Signaling by the Engulfment Receptor Draper: A Screen in *Drosophila melanogaster* Implicates Cytoskeletal Regulators, Jun N-Terminal Kinase, and Yorkie

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ABSTRACT Draper, the *Drosophila melanogaster* homolog of the Ced-1 protein of *Caenorhabditis elegans*, is a cell-surface receptor required for the recognition and engulfment of apoptotic cells, glial clearance of axon fragments and dendritic pruning, and salivary gland autophagy. To further elucidate mechanisms of Draper signaling, we screened chromosomal deficiencies to identify loci that dominantly modify the phenotype of overexpression of Draper isoform II (suppressed differentiation of the posterior crossvein in the wing). We found evidence for 43 genetic modifiers of Draper II. Twenty-four of the 37 suppressor loci and 3 of the 6 enhancer loci were identified. An additional 5 suppressors and 2 enhancers were identified among mutations in functionally related genes. These studies reveal positive contributions to Drpr signaling for the Jun N-terminal Kinase pathway, supported by genetic interactions with *hemipterous*, *basket*, *jun*, and *puckered*, and for cytoskeleton regulation as indicated by genetic interactions with *rac1*, *rac2*, *RhoA*, *myoblast city*, *Wiskcott–Aldrich syndrome protein*, and the formin CG32138, and for *yorkie* and *expanded*. These findings indicate that Jun N-terminal Kinase activation and cytoskeletal remodeling collaborate in Draper signaling. Relationships between Draper signaling and Decapentaplegic signaling, insulin signaling, Salvador/Warts/Hippo signaling, apical-basal cell polarity, and cellular responses to mechanical forces are also discussed.

IN *Drosophila*, the transmembrane protein Draper has been shown to be required for a number of processes that involve the recognition and clearance of cellular debris. For example, Draper plays roles in the elimination of apoptotic cells by hemocytes and macrophage (Manaka *et al.* 2004), and is required for glial clearance of apoptotic neurons in the developing nervous system of *Drosophila* embryos (Freeman *et al.* 2003). Draper has also been shown to play a role in the engulfment of apoptotic larval axons by glia, termed axon pruning, during morphogenesis (Awasaki *et al.* 2006). In response to injury, severed axons are removed in a Draper dependent manner in a process termed Wallerian degeneration (MacDonald *et al.* 2006). Furthermore, *draper* (*drpr*) mutant flies display defects in the phagocytosis of bacteria (Cuttell *et al.* 2008) and Draper mediated engulfment has been linked to the process of cell competition (Li and Baker 2007), although the latter is controversial (Lolo *et al.* 2012). In a recent study Draper was shown to activate autophagy during cell death in *Drosophila* salivary glands (McPhee and Baehrecke 2010).

Genetics of engulfment of cell corpses following programmed cell death was first characterized in *Caenorhabditis elegans*, where two *ced* (cell death abnormality) pathways were identified. The *drpr* homolog *ced-1* is part of the Ced-1, 6, 7 pathway and encodes a receptor that recognizes and engulfs dying cells (Reddien and Horvitz 2004). *ced-6* encodes an adapter protein for Ced-1 signaling; *ced-7* encodes a putative transporter protein that appears to play a role in both the dying and the engulfing cells (Reddien and Horvitz 2004). The second, Ced-2, 5, 10, 12 pathway was initially thought to act in parallel to mediate the cytoskeletal rearrangement required for engulfment. More recently, evidence that the Ced-1, 6, 7 pathway also feeds into Ced-10/Rac to some extent has appeared (Kinchen *et al.* 2005; Cabello *et al.* 2010). Ced-2, 5,

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10 constitute an adapter complex thought to act downstream of integrins (Hsu and Wu 2010). The *Drosophila* homologs of *ced-2*, *5*, and *12* are *Crk*, *mbc* (or *DOCK180*), and *ELMO*, respectively. The *ced-10* homolog is *Rac1*. These pathways are also conserved in vertebrates (Kinchen 2010).

Many questions concerning signaling downstream of Draper remain. The adapter protein Ced-6 interacts via Draper's intracellular NPXY motif and the N-terminal phosphotyrosine binding (PTB) domain of Ced-6 (Su *et al.* 2002; Awasaki *et al.* 2006). Another protein that has been shown to mediate Draper signaling is Shark, a nonreceptor tyrosine kinase belonging to the *Syk* family. Shark is required for Draper function during the process of Wallerian degeneration in which axonal debris is phagocytosed by glia following injury. The interaction between Shark and Draper is mediated by an immunoreceptor tyrosine-based activation motif (ITAM) contained within the intracellular domain of Draper proteins (Ziegenfuss *et al.* 2008). How Shark and Ced-6 function to transduce Draper activation into the cellular process of engulfment remains incompletely known, although there appears to be a role for calcium signaling (Cuttell *et al.* 2008; Fullard *et al.* 2009). Three alternative isoforms of *Draper* (DrprI, DrprII, and DrprIII) have been reported (Freeman *et al.* 2003). The extracellular domain of DrprI contains 15 atypical Epidermal Growth Factor (EGF) repeats, a transmembrane domain, and an intracellular domain. The extracellular domains of DrprII and DrprIII are shorter and contain only 5 EGF motifs. The intracellular domain of DrprII contains an additional 11 amino acids (aa) compared to DrprI whereas the intracellular domain of DrprIII is truncated by a deletion of 30 aa from the C terminus. Despite these differences, the intracellular domain of each of the Draper isoforms contains a conserved NPXY motif that interacts with Ced-6. The DrprI ITAM domain that interacts with Shark is replaced by other ITAM-like sequences in DrprII, but is absent from DrprIII. The specific roles of these isoforms have not been distinguished in most aspects of Drpr function, but in the case of the glial response to axonal injury, Logan *et al.* (2012) have recently found that DrprI promotes engulfment of axonal debris through its ITAM domain, whereas DrprII inhibits the engulfment function of glia through a DrprII-specific immunoreceptor tyrosine based inhibitory motif (ITIM). They hypothesized that DrprII negatively regulates DrprI signaling to terminate reactive glial responses, allowing glia to return to a resting state. In recent years, two ligands for Draper have been proposed, namely the ER protein Pretaporter (Kuraishi *et al.* 2009) and the membrane phospholipid phosphatidylserine (Tung *et al.* 2013).

The mechanisms of engulfment that depend on Draper and its homologs are important for development, neuronal remodeling, immunity, nutritional responses, vertebrate vision, and implicated in multiple diseases (Wu *et al.* 2006; Coleman and Freeman 2010; Elliott and Ravichandran 2010). Here, we describe the results from a modifier screen that utilized a gain-of-function phenotype produced by overexpression of DrprII to identify novel components of the Draper pathway in *Drosophila*.

Materials and Methods

Fly crosses were maintained at 25°.

Modifier screening with deficiencies and internal controls

The screen was performed by mating UAS–drprII; en–Gal4 UAS–GFP/CyO virgins to males from the Dros Del and Exelixis deficiency collections (Parks *et al.* 2004; Ryder *et al.* 2004; Ryder *et al.* 2007). Each genotype was assessed at least three times independently. Typically, suppressors were identified in females, enhancers in males; deficiencies that were synthetically lethal with en > DrprII were classified as enhancers (see *Results*). Since the penetrance of the crossveinless phenotype decreased when vials became overcrowded, no more than three males and three females were crossed and transferred at intervals of 1–3 days. As an internal control, the effect of each deficiency was compared to its balancer siblings within the same vial. Since the TM3 chromosome used to balance certain deficiencies in the Exelixis collection was itself found to suppress the phenotype, results from these deficiencies were disregarded.

Secondary screening with single gene mutants

Mutant and transposon insertion lines were used to assess interactions with individual genes contained within the loci identified by deficiencies. These strains were derived from multiple sources and, as such, differed in genetic background. The specificity of genetic interactions observed with insertions of *p{EPgy2}*-elements (EY elements) (Bellen *et al.* 2004) into the *rac1*, *CG32138*, psr, and *wasp* genes was supported by the lack of interaction shown by EY insertions in seven other loci. The specificity of genetic interactions observed with *Mi{ET1}* insertions (MB insertions) (Bellen *et al.* 2011) into the *fer2* and *crb* genes was supported by the lack of interaction shown by MB insertions in seven other loci. The specificity of genetic interactions observed with *Mi{MIC}* insertions (MI insertions) (Venken *et al.* 2011) into the *plx* and *osm-1* genes was supported by the lack of interaction shown by MI insertions in four other loci. Since the ex^{NY1} mutation was induced in our laboratory (Tyler *et al.* 2007), we were able to confirm that its genetic background did not modify the DrprII overexpression phenotype

Wing mounting and photography

Adult wings were mounted in DPX mountant from Fluka and photographed using a Zeiss Axioplan inverted microscope equipped with a Nikon Digital Sight DsRi1 camera.

Immunohistochemistry

Antibody labeling was performed as described Firth *et al.* (2006). Images were recorded using a Leica SP2 confocal microscope and processed with ImageJ and Adobe Photoshop. Primary antibodies: mouse anti-β-galactosidase was mAb40-1a from the Developmental Studies Hybridoma Bank, mouse anti-phospho-JNK and rabbit anti-cleaved caspase 3 (Cell

Signaling Technologies), and rat anti-GFP (Nacalai Tesque Inc). Secondary antibodies were multilabeling antibodies from Jackson ImmunoResearch Laboratories.

