RESEARCH ARTICLE

MIG-10 (lamellipodin) has netrin-independent functions and is a FOS-1A transcriptional target during anchor cell invasion in C. elegans

Zheng Wang, Qiuyi Chi and David R. Sherwood*

ABSTRACT

To transmigrate basement membrane, cells must coordinate distinct signaling activities to breach and pass through this dense extracellular matrix barrier. Netrin expression and activity are strongly associated with invasion in developmental and pathological processes, but how netrin signaling is coordinated with other pathways during invasion is poorly understood. Using the model of anchor cell (AC) invasion in C. elegans, we have previously shown that the integrin receptor heterodimer INA-1/PAT-3 promotes netrin receptor UNC-40 (DCC) localization to the invasive cell membrane of the AC. UNC-6 (netrin)/UNC-40 interactions generate an invasive protrusion that crosses the basement membrane. To understand how UNC-40 signals during invasion, we have used genetic, site of action and live-cell imaging studies to examine the roles of known effectors of UNC-40 signaling in axon outgrowth during AC invasion. UNC-34 (Ena/VASP), the Rac GTPases MIG-2 and CED-10 and the actin binding protein UNC-115 (abLIM) are dedicated UNC-40 effectors that are recruited to the invasive membrane by UNC-40 and generate F-actin. MIG-10 (lamellipodin), an effector of UNC-40 in neurons, however, has independent functions from UNC-6/UNC-40. Furthermore, unlike other UNC-40 effectors, its expression is regulated by FOS-1A, a transcriptional target during anchor cell invasion in C. elegans. UNC-6/UNC-40 interactions are also dependent on the integrin INA-1/PAT-3. These studies indicate that MIG-10 has distinct functions from UNC-40 signaling in cell invasion, and demonstrate that integrin coordinates invasion by localizing these molecules to the cell-basement membrane interface.

KEY WORDS: MIG-10, Netrin, Integrin, Cell invasion

INTRODUCTION

During development cells must navigate through complex cellular and extracellular matrix environments to disperse, form connections and generate tissues. One of the key barriers that cells encounter is basement membrane, a thin, dense, highly conserved sheet-like matrix that underlies all epithelia and surrounds most tissues (Hynes, 2012; Kalluri, 2003). To overcome this barrier, invasive cells generate and polarize a specialized cell membrane to breach basement membrane (Guo and Giancotti, 2004; Machesky et al., 2008; Ziel et al., 2009). Metastatic cancer cells are thought to utilize the same mechanisms to enable their spread (Rowe and Weiss, 2008). An understanding of how cells traverse basement membranes is limited, however, because of the challenge of recapitulating this behavior faithfully in vitro and the difficulty of studying cell-basement membrane interactions in native tissue environments (Even-Ram and Yamada, 2005; Hagedorn and Sherwood, 2011; Hotary et al., 2006; Nourshargh et al., 2010; Wang et al., 2006).

The C. elegans gonadal anchor cell (AC) is a uniquely differentiated cell that invades the juxtaposed gonadal and ventral epidermal basement membranes to initiate uterine-vulval connection during larval development (Ihara et al., 2011; Sharma-Kishore et al., 1999; Sherwood and Sternberg, 2003). The highly stereotyped manner of AC invasion and amenability to genetic and visual analysis have recently been utilized to facilitate experimental examination of cell-basement membrane interactions underlying invasion (Hagedorn and Sherwood, 2011). Polarization of the AC towards the basement membrane is regulated by the integrin receptor INA-1/PAT-3, a heterodimer composed of the α-subunit INA-1 paired with the β-subunit PAT-3, which is thought to bind to the basement membrane protein laminin (Baum and Garriga, 1997; Hagedorn et al., 2009). Integrin activity regulates the targeting of the netrin receptor UNC-40 (DCC) to the invasive cell membrane. UNC-40 protein orientation is refined by UNC-6, which is secreted from the ventral nerve cord and accumulates in the basement membrane under the AC (Ziel et al., 2009). UNC-6 (netrin) activation of UNC-40 is further required to generate an invasive protrusion that crosses the basement membrane and intercalates into the neighboring vulval tissue (Hagedorn et al., 2013). Netrin and integrin are strongly associated with invasive cellular activity in development and diseases such as metastastic cancer (Dessersollier and Cheres, 2010; Dumartin et al., 2010; Guo and Giancotti, 2004; Kaufmann et al., 2009; Lambert et al., 2012; Nguyen and Cai, 2006), suggesting that these pathways are conserved regulators of cell invasion through basement membrane.

Although both the unc-6 and unc-40 genes are essential for the formation of a large invasive protrusion, netrin signaling is not required to breach basement membrane. Loss of unc-6 and unc-40 slightly delays, but does not inhibit the ability of the AC to create gaps in the basement membrane (Hagedorn et al., 2013; Ziel et al., 2009). Breaching the basement membrane is dependent on the C. elegans ortholog of the vertebrate Fos family transcription factor, FOS-1A, which regulates the expression of genes that mediate basement membrane removal (Shekarabi et al., 2005). In fos-1a mutants, the AC generates a protrusion that flattens at an intact basement membrane (Sherwood et al., 2005). The effectors acting downstream of FOS-1A and UNC-6/UNC-40 signaling as well as the mechanisms that coordinate their activity at the invasive cell membrane are poorly defined.

