The wavy mutation maps to the Inositol 1,4,5-trisphosphate 3-kinase 2 (IP3K2) gene of Drosophila and interacts with IP3R to affect wing development

Derek M. Dean*¹, Luana S. Maroja*, Sarah Cottrill*, Brent E. Bomkamp*, Kathleen A. Westervelt*, and David L. Deitcher†

*Dept. of Biology, Williams College, Williamstown, MA 01267
†Dept. of Neurobiology and Behavior, Cornell University, Ithaca, NY 14853

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\(^1\)Corresponding author: 59 Lab Campus Drive, Dept. of Biology, Williams College, Williamstown, MA 01267. Phone: 413-597-2004 Email: ddean@williams.edu
ABSTRACT

Inositol 1,4,5-trisphosphate (IP$_3$) regulates a host of biological processes from egg activation to cell death. When IP$_3$-specific receptors (IP3Rs) bind to IP$_3$, they release calcium from the ER into the cytoplasm, triggering a variety of cell type- and developmental stage-specific responses. Alternatively, inositol polyphosphate kinases can phosphorylate IP$_3$; this limits IP3R activation by reducing IP$_3$ levels, and also generates new signaling molecules altogether. These divergent pathways draw from the same IP$_3$ pool yet cause very different cellular responses. Therefore, controlling the relative rates of IP3R activation versus phosphorylation of IP$_3$ is essential for proper cell functioning. Establishing a model system that sensitively reports the net output of IP$_3$ signaling is crucial for identifying the controlling genes. Here we report that mutant alleles of wavy (wy), a classic locus of the fruit fly Drosophila melanogaster, map to IP$_3$ 3-kinase 2 (IP3K2), a member of the inositol polyphosphate kinase gene family. Mutations in wy disrupt wing structure in a highly specific pattern. RNAi experiments using GAL4 and GAL80ts indicated that IP3K2 function is required in the wing discs of early pupae for normal wing development. Gradations in the severity of the wy phenotype provide high-resolution readouts of IP3K2 function and of overall IP$_3$ signaling, giving this system strong potential as a model for further study of the IP$_3$ signaling network. In proof of concept, a dominant modifier screen revealed that mutations in IP3R strongly suppress the wy phenotype, suggesting that the wy phenotype results from reduced IP$_4$ levels and/or excessive IP3R signaling.
INTRODUCTION

The *Drosophila* wing has proven to be a reliable model for addressing research questions throughout the field of developmental biology. Its stereotyped, easily recognized, planar network of cuticular hairs and veins, external location, and dispensability for survival allow for efficient scoring of structural abnormalities, yet the tissue goes through a dynamic process to arrive at its adult form, providing opportunities to study several different developmental phenomena. Wing precursor cells are initially derived during early embryogenesis as epithelial cells on the ventrolateral margins of the second thoracic segment invaginate, forming common primordia for the wings and legs of that segment. Shortly thereafter, subsets of cells from these two primordia migrate dorsally to form a pair of wing imaginal discs (Cohen et al. 1993). Throughout the larval stages, cells of these imaginal discs undergo patterned proliferation, and positional information is integrated to specify the regions of the prospective wings and their vein boundaries (Milan et al. 1996a; Biehs et al. 1998; Klein 2001; Cavodeassi et al. 2002; Crozatier et al. 2002; Crozatier et al. 2004). Pupal stages are marked by eversion of the discs, an eventual cessation of cell proliferation, organization of epithelial cells into hexagonal arrays, the formation of a single cuticular hair at the distal vertex of each cell, and refinement of vein positions (Wong and Adler 1993; Milan et al. 1996b; Classen et al. 2005; Blair 2007; Classen et al. 2008; Taylor and Adler 2008). Shortly after adult eclosion, the wings, which remain folded during metamorphosis, are expanded by an increase in hemolymph pressure. At this point epithelial cells switch to a mesenchymal identity, delaminate from the overlying cuticular tissue, undergo programmed cell death, and are resorbed into the thorax (Kiger et al. 2007; Link et al. 2007; Natzle et al. 2008). During and after this process, the cuticular portions of
the wing are left behind and intact, providing a clear readout of developmental perturbations that may have occurred anytime between embryogenesis and adult wing expansion. Therefore, the *Drosophila* wing acts as an accessible, one-stop destination to study a diverse array of cellular processes including fate determination, proliferation, morphogenesis, adhesion, polarity, migration, and programmed death.

The molecular genetic tractability of *Drosophila* has further facilitated inquiries into wing development. Researchers have employed a number of approaches successfully, including misexpression studies using wing-specific GAL4 drivers, RNAi, gene expression profiling of the developing wing, bioinformatics, and genetic interaction screens (RORTH et al. 1998; REN et al. 2005; DU et al. 2011; Dui et al. 2012). These approaches have helped us better understand signal transduction networks that have been highly conserved throughout evolution [e.g. wingless (Wnt), Notch (EGF), Hedgehog, and decapentaplegic (TGF-ß)], as well as how these networks interact to affect development (CASSO et al. 2011; SWARUP AND VERHEYEN 2012; KWON et al. 2013; YANG et al. 2013; HARTL AND SCOTT 2014).

Past studies have suggested that IP$_3$ (inositol 1,4,5-trisphosphate) signaling could join the list of highly conserved signal transduction networks that are modeled by the developing *Drosophila* wing. A specific heteroallelic combination of mutations for the IP$_3$ receptor gene *IP3R* has been reported to cause mild wing crumpling, and more combinations of *IP3R* alleles have been shown to affect wing “posture” (i.e. the angle at which wings are held from the body), flight behavior, and the physiology of the flight circuit (BANERJEE et al. 2004; BANERJEE et al. 2006; AGRAWAL et al. 2009; VENKITESWARAN AND HASAN 2009; AGRAWAL et al. 2010). *IP3R* has been shown to act in the nervous system to affect wing posture and flight behavior/neurophysiology; however, the mechanism by which
IP3R affects the morphology of the wing itself has remained unclear, and to our knowledge, no other IP3 signaling genes have been reported to affect Drosophila wing development.

In eukaryotes, the ER lumen typically stores Ca²⁺, and IP3R is a Ca²⁺ channel located on the ER membrane. When IP3 binds to IP3R the channel opens, releasing Ca²⁺ into the cytoplasm (Figure 1). This Ca²⁺ release goes on to affect many different cellular processes including gamete activation, fertilization, proliferation, contraction, secretion, immune cell activation, and apoptosis (Xia and Yang 2005; Berridge 2009; Leanza et al. 2013; Ramos and Wessel 2013; Roderick and Knollmann 2013; Ambudkar 2014; Ivanova et al. 2014; Kaneuchi et al. 2015; Nohara et al. 2015; Shah et al. 2015; Vervloesem et al. 2015).

Alternatively, IP3 may be phosphorylated at the other positions around its hexagonal carbon ring, generating IP4, IP5, or IP6. In Drosophila (Figure 1), phosphorylation of IP3 can be accomplished by the IP3K2 enzyme (IP3 3-kinase 2, the focus of this study), IP3K1 (IP3 3-kinase 1), or by sequential action of the Ipk2 and Ipk1 enzymes (inositol polyphosphate kinases). IP3K2, IP3K1, and Ipk2 all convert IP3 to IP4 (inositol 1,3,4,5-tetakisphosphate). IP3K2 may be affected by IP3R-mediated Ca²⁺ release from the ER: Calmodulin (Cam) binds to IP3K2 in a Ca²⁺-dependent manner, strongly upregulating IP3K2 activity (Seeds et al. 2004; Lloyd-Burton et al. 2007; Shah et al. 2015). However, unlike the IP3K enzymes of several other organisms that have been investigated, Drosophila IP3K2 and IP3K1 are not believed to provide IP4 for the synthesis of IP5 and IP6 (Seeds et al. 2004). Ipk2, on the other hand, can convert IP3 to one of two forms of IP4 (the aforementioned or phosphorylating at the 6’ instead of 3’ position, yielding inositol 1,4,5,6-tetakisphosphate), and then subsequently convert either IP4 isoform to IP5 (inositol 1,3,4,5,6-pentakisphosphate). Ipk1 then phosphorylates IP5 at the 2’ position, making IP6
(Seeds et al. 2004). In summary for Drosophila, possible fates for IP₃ include: (1) its binding to IP3R to trigger the release of Ca²⁺ from the ER, (2) its phosphorylation by IP3K2 or IP3K1 to form IP₄, or (3) its conversion to more highly phosphorylated species by the Ipk2/Ipk1 module (Figure 1).