Genetic strains

bsk^{2} (Sluss *et al.* 1996)
pten^{C076}, *CG32138*^{EY03931}, *wasp*^{EY06238}, *rac1*^{EY05848}, *sec23*^{EY06757}, *aPKC*^{EY22946}, *psr*^{EY07193}, *ced-6*^{KG04702}, *mbc*^{EY01437}, *CG16791*^{DG25603} (Bellen *et al.* 2004)
tara^{1} (Fauvarque *et al.* 2001)
14-3-3-ε^{EP3578} (Rorth 1996)
mad$^{8-2}$ (Wiersdorff *et al.* 1996)
l(2)gl^{4} (Mechler *et al.* 1985)
l(3)76bdr^{1} (Zhu *et al.* 2005)
osm-1^{1MI03576}, *plx*^{MI02460} (Venken *et al.* 2011)
how^{24B} (Fyrberg *et al.* 1997)
fer2^{MB09480}, *crb*^{MB08251}, *med*^{MB08684} (Bellen *et al.* 2011),
vps28^{k16503}, *aPKC*^{k06403}, *akt1*^{04226} (Spradling *et al.* 1999),
E(Pc)1^{bw1} (Moazed and O'farrell 1992)
ex^{NY1}, *fat*^{NY1} (Tyler *et al.* 2007)
ex^{e1} (Boedigheimer and Laughon 1993)
drpr $^{\Delta 5}$ (Freeman *et al.* 2003)
Rho1$^{E3.10}$ (Halsell *et al.* 2000)
yki $^{\Delta 5}$ (Huang *et al.* 2005)
elmo^{KO} (Bianco *et al.* 2007)
lid^{10424} (Gildea *et al.* 2000)
jun^{2} (Hou *et al.* 1997)
rac $^{2\Delta}$ (Ng *et al.* 2002)
cul-3^{gft} (Ou *et al.* 2002)
puc^{H246} (Salzberg *et al.* 1994)
pucLacz^{E69} (Ring and Martinez Arias 1993)
hep^{r75} (Glise *et al.* 1995)
put^{135} (Ruberte *et al.* 1995)
shn^{1B} (Arora *et al.* 1995)
tkv^{a12} (Szidonya and Reuter 1988)
crq^{KG01679} (Bellen *et al.* 2004)
kay ^{VK00037} (Spokony and White, personal communication, to Flybase 2012.5.22)
shark^{2} (Tran and Berg 2003)
mnt^{1} (Loo *et al.* 2005)
max^{1} (Steiger *et al.* 2008)
UAS–DrprII.FLG (gift of R. Biswas and E. R. Stanley)
UAS–DrprI.HA and UAS–DrprIII.HA (Logan *et al.* 2012)
UAS–DraperRNAi (MacDonald *et al.* 2006)
UAS–exRNAi^{III} (Dietzl *et al.* 2007)

Results

Characterization of the DraperII overexpression phenotype

Overexpression of UAS–Draper II in posterior compartments under the control of en–Gal4 resulted in an absence of posterior crossvein (pcv) in adult wings (R. Biswas and E. R. Stanley, personal communication) (Figure 1, A and B). This phenotype was highly penetrant in female flies, with 83% of wings displaying defective posterior crossveins. The phenotype was significantly less penetrant in males (23% of wings affected) (Figure 1, B and G). This phenotype was suppressed by coexpression of a Draper-RNAi construct (Figure 1D). It was also suppressed by a single copy of the *drpr*$^{\Delta 5}$ null allele (Figure 1C), although in this case we lack any control that distinguishes whether the *drpr* mutation or genetic background is responsible for the interaction. Overexpression of other Drpr isoforms was without effect, in our hands.

To test whether the en > DrprII phenotype was sensitive to known components of Drpr signaling, dominant effects of mutant and *P*-element insertion lines were evaluated. Consistent with the notion that this phenotype did depend on physiological mediators of *drpr* signaling, a mutant allele of *ced-6* (*ced-6*^{KG04702}) dominantly suppressed the en > DrprII phenotype (Figure 1E) with 25% of wings showing defective crossveins compared to 83% in controls (Figure 1G). The other canonical member of the Ced-1 pathway in *Caenorhabditis elegans*, *ced-7*, lacks any clear ortholog in *Drosophila* and so could not be tested. More recently, the cytoplasmic tyrosine kinase Shark has been established as a transducer of Draper signaling in *Drosophila* (Ziegenfuss *et al.* 2008). The mutant allele *shark*^{2} dominantly suppressed the en > DrprII phenotype (Figure 1F) with 40% of wings showing defective crossveins compared to 83% in controls (Figure 1G). Since the effect of en > DrprII on the posterior crossvein depended on the dose of these genes known to act positively in the Drpr pathway and that encode proteins that interact physically with Drpr, the en > DrprII phenotype could provide a sensitized assay for dependence of Drpr function on other genes.

A deficiency screen for dominant modifiers of the en > DrprII phenotype

We screened through 414 chromosomal deficiency stocks from the Dros Del and Exelixis collections to identify genomic regions that exerted a dominant effect on the en > DrprII crossveinless phenotype. Together, the DrosDel and Exelixis deficiency collections provide 78% coverage of *Drosophila* euchromatin (Cook *et al.* 2012), of which most of the autosomal deficiencies were used here, reflecting ~60% coverage. In addition to identifying loci that modify the en > DrprII phenotype, our screen also identified deficiencies that were dominantly lethal in combination with en > DrprII.

This first round of screening identified a total of 59 modifier deficiencies. To confirm these interactions, and to refine the genomic regions containing the putative *drprII* interacting loci, we tested 160 additional deficiencies that overlap those identified in the primary screen, identifying a further 37 modifier deficiencies. Together, these 96 deficiencies and their overlaps defined 43 discreet genomic regions (Table 1).

Suppressors of Draper function identified using genetic deficiencies

Of the 43 modifying loci identified, 37 suppressed the en > DrprII phenotype. These included the two loci already

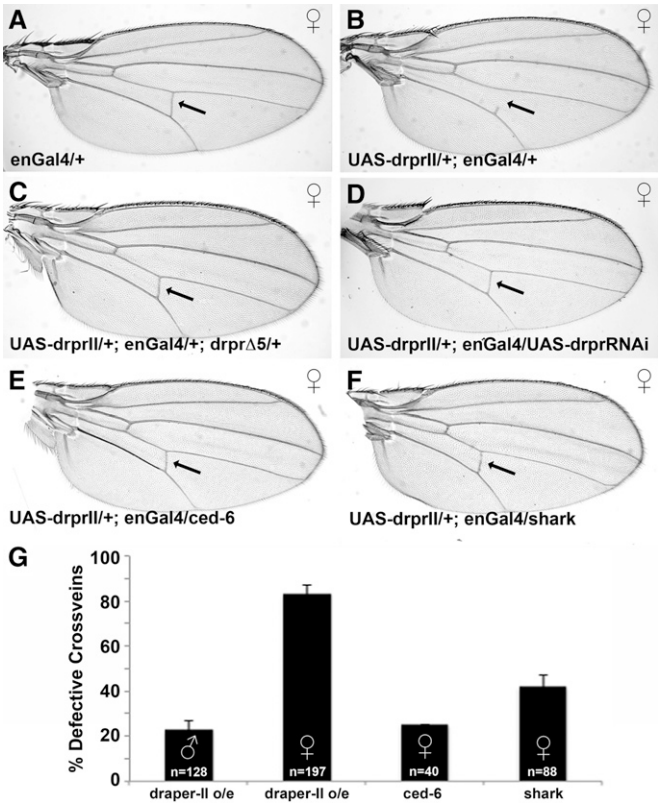

Figure 1 Overexpression of DrprII using the engrailed-Gal4 driver results in (B) a wing vein phenotype when compared to (A) controls. Removing one copy of (C) the endogenous draper gene suppresses the phenotype associated with DrprII overexpression, as does coexpression of (D) Draper–RNAi. Removing one copy of known downstream components of the Draper pathway, namely (E) *ced-6* and (F) *shark*, is also sufficient to suppress the phenotype. In all cases, female wings are shown. (G) The crossveinless phenotype due to DrprII overexpression is highly penetrant in the wings of female flies (83%) but less so in males (23%), and removal of a single copy of either ced-6 or shark reduces penetrance of the phenotype in females to 25 and 40%, respectively.

known to encode members of the Drpr pathway and for which suppression by point-mutated alleles had already been observed, namely *ced-6* and *shark*. To identify the individual gene, or genes, within the remaining 35 intervals, we tested a combination of *P*-element-insertion stocks and individual mutations in candidate genes and, from these studies, identified 22 other genes (corresponding to 20 of the intervals) that mimicked the suppression effects of their deficiencies. These en > DrprII suppressor loci were *lethal giant larvae* (*lgl*); *Mothers against Dpp* (*Mad*); *basket* (*bsk*) and *pten*, both contained within the 31B1 interval; *vps28*; *RhoA*; *Rac1*; *osm-1*; *CG32138*; *l(3)76bdr*; *sec23*; *pollux* (*plx*); *48 related 2* (*fer2*); *taranis* (*tara*); *14-3-3-ε*; *CG16791*; *held out wings* (*how*) and *phosphatidylserine receptor* (*psr*) both contained within the 94A1-94B5 interval; *myoblast city* (*mbc*); *crumbs* (*crb*), *wasp*; and *medea* (Figure 2). The suppressor loci and deficiencies that define them are listed in Table 1.

Ffifteen genomic intervals for which the suppressor locus (or loci) was not identified remained. In addition, analysis of the interval that contained *osm-1* indicated that a second suppressor, not yet identified, must reside within the interval 62B7–62B12. The intervals containing the 15 imputed but unidentified suppressors are listed in Table 1.

Enhancers of Draper function identified using genetic deficiencies

We utilized the observation that the posterior crossvein was defective in only 23% of en > DrprII males to identify enhancers. Three genomic regions that increased penetrance of the posterior crossvein defect in en > DrprII males were found (Table 1). Single loci that accounted for the enhancer activity of two of these three chromosomal regions were found (Figure 3).

Three overlapping deficiencies, Df(2L)ED385, Df(2L)ED354, and Df(2L)BSC353 behaved as enhancers. Two additional deficiencies that overlap the same region, Df(2L)ED299 and Df(2L)ED343, however, failed to modify the en > DrprII phenotype. Together, these findings pinpointed a region that contains a single gene, namely *little imaginal discs* (*lid*). Confirming this, a mutant allele (lid^{10424}) dominantly enhanced en > DrprII (Figure 3, B and B′), and coexpression of UAS–lid with UAS–DrprII suppressed the posterior crossvein phenotype (Figure 3C).

