Chapter 6

Functional Characterization of Kekkon3: BMP signaling and Kek5

ABSTRACT

The Kek family of LIG proteins, containing both LRRs and Ig domains, are emerging as mediators and modulators of various signaling pathways. For example, Kek1 has been shown to be an inhibitor of EGFR signaling, while Kek5 functions as a modulator of BMP signaling. Recently, Kek3 was shown to affect crossvein patterning and bristle development consistent with functions in common with Kek5. However, while misexpression of either Kek5 or Kek3 results in loss of crossveins, their misexpression exhibits opposing effects on bristle development. Kek5 misexpression results in bristle duplication in the scutellum, while misexpression of Kek3 results in loss of bristles. To better understand the role of Kek3 in crossvein patterning, preliminary studies were carried out to determine if Kek3 affected the expression of pMad. In addition, epistasis experiments undertaken to understand the relationship between Kek3 and Kek5 in bristle development, suggest that Kek3 acts downstream of Kek5.

INTRODUCTION

Kek family members share considerable sequence and structural similarity suggesting that they may have redundant biological roles. However, current data on Kek1 and Kek5 indicates that they function in distinct signaling pathways (Alvarado et al., 2004; Evans et al., 2009; MacLaren et al., 2004). More information needs to be obtained in order to understand whether all six members of the Kek family participate redundantly in a biological process or whether they each have different roles in distinct cellular processes.

Analysis of another member of the Kek family, *kek3*, revealed that: 1) as in the case of Kek5, misexpression of Kek3 using *ptcGal4* resulted in a complete loss of anterior crossvein with very high penetrance; 2) unlike misexpression of Kek5, which results in scutellar bristle duplication (an average of 13-15 bristles per fly at 28^{o}C), Kek3 misexpression using *ptcGal4* resulted in an opposite phenotype, i.e., loss of bristles (0-1 bristles per fly); and 3) while Kek5 shows membrane localization in the wing imaginal disc, Kek3 does not (Arata and Duffy, 2011). The above-mentioned findings indicated that Kek5 and Kek3 might function in the same biological processes.

Here I describe preliminary attempts to determine 1) if the loss of crossvein in Kek3 misexpression is due to modulation of BMP signaling and 2) the relationship between Kek3 and Kek5 in bristle development. As with Kek5, Kek3 misexpression affects pMad and dSRF expression in the presumptive anterior crossvein in pupal wings, indicative of an inhibitory role for Kek3 in BMP signaling. With respect to bristle development, my epistasis analysis suggests that Kek3 is downstream of Kek5.

RESULTS

Kek3 misexpression results in loss of anterior crossvein in pupal wings

Loss of crossveins in the *Drosophila* wing is one of the phenotypes associated with loss of BMP signaling. Recently it was shown that gain of function (GOF) of Kek3, a Kek family member causes crossvein defects similar to those seen with Kek5 (Figure 6.1) (Arata and Duffy, 2011). These defects ranged from a complete loss (86%) to truncation (13%) of the anterior crossvein when Kek3 was misexpressed with *ptcGAL4*. To determine if the defects in crossvein patterning were due to inhibition of BMP signaling, I examined the expression of the intracellular mediator of BMP signaling, Mad, which is phosphorylated upon activation of BMP signaling. For analyzing the affects on pMad and dSRF expression, Kek3 was utilized, which produced a high frequency of crossvein defects (Figure 6.1).

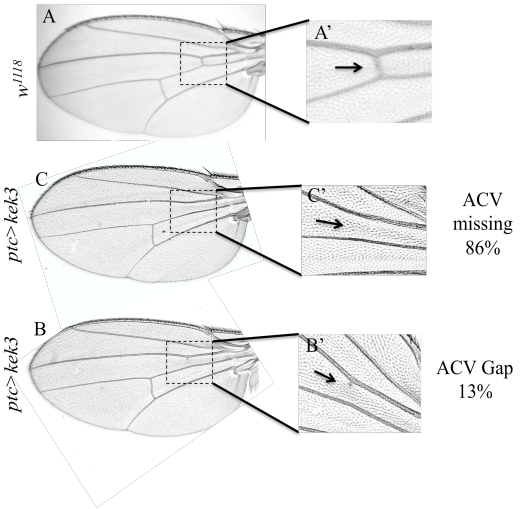

Figure 6.1: Misexpression of Kek3 causes cross vein defects. At 28°C misexpression of Kek3 with *ptcGAL4* results in anterior crossvein defects (>99%). ACV region is enlarged in A'-C'. Panels A and A' show the wild type ACV which was observed at a low frequency in *ptc>kek3* flies (1%).

