Redundant Canonical and Noncanonical Caenorhabditis elegans p21-Activated Kinase Signaling Governs Distal Tip Cell Migrations

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ABSTRACT p21-activated kinases (Paks) are prominent mediators of Rac/Cdc42-dependent and independent signaling and regulate signal transduction and cytoskeletal-based cell movements. We used the reproducible migrations of the Caenorhabditis elegans gonadal distal tip cells to show that two of the three nematode Pak proteins, MAX-2 and PAK-1, function redundantly in regulation of cell migration but are regulated by very different mechanisms. First, we suggest that MAX-2 requires CED-10/Rac function and thus functions canonically. Second, PIX-1 and GIT-1 function in the same role as PAK-1, and PAK-1 interaction with PIX-1 is required for PAK-1 activity; thus, PAK-1 functions noncanonically. The human Pak-Pix-Git complex is central to noncanonical Pak signaling and requires only modest Rac/CDC-42 input. Unlike the human complex, our results suggest that the C. elegans Pak-Pix-Git complex requires PAK-1 kinase domain activity. This study delineates signaling network relationships in this cell migration model, thus providing potential further mechanistic insights and an assessment of total Pak contribution to cell migration events.
Paks are critical regulators of cell movements and are important for the understanding of many diseases. However, the extensive redundancy inherent in six mammalian Pak genes suggests that Pak studies in simpler metazoans, which have fewer Paks, could shed important light on total Pak contribution to cell biology.

*Caenorhabditis elegans* contains three Pak proteins (Chen et al. 1996; Hofmann et al. 2004); Pak-1 shares all known sequence motifs with Group A Paks, Pak-2 is more similar to Group B Paks, and MAX-2, although closest in sequence identity to Group A Paks in the PBD and kinase regions, does not share other N-terminal regulatory sequence motifs typical of Group A Paks. Pak-1 binds CDC-42 and CED-10/Rac and during morphogenesis colocalizes with these GTPases at the plasma membrane of epithelial cells (Chen et al. 1996). Loss of MAX-2 causes modest disruptions of axonal pathfinding and loss of Pak-1 greatly enhances these defects, but loss of Pak-1 alone causes no obvious defects (Lucanic et al. 2006). Consistent with the frequently observed antagonism between Rac/Cdc42 and Rho, in Drosophila Pak and Rho are antagonistic (Vlahos and Harden 2010), and Pak participates with Rac and Cdc42 in migrations of epithelial sheets (Harden et al. 1999). Use of multiple signals and multiple GTPase effectors is complex in growth cone migration (Demenaro and Lundquist 2010; Lucanic et al. 2006; Lundquist et al. 2001; Norris et al. 2009; Quinn et al. 2008; Shakir et al. 2006, 2008; Soto et al. 2002; Struckhoff and Lundquist 2003) and epithelial morphogenesis (Gally et al. 2009; Patel et al. 2008; Zhang et al. 2011). Distal tip cell migration, analyzed here, may provide a simpler model for Pak pathway relationships.

In this study we analyze the roles of *C. elegans* Pak proteins in migration of the distal tip cells (DTCs) of the gonad. DTCs are somatic gonadal cells whose migratory path during larval development dictates the shape of the mature gonad (Hedgecock et al. 1987; Kimble and Hirsh 1979). Starting ventrally near the anterior/posterior (A/P) mid-point in the body, the two DTCs migrate anteriorly or posteriorly on the surface of the basement membrane, turn dorsally, then turn again to migrate back to the A/P mid-point, thus forming a highly reproducible inverted-U shape. During migration, the DTC remains connected to its gonad arm, and the gonad proceeds with germline development. Because the precise route of DTC migration can be inferred after the fact by the final shape of the mature gonad (Nishiwaki 1999), DTC migration is an excellent system for studying the genetic regulation of cell pathfinding and migration.

Here we describe striking synergy between *max-2* and *pak-1* in DTC migration. Loss of both results in pathfinding errors and incompletely extended gonad arms, suggesting that these redundant Paks are critical for both proper directional control of migration and migration itself. Genetically, we show that CED-10/Rac is likely to function upstream of MAX-2, whereas the Pix-1/Pix and GIT-1/Git orthologs are required for Pak-1 branch activity. Similar genetic relationships have been previously described (Lucanic and Cheng 2008), although we arrive at somewhat-different mechanistic conclusions. We also show that the CED-2, -5, F-12 Dock/ELMO noncanonical RacGEF complex is likely to activate CED-10 in DTC migration. Despite the apparent role of Pak-1 in a Pak-Pix-Git complex, which was previously described as being kinase-independent, our data suggest that Pak-1 kinase domain activity is required for Pak-1 branch function. Furthermore, genetic analysis suggests a modest contribution of CED-10/Rac to Pak-1 branch activity, suggesting that unlike previously described, the Pak-Pix-Git complex is partially Rac-dependent. We also identify other events during *C. elegans* development that redundantly require MAX-2 and Pak-1.

### MATERIALS AND METHODS

**C. elegans culturing**

Handling, maintenance, and nomenclature of *C. elegans* strains were as described (Brenner 1974; Horvitz et al. 1979). All strains were derived from the N2 (Bristol) wild type and were cultured on *Escherichia coli* strain OP50. Animals were grown in a 23°C incubator, which we found to be the optimal temperature for bacterially mediated RNAi. A summary of key mutations used in this study is shown in Table 1.

**Scoring DTC migration phenotypes**

Hermaphrodites were grown at 23°C and scored at the late L4 stage on 3% agar pads using a Nikon microscope equipped with Differential Interference Contrast (DIC) Nomarski optics. The DTC migration path was inferred from the final position of the two fully developed gonad arms, and for data storage for each animal, the shape of both gonad arms was sketched. For each gonad arm, the entire trajectory was tracked, including Z axis changes. Using this technique we found that we could reproducibly distinguish between folded-over and truncated gonad arms. In previous studies authors suggest that each arm migrates independently of the other, so that each arm constitutes an individual data point (Nishiwaki 1999).

Scoring DTC migration revealed two general phenotypic classes: (1) pathfinding defects, where the DTCs migrated the normal distance but made inappropriate turns; and (2) truncation defects, where the DTC migration stopped immediately after the dorsal turn. In addition, some DTCs exhibited both pathfinding and truncation defects. A DTC was scored as wild type if it extended from the vulva, turned dorsally, then returned to the midline, forming a “U” shape in one plane. Also scored as wild type were gonads with minor shape

| Table 1 Mutant alleles used in this study and their effects on protein function |
|-----------------------------|---------------------|---------------------|---------------------|---------------------|
| Gene | Allele | Lesion | Proposed Functional Effect | Reference |
|---|---|---|---|---|
| pak-1 | ok448 | Exon 7-10 Δ (out of frame) | Kinase deleted, regulatory intact | Lucanic et al. 2006 |
| | tm403 | Exon 1-7 Δ (in-frame) | Regulatory sequences deleted, kinase domain intact (if or gf?) | Zhang et al. 2011 |
| max-2 | n162 | Exon 1-4 Δ (incl. ATG/starting Met) | Null for full-length isoform | Lucanic et al. 2006 |
| | sy2 | G340E | Loss of kinase function | Lucanic et al. 2006 |
| pak-2 | ok332 | Exon 1-7 Δ | Reduced function | Fujiki et al. 2010 |
| ced-10 | n1993 | V190G | Altered function (gf or dhn) | Reddien and Horvitz 2000; Shakir et al. 2006 |
| | n3246 | G60R | Putative null | Zilpin et al. 1997 |
| mig-2 | mu28 | W60 stop | Putative null | Lucanic and Cheng 2008 |
| pix-1 | ok982 | Exon 12-14 Δ | Probable strong loss | Lucanic and Cheng 2008 |
| | gk416 | Exon 1-4 Δ | Probable strong loss | Lucanic and Cheng 2008 |
| git-1 | tm1962 | Exon 8-9 Δ | Probable strong loss | Lucanic and Cheng 2008 |
perturbations that appear at low frequency in all genotypes observed, including the wild type. Included in this category are gonads in whom the distal arm dips ventrally at the end of its migration, distal arms whose migration is slightly short, and minor dorsal extension defects that cause slight ripples in the resulting gonad. We also note that all strains derived from the N2 Bristol wild type had a baseline of 10–15% pathfinding defects, although no truncation defects were observed.

We hypothesize that this phenomenon is not restricted to the N2 strains had rare animals with significant differences in the A/P midline, although not a significant distance.

A truncation defect describes gonads with an early termination of migration. This phenomenon can be broken down into three categories: (1) A short arm, where the gonad extends, but makes no dorsal turn; (2) a dorsal "hockey stick," where the gonad stops just after the dorsal turn; and (3) no arm, where the gonad does not extend at all. The large majority of truncations observed were the "hockey stick" variety, where the DTC makes the dorsal turn but stops. Many truncated gonads were observed without sufficient room in the truncated gonad to contain the germline proliferation therein.

