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FOS-1 Promotes Basement-Membrane Removal during Anchor-Cell Invasion in *C. elegans*

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Summary

Cell invasion through basement membranes is crucial during morphogenesis and cancer metastasis. Here, we genetically dissect this process during anchor-cell invasion into the vulval epithelium in *C. elegans*. We have identified the *fos* transcription factor ortholog *fos-1* as a critical regulator of basement-membrane removal. In *fos-1* mutants, the gonadal anchor cell extends cellular processes normally toward vulval cells, but these processes fail to remove the basement membranes separating the gonad from the vulval epithelium. *fos-1* is expressed in the anchor cell and controls invasion cell autonomously. We have identified ZMP-1, a membrane-type matrix metalloproteinase, CDH-3, a Fat-like protocadherin, and hemicentin, a fibulin family extracellular matrix protein, as transcriptional targets of FOS-1 that promote invasion. These results reveal a key genetic network that controls basement-membrane removal during cell invasion.

Introduction

Regulated cell invasion through basement membranes occurs in normal developmental processes such as trophoblast implantation, organogenesis, and angiogenesis (Cross et al., 1994; Affolter et al., 2003; Pepper, 1997). During tumor progression, cancer cells also acquire the ability to invade through basement membranes, enabling them to metastasize to distant tissues (Egeblad and Werb, 2002). Cell invasion involves coordination of multiple individual cellular behaviors, including attachment of the invading cell to the basement membrane, breaching of the matrix components, and the acquisition of motility (Stetler-Stevenson et al., 1993). Cell invasive activity appears to be stimulated and targeted by diffusible chemoattractants (Starz-Gaiano and Montell, 2004; Balkwill, 2004). Despite its critical importance in development and cancer, the genetic pathways underlying cell-invasive behavior remain poorly understood (Hanahan and Weinberg, 2000). Elucidation of these mechanisms requires in vivo models where the dynamic interactions of the invading cell, basement membrane, and tissue being invaded can be clearly visualized and genetically dissected.

Anchor-cell (AC) invasion in *Caenorhabditis elegans* is a simple model of regulated cell-invasive behavior (Sherwood and Sternberg, 2003). Connection of the developing uterus and vulva in *C. elegans* is initiated by the uterine AC, which extends an invasive basolateral process across the basement membranes separating both tissues, and then moves between the center of the 1° vulval lineage cells. AC invasion is predominantly stimulated by a diffusible cue generated by the 1° vulval cells. AC invasion also involves the localized breakdown of the basement membrane under the AC and the generation of a mesenchymal-like basolateral protrusion. The molecular mechanisms that promote and integrate these behaviors are not known.

Fos proteins are basic region-leucine zipper (bZIP) transcription factors that bind to Jun or other bZIP proteins to create the AP-1 dimer complex, which regulates gene expression (Chinenov and Kerppola, 2001). Vertebrates have four *fos* genes (*c-fos*, *fosB*, *fra-1*, and *fra-2*). Members of this family are overexpressed in a number of metastatic cancers, including breast (Zajchowski et al., 2001), prostate (Aoyagi et al., 1998), osteosarcoma (Gamberi et al., 1998), thyroid (Kataki et al., 2003), and squamous cell lung carcinoma (Volm et al., 1993), suggesting that Fos proteins are associated with metastasis in some cancers. Moreover, pre-malignant papillomas in *c-fos*-deficient mice are incapable of progressing to malignant skin carcinomas (Saez et al., 1995). Ectopic expression of *c-fos*, *fra-1*, and the retroviral oncogene *v-fos* in cell lines can stimulate cell-invasive behavior through reconstituted extracellular matrices in vitro (Reichmann et al., 1992; Kustikova et al., 1998; Hennigan et al., 1994). Thus, Fos proteins may promote metastasis in part by fostering cell-invasive behavior. Whether Fos proteins are actually required for cell invasion and if they regulate invasion during normal developmental processes is not known.

