Chapter 2

Analysis of Kekkon5's role in the modulation of BMP signaling

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Abstract

Spatio-temporal regulation of cellular communication is critical for proper animal development. In the case of the Bone Morphogenetic Protein (BMP) pathway, a number of extracellular molecules contribute to ensuring appropriate ligand distribution and pathway activity. This chapter investigates the role of Kek5, a transmembrane protein of the LIG family that is a novel regulator of BMP signaling. Altered Kek5 expression affects crossvein development, observed directly at the level of pMAD and DSRF expression in pupal wings, and loss of *kek5* suppresses inhibition of BMP signaling. In addition, misexpression of Kek5 mimics that of the BMP modulator Sog, and can suppress activation of BMP pathway by misexpression of ligand, but not activated BMP receptors.

INTRODUCTION

Cellular communication is important for proper animal development, and this communication is achieved through a number of signal transduction pathways, such as those involving Bone Morphogenetic Protein (BMP), Epidermal Growth Factor Receptor (EGFR) and Wingless (Wnt). It is important that there is a precise spatio-temporal control of these pathways, which in the case of BMP signaling is achieved through an ever-growing number of extracellular molecules. In *Drosophila* wing, the study of posterior crossvein (PCV) specification, a process requiring BMP signaling, has been instrumental in elucidation of novel regulators of the pathway.

Among the components that assist PCV specification include: Sog and Cv-Tsg2, which aid in the movement of BMP ligands from longitudinal veins to the crossvein competent region by direct binding; Tlr, a metallo-protease that releases the ligands from the Sog complex; Crossveinless-2 (Cv-2), which aids in transfer of ligands to the receptors; Larval translucida (Ltl), an extracellular LRR protein that modulates BMP signaling in PCV by interacting with Cv-2; and Crossveinless-d (Cv-d), a vitellogenin–like lipoprotein that aids in the diffusion of BMP possibly though a lipid-BMP-lipoprotein mediated complex (Conley et al., 2000; Shimmi et. al., 2005; Serpe et al., 2005; O'Connor et al., 2006, Serpe et al., 2008; Szuperak et al., 2011; Chen et al., 2012). Here we describe a novel regulator of BMP signaling, Kekkon5, a transmembrane protein belonging to the LIG family of molecules. The LIG family of proteins is unique due to the presence of both Leucine rich repeats (LRR) and Immunoglobulin (Ig) domain in one protein (MacLaren et al., 2004).

Current work suggests a role for LIG proteins in the mediation and modulation of various signal transduction pathways. For example, the founding member of the Kekkon family,

Kekkon1, is an inhibitor of the Epidermal Growth Factor Receptor (EGFR) pathway (Alvarado et al., 2004a, b). Despite structural similarities among Kek family members, evidence suggests that they have distinct biological roles. For example, Kek2 has been proposed to be an adhesion molecule regulating synaptic growth while earlier work done in our lab on Kek6 suggests a barrier function for Kek6; consistent with this hypothesis Kek6 is present at the tricellular junctions (Arata and Duffy, 2011; Guan et al., 2005). Yet another Kek family member, Kek5 has been proposed to modulate BMP signaling and function in cell adhesion (Evans and Duffy, 2006). Evans, 2006 reported that loss of Kek5 activity results in perturbations to crossvein patterning (also, reduced viability and scutellar bristle duplication) and the presence of wing blisters, suggestive of roles in BMP signaling and cell adhesion, respectively (Evans and Duffy, 2006). Complementary gain-of-function studies with Kek5 showed related effects on crossvein patterning and wing development, consistent with the proposed roles.

The goal of this thesis was to gain a greater understanding of Kek5's function, confirm (or not) these postulated roles, and provide mechanistic insight underlying any such roles. Using loss-of-function (LOF), gain-of function (GOF) and structure/function analyses I have confirmed a role for Kek5 in BMP signaling, and implicated Kek5 in adherens junction biology and cell morphology.

Kek5 was proposed to act as a modulator of BMP signaling based on indirect evidence following genetic studies in crossvein patterning. Due to the indirect nature of the evidence, this proposed role remained to be confirmed (Evans and Duffy, 2006). More critically, the chromosome on which the *kek5* null allele was generated was found to contain a secondary mutation that also altered BMP signaling, complicating the interpretation of Kek5's proposed role. Work in this chapter focused on confirming the role of Kek5 as a modulator of BMP

signaling, through loss-of-function studies with a “clean” *kek5* null allele lacking the secondary mutation, assaying for direct effects on BMP signaling at the level of phosphorylated MAD (pMAD), and epistastis tests.

RESULTS

Expression of kek5

As previously described, *kek5* encodes a transmembrane protein of the LIG family and is broadly expressed throughout development (Evans and Duffy, 2006; Evans *et al.*, 2009) (Figure 2.1 and 2.2). Due to the putative role of Kek5 in crossvein patterning, expression of Kek5 was examined in more detail in pupal wings, during the temporal period in which crossvein specification occurs. In the pupal wing,