The N2 wild-type strain showed minor pathfinding defects at low penetrance. We examined N2 Bristol stocks from several different laboratories and the CB4856 wild isolate from Hawaii and observed defects at average penetrance of 12%. Most defects were minor, but all strains had rare animals with significant pathfinding defects. We analyzed plates made in different laboratories and incubators in different buildings and never saw an appreciable difference in basal defect rates.

We hypothesize that this phenomenon is not restricted to the N2 strain and is a general feature of *C. elegans* DTC migration.

**Bacterially mediated RNAi protocol**

RNAi methods were derived from described protocols (Kamath et al. 2003; Timmons et al. 2001). HT115 bacteria, transformed with L4440 plasmid containing coding region for the gene of interest, were plated for no more than 16 hr on LB media containing tetracycline and ampicillin. Overnight cultures in LB plus ampicillin were grown for no more than 12 hr (longer growth could decrease RNAi efficacy). A total of 80 µL of each feeding RNAi culture was spotted onto two NG plates containing 1 mM IPTG and 50 µg/mL carbenicillin. Depending on the fecundity of the strain, four to eight late L4 hermaphrodites were picked to a plate for each feeding RNAi clone. Animals were picked to RNAi plates using mineral oil to minimize transfer of contaminating OP50 bacteria. Animals were transferred after 24 hr to the second plate from the same feeding RNAi culture. L4 hermaphrodite progeny were scored 48 hr after the transfer.

Every experiment included positive and negative controls of pop-1 and gfp-directed RNAi, respectively. In our hands under optimal conditions, pop-1-directed RNAi induced 100% embryonic lethality. Experiments where animals escaped pop-1-induced lethality were discarded. All RNAi experiments were compared with gfp-directed RNAi baselines to control for possible migration effects of engaging the RNAi response.

**pak-1 dominance test**

We tested potential dominance of pak-1 alleles by comparing max-2 (RNAi), pak-1(tm403)+ with max-2(RNAi); pak-1(ok448)+ animals. pak-1 is on the X chromosome, so we performed this experiment by crossing N2 males to tm403 homozygotes to generate tm403 hemizygous males, which were then crossed into dpy-8(e130); unc-6(e76). This second cross was performed on max-2 RNAi plates, and non-unc non-Dpy hermaphrodite L4 progeny were scored for DTC migration defects.

**Plasmids used**

Bacterially mediated RNAi used genomic or cDNA sequence insertions in the pPD129.36 (a.k.a. L4440) backbone. Those annotated with chromosomal locations were from the Ahringer library (Kamath et al. 2001). Pre-existing RNAi plasmids used were: gfp (Zand et al. 2011), pop-1 (I-1K04), pak-1 (X-3E04), max-2 (II-8F19), git-1 (X-5119), unc-22 (IV-6K06), and rac-2 (IV-7L24). Our constructed feeding RNAi plasmids, pEP27 (L4440-vced-10). We digested pEL319 (Lundquist et al. 2001) with BglII/Ncol to remove rfp-3, leaving only ced-10 cDNA, and then it was Klenow treated and blunted ligated. pEP1 (L4440-mig-2); we used DJR611/612 (ATGCCTTACCGTGCGAG CAG/GCGCCGAAATGACAGTGGG) from *C. elegans* genomic DNA to amplify a 970-bp region between exons 1 and 3 of mig-2 and cloned it into L4440 digested with NotI/Sall.

Markers and experimental plasmids for transgenics, i.e., pMH86 (dpy-20 (+)), pPD118.33 (Pmyo-2::gfp): The pJK590 plasmid (Pak-1::gal) (Belloch et al. 1999) uses the 3-kb sequence upstream of the gfp start codon (5’ tttataaatccat–caatgtgtcc 3’) and contains a modified BsmI site within the promoter to assist in cloning. pCM15.2 (Ppak-1:: cdc-42); We amplified cdc-42 cDNA from plasmid ykl443h by using DJR54/550 (ATGACAGATCAAGTGCGTCGTCG/TTAGAGAATATGCACCTTCCTICTC) and cloning it into pCM15.1 (Ppak-1:: chw-1) digested with AgeI/NotI (V. Muniz-Medina, C. J. Der, and D. J. Reiner, unpublished data). We mutagenized pCM15.2 with DJR517/518 to create pCM15.3 (Ppak-1::cdc-42 Q61L) and mutagenized pCM15.2 with DJR623/624 to generate pCM15.4 (Ppak-1::cdc-42 S17N). pCM13.1 (Ppak-1::cex-10): ced-10 cDNA was plasmid pEL319 using DJR545/546 (ATGCACCGATCAAATATGTCG TC/TTAGACACGTACCTTGCTT777TG) was cloned into to pCM14.1 digested with AgeI/NotI. We mutagenized pCM13.1 with DJR513/514 to create pEP3.2 (Ppak-1::cex-10 Q61L). pEP1.2 (Ppak-1:: pak-1(1++)): we amplified pak-1 cDNA from ykl1691a by using DJR631/629 (AGGGCTTCAGGAAATGAAAGGTCGGCTG/TAGGTTGCTACCATGCGGCGTGC) and cloning it into pCM14.1 digested with KpnI/NotI. We mutagenized pEP12.1 with DJR678/679 to generate the disrupted Pix binding mutant pEP12.2 (Ppak-1::pak-1(234A,235A)) and mutagenized pEP12.1 with DJR674/675 to generate the kinase dead mutant pEP12.3 (Ppak-1::pak-1(1224R)). pEP15.1 (Ppak-1:: pak-1(-)): We digested pPD118.25 with Nhel to remove gfp and then religated to create pEP10.1. We amplified pak-1 cDNA from ykl1691a by using DJR631/629 (as described previously) and cloned into pEP10.1 digested with KpnI/SpeI. Sequencing primers for pPD118.25 were DJR655/656 and for pJK590mut were DJR654/660. The sequences of plasmids and oligonucleotides are available upon request.

**Transgene generation**

Markers (concentrations) used included: pa2132 (unc-119 (+)) at 50 ng/µL; pMH86 (dpy-20 (+)) at 20 ng/µL; pPD118.33 (Pmyo-2::gfp) at 5 ng/µL; Ppak-1::cex-10 (or cdc-42) at 5 ng/µL; and Ppak-1::pak-1 at 5 or 10 ng/µL.

**Mutations used**

We used the following mutations: pak-1(ok448), pak-1(tm403), max-2 (n162), max-2 (cy2), mig-2 (mu38), pex-1 (ok892), pex-1 (gk416), git-1 (tm1962), ced-10 (h1993), ced-10 (n3246), ced-1 (e1750), ced-5 (n1812), ced-12 (n3261), unc-33 (e204), htn1[unc-5(h1040)], unc-29(c193).
RESULTS

PAK-1 and MAX-2 redundantly control DTC migration

To analyze the functions of Pak kinases during development, we disrupted Pak activity by RNAi-targeting one Pak gene in a background where another Pak gene was mutated. At a morphological level, double loss-of-function animals appeared wild type except for one phenotype: greater than 90% of animals displayed light patches in various parts of the body between the pharynx and anus (Figure 1, A and B). This patchy phenotype is often seen with defects in migration of the gonadal DTCs (Nishiwaki 1999). Normally, the two DTCs of the somatic gonad migrate in mirror image U-shapes. Each arm of the mature gonad occupies a lateral half of the body cavity, whereas the intestine, which appears dark in the dissecting microscope, occupies the other half. Thus, because animals on the plate generally are viewed from the side, in the wild type one observes the uninterrupted dark band of the intestine running the length of the animal, and the clear gonad arms are not visible. However, in the event of defective DTC pathfinding, portions of the gonad can overlap, occupying both the left and right planes, from the lateral perspective, and thus displace the intestine. This intestinal displacement results in clear patches that are easily detected by dissecting microscope, and this phenotype has been shown to be strong enough to isolate mutants in genetic screens (Nishiwaki 1999).

By DIC/Nomarksi microscopy, we recorded highly penetrant DTC migration defects in max-2; pak-1 double loss-of-function animals compared with few defects in the single mutant (Figure 1C). We observed a wide range of pathfinding defects, but with one exception (see Pak controls both DTC pathfinding and migration execution, below) no class of defect was more representative than another, and both the anterior and posterior gonad arms were affected equally. We analyzed two alleles of each gene in double loss-of-function combinations by targeting the appropriate clear patches caused by gonad overlap. (C) All pair-wise combinations of max-2 and pak-1 disruption show strong synthetic DTC migration defects, with no quantitative or qualitative differences between the genetic combinations (Figure 1C). As negative controls, single-mutant animals subjected to gfp-directed RNAi were not significantly different from animals grown without RNAi.