Two further deficiencies, Df(2R)ED2219 and Df(2R) ED2222, dominantly enhanced the en > DrprII phenotype in males. Testing mutants of individual candidate genes within this region identified *Enhancer of Polycomb* [*E(Pc)*] as a dominant enhancer of DrprII. The final imputed enhancer interval for which no single gene has yet been identified is included in Table 1. In addition to these enhancers of en > DrprII, a further three regions that were synthetically lethal in both males and females when combined with en > DrprII were identified. We interpret the synthetic lethality to indicate strong enhancement of en > DrprII that is not compatible with viability.

One such region contained a single gene where a mutant allele was dominant sythetic lethal with en > DrprII, *cullin 3* (*cul3*) (Table 1). The critical genes that lie within the remaining two synthetically lethal regions, 32D2–32D5 and 32D5–32E4, have yet to be identified (Table 1). As the two deficiencies that identified these loci [Df(2L) Exel6027 and Df(2L)Exel6028] abut one another precisely, it is possible that they might affect a single locus. A mutation in the single gene interrupted by both deficiency breakpoints, $CG6287^{MI06828}$, did not modify the en > DrprII phenotype, indicating either that one deficiency exerts a position effect on a gene uncovered by the other deficiency or that each deficiency uncovers a distinct modifier locus.

Modification of the en > DrprII phenotype is specific for Drpr function

Dominant modification of the en > DrprII phenotype may indicate a genetic interaction with DrprII, but could, in principle, reflect an effect on the expression of enGal4 or on the activity of the Gal4–UAS system. To differentiate these

Table 1 Modifying intervals were defined by deficiencies that modified the en > drprII crossvein phenotype and by overlapping deficiencies that did not modify the phenotype, when such deficiencies existed

Cytogenic location	Estimated sequence location	Effect	Deficiencies that modify the phenotype	Overlapping deficiencies that fail to modify the phenotype	Mapped gene(s)
21A1;21B1	2L:(-)204333;67365	Suppresses	Df(2L)ED50001	Df(2L)ED2809, Df(2L) ED5878	l(2)gl
22D4;22E1	2L:2222091;2362808	Suppresses	Df(2L)Exel7010	Df(2L)ED125, Df(2L) ED134, Df(2L) Exel7011	Not determined
23C5;23E3	2L:3056809;3302636–3302646	Suppresses	Df(2L)ED4651, Df(2L) ED4559, Df(2L) Exel7015	Df(2L)ED206	mad
25F1;25F2	2L:5594234;5658629	Suppresses	Df(2L)Exel6256	Df(2L)Exel7023, Df(2L) ED270	Not determined
26A1;26B2	2L:5980153;5981009	Suppresses	Df(2L)ED292, Df(2L) Exel6014, Df(2L) Exel7024	Df(2L)ED280	Not determined
26B2;26B2	2L:5982466;6000124	Enhances	Df(2L)ED385, Df(2L) ED354, Df(2L)BSC353	Df(2L)ED299, Df(2L) ED343	lid
31B1;31B1	2L:10220877;10276871	Suppresses	Df(2L)ED729	Df(2L)Exel7046	bsk, pten
32D2;32D5	2L:11067029;11155825	Lethal	Df(2L)Exel6027		Not determined
32D5;32E4	2L:11155825;11358603	Lethal	Df(2L)Exel6028		Not determined
35C5;35D1	2L:15264714;15332688	Lethal	Df(2L)ED800, Df(2L) ED1054, Df(2L)ED3, Df(2L)Exel8034, Df(2L) ED1050, Df(2L) ED1004, Df(2L) PZ06430-mr14	Df(2L)ED793, Df(2L) Exel6036, Df(2L) Exel8033, Df(2L) Exel7063	cul-3
36E2;36E6	2L:17903087;18151698	Suppresses	Df(2L)Exel7070	Df(2L)ED1196	Not determined
37E3;37E5	2L:19464056;19517610	Suppresses	Df(2L)ED1272	Df(2L)ED1226, Df(2L) ED1231, Df(2L) ED1303	Not determined
42A11;42A13	2R:2019519;2108037	Enhances	Df(2R)ED1552	Df(2R)ED1612	Not determined
43F8;44B3	2R:3849654;4019248	Suppresses	Df(2R)ED1725, Df(2R) ED1735, Df(2R) ED1742, Df(2R) Exel7094	Df(2R)Exel7095, Df(2R) ED1770	Vps28
44B8;44D5	2R:4061673;4543134	Suppresses	Df(2R)ED1742, Df(2R) Exel6057	Df(2R)ED1770	Not determined
45B4;45F1	2R:5095046;5440757	Suppresses	Df(2R)ED1791	Df(2R)ED1770	ced6
47F13;48A3	2R:7340485;7487611	Enhances	Df(2R)ED2219, Df(2R) ED2222	Df(2R)ED2155, Df(2R) ED2247	E(pc)
52D11;52E7	2R:11887814;12017662	Suppresses	Df(2R)ED2457		RhoA
52F6;53B1	2R:12176759;12274020	Suppresses	Df(2R)Exel6063	Df(2R)Exel7142	shark
61E2;62A2	3L:1035182;1478674	Suppresses	Df(3L)ED207, Df(3L) ED4196, Df(3L)ED202, Df(3L)ED4238	Df(3L)ED4177, Df(3L) Exel6086, Df(3L) Exel6087	rac1
62A3;62A6	3L:1546104;1586663	Suppresses	Df(3L)ED4256, Df(3L) ED4238, Df(3L)ED207		Not determined
62BD1;62D4	3L:21517444;2235407	Suppresses	Df(3L)ED4284. Df(3L) ED4287, Df(3L) Exel6089, df(3L) bsc365	Df(3L)Exel6088	osm-1
63C1;63C1	3L:3226338;3893148	Suppresses	Df(3L)ED4293	Df(3L)ED208, Df(3L) Exel6093	Not determined
70C15;70D2	3L:14030132;14070123	Suppresses	Df(3L)ED4528, Df(3L) ED4529, Df(3L) ED4534, Df(3L) ED4536	Df(3L)ED4502, Df(3L) ED4515,	CG32138
76A6;76B3	3L:19323668;19475272	Suppresses	Df(3L)ED4789, Df(3L) ED4799, Df(3L)ED228	Df(3L)Exel9046	Not determined

(continued)

Table 1, *continued*

Cytogenic location	Estimated sequence location	Effect	Deficiencies that modify the phenotype	Overlapping deficiencies that fail to modify the phenotype	Mapped gene(s)
76B5;76B8	3L:19415402;19576113	Suppresses	Df(3L)ED4789, Df(3L) ED4799, Df(3L)ED228, Df(3L)Exel9007, Df(3L) Exel9008, Df(3L) Exel9009	Df(3L)exel9011	l(3)76bdr
83B7;83B8	3R:1474504; 1480524	Suppresses	Df(3R)ED5187, Df(3R) ED5197		sec23
83B8;83D2	3R:1480524;1833866	Suppresses	Df(3R)ED5196, Df(3R) ED5197		plx
84C4;85C3	3R:2954004;4882413	Suppresses	Df(3R)ED5220, Df(3R) ED5221, Df(3R) ED5223, Df(3R) ED5230, Df(3R) ED5296		puc (enhancer) and likely undetermined suppressor(s)
87F6;88A4	3R:9470856;9809634	Suppresses	Df(3R)ED5622, Df(3R) ED5623, Df(3R) ED5642	Df(3R)ED5612, Df(3R) ED5613, Df(3R) ED5634, Df(3R) ED5644	Not determined
89B2;89B6	3R:11727155;11983178	Suppresses	Df(3R)Exel7328	Df(3R)Exel7327	fer2
89B7;89B12	3R:12038635;12131435	Suppresses	Df(3R)ED10639,	Df(3R)ED10642, Df(3R) Exel6269, Df(3R) Exel7330	taranis
89E11;90D1	3R:12882199;13769792	Suppresses	Df(3R)ED5780, Df(3R) ED5785		Not determined
90F4;91A5	3R:13993596;14223249	Suppresses	Df(3R)ED5815	Df(3R)Exel6179, Df(3R) Exel6180	14-3-3epsilon
91A5;91F4	3R:14224953;14991505	Suppresses	Df(3R)ED2, Df(3R) ED5911, df(3R)bsc473		Not determined
93D4;93E10	3R:17122221;17459227	Suppresses	Df(3R)ED6058, Df(3R) ED6052	Df(3R)ED10845, Df(3R) ED10838, Df(3R) ED6076	CG16791
94A1;94B5	3R:17868550;18413403	Suppresses	Df(3R)ED6085, Df(3R) ED6090, Df(3R) ED6093	Df(3R)ED6076, Df(3R) ED6096, Df(3R) ED6091	psr, how
95B1;95D1	3R:19598843;19768726	Suppresses	Df(3R)Exel9014		mbc
95D10;96A7	3R:19877370;20369665	Suppresses	Df(3R)ED6187		crb
97D2;98B5	3R:22624758;23731307	Suppresses	Df(3R)BSC686, Df(3R) ED6265, Df(3R) ED6237, Df(3R) ED6242, Df(3R) ED6255		Not determined
98E1;98F5	3R:24500683;24816740	Suppresses	Df(3R)Exel6210	Df(3R)Exel6209, Df(3R) Exel6211	wasp
99F2;99F7	3R:26215013;26291258–26339208	Suppresses	Df(3R)Exel6216	Df(3R)ED6332, Df(3R) Exel6215	Not determined
100C7;100E1	3R:27434853;27762273	Suppresses	Df(3R)ED6361	Df(3R)ED6362, Df(3R) ED50003	medea

Many other deficiencies that neither modified the en > drprII phenotype nor helped define flanking modifier regions are not tabulated. Other modifiers were identified later from studies of candidate mutations (see Table 2).

possibilities, identified modifier genes and intervals were tested for interaction with overexpression of a distinct gene, *scabrous* (*sca*). Ectopic expression of Sca gives rise to loss of wing margin, and these phenotypes are modified by the dose of genes in the Notch signaling pathway (Lee *et al.* 2000). Accordingly, expression of UAS–Sca using enGal4 gave rise to nicked posterior wing margins (Figure 4). We tested deficiencies, mutants, and *P*-element insertion lines corresponding to each of the loci identified in the Draper overexpression screen and found that none modified the en > Sca phenotype, indicating that modification of en > DrprII likely reflects genetic interaction with DrprII. Examples of mutations that were found to suppress or enhance the Draper II overexpression phenotype and one that was shown to be synthetic lethal with ectopic Draper II are shown (Figure 4, B–D).

