C. elegans mig-6 encodes papilin isoforms that affect distinct aspects of DTC migration, and interacts genetically with mig-17 and collagen IV

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The gonad arms of C. elegans hermaphrodites acquire invariant shapes by guided migrations of distal tip cells (DTCs), which occur in three phases that differ in the direction and basement membrane substrata used for movement. We found that mig-6 encodes long (MIG-6L) and short (MIG-6S) isoforms of the extracellular matrix protein papilin, each required for distinct aspects of DTC migration. Both MIG-6 isoforms have a predicted N-terminal papilin cassette, lagrin repeats and C-terminal Kunitz-type serine proteinase inhibitory domains. We show that mutations affecting MIG-6L specifically and cell-autonomously decrease the rate of post-embryonic DTC migration, mimicking a post-embryonic collagen IV deficit. We also show that MIG-6S has two separable functions – one in embryogenesis and one in the second phase of DTC migration. Genetic data suggest that MIG-6S functions in the same pathway as the Mig-17/ADAMTS metalloprotease for guiding phase 2 DTC migrations, and MIG-17 is abnormally localized in mig-6 class-s mutants. Genetic data also suggest that MIG-6S and non-fibrillar network collagen IV play antagonistic roles to ensure normal phase 2 DTC guidance.

KEY WORDS: C. elegans, Cell migration, Mig-17/ADAMTS, Mig-6/papilin, Collagen IV

INTRODUCTION

Little is known about the dynamic extracellular matrix (ECM) processes that are evoked to ensure the orchestrated cell movements and proliferation required to produce organs of the proper shape, size and organization. However, during the past decade, several proteinases, including matrix metalloproteinases (MMPs) and ADAM family metalloproteinases, have been found to be necessary for normal morphogenesis and for cancer metastasis by remodeling the ECM (Chang and Werb, 2001; McFarlane, 2003).

We are using genetics to understand the molecular mechanisms that regulate a readily monitored cell migration in vivo – the migration of the two C. elegans hermaphrodite distal tip cells (DTCs) (Nishiwaki, 1999; Su et al., 2000). The sequential three-phase migration pattern of these two cells determines the final shape of each of the two U-shaped hermaphrodite gonad arms (Fig. 1A). The DTCs are born post-embryonically near one another in the ventral mid-body during the first larval stage. Their phase 1 migration comprises a longitudinal migration in opposite directions away from the mid-body using the ventral body muscle basement membrane as a substratum for migration. During phase 2, the DTCs turn and migrate across the lateral epidermal basement membrane as a substratum for migration. During phase 3, the DTCs reorient again and migrate centripetally on the dorsal body muscles – back towards the mid-body – where they normally stop (Fig. 1A).

Among the first genes known to affect DTC migration is unc-6, which encodes a secreted repulsive cue (UNC-6/netrin) for axon and DTC migrations that occur along the dorsoventral (DV) axis, as well as unc-5 and unc-40/DCC, which encode receptors that mediate the repulsive effects of UNC-6/netrin (Chan et al., 1996; Hedgecock et al., 1987; Leung-Hagesteijn et al., 1992). In mutants of these genes, the DTCs frequently fail to initiate the phase 2 migration, but if they do initiate it, this migration appears normal. This raises the question of what molecular mechanisms are required to guide the phase 2 DTC migration after it is initiated by UNC-6, UNC-5 and UNC-40 activities?

We have found two major phenotypic classes of mig-6 mutant alleles. Class-l mutations [aka mig-l(l)] hinder the rate of DTC migration during all phases of its migration, whereas class-s mutations [aka mig-6(s)] alter DTC guidance during the second (ventral to dorsal) phase of its migration. The nature of mutational lesions, RNAi, and rescue by endogenous and cell specific expression all show the mig-6 class-s and class-l mutations affect the function of the two alternatively spliced mRNAs, mig-6S and mig-6L, which encode MIG-6S and MIG-6L proteins, respectively.

We cloned mig-6 and found that it is the previously reported c-ppn gene (registered as ppn-1 in Wormbase) of C. elegans (Kramerova et al., 2000), which is highly related to genes encoding the secreted multi-component ECM proteins Drosophila papilin and Manduca sexta lacunin (Kramerova et al., 2000; Nardi et al., 1999). Previous histological studies have shown that papilin and lacunin are constituents of basement membranes and suggest that they have roles in the morphogenesis of epithelial tissues. Single papilin orthologues are found in C. elegans and Drosophila genomes. In this report, we use the name mig-6, the first published name for this gene (Hedgecock et al., 1987) and now the official gene name in Wormbase.