Figure 1  max-2 and pak-1 are redundant for DTC migration. Bright-field photomicrographs of (A) max-2(nv162) and (B) max-2(nv162); pak-1(RNAi) late L4 animals. In both animals anterior is left and ventral is down. A black arrow indicates the semicircular clear patch formed by the developing vulva and uterus at the late L4 stage, and white arrows indicate inappropriate clear patches caused by gonad overlap. (C) All pair-wise combinations of max-2 and pak-1 disruption show strong synthetic DTC migration and gonad shape defects. Number in columns is the number of gonad arms scored. pak-1 single mutants on negative control gfp-directed RNAi were not significantly different from the wild type (5–15% defective in all experiments, see Figure 3B for example), whereas max-2 loss of function mutations may cause DTC migration defects that are slightly elevated; cy2 but not nv162 on gfp(RNAi) was significantly different than both ok448 and tm403 (P < 0.05). For all gfp vs. pak-1 or max-2 RNAi comparisons (paired columns), P < 0.0001 by Fisher’s exact test. No error bars are shown because these are pooled assays.
Alleles used in this study are listed in Table 1. *pak-1(ok448)* deletes most of the kinase domain and disrupts subsequent reading frame and should therefore be null for PAK-1 kinase function. *pak-1(tm403)* deletes most of the N-terminal regulatory sequences, including the PBD, the autoinhibitory domain, the Pix-binding site, the acidic region and many of the potentially regulatory PXXP SH2-binding sites, but frame is retained and a functional kinase should be made. Such a truncated protein could result in a gain-of-function or toxic product (see *pak-1(tm403)* may be weakly semidominant, below). *max-2* (nvi62) deletes the first four exons of the gene, including the start codon, and thus represents a null for full-length pak function. *max-2* (cy2) is a point mutation altering a conserved residue in the kinase domain, suggesting that it causes strong kinase loss of function (Lucanic et al. 2006). We note that both *pak-1* and *max-2* express multiple isoforms due to alternative splicing or use of alternative promoters, including shorter isoforms altogether lacking the N-terminal regulatory regions (wormbase.org WS228). Furthermore, mammalian Group A Paks have been shown to have kinase-independent functions (see Putative PAK-1 partners GIT-1 and PIX-1 are redundant with *MAX-2* below). Thus, mutations that eliminate certain domains of the protein may still retain developmental functions, and none of the mutations characterized thus far is likely to be null for all gene functions.

*pak-1(tm403)* may be weakly semidominant

The *pak-1(tm403)* allele deletes the N-term regulatory sequences in-frame and, due to de-regulation, might produce a gain-of-function or otherwise toxic protein. Thus *tm403* might be predicted to be semidominant. We tested this prediction genetically. *gfp(RNAi)*; *pak-1* (*tm403*)/+ and *gfp(RNAi)*; *pak-1* (*ok448*)/+ hermaphrodites had similar, wild-type levels of DTC migration defects. However, *max-2(RNAi)*; *pak-1* (*tm403*)/+ and *max-2(RNAi)*; *pak-1* (*ok448*)/+ were significantly different, with almost 27% of *max-2(RNAi)*; *pak-1* (*tm403*)/+ gonad arms having DTC migration defects compared with 17% in the control (Table 2; *P < 0.05*, Fisher’s exact test). These observations suggest that *pak-1(tm403)* might encode a toxic, semidominant protein. As additional support for this hypothesis, we were unable to transgenically rescue *max-2(RNAi)*; *pak-1* (*tm403*) DTC migration defects with wild-type *PAK-1* protein, whereas we could rescue *max-2(RNAi); pak-1* (*ok448*) (see PAK-1 is cell autonomous and requires kinase activity and PIX-1-interaction sequences, below). Despite this predicted difference in consequences of the two *pak-1* alleles, *pak-1* (*tm403*) and *pak-1* (*ok448*) homozygotes on *max-2(RNAi)* conferred DTC migration defects of similar strength (Figure 1C). Interestingly, the *ced-10* (n1993) hypomorphic allele and *n3246* weak gain-of-function allele (Shakir et al. 2006) on *pak-1* (*RNAi*) also have roughly similar affects on DTC migration [Figure 3; *ced-10* null is maternal effect lethal (Kinchen et al. 2005) and was not used for this analysis for technical reasons]. Because it is outside of the scope of this study, we have not pursued further the potentially toxic nature of *pak-1(tm403)*.

### Table 2 pak-1(tm403) but not pak-1(ok448) is weakly dominant

| Genotype                  | % DTC Defect (N) |
|---------------------------|------------------|
| *gfp(RNAi)*; *pak-1* (*ok448*)/+ | 10.2 (168)       |
| *gfp(RNAi)*; *pak-1* (*tm403*)/+ | 12.5 (160)       |
| max-2(RNAi); *pak-1* (*ok448*)/+ | 17.3 (228)       |
| max-2(RNAi); *pak-1* (*tm403*)/+ | 26.5 (148)       |

ok448/+ or tm403/+ animals were tested for dominant effects by plating on *gfp*-directed (control) or *max-2*-directed (experimental) RNAi and assessing frequency of DTC migration defects. *tm403/+; max-2(RNAi)* is significantly different than *tm403/+; gfp(RNAi)* (*P < 0.05*, Fisher’s exact test). DTC, distal tip cells; N, number of gonad arms scored.

Paks control both DTC pathfinding and migration execution

We observed two general classes of gonad morphology defects in *max-2*; *pak-1* double loss-of-function animals: pathfinding and migration execution (defects that are referred to here as “truncation.”) We hypothesize that these defects reflect two distinct functional roles for *max-2* and *pak-1* in DTC migration. First, a broad variety of pathfinding defects were observed (a typical example is shown in Figure 2B, but all possible permutations were observed at low frequency). No single defect was representative of the *max-2*, *pak-1* double, but instead many possible misguidance defects were represented, and aberrant DTC turns were observed at every stage of DTC migration. We hypothesize that these phenotypes reflect a general requirement for redundant *max-2* and *pak-1* function in communicating positional information from the extracellular environment into the migrating cell.

Second, a large subset of mutant animals had prematurely terminated DTC migrations, where migration was arrested/truncated just after the DTC made the dorsal turn halfway through the wild-type migration path (Figure 2, C and D). Generally, the resulting gonads were of otherwise normal shape, although we observed bulging of the gonad arm toward the distal end of the gonad (Figure 2D). Table 3 displays the frequencies of the two classes of phenotypes. We note that the high frequency of DTC truncations likely obscures underlying pathfinding defects, thereby depressing our estimate of pathfinding defects in genotypes with frequent truncation defects. We speculate that without the truncation defect, most DTCs would still have pathfinding defects. The fertility of truncated gonad arms could not be assessed, because (1) they were frequently paired in the same animal with non-truncated gonad arms, and (2) we observed severe sterility in all animals in which both Pak pathways were disrupted (see *PAK-1* and *MAX-2* are required for fertility, below), even in animals in which neither gonad arm was truncated.

Although a small minority of truncated gonad arms failed to make the dorsal turn, generally the point of migration arrest was reproducible; most truncated DTCs truncated immediately after the dorsal turn, resulting in a shape like a hockey stick. To test whether DTC migration rate was diminished, we scored DTCs at the young adult stage rather than the usual L4 stage to give DTCs additional time to migrate, but we observed no differences in the frequency of truncated gonads, nor were truncated gonads migrated any farther. Therefore, we speculate that loss of *max-2* and *pak-1* results not in generally compromised cell migration, which we predict would result in gonads truncated at many points but rather disrupts response to a specific migratory cue, *i.e.*, that which guides the DTC back to the A/P midpoint of the animal near the developing vulva.

Below we describe genetic analyses of other components of the MAX-2 and PAK-1 signaling pathways. In all cases in which we disrupted the two redundant pathways, we observed accompanying pathfinding defects and truncated migrations (Table 3). This observation suggests that the same genetic pathways control both processes.

Previous work described altered DTC morphology in *pak-1* single mutants (Lucanic and Cheng 2008). We scored *pak-1* (*ok448*) and *pak-1* (*tm403*) mutants blindly and were unable to detect reproducible DTC morphology defects relative to the wild type. We do not know the source of this discrepancy.

### CED-10/Rac and the Dock/ELMO RacGEF complex function upstream of MAX-2

Based on extensive mammalian work, we hypothesized that CED-10/ Rac would be required to activate PAK-1 and/or MAX-2, and we
tested this model genetically by pair-wise knockdown of putative pathway components. Both ced-10 mutations examined had synthetic interactions with pak-1 but not max-2-directed RNAi (90% vs. 45–60%; Figure 3A). pak-1(ok448) and pak-1(tm403) animals had moderate synthetic defects when grown on ced-10-directed RNAi, whereas max-2(nv162) was not enhanced (~40% and 60% vs. <20%; Figure 3B). ced-10-directed RNAi was only modestly effective in all contexts, even though we included rrf-3 sequences in the RNAi vector to hypersensitize animals to ced-10-directed RNAi (Simmer et al. 2002).

