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An UNC-40 pathway directs postsynaptic membrane extension in *Caenorhabditis elegans*

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The postsynaptic membrane of the embryonic neuromuscular junction undergoes a dramatic expansion during later development to facilitate the depolarization of larger muscles. In *C. elegans*, the postsynaptic membrane resides at the termini of plasma membrane extensions called muscle arms. Membrane extension to the motor axons during larval development doubles the number of muscle arms, making them a tractable model to investigate both postsynaptic membrane expansion and guided membrane extension. To identify genes required for muscle arm extension, we performed a forward screen for mutants with fewer muscle arms. We isolated 23 mutations in 14 genes, including *unc-40*/*Dcc*, which encodes a transmembrane receptor that guides the migration of cells and extending axons in response to the secreted UNC-6/Netrin spatial cue. We discovered that UNC-40 is enriched at muscle arm termini and functions cell-autonomously to direct arm extension to the motor axons. Surprisingly, UNC-6 is dispensable for muscle arm extension, suggesting that UNC-40 relies on other spatial cues to direct arm extension. We provide the first evidence that the guanine-nucleotide exchange factor UNC-73/Trio, members of the WAVE actin-polymerization complex, and a homolog of the focal adhesion complex can function downstream of UNC-40 to direct membrane extension. Our work is the first to define a pathway for directed muscle membrane extension and illustrates that axon guidance components can play key roles in postsynaptic membrane expansion.

KEY WORDS: UNC-40, UNC-73, WAVE, Dcc, Trio, GEX-2, GEX-3, UNC-95, Muscle arms, Axon outgrowth, *Caenorhabditis elegans*

INTRODUCTION

The postsynaptic membrane is not a passive partner in the development of the neuromuscular junction (NMJ). For example, the striated muscles of both flies and mammals extend filopodial-like membranes, called myopodia, near incoming growth cones just prior to synaptogenesis (Misgeld et al., 2002; Ritzenthaler et al., 2000). In *Drosophila*, myopodial extension is thought to be an obligatory target-recognition step that precedes NMJ formation (Ritzenthaler and Chiba, 2003). As development continues, the postsynaptic membrane actively expands over 50-fold in flies and also expands in mammals, possibly to accommodate tissue growth (Guan et al., 1996; Lahey et al., 1994; Slater, 2007). Without postsynaptic membrane expansion, electrical conductance through the NMJ decreases (Gorczyca et al., 2007). Thus, the dynamic nature of the postsynaptic muscle membrane is important for both the formation and maintenance of the NMJ during animal development.

The postsynaptic membrane of the nematode *C. elegans* is readily visible in living animals because of their transparency and anatomical simplicity. The 95 mononucleate body wall muscles required for *C. elegans* locomotion and feeding are arranged in four longitudinal quadrants: two that flank the dorsal nerve cords and two that flank the ventral nerve cords (see Fig. 1) (Sulston and Horvitz, 1977). Within each quadrant is a distal row of muscles that is furthest from the nerve cord and a proximal row that is closest to the cord. The cell bodies of motoneurons that control body muscle contraction reside exclusively within the ventral nerve cords, some of which extend a commissural axon to populate the dorsal nerve cord. Typical of nematodes, the motor axons of *C. elegans* are not arborized, but instead develop presynaptic specializations en passant (White et al., 1986). Most body muscles must therefore extend plasma membrane processes, called muscle arms, to the motor axons to make a NMJ. The postsynaptic machinery resides at the termini of these muscle arms (White et al., 1986). In several axon guidance mutants, the motor axons fail to complete their circumferential migration to the dorsal midline and instead extend along the lateral body wall (Hedgecock et al., 1987). In these animals, the muscle arms of the dorsal muscles extend to the errant lateral motor axons (Hedgecock et al., 1990), demonstrating that muscle arm extension is likely to be guided to motor axons by a chemotropic cue that has yet to be identified.

We have shown that *C. elegans* muscle arms more than double in number during early larval development, and that their extension to the nerve cord is both stereotypical and dependent upon the remodeling of the actin cytoskeleton (Dixon and Roy, 2005). These observations, together with the ability to visualize adult muscle arms in living animals (Dixon and Roy, 2005; Hedgecock et al., 1990), make muscle arm extension a genetically tractable system in which to investigate both guided membrane extension and postsynaptic membrane expansion. However, despite the discovery of nematode muscle arms 200 years ago (Rudolphi, 1808), a mechanistic understanding of their development has been lacking until now.

To better understand muscle arm extension, we performed a forward genetic screen for *C. elegans* mutants with fewer muscle arms, a phenotype we call muscle arm development defective, or Madd. As is typical with studies of axon guidance, we infer defects in muscle arm extension by examining the postsynaptic membrane in young adults. We isolated 23 Madd mutants representing 14 genes, ten of which we have identified. A key gene identified through this screen is *unc-40*, which encodes the homolog of Deleted in colorectal carcinoma (Dcc) and neogenin in vertebrates. UNC-40/Dcc is characterized as a single-pass type I transmembrane protein receptor for the UNC-6/Netrin ligand (Chan et al., 1996;

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Hedgecock et al., 1990; Ishii et al., 1992; Keino-Masu et al., 1996; Serafini et al., 1994). UNC-40 guides the migration of axonal growth cones and cells towards increasing concentrations of UNC-6, which is enriched at the ventral midline of *C. elegans* (Wadsworth et al., 1996). When coupled with the UNC-5 co-receptor, UNC-40 mediates migration away from UNC-6 (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992). Intriguingly, the global asymmetric distribution of UNC-6 polarizes the sub-cellular localization of UNC-40 to the ventral side of neuronal cell bodies during ventrally directed axon outgrowth (Adler et al., 2006). Thus, the sub-cellular localization of UNC-40 is likely to be paramount in determining the direction of membrane outgrowth and, ultimately, the direction of cell and growth cone migrations.

In addition to UNC-40, our forward genetic screen led to the discovery of nine additional gene products not previously known to regulate muscle arm extension. We ordered many of these components relative to UNC-40 and provide the first evidence that *unc-73*, the genes encoding WAVE complex members, and the focal adhesion homolog *unc-95*, function downstream of UNC-40. Our work demonstrates that many genes required for guided cell and growth cone migration play related roles in directing muscle arm outgrowth, and in turn are crucial for the expansion of the postsynaptic membrane in *C. elegans*.

MATERIALS AND METHODS

Nematode strains, transgenics and photomicroscopy

Unless otherwise indicated, nematode strains were cultured at 20°C according to standard protocols. All muscle arm counts were performed in the background of *trIs30* as previously described (Dixon and Roy, 2005), and muscle arm numbers were counted from muscle 11 in the ventral left quadrant (V_L11) and muscle 15 in the dorsal right quadrant (D_R15) in 30 individual young adults. We chose these muscles because they are easily recognized. All mutants were obtained from the *Caenorhabditis* Genetics Center, except those designated with a RP or *tr* prefix, which were generated in our laboratory.

HSN axons were visualized with either the *zdIs13[tph-1p::GFP]IV* (Clark and Chiu, 2003) or *mgIs42[tph-1::GFP, pRF4(rol-6(su1006))]* (Sze et al., 2000) transgene as indicated. Microinjection of nematodes was performed following standard procedures (Mello et al., 1991). UNC-40 was tagged with YFP by replacing the *unc-40* stop codon with a YFP cassette (a kind gift from Andrew Fire, Stanford University, USA).

Worms were anaesthetized in 2-10 mM levamisole (Sigma) in M9 solution (Lewis and Fleming, 1995) and mounted on a 2% agarose pad in preparation for photomicroscopy. We used a Leica DMRA2 HC microscope with standard Leica filter sets for GFP, YFP, CGFP and DsRed epifluorescence for all pictures. Muscle arms were counted from photographs taken using a 20× or 40× dry objective. Localization analyses used a 63× oil-immersion objective.

Forward genetic screen, complementation tests and molecular mapping

A semi-clonal forward genetic screen for Madd mutants was performed by incubating a mixed-stage population of RP112 *trIs25 [pPRRF138.2(him-4p::MB::YFP), pPRZL47(F25B3.3p::DsRed2), pRF4(rol-6(su1006)]; rrf-3(pk1426)* animals in 50 μM ethyl methanesulfonate (EMS) for 4 hours as previously described (Brenner, 1974). *him-4p* drives expression in select distal body muscles within each quadrant, *F25B3.3p* drives expression pan-neuronally, and pRF4 induces a rolling phenotype so that a portion of the dorsal or ventral midline is always presented to the observer. Two or three resulting F1s were then dispensed into each well of an OP50 *E. coli*-seeded 12-well plate using the COPAS (Complex Object Parametric Analyzer and Sorter) Biosort (Union Biometrica). Adult F2s were screened 4 days later for Madd mutants using a Leica MZFLIII epifluorescence dissection microscope with a 2× objective.

The Madd mutations isolated in our screen were bulk mapped to a chromosome interval using snip-SNP mapping (Wicks et al., 2001). Complementation tests were performed by first crossing the *trIs30* transgene (Dixon and Roy, 2005) into strains carrying canonical alleles of candidate genes (*gene-X*, for example) that had similar phenotype and mapped within the same interval as our *tr* mutant of interest. Resulting *gene-X*/+; *trIs30*/+ males were then crossed to our Madd mutant. The fraction of resulting *trIs30*/+ Madd progeny was noted, and the number of muscle arms extended by V_L11 and D_R15 was determined for at least 30 Madd animals. We identified mutations by sequencing candidate genes from two individuals (Génome Québec Innovation Centre). Although we have mapped *tr50* to a 1.5 cM interval surrounding the *unc-60* locus and found that it fails to complement the *unc-60B(su158)* null mutant, we have not been able to find the mutation in *unc-60B* (or *unc-60A*) coding sequence. Our assertion that *tr50* is an allele of *unc-60B* is therefore tentative.

UNC-40 overexpression

Ectopic myopodia were induced by injecting *pPRKC294(him-4p::UNC-40::YFP)* (50 ng/μl) with co-injection markers *pPRGS317(him-4p::Mb::CFP)* (20 ng/μl) and *pPR1.1(unc-25p::DsRed2)* (10 ng/μl) into the various control and experimental strains. Myopodia were counted from the outer row of muscles (numbers 9-19) on the dorsal right quadrant of 15 independent F1 transgenic progeny. To examine the UNC-40::YFP-induced myopodia in a background compromised for *wve-1*, the injection mixture described above was injected into the RNAi-hypersensitive mutant *rrf-3(pk1426)*. *wve-1(RNAi)* [or *negative control(RNAi)*]-inducing bacteria were then fed to injected nematode parents as previously described (Timmons and Fire, 1998).

