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# The *Caenorhabditis elegans* P21-activated kinases are differentially required for UNC-6/netrin-mediated commissural motor axon guidance

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P21 activated kinases (PAKs) are major downstream effectors of rac-related small GTPases that regulate various cellular processes. We have identified the new PAK gene *max-2* in a screen for mutants disrupted in UNC-6/netrin-mediated commissural axon guidance. There are three *Caenorhabditis elegans* PAKs. We find that each *C. elegans* PAK represents a distinct group previously identified in other species. Here we examine their roles in the postembryonic migration of the P cell neuroblasts and the axon guidance of the ventral cord commissural motoneurons (VCCMNs). We find that the two PAKs, *max-2* and *pak-1*, are redundantly required for P cell migration and function with UNC-73/Trio and the rac GTPases (CED-10 and MIG-2). During axon guidance of the VCCMNs, PAK-1 also acts with the rac GTPases, CED-10 and MIG-2, and is completely redundant with MAX-2. Interestingly, we find that unlike MAX-2 activity during P cell migration, for motoneuron axon guidance *max-2* is also required in parallel to this PAK-1 pathway, independent of rac GTPase signaling. Finally, we provide evidence that MAX-2 functions downstream of the UNC-6/netrin receptor UNC-5 during axon repulsion and is an integral part of its signaling.

**KEY WORDS: Axon guidance, Netrin, *unc-73*, rac, P21-activated kinase**

## INTRODUCTION

Neuronal connectivity is achieved by guidance of axonal growth cones to their targets through various environmental cues (Dickson, 2002; Tessier-Lavigne and Goodman, 1996). Cellular interpretation of these cues requires the presence of proper cell surface receptors and the execution of complex signaling pathways inside the growth cones. In *Caenorhabditis elegans*, analysis of the factors required for axon guidance has led to the discovery of the highly conserved UNC-6/netrin signaling pathway (Hedgecock et al., 1990; Ishii et al., 1992; Serafini et al., 1994). UNC-6/netrin functions as both an attractant and a repellent. The receptor UNC-5 is required for repulsion from UNC-6/netrin, while the UNC-40 receptor is required for both repulsion and attraction (Colavita and Culotti, 1998).

During development, the ventral cord commissural motoneurons (VCCMNs), which include DA, DB, DD, VD and AS neurons, are born on the ventral midline of the animal. They express both UNC-5 and UNC-40, and their commissural axons are thereby repelled by UNC-6/netrin located on the ventral side of the animal (Wadsworth, 2002). These commissures migrate circumferentially around the animal along the dorsoventral axis. The putative chemoattractant UNC-129, a TGFβ homolog, is thought to direct these axons into the dorsal cord (Yu and Bargmann, 2001). They then extend along the dorsal cord, innervating their target muscle cells. Collectively these VCCMNs function to control forward and reverse locomotion of the animals. Among them, the cholinergic (DA, DB and AS) motoneurons activate the dorsal muscles of the animals, while the GABAergic (DD and VD) motoneurons coordinate firing of the ventral and dorsal muscles (White et al., 1986). While the attractive and repulsive molecules that direct these neurons are fairly well understood, much less is known about the subcellular signaling events that interpret these cues. We have taken advantage of this system and used forward genetics to identify new components in this conserved signaling pathway (Huang et al., 2002). We have identified max-2, a *C. elegans* p21-activated kinase (PAK) that is necessary for the dorsal guidance of the VCCMN axons.

PAKs are a group of highly conserved signaling molecules that control cytoskeletal dynamics. Previous studies have demonstrated that PAKs are effectors of the rac/cdc42 subfamily of rho type GTPases. PAKs were initially discovered through their ability to bind activated (GTP-bound) forms of these small GTPases (Manser et al., 1994). Each PAK consists of an N-terminal GTPase-binding domain and a C-terminal serine/threonine kinase domain. The nominal activation pathway for conventional PAKs has been described, in which an activated rac/cdc42 molecule binds a PAK dimer, allowing relief from its self-inhibition and subsequent activation of its kinase domain (Bokoch, 2003). This activated PAK then goes on to target other downstream effectors, which include multiple regulators of the cytoskeleton, such as myosin light chain kinase and LIM kinase. In yeast, PAKs are required for invasive and vegetative growth (Hofmann et al., 2004). In *Drosophila*, Pak1 is required for photoreceptor axon guidance (Hing et al., 1999). In humans, a deficiency of PAK3 results in non-syndromic mental retardation, attributed to a loss of dendritic complexity (Allen et al., 1998).

Here, we describe and characterize the three *C. elegans* PAKs. We find that two *C. elegans* PAKs function redundantly in P cell migrations with UNC-73/Trio and the rac GTPases, but are differentially required for UNC-6/netrin-mediated commissural motor axon guidance. We provide evidence that while PAK-1 is completely redundant with MAX-2 and functions with the rac GTPases during axon guidance, the newly identified *max-2* is additionally required in a rac-independent pathway. Finally, we show that MAX-2 functions downstream of the receptor UNC-5 during UNC-6/netrin-mediated axon repulsion.

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## MATERIALS AND METHODS

### *C. elegans* and culture methods

Worm cultures were maintained as described (Brenner, 1974). For RNAi experiments, dsRNA was microinjected into the intestine or gonad of young adult animals as described (Fire et al., 1998). The following commercially available RNAi clones (Ahringer Library Clones unless otherwise specified) were used in this study: I 2J23, II 8F19, X 3E04, C09B8.7 (Open Biosystems).

The following mutant alleles were used in these studies: LG I: *unc-40*(*e1430*), *dpy-5*(*e61*), *unc-73*(*gm33*), *unc-73*(*e936*), *unc-73*(*rh40*); LG II: *max-2*(*cy2*), *max-2*(*nv162*), *rol-1*(*e91*); LG III: *pag-1*(*ls2*); LG IV: *unc-5*(*e53*), *dpy-20*(*e1282*), *unc-129*(*ev557*), *eri-1*(*mg366*); LG V: *pak-2*(*ok332*), *unc-34*(*e566*), *him-5*(*e1490*), *max-1*(*cy2*); *evIs41*[*Pmec-7::lacZ*, *Pmec-7::unc-5*]; LG X: *oxIs12*[*Punc-47::GFP*, *lin-15*(+)], *pak-1*(*ok448*), *unc-6*(*ev400*), *unc-6*(*ju152*), *mig-2*(*mu28*).

### Genetic mapping

*max-2*(*cy2*) was mapped to LG II by standard SNP methods (Wicks et al., 2001). Mapping analysis placed *max-2*(*cy2*) between SNPs Y38F1A(41,752) and W09E7(4,859). dsRNA of open reading frames (ORFs) from within this genomic region were tested for their ability to elicit a phenotype (Fraser et al., 2000). Only dsRNA from the predicted ORF Y38F1A.10 was found to cause axon guidance defects. Sequencing of *max-2*(*cy2*) genomic DNA found a G-A transition at Y38F1A (69,599 bp).

### Deletion library screening

The *max-2*(*nv162*) allele was isolated by PCR screening of a *C. elegans* UV/TMP mutagenized deletion library using the following primers: nv162.f1, CTCTCTCTCAACCCGGCAGGAAG; nv162.f1', CCGGCAGGAAGACTATATGACTC; nv162.r1, TGTCGTCGCTCTCTCACGCACCT; and nv162.r1', CACAAAGAGGGAAGAAGATCCTC. Genotyping *max-2*(*nv162*) was subsequently performed by using the inner (nv162.f1' and nv162.r1') primers along with primer nv162.r2 CCTTCTTCTGATCGGCAAGACTG, which lies inside the *max-2*(*nv162*) deletion.

### Molecular biology

Cloning of DNA and generation of transgenes were accomplished by standard techniques (Hobert, 2002; Shevchuk et al., 2004). cDNAs were generated by RT-PCR from total N2 RNA samples. One *max-2* cDNA was generated by ligating the 3′ end of YK651h1 (gift from Yuji Kohara) onto the 5′ end of one of our cDNAs to generate a full-length *max-2* cDNA free of sequence changes. This was then cloned into a pBluescript SK(+) vector to give the clone pHJ101. To generate the *max-2* rescue construct, a full-length *max-2* cDNA was fused to the promoter region of *max-2* with PCR fusion techniques. Briefly PCR was used to generate a partial *max-2* cDNA (lacking the first two exons) out of pHJ101 with primers PMM.f1 (CAGAAGTTCAGCGGACTGCCGCAA) and PBK.r1 (CAGCTATGACCATGATTACGCCA). The following primers ex.f (GTTGCGTGCATATTCCGAACAGT) and ex.r (TTGCGGCAGTCCGCTGAACTTCTG) (complimentary to PMM.f1) were used to generate a *max-2* promoter region through the beginning of the third exon from genomic DNA. The resulting two PCR fragments were used as a template to generate the *max-2* rescue construct by PCR with primers mx2r.f' (GCTTGTCGAGTGTGCAATTTGTC) and mx2r.r' (AATGGTTCAATTCTACAGTTTATAGATT). This PCR fusion product was cloned into a TOPO XL vector to yield pHJ102.