An atypical Rac-specific Dock/ELMO GEF complex has been described in both mammals and nematodes, and the corresponding CED-2/5/12 complex activates CED-10/Rac in cell corpse engulfment. Single mutants for each of these genes, as well as ced-10, have been described as having moderately penetrant DTC migration defects (Gumienny et al. 2001; Reddien and Horvitz 2000; Wu and Horvitz 1998). We analyzed mutations in the Dock/ELMO complex in combination with loss of pak-1 or max-2. Double-mutant combinations between ced-5 or ced-12 and pak-1 yielded animals with the patchy bright-field phenotype indicative of DTC migration defects, whereas double-mutant combinations between ced-5 or ced-12 and max-2 were no different than single mutants (Table 4). We validated these observations with pak-1 and max-2-directed RNAi: loss of ced-2, ced-5, or ced-12 conferred strong synthetic DTC migration defects in combination with pak-1(RNAi) but not max-2(RNAi). These results suggest that the RacGEF complex required for CED-10 activation in cell corpse engulfment also functions in the same pathway as CED-10 in DTC migration because it has the same genetic interactions with max-2 and pak-1. Given published data about the Dock/ELMO complex, we propose that this RacGEF activates CED-10 to control DTC migration, and our genetic data argue that CED-10 then signals principally through MAX-2/Pak. That CED-10/Rac signals through MAX-2 but not PAK-1 was previously deduced, although the analysis was not extended to the Dock/ELMO complex and did not include multiple alleles of pak-1, max-2, and ced-10 (Lucanic and Cheng 2008).

The synergy between loss of ced-5/12 or CED-10 and loss of PAK-1, but not MAX-2, suggests that the CED-10/Rac protein signals primarily through MAX-2, whereas PAK-1 functions in a parallel signaling branch. However, one observation complicates this simple interpretation. CED-10/Rac was previously described as being required for DTC migration (Wu and Horvitz 1998), and we found that ced-10(n1993) or ced-10(n3246) grown on gfp-directed RNAi had DTC migration defects well above background levels (Figure 3A). Moderate defects in DTC migration were previously described for mutations disrupting the Dock/ELMO RacGEF (Gumienny et al. 2001; Reddien and Horvitz 2000; Wu and Horvitz 1998). We similarly observed mismigrated DTCs in ced-2(n1994), ced-2(e1752), ced-5(n1812), ced-12(tp2), and ced-12(n3261) backgrounds. Loss of these proteins had similar effects on DTC truncation (Table 3) (Gumienny et al. 2001; Reddien and Horvitz 2000; Wu and Horvitz 1998). The contrast in frequency of gonad arm truncation in pak-1 or max-2 single mutants compared with ced-2, -5, -10, and -12 mutant animals leads us to hypothesize that CED-10 and its GEF are required in both Pak pathway branches, though probably to differing degrees.

### Table 3 Disruption of both pak-1 and max-2 branches is required for significant DTC truncation defects

| Row | Genotypes                  | % Pathfinding | % Truncation | N  |
|-----|----------------------------|---------------|--------------|----|
| 1   | N2 on GFP                  | 11.8          | 0.0          | 160|
| 2   | pak-1(ok448); gfp(RNAi)    | 13.0          | 0.0          | 158|
| 3   | pak-1(ok448); max-2(RNAi)  | 29.4          | 73.1         | 160|
| 4   | pak-1(tm403); gfp(RNAi)    | 11.0          | 0.0          | 154|
| 5   | pak-1(tm403); max-2(RNAi)  | 1.0           | 91.8         | 146|
| 6   | max-2(nv162); gfp(RNAi)    | 14.6          | 1.2          | 158|
| 7   | max-2(nv162); pak-1(RNAi)  | 25.0          | 80.7         | 140|
| 8   | max-2(cy2); gfp(RNAi)      | 26.1          | 1.5          | 130|
| 9   | max-2(cy2); pak-1(RNAi)    | 6.0           | 97.1         | 140|
| 10  | ced-10(n1993); gfp(RNAi)   | 38.1          | 18.1         | 160|
| 11  | ced-10(n1993); pak-1(RNAi) | 14.3          | 92.2         | 154|
| 12  | ced-10(n3246); gfp(RNAi)   | 38.7          | 42.5         | 106|
| 13  | ced-10(n3246); pak-1(RNAi) | 3.0           | 98.0         | 100|
| 14  | pix-1(ok982); gfp(RNAi)    | 16.3          | 0.0          | 80 |
| 15  | pix-1(ok982); max-2(RNAi)  | 1.3           | 96.3         | 80 |
| 16  | git-1(tm1962); gfp(RNAi)   | 7.5           | 0.0          | 80 |
| 17  | git-1(tm1962); max-2(RNAi) | 12.5          | 81.3         | 80 |

Each gonad arm evaluated was independently scored for defects in pathfinding or truncation, so in principle the pathfinding and truncation columns can total greater than 100% (e.g., see row 9). Greater frequency of truncation defects correlates with lower frequency of pathfinding defects, presumably because defects in distal arm migration were more frequent than defective proximal arm migration, and thus truncated DTC migrations obscure underlying pathfinding defects. There is some overlap between categories (i.e., truncated gonad arms can be mis-migrated) and thus total percent defect can be greater than 100% (e.g., rows 7, 9, 11, 13). DTC, distal tip cells; N, number of gonad arms scored.
migration. Paks are generally thought of as effectors of Rho family small GTPases, specifically Rac and Cdc42. *C. elegans* contains a single Cdc42 homolog, CDC-42, and two Rac homologs, CED-10 and RAC-2. CDC-42 and CED-10 are very highly conserved with their mammalian counterparts and are absolutely conserved in the Switch 1 effector-binding region, but RAC-2 contains divergent residues in the Switch 1 region. Based on genetic analysis, CED-10 appears to be the major Rac homolog in *C. elegans*, whereas RAC-2 may play a minor, redundant role (Lundquist et al. 2001). In addition, mig-2 encodes a putative homolog of mammalian RhoG, a Cdc-42- and Rac-related but functionally distinct member of the Rho family that generally interacts with effectors different than those of Rac or Cdc42 (deBakker et al. 2004; Wennerberg and Der 2004).

**Figure 3** CED-10 acts primarily through MAX-2 and not PAK-1. Number of gonad arms assayed is shown in each column. (A) Both ced-10 alleles examined had significant baseline DTC defects and showed strong synthetic defects with pak-1 but not max-2-directed RNAi. (B) The wild-type, pak-1 and max-2 animals were grown on ced-10-directed RNAi. RNAi of ced-10 induces an additional defect in the N2 wild-type background that is significant (*P* < 0.001), but in all of our experiments the effects of ced-10-directed RNAi were significantly weaker than defects seen for ced-10 hypomorphic n1993 and weak gain-of-function n3246 alleles. The two pak-1 alleles but not max-2(nv162) are synthetic with ced-10(RNAi). (C) RNAi of pak-1, max-2, or ced-10 enhance the previously described DTC migration defect of mig-2(mu28) (*P* < 0.003), arguing that mig-2 functions in parallel to the two Pak pathways. (D) Mutations in git-1 and pak-1 are strongly synthetic with RNAi targeting max-2 but not pak-1. (E) Mutations in max-2 but not pak-1 are synthetic with RNAi targeting git-1. Statistics were generated via Fisher’s exact test. No error bars are shown because these are pooled assays.