RESULTS

A forward genetic screen for muscle arm development-defective (Madd) mutants

To identify genes required for muscle arm extension to the nerve cords, we first constructed a strain called RP112 that facilitates the visualization of muscle arms (see Materials and methods). RP112 expresses membrane-anchored YFP in select muscles of only the distal row of body wall muscles, providing an unobstructed view of the longer arms extending from these muscles (Fig. 1) (Dixon and Roy, 2005). RP112 animals were mutagenized and resulting F2 animals were screened in a semi-clonal manner for individuals that had altered or fewer muscle arms, a phenotype we call muscle arm development-defective (Madd). In addition to fewer muscle arms, several of these mutants had noticeably wider arms, suggesting that these mutant genes might also play a role in regulating muscle arm morphology. In total, 23 Madd mutants were isolated, representing 14 mutant genes, ten of which we identified (Table 1).

Four of the Madd mutants are allelic to two genes (*unc-54* and *unc-60B*) that we previously characterized through a candidate analysis (Dixon and Roy, 2005) (Table 1). For the remaining 19 mutants, we aimed to determine which might have muscle arm extension defects solely because of mispositioning of their motor axon targets and to eliminate these mutants from further consideration. The dorsal cord of mutants with severe circumferential axon extension defects is deficient in motor axons, whereas the ventral cord remains relatively intact (Hedgecock et al., 1990). Hence, mutants that are Madd as a secondary consequence of axon extension errors are expected to have dramatically fewer muscle arms extending from the dorsal muscles as compared with the ventral muscles. By contrast, mutant genes that play a primary role in muscle arm extension are expected to confer defects in both dorsal and ventral muscle arm extension, irrespective of circumferential axon guidance defects. We found that dorsal muscle arm extension was dramatically more defective than ventral arm extension in *tr105*, *tr114* and

tr126 mutants (Fig. 1 and see Fig. S1 in the supplementary material). We found that *tr114* is a novel missense mutation in *unc-33*, which encodes a homolog of Crmp2 (Dpysl2), a protein that facilitates tubulin polymerization (Fukata et al., 2002). *tr126* is a novel missense mutation within the serine/threonine kinase domain of the conserved kinase encoded by *unc-51* (Ogura et al., 1994) (Table 1, see Fig. S2 in the supplementary material). *tr105* remains uncloned. Both *unc-33* and *unc-51* are required for proper axon extension (see Table S1 in the supplementary material) (Hedgecock et al., 1985; McIntire et al., 1992; Ogura et al., 1994). We tentatively conclude that *unc-33*, *unc-51* and *tr105* do not play a primary role in muscle arm extension and are not considered further here.

For the remaining 16 mutants, ventral muscle arm extension was at least as defective as dorsal muscle arm extension, suggesting that the corresponding genes play a primary role in muscle arm extension. Below, we present a detailed analysis of the role of four of these genes, *unc-40*, *unc-73*, *unc-95* and *gex-2*, in muscle arm

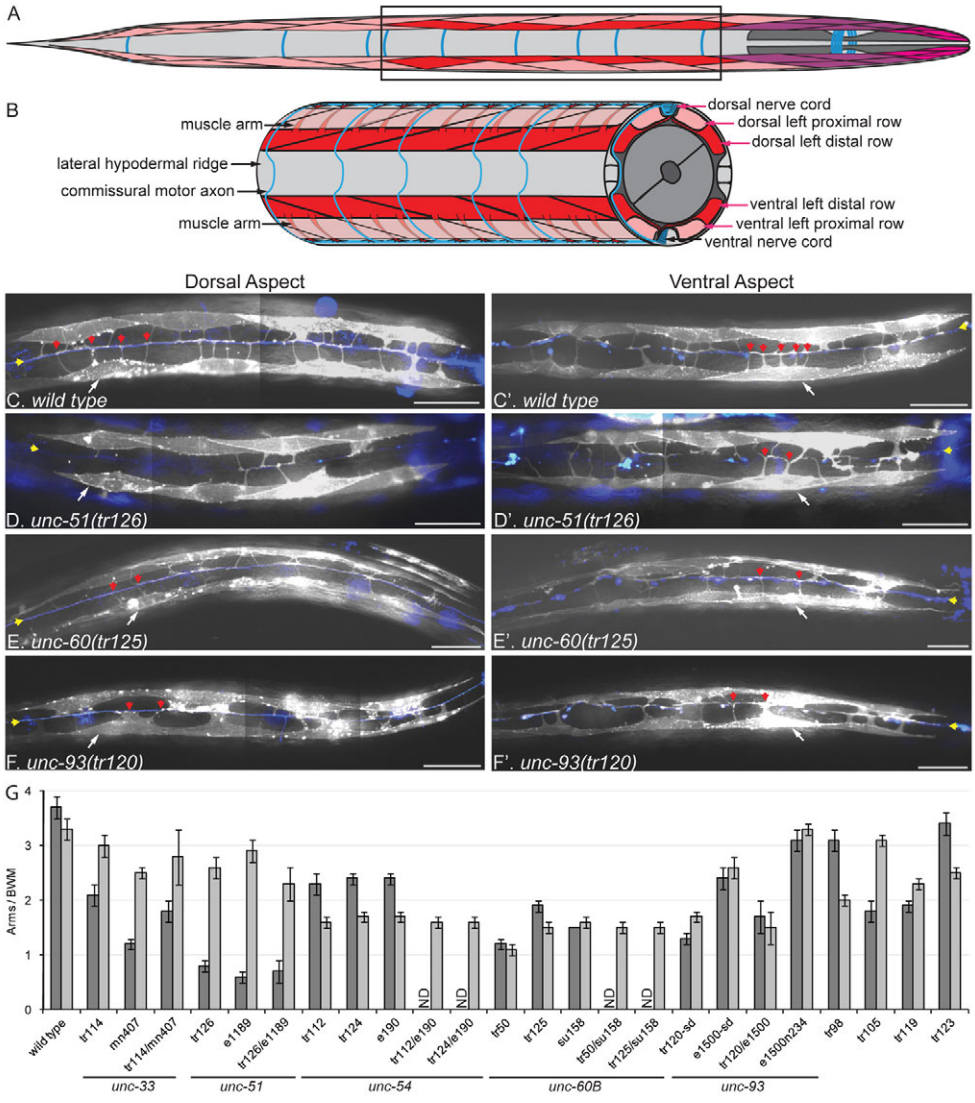

Fig. 1. An overview of selected mutants isolated from our screen. (**A**) Schematic of the right-hand side of an adult *C. elegans* hermaphrodite. The body wall muscles are depicted as rhomboids and those that express *him-4* at high levels are indicated in red. The box indicates the region shown in B. Anterior is to the right in all panels. In A and B, dorsal is up. (**B**) Select details of *C. elegans* neuromuscular anatomy. (**C-F'**) Fluorescent micrographs of the four distal muscles of dorsal left and right quadrants (left column) and the four distal ventral left and right muscles (right column) that express the *him-4p*::membrane-anchored YFP muscle arm reporter from the *trIs30* integrated transgenic array. The genotype is indicated. Motoneuron cell bodies and/or axons are false-colored blue. The nerve cord is indicated with a yellow arrowhead. In micrographs of dorsal muscles, left is up. In micrographs of the ventral muscles, left is down. Dorsal right muscle 15 (D_R15) is indicated with a white arrow in the left-hand column, and ventral left muscle 11 (V_L11) is indicated with a white arrow in the right-hand column (Dixon and Roy, 2005). The muscle arms of D_R15 and V_L11 were counted for all analyses herein and are indicated with red arrowheads. Scale bars: 50 μm. (**G**) Summary of the number of D_R15 (dark gray) and V_L11 (light gray) muscle arms per muscle for the indicated genotypes. The *unc-93* alleles *tr120* and *e1500* are semi-dominant (sd) with respect to uncoordinated movement, but not muscle arm extension (see Table S1 in the supplementary material), and are likely to be gain-of-function alleles (Levin and Horvitz, 1992). *unc-93(e1500n234)* is a loss-of-function allele. For muscle arm numbers for heterozygous controls, see Table S1 in the supplementary material. ND (not determined) indicates that only V_L11 muscle arms were counted. Error bars indicate s.e.m.

Table 1. The 23 Madd mutants recovered in our screen for genes required for muscle arm extension

	Complementation group	Allele	LG*	Map position*	Failed to complement†	Homolog	Mutation‡
1	*gex-2*	*tr116*	IV	< –16	*ok1603*	Sra1/p140/Cyfip1	R420Stop (c5009t)
2	*madd-2*§	*tr64*	V				
3		*tr96*					
4		*tr101*					
5		*tr103*					
6		*tr113*					
7		*tr129*					
8	*unc-33*	*tr114*	IV	–5 < +1	*e204*	Crmp2/Dpysl2	R502H (g6504a)
9	*unc-40*	*tr63*	I	–0.7 < 1.0	*n324*	Dcc/neogenin	Intron 6 splice donor (g4869a)
10		*tr115*		> –1.7	*n324*		W1107Stop (g8867a)
11		*tr121*		–4.5 < 1.0	*n324*		Exon 8 splice donor/D426N (g5765a)
12	*unc-51*	*tr126*	V	+19.4 > +25.2	*e369*	Ulk2	I59T (t1240c)
13	*unc-54*	*tr112*	I	LGI	*e190*	MHC-B/Myh7	–
14		*tr124*		LGI	*e190*		–
15	*unc-60B*	*tr125*	V	LGV	*su158*	Cofilin/ADF	G44E (g2241a)
16		*tr50*		–19.1 < –17.6	*su158*		–
17	*unc-73*	*tr117*	I	–4.5 < 1.0	*e936*		E1335K (g9486a)
18	*unc-93*	*tr120*^{sd}	III	–7.4 < –2	*e1500*	UNC-93	G388R (g2476a)
19	*unc-95*	*tr61*	I	LGI	*su33*	UNC-95	Intron 1 splice acceptor (g1693a)
20	–	*tr98*^{d}	I	–6.2 < –4.5	–	–	–
21	–	*tr105*	I	+4 < +23.5	–	–	–
22	–	*tr119*	I	+1.9 < +4	–	–	–
23	–	*tr123*	I	–4.8 < –1.6	–	–	–