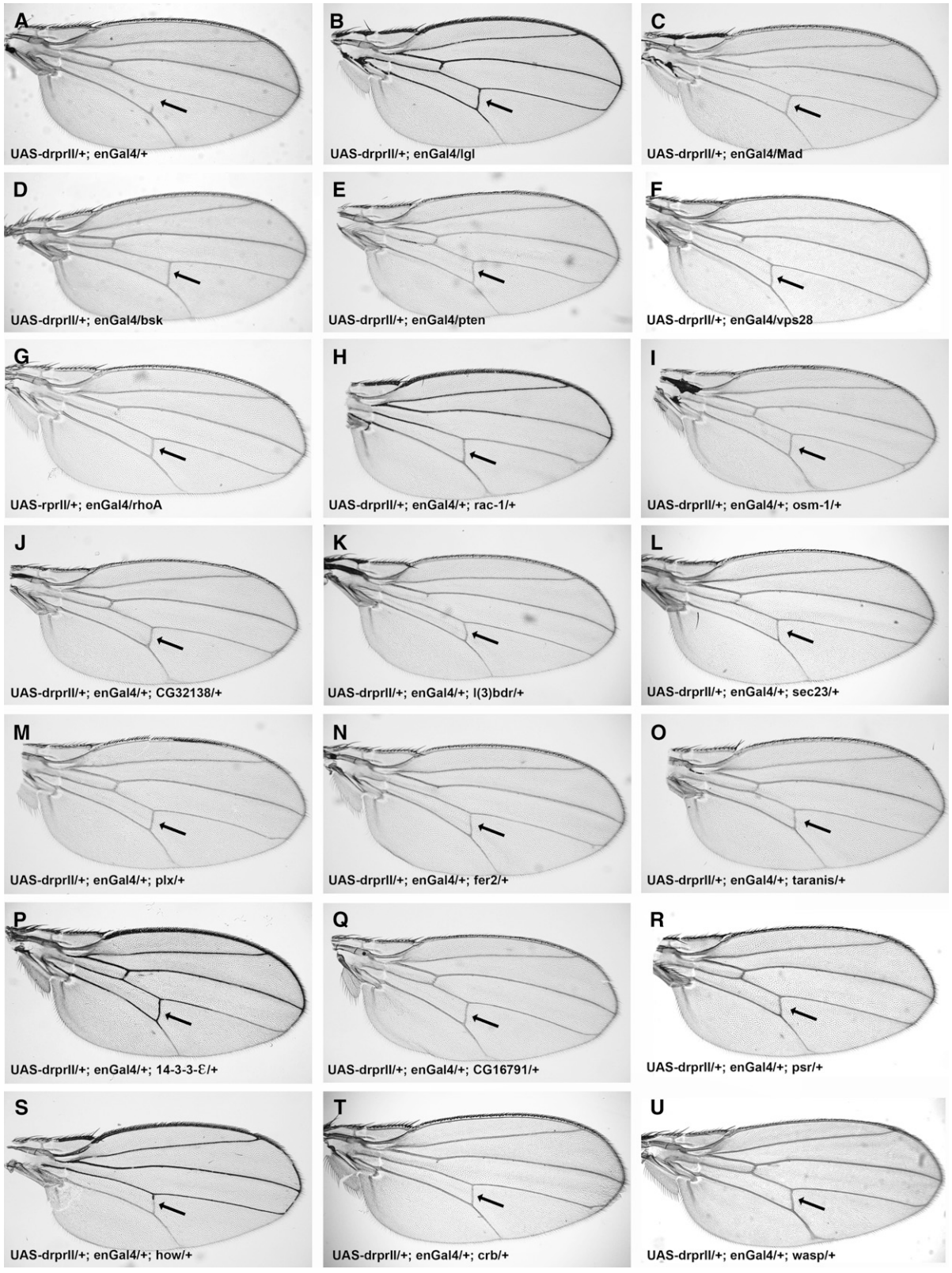

Figure 2 Dominant modification of (A) the enGal4>Draper-II over expression phenotype using mutant or P-element insertion lines of (B) *lgl*, (C) *Mad*, (D) *bsk*, (E) *pten* (F) *vps28* (G) *rhoA*, (H) *rac-1*, (I) *osm-1*, (J) *CG32138*, (K) *l(3)bdr*, (L) *sec23*, (M) *plx*, (N) *fer2*, (O) *tara*, (P) *14-3-3-ε*, (Q) *CG16791*, (R) *psr*, (S) *how*, (T) *crb* and (U) *wasp*. In all cases, wings from females are shown.

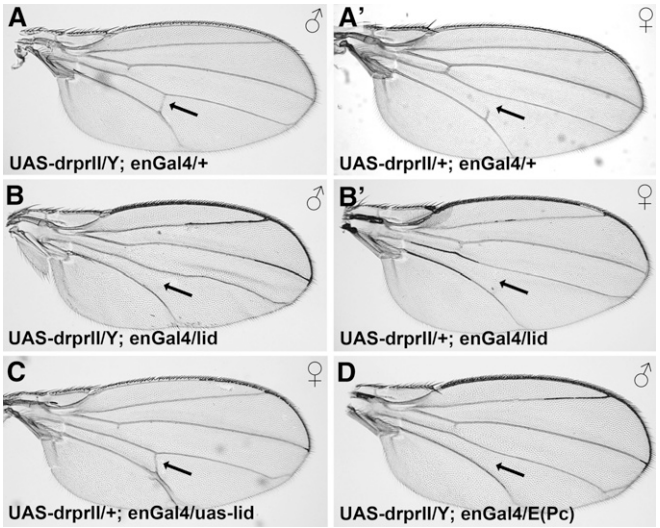

Figure 3 The pcv phenotype associated with Draper II overexpression shows (A) weaker penetrance in the wings of male flies when compared to wings of females (A′). Removal of one copy of *lid* dominantly enhanced the Draper II overexpression phenotype in both (B and B′) males and females. (C) Coexpression of Lid suppressed the en > Draper II phenotype. (D) The Draper II overexpression phenotype was also dominantly enhanced by removing a single copy of *E(pc)*.

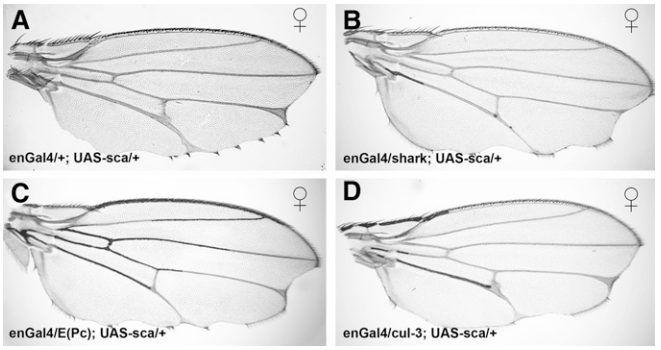

Figure 4 Ectopic expression of *scabrous* with enGal4 gives rise to a nicked wing margin phenotype (A). Deficiencies or specific genes identified in our Draper II overexpression modifier screen were also assayed for their effect on en > Sca. Results are shown for (B) *shark*^{2}, (C) *E(pc)*^{1}, and (D) *cul-3*^{gft}.

The JNK pathway modifies Draper signaling

Among the genes identified in the modifier screen was *basket* (*bsk*), encoding the *Drosophila* c-Jun N-terminal kinase (JNK). To determine the extent to which the JNK pathway might be involved in Draper function, other components of the JNK pathway were tested (Figure 5, C–F and Table 2). The en > DrprII phenotype was also dominantly suppressed by mutations of either the JnKK *hemipterous* (*hep*^{r75}) or *jun* (*jun*^{2}). None of these loci had been included among deficiencies tested in the primary screen; however, subsequent experiments identified a deficiency that uncovers the *hep* locus as suppressing the en > DrprII phenotype, Df(1)ED7170. Neither of two deficiencies that uncovered the *fos* locus modified en > DrprII, and a mutant allele (*kay*$^{T:Avic\GFP\-SF,T:Zzzz\FLAG}$) was also without effect. The *puckered* (*puc*) gene is a transcriptional target of JNK signaling and encodes a phosphatase that acts in a feedback loop to inhibit *bsk*. As would be predicted, each of two *puc* alleles (*puc*^{H246} and *puc-Lacz*^{E69}) dominantly enhanced the en > DrprII phenotype, while coexpression of UAS–puc along with UAS–DrprII suppressed the en > DrprII phenotype (Figure 5D). Surprisingly, the *puc* locus is contained in a deletion that suppressed en > DrprII in the primary screen. As this interval (84C4–85C3; Table 1), contains >300 genes, it is possible that the deficiency exhibits a compound effect due to another modifier in addition to *puc*. This remains unconfirmed at present, however, and as the two *puc* mutant alleles are in uncontrolled genetic backgrounds, identification of *puc* as a DrprII modifier is subject to this caveat.

To further assess the effect of ectopic Draper on the JNK pathway, we tested the effect of DrprII overexpression in a fly line containing a *puc* enhancer trap, *puc-LacZ*^{E69}. Overexpression of DrprII in the posterior compartment of wing imaginal discs leads to a marked elevation of puc-LacZ expression when compared to controls (Figure 6, A and B). Furthermore, DrprII overexpression leads to elevated levels of phosphorylated-JNK when compared to controls (Figure 6, C and D).

