POSITIVE FEEDBACK REGULATION OF *WISHFUL THINKING* SHAPES BONE MORPHOGENETIC PROTEIN SIGNALING IN THE FOLLICULAR EPITHELIUM

by

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ABSTRACT OF THE THESIS

Positive feedback regulation of *wishful thinking* shapes Bone Morphogenetic Protein signaling in the follicular epithelium

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Morphogenesis is preceded by extensive gene patterning that is regulated by a small number of signaling pathways. One such pathway, the Bone Morphogenetic Protein (BMP) pathway, is an essential developmental regulator in many organisms throughout Eukarya, including *Drosophila melanogaster*. An established system to study patterning that is regulated by cell-to-cell signaling is *D. melanogaster* oogenesis. A two-dimensional monolayer of follicle cells (FCs) surrounds the developing oocyte, becomes patterned and later folds into eggshell structures; this process is regulated, in part, by BMP signaling. While BMP signaling in the FCs is associated with the patterning of the type I receptor, *thickveins* (*tkv*), the cooperating type II receptor remains unknown. Here, I aimed to establish its identity. Though previously only reported during embryonic neurogenesis, I found that the type II receptor, *wishful thinking* (*wit*), is expressed in an evolutionary conserved pattern in the FCs of species that diverged over 45 million years. Also, WIT is self-regulated by a positive feedback loop, which I demonstrate works to sharpen the BMP signaling gradient. Of importance, null clones of

WIT coincide with a cell autonomous loss of BMP signaling, monitored by the phosphorylated form of the intercellular signaling molecule MAD (P-MAD). Loss of WIT changes the patterning of Broad, a marker for dorsal appendages, affecting eggshell morphology. These results demonstrate an active role for WIT in non-neuronal tissue, and establish WIT as the type II receptor working in concert with TKV during FC patterning.

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INTRODUCTION

Tissue development follows an extensive patterning of genes. These patterns are controlled by gradients of morphogen, or ligand, within the tissue. Morphogens diffuse from a localized source and naive cells respond to the gradient in a dose-dependent manner. By integration of multiple gradients, cells express genes specific to their spaciotemporal positioning (Kerszberg and Wolpert, 2007). The motivation for studying these developmental pathways is that they are highly conserved across animals. Also, malregulation is often associated with various developmental diseases (Massague, 2008; Ybot-Gonzalez et al., 2007).

The Bone Morphogenetic (BMP) pathway is a well conserved developmental pathway, serving as a morphogen within various aspects of development (Hogan, 1996; Wu and Hill, 2009). It belongs to the highly conserved TGF-beta super-family of signaling molecules. The BMP ligand diffuses through the tissue and establishes a gradient (Affolter and Basler, 2007). This ligand is perceived by cells expressing BMP type I and type II receptors. Upon ligand binding, the constitutively activated type II receptor forms a complex with the type I receptor, which is in turn phosphorylated. The type I receptor then activates a cytosolic signaling molecule, R-Smad (mother against Dpp in *Drosophila*, P-MAD). P-MAD associates with a coSmad (medea in *Drosophila*, MED). Together this complex translocates to the nucleus and serves as transcription factors for target genes (Affolter and Basler, 2007; Baker and Harland, 1997; Massague et al., 2000; Parker et al., 2004). While the BMP intercellular signaling cascade is well

understood (Fig. 1), the coordination between multiple components in the tissue remains largely unknown.

Drosophila melanogaster oogenesis is a well established system for studying signaling and patterning of the fly eggshell (Berg, 2005). A cluster of sixteen cells forms a cyst, with one cell destined to become the oocyte. The other fifteen cells, called nurse cells, provide the developing oocyte with essential mRNAs and proteins (Horne-Badovinac and Bilder, 2005). Approximately one-thousand somatic epithelial cells surround the oocyte and become patterened approximately half way through the three day process of eggshell development (Spradling, 1993). Two main signaling pathways are involved in follicle cell patterning, the epidermal growth factor receptor (EGFR) pathway (Neuman-Silberberg and Schupbach, 1993; Neuman-Silberberg and Schupbach, 1994; Queenan et al., 1997) and BMP (Deng and Bownes, 1997; Twombly et al., 1996). Following an extensive patterning of genes, the follicle cells go on to form the protective eggshell, including two dorsal appendages, breathing tubes for the developing embryo (James and Berg, 2003; Ward and Berg, 2005; Ward et al., 2006). This system can be utilized to study the type II receptor of the BMP signaling pathway and its function during eggshell patterning and morphogenesis.

The dynamics of BMP signaling were recently established during oogenesis. Early BMP signaling, monitored by phosphorylated Mad (P-MAD), displays only AP polarity. Later, P-MAD acquires a clear DV polarity. These dynamics are associated with the dynamic expression of the type I receptor, Thickveins (TKV). The BMP2/4 homolog, Decapentaplegic (Dpp), is secreted from the anterior and is perceived by follicle cells expressing TKV uniformly (Dobens and Raftery, 2000; Twombly et al.,

1996). The expression pattern of TKV later shifts to two dorsolateral domains on either side of the dorsal midline, which reflects the two BMP signaling domains (Yakoby et al., 2008b).

While Tkv is necessary for BMP signaling, it is not sufficient, since a heteromeric complex of type I and type II receptors is required for signaling (Parker et al., 2004). A screen for genes expressed during oogenesis yielded the pattern of *wishful thinking* (*wit*), a BMP type II receptor (Yakoby et al., 2008a). This was particularly interesting, given that type II receptors are usually uniformly expressed in other tissues. In addition, Wit was previously only known to be involved in axon guidance during embryogenesis. Here I show that this pattern is also reflected by the protein expression of WIT. This pattern is conserved over 45 million years of evolution and is regulated in a positive feedback loop. Loss of WIT corresponds to a loss in BMP signaling, affects patterning of a known dorsal appendage marker, Broad, and changes the morphology of the dorsal appendages and eggshell.

MATERIALS AND METHODS

Genetics

The following stocks were used in this study: wild-type OreR, 55B-gal4 E4-Gal4 and CY2-Gal4 (Queenan et al., 1997), CY2-UAS-*mae(edl)* (a gift from J. Duffy), UAS-*dpp* (a gift from T. Schüpbach), UAS-*dad* (Tsuneizumi et al., 1997), UAS-wit^{2A} (Chang et al., 2008) and UAS-*tkv** (Lecuit et al., 1996). The FLP/FRT mitotic recombination system (Duffy et al., 1998; Xu and Rubin, 1993) was used to generate clones of mutant follicle cells, marked by the absence of GFP. Analysis of Dpp input was conducted with the Mad^{12} allele: FRT^{40A} Mad^{12}/FRT^{40A} ubiGFP;GR1-Gal4 UAS-FLP (a gift from R. Padget). The role of Wit in Dpp Signaling was conducted with the Wit^{G15} allele: FRT^{79} Wit^{G15}/FRT^{79} ubiGFP;e22c-Gal4 UAS-FLP (a gift from M. O'Connor). The *wit*RNAi 865GD & 103808KK lines (VDRC) were balanced together to obtain dosage at levels significant enough to obtain measurable affects. Flies were grown on cornmeal agar; all crosses were completed at 23°C.