DTC migration defects and consequent gonad phenotypes of gon-1 and mig-17 mutants, which both encode secreted ADAMTS metalloproteinases (Blelloch and Kimble, 1999; Nishiwaki et al.,

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MATERIALS AND METHODS

Mutant strains

mig-6 class-l alleles: oz alleles were provided by Dr T. Schedl, Washington University; mig-6(e1931) was provided by the Caenorhabditis Genetics Center.

mig-6 class-s alleles: mig-6(ev701) was obtained from an EMS-induced (Brenner, 1974) screen for dominant DTC mutants. Other mig-6(s) alleles were obtained in F2 screens. A heterozygote of mig-6(ev788) was isolated by sib-selection from a formaldehyde-induced deletion library (Johnsen and Baillie, 1988). ev788 deletes nucleotides 490 in exon 4 to 1543 in exon 6 (gaattcgaacttctta...tgaagaagaggctgca). Class-s and null alleles were out-crossed four times and dpy-11(e224) mig-6 doublets were made and balanced by translocation eT1 (III, V) or nT1(qIs51) (IV, V) (Edgley et al., 2006). dpy-11 mig-6(ev788)/nT1[qIs51] segregates non-Dpy heterozygous worms with DTC defects and arrested embryos. All other strains were provided by the Caenorhabditis Genetics Center.

Genetic mapping and rescue of mutant phenotypes

mig-6 was mapped between two deficiencies, dD35 and dD20, and 30 cosmids clones (kind gifts from the Sanger Centre) in this region were tested for their ability to rescue the mig-6(s) and mig-6(l) mutants by injecting 10 μg/ml of each DNA with 50 μg/ml of sur-5::gfp co-transformation marker.

In rescue experiments designed to identify the mig-6 gene, class-s mutant DTC defects were scored as clear patches in the body – typically caused by altered DTC migration patterns, which rarely occur in wild-type animals (Hedgecock et al., 1987). In all other rescue experiments, three classes of DTC defects (see Results) were scored using Nomarski optics to examine the shape of gonad arms.

To score rescue of lethality and sterility, lines heterozygous for the transgene and for dpy-11(e224) mig-6 were established (dpy-11(e224) was used in initial rescue experiments and later found to further reduce viability). Homozygous dpy-11 mig-6(s) segregants were scored as rescued for lethality (designated ‘+’) if broods of transgenic animals were greater than 100 (broods of non-transgenic siblings were always less than 100). Homozygous dpy-11 mig-6(l) segregants are like mig-6(l) homozygotes in that they normally have no progeny (n=500 individuals); therefore, when a strain segregated dpy-11 mig-6(l) homozygotes that in the presence of the transgene produced any progeny at all (typically 20% of transgenic animals did this), the transgene was scored as rescuing (designated ‘+’).

Genetics

Animals doubly heterozygous for emb-9 and mig-6(ev701) were made by crossing dpy-11 mig-6(ev701)/nT1[qIs51](V, V) to unc-36(e251)emb-9(g23cg46)/c1 dpy-19 (e1259) III. F1 non-GFP-expressing male progeny were established (unc-36(e251) emb-9(g23cg46)/++; dpy-11 mig-6(ev701)/++) were crossed into dpy-11 mig-6(l) III. F1 Unc L4 larvae [unc-36(e251) emb-9(g23cg46)/c1; dpy-11 mig-6(ev701)/c1 [let-500(e2165)]V were cloned and the genotype was confirmed by outcrossing to N2 males and finding that F1s segregate Dpy and non-Dpy non-Unc animals with phase 2 DTC migration defects. Control strain unc-36(e251)/c1; dpy-11 mig-6(ev701)/c1 [let-500(e2165)]V was made by the same protocol. dpy-5 unc-13/I+/I-; dpy-11 mig-6(e1931)/+ I- males were crossed into smg-1(r861) I; dpy-11 mig-6(e1931)/+ V smg-1 self-progeny were homozygous and their Dpy progeny were checked for sterility and DTC defects. Ten out of 200 Dpy progeny were non-sterile and confirmed dpy-11 mig-6(e1931)/dpy-11 + recombinants.