Anti-dSRF stains the intervein region in a manner complementary to anti-pMad, which delineates the presumptive longitudinal veins (LVs) and crossveins (CVs), providing a more direct assay for analyzing the affects of Kek3 on BMP pathway. Two developmental time points were examined, 24hr and 30hr after puparium formation (APF). At 24h APF BMP signaling pathway is solely responsible for vein formation whereas at 30hr APF, EGFR signaling is involved (Blair, 2007).

As mentioned earlier, in wild type, pMad delineates the presumptive LVs and CVs while dSRF is present in the intervein region. In a preliminary analysis, the loss of ACV in *ptc>kek3* appears to be due to the loss of pMad expression from the presumptive crossvein region. I also observed a lack of down regulation of dSRF in the ACV region (Figure 6.2).

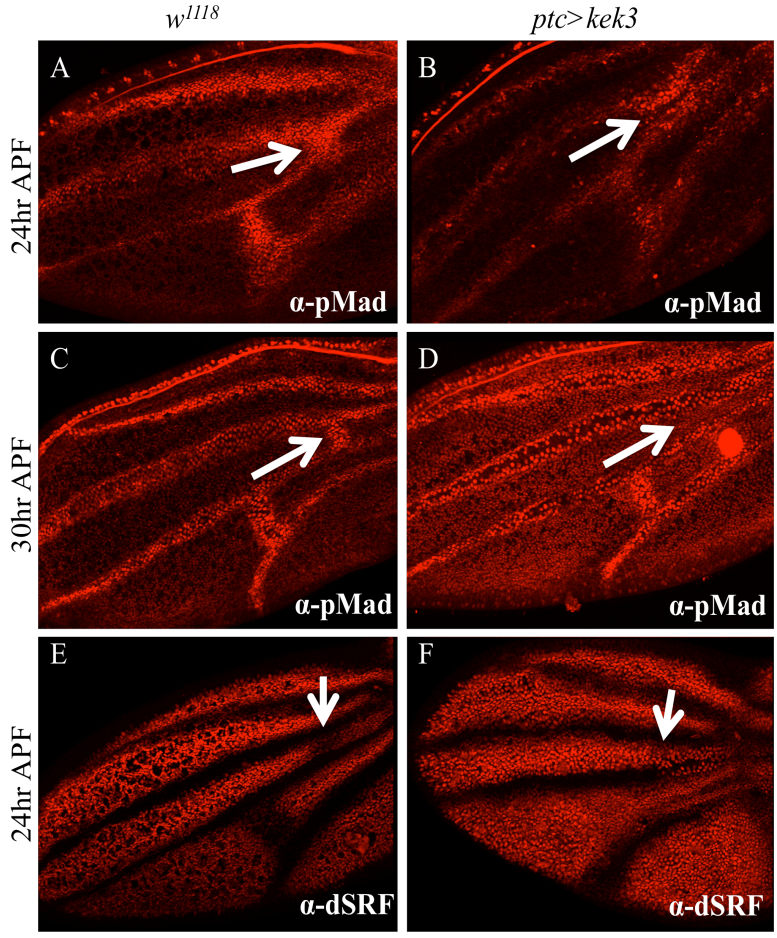

Figure 6.2: Kek3 misexpression alters BMP pathway activation and dSRF expression in the pupal wing. α-pMad outlines the presumptive vein and crossveins (A, C) while α-dSRF is upregulated in the intervein region but down regulated in the veins in wild type pupal wings (E). Kek3 misexpression affects both pMad and dSRF expression in the pupal wings (B, D, F). Arrows point to the ACV region. $Kek3^{M13\text{-}1Ma}$ transgenic line was used in this assay.

Epistasis analysis of Kek5 and Kek3 in bristle biology

While the crossvein phenotype of Kek5 was mimicked by Kek3 misexpression, the scutellar bristle duplication phenotype was found to be opposing. Misexpression of Kek3 resulted in a loss of scutellar bristles from four (present in wild type flies) to zero-one in Kek3 transgenic lines (Arata and Duffy, 2011).

Since Kek5 and Kek3 show opposite effects on bristle development, an epistasis experiment was performed to better understand their relationship. Co-misexpression progeny had no scutellar bristles suggesting that Kek3 was epistatic to Kek5. In addition, co-misexpression (*ptc>kek5, kek3*) progeny were not viable (pharate lethality) and had to be dissected out of their pupal cases to analyze the scutellar bristles (Fig 6.3).