### Scoring of animals

DD and VD commissural defects were scored as previously described (Huang et al., 2002). Briefly, the number of wild-type or defective commissures from a sample population was counted and percent defects were determined by summing all the defective commissures and dividing this by the number of commissures expected in the same number of wild-type animals (17 commissures/animal). A commissure was deemed to fail to reach the dorsal cord only if no part of the commissure could be observed to connect to the dorsal cord. For constitutively active rac experiments, the number of animals scored (*n*) was the combination of at least two independently generated lines. All lines assayed showed the same trends. Constitutively activated rac [*rac*(*GF*)] lines were crossed into the mutant [*pak-1*(*ok448*) or *max-2*(*cy2*)] background and *rac*(*GF*)*;wild type* and *rac*(*GF*)*;mutant* animals were isolated. The *rac*(*GF*)*;wild type* and *rac*(*GF*)*;mutant* animals were scored as first cousins. For *rac*(*GF*) RNAi experiments transgenic lines were generated in an *eri-1*(*mg366*)*IV;oxIs12X* background. Transgenic animals were picked to a single plate and approximately half were injected with dsRNA. The progeny of the injected and non-injected animals were then scored. The rac(GF) constructs were generously donated by Erik Lundquist, and each contains a rac gene with the equivalent to the canonical G12V mutation under the control of the *unc-115* promoter (Struckhoff and Lundquist, 2003).

P cell migration defects were scored by counting the number of laterally displaced VD neurons. VD neurons were identified by the expression of GFP in an *oxIs12*[*Punc-47::GFP*] background and by their gross morphology. VD neurons were counted as being laterally displaced if the cells were more than two cell widths from the ventral cord or had no visible connection to the ventral cord.

For the suppression of ectopic UNC-5 experiments, animals were fixed and stained as described (Colavita and Culotti, 1998). Touch receptor cells were scored as reaching the dorsal cord if any part of the neuron reached the dorsal cord. Only the anteriormost touch receptor cells (the ALMs and the AVM) were scored. Only animals in which all of the anterior touch receptor cells could be seen to extend long axons (or were directed into the DC) were scored.

## RESULTS

### *max-2* is required for commissural axon guidance of the VCCMNs and represents a gene with homology to p21-activated kinases

In a forward genetic screen for *C. elegans* mutants in motor axon guidance we have identified *max-2*(*cy2*) (Huang et al., 2002). *max-2*(*cy2*) animals have defects in the guidance of the axons of the VCCMNs (Fig. 1). All *max-2* animals show some percent of misguided commissures, yet have only subtle defects in locomotion and appear superficially wild type. Newly hatched *max-2* animals exhibit defects in the embryonically connected DA, DB and DD motoneurons (Fig. 1D,H), as well as in the postembryonic VD motoneurons (Fig. 1F). In *max-2* mutant animals, many motoneuron commissures turn either anteriorly or posteriorly before reaching the dorsal cord (Fig. 1D,F,H). Those commissures that fail to reach the dorsal cord often possess the length necessary to reach the dorsal cord had they been guided in the proper direction. This is consistent with *max-2* being required for the dorsal guidance of these motoneurons and not solely required for axonal outgrowth.

We mapped the *max-2*(*cy2*) mutation to an approximately 200 kb region on chromosome II (Fig. 2A). We next used a reverse genetics candidate screen to identify the *max-2* gene. dsRNA from only one of the genes in this area elicited an axon guidance phenotype. This predicted ORF Y38F1A.10 has homology to PAKs (Hofmann et al., 2004). A partial cDNA clone (a gift from Yuji Kohara) was found to span this predicted ORF and an adjacent one (F18A11.4) indicating that they are, in fact, a single gene (Fig. 2A boxed region). Comparison with a syntenic region of *C. briggsae* and knowledge of published PAK sequences allowed us to predict a probable ORF consisting of parts of these two annotated ORFs. Isolation and analysis of cDNAs confirmed this and demonstrated the genomic organization of the *max-2* gene (Fig. 2B). A minigene consisting of a 5 kb upstream element fused to a *max-2* cDNA (Fig. 2B) was found to rescue the *max-2*(*cy2*) defect (Fig. 2C).

### *max-2*(*cy2*) and *max-2*(*nv162*) are likely null alleles

The *max-2*(*cy2*) allele has a missense mutation in the ATP-binding region of the kinase domain. The resulting amino acid sequence change is expected to convert a highly conserved glycine to a glutamate (Fig. 2B, Fig. 4A). This glycine is the third in a group of glycines (gly-X-gly-X-X-gly) that are in the conserved region of

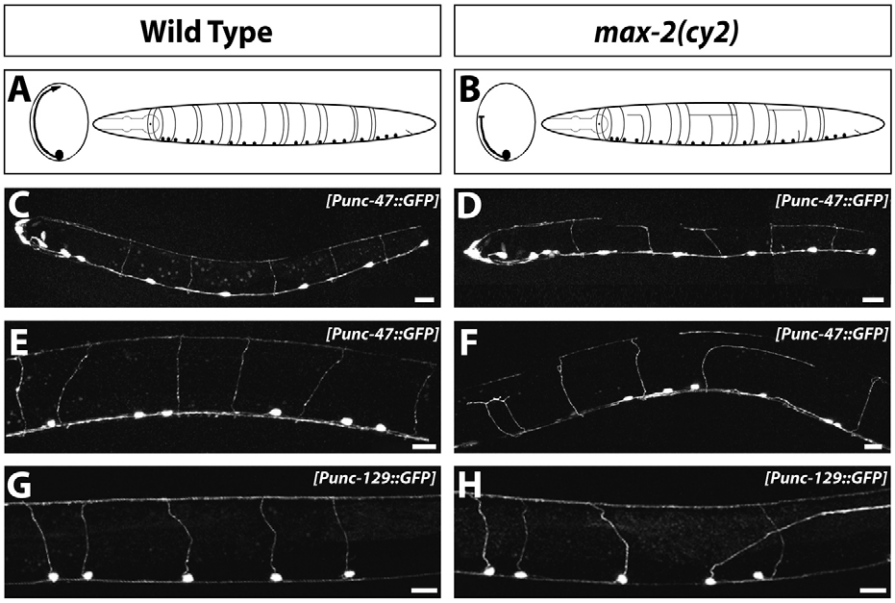


**Fig. 1. *max-2* is required for ventral cord commissural motoneuron guidance.** (**A**,**B**) A schematic of the DD and VD cell bodies (solid dots) and commissural axons in wild-type (A) and *max-2*(*cy2*) (B) animals. In each panel, a cross-section is on the left and a lateral view of the entire animal is on the right. (**C-H**) Confocal images of representative animals: anterior is to the left and dorsal is up. (C,D) First larval stage (L1) animals before the formation of the VD neurons. In wild-type animals (C), the dorsal commissures of the DD neuron all reach and enter the dorsal cord, but the DD commissures in *max-2*(*cy2*) animals (D) often fail to reach the dorsal cord. (E,F) L4 animals, after the migration of the VD commissures. In wild-type animals, all commissures reach the dorsal cord (E). In *max-2*(*cy2*) animals, many commissures fail to reach the dorsal cord (F). All animals in C-F are in the *oxIs12*[*Punc-47::GFP*]*X* background to visualize the DD and VD motoneurons. (G,H) *max-2* animals have defects in the DA and DB motoneurons. Shown here are examples of commissural axons in wild-type (G) and *max-2*(*cy2*) animals (H) at early L4 stage. All animals in G,H are in the *evIs82*[*Punc-129::GFP*] background to visualize the DA and DB neurons. Scale bar: 10 μm.

kinase subdomain I (Hanks et al., 1988). This change probably impairs the kinase activity by altering the structure of the ATP-binding pocket. To further examine the function of *max-2*, we analyzed the phenotype of *max-2*(*RNAi*) animals and isolated an additional mutant allele *max-2*(*nv162*). We found that RNAi of *max-2* caused a weaker but similar phenotype than either of our two mutants (Fig. 2D).