An understanding of netrin signaling has primarily been derived from studies in neuronal cells (Gitai et al., 2003; Lebrand et al., 2004; Li et al., 2002a; Quinn et al., 2008; Shekarabi et al., 2005). Thus, to

Received 9 August 2013; Accepted 9 January 2014
further elucidate how netrin signaling promotes AC invasion, we initiated genetic interaction and quantitative imaging studies in the AC on known neuronal effectors of UNC-6/UNC-40. We found that most known neuronal effectors are localized to the invasive cell membrane by UNC-40 and act downstream of UNC-40 signaling during AC invasion. Notably, MIG-10 (lamellipodin), an UNC-6/UNC-40 effector during axon outgrowth and synapse formation (Adler et al., 2006; Chang et al., 2006; Quinn et al., 2006; Stavoe and Colón-Ramos, 2012), was localized and functioned independently of UNC-40. In addition, mig-10 is transcriptionally regulated by FOS-1A, implicating MIG-10 activity in basement membrane removal. Like UNC-40 (DCC), MIG-10 was also dependent on integrin for localization to the invasive membrane. Together, these results suggest that MIG-10 (lamellipodin) and UNC-6/UNC-40 (netrin signaling) have distinct functions in basement membrane breaching and invasive protrusion formation, respectively, and that integrin targets their localization to the invasive cell membrane.

**RESULTS**

**Effectors of UNC-40 (DCC) in axon guidance promote AC invasion**

We have previously shown that UNC-6 (netrin) secreted from ventral nerve cord (VNC) orients UNC-40 (DCC) to the AC-basement membrane interface prior to invasion (Ziel et al., 2009). Furthermore, UNC-6 activation of UNC-40 generates a protrusion that crosses the basement membrane (Hagedorn et al., 2013) (Fig. 1A). We have also observed that UNC-34, the C. elegans ortholog of vertebrate Ena/VASP, and two Rac GTPases, MIG-2 and CED-10, are polarized by UNC-6 to this same region. Loss of unc-34 and the combined loss of mig-2 and ced-10 lead to defects in invasion (Ziel et al., 2009). Ena/VASP proteins and Rac GTPases are known downstream effectors of UNC-6/UNC-40 signaling in axon pathfinding and outgrowth (Chang et al., 2006; Gitai et al., 2003; Lebrand et al., 2004; Li et al., 2002b; Shekarabi and Kennedy, 2002), suggesting that netrin signaling might use similar effectors during AC invasion.
To determine whether UNC-40 engages the same downstream effectors to promote invasion as it does to guide axons, we examined AC invasion in strains with mutations in unc-34, ced-10, the human ABLIM/limain ortholog unc-115, and the lamellipodin ortholog mig-10 (Lundquist et al., 1998; Manser et al., 1997; Reddien and Horvitz, 2000; Withee et al., 2004; Yu et al., 2002; Zipkin et al., 1997). These genes are established downstream mediators of UNC-40 signaling during axon outgrowth and pathfinding (Chang et al., 2006; Gitai et al., 2003; Quinn et al., 2008). Another Rac GTPase, MIG-2, which often acts redundantly with CED-10 in neuronal development (Demarco et al., 2012; Shakir et al., 2008), was also included for analysis. All alleles examined were putative null mutations except for ced-10(n1993), which is a partial loss-of-function mutation (Reddien and Horvitz, 2000). We scored AC invasion in all assays at the P6.p four-cell stage when basement membrane invasion is completed in wild-type animals (Fig. 1B), and later at the P6.p eight-cell stage (Sherwood and Sternberg, 2003). Of these candidate effectors, we confirmed that unc-34, and the two Rac GTPase genes ced-10 and mig-2 that act redundantly, promote AC invasion (Fig. 1C; Table 1) (Ziel et al., 2009). We found that loss of a third Rac GTPase, rac-2, did not perturb invasion or enhance mig-2 or ced-10 defects (data not shown). Animals lacking unc-115(ky275) showed a partial block in AC invasion in 8% of ACs examined (Fig. 1D; Table 1), whereas loss of mig-10(ok2499) caused a partial invasion in 6% of ACs observed (Table 1). These results indicate that the effectors of UNC-6/UNC-40 in axon development also promote AC invasion.

**Ena/VASP (unc-34), Rac GTPases (ced-10 and mig-2) and UNC-115 act within the UNC-6/UNC-40 pathway during invasion**

To test whether these candidate effectors act within the netrin pathway to regulate invasion, we made double mutant combinations of unc-40 with each of these genes (Table 1). If these potential effectors function linearly with UNC-40, the double mutants would be predicted to display phenotypic defects similar to those in the unc-40 single mutants (Wang and Sherwood, 2011). An enhancement of the unc-40 invasion defect, however, would indicate a function outside of UNC-40 signaling. Loss of unc-34, ced-10, mig-2 and unc-115 did not significantly enhance AC invasion defects caused by unc-40, suggesting that these genes function within the UNC-6/UNC-40 (netrin) pathway during AC invasion (Table 1). By contrast, loss of mig-10 (lamellipodin) strongly enhanced both unc-40 and unc-6 defects (Table 1), indicating that mig-10 has functions outside of UNC-40 signaling.

