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Disruption of the protein kinase N gene of Drosophila melanogaster Results in the Recessive delorean Allele (pkn^{dln}) With a Negative Impact on Wing Morphogenesis

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ABSTRACT We describe the delorean mutation of the Drosophila melanogaster protein kinase N gene (pkn^{dln}) with defects in wing morphology. Flies homozygous for the recessive pkn^{dln} allele have a composite wing phenotype that exhibits changes in relative position and shape of the wing blade as well as loss of specific vein and bristle structures. The pkn^{dln} allele is the result of a P-element insertion in the first intron of the pkn locus, and the delorean wing phenotype is contingent upon the interaction of insertion-bearing alleles in trans. The presence of the insertion results in production of a novel transcript that initiates from within the 3' end of the P-element. The delorean-specific transcript is predicted to produce a wild-type PKN protein. The delorean phenotype is not the result of a reduction in pkn expression, as it could not be recreated using a variety of wing-specific drivers of pkn-RNAi expression. Rather, it is the presence of the delorean-specific transcript that correlates with the mutant phenotype. We consider the delorean wing phenotype to be due to a pairing-dependent, recessive mutation that behaves as a dosage-sensitive, gain of function. Our analysis of genetic interactions with basket and nemo reflects an involvement of pkn and Jun-terminal kinase signaling in common processes during wing differentiation and places PKN as a potential effector of Rho1’s involvement in the Jun-terminal kinase pathway. The delorean phenotype, with its associated defects in wing morphology, provides evidence of a role for PKN in adult morphogenetic processes.

KEYWORDS PKN wing morphology Rho effector signal transduction JNK pathway

A cell’s ability to receive information and respond appropriately requires a process of signal transduction, whereby an external stimulus is coupled to transducers that relay information in from the cell surface. Signaling pathways often connect stimuli with specific protein modifications that ultimately alter protein function to mediate the desired response. Any given cell type uses many pathways, often with cross talk between them. Despite such complexity, components of signal transduction pathways have been highly conserved (Pires-da-Silva and Sommer 2006).

Rho family GTPases are one such conserved family of proteins, which belong to the Ras-like GTPase superfamily (Wennerberg et al. 2005; Bourreux et al. 2007), acting as signal transducers that regulate and integrate a wide range of cellular processes, including actin reorganization, cell adhesion, cell polarity, cell-cycle progression, and apoptosis (Coleman and Olson 2002; Bustelo et al. 2007; Guilluy et al. 2011; Spiering and Hodgson 2011; Ridley 2012). Rho GTPases are monomeric guanosine-5’-triphosphate (GTP)-binding proteins (G proteins) that act as molecular switches, cycling between a GDP-bound inactive state and GTP-bound active state in response to external stimuli. In their active GTP-bound configuration, Rho GTPases bind to and activate downstream effectors. More than 60 effector proteins have been identified based on their interaction with the three

Protein kinase N (PKN) is a downstream effector of both Rac1 and Rhos that interacts directly with these GTPases in their active, GTP-bound state (Vincent and Settleman 1997; Lu and Settleman 1999). Activation by either Rac1 or Rhos occurs via association with distinct regulatory sequences found at the N-terminus of PKN, known as HR1 repeats. HR1 repeats exhibit similarity to the leucine zipper structural motif and adopt an antiparallel coiled-coil structure (Maasaki et al. 1999). Within its kinase domain, PKN is highly similar to protein kinase C (Mukai and Ono 1994) and, as such, is a member of the larger AGC kinase subfamily of serine-threonine protein kinases (Pearce et al. 2010). PKN homologs are found in invertebrates and vertebrates (Palmer et al. 1995; Kitagawa et al. 1995; Mukai et al. 1995; Ueno et al. 1997; Stapleton et al. 1998). In mammals, PKN has demonstrated involvement in the regulation of cytoskeletal reorganization (Mukai et al. 1997; Vincent and Settleman 1997), cell adhesion (Calautti et al. 2002), cell-cycle regulation (Mukai 2003), and tumorigenesis (Metzger et al. 2003; Leenders et al. 2004). There are three mammalian paralogs of PKN (Mukai 2003), presented in the literature under various names (PKN1, PKN2, PKN3, PKNα, PKNβ, PKNγ, PRK1, PRK2). PKN1 and PKN2 are expressed widely, whereas the expression of PKN3 is more restricted (Mukai and Ono 2006; Lachmann et al. 2011) and studied more often in cultured cells (Mukai et al. 1996). Although there is a great deal of information about PKN function from cultured cells, few whole-animal studies have been conducted. Furthermore, because the three types of mammalian PKN exhibit overlapping patterns of expression, it is sometimes difficult to know which paralog is functionally relevant or whether functional redundancy is involved. Therefore, we sought a simpler system in which to study PKN function.

The fruit fly Drosophila melanogaster offers a superb system to study the normal developmental role of Rho family effectors (Johnzdow et al. 2004). The protein structure of the single Drosophila PKN is very similar to its well-characterized mammalian orthologs, containing an N-terminal region with three HR1 repeats, followed by a C2 domain related to the calcium-dependent membrane-targeting domain of protein kinase C and finally a C-terminal kinase domain (Ueno et al. 1997). In Drosophila, protein kinase N (pkn) has been implicated in the process of dorsal closure during embryogenesis, involving the migration and fusion of epidermal cells that resembles wound healing (Lu and Settleman 1999). Loss of PKN results in dorsal closure failure in ~10% of homozygous pkn06736 individuals (Lu and Settleman 1999). Regulation and rearrangement of the cytoskeleton is required as cells change shape and alter contacts with other cells during dorsal closure. It is known that Rho GTPase-dependent signaling pathways participate in dorsal closure, because both Rac1 and Rhos loss-of-function mutants exhibit defects in the processes required for dorsal closure, including actin reorganization, cell movement, and cell-cell contact (Harden et al. 1999; Baek et al. 2010; Bahri et al. 2010). However, the exact mechanism by which PKN acts in its capacity as a Rac/Rho target effector in fly development has yet to be determined.

The loss-of-function, dorsal closure phenotype associated with pkn06736 is relatively mild (~10% of embryos with the phenotype) and may reflect functional redundancy with components of the Jun-terminal kinase (JNK) pathway, which is also required for dorsal closure in Drosophila. Previous work has suggested that in its capacity as a Rho1 effector, PKN is involved in a JNK-independent instructive signal for dorsal closure (Lu and Settleman 1999). PKN function is required for viability in Drosophila, as evidenced by a multiphasic lethal phenotype that fails to generate homozygous pkn06736 adults (Lu and Settleman 1999). However, this does not provide the insight needed to determine the nature of PKN’s effector function. For this reason, we sought to analyze additional mutant alleles of the pkn gene to better understand the function of the PKN protein. Here we describe a new allele of Drosophila pkn we called delorean (pkn06736), which was identified from the Kiss P-element mutagenesis stock collection of P[lacW] transposon insertions on the second chromosome (Torok et al. 1993). The delorean phenotype, with its associated defects in wing morphology, provides evidence for a novel role of PKN in morphogenetic processes and provides new insights into the effector function of PKN.

MATERIALS AND METHODS

Fly stocks and genetics

Fly stocks were maintained at 25°C on hydrated fly food (Carolina Biological Supply) supplemented with live yeast. Flies were anesthetized using carbon dioxide. Where heteroallelic interactions were studied for phenotypic analysis, matings between strains were carried out at 25°C using reciprocal crosses.