*The linkage group (LG) (i.e. chromosome) and map position are shown. Only the linkage group is shown if mapping did not proceed beyond bulk segregant analysis.
†The allele used in the complementation test is shown.
‡The mutant residue is shown followed by the mutant nucleotide in brackets, which is relative to the adenine of the predicted start codon in the genomic sequence (WormBase release 187). For *tr117* and *tr125*, the mutant nucleotides are with respect to F55C7.7b and C38C3.5c.1, respectively. *tr63* carries a mutation in the invariant first base of the splice donor of intron 6, which is likely to result in the translation of 11 additional codons within intron 6 before a stop codon is reached, truncating the protein between the second and third IG domains. Similarly, *tr121* is mutant in the last nucleotide of exon 8 and may also disrupt splicing (Farrer et al., 2002). For those aberrantly spliced *tr121* transcripts, a stop codon is present 99 codons into intron 8, resulting in a predicted truncated protein between the fourth IG domain and the first fibronectin type III domain. For those transcripts without altered splicing, a D426N mutation is created between the fourth IG domain and the first fibronectin type III domain.
§Details of the six *madd-2* alleles isolated in our screen will be presented elsewhere.

extension. The role of *unc-93* in muscle arm extension remains poorly understood, and a detailed characterization of the *madd-2* complementation group will be presented elsewhere.

unc-40, but not *unc-6*, is necessary for muscle arm extension

Complementation tests between four of our mutants that mapped to the middle of chromosome I revealed that *tr63*, *tr115* and *tr121* are likely to be alleles of the same gene (Table 1, see Table S1 in the supplementary material), which we initially referred to as *madd-1*. Because *unc-40* lies within the map interval of *madd-1* and shares all of the observed behavioral phenotypes of *madd-1* (our unpublished observations) (Hedgecock et al., 1990), we examined the muscles of *unc-40(n324)* null mutants and found them to be Madd (Fig. 2). We then tested whether *madd-1* mutants fail to complement the Madd phenotype of *unc-40(n324)*, and discovered that *tr63*, *tr115* and *tr121* are allelic to *unc-40* (Fig. 2, see Table S1 in the supplementary material). We found single mutations in the *unc-40* gene for each of our three *unc-40* alleles (Table 1). We conclude that *madd-1* is *unc-40* and that it is required for normal muscle arm extension.

We observed that *unc-40* mutants have dramatically fewer ventral muscle arms than controls (Fig. 2), indicating that the muscle arm extension defects of *unc-40* mutants are not a secondary consequence of axon guidance errors (see above). By contrast, ventral muscle arm extension in both *unc-5* and *unc-6* mutants was indistinguishable from that of wild-type controls (Fig. 2, see Fig. S2 in the supplementary material). These results suggest that UNC-40, but not UNC-5 or their canonical ligand UNC-6, is necessary for muscle arm extension.

UNC-40 directs muscle arm extension to motor axon targets cell-autonomously

We investigated where UNC-40 is required to regulate muscle arm extension. Given the role of UNC-40 in the development of the nervous system, we tested whether *unc-40* could rescue the Madd phenotype of *unc-40* null animals when expressed throughout the nervous system. Pan-neuronal expression of an UNC-40::GFP fusion protein rescued the commissural axon guidance defects of *unc-40(n324)* mutants (see Table S1 in the supplementary material), but not the dorsal and ventral muscle arm extension defects (Fig. 2), further demonstrating that the Madd phenotype of *unc-40* mutants is not secondary to neuronal defects. We then tested whether UNC-40::YFP expression in muscles would rescue the Madd phenotype of *unc-40* null mutants. Indeed, muscle expression of UNC-40::YFP from either extra-chromosomal transgenic arrays, or a chromosomally integrated array (called *trIs34*), rescued the muscle arm extension defects of *unc-40(n324)* animals (Fig. 2, see Table S1 in the supplementary material). We therefore conclude that *unc-40* acts cell-autonomously to regulate muscle arm extension.

Next, we investigated the spatial expression pattern of two functional UNC-40 reporters. First, we examined UNC-40::GFP expression driven by *unc-40* promoter and enhancer elements (Chan et al., 1996) and observed UNC-40::GFP expression in body

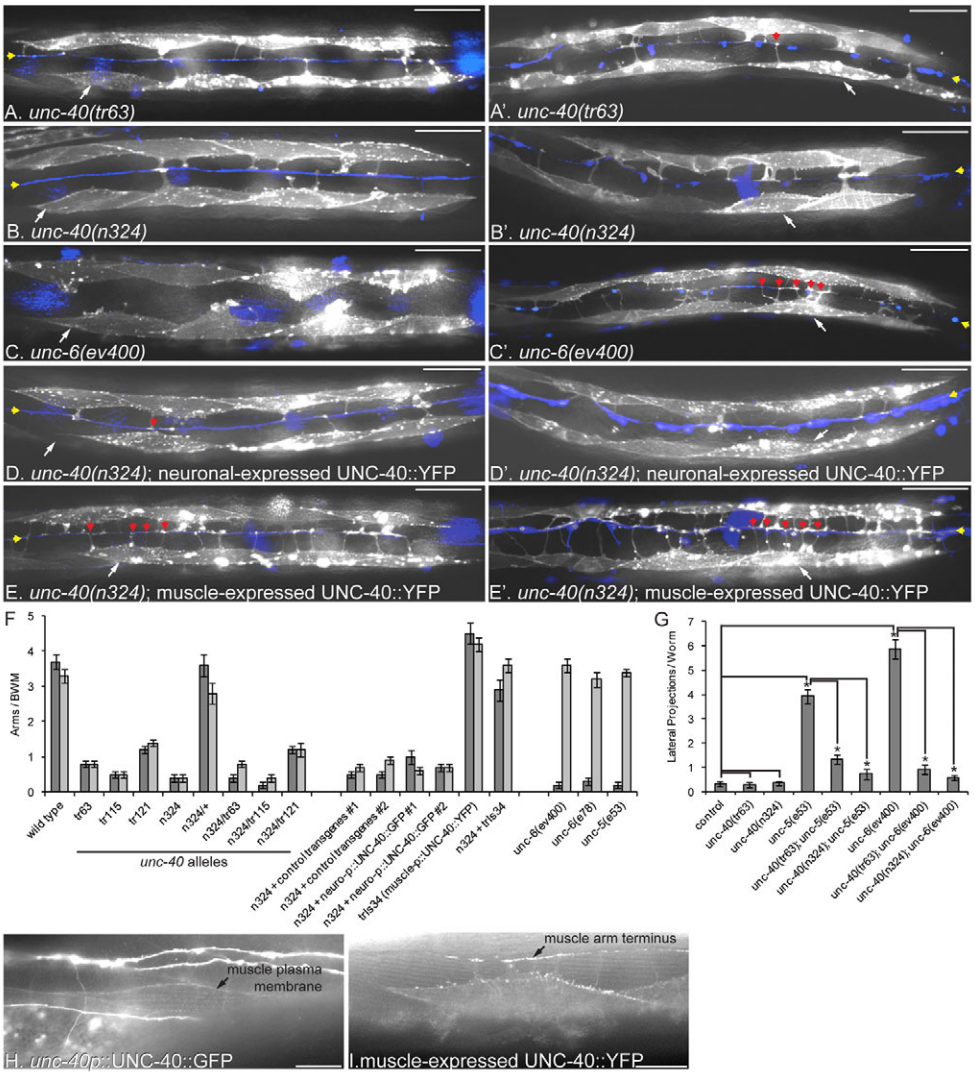

Fig. 2. *unc-40* functions cell-autonomously to regulate muscle arm extension. (**A-E'**) Dorsal (A-E) and ventral (A′-E′) views of muscle arms of *C. elegans* animals of the indicated genotypes. The annotation is the same as in Fig. 1. (D,D′) UNC-40::YFP was expressed in the nervous system using the *unc-119* promoter (neuro-p in F). The white arrow in D points to the area where the D_R15 muscle is normally positioned, but is not fluorescing in this animal. (E,E′). UNC-40::YFP was expressed in the distal muscles using the *him-4* promoter (muscle-p in F). (**F**) The average number of muscle arms extended by the indicated genotypes. The control transgenes are the DNAs common to all injection mixtures without the experimental *unc-40*-related transgene (see Materials and methods). Aside from *trls34*, the transgenic arrays are maintained extra-chromosomally, independent lines of which are indicated with a number. Additional annotation is the same as for Fig. 1G. (**G**) The number of lateral muscle membrane extensions for the indicated genotypes, illustrating that *unc-40* mutations suppress misdirected muscle arm extension to misguided lateral axons resulting from an *unc-5* or *unc-6* mutant background. Asterisks indicate significantly more defects than the controls ($P<0.001$) as indicated by the lines above the bars. The error bars in F and G represent s.e.m. (**H**) UNC-40::GFP expression directed by the *unc-40* promoter. Localization to the muscle plasma membrane is indicated. (**I**) Muscle-specific expression of functional UNC-40::YFP, driven by the *him-4* promoter. Enrichment at the muscle arm terminus is indicated. Scale bars: 50 μm in A-E′; 10 μm in H,I.

muscles (Fig. 2H). Because the sub-cellular localization of UNC-40::GFP in muscle cells is confounded by the fluorescence of surrounding cells, we examined the localization of a functional UNC-40::YFP fusion protein that is specifically expressed in the distal body muscles. We found that UNC-40::YFP localizes to the plasma membrane of muscles and is enriched on both myopodial-like protrusions and at muscle arm termini (Fig. 2I). When expressed in muscles at obviously lower levels, the localization of UNC-40::YFP was restricted to the muscle arm termini (see Fig. 3E′ and Fig. 5G′). The spatial expression pattern of the functional UNC-40 reporters is consistent with a cell-autonomous role for *unc-40* in muscle arm development.