Microscopy

Worms were observed and photographed using a Leica DMRA2 microscope equipped with Hamamatsu ORCA-ER digital camera. Lengths along the core of gonadal arms from the tip of anterior gonad were measured using OpenLab software (Improvision).

cDNA and minigene constructs

To make a full-length cDNA of mig-6, the 5’ upstream region was amplified by PCR from a wild-type cDNA pool using an SL1 primer and a reverse primer in exon 5, then spliced to the partial cDNA yk5c8 (from the
National Institute of Genetics, Japan). For mig-6L, the above fragment was then combined with partial cDNAs yk5c8 and yk257g8. A mig-6S genomic/cDNA minigene construct (pZH125) was made by splicing yk5c8 (exons 5 to 11a) and a Kpn-I-BsrGI genomic fragment spanning a 5 kb region 5′ upstream to exon 5. The mig-6L genomic/cDNA minigene (pZH117) was made by splicing yk3e8 (exons 8 to 11), yk257g8 (exon 11 to exon 18) and a Kpn-I-BsaBI genomic fragment spanning a 5 kb region 5′ upstream to exon 8. To produce heterologous promoter driven minigenes, 3.0 kb of the A210 bp genomic PCR fragment 5′ adjacent to and including the start codon was subcloned into the KpnI site of yk3e8 corresponding to the 3′ UTR of mig-6. This was used to make the 5′ regulatory region of the mig-6S genomic/cDNA minigene. cDNA fragments were PCR amplified using T7-tagged gene-specific primers and the M13-20 universal primer. Primer sequences are available on request. dsRNA preparations (0.2-0.5 mg/ml) were injected into intestine or primers and the M13-20 universal primer. Direct sequencing of PCR amplified cDNA fragments. Standard procedures were used. Direct sequencing of PCR amplified cDNA fragments. Molecular biology

Expression of MIG-17

A mig-17 promoter-driven translational fusion between mig-17 and gfp (Nishiwaki et al., 2000) and an unc-119(+)-to-unc-119(c2498) by microparticle bombardment (Praitis et al., 2001). By cloning non-Unc dauer worms, several mig-17::gfp integrated transgenic lines were established and passed into appropriate strains using standard genetic techniques.

Immunohistochemistry

For whole-mount immunostaining, worms were fixed in Bouin’s fixative (Duerr, 2006). Frozen sections of synchronized early L4 worms were prepared according to Kubota et al. (Kubota et al., 2006). Embryos were permeabilized with alkaline hypochlorite and fixed with 3% paraformaldehyde (Miller and Shakes, 1995). For blocking and antibody dilution, PBS containing 0.5% Triton X-100 and 1% bovine serum albumin (Sigma) was used.

RESULTS

Specific aspects of DTC migration are differentially affected by two distinct classes of mig-6 mutations

In class-I mig-6 mutants (e1931, oz113, oz159 and oz90) originally described by Hedgecock et al. (Hedgecock et al., 1987), DTC migration is extremely slow relative to the wild type, resulting in foreshortened gonad arms (Fig. 1B,C). The rate of DTC migration found in the mig-6(l) mutants is about one-third of the wild-type rate (14 versus 38 micrometers per hour) (Fig. 1C). DTCs in mig-6(s) mutants nevertheless initiate phase 2 migrations toward the dorsal muscles. However, the ability of the DTCs to reach the dorsal muscle with normal timing is impeded in these mutants. The distal region of the gonad does not grow in diameter and looks like a small appendage to the bulbus proximal arm (Fig. 1B). mig-6(l) mutant animals are also completely sterile. These phenotypes are fully penetrant and recessive for all four mig-6(s) alleles.

We also identified several semi-dominant, highly penetrant class-s alleles of mig-6 in which the DTCs migrate at an approximately normal rate but have specific defects in phase 2 migrations. In mig-6(s) mutants, the DTCs appear to detach from ventral muscle bands at the onset of their phase 2 migration, but their subsequent migration usually follows one of three patterns in addition to the

<table>
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<th>Phase 2D</th>
<th>Phase 2V</th>
<th>Phase 2M</th>
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<th>Phase 2V</th>
<th>Phase 2M</th>
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<td>9</td>
<td>78</td>
<td>87</td>
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</table>

*Number of animals scored.
†Putative null, ev788, homozygotes of which are lethal. eT1 is a balancer.
n.d., not determined. The total phase 2D, 2V, and 2M defects is 4%.
The two predicted transcripts share the same exon structure and open reading frame from exon 1 into exon 11, but differ in their 3’ extensions. \textit{mig-6S} includes the whole of exon 11, which encodes a stop codon and about 320 base pairs of 3’ untranslated mRNA, whereas \textit{mig-6L} includes only part of exon 11 as this exon is spliced to exon 12 using an alternative donor site.