The stock yw6723, F[w65mc = lacW]/(2)k06808/CyO (referred to as P[lacW]k06808) was obtained from the Kiss P-element mutagenesis stock collection (Torok et al. 1993). The basket (bsk1) pkn06736 chromosome was generated in our lab by recombining bsk1 (using Bloomington stock #3088) and pkn06736 alleles. All other stocks used in this study were obtained from Bloomington and Umea stock centers or created in our lab. The P[lacW]k06808 insertion chromosome was initially outcrossed for eight generations to a Bloomington stock isogenic for chromosome 2 (yw6723, iso-2) and rebalanced over yCyO (kindly provided by Karen Blochlinger). Subsequently, the P[lacW]k06808 insertion chromosome was outcrossed for >40 generations to a w1118 strain (w1118, iso-2) and maintained by selection for the mini-white marker of P[lacW].

Transposon excision

P[lacW]k06808/CyO individuals were crossed to w+; Sp/CyO; Δ2-3 Dr/TM6. Dysgenic P[lacW]k06808/CyO; Δ2-3 Dr/+ offspring were mated to yw/FM6; Pn+/CyO flies. Individual white-eyed yw/+/Y; ΔP/CyO males were backcrossed to P[lacW]k06808/CyO virgins to test for genetic interaction and to establish individual balanced revertant stocks.

Wing morphology analysis

Wings were dissected dry, transferred to glass microscope slide with isopropanol and mounted in AquaPolymount (Polysciences, Inc.). Anterior wing margin bristles and sensillae were viewed under brightfield optics using a Nikon E200 microscope at 400x magnification. For each wing, we counted the number of twin campaniform sensillae, stout bristles, dorsal recurved bristles, ventral recurved bristles, ventral slender bristles, and domed sensillae on longitudinal vein L3 and on the anterior crossvein. Sample size for each genotype varied from 5 to 32. Median values were subjected to a Kruskal-Wallis test.
Determination of delorean homozygote viability

Virgins of the genotype pkrtn+/CyO, P[w+;m]act5C-GFP]JMR1 pkn+ were mated to pkrtn+/pkrtn males and then allowed to lay eggs on a collection plate (3% agar, 20% apple juice, 10% sucrose) for 4–6 hr at 25°C. Immature eggs were removed and embryos were allowed to develop. First instar larvae were sorted for the presence or absence of the green fluorescent protein (GFP) marker associated with the CyO balancer, placed on Carolina food supplemented with yeast at 25°C and observed throughout development to adulthood. We would expect that if pkrtn+/pkrtn homozygotes are 100% viable they should represent half of the progeny from these matings.

Polytene chromosome in situ hybridization

In situ hybridization was carried out essentially according to Pardue (1986) with the following exceptions: Salivary glands were dissected from wandering third instar larvae in 50% acetic acid and squashed in 45% acetic acid, 18% lactic acid on glass microscope slides. Hybridization was performed overnight at 55°C in hybridization solution (0.6 M NaCl; 50 mM NaPO4, pH 7.2; 500 µg/mL salmon sperm DNA; 5× Denhardt’s solution) containing 3 µg/mL digoxigenin-labeled Xbal-linearized Plac/W probe (kindly provided by Ed Giger). Bound probe was detected using an alkaline phosphatase-based detection kit (Boehringer Mannheim). Chromosomes were counterstained with 4’,6-diamidino-2-phenylindole (DAPI). The insertion sites of the transposons associated with the "fl" and "pkndln" phenotype were mapped to a collection plate (3% agar, 20% apple juice, 10% sucrose) for 4–6 hr at 25°C through development to adulthood. We would expect that if pkrtn+/pkrtn homozygotes are 100% viable they should represent half of the progeny from these matings.

Genomic analysis of transposon insertion alleles and derivative alleles

Genomic DNA (gDNA) was prepared from 50 homozygous P[lacW] k06808 adults according to Ashburner (1989). Frozen flies were homogenized in buffer (0.1 M Tris-HCl, pH 9; 0.1 M ethylenediaminetetraacetic acid; 1% sodium dodecyl sulfate; 0.5% diethylpyrocarbonate). The homogenate was incubated at 65°C for 30 min, clarified with 1 M potassium acetate, and precipitated with isopropanol. Then, 1 µg of gDNA was digested individually with restriction endonucleases BamHI or EcoRI (New England Biolabs) and phenol/chloroform extracted and ethanol precipitated. Digested gDNA preparations were individually diluted to 1 µg/mL in TE (0.01 M Tris-HCl, pH 8.0; 0.001 M ethylenediaminetetraacetic acid), self-ligated with T4 ligase (Promega), and transfected into XL-1 Blue competent E. coli cells (Stratagene). Transformants were plated on LB containing 40 µg/mL ampicillin. Two positive colonies of each type were individually grown in LB-Amp broth. Plasmid DNA was prepared by alkaline lysis, purified through a QIAGEN-tip 100 column (QIAGEN), and sequenced with primers near the ends of P[lacW]. Primer sequences were EcoR1: 5’TGGTAAGTGGATGTCTCTTGC3’, BamL1: 5’TTCCTCTCAACAGCAAAAC3’ and BamR1: 5’GGCTGCGATTTTGTGA TGCTC3’. DNA sequencing was performed by the Iowa State University DNA Sequencing Facility.

The insertion sites of the transposons associated with the P[lacW] k06808 and P[PZl(2)l(2)G2322] strains were determined using the standard inverse polymerase chain reaction (PCR) protocol available from the Berkeley Drosophila Genome Project (http://www.fruitfly.org/about/methods/inverse.pcr.html) with the following specifications. The restriction endonuclease HinP11 (New England Biolabs) was used to digest gDNA with the P5′ region of the transposon amplified using primers Plac4 5’ACTGTGCGTATTGCTCGTCTTTG3′ and Plac1 5’CACCACAGCTGCTCCACACAA3’. The P3′ region of the transposon was amplified using primers Prly 5’CCCTAG CATGGCGTGTTGGATGA3’ and Prly 5’CTTGCGGCGGG ACCACCTTTAGTTAT3’. Sequence was obtained from the amplified P5′ product using Splac2 5’GAATTTAGCTGGGTGTTTACAA3’ or from the amplified P3′ product using one of the original PCR primers. DNA was sequenced on an Applied Biosystems 3130xl Genetic Analyzer at the Amnis Water Resources Institute Sequencing Facility of Grand Valley State University.

Analysis of imprecise excision derivatives used gDNA prepared from homozygous adults as described previously. Then, 5 µL of gDNA was amplified using LongAmp Taq polymerase (New England Biolabs) with 0.4 µM oligonucleotide primers in an Eppendorf Mtercycler. PCR conditions were as follows: initial denaturation at 98°C for 5 min followed by 35 cycles of 98°C for 40 sec, 55°C for 40 sec, 9 min. Primers used were plac4 forward 5’ACTGCGTTGATGGTGACTTGG 5’TTCTATGTATGTTAT3’, placWFor1 5’CAGCGGGACTATTCTGCAAC3’, Ex1For2 5’GGAAAGCAGTGGCGGCGTTATG3’, Ex2Rev2 5’GATG CCGTAGCTCTGGTGGT3’, and plac7 5’CGTGGGTGTCAGCTC GTGC3’. PCR products were size selected in an agarose gel, purified using MinElute columns (QIAGEN), and sequenced as described previously.