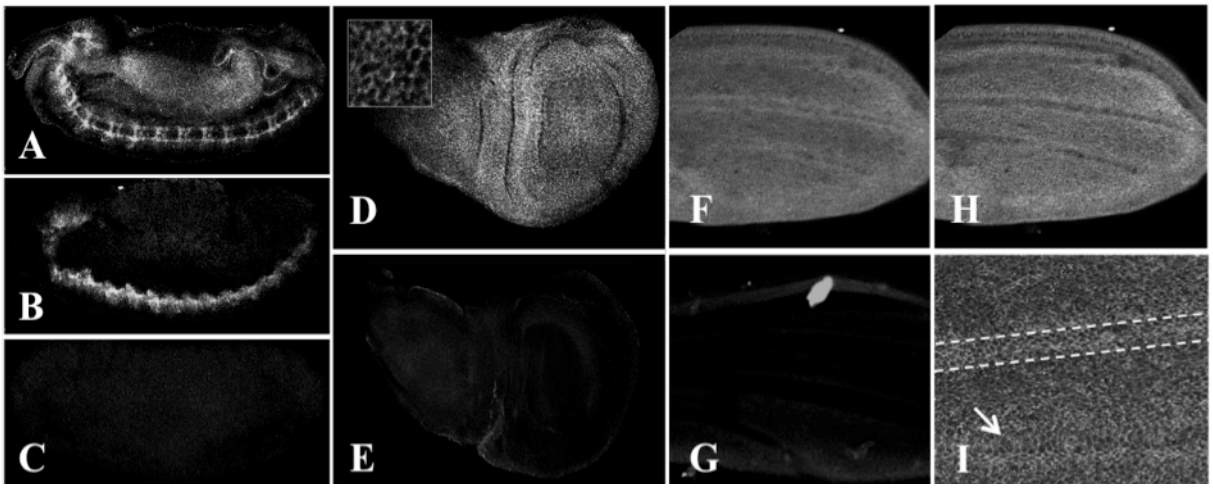

Figure 2.1: Kek5 is broadly expressed in *Drosophila*. Kek5 antiserum staining is shown in wild type embryos (A, B), wing disc (D and inset), and pupal wings (F, H, I). Staining is undetectable in embryos (C), wing discs (E), and pupal discs (G) in the null mutant *kek5*^{fe148}. Expression is membrane localized (D inset). F and H are different optical sections and I is a magnified view with the vein and intervein boundaries indicated (dashed line and arrow, respectively) demonstrating Kek5 is expressed in both regions. (From Evans *et al.*, 2009).

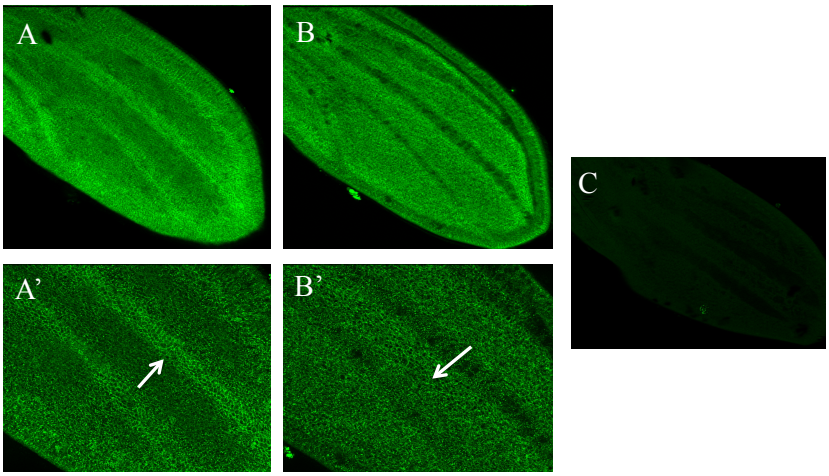

Figure 2.2: Kek5 is expressed in vein and intervein regions. Kek5 antiserum shows expression of Kek5 in pupal wings (wild type - A, A', B, B', *kek5*^{fe148} – C). In the pupal wings Kek5 is present in both vein and intervein (arrow). A and B are different focal planes of the same pupal wing and A' and B' are magnified views of A and B.

Kek5 expression was detected in both vein and intervein regions (Figures 2.1 and 2.2).

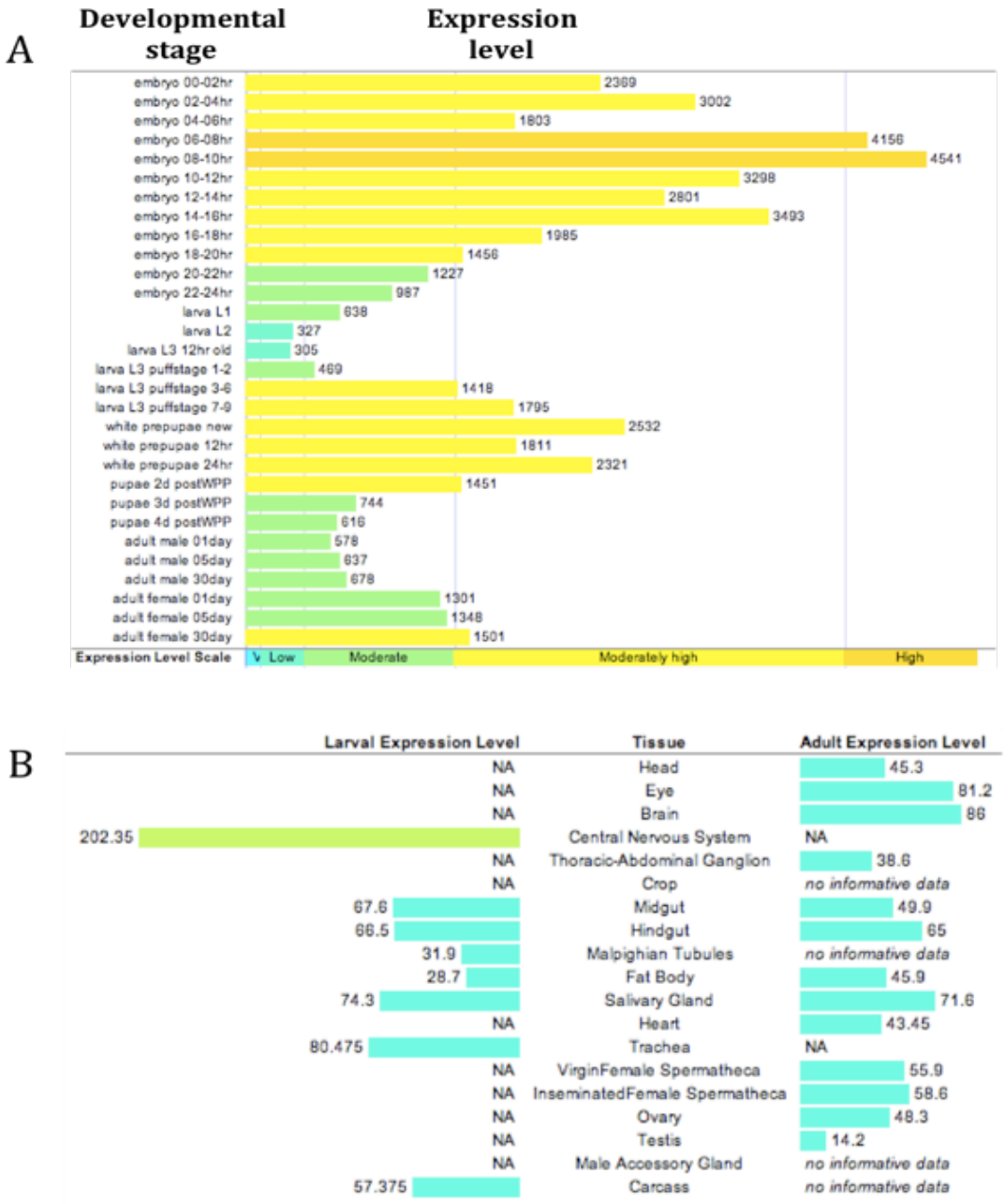

Figure 2.3: RNA expression profile of Kek5. RNA seq based expression profiling of *kek5* (A-whole animal, B-tissue) as reported in flybase.

