Development 128, 3533-3542 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV8795

four-jointed interacts with *dachs, abelson* and *enabled* and feeds back onto the *Notch* pathway to affect growth and segmentation in the *Drosophila* leg

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Accepted 26 June 2001

SUMMARY

The molecular basis of segmentation and regional growth during morphogenesis of *Drosophila* legs is poorly understood. We show that *four-jointed* is not only required for these processes, but also can direct ectopic growth and joint initiation when its normal pattern of expression is disturbed. These effects are non-autonomous, consistent with our demonstration of both transmembrane and secreted forms of the protein in vivo. The similarities between *four-jointed* and *Notch* phenotypes led us to further investigate the relationships between these pathways. Surprisingly, we find that although *four-jointed* expression is regulated downstream of Notch activation, *four-jointed* can induce expression of the Notch ligands, Serrate and Delta, and may thereby participate in a feedback loop with the Notch signaling pathway. We also show that *four-jointed* interacts with *abelson, enabled* and *dachs*, which leads us to suggest that one target of *four-jointed* signaling is the actin cytoskeleton. Thus, *four-jointed* may bridge the gap between the signals that direct morphogenesis and those that carry it out.

Key words: Four-jointed, Abelson, Enabled, Dachs, Serrate, Delta, Leg segmentation, *Drosophila melanogaster*

INTRODUCTION

While the establishment of anterior-posterior and dorsal-ventral axes in the *Drosophila* embryo and imaginal discs have been extensively studied (Lawrence, 1992), much less is known about the establishment of the proximal-distal (PD) axis. In the leg imaginal disc, the early steps in axis initiation have been largely elucidated, but later steps of leg segmentation and growth remain poorly understood (Brook et al., 1996; Couso and Bishop, 1998; Irvine, 1999). Previous studies have demonstrated that activation of the Notch (N) receptor controls two important morphogenetic events in the leg: growth and segment boundary formation. The N ligands, Serrate (Ser) and Delta (Dl), are expressed in a series of concentric rings, one ring per segment, along the PD axis of the developing leg. Loss of N signaling by removal of N or its ligands results in a failure in the formation of segment boundaries and in the reduction of leg growth (Shellenbarger and Mohler, 1978; Parody and Muskavitch, 1993; Speicher et al., 1994; de Celis et al., 1998; Bishop et al., 1999; Rauskolb and Irvine, 1999). Furthermore, N signaling is sufficient to promote leg segmentation and growth as ectopic N signaling induces the formation of ectopic segment boundaries and local cell proliferation (de Celis et al., 1998; Bishop et al., 1999; Rauskolb and Irvine, 1999).

It is likely that N signaling controls morphogenesis through the activation of one or more effector pathways, yet the exact nature of these downstream pathways remains unknown. In the leg, several genes have been shown to be regulated by N signaling*: four-jointed (fj), nubbin*, *odd skipped*, *big brain* and *AP-2* (de Celis et al., 1998; Rauskolb and Irvine, 1999; Kerber et al., 2001). Of these, only *fj*, *nubbin* and *AP-2* have been shown to be required for any aspect of limb development (Villano and Katz, 1995; Brodsky and Steller, 1996; Cifuentes and Garcia-Bellido, 1997; Kerber et al., 2001). Furthermore, since N is activated at every segment boundary, it is likely that distinct pathways become activated downstream of N signaling to confer more specific patterns of growth and identity to each leg segment. Thus, to fully understand the processes of segmentation and growth, it is necessary not only to identify the genes that are regulated by N, but also to characterize their roles during leg development in greater detail.

Fj is regulated by N activation in the leg, eye and wing, and thus may be an important mediator of N function in a wide range of tissues (Rauskolb and Irvine, 1999; Zeidler et al., 1999; Papayannopoulos et al., 1998; C. R., unpublished observations). Although *fj* codes for a conserved protein (Ashery-Padan et al., 1999), the sequence of Fj is novel and therefore offers no clues as to its biochemical functions. In vitro analysis has suggested that *fj* encodes a type-II transmembrane protein that can be cleaved to release the C-terminal domain as a secreted peptide (Villano and Katz,

1995). Mosaic analysis in the leg (Tokunaga and Gerhart, 1976), eye (Zeidler et al., 1999), wing and abdomen (Zeidler et al., 2000) have indicated that *fj* can act non-autonomously, consistent with a secreted signaling ligand, although secretion has not been demonstrated directly in vivo. Moreover, no studies to date have identified the molecular components of the effector pathway through which Fj acts.

The expression pattern and mutant phenotype of *fj* is consistent with a role in growth and segmentation of the leg. The *Drosophila* leg is composed of nine segments. The five most distal segments (those furthest from the body) constitute the tarsus and moving proximally, the tibia, femur, trochanter and coxa. Fj is expressed in a series of concentric rings in most or all segments of the developing leg and is required for the growth of the femur, tibia and tarsal segments 1-3. In addition, *fj* mutants lack the joint between tarsal segments 2 and 3 (Waddington, 1943; Tokunaga and Gerhart, 1976; Villano and Katz, 1995; Brodsky and Steller, 1996). However, especially in the tarsus, its expression is clearly associated with forming segment boundaries, raising the possibility that a requirement for *fj* in the segmentation of additional leg segments may be masked by genetic redundancy, as has been previously suggested for the eye (Zeidler et al., 1999).

Fj is not only required for segmentation and growth of the *Drosophila* leg, but also for PD growth in the wing, ommatidial polarity in the eye, and epithelial planar polarity in the wing and abdomen (Waddington, 1943; Villano and Katz, 1995; Brodsky and Steller, 1996; Zeidler et al., 1999; Zeidler et al., 2000). Importantly, all of these processes not only require *fj* function, but also involve dynamic changes in the actin cytoskeleton (Fristrom and Fristrom, 1993; Eaton, 1997; Mlodzik, 1999). We were therefore interested to determine whether Fj was involved in the instructions and/or in the execution of the morphogenetic events that it affects.