RAPD amplification of 5’ cDNA ends (5’ RACE)

The pkrtn+ and Df(2R) w45-30n chromosomes were rebalanced separately over CyO, P[w+;m]act5C-GFP]JMR1, and then the lines were mated together. Third instar larvae were sorted for the presence or absence of the GFP marker associated with the CyO balancer, placed on Carolina food supplemented with yeast at 25°C, and allowed to pupate. One-day-old pupae were homogenized in buffer RLT (QIAGEN), lysates were passed through QiaShredder columns (QIAGEN), and total pupal RNA was purified over RNaseasy columns (QIAGEN). RNA samples were treated with DNase (New England Biolabs) and repurified over RNasey columns. Then, 7 µL of each RNA sample was subjected to RNA ligase-mediated rapid amplification of 5’ cDNA ends (RLM-RACE) according to manufacturer’s protocol (Invitrogen GeneRacer kit). RNAs were reverse transcribed with a primer that binds within exon 7 of pkn (5’TCCGGACTCGAGTGAGTGGAGG-3’) then amplified with the GeneRacer 5′ primer (5′-CGACTCGAGGAC GAGGACGTGA-3’) and a primer that binds within exon 5 of pkn (5′-AATGGCCTCCTGATCTCTCT-3’). PCR products were cloned into pCR4-TOPO (Invitrogen) and transfected into One Shot TOP10 E. coli cells (Invitrogen) with selection on carbenicillin (0.075 mg/mL). Recombinant plasmid DNA was sequenced as described previously. Sequence data from this article have been deposited with NCBI GenBank under accession no. KJ201875.

RESULTS

delorean mutants have defects in wing development

In a screen of a second chromosome P-element insertion collection (Torok et al. 1993), we identified one line, y w; P[lacW] k06808/CyO, with a mutant phenotype that we named delorean. Flies homozygous for the P[lacW] k06808 insertion are unable to fly and their wings are held up from the thorax and curve ventrally (Figure 1, B and C), similar to the open doors of a Delorean automobile. The extent of curvature is variable, from a slight smooth curve to a severe folded-over phenotype. The anterior wing margin is also defective: there are gaps in marginal tissue, excessive spacing between stout mechanosensory bristles (Figure 1, E and G), and fewer sensilla and bristles relative to heterozygous siblings (Table 1). Occasionally, two or three stout bristles share
a common socket, and sometimes a stout bristle appears in the margin tissue without a socket (Supporting Information, Figure S1A, Figure S1B, and Figure S1D). A total of 95% of homozygous delorean flies analyzed (n = 67) had one or more double-stout bristle clusters, and 15% had triple-stout bristle clusters. We also noted defects in wing veins in homozygous delorean flies such as ectopic vein material surrounding the longitudinal vein L2 (Figure 1E) and/or extending from the posterior crossvein (Figure S1C) (females 69.0%, SD 11.8%, n = 163 and males 56.9%, SD 18.7%, n = 116).

A quantitative analysis focusing on specific anterior wing margin structures (Figure S1E) of the delorean wing phenotype was conducted (Table 1). Three of the traits analyzed showed significant differences between delorean homozygotes and either their heterozygous siblings or wild-type flies (w1118), regardless of sex. Reduced numbers of twin campaniform sensillae, stout bristles, and ventral recurved bristles were observed in delorean homozygotes (Table 1). We did not detect significant differences in the average number of slender bristles, dorsal recurved bristles, sensillae on vein L3 nor the anterior crossvein. Therefore, some sensory structures are affected by the delorean mutation whereas others are not. We consider the delorean wing phenotype to be a composite of a held-up position, ventral curvature, reduced sensory structures, and venation defects, suggesting that the affected gene plays diverse roles in wing development.

The delorean phenotype results from a mutation in the pkn gene

To determine the cause of this interesting phenotype, we defined the insertion site of the P[lacW] transposon. Polytenomes from the original w; P[lacW]808080/CyO stock were analyzed using in situ hybridization with a DNA probe containing sequence from the mini-white

Table 1 Quantitative analysis of wing sensory structures

<table>
<thead>
<tr>
<th>Genotype</th>
<th>W1118</th>
<th>pkn&lt;sup&gt;nh&lt;/sup&gt;</th>
<th>pkn&lt;sup&gt;mh&lt;/sup&gt;</th>
<th>pkn&lt;sup&gt;mh&lt;/sup&gt;; Dp(2;3) &lt;i&gt;pake&lt;/i&gt; / &lt;i&gt;pake&lt;/i&gt;</th>
<th>pkn&lt;sup&gt;nh&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twin sensillae</td>
<td>1.86 ± 0.38 (7)</td>
<td>2.0 ± 0 (8)</td>
<td>1.07 ± 0.7 (15)</td>
<td>2.0 ± 0 (7)</td>
<td>2.0 ± 0 (8)</td>
</tr>
<tr>
<td>Stout</td>
<td>2.0 ± 0 (7)</td>
<td>2.0 ± 0 (8)</td>
<td>1.41 ± 0.61 (32)</td>
<td>1.83 ± 0.41 (6)</td>
<td>2.0 ± 0 (13)</td>
</tr>
<tr>
<td>Ventral recurved</td>
<td>77.6 ± 3.02 (8)</td>
<td>82.1 ± 2.17 (8)</td>
<td>48 ± 13.9 (15)</td>
<td>85.9 ± 1.64 (8)</td>
<td>82.0 ± 3.16 (10)</td>
</tr>
<tr>
<td></td>
<td>73.8 ± 2.92 (8)</td>
<td>76.2 ± 3.22 (10)</td>
<td>53 ± 12.1 (32)</td>
<td>73.5 ± 6.16 (6)</td>
<td>74.3 ± 2.59 (13)</td>
</tr>
<tr>
<td></td>
<td>17.9 ± 0.9 (7)</td>
<td>18.0 ± 1.31 (8)</td>
<td>6.57 ± 2.9 (12)</td>
<td>17.3 ± 2.34 (6)</td>
<td>17.1 ± 1.45 (10)</td>
</tr>
<tr>
<td></td>
<td>17.6 ± 1.99 (7)</td>
<td>18.0 ± 1.22 (9)</td>
<td>5.69 ± 2.35 (29)</td>
<td>15.5 ± 2.38 (4)</td>
<td>14.2 ± 2.33 (9)</td>
</tr>
</tbody>
</table>

