR) and these cells will form the roof (top) of the DAs (Deng and Bownes, 1997; Tzolovsky *et al.*, 1999) (Fig. 4B).

Expression patterns for both *br* and *rho* were previously found to depend on both the EGFR and BMP signaling pathways (Deng and Bownes, 1997; Berg, 2005; Ward *et al.*, 2006; Yakoby *et al.*, 2008). Interestingly, patterning of *br* and *rho* involves a complex network of interactions between both the EGFR and BMP signaling pathways (Chen and Schupbach, 2006; Shravage *et al.*, 2007; Yakoby *et al.*, 2008). Specifically, FCs that form DAs are patterned by sequential actions of feedforward and feedback loops involving the EGFR and BMP pathways (Fig. 5) (Yakoby *et al.*, 2008). In this model, BR expression is controlled in space by a feedforward loop and in time by a BMP negative feedback loop (Yakoby *et al.*, 2008). In this work, the validity of this model was tested in *Drosophila* species with variations in DAs.

Not surprisingly, changes in components of signaling pathways cause dramatic morphological changes. For instance, Darwin's finches are a group of 14 closely related finch species that have evolved a variety of beak morphologies to exploit food sources. In this case, the levels of BMP4 correlated with variations in beak size and shape. Therefore, a change in a single component is responsible for this famous example of evolutionary adaptation (Abzhanov *et al.*, 2004). Morphological modification is not limited to quantitative changes in morphogen levels, other components in signaling pathways may also be responsible for morphological variations. For instance, *D. melanogaster* contains two homologous flight structures, the wing and haltere. Both structures have different shapes, sizes and functions and yet, these differences were attributed to changes in the distribution of the BMP type I receptor, Thickveins (TKV), during development (Crickmore and Mann, 2007; Crickmore and Mann, 2008). In *D. melanogaster*, formation of the amnioserosa, an extra embryonic tissue produced by the

dorsal ectoderm, is regulated by BMP signaling (Ashe and Levine, 1999; Eldar *et al.*, 2002). Interestingly, in the mosquito *Anopheles gambiae*, the dorsal ectoderm expands into two separate tissues, the amnion and serosa. In this case, the reduced levels of the negative BMP regulator Short-gastrulation (SOG) accounts for the expansion of BMP signaling and for the broadening of the dorsal ectoderm domain along the DV axis (Goltsev, *et al.*, 2007).

Within the genus *Drosophila*, nature has provided thousands of species with all types of eggshell morphologies (Fig. 6). These variations include DA shapes, DA positions, and the number of DAs present on an eggshell (Hinton, 1981; Perrimon and Duffy, 1998). The Matsuno group recently established that the number of DAs found in different species was determined by the number of EGFR signaling domains (Kagesawa, 2008). However, genetic modifications to BMP signaling dramatically altered eggshell morphologies in *D. melanogaster*, suggesting BMP signaling plays a role in DAs variation (Yakoby *et al.*, 2008; Shravage *et al.*, 2007; Deng and Bownes, 1997; Dobens and Raftery, 1998).

Cell signaling through morphogen gradients has been established to be responsible for morphogenesis (Wolpert, 1989; Moussian and Roth, 2005; Ashe and Briscoe, 2006). Signaling pathways are conserved from worms to humans and are used in many tissues within and between organisms. While an increasing knowledge is available for each pathway, the unique tissue-specific function has sparsely been addressed. Therefore, changes to morphologies throughout evolution most likely reflect changes in signaling patterning and components within these pathways. Finding the

components accountable for tissue specific differentiation is a fundamental requirement for understanding morphological variations (De Robertis, 2008; Carroll, 2008).

Variation in eggshell morphologies should reflect changes in cell signaling. Here, it was hypothesized that variations in DAs should reflect changes in BMP signaling. This question was addressed by analyzing BMP signaling in the FCs of 16 different *Drosophila* species.

Our results demonstrate that late BMP signaling for all species acquired distinct dorsal-ventral polarities that clustered into five diverse patterning groups. By using genetic tools, the BMP type I receptor *thickveins* (*tkv*) accounted for BMP signaling diversification. Based on signaling diversity and *tkv*'s accountability, computational modeling was applied and predicted patterns of *tkv*. Our results indicate that spatial changes to *tkv* guides diversification of late BMP signaling for most species. However, in species belonging to the *D. virilis-repleta* radiation, *tkv* can only partially account for BMP signaling diversity and for that radiation, the model proposes another receptor to be involved in guiding BMP signaling.

Material and Methods

Flies: The following *Drosophila* species were used in this study: *D. busckii*, *D. guttifer*, *D. funebris*, *D. mojavensis*, *D. nasuta*, and *D. willistoni* (The San Diego Stock Center), *D. borealis*, *D. ezoana*, *D. littoralis*, *D. mercatorum*, *D. nebulosa*, *D. pseudoobscura*, and *D. yakuba* (a gift from D. Stern), *D. virilis*, *D. phalera* (a gift from J. Duffy), and *D. melanogaster* (wild-type *OreR*); *D. busckii* and *D. guttifer* were maintained on molasses supplemented Wheeler-Clayton media. All other fly species were maintained on standard cornmeal media at room temperature. Activated yeast was added to the fly food 24 hours prior to dissections and egg collections. Additional fly stocks used in this study were rho-GAL4 (a gift from C. Berg), CY2-GAL4 (Queenan, 1997) br-GAL4 (a gift of H. Cui and L. Riddiford), UAS-*tkv* RNAi (VDRC), UAS-*tkv*1-3B3 (a gift from M. O'Connor).

Immunoassay: Ovary dissection and fixations were carried out as previously described (Pacquelet and Rorth, 2005). Ovaries were dissected using maintained ice cold graces media. The primary antibodies used were mouse anti-BR core (25E9.D7; 1:100, DSHB), rabbit anti-phosphorylated-Smad1/5/8 (1:3500, a gift from D. Vasilias, S. Morton, T. Jessell and E. Laufer) (Yakoby *et al.*, 2008). DAPI (1:10,000) was used to stain for nuclear DNA. The secondary antibodies used were 488 anti-mouse and 568 anti-rabbit (Invitrogen) were used (1:1000).

***in situ* hybridization:** An *in situ* hybridization protocol was carried out as previously described (Yakoby *et al.*, 2008; Wang *et al.*, 2006). Ovaries were dehydrated and rehydrated using 33%, 66%, and 100% methanol and washed using .2% Tween-20 in

PBS. Ovaries were permeabilized using three thirty minute washes of RIPA solution followed by a post-fix of .2% glutaraldehyde and 4% formaldehyde. Ovaries were pre-hybridized for three hours in pre-hybridization solution followed by an overnight hybridization at 65 degrees Celsius using probe dilutions of 1:5 in hybridization buffer. Ovaries were blocked for 1 hour using 1% BSA solution. Alkaline phosphate conjugated anti-digoxigenin antibody was used at 1:2000 in 1% BSA solution for 2 hour or overnight at 4 degrees Celsius. Samples were developed using 1ml of alkaline phosphate buffer with 6.6µl NBT and 3.3µl BCIP.

Microscopy: Egg chambers were imaged using a Leica DM2500 compound microscope. Eggs for SEM imaging were collected for 20 minutes from agar plates and placed on SEM stubs using double-sided carbon tape. Eggs were briefly frozen in liquid nitrogen and lyophilized for two hours. Eggs were then coated with gold/palladium for 45 seconds and imaged using a LEO 1450EP at high vacuum pressure ($<10^{-5}$).

RNA extraction: RNA was extracted from ovaries using the RNeasy Mini Kit (Qiagen). cDNA for each species was made using the Taqman kit (Roche) as previously described (Yakoby *et al.*, 2008; Goentoro *et al.*, 2006).

***tkv* amplification:** A partial region of the *tkv* gene was amplified from *D. willistoni*'s cDNA using forward 5'GGAGAATGGCGGACTATTGA 3' and reverse 5'CGTGTGTTCTGGGCAATATG 3' primers. All other partial *tkv* genes were amplified from all other species using forward 5'AGYAAAYGGHACCTGCGAGAC 3' and reverse

5' GYGKATTCTGYGCAATGTGRAT 3' primers. Polymerase Chain Reaction (PCR) was done using MJ Mini (BioRad) thermocycler.

***tkv* Cloning:** PCR products were cloned using the StrataClone PCR Cloning kit (Stratagene). Plasmid extraction was performed by using QIAprep spin Miniprep Kit (Qiagen). Isolated genes were sequenced (GeneWiz) and blasted against sequenced species using FlyBase.

Genetic Tools: The GAL4/UAS system was used to express *tkv* and *tkv* RNAi in a specific domain. A fly with a specific domain expression of the GAL4 transcription factor was crossed with another fly with the gene of interest under the control of the upstream activating sequence (UAS). This created an F1 generation that expressed the gene of interest in a specific tissue domain (Fig. 7; Reviewed by Johnston, 2002). The specific use of the GAL4/UAS system is described as followed. Expression of *tkv* in floor cells was done by UAS-*tkv*1-3B3 driven by the floor domain driver *rho*-GAL4. Expression of *tkv* along the anterior was done by UAS-*tkv*1-3B3 driven by the uniform driver CY2-GAL4. Depletion of *tkv* in the roof domain was done by UAS-*tkv* RNAi driven by the roof domain driver br-GAL4. All crosses were done at room temperature.

