

## *Drosophila* Rac1 controls motor axon guidance

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Accepted 13 November 1997; published on WWW 13 January 1998

### SUMMARY

Previous genetic studies of intersegmental nerve b development have identified several cell-surface proteins required for correct axon guidance to appropriate target muscles. Here we provide evidence that the small GTPase Drac1 also plays a key role in this guidance process. Neuronal expression of the dominant negative mutation Drac1(N17) causes axons to bypass and extend beyond normal synaptic partners. This phenotype is consistently

reproduced by pharmacological blockade of actin assembly. Genetic interactions between Drac1(N17) and the receptor-tyrosine phosphatase Dlar suggest that intersegmental nerve b guidance requires the integration of multiple, convergent signals.

Key words: *Drosophila*, Nerve, Axon guidance, Drac1, Rac1, Motor axon, Muscle

### INTRODUCTION

In order to reach appropriate target tissues in the developing embryo, neuronal growth cones must navigate through a complex cellular terrain in response to various guidance cues. This process requires an interplay between information outside the growth cone and intracellular motility machinery that drives the growth cone forward. Although rapid progress has been made in our knowledge of extracellular axon guidance signals and their receptors (reviewed by Tessier-Lavigne and Goodman, 1996), the biochemical pathways that convey and integrate this information within the growth cone are poorly understood.

Analysis of motor axon guidance in the *Drosophila* embryo provides a convenient system to identify molecules that control growth cone behavior in vivo (reviewed by Bate and Broadie, 1995; Keshishian et al., 1996). Motor growth cones must pathfind across a series of different surfaces and navigate a number of important choice points along the way to correct muscle targets (Van Vactor et al., 1993). Choice points are special landmarks in the developing embryo where growth cones reorganize their cell contacts, shifting from one cellular substratum to another, presumably in response to a combination of cues presented on the cells or extracellular matrix that they encounter in these regions. Using both classical and molecular genetics to examine one particular choice point, a number of genes encoding cell surface and secreted proteins have been shown to be necessary for specific motor growth cones (ISNb) to enter their correct target domain (Van Vactor et al., 1993; Lin and Goodman, 1994; Desai et al., 1996; Fambrough and Goodman, 1996; Krueger et al., 1996). Although different receptor-like proteins control each step in this process, none of the known choice point genes encodes an intracellular signaling molecule capable of linking cell surface to the motility machinery within the growth cone.

Growth cone exploration and response to the extracellular environment is believed to occur primarily at the leading edge membrane, a dynamic structure supported by a complex cytoskeletal architecture composed largely of actin microfilaments and associated proteins (reviewed by Smith, 1988; Stossel, 1993; Mitchison and Cramer, 1996). Studies of growth cone behavior in culture and in situ suggest that focal actin reorganization at the point of contact between the leading edge and other attractive cell surfaces plays an important role in guidance (O'Connor and Bentley, 1993; Lin and Forscher, 1993). Complete disruption of actin structures with high doses of cytochalasin stops growth cone advance (Yamada et al., 1971). However, when actin function is compromised with low doses of toxin, growth cones continue to extend but lose directional specificity (Letourneau and Marsh, 1984; Bentley and Toroian-Raymond, 1986; Chien et al., 1993). These observations suggest that guidance cues exert their effects by controlling actin assembly.

The small GTPases Cdc42, Rac and Rho control actin cytoarchitecture in leading edge structures (reviewed by Hall, 1990; Chant and Stowers, 1995; Luo et al., 1997) and coordinate cell polarity and morphogenesis (reviewed by Drubin and Nelson, 1996). Modeled on studies of Ras, dominant interfering mutations (Diekmann et al., 1991; Ridley et al., 1992) reveal that Cdc42, Rac and Rho control distinct actin-based motility behaviors (Ridley et al., 1995; Nobes and Hall, 1995a). In *Drosophila*, homologues of Rac and Cdc42 are involved in dorsal closure (Harden et al., 1995), wing bristle formation (Eaton et al., 1995), myoblast fusion and sensory neuron morphogenesis (Luo et al., 1994). Recently, constitutively active GTPase mutants were shown to block *Drosophila* motor axon outgrowth (Sone et al., 1997), presumably via massive and inappropriate actin polymerization (Luo et al., 1994). This indicates that GTPase effectors can be activated in motor growth cones, however, the

normal roles of Rac and Cdc42 in motor growth cones were not addressed with loss-of-function mutations. To better understand how these GTPases function in motor growth cones, we examined dominant loss-of-function phenotypes during neuromuscular development. These phenotypes suggest that motor growth cones require Rac activation for directional specificity and target recognition, whereas Cdc42 is required for motor axon extension.

## MATERIALS AND METHODS

### Genetics

Gal4 expression was controlled by the neuronal specific P[GAL4-C155] (Lin and Goodman, 1994), P[GAL4-1407] (Luo et al., 1994), or P[GAL4-elav] driver chromosomes (1407 was obtained from Dr Y. N. Jan; the others were obtained from Dr C. S. Goodman). Wild-type, (N17), (L89) and (V12) mutant P[UAS-Drac1], as well as P[UAS-Dcdc42] reporter lines are described in Luo et al. (1994). *Dlar*-P[Drac (N17.1)] double mutant combinations were created by crossing flies heterozygous for the *Dlar* alleles and homozygous for either P[UAS-Drac(N17.1)] or P[GAL4-C155]. All flies were maintained at 25°C.

### Immunocytochemistry and evaluation of phenotype

Motor axon pathways were visualized using mAb 1D4 as previously described (Van Vactor et al., 1993). mAb 3C10 (anti-even-skipped) and mAb In 4D9B1F1 (anti-engrailed) were used to assess cell fate and patterning in the CNS (obtained from Dr Goodman). mAb 22C10 (Zipursky et al., 1984) was used to assess PNS development. When homozygous mutants could not be made, the correct genotypes were chosen by staining with anti- $\beta$ -galactosidase antibodies (Sigma) to recognize embryos carrying *lacZ*-expressing balancer chromosomes (CyO-P[actin5C-LacZ]). Prior to evaluation, stage 17 embryos of the appropriate genotypes were selected and filleted under a dissection microscope. Embryos were staged according to Campos-Ortega and Hartenstein (1985). Each genotype was coded and scored blind. When some ISNb axons bypassed the choice point and others entered correctly, the segment was scored as a 'partial bypass'; when all axons bypassed, with no evidence of appropriate target domain entry, then the segment was scored as 'full bypass' (as scored in Krueger et al., 1996; note: total bypass = full + partial). Since the 'full bypass' phenotype is more accurate for quantitative comparisons, we scored only full bypass when evaluating genetic interactions.