The allele *max-2*(*nv162*) was found by screening a deletion library for N-terminal deletions in the *max-2* gene. The *max-2*(*nv162*) allele contains a deletion that removes all of exons 1-4, including the start codon (Fig. 2B). The first in frame methionine would lead to an interrupting stop codon before any wild-type protein could be made. The next in frame methionine does not occur until the middle of the kinase domain. *max-2*(*nv162*) is therefore almost certainly a null.

To test this and to compare the defects of these two mutant alleles, we scored the severity of each mutant defect in the dorsal guidance of the DD and VD neurons. We found that the phenotypes of the two mutants were of similar nature and severity (Fig. 2D). Next we scored the severity of *max-2*(*cy2*)*/max-2*(*nv162*) animals and found that they were not significantly different from animals homozygous for either allele (data not shown). Taken together, these results indicate that both *max-2*(*cy2*) and *max-2*(*nv162*) are likely genetic nulls.

## *max-2* expression is required in neurons for axon guidance

To examine the expression of *max-2*, we generated promoter-GFP fusions (Chalfie et al., 1994; Fire et al., 1990). GFP expression is nearly ubiquitous in the early embryo. At early comma stage expression becomes intensely focused in the anterior of the embryo (Fig. 3A). Strong expression is observed from the pharynx and some unidentified head neurons beginning around the 1.5-fold stage. After hatching, GFP expression is present in the ALM and PLM neurons (Fig. 3D). Beginning in late L1 stages expression comes on in the PVD neurons and some time later in the AVM (Fig. 3D,E). Despite using the same upstream DNA elements utilized for our rescue constructs with these promoter-GFP constructs, we did not observe significant expression of GFP in VCCMNs.

To test whether *max-2* functions cell autonomously to guide motoneurons we expressed *max-2* cDNAs under the control of tissue-specific promoters. There are 26 GABAergic neurons, 19 of these (six DD and 13 VD) send commissures dorsally and have defects in *max-2* mutants. We used promoters from the characterized *unc-25* and *unc-47* genes (Jin et al., 1999; McIntire et al., 1997) to drive expression of a *max-2* cDNA specifically in the GABAergic neurons of *max-2*(*cy2*) animals. Both these promoter-cDNA fusions could rescue the defects of *max-2*(*cy2*) mutants (Fig. 3F). We conclude that MAX-2 functions cell autonomously in these neurons.

## The *C. elegans* genome contains three p21-activated kinases

A database search of the *C. elegans* genome demonstrates the existence of three *C. elegans* PAKs, including *max-2*. *Caenorhabditis elegans pak-1* has previously been cloned by degenerate PCR and has been shown to co-localize with CED-10 and CDC-42 at late embryonic stages (Chen et al., 1996). Few data exist on the other PAK (C45B11.1), which we find is SL1 trans-spliced and will be hereafter referred to as *C. elegans pak-2*. Presented in Fig. 4A is a kinase domain alignment of the three *C.*

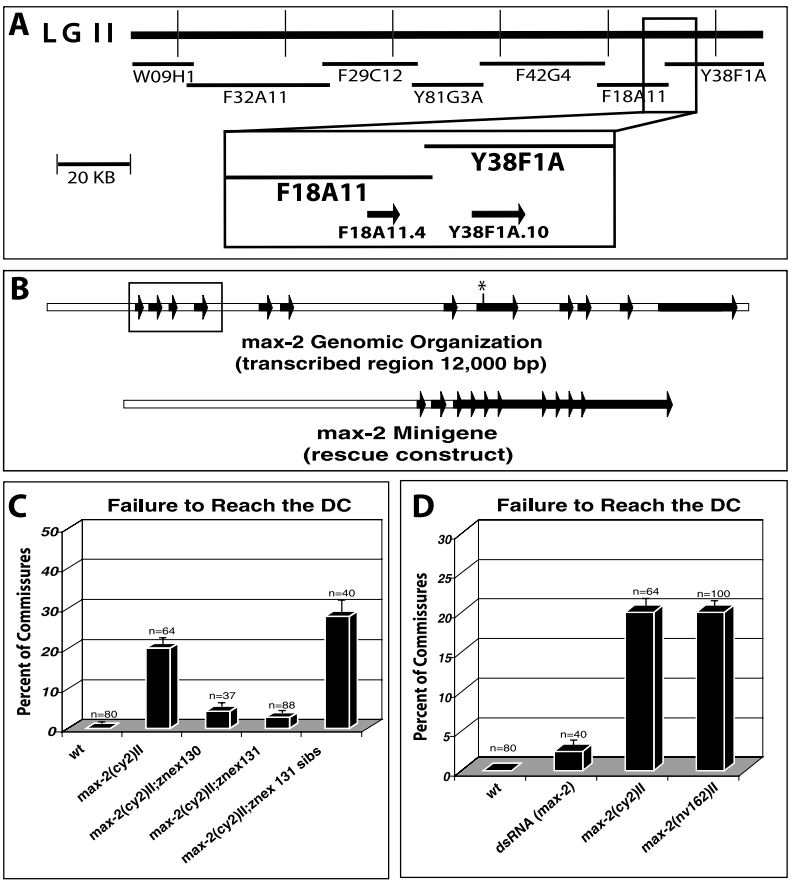


**Fig. 2. *max-2* is a single gene that includes the two predicted ORFs F18A11.4 and Y38F1A.10.** (**A**) A diagram of a genomic region on the distal right arm of chromosome II (LGII) that contains the *max-2* gene. Cosmid and YAC coverage in this region are diagrammed with black bars. The boxed region of the schematic is enlarged and shows the region that contains the two predicted ORFs F18A11.4 and Y38F1A.10 (boxed region is not to scale). (**B**) A diagram of the *max-2* genomic organization (upper) and the minigene construct (lower) used for the rescue experiments in (**C**). The boxed area indicates the region deleted in the *max-2*(*nv162*) allele, and the asterisk indicates the site of the point mutation identified in the *max-2*(*cy2*) allele. (C) *max-2* mutants are rescued by injecting the rescue construct depicted in B. The graph shows the percentage of the DD and VD motor commissures that fail to reach the dorsal cord (DC). Two independent transgenic lines expressing the minigene construct *znex130-131*[*Pmax-2::max-2*(*cDNA*)] are shown here; non-transgenic siblings that lack the transgene are also shown for *znex131*. (**D**) *max-2*(*cy2*) and *max-2*(*nv162*) show similar severity in the defects of dorsal guidance of commissural axons. Injecting double-stranded RNA (dsRNA) of the *max-2* gene causes less severe axon guidance defects that are otherwise similar to the *max-2* mutants. The numbers (*n*) of animals used for each experiment are shown; bars represent the standard error (C,D).

*elegans* PAKs and some closely related PAKs from other species. The overall structural differences of the *C. elegans* PAKs are also compared in Fig. 4B. These differences are striking with respect to the site and number of the putative SH3-binding motifs. An analysis of the PAK sequences from worms, flies and humans demonstrates that *C. elegans* PAK-1 is most closely related to group I/A PAKs and *C. elegans* PAK-2 is most closely related to group II/B PAKs (Fig. 4C). The kinase domain of MAX-2 is most closely related to group I/A PAKs (Fig. 4A), while the N-terminal region is more divergent than classic group I/A PAKs (Zhao and Manser, 2005). In this respect, MAX-2 is most similar to *Drosophila* DmPAK3.

## MAX-2 and PAK-1 function with partial redundancy to guide the VCCMNs

The motor axon defect of *max-2*(*cy2*) is the first mutant phenotype reported for any of the *C. elegans* PAKs. To begin to understand the in vivo functions of the other *C. elegans* PAKs, we created promoter-GFP fusions to examine their expression (see Fig. S1 in the supplementary material). We confirmed previous studies that *pak-1* is expressed in the VCCMNs (Iino and Yamamoto, 1998). *pak-1* was also expressed in the migrating distal tip cell (DTC), in the developing uterus and later in the vulval muscle cells (see Fig. S1 in the supplementary material, and data not shown). *pak-2* did not appear to be neuronally expressed (see Fig. S1 in the supplementary material). To further address whether all three *C. elegans* PAKs are involved in UNC-6/netrin axon guidance, we analyzed *pak-1* and *pak-2* mutants for neuronal defects similar to those found in *max-2* mutants. *pak-1*(*ok448*) and *pak-2*(*ok332*) mutant alleles were obtained from the *C. elegans* Gene Knock Out Consortium. Both these mutants are expected nulls. The *pak-1*(*ok448*) allele has a deletion that removes the majority of the kinase coding region and results in a frame shift that is expected to cause a premature stop codon. The *pak-2*(*ok332*) allele has a deletion that removes the start codon. Both these PAK mutants appear superficially wild type and we have found them to be wild type for DD and VD axon guidance (Fig. 5E).

