© 2014. Published by The Company of Biologists Ltd | Development (2014) 141, 4110-4114 doi:10.1242/dev.110171

RESEARCH REPORT

TGF-α ligands can substitute for the neuregulin Vein in *Drosophila* development

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ABSTRACT

ErbB receptors, including the epidermal growth factor receptor (Egfr), are activated by EGF ligands to govern cell proliferation, survival, migration and differentiation. The different EGF-induced cell responses in development are regulated by deployment of multiple ligands. These inputs, however, engage only a limited number of intracellular pathways and are thought to elicit specific responses by regulating the amplitude or duration of the intracellular signal. The single *Drosophila* Egfr has four ligands: three of the TGF-α-type and a single neuregulin-like called *vein* (*vn*). Here, we used mutant combinations and gene replacement to determine the constraints of ligand specificity in development. Mutant analysis revealed extensive ligand redundancy in embryogenesis and wing development. Surprisingly, we found that the essential role of *vn* in development could be largely replaced by expression of any TGF-α ligand, including *spitz* (*spi*), in the endogenous *vn* pattern. *vn* mutants die as white undifferentiated pupae, but the rescued individuals showed global differentiation of adult body parts. Spi is more potent than Vn, and the best morphological rescue occurred when Spi expression was reduced to achieve an intracellular signaling level comparable to that produced by Vn. Our results show that the developmental repertoire of a strong ligand like Spi is flexible and at the appropriate level can emulate the activity of a weak ligand like Vn. These findings align with a model whereby cells respond similarly to an equivalent quantitative level of an intracellular signal generated by two distinct ligands regardless of ligand identity.

KEY WORDS: Vein, Spitz, Egfr, Neuregulin, Wing, *Drosophila*

INTRODUCTION

Drosophila Egfr is the sole homolog of the vertebrate family that includes Egfr/ErbB1, neu/ErbB2, ErbB3 and ErbB4. Perturbation of the pathways, which have key roles in proliferation and differentiation during development and homeostasis, results in major developmental defects and problems in human health, including heart disease, schizophrenia and cancer [reviewed by, for example, Mei and Xiong (2008); Sanchez-Soria and Camenisch (2010); Yarden and Pines (2012)]. In mammals there are 11 EGF-like ligand genes (Groenen et al., 1994; Yarden, 2001). *Drosophila* has four EGF ligands, representing two major classes: the TGF-α ligands *spitz* (*spi*), *gurken* (*grk*) and *Keren* (*Krn*), and the neuregulin-like (NRG) ligand *vein* (*vn*) (Rutledge et al., 1992; Neuman-Silberberg and Schüpbach, 1993; Schnepp et al., 1996; Reich and Shilo, 2002; Urban et al., 2002).

Deployment of the four ligands, which operate both in distinct and in overlapping processes, contributes significantly to *Drosophila* development (Shilo, 2003). *grk* has a maternal role (Schüpbach, 1987), whereas *spi* has a major role in zygotic development and mutants die as embryos with ventral defects (Mayer and Nüsslein-Volhard, 1988). *vn* mutants have milder ventral defects and die at the pupal stage (Simcox, 1997). *spi; vn* double mutants have more than an additive phenotype, consistent with redundant ligand function (Schnepp et al., 1996). Further redundancy is suggested by the finding that *Krn* single mutants are viable and fertile adults, but functions in the eye, gut, ovary and brain are revealed when other ligands are compromised, animal physiology is altered or when *Krn* is expressed ectopically (Yang and Baker, 2003; McDonald et al., 2006; Brown et al., 2007; Jiang and Edgar, 2012; Rahn et al., 2013). Ectopic *Krn* expression can restore MAPK signaling in a *spi* mutant, thereby further demonstrating functional redundancy of the TGF-α ligands (Reich and Shilo, 2002). Some roles, by contrast, are ligand specific; for example, *vn* has a major role in wing development, whereas *spi* or *Krn* are not required (Simcox et al., 1987; Simcox, 1997; McDonald et al., 2006).