### Microinjection of cytochalasin D

Carefully staged, wild-type (Canton-S) embryos were dechorionated and aligned on tape under a thin layer of Halocarbon oil (series 700); these were injected on an inverted microscope with diamond-beveled micropipettes. Cytochalasin D (CD; Sigma) was dissolved in DMSO prior to dilution in standard embryo injection buffer (0.1 mM phosphate buffer, pH 6.8, 5 mM KCl). The final concentration of DMSO was 0.01% in all samples, including buffer controls. Non-toxic green food dye was added to help visually evaluate the success of injections; this had no effect on ISNb development in control injections. In order to access the internal core of the developing embryo without disturbing morphogenesis, fixed volumes of sample were injected into the perivitelline space at embryonic stage 13. By stage 15, the injected sample was incorporated into the gut cavity, providing a central source of membrane permeable toxin to diffuse throughout the embryo during the key stages of ISNb pathfinding. At estimated final CD concentrations approaching 1.0 mg/ml, muscle development was often abnormal; thus phenotypic analysis was restricted to concentrations at or below 0.5 mg, where the intersegmental nerve (ISN) and muscle development appeared completely normal. Injected embryos were allowed to mature to stage

17, rinsed from the slide surface and stained with mAb 1D4. Muscle morphology was evaluated with Nomarski optics.

## RESULTS

### ISNb growth cones require Cdc42 for extension and Rac for guidance to appropriate targets

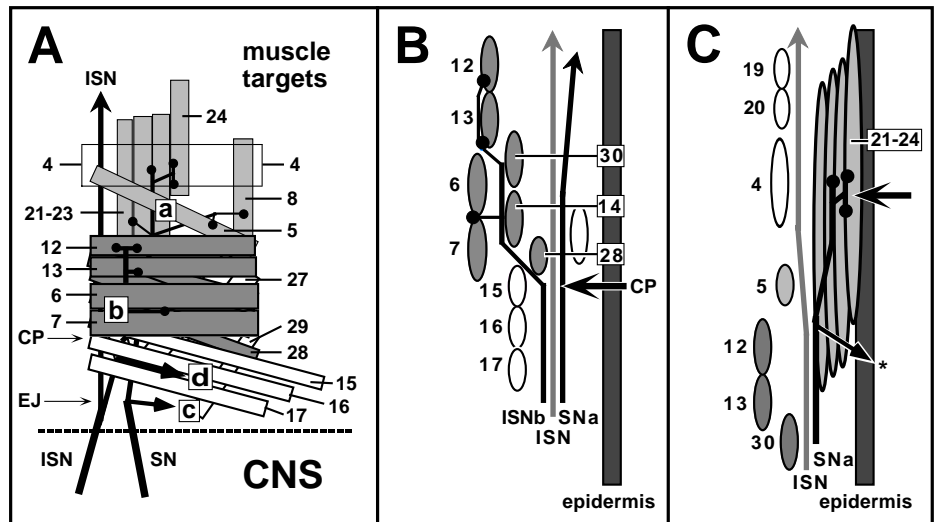
To disrupt GTPase function in neurons during axon pathfinding, without affecting the development of other tissues or previous embryonic stages, dominantly activated (V12) and dominant negative (N17 and L89) GTPase mutations were placed under the control of the yeast transcriptional activator GAL4 (Luo et al., 1994; see Materials and Methods). Combination of a neuronal GAL4 'driver' with a GTPase cDNA 'reporter' under control of the GAL4 upstream activator sequence (UAS) results in specific cDNA expression (Brand and Perrimon, 1993); three different neuron-specific GAL4 drivers were used (see Materials and Methods). Although gain-of-function and loss-of-function GTPase mutations have similar effects on sensory axon outgrowth (Luo et al., 1994), they have distinct effects on motor axon pathways, which reveal a role for Rac in executing specific guidance decisions (see below).

In each abdominal hemisegment, *Drosophila* motor growth cones exit the central nervous system (CNS) via two pathways: the segmental (SN) and intersegmental (ISN) nerve roots (Fig. 1). In the periphery, motor axons divide into five major nerve branches, each destined for a different region of body wall muscle (Johansen et al., 1989). Intersegmental nerve b (ISNb, previously known as SNb) development has been most extensively characterized, in both cellular and genetic terms. To reach its correct muscle targets after emerging from the CNS as part of the ISN, ISNb defasciculates at the exit junction to form a distinct bundle of nine axons, and then leaves the ISN axon pathway to explore ventral muscle surfaces at the ventral choice point (Fig. 1A). Target entry at the ventral choice point is very precise: after leaving the common motor pathway, wild-type ISNb axons always follow the internal surface of muscle 14 in order to enter the ventral domain (Van Vactor et al., 1993) (Fig. 1B).

Neuronal-specific expression of either *Drac1*(V12) or *Dcdc42*(V12) causes ISNb motor growth cones to arrest outgrowth just as axons begin to explore the periphery [Fig. 2B shows the *Drac1*(V12) phenotype; the *Dcdc42*(V12) phenotype is identical] (also see Sone et al., 1997). This highly penetrant motor phenotype (96%,  $n=134$  abdominal segments under the control of the C155-GAL4 driver) is analogous to the growth cone arrest observed in sensory neurons when they express the same GTPase mutations (Luo et al., 1994). The growth cone arrest seen in the V12 backgrounds suggests that hyperactivation of different GTPase pathways disrupts leading edge motility. However, in order to determine when and where GTPases normally act in motor axon guidance, it is necessary to examine loss-of-function phenotypes.

Neuronal-specific expression of dominant negative mutations reveal a difference in the function of *Dcdc42* and *Drac1*. Expression of *Dcdc42*(N17) has a more subtle effect on axonal development than *Drac1*(N17); we only observed a *Dcdc42*(N17) phenotype under the strongest GAL4 driver (elav-GAL4). When UAS-*Dcdc42*(N17) is combined with

**Fig. 1.** Motor axon pathways in the *Drosophila* embryo. (A) The normal anatomy of the motor branches seen in an abdominal segment (A2-7) is shown in schematic form. Only ventral oblique (white), ventral longitudinal (dark gray) and lateral (light gray) muscle domains are diagrammed (the position of the dorsal muscle 4 is indicated for comparison to the dorsal projection of SNa). Motor axons emerge from the CNS in two pathways, ISN and SN, before dividing into five peripheral branches. The exit junction (EJ) where ISNb defasciculates, and the choice point (CP) where ISNb enters the ventral domain are marked. (B) The ventral target domain is shown in cross-sectional schematic. ISNb axons enter their target region at the ventral choice point (large arrow), where these axons diverge from the common motor pathway (including ISN, SNa and ISNb). (C) The lateral target domain is shown in cross-sectional schematic. The dorsal projection of SNa innervates muscles 21-24 in a characteristic position (large arrow) that lies beneath the dorsal muscle 4 (white). The dorsal projection of SNa extends to contact



elav-GAL4, ISNb axons stop short of their most distal target muscle (muscle 12) in 8% of abdominal hemisegments (Fig. 2C). Interestingly, the *Dcdc42(N17)* ISNb phenotype closely resembles the growth cone arrest observed in profilin mutants (D. Van Vactor and C. S. Goodman, unpublished observations). In contrast, inhibition of neuronal *Drac1* function with *Drac1(N17)* altered the trajectory of motor axons but did not block their extension (see below). The fact that *Cdc42* and *Rac* phenotypes do not overlap suggests that each *N17* dominant negative effect is specific.

