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

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ABSTRACT Inositol 1,4,5-trisphosphate (IP3) regulates a host of biological processes from egg activation to cell death. When IP3-specific receptors (IP3Rs) bind to IP3, 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 IP3; this limits IP3R activation by reducing IP3 levels, and also generates new signaling molecules altogether. These divergent pathways draw from the same IP3 pool yet cause very different cellular responses. Therefore, controlling the relative rates of IP3R activation vs. phosphorylation of IP3 is essential for proper cell functioning. Establishing a model system that sensitively reports the net output of IP3 signaling is crucial for identifying the controlling genes. Here we report that mutant alleles of wavy (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 IP3 signaling, giving this system strong potential as a model for further study of the IP3 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 IP4 levels, and/or excessive IP3R signaling.

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 (Milán et al. 1996a; Biels et al. 1998; Klein 2001; Cavodeassi et al. 2002; Crozatier et al. 2002, 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; Milán et al. 1996b; Classen et al. 2005, 2008; Blair 2007; Taylor and Adler 2008). Shortly after adult eclosion, the wings which

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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; Nattle 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-β)], 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 IP₃ (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 IP₃ 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, 2006; Agrawal et al. 2009, 2010; Venkiteswaran and Hasan 2009). 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; 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 a stock mutant for the wavy (wy) locus. Flies mutant for wy exhibit wings that are bent and crumpled in a highly specific pattern (Nachtstein 1928; Parker 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 the 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 wy¹ (Bloomingston Stock Center #168, or BL 168: wy¹; Nachtstein 1928), wy² (BL 192: wy²; P{EP}), and wy³⁶ alleles (BL 1294: t¹ v¹ m⁷⁶ wy³⁶; Lindsley and Zimm 1992) were used in this study. wy² was reintroduced into a w¹ background, and a w wy stock was established to facilitate mapping and the tracking of w²-labelled constructs during crosses. w¹, f, and the w³ transgene insertion lines P{EP}[WH]CG12090(95378) (BL 18906: w¹ wy¹ Bac[WH]CG12090(95378), and P{EP}[Tango2G517L] (BL32580: w P{EP}[Tango2G517L]) were used for three-point replacement mapping (Bellen et al. 2004; Thibault et al. 2004). For complementation tests, wy¹, wy², or wy³⁶ females were crossed to males carrying either of two duplications Dp(1;3)DC267 (BL 30384: w¹ wy¹, Dp(1;3)DC267, PBac[DC267]VK00033) or Dp(1;3)DC268 (BL 30385: w¹ wy¹, Dp(1;3)DC268, PBac[DC268]VK00033), and F1 w¹/wy¹;
IP3K2 activity by binding to the enzyme in a calcium-dependent fashion (Lloyd-Burton et al. 2007). See Introduction for additional molecular details. In this report, we present evidence that IP3K2 is encoded by the \textit{BSC766}, \textit{w1118}\textsuperscript{P{XP-U}Exel6245/FM7c}\textsuperscript{fl}{wy1 w1} locus, and that a balance between IP3K2 and IP3R functioning is necessary for normal wing morphology.

Phenotypic assessment and microscopy
A numerical scale was devised to quantify \textit{wy} penetrance and expressivity throughout this study (see Results 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 IP3K2 alleles and DNA sequencing
All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Individual \textit{wy} (from a \textit{y w}\textsuperscript{1} strain, BL 1495), \textit{wy} \textit{w2}\textsuperscript{zen}, and \textit{wy} adult male flies were first frozen in 1.5 ml Eppendorf tubes, then each was ground within their tube in 50 \textmu l of standard fly “squishing” buffer [10 mM Tris (pH 8), 1 mM EDTA, 25 mM NaCl, 200 \mu g/ml Proteinase K]. Crushed flies were incubated for 30 min at 37°C to digest fly tissue, then at 94°C for 3 min to denature the Proteinase K. Segments of the \textit{IP3K2} gene were PCR-amplified from DNA extract using GoTaq Flexi DNA polymerase (Promega; 1 \mu l DNA extract per 19 \mu l 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 Supporting Information, Table S1. Sequence outputs were analyzed using the MEGA5 software (Tamura et al. 2011). Sequences from at least two individual flies of each genotype were analyzed in order to resolve ambiguities.