Anesthetized adult flies were sorted by sex, wings were dissected and mounted flat, and twin campaniform sensillae, stout mechanosensory bristles, and ventral recurved chemosensory bristles were counted. Average number is given along with SD and number of wings scored (sample size). Because female wings are larger and have more bristles than males, we scored sexes separately. Top row for each trait gives values for females; bottom row for each trait gives values for males. Although it is not listed, all genotypes are in a w1118 background. Wild-type wings have normally two twin campaniform sensillae (TCS) at the proximal end of longitudinal vein L1. Wings of flies homozygous for the pkn<sup>nh</sup> insertion have two, one, or zero TCS (female average ~57% of wild-type, P < 0.05; male average ~70% of wild-type, P < 0.05), ~66% of the wild-type number of stout bristles (P < 0.05), and ~33% of the wild-type number of ventral recurved bristles (P < 0.05). Flies homozygous for the pkn<sup>mh</sup> insertion that also carry a duplication of pkn show wild-type values of sensillae and bristles indicating a rescue of the delorean mutation. Flies hemizygous for the pkn<sup>nh</sup> insertion have wild-type values indicating the pkn<sup>nh</sup> allele is haplosufficient. pkn, protein kinase N.
component of the P[lacW] transposon. The only hybridization signal found outside of the white gene on the X chromosome was at region 45C on chromosome 2 (data not shown). This indicates that the P[lacW] transposon is present only once in the y w; P[lacW] k06808/CyO genome. We next characterized the insertion site using gDNA from homozygous y w; P[lacW]k06808 individuals. A total of 7 kb of gDNA flanking both sides of the P[lacW] insertion site was cloned. We sequenced three distinct clones and obtained 2.7 kb of genomic sequence flanking the P[lacW] insertion site. A BLAST search of the Drosophila genome identified this region as the 5’ end of the pkn gene, which maps to 45C. Sequence analysis revealed that the P[lacW] transposon is inserted in the first intron of pkn (at nucleotide position 5172249 of the Drosophila genomic sequence; http://www.flybase.org) and its orientation is identical to the transcribed strand (Figure 2A). Thus, P[lacW]k06808 is an allele of the pkn gene, and we designate this allele as pkn\textsuperscript{dis}. To verify that this insertion causes the delorean phenotype, we outcrossed the original y w; P[lacW]k06808/CyO stock to w\textsuperscript{11182} iso-2 for more than 40 generations and have maintained the pkn\textsuperscript{dis} allele as an unbalanced stock by following the mini-white marker of the P[lacW] transposon. Flies from these stocks that are made homozygous for pkn\textsuperscript{dis} allele display the complete delorean phenotype described previously. These findings indicate that the delorean phenotype is associated with the presence of the P[lacW] element in the pkn gene on the second chromosome and is not the result of other mutations in the genetic background of the original P-element insertion line from the Kiss collection. We also recovered 36 lines that had lost the mini-white\textsuperscript{+} eye color marker associated with the P[lacW] transposon of pkn\textsuperscript{dis} after a standard transposase-mediated mobilization procedure (see the Materials and Methods). When these lines were examined, most (34) exhibited a wild-type phenotype when heterozygous with either the pkn\textsuperscript{dis} allele or a deficiency of the pkn gene region (data not shown), suggesting that there had been a precise excision of the P[lacW] transposon. This further confirms that the delorean phenotype is caused by the P[lacW] transposon insertion in the pkn gene and that loss of this insertion returns the phenotype to wild type. To see whether a wild-type allele of pkn and the surrounding region can rescue this phenotype, we used a duplication of cytological region 44B–46D on chromosome 3 (derived as a separable component of Tp(2;3)jve\textsuperscript{1,18}; http://www.flybase.org). pkn\textsuperscript{dis}/pkn\textsuperscript{dis}; Dp(2;3)jve\textsuperscript{1,18}+/+ wings do not exhibit ventral curvature and flies no longer have anterior wing margin bristle defects or ectopic veins associated with the delorean phenotype (Figure S1F, Table 1), indicating that a single dose of region 45C suppressed these aspects of the delorean phenotype. Taken together, these results demonstrate that the delorean phenotype is due to the transposon insertion in pkn. The delorean phenotype is not a simple recessive, loss-of-function mutation The previously characterized, loss-of-function pkn\textsuperscript{06736} allele has demonstrated multiphasic lethal phenotype (Lu and Settlemeyer 1999), and we wanted to examine whether pkn\textsuperscript{dis} also affected viability. The delorean mutation does not cause a reduction in viability when pkn\textsuperscript{dis}/pkn\textsuperscript{dis} offspring are compared with pkn\textsuperscript{dis}/pkn\textsuperscript{+} siblings from a cross of pkn\textsuperscript{dis}/pkn\textsuperscript{+} mothers to pkn\textsuperscript{dis}/pkn\textsuperscript{dis} fathers. We saw no significant difference from the expected equal proportions

![Figure 2](image-url)
(47.3 ± 2.02% pknR/−/pknrR/− offspring compared with 52.7 ± 2.02% pknR/−/pkn+ offspring, P > 0.05). Thus, the pknR/− allele is adult viable, and its effect on the expression of the pkn gene must be fundamentally different than that of the pkn+ allele.

In our analysis of the composite delorean wing phenotype, we noted that wing defects are only seen in homozygous pknR/− flies. Heterozygous pknR/−/pkn+ flies have wild-type wings (Figure 1, A, D, and F and Table 1). We wanted to further characterize the nature of the delorean mutation and examined the phenotype of the pknR/− allele when hemizygous with deficiencies that lack region 45C, as well as in combination with other alleles of pkn. Deficiency Df(2R)w45-30n lacks regions 45A-45E, thereby removing the complement null alleles of the pkn gene. We designate these alleles as pknR/−/− and pkn−/− (Table 2 and data not shown). Furthermore, pknR/− complements the pkn alleles pknR/−/− and pkn−/− (Table 2 and data not shown). These data suggest that the delorean phenotype is recessive, as it takes two copies of the pknR/− allele to give the full delorean wing phenotype. In addition, we did see occasional pknR/−/Df(2R)w45-30n individuals that carry their wings held up from the thorax similar to pkn−/− homozygotes (31.5% for males; 19.0% for females) consistent with the idea of a dose-sensitive, gain-of-function.

To further elucidate the nature of the pknR/− allele we decided to look at the phenotypic consequence of reducing pkn expression in wing tissue. We used the GAL4/UAS ectopic expression system to drive tissue-specific production of a pkn double-stranded RNA that would specifically degrade pkn messenger RNA by RNA interference. Several different wing-specific GAL4 drivers were examined (Table S1), each in combination with the same UAS-pkn-RNAi transgene on the third chromosome. This UAS-pkn-RNAi construct causes lethality when a GAL4 driver that is ubiquitously expressed due to the presence of either the α-tubulin or actin5C promoter is used (data not shown). This is expected, given the multiphasic lethality associated with the null allele, pknR/−/− (Lu and Settleman 1999), and indicates that loss of pkn RNA can be achieved using this system. The reduction of pkn using wing-specific GAL4 drivers does not generate a mutant phenotype (Figure S2). All wings examined exhibit wild-type anterior wing margin bristle numbers consistent with values reported for Canton-S (Table S2; Hartenstein and Posakony 1989). For this reason, we are able to rule out the possibility that the delorean phenotype is due to a loss of function mutation in the pkn gene.

The delorean phenotype is shared by other transposon insertions in the first intron of pkn

There are several transposon insertions in region 45C (Figure 2B) for which stocks are available. Two of these transposon insertions, P[lacW]ll(2)k11209 and P[PZ]ll(2)rG232, are in close proximity to P[lacW]k06808 within a ~140-bp region of the first intron of the pkn gene. We designate these alleles as pknR/−/− and pkn−/−, respectively (l(2)k11209 and l(2)rG232 were mapped to 45C in Spradling et al. 1999; we mapped l(2)rG232 to genomic position 5172186). We note that the insertions of P[lacW]ll(2)k11209 in pknR/−/− and P[lacW]k06808 in pkn−/− are in the same orientation such that the 5’ terminal and 3’ terminal of the P[lacW] element have the same relative orientation as transcription from the pkn gene (Figure 2A). The P-element of P[PZ]ll(2)rG232 is in the opposite orientation. Individuals homozygous for the pknR/−/− and pkn−/− alleles exhibit a similar delorean wing phenotype, and all are able to complement null alleles of the pkn gene (Table 2). Both pknR/−/− and pkn−/− fail to complement pkn−/− as heterozygous individuals have a delorean wing phenotype similar to that of pknR/− homozygotes (Table 2). We conclude that these P-element insertion alleles exhibit a delorean phenotype because of a similar disruption of pkn gene expression. In addition, the close proximity of the insertion sites indicates that the relative location of the insertions within the first intron is a more important determinant of the mutant phenotype than the type and orientation of the transposon.