Genes that when mutant interrupt segmentation of the leg and growth of the wing in a manner similar to Fj are rare and are candidates for members of a Fj signaling pathway. Loss-of-function mutations in *dachs (d)* produce phenotypes in the leg and wing that resemble those of *fj* (Waddington, 1943). By contrast, over-expression of the *abelson* (also known as *abl oncogene; abl*) gene using the UAS-Gal4 system produces truncations and fusions of the tarsal segments of the leg (F. Michael Hoffmann, per comm; G. R. B. and F. N. K., unpublished data) and wing vein phenotypes (G. R. B. and F. N. K., unpublished observations) similar to loss of *fj* expression. *Drosophila* Abl, a homolog of the mammalian *c*-Abl proto-oncogene, encodes a cytoplasmic tyrosine kinase (Henkemeyer et al., 1988). A major substrate for Abl kinase activity is the Ena (also known as Enb) gene product, which is a member of the Ena/Mena/VASP family (Gertler et al., 1990; Gertler et al., 1995). Ena homozygotes are embryonic lethal and imaginal phenotypes have not been analyzed. Interestingly, both Abl and all members of the Ena family bind directly to F-actin and to genes that influence actin polymerization (Hoffmann, 1991; McWhirter and Wang, 1991; Wang, 1993; Gertler et al., 1995; Gertler et al., 1996; Reinhard et al., 1995; Bachmann et al., 1999). Moreover, both have been shown to be regulators of actin dynamics in diverse tissues (Gertler et al., 1996; Plattner et al., 1999; Lewis et al., 1996; Bear et al., 2000; Korey and Van Vactor, 2000; Lanier and Gertler, 2000; Vasioukin et al., 2000). Together, these data led us to investigate the relationships between these genes and *fj*.

We have explored the biochemistry of Fj, its place in the segmentation and growth hierarchy, and its effector pathway. Our results indicate that Fj can act as a secreted signaling molecule, consistent with our biochemical data. We show that *fj* is sufficient for joint initiation and growth in the leg, placing it high in the hierarchy of leg segmentation and growth along the PD axis. Surprisingly, we find that Fj and N signaling participate in a feedback loop, which has implications for how N activation may be regulated in other tissues. We also used genetic interactions to identify candidates for a Fj effector pathway, which lead us to suggest that actin may be one target of *fj* signaling.

MATERIALS AND METHODS

Genetics

Unless otherwise mentioned, all mutations are as described in FlyBase (1999). The wild-type control stock was OregonR (OrR). $d^{nc104.6}$ and d^{210} are strong alleles of *d* kindly provided by Eric Liebl and F. Michael Hoffmann. Flies carrying the reporter construct I-2.2 have been described previously (Bachmann and Knust, 1998). *Fj-lacZ* (fj^{p1}) and fj^{d1} were described by Brodsky and Steller (Brodsky and Steller, 1996). For the genetic interactions described in Fig. 6, the phenotypes of males and females of the fj^{N7} stock and of selected genetic interaction stocks were first analyzed separately. No significant differences were found. Thereafter, data from males and females were combined to generate the table in Fig. 6A.

Generation of Fj antibodies, western blots and molecular biology

Unless otherwise stated, all standard molecular techniques were as described by Sambrook et al. (Sambrook et al., 1989). To generate the anti-FjC antibody, the C-terminal domain of the *fj* cDNA (beginning at the *Bgl*II site immediately following the transmembrane domain and including the C-terminal 460 amino acids of the Fj protein) was cloned into the pET 28b expression vector (Novagen) in-frame to a 6xHis tag at the N terminus. The protein was expressed in bacteria and purified over a Ni-charged resin, as per the Novagen protocol. Considerable proteolysis of the protein occurred. The predominant fragment at 20 kDa (Fig. 1B: lane 1, arrow) was used for injection into rabbits. Serum was recovered that recognized the full-length C-terminal domain expressed in bacteria (Fig. 1B), as well as Fj protein produced from *fj* mRNA in an in vitro translation system (data not shown). Serum was further affinity-purified against the bacterial Fj products and used at a concentration of 1:1000 on western blots using goat anti-rabbit secondary antibody (BioRad) at 1:30,000 and ECL chemiluminescence visualization (ECL labs), as per the manufacturer's protocol. For western blots of larval tissue, the anterior third of third instar larvae, including the cephalic complex and associated discs, was isolated, homogenized, and incubated with Concanavalin A (ConA) Sepharose beads (Pharmacia Biotech) to enrich the glycoprotein fraction before loading on SDS-PAGE.

Construction of stable S2:*fj* cell lines

A 2.2 kb fragment from the *fj* cDNA containing the entire *fj* open reading frame (ORF), but with most of the 5′-UTR removed, was cloned into the pCaSpeR4-hs vector (Pirrotta, 1988) behind an hsp70 promoter (creating pHS-fj^{+}). 12 μg of purified DNA was co-transfected into 4.5×10^{6} S2 cells (Schneider, 1972) with 3 μg pPC4 (Jokerst et al., 1989) using the lipofectin method (BRL), as per the manufacturer's protocol. Cells resistant to α-amanitin (Boehringer)

were sub-cloned by limiting-dilution cloning, and positive colonies (S2:*fj* clones) were identified by incubation with anti-FjC. In subsequent experiments, three highly expressing lines (D, A4 and 11) all gave comparable results.

Subcellular fractionation of S2 cells

S2 or S2:*fj* cells were grown in 24-well plates to confluence in Schneider's *Drosophila* medium with L-glutamine (GIBCO-BRL) containing 10% fetal calf serum (FCS; Hyclone) at 1 ml medium/well. After a 30 minute heat shock at 37°C, they were incubated at 25°C for 30 minutes, washed in pre-warmed Schneider's medium without FCS, and incubated with 200 μl Schneider's without FCS for 5 hours. From a single well, cells were separated from supernatant by centrifugation at 400 ***g*** for 5 minutes. Cells were solubilized in Laemmli sample buffer (LSB; Laemmli, 1970). The supernatant was precipitated with trichloroacetic acid (TCA) and solubilized in LSB. Alternatively, the contents of six wells were combined, cells were separated from supernatant as above, washed in Schneider's, and used for subcellular fractionation as described by Hortsch (Hortsch, 1994), with an initial volume of 200 μl. Supernatant fractions were concentrated by TCA precipitation and both supernatants and pellets were resuspended in LSB. Aliquots from all fractions were loaded on SDS-PAGE gels, western blotted, and analyzed with anti-FjC.

Generation of transgenic lines and heat shock analysis

pHS-fj^{+} was used for germline transformation into w^{1118} embryos, as described by Spradling (Spradling, 1986). Eight lines were recovered, which showed varying degrees of *fj* expression and phenotype after heat shock. The line designated P60 was used in the analysis presented. Transgenic flies containing two copies of an insertion of *pHS-*fj^{+} were subjected to an approximately 30 minute heat-shock every 6 hours, starting at 24-42 hours after egg laying (AEL) and terminating 72-90 hours AEL, at 25°C using a water bath system. They were then allowed to develop through eclosion at 29°C.