**Effectors of UNC-6/UNC-40 are expressed and function within the AC**

We have previously shown that mig-2 and ced-10 are expressed and function in the AC to promote invasion (Ziel et al., 2009). To determine where unc-34 and unc-115 function, we examined their expression and site of action. Examination of transgenic animals expressing the 5′ cis-regulatory elements of unc-34 and unc-115 fused to GFP revealed expression in the AC throughout the invasion process (Fig. 1E,F). Supporting the notion that UNC-34 and UNC-115 function in the AC, AC-specific expression of full-length GFP–tagged UNC-34 and UNC-115 (cdh-3>GFP::unc-34 and cdh-3>GFP::unc-115) rescued invasion defects caused by their corresponding mutations (Table 1; Fig. 1G,H). We conclude that effectors of UNC-6/UNC-40 signaling function within the AC during invasion.

**Effectors of UNC-6/UNC-40 act downstream of the UNC-40 receptor in the AC**

Overexpression of UNC-40 in muscles induces randomly directed myopodial extensions in C. elegans, suggesting that increased levels of UNC-40 are active, but override polarity cues from UNC-6. Loss of effectors of UNC-40 in muscle arm extension suppresses this phenotype, confirming their function downstream of UNC-40 (Alexander et al., 2009). We similarly found that UNC-40 overexpressed in the AC induced randomly directed protrusions. To determine whether the effectors we identified act downstream of the UNC-40 receptor, we thus determined if their loss suppressed ectopic protrusion formation. As unc-115 appears to act downstream of ced-10 in neurons (and in the AC, see below), we examined ced-10 as a proxy for unc-115. Individual loss of unc-34, mig-2 and ced-10 dramatically suppressed the length of the ectopic protrusion (Fig. 2A-G), indicating that these effectors can act downstream of UNC-40 in the AC. Further supporting a downstream function, we found that UNC-40::GFP was polarized normally in unc-34 mutants and in mig-2(mu28) animals treated with ced-10 RNAi (Fig. 2H-K).

**Effectors of UNC-6/UNC-40 function within two branches to regulate AC invasion**

Genetic and molecular studies in C. elegans have indicated that two distinct pathways act downstream of UNC-40 signaling during axon outgrowth and turning (Gitai et al., 2003). One pathway is mediated by UNC-34, and the other is composed of CED-10 and UNC-115. Consistent with a similar organization in the AC, the unc-34 mutant invasion defects were enhanced by loss of unc-115 as well as reduction of ced-10 by RNAi (Table 1). Furthermore, loss of mig-2 also enhanced unc-34 mutants (Table 1). These results suggest UNC-34 also functions in a distinct branch downstream of UNC-40 signaling in the AC (summarized in Fig. 1I).

We then examined genetic interactions between mig-2, ced-10 and unc-115. Loss of mig-2 significantly enhanced the unc-115 mutant phenotype, indicating parallel activities. By contrast, ced-10(n1993) did not enhance loss of unc-115, suggesting that ced-10 and unc-115 function in a linear pathway. These results are consistent with studies on axon pathfinding and outgrowth, suggesting that the actin binding protein UNC-115 acts downstream of CED-10 and in parallel to MIG-2 (Fig. 1I) (Demarco and Lundquist, 2010; Gitai et al., 2003; Struckhoff and Lundquist, 2003).

**Effectors of UNC-40 promote F-actin formation at the invasive cell membrane of the AC**

Ena/VASP proteins, Rac GTPases and abLIM/limain are known regulators of actin cytoskeletal signaling (Bear and Gertler, 2009; Burridge and Wennerberg, 2004; Struckhoff and Lundquist, 2003). This suggests that UNC-34 (Ena/VASP), the Rac GTPases MIG-2 and CED-10, and UNC-115 (abLIM) may help organize the F-actin network downstream of UNC-40 signaling during invasion. We thus examined the localization and volume of the integrated fluorescence intensity of the F-actin probe mCherry::moeABD in animals harboring mutations in these genes. Compared with wild-type ACs where F-actin was tightly polarized to the invasive cell membrane, we found that 22% of the total amount of F-actin was mislocalized to the apical and lateral membranes of ACs in unc-34 mutants (Fig. 3A,B,E). In addition, the overall volume of F-actin in unc-34 mutants was reduced by ~50% (Fig. 3F). Reduction of the activity in the Rac GTPase branch of UNC-40 signaling [mig-2(mu28);ced-10(RNAi)] did not alter the polarity of F-actin, but led to a 65% reduction of F-actin volume (Fig. 3A,C,E,F). Notably, loss of mig-10, which has functions outside UNC-40 signaling in the AC, did...
UNC-40 polarizes its effectors to the invasive cell membrane

UNC-6 (netrin) orients UNC-40, F-actin and the actin regulators UNC-34, CED-10 and MIG-2 to the invasive cell membrane of the

not affect F-actin polarity or volume (Fig. 3D-F). Taken together, these results indicate that dedicated effectors of UNC-40 signaling function to promote F-actin formation, with UNC-34 also having a role in regulating F-actin polarity.