Results

BMP signaling is dynamic and diverse across species

Sixteen species of *Drosophila* were screened for BMP signaling dynamics in the FCs of developing egg chambers. In all species, BMP signaling during early oogenesis had only anterior-posterior polarity and appeared along the NC-FCs border (Figs. 8-13). Interestingly, later BMP signaling gained DV polarity in all species (Figs. 8-13). Using Broad (BR) expression as a reference to mark the roof domain, analyses of late BMP signaling patterns established five unique BMP signaling patterning groups. The groups were determined based on, or in a combination of, three FC domains, Roof, Floor, and Anterior (Fig. 4A). A representative for each signaling group is seen in Figure 8.

In species belonging to group I, BMP signaling appeared in an anterior stripe that lacked the dorsal domain and in two dorsolateral patches on both sides of the midline on the roof domain (Figs. 8B, B', 9). In group II, signaling appeared in an anterior stripe that lacked the dorsal domain and in two dorsolateral patches on both sides of the midline on the roof and floor domains (Figs. 8D, D', 10). Species belonging to group III had no dorsolateral extension of BMP signaling and only had a dorsal midline clearing (Fig. 8 F, F', 11). Group IV species had BMP signaling maintained in the anterior and in the roof domain (Figs. 8 H, H', 12). Group V had BMP signaling in a combination of all three domains, the anterior, roof, and floor (Figs. 8 J, J', 13).

***tkv* is sufficient for BMP signaling**

The expression pattern of *tkv* is necessary for BMP signaling (Yakoby *et al.*, 2009). Here, expression of *tkv* in the floor domain in *D. melanogaster* was sufficient to

induce BMP signaling on the floor domain, similar to species in group II (Figs. 14 A, A', B, B', 8). Depletion of *tkv* from the roof domain by ectopic expression of *tkv* RNAi removed BMP signaling from the roof domain in *D. melanogaster*, similar to species in group III (Figs. 14 C, C', D, D', 8). By expressing *tkv* in all FCs in *D. melanogaster*, BMP signaling was sufficiently maintained in the dorsal anterior, similar to species in group IV and V (Figs. 14 E, E', F, F', 8). Patterns of ectopic *tkv* expressions are presented in Figure 15.

Computational predictions of *tkv* patterns

A computational model was formulated to predict BMP signaling patterns based on receptor distribution (Lembong *et al.*, 2008). Here, the inverse approach was used to predict a BMP receptor given the pattern of BMP signaling. Using the recently established five unique patterns of BMP signaling (Fig. 8) and the sufficiency of *tkv* to diversify signaling (Fig. 14), the model predicted *tkv* patterns that corresponded to the diverse late BMP signaling patterns (Fig. 16). This modeling was carried out in collaboration with Jitendra Kanodia (Princeton University).

Expression of *tkv* across *Drosophila* species

In *D. melanogaster*, *tkv* controls the dynamics of P-MAD (Yakoby *et al.*, 2008). Therefore, we hypothesized that this to be the case for other species. Testing computational predictions required the cloning of *tkv* from all 16 species and screening for *tkv* patterns. In all species, *tkv* expression during early oogenesis was uniform (Fig. 17-21). During late stages however, *tkv* expression patterns were diverse across *Drosophila* species (Figs. 17-21). In species belonging to group I, the dorsal midline was

cleared and *tkv* appeared in dorsolateral patches that correspond to the roof domain (Figs. 17B, 18). In species belonging to group II, *tkv* cleared from the dorsal anterior but appeared in the floor and roof domains (Figs. 17 D, 19). Species belonging to group III had no dorsolateral extension of *tkv* and only had a dorsal midline clearing (Figs. 17F, 20). Group IV and V species maintained uniform *tkv* expression even in late stages of oogenesis (Figs. 17H, J, 21). Of note, since fluorescent *in situ* hybridization did not work in the FCs, the distances between *tkv* and BR patches were measured by staining with the nuclear DNA marker DAPI. In all cases with BMP signaling on the floor domain, the number of cells between BR patches was always greater than the number of cells between *tkv* patches.

Discussion

BMP signaling is diverse among *Drosophila* species

Although BMP signaling dynamics have been previously described for *D. melanogaster*, here I provide an evolutionary angle to BMP signaling dynamics stretching ~45 million years of evolution (Yakoby *et al.*, 2008). Interestingly, for all 16 species, early BMP signaling is restricted to the NCs-FCs border as an anterior stripe (Figs. 8-13). As previously described and supported here, this pattern is achieved by the anterior secreted DPP and the early uniform expression of *tkv* (Dobens and Raftery, 1998; Berg, 2005; Peri and Roth, 2000; Shravage *et al.*, 2007). Since early BMP signaling is an important regulator of the eggshell's anterior structures such as DAs and the operculum, conserved early patterning across species is not surprising (Berg, 2005; Shravage *et al.*, 2007; Yakoby *et al.*, 2008).

In all species examined, late BMP signaling acquired clear DV polarities. Analyses of these DV signaling patterns following the three domains, anterior, floor and roof, clustered all signaling patterns into five unique groups (Fig. 8). In *D. melanogaster*, BMP signaling dynamics is regulated by *tkv* which is controlled by the dynamic expression of BR (Fig. 5). In addition, *tkv* is also regulated in a positive feedback loop by P-MAD (Yakoby *et al.*, 2008; Lembong *et al.*, 2009). In this model, P-MAD and BR are present in the same domain, the roof cells. Interestingly, this pattern was restricted to only four *Drosophila* species and in other species it was surprising to find P-MAD in domains that did not overlap the roof cells. These groups are specifically addressed and discussed in the following sections.

***tkv* accounts for the diversification of BMP signaling**

In *D. melanogaster*, the pattern of *tkv* guides BMP signaling dynamics (Yakoby *et al.*, 2008). Therefore, we hypothesized that changes in the distribution of *tkv* is responsible for diversity in BMP signaling across 45 million years of evolution and tested this hypothesis by spatially changing *tkv* expression in *D. melanogaster*. In group II, where P-MAD occupies the floor and the roof domains (Fig. 8D'), *tkv* was ectopically expressed onto the floor domain in *D. melanogaster* (Fig. 15a). By changing the spatial expression of *tkv*, BMP signaling was detected in the floor domain, similar to those species in group II (Fig. 14A, A', B, B'). Spatial changes in *tkv* in these species (Figs. 17D, 19) suggest that, in addition to BR and P-MAD, another regulator must be instructing *tkv* expression on the floor domain. Furthermore, the floor domain is exposed to high levels of EGFR signaling and therefore, this potential regulator may be a downstream target of EGFR signaling; further adding to a complex network of interacting pathways. Such potential candidates include the transcription factors Jra and Fos/Kayak (Dequier *et al.*, 2001; Tran and Berg, 2003).

In group III, where P-MAD is cleared from both the dorsal midline and roof domain (Fig. 8F'), *tkv* is decoupled from BR control. To test this, *tkv* was depleted from the roof domain using a *tkv* RNAi in *D. melanogaster* (Fig. 15b). Consistent with the above speculation, removing *tkv* expression from the roof domain eliminated BMP signaling similar to species in group III (Fig. 14C, C', D, D'). The pattern of *tkv* in group III (Figs. 17F, 20) species becomes interesting since a different model must be suggested.

While the lack of dorsolateral P-MAD can be explained by the lack of *tkv* and that BR no longer controls *tkv*, my model fails to explain the dorsal anterior repression of P-MAD/*tkv* in this group. In order for *tkv* to be removed from both the dorsal midline and the roof domain, *tkv* must be decoupled from BR control and repressed in the midline by a repressor. One potential candidate that has been previously described as a midline repressor in the FCs is Pointed (PNT1) (Zartman *et al.*, 2009; Lembong *et al.*, 2009; Boisclair-Lachance *et al.*, 2009; Yakoby *et al.*, 2008). Therefore, in three DAs species, the lack of BMP signaling in the midline may be under direct control of PNT1 acting on *tkv*.

Although *tkv* can account for the dynamics of BMP signaling in groups I, II, and III, the network controlling *tkv* diverged considerably. Specifically, changes to *tkv*'s regulation throughout evolution may be due to modifications in transcription factor binding sites of *tkv*'s enhancers, and thus, must be further investigated.

The *Drosophila virilis-repleta* radiation

Remarkably, in an entire phylogenetic branch (i.e. the *virilis-repleta* radiation; Fig. 6), *tkv* cannot account for the pattern of BMP signaling; in these species, *tkv* remained uniform during late stages of oogenesis (Figs. 17H, J, 21). Interestingly, all species in group IV and V maintained late BMP signaling along the anterior. Since uniform expression of *tkv* accounts for a similar pattern in early oogenesis, genetic tools were used to uniformly express *tkv* in the FCs in *D. melanogaster* (Fig. 15c) (Queenan *et al.*, 2007). Strikingly, BMP signaling was maintained in an anterior stripe similar to those species in groups IV and V (Figs. 14E, E', F, F'). Thus, the anterior stripe of late

BMP signaling in these groups can be explained by the uniform expression of *tkv*. What cannot be explained by *tkv* expression is the dorsolateral pattern of BMP signaling.