We and others have previously observed that dorsal muscle arms extend to the misguided commissural motor axons that are positioned along the sides of *unc-5* mutant animals (Dixon et al., 2006; Hedgecock et al., 1990). We found that *unc-5* and *unc-6* mutants have ~4-6 muscle arms per side that project into the lateral space (Fig. 2G). By contrast, the number of lateral muscle arms in *unc-40* mutants did not differ significantly from controls ($P>0.05$) (Fig. 2G). If *unc-40* is indeed required for muscle arm extension towards motor axons, then disrupting *unc-40* should suppress lateral muscle arm extension in mutants with commissural axon guidance defects. If, on the other hand, *unc-40* is required for muscle arm extension towards some other target at

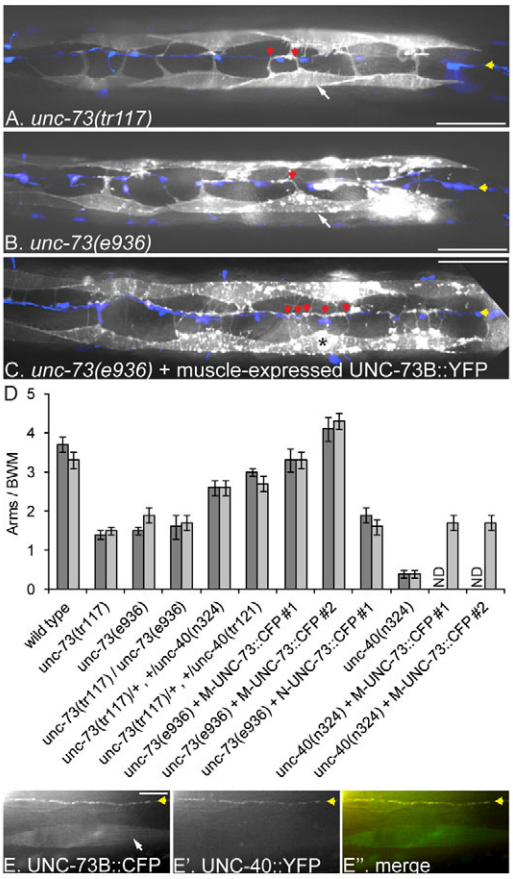

Fig. 3. UNC-73 is necessary for muscle arm extension and co-localizes with UNC-40 at muscle arm termini. (**A**,**B**) The ventral muscle arm extension defects of *C. elegans unc-73* mutants. (**C**) Muscle-specific expression of UNC-73B::YFP rescues the muscle arm extension defects of *unc-73* mutants. The annotation for A-C is as described in Fig. 1C-F′, except that only the ventral quadrants are shown. V_L11 is indicated with either a white arrow or an asterisk. (**D**) Summary of muscle arm extension in the background of *unc-73*-related mutants. The annotation for D is the same as that for Fig. 2F, except that M-UNC-73 and N-UNC-73 represent muscle-expressed and neuronally expressed UNC-73 using the *him-4* and *unc-119* promoters, respectively. ND, not determined. (**E-E″**) UNC-73B::CFP specifically expressed in muscles in the background of the *trIs34* integrated transgene that expresses UNC-40::YFP specifically in muscles. The localization of fusion proteins to the muscle arm termini is indicated with a yellow arrowhead. Scale bars: 50 μm in A-C; 10 μm in E-E″.

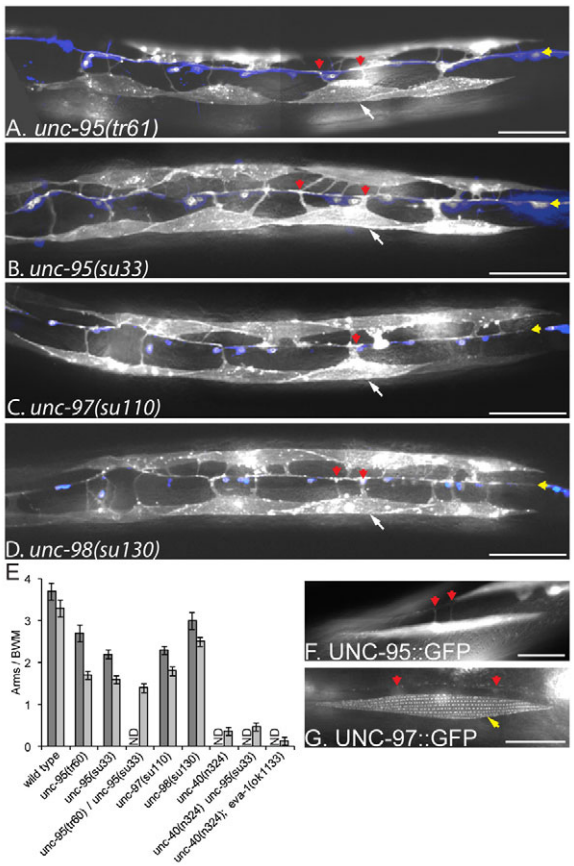

Fig. 4. Dense body components are necessary for muscle arm extension. (**A-D**) The ventral muscle arms of *C. elegans* mutants with disrupted dense body components. The annotation is the same as that for Fig. 1C-F′. (**E**) A summary of the number of muscle arms for the indicated genotypes. The annotation is the same as that for Fig. 1G. For heterozygous control counts, see Table S1 in the supplementary material. ND, not determined. (**F**,**G**) Functional UNC-95::GFP (Broday et al., 2004) (F) and UNC-97::GFP (Hobert et al., 1999) (G) fusion proteins can be seen in the muscle arms (red arrows). Scale bars: 25 μm in A-D; 50 μm in F,G.

or near the midline, *unc-40* mutations should not affect muscle arm extension to the errant motor axons of *unc-5* and *unc-6* mutants. We found that *unc-40(n324)* suppresses the lateral muscle arm extensions of *unc-5(e53)* and *unc-6(ev400)* mutants ($P<0.001$) (Fig. 2G). These observations support the idea that UNC-40 directs muscle arm extension towards motor axon targets, irrespective of their anatomical position.

UNC-73 regulates muscle arm extension cell-autonomously and co-localizes with UNC-40 at muscle arm termini

Animals homozygous for *tr117* resemble *unc-40* mutants in that they are Madd, short, uncoordinated and have commissural axon guidance defects (Fig. 3, see Table S1 in the supplementary material). *tr117* maps to an interval on chromosome I and fails to complement *unc-73(e936)* (Table 1, Fig. 3). *unc-73* is a complex locus that encodes at least eight isoforms of UNC-73 (a homolog of Trio), which is needed for guided cell migrations and axonal pathfinding (Forrester and Garriga, 1997; Hedgecock et al., 1987; Steven et al., 1998; Steven et al., 2005). In addition to other domains, *unc-73* encodes tandem Rho guanine-nucleotide exchange factor (GEF) domains (Steven et al., 1998). Sequencing of *unc-73(tr117)* revealed a missense mutation in the first RhoGEF domain (Table 1). Consequently, we investigated whether a muscle-expressed CFP-tagged UNC-73B isoform lacking the second RhoGEF domain (a kind gift from Rob Steven, The University of Toledo, OH, USA) could rescue the Madd phenotype of *unc-73(e936)* mutants, and found that it could (Fig. 3). This demonstrates that UNC-73 functions cell-autonomously and that the first of the two RhoGEF domains is necessary to regulate muscle arm extension.

We next examined the sub-cellular localization of the functional UNC-73B::CFP fusion protein when expressed in the muscles. We found that UNC-73B::CFP localizes diffusely throughout the cytoplasm, but is obviously and consistently enriched at the muscle arm termini, where it co-localizes with UNC-40::YFP (Fig. 3E). This suggests that UNC-40 and UNC-

73 might function together to direct muscle arm extension. Our observation that *unc-73(tr117)*/+, +/*unc-40* transheterozygotes have fewer muscle arms than controls (Fig. 3D) further supports this model because non-allelic non-complementation often occurs between components within the same complex (Yook et al., 2001).

Dense body components are necessary for muscle arm extension

We hypothesized that *tr61* is allelic to *unc-95* because of their overlapping map positions on the right arm of chromosome I and because of their similar locomotory and egg-laying defects. Indeed, *tr61* fails to complement the *unc-95(su33)* null allele and sequencing revealed a splice acceptor mutation in *unc-95* in *tr61* mutants (Table 1, Fig. 4). To investigate whether *unc-95* acts in the *unc-40* pathway to regulate arm extension, we made an *unc-40(n324) unc-95(su33)* double mutant. If *unc-95* regulates arm extension independently of the *unc-40* pathway, then *unc-95(su33)* should enhance the Madd phenotype of *unc-40* null mutants. We found that *su33* fails to enhance *unc-40(n324)*. Through an independent line of investigation, we found that *eva-1(ok1133)* enhances the muscle arm defects of the *unc-40(n324)* null ($P<0.05$) (Fig. 4), demonstrating that it is possible to reveal pathways that may act in parallel to *unc-40* in muscle through genetic analyses. Together, these results suggest that *unc-95* functions in the *unc-40* pathway to regulate muscle arm extension.

unc-95 encodes a LIM-domain-containing protein that localizes to dense bodies, nuclei and muscle arms of body wall muscles (Broday et al., 2004). *C. elegans* dense bodies are analogous to vertebrate focal adhesions and connect the contractile apparatus to the extracellular matrix (ECM), facilitating force transduction (Lecroisey et al., 2007). We previously reported that disrupting the dense body component *pat-2*, which encodes α-integrin, also results in muscle arm extension defects when compromised (Dixon et al., 2006). These findings prompted us to investigate the role of other dense body components in muscle arm extension, including *unc-97*, which encodes a Pinch (Lims1) ortholog (Hobert et al., 1999), and *unc-98*, the product of which physically interacts with UNC-97 (Mercer et al., 2003). We found that *unc-97* and *unc-98* mutations each confer a Madd phenotype (Fig. 4). By contrast, mutation of the M-line component UNC-89 did not affect muscle arm extension (see Table S1 in the supplementary material), suggesting that the muscle arm extension defects of *unc-95*, *unc-97* and *unc-98* are unlikely to be a secondary consequence of sarcomeric disruption. Upon examining the subcellular localization of functional UNC-95 and UNC-97 fusion proteins (Broday et al., 2004; Hobert et al., 1999), we found that both were present in muscle arms, but at much reduced levels compared with their levels in the cell body of the muscle (Fig. 4). Together with the previous findings that *unc-95* and *unc-98* are specifically expressed in muscle (Broday et al., 2004; Mercer et al., 2003) and that *unc-97* is expressed only in muscles and six mechanosensory neurons (Hobert et al., 1999), our observations suggest that *unc-95* and other dense body components likely function cell-autonomously to regulate muscle arm extension.