The double PAK mutants *max-2*(*cy2*)*;pak-1*(*ok448*) were severely defective for DD and VD axon guidance (Fig. 5D,E). These animals were also uncoordinated, defective in egg laying and in DTC migrations, and exhibited ventral enclosure defects. RNAi was used to confirm these results independently (data not shown). *max-2*(*cy2*)*;pak-2*(*ok332*) animals were superficially wild type and did not have DD and VD defects significantly worse than *max-2*(*cy2*) animals (Fig. 5E). *pak-1*(*ok448*)*;pak-2*(*ok332*) animals had defects in embryogenesis and exhibited L1 lethality, but escapers were wild type for VCCMN guidance and appeared relatively coordinated. We conclude that PAK-2 does not play a role in the axon guidance of the VCCMNs, while *max-2* and *pak-1* function with some redundancy to control axon guidance.

## PAK mutants are phenotypically similar to *unc-73/Trio* and *rac* mutants

PAKs are activated by racs. Racs are rho family GTPases, implicated in controlling cell migrations and axon guidance by acting as molecular switches that can relay and amplify cellular signals (Hall, 1998; Lundquist, 2003; Luo et al., 1997). Racs are activated by guanine exchange factors (GEFs). Of the rac activators, which coordinate rac activity during axon guidance, UNC-73/Trio is the best known (Steven et al., 1998). In *C. elegans*, *unc-73/Trio* mutants have major defects in cell migrations, axon

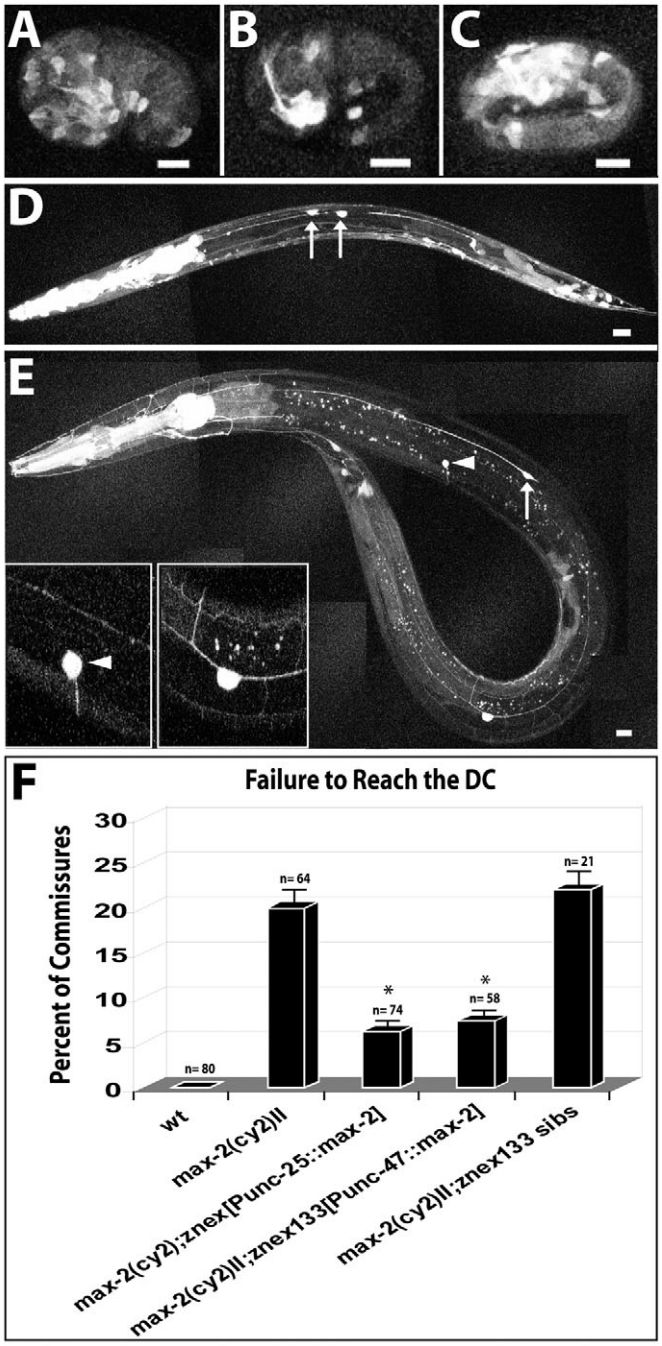


**Fig. 3. *max-2* expression is dynamic and is required cell autonomously for dorsal commissure guidance.** (**A-E**) The expression pattern of *max-2* from *znex135*[*Pmax-2::GFP*] transgenic animals. Promoter element (5′-3′) contains the 5 kb region from 5557 to 262 on cosmid F18A11. (A-C) At the comma and later embryonic stages, GFP expression is mainly in the anterior region of the embryo and along the ventral cord. (D) After hatching, GFP is expressed in the pharynx and several head and tail neurons. Expression of GFP in the ALM neurons (arrow) and the postembryonic PVD neuron can also be seen. (E) A young adult animal, showing GFP expression in the postembryonic AVM neuron (arrow and left inset) and the elaborate dendritic connections of a mature PVD neuron (right inset). (**F**) A *max-2* cDNA expressed in the DD and VD motoneurons cell-autonomously rescues the *max-2*(*cy2*) defects in these neurons. For the *Punc-25::max-2* rescue, the data presented are the combined data from four independently generated transgenic lines. A *Punc-47::max-2* rescue line (*znex133*) and its non transgenic siblings (*znex133 sibs*) are also shown here. The *unc-25* promoter used for this experiment contained 2 kb of the 5′ region through the start codon. The *unc-47* promoter used for this experiment contained 1.7 kb of the region 5′ to the start codon through the 50th codon. The numbers (*n*) of animals used for each experiment are shown; bars represent the standard error in F. Asterisks indicate a *P*-value of less than 0.001 (Student's *t*-test). In all pictures, anterior is to the left and dorsal is up. Scale bar: 10 μm.

outgrowth and axon guidance. The three *C. elegans* racs (MIG-2, CED-10 and RAC-2) are activated during multiple developmental processes by the GEF UNC-73/Trio (Lundquist et al., 2001; Wu et al., 2002; Zipkin et al., 1997). Consistent with *C. elegans* PAKs being used in this UNC-73/Trio-rac pathway, we found that *max-2*(*cy2*)*;pak-1*(*ok448*) double mutants were phenotypically very similar to *unc-73* mutants and also *ced-10*(*n1993*)*;mig-2*(*mu28*) double rac mutant animals with respect to their mutant phenotypes in DD and VD axon guidance and also in P cell migrations (Fig. 5 and Table 1). We chose to examine in detail the roles that the *C. elegans* PAKs play during these two different cellular processes. We first present data on PAK function in P cell migrations and then examine PAK function in the UNC-6/netrin VCCMN axon repulsion pathway.

## The *C. elegans* PAKs function redundantly in the UNC-73/Trio-RAC pathway to guide P cell migrations

Immediately after hatching, the 12 P cells migrate from their lateral positions down into the ventral cord and subsequently divide, generating the P cell lineage. A failure in this migration can lead to the ectopic placement of cells in the P cell lineage. Among the P cell descendents are the VD neurons (Sulston and Horvitz, 1977). We used ectopically placed VD neurons as an indicator of defects in P cell migrations. Two racs (CED-10 and MIG-2) are implicated in acting with UNC-73 during the migration of the P cells (Spencer et al., 2001; Wu et al., 2002). Both *unc-73* and double rac mutants have defects in P cell migrations. We found that, while no single PAK mutant exhibited defects, the double PAK mutant *max-2*(*cy2*)*;pak-1*(*ok448*) had defects in P cell migrations similar to those of the *unc-73* mutants and the *ced-10*(*n1993*)*;mig-2*(*mu28*) double rac mutants (Fig. 5B-D,F and Table 1). As with the racs, we found that the PAKs, *max-2* and *pak-1*, were completely redundant with each other for this process.