Histology, immunohistochemistry and the generation of *fj* flip-out clones

Adult legs were prepared and mounted as previously described (Villano and Katz, 1995). Antibody stains were done as described previously (Rauskolb and Irvine, 1999) using the following antibodies: rat anti-Ser (Papayannopoulos et al., 1998), mouse anti-Dl (C594.9B; Developmental Studies Hybridoma Bank), and goat anti-β-galactosidase (Biogenesis). Secondary antibodies from Jackson ImmunoResearch Laboratories (Cy3-conjugated) or Molecular Probes (Alexa Fluor 488) were used. In the *patched* (*ptc*)-Gal4 UAS-*fj* experiment the larvae were raised to third instar or adulthood at 18°C, while experiments with all other drivers were carried out at 25°C. Flip-out clones were induced at 48-72 hours AEL using an AyGal4 UAS-GFP chromosome with the flip-out Gal4 technique, whereby clones will be marked in adult legs by the loss of the $yellow^{+}$ marker from the flip-out cassette (Struhl et al., 1993; Ito et al., 1997). UAS-*fj* on the first chromosome (with *decapentaplegic* (*dpp*)-Gal4 and 69B-Gal4 drivers) and on the third chromosome (with *ptc*-Gal4, *engrailed* (*en*)-Gal4, *dpp*-Gal4, and flip-out clones) were used in these experiments (Zeidler et al., 1999).

RESULTS

Four-jointed is found in both transmembrane and secreted forms

To understand Fj function, we first sought to clarify whether Fj exists in vivo as a secreted or a transmembrane protein. *Drosophila* S2 cell lines containing the *fj* cDNA under the control of a heat-shock promoter were generated (S2:*fj*). Homogenates from these cells and from the parent S2 line were analyzed on western blots using antibodies generated against

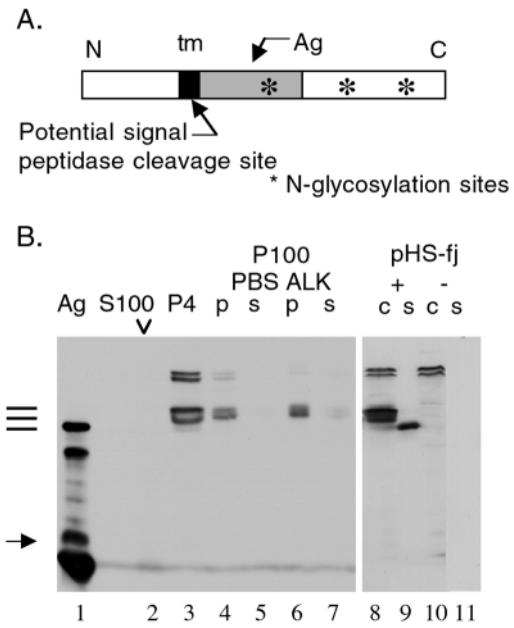

Fig. 1. Subcellular fractionation of Fj in cultured cells. (A) Schematic diagram of the Fj protein, showing the relevant domains referred to in the text. Tm, transmembrane domain; Ag, approximate extent of the antigen used to generate anti-FjC. (B) Western blot analysis of Fj expression. Lane 1: expression of the C-terminal domain of Fj in bacterial cells. The slowest migrating band, of M_r 63.5×10^3, represents the intact C-terminal domain. Considerable protein degradation occurs. The arrow indicates a fragment of approximate M_r 20×10^3, used as the antigen (Ag) to generate anti-FjC. Lanes 2-7: subcellular fractionation of heat-shocked S2:*fj* cells containing a HS-*fj* cDNA insertion. S100, 100,000 ***g*** supernatant; P4, 4000 ***g*** pellet; P100, 100,000 ***g*** pellet; pellet (p) and supernatant (s) after treatment of the P100 pellet with either PBS or 0.1 M Na_2CO_3 (ALK). Lanes 8-11: expression of Fj in intact, washed cells (c) and extracellular medium (s) of S2 cells with (+) or without (–) the HS-*fj* cDNA (pHS-*fj*) insertion. The lines at the far left designate Fj-specific bands. The blot was developed with anti-FjC.

Fj (Fig. 1A). Antisera detected three major bands that were present in the S2:*fj* cells after heat-shock but were absent from the S2 parent line (Fig. 1B). In cell fractionation experiments of the S2:*fj* cells, the two more slowly migrating forms behaved as integral membrane proteins, as they segregated with both the heavy and the light membrane fractions and could not be washed off the membranes by alkaline treatment. Their sizes were consistent with the full-length protein predicted from sequence analysis. By contrast, the smallest polypeptide was secreted into the medium and co-migrated on SDS-PAGE with the intact C-terminal domain (Fig. 1B).

To detect Fj reliably in larval tissue, glycoproteins were first concentrated by precipitation with ConA Sepharose beads before loading on the gel for western blot analysis. Under these conditions, three polypeptides of the same size seen in cultured cells were also detected in homogenates from wild-type third instar larvae but were absent from homogenates produced from larvae homozygous for mutant alleles of *fj* (Fig. 2A). Just as the in vitro forms were shown to be altered by endoglycosidase H treatment and therefore N-glycosylated (Villano and Katz, 1995), so all three larval forms could be bound by ConA and

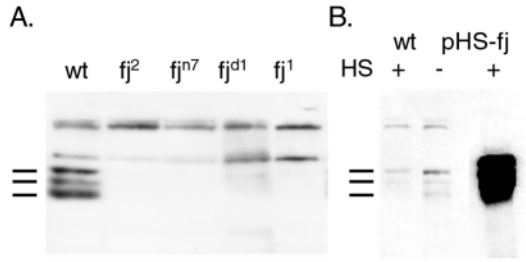

Fig. 2. Western blot analysis of Fj expression in larval tissue. ConA-concentrated extracts from third instar larvae of wild-type flies compared with (A) *fj* mutant flies and (B) transgenic line P60 containing the pHS-fj^{+} transposon, with or without a 30 minute heat shock. The blots were stained with anti-FjC. All lanes in A and B contain extracts derived from equal numbers of larvae, but blot B was developed for a shorter period of time. The three forms of the Fj protein are indicated by lines to the left of the blots.

therefore contained asparagine-linked core glycosylation. To avoid any artifact due to ConA selection, we also evaluated the stoichiometry of the three protein forms in whole homogenates (data not shown). While the relative abundance of the three forms is somewhat variable, under all conditions the largest transmembrane form is present in equal or up to five-fold excess of the secreted form. Thus a significant fraction of Fj protein remains membrane-bound, although some protein molecules are cleaved and secreted.