### Table 4 The Dock/Elmo RacGEF complex is redundant with pak-1 but not max-2

| Genotypes     | +       | ced-10(n1993) | ced-5(n1812) | ced-12(n3261) | ced-1(e1735) |
|---------------|---------|--------------|--------------|--------------|--------------|
| pak-1(ok448)  | fertile | fertile, wk patchy | fertile, wk patchy | fertile, wk patchy | fertile |
| pak-1(tm403)  | fertile | sterile, str patchy | sterile, str patchy | sterile, str patchy | fertile |
| max-2(nv162)  | fertile | sterile, str patchy | sterile, str patchy | sterile, str patchy | fertile |
| max-2(cy2)    | fertile | sterile, Unc | sterile, wk patchy | sterile, wk patchy | fertile |

Like ced-10(n1993), ced-5(n1812) and ced-12(n3261), which encode components of the Dock/Elmo RacGEF complex, caused synthetic DTC migration and fertility defects in combination with pak-1, but not max-2. Animals were scored by inspection under dissecting microscope: “Str” is “strong,” “wk” is “weak,” and “Unc” is “uncoordinated.” ced-1(e1735), which regulates cell corpse engulfment through a different pathway, was included as a negative control. As described, max-2 mutations also cause a severe uncoordinated phenotype in the ced-10(n1993) background (Lucanic and Cheng 2008), but not in ced-5 and ced-12 backgrounds (our observations), arguing that a GEF other than CED-2/5/12 functions to activate CED-10 in neurite outgrowth. ND, not determined.
Previous studies (Lucanic and Cheng 2008; Lundquist et al. 2001) suggest that RAC-2 plays a modest role in DTC migration, but our results are weak or negative. This discrepancy in a relatively mild phenotype could be due to experimental differences.

cdc-42-directed RNAi confers strong embryonic lethality, and surviving animals are grossly defective in gonad organization as well as showing disruption of early somatic gonad polarity (Welchman et al. 2007). Previously, cdc-42-directed DTC tissue-specific RNAi did not perturb DTC migration on its own. However, cdc-42+pix-1 but not cdc-42+max-2 double DTC-specific RNAi caused a weak synthetic phenotype (Lucanic and Cheng 2008). The strength of this effect was weaker than the strong synthetic phenotype observed between pak-1 and max-2, perhaps due to variability in RNAi efficacy from gene to gene. Alternatively, perhaps contribution of CDC-42 to MAX-2 is modest. But the conclusion was that CDC-42 plays a role in parallel to the Pak-Pix-Git complex, perhaps upstream of MAX-2 (Lucanic and Cheng 2008).

To test this hypothesis with a different methodology, we expressed mutant CDC-42(T17N) protein specifically in DTCs using the lag-2 promoter, which drives expression in DTCs and not surrounding cells (Blelloch et al. 1999). This 17N mutation is well characterized in Ras and Rho small GTPases as conferring potent dominant-negative activity, and the main caveat with this reagent is that it could be overly strong by sequestering GEFs required for activation of other Rho family members in addition to CDC-42. DTC-specific CDC-42 (T17N) caused no DTC migration defects and was not pursued further (data not shown).

We also tested the MIG-2/RhoG contribution to Pak-mediated DTC migration. RhoG does not bind and activate Pak proteins in mammalian cells (Wennerberg et al. 2002), but by yeast two-hybrid assay MIG-2/RhoG may interact with MAX-2/Pak (Fujiki et al. 2010). Along with the previously observed moderate DTC migration defect in mig-2 null mutant animals (Lundquist et al. 2001), we found that loss of max-2, pak-1, or ced-10 weakly enhanced mig-2 mutant defects (Figure 3C), but not with the very strong synergy observed between the max-2 and pak-1 pathway branches. Although in general ced-10 (RNAi) efficacy was modest, pak-1- and max-2-directed RNAi conferred strong loss-of-function phenotypes. Thus, the weak interaction between mig-2 and pak-1 or max-2 argues that mig-2 probably does not play a significant role in either of these pathways, but rather is involved in a different aspect of DTC migration. This observation is noteworthy because MIG-2/RhoG has been described as functioning upstream of the Dock/ELMO RacGEF that activates CED-10/Rac in nemotodes and mammalian apoptotic cell corpse engulfment (deBakker et al. 2004) and thus might have been predicted to functionally similar to this group of proteins. To summarize, MIG-2/RhoG functions in DTC migration, but does not have clearly interpretable genetic interactions with other signaling components.

If molecules function in a linear pathway, ectopic activation of the upstream component might allow genetic dissection of pathway colinearity. We expressed wild-type or Q61L-mutated CED-10 specifically in DTCs using the lag-2 promoter. Q61L mutations or their equivalent activate all Rho and Ras small GTPases by inactivating their intrinsic GTPase catalytic activity, thus locking the protein in the GTP-bound active state. Ectopic CED-10(Q61L) caused defective DTC migrations (Figure 4A), arguing that mutationally activated CED-10 protein has biological activity in migrating DTCs. However, loss of pak-1 or max-2 failed to abrogate the activated CED-10 phenotype (Figure 4A), arguing that CED-10 does not exclusively use a single Pak effector pathway, but rather contributes to both, consistent with our arguments based on the aforementioned ced-2/5/10-12 single mutants.

Similarly, ectopic expression of CDC-42(61L) caused DTC migration defects. Alternatively, CED-10 or CDC-42 could engage effectors other than Paks when ectopically expressed in DTCs, but endogenous protein does not do so. This result may seem to contradict our observation that CDC-42(dn) causes no DTC defect, but we argue that ectopic CDC-42 is capable of engaging Pak effectors and may do so elsewhere during development. PAK-1 loss weakly suppressed the total DTC defect but not specifically pathfinding or truncation, while MAX-2 loss weakly suppressed the truncation defect (Figure 4B). We hypothesize that ectopic, activated CDC-42 can engage both Pak proteins simultaneously, and thus can only be suppressed weakly by loss of single Paks.

Putative PAK-1 partners GIT-1 and PIX-1 are redundant with MAX-2

Our pair-wise genetic analysis suggests that PAK-1 has a modest requirement for CED-10/Rac activation. This conclusion is consistent with a previously described, partially GTPase-independent function of

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**Figure 4** Activated CED-10/Rac and CDC-42 disrupt DTC migration. (A) DTC-directed expression of gain-of-function (gf), mutationally activated CED-10(Q61L) causes pathfinding and truncation defects. (B) DTC-directed expression of gain-of-function, mutationally activated CED-10(Q61L) also causes pathfinding and truncation defects. Loss of either pak-1 or max-2 fails to rescue the activated CED-10 defects, whereas the loss of pak-1 suppresses the truncation but not pathfinding defect of activated CDC-42, and loss of max-2 modestly suppresses both defects of activated CDC-42. Statistics were generated via Fisher’s exact test, and only significant differences are shown. No error bars are shown because these are pooled assays.
Group A Paks in cell culture. In addition to its role as a Rac/Cdc42 effector, Pak can function as a scaffold for a protein complex that includes Git1 and Pix (Loo et al. 2004). This Pak-Pix-Git complex may not require GTPase input for activation, though Cdc42 and Rac are thought to help target the complex to discrete subcellular compartments, and thus contribute somewhat to optimal complex activity. It is also notable that the mammalian Pak-Pix-Git complex does not require activity of the Pak kinase domain. The signaling output of this complex is unclear, though clearly biologically important (Zhang et al. 2011).

The C. elegans genes git-1 and pix-1 are well-conserved orthologs of Git1 and Pix, respectively (Dyer et al. 2010; Luican and Cheng 2008). To test whether the putative GTPase-independent role of Pak-1 in DTC migration is GIT-1- and PIX-1-dependent, we used pairwise genetic knockdowns like those used to characterize PAK-1 and MAX-2 parallelism. We found that outcrossed strains containing git-1(tm1962), pix-1(gk416), or pix-1(ok982) conferred no DTC defects alone (or on gfp-directed RNAi; Table 3) and were strongly synthetic with max-2(RNAi) but not pak-1(RNAi). Conversely, mutants for max-2 showed strong synthetic defects in combination with git-1-directed RNAi, but pak-1 mutants did not (Figure 3, D and E). Furthermore, mutants for ced-10 were synthetic with git-1(RNAi) (data not shown). These results indicate that git-1 and pix-1 are redundant with max-2 and ced-10 but not pak-1, and therefore we hypothesize that Pak-1 functions as part of a Pak-Git-Pix complex described in other systems.

**Pak-1 is cell autonomous and requires kinase activity and Pak-1-interaction sequences**

Because a major function of mammalian Pak proteins is to regulate the cytoskeleton, we hypothesized that PAK-1 functions cell autonomously in the DTCs to control their pathfinding and extension. To test this model, we expressed the wild-type pak-1A isoform cDNA behind the lag-2 promoter, which drives expression in DTCs but not surrounding cells (Bléloch et al. 1999). We crossed Plag-2::pak-1(+) containing transgenes marked with Pmyo-2::gfp into the pak-1(ok448) background, which we predict to be null for Pak-1 kinase function. We then subjected the strain to max-2-directed RNAi and scored array-bearing vs. nonarray-bearing animals (the latter provided an internal control for RNAi efficacy and a baseline for statistical comparison to sibling array-bearing animals). The DTC-directed expression of the pak-1(+) cDNA, injected at 5 ng/mL, rescued the synthetic DTC migration defect with max-2, although not fully (Figure 5A). To test whether transgene dose was relevant, we also injected at 10 ng/mL, but the rescue results were similar to those at the lower concentration. Therefore, we conclude that a significant portion of PAK-1 function in DTC migration is cell-autonomous, as predicted. We speculate that rescue is incomplete because PAK-1 functions in a multiprotein complex, where stoichiometry may be important for function. Thus, perhaps transgenic overexpression of PAK-1 titrates crucial PAK-1-binding proteins away from the complex, thus compromising function and preventing full rescue. Alternatively, perhaps not all PAK-1 function is cell autonomous, or a different isoform provides some critical PAK-1 function in the DTCs.