Here, we investigated the requirement for ligands by examining mutants lacking combinations of genes, and for ligand specificity by determining the ability of one ligand to replace another. We found that Egfr signaling is fully ligand dependent in embryogenesis and that all zygotic ligands play a role in embryo and wing development. Surprisingly, each of the TGF-α ligands could largely replace the essential role of *vn* in development when the active ligand was expressed in the *vn* pattern. Our results support the idea that stimulating the pathway to a particular level, and not the specific ligand bound, is important.

RESULTS AND DISCUSSION

Egfr-mediated embryonic patterning is dependent on function of all three zygotically active ligands

As single mutants, only *spi* and *vn* have defects in embryonic ventral patterning, and the double mutant is more severe (Schnepp et al., 1996) (Fig. 1A-C). Here, we found that *Krn* plays a redundant role with *spi* in embryonic cuticle patterning. *spi; Krn* double mutants had a similar phenotype to *spi; vn* double mutants (Fig. 1C,D). The triple mutant *spi; vn; Krn* was much more extreme than either double mutant and was indistinguishable from an *Egfr* null mutant (Fig. 1E,F). Both mutants lack ventral denticle belts and head structures. As expected, zygotic removal of the maternal ligand *grk* (*spi, grk; vn, Krn*) had no additional effect (Fig. 1G).

The equivalent phenotypes of mutant embryos lacking the receptor or the ligands suggest that there is no signaling through Egfr in the absence of a ligand (Fig. 1E-G). Ligand-independent signaling has been reported for overexpression of Egfr in *Drosophila* and in mammalian cells, where the high levels of Egfr are thought to cause spontaneous dimerization and signaling (Schweitzer et al., 1995; Nagy et al., 2010; Endres et al., 2013). Our results suggest that, at the normal physiological level of the receptor in the fly embryo, all signaling is ligand dependent. The

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Received 16 March 2014; Accepted 1 September 2014