Using computational modeling, the pattern of a BMP receptor pattern was predicted based on signaling patterns in these groups. Experimental results support that the anterior region of BMP signaling is explained by *tkv*, and thus the computational model predicts another BMP receptor to be dorsolaterally patterned. These results suggest that during evolution another receptor may have gained control to pattern BMP signaling. An additional BMP type I receptor *saxophone* (*sax*) has been previously described to affect eggshell patterning and morphogenesis, and thus is a potential candidate for controlling BMP signaling in these species (Twombly *et al.*, 1996).

Other BMP signaling components such as, Short-gastrulation (SOG), have been found to regulate the expansion of BMP signaling in other tissues (Ashe and Levine, 1999; Goltsev *et al.*, 2007). In addition, ligand combinations such as Glass Bottom Boat (GBB) and DPP heterodimers have been found to affect BMP signaling distribution in tissues such as the wing (Bangi and Wharton, 2006). Since computational predictions in the *D. virilis-repleta* radiation also proposed an increase in ligand migration in the presence of receptor (Fig. 16), mechanisms found in other tissues may act in these species and may be responsible for guiding BMP signaling. Such regulating mechanisms should be further addressed to study strategies in signaling diversification.

Variations in eggshell morphologies reflected diversity in BMP signaling during oogenesis across *Drosophila* species. Systematic analyses of BMP signaling diversity revealed five diverse signaling patterns. Based on our analyses, the type I BMP receptor *thickveins* (*tkv*) accounted for diversity and, in most species, guided BMP signaling

diversification. In species belonging to the *D. virilis-repleta* radiation, *tkv* accounted for and guided late BMP signaling diversity in the dorsal-anterior domain. Our results establish *tkv* as a major regulator of BMP signaling diversification during oogenesis across *Drosophila* species.

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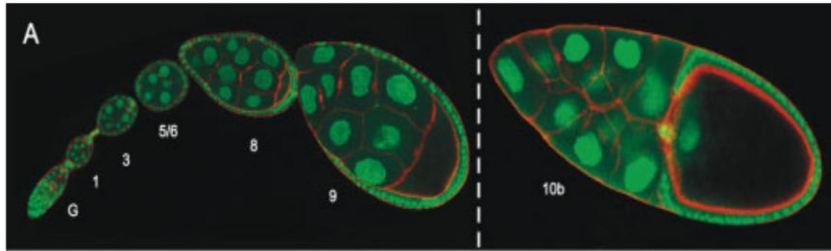
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Figures

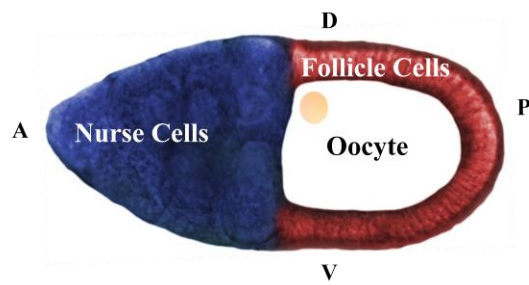
Figure 1

A



(Horn-Badovinac and Bilder, 2005)

B



C

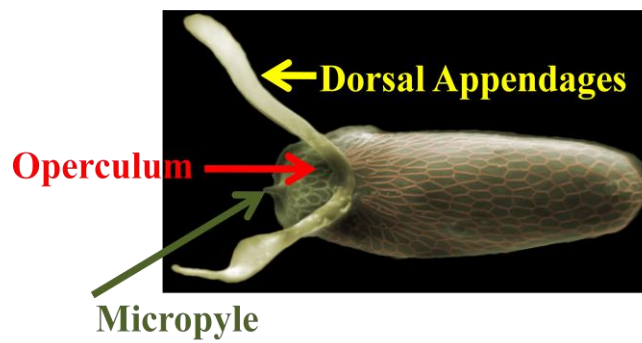


Figure 1: Oogenesis in genus *Drosophila*. **(1A)** An example of the morphologically defined stages an egg chamber undergoes during oogenesis. **(1B)** A single egg chamber at stage 10b is shown with its defining features and orientation. At the most anterior region, “A”, are the nurse cells (NCs). To the right of the NCs is the developing oocyte; the location of the oocyte’s nucleus defines dorsal, “D”. Surrounding the oocyte is a two-dimensional tissue layer of follicle cells (FCs). **(1C)** A fully developed egg of *Drosophila melanogaster*; the eggshell’s main structures are labeled.

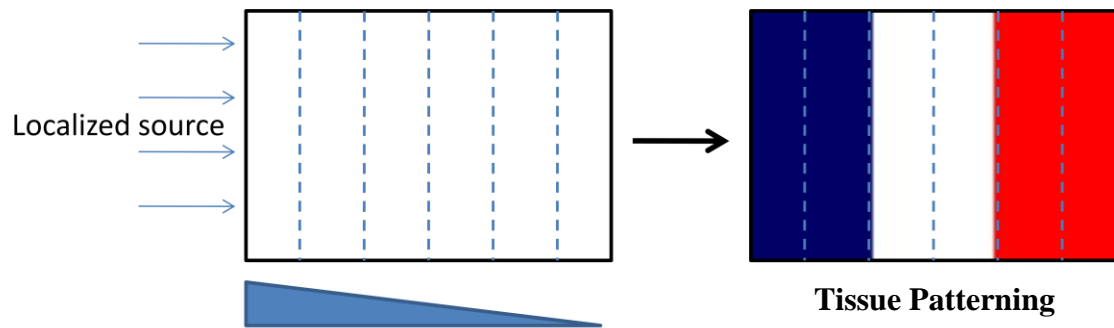
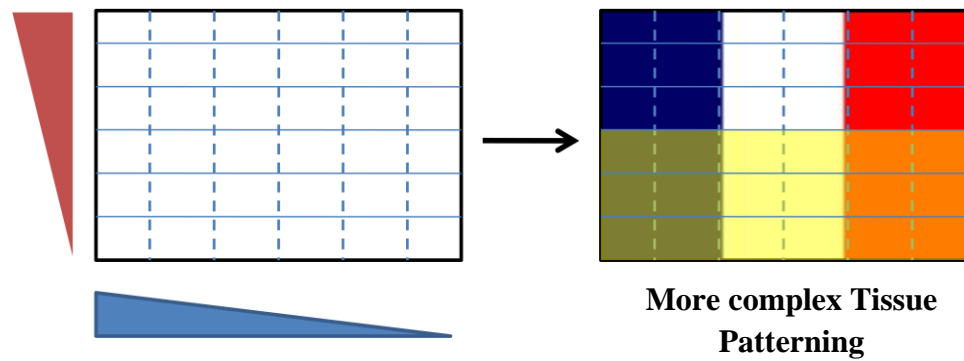
Figure 2:**A****B**

Figure 2: Morphogen gradients. (2A) A morphogen is secreted from a localized source and creates a concentration gradient that is interpreted by cells within a tissue causing non-uniform gene expression. **(2B)** An interaction of multiple morphogens allows for more complex tissue patterning.

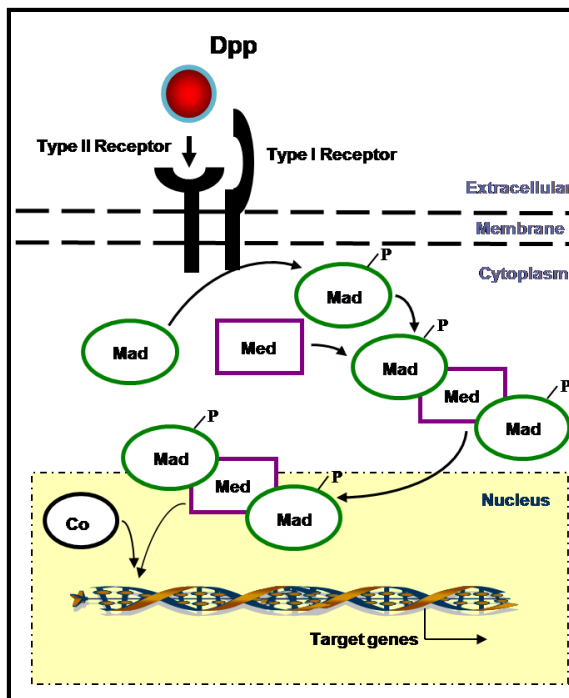
Figure 3**A****B**

Figure 3: DPP and BMP signaling during oogenesis. (3A) *dpp* transcription is limited to the anterior most region of the FCs (Twombly, *et al.*, 1996). (3B) A diagram summarizing BMP signaling in *Drosophila*. DPP binds to heteromeric receptor complex which then causes an intracellular cascade event. The type I receptor phosphorylates SMAD to make P-MAD, two P-MADs bind to one Medea (MED), and with other proteins enter the nucleus to control gene expression.