We similarly tested Plag-2::pak-1(+) rescue of pak-1(tm403) the in-frame truncation of the PAK-1 N-terminal regulatory sequences that is weakly dominant (see above). We found that DTC-directed expression of pak-1(+) failed to rescue pak-1(tm403) (Figure 5A). The transgene was used for the same rescue experiments with pak-1(ok448), so we are confident that wild-type protein is expressed. This result reinforces our hypothesis that pak-1(tm403) encodes a toxic, perhaps dominant-negative or gain-of-function protein product.

We further used DTC-directed rescue of Pak-1 to test the molecular requirements of PAK-1 in the putative Pak-Git-Pix complex. If PAK-1 functions as part of such a complex, the Pix-binding domain of PAK-1, which is highly conserved with its mammalian counterparts, should be required for rescue. Therefore, we constructed a Plag-2::pak-1(A) R234A, P235A "Pix dead" plasmid, guided by previous binding studies of human Pak1 and Pak interactions, in which residues of PAK-1 essential for Pix-binding were mutated (Daniels et al. 1999). Using the same assay as for pak-1(+) rescue, we found that the "Pix dead" construct failed to rescue the synthetic max-2(RNAi); pak-1(ok448) DTC migration phenotype (Figure 5B), arguing that PAK-1 binding to PIX-1 is necessary for PAK-1 function in DTCs. Together with the requirement for pak-1 and git-1 for pak-1 branch activity, this result indicates that PAK-1 functions in a complex with PIX-1 and GIT-1 to regulate DTC migration.

Previous mammalian studies suggest that the kinase domain of Pak is dispensable for Pak function with the Pak-Git-Pix complex and the Pak scaffolding activity for phosphatidylinositol 3 kinase and PDK (Higuchi et al. 2008). We previously predicted that DTC-directed expression of pak-1 cDNA with a mutated kinase domain would still be able to rescue the max-2(RNAi); pak-1(ok448) synthetic DTC migration defect. We constructed a Plag-2::pak-1 K324R “kinase dead” (KD) plasmid, guided by previous biochemical and functional assays...
of human Group A Pak proteins (Manser et al. 1997; Sells et al. 1997). Surprisingly, we found that DTC-directed expression of pak-1(k324R) failed to rescue (Figure 5B). Therefore, we hypothesize that the Pak-Pix-Git complex requires Pak kinase activity to perform its function in DTC migration.

**Pak proteins are required for embryonic morphogenesis**

To further characterize Pak-dependent DTC migration defects, we built pair-wise double mutant strains with mutations in pak-1(ok448 and tm403) and max-2(nv162 and cy2). All allelic combinations were lethal or subviable and therefore impossible to maintain in culture, with severely compromised fertility (see **PAK-1 and MAX-2 are required for fertility**, below) and frequent embryonic morphogenesis defects. This latter defect was not examined in great detail, but cursory inspection revealed embryos in which the epithelium had failed to enclose, thus resulting in gross disorganization of tissue types and gut cells on the exterior of the embryo (Soto et al. 2002). We cannot rule out defects in cell fate, but differentiated pharynx and gut were visible. Rare surviving embryos that hatched had variable surface bulges consistent with morphogenetic defects in ventral enclosure or elongation (Chisholm and Hardin 2005). In addition, these rare, surviving double-mutant animals had severe locomotion defects, whereas pak-1 single-mutant locomotion was wild type, and max-2 single-mutant locomotion was only mildly defective (Lucanic et al. 2006). The lethality and locomotion defects were maternally rescued; double-mutant locomotion was only mildly defective (Lucanic et al. 2005) whereas elongation (Chisholm and Hardin 2005). In addition, these rare, surviving double-mutant animals had severe locomotion defects, whereas pak-1 single-mutant locomotion was wild type, and max-2 single-mutant locomotion was only mildly defective (Lucanic et al. 2006). The lethality and locomotion defects were maternally rescued; double-homozygous mutant F2 progeny from a double-heterozygote parent could be readily selected based on the patchy phenotype from DTC migration defects. These patchy animals moved well and had no morphogenetic defects, although they had few or no progeny. We also observed maternal rescue of morphogenetic and locomotion defects when the parent was homozygous mutant for one Pak, and heterozygous mutant for the other Pak, arguing that both max-2 and pak-1 are maternally rescued. We conclude that early developmental events like embryonic morphogenesis and axon pathfinding rely on maternal PAK-1 and MAX-2 contribution, whereas later developmental events like DTC migration rely on zygotic gene product.

During these experiments, we found that only one of the four possible pair-wise double mutants yielded viable progeny, albeit at a very low rate. max-2(cy2); pak-1(tm403) animals had 2–3 progeny per generation, enough that the strain could be maintained but not frozen. Double mutant animals for other allelic combinations were isolated, but the strains were inviable. However, upon picking many double-mutant animals for each genotypic combination, rare healthier clones of animals arose after a variable number of generations, and the resulting strains were qualitatively more robust. These healthier clones presumably resulted from spontaneous second site mutations that compensated for the severity of the max-2; pak-1 double-mutant embryonic lethality and sterility. Additionally, background enhancer mutations were lost, though outcrossed strains were used for the constructions. Unlike the previous Pak mutant study (Lucanic and Cheng 2008) we did not analyze double-mutant DTC phenotypes of these healthier isolates because we were concerned about the potential caveats of additional mutations in unknown genes. Because the healthier clones arose at relatively high frequency, we predict that a large group of genes can be mutated to weakly ameliorate the max-2; pak-1 embryonic lethality and sterility.

The fact that cy2; tm403 can be cultured without second site mutations whereas other allelic combinations could not argue that these two alleles are not null (see above). We note that pak-1(tm403) and max-2(cy2) have the most severe degree of gonad arm truncation when in combination with RNAi of the redundant Pak gene (Table 3, lines 5 and 9, respectively). Perhaps this observation indicates that both tm403 and cy2 encode proteins that are toxic for full-length DTC migration but are less severe than putative null alleles for embryonic morphogenesis. Alternatively, unlike max-2(nv162), which disrupts the translational start of the predicted long but not short isoform, max-2(cy2) disrupts kinase domain function, presumably in both isoforms. So perhaps the MAX-2 kinase domain is required in multiple isoforms during DTC migration, but not as strongly required for embryonic development and fertility.

**PAK-1 is a clear ortholog of mammalian Group A Paks** (Pak1, 2, 3), with all known mammalian sequence motifs conserved in the C. elegans protein (Chen et al. 1996; Hofmann et al. 2004). By sequence analysis, MAX-2 is Group A-like, but stereotypical Group A Pak sequence motifs are missing, notably the PXPF SH2 domain recognition motifs and the PIX binding domain (Lucanic et al. 2006). Additionally, the MAX-2 PBD, required for binding by activated Rac or Cdc42, is less conserved than that of PAK-1 and the mammalian Group A Pak proteins. C. elegans also contains a protein, pak-2, that is an ortholog of mammalian Group B Pak proteins (Hofmann et al. 2004; Lucanic et al. 2006). To examine the total contribution of Pak proteins to embryogenesis, we analyzed single, double, and triple Pak loss of function. We observed that pak-2(ok332) single mutants and pak-1(ok448) single mutants had significant but relatively low-level embryonic lethality (P < 0.0001; Figure 6). We constructed a pak-2(ok332); pak-1(ok448) double-mutant strain, and observed significantly more lethality than either the pak-1 or pak-2 single-mutant strains (P < 0.0001). Growth of pak-1(ok448) single-mutant animals on max-2-directed RNAi caused no change in baseline lethality, but growth of pak-2(ok332) single mutant or pak-2(ok332); pak-1(ok448) double-mutant animals on max-2-directed RNAi significantly suppressed lethality compared with the gfp-directed RNAi control group (P < 0.0001 and P = 0.04, respectively; Figure 6). Together, these observations suggest that pak-1 and pak-2 are redundant for embryonic morphogenesis and fertility, and perhaps that max-2 antagonizes pak-2 in this process. Since pak-2 did not contribute to DTC migration, we did not further pursue pak-2 analysis.

Additionally, pak-2(ok332); pak-1(ok448) double-mutant animals were substantially smaller than wild type as confirmed by blind phenotype scoring. Neither pak-1(ok448) nor pak-2(ok332) single mutant animals were of abnormally small stature, suggesting that pak-1 and pak-2 are redundant for regulation of body stature. Body stature is controlled by TGF-beta signaling (Savage-Dunn 2005), so perhaps pak-1 and pak-2 function in that process.