Two transcripts of approximately the predicted sizes are detected by Northern blot analysis (Fig. 3C). RNAase protection experiments show that both transcripts are abundant in embryos and less abundant during all larval stages (see Fig. S1 in the supplementary material).

As shown in Fig. 3D, the predicted MIG-6 proteins bear a common signal sequence for secretion at their N terminus, but no predicted transmembrane domain, suggesting they are secreted. The signal peptide is followed by thrombospondin type 1 (TSP1) repeats (also known as TSRs), the first two of which are separated by a cysteine rich spacer region (CRI), then a series of cysteine-rich lagrin repeats (CRII) and six predicted Kunitz-type serine proteinase inhibitor (KU) domains. The C terminus of MIG-6L contains five additional Kunitz domains and a single predicted immunoglobulin (Ig) C2-type domain (Fig. 3D). Secreted basement membrane proteins with similar domain organization as MIG-6 are a lacunin from the moth \textit{Manduca sexta} (Nardi et al., 1999), and papilins from \textit{Drosophila} (Kramerova et al., 2000) and the nematode \textit{Haemonchus contortus} (Skuce et al., 2001). Genes encoding papilin related proteins with similar overall domain organization are also found in human and mouse genomes. These proteins differ in the number of repeats of a given type of domain, having, for example, only a single KU domain in the mammalian version.

In \textit{Drosophila}, alternative papilin splice forms are known that differ only in the number of KU and IgC2 domains they encode (Kramerova et al., 2003). Whether mammalian papilin-like genes encode multiple mRNAs and proteins is unknown. The N-terminal TSP1 repeats and the intervening cysteine rich CRI domain, together termed the ‘papilin cassette’, have highest evolutionary conservation among related proteins (38% identity and 54% similarity between MIG-6 and \textit{Drosophila} papulin, and 36% identity and 50% similarity between MIG-6 and human or mouse papulin) (Fig. 3D). Comparisons among these related molecules have been described previously (Kramerova et al., 2000; Nardi et al., 1999).

MIG-6L and MIG-6S have separate functions in DTC migration as determined by isoform-specific RNAi

To explore stage-specific requirements for the two \textit{mig-6} transcripts, we compared RNAi of specific transcripts by injection (affecting embryonic and post-embryonic expression in the subsequent generation), and by feeding starting as L1 larvae (affecting post-embryonic and post-embryonic expression in the subsequent generation) (Table 2). RNAi of \textit{mig-6L} by either method phenocopies the \textit{mig-6(s)} mutant DTC defects (Fig. 1B). This suggests that \textit{mig-6L} alone normally encodes class-I functions that are required post-embryonically for a wild-type rate of DTC migration.

Although ~60% of self-fertilization progeny of \textit{mig-6(s)} homozygotes survive to adulthood, RNAi of \textit{mig-6S} by injection causes severe embryonic and early larval lethality allowing only about 1-2% of animals to survive to adulthood. Thus, RNAi is more potent at reducing embryonic MIG-6S activity than are any of the \textit{mig-6(s)} mutations, probably because they all cause amino acid substitutions that primarily affect postembryonic DTC migrations (see below). This \textit{mig-6S} RNAi effect is also indistinguishable from the effect of injecting dsRNA that targets both transcripts (Table 2),

wild-type pattern (Table 1). For the phase 2D pattern, DTCs migrate diagonally until they reach the dorsal body muscles, which they then follow back to the mid-body region (Fig. 2A). For the phase 2V pattern, DTCs start the phase 2 ventral to dorsal migration onto the lateral epidermis normally, but quickly return to the ventral body muscles and complete phase 3 by migrating centripetally along these muscles back towards the mid-body (Fig. 2B). This causes gonad arm defects roughly reminiscent of those observed in mutants of \textit{unc-5}, \textit{unc-6} and \textit{unc-40} (Hedgecock et al., 1990). Finally, for the phase 2M category, the DTCs, after a diagonal phase 2 migration as in phase 2D defects, migrate towards mid-body but meander along the DV axis of the lateral epidermis as they do (Fig. 2C). We suggest that this phenotype results from loss of precise DTC guidance along the DV axis of the lateral epidermis during the phase 2 migration.

\textit{mig-6(s)} mutations are semi-dominant and, as heterozygotes, show the same spectrum of DTC migration patterns as homozygotes. In class-s heterozygotes, phase 2V and 2D patterns are most prevalent, whereas meandering gonads (phase 2M) are less frequent (especially anterior arms) than in homozygotes (Table 1). Thus, we consider the meandering gonad phenotype to be caused by a greater loss of \textit{mig-6} function. This is supported by \textit{mig-6S}-specific RNAi experiments (see below).