Expression of *Drac1(N17)* caused ISNb axons to bypass ventral muscle targets (Fig. 2D,E; see arrowheads). Instead of turning to grow into the ventral muscle domain along the internal surface of muscle 14, *Drac1(N17)* ISNb axons extended along the exterior surface of this muscle and followed ISN axons towards dorsal muscles. Although the qualitative nature of these phenotypes remained constant in different GAL4 driver-*Drac1(N17)* combinations, each genotype yielded a different frequency of defects, suggesting a dose-dependent relationship (Fig. 3A). The presence of neuronally expressed GAL4 or the *Drac1(N17)* transgenes by themselves had no effect on ISNb development (Fig. 3A). As a control, wild-type *Drac1* was expressed in the same manner; no defects in motor axon pathways resulted. Thus, the *Drac1(N17)* phenotypes resulted from the dominant negative mutation and not simply overexpression of *Rac* protein. The cell fate and patterning of neural soma within the CNS was not altered in the GAL4-*Drac1(N17)* embryos, as assessed with anti-even skipped and anti-engrailed antibodies (data not shown).

Neuronal *Drac1(N17)* expression did not cause ISNb growth cone arrest. ISNb axons that enter the ventral domain correctly in *Drac1(N17)* embryos reach all of their targets. Even under the strongest GAL4 driver, growth cone arrest did not increase above background levels observed in the P[UAS-*Drac1(N17.1)*] line alone (maximal at 3%). Furthermore, when ISNb bypassed ventral muscles (as often as 73% in the *elav-GAL4;Drac1(N17)* genotype), these growth cones tended to

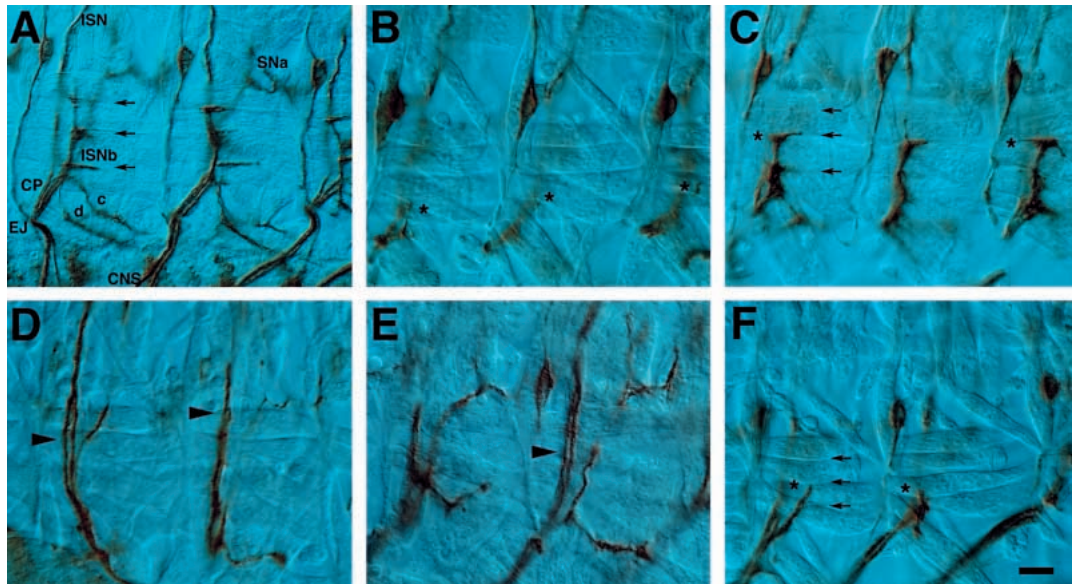
extend further than their normal counterparts, suggesting that outgrowth and motility were intact even though directional guidance was compromised.

The genetic analysis of *Drosophila* neuromuscular development has identified several proteins required for ISNb axon guidance (Van Vactor et al., 1993; Lin and Goodman, 1994; Desai et al., 1996; Fambrough and Goodman, 1996; Krueger et al., 1996). Close examination of the ISNb phenotypes in different mutants reveals two steps in choice point guidance: the defasciculation of ISNb from ISN at the exit junction, and target domain entry at the ventral choice point (see Krueger et al., 1996 for a discussion of this distinction). In *Drac1(N17)* embryos, misguided ISNb axons were most frequently observed as a fascicle distinct from the ISN (Fig. 2D,E). The frequent presence of two separate axon fascicles in the ISN focal plane indicates that failure in target entry does not represent a failure to defasciculate from the common pathway. In this regard, the *Drac1(N17)* phenotype most closely resembles the phenotype of only one other known protein: the receptor-tyrosine phosphatase *Dlar* (Krueger et al., 1996).

### SNa axons also require *Rac* to reach correct targets

As expected, the effects of *Drac1(N17)* are not specific to ISNb; a related phenotype was observed in the SNa projection to lateral muscle targets. The dorsal branch of SNa normally forms terminal contacts with muscles 21-24 in a characteristic region of muscle surface underneath or proximal to the non-target dorsal muscle 4 (Figs 1A,C, 4A). In *Drac1(N17)* embryos, the dorsal projection of SNa often bypassed this stereotypic target region on lateral muscles (Fig. 3B). In many such cases, SNa axons failed to make normal target contacts and extended beyond lateral muscles to make ectopic contacts onto a variety of cell surfaces. Ectopic contacts were observed on the sheath cells of lateral sensory neurons (chordotons), on ISN axons or often on non-target muscles in the dorsal region (including muscles 11, 19 and 20; Fig. 4B,C). This

**Fig. 2.** GTPase mutations alter intersegmental nerve b development. (A) The normal anatomy of the motor branches is shown in a wild-type embryo. ISNb innervates the ventral longitudinal muscles after separating from the ISN at the choice point. Within the ventral muscle domain, ISNb is in a different focal plane than the ISN. Three small arrows mark the clefts between muscles where synaptic contacts initiate. Structures featured in Fig. 1 are marked for comparison. (B) ISNb growth cone arrest at contacts with muscle 14 (asterisks) are shown in three abdominal segments of a P[w+;C155-GAL4];P[UAS-Drac1(V12)] embryo. (C) Late ISNb growth cone arrest at contacts with muscle 13 (asterisks) are shown in two abdominal segments of a P[w+;elav-GAL4];P[UAS-Dcdc42(N17)] embryo. The third segment shows an abnormally small contact to muscle 12. (D) ISNb axons extend past the ventral longitudinal muscles in P[w+; GAL4-C155]; P[w+; Rac1-N17.1] embryos. These abnormal ISNb axons form a distinct fascicle separate from the ISN which remains in the ISN focal plane underneath the ventral longitudinal muscles (arrowheads). (E) A second example of the P[w+; GAL4-C155]; P[w+; Rac1-N17.1] phenotype is shown. ISNb axons form a distinct fascicle (arrowhead) that follows the ISN beyond ventral muscles. (F) Early ISNb growth cone arrest (asterisks) is observed in P[w+;C155-GAL4];P[UAS-Drac1(L89)] embryos, never reaching distal muscle targets. Scale bar represents approximately 15 microns. In this and all subsequent micrographs, anterior is left.