**PAK-1 and MAX-2 are required for fertility**

We observed that max-2; pak-1 double-mutant animals were mostly sterile. We similarly observed that max-2 or pak-1 single-mutant animals grown on RNAi targeting the reciprocal Pak-encoding gene were mostly sterile. Aside from aberrant pathfinding and shortened gonad arms, these gonads looked mostly normal by DIC. The only defect we observed was that many of the mature oocytes were smaller than their wild-type counterparts. Late L4 single mutant animals picked to the wild-type background were themselves quite fertile. When the extreme pairs max-2; pak-1 and pak-1; pak-2 were crossed, the resulting F1 animals were sterile to F2 generation, suggesting that neither max-2; pak-1 nor pak-1; pak-2 were necessary for fertility. The only fertility defect we observed was a defect in germline development in which both max-2; pak-1 and max-2; pak-2 were sterile. Since fertility is controlled by TGF-beta signaling (Savage-Dunn 2005), we conclude that neither max-2; pak-1 nor max-2; pak-2 were necessary for fertility.

To determine the cause of the extensive sterility, we fixed whole animals at various stages from L4 to mid-adult and stained them with DAPI to visualize DNA. We thus analyzed all pair-wise max-2; pak-1 mutant-RNAi combinations and observed no genotypic differences. As controls, we compared double loss of function to single mutants

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grown on bacteria expressing $gfp$-directed dsRNA. General gonad architecture in the double mutants was less well organized than in $gfp$-directed RNAi controls, particularly in truncated gonad arms (we could occasionally compare truncated and nontruncated in the same animal). However, in most gonad arms we observed the typical wild-type pattern of distal mitotic proliferation, followed by a clear transition zone, followed by the meiotic region of the gonad and oocyte arrest in diakinesis (Greenstein 2005; Kimble and Crittenden 2005).

We observed that many gonad arms in mature adults completely lacked sperm nuclei in the spermathecae. This phenotype was incompletely penetrant; although the majority of gonad arms lacked sperm, animals in which one arm had sperm whereas the other did not were relatively common, and we observed several animals in which both gonad arms had sperm whereas the other did not. Lethality in wild-type backgrounds is not shown here but is generally <0.5%. Each pair of columns includes $gfp$ or max-2-directed RNAi. Sample size indicated in columns. Statistics were generated via Fisher’s exact test. No error bars are shown because these are pooled assays.

We have thus identified a spermatogenesis defect caused by loss of max-2 and pak-1, but we do not believe that this defect is the sole source of sterility in these animals. Instead, we speculate that loss of max-2 and pak-1 could compromise the motility of sperm. C. elegans sperm are amoeboid, and active crawling behavior is conducive to fertilization. Consistent with this possibility, we also observed that the sperm in double loss-of-function animals were frequently more diffusely localized than in single mutants, with sperm frequently found in the uterus in spite of the absence of fertilized eggs there (Figure 7). But therefore a sperm motility defect is not sufficient to explain the degree of sterility, suggesting additional defects. We conclude that the sterility phenotype of max-2, pak-1 animals is likely to be complex, comprising a partial spermatogenesis defect, perhaps a sperm migration defect, and presumably additional defects. Consequently, only rarely is an animal able to produce a handful of fertile progeny.

**DISCUSSION**

We have characterized two Pak pathways that are required for proper migration and extension of DTCs during gonadal development. Loss of both pathways causes essentially 100% defective gonad morphology, and results in both DTC pathfinding defects and short migrations (truncation). Apparently both general classes of phenotypes are governed by the same genetic pathways (Figure 8), and thus may be mechanistically distinct processes that share the same signaling modules.

Disruption of individual pathway branches causes little or no defect, indicating extensive redundancy between the two pathways. This twofold}

![Figure 6](https://academic.oup.com/g3journal/article/3/2/181/6025721)

**Figure 6** *C. elegans* Pak proteins (PAK-1, MAX-2, and PAK-2) contribute to embryonic development. pak-1 and pak-2 function redundantly in embryonic development, and max-2(RNAi) suppresses the pak-2 mutant defect alone or in conjunction with pak-1. Lethality in wild-type backgrounds is not shown here but is generally <0.5%. Each pair of columns includes $gfp$ or max-2-directed RNAi. Sample size indicated in columns. Statistics were generated via Fisher’s exact test. No error bars are shown because these are pooled assays.

![Figure 7](https://academic.oup.com/g3journal/article/3/2/181/6025721)

**Figure 7** Loss of max-2 and pak-1 disrupts spermatogenesis and perhaps other aspects of fertility. (A, B) Wild-type animal. (C, D) max-2(nv162); pak-1(RNAi) animal. Both animals are shown from a nearly ventral view. DAPI fluorescence (A, C) and DIC (B, D). The white arrowheads indicate the location of sperm nuclei, white arrows indicate the cluster of bivalents in mature oocytes, black arrows indicate the vulva, and the black arrowhead indicates a fertilized embryo in its eggshell. The double Pak loss of function animal (below) shows sperm nuclei (scattered, small, intense spots) in the left-hand spermatheca, whereas the right-hand spermatheca is empty and no sperm nuclei are visible. Scale bar is 20 μm.
redundancy and the robust phenotypes when both pathways are lost allows for thorough dissection of the two pathway branches, and provides a fertile avenue for future research into Pak pathway functions.

We present a model of Pak pathway composition in DTC migration based on our genetic analyses (Figure 8). One pathway features the divergent Group A Pak, MAX-2, functioning like a canonical Group A Pak, perhaps surprising given that MAX-2 lacks Group A regulatory sequences other than the PBD. Our results in conjunction with a wealth of mammalian Pak studies (reviewed in Arias-Romero and Chernoff 2008; Bokoch 2003; Hofmann et al. 2004) suggest that MAX-2 is bound and activated by GTP-bound CED-10/Rac. Furthermore, in DTC migration CED-10 is activated by the Dock/ELMO RacGEF complex of CED-2/CED-5/CED-12, consistent with their similar relationship in cell corpse engulfment (Wu and Horvitz 1998). We have not identified the upstream signals that control RacGEF activation in this process, though a role for integrins has been reported (Lucanic and Cheng 2008). Our data also suggest that MIG-2/RhoG, which activates the Dock/ELMO atypical RacGEF complex in human and C. elegans cell corpse engulfment (deBakker et al. 2004), does not activate the CED-2/CED-5/CED-12 Dock/ELMO RacGEF complex in DTC migration. We place MIG-2/RhoG in parallel to the Paks and CED-11/Rac because of the apparent mutant additivity. Formally, MIG-2/RhoG may function downstream of the RacGEF, but this relationship is unclear. We also do not know through which effectors MAX-2 and PAK-1 signal in DTC pathfinding and extension.

The canonical PAK-1, in contrast, does not appear to have an absolute requirement for GTPase input for activity, though our analysis suggests some contribution of CED-10/Rac to PAK-1 branch activity. PIX-1 and GIT-1, orthologous to the human Pix exchange factor and Git G-protein coupled receptor kinase interactor, respectively, are equally required in the PAK-1 pathway branch, and thus we have identified a putative Pak-Pix-Git complex. Our finding that ectopic PAK-1 mutated in the Pix-binding motif can no longer rescue the pak-1 mutant phenotype corroborated this model.

Regulation of Pak-Pix-Git complex activity

Another group has reported conclusions consistent with some but not all of our data (Lucanic and Cheng 2008). They describe pak-1 mutant defects in the morphology of DTCs that we were unable to replicate in blind assays. The human Pak-Pix-Git complex has been reported to function independently of kinase activity, and thus the role of Pak is thought to be that of a scaffold for nucleating the complex (Li et al. 2003). However, our KD PAK-1 construct failed to rescue the pak-1 synthetic mutant phenotype compared to successful rescue by a PAK-1(+)+ control. Although we cannot confirm that transgenic protein is expressed, the wild-type PAK-1 did rescue the double loss of function, and the Pix- and kinase-dead have been validated in human proteins. Thus, our results argue that a functional kinase domain is required in part for full Pak-Pix-Git complex activity. Alternatively, perhaps there is more than one PAK-1-mediated activity present in DTCs. For example, a major PAK-1 function in DTCs may be that of the scaffold in the Pak-Pix-Git complex, but there is also conventional Pak kinase activity. Contradicting this model is the observation that the requirement of PIX-1 and GIT-1 are equally as strong as the requirement for PAK-1 in DTC migration (Figures 1C and 3D).

Lucanic and Cheng (2008) also weakly rescued pak-1 gonad morphology defects, which we cannot reproduce, with a GTPase-interaction dead PAK-1 protein, suggesting that PAK-1 activities are Rac- and CDC-42-independent (Lucanic and Cheng 2008). However, our genetic data argue that CED-10/Rac contributes significantly to the PAK-1 signaling branch, raising the possibility that the Pak-Pix-Git complex is not entirely Rac-independent.