\textbf{mig-6 encodes two predicted ECM proteins with putative matrix binding and proteinase inhibitory domains}

We mapped \textit{mig-6} to a short region of LGV and rescued the DTC phenotypes with two overlapping cosmids (\textit{C32A1} and \textit{C37C3}) then with gene \textit{C37C3.6} (Fig. 3A). \textit{C37C3.6} encodes at least two mRNAs, a short (\textit{C37C3.6a}) and a long (\textit{C37C3.6b}) form, herein designated \textit{mig-6S} and \textit{mig-6L}, respectively (Fig. 3B). The predicted 3’ ends of these transcripts are available as EST cDNA clones (Y.K., National Institute of Genetics, Mishima, Japan).
suggested that the null phenotype is lethal. To further examine this possibility, we isolated a predicted null allele (Fig. 3D) by formaldehyde-induced direct deletion (Materials and methods). *mig-6(ev788)* is deleted for a genomic segment ranging from nucleotide 490 in exon 4 to 1543 in exon 6. All possible splicing of the mutant transcript is predicted to produce an early out-of-frame protein. As predicted from the RNAi experiments, all *ev788* homozygotes are embryonic or early larval lethals.

RNAi of *mig-6* by feeding, which should affect post-embryonic functions of MIG-6 in the same generation, phenocopies the phase 2D and 2V DTC defects predominant in class-s heterozygotes but at a lower penetrance and with rare meandering DTC defects (Table 2). The reduced penetrance and severity (i.e. rarity of phase 2M defects) may occur because of incomplete disruption of the *mig-6S* message by this method. In fact, predicted enhancement of RNAi efficacy by feeding in an *rrf-3* mutant background (Simmer et al., 2002) enhances the frequency of meandering DTCs (Table 2). These results suggest that *mig-6S* alone encodes class-s functions required post-embryonically for properly executing the second phase DTC migration and verify that meandering DTC defects reflect a greater loss of *mig-6S* protein.

Because ~40% of the fertilized eggs laid by homozygous class-s hermaphrodites die in embryogenesis and as L1 larvae (see Table S1 in the supplementary material), it was surprising to find that 132 of 526 progeny (25%) from class-s mig-6(ev701) heterozygotes were viable mutant homozygotes that became adults, the majority of

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**Table 2. Transcript-specific RNA interference**

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<th>RNAI method</th>
<th>Target</th>
<th>Genotype</th>
<th>Development</th>
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<th>Class-s</th>
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<td>Wild type</td>
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<td>Normal</td>
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<td>0%(A), 6%(P)*</td>
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*Phase 2V and D defects are predominant.  †Phase 2M defect is predominant.
which had meandering DTC defects. This suggests that development of these mutants is largely normal but is defective in a way that specifically affects post-embryonic DTC migrations.

**mig-6(l) mutations affect only MIG-6L KU and IgC2 function, whereas mig-6(s) mutations affect ancillary domains**

We determined molecular lesions for eight mig-6 alleles. Three class-l mutations introduce a premature stop codon predicted to delete the MIG-6L-specific KU repeats and the C-terminal IgC2 motif (Fig. 3D). Combined with the finding that RNAI silencing of mig-6L produces a class-l mutant phenotype and that a clone including only mig-6S (pZT196) is not sufficient to rescue mig-6(e1931) class-l mutants (Fig. 3B), we conclude that MIG-6L is solely responsible for the DTC migration functions that are defective in mig-6(l) mutants.

In *C. elegans* early stop codons, like those observed for the class-l alleles, may trigger nonsense-mediated decay of mRNAs. However, the *smg-1(r861)* mutation, which is known to suppress this decay (Blelloch and Kimble, 1999), did not suppress the *mig-6(e1931)* class-l DTC migration phenotype, as *smg-1(r861); dpy-11 mig-6(e1931)* was 100% sterile (n=190) and had class-l mutant DTC defects. The tested allele (*e1931*) is a nonsense mutation that eliminates the last three Kunitz domains and the IgC2 domain (Fig. 3D), suggesting that at least one of these Kunitz domains and/or IgC2 is essential for MIG-6L function.