Due to the established role of JNK signaling in mediating apoptosis (Dhanasekaran and Reddy 2008) we also tested whether the Draper overexpression phenotype might be dependent upon cell death. Although DrprII overexpression leads to more cleaved caspase-3 compared to controls (Figure 6, E and F), the Df(3L)H99 chromosomal deletion that lacks three apoptosis-inducing genes, *reaper*, *head involution defective* (*hid*), and *grim* had no effect on the crossveinless phenotype (Figure 5G) (Goyal *et al.* 2000). Similarly, a deficiency that uncovers the gene encoding the *Drosophila* Inhibitor of Apoptosis Protein 1 (IAP1) [Df(2r)ED2436] had no effect (data not shown). Ectopic expression of UAS–IAP1 (Figure 5H) or UAS–p35 and UAS–DroncDN also failed to modify the phenotype (data not shown). Taken together, these data suggest that the crossveinless phenotype that arises following DrprII overexpression, although dependent on JNK activity, is not dependent on cell death.

Interactions between Draper and the DPP pathway

Our screen identified *mad*, a transcription factor that regulates gene expression in response to Dpp signaling, as a dominant suppressor of en > DrprII. A deficiency, Df(3R) ED6361, uncovering the gene encoding the Mad interacting protein Medea also suppressed the Draper overexpression phenotype. In addition, removal of *medea* using a *P*-element insertion line (*med*^{MB08684}) suppressed the phenotype; however, another allele of *med* (*med*^{1}) failed to dominantly modify Draper (Table 2).

Other components of the Dpp pathway were examined to determine to what extent this pathway might be involved in Draper function. The mutant allele *shn*^{1B} suppressed the en > DrprII phenotype (Figure 5I and Table 2). However, *shn* was uncovered by two deficiencies tested in the screen:

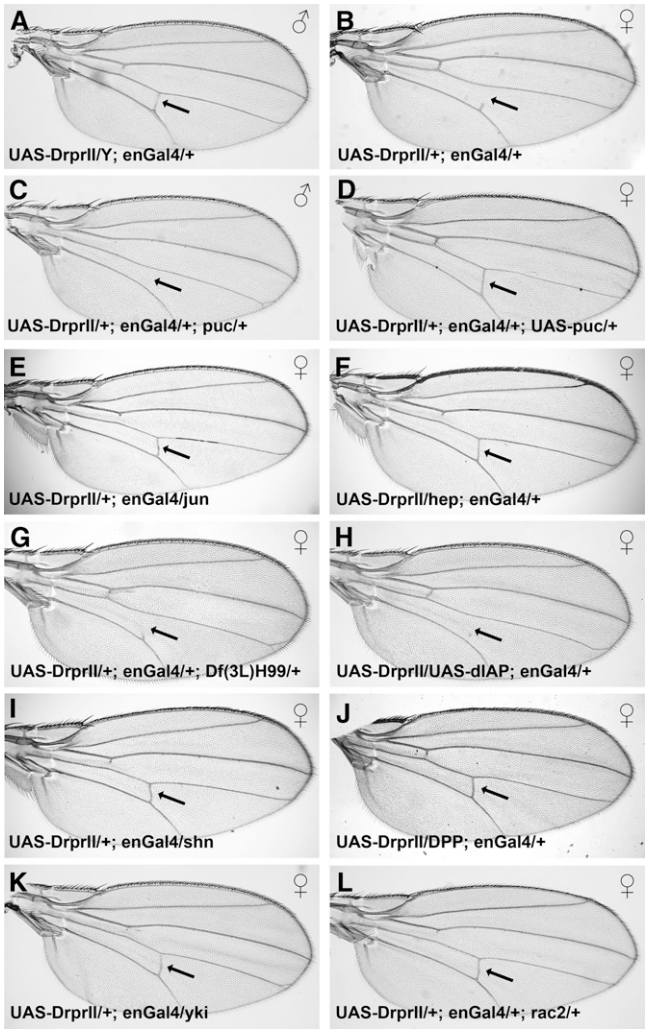

Figure 5 Testing components of putative pathways identified in our screen for modifiers of Draper function. (C) Removal of *puc* enhances the crossveinless phenotype in the wings of male flies. Conversely, coexpression of puc along with Draper II suppresses the Draper overexpression phenotype in (D) females, as does removal of (E) *jun* and (F) *hep*. (I and J) A mutant allele of the Dpp pathway component *shn* (shn^{1B}) also suppresses, as does a wing-specific allele of *Dpp* (Dpp^{dr}). (K) *yki* ($yki^{\Delta 5}$) dominantly suppresses the en > DrprII phenotype as does (L) *Rac2* ($rac^{2\Delta}$).

Df(2R)ED2155, which did not suppress en > DrprII, and Df(2R)ED2219 enhanced en > DrprII in males, because, as reported above, it uncovers *E(pc)*. A mutant allele of *dpp* (dpp^{dr}) also suppressed the en > DrprII phenotype (Figure 5J). Mutant alleles of neither the Dpp receptor proteins Tkv (tkv^{a12}) nor Punt (put^{135e}) modified en > DrprII. No deficiency uncovering *tkv* was included in the primary deficiency screen; two deficiencies uncovering *punt* [Df(3R)ED5644 and Df(3R)ED10555] each failed to modify en > DrprII.

To further assess the effect of ectopic Draper on the Dpp pathway we tested the effect of Draper II overexpression on expression of the Dpp target gene *spalt major* (*salm*) in the wing disc. No effect of DrprII overexpression was seen (data not shown). Thus, despite the recovery of *mad* and *dpp* as suppressors of en > DrprII, it was not clear whether the effects of DrprII overexpression depend on the Dpp signaling pathway as a whole.

Interactions between Draper and the insulin receptor pathway

One gene that we identified as a suppressor of the DrprII overexpression phenotype was *pten* (*phosphatase and tensin homolog*). Pten is a tumor suppressor and negative regulator of insulin signaling (Goberdhan *et al.* 1999). As such, we wondered whether loci that contain components of the insulin signaling pathway interacted with DrprII. Deficiencies that uncovered *dTor* [Df(2L ED784)], *s6k* [Df(3L)Exel6107], *rheb* [Df(3R)ED10257 and Df(3R)exel6144], and *foxo* [Df(3R)ED5634 and Df(3R)ED5644] failed to dominantly modify the phenotype. A deficiency that uncovered *akt1* [Df(3R)exel7328] suppressed, but this interval also contained the suppressor *fer2*, which may be responsible. Subsequent analysis with a *P*-element insertion line ($akt1^{04226}$) showed no interaction with en > DrprII. Similarly, a deficiency uncovering *chico* [Df(2L)729] suppressed, but this interval also contained *bsk* and *pten* itself. A deficiency including the *insulin-like receptor* (*InR*) gene [Df(3R)ED6058] suppressed en > DrprII, but this deficiency also contained the gene *CG16791* that is sufficient to explain the interaction. Taken together, the evidence did not strongly implicate insulin signaling in the crossveinless phenotype caused by DrprII overexpression.

Apical-basal polarity genes and components of the Salvador/Warts/Hippo pathway are modifiers of Draper

The deficiency screen identified the interval 21A1–21B1 (Table 1) as containing a gene, or genes, that suppress the en > DrprII phenotype. Analysis using mutant lines identified the gene responsible as *lgl*. *lgl* is a member of the apical-basal polarity genes that are responsible for regulating the polarity and proliferation of epithelial cells, along with *discs large* (*dlg*) and *scribble* (*scrib*) (Humbert *et al.* 2003). However, neither of the deficiencies used in the primary screen that uncovered *dlg* or *scrib* nor point mutations in these genes had any affect on the crossveinless phenotype of en > DrprII.

Our screen also identified *crumbs* (*crb*) as a modifier of en > DrprII. The Crumbs protein is essential for the biogenesis of the adherens junction and the establishment of apical polarity in ectodermally derived epithelial cells. In addition to suppression of en > DrprII by a deficiency [Df(3R) ED6187] and a *P*-element insertion ($crb^{[MB08251]}$), we also found that coexpression of UAS–Crb with UAS–DrprII was synthetically lethal. The genetic interactions between DrprII and both *crb* and *lgl* are potentially linked, because Grzeschik *et al.* (2010) have shown that *crumbs*, along with *lgl* and *aPKC*, can regulate the Salvador/Warts/Hippo (SWH) pathway. Specifically, depletion of Lgl leads to upregulation of targets of the SWH pathway, a result that is mimicked following overexpression of Crumbs or aPKC. The *aPKC* locus was

Table 2 List of all identified modifier genes, including the alleles and deficiencies tested

Modifier locus	Modifier allele(s)	Modifier deficiencies
		Suppressors
l(2)gl	*l(2)gl^{4}*	Df(2L)ED50001
mad	*mad$^{8-2}$*	Df(2L)ED4651, Df(2L)ED4559, Df(2L)Exel7015
bsk	*bsk^{2}*	Df(2L)ED729
pten	*pten^{C076}*	Df(2L)ED729
Vps28	*vps28^{k16503}*	Df(2R)ED1725, Df(2R)ED1735, Df(2R)ED1742, Df(2R)Exel7094
ced6	*ced-6^{KG04702}*	Df(2R)ED1791
RhoA	*Rho1$^{E3.10}$*	Df(2R)ED2457
shark	*shark^{2}*	Df(2R)Exel6063
rac1	*rac1^{EY05848}*	Df(3L)ED207, Df(3L)ED4196, Df(3L)ED202, Df(3L)ED4238
osm-1	*osm-1^{1MI03576}*	Df(3L)ED4284. Df(3L)ED4287, Df(3L)Exel6089, df(3L)bsc365
CG32138	*CG32138^{EY03931}*	Df(3L)ED4528, Df(3L)ED4529, Df(3L)ED4534, Df(3L)ED4536
l(3)76bdr	*l(3)76bdr^{1}*	Df(3L)ED4789, Df(3L)ED4799, Df(3L)ED228, Df(3L)Exel9007, Df(3L)Exel9008, Df(3L)Exel9009
sec23	*sec23^{EY06757}*	Df(3R)ED5187, Df(3R)ED5197
plx	*plx^{MI02460}*	Df(3R)ED5196, Df(3R)ED5197
fer2	*fer2^{MB09480}*	Df(3R)Exel7328
taranis	*tara^{1}*	Df(3R)ED10639,
14-3-3-ε	*14-3-3-ε^{EP3578}*	Df(3R)ED5815
CG16791	*CG16791^{DG25603}*	Df(3R)ED6058, Df(3R)ED6052
psr	*psr^{EY07193}*	Df(3R)ED6085, Df(3R)ED6090, Df(3R)ED6093
how	*how^{24B}*	Df(3R)ED6085, Df(3R)ED6090, Df(3R)ED6093
mbc	*mbc^{EY01437}*	Df(3R)Exel9014
crb	*crb^{MB08251}*	Df(3R)ED6187
wasp	*wasp^{EY06238}*	Df(3R)Exel6210
medea	*med^{MB08684}*	Df(3R)ED6361
yki	*yki^{Δ5}*	
jun	*jun^{2}*	
hep	*hep^{r75}*	
rac	*rac ^{2Δ}*	
shn	*shn^{1B}*	
		Enhancers
E(pc)	*E(Pc)1^{bw1}*	Df(2R)ED2219, Df(2R)ED2222
lid	*lid^{10424}*	Df(2L)ED385, Df(2L)ED354, Df(2L)BSC353
puc	*puc^{H246}*, *pucLacz^{E69}*	Df(3R)ED5220, Df(3R)ED5221, Df(3R)ED5223, Df(3R)ED5230, Df(3R)ED5296 (these deficiencies suppress and therefore likely uncover a distinct suppressor locus)
		Synthetic lethal
cul-3	*cul-3^{gft}*	Df(2L)ED800, Df(2L)ED1054, Df(2L)ED3, Df(2L)Exel8034, Df(2L)ED1050, Df(2L)ED1004, Df(2L) PZ06430-mr14
ex	*ex^{NY1}, ex^{e1}*	