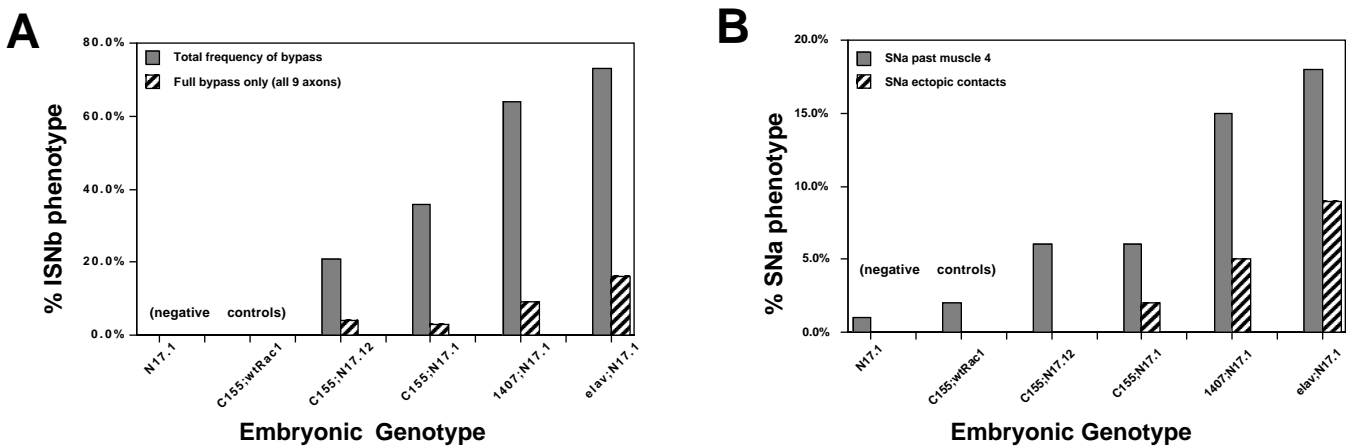


particular SNa phenotype has not been described in any previous mutant background; however, it appears analogous to the ISNb bypass phenotype where motor growth cones extend beyond normal target muscles. In addition, Drac1(N17) causes subtle disruption of interneuronal pathways within the ventral nerve cord (data not shown); misguided CNS axons could not

be followed individually with mAb 1D4 due to the complexity of the central neuropil.

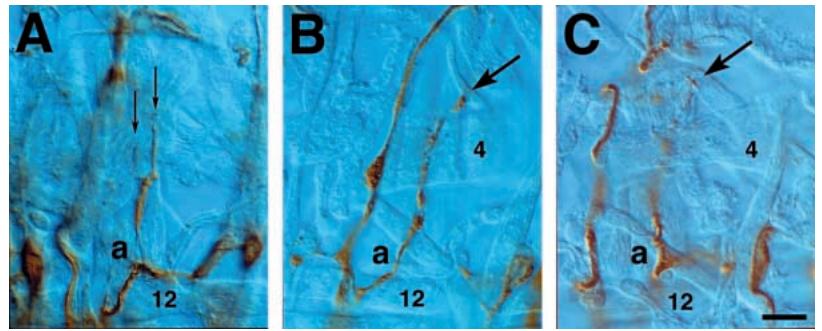
### The Drac1(L89) dominant mutation displays complex behavior

The N17 dominant loss-of-function mutation is well



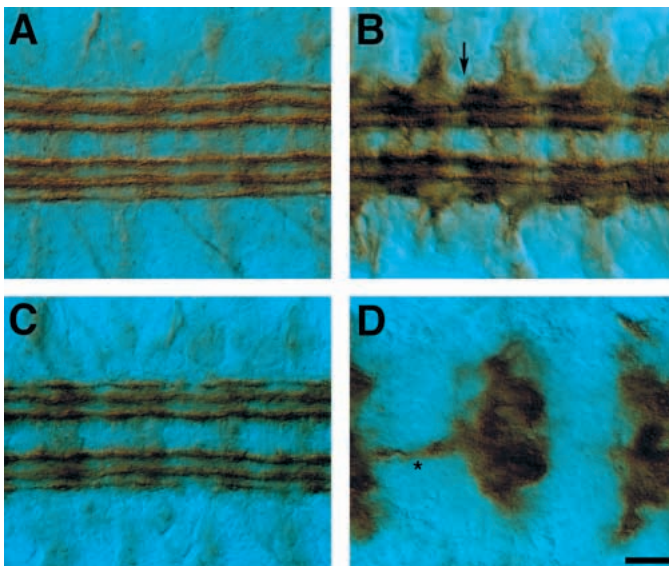
**Fig. 3.** Quantitation of Drac1(N17) motor axon phenotypes. (A) ISNb bypass phenotypes are displayed in this histogram as percentages of all segments scored. Segments showing any evidence of ISNb bypass are shown in gray, whereas segments showing full bypass are shown in stripes (see Results). (B) SNa axon phenotypes are displayed in this histogram as percentages of all segments scored. Segments in which SNa dorsal projection extend beyond the distal edge of muscle 4 are shown in gray. Those SNa axons that extend beyond the target site to make ectopic contacts onto dorsal muscles, sensory sheath cells or ISN axons are shown in stripes. The number of abdominal segments scored in each genotype ( $n$ ) was: 214 for N17.1, 225 for C155;wtDrac1, 242 for C155;N17.12, 206 for C155;N17.1, 205 for 1407;N17.1, and 196 for elav;N17.1.

**Fig. 4.** Segmental nerve a bypasses targets in *Drac1(N17)* embryos. (A) The normal anatomy of the SNa motor branch is shown in a wild-type embryo (marked a). SNa innervates the lateral muscles 21-24 at characteristic sites in the clefts between these targets (thin arrows) The position of the ventral muscle 12 is shown for positional reference. (B,C) Two examples of SNa phenotypes in  $P[w+; GAL4-1407]$ ;  $P[w+; Rac1-N17.1]$  embryos. The dorsal branch of SNa extends beyond muscle 4 (marked) to make ectopic contacts (large arrow) on dorsal muscles 20 (B) or 19 (C) Such dorsal muscle contacts are never observed in wild-type embryos. Scale bar represents approximately 10  $\mu$ m.

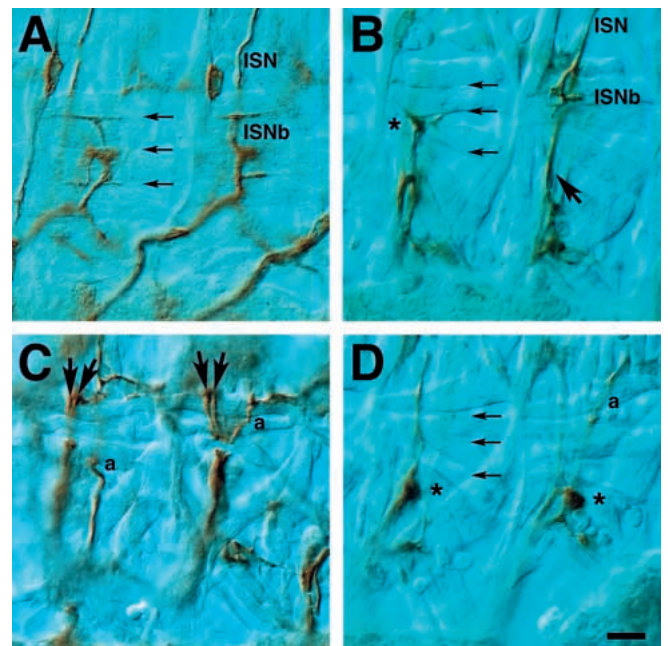


characterized, reducing GTPase affinity for GTP tenfold relative to GDP binding (Feig and Cooper, 1988; Ridley et al., 1992). N17 is thought to block endogenous GTPase by competing for the binding of cellular activators (e.g. exchange factors). A second class of Rac dominant mutation (L89), inspired by a Ras(F89) allele (Han and Sternberg, 1991), was previously shown to block sensory axon outgrowth (Luo et al., 1994). Although the biochemical effects of the L89 mutation have not been determined, Luo et al. (1994) showed that *Drac1(N17)* and *Drac1(L89)* have differential effects, depending on the cells in which they are expressed (e.g. neuron

or muscle). For ISNb motor axons, we found that *Drac1(L89)* has effects similar to both *Drac1(V12)* and *Drac1(N17)*. Expression of *Drac1(L89)* under the neuronal GAL4 drivers described above resulted in a consistent ISNb growth cone



