Chapter 3

Structure/Function Analyses of Kek5 in BMP signaling

ABSTRACT

Kek family members belong to the LIG family of transmembrane proteins having both Leucine rich repeats (LRRs) and immunoglobulin (Ig) domain. In addition to these extracellular domains, family members have multiple and distinct conserved sequence motifs in their intracellular (IC) domains. Prior structure/function work has shown that the IC domain of Kek1 is only required for localization and is not directly involved in Kek1's regulation of EGFR signaling. Kek5, in contrast, has been shown to modulate BMP signaling, and structure/function studies have implicated the LRRs in this activity. Using a series of Kek5 extracellular and intracellular variants, an extensive structure/function analysis was undertaken to define additional sequence elements important for Kek5's activity in BMP signaling. Within the IC domain, I show that motifs 4 and 5 of Kek5 are important for its role in BMP signaling.

INTRODUCTION

Kek5 plays a role in modulation of BMP signaling and is involved at the adherens junctions as elaborated in the next chapter. This is in contrast to the founding member of the Kek family, Kek1, which is an inhibitor of the EGFR signal transduction pathway. Considerable understanding of the mechanism of this inhibition was gained from generation of various chimeric Kek1-Kek2 swaps and deletion variants. It was found that Kek1 inhibits the EGFR pathway in a bipartite fashion where the LRRs of Kek1 are required for binding to EGFR while the juxta/transmembrane region is crucial *in vivo* for inhibition of signaling (Alvarado et al., 2004a, b; Derheimer et al., 2004).

Much like the structural approach taken for Kek1, Kek5 has been analyzed by generation of structural variants, since it also contains 7 LRRs and 1 Ig domain in the extracellular region and several conserved motifs in the cytoplasmic region. Given the diversity of phenotypic effects displayed by alteration in the levels of Kek5, it is conceivable that disparate Kek5 domains/motifs may contribute to different phenotypes.

Previously the LRRs of Kek5 were shown to be important for their ability to induce crossvein defects (Evans *et al.*, 2009). It was also shown that removal of the entire IC domain except the last 6 amino acids ($Kek5^{\Delta IC+P}$) dramatically reduced Kek5 function in BMP signaling (Evans *et al.*, 2009). This points to the importance of sequence elements in the IC domain for Kek5 function. The IC domain consists of 6 recognizable conserved motifs (1-6); motif 1 being closest to the transmembrane region and motif 6 (also the PDZ domain binding site) at the C-terminal end of Kek5. Through the generation of a series of IC deletions I show that IC motifs 4 and 5 play a role in BMP signaling, specifically induction of cross vein defects.

RESULTS

To better understand the relationship between Kek5 structure and its function in BMP signaling six additional Kek5 IC variants were generated (see Material & Methods; Figure 3.1). These variants target the conserved motifs within the IC domain and include both single and multi-motif deletions. In addition to the deletion variants, C-terminal PDZ domain binding site swaps between Kek5 and Kek6 were also assessed for function.

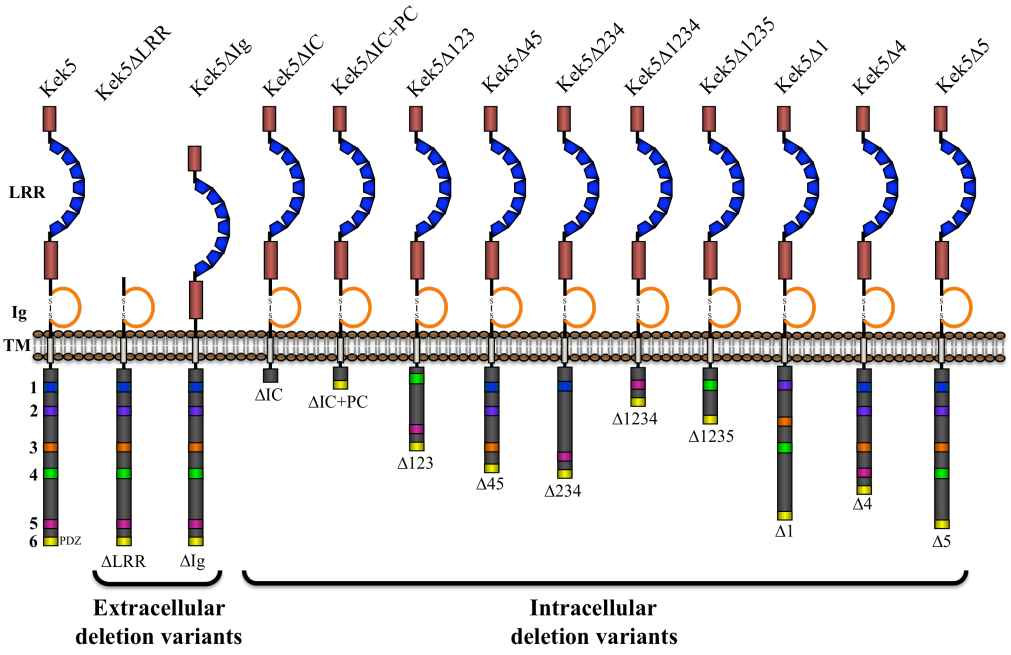

Figure 3.1: Schematic representation of Kek5 deletion variants. Both extracellular and intracellular deletion variants of Kek5 are represented. LRR - Leucine rich repeat, Ig - Immunoglobulin domain, T M- Transmembrane region, 1-6 denotes the conserved intracellular motifs (Evans et al., 2009). Note: ΔLRR and ΔIg variants were generated by N. Klemens; Δ123, Δ45 and ΔIC+PC generated by C. Ernst.