See Table 1 for chromosome intervals inferred to contain modifiers that are not yet identified.

not covered by any of the deficiencies tested in our screen, however, and two different alleles of *aPKC* (*aPKC^{k06403}* and *aPKC^{EY22946}*) failed to dominantly modify the Draper II overexpression phenotype.

Since Crumbs regulates SWH signaling via the FERM-domain protein Expanded (Chen *et al.* 2010; Ling *et al.* 2010; Robinson *et al.* 2010), we next tested whether components of the SWH pathway had any effect. Mutants and deficiencies affecting *salvador*, *warts*, *hippo*, and *merlin* had no dominant effect on the en > DrprII phenotype nor did the mutation *fat^{NY1}*. A mutant allele of *yki* (*yki^{Δ5}*) suppressed the phenotype (Figure 5K and Table 2), whereas coexpression of UAS–Yki enhanced it (data not shown). By contrast, two mutant alleles for *ex* (*ex^{NY1}* and *ex^{e1}*) were lethal in combination with ectopic DrprII, and coexpression of dsRNAi for *ex* enhanced the phenotype. No interaction was seen with the hypomorph *ex^{AP49}*, however (Table 2). We also assessed the affect of en > DrprII on expression levels of Fat and Ex protein and on an *ex-LacZ* enhancer trap line but saw no effects (data not shown).

lid is a modifier of Draper

The histone demethylase *lid* was identified as an enhancer that dominantly increased penetrance of the crossveinless phenotype in en > DrprII male flies (Figure 3, B and B′). Consistent with this, we found that co-overexpressing UAS–Lid with UAS–DrprII restored more normal development of the posterior crossvein to female flies (Figure 3C). Lid is required for the cell growth induced by ectopic dMyc expression (Secombe *et al.* 2007). The null allele *myc^{4}* was found to dominantly suppress en > DrprII, which was surprising as *lid* was an enhancer. The *myc* gene is X linked and was not tested

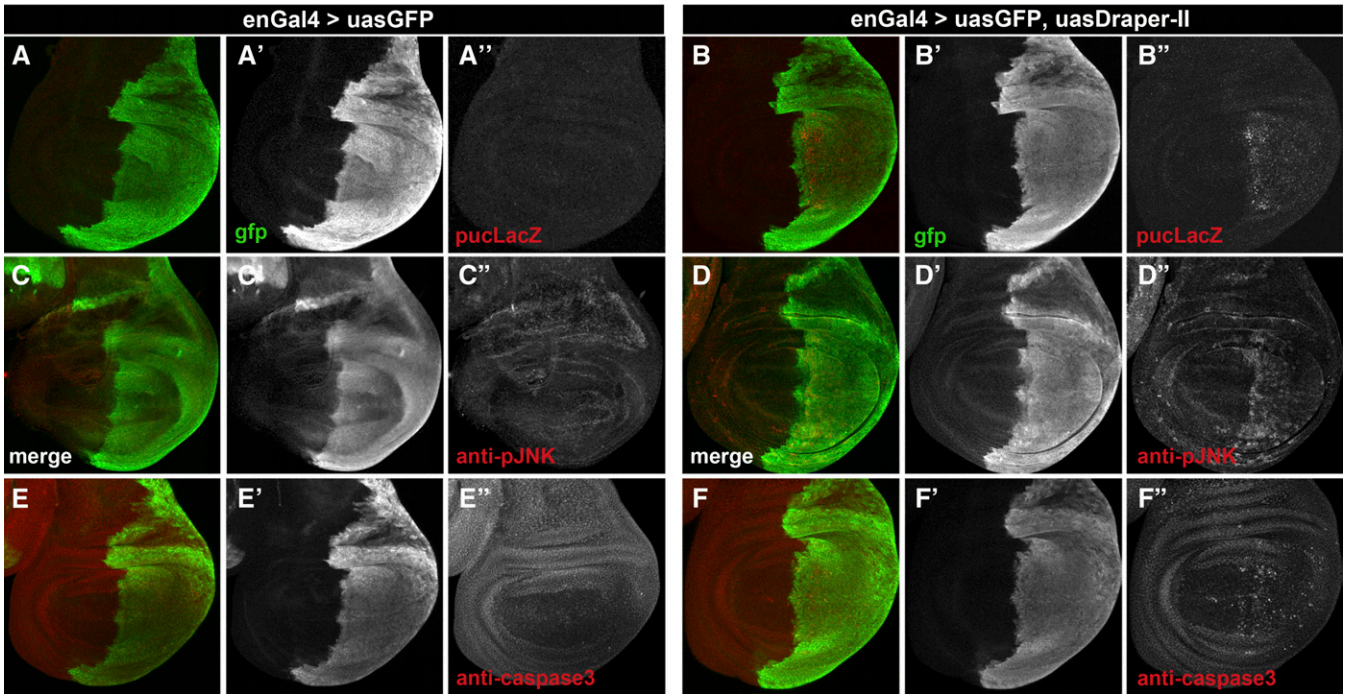

Figure 6 When compared to controls (A–A'') en > DrprII leads to elevated levels of puc expression as seen by the increased activity of a puc–lacZ enhancer trap line (*pucLacZ*^{E69}) in the posterior compartment of wing imaginal discs (B–B''). Increased levels of phosphorylated-JNK are also observed in en > DrprII *vs.* controls (compare C–C' to D–D''). Draper II overexpression also leads to increased levels of cells in the posterior compartment that stain positive for the apoptotic marker, cleaved-caspase 3 (compare E'' to F'').

in the primary deficiency screen; however, subsequent experiments showed that a deficiency uncovering the *myc* locus, Df (1)Exel6233, failed to modify the DrprII overexpression phenotype, suggesting that *myc* does not interact with the DrprII pathway. Mutant alleles of *max* (*max*^{1}) and *mnt* (*dmnt*^{1}), respectively an agonist and antagonist of Myc, had no effect on en > DrprII. Since *lid* encodes a chromatin modification enzyme that may affect expression of many genes, it is possible that *lid* interacts with DrprII by a route independent of its role in *myc*-dependent cell growth.

Rac, Rho, and the cytoskeleton

Two genes identified as suppressors of en > DrprII, *rac1* and *mbc*, are homologs of the *C. elegans* genes *ced10* and *ced5*, respectively. Suppression by loss of *rac1* was observed with deficiencies of the 61E1–62A2 region (Table 1) as well as the *P*-element insertion line *rac1*^{EY05848}. Deficiencies and a point mutant affecting the *mbc* (*ced5*) gene also suppressed. Neither a deficiency uncovering the *ELMO* locus (the *ced-12* homolog) nor a mutant allele of *ELMO* (*ELMO*^{KO}) modified the en > DrprII phenotype; the *Crk* (*ced2*) gene lies on chromosome 4 and its interactions with DrprII overexpression remain untested.

In addition to *rac1* and *mbc*, two other suppressors that were identified in the deficiency screen, *rhoA* and *wasp*, also play important roles in cytoskeleton regulation, and a mutation of *rac2* also suppressed (Figure 5L and Table 2). In addition, we identified the suppressor locus *CG32138* that encodes a homolog of the human formin genes that have been implicated in actin cytoskeleton regulation (Table 1) (Bai *et al.* 2011). We assessed the effect of en > DrprII on the actin cytoskeleton using phalloidin staining of en > DrprII wing discs but observed no differences from controls (data not shown).

Interactions between Drpr isoforms

DrprII was the only isoform with a morphological phenotype when overexpressed in the wing. By contrast, Drpr I is necessary and sufficient for glial engulfment of axon fragments *in vivo*, in which DrprII plays a downregulatory role because of its distinct intracellular domain (Logan *et al.* 2012). No positive or negative contribution of DrprIII to glial activation has been reported. To explore the relationship of DrprI and DrprIII to ectopic DrprII in wing patterning, the isoforms were coexpressed under enGal4 control. Both DrprI and DrprIII suppressed the en > DrprII phenotype, with statistical significance in female flies (Figure 7A).