**Fig. 5.** A *Drac1(L89)* phenotype is enhanced by wild-type *Drac1*. (A) The normal anatomy of CNS pathways visualized with mAb 1D4 at stage 17 is observed in a  $P[C155-GAL4];P[UAS-wtDrac1]$  embryo. (B) Neuronal-specific expression of *Drac1(L89)* with C155-GAL4 disrupts CNS axon pathways slightly. Although all three fascicles are present, gaps between segments suggest failure in axon extension (arrow). (C) Co-expression of wild-type *Drac1* and *Drac1(N17)* with C155-GAL4 yields near-normal morphology of CNS pathways. Although some abnormalities can be seen in occasional *Drac1(N17)* segments, this phenotype is not enhanced by wild-type *Drac1*. (D) Co-expression of wild-type *Drac1* and *Drac1(L89)* with C155-GAL4 causes a massive failure in axon extension, dramatically enhancing the *Drac1(L89)* effect. Very few mAb 1D4-positive axons extend from one segment to another in these embryos. Axons that do extend follow abnormal trajectories (asterisk). Scale bar represents approximately 8  $\mu$ m.



**Fig. 6.** Cytochalasin D disrupts intersegmental nerve b guidance. (A) The normal anatomy of the motor branches is shown in a wild-type embryo, filleted to lay the periphery flat. ISNb innervates the ventral longitudinal muscles after separating from the ISN at the choice point, making synaptic contacts at the clefts between each muscle (small arrows). (B) An example of an embryo injected with a low dose of cytochalasin D (approximately 0.1  $\mu$ g/ml CD). Occasional late ISNb growth cone arrest is observed (asterisk), however ISNb bypass is prevalent. In this example of fusion bypass (large arrow), ISNb growth cones turn back to contact potential targets after extending beyond the most distal target. Normal innervations are missing (small arrows) (C) A second example of 0.1  $\mu$ g/ml CD shows the parallel bypass seen in these embryos (each pair of large arrows indicates the positions of ISN and ISNb as they extend past muscle 12 in each segment. Due to the plane of focus, SNa can be seen in both segments (marked a). (D) At higher doses of toxin, ISNb growth cones often stop at the entry to the ventral domain (asterisks), before reaching characteristic sites of innervation (small arrows). SNa is visible in one segment. Scale bar represents approximately 15  $\mu$ m.

arrest; this arrest ranged from 6% to 68% ( $n=172$  hemisegments or greater), depending on the combination of GAL4 driver and UAS reporter. Drac1(L89) ISNb axons stop at different points along their normal trajectory to ventral muscles (Fig. 2F). Though the growth arrest phenotype is prevalent in Drac1(L89), we also observed an ISNb bypass phenotype in some segments (ranging from 7% to 16%). Drac1(L89) also caused disruption of CNS axon pathways, consistent with growth cone arrest (Fig. 5B).

Since the Drac1(L89) phenotype shared elements of both loss-of-function and gain-of-function, we asked whether these phenotypes could be rescued by co-expression of wild-type Drac1. Luo et al. (1994) showed that co-expression of wild-type Drac1 could rescue a very weak Drac1(L89) sensory axon phenotype as evidence of a loss-of-function. However, when we examined embryos that co-expressed wild-type Drac1 and Drac1(L89.6) with the mAb 1D4 marker, we found that the majority of embryos displayed a dramatic enhancement of the growth cone arrest phenotype (62%,  $n=222$  segments; Fig. 5D). The three characteristic mAb 1D4-positive interneuronal pathways that form along the longitudinal connectives could not be seen in these segments; there was a massive failure in axonal extension. The few axons that did extend into the periphery in these embryos were highly disorganized; we were unable to identify specific nerve branches in these cases. The dramatic enhancement of axon extension phenotypes in embryos that co-expressed wild-type and (L89) forms of Drac1 indicates that Drac1(L89) is not a simple loss-of-function allele. In contrast, co-expression of wild-type Drac1 and Drac1(N17) showed no enhancement of the N17 phenotype (Fig. 5C). Similar results have been obtained from comparisons of Drac1(N17) and Drac1(L89) during dorsal closure (N. Harden, personal communication), suggesting that Drac1(L89) acts through a different mechanism than N17 and V12. In fact, several dominant alleles of Ras have been described that display combined loss- and gain-of-function phenotypes (Han and Sternberg, 1991). For this reason, Drac1(L89) was not used in the double mutant analysis with Dlar and Beaten Path (see below and discussion).

### Low doses of cytochalasin D mimic GTPase phenotypes

Since GTPase signaling is known to control processes other than motility behaviors (e.g. Lamarche et al., 1996), we wondered whether the Drac1 loss-of-function phenotype was correlated with a defect in cytoskeletal assembly. Unfortunately, due to the small size of *Drosophila* motor growth cones and the fact that they extend between actin-rich muscle fibers, we have been unable to obtain high-resolution views of motor growth cone cytoskeleton in vivo by conventional or confocal fluorescence microscopy. As an alternative approach, we compared the GTPase phenotypes to the effects of a pharmacological agent that blocks actin assembly. In order to examine a range of toxin effects without disturbing the development of muscle targets or the common motor pathway, we took advantage of the fact that ISNb growth cones are last to reach their targets. Different concentrations of membrane permeable cytochalasin D (CD) were microinjected into developing embryos after the ISN pathway had extended into the periphery and muscle targets were patterned correctly,

but before ISNb axons had begun to explore the ventral choice point (see Materials and Methods).

At low doses of CD, many ISNb projections were normal (48% normal at 0.05 mg/ml or 0.1  $\mu$ M estimated final concentration,  $n=234$  abdominal hemisegments). At this low concentration of toxin, where CD is known to inhibit membrane ruffling (Cooper, 1987), many ISNb branches displayed bypass phenotypes (32%). Interestingly, both the fusion bypass (failure to defasciculate, as seen in *beaten path*) and the parallel bypass (failure in target entry, as seen in *Dlar*) phenotypes were observed (Fig. 6B,C). This suggests that actin assembly is essential for both phases of ISNb guidance. Bypassing axons often reached back from an abnormal trajectory to contact ventral targets, illustrating that the bypassing axons were indeed ISNb (Fig. 6B).

ISNb branches that did enter the ventral domain often stopped early as observed in Dcdc42(N17) embryos (20%; Fig. 6B). At this low dose, filopodia remained visible on ISNb growth cones (lamellipodia are difficult to distinguish in such small growth cones). No ISNb defects were observed in control injections (data not shown).