We found that either *max-2*(*cy2*) or *pak-1*(*ok448*) only slightly increased the P cell migration defect of a weak *unc-73* allele [*unc-73*(*rh40*)]. Interestingly, a double mutant of *max-2*(*cy2*) and a strong allele of *unc-73* [*unc-73*(*gm33*)] did not increase the average number of ectopic P cells per animal found in the *unc-73*(*gm33*) allele alone (Table 1). It is important to note that null alleles of *unc-73* are lethal, which precludes a definite conclusion that the PAKs act entirely with UNC-73 during P cell migrations. However, our observation that a loss of *max-2* did not enhance the strong *unc-73* allele, along with the weak enhancement observed in *PAK;unc-73*(*rh40*) double mutants, indicates that the two *C. elegans* PAKs (MAX-2 and PAK-1) act with UNC-73 in guiding P cell migrations. Finally, we found that any combination of *rac;PAK* double mutants either did not enhance, or only weakly enhanced, the defects of the single mutants (Table 1). This indicates that the PAKs function linearly with the racs to guide this process.

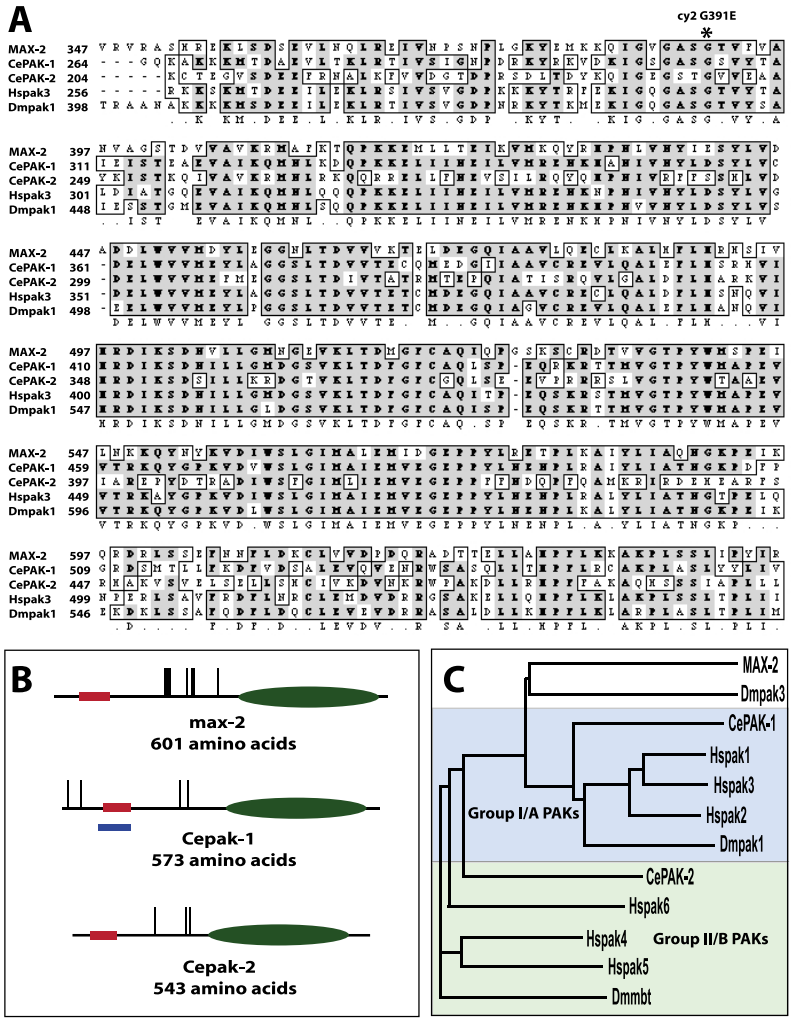


**Fig. 4. Sequence analysis of the three *C. elegans* p21-activated kinases.** (**A**) An amino acid sequence comparison of the kinase domains from the three *C. elegans* PAKs and representative PAKs from other species. The asterisk indicates the site of the *max-2*(*cy2*) mutation. (**B**) A diagram of the structural organization of the three *C. elegans* PAKs. The red boxes indicate the P21-binding domain, the blue box indicates the site of a clearly conserved autoinhibitory domain, the green ovals indicate the kinase domain and the vertical lines indicate the sites of putative SH3-interacting (PXXP) domains. (**C**) An unrooted phylogenetic tree of the entire amino acid sequence of all the known PAKs from *C. elegans* (Ce), *D. melanogaster* (Dm) and *H. sapiens* (Hs). The group I/A and group II/B PAKs are boxed with blue and green, respectively. This tree was generated with the ClustalW alignment tool from the European Bioinformatics Institute (www.ebi.ac.uk/clustalw/). Sequences for *max-2* and *pak-2* were deposited in GenBank as DQ523832 and DQ523831, respectively.

## The *C. elegans* PAKs function differentially to control *UNC-6*/netrin-mediated VCCMN axon guidance

Of all the *C. elegans* rac and PAK single mutants, only *max-2* animals had significant defects in UNC-6/netrin-mediated VCCMN axon guidance (Fig. 6A). To determine if these molecules act together or in parallel, we systematically analyzed double mutant combinations. Double mutants of either of the two rac genes (*ced-10* or *mig-2*) with *pak-1* did not cause defects in VCCMN guidance greater than any single mutant (Fig. 6A). This is consistent with PAK-1 and the racs acting linearly in this VCCMN guidance pathway. Surprisingly, we found that *max-2* double mutants with either of these two rac genes greatly enhanced the VCCMN defects of *max-2* (Fig. 6A). This indicates that MAX-2 functions in parallel to the racs and PAK-1 during VCCMN guidance.

It has previously been reported that the racs function with (and probably downstream of) UNC-73 to guide the DD and VD motoneuron axons (Wu et al., 2002). We found that double pak mutants were phenotypically similar to *unc-73* mutants, yet single PAK mutants enhanced non-lethal *unc-73* alleles for DD and VD axon guidance defects (Fig. 5B,D, Fig. 6A and data not shown). This has previously been reported for the racs as well (Wu et al., 2002).

To further address whether this parallel rac-independent pathway exists, we tested whether a loss of function in PAK gene activity could suppress the defects resulting from constitutively active racs. We hypothesized that the defects caused by a constitutively active molecule would be decreased when one of its downstream effectors was lost, or conversely the loss of parallel activity would cause an increase in these defects. We generated transgenic animals that express constitutively active *C. elegans* racs under a pan-neuronal promoter (Struckhoff and Lundquist, 2003). We found that loss of function in *pak-1*(*ok448*) significantly suppressed the axon guidance defects caused by the constitutively active rac, MIG-2 (Fig. 6B). We also found consistent but non-significant suppression of the defects caused by constitutively active CED-10 when we used RNAi to knock out *pak-1* function. Generally, RNAi yields a weaker phenotype than null mutants, particularly in neurons. For this reason we cannot conclude that PAK-1 does not function downstream of CED-10. However, our results are consistent with PAK-1 acting downstream of at least some of the rac GTPases.

Unlike *pak-1*, a loss of function in *max-2* considerably enhanced the defects caused by constitutively active racs in all cases (Fig. 6C). This indicates that *max-2* functions in a rac-independent pathway during commissural axon guidance. It is important to note that this does not exclude MAX-2 from functioning downstream of the racs as well. In our assay, the loss of MAX-2 parallel activity may have masked any suppression [of the *rac*(*GF*) induced defects] that was occurring. Collectively, our results indicate that PAK-1 functions with the racs, downstream