Phosphorylation of IP₃ has been shown to have functional consequences in eukaryotes. A number of proteins with IP₄ or IP₆-specific binding have been isolated (Donie et al. 1990; Theibert et al. 1991; Fukuda and Mikoshiba 1997; Xia and Yang 2005; Fain 2013). In mammals, IP₄ appears to mediate Ca²⁺ transport into the intermembrane space of the nuclear envelope, as well as into the cytoplasm from outside the cell, and IP₆ has been shown to act as a cofactor for enzymes involved in DNA damage repair and RNA editing (Humbert et al. 1996; Hanakah et al. 2000; Ma and Lieber 2002; Byrum et al. 2004; Macbeth et al. 2005; Xia and Yang 2005; Malviya and Klein 2006). The distinct receptors for—and functions of—the various inositol species suggest that coordinating their relative levels would be important in cellular functioning. Drosophila genetics provides an excellent toolbox to investigate this possibility, the Drosophila wing is a proven model system for investigating signal transduction in general, and wing morphology is at least mildly affected by IP3R function, suggesting that IP₃ signaling plays a role in wing development (Banerjee et al. 2004).

Many classical mutations affecting the wings have not been mapped to their respective genes—this is presumably because a large number of Drosophila mutations, particularly viable alleles that affect external tissues such as the wing, were relatively easy to isolate and maintain in stocks long before molecular techniques were developed, creating a backlog. From such collections, we obtained stocks mutant for the wavy (wy)
locus. Flies mutant for wy exhibited wings that were bent and crumpled in a highly specific pattern (NACHTSHEIM 1928; PAKER 1935; LINDSLEY AND ZIMM 1992). Here we report the mapping of available wy alleles to IP3K2 (CG1630). This gene encodes the IP3 3-kinase 2 enzyme described above (Figure 1; LINDSLEY AND ZIMM 1992; SEEDS et al. 2004; LLOYD-BURTON et al. 2007). We also characterize the developmental window during which wy function is required to specify wing morphology. Finally, we describe strong genetic interactions between wy and IP3R, suggesting a possible mechanism by which the IP3 signaling network affects wing morphology, i.e. by balancing IP3R and IP3K2 activity. These findings help establish IP3 signaling as another highly conserved genetic network that is effectively modeled by the Drosophila wing.
MATERIALS AND METHODS

**wy alleles, mapping, complementation tests:** The *wy* (Bloomington Stock Center #168, or BL 168: *wy*; Nachtsheim 1928), *wy* (BL 192: *y*² *wy*² *g*²; Parker 1935), and *wy*⁷⁴i alleles (BL 1294: *t*¹ *v*¹ *m*⁷⁴i *wy*⁷⁴i; Lindsley and Zimm 1992) were used in this study. *wy*² was recombined into a *w*⁻¹ background and a *w* *wy*² *f* stock was established to facilitate mapping and the tracking of *w*⁻¹-labeled constructs during crosses. *wy*², *f*, and the *w*⁻¹ transgene insertion lines PBac{WH}CG12096⁶⁰⁵⁷⁸² (BL 18906: *w*¹¹¹⁸ Bac{WH}CG12096⁶⁰⁵⁷⁸²) and P[EP]Tango²⁶⁵¹⁷ (BL32580: w P[EP]Tango²⁶⁵¹⁷) were used for 3-point recombination mapping (Bellen et al. 2004; Thibault et al. 2004). For complementation tests, *wy*¹, *wy*², or *wy*⁷⁴i females were crossed to males carrying either of two duplications Dp(1;3)DC267 (BL 30384: *w*¹¹¹⁸; Dp(1;3)DC267, PBac{DC267}VK00033) or Dp(1;3)DC268 (BL 30385: *w*¹¹¹⁸; Dp(1;3)DC268, PBac{DC268}VK00033) and F1 *wy*/Y; Dp(1;3)/+ males were scored for the *wy* phenotype. In a second set of complementation tests, *wy*¹, *wy*², or *wy*⁷⁴i males were crossed to females carrying either of two deletions [Df(1)BSC766 (BL 26863: Df(1)BSC766, *w*¹¹¹⁸/Binsinscy) or Df(1)Exel6245 (BL 7718: Df(1)Exel6245, *w*¹¹¹⁸ P{XP-U}Exel6245/FM7c)] and F1 Df/*wy* females were scored for the *wy* phenotype (Parks et al. 2004; Venken et al. 2010; Cook et al. 2012).

**Phenotypic assessment and microscopy:** A numerical scale was devised to quantify *wy* penetrance and expressivity throughout this study (see Results text for a description of this scale). Wings were scored under a Leica dissecting scope, and photographs were taken using a NEX-3N-alpha camera body (Sony) attached to the microscope eyepiece with a T-Ring for Sony E Mount and 2-Inch Universal T Adapter (CNC Parts Supply, Inc.)
PCR of \textit{IP3K2} alleles and DNA sequencing: All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Individual \textit{wy}\textsuperscript{*} (from a \textit{y}\textsuperscript{l} \textit{w}\textsuperscript{l} strain, BL 1495), \textit{wy}\textsuperscript{74i}, and \textit{wy}\textsuperscript{2} adult male flies were first frozen in 1.5 ml Eppendorf tubes, then each was ground within their tube in 50 µl of standard fly “squishing” buffer [10 mM Tris (pH 8), 1 mM EDTA, 25 mM NaCl, 200 µg/ml Proteinase K]. Crushed flies were incubated for 30 minutes at 37°C to digest fly tissue, then at 94°C for 3 minutes to denature the Proteinase K. Segments of the \textit{IP3K2} gene were PCR amplified from DNA extract using GoTaq Flexi DNA polymerase (Promega; 1 µl DNA extract per 19 µl of standard reaction mix). PCR products were run through a 0.8% low-melt agarose gel to separate them from unincorporated primers, slabs containing the PCR products were excised from the gel, and products were purified from the agarose using the QIAquick Gel Extraction Kit (Qiagen). Purified PCR products were sent to the Cornell University Biotechnology Resource Center (Ithaca, NY) for sequencing using their recommended protocols. PCR/sequencing primers are described in the Supporting Information (Table S1). Sequence outputs were analyzed using the MEGA5 software (Tamura \textit{et al.} 2011). Sequences from at least two individual flies of each genotype were analyzed in order to resolve ambiguities.

\textbf{Rescue construct, RNAi of \textit{IP3K2}, GAL4 driver, and GAL80\textsuperscript{ts}:} To assemble the rescue construct, a \textit{NotI-AvrII} fragment containing the \textit{IP3K2} open reading frame (restriction enzymes from New England Biolabs) was extracted from the RE35745 cDNA clone (GenBank accession number AY084158; Stapleton \textit{et al.} 2002; Hoskins \textit{et al.} 2011), ligated with T4 DNA ligase (New England Biolabs) into pUAS-c5-attB (Daniels \textit{et al.} 2014) in order to place the \textit{IP3K2} cDNA downstream of a UAS site, and finally sent to Bestgene for transformation in a \textit{w} background (Chino Hills, CA). Two independent insertion lines were
obtained, both on the third chromosome at the 68A4 location. The manuscript refers to these rescue lines as UAS-IP3K2.