In situ hybridization

Localization of the *wit* gene was previously described in a screen of genes involved in *D. melanogaster* oogenesis (Yakoby et al., 2008a). The *wit* gene was cloned from a cDNA library generated with the Stratagene cDNA Synthesis Kit. Degenerate primers were designed by hand using a multiple sequence alignment of several sequenced *Drosophila* species, *D. melanogaster*, *D. pseudoobscura*, and *D. virilis*. Forward primer: CCATCATSCGATCRTCGT. Reverse primer: AGCGBATGCARGAGATGT. In situ

hybridization was performed as previously described (Wang el al., 2006), but without the RNase digestion step.

Immunofluorescence and microscopy

Dissection and fixation was conducted as reported elsewhere (Pacquelet and Rorth, 2005). Primary antibodies: mouse anti-Wit (23C7; 1:500, DSHB), rabbit anti-phosphorylated-Smad1/5/8 (1:3000, a generous gift from D. Vasiliauskas, S. Morton, T. Jessell and E. Laufer), mouse anti-betagalactosidase (1:1000, Promega), and sheep anti-GFP (1:2000, Biogenesis). Secondary antibodies: Alexa Fluor (1:2000, Molecular Probes), and DAPI (1:10000). Images were captured with a Leica DM3000 Compound Microscope. Scanning electron microscopy was conducted with a Leo 1450EP SEM. Images were processed with ImageJ (Rasband, 1997-2009) and Gimp (GNU Image Manipulation Program, 1995-2008).

Quantification of BMP signaling

Signaling was quantified and related to a specific developmental stage. Signaling was recorded as the number of cells that contain detectable signaling, continuously from the anterior. These quantifications were performed in both the dorsal and ventral domains for each stage. For each domain/stage n = 10. Standard error was calculated and laid over a bar graph depicting these quantifications.

RESULTS

wit and WIT are expressed during *Drosophila* oogenesis of various species

In situ hybridization was performed to characterize the expression of *wit*. The gene was determined to display patterning dynamics throughout stages 10-11 of oogenesis. *Wit* was first expressed at stage 10A in a ring along the anterior of the FCs (Fig. 2A). At stage 10B, expression decreased from the most dorsal region and become undetectable by stage 11 (Fig. 2B,C). The WIT protein strongly correlated with these dynamics (Fig. 2E-F). The initial pattern of WIT in the anterior domain has been conserved over 45 million years of speciation, present in *D. busckii* and *D. virilis* (Fig. 3). Interestingly, the pattern of WIT correlated with signaling dynamics. The dorsal expansion of signaling at stage 10B correlated with the loss of detectable dorsal WIT. At stage 11, signaling was no longer detectable in the anterior, and correlated with the dorsal loss of WIT (Fig 4).

WIT is patterned by positive feedback on BMP signaling

WIT was tested as a downstream target of signaling. To investigate this, the Dpp ligand was ectopically expressed in the developing egg chamber's posterior end. This resulted in detectable signaling in the posterior, as well as ectopic WIT within the same domain (Fig. 5A-C). Using the inverse strategy, a known inhibitor of signaling, DAD, was expressed throughout the egg chamber. This resulted in loss of the endogenous anterior signaling, and coincided with a loss of WIT (Fig. 5D-F).

Null clones of MAD, as essential intercellular signaling molecule for BMP signaling, resulted in the cell autonomous loss of WIT expression along the anterior. Clones of BRK, however, did not result in ectopic WIT. All clonal populations of cells were tracked by loss of GFP (Fig. 6).

Positive feedback on receptors refined the BMP signaling gradient

In *D. melanogaster*, the initial stage of BMP signaling has only AP polarity. At stage 10B, signaling began to buckle away from the anterior, resulting in a wider number of cells with detectable P-MAD. By stage 11, signaling was undetectable in the anterior, and had moved onto two dorsolateral patches (Fig. 4A-C). During stage 10B, the ventral domain appeared to refine closer to the anterior, but was insignificant, relative to stage 10A. At stage 11, the ventral side continued to refine, such that the amount of cells with detectable P-MAD decreased. This was quantified and graphed in order to compare signaling during different developmental stages (Fig. 7OreR).

When dorsal midline formation was abolished by expressing CY2>*mae*, the dorsal signaling did not clear from the anterior, and could still be detected in a stage 11 egg chamber. This continued refinement resulted in a dorsal anterior gradient that continued to refine. The perturbed system resulted in stage 11 egg chambers that had equal dorsal and ventral signaling, which was indistinguishable from that of the WT ventral domain (Fig. 7CY2>*mae*).

The continued refinement of signaling in the dorsal domain of the perturbed egg chamber was evident when following dynamics of WIT in WT and CY2>*mae*

backgrounds. WIT expression in CY2>*mae* was indistinguishable from WT ventral expression at the same stage (Fig. 8).

Wit overexpression results in ectopic signaling, defects in patterning and morphogenesis

Expression of WIT in the posterior egg chamber resulted in ectopic BMP signaling in this domain (Fig. 9). Expression of WIT throughout the egg chamber resulted in suppressed levels of Broad in the dorsal FCs, due to the expansion of the dorsal midline. This led to defects in eggshell morphology, including an increased operculum in addition to the lack of dorsal appendages (Figure 10).

Wit is required for BMP signaling in oogenesis

Early signaling appears in an anterior band. Thus, necessary signaling components within this domain result in loss of P-MAD. Null clones of WIT, in the anterior, result in cell autonomous loss of BMP signaling (Fig. 11A). These clones were tracked by loss of GFP, shown in the merge (Fig. 11B). An inset is shown, which highlights a small clone, as well as clear loss of signaling within these cells (Fig 11B').

Wit reductions result in defects in patterning and morphology

Depletion of *wit* by overexpressing *wit*RNAi resulted in the invasion of the Broad domain into the anterior as well as a reduced midline (Fig. 12A,B). This correlates with the phenotype of slightly reduced length dorsal appendages, which lie more parallel to the eggshell (Fig. 12C,D).

DISCUSSION

A type II receptor is required for BMP signaling

Our understanding of BMP dynamics during *Drosophila melanogaster* oogenesis is currently explained by the dynamic patterning of the type I receptor, TKV. Early BMP signaling is based on the anteriorally expressed Dpp interacting with the uniformly expressed TKV, in order to produce an anterior to posterior gradient of signaling. Later, TKV clears from this domain and follows the expression of Broad, which becomes highly expressed in two dorsolateral patches on either side of the dorsal midline (Deng and Bownes, 1997; Yakoby et al., 2008b). While this mechanism adheres to experimental evidence, it still does not account for the type II receptor functioning during oogenesis. The phosphorylation cascade dictates that type II receptors are expressed in order to form a complex with the ligand-type I receptor complex. This is especially important, since the entire complex is taken into the cell by endocytosis, signals, and is targeted for degradation (Parker et al., 2004). I aimed to establish the identity of the type II receptor functioning in the FCs.