Table 1. Genetic analysis of the netrin pathway, mig-10, integrin and the FOS-1 pathway during AC invasion

<table>
<thead>
<tr>
<th>Genotype/treatment</th>
<th>AC invasion*</th>
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<tbody>
<tr>
<td></td>
<td>P6.p 4-cell stage (mid-to-late L3 stage)</td>
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<tr>
<td></td>
<td>Full invasion (%)</td>
</tr>
<tr>
<td>UNC-6/UNC-40 pathway effectors</td>
<td></td>
</tr>
<tr>
<td>Wild type (N2)</td>
<td>100</td>
</tr>
<tr>
<td>unc-40(e271)</td>
<td>5</td>
</tr>
<tr>
<td>unc-6(ev400)</td>
<td>14</td>
</tr>
<tr>
<td>unc-34(gm104)</td>
<td>44</td>
</tr>
<tr>
<td>unc-34(e951)</td>
<td>33</td>
</tr>
<tr>
<td>mig-2(mu28)</td>
<td>100</td>
</tr>
<tr>
<td>ced-10(n1993)</td>
<td>98</td>
</tr>
<tr>
<td>mig-2(mu28);ced-10(RNAi)</td>
<td>62</td>
</tr>
<tr>
<td>unc-115(ky275)</td>
<td>92</td>
</tr>
<tr>
<td>mig-10(ok2499)</td>
<td>94</td>
</tr>
<tr>
<td>mig-10(ct41)</td>
<td>98</td>
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<tr>
<td>UNC-40 polarizes its effectors to the invasive cell membrane</td>
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<tr>
<td>UNC-6(unc-40) pathway effectors</td>
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<tr>
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<td>100</td>
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<tr>
<td>mig-10(ct41)</td>
<td>98</td>
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*Normal invasion, partial invasion and no invasion were defined by the degree to which the BM underneath of the AC was interrupted (see Fig. 1). BM integrity was examined using DIC optics as previously shown (Sherwood and Sternberg, 2003).

Statistical comparison of scoring results (Fisher’s exact test; full invasion versus defective invasion, unless noted otherwise):

- Compared with unc-40(e271), P>0.1 at the P6.p four-cell stage;
- Compared with unc-40(e271), P<0.01 at the four-cell stage (partial invasion versus no invasion);
- Compared with unc-40(e271), P<0.01 at the four-cell stage (full invasion versus no invasion);
- Compared with unc-34(gm104), P<0.01 at the four-cell stage (partial invasion versus no invasion);
- Compared with unc-34(gm104), P<0.01 at the four-cell stage (full invasion versus no invasion); compared with unc-40(e271), P<0.01 at the four- and eight-cell stages;
- Compared with unc-34(gm104), P<0.01 at the four-cell stage; compared with corresponding double mutants, P>0.07 (partial invasion versus no invasion); P<0.01 at the eight-cell stage, indicating a partial rescue;
- Compared with unc-34(gm104), P<0.01 at the four- and eight-cell stages, indicating a rescue; compared with corresponding double mutants, P>0.07 (partial invasion versus no invasion); P<0.01 at the eight-cell stage, indicating a partial rescue;
- Compared with unc-34(gm104), P<0.01 at the four-cell stage; compared with corresponding double mutants, P>0.07 (partial invasion versus no invasion); P<0.01 at the eight-cell stage, indicating a partial rescue;
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- Compared with unc-40(e271), P<0.01 at the four-cell stage; compared with corresponding double mutants, P>0.07 (partial invasion versus no invasion); P<0.01 at the eight-cell stage, indicating a partial rescue;
AC prior to invasion (Ziel et al., 2009). To provide a more complete understanding of the localization of UNC-40 effectors, we first determined the subcellular localization of UNC-115 in the AC. Similar to UNC-34, CED-10 and MIG-2, AC-specific expression of GFP-tagged UNC-115 showed that UNC-115 was first localized to the basal invasive membrane, approximately 5 hours prior to invasion and its polarity increased throughout AC invasion (Fig. 4A,I). We next determined whether UNC-40 functions to localize its effectors at the invasive cell membrane. Consistent with their genetic placement downstream of unc-40, UNC-115 and UNC-34 showed an approximate 50% reduction in polarity in unc-40 mutants (Fig. 4A,B,E,F,I). The Rac GTPases CED-10 and MIG-2 had an approximate 70% reduction in polarity (Fig. 4C,D,G-I). We conclude that UNC-40 promotes the localization of its dedicated effectors to the invasive cell membrane of the AC.