In contrast to class-l alleles, all class-s alleles are missense mutations that affect a TSP-1 repeat or a CR1 domain in the papilin cassette (Kramerova et al., 2000), or a cysteine-rich lagrin repeat (Fig. 3). Previous studies found that the ‘papilin cassette’ and the mutations that affect a TSP-1 repeat or a CR1 domain in the papilin result from a greater than 50% (half dose) loss of ev701/+ penetrance of defects in class-s heterozygotes (e.g. phenotypes (Table 1). If *in situ* hybridization with a they encode, are responsible for the differences in their function. In principle, it is possible that the differential expression of L have different tissue specific expression profiles

In principle, it is possible that the differential expression of mig-6S and mig-6L, as opposed to different inherent abilities of the proteins they encode, are responsible for the differences in their function. In situ hybridization with a mig-6L-specific probe shows that mig-6L is restricted to the DTCs (Fig. 4A) and what are likely to be coelomocytes (not shown, but see Fig. 4E); however, a probe that detects both transcripts revealed additional expression in body wall muscles (Fig. 4B). Transcriptional reporters for mig-6 revealed expression in DTCs, body wall muscles, CAN neurons, head mesodermal cells, GLR cells and coelomocytes (Fig. 4C-E). This expression pattern is consistent with the in situ hybridization data; however, it does not distinguish whether mig-6S is normally expressed in the DTCs, as we know to be the case for mig-6L.

**Developmental change in MIG-6 localization**

MIG-6S localizes to embryonic muscles (Fig. 4F). In double immunostaining experiments with anti-myosin and anti-MIG-6 antisera, MIG-6 appears to be localized near the muscle surface (not shown). In the mig-6(ev788) putative null allele there is no expression by muscle (Fig. 4G) even though the epitope is near the N terminus and therefore little else is predicted to be encoded by this allele (Fig. 3D) (and Materials and methods).
MIG-6 in larvae also localizes to intestine, pharynx and gonad basement membranes (Fig. 4H-J). There is no obvious abnormal localization (either intracellular or extracellular) of MIG-6 proteins in mig-6(s) mutants (Fig. 4K,L); however, functionally relevant concentration differences could have been missed by this technique.

MIG-6S functions cell non-autonomously and and MIG-6L functions cell autonomously in DTCs to regulate their migration

A genomic DNA construct (pZH95), including regulatory sequence and sequence encoding both mig-6 transcripts rescued the lethality and DTC migration defects of the null allele to near wild-type levels (see Table S2 in the supplementary material). A genomic/cDNA minigene construct (pZH125) encoding MIG-6S could not rescue class-l mutant sterility (not shown) or DTC defects (Table 3), but could partially rescue viability and DTC defects of mig-6(s) homozygotes (slightly) and mig-6(s) heterozygotes (substantially) (Table 3). A genomic/cDNA minigene encoding MIG-6L (pZH117) partially rescued the DTC defects of the class-l homozygotes, but not homozygous class-s DTCs or lethality (Table 3); however, substantial rescue of class-s heterozygote DTC defects by mig-6(L) did occur (Table 3). These data indicate that when expressed under the control of nearly identical endogenous regulatory sequences, MIG-6S cannot substitute for MIG-6L but MIG-6L can partially substitute for MIG-6S in guiding phase 2 DTC migrations.

To explore the significance of mig-6 expression in DTCs and in muscles, we engineered mig-6S or mig-6L minigenes under the control of either of two heterologous 5′ regulatory regions. The lag-2 promoter (lag-2p) drives expression primarily in the hermaphrodite DTCs (Blaloch and Kimble, 1999; Nishiwaki et al., 2000), whereas the myo-3 promoter (myo-3p) drives expression in all of the body-wall muscles (Okkema et al., 1993). For each transgene, rescuing activity was examined in class-s and class-l heterozygous for mig-6(s), suggesting that MIG-6S does not significantly affect the overall level of MIG-17, but does prevent over-accumulation in variable regions of the gonad arms. Whether this is a direct or an indirect effect of mutant MIG-6S proteins on MIG-17 is unknown.

Intergenic non-complementation between mig-6 class-s and mig-17 mutations suggests they act in the same pathway to promote normal phase 2 DTC migrations

Mutants of mig-17 display DTC defects that mimic DTC defects of mig-6(s) homozygotes, but at lower penetrance. Although the mig-17 mutant defects are recessive, animals that are doubly heterozygous for mig-6 class-s alleles ev701 or k177, and the mig-17 putative null allele, k174, show higher penetrance (Fig. 5A) and expressivity (higher frequency of type 2M DTC migrations) of defects than respective mig-6(s) heterozygotes. This intergenic non-complementation suggests that MIG-6 functions in the same pathway (but see Yook et al., 2001) as MIG-17, a known ADAMTS metalloproteinase that is required (like MIG-6S) to guide phase 2 DTC migrations.