RNAi experiments of IP3K2 were conducted with a stock from the Vienna Stock Center (VDRC v19159: P{GD8778}v19159/TM3 Sb), hereafter referred to as RNAi-IP3K2 (Dietzl et al. 2007).

We used nub-GAL4 (BL 25754: P{UAS-Dcr-2.D}1, w^{1118}; P{GawB}nubbin-AC-62), a wing disc-specific driver, for our RNAi experiments (Brand and Perrimon 1993; Azpiazu and Morata 2000). The Tub-GAL80ts construct (from BL 7108: w; P{tubP-GAL80ts}10; TM2/TM6B, Tb^{1}), which was employed for temperature-sensitive deactivation of nub-GAL4, was recombined onto the same chromosome as nub-GAL4 and a w stock was established that was also homozygous for both insertions but did not contain the P{UAS-Dcr-2.D}1 construct (also see Fly culturing below; Ferris et al. 2006; Baena-Lopez et al. 2009; Rodriguez et al. 2012).

**Dominant genetic modifier screen**: We tested several IP3-signaling loci for genetic interactions with wy. The following alleles were obtained: (1) IP3K1^{KG02192}, a P-insertion within an intron of IP3K1, (BL 14263: y^{1}; P{SUPor-P}IP3K1^{KG02192}/CyO; ry^{506}; Bellen et al. 2004); (2) “Df-Ipk2”, a deletion spanning multiple genes including Ipk2 [BL 9190: w^{1118}; Df(2L)ED49/SM6a; Ryder et al. 2007]; (3) Cam^{n39}, a deletion of Cam resulting from an imprecise P-element excision (BL 6806: y^{1} w; Cam^{n39}/CyO, y^{+}; Heiman et al., 1996), (4) Cam^{7}, an EMS-induced point mutation (V91G) in the N-terminal helix region of the gene (BL 8140: y^{1} w; I^{1} Cam^{7}/CyO, y^{+}; Nelson et al. 1997); (5) IP3R^{90B.0}, a deletion of the IP3R gene generated by an imprecise excision of a P-element (BL 30737: IP3R^{90B.0}/TM6B Tb^{1}; Venkatesh and Hasan 1997); and (6) IP3R^{ug3}, an EMS-induced point mutation (S224F) in the
IP₃-binding domain (BL 30738: IP₃R<sup>mut3</sup>/TM6B Tb; Joshi <i>et al.</i> 2004). Genetic modifier experiment crosses are described in the footnotes of Tables S3 and 2.

**Fly culturing, and its modification for GAL4- and GAL80<sup>ts</sup>-based experiments:**
Flies were reared on a modified yeast/dextrose/cornmeal diet that is described in the Supporting Information (File S1). Unless otherwise noted, culture maintenance and experimental conditions were at 25°C under a 12h light: 12h dark cycle in an incubator humidified to maintain conditions at 60-80% relative humidity.

Culturing was also modified for the GAL80<sup>ts</sup> experiments. <i>nub-GAL4</i> Tub-GAL80<sup>ts</sup> females were mated to RNAi-<i>IP3K2</i> males, and vials containing the progeny from these crosses were incubated at either 18°C to minimize expression of RNAi-<i>IP3K2</i> or 29°C to express RNAi-<i>IP3K2</i> at high levels (Ferris <i>et al.</i> 2006; Baena-Lopez <i>et al.</i> 2009; Rodriguez <i>et al.</i> 2012). Shifts from one temperature to the other were conducted at different developmental stages throughout the life cycle, and the wings of adult F1 flies were scored. A more detailed description of this experimental design is found in the Results text and in the Figure 4 caption.

**Data and reagent availability:** The sequence assemblies for the <i>IP3K2</i> loci of <i>y¹ w¹</i>, <i>wy²</i>, and <i>wy⁷⁴i</i> flies are deposited in Genbank under accession numbers KT732028, KT732029, and KT732027 respectively. The <i>w wy² f</i> and <i>w</i>; <i>UAS-IP3K2</i> fly stocks and <i>UAS-IP3K2</i> construct are available upon request. All other fly stocks and reagents are commercially available.
RESULTS

Characterization of the wavy (wy) phenotype: We first examined the three classic mutant strains available from the Bloomington Center—wy¹, wy², and wy⁷⁴♭—to confirm and expand on their published phenotypic descriptions. As previously reported, the wings of wy mutants were severely deformed in a very specific pattern. In the most extreme cases, the wings of wy mutants exhibited all three of the following phenotypes (Figure 2A): (1) a wave-like buckle at a specific location along the costal vein, just distal to its intersection with the subcostal region, (2) an upturn at the most distal margin of the wing, and (3) an overall morphology that is shriveled but patterned in a manner that it is readily distinguishable from non-specific, mechanical wing damage or from wings that fail to inflate after adult eclosion (example of failed inflation shown in LAHR et al. 2012). However, in many other cases, mutant flies exhibited a subset of these phenotypes, and strikingly, only certain subsets were seen. We developed a numerical scale (0-3) to reflect the hierarchical pattern that we observed among the phenotypes and to quantitatively compare the genotypes analyzed in this study (Figure 2B-E): a score of “0” indicated a phenotypically wild type wing (never observed in the original wy mutants), “1” a costal buckle only, “2” a costal buckle along with a distal upturn, and “3” a costal buckle, distal upturn, and shriveled morphology. No other combinations of phenotypes were seen (e.g. distal upturn or shriveled wings without the other two phenotypes). The two wings of a fly were given a collective score because in >99% of flies examined, there was symmetrical penetrance and expressivity, and so both wings would have been given the same score if they had been scored individually. In the rare instance when a phenotypic mismatch was
seen between the two wings of a fly, the wings would have always received scores within 1 of each other, and the fly was given the lower of the two scores.

On average, \( \text{wy}^{74i} \) had the most severe phenotype, followed by \( \text{wy}^2 \) and \( \text{wy}^l \) (Table 1A). All three alleles were fully recessive, and no significant sexual dimorphism was observed within any strain. The three mutant strains we obtained have been described by different researchers in publications that were separated by significant spans of time, and we could not find explicit confirmation in the literature that all three alleles map to the same locus (Nachttsheim 1928; Parker 1935; Lindsley and Zimm 1992). Therefore, we crossed all three \( \text{wy} \) strains to each other and examined the wings of heteroallelic F1 females. All three alleles fail to complement one another, supporting the hypothesis that they map to the same locus (Table 1B). The \( \text{wy}^l \) phenotype became significantly more severe when in a heteroallelic combination with \( \text{wy}^2 \) or \( \text{wy}^{74i} \), and the \( \text{wy}^2 \) phenotype became significantly more severe over \( \text{wy}^{74i} \). These experiments also provided further validation for our numerical scale, since the hierarchical nature of the phenotypes shown in Figure 2B-E was still seen, even in these mixed genetic backgrounds.

We did not observe the lengthened abdomens that were previously reported of \( \text{wy}^l \) mutants in any of our \( \text{wy} \) mutant strains (Nachttsheim 1928)

**Mapping the \( \text{wy} \) locus:** Standard 3-point cross mapping using \textit{forked} (\textit{f}) and various \textit{w\textsuperscript{*}}-carrying transposable element insertions within the \( \text{wy} \) region as reference points placed \( \text{wy}^2 \) between PBac\{WH\}CG12096/\textsuperscript{05782} (13,159,870) and P\{EP\}Tango2\textsuperscript{6517} (13,617,116). This was followed by finer resolution mapping using complementation assays between \( \text{wy} \) alleles and a series of defined deletions and duplications (Parks \textit{et al.} 2004; Venken \textit{et al.} 2010; Cook \textit{et al.} 2012). The deletions Df(1)BSc766 and Df(1)Exel6245
failed to complement \textit{wy}^i, \textit{wy}^2, and \textit{wy}^74i, and the duplications Dp(1;3)DC267 and Dp(1;3)DC268 fully complemented these same \textit{wy} alleles. The overlapping region between these deletions and duplications implicates \textit{IP3K2} (\textit{IP}_3 \textit{kinase 2}) as the \textit{wavy} gene (Figure 3A).