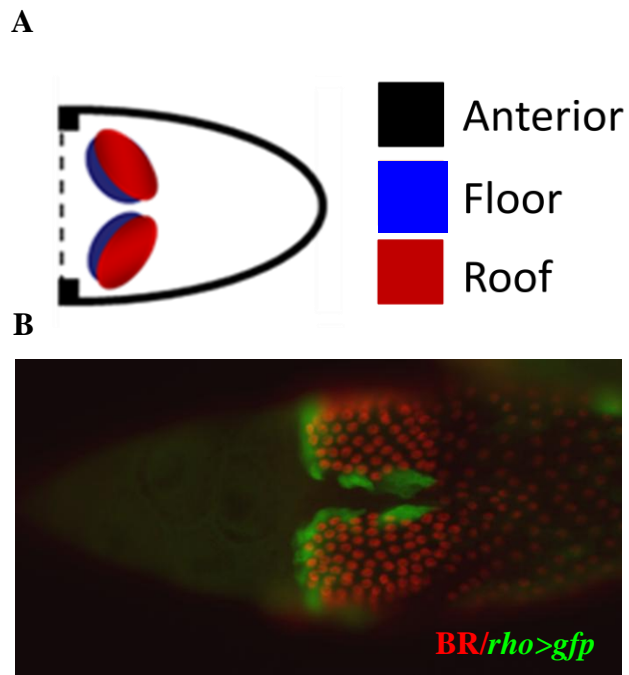
Figure 4

Figure 4: Two non-overlapping populations of cells give rise to the DAs. (4A) A cartoon depiction showing the boundaries between two cell populations that are involved in forming the dorsal appendages (DAs). **(4B)** An egg chamber with the two non-overlapping domains marked; **Green** marks the floor cells and **Red** marks the roof cells.

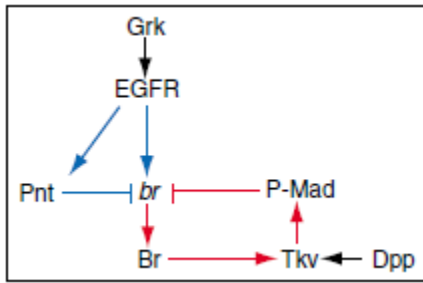
Figure 5:

Figure 5: Model of EGFR and BMP signaling interactions during DA formation. In this model, EGFR signaling initiates *br* and Pointed (PNT) expression, PNT being expressed in higher levels of EGFR signaling. PNT acts as a repressor to *br* and thus spatially controls *br* expression in a feedforward loop. Sequentially, BR induces the BMP receptor *tkv* activating a negative feedback loop through P-MAD that represses *br* expression in time (Yakoby *et al.*, 2008).

Figure 6:

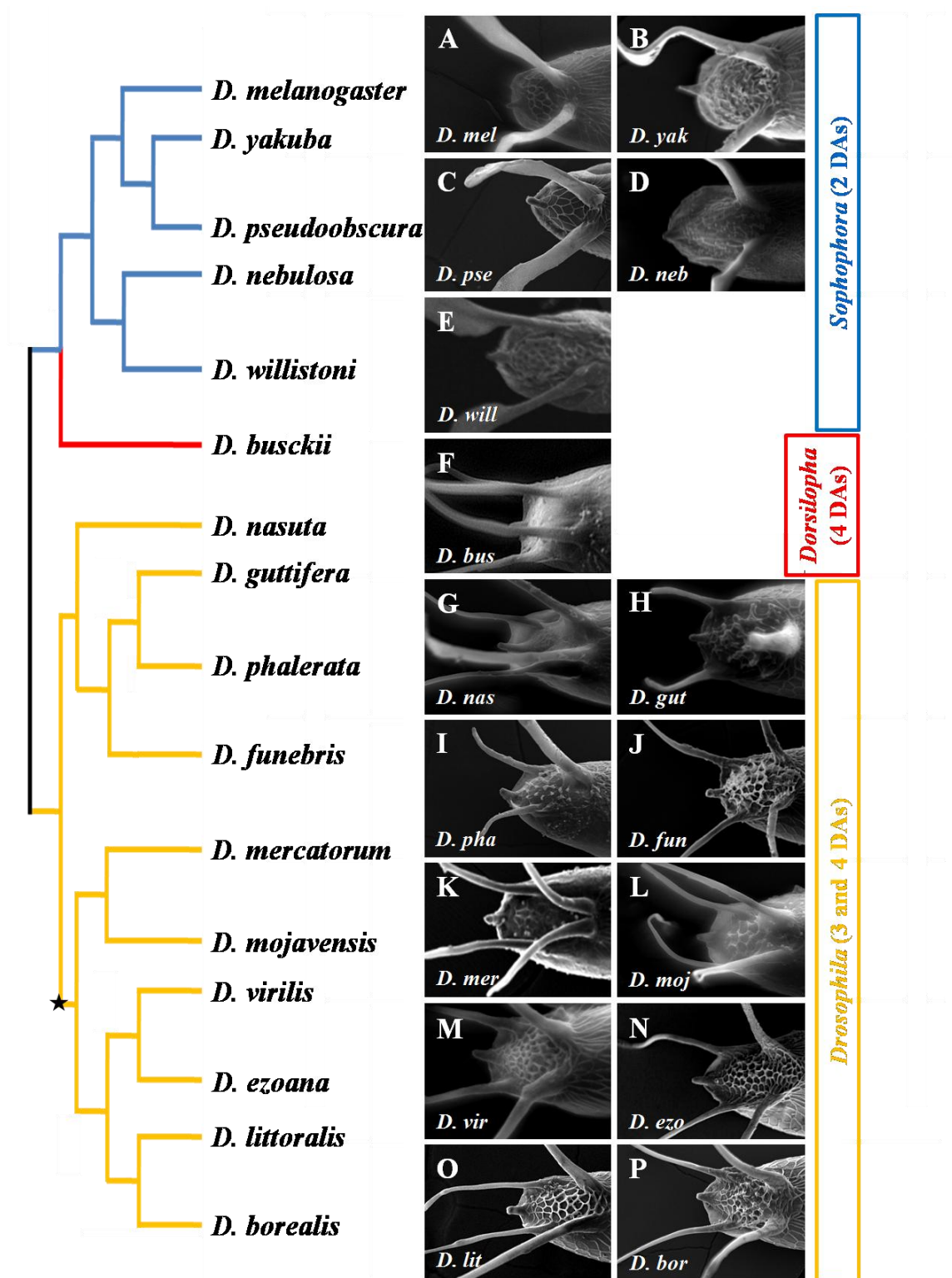
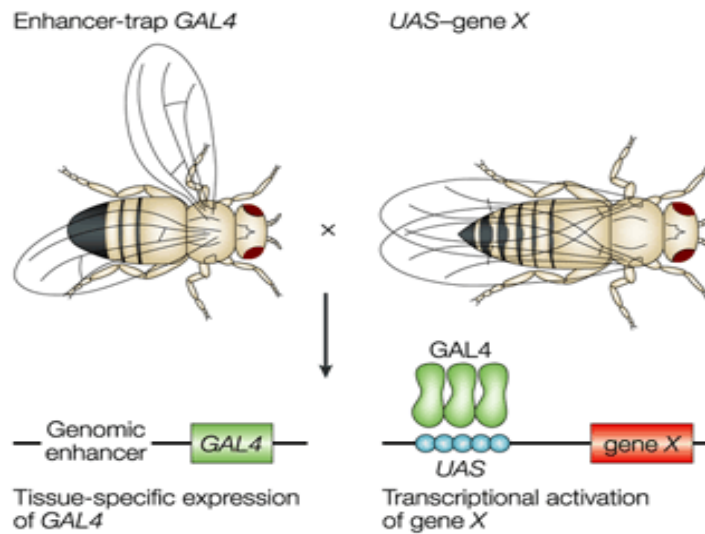


Figure 6: Variety of eggshell morphologies in three subgenera of *Drosophila*.

Subgenus *Sophophora*, represented by *D. melanogaster*, *D. yakuba*, *D. pseudoobscura*, *D. nebulosa*, and *D. willistoni*, contain only two DAs (blue, A-E). *D. busckii* of subgenus *Dorsilopha* has four DAs (red, F). Subgenus *Drosophila* contains the widest range of dorsal appendage numbers (yellow, G-P). Species such as *D. guttifera* and *D. phalerata* have three DAs (H, I), and *D. funebris*, *D. mercatorum*, *D. mojavensis*, *D. virilis*, *D. ezoana*, *D. borealis*, and *D. littoralis* have four dorsal appendages (J-P) (SEM micrographs, dorsal views, anterior to the left) (Phylogenetic tree based on Flybase and Kagesawa *et al.*, 2008)

Figure 7:

(Johnston, 2002)

Figure 7: A diagram of the GAL4/UAS system. This diagram explains how the GAL4/UAS system is used to express a gene of interest in a specific domain. A fly with a specific domain expression of the GAL4 transcription factor is crossed with another fly that has a gene of interest under the control of the upstream activating sequence (UAS), in this case *tkv* and *tkv* RNAi. This will create an F1 generation that will express a gene of interest in a specific tissue domain (reviewed by Johnston, 2002).