Members of the predicted WAVE complex are required for muscle arm extension

The *tr116* homozygotes isolated from our screen are sterile, have a protruding vulva (Pvl), and map to the distal left arm of chromosome IV, as do *gex-2* mutants (Soto et al., 2002). We found that the *gex-2(ok1603)* deletion confers a phenotypic profile similar to that of

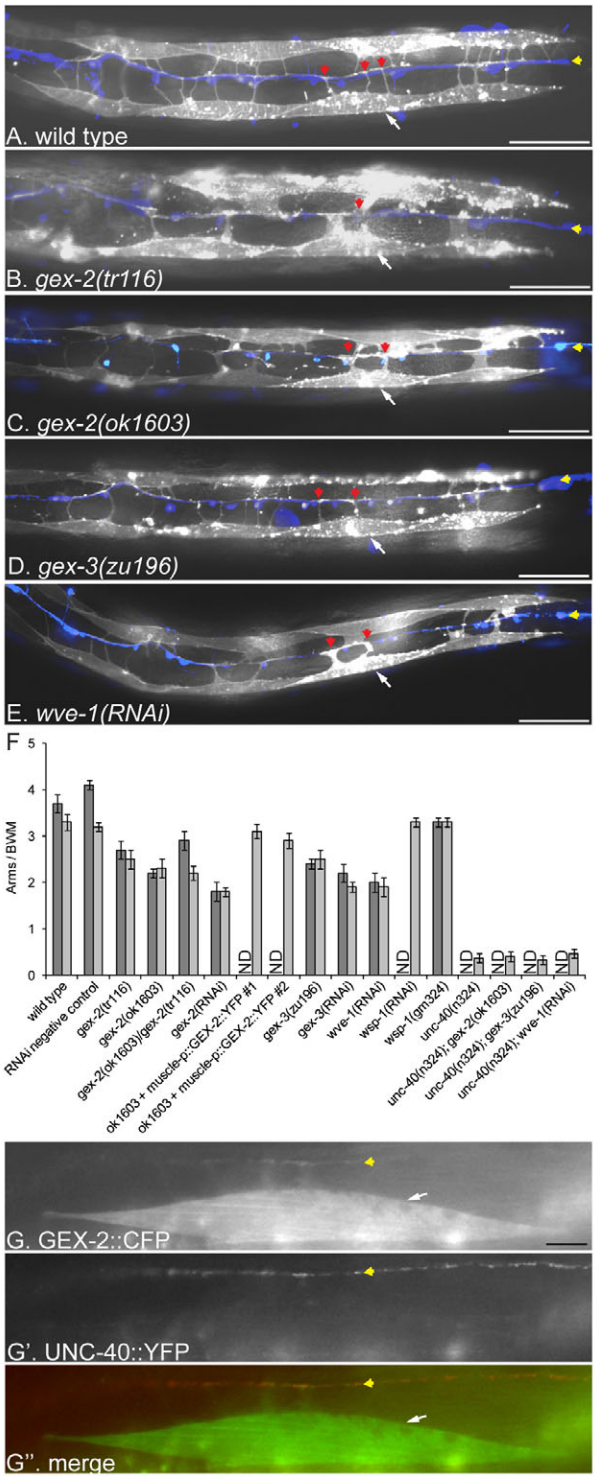

Fig. 5. Members of the WAVE complex are required for muscle arm extension. (**A-E**) The genotype or RNAi treatment is indicated; the annotation is the same as Fig. 1C-F′. For E, *rrf-3(pk1426) C. elegans* were treated with *wve-1(RNAi)*. For *rrf-3(pk1426)* control animals, see Fig. S2 in the supplementary material. (**F**) Summary of the muscle arm numbers in WAVE-related backgrounds. The annotation is the same as for Fig. 1G. ND, not determined. (**G-G"**) GEX-2::CFP specifically expressed in muscles in the background of the *trIs34* integrated transgene that expresses UNC-40::YFP specifically in muscles. The localization of fusion proteins to the muscle arm termini is indicated with a yellow arrowhead. The muscle cell body is indicated with a white arrow. Scale bars: 50 μm in A-E; 5 μm in G-G".

tr116 animals and that *ok1603* fails to complement the sterile, Pvl and Madd phenotypes of *tr116* (Fig. 5 and data not shown). Furthermore, *gex-2(RNAi)* also induced muscle arm extension defects (Fig. 5). Sequencing *gex-2* in *tr116* mutants revealed a nonsense mutation in codon R420 (Table 1). We conclude that *gex-2* is required for proper muscle arm extension.

gex-2 encodes the *C. elegans* ortholog of Rac1-associated protein 1 (also known as Sra1, p140, Pir121, Cyfip1) (Soto et al., 2002). In mammals, Sra1, together with Nck-associated protein 1 (also known as Nap1, Nckap1, Kette, Hem2), inhibits WAVE activity and protects it from degradation within a multi-subunit complex (Kunda et al., 2003). WAVE (Wasf3) is a member of the WASP family of proteins, which promote membrane extension by stimulating the Arp2/3 (Actr2/3) actin-polymerization complex (Weaver et al., 2003). The interaction of activated Rac1 with Sra1 dissociates the WAVE complex, which frees WAVE to stimulate the Arp2/3 complex (Eden et al., 2002; Miki et al., 1998; Steffen et al., 2004). By contrast, WASP (Wasl) is auto-inhibited and only able to stimulate the Arp2/3 complex upon binding activated Rho, Cdc42, or other positive regulators (Abe et al., 2003; Symons et al., 1996; Weaver et al., 2003). The *C. elegans* orthologs of Nap1, WAVE and WASP are encoded by *gex-3*, *wve-1* and *wsp-1*, respectively (Sawa et al., 2003; Soto et al., 2002; Withee et al., 2004), which have recently been shown to play a role in axon guidance (Shakir et al., 2008). Consistent with our finding that *gex-2* is required for muscle arm extension, we found that animals compromised for *gex-3* and *wve-1* function have fewer muscle arms than do controls (Fig. 5, see Table S1 in the supplementary material). By contrast, compromising *wsp-1* by RNAi or mutation had no effect on muscle arm extension (Fig. 5).

Previous work showed ubiquitous expression of GEX-2 after the 100-cell stage of embryogenesis (Soto et al., 2002). When specifically expressed in muscles, GEX-2::CFP localized diffusely within the cell body of body wall muscles, was slightly enriched at the arm termini relative to the arm stalks, and co-localized with UNC-40::YFP at the muscle arm termini (Fig. 5G). We found that GEX-2::YFP rescues the Madd phenotype of *gex-2(ok1603)* mutants (Fig. 5), demonstrating that *gex-2* functions cell-autonomously to regulate muscle arm extension. Similar to the function of Sra1, Nap1 and WAVE in mammals, we speculate that GEX-2, GEX-3 and WVE-1 regulate actin polymerization at the leading edge of the body muscles to drive muscle arm extension.

The predicted WAVE complex functions in an UNC-40 pathway

Because both *gex-2* and *unc-40* function cell-autonomously to direct muscle arm extension to the midline and because *gex-3* and *wve-1* are also necessary for normal muscle arm extension, we hypothesized that *gex-2*, *gex-3* and *wve-1* act in an *unc-40* pathway. We took two approaches to test this hypothesis. First, we asked whether compromising the function of any one of these genes could enhance the muscle arm extension defects of the *unc-40* null and found that they could not (Fig. 5). Second, we examined the role of *gex-2*, *gex-3* and *wve-1* in the membrane extension of a second cell type that requires UNC-40. The bilaterally symmetrical pair of hermaphrodite-specific neurons (HSNs) flank the vulva and extend an axon ventrally during the fourth larval stage (Adler et al., 2006). Upon meeting the ventral midline, the HSN axons turn and extend longitudinally towards the head (see Fig. 6A). Consistent with previous observations (Adler et al., 2006; Desai et al., 1988), we found that the HSN axons of *unc-40* null animals are misguided in both their initial outgrowth and their ultimate extension to the ventral midline (Fig. 6, see Table S2 in the supplementary material). Here, we consider outgrowth as misguided if the adult HSN axon is not directed ventrally within two HSN-cell diameters from the HSN cell body (see Fig. 6). An extensive analysis of HSN cell body position revealed little to no correlation with the direction of HSN axon outgrowth (see Fig. S3 in the supplementary material). We found that the outgrowth of the HSN axons in animals compromised for *gex-2*, *gex-3* or *wve-1* is occasionally misdirected (Fig. 6, see Table S2 in the supplementary material). Driving GEX-2::YFP expression in the HSNs from the *unc-86* promoter (Baumeister et al., 1996) rescued the outgrowth defects of *gex-2(ok1603)* mutants (Fig. 6), suggesting that the WAVE complex functions cell-autonomously to direct HSN axon outgrowth. Disrupting *gex-2*, *gex-3* or *wve-1* failed to enhance the outgrowth guidance defect of the *unc-40(n324)* null mutant (Fig. 6). By contrast, the *slt-1(ok255)* control enhanced the outgrowth defects of the *unc-40* null mutant (Fig. 6). Because the muscle arm extension and HSN axon outgrowth defects of the *unc-40* null mutant are not enhanced by the disruption of *gex-2*, *gex-3* or *wve-1* function, we conclude that *gex-2*, *gex-3* and *wve-1* function in the *unc-40* pathway to direct membrane extension from both muscles and neurons.

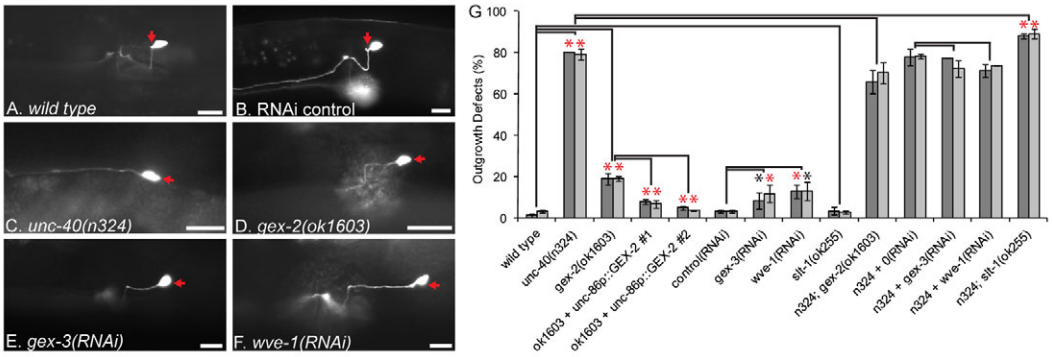

Fig. 6. Members of the WAVE complex direct HSN axon outgrowth in an UNC-40 pathway. (**A-F**) Examples of *C. elegans* HSNl axons, visualized with the *zdIs13[tph-1p::GFP]* array in the indicated genetic or RNAi background. Ventral is down and anterior is to the left. The red arrowhead indicates the direction of HSNl axon outgrowth. Scale bars: 10 μm. (**G**) The percentage of HSNl (dark gray bars) and HSNr (light gray bars) axons that fail to extend ventrally within two cell diameters of the cell body. Asterisks indicate significantly more defects than the controls indicated by the lines above the bars (red, $P<0.05$; black, $P<0.1$). Error bars indicate s.e.m. For further details, see Fig. S3 in the supplementary material.