Identifying non-complementing deletions also provided an opportunity to genetically characterize the available mutant alleles. Over the noncomplementing deletion Df(1)Exel6245, \textit{wy}^i hemizygotes exhibit a more severe phenotype than homozygotes, while \textit{wy}^2 and \textit{wy}^74i hemizygotes have a slightly less severe phenotype than homozygotes (Table 1B).

**Sequencing the \textit{wy}^2 allele, rescue, and RNAi:** The majority of DNA magnified in Figure 3B was sequenced for \textit{wy}^r (from the \textit{y}^i \textit{w}^i strain) \textit{wy}^2, and \textit{wy}^74i flies. Consistent with the hypothesis that \textit{IP3K2} is the \textit{wavy} gene, \textit{wy}^2 flies have a 5 bp deletion in the open reading frame of \textit{IP3K2} downstream of the calmodulin-binding site and catalytic domain, presumably causing a frameshift and premature stop codon (Figure 3B; Genebank accession number KT732029). In the case of \textit{wy}^74i, no mutation was identified in our sequencing of the majority of the region shown in Figure 3B. It is therefore possible that the \textit{wy}^74i mutation is in an upstream exon or expression regulatory region.

Flies mutant for \textit{wy}^i or \textit{wy}^2 were fully rescued and \textit{wy}^74i flies were significantly rescued by a single copy of a UAS-\textit{IP3K2} rescue construct, even without a GAL4 driver, probably due to low levels of leaky expression from the transgene (Table 1C). These results were seen with both of our rescue construct insertions.

To further confirm the identity of the \textit{wavy} gene as \textit{IP3K2} and to determine if the gene acts within the developing wing itself, we crossed flies carrying the RNAi-\textit{IP3K2}
construct to *nub*-GAL4, which expresses GAL4 throughout the prospective wing blade of the wing disc (Brand and Perrimon 1993; Azpiazu and Morata 2000). The *nub*-GAL4-driven RNAi-IP3K2 recapitulated the *wy* phenotype (Table 1D).

**Temporal requirement for IP3K2 function:** We next sought to determine the point of development at which *IP3K2* function is required for affecting adult wing morphology using the GAL4-GAL80ts system (Ferris et al. 2006; Baena-Lopez et al. 2009; Rodriguez et al. 2012). Given that the UAS-*IP3K2* construct did not require a GAL4 driver to rescue *wy* mutants, yet a copy of the RNAi-*IP3K2* construct did require a GAL4 driver to phenocopy *wy*, we shifted our focus to RNAi for these experiments so as to control GAL4-driven construct expression with GAL80ts and temperature shifts (see the Figure 4 caption for a detailed description of the experimental design). Control flies that were reared at 29°C exhibited the *wy* phenotype, presumably due to dysfunctional GAL80ts and consequent functioning of *nub*-GAL4 to express the RNAi-*IP3K2* construct. Control flies reared at 18°C did not phenocopy *wy* at all, presumably because GAL80ts was able to repress GAL4 and because GAL4 is generally less active at this lower temperature (Duffy 2002; Ferris et al. 2006; Baena-Lopez et al. 2009; Rodriguez et al. 2012). Reciprocal shifts from 29°C to 18°C and from 18°C to 29°C at various points during the life cycle revealed a dramatic reduction in the frequency of the *wy* phenotype if flies had experienced their Stages P1-P3 (white puparium-buoyant, see Bainbridge and Bownes 1981) at 18°C (low RNAi-*IP3K2* expression) as opposed to 29°C (high RNAi-*IP3K2* expression). This suggests that *IP3K2* function is required in the prospective wing blade for wing development during early pupal life.
**Genetic interaction assays between wy and other IP3 signaling loci:** Given that *IP3K2* encodes an IP3 3-kinase, other components of IP3 signaling may interact with *IP3K2* to affect wing development. To investigate this hypothesis, we tested whether mutations in several different IP3 pathway loci dominantly modify the wy phenotype, reasoning that such a sensitive interaction would indicate a strong functional relationship. The wy^2^ allele was used for the primary screen because of its intermediate phenotype and therefore presumed versatility in detecting both genetic enhancers and suppressors. *IP3K1, lpk2, Cam,* and *IP3R* were selected as candidate interactors because the proteins encoded by these loci have strong biochemical associations with IP3K2: IP3K1 and lpk2 also use IP3 as a substrate, Cam binds to and regulates IP3K2, and IP3R binds IP3 (Banerjee et al. 2004; Seeds et al. 2004; Lloyd-Burton et al. 2007).

In a wy^r^ background, the *IP3K1*^{KG02192}, *Df-lpk2, Cam^n339, Cam^7, IP3R^90B.0, and IP3R^ug3* alleles were all homozygous lethal mutations and had no discernible effect on wing structure in the heterozygous condition. *IP3R^sa54/IP3R^ug3* mutants had been reported to be a viable heteroallelic combination that exhibited mild crumpling at the margins of their wings, but we were unable to obtain the *IP3R^sa54* allele to reproduce these results (Banerjee et al. 2004). In our genetic modifier screen, *IP3K1*^{KG02192}, *Df-lpk2, Cam^n339, and Cam^7* did not dominantly modify wy^2^ wing scores (Table S3), but *IP3R^90B.0* and *IP3R^ug3* did (Table 2), strongly suppressing the wy^2^ phenotype relative to controls (for all modifier tests, controls were wy^r^/+ siblings from the same cross—cross schemes described in the footnotes of Tables S3 and 2). Further tests showed that the phenotypes of wy^74i^ and especially wy^i^ flies were also dominantly suppressed by both *IP3R* alleles—in fact, the wings of virtually all wy^i^; *IP3R^ug3/+* and wy^i^; *IP3R^90B.0/+* flies were phenotypically wild type (Table 2). In
summary, all three mutant alleles of \textit{wy} were dominantly suppressed by both mutant alleles of \textit{IP3R}.
wavy maps to IP$_3$ 3-kinase 2: In this study, we present strong evidence that mutations in wavy (wy), the first of which was described nearly 90 years ago (NACHTSHEIM 1928), are alleles of IP3K2 (IP$_3$ 3-kinase 2). The 3-point recombination mapping along with complementation analyses using molecularly defined deletions and duplications mapped wy down to the IP3K2 gene (Figure 3A). Sequencing of the IP3K2 gene of wy$^d$ flies revealed a 5 bp deletion in its open reading frame, putatively causing a frameshift mutation (Figure 3B; Genebank accession number KT732029), and although mutant sequences have not yet been identified for the other two alleles, wy$^d$ fails to complement wy$^I$ and wy$^{74i}$ (Table 1B), suggesting that they are alleles of the same locus. A UAS-IP3K2 construct rescues all three wy alleles (Table 1C), and RNAi of IP3K2 using the wing disc-specific driver nub-GAL4 phenocopies wy (Table 1D).