Figure 8:

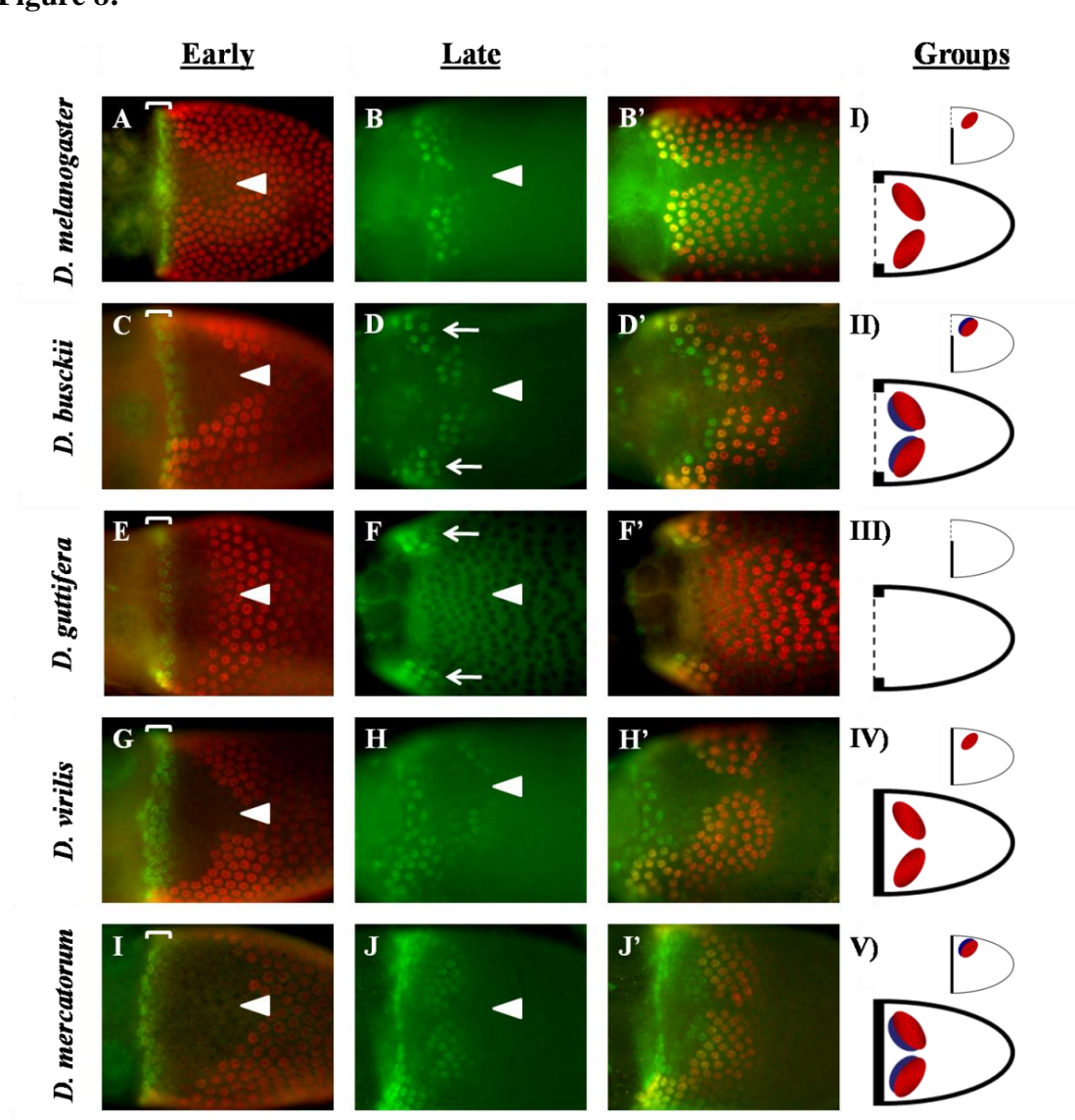
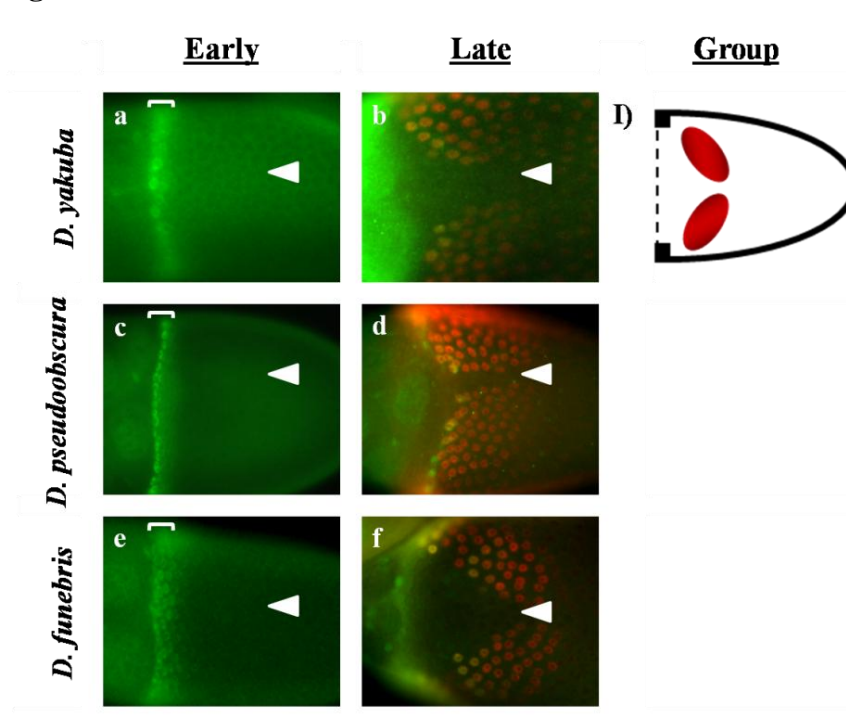


Figure 8: Diversity of BMP signaling in *Drosophila* species. Initial BMP signaling (**Green**) was an anterior stripe (white brackets) along the nurse cells oocyte border in all species (**A, C, E, G, and I**). Later BMP signaling diverged into five unique groups; each pattern appears with and without an overlap with the roof marker BR (**Red**). (**B, B'**) In the first group, represented by *D. melanogaster*, late BMP signaling was removed from the dorsal midline and also appeared in two dorsolateral patches on the roof domain (cartoon group I in red) (lateral cartoons show remaining ventral BMP signaling). (**D, D'**) In the second group, represented by *D. busckii*, late BMP signaling cleared from the dorsal midline and appeared in two dorsolateral patches on both the roof (red) and floor (green) domains (cartoon group II roof- red, floor-blue). (**F, F'**) In group three, represented by *D. guttifera*, signaling cleared from the dorsal midline and was absent from the roof/floor domains (cartoon group III black). (**H, H'**) Signaling in group four, represented by *D. virilis*, remained in the dorsal anterior and extended onto the roof domains (cartoon group IV). (**J, J'**) The fifth group represented by *D. mercatorum*, signaling remained in the dorsal anterior, and also appeared in the roof and floor domains (cartoon group V). White arrowhead denotes the dorsal midline; all images are dorsal up and anterior to the left.

Figure 9:



P-MAD (Green); BR (Red)

Figure 9: Group I, *Drosophila* species with late BMP signaling in only the roof

domain. Initial BMP signaling began as an anterior stripe (white brackets) along the

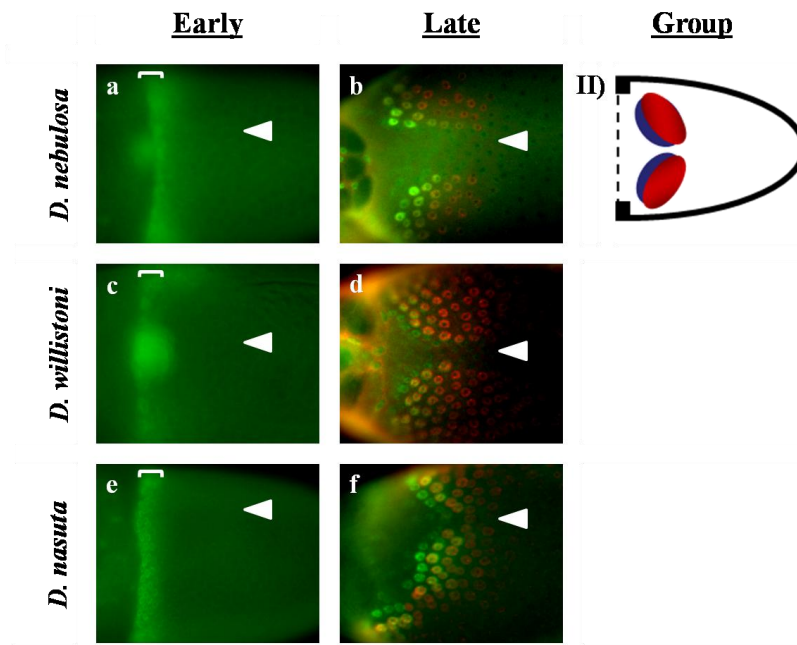
nurse cells oocyte border (**a, c, and e**). Late BMP signaling in *D. yakuba*, *D.*

pseudoobscura, and *D. funebris*, was removed from the dorsal midline and appeared in

dorsolateral patches that overlap with roof cells (**b, d, and f**) (cartoon group I, red

domain). White arrowhead denotes the dorsal midline, all images dorsal up and anterior