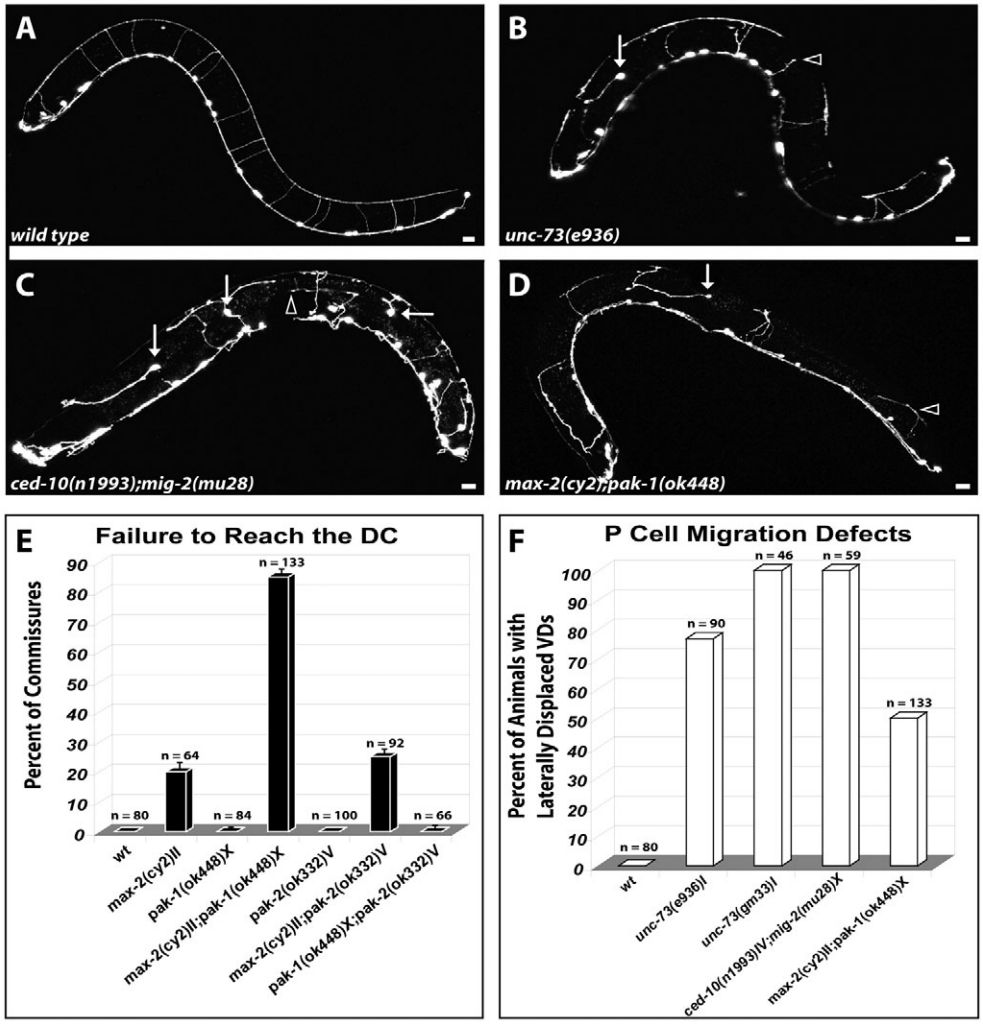


**Fig. 5. *max-2;pak-1* double mutants are phenotypically similar to *unc-73* and double rac mutants.** (**A-D**) Representative morphology of the DD and VD neurons in *unc-73*, rac and PAK mutants. Arrows point to displaced VD neurons and open arrowheads point to abnormal commissures. (A) A wild-type animal. (B) An *unc-73*(*e936*)*I* animal. (C) A *ced-10*(*n1993*)*IV;mig-2*(*mu28*)*X* animal. (D) A *max-2*(*cy2*)*II;pak-1*(*ok448*)*X* animal. (**E**) DD and VD commissural guidance defects in PAK mutants. (**F**) P cell migration defects in PAK, rac and *unc-73* mutants. The numbers (*n*) of animals used for each experiment are shown; bars represent the standard error in E,F. All animals are in the *oxIs12*[*Punc-47::GFP*]*X*. Scale bar: 10 μm.

of MIG-2 (at least) and that MAX-2 functions in parallel (at least partly) to this UNC-73-rac-PAK-1 pathway to guide the VCCMN axons.

## MAX-2 likely acts with UNC-6/netrin during DD and VD commissural axon guidance

As *max-2* mutants exhibited defects specifically in an axon guidance process controlled by UNC-6/netrin, we analyzed *max-2* genetic interactions with genes that are implicated in this pathway. Mutations in the dorsally expressed orphan ligand *unc-129* (Colavita et al., 1998) exhibited an additive enhancement in a *max-2* background. However, *max-1* and *unc-34* mutants were dramatically enhanced in a *max-2* background (Fig. 6D). Double mutants of *unc-40*/DCC (the UNC-6/netrin receptor) and *max-2* exhibited synergistic genetic interactions (Fig. 6D). This indicates that the two (*unc-40* and *max-2*) are required in parallel but interconnected signaling pathways. Null mutants of *unc-6*/netrin and *unc-5* have VCCMN axon guidance defects that are absolutely severe. Because of this, *unc-6* and *unc-5* double mutant combinations with *max-2* were less than informative. As an alternative we tested *max-2* dosage interactions with these genes. *max-2* mutants synergized with a hypomorphic allele of *unc-6* to nearly complete severity [from 20% failure in *unc-6*(*ju152*) to 75% in *max-2*(*nv162*);*unc-6*(*ju152*)]. Additionally, we found that *max-2* mutants were dramatically enhanced when they had only a single copy of *unc-6* or *unc-5*. A much weaker enhancement was observed when only a single copy of *unc-40* was present (Fig. 6D). Collectively, our data indicate that MAX-2 functions with UNC-6/netrin and its repulsion receptor UNC-5 in parallel to UNC-40 to mediate commissural motor axon guidance.

## The axon guidance defects caused by ectopic expression of the UNC-6/netrin receptor UNC-5 are partially suppressed by a loss of *max-2*

To test directly whether *max-2* functions downstream of UNC-5 and UNC-6/netrin, we determined whether a loss of *max-2* function could suppress the axon guidance effects of ectopically expressed UNC-5. Previously, Hamelin and colleagues (Hamelin et al., 1993) demonstrated that if UNC-5 is ectopically expressed in the touch receptor cells their axonal processes are re-routed away from UNC-6/netrin. This effect absolutely requires UNC-6/netrin and demonstrates that the machinery necessary for UNC-5 signaling is present in these cells. This is the basis for a classic screen that has identified many genes required for UNC-5 repulsion (Colavita and Culotti, 1998). Our *max-2* expression studies indicate that MAX-2 is present in several of these touch receptor cells. In particular, high expression was observed in the anteriormost touch receptor cells (the ALMs and the AVM) (Fig. 3D,E). We reasoned that MAX-2 might be part of the signaling machinery usurped by the ectopically expressed UNC-5, to misguide these axonal projections.

**Table 1. MAX-2 and PAK-1 function with redundancy in the UNC-73-rac pathway to control P cell migrations**

| Genotype | Percentage of animals with laterally displaced VDs* | Average number of laterally displaced VDs/animal† | *n* |
|---|---|---|---|
| *wt* | 0 | 0 | 80 |
| *max-2(cy2)II* | 0 | 0 | 64 |
| *pak-1(ok448)X* | 0 | 0 | 84 |
| *max-2(cy2)II;pak-1(ok448)X* | 50 | 0.71 | 133 |
| *unc-73(rh40)I* | 27 | 0.38 | 52 |
| *max-2(cy2)II;unc-73(rh40)I* | 35 | 0.42 | 122 |
| *pak-1(ok448)X;unc-73(rh40)I* | 31 | 0.45 | 96 |
| *unc-73(gm33)I* | 100 | 4.8 | 46 |
| *max-2(cy2)II;unc-73(gm33)I* | 100 | 4.8 | 58 |
| *ced-10(n1993)IV* | 0 | 0 | 100 |
| *mig-2(mu28)X* | 0 | 0 | 80 |
| *ced-10(n1993)IV;mig-2(mu28)X* | 100 | 3.4 | 59 |
| *max-2(cy2)II;ced-10(n1993)IV* | 0 | 0 | 100 |
| *max-2(cy2)II;mig-2(mu28)X* | 6.3 | 0.11 | 95 |
| *pak-1(ok448)X;ced-10(n1993)IV* | 5.5 | 0.05 | 55 |
| *pak-1(ok448);mig-2(mu28)X* | 1.5 | 0.01 | 68 |

*The number of animals with a laterally displaced VD neuron divided by the total number of animals scored.
†Animals were scored for the number of laterally displaced VD neurons and the sum of these numbers was divided by the total number of animals.

*max-2*(*cy2*) was crossed into the ectopic UNC-5 expressing strain NW798 (a gift from Joseph Culotti). *max-2*(*cy2*) mutants suppressed the guidance effects caused by the ectopic UNC-5 (Fig. 7A-C). We also found that in the *max-2* mutant background the touch cell receptor axons that reach the dorsal cord take a less direct route, often stretching anteriorly for approximately half their length before turning toward the dorsal cord. The lack of absolute suppression observed from a loss of *max-2* was quite similar to the defects observed in *max-2* mutants. In both cases an apparently relatively small portion of UNC-5 signaling was lost. Our ectopic UNC-5 suppression results are therefore highly correlative to the situation found with the guidance of the VCCMNs, where a loss of *max-2* is far less severe than a loss of *unc-5*. This confirms previous observations that there are multiple signaling pathways downstream of UNC-5, in UNC-6/netrin-induced axonal repulsion, and implicates MAX-2 as being integral to at least one of these cascades.