Fj is sufficient for joint formation and growth in the leg

Fj is expressed in a series of concentric rings in the developing leg and its expression in the tarsus is tightly associated with forming segment boundaries (Villano and Katz, 1995; Fig. 5A,B). *Fj* is necessary for the formation of the T2/3 joint and is required for growth of the femur, tibia and tarsal segments 1-3. If Fj is a key regulator of these morphogenetic processes, we might expect that Fj would also be sufficient to initiate joint formation and growth in the leg. Furthermore, since *fj* is expressed in only a subset of the cells within each leg segment, we also investigated whether distinct domains of *fj* expression are indeed important for proper leg development.

Ubiquitous expression of *fj* in the developing legs was accomplished by either inducing HS-*fj* (Figs 2B, 3C) or by driving UAS-*fj* (Zeidler et al., 1999) with 69B-Gal4 (Brand and Perrimon, 1993; Fig. 3D). We found that ectopic *fj* expression is capable of inducing the formation of ectopic joint-like structures, which resemble the partial joints seen at T2/3 in *fj* hypomorphic alleles (Fig. 3B). In flies homozygous for the HS-*fj* insertion, 80.5% (n=41) of legs contained such ectopic partial joints. These appeared as donut-shaped invaginations in T3 (Fig. 3C) or, much less frequently, in T2 and T4 (data not shown), usually centered approximately equidistant from the flanking joints. These invaginations resembled the ball-and-socket structure of a normal tarsal joint (Held et al., 1986) and, like normal joints, contained only bare cuticle. In the HS-*fj* flies, these ectopic structures occurred with minimal loss of growth in the segment and no loss of endogenous joints (Fig. 3C). These results suggest that *fj* is sufficient to initiate joint formation in the tarsus and that this capacity is largely independent of growth control in the segment. The UAS-*fj*

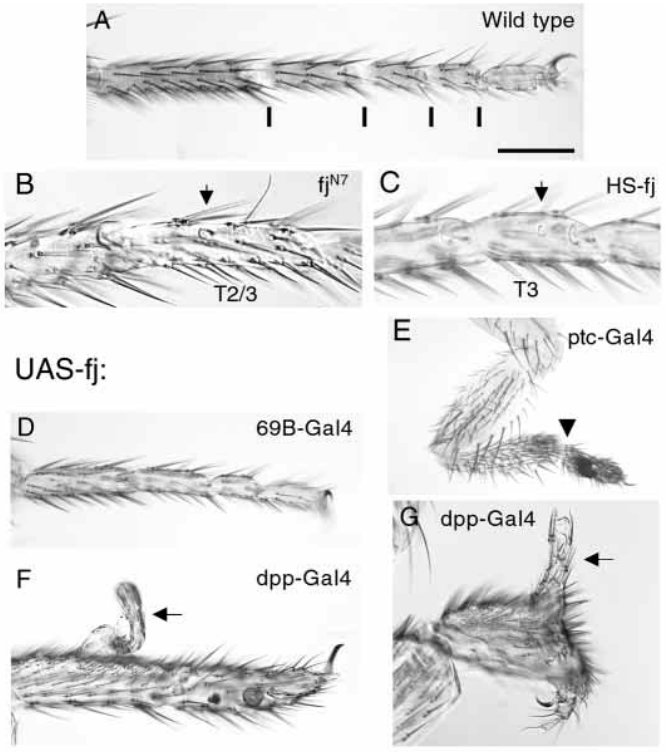

Fig. 3. Leg patterning defects resulting from ectopic expression of *fj*. A,D,F, and G are adult female complete tarsi. E is an adult male leg showing the tarsus, tibia and femur. (A) Wild type. The lines indicate the joints separating the five tarsal segments. (B) fj^{N7}. An incomplete joint is present at T2/3 (arrow). (C) HS-*fj*. An ectopic joint-like structure is present in the center of the T3 segment (arrow). (D-G) UAS-*fj* driven by: (D) 69B-Gal4, with truncations and loss of the T2/3 joint; (E) *ptc*-Gal4, with significant truncation and segment fusions of the tarsus. The arrowhead points to the juncture between the tarsus and the tibia. The sex comb is obvious on the tarsal remnant. (F,G) *dpp*-Gal4, with representative leg truncation and segment fusions, cuticular abnormalities, and outgrowths (arrows). The outgrowth in F appears to be segmented and both outgrowths contain bristles. The *dpp*-Gal4 stock alone has no detectable phenotypes (data not shown). Bar in A represents 50 μm in A,D,F, and G; 10 μm in B and C; and 100 μm in E.

driven expression caused more widespread ectopic joint-like structures in the tarsus, additional loss of PD growth within the tarsal segments, and loss of the T2/3 joint (Fig. 3D).

Patterned misexpression of *fj* across multiple segment boundaries caused more dramatic results. Expression of *fj* along the AP compartment boundary using UAS-*fj* and *ptc*-Gal4 (Hinz et al., 1994) resulted in legs with reduced leg growth and segmental fusions, as had been previously reported by Zeidler et al. (Zeidler et al., 2000). The effects were particularly dramatic in the tarsus (Fig. 3E). A similar effect was seen when *dpp*-Gal4 (Staehling-Hampton et al., 1994) was used as the driver. In addition, these animals displayed occasional outgrowths from the leg (Fig. 3F,G). Some of these outgrowths appeared to be segmented (Fig. 3F) and most contained at least some bristles. Similar outgrowths and truncations were seen when expression was driven with *en*-Gal4, which is expressed within the posterior compartment of the leg disc (data not shown). Together, these results suggest that the endogenous pattern of *fj* expression

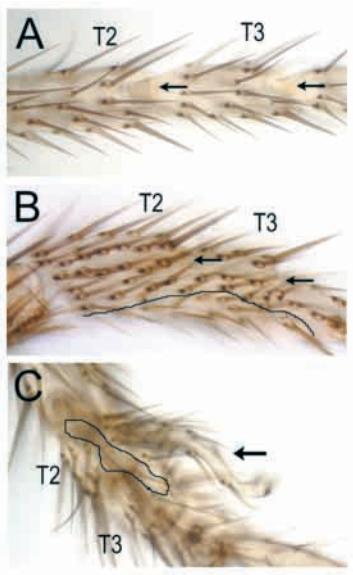

Fig. 4. Fj affects segmentation and growth non-autonomously. Portions of tarsi of adult legs containing flip-out clones expressing *fj*. The *fj*-expressing clones are marked with *yellow* and are outlined. (A) Wild type. Arrows indicate that joints are visible around the circumference of the leg. (B) *fj*-expressing clone in T2-T4. Arrows denote the extent of joint structures, which do not form around the circumference of the leg. Note that joint formation is inhibited far from the cells expressing *fj*. *(C) fj*-expressing clone in T2-T3. A leg outgrowth is induced nonautonomously (arrow); the outgrowth does not include any *yellow* bristles.

is critical to its function in both growth control and segmentation of the leg.