We report here that an isoform of the *C. elegans fos* transcription factor ortholog *fos-1* is a cell-autonomous regulator of AC invasion. Visualization of AC behavior in *fos-1* mutants revealed that *fos-1* specifically promotes basement-membrane removal during AC invasion and does not regulate the ability of the AC to extend mesenchymal-like protrusions or respond to the invasion cue from the 1° vulval cells. We also show that ZMP-1, a membrane-type matrix metalloproteinase, CDH-3, a Fat-like protocadherin, and hemicentin, a fibulin family extracellular matrix protein, are downstream transcriptional targets of *fos-1* that have subtle roles in promoting basement-membrane removal.

Results

AC Invasion into the Vulval Epithelium

AC invasion was visualized in animals containing a *cdh-3*::CFP reporter gene. *cdh-3* is a cadherin family mem-

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ber expressed strongly in the AC (Pettitt et al., 1996). In wild-type animals, the gonadal AC initiates invasion ventrally toward the 1°-fated daughters of the vulval precursor cell (VPC) P6.p in the mid-L3 larval stage (Sherwood and Sternberg 2003; Table S1). By the late-L3 stage, the basolateral portion of the AC breaches the gonadal and ventral epidermal basement membranes and contacts the two central 1°-fated P6.p granddaughters (Figure 1A). Visualized under Nomarski optics, AC invasion interrupts the clear line formed by the juxtaposed gonadal and ventral epidermal basement membranes (Figure 1A) and establishes an attachment that halts the independent sliding of the uterine and vulval tissues. AC invasion concludes 3.5 hr later at the early-L4 stage when the basolateral portion of the AC moves between the central P6.p great-granddaughter cells.

A Mutation in *fos-1* Disrupts AC Invasion

Ablation of the AC just prior to invasion abolishes uterine-vulval attachment and results in an everted/protruded vulva (Evl/Pvl) phenotype in the adult (Kimble, 1981; Seydoux et al., 1993). We thus examined known Evl and Pvl mutants and found that the previously uncloned mutant *evl-5(ar105)* (Seydoux et al., 1993), renamed *fos-1*, has an invasion defect. In the mid-L3 stage in *fos-1* mutants, the AC is positioned normally in the gonad and is in contact with the gonadal basement membrane. However, at the late-L3 stage when invasion normally occurs, none of the ACs in *fos-1* mutants had invaded (P6.p four-cell stage; Table 1). A distinct line separated the gonad from the vulval epithelium and these tissues slid alongside one another freely (Figure 1B). Later, during the early-L4 stage, 25% of ACs in *fos-1* mutants had extended a narrow invasive process into the P6.p great-granddaughters (P6.p eight-cell stage; Table 1). Thus, ACs in *fos-1* mutants invade only occasionally in a delayed and partial manner.

ACs in *fos-1* Mutants Are Defective in Their Ability to Breach the Basement Membrane

AC invasion requires the 1° vulval lineage, which produces a cue that stimulates AC invasion toward these cells (Sherwood and Sternberg, 2003). In wild-type animals, the VPC directly ventral to (below) the AC, P6.p, adopts the 1° fate in response to the growth factor LIN-3, expressed in the AC (Moghal and Sternberg, 2003). To determine if the 1° vulval lineage is altered in *fos-1* mutants, we examined the expression of an *egl-17*::YFP transgene. *egl-17* encodes a fibroblast growth factor with no apparent role in AC invasion, and its promoter drives expression specifically in 1°-fated VPCs and their descendants (Burdine et al., 1998). The P6.p cell and its descendants expressed *egl-17* normally in *fos-1* mutants (>20 animals examined for each stage; Figures 1A and 1B), indicating that the 1° vulval lineage is specified appropriately.