Prior structural analysis of Kek5 pointed toward the importance of the LRRs and lack of requirement for the Ig domain for the BMP associated function of Kek5 (Evans et al., 2009). The work by Evans also showed that the IC C-terminal PDZ domain binding site was important for Kek5 expression/localization and consequently function (Evans et al., 2009). Re-evaluation of

the data on the deletion variant Kek5$^{\Delta IC+P}$ revealed the importance of the IC region beyond the PDZ domain binding site of Kek5 in BMP signaling (Ernst and Duffy, 2010). Detailed work on the IC region of Kek5, which is ~500aa long and contains 6 evolutionarily conserved motifs, was initiated with the generation of N and C-terminal intracellular deletion variants, Kek5$^{\Delta 123}$ and Kek5$^{\Delta 45}$ (Ernst and Duffy, 2010). Here this is extended by the generation of single domain (Kek5$^{\Delta 1}$, Kek5$^{\Delta 4}$ and Kek5$^{\Delta 5}$) and additional multi-domain (Kek5$^{\Delta 234}$, Kek5$^{\Delta 1234}$, Kek5$^{\Delta 1235}$) deletion variants. Schematic representation of various Kek5 deletion variants can be seen in Figure 3.1. All the constructs were tested for effects on viability, crossveins formation (BMP signaling), Armadillo upregulation (adherens junctions), extrusion and large cell morphology (Chapter 4); all the different phenotypes associated with Kek5. In this chapter I describe the results predominantly pertaining to Kek5's activity in BMP signaling. The majority of the misexpression studies were done using *ptcGAL4* driver, which drives expression in the A/P boundary in the fly wing. Results obtained for all the transgenic lines for each Kek5 variant tested can be found in Appendix A.

Characterization of Kek5 extracellular functionality in BMP signaling – Kek5$^{\Delta LRR}$ and Kek5$^{\Delta Ig}$

As observed previously, deletion of the LRRs resulted in the loss of Kek5 activity in BMP signaling (crossvein patterning). Misexpression of Kek5$^{\Delta LRR}$ did not result in loss of anterior crossvein (ACV) (Evans et al., 2009). Closer examination of the localization pattern of Kek5$^{\Delta LRR}$ revealed this variant exhibited aberrant localization; membrane localization was disrupted, while what appeared to be vesicular staining predominated (Figure 3.2). Thus, reduced activity for this variant is likely to simply reflect its disrupted localization. Unlike deletion of the

LRRs, removal of the Ig domain did not have any significant effect on activity with respect to crossvein defects (97% ACV defect in Kek5$^{\Delta Ig}$ versus 100% ACV defect in full length Kek5) and localized properly (Evans et al., 2009). Consistent with its retained activity, this variant demonstrates that the Ig domain is not essential for Kek5's role in BMP signaling (Figure A10 C).