How the available wy alleles might affect IP3K2 function: wy$^I$/Df flies have a more severe phenotype than wy$^I$/wy$^I$ flies, suggesting that wy$^I$ is a hypomorphic allele. On the other hand, wy$^d$ and wy$^{74i}$ become less severe in the hemizygous condition, yet both are fully recessive (Table 1B). Hence, wy$^d$ and wy$^{74i}$ do not neatly fall into any classic mutant category (MULLER 1932; WILKIE 1994). However, the UAS-IP3K2 construct fully rescues wy$^d$ and significantly alleviates the wy$^{74i}$ phenotype, and RNAi-IP3K2 expression in the wing disc causes a phenotype that resembles those of both wy$^d$ and wy$^{74i}$ (Table 1C,D). Finally, both alleles are fully complemented by the duplications shown in Figure 3A. Taken together, these data suggest that wy$^d$ and wy$^{74i}$ are both strong loss of function alleles, and that their wing scores were somewhat reduced by the genetic background of the deficiency line. A molecular null allele of IP3K2 was recently generated (Nelson et al. 2014). Although
no mention was made of a wing-related phenotype, the reporting manuscript was wholly focused on the function of IP3K2 in the salivary glands. Analysis of how this null allele affects wing development would provide further insight into the nature of the wy² and wy⁷⁴i alleles and, of course, be necessary to understand the consequences of completely removing gene function.

The frameshift mutation we found in wy² lies well downstream of the regions encoding a calmodulin-binding domain and the active site (Figure 3B). Therefore, wy² may have some IP3K2 activity in spite of its strong phenotype. Consistent with this hypothesis, wy² is fully rescued by UAS-IP3K2 without any GAL4 driver, while UAS-IP3K2 only partially rescues the more severe wy⁷⁴i allele (Table 1C). Even if wy² had some residual function, the protein encoded by wy² would be truncated by a premature stop codon, and so its conformation, interactions with regulating proteins such as calmodulin (Lloyd-Burton et al. 2007), and/or stability may be significantly affected. Enzymatic assays of the altered IP3K2 enzyme encoded by wy² may provide further insight into how this allele affects enzyme activity, stability, and regulation. Similar studies could be done with proteins encoded by the wy¹ and wy⁷⁴i alleles once they have been molecularly defined.

**IP3K2 function is required in the developing wing blade during early pupal life:** Controlled expression of the RNAi-IP3K2 construct using nub-GAL4, Tub-GAL80ts, and temperature shifts during specific developmental windows revealed a requirement for IP3K2 function in the wing disc during pupal stages P1-P3 (Figure 4; Table S2 in the Supporting Information). This developmental window may provide clues into the cellular process that IP3K2 is involved in. As described in the Introduction and in Figure 1, IP₃ signaling can regulate IP3R-mediated Ca^{2+} release from stores in the ER, and elsewhere in
the literature, there is evidence that intracellular calcium signaling is involved in the development of the pupal wing of insects. Cytoplasmic calcium waves have been documented in the pupal wings of the butterfly Junonia orithya, and these waves are halted by pharmacological inhibition of ER Ca\(^{2+}\)-ATPase, an enzyme responsible for initially sequestering Ca\(^{2+}\) in the ER before release occurs. Data suggest that these calcium waves are involved in wing eyespot development in Junonia orithya (Ohno and Otaki 2015).

Calcium waves have also been induced in Drosophila larval wing discs in response to laser-induced wounding (Narciso et al. 2015).

The Drosophila calmodulin (Cam) protein binds and regulates the activity of IP3K2 in a Ca\(^{2+}\)-dependent fashion (Figure 3B; Lloyd-Burton et al. 2007). Many loss of function alleles in Cam are lethal before adulthood, but some viable alleles result in ectopic wing veins (Nelson et al. 1997). While we did not observe this ectopic vein phenotype with loss of IP3K2 function or with our genetic interaction experiments, Cam has a broad spectrum of functions, and so the ectopic vein phenotype may be due to a process unrelated to IP\(_3\) signaling. Relevant here, however, is that the wing vein positions are refined during the P1-P3 stages, the stages at which we found IP3K2 function is required in the wing (Blair 2007; Figure 4; Table S2 in the Supporting Information). In addition, it is at least known that Cam mRNA is expressed at very high levels in wing discs that were cultured shortly before the P1 stage (Chehabas et al. 2011). Therefore Cam, a Ca\(^{2+}\)-dependent regulator of IP3K2 activity, may be active in the wing disc during the same developmental window that IP3K2 function is required (Figure 4). Although our dominant modifier screen did not detect an interaction between wy and Cam (Table S3), a single copy of a Cam mutation may not have reduced function enough to see an effect.
Similarly, we did not detect dominant modification of wy by mutations in IP3K1 or Ipk2 (Table S3). Both IP3K1 and Ipk2 encode enzymes that are specific for the IP3K2 substrate (IP$_3$), and similar to Cam, both genes are expressed at moderate to high levels in the cultured wing discs of wandering larvae (Seeds et al., 2004; Cherbas et al. 2011). Therefore, IP3K2, IP3K1, and Ipk2 might compete for the same IP$_3$ pool in the wing discs and/or exhibit redundant functions. Importantly, however, our experiments indicate a requirement for IP3K2 function at the P1-P3 stages—not during but shortly after the wandering phase (Figure 4), and to our knowledge detailed expression patterns are not available for IP3K2, Cam, IP3K1, Ipk2, or IP3R in wing discs during these developmental stages. Therefore, future experiments should characterize expression of and more extensively test interactions between these IP$_3$ signaling pathway genes, focusing on the early pupal wing disc and using stronger losses of gene functions than were present in our dominant modifier screen.

While we have determined a spatiotemporal requirement for IP3K2 function and an interacting locus (IP3R), our data do not identify a cellular mechanism underlying the wy phenotype. The developmental events that normally occur in the wing disc during the P1-P3 stages may provide clues into this aspect of IP3K2 function. For example, wing bristle precursors at the anterior margin of the wing are proliferating during early pupal life, while cells of the prospective wing blade are mitotically quiescent until shortly after P3 (Milán et al. 1996b). Interestingly, the anterior margin is the general region of the wing that most consistently exhibits a phenotype in wy flies (i.e. the “costal buckles” shown in Figure 2A,C-E). IP$_3$ signaling is involved in the cell proliferation of multiple systems, and in Drosophila, IP3R has been shown to be required for the cytokinesis of spermatocytes (Wong et al. 2005;
Therefore, it is conceivable that mutations in \textit{wy} disrupt cell cycle regulation in the pupal wing.

Another possible function for \textit{IP3K2} in the wing comes from reports of its function in another \textit{Drosophila} tissue. The micro-
RNA \textit{miR-14} induces autophagy of the salivary glands during early pupal life by targeting \textit{IP3K2} (Nelson et al. 2014). The consequent downregulation of \textit{IP3K2} is thought to increase the amount of IP$_3$ available to \textit{IP3R}, \textit{IP3R} is activated as a result, and autophagy is induced, at least in part by Ca$^{2+}$ release from the ER. This same study suggested that \textit{Atg6}, an autophagy-inducing gene that encodes a component of the Vps34 phosphatidylinositol 3-kinase (PI3K) complex III, acts in the same pathway as \textit{miR-14}. In another study, \textit{Atg6} was shown to be required for autophagy in the pupal wing of \textit{Drosophila} (Lorincz et al. 2014). These findings suggest the intriguing possibility that \textit{IP3K2} and \textit{IP3R} regulate autophagy in the developing wing, perhaps by interactions with the \textit{Atg6/miR-14} module.

\textbf{Modeling the interactions between \textit{wavy} and \textit{IP3R}:} In past studies, mild wing crumpling in \textit{IP3Rsas}$^{54}$/\textit{IP3R}$^{ugs}$ mutants hinted at the involvement of the IP$_3$ signaling network in wing development, but further analysis was presumably hindered because other \textit{IP3R} allele combinations were either lethal or had normal wing morphology (Banerjee et al. 2004). \textit{wy} provides an alternative entry point to \textit{IP3R}, and has useful qualities for investigating how IP$_3$ signaling affects wing morphology: (1) flies with strong loss of \textit{IP3K2} function have good viability, and (2) the \textit{wy} phenotype has several discrete features to it—costal buckling, upward curling, and overall crumpling—that are easily scored and consistently appear in a hierarchical pattern (Figure 2B-E; Table 1). This makes
the wy phenotype an efficient, precise indicator of the levels of gene and IP₃ pathway function, and therefore a sensitive gauge for identifying genetic interactors.