We also examined smaller, randomly positioned clones ectopically expressing *fj* to address whether *fj* affects leg growth and segmentation non-autonomously, as would be predicted if Fj acts as a signaling molecule. Smaller patches of *fj*-expressing cells were produced using the flip-out actin-Gal4 technique (Struhl et al., 1993; Ito et al., 1997), and such clones were marked by the cuticular marker *yellow*. Clones expressing *fj* that spanned a segment border resulted in the fusion of most segments, consistent with our results above. Effects were most dramatic in the tarsus, with a fusion of tarsal segments and accompanying reduced growth (data not shown). Although in all examples an autonomous influence of *fj* was observed, in many instances we also observed apparent non-autonomy, in which joint structures were lost both within and adjacent to the clone (compare Fig. 4A and B; see also, Discussion).

In addition to the disruptions in leg segmentation and growth observed with larger clones, occasional outgrowths from the leg were found (Fig. 4C). Importantly, these outgrowths were entirely composed of wild-type tissue, while the *fj*-expressing clone neighbored the outgrowth. Together, our results strongly argue that *fj* is a key regulator of leg segmentation and growth, and that *fj* can function non-autonomously in these processes.

Fj can regulate Ser and Dl expression

Fj is regulated downstream of N signaling and many of the phenotypes observed with ectopic *fj* expression are similar to those seen upon ectopic activation of N (de Celis et al., 1998; Bishop et al., 1999; Rauskolb and Irvine, 1999). It is possible that these similarities might derive from a common molecular cause. For example, deregulation of N signaling may cause a deregulation of *fj* expression, which would then disrupt normal morphogenesis. Alternatively, as *fj* is known to have a transcriptional feedback on its own expression (Zeidler et al., 1999), perhaps it also participates in a feedback loop onto the N pathway such that misexpression of *fj* actually results in misexpression of activated N. The most likely target for such feedback would be the N ligands, as N is expressed widely in the disc but only becomes activated at the restricted positions of ligand expression (de Celis et al., 1998; Bishop et al., 1999; Rauskolb and Irvine, 1999).

To investigate whether Fj feeds back onto the N signaling

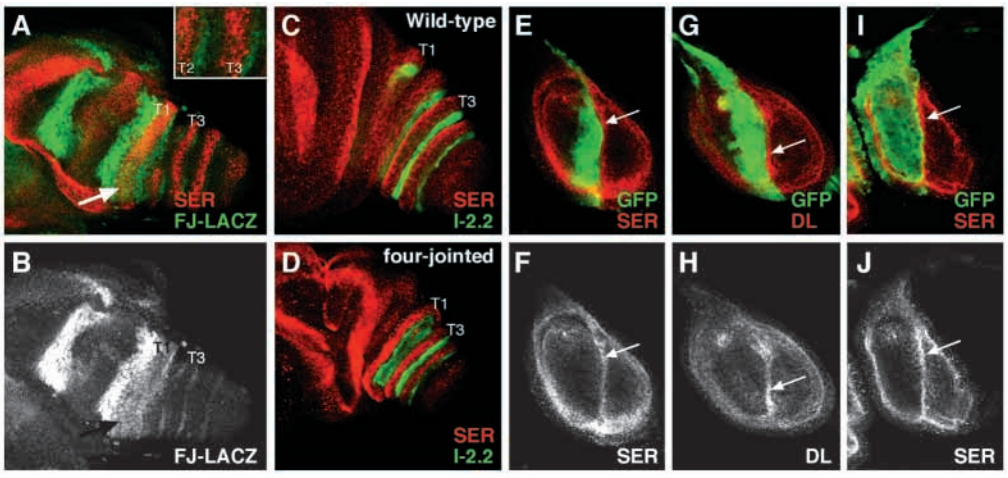

Fig. 5. Fj regulates N ligand expression. (A,B) Comparison of *fj-lacZ* (green) and Ser (red) expression in everting pupal leg. Expression is complementary in tarsal segments T2-4 (inset of T2 and T3) while expression overlaps in T1, where *fj*-β-gal expression is lower in cells also expressing Ser (arrow). (C,D) Ser expression (red) in wild-type (C) and fj^{d1} (D) everting pupal legs. To help identify the tarsal segments, we used a reporter gene construct, I-2.2 (green; Bachmann and Knust, 1998), which is expressed in T1-4 in a pattern complementary to endogenous Ser. Ser expression is dramatically reduced in T2 of fj^{d1} while I-2.2 expression remains. (E-H) *ptc*-Gal4 UAS-GFP UAS-*fj* mid-third instar leg discs. *ptc*-Gal4 drives expression of GFP (green) and *fj* in a stripe along the anterior side of the AP compartment boundary. (E,F) Ser (red in E and white in F) expression is induced in cells adjacent to those expressing *fj* (arrows). (G,H) Dl (red in G and white in H) expression is also induced in cells adjacent to *fj*-expressing cells (arrows). (I,J) *dpp*-Gal4 UAS-GFP UAS-*fj* mid-third instar leg discs. *dpp*-Gal4 drives expression of GFP (green) and *fj* in the anterior compartment. Ser (red in I and white in J) is induced non-autonomously in posterior cells abutting those expressing high levels of *fj* (arrows). Ser and Dl were visualized by antibody staining.

pathway, we examined the expression of the N ligands Ser and Dl in leg discs in which *fj* was ectopically expressed along the AP axis using *ptc*-Gal4-driven expression of UAS-*fj* (Fig. 5E-H). Such misexpression of *fj* results in severe truncation of the tarsus (Fig. 3E). *ptc* is expressed at highest levels along the AP boundary, with graded expression in the anterior compartment of the disc (Fig. 5E,G). We found that ectopic *fj* expression induced the expression of both Ser and Dl along the posterior edge of the *fj*-expressing stripe, and did so largely non-autonomously. The non-autonomy is consistent with the aforementioned biochemical data, and provides further evidence that Fj acts as a signaling molecule. Furthermore, the ectopic expression of Ser and Dl, leading to ectopic activation of N, could account for some of the observed effects of ectopic *fj* expression on leg development.