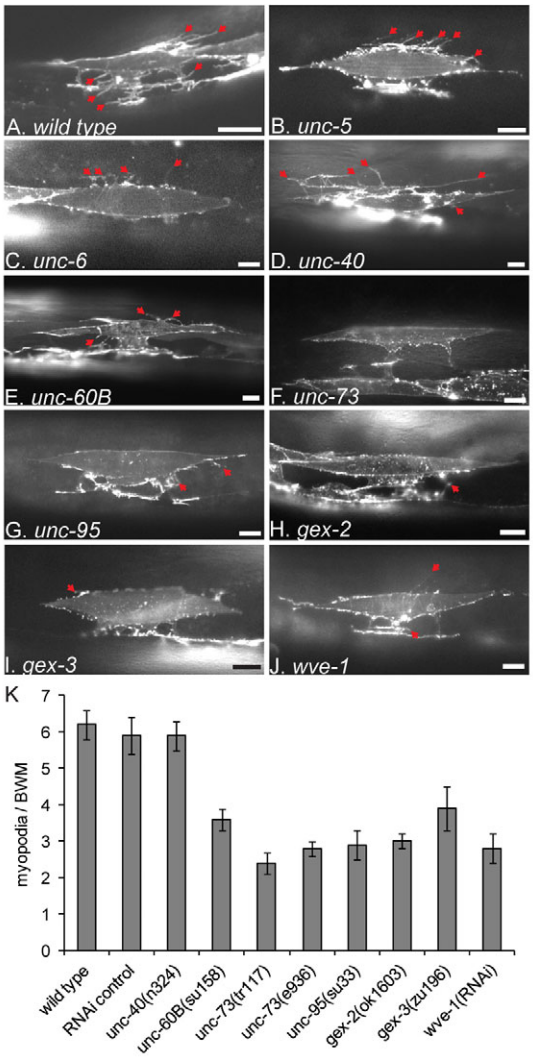

Fig. 7. UNC-40-induced myopodia are suppressed by mutations in cytoskeletal regulators and in *unc-95*. (**A-J**) Transgenic arrays created with 25-fold more UNC-40::YFP transgene than that used for rescue experiments induce fine membrane protrusions called myopodia (red arrowheads). Examples of body wall muscles extending myopodia induced by overexpressed UNC-40::YFP. The UNC-40::YFP is used to visualize the body wall muscle plasma membrane (white). The alleles or RNAi treatments used are indicated in K. Scale bars: 10 μm. (**K**) Summary of the number of myopodia observed per body wall muscle of the indicated genotype. All alleles used in this analysis are null except for those of *unc-73*. Myopodia counts for *gex-2* and *gex-3* mutant *C. elegans* were performed in homozygous offspring of heterozygous parents because *ok1603* and *zu196* are maternal-effect lethal. Error bars indicate s.e.m.

UNC-73 functions downstream of UNC-40 in muscle

To determine whether the genes we identified through our screen are likely to function upstream or downstream of *unc-40*, we exploited an UNC-40 gain-of-function phenotype in muscles, similar to the means by which candidate genes were ordered relative to *unc-40* in neurons (Gitai et al., 2003; Levy-Strumpf and Culotti, 2007). During our *unc-40* rescue experiments, we discovered that transgenic animals created with high concentrations of the UNC-40::YFP transgene exhibit ectopic plasma membrane extensions from the muscles (see Materials and methods). These extensions, which we refer to as myopodia, project randomly and are thinner than muscle arms (Fig. 7). In these animals, UNC-40::YFP was localized along the entire plasma membrane of the muscles (Fig. 7). We found that *unc-60B*, *unc-73*, *unc-95* and members of the predicted WAVE complex were each required for the UNC-40::YFP-mediated ectopic myopodial extension, but did not obviously disrupt UNC-40::YFP localization to the plasma membrane (Fig. 7). These observations indicate that *unc-73* and the other genes may act downstream of *unc-40* in muscles. To further investigate this model, we tested whether increased UNC-73 activity might suppress the muscle arm extension defects of *unc-40(n324)* null mutants. From our test of *unc-73* cell autonomy, we observed that an extra-chromosomal array carrying the *him-4p*::UNC-73B::CFP transgene conferred supernumerary muscle arms (see Fig. 3D). We found that these UNC-73::CFP-expressing arrays could indeed suppress the muscle arm defects of the *unc-40(n324)* null mutant ($P<0.05$) (Fig. 3D). The most parsimonious interpretation of these results is that *unc-73*, and perhaps *unc-60B*, *unc-95* and members of the WAVE complex, function downstream of *unc-40* in muscles.

DISCUSSION

Previously, only three genes were known to function in muscle to regulate arm extension: *lev-11* (tropomyosin), *unc-54* (muscle myosin heavy chain B) and *unc-60B* (ADF/Cofilin) (Dixon and Roy, 2005). Here, we isolated 23 muscle arm development-defective (Madd) mutants, representing a total of 14 genes based on phenotype, map position, complementation tests and sequencing data. Satisfyingly, both *unc-54* and *unc-60B* (Dixon and Roy, 2005), were identified in our screen. Of the remaining 12 genes, four are not yet cloned and the role of *unc-93* (a potassium channel subunit) in muscle arm extension remains poorly understood. We infer that the Madd phenotype of three mutants isolated in our screen, namely *unc-33* (a Crmp2 homolog), *unc-51* (a serine/threonine kinase) and *tr105*, is secondary to commissural axon extension errors because only dorsal muscles have dramatic defects in muscle arm extension in these mutants. It remains possible, however, that in addition to playing a role in commissural axon guidance, *unc-33*, *unc-51* and *tr105* also play a more prominent role in dorsal, but not ventral, muscle arm extension. We demonstrated that the five remaining genes, *gex-2* (encoding a Sra1 homolog), *unc-40* (Dcc), *unc-73* (Trio/RhoGEF), *unc-95* (a LIM-domain protein) and *madd-2* (M.A. and P.J.R., unpublished), are necessary for both dorsal and ventral muscle arm extension. The analysis of these Madd mutants led to the identification of four additional genes that are necessary for muscle arm extension: *gex-3* (a Nap1 homolog), *unc-97* (a Pinch ortholog), *unc-98* (an UNC-97-interacting protein), and *wve-1* (WAVE). We demonstrated that *gex-2*, *unc-40* and *unc-73* function autonomously in muscles to regulate arm extension, and that *unc-95*, *unc-97* and *unc-98* are likely to do the same based on their expression pattern and function (Broday et al., 2004; Hobert et al., 1999; Mercer et al., 2003). Through analyses of these genes, we define the pathway that regulates muscle membrane extension: UNC-40 directs muscle arm extension to the motor axons and UNC-60/ADF/Cofilin, UNC-73/Trio, UNC-95 and members of the predicted WAVE complex mediate UNC-40-directed muscle membrane extension.

Focal adhesion homologs are required for muscle arm extension

We identified multiple components of the dense body complex that are required for muscle arm extension, including PAT-2/α-integrin (Dixon et al., 2006), UNC-95, UNC-97 and UNC-98. In contrast to

the localization pattern of functional UNC-40 and UNC-73 reporters, we observed only weak localization of functional UNC-95 and UNC-97 reporters within muscle arm stalks and termini. Examining animals during the period of muscle arm extension also failed to reveal enrichment of UNC-95 and UNC-97 reporters at the leading edge of the muscle membrane (data not shown). These observations raise the possibility that the Madd phenotype of dense body mutants might be a secondary consequence of other defects within the soma of muscle cells, such as disarrayed sarcomeres or disengagement from the body wall (Lecroisey et al., 2007). This model, however, is not supported by our observation that mutation of *unc-89*, a gene required for sarcomere organization (Small et al., 2004), results in normal muscle arm extension. By contrast, a model whereby dense body components function at the leading edge to facilitate muscle arm extension is supported by three additional observations. First, several orthologs of dense body components, such as Pinch and the integrins, function within focal adhesion complexes at the leading edge of migrating cells to anchor the extending plasma membrane to the surrounding ECM and promote further membrane extension (DeMali et al., 2002; Serrels et al., 2007; Tu et al., 1999). Second, *unc-95* fails to enhance the muscle arm defects of the *unc-40* null. If the Madd phenotype of dense body mutants was a non-specific consequence of other muscle functions gone awry, dense body mutants would be expected to dramatically enhance the muscle arm defects of the *unc-40* null mutant. Because the opposite is true, *unc-95* is likely to function in the *unc-40* pathway, which has no known role in muscle aside from directing muscle arm extension. Finally, *unc-95* is required for UNC-40-mediated myopodial extensions. Together, these results argue that these focal adhesion homologs play a primary role in muscle arm extension.

UNC-40 directs muscle arm extension independent of UNC-6

Several lines of evidence demonstrate that UNC-40 functions in muscle to direct muscle arm extension towards motor axon targets. First, muscle-specific expression of UNC-40 rescues the Madd phenotype of *unc-40* mutants. Second, the sub-cellular localization of UNC-40 within muscle cells is a key factor in directing membrane outgrowth. When expressed at relatively low levels in muscles from the *trIs34* transgenic array, the UNC-40 fusion protein can rescue the Madd phenotype of *unc-40* mutants and localizes exclusively to the muscle arm termini. When expressed at relatively high levels, the UNC-40 fusion protein is distributed along the entire plasma membrane of the muscle cells and induces ectopic myopodia in a non-directed fashion. Third, *unc-40* is required for the redirection of muscle arms to mislocalized motor axon targets in an *unc-5* or *unc-6* mutant background. We conclude that UNC-40 directs the extension of muscle arms towards their motor axon targets. Furthermore, the abundance of UNC-40 at the plasma membrane is key to its ability to promote outgrowth, adding to the previous observation that spatial cues are likely to direct axonal extension by polarizing the distribution of UNC-40 towards the leading edge of the plasma membrane (Adler et al., 2006).

We found that the Netrin ortholog UNC-6, the canonical ligand for UNC-40, is dispensable for muscle arm extension. The simplest interpretation of this result is that UNC-40 is either responding to a non-Netrin cue, or relies on parallel pathways to provide polarity information to direct muscle arm extension to the nerve cord. Other guidance events that require UNC-40, but not UNC-6, have been described previously. These include the extension of the AVM axon along the anterior-posterior axis (Yu et al., 2002), and the posterior-directed migration of the Q_L neuroblast (Honigberg and Kenyon, 2000). We are currently investigating the mechanism by which UNC-40 directs muscle arm extension and will determine whether other parallels exist between muscle arm extension, AVM axon guidance and Q_L neuroblast migration. Regardless, the role of UNC-40 in directing muscle arm extension prompts the exciting question of whether a similar pathway facilitates the expansion of the postsynaptic membrane during the development of other animals such as flies and mammals.