To determine if the 1° vulval lineage generates an invasion cue and whether the AC has normal mesenchymal properties and can respond to this cue in *fos-1* mutants, we ablated during the L2 stage all VPCs except for the P8.p cell, located posterior to the P6.p cell. Under these conditions the isolated P8.p usually takes on the 1° vulval fate (Sternberg and Horvitz, 1986). To visualize the response of the AC to displaced 1° vulval cells, we observed AC behavior in late-L3 animals containing both the AC marker *cdh-3*::CFP and the 1° vulval fate marker *egl-17*::YFP. In wild-type animals, the AC responds to isolated P8.p descendants by directing a basolateral invasive process through the basement membrane and then toward these 1°-fated cells (Figure 1C; Sherwood and Sternberg, 2003). In *fos-1* mutants, a basolateral process was extended toward the isolated 1°-fated P8.p descendants in all cases (23/23 animals, Figure 1D). Unlike wild-type animals, however, these processes tracked along and accumulated at the site of the gonadal basement membrane rather than crossing this barrier (23/23 animals, Figure 1D).

To determine if ACs in *fos-1* mutants are able to remove the basement membrane, we examined the localization of MitoTracker Red dye, which we have found stains basement membrane in living animals (see Experimental Procedures). We also analyzed the distribution of the basement-membrane protein laminin in fixed animals (Huang et al., 2003). In wild-type animals, the basement membranes are always removed under the AC by the P6.p four-cell stage (Figures 1E and 1G; Sherwood and Sternberg, 2003). By contrast, the basement membrane remained intact under the AC in all *fos-1* mutants at this time (Figures 1F and 1H; >20 animals examined for each marker). Although it is difficult to differentiate the tightly juxtaposed gonadal and ventral epidermal basement membranes, in cases where they were separated (n = 4), neither showed disruptions. Taken together, these results demonstrate that *fos-1* is specifically required to promote basement-membrane removal during AC invasion.

fos-1 is the *C. elegans* Ortholog of the Vertebrate *fos* Transcription Factor Gene Family

The *fos-1* gene had been mapped to chromosome V between *unc-46* and *dpy-11* (Seydoux et al., 1993). Through additional genetic mapping and transformation rescue of the invasion phenotype, we identified *fos-1* as the predicted gene F29G9.4. cDNA sequences from the *C. elegans* EST collection (Y. Kohara) and RT-PCR analysis revealed that *fos-1* generates two transcripts from distinct start exons (Figure 2A). The *fos-1a* transcript contains a start ATG codon within the unique first exon and encodes a predicted 467 amino acid protein. The *fos-1b* transcript start codon is in the shared third exon and encodes a predicted 331 amino acid protein (Figures 2A and 2B).

fos-1 is an ortholog of the *fos* bZIP transcription factor family (Tatusov et al., 2003). The FOS-1 protein is similar to other Fos proteins in the basic DNA binding domain, contains a leucine zipper region, and has a carboxyl terminus rich in serine and threonine residues, which are thought to be important phosphorylation sites (Figures 2B and 2C). A notable distinction within the FOS-1 sequence is the presence of the basic amino acid lysine in the third leucine repeat instead of a hydrophobic amino acid such as leucine. A maximum parsimony analysis of bZIP transcription factors revealed that *fos-1* is the only *fos* gene family member in *C. elegans* and that the duplication of these genes in verte-