The asymmetry of induction only along the border of highest expression raised the possibility that induction might only occur at sharp boundaries of expression, such as that on the posterior edge of the *ptc* domain. To test this, we expressed UAS-*fj* with two additional drivers, *dpp*-Gal4 and *en*-Gal4, which are both expressed at somewhat lower levels than *ptc*-Gal4 (C. R., unpublished observations). *dpp*-Gal4 is expressed within the anterior compartment of the leg disc (Morimura et al., 1996; Fig. 5I), while *en*-Gal4 (FlyBase, 1999) is expressed in the posterior compartment with a sharp boundary of expression along the AP border (as confirmed in these experiments). Misexpression of *fj* under either driver produced truncations of the tarsus as well as apparent outgrowths and/or bifurcations of the distal leg (Fig. 3F,G and data not shown). As with *ptc*-Gal4, both *dpp*- and *en*-Gal4-driven expression of *fj* induced expression of Ser in cells neighboring those expressing high levels of *fj*: at the posterior edge of the *dpp* domain (Fig. 5I,J) and at the anterior edge of the *en* domain (data not shown). Similar non-autonomous induction of Dl was observed with these drivers (data not shown).

We also investigated whether *fj* is required for normal Ser expression. We examined Ser expression in pupal leg discs homozygous mutant for *fj*. Expression of Ser is unaffected in all leg segments except for one; Ser expression is significantly reduced in the second tarsal segment (compare Fig. 5C and D). This finding is consistent with the observation that *fj* mutants have a partial or complete lack of the joint between the second and third tarsal segments and reduced growth within the fused segment.

Since *fj* induces Ser expression non-autonomously, it was of interest to examine their endogenous expression patterns during development of the leg. Consistent with the inductive behavior we have observed, *fj* and Ser appear to be expressed in adjacent but largely non-overlapping stripes in tarsal segments 2-4 in the developing leg disc (Fig. 5A,B).

Together, these results suggest that there is a feedback loop between N ligand expression and the N target gene *fj*. *Fj* appears to be necessary for the initiation, upregulation, or maintenance of Ser expression. We note, however, that although *fj* is expressed in every tarsal segment, Ser expression is only affected by loss of *fj* in tarsal segment 2.

Dachs, *abl, ena and Ser* are dominant enhancers of *fj*

To begin to understand how Fj signaling affects such diverse processes as leg segmentation and growth, ommatidial rotation,

A.

Genotype	n	% Complete	% Partial	% Fusion
fj^4 / fj^4	109	34	66	0
$fj^4 / d^{nc104.6}\, fj^4$	105	0	100	0
fj^{N7} / fj^{N7}	278	0	96.7	3.3
$fj^{N7} / d^{210}\, fj^{N7}$	77	0	64.9	35.1
$fj^{N7} / fj^{N7}\, ena^{23}$	146	0	25.1	74.9
$fj^{N7} / fj^{N7}\, ena^{210}$	117	0	76.9	23.1
fj^{N7} / fj^{N7} ; $abl^{-} / +$	109	0	25.6	74.4
fj^{N7} / fj^{N7} ; $Ser^{RX106} / +$	110	0	57.3	42.7
fj^{N7} / fj^{N7} ; $Ser^{94C} / +$	104	0	76.2	23.8

B.

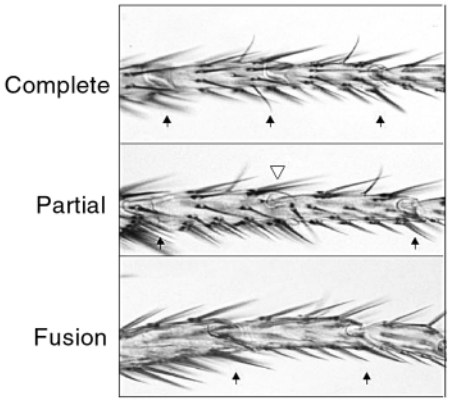

Fig. 6. Dominant enhancement of the *fj* phenotype. (A) Table showing genetic interactions with *fj* hypomorphic alleles. *N*, number of legs analyzed. *Abl*$^{-}$, *Df(3L)stj7*, a deletion that removes the *abl* gene. All interactions are statistically significant at $P<0.001$ by the χ^2 test (Devore and Peck, 1997). (B) Illustration of 'complete' joints (black arrows), 'partial' joints (white arrowhead), and 'fusion' phenotypes.

and epithelial planar polarity, we sought to identify other genes with which Fj interacts. Some of the effects of Fj are likely to be due to its feedback onto the N signaling pathway, as described above, and this would presumably require a Fj signal transduction pathway. In addition, it is likely that Fj also functions independently of its regulation of Ser and Dl, as the loss-of-function and gain-of-function phenotypes of N ligands and Fj are not identical.

Similar mutant phenotypes may indicate that the genes causing them may act in the same molecular pathway. As described in the Introduction, *d* and *abl* mutant phenotypes imitate those of *fj*, thus both of these genes are attractive candidates for the *fj* signaling pathway. A major substrate for Abl kinase activity is the Ena gene product (Gertler et al., 1995). *Ena* homozygotes are embryonic lethal and imaginal phenotypes are not known. However, Abl and Ena appear to function in the same pathway in *Drosophila* (Lanier and Gertler, 2000). Finally, given the molecular epistatic interactions we have observed between *fj* and *Ser*, above, we also wished to test whether *fj* and *Ser* interact genetically.

In our experiments we addressed whether strong alleles of *d*, *abl*, *ena* or *Ser* could genetically interact with *fj* mutations. To test the relationships of these genes, we made use of two hypomorphic alleles of *fj*, fj^4 (Villano and Katz, 1995) and fj^{N7}. The majority of legs of fj^{N7} flies retain partial joints of a ball

Table 1. Interactions between *abl* and *fj* reveal a function for Fj at the T1/2 segment boundary

fj^2/fj^2; $abl^-/+$	*n*	% Flies with 3-jointed tarsi
No transgene	176	31.0
$Tnabl^+$	154	1.3
$Tnabl^{K\text{-}N}$	176	25.4

Quantitation of the phenotypes of fj^2; *abl*/+ flies in the absence (no transgene) or presence of a wild-type ($Tnabl^+$) or kinase-dead ($Tnabl^{K\text{-}N}$) *abl* transgene. The fj^2 allele is a strong allele of *fj* that gives complete fusions at T2/3 but does not by itself affect any other joint (Villano and Katz, 1995). *Df(3R)stj7*, a deficiency that deletes *abl*, was used in these experiments. Flies were scored as 'three-jointed' if they displayed one or more legs showing partial joints or complete fusions at T1/2. In all combinations, the T2/3 segments were fused. *Abl* shows a similar interaction with the fj^{d1} allele (data not shown).

and socket morphology at the juncture between T2 and T3 (Fig. 6B), while fj^4 produces larger partial joints or complete joints at the T2/3 boundary (Villano and Katz, 1995). Introduction of one mutant copy of *d*, *abl*, *ena*, or *Ser* into these backgrounds significantly increased the severity of the hypomorphic phenotypes (Fig. 6A), while each of these genes by itself is wholly recessive in the leg (FlyBase, 1999 and data not shown). Thus, *d*, *abl*, *ena*, and *Ser* act as dominant enhancers of *fj*, suggesting these genes may be part of a common pathway or network.