## DISCUSSION

We have identified MAX-2 as a p21-activated kinase. There are three PAK genes in the *C. elegans* genome. Sequence analysis indicates that each *C. elegans* PAK is representative of a distinct group of PAKs previously identified in other species. Importantly, none of the *C. elegans* PAK mutants is embryonic lethal. With these mutants, we can therefore perform in vivo genetic analysis on phenotypes to study specific PAK functions. Our genetic and expression pattern studies demonstrate that *pak-1* and *max-2* are key players in regulating cell migration and axon guidance: they function redundantly as rac effectors in P neuroblast migration and differentially for VCCMN axon guidance. We conclude that these *C. elegans* PAKs function both redundantly and independently to regulate multiple cellular processes.

### Each of the three *C. elegans* PAKs represents a distinct group of PAKs

In mammals, the PAK family is divided into two subgroups (I/A and II/B) based on structural differences. Members from both PAK groups are implicated in neuronal morphogenesis. The human PAK3, a group I/A PAK, is mutated in an X-linked mental retardation (Allen et al., 1998). Knockout mice deficient for PAK4, a group II/B PAK, show clear neuronal migration defects (Qu et al., 2003).

*Drosophila melanogaster* has three PAKs. The *Drosophila* group I/A PAK (Pak1; previously know as Dpak1) has been shown to be required along with NCK/Dock and UNC-73/Trio, for the guidance of photoreceptor axons (Hing et al., 1999; Newsome et al., 2000) and has also been shown to function downstream of the chemorepellent slit in midline axon guidance (Fan et al., 2003). The *Drosophila* group II/B PAK (Mbt), is believed to regulate neuronal morphogenesis, rather than axon guidance (Melzig et al., 1998). A third *Drosophila* PAK (Pak3) has sequence similarity to both group I/A and group II/B PAKs (Mentzel and Raabe, 2005). In vivo functional data on *Drosophila* Pak3 have yet to be reported.

We found that there are three PAKs in the *C. elegans* genome. Humans and mice contain six PAKs. Interestingly, we have found that the *C. elegans* PAKs consist of single representative members from the two mammalian PAK groups (I/A and II/B) and also a third more divergent member of the PAK family. This phenomenon has previously been reported of *D. melanogaster* (Mentzel and Raabe, 2005). The differences in PAK representatives between mammals and invertebrates may imply that an expanded repertoire of PAKs in both groups can replace the functions of more divergent PAKs. The expansion probably suggests a specialization of PAK activity for members within each group. Studying and comparing PAK functions will lead to a better understanding of the signaling specificity of different PAK member activities in vivo.

### A model for PAK activity in P cell migration and VCCMN axon guidance

The *C. elegans* PAKs function redundantly in the UNC-73/Trio-rac pathway to control the migration of P cells. Mutations in the individual PAK genes did not lead to defects. However, double mutants of *pak-1* and *max-2* did have defects in P cell migration. PAK mutants only weakly enhanced *unc-73*(*rh40*). Mutations in *max-2* did not enhance the defects of a strong *unc-73*/Trio allele. These data indicate that these PAKs function with UNC-73/Trio (see Fig. S2 in the supplementary material). Loss of either of two of the rac genes (*mig-2* or *ced-10*) in either a *max-2* or *pak-1* mutant background either weakly enhanced or had no defect greater than any of the single mutants. These data indicate that the PAKs function with the racs during this process. By examining the average number of ectopic cells per animal, we noted that in the double PAK mutant the severity

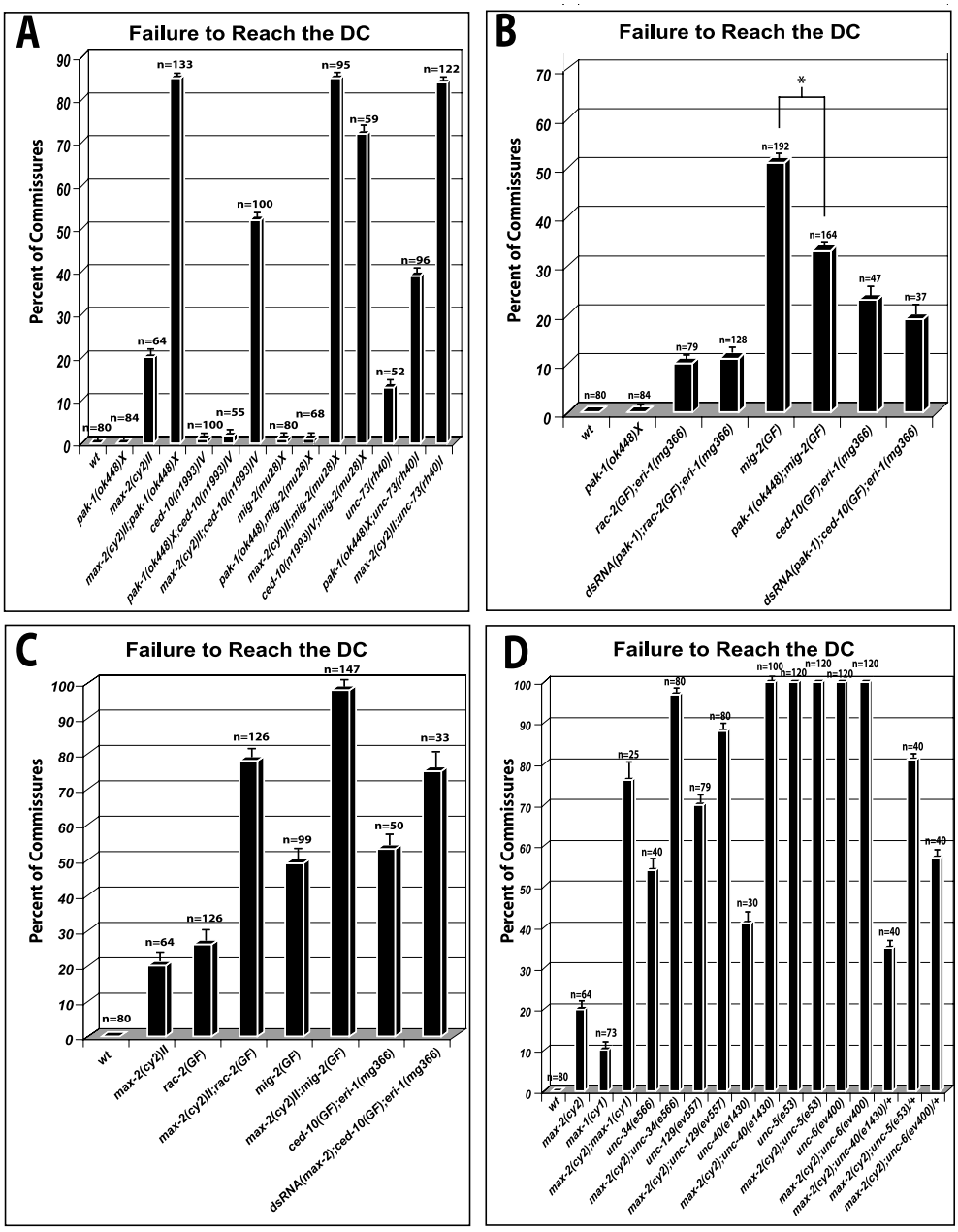


**Fig. 6. *pak-1* and *max-2* are differentially required for commissural axon guidance.** Genetic interaction studies on the DD and VD commissural guidance defects in PAK mutants and other mutants in genes that are known to play roles in the *unc-6*/netrin commissural guidance pathway. (**A**) Axon guidance defects of rac mutants are greatly enhanced by a loss of *max-2* but not by a loss of *pak-1*. (**B**) The defects caused by constitutively active *rac*(*GF*)*s* can be suppressed by the loss of *pak-1*. The asterisk denotes a *P*-value less than 0.001 (Student's *t* test). (**C**) The defects caused by constitutively active *rac*(*GF*)*s* are enhanced by a loss of function in the *max-2* gene. The *max-2* data was also independently confirmed with dsRNA as in the *ced-10*(*GF*) result. (**D**) *max-2*(*cy2*) enhances the defects of mutants from genes that are known to play roles in the *unc-6*/netrin commissural guidance pathway. The numbers (*n*) of animals used for each experiment are shown; bars represent the standard error.

of the P cell migration defects were less than that of the double *rac* mutant, which in turn was less severe than the strong *unc-73*(*gm33*) allele (Fig. 5F and Table 1). This is consistent with a model in which a fraction of the P cell migration activity of UNC-73 is controlled by the racs, and a fraction of this rac activity is controlled by the PAKs (see Fig. S2 in the supplementary material). This supports previous findings that UNC-73/Trio also stimulates rho activity (in addition to racs), which in turn activates the rho kinase (LET-502), facilitating the migration of P cells (Spencer et al., 2001).