At high CD concentrations, few ISNb branches were normal (12% normal at 0.5 mg/ml or 1  $\mu$ M estimated final concentration,  $n=272$  abdominal hemisegments); far fewer ISNb branches bypassed the choice point (19%) than displayed growth cone arrest (69%). Growth cone arrest at high concentrations was often observed at, or prior to, the ventral choice point (Fig. 6D). The tips of axons in these embryos typically lacked filopodia. Previous experiments with CD in vitro have suggested that axon extension can proceed in the absence of actin assembly (Letourneau and Marsh, 1984); this is not the case for ISNb motor axons in vivo. Although toxin effects on peripheral cell types (ie. muscle) were a concern, the ventral muscles appeared morphologically normal after late stage injections of 0.05-0.5  $\mu$ g/ml CD (see Materials and Methods). The prevalence of ISNb bypass phenotypes at the low toxin dose supports a model where target entry requires active cytoskeletal assembly downstream of Rac activation.

### Genetic interactions reveal distinct pathways and convergent signals

Of all the proteins currently known to control ISNb guidance, Drac1 and Dlar are most likely to mediate target entry as opposed to defasciculation, because both display the parallel bypass phenotype. To better understand the relationship between Dlar and Drac1, we examined double mutant embryos. The penetrance of full ISNb bypass in *Dlar* hemizygous (*Dlar*<sup>L3.2/+</sup>), partial loss-of-function (*Dlar*<sup>bypass/Dlar</sup><sup>bypass</sup>) and null (*Dlar*<sup>5.5/Dlar</sup><sup>L3.2</sup>) genotypes is 0%, 7% and 21%, respectively (see Materials and Methods for scoring criteria). However, when combined with C155-GAL4;Drac1(N17.1) which displays 3% full bypass alone, all three Drac1(N17)-Dlar compound mutants display a penetrance twofold to threefold higher than expected if the defects were simply additive (Table 1). Thus, Rac function in ISNb axons is quite sensitive to the dosage of Dlar protein. This synergistic genetic interaction suggests that Rac and Dlar function together, though not in a simple linear pathway. The increase in penetrance observed in the Drac1(N17)-*Dlar*<sup>null</sup> combination, far above the maximum frequency of defects seen in the complete absence of Dlar, suggests that a Dlar-

**Table 1. Genetic interactions between Rac1(N17) and Dlar**

Genotype	ISNb bypass ( <i>n</i> )		
C155-GAL4;Drac1(N17.1)	3%	(206)	weak <i>Rac</i>
C155-GAL4; <i>Dlar</i> <sup>13.2/+</sup> ; Drac1(N17.1)	<b>10%</b>	(176)	double mutant
<i>Dlar</i> <sup>13.2/+</sup>	0%	(319)	one copy <i>Dlar</i> <sup>+</sup>
C155-GAL4;Drac1(N17.1)	3%	(206)	weak <i>Rac</i>
C155-GAL4; <i>Dlar</i> <sup>bypass</sup> / <i>Dlar</i> <sup>bypass</sup> ; Drac1(N17.1)	<b>26%</b>	(452)	double mutant
<i>Dlar</i> <sup>bypass</sup> / <i>Dlar</i> <sup>bypass</sup>	7%	(221)	hypomorphic <i>Dlar</i>
C155-GAL4; Drac1(N17.1)	3%	(206)	weak <i>Rac</i>
C155-GAL4; <i>Dlar</i> <sup>13.2</sup> / <i>Dlar</i> <sup>5.5</sup> ; Drac1(N17.1)	<b>49%</b>	(144)	double mutant
<i>Dlar</i> <sup>13.2</sup> / <i>Dlar</i> <sup>5.5</sup>	21%	(149)	null <i>Dlar</i>
C155-GAL4; Drac1(N17.1)	3%	(206)	weak <i>Rac</i>
C155-GAL4; <i>beat</i> <sup>2</sup> / <i>beat</i> <sup>3</sup> ; Drac1(N17.1)	<b>80%</b>	(434)	double mutant
<i>beat</i> <sup>2</sup> / <i>beat</i> <sup>3</sup>	69%	(351)	hypomorphic <i>beat</i>

Note: The ISNb phenotype scored in this experiment is “full bypass” where all 9 SNb axons extend beyond the ventral muscles on the external side of muscle 14 (see Results). The *n* represents the number of individual embryonic abdominal segments scored. The 5.5 and 13.2 alleles of *Dlar* are described by Kreuger et al. (1996); the *Dlar*<sup>bypass</sup> allele is described by D. Lin, P. Matubang, D. Van Vactor and C.S. Goodman, Soc. Neurosci. Abs., 1994).

independent pathway mediated by Drac1 contributes to ISNb target entry.

To address the specificity of Rac’s role in target entry, we examined embryos deficient in both Rac and the defasciculation factor Beaten Path. In *beat*<sup>2</sup>/*beat*<sup>3</sup> embryos, we find a 69% penetrance of full ISNb bypass (consistent with Fambrough and Goodman, 1996) (Table 1). However in C155; *beat*<sup>2</sup>/*beat*<sup>3</sup>; Drac1(N17.1) double mutant embryos, the penetrance of ISNb bypass increases only to 80% (Table 1). This small increase in penetrance contrasts the synergy seen between Drac1(N17) and *Dlar*.

## DISCUSSION

The signaling pathways that growth cones use to interpret their environment are not well understood. Rho-family GTPases such as Cdc42 and Rac control distinct actin-dependent motility behaviors in response to extracellular cues, making them attractive candidates to mediate growth cone guidance (Reviewed by Nobes and Hall, 1995b; Machesky and Hall, 1996). We have shown that whereas neuron-specific disruption of Dcdc42 blocks motor axon extension, the equivalent perturbation of Drac1 alters directional specificity without inhibiting extension. Although previous studies have shown that constitutively active Drac1 and Dcdc42 can disrupt motor pathways (Sone et al., 1997; see Results), our results provide the first evidence that Drac1 is required for directional guidance in vivo.

The effects of Dcdc42(N17) are subtle, which may indicate that Cdc42 plays only a minor role in axonogenesis, as suggested by recent experiments in chick neurons (Jin and Strittmatter, 1997). The effects of Drac1(N17) are more dramatic, causing different groups of motor axons to bypass their normal target regions. Since the Drac1(L89) mutation

displays features consistent with some gain-of-function activity, we base our interpretation of Rac function on the well-characterized N17 loss-of-function mutation. Our observation that partial disruption of actin assembly consistently mimics the N17 effect further supports the model that Rac conveys guidance information from cell surface to cytoskeleton.

The correct entry of ISNb axons into the ventral muscle domain requires a very precise turn at contacts with specific muscle surfaces; errors are rarely made in wild-type embryos. Genetic analysis of ISNb development has identified several proteins that control different aspects of this guidance process. For example, the adhesion molecule Fasciclin II and the secreted, anti-adhesion protein Beaten path control the defasciculation of ISNb and other axons from the common motor pathway that emerges from the central nervous system (Lin and Goodman, 1994; Fambrough and Goodman, 1996). The receptor-like tyrosine phosphatase (RPTase) Dlar is required for target entry, but not for defasciculation (Krueger et al., 1996). So far, Rac is the only intracellular signaling molecule that has been shown to function in the target entry pathway. Presumably Rac mutations were not recovered in a previous neuromuscular screen of the third chromosome (Sink et al., 1993) due to requirements early in embryogenesis (e.g. Harden et al., 1995).