If our model were true, then the canonical PAK-1 activity would be predicted to be GTPase-dependent. Our observations suggest that CED-10 plays a moderate role in PAK-1 branch activity in addition to its strong requirement for MAX-2 branch activity. First, even hypomorphic loss of CED-10 (or the CED-2/CED-5/CED-12 components of the atypical RacGEF) function confers a substantial DTC migration defect, while strong loss of single Pak proteins caused little (e.g., MAX-2) or no (e.g., PAK-1) defect. If CED-10 signaled solely through MAX-2, then the ced-10 loss-of-function (lf) phenotype should be comparable to that of max-2 mutants. Second, ced-10(lf) confers a substantial truncation defect, whereas we typically see substantial truncation with loss of both pathways (see Table 2). Third, ectopically expressed activated CED-10 cannot be suppressed by loss of either pak-1 or max-2, thus arguing that ectopic activated CED-10/Rac inappropriately engages both pathways. The parsimonious explanation of these observations is that CED-10 contributes to PAK-1 branch activity, and thus loss of CED-10 strongly disrupts the MAX-2 branch in addition to weakly disrupting the PAK-1 branch. However, we cannot determine whether that contribution is through targeting the Pak-Pix-Git complex to a specific subcellular compartment to facilitate its activity (Brown et al. 2002), or whether CED-10 additionally activates canonical PAK-1 kinase-dependent activities.

An alternative possibility to our model is that CED-10 has roles in DTC migration independent of Pak proteins, perhaps by using additional effector proteins. CED-10 has previously been shown to use diverse effectors in axon pathfinding and hypodermal enclosure.

![Figure 8](https://academic.oup.com/g3journal/article-lookup/doi/10.1371/journal.pgen.1008357.g008)
Our observations suggest that although CDC-42 has no nonredundant contribution to DTC migration, ectopic-activated CDC-42 is capable of engaging both PAK-1 and MAX-2. While this isn’t necessarily expected of the divergent MAX-2, we predict that CDC-42 can engage the well-conserved PAK-1 as an effector in some biological processes. The PAK-1 PBD is well conserved with human Group A Pak proteins, and the CDC-42 core effector-binding region is 100% identical to that of human Cdc42 (YVPTFVDNS).

Other studies have found a modest role for RAC-2 in DTC migration (Lucanic and Cheng 2008; Lundquist et al. 2001), but we saw minimal or no synthetic interactions. RAC-2 is thought to have minor redundancy with CED-10 in various processes (Lundquist et al. 2001), but it is unclear whether RAC-2 uses the same effectors as CED-10. We note that while the core effector-binding region of CED-10 is 100% conserved with human Rac1, Rac2 and Rac3 (YVPTFDNS), RAC-2 has nonconservative variants in two residues (Y197F) and mutation of many of these residues in human Rac1 disrupt binding of Pak and other effectors (Westwick et al. 1997). Thus, it is likely that RAC-2 is a divergent Rac protein that uses a somewhat-different spectrum of effectors than CED-10 and human Racs.

Finally, we find no evidence for the MIG-2/RhoG homolog functioning in either the MAX-2 or PAK-1 branch. MIG-2 clearly plays a role in regulating DTC migration, but we propose that this role is through additional pathways. The nematode Actin homolog, CACN-1, was shown genetically to interact in the MIG-2 pathway in DTC migration and functions in parallel to CED-10, PAK-1 and MAX-2, so we echo the model that MIG-2/RhoG functions in parallel to PAK-1 and MAX-2 signaling (Tannoury et al. 2010). By yeast two-hybrid assay MIG-2 has been found to interact with MAX-2 (Fujiki et al. 2010). This result is unexpected but perhaps reflects the atypical MAX-2 PBD. Beyond this yeast two-hybrid result, the defined effectors for RhoG proteins are the ELMO atypical GEF family, phosphatidylinositol 3 kinase, phospholipase D, and kinectin (deBakker et al. 2004; Katoh et al. 2000; Vignal et al. 2001; Wennerberg et al. 2002; Yamaki et al. 2007). The core effector-binding sequence in the Switch 2 region of Rac (YVPTFDNY) and Cdc42 (YVPTFDNY) are absolutely conserved between C. elegans and mammals, although as noted nematode RAC-2 has divergent changes. MIG-2 (YVPTFDNY) and RhoG (YVPTFDNY) have one conservative residue difference (V to I), making the worm MIG-2 effector binding sequence identical to Rac while the RhoG effector binding sequence is identical to Cdc42. Therefore, at the sequence level it is plausible that MIG-2 binds MAX-2, though inconsistent with our DTC migration genetics.

Two receptor systems have been shown to regulate DTC migration. Loss of integrin function disrupts DTC migration (Baum and Garriga 1997; Cram et al. 2003; Lee et al. 2001; Meighan and Schwarzbauer 2007), but loss of integrin has no additive effects when combined with loss of Pak function (Lucanic and Cheng 2008). The netrin signaling system also regulates DTC migration (Hedgecock et al. 1987, 1990), and loss of Pak is additive with loss of Ntrins (Lucanic and Cheng 2008). These results argue that Paks function downstream of integrins, but not Ntrins. No putative downstream partners of MAX-2 or PAK-1 have been identified. An RNAi screen has identified many other factors regulating DTC migration (Cram et al. 2006), and the roles of many are not understood.

Rac1-dependent activation of Pak1 scaffolding for PDK-Akt activity was described (Higuchi et al. 2008), but this study does not state whether Pix and Git are part of this scaffold. We tested RNAi-directed at the C. elegans orthologs of PDK and Akt, but observed no synergy with max-2 (data not shown).

**Paks are redundant in other nematode biological processes**

We found that MAX-2 and PAK-1 are also redundant for additional biological functions. These redundant Paks are required for at least one, and probably multiple diverse roles in germline development and function. Clearly sperm development is disrupted in the double loss of function, but we argue that other functions are disrupted as well to account for the entire sterile phenotype in the double loss of function.

We found a complex relationship among the C. elegans Pak proteins in embryonic morphogenesis. Simplicistically, we hypothesize that max-2 antagonizes the effect of the Group B Pak, pak-2, but embryonic morphogenesis is a very complex process involving diverse movements of differing cell types (Zhang et al. 2011), and in principle these proteins could even function in separate cells. The interaction and regulation of these Pak proteins in embryogenesis will be the interesting subject of future studies. However, we were surprised that one of the most complicated series of morphogenetic events in the nematode, the development of the male tail, was not perturbed by loss of max-2 and pak-1. Specifically, we found that pak-1(ok448); pak-2 (RNAi) and pak-1(tm403); max-2(RNAi) males had wild-type spacing and morphology of sensory rays and other male tail structures (data not shown).

We did, however, observe moderate defects in vulval morphogenesis in max-2; pak-1 double mutant animals. The vulva frequently was protruding in double-mutant adults, and in many L4 animals the “Christmas tree” invaginating vulva is asymmetrical. This result is not surprising, because CED-10 has been previously implicated in vulval morphogenesis (Kishore and Sundaram 2002), and CDC-42 has been implicated in vulval precursor cell polarity and induction (Welchman et al. 2007). Either could use one or both Pak proteins to propagate their signal. In addition to the potential role in cytoskeletal regulation during vulval morphogenesis, it is interesting to consider Paks as a possible MAPKKKK or MAPKKK (MAP4 kinase or MAP3 kinase), since Paks have been described as phosphorylating Raf kinase and MEK. LET-60/Ras regulates vulval fate through Raf-Mek-Erk signaling, and we found no role for PAK-1 and MAX-2 in regulation of this pathway (data not shown). However, LET-60 also regulates post-specification vulval morphogenesis through Raf-Mek signaling, and perhaps Paks regulate Raf-MEK-ERK activity in this process.

In conclusion, our study raises two substantial mechanistic questions. First, is the Pak-Pix-Git complex kinase independent? We find that DTC-directed expression of putative kinase-dead PAK-1 fails to rescue loss of pak-1 compared with rescuing wild-type construct. This observation argues that the PAK-1 function is in part dependent on kinase activity, in contrast to reports on the mammalian Pak-Pix-Git complex. Second, phenotypes caused by loss of CED-10 or components of its RacGEF, CED-2/S12, confer substantially stronger migration defects than loss of either PAK-1 or MAX-2 alone, suggesting that CED-10/Rac functions in both pathway branches. One interpretation of these data are that the Pak-Pix-Git complex relies to some degree on Rac activity, perhaps by membrane targeting of the complex, and on some kinase activity. Alternatively, perhaps PAK-1 performs both canonical and non-canonical functions in DTC migration. However, this interpretation is not consistent with the requirement of PIX-1 and GIT-1 activity that is equally as strong as the PAK-1 requirement.
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