Development (2014) 141, 4110-4114 doi:10.1242/dev.110171

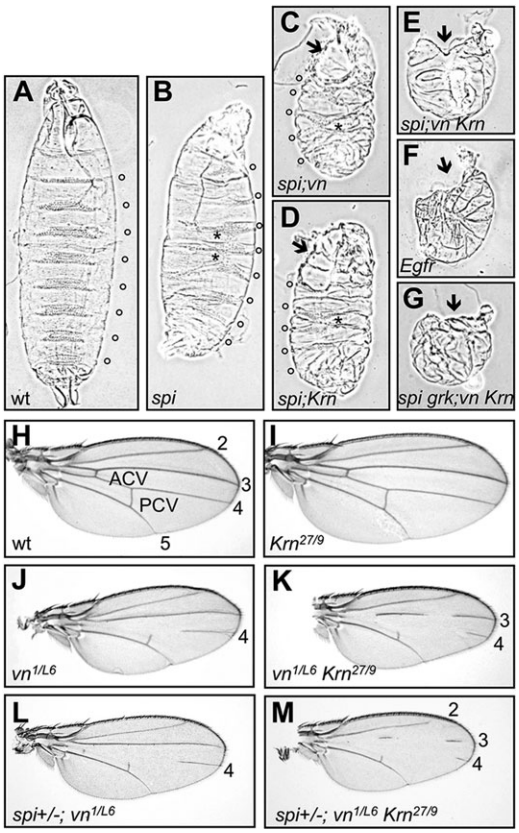

Fig. 1. Redundant roles for EGF ligands in embryogenesis and wing development. (A) Wild-type embryo, with eight denticle belts (open circles). (B) *spi*^{2A} mutant embryo, with eight narrow denticle belts (open circles), some of which are fused (*). (C) *spi*^{2A}*; vn*^{L6} and (D) *spi*^{2A}*; Krn*^{27} double mutant embryos, with a reduced number of denticle belts (open circles), fusions (*) and an anterior hole (arrow). (E) *spi*^{2A}*; vn*^{L6}*, Krn*^{27} triple mutant, (F) *Egfr*$^{3F18/F24}$ receptor null and (G) *spi*^{2A}*, grk*^{HF}*; vn*^{L6}*, Krn*^{27} quadruple mutant. The embryos are very short with no denticle belts and a large anterior hole (arrow). (H) Wild-type wing, with four major longitudinal veins (L2-5) and two crossveins, anterior crossvein (ACV) and posterior crossvein (PCV). (I) *Krn*$^{27/9}$ wing with a normal vein pattern. (J) *vn*$^{1/L6}$ hypomorphic mutant wing with loss of part of L4 and the ACV. Very few flies (7%, *n*=55) lack parts of L3. (K) *vn*$^{1/L6}$*, Krn*$^{27/9}$ wing, with more frequent (52%, *n*=64) loss of parts of L3. (L) *spi*$^{2A/+}$*; vn*$^{1/L6}$ wing, with similar vein loss as *vn*$^{1/L6}$. (M) *spi*$^{2A/+}$*; vn*$^{1/L6}$*, Krn*$^{27/9}$ wing, with frequent and more extensive loss of L3 (90% *n*=65).

mutant combinations, however, suggest extensive redundancy between the ligands, consistent with roles that are only revealed when multiple ligands are compromised.

Wing vein patterning requires the function of all three zygotic ligands

vn has a role in patterning of the wing blade into territories of veins and interveins (Sturtevant and Bier, 1995; Simcox et al., 1996). No role has been found for *spi* or *Krn* in wing patterning (Simcox, 1997; Guichard et al., 1999; Nagaraj et al., 1999; McDonald et al., 2006). Reducing activity of Egfr or the protease Rhomboid (Rho), which is required to process the TGF-α ligands, causes vein loss, and *rho vn* double mutants lack all veins (Sturtevant et al., 1993; Sturtevant and Bier, 1995; Guichard et al., 2000; Ghiglione et al., 2002; Urban et al., 2002). This suggests that either or both *spi* and *Krn* could have redundant roles in wing development. We found that eliminating *Krn* function exacerbated the vein-loss phenotypes in *vn* mutants (Fig. 1H-K). Reducing *spi* levels had no observable effect on *vn* phenotypes (Fig. 1L; supplementary material Fig. S1). The most extreme phenotype resulted from reduction of function in all three genes (Fig. 1M), thereby suggesting that all three contribute to wing vein patterning.