## Actin: motility and guidance are separable functions

Dissecting the relationship between guidance signals that instruct the growth cone and the motility apparatus that implements this information is difficult, because cytoskeletal reorganization is essential for two vital roles in axonal development: axonal extension and guidance. Since different forms of actin cytoskeleton are presumably controlled by different intracellular signals, we would expect that cytochalasin D (CD) should generate a composite phenotype. In fact, at low toxin doses, we observed failures in both ISNb defasciculation and target entry. However, different doses of toxin favor distinct axonal phenotypes: bypass of ventral targets at low doses and growth cone arrest at high doses. This indicates that forward motility and directional specificity demand slightly different levels of actin assembly or polymerization. For example, the predominance of bypass at low CD suggests that the normal response of ISNb growth cones to their target domain (mediated by *Dlar*, *Drac1* and other genes) requires a high level or rate of actin assembly. This is consistent with the observation that growth cone interactions that define directionality often induce large and immediate accumulations of F-actin at the site of cell contact (O’Connor and Bentley, 1993; Lin and Forscher, 1993).

## Rac: cell polarity and the growth cone

From budding yeast to the *Pelvetia* zygote, cell polarity relies on focal assembly of actin-based cytoskeletal components, often in response to extracellular cues (Reviewed by Drubin and Nelson, 1996; see also Kropf, 1994). Cell polarity in the *Drosophila* wing epithelium is oriented by cell-cell communication and is manifested by a burst of actin polymerization at the apex of each cell that leads to the formation of a tiny actin-filled wing hair (Wong and Adler, 1993). Whereas inhibition of Cdc42 stops wing hair elongation, disruption of Rac prevents the coherent focus of actin and randomizes hair initiation, mimicking the phenotypes

of mutations in genes (e.g. for the transmembrane receptor Frizzled) that transmit the patterning information (Eaton et al., 1995, 1996). The roles of Cdc42 and Rac in motor growth cones may be analogous. Dcdc42(N17) blocks motor axon extension, while Drac1(N17) interferes with directional guidance. At a choice point, cytoskeletal asymmetry induced by extracellular cues may drive the growth cone in a new direction. Activation of Rac is known to stimulate actin assembly and the formation of lamellipodial membrane ruffles (Nobes and Hall, 1995a). Recent evidence suggests that lamellipodia play a dominant role in growth cone turning behavior in vitro (Wang and Jay, 1997). However, this model assumes an asymmetric activation of Rac at the leading edge. Since removal of all somatic muscle results in the failure of ISNb, and other motor axons, to leave the common motor pathway (Sink, H. and Goodman, C.S., personal communication), we speculate that Rac responds to localized mesodermal signals in choice point regions in order to orient growth cone movement.

### Rac and the ISNb guidance mechanism

The similarity of the Drac1(N17) and *Dlar* phenotypes suggests that Drac1 functions specifically in the target entry process, as opposed to the defasciculation of ISNb. This is supported by the strong dose-sensitive relationship between *Dlar* and Drac1. Accordingly, there is no dramatic interaction between Drac1 and *beaten path*. However, the interaction between Drac1(N17) and *Dlar* null mutations further suggests that ISNb target entry requires multiple, convergent signals. The existence of multiple inputs during target entry may explain why null mutations in *Dlar* (and other choice point genes) are not completely penetrant on their own (Desai et al., 1996; Krueger et al., 1996; Korey et al., 1997).

*Dlar* is a member of an RPTase sub-family expressed in the developing nervous system, including the human proteins LAR, HPTP $\delta$  and HPTP $\sigma$  (reviewed by Van Vactor, 1998). Recently, the highly conserved D2 PTPase domain of human LAR was shown to interact with a novel protein, Trio, that contains a Rac-specific guanine nucleotide exchange factor domain (Debant et al., 1996). This suggests a model for RPTase function where activation of the receptor leads to cytoskeletal reorganization via recruitment and activation of the small GTPase pathway. Although a *Drosophila* Trio counterpart exists (J. Bateman and D. Van Vactor, unpublished observations) and human Trio avidly interacts with the *Dlar* cytoplasmic domain (N. Kaufmann and D. Van Vactor, unpublished observations), the functional relationships between DTrio and the *Drosophila* RPTases remain to be elucidated.

We wish to thank Ryn Miake-Lye, John Flanagan, Rong Li, Liqun Luo and Tim Mitchison for helpful comments and discussions regarding this manuscript. We thank Corey Goodman for providing the C155 and *elav-GAL4* drivers, in addition to various antibody stocks. We thank Liqun Luo and Yuh Nung Jan for providing *Drosophila* stocks carrying dominant mutations in Rac1 and Cdc42 under the control of *GAL4 UAS*, in addition to the 1407 *GAL4* driver. Part of this work was supported by The Esther A. and Joseph Klingenstein Fund for Neuroscience and NIH grant NS35909. Nancy Kaufmann was supported by a NIH/NRSA predoctoral traineeship and Zachary Wills by a National Eye Institute Graduate Fellowship.