Our results along with the biochemical relationship between IP3K2 and IP3R suggest that IP₄ levels and/or IP₄-independent IP3R signaling affect wing development (Figure 5A). Previous studies of Drosophila S2 cell cultures have found that loss of IP3K2 function can contribute to expansion of the IP₃ pool. This suggests that IP₃ is being steadily produced from IP3K2-independent sources (e.g. phospholipase C), and so in control cells, the forward reaction for IP3K2 (IP₃→IP₄) is predominant (Seeds et al. 2004). If we assume that the forward reaction is predominant in the pupal wing tissue as well (which would require direct confirmation in future studies), then loss of wy function would be expected to expand the IP₃ pool and decrease levels of IP₄. If this were the case, the wy phenotype may be due to insufficient levels of IP₄ (Figure 5B), and/or an excess of IP₃ that hyperactivates IP3R (Figure 5C). In both cases, a partial loss of IP3R function could potentially alleviate the wy mutant phenotype. Both models assume that IP3K2 catalytic activity is required to affect wing morphology. Although we have not tested this model directly, the strong genetic interaction between wy and IP3R (Table 2) and the biochemical relationship between their encoded proteins support this assumption. In addition, IP3Ks are typically cytoplasmic (Xia and Yang 2005), and IP3K2 has been shown to localize to the cytoplasm when expressed in HeLa cells (Lloyd-Burton et al. 2007). Therefore, IP3K2 protein is likely to be expressed in the proper subcellular compartment in order to have the hypothesized interaction with ER-bound IP3R (i.e. drawing from the same pool of IP₃).

In summary, this study maps a classic mutant phenotype to a single gene and helps establish Drosophila wing development as an effective system to study IP₃ signaling. Future
experiments should investigate possible cellular mechanisms underlying the wy phenotype (e.g. the potential roles of Ca^{2+} release from the ER and possible effects of wy on cell proliferation and autophagy), as well as continue to test and refine models of how IP3K2 interacts with other components of the IP_3 signaling network to build a wing.
ACKNOWLEDGEMENTS

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Yang, L., F. Meng, D. Ma, W. Xie and M. Fang, 2013 Bridging Decapentaplegic and Wingless signaling in Drosophila wings through repression of naked cuticle by Brinker. Development 140: 413-422.
**Figure 1** Some key components of IP$_3$-related signaling in *Drosophila*. Enzyme names are boxed and an encircled “P” denotes an inorganic phosphate group. IP$_3$ (inositol 1,4,5-trisphosphate, top center) may undergo the following fates: (1, left) bind to the IP$_3$-gated calcium channel IP$_3$R (IP$_3$ receptor), causing IP$_3$R to open and release calcium that was sequestered in the ER lumen, (2, top right) be phosphorylated by IP3K2 (IP$_3$ 3-kinase 2) to form IP$_4$ (inositol 1,3,4,5-tetrakisphosphate), or (3, bottom right) be more highly phosphorylated by Ipk2 (inositol polyphosphate kinase 2) and subsequently Ipk1 (inositol polyphosphate kinase 1) to yield IP$_6$. Only forward reactions are shown because previous studies suggested that these reactions predominate in fly cells (*Seeds et al.* 2004). IP3K1 catalyzes the same reaction as IP3K2 but is not the primary focus of this study and therefore not shown. Calmodulin (Cam), also not shown, increases IP3K2 activity by binding to the enzyme in a calcium-dependent fashion (*Lloyd-Burton et al.* 2007). See Introduction text for additional molecular details. In this report, we present evidence that IP3K2 is encoded by the *wavy* (*wy*) locus and that a balance between IP3K2 and IP3R functioning is necessary for normal wing morphology.
Figure 2 Mutations in *wavy* (*wy*) disrupted wing morphology. (A) Wild type (left) and *wy*74i flies (right). Note that the *wy*74i flies obtained from the Bloomington Center (BL#1162) were also mutant for the eye color gene *vermillion* (*v*). In the most extreme cases, a mutation in *wy* caused the following three phenotypes: (1) a wave-like buckle (black arrow) at a specific location midway along the costal vein, just distal to the intersection of this vein with the first longitudinal vein, (2) an upturn at the most distal margin of the wing (blue arrow), and (3) a generally shriveled appearance with a pattern. However, *wy* mutants often displayed only specific subsets of these phenotypes. (B-E) Numerical scoring system reflecting the subsets of *wy* phenotypes that were observed. (B) Phenotypically wild type wings received scores of “0” (note that none of the original mutant strains received a wild type score; see Table 1A). (C) A wing with a costal buckle as the only apparent abnormality (black arrow) received a score of “1”. (D) A wing with a costal buckle and distal upturn was scored as a “2”. (E) A wing with a costal buckle, distal upturn, and overall shriveled appearance received a score of “3”. No other combinations of these three phenotypes were observed throughout our experiments.
Figure 3 IP3K2 is the wavy gene. (A) Image depicts the 11E9-11 region of the X chromosome, after an image generated using the Flybase GBrowse tool (St Pierre et al. 2014). From top, dark blue rectangles represent gene boundaries, with arrowheads indicating directions of transcription. Known transcripts are displayed immediately below gene boundaries, with tan representing coding sequence, gray representing non-coding sequence, lines representing introns, and again arrowheads indicating directions of transcription. Red rectangles indicate sequences deleted in the Df(1)BSC766 and Df(1)Exel6245 stocks. These deletions fail to complement wy1, wy2, and wy74i. On the other hand, the sequences duplicated in the Dp(1;3)DC267 and
Dp(1;3)DC268 stocks (light blue rectangles) fully complement all three wy alleles. The unshaded region in the middle of the figure highlights the overlap between all four of these deleted and duplicated sequences; \textit{IP3K2} is the only gene that expresses full length transcripts and predicted coding sequences from this shared segment. In further support of \textit{IP3K2} being the \textit{wavy} gene, the \textit{wy}^2 allele contains a 5 bp deletion (Genebank accession number KT732029) just downstream of a conserved inositol polyphosphate kinase (Ipk) domain (further magnified image at bottom right, dark brown segment). Rescue and RNAi experiments also indicated that \textit{IP3K2} is the \textit{wavy} gene (see Table 1C,D). Bottom left, a scale bar for the top, low-magnification portion of the figure panel. (B) Putative amino acid sequence of the four contiguous coding exons which are shared by all known \textit{IP3K2} transcript isoforms (\textit{i.e.} the enlarged, brown exons featured at the bottom right of (A)).