To see whether DrprI and DrprIII could modify the interactions of DrprII with other genes, DrprII was coexpressed with these isoforms in backgrounds heterozygous for enhancers of the DrprII phenotype. Consistent with the antagonism reported above, both DrprI and DrprIII expression prevented heterozygosity for *lid* from enhancing en > DrprII (Figure 7A). Results with *E(pc)* or *cul3* were more complicated: heterozygosity for these loci produced novel phenotypes in en > drprI and en > drprIII flies, and these phenotypes were epistatic when drprII was coexpressed. In detail, flies overexpressing DrprI and heterozygous for *E(Pc)* usually did not survive, but rare escapers exhibited vein

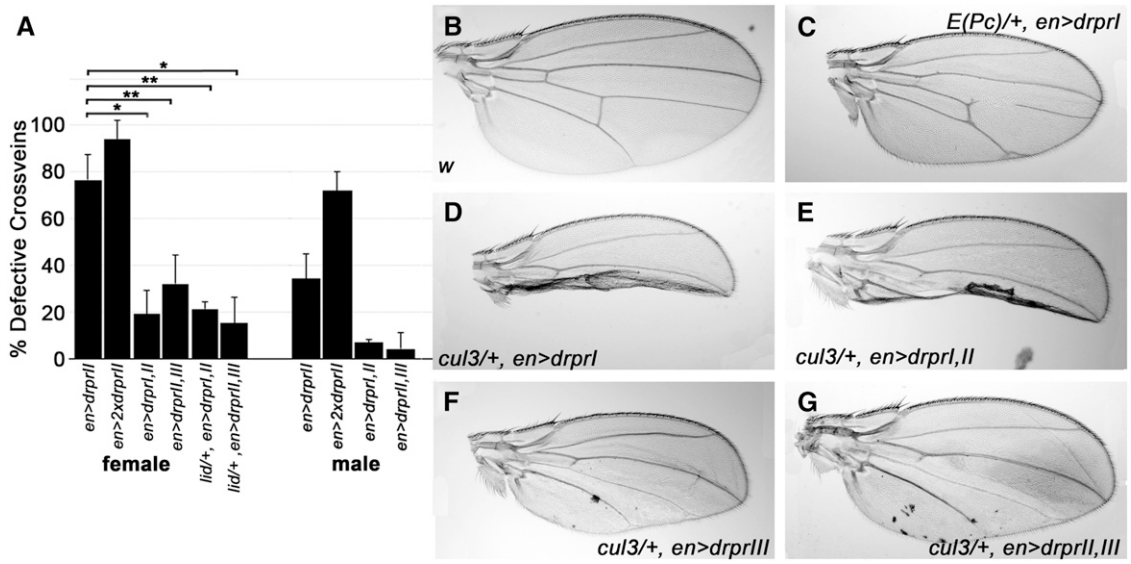

Figure 7 Interactions between Drpr isoforms. (A) Absence of posterior crossveins quantified in flies expressing combinations of Drpr isoforms. All statistically significant differences are indicated (Students *t*-test: *, $P < 0.05$; **, $P < 0.01$). These experiments made use of two UAS–drprI insertions and three UAS–drprIII insertions. Since results were similar with each, the mean and observed standard error of the results with distinct insertions is shown here. The *lid*/+ genotypes were heterozygous for Df(2L)ED385. (B) Wing from normal male fly ($w^{11\text{-}18}$). (C) Male en > DrprI wing, also heterozygous for Df(2R)ED2219. Only rare male escapers were seen for this genotype. (D) Male en > DrprI wing, also heterozygous for $cul3^{gft}$. (E) Female sibling of the fly in D, also heterozygous for the X-linked UAS-DrprII transgene. (F) Male en > DrprIII wing, also heterozygous for $cul3^{gft}$. (G) Female sibling of the fly in F, also heterozygous for the X-linked UAS–DrprII transgene.

defects distinct from those caused by DrprII (Figure 7, B and C). Flies overexpressing DrprIII and heterozygous for *E(Pc)* did not survive. Flies overexpressing DrprI and heterozygous for *cul3* largely lacked the posterior compartment of the wing (Figure 7, D and E). Flies overexpressing DrprIII and heterozygous for *cul3* exhibited fully penetrant vein, growth, and other defects in the posterior compartment (Figure 7, F and G).

Discussion

Genetic modification of draper II overexpression

The paradigm for genetic modifier screens in *Drosophila* has been to employ either gain- or loss-of-function genotypes in the pathway of interest that generate a sensitized phenotype whose penetrance or expressivity thereby becomes dependent on the copy number of genes in the same or related pathways. Variants of this approach were instrumental in establishing the main lines of the receptor tyrosine kinase/ras signaling pathways (Simon *et al.* 1991; Doyle and Bishop 1993; Karim *et al.* 1996) and in many other screens.

To screen an externally visible phenotype reflecting Drpr activity, we made use of the observation that overexpression of UAS–DrprII in posterior compartments under the control of en–Gal4 eliminated the posterior crossvein from adult wings with variable penetrance (Figure 1). Previous reports have described a role for Rho–GTPases in crossvein formation (Denholm *et al.* 2005). Our data indicate that RhoA, as well as Rac1 and Rac2, are required for the crossvein defect caused by Draper II overexpression (see below). The modifier regions defined through deficiency screening are listed in Table 1, and all the modifier loci and alleles identified by any method are listed in Table 2.

Recent studies of glial responses to axon damage indicate that DrprII uses its isoform-specific ITIM domain to terminate the DrprI response, allowing glia to return to a resting state (Logan *et al.* 2012). Such downregulation plays a positive role in the long term, facilitating multiple responses to successive nerve injuries (Logan *et al.* 2012). DrprI and DrprII share the interaction domain for Ced-6, and although the DrprI ITAM domain that interacts with Shark is absent from DrprII, it is replaced by other ITAM-like sequences (Logan *et al.* 2012). In the case of overexpression in the wing, we found that the en > DrprII phenotype depended positively on the adapter proteins Ced-6 and Shark, which act positively in Drpr signaling. Therefore, other modifiers of the en > DrprII phenotype are candidates to contribute to Drpr signaling processes, at least those that depend on Ced-6 and Shark. It is also possible that the en > DrprII phenotype may be modified by genes that depend on the ITIM domain and play inhibitory roles in physiological Drpr signaling. In addition, the en > DrprII phenotype may not be sensitive to any genes that interact exclusively with DrprI or DrprIII.

The notion that modifiers of en > DrprII may be relevant to function of the other isoforms is supported by the finding that wings overexpressing DrprI and DrprIII were no longer normal in the presence of mutations that enhance the DrprII phenotype, such as *E(Pc)* and *cul3* (Figure 7, C, D, and F). Since overexpression of DrprI or DrprIII suppressed the en > DrprII phenotype (Figure 7A); however, it is difficult

to provide a simple model of the isoform relationships that accounts for all the observations.

Interactions with the Ced-2,5,10,12 engulfment pathway and the cytoskeleton

In *C. elegans*, the Ced-2, 5, 10, 12 pathway (in *Drosophila*: *Crk*, *mbc*, *Rac1*, and *dCed-12*, respectively) regulates cytoskeletal rearrangements in the engulfing cells that are required for formation of the phagocytic cup (Ellis *et al.* 1991; Albert *et al.* 2000; Chimini and Chavrier 2000; Gumienny *et al.* 2001; Fullard *et al.* 2009; Kinchen 2010). In contrast, the Ced-1, 6, 7 pathway (including *drpr* and *dCed-6*) is thought to recognize apoptotic cells (Liu and Hengartner 1998; Wu and Horvitz 1998; Zhou *et al.* 2001; Awasaki *et al.* 2006), remodel cell membranes during phagocytosis (Yu *et al.* 2006), and function in phagosome maturation (Kurant *et al.* 2008; Yu *et al.* 2008; Fullard *et al.* 2009; Kinchen 2010). Although somewhat independent, coordination between these pathways is likely to be important, and more recent studies have suggested that the Ced1, -6, -7 pathway feeds in to Ced-10/Rac (Kinchen *et al.* 2005; Cabello *et al.* 2010).

Our findings suggest that the Draper signaling pathway is related to Rac activity in *Drosophila* as well, such that DrprII overexpression can be phenotypically silenced by reduced function of *rac1* and *mbc*. Other modifiers are also regulators of the cytoskeleton (Table 2). Like Rac, RhoA is a member of the small GTPase family that regulates the cytoskeleton (Van Aelst and D'souza-Schorey 1997; Ravichandran and Lorenz 2007). WASp is a well-known cytoskeletal regulator involved in the transduction of signals from receptors on the cell surface to the actin cytoskeleton and required for phagocytosis (Rohatgi *et al.* 1999; Badour *et al.* 2003; Takenawa and Suetsugu 2007; Veltman and Insall 2010). Our screen identified another gene, *CG32138*, which is a homolog of the human formin genes *FMNL1*, *FMNL2*, and *FMNL3* and is implicated in actin cytoskeleton regulation and cellular migration (Liu *et al.* 2010; Bai *et al.* 2011). The *osm1* gene is predicted to constitute a component of the cytoskeleton (Goldstein and Gunawardena 2000) required for the formation and function of cilia (Avidor-Reiss *et al.* 2004; Laurencon *et al.* 2007). The *pten* gene is also implicated in cytoskeletal regulation (Goberdhan and Wilson 2003; Li *et al.* 2005), as well as in apical-basal polarity (Von Stein *et al.* 2005),

Interactions with JNK signaling

DrprII overexpression increased JNK signaling levels, and multiple members of the JNK pathway modified the effects of DrprII (Figure 6 and Table 2). Since this study was undertaken, another study has shown that Draper functions upstream of the JNK pathway during follicle cell engulfment in the *Drosophila* ovary (Etchegaray *et al.* 2012). In addition, *shark* is required for JNK activity during embryonic dorsal closure, even though it is not known whether Drpr is involved in this process (Fernandez *et al.* 2000). These findings strongly support a link between Drpr signaling and JNK activation. JNK signaling can be pro-apoptotic (Igaki 2009). Although ectopic Draper II increased staining for the pro-apoptotic marker, cleaved caspase-3, reduced dose of the pro-apoptotic genes *reaper*, *hid*, and *grim* did not modify the en > DrprII phenotype, nor did overexpression of the anti-apoptotic protein IAP1 (Figure 5, G and H). Taken together, these data suggest that the crossveinless phenotype depends on a nonapoptotic function of the JNK pathway.