The *wit* gene was previously found in a screen of genes expressed during *D. melanogaster* oogenesis (Yakoby et al., 2008a). Interestingly, this gene has previously only been described during neurogenesis, implicated in axon guidance in the developing neuromuscular junction (Aberle et al., 2002; Marques et al., 2002). In the FCs, *wit* expression is correlated with the dynamics of BMP signaling. In addition, I established that the WIT protein expression strongly correlates to this gene patterning. WIT clears from the dorsal half of a stage 10B developing egg chamber. I propose that reduction in

signaling results in the reduction of both WIT and TKV. As a consequence, ligand is no longer restricted to the anterior. Thus, signaling can be found buckling away from the anterior within this domain. The early anterior band pattern of WIT is conserved through *D. virilis*, representing 45 million years of speciation from *D. melanogaster*. This pattern is also found in *D. busckii*, accounting for three different subgenera. Given that this patterning is conserved throughout evolution, this suggests an importance of WIT towards the function of BMP signaling. Particularly, the early band of WIT suggested WIT as a potential downstream target of BMP signaling.

WIT is patterned by BMP signaling

WIT was tested as a target of signaling through several approaches. The Dpp ligand was ectopically expressed in the posterior of a developing egg chamber, a domain that does not consist of any BMP signaling in the wild type *D. melanogaster*. This resulted in detectable signaling in this domain, and of particular interest, WIT. The formation of signal in this domain is explained by the early low level uniform expression of both *tkv* and *wit*. The presence of ectopic ligand simply drives the interaction between these two receptors and drives the system further, in a positive feedback, since TKV and potentially WIT are downstream targets. However, since this is a non-endogenous domain for signaling, it was important to test the effects of loss of endogenous signaling in the anterior. This was accomplished by overexpression of DAD, a known antagonist of signaling. This perturbation resulted in loss of any detectable signaling, as well as loss of WIT. It still is not clear though if this was a cell autonomous effect.

To address this point, I generated null clones for MAD, using FLP/FRT mediated meitotic recombination (Duffy et al., 1998; Xu and Rubin, 1993), monitored by GFP. Clones along the anterior resulted in a cell autonomous loss in signaling, detected by the loss of P-MAD and more importantly, this was also the case for WIT. In a sea of cells rendered incapable of conferring the intercellular BMP signal, two wild type cells remained. This island of cells expressed WIT, unlike the surrounding perturbed cells (Fig. 6A-D). The genetic mechanism controlling expression of WIT by BMP became an important question.

BMP signaling targets have been shown to be products of induction or of repression of a repressor. The first scenario is where high levels of P-MAD correlate to the expression of target genes, such as Dad (Tsuneizumi et al., 1997). The latter occurs when targets are repressed by a gene known as Brinker. Brinker is described as a transcriptional repressor of BMP target genes. P-MAD alleviates this repression by turning off the expression of Brinker (Bangi and Wharton, 2006; Winter and Campbell, 2004). I tested if this is the case with WIT expression in the ovary by generating null clones of Brinker away from the anterior. If WIT expression was the product of de-repression of Brinker, ectopic WIT should be detected in clones. This was not the case, as WIT expression was not detected in any clones of Brinker that were generated (Fig. 6E,F). Therefore, WIT expression is not dependant on repression of Brinker. Therefore is likely due to induction by BMP signaling. Since WIT is regulated in a positive feedback loop by BMP, I reasoned that there was likely a role for the feedback. In fact, the type I receptor, TKV, was also regulated in a similar way, as predicted by a mathematical model of BMP signaling in oogenesis (Lembong et al., 2008; Lembong et

al., 2009). Therefore I aimed to further investigate the role of positive feedbacks on receptors within this system.

Positive feedback on receptors works to refine the BMP signaling gradient

Uri Alon, as well many others, has proposed a need for establishing a robust morphogen gradient throughout a tissue. This is especially important during development, given that development involves gradients that must remain undisrupted by the external environment, as well as compensate for mutations affecting gene dosage. This is possible by a self regulating network motif that creates a morphogen gradient that follows a power law, as opposed to an exponential decay. This is accomplished in one of two ways, negative feedback at the level of an inhibitory receptor, or positive feedback at the level of a receptor (Alon, 2007). With BMP, the case seems to be the latter. Further, it was predicted that over time, this feedback will actually shift the gradient towards the localized source.

Since BMP signaling is known to clear from the dorsal side, focus was placed on the wild type ventral domain of signaling. For the first time, the signaling gradient was quantified, by counting the number of cells with a continuous level of detectable P-MAD, beginning at the anterior. There is no significant difference between the dorsal and ventral domains at stage 10A, with signaling being the width of two to three cells (Fig. 7). The lack of any DV polarity at this stage supports the idea that early BMP signaling is independent of other signaling pathways (Berg, 2005; Shravage et al., 2007). By stage 10B, the dorsal signal expands and buckles away from the anterior, to between four and five cells. At this time, the ventral signaling begins to narrow and is marginally different

in significance from the WT stage 10A ventral, at a fairly regular two cells. By stage 11, signaling is almost finished clearing from the dorsal anterior domain. However, ventral signaling refines to a number of cells significantly less than stage 10A, between one and two cells.

Given that there was a significant difference between stage 10A and 11 on the ventral side, I reasoned that I should witness a similar refinement if the dorsal clearing was canceled via a genetic perturbation. As predicted, I found no significant difference between WT ventral and the dorsal side of egg chambers that lacked a dorsal clearing. By canceling midline repression, the dorsal side was allowed to continue refining, much like the endogenous ventral side. Of particular importance, the same is evident for the pattern of WIT, as it is a target for BMP signaling. WIT remains on the dorsal side in the perturbed system and also refines, similar to WT ventral side. This pattern is substantially different from the wider pattern of WIT at stage 10A.

Wit affects eggshell signaling, patterning, and morphogenesis

Overexpression of WIT resulted in ectopic signaling wherever it was expressed (Fig. 9). This is likely caused by placing the type II receptor well beyond the low level background that is present in the wild type. This results in a concentration of type II receptors that is so high that it enables interaction with the type I receptor in a ligand-independent manor. By driving expression of WIT uniformly in the follicle cells, Broad appears in a pattern that resembles constitutively active type I receptors or overexpression of Dpp (Deng and Bownes, 1997; Shravage et al., 2007; Twombly et al., 1996; Yakoby et

al., 2008b; Zartman et al., 2008). The midline between Broad was greatly expanded and the level of Broad was maintained at basal levels (Fig. 10A). This directly corresponds to the eggshell morphology. With a large midline, the operculum is expanded to almost half of the egg chamber. Since high levels of Broad mark the roof of the dorsal appendages (Berg, 2005; Deng and Bownes, 1997), without this high expression no appendages are formed (Fig. 10B). This however only shows effect of overexpressed WIT on BMP signaling. More importantly, I aimed to test the necessity of endogenous levels of WIT during WT oogenesis.

Wit is required for BMP signaling, patterning, and morphogenesis of the *Drosophila melanogaster* eggshell

Given the conserved pattern and positive feedback, I aimed to establish if WIT was involved in BMP signaling. Indeed, clones of null WIT along the anterior resulted in a cell autonomous loss of signaling (Fig. 11). RNAi was utilized to reduce levels of *wit* transcript throughout the egg chamber. Of note, a single copy of *wit*RNAi was not sufficient to reduce BMP signaling; therefore, I used two copies in order to produce a substantial effect on patterning and morphology. This is either due to the weakness of the RNAi lines or the strength of the positive feedback on WIT. Since I did not quantify the levels of *wit*RNAi or *wit* expression in these backgrounds, either case is possible. I found a shift in Broad in the opposite direction of BMP overexpression. Broad appears to shift anteriorally, with more cells expressing high levels of Broad. In addition, the midline was repressed and the Broad patches shift closer to each other. This highlights the negative regulation of BMP on Broad. These changes are also accompanied by changes

in eggshell size and shape. This suggests that BMP signaling may also play a role in eggshell morphology, even further away from the anterior. Dorsal appendages appear to be shorter and more forward facing. This effect is potentially the result in expression defects of extracellular matrix proteins shown to play a role in dorsal appendage formation, which have inputs from both EGFR and BMP, such as Cad74A (Zartman et al., 2008).