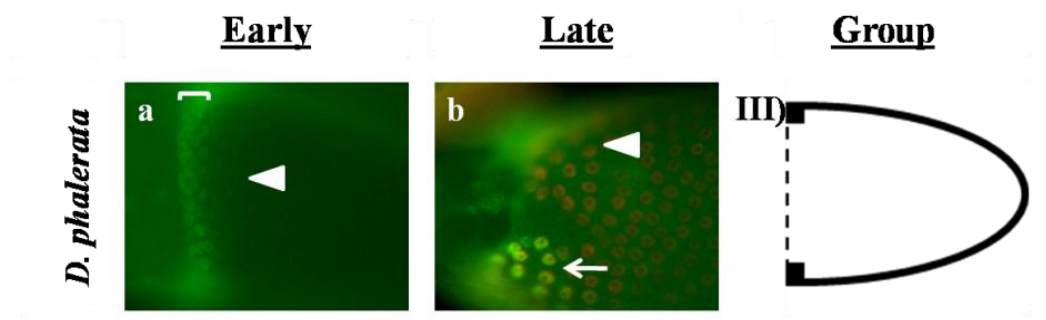
to the left.

Figure 10:

P-MAD (**Green**); BR (**Red**)

Figure 10: Group II, *Drosophila* species with late BMP signaling in both roof and floor domains. Initial BMP signaling began as an anterior stripe (white brackets) along the nurse cells oocyte border (**a, c, and e**). Late BMP signaling in *D. nebulosa*, *D. willistoni*, and *D. nasuta* cleared from the dorsal midline and appeared in the roof cells (red) and adjacent floor (green) domain (**b, d, and f**) (cartoon group II, red-roof and blue-floor domains). White arrowhead denotes the dorsal midline, all images dorsal up and anterior to the left.

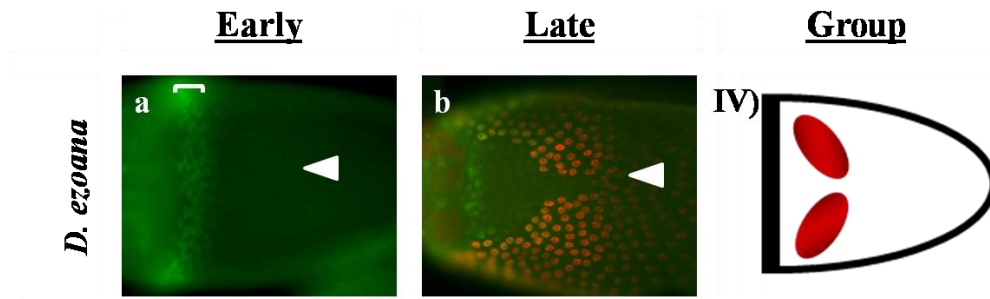
Figure 11:



P-MAD (Green); BR (Red)

Figure 11: Group III, *Drosophila* species lacking a dorsolateral extension of lateBMP signaling. Initial BMP signaling began as an anterior stripe (white bracket) along the nurse cells oocyte border (a). Late BMP signaling in *D. phalerata* cleared from the dorsal midline and remains absent in roof and floor cells (b) (cartoon group III, black). White arrowhead denotes the dorsal midline; white arrow refers to anterior signaling outside the midline, all images dorsal up and anterior to the left.

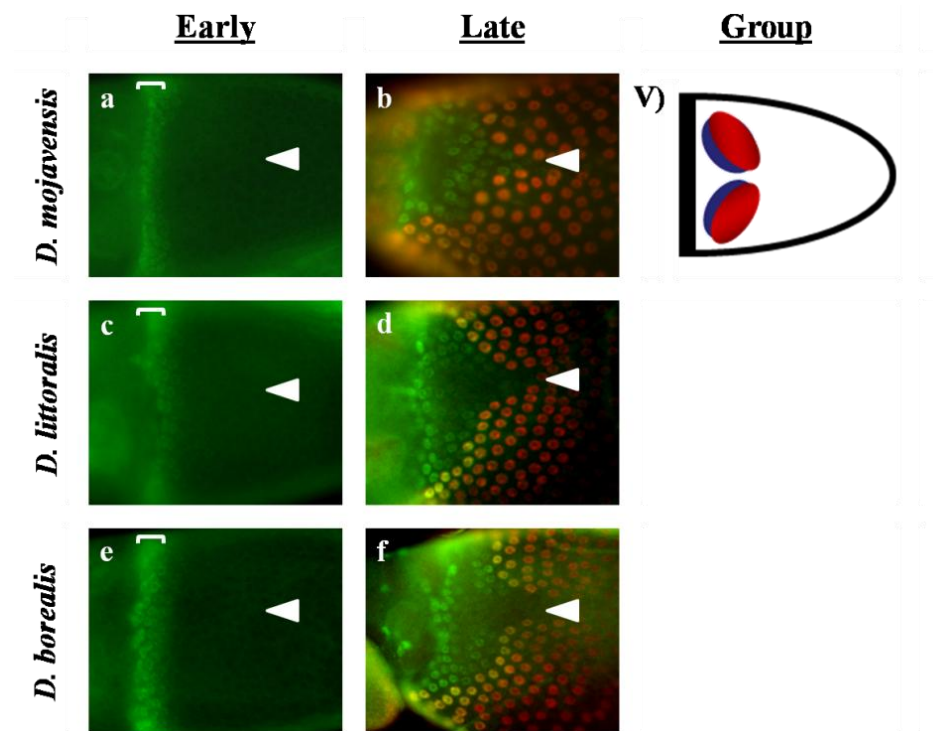
Figure 12:



P-MAD (**Green**); BR (**Red**)

Figure 12: Group IV, *Drosophila* species with late BMP signaling in the dorsal-anterior and roof cells. Initial BMP signaling began as an anterior stripe (white bracket) along the nurse cells oocyte border (**a**). Late BMP signaling in *D. ezoana* remained in the dorsal-anterior and appeared in the roof domain (**b**) (cartoon group IV, black-anterior and red-roof). White arrowhead denotes the dorsal midline, all images dorsal up and anterior to the left.

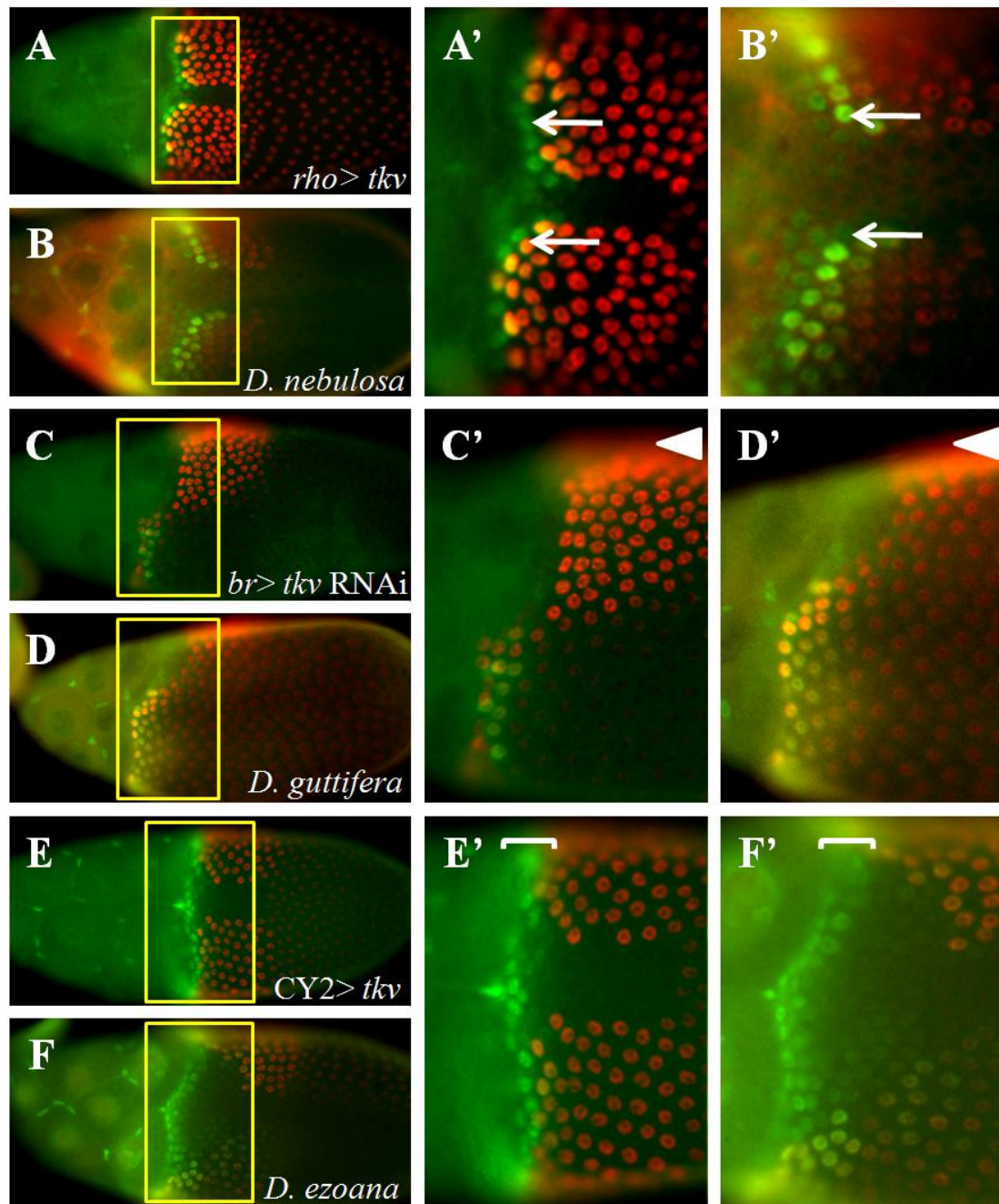
Figure 13:



P-MAD (**Green**); BR (**Red**)

Figure 13: Group V, *Drosophila* species with late BMP signaling in the dorsal-anterior, roof and floor domains. Initial BMP signaling began as an anterior stripe (white brackets) along the nurse cells oocyte border (**a, c, and e**). Late BMP signaling in *D. mojavensis*, *D. littoralis*, and *D. borealis* was maintained in the dorsal-anterior and appeared in both the roof and floor domains (**b, d, and f**) (cartoon group V, black-anterior, red-roof and floor-blue). White arrowhead denotes the dorsal midline, all images dorsal up and anterior to the left.

Figure 14:



P-MAD (Green); BR (Red)

Figure 14: Variation in *tkv* patterning accounts for BMP signal diversity. (A, A')

Expression of *tkv* in floor domain in *D. melanogaster* was sufficient to induce signaling on the floor domain. **(B, B')** This pattern was similar to natural signaling in *D. nebulosa* (white arrows point to the floor domain of P-MAD). **(C, C')** Depleting *tkv* from roof domain eliminated signaling from this domain (arrowhead). **(D, D')** This pattern was similar to species represented by *D. guttifera*. **(E, E')** Uniform expression of *tkv* maintained P-MAD in the dorsal-anterior region (bracket). **(F, F')** This pattern was similar to species represented by *D. ezoana*. Dorsal views (**A, B, E, and F**), lateral view (**C, D**). In all images anterior is to the left.

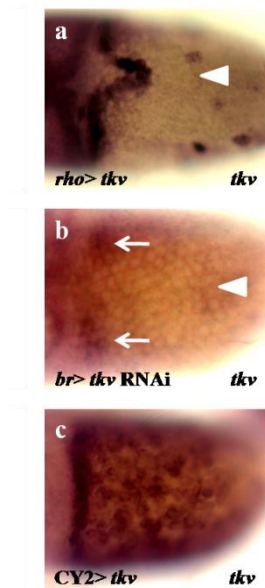
Figure 15:

Figure 15: Ectopically expressed *tkv* patterns. *tkv* driven by the *rho*-GAL4 driver expressed *tkv* on the floor domain (a). *tkv* was removed from the roof domain by expressing a *tkv* RNAi with the *br*-GAL4 driver (b), white arrows point to anterior expression of *tkv* outside the dorsal midline. *tkv* was expressed uniformly by using the CY2- GAL4 driver (c). White arrowhead denotes the dorsal midline, anterior to the left.

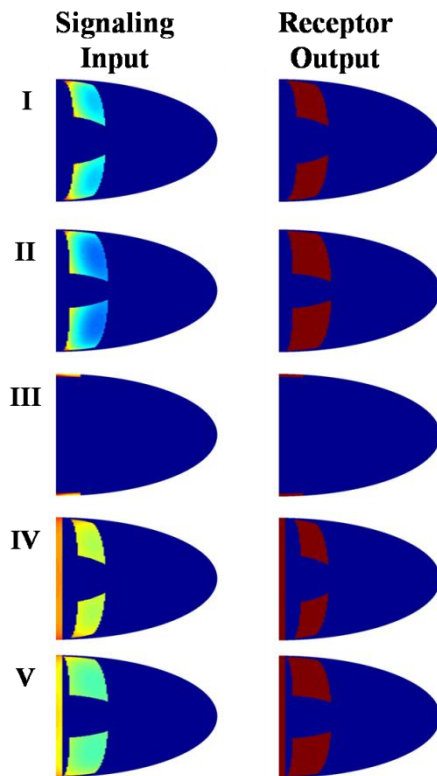
Figure 16:

Figure 16: Computational predictions of BMP receptor patterning. Two-dimensional model of BMP signaling in five species of *Drosophila* which represent the five BMP signaling patterning groups (Fig. 8). Ligand is secreted from the anterior (left side) and captured by nonuniform expression pattern of a receptor. In this case, the simulation recapitulates the corresponding patterns of P-MAD by altering the distance in which DPP can travel in the presence of a receptor; Group I: 1-2 cells, groups II and III: 2-3 cells, and groups IV and V: 4-5 cells; suggesting that in the presence of a receptor, DPP can diffuse to different ranges before internalization and degradation upon binding to the receptor. Based on the recapitulated patterns of BMP signaling, the patterns of *tkv* were simulated to reflect the spatial distribution of signaling. In all images, dorsal is up and anterior is to the left.

Figure 17:

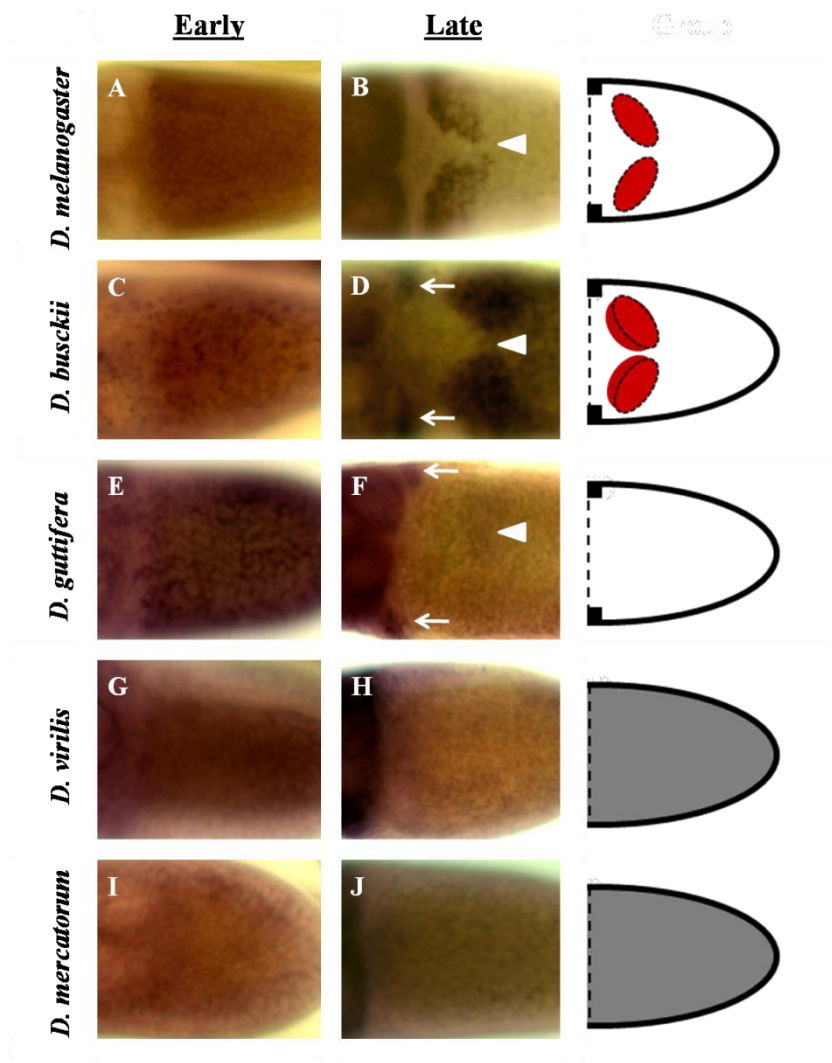


Figure 17: Expression of *tkv* is diverse across *Drosophila* species. Early *tkv* in all species was uniformly expressed (**A, C, E, G, and I**). Late *tkv* expression patterns diverged into three unique groups represented by (**B**) *D. melanogaster*, (**D**) *D. busckii*, and (**F**) *D. guttifera*. In species represented by *D. melanogaster*, *tkv* was expressed on the roof domain and in an anterior band without its dorsal domain (**B, and cartoon**). In the *D. busckii* group, *tkv* was expressed in the roof cells and in the floor domain (**D, and cartoon**). Expression of *tkv* in the *D. guttifera* group was restricted to the anterior without the dorsal domain (**F, and cartoon**). For the two groups represented by *D. virilis* and *D. mercatorum*, *tkv* remained uniformly expressed (**H, J, and cartoons**). White arrowhead denotes dorsal midline. White arrow points to anterior expression of *tkv* in **D** and **F**. In all images, dorsal is up and anterior is to the left.