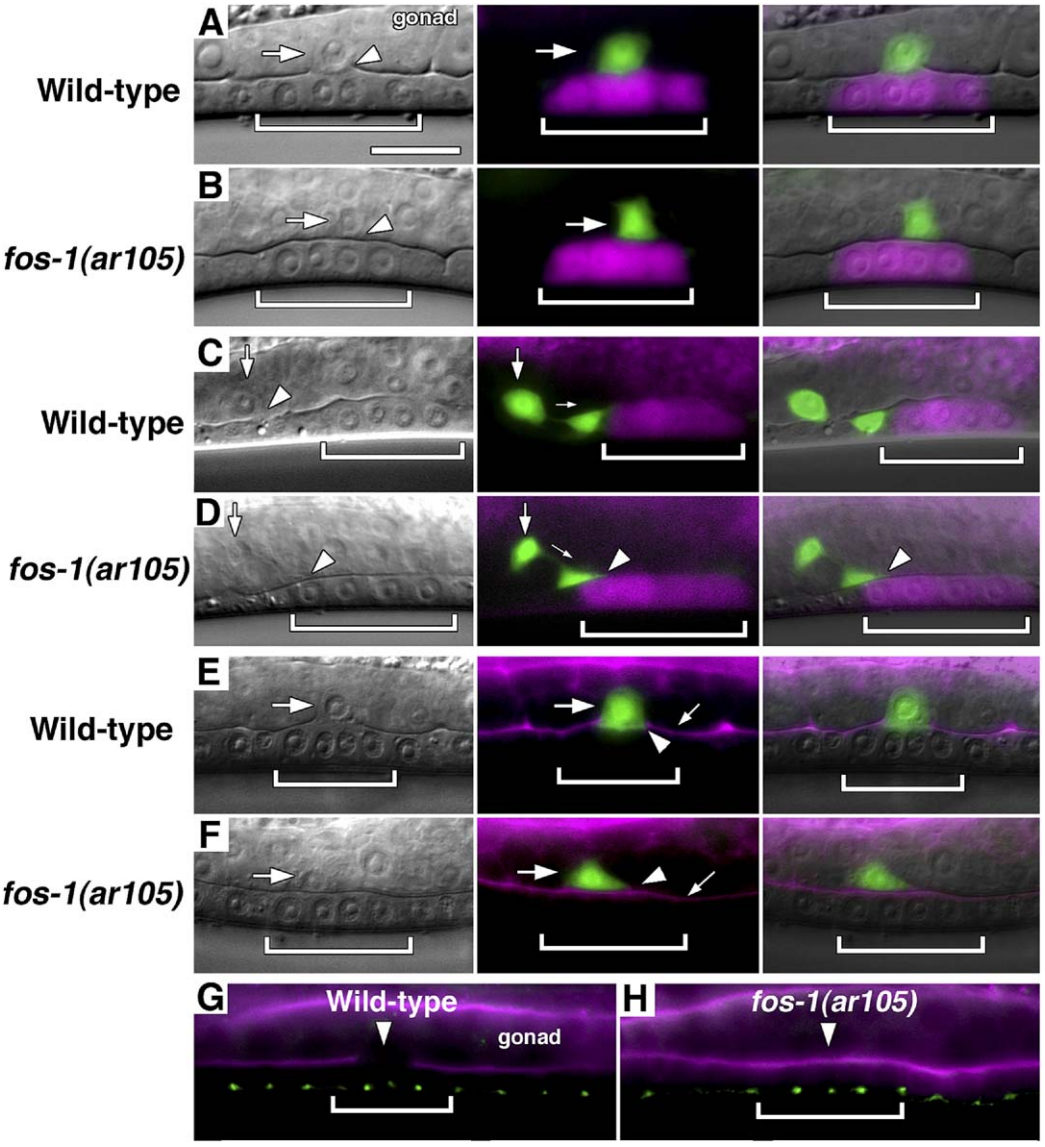

Figure 1. *fos-1* Is Required for Basement-Membrane Removal during AC Invasion

All animals were viewed at the late-L3 stage; anterior left; ventral down; arrow, AC; arrowhead, invasion area; bracket, 1°-fated vulval cells.

(A) Nomarski (left), fluorescence (center), and overlaid (right) images of a cross-section through the central gonad and vulval region of a wild-type animal are shown. The AC (expressing *cdh-3*::CFP in green) has crossed the gonadal and ventral epidermal basement membranes (left) and is attached to the inner 1°-fated P6.p descendants (expressing *egl-17*::YFP in purple).