Concluding statements

I have shown that WIT is both necessary and sufficient to confer BMP signaling and patterning of target genes. WIT is patterned in an evolutionary conserved anterior band in the follicle cells of *Drosophila* species. The pattern likely arises due to the positive feedback at the level of receptors. Since I show that WIT is necessary for signaling, it is likely that low uniform levels of WIT expression are present before being fed back on. Indeed, with hindsight, this is apparently the case with uniform low levels of gene detected in the *in situ* hybridizations. A simple model shows the early patterning of receptors and signaling, as well as diagrams the established positive feedbacks on receptors (Fig. 13). I make no conclusions about the other type II receptor, PUT. In fact, others have described the need for this other type II receptor as well (Brummel et al., 1994; Childs et al., 1993; Crickmore and Mann, 2006; Gibson and Perrimon, 2005; Haerry et al., 1998; Ruberte et al., 1995; Twombly et al., 1996). It might be the case that levels of both type II receptors are required before the positive feedback on WIT occurs. Future work will be required to address whether or not the two type II receptors are functioning together in a complex with TKV.

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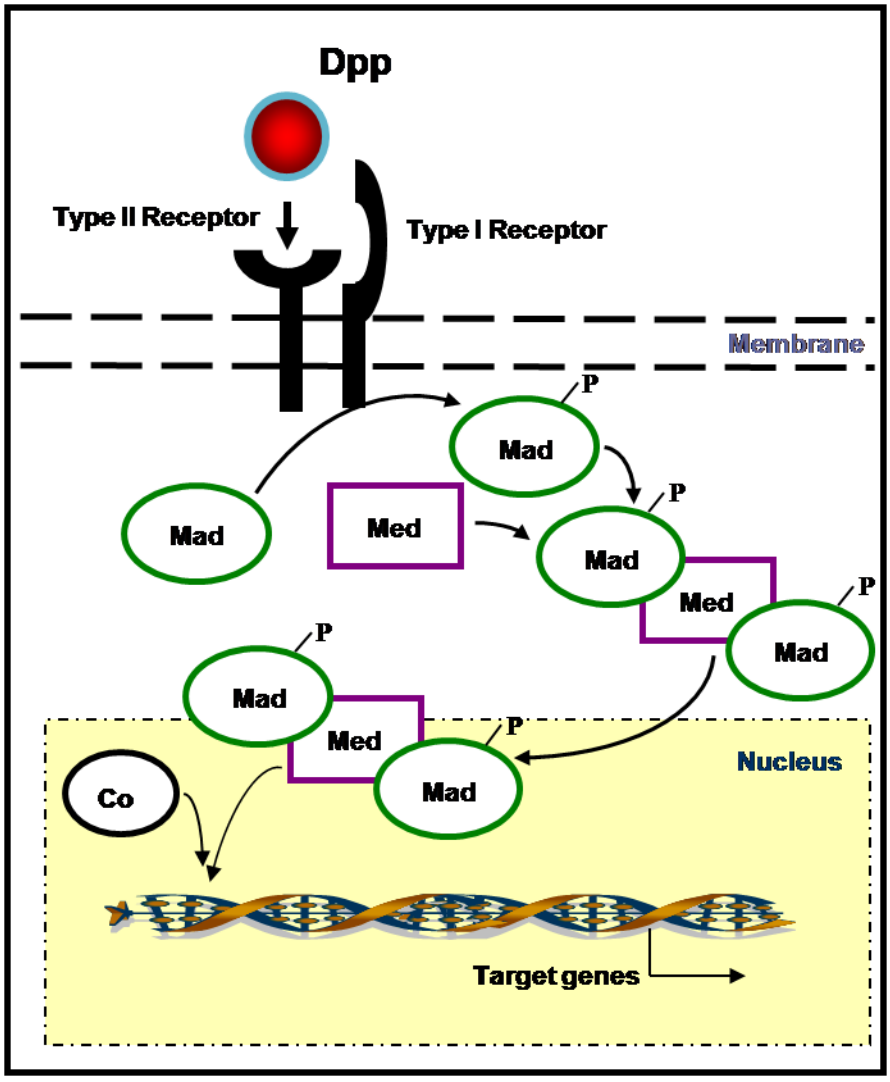

Figure 1. The BMP pathway. Depicted is a diagram of the signaling cascade in BMP signaling. P-MAD was used to monitor BMP signaling.

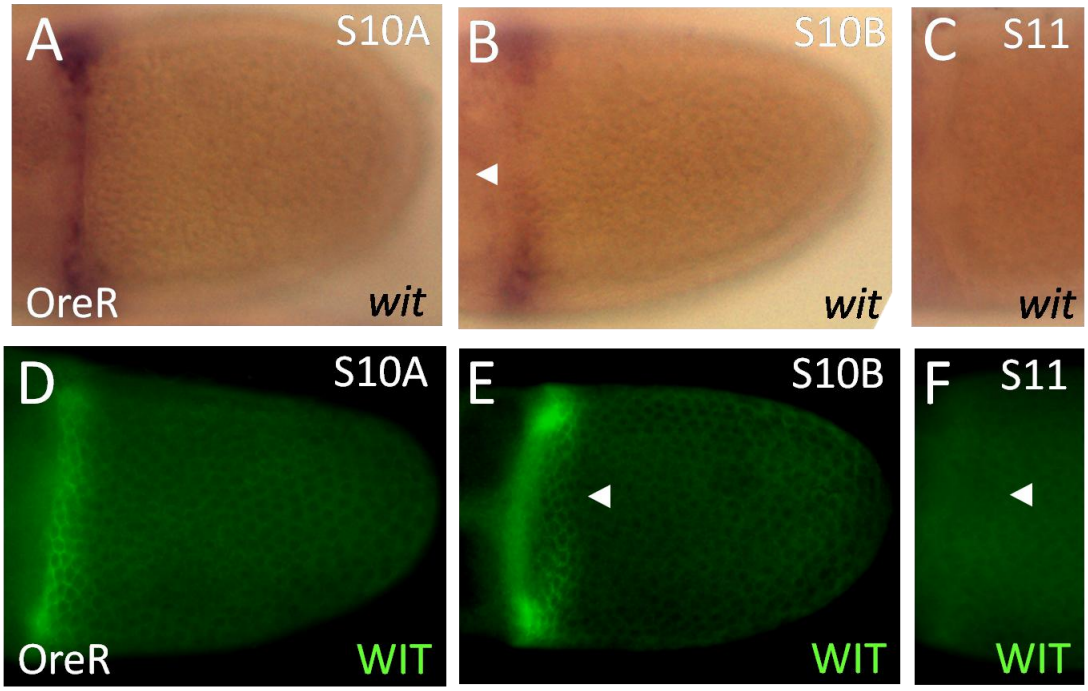

Figure 2. *wit* / WIT are dynamically patterned. *wit* was expressed in an anterior band in the follicle cells during stage 10A (**A**), at stage 10B (**B**) cleared from the dorsal side (marked by triangle), and was undetectable by stage 11 (**C**). The WIT protein strongly followed this transition (**D-F**).