Table 3. Rescue of mig-6 mutant classes by heterologous promoter driven mig-6 minigenes

<table>
<thead>
<tr>
<th>Transgene</th>
<th>% Rescue of DTC migration defects*</th>
<th>Rescue of lethality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>class-l (e1931)</td>
<td>class-s/+ (ev701/+)</td>
</tr>
<tr>
<td>mig-6p::mig-6S (pZH125)</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>mig-6p::mig-6L (pZH117)</td>
<td>67</td>
<td>80</td>
</tr>
<tr>
<td>lag-2p::mig-6S (pZH116)</td>
<td>0</td>
<td>59</td>
</tr>
<tr>
<td>lag-2p::mig-6L (pZH118)</td>
<td>45</td>
<td>51</td>
</tr>
<tr>
<td>myo-3p::mig-6S (pZH135)</td>
<td>0</td>
<td>58</td>
</tr>
<tr>
<td>myo-3p::mig-6L (pZH145)</td>
<td>0</td>
<td>42</td>
</tr>
</tbody>
</table>

*The percent rescue was calculated by (Pc–Pt)/Pc, where Pc and Pt means the percentages of worms that show the defects in the mutants without the transgene and with the transgene, respectively. For each experiment, at least 100 animals were scored. DTC defects (as in Figs 1 and 2) and lethality were scored as described in the Materials and methods.

The mig-6 class-l gonad phenotype is mimicked by mutations in the emb-9 collagen IV gene

Previous anecdotal reports of the effects of collagen mutations on DTC migration (Cassada et al., 1981) prompted us to examine collagens as possible targets of MIG-6 activity. Collagen type IV is one of the major components of specific basement membranes in C. elegans and other animals. Two EMB-9/collagen IV alpha 1 chains and one LET-2/collagen IV alpha 2 chain normally form a single heterotrimeric collagen. Glycine substitution mutants in Gly-X-Y repeats of the EMB-9 or LET-2 exhibit temperature-sensitive embryonic lethality, caused by reduced secretion and assembly of collagen type IV into basement membranes (Gupta et al., 1997). We
exposed the emb-9(g34ts) mutants to the non-permissive temperature for 8 hours of L2 to L3 larval development at the time when the DTCs initiate their phase 1 migration. emb-9(g34ts) hermaphrodite animals so treated have a normal morphology, but their gonads phenocopy the mig-6(l) mutant gonad morphology, suggesting a possible functional/regulatory connection between MIG-6L and EMB-9 (Fig. 6A,B).

**mig-6 class-s mutations are partially rescued by a reduction in functional collagen IV**

Given the above data, we attempted to examine the relationship of MIG-6S with collagen IV function. Suppression of the phase 2 DTC defect was partial when RNAi was used to reduce EMB-9/collagen IV in mig-6(ev701) heterozygotes, but effectively complete when the wild-type dose of emb-9 was halved in heterozygotes for the putative null (Gupta et al., 1997) allele emb-9(g23cg46) (Fig. 6C). A lesser but substantial suppression was also caused by let-2 temperature-sensitive mutations (Fig. 6C). Thus, a reduction in collagen IV protein (by RNAi or a heterozygous null) or a reduction in collagen IV folding and secretion (caused by point mutation) can suppress mig-6 class-s DTC defects. These results indicate that collagen IV acts antagonistically to MIG-6S in an extracellular mechanism that affects phase 2 DTC migration. We did not observe any difference in EMB-9 distribution between wild type and mig-6 or mig-17 mutants using anti-EMB-9 antibodies for immunostaining; however, functionally relevant concentration differences could have been missed by this technique.

**DISCUSSION**

**MIG-6/papilin is essential for embryogenesis, DTC migration and gonadogenesis**

Papilin is a basement membrane protein that is essential for embryonic development, as shown by RNAi knockdown experiments in *Drosophila* and *C. elegans* (Kramerova et al., 2000). In the present study, we identified two different classes of MIG-6/papilin proteins and analyzed their functions by class-specific mutations and RNAi. Our results indicate that both of the MIG-6/papilin isoforms, MIG-6S and MIG-6L, function specifically to regulate DTC migration and MIG-6S also affects embryogenesis. There are several reasons to believe that the DTC and embryonic functions of the MIG-6 proteins are separate. First, the class-specific RNAi by feeding, which affects MIG-6 post-embryonic function in the same generation, can produce either class-s or class-l mutant DTC phenotypes. Second, all DTC phenotypes can be rescued to some degree by post-embryonic expression of one or the other of the two isoforms. Finally, the DTC function of MIG-6S proteins is genetically separable from the observed function of MIG-6S in embryogenesis as class-s mutant heterozygotes have normal sized broods with one-quarter mutant homozygous progeny that manifest DTC defects. Taken together, these results indicate that mutations that affect either MIG-6 isoform can cause specific defects in post-embryonically derived basement membrane structures required for DTC migration without affecting basement membrane functions required for embryogenesis.