UNC-73 acts upstream of UNC-40 in neurons, but downstream of UNC-40 in muscles

Seven lines of evidence suggest that UNC-73 acts with UNC-40 to direct muscle arm extension. First, loss-of-function mutations in each gene reduce the number of muscle arms that extend to the motor axons. Second, both genes function cell-autonomously in muscles to regulate arm extension. Third, alleles of *unc-73* show mild non-allelic non-complementation with the *unc-40* null allele. Fourth, functional UNC-73 and UNC-40 reporters co-localize at the muscle arm termini. Fifth, *C. elegans* UNC-73 and UNC-40 and the respective orthologs from *Drosophila* physically interact (Forsthoefel et al., 2005; Watari-Goshima et al., 2007). Sixth, we showed that *unc-73* is necessary for UNC-40 gain-of-function activity in muscles. Finally, transgenically increasing the gene dose of *unc-73* can suppress the muscle arm extension defects of the *unc-40* null mutant. Together, these observations provide the first evidence that UNC-73 can function downstream of UNC-40.

We also presented evidence that the first of the two RhoGEF domains of UNC-73 is necessary for muscle arm extension. First, our *tr117* allele is a missense mutation within the RhoGEF-1 domain. Second, UNC-73B has only the first of the two RhoGEF domains and is sufficient to rescue the muscle arm extension defects of *unc-73* mutant animals. The RhoGEF-1 domain of UNC-73 stimulates Racs (Steven et al., 1998), whereas the RhoGEF-2 domain stimulates Rho (Spencer et al., 2001). This is especially intriguing because we found that *C. elegans* WAVE complex members, but not the WASP ortholog, are similarly required for muscle arm extension. Rac GTPases stimulate Arp2/3-mediated actin-based membrane extension via the WAVE complex (Miki et al., 1998), whereas Rho and Cdc42 stimulate membrane extension via the WASPs (Abe et al., 2003; Symons et al., 1996). We therefore propose that UNC-73 acts downstream of UNC-40 to stimulate actin-based muscle arm extension via Rac-mediated WVE-1 activity.

Given that UNC-73 can stimulate small GTPases that are well characterized as regulators of actin polymerization in migrating cells (Raftopoulou and Hall, 2004), the naïve expectation is that UNC-73 functions downstream of guidance receptors, which is consistent with our findings. However, several independent investigations have recently shown that UNC-73 may function upstream of guidance receptors, including UNC-40, to regulate their activity in guiding neuronal migrations and axonal extension (Gitai et al., 2003; Levy-Strumpf and Culotti, 2007; Watari-Goshima et al., 2007). Although the mode of UNC-73 action in these mechanosensory neurons is not yet clear, UNC-73 might regulate receptor abundance or localization through receptor trafficking (Levy-Strumpf and Culotti, 2007; Watari-Goshima et al., 2007). However, we found that *unc-73* activity in muscles is dispensable for the localization of a functional UNC-40 fusion protein to the muscle plasma membrane and arm termini (see Fig. 7F). Hence, *unc-73* is likely to play a different role within the *unc-40* pathway in *C. elegans* neurons as compared with muscle cells.

Finally, the perdurance of UNC-40 and UNC-73 proteins at the muscle arm termini of adults is intriguing. It suggests that these proteins might not only function in directing muscle arm extension, but may also play a role at the mature postsynaptic membrane of the NMJ. Alternatively, these proteins might perdure at the termini without consequence after directing muscle arm extension. Regardless, the continued localization of UNC-40 and UNC-73 implies that the machinery necessary for polarized localization in muscles perdures in adults and could be exploited to identify genes required for the localization of guidance components to the leading edge of the extending membrane.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/6/911/DC1

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Table S1. The Madds of mutants recovered in the forward genetic screen

A	Genotype	Muscle arm # (dorsal right)*	Muscle arm # (ventral left)†	Dorsal comms (%)‡	*P*-value§
1	*control(trIs30)*	3.7±0.2	3.3±0.17	18.4±0.67	
2	*rrf-3(pk1426)*	3.7±0.2	3.1±0.1	16.5±0.4	0.22 (1)
	gex-2				
3	*tr116*	2.7±0.2	2.5±0.1	16.1±0.4	<0.001 (1)
4	*tr116/+*	4.0±0.2	3.1±0.1	–	–
5	*ok1603*	2.2±0.1	2.3±0.1	18.7±0.5	<0.001 (1)
6	*ok1603/+*	3.9±0.1	3.2±0.2	–	–
7	*tr116/ok1603*	2.9±0.2	2.2±0.2	–	<0.001 (1)
8	*gex-2(RNAi);rrf-3(pk1426)*	1.8±0.2	1.8±0.1	7.9±0.5	<0.001 (2)
9	*gex-2(ok1603); Ex[him-4p::GEX-2::YFP] line 1*	–	3.1±0.2 (n15)	–	0.18 (1)
10	*gex-2(ok1603); Ex[him-4p::GEX-2::YFP] line 2*	–	2.9±0.2 (n15)	–	0.05 (1)
11	*unc-40(n324); gex-2(ok1603)*	0.27±0.08	0.40±0.10	5.97±0.37	0.40 (26)
	unc-33				
12	*tr114*	2.1±0.2 (n23)	3.0±0.2 (n21)	15.7±0.6	0.11 (1)
13	*tr114/+*	3.8±0.1 (n27)	3.3±0.2 (n28)	–	–
14	*e204/+*	3.8±0.2	3.1±0.1	–	–
15	*tr114/e204*	2.5±0.3	2.9±0.2 (n8)	–	0.11 (1)
16	*mn407*	1.2±0.1	2.4±0.1	14.5±0.5	<0.001 (1)
17	*mn407/+*	4.3±0.2	3.4±0.1	–	–
18	*tr114/mn407*	1.8±0.2 (*n*=16)	2.8±0.5 (*n*=4)	–	0.14 (1)
19	*unc-40(n324); unc-33(tr114)*	0.33±0.09	0.33±0.11	3.37±0.32	0.41 (26)
	unc-40				
20	*tr63*	0.8±0.1	0.8±0.1	9.0±0.4	<0.001 (1)
21	*tr63/+*	3.3±0.1	2.8±0.2	–	–
22	*tr115*	0.5±0.1	0.5±0.1	10.9±0.4	<0.001 (1)
23	*tr115/+*	3.5±0.1	3.0±0.2	–	–
24	*tr121*	1.2±0.1	1.4±0.1	14.6±0.5	<0.001 (1)
25	*tr121/+*	3.5±0.1	3.2±0.1	–	–
26	*n324*	0.37±0.13	0.37±0.09	3.90±0.31	<0.001 (1)
27	*n324/+*	3.6±0.3 (n10)	2.8±0.3 (n8)	–	–
28	*tr63/n324*	0.4±0.1 (n23)	0.8±0.1 (n26)	–	<0.001 (1)
29	*tr115/n324*	0.2±0.1 (n27)	0.5±0.1 (n27)	–	<0.001 (1)
30	*tr121/n324*	1.2±0.1 (n15)	1.3±0.2 (n10)	–	<0.001 (1)
31	*trIs34(him-4p::UNC-40::YFP)*	4.5±0.3	4.2±0.2	–	0.0036 (1)
32	*unc-40(n324); trIs34*	2.9±0.3	3.6±0.2	–	0.075 (1)
33	*unc-40(n324); Ex[control]#1*	0.4±0.1	0.5±0.1	5.9±0.3	0.25 (26)
34	*unc-40(n324); Ex[control]#2*	0.4±0.1	0.3±0.1	5.0±0.4	0.30 (26)
35	*unc-40 (n324); Ex[him-4p::UNC-40::YFP]#1*	3.6±0.3	3.6±0.2	–	<0.001 (26)
36	*unc-40 (n324); Ex[him-4p::UNC-40::YFP]#2*	3.3±0.3	3.4±0.2	–	<0.001 (26)
37	*unc-40 (n324); Ex[unc-119p::UNC-40::GFP]#1*	1.0±0.2	0.6±0.1	18.5±0.7	0.06 (26)
38	*unc-40 (n324); Ex[unc-119p::UNC-40::GFP]#2*	0.7±0.1	0.7±0.1	19.1±0.5	0.02 (26)
	unc-51				
39	*tr126*	0.8±0.1	2.6±0.2 (n23)	–	<0.01 (1)
40	*tr126/+*	4.6±0.2	3.1±0.1	–	–
41	*e369/+*	4.6±0.3	3.9±0.2 (n14)	–	–
42	*tr126/e369*	0.6±0.1	2.3±0.2 (n14)	–	<0.001 (1)
43	*e1189*	0.6±0.1	2.9±0.2	2.9±0.3	0.09 (1)
44	*e1189/+*	3.9±0.2	3.1±0.1 (n15)	–	–
45	*tr126/e1189*	0.7±0.2	2.3±0.3 (n6)	–	0.01 (1)

B	Genotype	Muscle arm # (dorsal right)*	Muscle arm # (ventral left)†	Dorsal comms (%)‡	*P*-value§
1	*control(trIs30)*	3.7±0.2	3.3±0.2	18.4±0.67	
2	*rrf-3(pk1426)*	3.7±0.2	3.1±0.1	16.5±0.4	0.22 (1)
	unc-54				
46	*tr112*	2.3±0.2	1.6±0.1	14.9±0.4	<0.001 (1)
47	*tr112/+*		3.9±0.1	–	–
48	*tr124*	2.4±0.1	1.7±0.1	14.6±0.5	<0.001 (1)
49	*tr124/+*	–	3.4±0.1	–	–
50	*e190*	2.4±0.1	1.7±0.1	15.1± 0.4	<0.001 (1)
51	*e190/+*	–	3.3±0.1	–	–
52	*tr112/e190*	–	1.6±0.1	–	<0.001 (1)
53	*tr124/e190*	–	1.6±0.1	–	<0.001 (1)
	unc-60B				
54	*tr50*	1.2±0.1	1.1±0.1	15.6±0.5	<0.001 (1)
55	*tr50/+*	–	3.7±0.1	–	–
56	*tr125*	1.9±0.1	1.5±0.1	15.3±0.5	<0.001 (1)
57	*tr125/+*	–	3.7±0.1	–	–
58	*su158*	1.5±0.1	1.6±0.1	15.2±0.4	<0.001 (1)
59	*su158/+*	–	3.8±0.1	–	–
60	*tr50/su158*	–	1.5±0.1	–	<0.001 (1)