Abl unmasks a requirement for *fj* at the T1/2 joint

Loss-of-function *abl* alleles are recessive and heterozygous *abl* flies have normal leg morphology. However, when one copy of *abl* was removed in a strong *fj* background, we observed the complete or partial loss of an additional tarsal joint at T1/2 on one or more legs in one third of the animals (Table 1). A wild-type *abl* transgene ($Tnabl^+$; Henkemeyer et al., 1990) could restore this joint, confirming that *abl* was indeed responsible for the interaction. To test if kinase activity was required for *abl* activity at this joint, we introduced a transgene with an inactive kinase ($Tnabl^{K\text{-}N}$; Henkemeyer et al., 1990) into the same genetic background. This transgene was unable to rescue the interaction, suggesting that *abl* kinase activity is indeed required. These results suggest that *abl* and *fj* participate in redundant pathways in the leg. Moreover, they demonstrate that *fj* is required at segment boundaries other than T2/3, which is the only boundary lost in *fj* null mutants. More widespread activity is consistent with the expression of *fj* at additional segment boundaries in the leg (Villano and Katz, 1995; Brodsky and Steller, 1996). Together with the genetic interactions described above, these results also reveal a concealed function for *abl* in leg morphogenesis.

DISCUSSION

What role might Fj play in the cascade of events leading to segment boundary initiation and growth? Fj is required for joint formation and growth in a restricted set of segments in the *Drosophila* leg. We have shown that its pattern of expression is critical to its function and that, under appropriate circumstances, *fj* can direct ectopic growth and joint initiation in the leg. While the consequences of loss of *fj* activity are more restricted than those of the N pathway, the similarities in some of its phenotypes led us to investigate further the relationships between these pathways. We have found that Fj participates in a feedback loop with N signaling. In addition, it interacts with genes whose products may directly regulate actin assembly. Thus, Fj may bridge the gap between the signals that direct morphogenesis and those that carry it out.

Fj participates in a feedback loop with N signaling

Localized expression of the N ligands and Fringe results in the local activation of N within each developing leg segment (de Celis et al., 1998; Bishop et al., 1999; Rauskolb and Irvine, 1999). This local activation of N controls the formation of segment borders (joints) and induces leg growth, presumably by regulating the expression of different sets of target genes that execute these morphological events. Identifying the target genes regulated downstream of N is crucial to any molecular understanding of how leg segmentation and growth occur. There will ultimately be some downstream genes involved specifically in forming the joints between segments and others involved in tissue growth. However, our results with Fj indicate that the regulation of leg segmentation and growth is more complex.

The Fj and N signaling pathways appear to affect each other's activity. Fj is regulated downstream of N signaling in the leg (Rauskolb and Irvine, 1999). However, we have presented three pieces of evidence that together suggest that *fj* interacts with components of the N signaling pathway and is capable of altering their expression. First, our studies demonstrate that ectopic *fj* has an inductive effect; both Ser and Dl are induced non-autonomously by cells expressing *fj*. Second, Ser expression is greatly reduced in tarsal segment 2 of *fj* mutant pupae. While this may be an indirect effect of an earlier loss of cells in that segment, it is also consistent with our induction results, suggesting that a failure to initiate or maintain Ser (and possibly Dl) expression in T2 contributes to the fusion of tarsal segments 2 and 3 observed in *fj* mutant legs. Clearly Fj is not necessary to regulate Ser expression in other segments (as Ser is still expressed outside of T2), nor are other boundaries lost in the mutant. Third, Ser acts as a dominant enhancer of *fj*, and thus an interaction between these genes is required to fulfill *fj* function. Together these data argue that Fj and N act in a feedback loop, which, we suggest, may help to refine their respective domains of expression and sharpen signaling events to the segment boundary. By the end of the third instar, the work of designating segment boundaries and growth in the tarsus is largely completed, and *fj* mRNA disappears abruptly at this time (Villano and Katz, 1995). It is possible that this rapid turn-off is due to inhibition by N, which is capable of turning off *fj* expression during this interval (Rauskolb and Irvine, 1999). In any case, a similar feedback regulatory loop has recently been described in the wing between N and its downstream target genes *wingless* and *cut* (Neumann and Cohen, 1996; de Celis and Bray, 1997; Micchelli et al., 1997) and such feedback regulatory interactions may well be a common feature of N function. Fj is also regulated by N in the *Drosophila* eye and wing (Papayannopoulos et al., 1998; Zeidler et al., 1999; C.R. unpublished observations), two tissues in which N signaling plays important morphological roles, and it would be

interesting to examine whether Fj is capable of influencing N ligand expresssion in those tissues. Furthermore, the N signaling cascade is deployed in a wide range of tissues not only in *Drosophila*, but also in vertebrates. A vertebrate *fj* homolog has been identified (Ashery-Padan et al., 1999); it will be interesting to determine whether Fj also functions as a mediator of N function during vertebrate development.

Multiple forms of Fj protein and non-autonomy of Fj function

We have shown that Fj protein isolated from *Drosophila* cell lines or larval tissues exists in both integral membrane and secreted forms. The predicted molecular structure of Fj suggests that it is a type II transmembrane protein, with two potential signal peptidase cleavage sites near the end of the transmembrane domain whose use would be consistent with the size of the secreted product we observe (Villano and Katz, 1995). However, we find that not all Fj is secreted, as two integral membrane forms remain present in all our preparations. While there are many examples of growth factors that have both transmembrane and secreted forms (Massague and Pandiella, 1993), all of these appear to undergo cleavage at or near the cell surface, including N and its ligand Dl (Artavanis-Tsakonas et al., 1999). If signal peptidase is indeed responsible for Fj cleavage (and this seems likely, as Fj is cleaved even in an in vitro microsomal preparation; Villano and Katz, 1995), it is unusually inefficient. Alternatively, it is possible that, in vivo, Fj is instead cleaved by a protease later in the secretory pathway or in response to signaling. This will need to be resolved in future experiments.