**MIG-6L/papilin may act with collagen IV to promote a normal rate of DTC migration**

We have found that the two isoforms of MIG-6/papilin are made by different cell types in *C. elegans* and normally have distinctly different functions in DTC migration. Class-l mutations, which eliminate the three C-terminal KT and the single IgC2 C-terminal domains (predicted to only affect MIG-6L), appear to specifically reduce the rate of DTC migration to about one-third that of the wild type. Other more subtle gonad defects are likely in class-l mutants as they are sterile and their distal gonad arms have a greatly reduced cross-sectional diameter compared with the proximal arm.

There are several reasons to believe that mig-6(l) mutations primarily affect the gonad basement membrane. Most importantly, MIG-6L is expressed by DTCs as they migrate and can act cell autonomously in larvae to promote DTC migration even though it is predicted to be a secreted protein. It is possible that the MIG-6L
Fig. 6. Effect of collagen IV mutations. (A, B) Temperature-sensitive collagen IV mutant emb-9(g23) cultured at non-permissive temperature from L2 to L4 larval stages showed slow gonad elongation (B), which is similar to class-

specific C-terminal region may include a retention signal or a motif (e.g. one or more KU domains and/or the IgC2 domain) that help localize it to the gonad basement membrane.

Because emb-9/collagen IV alpha 1 have been suggested to affect DTC migration (Cassada et al., 1981), we further characterized the DTC migration defects caused by inactivating emb-9 during DTC migration. The phenotypic similarity of post-embryonically induced DTC defects in emb-9 and in mig-6(s) mutants is striking. As both MIG-6L and EMB-9 are produced by the DTC, it is tempting to speculate that MIG-6L promotes post-embryonic collagen IV function, and that it could do so by downregulating the activity of proteinases that degrade collagen IV as is known for ADAMTS mutants ([emb-9;collagen IV alpha 2 mutations, clearly suppresses mig-6(s) mutant DTC defects. Thus, genetic reduction of mutant or wild-type forms of collagen IV are both able to suppress mig-6(s) defects, suggesting that losing mutant forms of collagen IV [which are abnormally retained in the endoplasmic reticulum (Gupta et al., 1997) and might have caused co-retention of MIG-6 mutant protein] does not contribute to the suppression. The suppression observed is therefore reminiscent of the ability of fbl-1 (fibulin) mutations to suppress gon-1/adamts proteinase and mig-17 mutant DTC defects (Hesselson et al., 2004; Kubota et al., 2004) and suggests that secreted forms of collagen IV and MIG-6S act antagonistically, either in parallel mechanistic pathways (like fibulin and GON-1) or in the same pathway.

The genetic and phenotypic characterization of mig-6, mig-17 and collagen IV mutants reported here shows that MIG-6S and MIG-17 act antagonistically with secreted collagen IV to guide phase 2 DTC migrations and suggests that MIG-6L and collagen IV act together to promote a normal rate of DTC migration. We speculate that the abnormal distribution of MIG-17 in mig-6(s) mutants causes an overabundance of normally or abnormally localized collagen IV, which hinders phase 2 DTC guidance. This model predicts that reducing the collagen IV level or altering its

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supramolecular structure could also rescue the DTC migration defects of mig-17 mutants. In fact, one of the suppressors of mig-17 is a let-2 allele that has a mutation in the non-collagenous (NC) domain of the collagen IV alpha 2 chain (Ohkura and Nishiwaki, personal communication). NC domains are involved in forming a supramolecular network among triple helical protomers and this allele may have lost the ability to form this network. According to this model, EMB-9/collagen IV could be a target for this suppressor, and the former pair would negatively regulate it or antagonize it in some other way. We therefore speculate further that collagen IV levels in gonad basement membrane may need to be regulated oppositely in phase 1 and phase 2 DTC migration, and that this is accomplished by the two isoforms of mig-6.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/9/1442/DC1

References