In addition to a survey of Kek5 expression with antiserum, genome wide RNA expression studies by the *Drosophila* community reveal broad expression profile for *kek5* with two peaks; one during embryonic development (6-10hrs) and another during early pupal stages (Figure 2.3).

In addition, as with other members of the Kekkon family, Kek5 expression shows enrichment in the central nervous system (Evans and Duffy, 2006; Evans *et al.*, 2009) (Figure 2.1 and 2.3).

Confirming kek5 loss-of-function phenotypes

Previously, studies on *kek5* pointed to a role in BMP signaling (Evans and Duffy, 2006). In particular, LOF studies with the null allele, $kek5^{fe148}$, showed defects in posterior crossvein patterning, including loss of crossvein and crossvein duplications (30% defects). Reduced viability (~60%), wing blisters (~4%), and leg defects (<5%) were also observed (Evans and Duffy, 2006). As BMP signaling is required for crossvein specification, the effects attributed to loss of *kek5* were consistent with a role in BMP signaling (Blair, 2007; O'Connor et al., 2006).

The null allele, $kek5^{fe148}$, used in these initial studies was generated through FLP-mediated mitotic recombination between two FRT containing piggyBac elements flanking the *kek5* coding region (*PBac{WH}f04013* and $PBac\{RB\}CG32533^{e00904}$), which deleted the entire coding region (Evans and Duffy, 2006). It was subsequently discovered that the *kek5* null chromosome generated and tested in these studies had a putative mutation in the BMP pathway inhibitor *brinker (brk)* (J. Duffy, unpublished). Additional work showed that this mutation was present in the piggyBac line, $PBac\{RB\}CG32533^{e00904}$ that served as a progenitor for the *kek5* null allele.

Given that the phenotypes associated with $kek5^{fe148}$ were BMP related the possibility existed that the defects observed in the $kek5^{fe148}$ flies were in fact not due to loss of *kek5* activity, but rather to the presence of this linked mutation. To address this, the putative *brk* mutation was removed from the $kek5^{fe148}$ null chromosome by recombination, which allowed the effect of eliminating solely *kek5* to be addressed ($kek5^{JDR3fe148}$ or $kek5^{JDR3}$, J. Duffy, unpublished).

Table 2.1: Comparison of wing defects in original and recombinant *kek5* null chromosome

Genotypes	Crossvein defects (%)		Blisters (%)	
	$kek5^{fe148}$	$kek5^{JDR3}$	$kek5^{fe148}$	$kek5^{JDR3}$
$kek5^{-}$	28.3	28.0%	3.9	2.5%
$kek5^{-}$ /Df(1)JA27	67.5	64.75	9.0	10.2
$kek5^{-}$ /Df(1)Exel7468	56.1	47.65	1.5	5.6

Note: The crosses were done at 25°C. Females (N=>100) from these crosses were scored for wing defects. Wings with blisters were excluded from crossvein defect assessment as blisters obscured the crossveins. $kek5^{fe148}$ columns represent the data obtained previously in the lab (Evans and Duffy, 2006).

Phenotypic analysis of homozygous $kek5^{JDR3}$ adults indicated that both wing and leg defects were observed in frequencies similar to those observed with the original null chromosome brk^{*}, $kek5^{fe148}$ (Table 2.1 & Figure 2.4). The $kek5^{JDR3}$ adults displayed 28% wing defects and 2% blisters. The wing defects included extra vein material and truncated PCVs (Figure 2.4) and meandering PCV (not shown). When $kek5^{JDR3}$ was in trans with two large deficiencies that remove *kek5*, *Df(1)JA27* and *Df(1)Exel7468*, an enhancement in the frequency of defects was observed (Table 2.1). In

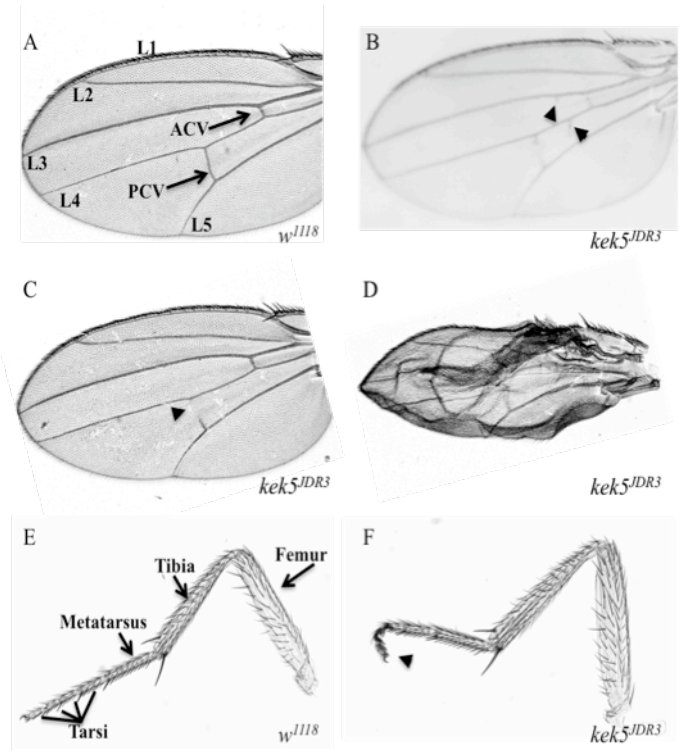

Figure 2.4: Phenotypes displayed by *$kek5^{JDR3}$*. $kek5^{JDR3}$ displays various types of wing defects including a meandering PCV (B), gaps in PCV (C) and wing blisters (D) when compared to a wild type wing (A). In addition to the wing defects, anomalies are also observed in the legs (E & F). Arrowheads point to the defects (B, C & F).

addition to the wing defects, leg defects at <5% frequency were also observed, similar to that seen with *kek5*^{fe148} (Figure 2.4).