**mig-10b, but not dedicated UNC-40 effectors, are transcriptionally regulated by FOS-1A**

Loss of mig-10 strongly enhanced the invasion defects in unc-40 and unc-6 mutants (Table 1), suggesting that MIG-10 has functions outside UNC-6/UNC-40 signaling that contribute to invasion. We were next interested in understanding how MIG-10 regulates AC invasion. The mig-10 gene encodes three protein isoforms, MIG-10A-C (Manser et al., 1997; Quinn et al., 2006; Stavoe et al., 2012). Using reporters consisting of upstream 5’ cis-regulatory elements (CRE) to drive GFP (3.5 kb, 2.9 kb and 4 kb immediately upstream of ATG start codons of mig-10a, mig-10b and mig-10c, respectively), we found that the 5’ CRE of mig-10a drove expression in uterine cells surrounding the AC, but was not detectable in the AC (Fig. 5A). mig-10c was expressed in ventral uterine cells and neurons of the ventral nerve cord, and was also absent from the AC (Fig. 5B). Notably, the 5’ CRE for mig-10b was specifically
expressed in the AC throughout invasion (Fig. 5C), suggesting that the mig-10b isoform regulates AC invasion. Consistent with this idea, AC-specific expression of MIG-10B had rescuing activity in the unc-6; mig-10 double mutant (we utilized mig-10 enhancement of the unc-6 mutant phenotype as a more sensitive basis to determine rescue; Table 1; Fig. 5I). Thus, MIG-10B functions in the AC to promote invasion.

Outside of UNC-6/UNC-40 signaling, the transcriptional regulator fos-1p plays a distinct role in AC invasion. FOS-1A regulates the expression of genes that promote breaching of the basement membrane, but it does not regulate protrusion formation (Sherwood et al., 2005). To test if mig-10b functions in the FOS-1A pathway, we examined the expression of mig-10b in animals treated with fos-1 RNAi. Strikingly, loss of fos-1 led to a complete absence of detectable mig-10b expression in the AC (n=19/19 animals; Fig. 5D). FOS-1A is thought to regulate diverse targets that function together to promote basement membrane removal (Sherwood et al., 2005). Loss of these genes leads to additive invasion defects. Consistent with MIG-10B acting as a functional target of FOS-1A, loss of mig-10 enhanced the invasion defect of the matrix component hemicentin (him-4), a FOS-1A transcriptional target that promotes basement membrane removal (Table 1) (Sherwood et al., 2005).

We next determined whether the expression of the dedicated netrin pathway effectors are controlled by FOS-1A. The expression of the Rac GTPase mig-2 in the AC is not regulated by FOS-1A (Sherwood et al., 2005). Similarly, we found that ced-10, unc-115 and unc-34 were still expressed in the AC after loss of fos-1 (n≥10 animals examined for each; supplementary material Fig. S1). These results are consistent with our genetic studies, indicating that these effectors function specifically within the netrin pathway to promote invasion. Taken together, these data suggest that mig-10b is a component of the FOS-1A transcriptional pathway, which promotes basement membrane breaching.

**MIG-10B localization to the invasive membrane is independent of UNC-40, but requires integrin**

We next examined a functional translational fusion of GFP to MIG-10B to determine where it is localized in the AC. Consistent with a role in promoting basement membrane breach, MIG-10B was strongly polarized to the invasive cell membrane prior to invasion (Fig. 5E,J). Unlike dedicated unc-6 and UNC-40 effectors, however, MIG-10B polarization was not dependent on UNC-40 (Fig. 5F,J; supplementary material Fig. S2). In both the HSN and AIY neurons, MIG-10 localization is regulated through netrin signaling by the Rac GTPase CED-10 (Quinn et al., 2008; Stavoe and Colón-Ramos, 2012). Consistent with netrin-independent localization, MIG-10B was polarized normally at the invasive cell membrane of the AC in ced-10 (n=993) mutant animals (supplementary material Fig. S3). These results indicate that another polarity pathway plays a primary role in directing the polarized localization of MIG-10B in the AC.

The only other known polarity system regulating invasive membrane polarization during AC invasion is the integrin heterodimer INA-1/PAT-3 (Hagedorn et al., 2009). INA-1/PAT-3 functions upstream of UNC-40 (DCC) and is required for UNC-40 targeting to the invasive cell membrane (Hagedorn et al., 2009). We thus examined whether MIG-10B localization is dependent on INA-1/PAT-3 activity. Null mutations in ina-1 cause L1 larval lethality (Baum and Garriga, 1997). We therefore examined animals containing a hypomorphic, viable mutation in ina-1: ina-1(gm39) (Baum and Garriga, 1997). ina-1(gm39) mutants had a 50% reduction in MIG-10B polarity (Fig. 5G,J), demonstrating that INA-1 mediates MIG-10B localization to the invasive membrane. To determine if INA-1/PAT-3 functioned cell autonomously to promote MIG-10B localization, we examined MIG-10B in animals expressing a previously characterized dominant-negative integrin specifically driven in the AC (zmp-1>HA-ftail) (Hagedorn et al., 2009).
which has a UNC-40/UNC-6 independent function as a FOS-1A target promoting basement membrane removal. Further supporting the idea that integrin localizes signaling molecules at the invasive membrane, INIA-1/PAT-3 is also required for the localization of the UNC-40 effector MIG-2 (Hagedorn et al., 2009) and the UNC-40 effectors UNC-34 and UNC-115 (supplementary material Fig. S5). These studies support a model in which INIA-1/PAT-3 targets the localization of multiple signaling molecules to the invasive membrane where they function during invasion.