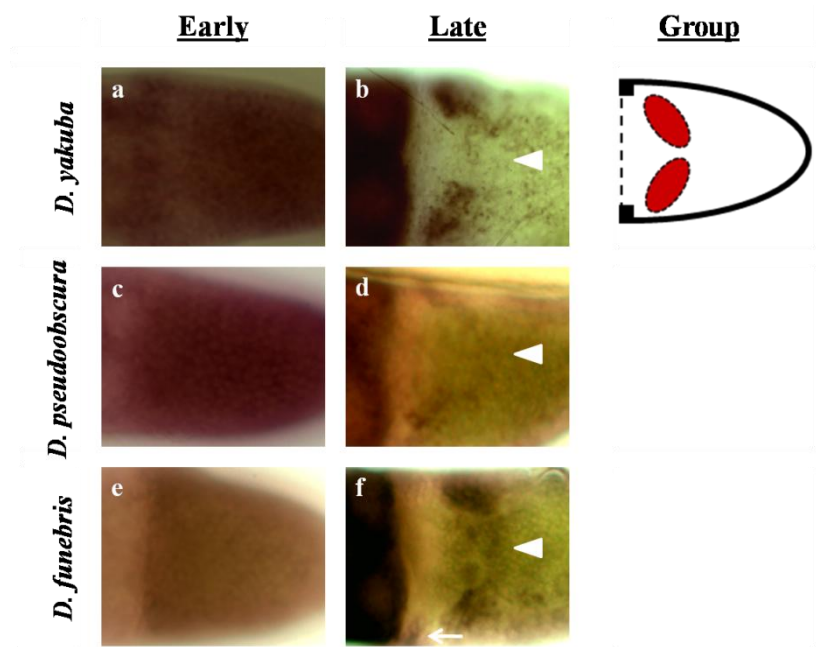
Figure 18:

Figure 18: *Drosophila* species with late *tkv* in roof domain. Initial *tkv* was expressed uniformly (**a, c, and e**). Late *tkv* for *D. yakuba*, *D. pseudoobscura*, and *D. funebris* was removed from the dorsal midline and appeared in the roof domain (**b, d, and f**) (cartoon red domain). White arrowhead denotes the dorsal midline, white arrow refers to anterior *tkv* expression outside the midline (cartoon black squares). In all images, dorsal is up and anterior is to the left.

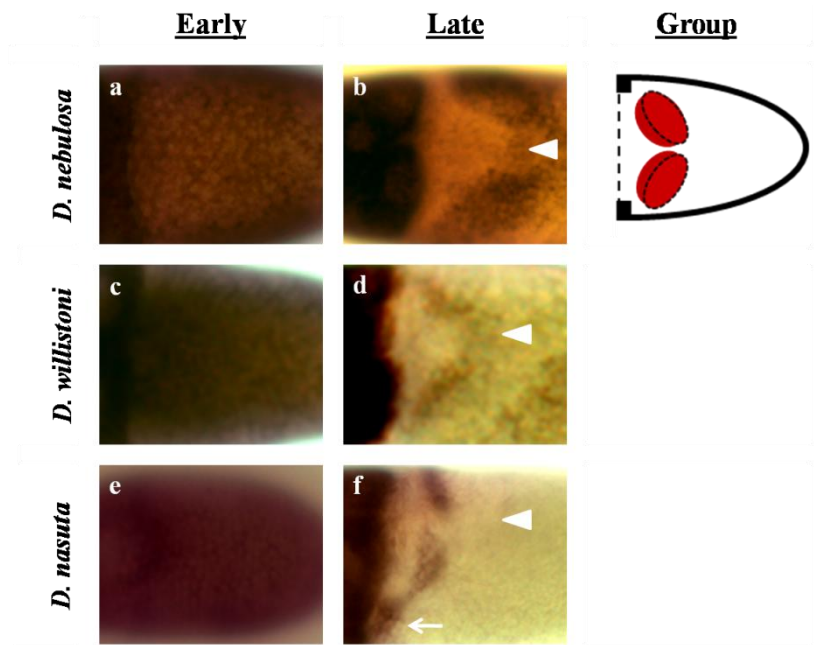
Figure 19:

Figure 19: *Drosophila* species with late *tkv* in roof and floor domains. Initial *tkv* was expressed uniformly (**a, c, and e**). Late *tkv* for *D. nebulosa*, *D. willistoni*, and *D. nasuta* is removed from the dorsal midline and appeared in the roof and floor domains (**b, d, and f**) (cartoon roof-red outlined in black). White arrowhead denotes the dorsal midline, white arrow refers to anterior *tkv* expression outside the midline (cartoon black squares). In all images, dorsal is up and anterior is to the left.

Figure 20:

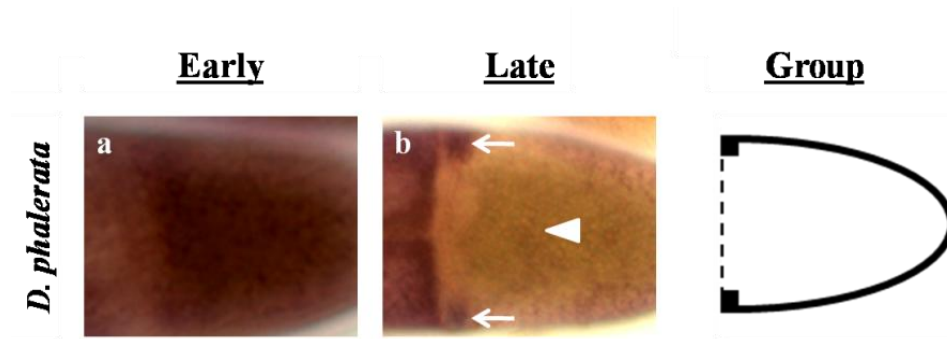


Figure 20: *Drosophila* species with late *tkv* restricted to the anterior without the dorsal domain. Initial *tkv* was expressed uniformly (**a**). Late *tkv* for *D. phalerata* was removed from the dorsal midline and appeared restricted to the anterior (**b**). (cartoon black squares). White arrowhead denotes the dorsal midline; white arrow refers to anterior *tkv* expression outside the midline. In all images, dorsal is up and anterior is to the left.

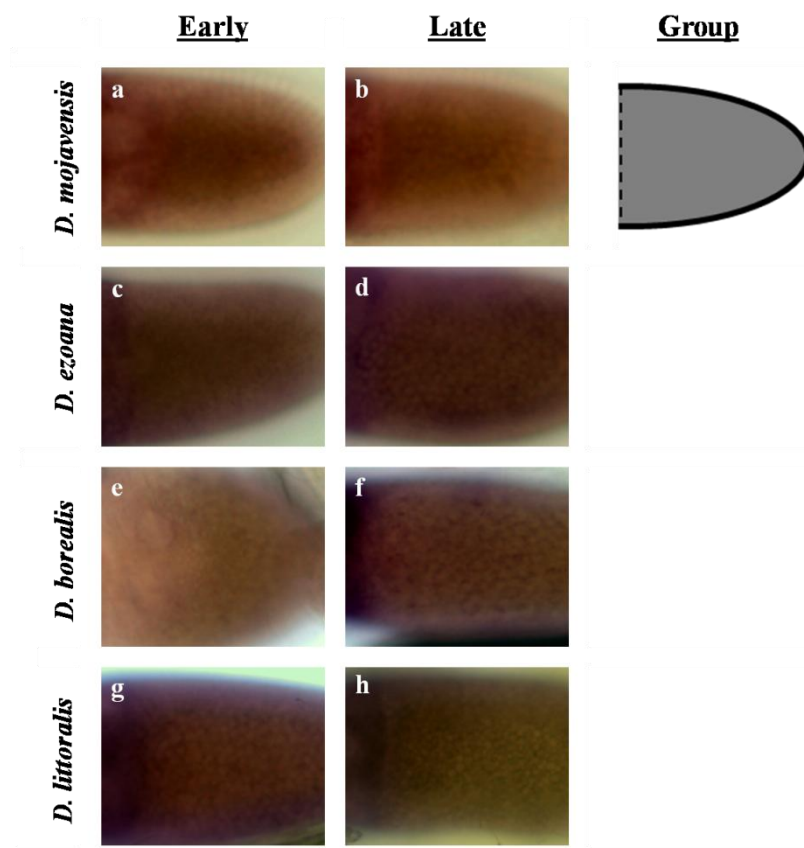
Figure 21:

Figure 21: *Drosophila* species with late *tkv* uniform expressions. Initial *tkv* was uniformly expressed (**a, c, e, and g**). Late *tkv* for *D. mojavensis*, *D. ezoana*, *D. littoralis* and *D. borealis* remained uniform (**b, d, f, and h**) (cartoon gray).