These results thus confirmed that the adult phenotypes initially associated with loss of *kek5* activity were indeed due to loss of *kek5* and not a result of reduced *brk* activity. Consequently, *kek5*^{JDR3} was used as the *kek5* null stock for all additional experiments.

Gain of function kek5 phenotypes

Simultaneous with the re-evaluation of the recombinant null allele, the phenotypic effects associated with *kek5* misexpression were also examined. Prior work using the GAL4/UAS system to misexpress *kek5* had revealed a number of distinct adult phenotypes, including loss of anterior and posterior crossveins, scutellar bristle duplications, stumpy legs, blistered wings, growth defects, wing

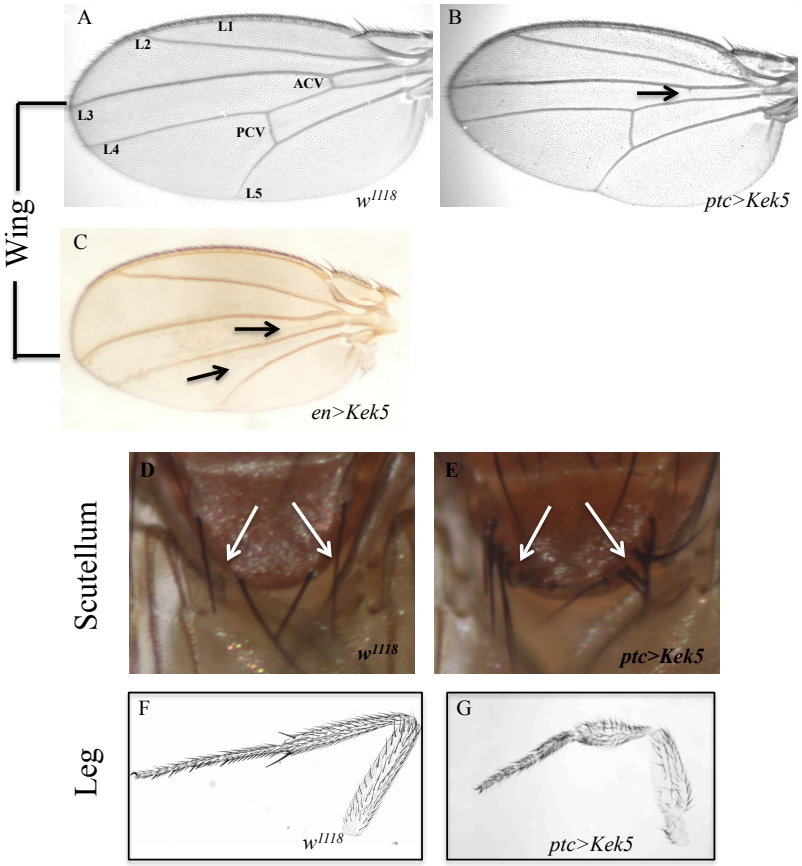

Figure 2.5: Adult phenotypes associated with misexpression of Kek5. Misexpression of *kek5* in wing results in crossvein defects (A-C). Defects are also observed in the scutellum (D, E) where bristle duplication is seen and in the legs (F, G). Black arrows indicate wing defects while white arrows points to the scutellar bristles.

curling, and rough eyes (Evans and Duffy, 2006; Ernst and Duffy, 2010). As expected, phenotypic effects were consistent with those observed previously (Figure 2.5). Thus both loss and gain of function Kek5 activity results in a variety of adult phenotypes, including those consistent with involvement in BMP signaling and cell adhesion.

Kek5 modulates BMP signaling in the wing

As discussed earlier, crossveins are dependent on BMP signaling. Reduced activity in BMP pathway components that contribute to ligand distribution, *sog*, *cv* and *tlr*, for example, exhibit crossvein specific phenotypes, including both loss and gain of crossveins. As now confirmed, *kek5* mutants also exhibit both loss and gain of crossvein tissue raising the possibility that *kek5* might function in the BMP pathway during crossvein development. Moreover, misexpression of Kek5 leads to loss of crossveins, consistent with inhibition of BMP signaling. If Kek5 functions as an inhibitor then its misexpression should suppress phenotypes associated with activation of the BMP pathway, while loss of Kek5 activity would be expected to suppress phenotypes associated with inhibition of the BMP pathway.

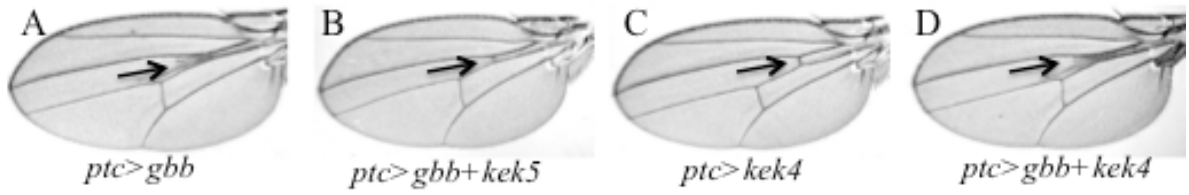

Figure 2.6: Kek5 suppresses Gbb dependent activation of BMP signaling. Activation of BMP signaling, *ptc>gbb*, results in the production of ectopic veins (A) which can be suppressed by *kek5* (B), but not *kek4* (D) misexpression (Evans and Duffy, 2006). Arrows point toward the ACV region.

Activation of BMP pathway in the wing through excess production of the ligand Gbb, *ptc>gbb* results in ectopic veins (Evans and Duffy, 2006; Figure 2.6 A). Consistent with a role as an inhibitor of BMP signaling, misexpression of Kek5 is capable of suppressing the ectopic veins

produced by excess Gbb expression (Evans and Duffy, 2006; Figure 2.6 B). To extend this, the ability of Kek5 to suppress ectopic veins produced in response to another key BMP ligand, Dpp was also analyzed (Figure 2.7).

Lethality associated with misexpression of *dpp* with the *ptcGAL4* (driving expression in the A/P boundary in the wing disc) driver prevented epistatic analysis with this driver. Use of a different driver, *GMRGAL4* (typically used as a driver for misexpression in the eye, however has been reported to have a broad expression pattern (Li et al., 2012)), for *dpp* misexpression (*GMR>dpp*) resulted in viable adults with ectopic veins throughout the wing (Figure 2.7). In contrast to suppression of Gbb, misexpression of *kek5* in the *GMR>dpp* background did not appear to suppress the ectopic veins resulting from excess Dpp (Figure 2.7). In this case it is possible that: 1) Kek5 doesn't affect Dpp mediated activation of BMP signaling or 2) this stronger ectopic vein phenotype cannot be suppressed by Kek5.