Loss of UNC-6/UNC-40 (netrin) signaling does not control INIA-1/PAT-3 localization at the invasive membrane, consistent with a role for UNC-40 downstream of integrin activity (Hagedorn et al., 2009). We thus next examined whether FOS-1A activity regulates INIA-1/PAT-3 localization. Similar to Drosophila and vertebrates, the C. elegans α-INIA-1 and β-PAT-3 subunits require association within the secretory apparatus to be transported to the cell surface (Hagedorn et al., 2009; Leptin et al., 1989; Marlin et al., 1986). Therefore, we examined the expression of an INIA-1/PAT-3 reporter from worms expressing genomic DNA encoding ina-1 and genomic DNA encoding pat-3 tagged with GFP. INIA-1/PAT-3::GFP was transported to the surface of ACs and polarized to the invasive cell membrane (Fig. 6A,C). Loss of fos-1 did not reduce or alter the polarized localization of INIA-1/PAT-3::GFP (Fig. 6B,C). These results indicate that FOS-1A activity does not regulate INIA-1/PAT-3 expression or localization at the invasive membrane.

To further test the idea that integrin regulates multiple signaling activities at the invasive cell membrane, we examined genetic interactions between mig-10b and integrin. A synergistic interaction would be consistent with INIA-1/PAT-3 (integrin) regulating the activity of other signaling pathways (such as netrin) that are crucial to invasion (Pérez-Pérez et al., 2009). Supporting this hypothesis, animals harboring a null mutation of mig-10, treated with ina-1(RNAi) had a defect in AC invasion greater than the additive loss of mig-10 and ina-1(RNAi) (Table 1). Taken together, these results support the notion that INIA-1/PAT-3 mediates the localization and function of distinct signaling pathways at the invasive cell membrane of the AC.

### DISCUSSION

The signaling mechanisms that cells utilize to cross basement membranes are poorly understood. Using the model of AC invasion in C. elegans, we reveal downstream effectors of two pathways that are conserved regulators of invasion: the netrin pathway, which organizes a cellular protrusion that crosses the basement membrane; and the FOS-1A transcription pathway, which regulates the expression of genes that promote basement membrane breaching. Furthermore, we show that the integrin receptor INIA-1/PAT-3 localizes effectors of these pathways to the invasive front, suggesting that integrin coordinates distinct cellular behaviors that contribute to invasion (summarized in Fig. 7).

**UNC-6/UNC-40 effectors during AC invasion**

Although the UNC-6 (netrin)/UNC-40 (DCC) pathway mediates many diverse morphogenetic processes (Adler et al., 2006; Colón-Ramos et al., 2007; Hedgecock et al., 1990; Lai Wing Sun et al., 2011; Teichmann and Shen, 2011; Ziel et al., 2009), how netrin signals in cells other than neurons remains poorly understood. We have found that the effectors downstream of the netrin receptor UNC-40 during AC invasion are largely shared and show a similar genetic organization to those identified in C. elegans neuronal pathfinding and outgrowth. Specifically, the pathway downstream of UNC-40 signaling in AC invasion also has at least two branches:
one containing UNC-34 and the other composed of the Rac GTPase CED-10 and the actin-binding protein UNC-115 (Chang et al., 2006; Demarco and Lundquist, 2010; Giti et al., 2003; Teichmann and Shen, 2011). Our data also suggest that the Rac GTPase MIG-2, which appears to act redundantly with CED-10 (Demarco et al., 2012), is in this branch. We show that these effectors function in the AC, UNC-40 directs their localization to the invasive cell membrane of the AC, and that they promote F-actin formation, which is probably necessary to generate an invasive protrusion that penetrates the basement membrane. The shared downstream effectors of UNC-40 between neurons and the AC suggest that this may be a core set of UNC-40 effectors and that UNC-40 has similarities in how it signals in diverse contexts.

**MIG-10B is a target of FOS-1A regulation in the AC**

MIG-10 is a member of the MRL (MIG-10, RIAM and lamellipodin) family of multi-adaptor proteins that mediate cell adhesion and migration (Coló et al., 2012; Lafuente et al., 2004). Recent studies examining the three isoforms (A-C) of MIG-10 in *C. elegans* have revealed that their unique N-terminal regions influence their localization and function (Stavoe et al., 2012). In response to UNC-6 (netrin), MIG-10A and MIG-10B are asymmetrically localized and are thought to organize the cytoskeleton to mediate axon outgrown and guidance (Adler et al., 2006; Chang et al., 2006; Quinn et al., 2008). Furthermore, UNC-6 and the active zone proteins SYD-1 and SYD-2 localize MIG-10B to presynaptic sites, which in turn direct synaptic vesicle clustering (Stavoe et al., 2012). Our genetic and cell biological results revealed that the *mig-10b* isoform is specifically expressed in the AC, but that it is not a dedicated effector of UNC-40 signaling. Importantly, our data does not rule out a function for MIG-10B acting as an effector of UNC-40, but it does show that it has functions other than netrin signaling. Our data point to an UNC-40-independent role for MIG-10B as a regulator of basement membrane removal. Unlike dedicated UNC-40 effectors, *mig-10b* expression in the AC was dependent on the transcription factor FOS-1A, which controls the expression of genes that promote basement membrane removal. A further distinction from dedicated UNC-40 effectors was that MIG-10B localization to the invasive membrane was not dependent on UNC-40. Instead MIG-10B was targeted to the invasive cell membrane by the extracellular matrix receptor INA-1/PAT-3 (integrin). Interestingly, the earliest characterization of *mig-10* mutant animals suggested that MIG-10 might have functions in cell-matrix interactions during excretory canal outgrowth and cell migration (Manser and Wood, 1990). Furthermore, MIG-10 is known to have functions other than a dedicated effector of netrin signaling (Quinn et al., 2006). It is not well understood how MRL proteins localize to specific membrane regions (Coló et al., 2012; Stavoe et al., 2012). The unique N-terminus of MIG-10B is predicted to adopt an amphipathic α-helix conformation, which mediates binding of the vertebrate MRL protein RIAM to talin (Coló et al., 2012; Lee et al., 2009). Given that talin links integrin to the actin cytoskeleton, it is possible that...
MIG-10B is targeted to the invasive cell membrane of the AC by a close association with INA-1/PAT-3. Alternatively, integrins are known to regulate the cytoskeleton (Legate et al., 2009), as well as vesicular trafficking (Caswell et al., 2009), both of which might promote the localization of MIG-10B to the invasive membrane.