It still remains to be determined which forms of Fj have functional significance. Certainly, the non-autonomous effects of Fj in the wing, eye and abdomen, all implicate secreted Fj as biologically relevant (Zeidler et al., 1999; Zeidler et al., 2000). Indeed, in vertebrates Fj appears to be produced as a wholly secreted protein with the transmembrane domain serving as an N-terminal signal sequence (Ashery-Padan et al., 1999). In the gain-of-function clones described here, the induction of outgrowths composed of wild-type tissue similarly supports a non-autonomous role for Fj in the leg, although the non-autonomous influence may be indirect, for example by the early induction of a second growth factor. The failure of joint formation by wild-type tissue adjacent to our clones may also provide examples of non-autonomy. Alternatively, however, it is possible that an inhibition of joint formation within the clone mechanically inhibited nearby cells from forming joints. Tokunaga and Gerhart (Tokunaga and Gerhart, 1976) observed a similar local inhibitory influence on heterozygous cells adjacent to loss-of-function *fj* clones. Interestingly, the opposite was not observed: wild-type tissue was never seen to rescue joint formation within mutant clones. Again, this may represent the competing influences of induction from outside the clone and mechanical inhibition from within the clone. Thus the cooperative nature of joint development makes any determination of local non-autonomy ambiguous.

Local suppression of joint formation adjacent to our clones may also explain why ectopic joints were not seen along the borders of our flip-out clones, which produce sharp boundaries of expression that might be expected to resemble the normal patterning of *fj* expression. By contrast, ectopic joints were produced when *fj* was uniformly elevated in a wild-type background. While the significance of this remains unclear, we note that these ectopic structures tended to form in the center of the segment, where endogenous activity (and thus presumably additive activity) is lowest. Thus, in addition to the patterning of *fj* expression, the absolute level of Fj may determine whether joint initiation is permissible in any region of the disc.

We also note that *fj* induces expression of Ser and Dl in cells neighboring those ectopically expressing high levels of *fj*, again consistent with a non-autonomous activity. The induction of these ligands only on the posterior border of the *ptc*-driven clone suggested that the relevant signal might be a sharp boundary of expression, and our results with two additional drivers are consistent with such an interpretation. A similar dramatic effect of ectopic boundaries of *fj* expression has been observed in studies of *fj* activity in planar polarity of the eye (Zeidler et al., 1999). The requirement for a sharp boundary would also explain why Fj failed to induce expression of these ligands in the cells also expressing *fj*. Alternatively or additionally, high levels of Fj expression, such as that produced in our clonal analysis, may interfere with reception of the Fj signal, similar to what is observed with the N ligands Ser and Dl (termed autonomous inhibition; Panin and Irvine, 1998). Mechanistically this could occur if high level expression of the transmembrane-anchored Fj inhibited a cell from receiving the secreted Fj signal from a neighboring cell. This may be a mechanism by which to further regulate Fj signaling.

fj genetic interactions suggest that one consequence of Fj signaling may be alteration of the actin cytoskeleton

In addition to Fj's effects on N signaling, our genetic studies have shown that *fj* interacts with *d*, *abl* and *ena*. Both *abl* and *ena* have been shown to play significant roles in microfilament dynamics, including effects on cell shape change, intercellular adhesion, motility, and actin cytoskeletal rearrangements in response to extracellular ligands (Gertler et al., 1996; Lewis et al., 1996; Plattner et al., 1999; Bear et al., 2000; Korey and Van Vactor, 2000; Lanier and Gertler, 2000; Vasioukin et al., 2000). Moreover, we have shown that *fj* also interacts with *d*. We have recently cloned *d* and shown that it encodes an unconventional myosin with a well-conserved actin-binding domain (W. L. Hu, G. Minihan, G. R. B., H. Hayter, E. Liebl and F. N. K., unpublished). Together, these interactions strongly suggest that one outcome of *fj* signaling is an alteration of the actin cytoskeleton. This is consistent with the morphogenetic processes affected by *fj*, both in the leg and in other tissues. Thus, in addition to a potential instructive role through interactions with the N pathway, Fj may also initiate some of the morphogenetic work that depends on actin and is necessary to carry out these instructions.

Redundancy in Fj signaling

We have shown that Abl kinase function partially masks a requirement for *fj* at the T1/2 segment boundary. While *fj* is expressed at all tarsal segment boundaries, it appears to be required for segmentation only at T2/3, although rare instances of partial loss of T1/2 have been observed (Tokunaga and Gerhart, 1976; Held et al., 1986). However, in the absence of one copy of *abl*, additional loss of the T1/2 boundary is seen in a third of all animals. We interpret this to mean that additional information, modified by or working through *abl*,

acts together with *fj* to elaborate that boundary. Most *abl* homozygous flies have normal legs, although we have occasionally observed severe truncations of the legs in individual *abl* flies (unpublished observations). In addition, over-expression of *abl* causes tarsal truncations and segment fusions. A likely target of Abl activity is Ena. However, Abl is not the only tyrosine kinase that phosphorylates Ena (Gertler et al., 1995), and this multiple regulation may in part explain the variability of *abl* loss-of-function phenotypes. A critical test of whether Ena is indeed part of a common pathway for the morphogenetic work of segmentation at all leg segment boundaries will be to examine loss-of-function *ena* clones in the leg. Alternatively, the common pathway at each segment boundary may be the actin cytoskeleton itself, with multiple inputs providing redundancy, and overlapping position-specific regulators competing or cooperating to regulate the state of actin dynamics in each cell. Indeed, in the larger sense, Fj itself may serve to coordinate information provided by multiple signaling pathways (Notch, Jak/Stat, and Wingless, which all regulate *fj*: Papayannopoulos et al., 1998; Rauskolb and Irvine, 1999; Zeidler et al., 1999) with alterations in the actin cytoskeleton that ultimately have morphological consequences.

We thank the following for stocks: F. Michael Hoffmann, Martin Zeidler, Hermann Steller, Alfonso Martinez Arias, David Van Vactor, Elisabeth Knust, and the Bloomington Stock Center. We thank Helmut Kramer for help with cell transfections and germline transformation, Genevieve Pfluger and Joe Cole for technical assistance, and Bruce Riley and members of the Katz laboratory for helpful comments on the manuscript. C.R. thanks Ken Irvine for his generosity. This work was supported by NSF grant IBN 9724930 (to F. K.) and by NIH grant 1 R01 GM63057 (to C. R.).

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