Green box highlights a tryptophan residue that is necessary for calmodulin binding (Lloyd-Burton \textit{et al.} 2007). Underlined, bold sequence represents a domain that is highly conserved by the Ipk superfamily that includes IP3K enzymes. Orange box highlights a PxxxDxKxG motif, which is a key characteristic of the active site (Lloyd-Burton \textit{et al.} 2007). Yellow box outlines the location and effect of the \textit{wy}^2 mutation: a frameshift that changes the sequence of 6 amino acids then inserts a premature stop codon.
Figure 4 Identifying the critical stage for IP3K2 function in the developing wing using the GAL4-GAL80ts system and RNAi. nub-GAL4 Tub-GAL80ts females were mated to RNAi-IP3K2 males, and their nub-GAL4 Tub-GAL80ts/+; RNAi-IP3K2/+ progeny were reared at either 29°C to express the RNAi construct at high levels or at 18°C to minimize its expression. Control groups (left) were reared at either 29°C or 18°C for their entire life cycle and experimental groups (right) were initially reared at one temperature or the other, shifted from 18°C to 29°C (solid line) or from 29°C to 18°C (dotted line) during a specific developmental window, then maintained at the second temperature for the remainder of their life cycles. Developmental windows during which temperature shifts were administered (x-axis) were designated based on published descriptions of the fly life cycle (Bainbridge and Bownes 1981; Bate et al. 1993): (1) Emb, embryos, (2) Larv, first through mid-third instar larvae, (3) Wand, late third instar-wandering larvae, (4) Pup, white puparium formation-buoyant (P1-P3), and (5) Meta, metamorphosis from head eversion to meconium stages (P4-P15). y-axis indicates wy phenotype scoring as described in Figure 2B-E and the Results text. Average wing scores are shown with error bars depicting the standard errors of the means. *, p<0.05 for the Fisher’s exact tests comparing the marked experimental group to each of the two controls on the far left. (For all unmarked experimental groups, p>0.05 when tested against one of these two controls, and p<<0.05 when tested against the other control.) Raw data for this experiment are presented in the Supporting Information (Table S2).
Figure 5 Potential models for how IP3K2 affects wing morphology and how mutations in IP3R dominantly suppress the wy phenotype. (A) The strong genetic interaction between wy and IP3R and what is known about the biochemical functions of their encoded proteins suggest that wing morphology as assessed in this study may be affected by IP₄ signaling, IP₄-independent IP3R signaling, or an integration of both signals. Question marks indicate uncertainty about the relative importance of these two signals. By extension, the wy phenotype may be caused by (B) reduced IP₄ levels and/or (C) excessive IP3R activation that triggers IP4-
independent signals (e.g. increased Ca2+ release from the ER). (B) If the wy phenotype is solely caused by reduced IP4 levels, then IP3R would be expected to further inhibit accumulation of IP4 by inhibiting residual activity of mutant IP3K2 enzyme ("Wavy"), for example, by usurping the IP3 substrate. A mutant copy of IP3R would be expected to make more IP3 available to IP3K2, increasing IP4 formation and suppressing the wy phenotype. Increased levels of IP3 are shown here due to loss of IP3K2 function. However, this model would hold whether or not IP3 actually accumulates in the wing discs of wy mutants, because in either case, loss of IP3R function could increase the amount of substrate available to the mutant IP3K2 enzyme. (C) Model if increased IP3R signaling triggers downstream, IP4-independent events to cause the wy phenotype. Here, IP3 is assumed to accumulate due to loss of IP3K2 function, as suggested by studies in Drosophila S2 cells (Seeds et al. 2004); this accumulation of IP3 would be expected to hyperactivate IP3R, increasing calcium release from the ER. A partial loss of IP3R function would reduce this excessive IP3R signaling, suppressing the wy phenotype. (B) and (C) represent extreme models that exclude one factor or the other, but a hybrid model is also possible where both IP4 signaling and IP4-independent IP3R signaling play significant roles in wing development.
### Table 1: Wing scores of wy mutant strains, complementation analysis, rescue, and RNAi

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<th>Genotype</th>
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<th>% with each wing score</th>
<th>Average wing score</th>
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<td><strong>A. Mutant strains</strong></td>
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<tr>
<td>wy(^i)</td>
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<td>wy(^j)</td>
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<td>wy(^74i)</td>
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<td>wy(^i)/wy(^j)</td>
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</tr>
<tr>
<td>wy(^74i)/Df</td>
<td>20</td>
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</tr>
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<td><strong>C. Rescue(^c)</strong></td>
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<tr>
<td>wy(^i); UAS-IP3K2/+</td>
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</tr>
<tr>
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<td>nub-GAL4/+</td>
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<tr>
<td>RNAi-IP3K2/+</td>
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<tr>
<td>nub-GAL4/+; RNAi-</td>
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</table>
See first section of Results text and Figure 2B-E for a detailed description of the scoring system.

“Df” represents the deficiency carried by the Df(1)Exel6245 stock (BL#7718). Details for these crosses are in the Materials and Methods.

One copy of a UAS-IP3K2 transgene on chromosome 3 was crossed into wy mutant backgrounds (denoted as “wy; UAS-IP3K2/+”, with the “+” indicating that the other third chromosome has no rescue construct insertion). The results from only one of our two UAS-IP3K2 insertions are reported here, but the other UAS-IP3K2 insertion yielded identical results.

For these experiments, wy fly flies with one copy of the nub-GAL4 construct only (“nub-GAL4/+”, F1 Sb males from a cross between RNAi-IP3K2/TM3 Sb females and Dcr-2; nub-GAL4 males), one copy of RNAi-IP3K2 only (“RNAi-IP3K2/+”, F1 Sb+ males from a cross between RNAi-IP3K2/TM3 Sb females and y¹ w¹ males), and both in combination (“nub-GAL4/+; RNAi-IP3K2/+”, F1 Sb+ males from a cross between RNAi-IP3K2/TM3 Sb females and Dcr-2; nub-GAL4 males) were scored and compared. As with c, the “+” denotes a chromosome with no construct insertion. Qualitatively similar results were obtained with female progeny from these same crosses.

*p<10⁻¹⁴, Fisher’s exact test comparing each true breeding mutant strain to the other two strains listed in Section A of this table.

**p<10⁻⁷, or in the case of wy²/wy⁷⁴, p<0.05, Fisher’s exact test comparing flies with a heteroallelic combination to those only carrying the corresponding milder allele from Section A of this table (e.g. wy¹/wy⁷⁴ compared to wy¹).

***p<10⁻⁶, Fisher’s exact test comparing wy/Df or wy; UAS-IP3K2/+ flies to the corresponding wy control from Section A of this table (e.g. wy¹/Df or wy¹; UAS-IP3K2/+ compared to wy¹).

****p<10⁻¹⁸, Fisher’s exact test comparing nub-GAL4/+; RNAi-IP3K2/+ to its controls nub-GAL4/+ and RNAi-IP3K2/+ in Section D of this table.
TABLE 2 Testing for dominant modification of the wy phenotype by loss of function in the IP₃ receptor gene

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>% with each wing score</th>
<th>Average wing score</th>
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<td>wy&lt;sup&gt;74i&lt;/sup&gt;; +/+</td>
<td>17</td>
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</tbody>
</table>

<sup>a</sup>See first section of Results text and Figure 2B-E for a detailed description of the scoring system.

<sup>b</sup>Each pair of rows compares F1 male siblings from the following cross:

(wy/wy; +/+ females) X (wy<sup>y</sup>/Y; [IP₃R mutant allele]/TM6B Tb<sup>y</sup>, IP₃R<sup>y</sup> males)

The wings of wy/Y; [IP₃R mutant allele]/+, F1 males (experimental group, upper row within each pair) were compared to those of their wy/Y; +/TM6B Tb<sup>y</sup>, IP₃R<sup>y</sup> F1 male siblings (control group, lower row within each pair—
note that in the table, TM6B Tb\textsuperscript{1}, IP3R\textsuperscript{T} is shortened to a second + for simplicity). These comparisons tested whether mutations in IP3R can dominantly modify wy while controlling for the genetic background of the other chromosomes. While the change in genetic background or even the TM6B balancer may have had some effect on the distribution of wy wing scores, mutations in IP3R had a far more pronounced effect, lowering scores significantly relative to their sibling controls and even more dramatically relative to the original strains (compare control and experimental values in this table to Table 1A), the hierarchical nature of the phenotypes was once again preserved, and no novel phenotypes were seen.

\*p<10\textsuperscript{-4}, Fisher’s exact test vs. sibling controls in the row immediately below the marked row.