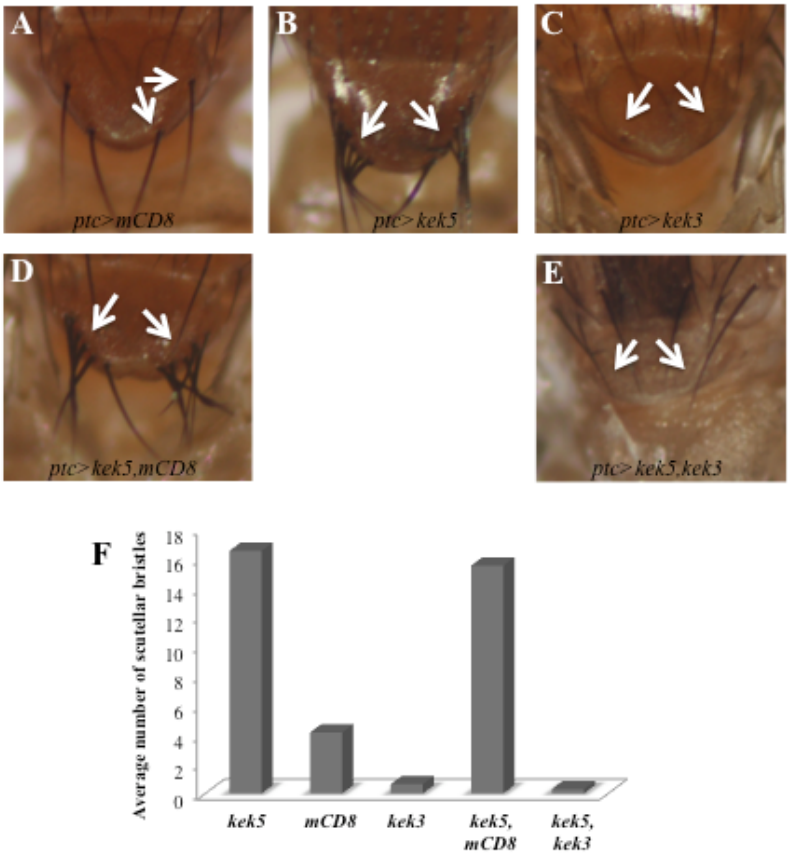

Figure 6.3 Kek3 is epistatic to Kek5. Misexpression of Kek5 with *ptcGAL4* results in bristle duplication while misexpression of a stronger Kek3 line ($Kek3^{MDA2}$) results in a reduction in the number of bristles (A-C, F). Misexpression of both Kek5 and Kek3 results a decline in the number of scutellar bristles (E, F). Between 25-50 flies were scored for each genotype, however due to pharate lethality of the *kek5, kek3* double, only 6 flies were analyzed after removal from the pupal cases.

DISCUSSION

Prior work done on the *Drosophila* LIGs suggests a functionally divergent role for the various Kek family members, despite their structural similarity. For example, Kek1 has been shown to inhibit EGFR signaling, while Kek5 has been shown to be a modulator of BMP signaling and cellular architecture. More recent work on Kek3 suggested a role in BMP signaling. To address this, I have carried out a preliminary analysis of pMad expression, a direct readout of BMP signaling in the pupal wings of wings misexpressing Kek3. In addition, the relationship of Kek3 and Kek5 in bristle patterning was examined through epistasis.

Phenotypic comparison of Kek5 and Kek3

Kek5 and Kek3 misexpression exhibit similarities and differences in the phenotypes displayed (Table 6.1). Even though there is no data available on the loss of *kek3* (due to lack of availability of classical mutants), misexpression of Kek3 has an effect on crossvein development similar to Kek5. Examination of pMad suggests that both Kek3 and Kek5 affect crossvein development by inhibiting BMP signaling (Figure 2.10, and 6.2).

Table 6.1: Comparison of Kek5 and Kek3 misexpression phenotypes

Phenotype	Kek5	Kek3
Crossvein defect	Yes	Yes
Extrusion	Yes	Yes
Arm upregulation	Yes	No
Scutellar Bristles	duplication	reduction
Localization in the wing	membrane	punctate

It will be interesting to determine if they act in concert with respect to BMP signaling (Figure 6.4, Model I) or through different mechanisms (Figure 6.4, Model II).

In contrast to the crossvein phenotype, Kek3 and Kek5 display opposing effects on scutellar bristles, loss versus gain, respectively. Co-misexpression of both Kek3 and Kek5 led to pharate lethality and retained the loss of bristles observed with the Kek3 misexpression. This epistasis analysis suggests that *kek3* is epistatic to *kek5* in bristle development. Although inhibition of BMP signaling has been shown to affect bristle development, the mechanism through which this is accomplished is yet to be elucidated (Wharton et al., 1999). Moreover, additional signaling pathways, including Notch and EGFR signaling, function in bristle patterning and it is currently unclear what pathway the bristle phenotypes of Kek5 and Kek3 reflects involvement in (Furman and Bukharina, 2008).