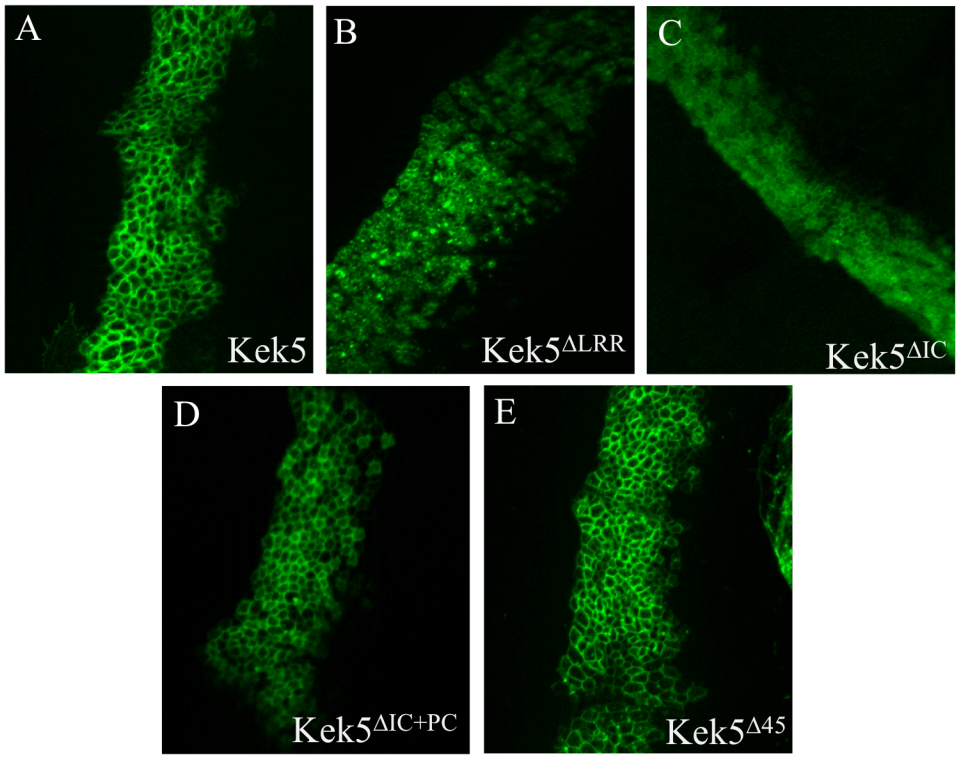

Figure 3.2: Localization of Kek5 deletion variants. GFP-tagged variants were misexpressed in the A/P boundary using *ptcGAL4*. Localization of variants to the membrane was normal (D, E) except Kek5$^{\Delta LRR}$ (B) and Kek5$^{\Delta IC+PC}$ (C). All the wing discs are oriented with their anterior side to the right and ventral side up.

Characterization of Kek5 intracellular functionality in BMP signaling

Misexpression of Kek5$^{\Delta IC}$ did not localize to the membrane or display any Kek5 phenotypes suggesting that membrane localization was required for activity (Evans et al., 2009; Figure 3.2). Retaining the C-terminal PDZ domain-binding site while deleting the rest of the IC

domain restored membrane localization of the protein, but nevertheless did not confer full activity to Kek5 (Evans and Duffy, 2006; Evans et al., 2009; Ernst and Duffy, 2010) (Figure 3.2, Figure A1). Misexpression of Kek5$^{\Delta IC+PC}$ resulted in a low percentage (<2%) of crossvein defects (compared to 100% defect with full length Kek5). The observation that deletion of most of the IC domain (Kek5$^{\Delta IC}$ or Kek5$^{\Delta IC+PC}$) resulted in loss of Kek5 activity clearly implied that elements within the IC domain are required for Kek5's activity. These findings were in contrast to the structural requirements of Kek1 where the entire intracellular domain (except the PDZ domain binding site) was dispensable for its role in inhibition of EGFR signaling (Alvarado et al., 2004a).

The IC domain of Kek5 has six evolutionarily conserved motifs with the C-terminal motif (6) being the PDZ domain binding site (Figure 3.1). Initial structure/function studies of the IC domain were initiated with the generation of N- and C- terminal IC deletions, Kek5$^{\Delta 123}$ and Kek5$^{\Delta 45}$ (Ernst and Duffy, 2010). Analysis of these two deletions indicated that these two halves probably have separable functions in Kek5 activity. In the following sections I describe results obtained from these deletions and the subsequent multi and single variants generated to test the specificity and requirement of various motifs in Kek5 function.

BMP signaling specifies crossveins in the *Drosophila* wing and Kek5 has been shown to modulate this process. Analysis of Kek5$^{\Delta 45}$, revealed the importance of motifs 4 and 5 in crossvein specification since Kek5's activity was reduced (Figure 3.3, Figure A4) and this reduction in activity was not due to poor membrane localization (Figure 3.2).

Since deleting domains 4 and 5 together (Kek5$^{\Delta 45}$) dramatically reduced the crossvein defects shown by Kek5, it was hypothesized that either IC4 or IC5 might be important for Kek5's role in inhibition of BMP signaling. To test this theory, two types of deletion variants were generated: 1) single domain deletion variants deleting only IC4 (Kek5$^{\Delta 4}$) or IC5 (Kek5$^{\Delta 5}$) and 2) multi-domain deletion variants deleting everything except IC4 (Kek5$^{\Delta 1235}$) or IC5 (Kek5$^{\Delta 1234}$) and retaining the PDZ domain to ensure proper membrane localization of the variant. As expected all these variants displayed appropriate membrane localization (Figure A10). Both the single domain deletions, IC4 and IC5, resulted in relatively high frequency of crossvein defects (~50%) when compared to Kek5$^{\Delta 45}$ (16%) (Figure 3.3, Figure A8 and A9). These results suggest that motifs 4 and 5 have non-redundant, additive roles in crossvein specification and BMP signaling (Figure 3.3).