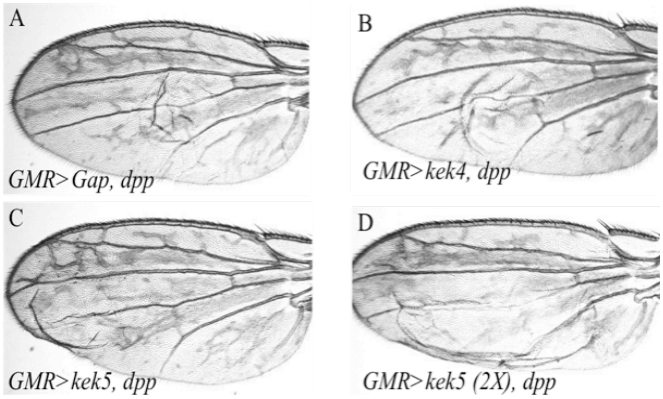

Figure 2.7: Kek5 does not suppress activation of BMP signaling achieved through excess ligand (UAS-dpp). Activation of BMP signaling, *GMR>dpp*, results in the production of ectopic veins throughout the wing (A), which is not suppressed by Kek4 (B), Kek5 (C) or 2X Kek5 (D). To account for any titration of GAL4, UAS-GAP is used along with Dpp (A).

As observed for Dpp, the effects of Kek5 on increased BMP signaling mediated by activated receptors were also not consistent with a simple function as an inhibitor. Rather than suppression, misexpression of *kek5* in the presence of constitutively activated receptors resulted in an additive effect (Evans et. al., 2009).

Misexpression of activated receptors, *ptc>tkvA* and *ptc>saxA*, induced severe phenotypes including excess vein material, fusion of longitudinal veins and loss of intervein region. Misexpression of *kek5* in this background was unable to suppress this phenotype instead resulted in additive effects - in addition to the vein defects, the wings were smaller characteristic of Kek5 misexpression (Evans et al., 2009).

As a complement to the *kek5* misexpression experiments, a series of loss-of-function experiments were also carried out to address its role in BMP signaling. If Kek5 acts as an inhibitor of BMP signaling, reducing its activity should rescue loss-of-function phenotypes resulting from compromised BMP signaling. Expression of dominant negative versions of either the BMP receptors ($tkv^{1A\Delta GS}$, $tkv^{\Delta GSK}$, $sax^{\Delta 1}$) or expression of the extracellular molecule Sog leads to compromised BMP signaling and alterations in vein patterning (Figure 2.8 and 2.9). Misexpression of dominant negative Tkv ($ap>tkv^{1A\Delta GS}$) resulted in large gaps in the longitudinal vein L4 in 91% of the wings scored (Class II,

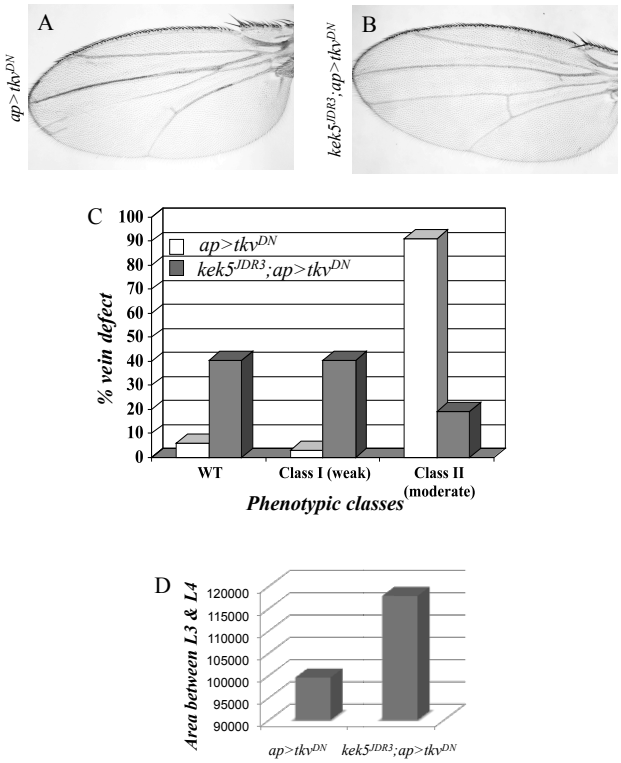

Figure 2.8: BMP inhibition is rescued by loss of *kek5*. Inhibition of BMP signaling with dominant negative Tkv (Tkv^{DN}) is suppressed by removal of *kek5* (A, B). Misexpression of tkv^{DN} produces large gaps in L4 (Class II moderate defects) in 91% of the wings and this is suppressed ~4.8 fold by removal of *kek5* (C). Removal of *kek5* also rescues the L3-L4 area defect. $tkv^{DN}=tkv^{1A\Delta GS}$ (D).

moderate phenotype) and reduced wing area between longitudinal veins L3 and L4 (Figure 2.8). Removing *kek5* from this background was able to rescue the L4 gap phenotype ~4.8 fold and the loss of wing area between L3 and L4 (Figure 2.8).

Likewise, loss of *kek5* was able to rescue phenotypes due to compromised BMP signaling through Supersog, an inhibitory variant of Sog (Figure 2.9). Misexpression of Supersog by *ptcGAL4* results in elimination of the intervein region between L3 and L4, including the PCV and removing *kek5* from this background was able to restore both the intervein region and PCV (Figure 2.9). While Supersog appears to principally inhibit BMP signaling, wild type Sog inhibits signaling locally, but promotes signaling at a distance. Consistent with this, misexpression of Sog with *ptcGAL4* results in a loss of ACV, as does Kek5, but unlike Kek5 also results in ectopic veins due to its role in promoting long range BMP signaling (Figure 2.9). As would be expected if Kek5 acts as an inhibitor, deletion of *kek5* in this background resulted in an enhancement of the Sog ectopic vein phenotype, while simultaneous misexpression of *kek5* caused a suppression of the Sog associated ectopic vein. Taken together all these loss and gain of function results support a role for Kek5 in modulating BMP signaling during wing vein patterning and development.