### Table 2 Quantitative analysis of wing sensory structures in various genetic combinations of pkn alleles

<table>
<thead>
<tr>
<th>Genotype</th>
<th>pknR/−/−</th>
<th>pkn−/−/−</th>
<th>pknR/−/−</th>
<th>pkn−/−/−</th>
<th>pknR/−/−</th>
<th>pkn−/−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twin sensillae</td>
<td>2.0 ± 0 (8)</td>
<td>2.0 ± 0 (8)</td>
<td>1.07 ± 0.7 (15)</td>
<td>1.36 ± 0.5 (11)</td>
<td>0.45 ± 0.69 (11)</td>
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</tr>
<tr>
<td>Stout</td>
<td>82.1 ± 2.17 (8)</td>
<td>76.2 ± 3.22 (10)</td>
<td>48 ± 13.9 (15)</td>
<td>64.27 ± 6.32 (11)</td>
<td>49.8 ± 4.21 (5)</td>
<td></td>
</tr>
<tr>
<td>Ventral recurved</td>
<td>18.0 ± 1.31 (8)</td>
<td>18.0 ± 1.22 (9)</td>
<td>6.57 ± 2.9 (12)</td>
<td>8.58 ± 3.34 (12)</td>
<td>nda</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>pknR/−/−</th>
<th>pkn−/−/−</th>
<th>pknR/−/−</th>
<th>pkn−/−/−</th>
<th>pknR/−/−</th>
<th>pkn−/−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twin sensillae</td>
<td>1.89 ± 0.33 (9)</td>
<td>1.89 ± 0.33 (9)</td>
<td>2 ± 0 (10)</td>
<td>0.86 ± 0.38 (7)</td>
<td>1.5 ± 0.71 (2)</td>
<td></td>
</tr>
<tr>
<td>Stout</td>
<td>81.2 ± 3.36 (10)</td>
<td>77.6 ± 6.38 (10)</td>
<td>76.6 ± 5.48 (10)</td>
<td>59.2 ± 5.07 (9)</td>
<td>75.8 ± 17.2 (4)</td>
<td></td>
</tr>
<tr>
<td>Ventral recurved</td>
<td>20 ± 1.83 (10)</td>
<td>17.5 ± 0.84 (6)</td>
<td>20 ± 1.47 (10)</td>
<td>7.67 ± 2.83 (9)</td>
<td>19.5 ± 1.73 (4)</td>
<td></td>
</tr>
</tbody>
</table>

Format is the same as in Table 1. Average number is given along with SD and number of wings scored (sample size). Top row gives values for females; bottom row gives values for males. All genotypes are in a w1118 background except for pknR/−/− homozygotes, which are in a yw6 background. The P-element insertion alleles, pknR/−/−, pkn−/−/−, and pkn−/−/−, are located in the same intron of the pkn gene (see Figure 2). Homozygotes of the three insertions show the delorean phenotype although to a variable extent. pkn, protein kinase N.

nda = not determined; could not be scored unequivocally because wings were too distorted.
A deletion derivative of the \textit{P}[lacW]k06808 insertion exhibits a less-severe \textit{delorean} phenotype

Most of the transposase-induced excisions of the \textit{P}[lacW] element of \textit{pkn} \textsuperscript{dim} resulted in reversion to wild type due to complete loss of the \textit{P}-element. However one excision event was imprecise and generated a line (designated \textit{pkn} \textsuperscript{dim} \textit{c}) that had lost the mini-white \textsuperscript{*} eye color marker associated with \textit{P}[lacW]. This \textit{pkn} \textsuperscript{dim} \textit{c} derivative was found to have a milder \textit{delorean} phenotype when heterozygous with \textit{pkn} \textsuperscript{dim} (Table 2). gDNA was extracted from individuals homozygous for \textit{pkn} \textsuperscript{dim} \textit{c} and the region surrounding the \textit{P}[lacW] insertion site was amplified by PCR. DNA sequencing of the PCR product revealed that the \textit{pkn} gDNA and most of the \textit{P}[lacW] element was intact, but a 1874-bp deletion had removed part of the mini-white \textsuperscript{*} cassette of \textit{P}[lacW], hence its white-eyed phenotype.

We found that homozygous \textit{pkn} \textsuperscript{dim} \textit{c} individuals have a less severe phenotype than \textit{pkn} \textsuperscript{dim} homozygotes (\textit{P} < 0.05, Table 2). However the \textit{pkn} \textsuperscript{dim} \textit{c} allele behaves similarly in its interaction with either the loss-of-function allele \textit{pkn} \textsuperscript{K6736} or with \textit{Df(2R)w45-30n} in heterozygous flies, resulting in a wild-type wing phenotype (Table 2). This deletion derivative of the \textit{P}[lacW]k06808 insertion also behaves as a recessive mutation, with the only difference between \textit{pkn} \textsuperscript{dim} \textit{c} and the original \textit{pkn} \textsuperscript{dim} allele being a reduction in the severity of the wing phenotype (Table 2). Of particular note with respect to the \textit{pkn} \textsuperscript{dim} \textit{c} allele is its ability to increase the expression of the intact mini-white \textsuperscript{*} marker of \textit{P}[lacW] present in \textit{pkn} \textsuperscript{dim} when flies are heterozygous for these alleles are examined (\textit{pkn} \textsuperscript{dim} \textit{c}/\textit{pkn} \textsuperscript{dim}, Figure 3A). This “trans-allelic” interaction does not require the presence of extensive homology between paired transposons or a precisely shared insertion site. This conclusion is based on the elevated expression of the mini-white \textsuperscript{*} marker of \textit{pkn} \textsuperscript{dim} when heterozygous with \textit{pkn} \textsuperscript{G232} (Figure 3B), an allele caused by the insertion of the \textit{P}[PZ] transposon that carries the \textit{rosy} gene as a marker and contains only the \textit{P}-element ends and the \textit{lacZ} gene in common with \textit{P}[lacW].

\textbf{Molecular analysis of expression from the \textit{delorean} allele}

To better understand the molecular basis of the \textit{delorean} phenotype, we wanted to determine the nature of transcription from the \textit{pkn} \textsuperscript{dim} allele. An examination of the modENCODE Temporal Expression Data (flybase; http://flybase.org/reports/FBgn0020621.html) informed our choice of the white pupae stage as the source of RNA to be analyzed. We reasoned that this stage of development had moderate levels of expression and would also be undergoing significant development of the wing discs. 5' RACE on total RNA isolated from 1-d-old pupae resulted in two products in \textit{pkn} \textsuperscript{dim} \textit{c} homozygotes and \textit{pkn} \textsuperscript{dim}/\textit{Df(2R)w45-30n} hemizygotes (Figure 4A). The smaller of the two products was found to comigrate with the wild-type product. Sequencing of the RACE products revealed that this smaller \textit{pkn} \textsuperscript{dim} \textit{c} product is equivalent to the wild-type transcript (Figure 4B), demonstrating that the intron containing the \textit{P}-element insertion can be recognized and excised in both \textit{pkn} \textsuperscript{dim} \textit{c} homozygotes and \textit{pkn} \textsuperscript{dim}/\textit{Df(2R)w45-30n} hemizygotes. Sequencing of the larger of the two \textit{pkn} \textsuperscript{dim} \textit{c}-derived RACE products indicates that transcription from the \textit{pkn} \textsuperscript{dim} \textit{c} allele initiates within the 3' end of the \textit{P}-element (P3; Figure 4C). The 5' end of the \textit{delorean}-specific transcript begins with 63 nucleotides derived from the P3' end of \textit{P[lacW]}, followed by 122 nucleotides of the first intron immediately downstream of the insertion site that are then spliced to the 5' end of exon 2. Thus, the \textit{pkn} transcripts in \textit{pkn} \textsuperscript{dim} \textit{c} flies represent a mixture of wild-type and aberrant messenger RNAs. The protein made using the \textit{delorean}-specific transcript is predicted to be identical to that made by the wild-type transcripts. These results confirm our genetic analyses characterizing the \textit{delorean} phenotype as a gain-of-function mutation.