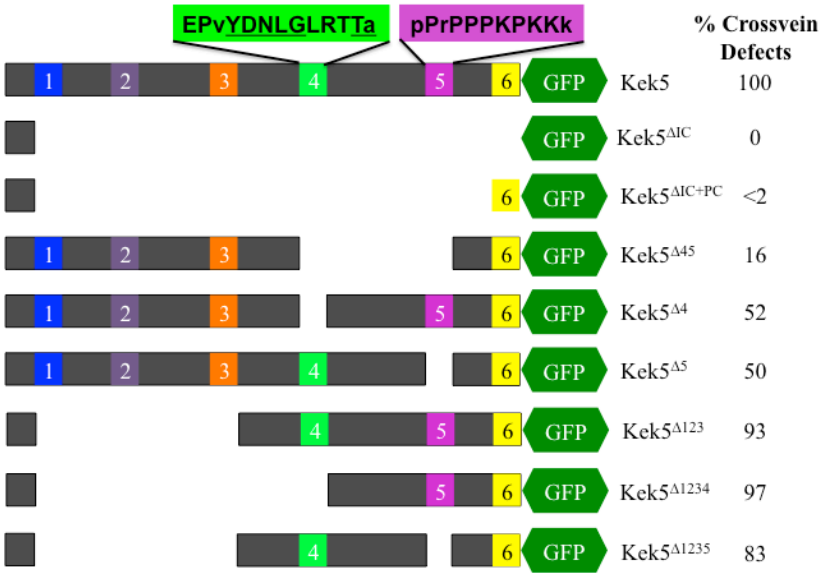

Figure 3.3: Intracellular motifs 4 and 5 are crucial for crossvein defects induced by Kek5. IC domain is represented as the black rectangle while motifs are represented in numbered colored boxes. All the constructs are GFP tagged (green hexagon). Percent crossvein defects are shown for each variant when crossed to *ptcGAL4*.

In a complementary scenario, if motifs 4 and 5 were both important for crossvein specification, then having just either of these domains individually in the intracellular region would provide considerable function to Kek5 in crossvein specification (BMP signaling). Misexpression of both the multi domain deletion variants, Kek5$^{\Delta 1234}$ and Kek5$^{\Delta 1235}$, with

ptcGAL4 resulted in significant lethality in most transgenic lines obtained for each variant. However, transgenic lines giving rise to viable adults with *ptcGAL4* were recovered for each variant and tested for effects on crossvein specification. These were found to have greater activity with respect to crossvein defects than that observed for the single domain deletions, Δ4 and Δ5 (Figure 3.3). These results suggest that removal of additional N-terminal IC motifs (Δ1-3) may cause Kek5 to be more active thus leading to a higher frequency of crossvein defects (and lethality in most cases) than for the single domain deletions. This was also true in the case of $Kek5^{\Delta 123}$, which with *ptcGAL4* caused lethality in most lines and had crossvein defects comparable to full length Kek5.

Table 3.1: C-terminal PDZ domain-binding site consensus sequence. (Hung and Sheng, 2002).

Class	Consensus
Type I	-X-S/T-X-Φ
Type II	-X-Φ-X-Φ
Type III	-X-D/E-X-Φ

X = any residue; Φ = hydrophobic residue

Lastly, the role of IC motif 6, the PDZ domain binding site, in Kek5 function in BMP signaling was investigated. Because IC 6 was previously shown to be important for localization, swaps rather than a deletion were used to test its requirement in BMP signaling. Kek6 was selected to generate the binding site swap because it has a type II binding site (FVSL) as opposed to Kek5, which contains a type I binding site (GTEV) (Arata and Duffy, 2011) (Table 3.1). If the type I binding site of Kek5 were specifically required for BMP signaling, then replacing it with the type II site from Kek6 would eliminate the activity of this Kek5 variant. Contrary to this, a Kek5 variant with the PDZ domain binding site of Kek6 ($Kek5^{K6PDZ}$) exhibited the same ability to inhibit crossvein formation as wild type Kek5 (data not shown). This supports the idea that the PDZ domain is a generic sequence element that acts principally to ensure correct Kek5 membrane localization (Figure A11).

Characterization of structure and functionality of Kek5 in scutellar bristle patterning

Loss of BMP signaling has been associated with effects on scutellar bristles, and misexpression of Kek5 also affects the number of scutellar bristles; scutellar bristles increase from 4 in wild type to about 15 in *ptc>kek5* (Evans et al., 2009; Wharton et al., 1999) (Figure 3.4). Concomitant with the analysis of crossvein patterning, Kek5 variants were also analyzed for effects on bristle patterning, thereby providing structure/function information on Kek5 with respect to this phenotype.

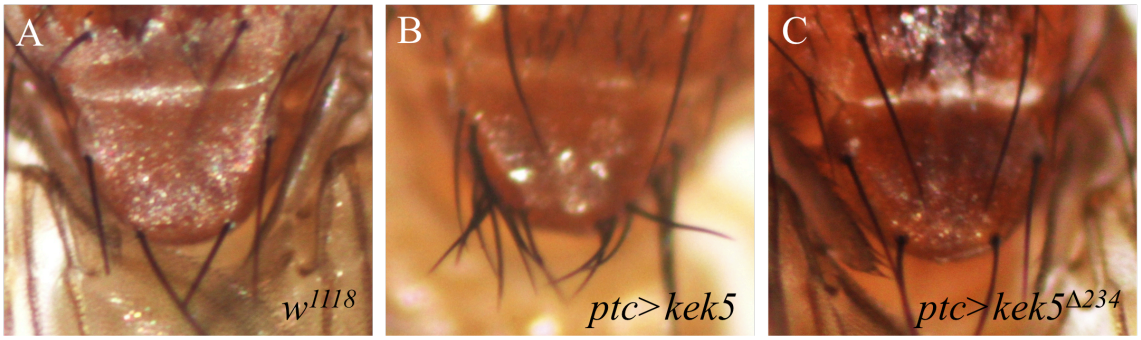

Figure 3.4: IC motifs 2-4 appear to be important for scutellar bristle duplication. Wild type flies have 4 scutellar bristles (A) and misexpression of Kek5 results in scutellar bristle duplication (B), which is abolished by deletion of IC motifs 2, 3 and 4 (C).