(B) In a *fos-1(ar105)* mutant, the AC fails to invade through the basement membranes and contact the 1°-fated P6.p descendants, although they express *egl-17*::YFP normally.

(C) A wild-type animal in which the P3.p-P7.p cells have been ablated and the isolated P8.p cell has given rise to four 1°-fated vulval cells that express *egl-17*::YFP is shown. These 1° vulval cells stimulate the AC to direct a basolateral process that invades across the basement membranes (left) and toward them (small arrow).

(D) In the *fos-1* mutant, the isolated 1°-fated P8.p descendants also generate the cue, stimulating the AC to extend a basolateral process toward these cells (small arrow). However, this process flattens and accumulates at the site of the basement membrane, indicating an inability to remove this barrier.

(E) MitoTracker Red staining of the basement membrane (purple) and *cdh-3*::CFP expression in the AC (green) show an AC in a wild-type animal whose basolateral process has removed the juxtaposed gonadal and ventral epidermal basement membranes (small arrow), crossed through the gap in these basement membranes, and is attached to the inner 1°-fated P6.p descendants.

(F) In a *fos-1* mutant, the AC fails to remove the underlying basement membrane.

(G) In a wild-type animal, laminin antibody staining (purple) is lost over the 1°-fated P6.p descendants (cell borders labeled in green with an antibody to the apical adherens protein AJM-1) where the AC invades.

(H) Laminin remains intact in *fos-1* mutants. Differences in intensity of laminin staining reflect uneven permeability of antibody staining.

The scale bar is 10 μm.

Table 1. Timing and Degree of AC Invasion into the Vulval Epithelium

	Number (Percentage) of ACs Showing Full, Partial, or No Invasion					
	P6.p Four-Cell Stage (Late-L3, 31.5 hr Post-Hatching)			P6.p Eight-Cell Stage (Early-L4, 35 hr Post-Hatching)		
Strain/Treatment	Full Invasion	Partial Invasion	No Invasion	Full Invasion	Partial Invasion	No Invasion
Wild-type (N2)	110 (100%)	0	0	100 (100%)	0	0
fos-1(ar105)	0	0	105 (100%)	0	28 (25%)	105 (75%)
RNAi treatment						
fos-1(RNAi); N2	9 (31%)	6 (21%)	14 (48%)	12 (30%)	10 (25%)	18 (45%)
fos-1(RNAi); *rrf-3*	1 (4%)	4 (15%)	21 (81%)	1 (2%)	13 (28%)	33 (70%)
fos-1(RNAi); *evl-5(ar105)*	0	0	20 (100%)	0	10 (25%)	30 (75%)
Transgenic rescue						
fos-1(ar105); *fos-1a*::YFP-TL	18 (85%)	2 (10%)	1 (5%)	21 (95%)	1 (5%)	0
fos-1(ar105); *fos-1a/b*::GFP-TL	21 (92%)	1 (4%)	1 (4%)	25 (100%)	0	0
fos-1(ar105); *pAC-fos-1a*::YFP	3 (9%)	20 (63%)	9 (28%)	53 (100%)	0	0
fos-1(ar105); *pAC-fos-1b*::CFP	0	0	23 (100%)	0	11 (22%)	39 (78%)
fos-1a target mutant phenotypes						
zmp-1(cg115)	100 (100%)	0	0	100 (100%)	0	0
cdh-3(pk87)	99 (98%)	1 (1%)	1 (1%)	100 (100%)	0	0
zmp-1(cg115) cdh-3(pk87)	96 (93%)	5 (5%)	2 (2%)	100 (100%)	0	0
him-4(rh319)	86 (83%)	5 (5%)	12 (12%)	100 (100%)	0	0
zmp-1(cg115) cdh-3(pk87); him-4(rh319)	77 (75%)	11 (11%)	14 (14%)	99 (98%)	2 (2%)	0
Triple-mutant controls						
unc-5(e53); dpy-11(e224); lon-2(e678)	100 (100%)	0	0	105 (100%)	0	0
che-3(sy172); dpy-17(e164); him-5(e1490)	100 (100%)	0	0	105 (100%)	0	0

brates occurred after their divergence from *C. elegans* (Figure 2D).