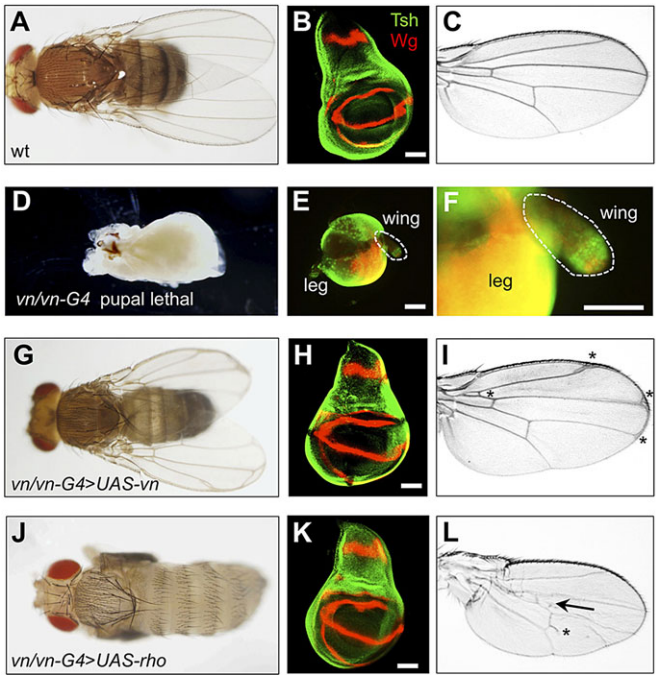

Fig. 2. Expression of *rho* rescues developmental abnormalities in *vn* mutants. *rho* encodes a protease that cleaves TGF-α ligands into an active form. (A) Wild-type adult fly, (B) wing disk and (C) wing. (D) *vn*^{L6}*/vn-Gal4* mutant pupa, (E) leg and wing disk and (F) higher magnification view of wing disk in E. *vn*^{L6}*/vn-Gal4* mutants die as pupae and have very small wing disks. (G) *UAS-vn; vn*^{L6}*/vn-Gal4* adult, (H) wing disk and (I) wing with a mild extra-vein phenotype consistent with overactivity of Egfr signaling (*). (J) *UAS-rho; vn*^{L6}*/vn-Gal4* pharate adult, (K) wing disk and (L) wing with fused L3 and L4 veins (arrow) and extra vein material (*). Wing disks were stained for Tsh (green) and Wg (red). Scale bars: 50 µm.

The TGF-α ligands can replace the essential function of *vn* in development

The *vn-GAL4* allele, generated as part of this study, has an amorphic phenotype and mutants die as white pupae with tiny wing disks (Fig. 2D-F; supplementary material Fig. S2). This allele, which is expressed in the endogenous *vn* pattern (supplementary material Fig. S3), allowed us to test whether ectopic activity of the TGF-α ligands rescued *vn* mutants.

Expression of *UAS-vn* with *vn-Gal4* (*UAS-vn; vn*^{L6}*/vn-Gal4*) rescued ~30% of *vn* mutants to adult flies with grossly normal patterning of the wing (Fig. 2G-I). Some extra vein material was observed, consistent with mild Egfr overactivity (Fig. 2I). Expression of *rho* (*UAS-rho; vn*^{L6}*/vn-Gal4*) rescued ~50% of *vn* mutants to pharate adults (differentiated adults that remain in the pupal case) with grossly normal wings (Fig. 3K,L). The region between veins L3 and L4 was fused and there was extra vein material, which correlates with the stripe of *vn* expression in the wing pouch (Simcox et al., 1996), suggesting that *vn* is uniquely suited to pattern this region correctly. *vn* is also required during the first and second instars for growth and global patterning of the early wing disk (Wang et al., 2000; Paul et al., 2013). Expression of *rho* replaced this function of *vn* and produced a disk of normal size and pattern (Fig. 2K). This suggests that one or more TGF-α ligands are present in the disk at the early stages, but remain in an inactive form

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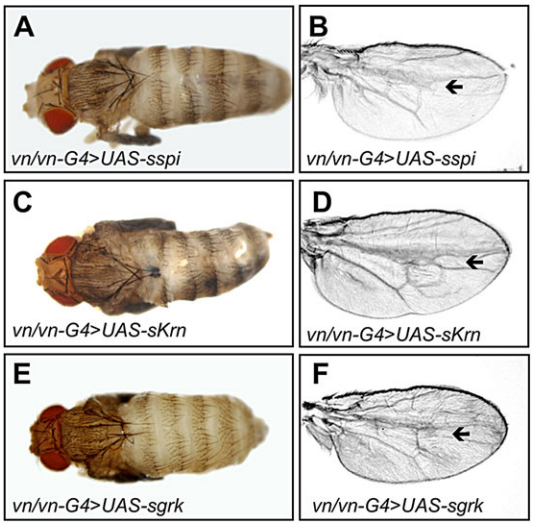

Fig. 3. Expression of any TGF-α ligand rescues developmental abnormalities in *vn* mutants. Expression of transgenes encoding the TGF-α ligands in the *vn* expression pattern rescues differentiation in *vn* mutant flies, including the body wall and wing. The flies are pharate adults that do not eclose. The wings have fused L3 and L4 veins and extra vein material (arrows). (A) *UAS-sspi; Gal80^{TS}; vn^{L6}/vn-Gal4* pharate adult and (B) wing (27°C). (C) *UAS-sKrn, vn^{L6}/vn-Gal4* pharate adult and (D) wing (17°C). (E) *UAS-sgrk, vn^{L6}/vn-Gal4* pharate adult and (F) wing (17°C). Gal80^{TS} is a repressor of Gal4 and reduces transgene expression (supplementary material Fig. S6).