As expected due to the aberrant localization of the LRR deletion variant, loss of the LRRs eliminated Kek5 activity in bristle patterning. In contrast, deletion of the Ig domain did not affect Kek5 activity as ectopic scutellar bristles were still observed. Thus, as seen in crossvein patterning, the Ig domain does not appear to be required for Kek5 function.

Deletion of the entire IC domain except the PDZ domain binding site, eliminated Kek5 activity with respect to scutellar bristle duplication suggesting that there are sequence elements in the IC region of Kek5 that are important for scutellar bristle duplication. Of all the remaining IC variants, only Kek5$^{\Delta 234}$ consistently displayed little to no scutellar bristle duplication (Figure 3.4).

This suggests that individually or in combination motifs 2, 3 and 4 are important for bristle duplication. Deletion of motif 4 in the case of variants Kek5$^{\Delta 45}$ and Kek5$^{\Delta 4}$ resulted in a modest reduction in the average number of scutellar bristles (~9, 10) suggesting that motif 4 might play a role in this phenotype. Supporting this was analysis of Kek5$^{\Delta 5}$, which did not seem to affect Kek5's ability to induce bristle duplication (~13). Analysis of the multi-motif deletion Kek5$^{\Delta 1234}$ however complicated this conclusion as it still conferred considerable function to Kek5 (~13). Interestingly, while Kek5$^{\Delta 234}$ has essentially no activity, the additional removal of only motif 1 resulted in an almost complete restoration of activity, suggesting the possibility of antagonistic or inhibitory interaction for motif 1 (Appendix A).

Characterization of the structure and functionality of Kek5 in viability

Misexpression of the Kek5 variants revealed a striking degree of lethality associated with specific variants. While misexpression of wild type Kek5 with *ptcGAL4* results in no lethality, for some variants more than 75% of the lines tested resulted in lethality when driven with *ptcGAL4*. This was true for most of the variants that deleted motifs 2 and 3 i.e. Kek5$^{\Delta 234}$, Kek5$^{\Delta 1234}$ and Kek5$^{\Delta 1235}$ (Figure 3.5, Figure 3.6, Appendix A). One possibility is that

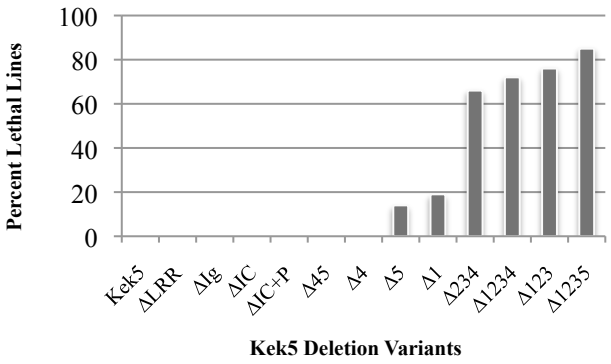

Figure 3.5: Lethality associated with Kek5 misexpression. The viability of all lines for each Kek5 variant was determined with *ptcGAL4*. In the case of Kek5, Kek5$^{\Delta LRR}$, Kek5$^{\Delta Ig}$, Kek5$^{\Delta IC}$, Kek5$^{\Delta IC+PC}$ or Kek5$^{\Delta 4}$ none of the lines tested with *ptcGAL4* caused lethality. In contrast, misexpression of Kek5$^{\Delta 234}$, Kek5$^{\Delta 1234}$, Kek5$^{\Delta 123}$ and Kek5$^{\Delta 1235}$ was associated with significant lethality.

motifs 2 and 3 may function in an inhibitory manner, and their deletion triggers increased or unregulated activity of Kek5, leading to lethality.

Figure 3.6: Schematic representation of the intracellular region of Kek5 variants resulting in lethality. Intracellular region is represented as the black rectangle while motifs are represented in numbered colored boxes. Grey striped box indicates the sequence elements common to the variants, misexpression of which leads to lethality with *ptcGAL4*.

DISCUSSION

Proteins have unique structural elements that impart to them different biological functions. Generating structural variants such as by generation of swaps or deletions one can perform functional analysis of a protein. Previously, we have performed such analyses on Kek1 and have shown that its LRRs and transmembrane region are important for its function in EGFR inhibition (Alvarado et al., 2004a). Similarly we have also shown the importance of LRRs and the PDZ domain binding sites in Kek5 function (Evans et al., 2009). Here we have performed a structure/function analysis of the IC domain of Kek5 by characterizing the activity of deletion variants. Our findings suggest that disparate motifs are required for various Kek5 associated phenotypes (Figure 3.7). Motifs 4 and 5 have an additive role in BMP signaling, while motifs 2, 3, and 4 may play a role in scutellar bristle duplication. Finally, motifs 2 and 3 may regulate the activity of Kek5 in an inhibitory manner, as deletion variants lacking these motifs are associated with increased lethality.