\textbf{The \textit{delorean} phenotype can be enhanced by loss of JNK pathway components}

To better understand the cell biological basis of the \textit{delorean} phenotype, we examined its genetic interaction with mutations that have been previously identified to interact with the \textit{pkn} loss-of-function allele \textit{pkn} \textsuperscript{K6736} during embryonic development. We looked specifically at the \textit{Drosophila} homolog of mammalian Jun-N-terminal kinase (D-JNK), encoded by \textit{bsk}. The loss-of-function \textit{bsk} \textsuperscript{1} allele increased the frequency of embryos with dorsal closure defects derived from \textit{pkn} \textsuperscript{K6736} germline clones (Lu and Settleman 1999). We found that the \textit{delorean} wing phenotype is enhanced upon reduction of D-JNK when we observed the anterior wing margin of \textit{delorean} homozygotes that had lost the \textit{bsk} \textsuperscript{1} allele (genotype: \textit{bsk} \textsuperscript{1} \textit{pkn} \textsuperscript{dim} / \textit{pkn} \textsuperscript{dim}, Figure 5). Compared with \textit{pkn} \textsuperscript{dim} \textit{c}/\textit{pkn} \textsuperscript{dim} homozygotes alone, the average numbers of twin sensillae (0.43 vs. 1.07), stout bristles (42.5 vs. 48), and ventral recurved bristles (5.86 vs. 6.57) in females are reduced in
This suggests that the delorean mutation causes a disruption in some aspect of wing morphogenesis that may be related to a function also mediated by the JNK pathway. The parallel nature of the interaction between a component of the JNK signaling pathway and the \( pkndln \) and \( pkn{06736} \) alleles as observed in two different morphogenetic processes prompted us to examine other participants in JNK signaling. We examined the \textit{Drosophila} Nemo-like kinase (\textit{nmo}) because of its potential role in the integration of signaling pathways involved in regulation of wing patterning (\textit{wg} and \textit{dpp} signaling) with JNK-mediated programmed cell death (Mirkovic et al. 2002). Defects associated with the anterior wing margin and wing crossveins are enhanced upon reduction of Nmo (Figure 6A, genotype: \( pkndln/pkndln; nmoP/nmo+ \)). We found that 50% of flies with the genotype \( pkndln/pkndln; nmoP/nmo+ \) exhibit complete or partial loss of anterior or posterior crossveins, or both. Loss of crossvein structure is not typically seen in \( pkndln/pkndln \) flies but is a reported consequence of expressing \textit{nmo} in the wing using an epidermal Gal4 driver (Verheyen et al. 2001; Mirkovic et al. 2002). The extreme loss of anterior wing margin material in \( pkndln/pkndln; nmoP/nmo+ \) flies made it difficult to complete our standard quantitative analysis without a bias in the samples analyzed. Nonetheless, it is clear that a reduction in the level of Nmo dramatically affects the delorean phenotype and our analysis of the wings from \( pkndln/pkndln; nmoP/nmo+ \) individuals suggests that the balance between JNK signaling and signaling pathways involved in patterning of the wing is also compromised in \( pkndln \) homozygotes.

We also wanted to explore interactions with Rho pathway components that have been found to be involved in wing morphogenesis. Expression of a kinase-deficient form of Lim kinase 1 (LIMKI1) results in wing and leg malformations that can be suppressed if the levels of Rho1 are reduced (Chen et al. 2004). This genetic interaction is not seen with PKN however as the loss-of-function allele \( pkndln \) does not have the same ability to suppress the effects of the kinase-deficient LIMKI1 (Chen et al. 2004). We examined whether \textit{LIMK}\textsubscript{EY08757} affected the delorean phenotype using flies of the genotype \( y{w}{67C}^{23}P[EPgy2]\textit{LIMK}\textsubscript{EY08757}; \textit{pkndln} / \textit{pkndln} \). We did not find...
a significant difference in phenotype relative to \textit{pkn}^{dln}/\textit{pkn}^{dln} homozygotes (Figure 5 and Table 3). This informs us that PKN’s effector function is not likely to be related to that of LIMK1.

**DISCUSSION**

**Characterization of the \textit{pkn}^{dln} allele**

We have characterized the \textit{delorean} mutation that causes defects in wing morphology due to a \textit{P}[lac\textit{W}] insertion in the \textit{protein kinase \textit{N} (\textit{pkn})} gene of \textit{Drosophila melanogaster}. An essential function of the \textit{pkn} gene is not disrupted by the \textit{P}[lac\textit{W}] insertion because individuals homozygous for the \textit{pkn}^{dln} allele can be recovered in expected numbers. Although the wing phenotype of heterozygous \textit{pkn}^{dln}/\textit{pkn}^{+} individuals is wild type in all respects, the effect of the transposon insertion cannot be classified as a loss-of-function mutation. This is shown by the wild-type wing morphology of individuals that are hemizygous for the \textit{pkn}^{dln} allele (\textit{pkn}^{dln}/\textit{D}(2R)w45-30n) and because the reduction of \textit{pkn} RNA in wing tissue using RNA interference has no phenotypic consequence. Moreover, it is clear that the \textit{delorean} wing phenotype is only seen when both alleles present contain a transposon insertion (or a derivative of this transposon) within a 140-bp region of the first intron of the \textit{pkn} gene. For these reasons, we consider the \textit{delorean} phenotype to be caused by a recessive mutation.

A model of the molecular structure of the \textit{pkn} gene based on annotated transcripts that have been identified to date indicates that there are a number of alternative transcription start sites as well as alternate exon usage (Figure 2A; [http://www.ifybase.org]). Of the four predicted alternative transcription start sites, there are three that would generate a transcript capable of encoding the amino-terminal domain of PKN required for both Rho binding and the ability to rescue the loss-of-function \textit{pkn}^{D736} allele (Lu and Settleman 1999). The position of the \textit{P}[lac\textit{W}] insertion of \textit{pkn}^{dln} reflects the bias that \textit{P} elements have for insertion near promoters and 5' regulatory regions (Berg and Spradling 1991) and would likely have an impact on one of the more well-supported transcripts (indicated by darker shading in Figure 2A) that uses the 5' most transcription start site. Our molecular analysis of the \textit{delorean} allele using 5' RACE aimed to determine how a transposon insertion within the first intron of the \textit{pkn} gene could generate a mutant phenotype. The presence of \textit{P}[lac\textit{W}] does not prevent the proper splicing of the first two \textit{pkn} exons and indicates that the predicted, wild-type transcript can be generated using the 5' most transcription start site. Our 5' RACE analysis also identified a transcript specific to the \textit{delorean} allele that is the result of initiation from within the 3' end of the \textit{P}[lac\textit{W}] transposon and includes intron sequence spliced to the second exon of the \textit{pkn} gene. The predicted PKN protein produced from the \textit{delorean} transcript is the same as that from the wild-type transcript. Given our determination that the composite \textit{delorean} phenotype is not the result of a loss-of-function mutation, it

**Table 3** Quantitative analysis of wing sensory structures of \textit{pkn}^{dln} homozygotes in combination with \textit{basket} or \textit{LIMK1} indicate that \textit{bsk} can enhance the severity of the \textit{delorean} mutation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>\textit{Genotype}</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{pkn}^{dln}</td>
<td>\textit{bsk pkndln}</td>
</tr>
<tr>
<td>Twin</td>
<td>1.07 ± 0.7 (15)</td>
</tr>
<tr>
<td>sensillae</td>
<td>1.41 ± 0.61 (32)</td>
</tr>
<tr>
<td>Stout</td>
<td>48 ± 13.9 (15)</td>
</tr>
<tr>
<td></td>
<td>53 ± 12.1 (32)</td>
</tr>
<tr>
<td>Ventrall</td>
<td>6.57 ± 2.9 (12)</td>
</tr>
<tr>
<td>recurved</td>
<td>5.69 ± 2.35 (29)</td>
</tr>
</tbody>
</table>