61	*tr125/su158*	–	2.0±0.1	–	<0.001 (1)
	unc-73				
62	*tr117*	1.4±0.1	1.5±0.1	13.2±0.42	<0.001 (1)
63	*tr117/+*	3.4±0.2	2.9±0.1	–	–
64	*e936*	1.5±0.1	1.9±0.2	6.6±2.1	<0.001 (1)
65	*e936/+*	3.8±0.2	3.5±0.1	–	–
66	*tr117/e936*	1.6±0.3	1.7±0.2	–	<0.001 (1)
67	*tr117/n324*	2.6±0.2 (n14)	2.6±0.3 (n13)	–	<0.01 (1)
68	*tr117/tr121*	3.0±0.1 (n27)	2.7±0.2 (n29)	–	<0.01 (1)
69	*unc-73(e936); Ex[him-4p::UNC-73::CFP] line 1*	3.3±0.3	3.3±0.2	–	<0.001 (64)
70	*unc-73(e936); Ex[him-4p::UNC-73::CFP] line 2*	4.1±0.3	4.3±0.2	–	<0.001 (64)
71	*unc-73(e936);Ex[unc-119p::UNC-73::CFP]line 1*	1.9±0.2	1.6±0.2	–	0.06 (64)
	unc-93[d]				
72	*tr120*	1.3±0.1	1.7±0.1	17.3±0.6	<0.001 (1)
73	*tr120/+*	3.6±0.1	3.9±0.1	–	–
74	*e1500*	2.4±0.2	2.6±0.2	16.1±0.6	0.003 (1)
75	*e1500/+*	3.8±0.2	3.9±0.2	–	–
76	*tr120/e1500*	1.7±0.3	1.5±0.3 (n6)	–	<0.001 (1)
77	*e1500n234*	3.1±0.2	3.3±0.1	18.3±0.5	0.21 (1)
	unc-95				
78	*tr60*	2.7±0.2	1.7±0.1	16.3±0.4	<0.001 (1)
79	*tr60/+*	–	3.4±0.1		–
80	*su33*	2.2±0.1	1.7±0.1	16.7±0.4	<0.001 (1)
81	*su33/+*	–	3.7±0.1 (n29)	–	–
82	*tr60/su33*	–	1.4±0.1	–	<0.001 (1)
83	*unc-40(n324) unc-95(su33)*	0.30±0.10	0.47±0.11	5.90±0.27	0.25 (26)
	Mutants not yet cloned				
84	*tr98*	3.1±0.2	2.0±0.1 (n27)	20.4±0.6	<0.001 (1)
85	*tr105*	1.8±0.2	3.1±0.1	4.3±0.3	0.186 (1)
86	*tr119*	1.9±0.1	2.3±0.1	16.0±0.4	<0.001 (1)
87	*tr123*	3.4±0.2	2.4±0.1	20.3±0.7	<0.001 (1)

C	Genotype	Muscle arm # (dorsal right)*	Muscle arm # (ventral left)†	Dorsal comms (%)‡	*P*-value§
1	*control(trIs30)*	3.7±0.2	3.3±0.2	18.4±0.67	
2	*rrf-3(pk1426)*	3.7±0.2	3.1±0.1	16.5±0.4	0.22 (1)
26	*n324*	0.37±0.13	0.37±0.09	3.90±0.31	–
88	*RNAi negative control*	–	3.7±0.1	–	0.22 (1)
	Other components				
89	*unc-5(e53)*	0.2±0.1	3.4±0.1	0.0±0.2	0.26 (1)
91	*unc-6(ev400)*	0.2±0.1	3.6±0.2	0.0±0.0	0.19 (1)
91	*unc-6(e78)*	0.3±0.1	3.2±0.2	0.1±0.0	0.38 (1)
92	*gex-3(zu196)*	2.4±0.1	2.4±0.2 (n15)	15.9±0.4	<0.01 (1)
93	*rrf-3(pk1426); gex-3(RNAi)*	2.2±0.2	1.9±0.1	8.3±0.5	<0.001 (2)
94	*unc-40(n324); gex-3(zu196)*	0.1±0.1	0.33±0.1	5.6±0.4	0.41 (12)
95	*rrf-3(pk1426); wve-1(RNAi)*	2.0±0.2	1.9±0.2	9.9±0.5	<0.001 (2)
96	*unc-40(n324); rrf-3(pk1426); wve-1(RNAi)*	0.4±0.1	0.47±0.1	4.77±0.36	0.26 (26)
97	*wsp-1(RNAi)*¶	–	3.3±0.1	–	0.02 (88)
98	*wsp-1(gm324)*	3.3±0.1	3.3±0.1	–	0.45 (1)
99	*rrf-3(pk1426); wsp-1(RNAi)*	–	3.5±0.1	–	0.32 (2)
100	*unc-89(e1460)*	3.4±0.1	3.6±0.1	–	0.06 (1)
101	*unc-97(su110)*	2.3±0.1	1.8±0.1	16.7±0.5	<0.001 (1)
102	*unc-98(su130)*	3.0±0.2	2.5±0.1	20.0±0.5	<0.001 (1)
103	*unc-40(n324); eva-1(ok1133)*	0.17±0.07	0.13±0.06	3.83±0.37	0.0186 (2)

This table does not include information on *madd-2*, which will be published elsewhere. All counts were performed on young adults raised at 20°C.
*The average number of muscle arms from dorsal right muscle number 15 (*n*=30, unless otherwise indicated).
†The average number of muscle arms from ventral left muscle number 11 (*n*=30, unless otherwise indicated).
‡The percentage of commissural axons on the right side that reach the dorsal nerve (*n*=30 animals).
§The *P*-value of the difference in the number of muscle arms for ventral left muscle number 11 in the experimental versus the control data point in the indicated row (in brackets). A Mann-Whitney test was used to derive *P*-values. –, not determined.
¶The muscle arm numbers of *trIs30; wsp-1(RNAi)* animals were examined in parallel to treating *trIs30; unc-34(e566)* animals with the same batch of *wsp-1(RNAi)*, which is used as a positive control for *wsp-1(RNAi)* activity as the *wsp-1(RNAi)*-treated *trIs30; unc-34(e566)* animals produced a synthetic lethal phenotype, as predicted (Withee et al., 2004).

Table S2. Genetic interactions of *unc-40* and members of the WAVE complex in directing HSN axon outgrowth, but not guidance to the midline

	Genotype†	HSNl axon outgrowth defects‡	HSNr axon outgrowth defects‡	HSNl axon guidance defects§	HSNr axon guidance defects§
1	*negative control*	2.0±1.2	4.0±1.2	0.0±0.0	1.3±1.3
2	*negative control(RNAi)*	4.0±1.2 (0.1, 1)	3.3±0.7 (0.3, 1)	0.0±0.0	0.7±0.7 (0.3, 1)
3	**negative control*	1.3±1.3	1.3±0.7	1.3±1.3	1.3±0.7
4	**unc-40(n324)*	59±9.4 (<0.01, 3)	66.0±1.2 (<0.001, 3)	50.7±7.8 (0.0017, 3)	56.0±3.1 (<0.001, 3)
5	*unc-40(n324)*	76.7±3.3 (<0.001, 1)	74.7±4.7 (<0.001, 1)	70.7±4.1 (<0.001, 1)	63.3±3.5 (<0.001, 1)
6	*unc-40(n324); negative control(RNAi)*	77.6±3.9 (<0.001, 2)	77.9±1.2 (<0.001, 2)	63.3±3.3 (<0.001, 2)	67.7±2.3 (<0.001, 2)
7	*gex-2(ok1603)*	18.9±2.8 (<0.01, 1)	18.9±1.1 (<0.001, 1)	6.6±3.3 (0.06, 1)	1.1±1.1 (0.45, 1)
8	*gex-2(ok1603); Ex[unc-86p::GEX-2::YFP] line 1*	7.8±1.1 (0.01, 7)	6.7±1.9 (<0.01, 7)	–	–
9	*gex-2(ok1603); Ex[unc-86p::GEX-2::YFP] line 2*	4.8±1.0 (<0.01, 7)	3.7±0.3 (<0.001, 7)	–	–
10	**gex-3(zu196)*	11.7±0.5 (<0.01, 3)	11.7±0.5 (<0.001, 3)	0.0±0.0 (0.25, 1)	0.0±0.0 (0.11, 1)
11	*gex-3(RNAi)*	11.1±4.0 (0.08, 2)	12.2±2.9 (0.02, 2)	0.0±0	0.0±0.0 (0.2, 2)
12	*wve-1(RNAi)*	12.7±3.3 (0.03, 2)	12.9±4.6 (0.06, 2)	0.0±0	0.0±0.0 (0.18, 2)
13	*unc-40(n324); gex-2(ok1603)*	65.7±5.7 (0.09, 5)	70.0±5.1 (0.3, 5)	56.7±5.3 (0.05, 5)	63.4±7.0 (0.49, 5)
14	*unc-40(n324); gex-3(RNAi)*	74.7±2.3 (0.3, 6)	73.0±3.1 (0.1, 6)	64.7±4.7 (0.41, 6)	62.7± 6.8 (0.26, 6)
15	**unc-40(n324); gex-3(zu196)*	90 (n=1×10)	80 (n=1×10)	80 (n=1×10)	60 (n=1×10)
16	*unc-40(n324); wve-1(RNAi)*	71.1±2.9 (0.12, 6)	73.3±0 (<0.01, 6)	70.2±8.6 (0.25, 6)	71.1±4.8 (0.28, 6)

The HSN cell bodies and axons of young adults were visualized with *zdIs13[tph-1::GFP] IV* (a gift from Oliver Hobert), except for those genotypes marked with an asterisk that were visualized with *mgIs42[tph-1::GFP + pRF4(rol-6(su1006))]*. Unless otherwise indicated, 30 animals were counted in three separate trials. All counts made at 20°C.

‡The percentage of HSNs extending an axon in a direction other than ventral for more than two cell diameters away from the cell body. Standard error of the mean follows the mean, followed by a *P*-value relative to the data in the indicated row. *P*-values were calculated using a *t*-test.

§The percentage of HSN axons that fail to ever extend ventrally. The annotation is the same as indicated above