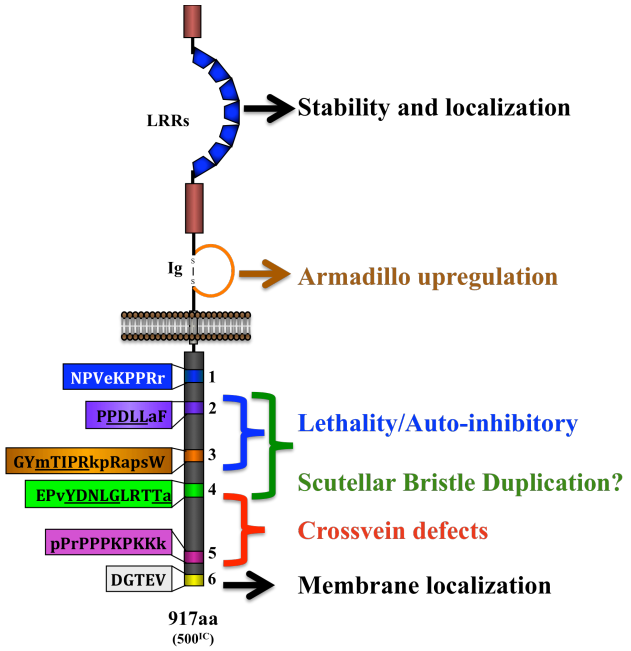

Figure 3.7: Different structural elements of Kek5 are responsible for disparate BMP signaling related phenotypes of Kek5. Extracellular LRRs are important for membrane localizations and stability of the protein. Intracellular motifs 2 and 3 seem to have an auto-inhibitory role. Motifs 4 and 5 are important for crossvein defects. Motifs 2-4 appear to be important for scutellar bristle duplication.

Intracellular motifs 4 and 5 are required for Kek5's role in BMP signaling

Deletion of motifs 4 and 5 together (Kek5$^{\Delta 45}$) results in a stronger loss of Kek5's ability to induce crossvein defects than deletion of either motif alone (Kek5$^{\Delta 4}$ and Kek5$^{\Delta 5}$). This suggests additive and non-redundant roles for these motifs. One simple model to explain this behavior is that a protein involved in BMP signaling might interact with both motifs 4 and 5. In the absence of one motif, this protein may be able to interact with the remaining motif to confer partial function to Kek5 (Figure 3.8). It is also possible that instead of a single protein as shown in the model above multiple proteins in a complex may act in a similar manner.

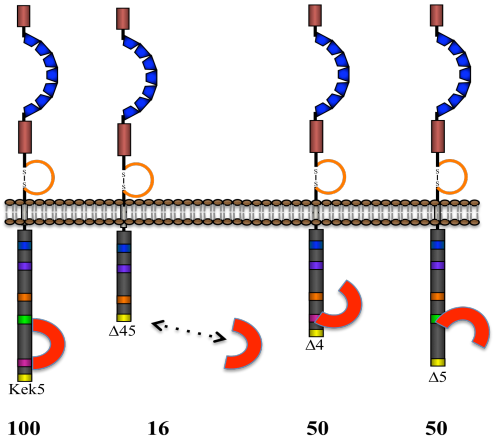

Figure 3.8: Model for an additive role of Kek5 motifs 4 and 5 in BMP signaling. Number below each variant indicates the average frequency of crossvein defects induced by that variant.

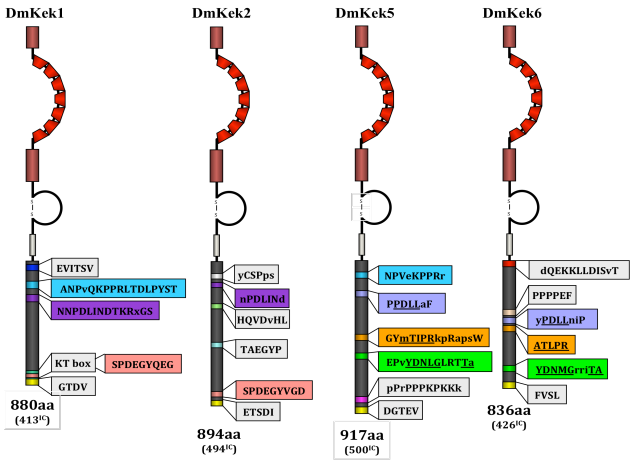

Figure 3.9: Conserved IC motifs in Kek family members. Sequence comparison of Kekkon family members reveal conserved cytoplasmic stretches/motifs shown in colored boxes. Similar motifs are represented in same colored (Evans and Duffy, 2006).