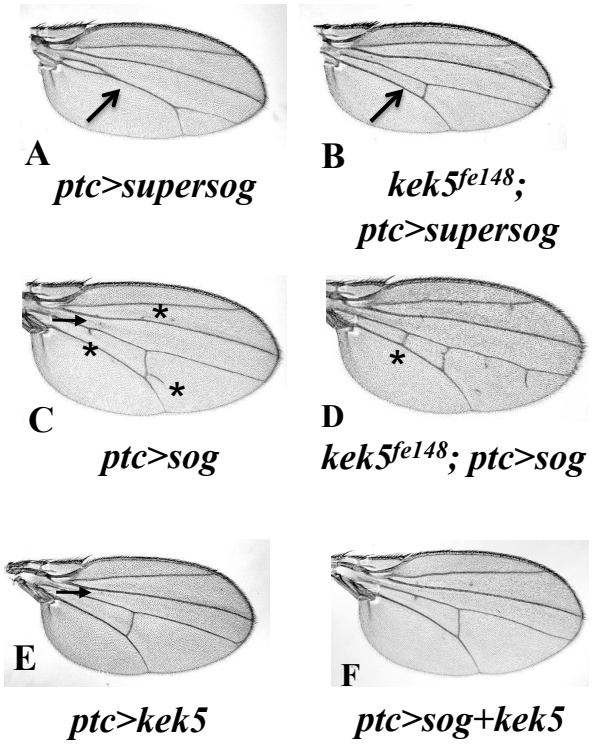

Figure 2.9: Effects of Kek5 on Sog mediated BMP perturbations. Misexpression of Supersog is suppressed by loss of *kek5* (A, B). Amount of ectopic vein material due to Sog misexpression is enhanced in *kek5* mutant background (C, D) while it is suppressed by simultaneous misexpression of Kek5 (F). * indicates ectopic vein material. (Evans, 2006)

Kek5 affects pMAD expression in the pupal wing

As demonstrated above, alterations in the levels of Kek5 results in crossvein defects, presumably through effects on BMP signaling. During pupal wing development BMP ligands from longitudinal veins diffuse to the crossvein region, thereby activating BMP receptors and triggering crossvein formation (Ralston and Blair, 2005). Crossvein specification is controlled solely by BMP signaling at 24hr APF (After Puparium Formation) while at 30hr APF, other signaling pathways like the EGFR pathway also contribute to crossvein specification (Blair, 2007). To confirm that *kek5* effects on crossvein patterning were due to modulation/loss of BMP signaling, phosphorylated Mad (pMAD), a direct readout of BMP signaling, was assayed at both 24hr and 30hr APF wings (After Puparium Formation) in pupal wings in which *kek5* expression had been increased or decreased.

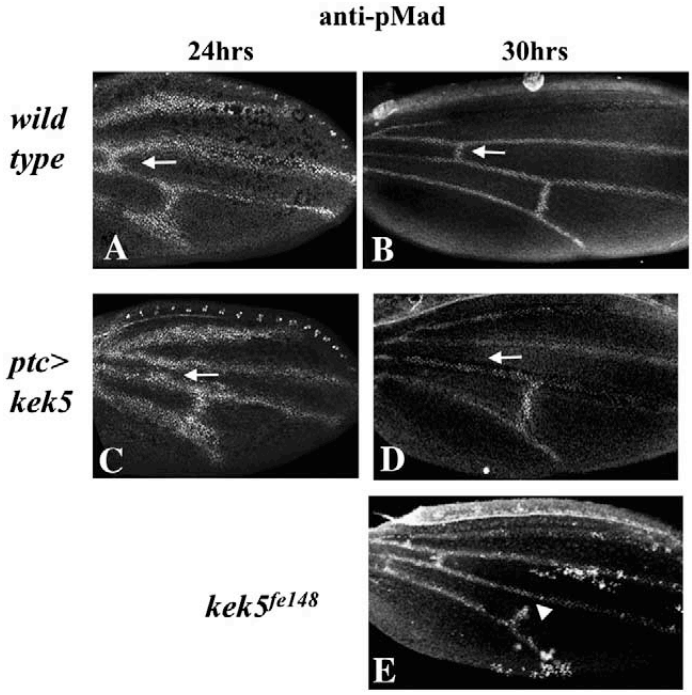

Figure 2.10: BMP pathway activation is altered by changes in Kek5 activity. Pupal wings at 24hr and 30hr APF are shown. Accumulation of pMAD in the pupal wings delineates the presumptive longitudinal and crossveins (ACV – arrows; A, B). When *kek5* is misexpressed in a region overlapping the ACV, pMAD expression is abolished and the ACV fails to form (arrow, C&D). In $kek5^{JDR3}$ mutant wings, pMAD expression is truncated in the presumptive PCV region leading to a gap in this crossvein (arrowhead).

Misexpression of *kek5* leads to loss of the ACV in adults and a loss of pMAD expression in the presumptive crossvein region of pupal wings at both 24hr and 30hr APF (Figure 2.10). Likewise, adults derived from a *kek5* null background exhibit PCV

defects (truncations) and a loss of pMad expression in the presumptive crossvein region of pupal wings at both 24hr and 30hr APF (Figure 2.10).

Just like pMAD delineates the presumptive vein tissue, *Drosophila* Serum Response Factor (dSRF), a MADS-box containing transcription regulator, exhibits a complementary pattern, demarcating the intervein region (Montagne et al., 1996) (Figure 2.11). When Kek5 was misexpressed, dSRF expression failed to be down regulated in the presumptive ACV (Figure 2.11). Consistent with the *kek5* null adult crossvein phenotypes, exhibit ectopic ACVs, there is a duplicated downregulation of dSRF in the ACV region (Figure 2.11).