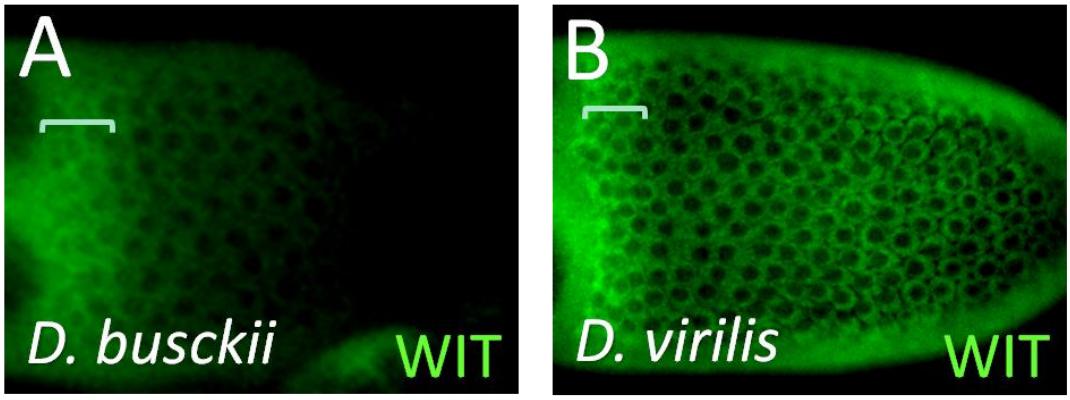

Figure 3. The anterior band of WIT was maintained throughout evolution. WIT was detected with the *D. melanogaster* antibody in a similar anterior pattern (marked by bracket) in two other *Drosophila* species. *D. virilis* represents ~45 million years of evolution (**A**-**B**).

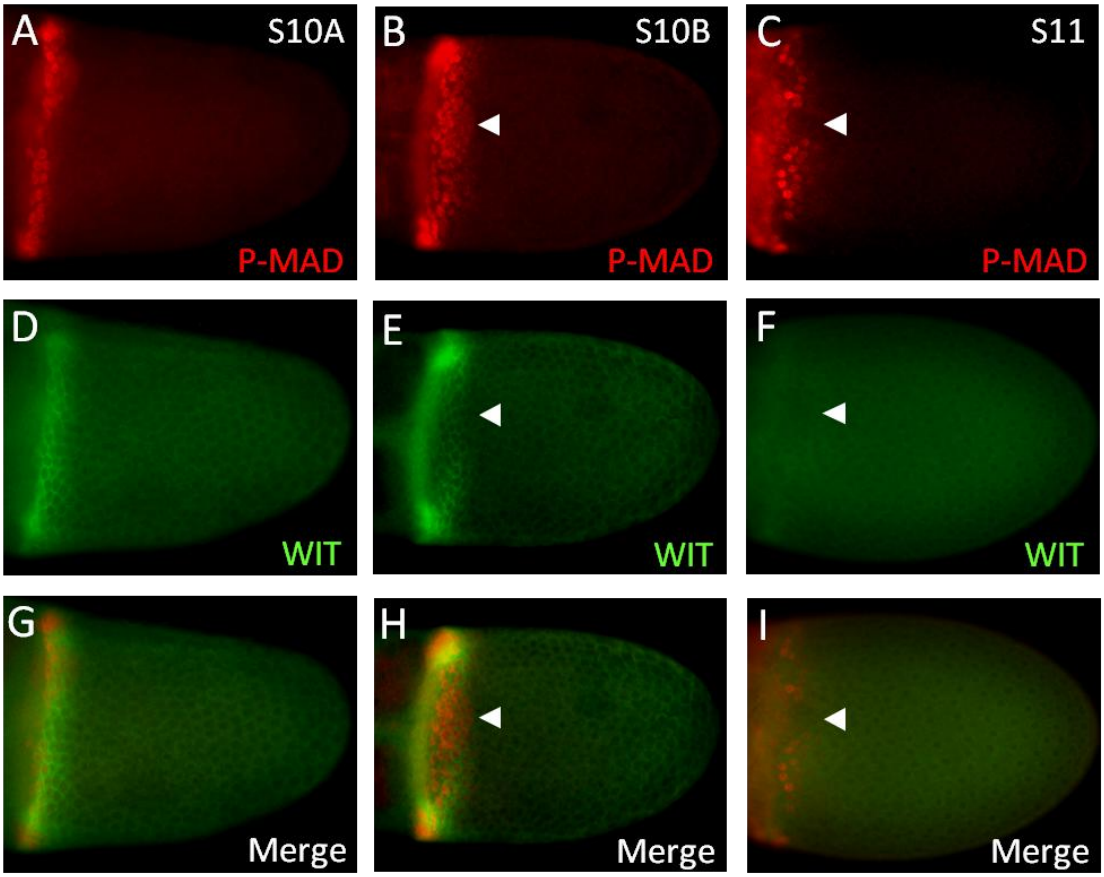

Figure 4. Patterning was correlated with known BMP dynamics. The band of WIT (**D**) was reflected by the band of P-MAD (**A**). As signaling buckled away from the anterior on the dorsal side (marked by triangle) (**B**), WIT cleared from this domain (**E**). By stage 11, P-MAD moved onto the patches clears in the anterior (**C**). WIT completely cleared from the dorsal side (**F**). Merges are shown for clarity (**G-I**).