Interestingly, while some motifs are shared among some Kek family members (i.e. motif 1 in Kek5 and Kek1), motifs 4 and 5 are only present together in Kek5, supportive of a combinatorial role (Figure 3.9). Although none of these conserved IC elements contain any previously described sequence elements, it is interesting to

note that such a sequence combination may be the reason for Kek5's involvement in these processes (Figure 3.9).

Kek5 and scutellar bristle development

Kek5 misexpression causes scutellar bristle duplication. Extra bristles are also seen in perturbations of various signaling pathways, including Notch, EGFR, and BMP (Furman and Bukharina, 2008; Wharton et al., 1999). We have shown that Kek5 is a modulator of BMP signaling. Together these facts suggest that Kek5's affect on scutellar bristle duplication might be mediated through modulating BMP signaling. In addition the structure/function analysis indicates a possible requirement for IC motifs 2, 3, and 4 in bristle duplication by Kek5. How these sequence elements contribute mechanistically to Kek5 activity remains to be determined, but their functional identification provides an important step in this direction.

Potential auto-inhibitory functions of IC motifs 2 and 3

It was surprising to discover that Kek5 variants where motifs 2 and 3 were deleted were associated with increased lethality. It would be interesting to clarify whether this potential auto-inhibitory function is due to a combined deletion of motifs 2 and 3, or deletion of either motif alone.

As in the case of the additive contribution of motifs 4 and 5, it is possible that motifs 2 and 3 also have an additive role. As shown in Figure 3.9 motifs 2 and 3 (and 4) are conserved in Kek6, however, to date analysis of Kek6 has not revealed any common activities between Kek6 and Kek5. Swapping motifs 2, 3, and 4 between Kek5 and Kek6 would allow any conservation of their activities between the two Keks to be directly tested.

MATERIALS AND METHODS

Drosophila genetics

All matings were carried out at 28^{o}C unless otherwise noted. Transgenic flies were generated using standard germline transformation techniques (Spradling, 1986). The variants generated were sent to Genetic services Inc. for injections. Injected embryos/larvae thus received were processed to generate various transgenic lines. All the transgenics hence generated were tested for expression and activity for each of the constructs.

Molecular cloning of Kek5 variants

Routine molecular cloning methods were used to generate the various Kek5 variants. The intracellular deletion variants were generated using two different approaches: stitching PCR products (Table 3.2 and Figure 3.10) and Site directed mutagenesis (Table 3.3 and Figure 3.11).

Table 3.2: Primers used to generate the multi-motif Kek5 deletions by stitching PCR

	Kek5$^{\Delta IC234}$ (or Kek5$^{\Delta 234}$)	Kek5$^{\Delta IC1234}$ (or Kek5$^{\Delta 1234}$)	Kek5$^{\Delta IC1235}$ (or Kek5$^{\Delta 1235}$)
5' Fragment	5’ W45	5’ W45	5’ W45
	3’ W137	3’ W139	3’ W135
3' Fragment	5’ W138	5’ W140	5’ W136
	3’ W22	3’ W22	3’W22

Note – Template used to generate Kek5$^{\Delta 234}$ and Kek5$^{\Delta 1234}$ was pUASTKek5GFP while pUASTKek5$^{\Delta 123}$GFP (generated by C. Ernst and me) was the template used to generate Kek5$^{\Delta 1235}$. Primer sequences can be found in Appendix B

For generation of the multi-motif deletion variants, 5’ and 3’ fragments were separately generated by PCR and sewed together in a stitching PCR reaction using the outside/external primers. GFP tagged Kek5 variants (in the Gateway system, Invitrogen) thus generated by this method include; Kek5$^{\Delta 123*}$, Kek5$^{\Delta 45*}$, Kek5$^{\Delta IC+PC*}$, Kek5$^{\Delta 234}$, Kek5$^{\Delta 1234}$, Kek5$^{\Delta 1235}$ (* Constructs generated by C. Ernst). Tables 3.1 and 3.2 include only information about the constructs I generated.