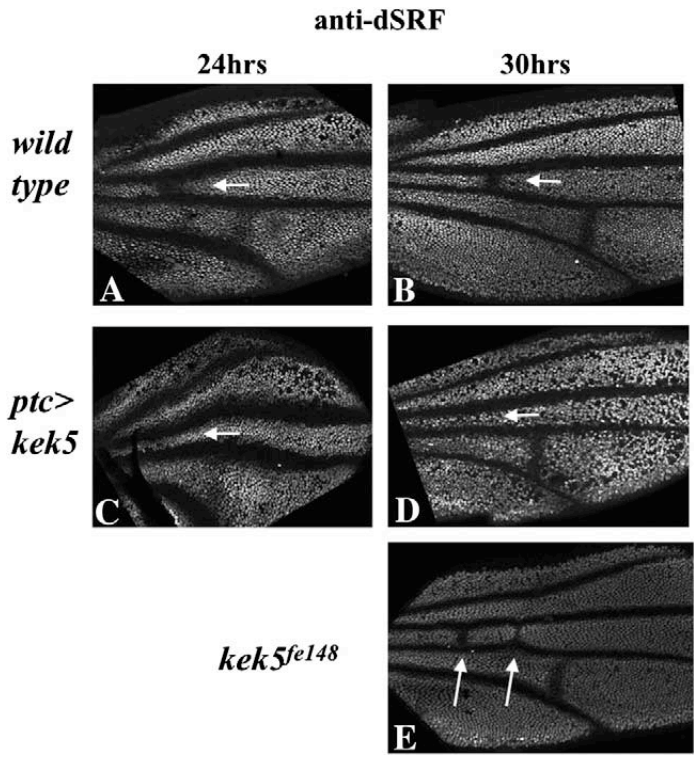

Figure 2.11: DSRF expression is altered by changes in Kek5 activity. Pupal wings at 24hr and 30hr APF stained with anti-dSRF antibody. Loss of dSRF in wild type pupal wings delineates the presumptive longitudinal and crossveins (arrows, A, B). When Kek5 is misexpressed in a overlapping the ACV, dSRF expression is not down regulated and the ACV fails to form (arrows, B, D). dSRF in *kek5* mutant wings, indicating the presence of an ectopic ACV (arrows, E).

Does Kek5 associate with BMP components?

Genetic and molecular evidence supports the idea that *kek5* functions in BMP signaling. Together these results are consistent with a mechanism whereby Kek5 acts both to decrease and increase BMP signaling. To gain insight to the mechanism of Kek5's action, biochemical studies were initiated using both extracellular components (ligands and extracellular modulators) and receptors of the BMP signaling pathway.

Co-immunoprecipitation (CoIP) and cell surface binding assays with Kek5 and extracellular components (Dpp, Gbb and Sog) have not provided any positive results (personal communication – M. Serpe and M. O'Connor, Madison, Wisconsin). To test for direct interactions between Kek5 and BMP receptors by co-IP, tagged (6XHis/V5) versions of the BMP receptors, Tkv, Put, Sax, and Wit were generated by Gateway cloning. These constructs were transfected in *Drosophila* S3 cells and receptor expression was confirmed (Figure 2.13). Currently, appropriate negative and positive controls have not been replicated, hindering the testing of a direct interaction between Kek5 and the BMP receptors. Specifically, it has been demonstrated that Kek1 inhibits and directly interacts with the EGFR, while Kek5 does not bind EGFR and has no function in this signaling pathway (Alvarado et al., 2004a; Evans and Duffy, 2006). Under current conditions, Kek5 associates with the EGFR, indicating additional troubleshooting is necessary for this assay to be viable for screening for Kek5 interactors.

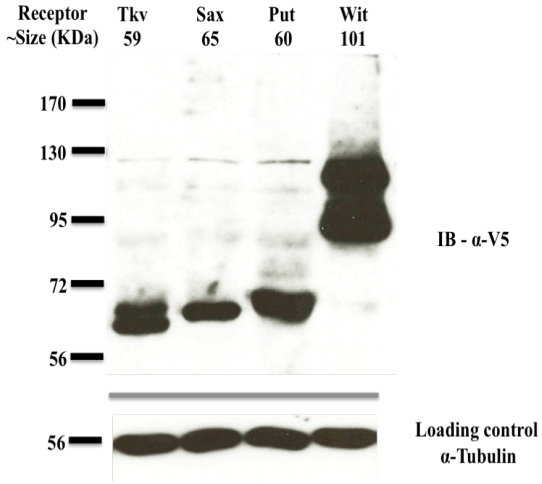

Figure 2.12: BMP receptors appropriately expressed in S3 cells. 6XHis/V5 tagged BMP receptors were generated using Gateway cloning and are appropriately expressed in *Drosophila* S3 cells as can be seen in the Western blot.

DISCUSSION

Appropriate signal transduction through BMP signaling is required for proper development of the metazoan body plan. This requires accurate spatial and temporal control of signaling, which is ensured by the interplay of a diverse set of molecules. Studies using *Drosophila* crossveins as a model system have revealed the identity of many such components (Conley et al., 2000; Ralston and Blair, 2005; Serpe et al., 2005; Shimmi et al., 2005; Vilmos et al., 2005; Szuperak et al., 2011; Chen et al., 2012). This work now confirms that Kek5, a LIG family member, is a novel regulator of BMP signaling.

Kek5 modulates BMP signaling

Changes in the level of Kek5 activity result in phenotypes associated with altered BMP signaling - crossvein patterning defects, effects on pMAD, and growth defects. Genetic studies support a role for Kek5 with extracellular BMP components. First, loss of Kek5 activity results in loss and gain of crossveins, complementary phenotypes consistent with an altered distribution of extracellular BMP signals. Second, misexpression of *kek5* is able to suppress Gbb mediated activation of BMP signaling, compatible with a role downstream of this ligand. Third, loss of *kek5* rescued the BMP inhibitory effects of a dominant negative version of the receptor Tkv, and the secreted inhibitor SuperSog, but enhanced long-range signaling by Sog, again indicative of a dual role. Finally, while loss of *kek5* enhanced long-range Sog effects, complementary *kek5* misexpression was able to suppress them. Together with the demonstration that the extracellular LRRs in Kek5 are required for its function, these data suggest a model in which Kek5 interacts with extracellular BMP components to mediate its role in BMP signaling (Evans et al., 2009).

However, additional data suggests a more complex picture for Kek5 function in BMP signaling. Misexpression of Kek5 was not able to suppress Dpp mediated activation of BMP signaling nor suppress the effects of activated BMP receptors. In addition, experiments with extracellular BMP pathway components (Dpp, Gbb and Sog) failed to detect any direct physical interactions (M. Serpe and M. O'Connor, personal communication). Moreover, a simple extracellular interaction model does not hold true given that the intracellular domain of Kek5 is required for activity. An alternative possibility is that that Kek5 interacts with the BMP receptors through both extracellular and intracellular sequence elements. Biochemical interaction studies with Kek5 and the BMP receptors would help directly evaluate such a model and remains to be assessed. However, it is important to consider that Kek5 may not interact with a single BMP pathway component in isolation, but rather could interact with multiple BMP pathway components.