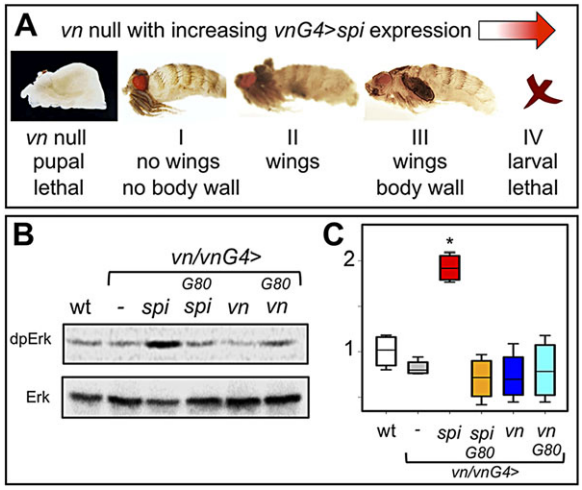

Fig. 4. Optimal rescue of *vn* mutants occurs when transgenic *spi* expression restores signaling close to wild-type levels. (A) *vn* mutants die as pupae (left image). *vn* mutant flies rescued with increasing expression of sSpi are shown from left to right: *UAS-sspi; Gal80^{TS}/+; vn^{L6}/vnGal4;* at (I) 17°C, (II) 25°C and (III) 25-29°C. sSpi is toxic at a high expression level and individuals died as larvae (*UAS-sspi; vn^{L6}/vnGal4* 17°C, see IV). (See also supplementary material Fig. S4.) (B) Western blot analysis and (C) quantification of dpErk (activated MAPK) levels relative to corresponding ERK levels, in the indicated genotypes (25°C). (wt): wild type. (-): *vn^{L6}/vnGal4; (spi): UAS-sspi, vn^{L6}/vnGal4. (G80 spi): UAS-sspi; Gal80^{TS}/+, vn^{L6}/vnGal4. (vn): UAS-vn1.1; vn^{L6}/vnGal4. (G80 vn): UAS-vn1.1; Gal80^{TS}/+; vn^{L6}/vnGal4*. The quantification shown in C is the dpErk level normalized to the corresponding Erk level (n=6 replicates). Only the dpErk level following high *sspi* expression *(UAS-sspi, vn^{L6}/vnGal4)* was significantly different than WT (*P<0.01). Statistical analysis was performed using a Wilcoxon rank-sum test. Gal80^{TS} is a temperature-sensitive inhibitor of Gal4 and hence reduces transgene expression.

because the processing factor *rho* is not normally expressed (Simcox, 1997; Zecca and Struhl, 2002).

Rho can cleave all three TGF-α ligands, and to determine whether expression of a particular ligand can replace the function of *vn*, we tested each ligand individually. Secreted forms were expressed to bypass the requirement for Rho (Urban et al., 2002). Expression of any TGF-α ligand rescued development of *vn* mutants to the pharate adult stage (Fig. 3; supplementary material Figs S4 and S5). The wings had regions of vein fusion and extra-vein material, characteristic of Egfr overactivity (Fig. 3).

The level of TGF-α transgene expression had a significant impact on the extent of rescue (supplementary material Fig. S4). To correlate rescue phenotypes with signaling, we examined in more detail the ability of secreted Spi (sSpi) to rescue *vn* mutants. Expression of sSpi at varying levels (supplementary material Fig. S6) produced different degrees of rescue in *vn* mutants (Fig. 4A). Adult head, leg and abdomen development were restored across a range of expression levels tested, suggesting that these body parts are relatively insensitive to the level of Egfr activity (Fig. 4A; supplementary material Figs S4 and S5). By contrast, a progression of rescue phenotypes was seen, as sSpi expression increased, from animals lacking all wing disk structures, to those with just wings and to optimal cases of animals with wings and a thoracic body wall (Fig. 4A I-III; supplementary material Fig. S7). At the highest Spi levels tested, the animals died as larvae (Fig. 4A IV). The Spi expression level that gave rise to optimal rescue correlated with a signaling output, as measured by MAPK phosphorylation (dpErk), which most closely matched to wild type (WT) (Fig. 4B,C). High levels of sSpi expression produced strongly elevated dpErk levels, which most likely accounted for the early death of these animals.