Rescue construct, RNAi of IP3K2, GAL4 driver, and GAL80\textsuperscript{ts}
To assemble the rescue construct, a \textit{NotI}–\textit{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 et al. 2002; Hoskins et al. 2011), ligated with T4 DNA ligase (New England Biolabs) into pUAS-c5-attB (Daniels et al. 2014) in order to place the \textit{IP3K2} cDNA downstream of a UAS site, and finally sent to Bestgene (Chino Hills, CA) for transformation in a \textit{w} background. Two independent insertion lines were obtained, both on the third chromosome at the 68A4 location. The manuscript refers to these rescue lines as UAS-\textit{IP3K2}.

RNAi experiments of \textit{IP3K2} were conducted with a stock from the Vienna Stock Center (VDRC v19159: P[GD7878]v19159/TM3 \textit{SB}, hereafter referred to as RNAI-\textit{IP3K2} (Dietzl et al. 2007).

We used \textit{nub-GAL4} (BL 25754: P[UAS-Dcr-2.\textit{D}]1, \textit{w}\textsuperscript{1118}P; \textit{GawB} \textit{nubbin-AC-62}), a wing disc-specific driver, for our RNAi experiments (Brand and Perrimon 1993; Azpiazu and Morata 2000). The \textit{Tub-GAL80\textsuperscript{ts}} construct (from BL 7108: \textit{w}; P[\textit{tubP-GAL80\textsuperscript{ts}}10; TM2/TM6B, \textit{Tb}]), which was employed for temperature-sensitive deactivation of \textit{nub-GAL4}, was recombined onto the same chromosome as \textit{nub-GAL4}, and a \textit{w} stock was established that was also homozygous for both insertions but did not contain the P[UAS-\textit{Dcr-2.\textit{D}]1 construct (also see \textit{Fly} culturing below; Ferris et al. 2006; Baena-Lopez et al. 2009; Rodriguez et al. 2012).

Dominant genetic modifier screen
We tested several \textit{IP3}\textsubscript{3} signaling loci for genetic interactions with \textit{wy}. The following alleles were obtained: (1) \textit{IP3K1} \textsubscript{1G02192}, a \textit{P}-insertion within an intron of \textit{IP3K1} (BL 14263: \textit{y} P[\textit{SUPor-P}\textit{IP3K1} \textsubscript{1G02192}/\textit{CyO}; \textit{ry} \textsubscript{106}; Bellen et al. 2004); (2) \textit{Df} [\textit{ipk2} \textit{fl}], a deletion spanning multiple genes including \textit{Ipk2} (BL 9190: \textit{w}\textsuperscript{1118}; Bellen et al. 2007);...
(3) Camn339, a deletion of Cam resulting from an imprecise P-element excision (BL 6806: y1 w; Camn339/CyO, y+; Heiman et al. 1996), (4) Cam7, an EMS-induced point mutation (V91G) in the N-terminal helix region of the gene (BL 8140: y1 w; J1 Cam7/CyO, y+; Nelson et al. 1997); (5) IP3R90B.0, a deletion of the IP3R gene generated by an imprecise excision of a P-element (BL 30737: IP3R90B.0/TM6B Tb1; Venkatesh and Hasan 1997); and (6) IP3Rug3, an EMS-induced point mutation (S224F) in the IP3-binding domain (BL 30738: IP3Rug3/TM6B Tb1; Joshi et al. 2004).

Fly culturing, and its modification for GAL4- and GAL80ts-based experiments

Flies were reared on a modified yeast/dextrose/cornmeal diet that is described in 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 GAL80ts experiments. nub-GAL4 Tub-GAL80ts females were mated to RNAi-IP3K2 males, and vials containing the progeny from these crosses were incubated at either 18°C to minimize expression of RNAi-IP3K2, or 29°C to express RNAi-IP3K2 at high levels (Ferris et al. 2006; Baena-Lopez et al. 2009; Rodriguez et al. 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 section and in the Figure 4 caption.