Another gene that we identified has also been implicated in JNK signaling, namely the gene that encodes the so-called phosphatidylserine receptor, *psr*. Apparently named in error, since it encodes a nuclear jumonji-domain protein, there is evidence that *psr* suppresses JNK signaling (Krieser *et al.* 2007). It was therefore unexpected that *psr* mutations dominantly suppressed the en > DrprII crossveinless phenotype, consistent with a positive role in JNK signaling.

Interactions with cell junctions and the Salvador/Warts/Hippo pathway

We found that the apical-basal polarity genes *lgl* and *crb* were modifiers of DrprII. Crumbs and Lgl can function together to regulate the SWH pathway (Grzeschik *et al.* 2010). Strikingly, mutations in the FERM domain protein gene *ex* were synthetically lethal in combination with ectopic Draper II. Many members of the SWH pathway showed no genetic interaction with DrprII, however, exceptions being *ex*, *yki*, and *14-3-3-epsilon*. 14-3-3-epsilon is important in nuclear localization of Yki and other proteins (Oh and Irvine 2008). Not only were the interactions between DrprII and *ex*, *yki*, and *14-3-3-epsilon* not shared by other SWH genes, they were opposite to those expected if *lgl* suppresses en > DrprII by activating SWH that pathway.

Recent work in mammalian cells establishes a link between YAP, the mammalian ortholog of Yki and the GTPases RhoA and Cdc42 (Dupont *et al.* 2011; Reginensi *et al.* 2013). This is thought to be part of a mechanosensory signaling system, by which cells interpret physical and mechanical cues from the microenvironment, and regulates YAP independently of the SWH pathway. If a similar pathway exists in *Drosophila*, the genetic interaction observed between *yki* and en > DrprII might be explained as a consequence of cytoskeleton remodeling and RhoA activity, independently of the core SWH pathway. Interestingly, although *ex* is well known as an upstream regulator of the SWH pathway, it can also bypass this pathway to interact with Yki directly (Badouel *et al.* 2009).

Interactions with other corpse engulfment genes

Previous studies of corpse engulfment by *Drosophila* S2 cells in culture have identified a distinct set of genes (Fullard *et al.* 2009; Kinchen 2010). It is thought that Draper triggers Ca^{2+} release from the endoplasmic reticulum via the ryanodine receptor 44F, which in turn leads to an influx of extracellular Ca^{2+} that depends on Ca^{2+} channels, the ER Ca^{2+} sensor dSTIM, and the junctophilin Undertaker/Retinophilin (Cuttell *et al.* 2008). The screen we performed included deficiencies that could have revealed interactions with *uta*, *orai*,

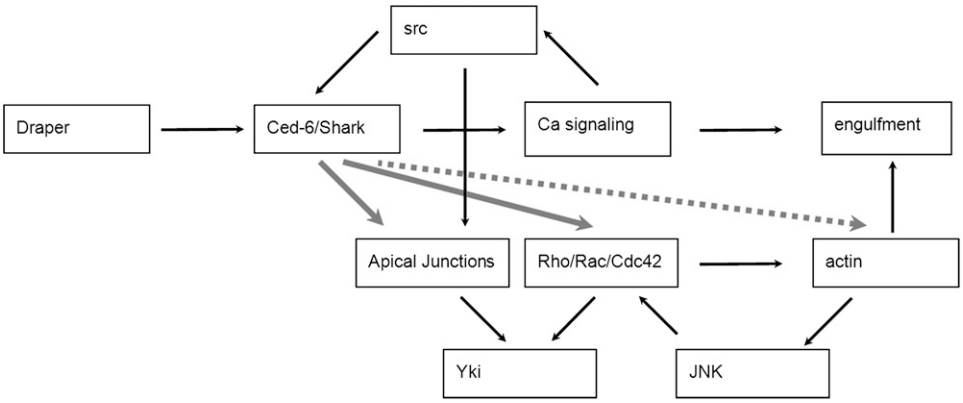

Figure 8 A scheme of interactions hypothesized to connect the receptor protein Draper to the execution of the engulfment process. Solid arrows represent connections established by previous studies (see *Discussion*). Shaded arrows highlight the predominant interactions indicated in this study of genetic modifiers. The arrow connecting Ced-6/Shark to actin is dotted because the results do not distinguish whether the Draper pathway affects actin only through the small GTPases or also independently of them. The most parsimonious explanation of JNK activity in response to Draper is shown, whereby JNK is activated indirectly via changes in the actin cytoskeleton. An additional, more direct connection between Draper and Ced-6 or Shark and JNK cannot be excluded. The contribution of Yki activity to engulfment, if any, remains uncertain at present.

and *rya-r44F*, as well as *six microns under* (*simu*) and *nimrod*, two other transmembrane proteins similar to Drpr that are implicated in corpse engulfment (Kurant *et al.* 2008), but we found no such interactions. In addition, no interactions were seen with *src42A* or *src64B*, although Src-family kinases are thought to be required for Shark to interact with Draper (Ziegenfuss *et al.* 2008). These negative findings indicate that genetic modification of DrprII overexpression does not detect all loci with related functions. Some of these genes might be specific for S2 cells or for signaling by DrprI or DrprIII. It is also possible that they are not dose sensitive in the DrprII overexpression background.

Our screen also identified *pollux* (*plx*). Evidence suggests that Plx interacts with integrins (Zhang *et al.* 1996). Integrins are receptors for apoptotic corpses in mammals and in the *C. elegans* Ced2, 5, 10, 12 engulfment pathway (D'mello and Birge 2010; Hsieh *et al.* 2012), but no apoptotic role for *Drosophila* integrins is known. Plx is homologous to the human TBC1D1 and TBC1D4 proteins and, as such, might function as a RabGAP (Laflamme *et al.* 2012).

One modifier that we identified, *CG16791*, was also identified in an RNAi screen for genes required for phagocytosis of the fungal pathogen *Candida albicans* by *Drosophila* S2 cells (Stroschein-Stevenson *et al.* 2006). Since little is known about this protein, which was not recovered in some other high-throughput screens for phagocytosis functions (Ellis *et al.* 1991; Kinchen *et al.* 2008; Lombardo *et al.* 2013), our data may bolster the evidence that *CG16791* is involved in phagocytosis.

Other genetic modifiers

Other genetic modifiers identified in our screen did not cluster together into known pathways (Table 2). Although we found *Dpp*, *Mad*, *medea*, and *shn* as modifiers of Draper II function, no interaction was seen with the Dpp receptors Tkv or Put. The modifier *how* encodes an RNA binding protein that can bind to dpp mRNA (Israeli *et al.* 2007). How plays roles in integrin-mediated cell adhesion (Walsh and Brown 1998) and in the maintenance of stem-cell proliferation in testes (Monk *et al.* 2010). Vps28 is a component of the ESCRT-I complex, which is required for trafficking of ubiquitylated proteins (Vaccari *et al.* 2009) and has been shown to play a role in autophagy (Rusten *et al.* 2007). Sec23 is part of a protein complex that plays a role in ER–Golgi protein trafficking (Paccaud *et al.* 1996). Fer2 is a little-characterized bHLH transcription factor. *tara* is a member of the trithorax group of genes and was also identified in screens for genes required for vein formation (Molnar *et al.* 2006) and growth control and patterning (Cruz *et al.* 2009). Another chromatin protein that modified en > DrprII was the jumonji domain-containing histone demethylase *lid* (Secombe and Eisenman 2007). *l(3)76bdr* encodes the ribosome associated listerin E3 ubiquitin protein ligase 1 (LTN1) that plays a role in controlling the proteasomal degradation of proteins (Bengtson and Joazeiro 2010).

Conclusions and model

Our studies demonstrate multiple genetic interactions between the DrprII pathway and both JNK signaling and cytoskeleton regulators including Rac, Rho, Ex, and Yki. The molecular mechanisms connecting these signaling pathways during engulfment remain uncertain. Numerous studies implicate JNK activity downstream of Rac signaling, also potentially mediated by the actin cytoskeleton (Tapon *et al.* 1998; Fanto *et al.* 2000; Boureux *et al.* 2005). During embryonic dorsal closure, JNK activity is itself required for cytoskeletal remodeling, a potential positive feedback (Sluss *et al.* 1996). In other contexts, it is thought that Rho activates JNK through its effects on the actin cytoskeleton and that JNK activity probably feeds back on the cytoskeleton in turn (Fernandez *et al.* 2014). Taken together, all these studies support JNK responding to and amplifying cytoskeletal rearrangements in a positive feedback loop.

Interestingly, Rho and JNK are significant effectors of Src signaling in tumorigenesis and are thought to act downstream of disruption of apical epithelial junctions by Src

activity (Enomoto and Igaki 2013; Fernandez *et al.* 2014). Although neither *src* locus was found here to modify en > DrprII, *src* family kinases are required for Shark to bind to Drpr (Ziegenfuss *et al.* 2008). Both the apical junction components *lgl* and *crb* interact with the SWH pathway (Grzeschik *et al.* 2010), which can also be activated by disrupting the actin cytoskeleton (Fernandez *et al.* 2011; Sansores-Garcia *et al.* 2011). In mammals, *cdc42* is thought to activate Yap independently of SWH signaling, in response to mechanical stress (Dupont *et al.* 2011; Wada *et al.* 2011; Reginensi *et al.* 2013). It is not known whether *yki* is activated during engulfment, or what role this might play if so, but both JNK and Yki have been implicated in compensatory proliferation in response to cell death (Ryoo *et al.* 2004; Worley *et al.* 2012). The role of JNK in compensatory proliferation was presumed to occur in the apoptotic cells, perhaps in the generation of proliferative signals from dying cells, but recently activity in the compensating cells has also been demonstrated (Fan *et al.* 2014). This would be consistent with a signal for compensatory proliferation being sent to JNK and Yki when Drpr recognizes apoptotic cells.

Together, the connections can be summarized into a tentative model of the potential pathways interacting with Drpr during engulfment, which could prove a useful guide to further studies aimed at elucidating the precise molecular mechanisms that coordinate cellular processes during engulfment (Figure 8). This model includes potential positive feedback loops involving Src and JNK that help connect Draper to actin remobilization in the engulfment process and a connection to growth regulators at apical cell junctions and in the nucleus.

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