Fos family transcription factors are conserved regulators of invasion and have been implicated in regulating a battery of genes that contribute to basement membrane breaching (Luo et al., 2010; Matus et al., 2013). It is likely that FOS-1A transcriptional targets weaken the presence of at least 24 integrin heterodimers in vertebrates, as well as the complexity of the tissues, has hindered experimental dissection of the functions of these receptors during cell invasion in vivo (Bader et al., 1998; Brockbank et al., 2005; Felding-Habermann, 2003; Sixt et al., 2006). The C. elegans genome encodes only two predicted integrin receptors (Kramer, 2005) and only one of these, INA-1/PAT-3, is expressed and functions in the AC (Hagedorn et al., 2009). Loss of ina-1 or pat-3 strongly blocks AC invasion, but only slightly reduces AC-basement membrane contact (Hagedorn et al., 2009). This observation indicates that INA-1/PAT-3 has roles in promoting invasion beyond AC-basement membrane adhesion. Previously, it has been shown that INA-1/PAT-3 functions upstream of UNC-40 to regulate the targeting of UNC-40 to the invasive cell membrane (Hagedorn et al., 2009). Our results here extend these findings to the fos-1a transcriptional target MIG-10B, which also requires INA-1/PAT-3 for invasive membrane localization (Fig. 7). The notion that integrin acts to coordinately regulate distinct signaling functions required for invasion is further supported by the synergistic genetic interactions between ina-1 and unc-40 shown previously (Hagedorn et al., 2009), as well ina-1 and mig-10, and unc-40 and mig-10 demonstrated here. These genetic interaction studies suggest that there is a strong cooperative function between integrin, FOS-1A and netrin pathways during invasion.

How might integrin, netrin and FOS-1A function together to promote invasion? It has recently been shown that the invasive protrusion directed by UNC-40 enhances basement membrane gap opening by physically displacing matrix as the protrusion expands and extends through the basement membrane opening (Hagedorn et al., 2009). Is it likely that FOS-1A transcriptional targets weaken the basement membrane by matrix remodeling, thus facilitating passage of the UNC-40-directed invasive protrusion. Thus, although each pathway has unique functions, successful invasion is dependent on both acting together. By localizing these pathways to the invasive cell membrane, integrin probably mediates the cooperative interactions between the FOS-1A targets and netrin effectors (and possibly other unidentified pathways). Together, these studies support the idea that integrin has a key scaffolding function within invasive cells that directs the trafficking or stabilization of distinct signaling molecules to the cell-basement membrane interface that act together to mediate the invasive process.

**MATERIALS AND METHODS**

**Worm handling and strains**

Worms were reared under standard conditions (Brenner, 1974). In the text and figures, we use a ‘>’ symbol for linkages to a promoter and use a ‘::’ symbol for linkages that fuse open reading frames. The following alleles and transgenes were used: qyEx196 [unc-115 > GFP], qyEx258 [unc-34 > GFP], qyEx359 [cdh-3 > unc-40::GFP(overexpressed); myo-2 > GFP], qyEx412 [cdh-3 > mig-10b(5X]]; GFP, myo-2 > GFP], qyIs183 [cdh-3 > mig-10b(5X]); GFP; myo-2 > GFP], qyIs182 [cdh-3 > GFP::unc-115], qyIs183 [cdh-3 > mig-10b(5X)]; GFP; myo-2 > GFP].
10b::GFP; cdh-3 > mChR], qyls220 [cdh-3 > GFP::mig-2], qyls221 [cdh-3 > GFP::ced-10], sls10246 [mig-10a > GFP], sls14214 [mig-10b > GFP], olaEx889 [mig-10c > GFP]; Linkage Group I (LGI): unc-40(e271); LGII: rrf-3(pk1426), qyls17 [zm-1 > mCherry]; LGIII: ina-1(gm39), mig-10(ct41), mig-10(ok2499); LGIV: mig-2(mu28); LGV: unc-34(gm104), unc-34(e951), qyls50 [cdh-3 > mCherry::moeABD]; LGX: unc-6(ev400), unc-115(ky275), mig-2(mu28), him-4(rh319).