The intrinsic activity of an EGF ligand correlates with its biological effect; thus, at saturating concentrations, a ligand with high intrinsic activity has a higher maximum biologic effect than a ligand with low intrinsic activity (Wilson et al., 2012). Spi, therefore, appears to have a higher intrinsic activity than Vn, because at higher levels Spi exceeded the required biologic response and was toxic (Fig. 4; supplementary material Fig. S4). Spi also has a higher affinity than Vn (Alvarado et al., 2010). Low affinity might limit the access of Vn to the receptor through negative cooperativity, which proposes that, when the first ligand binds, a conformational change occurs that occludes the second site, rendering it accessible only to a high affinity ligand (Macdonald and Pike, 2008; Alvarado et al., 2010). The biological role of a low-affinity ligand like Vn might therefore be limited, because some cell responses require signaling levels that can be evoked only by high levels of a high-affinity ligand (Krall et al., 2011).

The Ig-domain is not required for the essential function of *vn* in development

Even in cases of the best morphological phenotypes, rescue of *vn* mutants by *spi* was incomplete because the flies did not eclose. This could reflect the artificial Gal4-UAS expression system. Alternatively, the Ig-domain found in Vn and the vertebrate NRGs could confer a unique function. In mice, Ig-containing NRG isoforms are essential (Kramer et al., 1996). A form of Vn lacking the Ig-domain (Vn-ΔIg) rescued *vn* mutants to adults, thus demonstrating that Ig containing forms are not essential for viability (supplementary material Fig. S8A). The wings of these flies were abnormal and had notched margins (supplementary material Fig. S8B), a phenotype that was also seen when this transgene was expressed in a wild-type fly (Donaldson et al., 2004). The Ig-domain could mediate the role of Vn in another pathway independent of its role in Egfr signaling. The wing phenotype is reminiscent of *Notch* or *wingless* mutants (Couso et al., 1994; Price et al., 1997; Wang et al., 2000), and the Vn Ig-domain physically

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interacts with Hedgehog co-receptors (Özkan et al., 2013), making any of these pathways candidates for interacting with Vn via the Ig domain.

We found a chimeric ligand, in which the Spi EGF-domain had been swapped with the Vn EGF-domain (Schnepp et al., 1998), rescued a *vn* mutant. These animals developed to pharate adults with pattern defects consistent with Egfr overactivity (supplementary material Fig. S8C,D). The phenotypes were similar to those seen following rescue by sSpi, showing that linking the Spi EGF-domain with an Ig-domain does not significantly alter its activity relative to the receptor.

Conclusions

In summary, our results show a high degree of functional redundancy among Egfr ligands and, moreover, that low levels of a strong TGF-α ligand like Spi can largely replace the developmental function of a weaker ligand like Vn. This is not reciprocal, however, because the lower biological activity of Vn limits its ability to replace or emulate the activity of Spi (Schnepp et al., 1998; Golembo et al., 1999; Donaldson et al., 2004; McDonald et al., 2006; Jiang and Edgar, 2009). The results support the idea that the appropriate cell response will occur regardless of ligand identity, provided a certain quantitative level of signaling is met. This conclusion is based on signaling resulting from activity of the EGF domain, which all Egfr ligands possess. Other unique domains, such as the Ig-domain found in Vn and the neuregulins, might facilitate engagement of other pathways and hence regulate different targets.