The *C. elegans* PAKs act both rac-dependently and rac-independently in the guidance of VCCMN axons. In VCCMN axon guidance, as with the migrations of the P cells, the *rac* genes are reported to function downstream of the GEF UNC-73/Trio (Wu et al., 2002). Indeed, the phenotypes of double rac mutants and *unc-73/Trio* are remarkably similar (Fig. 5B,C). We found that, as has been reported for the racs, PAK mutants enhanced *unc-73* mutant defects for DD and VD axon guidance. This may indicate that there is another rac-GEF acting in parallel to UNC-73/Trio during this process (see Fig. S2 in the supplementary material). Although the *C. elegans* PAKs (*pak-1* and *max-2*) appeared to function in a typical manner with this GEF-rac pathway to guide the P cell migrations, the two did not function solely in this GEF-rac pathway for the dorsal guidance of commissural axons. While PAK-1 acts with the racs in guiding these axons, we found that MAX-2 has a unique role outside this pathway. Because *max-2* can act redundantly with *pak-1*, we conclude that *max-2* is probably also acting in part downstream of the racs in VCCMN guidance (see Fig. S2 in the supplementary material). Interestingly, *max-2* mutants enhanced *unc-73*/Trio axon guidance defects much more than *pak-1* mutants did (Fig. 6A). This is consistent with the rac-independent activity of MAX-2 acting in parallel to the GEF, UNC-73/Trio.

It is interesting to note that several lines of evidence indicate a subtle preference by the racs for the PAKs. We found that for P cell migrations double mutants of *ced-10* and *pak-1* were weakly

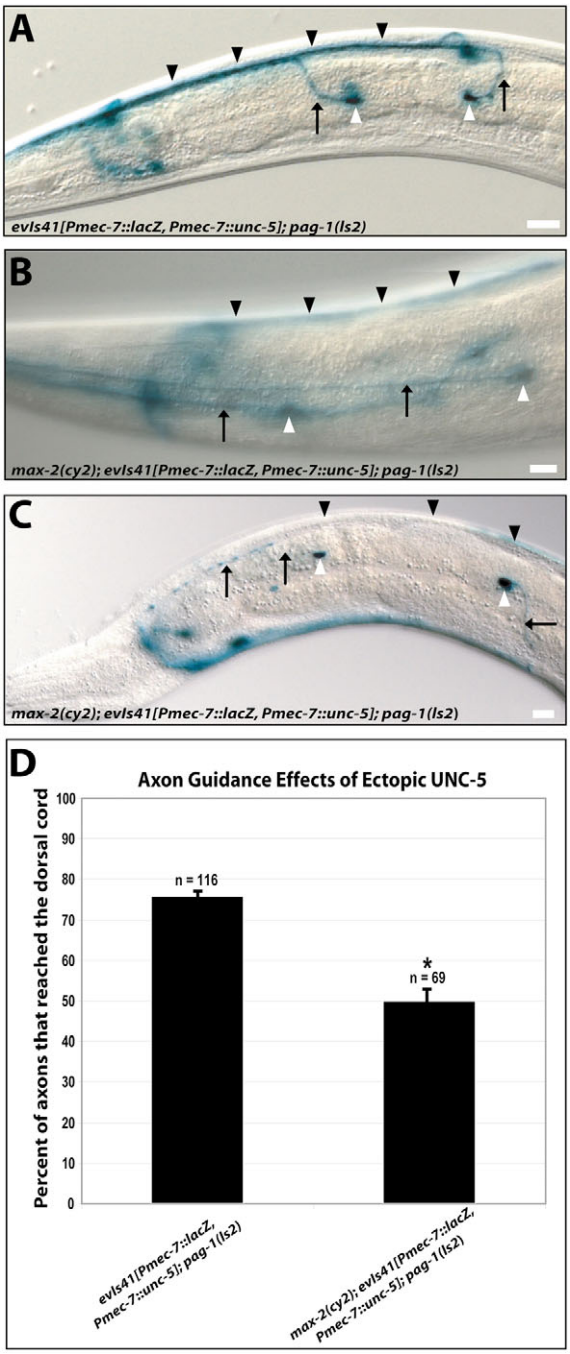


**Fig. 7. MAX-2 is required for the axon guidance effects induced by ectopic expression of UNC-5 in the anterior touch cell receptor neurons.** (**A-C**) DIC images of adult animals after fixation and staining for the presence of *lacZ*. In all images, dorsal is up and anterior is to the left. Black arrowheads mark the dorsal cord, white arrowheads mark touch receptor cell bodies and black arrows mark touch receptor cell axons. For clarity not all cell bodies and axons present are marked in each image. Animals [*evIs41*[*Pmec7::lacZ,Pmec7::unc-5*];*pag-1*(*ls2*)] that ectopically express UNC-5 and *lacZ* under the control of the mec-7 promoter have the anterior touch receptor cell axons (ALMs and AVM) guided to the dorsal cord (A). (B,C) In a *max-2* background, the guidance effects from ectopic expression of UNC-5 are partially suppressed. The image in B is two superimposed images from different focal planes of the same animal. Occasionally we observed ventral migration of the AVM axon in a *max-2* background, as shown in C. We never observed this in the ectopic UNC-5 strain alone. (**D**) A graphical representation of the quantification of ALM and AVM guidance in the two populations. The numbers (*n*) of animals used for each experiment are shown; bars represent the standard error. The asterisk denotes a *P*-value less than 0.001 (Student's *t*-test). Scale bar: 10 μm.

enhanced, while double mutants of *mig-2* and *pak-1* were not. We found that *max-2* had a reversed relationship (Table 1). Additionally, we found that a loss of *pak-1* significantly suppressed the defects caused by a constitutively active MIG-2 but did so to a lesser extent for CED-10. Conversely, a loss of *max-2* greatly enhanced the defects of constitutively active MIG-2 but did so to a lesser extent for CED-10 (Fig. 6). Finally, double mutants of *mig-2* and *max-2* were enhanced much more than double mutants of *ced-10* and *max-2* (Fig. 6A). A subtle opposite relationship could also be seen for *pak-1* (Fig. 6A). These data collectively argue that the preferred PAK for CED-10 is MAX-2, while the preferred PAK for MIG-2 is PAK-1. However, the fact that double mutants of *max-2;mig-2* or *pak-1;ced-10* do not equal a double PAK mutant indicate that either rac can use either PAK (Table 1).

## MAX-2 is involved in UNC-6/netrin-mediated commissural axon repulsion

The dorsal guidance of the VCCMN axon is controlled by the chemorepellent UNC-6/netrin and its receptor UNC-5. Null mutants of the two genes showed a complete absence of the commissurals. Although the absolute severity of null mutants precludes double mutant analysis, several lines of evidence indicate that MAX-2 acts downstream of UNC-6/netrin and UNC-5. First, the neurons that showed defects in *max-2* mutants appeared to be disrupted specifically in their dorsal commissural migrations. In *max-2* mutants the neuronal processes in the ventral cord of the VCCMNs were normal. Second, *max-2* mutants synergized with other mutants involved in this pathway. MAX-1 has been shown to mediate UNC-5 signaling in the VCCMN guidance. UNC-40 is another UNC-6/netrin receptor that is partially required in this guidance. Double mutants of *max-2* with *max-1* or *unc-40* showed profound commissural guidance defects, suggesting that *max-2* acts in parallel to both *max-1* and *unc-40*. Interestingly, *max-2* was also found to enhance a weak *unc-6* allele, suggesting that MAX-2 does not function downstream of UNC-6 with a simple linear relationship. Finally, *max-2* was required for axon guidance effects of ectopically expressed UNC-5. The axons of touch neurons were guided dorsally when UNC-5 was ectopically expressed in these cells. This phenotype absolutely requires UNC-6/netrin and is suppressed in *max-2* mutants. Taken together, our studies demonstrate that MAX-2 acts downstream of UNC-6/netrin and UNC-5 to regulate the dorsal guidance of VCCMN axons.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/22/4549/DC1

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