Data availability

The sequence assemblies for the IP3K2 loci of y1 w1, wy2, and wy74i flies are deposited in GenBank under accession numbers KT732028, KT732029, and KT732027 respectively. The w wy2 f and w; UAS-IP3K2 fly stocks and UAS-IP3K2 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 wy74i—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 is readily distinguishable from nonspecific, mechanical wing damage, or from wings that fail to inflate at 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 ever observed in any pairwise cross of *wy* alleles; and a score of "4" denoted a chromosome with no construct insertion. Qualitatively similar results were obtained with female progeny from these same crosses.

**Table 1** Wing scores of *wy* mutant strains (A), complementation analysis (B), rescue (C), and RNAi (D)

| Genotype            | n   | % With Each Wing Score | Average Wing Score |
|---------------------|-----|------------------------|--------------------|
| **A. Mutant strains** |     |                        |                    |
| *wy*                | 31  | 0.0                    | 83.9               |
| *wy*                | 32  | 0.0                    | 12.5               |
| *wy*                | 33  | 0.0                    | 36.4               |
| *wy*                | 25  | 0.0                    | 90.0               |
| *wy*                | 20  | 0.0                    | 100.0              |
| **B. Complementation analysis** |     |                        |                    |
| *wy*/*wy*           | 30  | 0.0                    | 33.3               |
| *wy*/*wy*           | 32  | 0.0                    | 12.5               |
| *wy*/*wy*           | 33  | 0.0                    | 36.4               |
| *wy*/*Dc            | 25  | 0.0                    | 90.0               |
| *wy*/*Df            | 25  | 0.0                    | 90.0               |
| *wy*/*Df            | 20  | 0.0                    | 100.0              |
| **C. Rescue**       |     |                        |                    |
| *wy*/*UAS-IP3K2/+   | 28  | 100.0                  | 0.0                |
| *wy*/*UAS-IP3K2/+   | 17  | 100.0                  | 0.0                |
| *wy*/*UAS-IP3K2/+   | 39  | 0.0                    | 100.0              |
| **D. RNAi**         |     |                        |                    |
| *nub-GAL4/+         | 47  | 100.0                  | 0.0                |
| *nub-GAL4/+         | 46  | 100.0                  | 0.0                |
| *nub-GAL4/+         | 26  | 0.0                    | 88.5               |

* See first section of Results and Figure 2, B–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* 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 F1 Dcr-2, *nub-GAL4* males) were scored and compared. As with footnote 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 < 10⁻⁵, or in the case of *wy*/*wy*, 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., *wy*/*wy* compared to *wy*).
* P < 10⁻¹⁰, 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., *wy*/*Df* or *wy*; UAS-IP3K2/+ compared to *wy*).
* 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.

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 is readily distinguishable from nonspecific, mechanical wing damage, or from wings that fail to inflate at 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, *wy*/*wy* had the most severe phenotype, followed by *wy*/*wy* and *wy*/*wy* (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 (Nachtsheim 1928; Parker 1935; Lindsay and Zimm 1992). Therefore, we crossed all three *wy* mutant strains with 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 *wy* phenotype became significantly more severe when in a heteroallelic combination with *wy*/*wy* or *wy*/*wy*, and the *wy* phenotype became significantly more severe over *wy*/*wy*. These experiments also provided further validation for our numerical scale, since the hierarchical nature of the phenotypes shown in Figure 2, B–E was still seen, even in these mixed genetic backgrounds.

We did not observe the lengthened abdomens that were previously reported of *wy* mutants in any of our *wy* mutant strains (Nachtsheim 1928).