MATERIALS AND METHODS

Drosophila genetics

Flies were raised on standard media (7.2g/L agar, 25g/L yeast, 47g/L cornmeal, 100g/L dextrose, 0.22% Tegosept; prepared at University of Massachusetts, Worcester) at room temperature as a routine. All the crosses were carried out at 28^{o}C unless otherwise noted. Targeted misexpression of Kek5 or other transgenes was carried out using the yeast derived UAS/GAL4 system (Duffy, 2002). GAL4 lines used were: *P{GawB}559.1* (or ptc-GAL4), *P{GawB}ap^{md544}* (or ap-GAL4), *P{en2.4-GAL4}e16E* (or en-GAL4), *P{GawB}CY2* (or CY2-GAL4), *P{GAL4}A9* (or A9-GAL4) (gift from Kristi Wharton). Deficiency stocks were ordered from the Bloomington Stock Center.

Imaging and Immunostainings/Immunohistochemistry

Drosophila wing discs (along with some head tissue for easier visualization during washes) were dissected in PBS, fixed in 4% formaldehyde in PEMP (0.1M PIPES, 1mM EDTA, 2mM $MgSO_4$ and 0.5% NP-40) for 15 minutes. The discs were then washed several times with antibody wash (150mM NaCl, 0.1% NP40, 100mM Tris-Cl pH7.4, 1mg/mL BSA). Samples were blocked (5%BSA in antibody wash) for 1-4 hours before addition of the primary antibodies. Primary antibodies were diluted in antibody wash. Upon addition of 100uL of the primary antibody dilution, samples were incubated at 4^{o}C overnight. Tissue was washed numerous times with antibody wash and incubated for 2 hours with 100uLs of secondary antibody also diluted in antibody wash (1:500). After incubation with the secondary antibody the samples were thoroughly washed with antibody wash before storing the samples in 50% Glycerol PBS (Boston BioProducts). Wing discs were separated from head tissue on a slide

using needles and a couple of drops of an anti-bleach agent (*SlowFade*® Gold antifade reagent, Invitrogen) was added to the tissue. A coverslip was then dropped on the slide after removal of the remaining head tissue. The samples were observed under a Zeiss Microscope (Imager.Z1). Fluorescent images were captured using the Axiocam and ApoTome using the Zeiss Axiovision program under 20X or 40X magnification.

Pupal Wing Disc Staining

For pupal wing dissections, white prepupae (0hr APF or After Puparium Formation) were picked and aged on a damp kimwipe in a small petridish (60mm) at 28^{o}C for 18hrs (for 24hrs time point) or 24hrs (for 30hrs time point). Development at 28^{o}C is approximately 1.25 times faster than at 25^{o}C (Ashburner, 1989). Pupae of the desired age were removed from the pupal casing and transferred to Ringer's solution (130mM NaCl, 5mM KCl and 1.5mM $MgCl_2$) in a glass dish. Ringer's solution was replaced with pre-chilled Brower's fixative upon removal of pupae from the pupal cases and the pupae were fixed for 4-5 hours at 4^{o}C. Brower's fixative is composed of 2% formaldehyde (16% EM grade formaldehyde, Polysciences Inc.) in 1.5X Brower's solution (150mM PIPES, 3mM $MgSO_4$, 1.4mM EGTA and 1.5% NP-40). Pupal wings were dissected out after transferring the fixed pupae to a small petri dish and washing them with ice cold PBST (PBS and 0.3% Triton X-100). Dissected pupal wings were transferred into a glass dish with cold PBST and washed with PBST before blocking the tissue for 1-4 hours with freshly prepared blocking solution (PBST and 5mg/mL BSA) at 4^{o}C. The pupal wings were then incubated with primary antibody (Mouse anti-DSRF and Rabbit anti-pMAD at 1:1000 and 1:5000 respectively, Table 4.3) overnight. Samples were incubated in secondary antibodies (1:500, Molecular Probe) for 2 hours after thoroughly washing them with cold PBST. Tissue was

stored in 70% glycerol PBS premixed with anti-fade reagent (*SlowFade,* Invitrogen). Pupal wings were imaged using a Zeiss Imager.Z1 microscope and image acquisition done using the Apotome and AxioImager.

Adult tissue analysis and Imaging

Wings from adult flies were dissected in 95% ethanol in a glass dish using tweezers (DUMONT tweezer, 110mm, # 5). The wings were dried on a piece of kimwipe and placed on a slide containing the mounting medium composed of 80% Canada balsam (Sigma) and 20% Methyl salicylate (Sigma). Slides were incubated on a hot plate (~65^{o}C) overnight before imaging on a Zeiss microscope (Imager.Z1) under bright field at 5X. Flies of the appropriate genotype were stored at -20^{o}C overnight for imaging of scutellar bristles. Imaging was done using a Canon T2i camera mounted on a Zeiss Stemi 2000 at 5X magnification.

Molecular cloning and expression of tagged BMP receptors

Tagged (6XHisV5) BMP receptors Thickveins (Tkv), Saxophone (Sax), Punt (Put) and Wishful thinking (Wit) were generated using Gateway cloning methodology (refer to Materials & Methods in Chapter 3). Tagged Tkv, Sax and Put were generated by PCR using cDNA clones ordered from DGRC. These purified PCR products were used to generate the pENTR clones, which were then used to generate the 6XHisV5 tagged versions of the receptors. Creation of tagged Wit was achieved using a pENTR Wit clone provided by Kristi Wharton (Table 2.2). Tagged constructs were expressed in *Drosophila* S3 cells and transient expression was achieved using co-expression of mtGAL4 (Klueg et al., 2002).

Table 2.2: Generation of tagged BMP receptors.

BMP Receptor	Starting DNA clone	Reference	Primers pair used
Thickveins (Tkv)	cDNA clone	GH25238	5'W370 & 3'W371
Saxophone (Sax)	cDNA clone	GH13369	5'W372 & 3'W373
Punt (Put)	cDNA clone	LP03259	5'W374 & 3'W375
Wishful thinking (Wit)	pENTR clone	Kristi Wharton	-

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