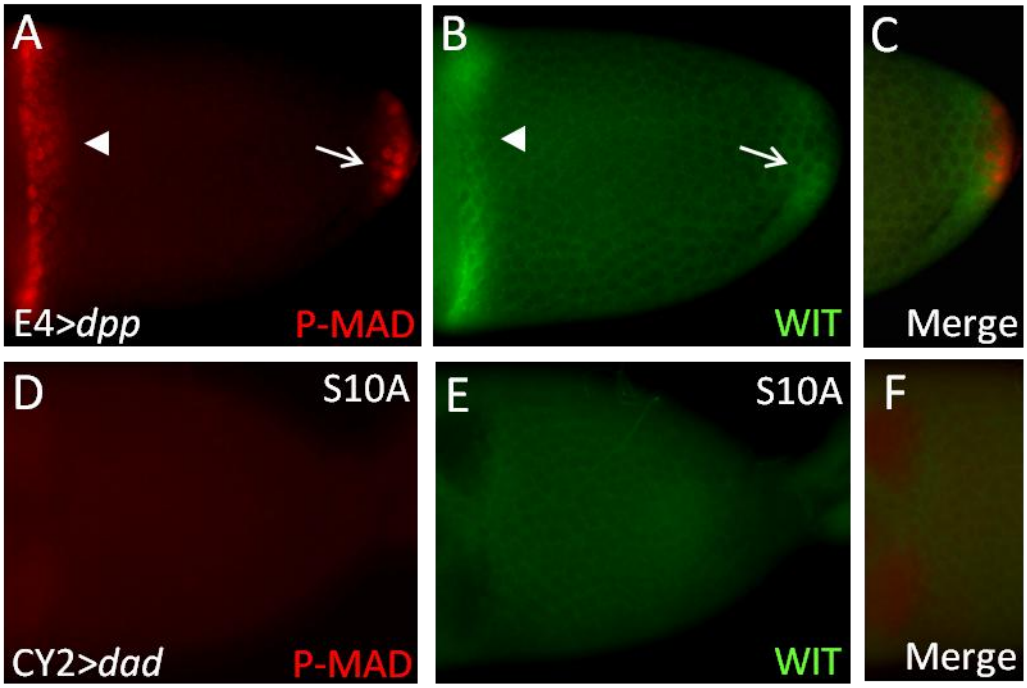

Figure 5. WIT was induced through positive feedback on BMP signaling. Ectopic ligand produced signaling in the posterior (**A**), and resulted in ectopic WIT (**B**). Overexpression of DAD, a signaling inhibitor, resulted in loss of endogenous signaling (**D**). This loss of signaling resulted in loss of WIT (**E**). Merges are shown for clarity (**C, F**).

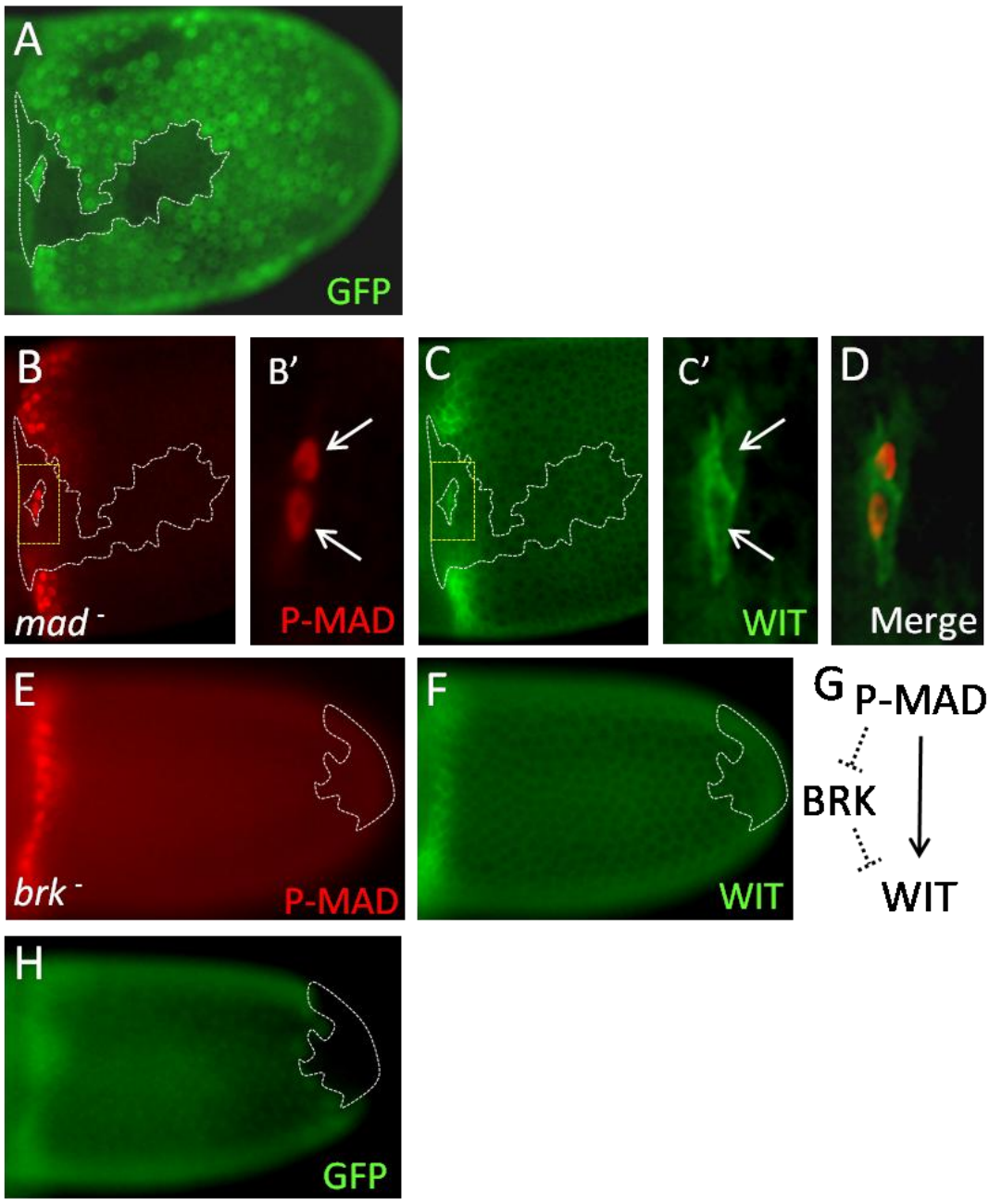

Figure 6. WIT was patterned through induction, not de-repression. Null clones of MAD (followed by loss of GFP (**A**) outlined in dotted white) resulted in loss of signaling (**B**). These cells also cell-autonomously lost WIT (**C**). Insets (marked by the yellow dotted rectangle) highlight two WT cells (arrows), which retained signaling as well as

WIT (**A', B'**). Merge is shown for clarity (**D**). Null clones of BRK (followed by loss of GFP (**H**) outlined in dotted white) did not result in ectopic signaling (**E**) or WIT (**F**). A simple model shows induction of WIT by BMP signaling, not by de-repression (**G**).