Format is same as in Table 1 and Table 2. Flies that are heterozygous for a loss-of-function allele of \textit{basket} (\textit{bsk}^+) in a \textit{pkn}^{dln} homozygous background have on average significantly fewer twin campaniform sensillae than flies that are homozygous for the \textit{pkn}^{dln} insertion alone and flies heterozygous for a loss of function allele of \textit{LIMK1} in a \textit{pkn}^{dln} homozygous background (\(P < 0.05\)). The same significant reduction is found when comparing average number of stout mechanosensory bristles in male flies but not in females. Average number of ventral recurved bristles was significantly different between \textit{bsk} \textit{pkn}^{dln} / \textit{pkn}^{dln} and \textit{LIMK1/+}; \textit{pkn}^{dln} flies (\(P < 0.05\)) but not between \textit{bsk} \textit{pkn}^{dln} / \textit{pkn}^{dln} and \textit{pkn}^{dln} \textit{pkn}^{dln} flies. Data were analyzed by Kruskal-Wallis tests followed by Tukey’s HSD.
is reasonable to assume that the identified delorean-specific transcript generated from the pkndln allele is the cause of the mutant phenotype. Our finding that reducing the levels of pkn transcript using RNAi has no effect on wing morphology is in support of this conclusion. For these reasons it is likely that the delorean phenotype is due to altered regulation or ectopic expression of the delorean-specific transcript. A similar situation has been described previously (LaFave and Sekelsky 2011) whereby initiation of transcription was found to occur from within a P-element. The authors conclude that an outcome of such cryptic transcription would likely be altered expression of the gene under study.

Our genetic analysis indicates that the pairing context of the pkndln allele is an important determinant of the delorean phenotype. This is demonstrated by our observations of the pkndln allele, an imprecise excision derivative of pkndln that has a deletion within the mini-white gene marker of P{lacW} and as a result white-eyed. Expression of the intact mini-white reporter in the pkndln allele is elevated in pkndln/pkndln heterozygotes as revealed by their eye phenotype, which is darker than pkndln/pkn+ individuals. Transcription from the mini-white gene is correlated with the degree of pigmentation (Silicheva et al. 2010). An increase in expression of the mini-white reporter may reflect the creation of a local chromosomal environment that is favorable for transcription in this heteroallelic state due to the presence of paired insertion-bearing alleles. The responsiveness of the mini-white gene to the chromatin environment is well known (Bier et al. 1989; Zhang and Odenwald 1995). It follows that the paired state of the delorean allele (or its derivative) as is found in pkndln/pkndln, represents an optimized conditions for expression of both the mini-white reporter and the delorean-specific pkn transcript that generates the mutant wing phenotype. We consider it reasonable that the delorean phenotype in its entirety results when maximum levels of the delorean-specific transcript are expressed and that this in turn is more likely to occur with the pairing of insertion-bearing alleles.

We have not used chromosomal rearrangements to specifically disrupt pairing and test for classic transvection effects in our analysis of the delorean phenotype. However, we do see suppression of the delorean phenotype in pkndln/pkndln; Dp(2;3)eye1.18 individuals that are wild type in all respects except for the held up position of their wings (also seen in pkndln homozygotes). The lack of rescue by an additional copy of the pkn gene present in Dp(2;3)eye1.18 is not unexpected in the case of a mutant phenotype caused by misexpression of a pkn transcript, as we have argued to be the case for the pkndln allele. In fact the observation that there is suppression at all argues the amount of misexpressed pkn has been reduced. The presence of an additional homologous pairing partner could influence the interaction between the pkndln alleles. Somatic pairing of homologous chromosomal regions is robust in Drosophila (Apte and Meller 2012) and the local chromosomal interactions that result are not only common (Chen et al. 2002; Mellert and Truman 2012) but with profound influence on expression (Sass and Henikoff 1999; Wu and Morris 1999; Ou et al. 2009). We postulate that in an unpaired state the pkndln allele in pkndln/pkndln; Dp(2;3)eye1.18 individuals would still be able to express the delorean-specific transcript, albeit at lower levels due to asynapsis (Goldborough and Kornberg 1996), and thereby with reduced phenotypic impact (i.e., result in a held-up wing phenotype but a wild-type anterior wing margin, for example). The pkndln allele would also be an “unpaired” state when hemizygous with a deficiency of the pkn gene. We observed that wings of pkndln/Df(2R) w45-30n hemizygotes are also on occasion held up vertically and a similar phenotype has been reported for pkndlnΔ2232/ Df(2R)wu45 hemizygotes (Zhang et al. 1996). The absence of the pkn gene region in trans to the insertion-bearing pkndln chromosome may favor a change in the topology of the region such that the transcription of a wild-type pkn product is favored over that of the delorean-specific transcript. Changes in topology have also been suggested to mediate the “enhancer bypass” mechanism underlying specific cases of transvection (Morris et al. 1998). The “held-up” wing phenotype seen in pkndln/pkndln; Dp(2;3)eye1.18 and pkndln/Df(2R) individuals would then reflect a sensitivity to low levels of misexpression from the pkndln allele that persists in an unpaired state (see Model, Figure 7). Thus we consider the delorean wing phenotype to be due to a pairing-dependent, recessive mutation that behaves as a dose-sensitive, gain-of-function due to misexpression.

Figure 6 The delorean mutation interacts with nemo. (A, A’) Dissected wing of pkndln/ pkndln; nmo/+ shows a more extreme delorean phenotype than wings of pkndln siblings without nmo+. Gaps in crossveins are indicated by arrows. (B, B’) Dissected wing of pkndln/ pkndln shows the delorean phenotype. (C, C’) Dissected wing of pkndln/pkn+; nmo+/nmo+ shows a wild-type phenotype. Left panels (A, B, C) are whole wings, scale bar = 0.2 mm, and right panels (A’, B’, C’) are close-ups of anterior wing margins, bar = 0.1 mm.
Comparative analysis of the delorean phenotype

We have described the wing phenotype associated with the \( pkn^{dm} \) allele as a composite of several morphological defects. Changes in the overall position and structure of the wing are seen in delorean heterozygotes as a upheld wing and a ventral curvature to the wing, respectively. The flightless, upheld wing phenotype of delorean flies resembles that of known Drosophila mutants with defects in flight muscles (vertical wings, Deak 1977; upheld, Deak et al. 1982; wings up A—a mutation in the muscle protein troponin, Barbas et al. 1993). The curved wing phenotype of \( pkn^{dm} \) homozygotes suggests a disruption of epithelial cell morphology or changes in cell growth that would alter the relationship between the dorsal and ventral surfaces of the adult wing. Also associated with the delorean phenotype is a reduction in sensory structures and venation defects. The loss of a subset of anterior wing margin bristles suggests a patterning defect whereby external sensory organ structures are transformed to internal structures. Errors in patterning could also explain the ectopic veins formed in delorean mutants as epithelial cells of the developing wing disc fail to make the appropriate choice between a vein or intervein fate. Such a varied response within a single tissue provides a unique opportunity to establish a mechanism by which misexpression of \( pkn \) mediates these apparently diverse effects and in turn determine the genetic interactions that are required for PKN involvement in wing morphogenesis.

Previous studies of the \( pkn \) gene in Drosophila have used a loss-of-function mutation that is the result of a \( P[PZ] \) transposon insertion in the fifth intron of the \( pkn \) gene generating the embryonic lethal, \( pkn^{6726} \) allele (Lu and Settlement 1999; see Figure 2A). The embryonic lethality of \( pkn^{6726} \) can be rescued with a \( pkn \) cDNA that is expressed using a heat shock promoter (Betsont and Settlement 2007). Rescued individuals exhibit a mild adult wing phenotype that exhibits considerable variability that may reflect a functional redundancy in PKN function at later stages of development. The absence of a loss-of-function phenotype due to functional redundancy has been argued to be the case for at least 60% of genes in Drosophila (Miklos and Rubin 1996) and places constraints on the use of the \( pkn^{6726} \) allele in an analysis of PKN function. For this reason, our analysis of the delorean wing phenotype was undertaken to better understand the molecular lesion associated with \( pkn^{dm} \) as it represents a unique allele of \( pkn \) that is adult viable and not the result of a loss-of-function mutation. Furthermore, our characterization of the molecular structure of \( pkn^{dm} \) indicates that this particular allele represents an opportunity to study the effects of misexpression of the \( pkn \) gene.