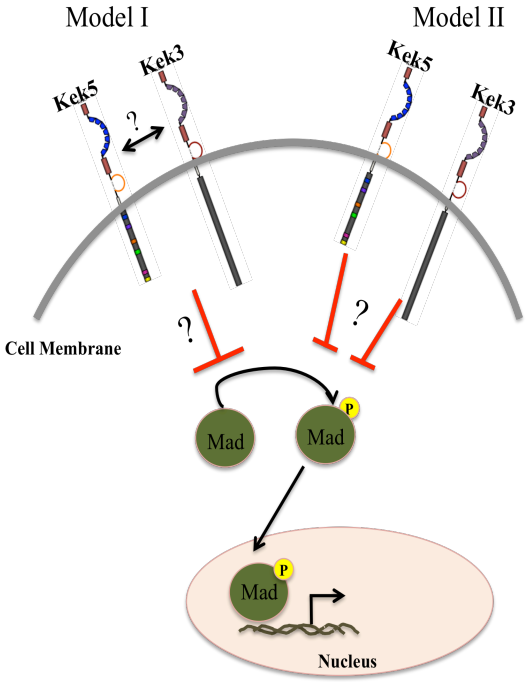

Figure 6.4: Both Kek5 and Kek3 inhibit BMP signaling. Activation of BMP signaling results in phosphorylation of Mad, the intracellular mediator of BMP signaling, which gets translocated into the nucleus to bring about transcriptional activation or repression of BMP target genes. It is possible that Kek5 and Kek3 either act in the same pathway to prevent Mad phosphorylation or they may do this through different pathways.

As a further tie to Kek5 activity, epithelial cell extrusion, a cellular phenotype observed with Kek5 misexpression is also seen upon Kek3 misexpression (Arata and Duffy, 2011). In

contrast, Kek3 misexpression has no affect on Arm expression at the adherens junction, which is upregulated in response to Kek5 misexpression.

Sequence comparison of Kek5 and Kek3

Phylogenetic analysis showed that Kek5 and Kek3 belong to two different evolutionary clades with Kek1/2/3 representing one clade and Kek4/5/6 another (MacLaren et al., 2004) When comparing Kek5 and Kek3 in different species, more sequence similarity exists in the extracellular region than in the intracellular region, which is consistent among the Kek family members (Evans and Duffy, 2006; MacLaren et al., 2004). Despite the overall divergent sequence of the cytoplasmic regions of the Kek molecules, closer examination revealed the presence of small conserved intracellular motifs (Evans and Duffy, 2006; MacLaren et al., 2004). However, sequence alignment of Kek5 and Kek3 reveals minimal level of conservation in the intracellular region with respect to the defined IC motifs of Kek5. Thus, none of the IC motifs required for Kek5 activity in BMP signaling, extrusion, and bristle duplications are present in Kek3. This suggests that the mechanisms underlying the common phenotypic effects of these two Kek family members may be different.

MATERIALS AND METHODS

Drosophila genetics

All the crosses were done at 28^{o}C. Misexpression was done using the GAL4/UAS system using *ptcGAL4* driver. *UAS-kek3*^{MDA2} and *UAS-kek3*$^{M13\text{-}1Ma}$ were used as the Kek3 transgenic lines (Arata and Duffy, 2011).

Immunohistochemistry

Pupal wing antibody stainings were performed according to the standard protocol as described in Chapter 2. Anti-dSRF (Mouse) and anti-pMad (Rabbit) antibodies were used at 1:1000 and 1:2500, respectively. The secondary antibodies, Alexa 568 were used at 1:500. The stained wings were mounted in 50% Glycerol-PBS with addition of ~10uL of anti-fade reagent (Slowfade, Invitrogen). Images were captured on a Zeiss Imager.Z1 microscope and image acquisition done using the Apotome and AxioImager at 20X magnification.

Adult tissue dissection and mounting

Adult scutellum was imaged on Zeiss microscope (Imager.Z1) under bright field at 5X magnification after mounting in a white raised surface.

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ACKNOWLEDGEMENTS

I want to thank Michelle Arata for cloning UAS-Kek3.GFP and mapping transgenic flies for the same. Michelle carried out the initial characterization and found that Kek3 affected crossvein development.

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