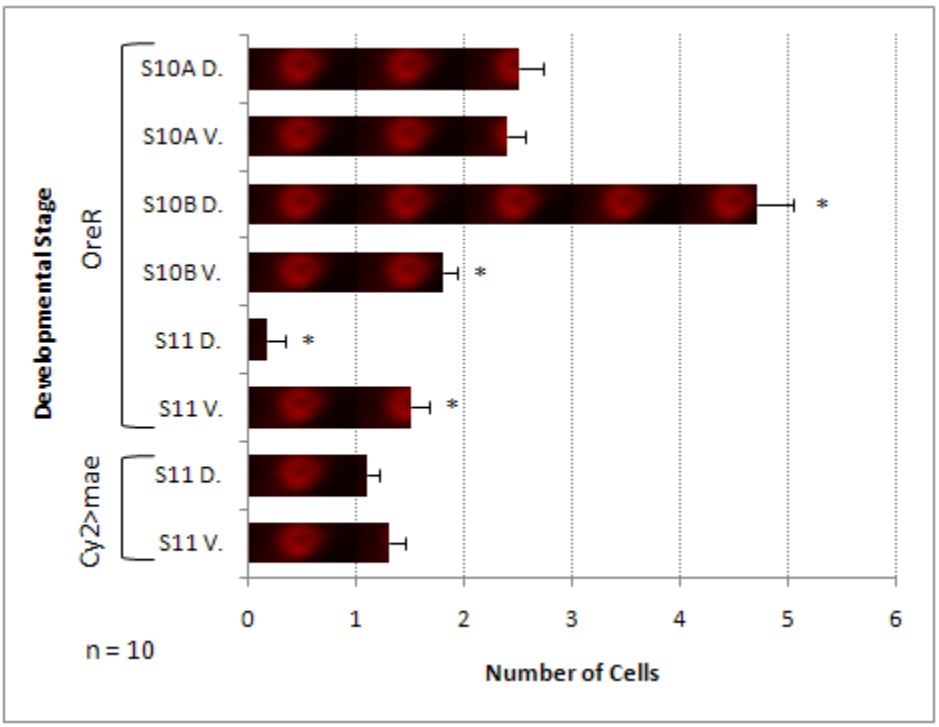

Figure 7. Positive feedbacks function to refine the signaling gradient. Graph depicting quantified cells with anterior signaling over developmental stages in different domains (D = dorsal, V = ventral, n = 10). Asterisk indicates significance from control. For WT (OreR), stage 10A ventral was used as a control. For CY2>*mae*, WT stage 11 ventral was used as a control. The WT ventral signaling was shown to narrow over time. Canceling the dorsal clearing with CY2>*mae* produced signaling refinement so that dorsal and ventral domain are indistinguishable.

T-Test	Comparison	
0.722185826	10AD&V	
2.26978E-07	10BD&V	*
0.176879648	10BV&11V	
0.000113914	11D&V	*
0.388009107	maeV&OV	
0.054371016	maeD&OV	
0.287763068	maeD&V	
0.010722475	10AV&10BV	*
0.001154587	10AV&11V	*

Table 1. T-tests on quantification of P-MAD. Significant differences are denoted by an asterisk. The WT ventral signaling narrowed over developmental time. Canceling of dorsal clearing resulted in dorsal and ventral domains that both had signaling refinement.

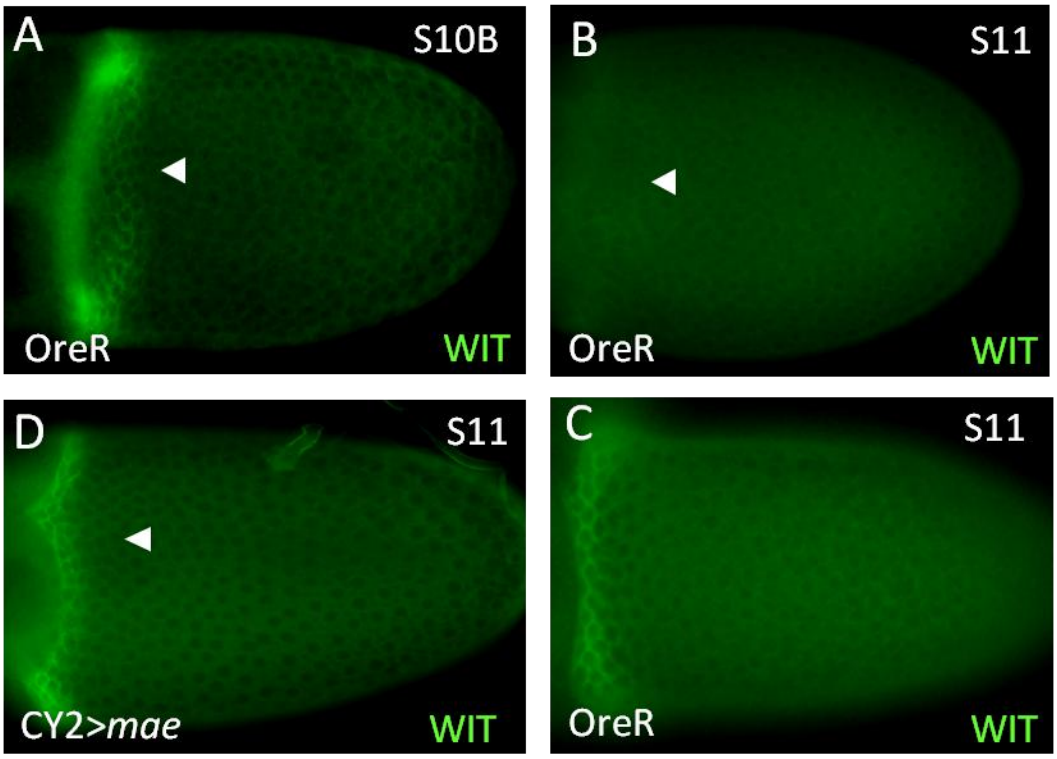

Figure 8. Canceling the dorsal clearing mimiced the WT ventral. The wild type dorsal expression is shown (**A,B**). Without clearing of BMP signal from the dorsal domain, WIT remained in stage 11 (**D**), much like wild type ventral at this stage (**C**).

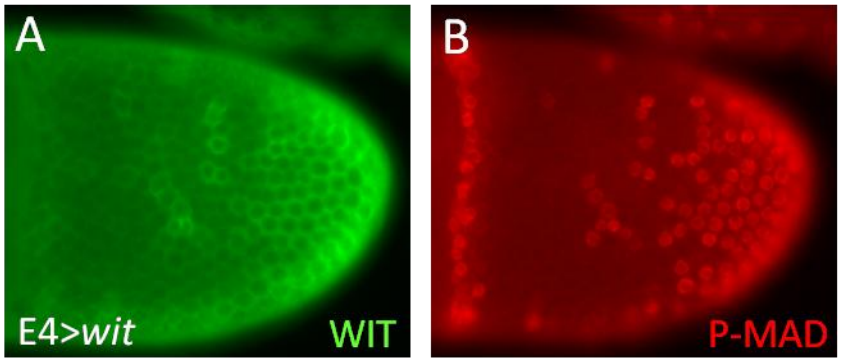

Figure 9. WIT is a limiting factor on BMP signaling. WIT was overexpressed in the posterior and was detected in this domain (**A**). This led to ectopic BMP signaling in the posterior (**B**).

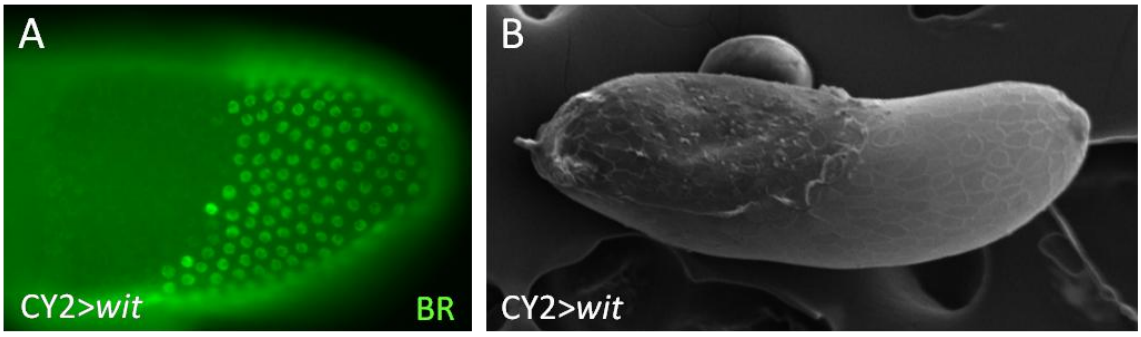

Figure 10. WIT overexpression affected patterning and morphogenesis. Uniform expression of WIT caused a large midline clearing of Broad. High levels of Broad, which usually become part of the dorsal appendages, were abolished (**A**). This was reflected in the eggshell morphology, where an extended midline resulted in a large operculum and lack of high levels of broad resulted in loss of appendages (**B**).