RNA interference
Double-strand RNA (dsRNA)-mediated gene interference (RNAi) was performed by feeding larvae with bacteria expressing dsRNA (Kamath et al., 2003). dsRNAi was targeted against ced-10, ina-1 and fos-1 to avoid larval lethality with genetic interactions, as well as sterility (Shakir et al., 2006; Sherwood et al., 2005). dsRNA targeting ced-10 was delivered by feeding mig-2(mu28), unc-34(gm104), or unc-40(e271); mig-2(mu28) to L4 larvae at 20°C; animals were allowed to grow to produce F1 progeny that were then analyzed. dsRNA targeting ina-1 was delivered by feeding rrf-3(pk1426) and rrf-3(pk1426); mig-10(ct41) to L1 larvae. dsRNA targeting fos-1 was delivered by feeding qylEx196 [unc-115 > GFP], qylEx258 [unc-34 > GFP], qyls28[cdh-10 > GFP::CED-10] and qyls43[pat-3::GFP; genomic ina-1] to L1 larvae.

Scoring of AC invasion and polarity measurement
AC invasion was scored by examining the integrity of the phase-dense line between the AC and the descendants of the P6.p vulval precursor cell as previously described (Sherwood et al., 2005). Quantitative measurements of polarity were performed by determining the ratio of the average fluorescence intensity from a five-pixel-wide line drawn along the invasive (basal) versus the noninvasive (apical and lateral) membranes of images of the AC, using ImageJ software (Hagedorn et al., 2009).

Microscopy, image acquisition and processing, and quantitative analysis of F-actin and MIG-10
Images were acquired using a Zeiss AxioImager microscope with a 100× Plan-APOCHROMAT objective and equipped with a Yokogawa CSU-10 spinning disc confocal scan head controlled by Vision software (Biovision Technologies), or using a Zeiss AxioImager microscope with a 100× Plan-APOCHROMAT objective and a Zeiss AxioCam MRm CCD camera controlled by Axiovision software (Zeiss Microimaging). Acquired images were processed using ImageJ 1.40 and Photoshop CS3 Extended (Adobe Systems). Three-dimensional reconstructions were built from confocal z-stacks, analyzed and exported using Imaris 7.4 (Bitplane). F-actin and MIG-10 volume was measured using the ‘isosurface rendering’ function of Imaris (Hagedorn et al., 2009).

Quantitative analysis of suppression on UNC-40-overexpression phenotype
The ACs from animals overexpressing cdh-3 > unc-40::GFP in wild-type, unc-34(gm104), ced-10(n1993) and mig-2(mu28) animals were imaged using 0.5 μm z-slice intervals on a confocal microscope. The z-stacks were then built into three-dimensional images. The number of protrusions on the apical and lateral membranes of ACs was determined and the length of protrusions was assessed using the Imaris measurement function.

Molecular biology and transgenic strains
Standard techniques were used to generate PCR fusion products (Hobert, 2002), plasmids and transgenic animals (Sherwood et al., 2005). Templates and specific PCR primers for promoters and genes, and transgenic extrachromosomal (Ex) lines and integrated strains (Is) generated in this study are listed in supplementary material Tables S1 and S2. To generate the transcriptional reporter for the unc-115 gene, the promoter region 3.9 kb upstream of the ATG start codon of the unc-115 gene was amplified. This promoter sequence was then fused in frame to the GFP coding sequence (vector pPD95.81) using PCR fusion. For the transcriptional reporter of the unc-34 gene, 4.2 kb upstream of the unc-34 coding sequence was PCR amplified and subcloned into pPD95.75 (GFP vector) at BamHI and Kpnl sites. The unc-115 cDNA amplified from N2 genomic DNA was PCR fused to the cdh-3 > GFP amplicon to generate cdh-3 > GFP::unc-115. AC-specific MIG-10B::GFP was generated by fusing the cdh-3 promoter (Sherwood et al., 2005) to a coding sequence for MIG-10B::GFP amplified from the unc-86 > mig-10b::GFP vector (obtained from C. Bargmann, The Rockefeller University). To generate AC-specific mig-10b(ΔN)::GFP, mig-10b(ΔN)::GFP was amplified from the unc-86 > mig-10b(ΔN)::GFP vector and then fused to the cdh-3 promoter. Transgenic worms were created by co-injection of expression constructs with the transformation marker pPD#MM016B (unc-119+), or the co-injection marker (myo-2 > GFP) or both into the germline of unc-119(ed4) mutants. These markers were injected with EcoRI-digested salmon sperm DNA and pBluescript II at 50 ng/μl as carrier DNA, along with the expression constructs, which were normally injected at 10-50 ng/μl. Integrated strains were generated as described previously (Sherwood et al., 2005).

Statistics
Statistical analysis was performed using Student’s t-tests and Fisher’s exact tests as indicated in the text.

Acknowledgements
We are grateful to E. A. Lundquist for the unc-115(ky275) mig-2(mu28) strain; the CCG for providing strains; D. A. Colon-Ramos for the olaEx889[mig-10c >GFP] strain; and C. I. Bargmann for the unc-86 > mig-10b::GFP plasmid.

Competing interests
The authors declare no competing financial interests.


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