![Figure 7](image_url)

**Figure 7** Pairing dependence of \( pkn^{dm} \) expression as a working model to explain the delorean phenotype. The model depicts the 5' end of the \( pkn \) gene showing only the affected region of the gene (first two exons and intron 1; refer to Figure 2). Thickness of arrows represents assumed relative levels of transcription. Transcription of the wild-type transcript (\( pkn^+ \)) begins at the first exon. Initiation of the delorean-specific transcript, referred to as \( pkn^{dm} \), from within the \( P \)-element generates a transcript that is either expressed ectopically or regulated differently than \( pkn^+ \). Relative levels of \( pkn^+ \) and \( pkn^{dm} \) transcripts are given with the “+” symbol. The schematic in the far right column depicts the position and shape of the wing for each genotype. (A) A reduction in the expression of wild-type \( pkn \) and an increase in the expression of \( pkn^{dm} \), occurs when the \( pkn^{dm} \) allele is homozygous. The level of \( pkn^{dm} \) expression is elevated as a result of pairing thereby causing the composite wing defects of the delorean phenotype. The level of wild-type transcript may be reduced due to the additional sequence in the form of the \( P \)-element in the first intron that must be removed by splicing. (B) Levels of the delorean-specific transcript are reduced in \( pkn^{dm}/pkn^+ \) heterozygotes due to the absence of \( P(\text{lacW}) \) in trans to the \( pkn^{dm} \) allele. The reduction in the level of \( pkn^{dm} \) transcript is below a threshold needed to generate a phenotype. (C, D) Our analysis of heteroallelic combinations of \( pkn \) alleles also lead us to the conclusion that a reduction in the level of delorean-specific transcript occurs in the absence of either homologous pairing in trans (C; \( pkn^{dm}/\text{Df}[2R] \)) or when pairing is disrupted (D; \( pkn^{dm}/pkn^+; \text{Dp}[2:3] \)). In the case of \( pkn^{dm}/\text{Df}[2R] \) there would be a reduction in the level of both \( pkn^{dm} \) and \( pkn^+ \) transcripts. Reduced levels of \( pkn^{dm} \) transcript can suppress the delorean phenotype. In the case of \( pkn^{dm}/pkn^{dm}; \text{Dp}[2:3] \), the transposed sequence present in the duplication (Dp) is able to disrupt pairing between the \( pkn^{dm} \) alleles due to homology resulting in decreased \( pkn^{dm} \) transcript levels. It is also likely that the level of wild-type \( pkn \) transcription is affected by the pairing state at the \( pkn \) gene. We consider it reasonable to infer that the relative levels of a delorean-specific transcript are involved in determining the extent of the delorean phenotype.
That the delorean phenotype represents a misexpression of PKN in affected tissues is supported by the observation of Betson and Settleman (2007) that overexpression of the kinase domain of PKN specifically in the wing margin generates a wing phenotype that is analogous to the anterior wing margin defects of delorean flies. Loss of sensory bristles along the anterior wing margin as well as alteration of posterior wing hairs was associated with expression of the PKN kinase domain in the wing margin (Betson and Settleman 2007). We note that posterior wing margin defects are not seen in delorean mutants and this may reflect an anterior wing-specific expression pattern of the pkn\textsuperscript{delorean} allele. Nonetheless, the correspondence of the anterior wing margin phenotypes demonstrates that misexpression of the delorean-specific transcript behaves similarly to the overexpression of the PKN kinase domain lacking the normal amino-terminal regulatory domains that mediate Rho-GTPase binding (Betson and Settleman 2007). The N-terminal region of PKN is autoinhibitory and it is thought to restrict the kinase activity of the catalytic domain in the absence of activators such as Rho1 (Yoshinaga et al. 1999). If the kinase activity of ectopically expressed pkn can be activated (by Rho1 or another putative activator Rac1), it would be equivalent to overexpression of the kinase domain. Thus misexpression of the pkn transcript, like overexpression of the kinase domain of PKN, can cause alterations in anterior wing margin morphogenesis.

Involvement of pkn in wing morphogenesis

Genetic interactions between pkn and the bsk and LIMK1 genes have previously been examined in the context of the loss-of-function allele pkn\textsuperscript{p6736} (Lu and Settleman 1999; Chen et al. 2004). Our examination reveals that the essential nature of these interactions is maintained in the context of the pkn\textsuperscript{delorean} allele and can be expanded to include the process of wing morphogenesis. We observed that reduction of Lim Kinase 1 has no significant effect on the delorean wing phenotype. Lack of a notable interaction between pkn\textsuperscript{p6736} and overexpression of LimK1 was previously observed and found to be in contrast to the considerable impact that loss of Rho1 had on suppression of the wing phenotype associated with overexpression of LimK1 (Chen et al. 2004). This finding is consistent with the notion that PKN is just one of multiple target effectors that function downstream of Rho1 in any given process or tissue, especially one that would involve a diverse array of responses as needed during wing morphogenesis.

Lu and Settleman (1999) found that reduced levels of a component of the JNK signaling pathway (bsk; D-JNK) causes an increase in the dorsal closure defects associated with loss of PKN function in germ-line clones. We see a similar enhancement of the delorean wing phenotype when the dose of bsk is reduced. This extends the observation that the function of the Rho1-PKN and JNK signaling cascades converge at some point in their function as regulators during embryogenesis (Lu and Settleman 1999) to processes occurring during wing morphogenesis. The enhancement of the delorean phenotype upon reduction of Nmo, the founding member of the Nemo-like kinase family of kinases, adds additional evidence in this regard. Nemo-like kinases coordinate the activity of multiple signals as they mediate cross talk between various pathways (Ishitani and Ishitani 2013). In such a capacity, Drosophila Nmo plays a key regulatory role as an antagonist of the Wingless signaling pathway during patterning of the developing wing (Zeng and Verheyen 2004). It has also been implicated in the regulation of the JNK pathway and programmed cell death (Mirkovic et al. 2002) probably by a process of “morphogenetic apoptosis,” whereby JNK-mediated apoptosis is activated to correct developmental patterning errors (Igaki 2009). Thus, simultaneous reduction of Nmo and misexpression of PKN could have disruptive effects on the integration of a number of signaling pathways involved in wing morphogenesis—Wingless, Rho1, JNK. The observed enhancement of the delorean phenotype upon reduction of a regulator (Nmo) and component (D-JNK) of the JNK pathway leads us to speculate about the possibility that PKN may be involved in mediating the previously established dynamic relationship between Rho1 and activation of apoptosis via the JNK pathway (Neisch et al. 2010). We note that in this role PKN might not be the only downstream effector target of Rho1 given the observation that dorsal closure defects in Rho1 mutants cannot be rescued by elevated levels of PKN (Lu and Settleman 1999). Nonetheless, our results add weight to the argument that PKN acts as an effector of Rho1 signaling and expands its possible function to wing morphogenesis.

The role of Pkn as a Rho1 effector

Rho GTPases are critical participants in the orchestration of modifications to cell architecture that must occur during tissue morphogenesis. The array of different biological processes that can be regulated by Rho GTPases coupled with the significant degree of coordination that occurs between them makes an analysis of Rho GTPase signaling pathways a challenge. One approach to understanding the function of Rho GTPases is to determine key effectors in a given signaling event, in a specific tissue. We report here on the development of such a system in Drosophila that will allow us to more fully examine the role of the Rho1-Pkn pathway in wing morphogenesis.

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