In the first example documenting distinct responses to different growth factors engaging MAPK signaling, the duration of signaling was shown to determine whether cells differentiated or proliferated (Marshall, 1995). The subsequent understanding of pathway architecture, including feedback loops, provides a deeper mechanistic view of how intracellular signaling through shared pathways produces different transcriptional responses (Nakakuki et al., 2010). Determining the amplitude and duration of signaling and specific transcriptional outputs induced by Spi and Vn will help shed light on potential differences between the ligands. These might account for the incomplete rescue of *vn* mutants by the TGF-α ligands and the patterning defects they induced in the wing. Nevertheless, the substantial rescue resulting from ligand substitution prompts the question of why multiple ligands have evolved in the fly when it seems that, in theory, fewer could suffice. Complex animals like the fly might have evolved a collection of high- and low-affinity ligands that are active in specific patterns and at different levels because this is more parsimonious than deploying a multitude of tissue-specific modulators to fine-tune the actions of fewer ligands.

MATERIALS AND METHODS

Fly stocks

The following mutant alleles were used: spi^{2A}, grk^{HF}, vn^{1}, vn^{L6}, Krn^{27}, Krn^{9}, $Egfr^{3F18}$ and $Egfr^{F24}$. The following transgenes were used: *vn-Gal4*, *sd-Gal4*, *UAS-vn1.1* (Schnepp et al., 1996); *UAS-vnΔIg* (Donaldson et al., 2004); *UAS-rho*, *UAS-mspi::GFP* (Tsruya et al., 2002); *UAS-sspi* (Schweitzer et al., 1995); *UAS-sKrn* (Urban et al., 2002); *UAS-sgrk* (Queenan et al., 1999); *UAS-vn::SpiEGF* (Schnepp et al., 1998); *UAS-*spi^{dsRNA} (TRiP 34645 and 28387); *UAS-*vn^{dsRNA} (VDRC 50358 and 109437) and $tubP\text{-}Gal80^{ts}$. Stocks with combinations of alleles were generated from single mutants, with the exception of a spi^{2A}, grk^{HF}/*CyO* stock that was a gift from Trudy Schüpbach (Princeton University, NJ, USA). The *vn-Gal4* allele was obtained using the method of Sepp and Auld (1999). The *vn-lacZ* element *(P[PZ,* ry^{+}*]* vn^{rF264}, FBti0005059) was replaced with the *P[GawB,* w^{+}*]* element in the *PG142/FM7* stock (a gift from Norbert Perrimon, Harvard Medical School, Boston, MA, USA). The *Gal4* element in the *vn-Gal4* allele is in the 5′-UTR, 590 bp upstream of the open reading frame. *Krn* recombinants were genotyped by PCR, as homozygotes have no phenotype. Primers were used to amplify across the deleted region (8 kb in WT) and gave a band of ∼1 kb for Krn^{27} or ∼1.2 kb for Krn^{9} (F: AGTCGGCGGCCGTCAATTCG, R: TCCTGGGGCTCCT-TGCGTGT).

Protein analysis

Immunoblotting and immunohistochemistry were conducted using standard protocols with the following antibodies: anti-Phospho-p44/p42 MAPK (Cell Signaling Technology, #9106; 1:1000), anti-ERK 2 (Santa Cruz Biotechnology, K-23; 1:2000), anti-Tshirt (a gift from Steven Kerridge, Developmental Biology Institute of Marseille, France; 1:500), anti-GFP (Sigma, #G 1544; 1:1000) and anti-Wingless (Developmental Studies Hybridoma Bank, 4D4; 1:166).

Acknowledgements

We thank our colleagues Henri Jasper, Denise Montell, Norbert Perrimon, Trudi Schüpbach and Jessica Treisman, the Bloomington Stock Center for fly stocks, and Cassandra Jones and Adeline Ding, high school students at the time, for technical help.

Competing interests

The authors declare no competing financial interests.

Author contributions

All authors performed experiments, analyzed data, prepared the figures and wrote the manuscript.

Funding

This work was funded by the National Science Foundation [IOS 090231 to A.S.], a Pelotonia Fellowship (C.L.A.) and a Seilhamer Fellowship (S.N.M.).

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.110171/-/DC1

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