### REFERENCES

- Bate, M. and Broadie, K. (1995). Wiring by fly: The neuromuscular system of the *Drosophila* embryo. *Neuron* **15**, 513-525.
- Bentley, D. and Toroian-Raymond, A. (1986). Disoriented pathfinding of pioneer neurone growth cones deprived of filopodia by cytochalasin treatment. *Nature* **323**, 712-715.
- Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.
- Campos-Ortega, J. A. and Hartenstein, V. (1985). *The Embryonic Development of Drosophila melanogaster*. New York: Springer-Verlag.
- Chant, J. and Stowers, L. (1995). GTPase cascades choreographing cellular behavior: movement, morphogenesis, and more. *Cell* **81**, 1-4.
- Chien, C.-B., Rosenthal, D. E., Harris, W. A. and Holt, C. E. (1993). Navigational errors made by growth cones without filopodia in the embryonic *Xenopus* brain. *Neuron* **11**, 237-251.
- Cooper, J. A. (1987). Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* **105**, 1473-1478.
- Debant, A., Serra-Pages, C., Seipel, K., O'Brien, S., Tang, M., Park, S. and Streuli, M. (1996). The multidomain protein Trio binds the LAR transmembrane tyrosine phosphatase, contains a protein kinase domain, and has separate rac-specific and rho-specific guanine nucleotide exchange factor domains. *PNAS* **93**, 5466-5471.
- Desai, C. J., Gindhart, Jr., J. G., Goldstein, L. S. B. and Zinn, K. (1996). Receptor tyrosine phosphatases are required for motor axon guidance in the *Drosophila* embryo. *Cell* **84**, 599-609.
- Desai, C. J., Krueger, N. X., Saito, H. and Zinn, K. (1997). Competition and cooperation among receptor tyrosine phosphatases control motoneuron growth cone guidance in *Drosophila*. *Development* **124**, 1941-1952.
- Diekmann, D., Brill, S., Garrett, M. D., Totty, N., Hsuan, J., Monfries, C., Hall, C., Lim, L. and Hall, A. (1991). Bcr encodes a GTPase-activating protein for p21<sup>rac</sup>. *Nature* **351**, 400-402.
- Drubin, D. G. and Nelson, W. J. (1996). Origins of cell polarity. *Cell* **84**, 335-344.
- Eaton, S., Auvinen, P., Luo, L., Jan, Y. N. and Simons, K. (1995). Cdc42 and Rac1 control different actin-dependent processes in the *Drosophila* wing disc epithelium. *J. Cell Biol.* **131**, 151-164.
- Eaton, S., Wepf, R. and Simons, K. (1996). Roles for Rac1 and Cdc42 in planar polarization and hair outgrowth in the wing of *Drosophila*. *J. Cell Biol.* **135**, 1277-1289.
- Fambrough, D. and Goodman, C. S. (1996). The *Drosophila* *beaten path* gene encodes a novel secreted protein that regulates defasciculation at motor axon choice points. *Cell* **87**, 1049-1058.
- Feig, L. A. and Cooper, G. M. (1988). Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. *Mol. Cell Biol.* **8**, 3235-3243.
- Hall, A. (1990). The cellular functions of small GTP-binding proteins. *Science* **249**, 635-640.
- Han, M. and Sternberg, P. W. (1991). Analysis of dominant-negative mutations of the *Caenorhabditis elegans* let-60 ras gene. *Genes Dev.* **5**, 2188-2198.
- Harden, N., Loh, H. Y., Chia, W. and Lim, L. (1995). A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in *Drosophila*. *Development* **121**, 903-914.
- Jin, Z. and Strittmatter, S. M. (1997). Rac1 mediates Collapsin-1-induced growth cone collapse. *J. Neurosci.* **17**, 6256-6263.
- Johansen, J., Halpern, M. E. and Keshishian, H. (1989). Axonal guidance and the development of muscle fiber-specific innervation in *Drosophila* embryos. *J. Neurosci.* **9**, 4318-4332.
- Keshishian, H., Broadie, K., Chiba, A. and Bate, M. (1996). The *Drosophila* neuromuscular junction: a model system for studying synaptic development and function. *Ann. Rev. Neurosci.* **19**, 545-575.
- Korey, C., Bascom-Slack, C., Scalice, D., Percival, B., Van Vactor, D. (1997). X-chromosome genes provide further insight into embryonic motor axon guidance. *Soc. Neurosci. Abs.* **23**, 1956.
- Kropf, D. L. (1994). Cytoskeletal control of cell polarity in a plant zygote. *Dev. Biol.* **165**, 361-71.
- Krueger, N. X., Van Vactor, D., Wan, H., Goodman, C. S., Gelbart, W. and Saito, H. (1996). The transmembrane tyrosine phosphatase DLAR controls motor axon guidance in *Drosophila*. *Cell* **84**, 611-622.
- Lamarque, N., Tapon, N., Stowers, L., Burbelo, P. D., Aspenstrom, P., Bridges, T., Chant, J. and Hall, A. (1996). Rac and Cdc42 induce actin



- polymerization and G1 cell cycle progression independently of p65<sup>PAK</sup> and the JNK/SAPK MAP kinase cascade. *Cell* **87**, 5129-529.
- Letourneau, P. C. and Marsh, L.** (1984). Growth of Neurites without filopodial or lamellipodial activity in the presence of Cytochalasin B. *J. Cell Biol.* **99**, 2041-2047.
- Lin, C. and Forscher, P.** (1993). Cytoskeletal remodeling during growth cone-target interactions. *J. Cell Biol.* **121**, 1369-1383.
- Lin, D. M. and Goodman, C. S.** (1994). Ectopic and Increased expression of Fasciclin II alters motoneuron growth cone guidance. *Neuron* **13**, 507-523.
- Luo, L., Liao, Y. J., Jan, L. Y. and Jan, Y. N.** (1994). Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Dev.* **8**, 1787-1802.
- Luo, L., Jan, L. Y. and Jan, Y. N.** (1997). Rho family small GTP-binding proteins in growth cone signalling. *Curr. Opin. in Neurobiol.* **7**, 81-86.
- Machesky, L. M. and Hall, A.** (1996). Rho: a connection between membrane receptor signaling and the cytoskeleton. *TIBS* **6**, 304-310.
- Mitchison, T. J. and Cramer, L. P.** (1996). Actin-based cell motility and cell locomotion. *Cell* **84**, 371-379.
- Nobes, C. D. and Hall, A.** (1995a). Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62.
- Nobes, C. D. and Hall, A.** (1995b). Rho, Rac, and Cdc42 GTPases: regulators of actin structures, cell adhesion and motility. *Biochem. Soc. Trans.* **23**, 456-459.
- O'Connor, T. P. and Bentley, D.** (1993). Accumulation of actin in subsets of pioneer growth cone filopodia in response to neural and epithelial guidance cues in situ. *J. Cell Biol.* **123**, 935-948.
- Pulido, R., Serra-Pages, C., Tang, M. and Streuli, M.** (1995). The LAR/PTPd/PTPs subfamily of transmembrane protein-tyrosine-phosphatases: Multiple human LAR, PTPd, and PTPs isoforms are expressed in a tissue-specific manner and associate with the LAR-interacting protein LIP.1. *PNAS* **92**, 11686-11690.
- Ridley, A. J. and Hall, A.** (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389-399.
- Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D. and Hall, A.** (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401-410.
- Ridley, A. J., Comoglio, P. M. and Hall, A.** (1995). Regulation of scatter factor/hepatocyte growth factor responses by ras, rac, and rho in MDCK cells. *Mol. Cell Biol.* **15**, 1110-1122.
- Sink, H., Van Vactor, D., Robinson, Y. M. and Goodman, C. S.** (1993). *Soc. Neurosci. Abs.*
- Smith, S. J.** (1988). Neuronal cytomotility: the actin-based motility of growth cones. *Science* **242**, 708-715.
- Sone, M., Hoshino, M., Suzuki, E., Kuroda, S., Kaibuchi, K., Nakagoshi, H., Saigo, K., Nabeshima, Y. and Hama, C.** (1997). Still life, a protein in synaptic terminals of Drosophila homologous to GDP-GTP exchangers. *Science* **275**, 543-547.
- Stossel, T. P.** (1993). On the crawling of animal cells. *Science* **260**, 1086-1094.
- Tessier-Lavigne, M. and Goodman, C. S.** (1996). The molecular biology of axon guidance. *Science* **274**, 1123-1133.
- Van Vactor, D.** (1998). Tyrosine phosphatases in the developing nervous system. *Curr. Op. Cell Biol.* (in press).
- Van Vactor, D., Sink, H., Fambrough, D., Tsou, R. and Goodman, C. S.** (1993). Genes that control neuromuscular specificity in Drosophila. *Cell* **73**, 1137-1153.
- Wang, S. S. and Jay, D. G.** (1997). *Am. Soc. Cell Biol. Abs.*
- Wong, L. and Adler, P.** (1993). Tissue polarity genes of *Drosophila* regulate the subcellular location of prehair initiation in pupal wing cells. *J. Cell Biol.* **123**, 209-221.
- Yamada, K. M., Spooner, B. S. and Wessells, N. K.** (1971). Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.* **49**, 614-635.
- Zipursky, S. L., Venkatesh, T. R., Teplow, D. B. and Benzer, S.** (1984). Neuronal development in the Drosophila retina: monoclonal antibodies as molecular probes. *Cell* **36**, 15-26.