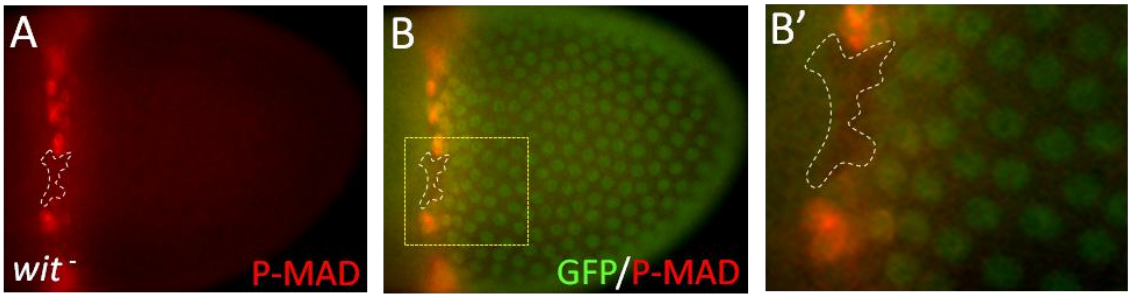

Figure 11. WIT was required for BMP signaling. Null clones of WIT (followed by loss of GFP, shown in merge (**B**), outlined in dotted white) resulted in loss of signaling (**A**). Inset is shown depicting the cell autonomous loss of signaling in clones of WIT (**B'**).

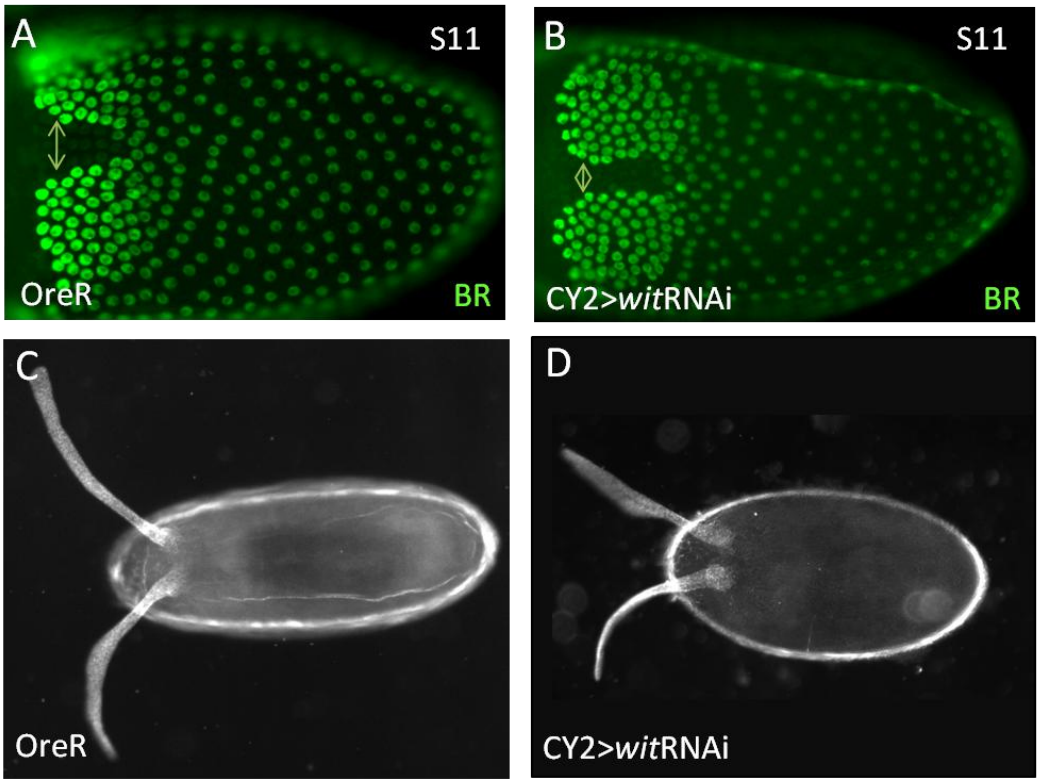

Figure 12. WIT was required for eggshell patterning and morphogenesis. WT patterning of Broad (**A**) and eggshell (**C**) are shown for comparison. Reductions in WIT resulted in invasion of Broad into the anterior and a reduction in the size of the dorsal midline (double arrow) (**B**). This correlated to eggshells with a reduced operculum, shorter more forward facing appendages (**D**).

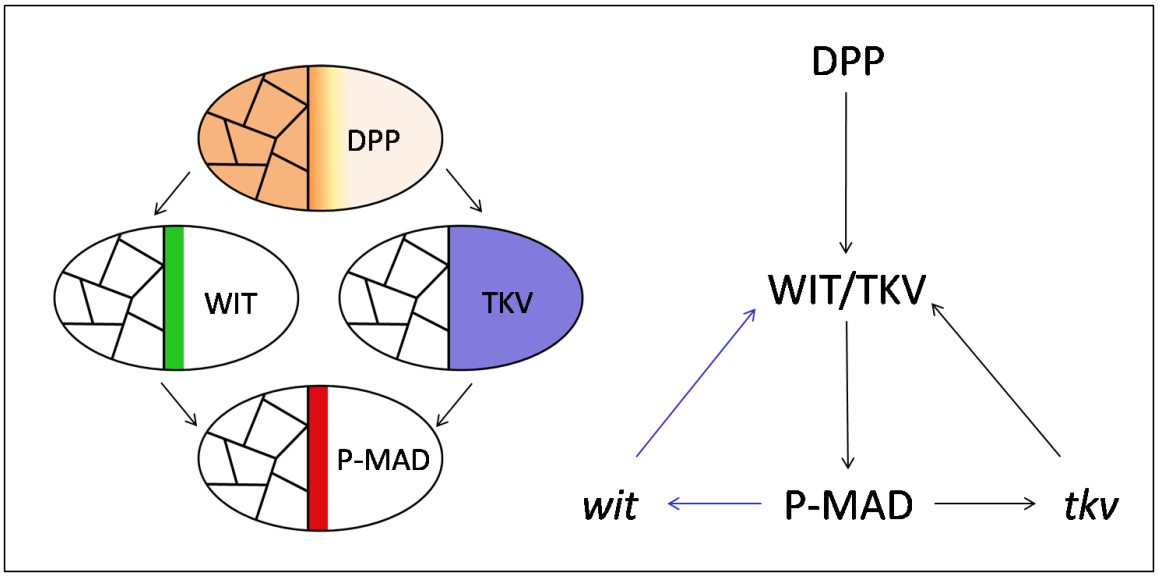

Figure 13. The model. The left model depicts an AP gradient of ligand, Dpp, diffusing over a uniform TKV and WIT. Strong feedbacks on WIT result in the anterior pattern of WIT and result in the anterior band of signaling. The right model adds WIT as a mediator in BMP signal perception, in addition to TKV. Both receptors are regulated in a positive feedback loop, in order to narrow the signaling gradient over time.