**Mapping the *wy* locus**

Standard three-point cross mapping using *forked* (*f*) and various *wy*-carrying transposable element insertions within the *wy* region as reference points placed *wy* between PBac(WH)_CG12090_{65876} (13,159,870) and P[EP]Tango2_{657} (13,617,116). This was followed by finer resolution mapping using complementation assays between *wy* alleles, and a series of defined deletions and duplications (Parks et al. 2004; Venken et al. 2010; Cook et al. 2012). The deletions Df(1)BSC766 and...
Df(1)Exel6245 failed to complement wy^y, wy^z, and wy^24, and the duplications Dp(1;3)DC267 and Dp(1;3)DC268 fully complemented these same wy alleles. The overlapping region between these deletions and duplications implicates IP3K2 (IP3-3-kinase 2) as the wavy gene (Figure 3A).

Identifying noncomplementing deletions also provided an opportunity to genetically characterize the available mutant alleles. Over the noncomplementing deletion Df(1)Exel6245, wy^y hemizygotes exhibit a more severe phenotype than homozygotes (Table 1B). Sequencing the majority of the region shown in Figure 3B. It is therefore possible that the wy^z mutation is in an upstream exon, or expression regulatory region.

Flies mutant for wy^y or wy^z were fully rescued, and wy^24 flies were significantly rescued by a single copy of a UAS-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 wavy gene as IP3K2, and to determine if the gene acts within the developing wing itself, we crossed flies carrying the RNAi-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-GAL80^ts 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 legend 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 GAL4, and because GAL4 is generally less active at this lower temperature (Duffy 2002; Ferris et al. 2009; Nachtsheim et al. 2004). In our genetic modifier screen, IP3K1KG02192, Df-iptk2, Cam11339, Cam7, IP3R080.0, and IP3R085 alleles were all homozygous lethal mutations, and had no discernible effect on wing structure in the heterozygous condition. IP3R084/IP3R085 mutants had been reported to be a viable heteroallelic combination that exhibited mild crumbling at the margins of their wings, but we were unable to obtain the IP3R084 allele to reproduce these results (Banerjee et al. 2004). In our genetic modifier screen, IP3K1KG02192, Df-iptk2, Cam11339, and Cam7 did not dominantly modify wy2 wing scores (Table S3), but IP3R080.0 and IP3R085 did (Table 2), strongly suppressing the wy2 phenotype relative to controls (for all modifier tests, controls were wy+/+ siblings from the same cross–cross schemes described in the footnotes of Table S3 and Table 2). Further tests showed that the phenotypes of wy3; and especially wy4 flies, were also dominantly suppressed by both IP3R alleles—in fact, the wings of virtually all wy3; IP3R084/+ and wy4; IP3R084/+ flies were phenotypically wild type (Table 2). In summary, all three mutant alleles of wy were dominantly suppressed by both mutant alleles of IP3R.

**DISCUSSION**

**wavy maps to IP3 3-kinase 2**

In this study, we present strong evidence that mutations in *wy* (wy), the first of which was described nearly 90 years ago (Nachtsheim 1928), are alleles of *IP3K2 (IP3 3-kinase 2)*. The three-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 wy2 flies revealed a 5-bp deletion in its open reading frame, putatively causing a frameshift mutation (Figure 3B; GenBank accession number KT732029), and,

| Table 2 Testing for dominant modification of the wy phenotype by loss of function in the IP3 receptor gene IP3R. |
|---|
| Genotype | n | % With Each Wing Score | Average Wing Score |
| wy2; IP3R080/+/+ | 25 | 28.0 72.0 | 0.0 0.0 | 0.0 0.0 |
| wy2; +/+ | 25 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 |
| wy2; IP3R080/0/+ | 25 | 8.0 40.0 | 52.0 0.0 | 0.0 0.0 |
| wy2; +/+ | 25 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 |
| wy1; IP3R080/+/+ | 25 | 100.0 0.0 | 0.0 0.0 | 0.0 0.0 |
| wy1; +/+ | 15 | 33.3 60.0 | 6.7 0.0 | 0.0 0.0 |
| wy1; IP3R080/0/+ | 25 | 96.0 4.0 | 0.0 0.0 | 0.0 0.0 |
| wy1; +/+ | 25 | 0.0 0.0 | 0.0 0.0 | 0.0 0.0 |
| wy2; IP3R084/+/+ | 25 | 0.0 0.0 | 100.0 0.0 | 0.0 0.0 |
| wy2; +/+ | 17 | 0.0 0.0 | 0.0 0.0 | 100.0 0.0 |

The wings of wyY; (IP3R mutant allele)/+, F1 males (experimental group, upper row within each pair) were compared to those of their wyY; +/TM6B Tb5; IP3R F1 male siblings (control group, lower row within each pair). Note that in the table, TM6B Tb5; IP3R 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.