\**p<0.05, Fisher’s exact test vs. sibling controls in the row immediately below the marked row.
Fly media

For every 100 ml of near-boiling distilled water, stir the following in sequentially:

- 11 g dextrose (Fisher Scientific)
- 1.5 g agar (Fisher Scientific)
- 5 g Baker’s yeast (Red Star)
- 5.2 g commercial cornmeal

Take diet off of heat and blend to cool below 60°C. While blending, add 750 µl of propionic acid (Sigma) and 500 µl of 15% p-hydroxybenzoic acid methyl ester (Sigma), the latter dissolved in 95% ethanol. After dispensing into vials or bottles and allowing time for diet to congeal, supplement with pinches of Baker’s yeast and cap.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP3K2-1S</td>
<td>5'-CGC TAT CAG GTA TTG CCA CTG ATG CAC-3'</td>
</tr>
<tr>
<td>IP3K2-2AS</td>
<td>5'-AGC GTT GGC ATT GCT GGC GC-3'</td>
</tr>
<tr>
<td>IP3K2-2S</td>
<td>5'-GCG CCA GCA ATG CCA ACG CT-3'</td>
</tr>
<tr>
<td>IP3K2-3S</td>
<td>5'-CCA TGG GTT CAA CTG GCC GG-3'</td>
</tr>
<tr>
<td>IP3K2-3AS</td>
<td>5'-CCG GCC AGT TGA ACC CAT GG-3'</td>
</tr>
<tr>
<td>IP3K2-4S</td>
<td>5'-GCC CCG CTA CAT ACA GCG TT'T G-3'</td>
</tr>
<tr>
<td>IP3K2-5AS</td>
<td>5'-GCC CCA AAC CTC CGT GTG GA-3'</td>
</tr>
</tbody>
</table>
### TABLE S2 Wing scores$^a$ of *nub*-GAL4 *Tub*-GAL80$^{15}/+; RNAi-IP3K2/+ flies under expressing (29°C) and non-expressing (18°C) conditions

<table>
<thead>
<tr>
<th>Incubation conditions$^b$</th>
<th>n</th>
<th>% with each wing score</th>
<th>Average wing score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>A. Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29°C</td>
<td>87</td>
<td>10.3</td>
<td>0.0</td>
</tr>
<tr>
<td>18°C</td>
<td>69</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>B. 29°C to 18°C shift</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Emb</em></td>
<td>18</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Larv</em></td>
<td>34</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Wand</em></td>
<td>68</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Pup</em></td>
<td>26</td>
<td>42.3</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Meta</em></td>
<td>67</td>
<td>29.9</td>
<td>10.4</td>
</tr>
<tr>
<td>C. 18°C to 29°C shift</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Emb</em></td>
<td>37</td>
<td>5.4</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Larv</em></td>
<td>95</td>
<td>10.5</td>
<td>4.2</td>
</tr>
<tr>
<td><em>Wand</em></td>
<td>41</td>
<td>12.2</td>
<td>0.0</td>
</tr>
<tr>
<td><em>Pup</em></td>
<td>20</td>
<td>0.0</td>
<td>75.0</td>
</tr>
<tr>
<td><em>Meta</em></td>
<td>39</td>
<td>100.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

$^a$See first section of Results text and Figure 2B-E for a detailed description of the scoring system.

$^b$See Figure 4 caption for a detailed description of the experimental design and developmental staging codes.

*p<0.05 for the Fisher’s exact tests comparing the marked experimental group to each of the two controls in Section A. (For all unmarked experimental groups, p>0.05 when tested against one of these two controls, and p<<0.05 when tested against the other control.)
TABLE S3 Testing for dominant modification of the wy phenotype by mutations in the IP₃ signaling pathway genes IP3K1, Ipk2, and Cam

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>% with each wing score</th>
<th>Average wing score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>A. IP₃ 3-kinase 1 (IP3K1)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wy²; IP3K1&lt;sup&gt;KG02192&lt;/sup&gt;/+</td>
<td>53</td>
<td>0.0</td>
<td>22.6</td>
</tr>
<tr>
<td>wy²; +/-</td>
<td>64</td>
<td>0.0</td>
<td>23.4</td>
</tr>
<tr>
<td><strong>B. Inositol polyphosphate kinase (Ipk2)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wy²; Df-Ipk2/+</td>
<td>41</td>
<td>0.0</td>
<td>2.4</td>
</tr>
<tr>
<td>wy²; +/-</td>
<td>38</td>
<td>0.0</td>
<td>5.3</td>
</tr>
<tr>
<td><strong>C. Calmodulin (Cam)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wy²; Cam&lt;sup&gt;n339&lt;/sup&gt;/+</td>
<td>83</td>
<td>0.0</td>
<td>24.1</td>
</tr>
<tr>
<td>wy²; +/-</td>
<td>64</td>
<td>0.0</td>
<td>12.5</td>
</tr>
<tr>
<td>wy²; Cam&lt;sup&gt;1&lt;/sup&gt;/+</td>
<td>61</td>
<td>1.6</td>
<td>14.8</td>
</tr>
<tr>
<td>wy²; +/-</td>
<td>39</td>
<td>0.0</td>
<td>12.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>See first section of Results text and Figure 2B-E for a detailed description of the scoring system.

<sup>b</sup>w wy² f females were crossed to y<sup>1</sup>; IP3K1<sup>KG02192</sup>/CyO; ry<sup>506</sup> males and w wy² f/Y; IP3K1<sup>KG02192</sup>/+ F1 males were collected. (This removed the CyO balancer from the crosses, allowing wy to be scored without obfuscation from the similar Cy trait.) These F1 males were crossed to w wy² f females, and w wy² f/Y; IP3K1<sup>KG02192</sup>/+ F2 males were compared to their w wy² f/Y; +/- male sibling controls. These F2 males were able to be distinguished because the IP3K1<sup>KG02192</sup> P-insertion carries the w<sup>+</sup> allele.

<sup>c</sup>w wy² f females were crossed to Df-Ipk2/S6a, y<sup>1</sup> w males to, as discussed in <sup>b</sup> above, remove the SM6a-associated Cy marker from the cross scheme. w wy² f/Y; Df-Ipk2/+ F1 males were crossed to w wy² f females,
and w wy<sup>2</sup> f/Y; Df-Ipk2/+ F2 males were compared to their w wy<sup>2</sup> f/Y; +/+ male sibling controls. These F2 males were able to be distinguished because transposable elements associated with the Df-Ipk2 deletion carry the w<sup>+</sup> allele (Ryder et al. 2007).

d<sup>y</sup> w; Act5C-GAL4, Cam<sup>+</sup>/CyO, y<sup>+</sup> females (BL 4414) were crossed to either y<sup>n</sup> w; Cam<sup>n329</sup>/CyO, y<sup>-</sup> or y<sup>n</sup> w; Cam<sup>+</sup>/CyO, y<sup>+</sup> males. y<sup>n</sup> w; Cam/Act5C-GAL4, Cam<sup>+</sup> F1 males were selected to, as discussed in b above, remove the CyO-associated Cy marker from the cross scheme. w wy<sup>2</sup> f/Y; Cam/Act5C-GAL4, Cam<sup>+</sup> F1 males were crossed to w wy<sup>2</sup> f females, and w wy<sup>2</sup> f/Y; Cam/+ F2 males were compared to their w wy<sup>2</sup> f/Y; Act5C-GAL4, Cam<sup>+</sup>/+ male sibling controls (for simplicity, controls represented as “wy<sup>2</sup>; +/+” in the table). These F2 males were able to be distinguished because the Act5C-GAL4 construct carries a w<sup>+</sup> allele.

*p>0.10, Fisher’s exact test vs. sibling controls in the row immediately below the marked row.