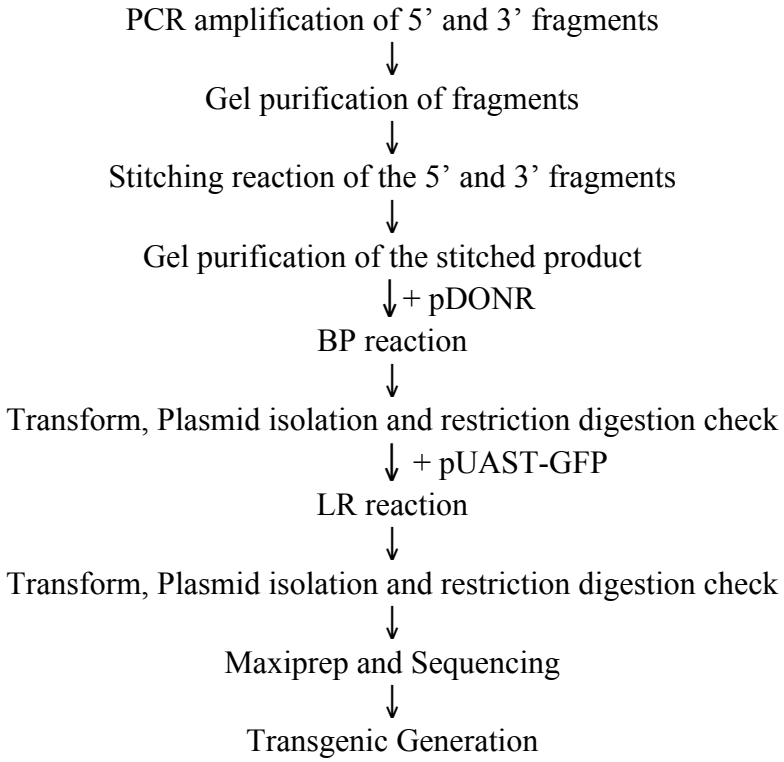

Figure 3.10: Flowchart depicting generation of multi-motif Kek5 deletion variants by stitching PCR products.

Single domain Kek5 deletion variants were generated using SDM (Quick change II Site-Directed Mutagenesis by Stratagene). Detailed procedure can be found in the Stratagene manual and a schematic is shown in Figure 3.11.

Table 3.3: Primers used to generate the single-motif Kek5 deletion variants by Site-Directed Mutagenesis (SDM)

$Kek5^{\Delta IC1}$ (or $Kek5^{\Delta 1}$)	$Kek5^{\Delta IC4}$ (or $Kek5^{\Delta 4}$)	$Kek5^{\Delta IC5}$ (or $Kek5^{\Delta 5}$)
5' W200	5' W202	5' W204
3' W201	3' W203	3' W205

Note – Template used for SDM reactions was pENTRKek5. Primer sequences can be found in Appendix B

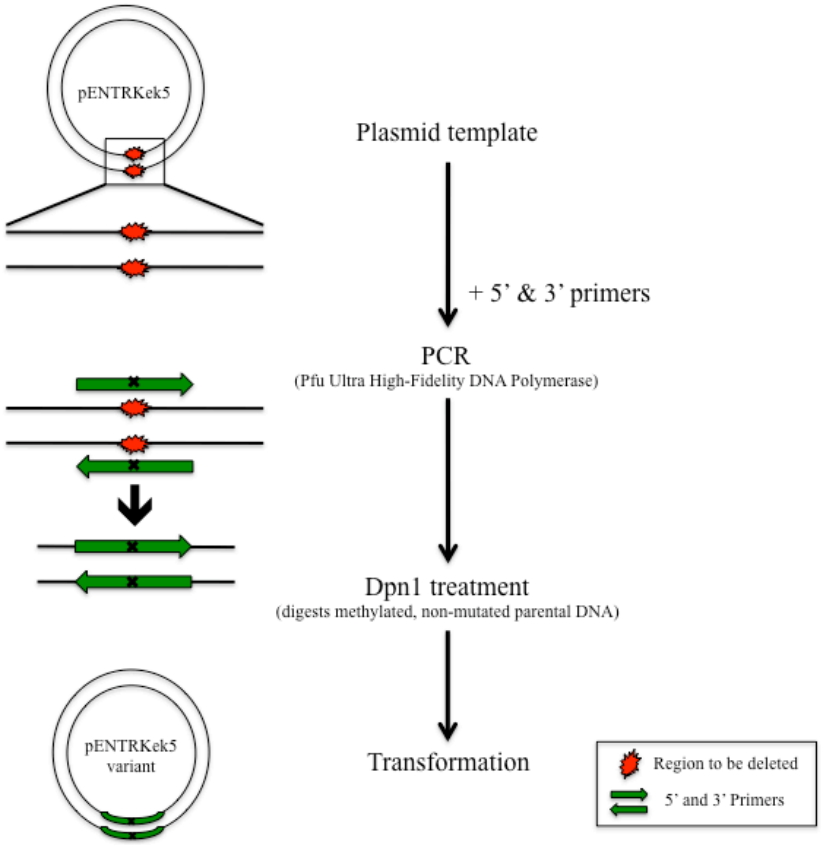

Figure 3.11: Scheme for generation of single-motif Kek5 deletion variants by SDM (Adapted from Quick change II Site-Directed Mutagenesis by Stratagene**)**

Kek5 variant localization analysis

Localization of the variants was determined in third instar wing discs from *ptc>kek5*^{variants} that had been stained for one of Armadillo. The discs were mounted in 50% PBS glycerol and 5uL of Slowfade anti-fade reagent. Imaging was done on Ziess Axio Imager equipped with Apotome at 40X.

ACKNOWLEDGEMENTS

Nick Klemens generated extracellular deletions of Kek5. $Kek^{\Delta 123}$, $Kek5^{\Delta 45}$ and $Kek5^{\Delta IC+PC}$ were generated by Christina Ernst. The Kek5/Kek6 PDZ domain binding site swaps were generated by Michelle Arata. I also want to sincerely thank Neil Silverman (University of Massachusetts Medical School) for suggesting the use of SDM for generating the single domain deletions.

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