*See first section of Results and Figure 2, B–E for a detailed description of the scoring system.
Each pair of rows compares F1 male siblings from the following cross: (wy/wy; +/+ females) X (wyY/y; IP3R mutant allele)/+; TM6B Tb5; IP3R +/+ males).

* P < 10^-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.**

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 wy2 allele was used for the primary screen because of its intermediate phenotype, and therefore presumed versatility in detecting both genetic enhancers and suppressors. *IP3K1*, *Ipk2*, *Cam*, and *IP3R* were selected as candidate interactors because the proteins encoded by these loci have strong biochemical associations with IP3K2: *IP3K1* and *Ipk2* 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 + background, the *IP3K1KG02192*, Df-iptk2, Cam11339, Cam7, IP3R080.0, and IP3R085 alleles were all homozygous lethal mutations, and had no discernible effect on wing structure in the heterozygous condition. IP3R084/IP3R085 mutants had been reported to be a viable heteroallelic combination that exhibited mild crumbling at the margins of their wings, but we were unable to obtain the IP3R084 allele to reproduce these results (Banerjee et al. 2004). In our genetic modifier screen, *IP3K1KG02192*, Df-iptk2, Cam11339, and Cam7 did not dominantly modify wy2 wing scores (Table S3), but IP3R080.0 and IP3R085 did (Table 2), strongly suppressing the wy2 phenotype relative to controls (for all modifier tests, controls were wy+/+ siblings from the same cross–cross schemes described in the footnotes of Table S3 and Table 2). Further tests showed that the phenotypes of wy3; and especially wy4 flies, were also dominantly suppressed by both IP3R alleles—in fact, the wings of virtually all wy3; IP3R084/+ and wy4; IP3R084/+ flies were phenotypically wild type (Table 2). In summary, all three mutant alleles of wy were dominantly suppressed by both mutant alleles of IP3R.
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 2, B–E and the Results section. 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 Table S2.

although mutant sequences have not yet been identified for the other two alleles, wy2 fails to complement wy1 and wy2wi (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/Df flies have a more severe phenotype than wy/wy flies, suggesting that wy is a hypomorphic allele. On the other hand, wy1 and wy2wi become less severe in the hemizygous condition, yet both are fully recessive (Table 1B). Hence, wy1 and wy2wi do not neatly fall into any classic mutant category (Muller 1932; Wilkie 1994). However, the UAS-IP3K2 construct fully rescues wy1 and significantly alleviates the wy2wi phenotype, and RNAi-IP3K2 expression in the wing disc causes a phenotype that resembles those of both wy1 and wy2wi (Table 1, C and D). Finally, both alleles are fully complemented by the duplications shown in Figure 3A. Taken together, these data suggest that wy1 and wy2wi 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 functionality of IP3K2 in the salivary glands. Analysis of how this null allele affects wing development would provide further insight into the nature of the wy1 and wy2wi alleles and, of course, be necessary to understand the consequences of completely removing gene function.

The frameshift mutation we found in wy lies well downstream of the regions encoding a calmodulin-binding domain and the active site (Figure 3B). Therefore, wy may have some IP3K2 activity in spite of its strong phenotype. Consistent with this hypothesis, wy is fully rescued by UAS-IP3K2 without any GAL4 driver, while UAS-IP3K2 only partially rescues the more severe wy2wi allele (Table 1C). Even if wy had some residual function, the protein encoded by wy 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 wy 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 wy2wi 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-GAL80Δ, and temperature shifts during specific developmental windows revealed a requirement for IP3K2 function in the wing disc during pupal stages P1–P3 (Figure 4 and Table S2). This developmental window may provide clues as to the cellular process that IP3K2 is involved in. As described in the Introduction and in Figure 1, IP3 signaling can regulate IP3R-mediated Ca2+ 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 Ca2+-ATPase, an enzyme responsible for initially sequestering Ca2+ in the ER before release occurs. Data suggest that these calcium waves are involved in wing eyespot development (Narciso et al. 2015). Calcium waves have also been induced in Drosophila larval wing discs in response to laser-induced wounding (Narciso et al. 2015).

The Drosophila Cam protein binds and regulates the activity of IP3K2 in a Ca2+-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 IP3 signaling. Relevant here, however, is that the wing vein positions are regulated during the P1–P3 stages, the stages at which we found IP3K2 function is required in the wing (Blair 2007; Figure 4 and Table S2). 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 (Cherbas et al. 2011). Therefore Cam, a Ca2+-dependent regulator of IP3K2 activity, may be active in the wing disc during the same developmental window in which 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 (IP3) 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 IP3 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. To resolve this ambiguity, future experiments should characterize expression of, and more extensively test interactions between, these IP3 signaling pathway genes, focusing on the early pupal wing disc, and using stronger losses of gene function 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 proliferate during early pupal life, while cells of the prospective wing blade are mitotically quiescent until shortly after P3 (Mílan 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 2 A and C–E). IP3 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; Berridge 2009; Leanza et al. 2013; Nohara et al. 2015). Therefore, it is conceivable that mutations in wy disrupt cell cycle regulation in the pupal wing.

Another possible function for IP3K2 in the wing comes from reports of its function in another Drosophila tissue. The micro-RNA mir-14 induces autophagy of the salivary glands during early pupal life by targeting IP3K2 (Nelson et al. 2014). The consequent downregulation of IP3K2 is thought to increase the amount of IP3 available to IP3R, IP3R is activated as a result, and autophagy is induced, at least in part by Ca2+ release from the ER. This same study suggested that Atg6, an autophagy-inducing gene that encodes a component of the Vps34 phosphatidylinositol 3-kinase (PI3K) complex III, acts in the same pathway as mir-14. In another study, Atg6 was shown to be required for autophagy in the pupal wing of Drosophila (Lórinicz et al. 2014). These findings suggest the intriguing possibility that IP3K2 and IP3R regulate autophagy in the developing wing, perhaps by interactions with the Atg6/mir-14 module.

Modeling the interactions between wavy and IP3R

In past studies, mild wing crumpling in IP3R+/-IP3R+/- mutants hinted at the involvement of the IP3 signaling network in wing development, but further analysis was presumably hindered because other IP3R allele combinations were either lethal or had normal wing morphology (Banerjee et al. 2004). wy provides an alternative entry point to IP3R, and has useful qualities for investigating how IP3 signaling affects wing morphology: (1) flies with strong loss of IP3K2 function have good viability; and (2) the 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 2, B–E and Table 1). This makes the wy phenotype an efficient, precise indicator of the levels of IP3K2 gene and overall 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 may provide clues into this aspect of IP3K2 function 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 assayed 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). If the wy phenotype is caused solely 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 IP4 substrate. A mutant copy of IP3R would be expected to make more IP4 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, IP4 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 IP4 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.
predominant in the pupal wing tissue as well (which would require direct confirmation in future studies), then loss of wv function would be expected to expand the IP$_3$ pool, and decrease levels of IP$_4$. If this were the case, the wv phenotype may be due to insufficient levels of IP$_4$ (Figure 5B), and/or an excess of IP$_3$ that hyperactivates IP$_3$R (Figure 5C). In both cases, a partial loss of IP$_3$R function could potentially alleviate the wv 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 wv and IP$_3$R (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 IP$_3$).

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 IP$_3$ signaling. Future experiments should investigate possible cellular mechanisms underlying the wv phenotype (e.g., the potential roles of Ca$^{2+}$ release from the ER and possible effects of wv 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.

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