The *ar105* Mutation in *fos-1* Specifically Disrupts the *fos-1a* Transcript

The *ar105* allele is associated with a single base transition that changes Gln119 (CAA) to a stop codon (TAA) in the predicted *fos-1a* transcript. This mutation does not affect the open reading frame of the *fos-1b* transcript (Figures 2A and 2B). The truncation of *fos-1a* occurs prior to the conserved basic DNA binding region and leucine zipper and thus suggests that the *ar105* mutation is a molecular null for *fos-1a*. Consistent with this notion, *fos-1* RNA interference treatment (RNAi; Fire et al., 1998) produced an AC-invasion defect similar to *fos-1(ar105)* mutants in wild-type animals and in the RNAi-hypersensitive strain *rrf-3* (Simmer et al., 2002) but did not increase the AC-invasion defect in *fos-1(ar105)* (Table 1). Thus, only the *fos-1a* transcript of *fos-1* promotes basement-membrane removal during AC invasion.

FOS-1A Is Expressed at High Levels in the AC during Invasion

The expression patterns of *fos-1a* and *fos-1b* were determined by generating transcriptional (TX) and translational (TL) transgene reporters that inserted YFP and CFP into the unique start exons of *fos-1a* and *fos-1b*, respectively (*fos-1a*::YFP-TX, -TL and *fos-1b*::CFP-TX, -TL; Figure 3A) . Transgenic animals containing the *fos-1a*::YFP-TX and -TL reporters showed the same expression pattern at all times examined, and *fos-1a*::YFP-TL protein localized to the nucleus (Figure 3B). *fos-1a*::YFP-TL also rescued the AC-invasion defect when expressed in *fos-1(ar105)* mutants (Table 1). *fos-1a* was expressed at the highest level in the AC and at lower levels in neighboring somatic gonad cells during AC invasion (Figure 3B). No expression of *fos-1a* was detected in vulval cells. In contrast, both the *fos-1b*::CFP-TX and -TL reporters directed expression in vulval cells and the AC (Figure 3C). *fos-1b*::CFP-TL also drove expression in neighboring uterine cells, whereas *fos-1b*::CFP-TX did not, suggesting the presence of a downstream uterine enhancer element in *fos-1b*::CFP-TL. Unlike *fos-1a* expression, levels of *fos-1b*::CFP-TL protein were not greater in the AC compared to neighboring cells (Figure 3C). Moreover, whereas the *fos-1a* reporters drove expression almost exclusively in the somatic gonad cells, the *fos-1b* reporters directed expression in nearly all cells at the L3 stage (Figure 3D). The different expression patterns and amino acid composition suggest that *fos-1a* and *fos-1b* may encode proteins with distinct activities. Furthermore, the high level of FOS-1A::YFP in the AC suggests a cell-autonomous role for *fos-1a* during invasion.

AC-Specific FOS-1A, but Not FOS-1B, Expression Rescues Invasion in *fos-1(ar105)* Mutants

To determine whether *fos-1a* can function cell autonomously in the AC to promote invasion, we expressed *fos-1a* in the AC using a 1.5 kb *cis*-regulatory region of the *cdh-3* gene (*pAC*), which directs expression solely in the AC from the L2 molt through the early-L4 stage (Kirouac and Sternberg, 2003). To follow expression of *fos-1a*, we inserted YFP at the C terminus of the *fos-1a* cDNA to create the fusion transgene *pAC-fos-1a*::YFP. Insertion of GFP into this C-terminal site in a *fos-1* genomic clone rescued the AC-invasion defect in *fos-1(ar105)* mutants (see *fos-1a/b*::GFP-TL; Table 1).

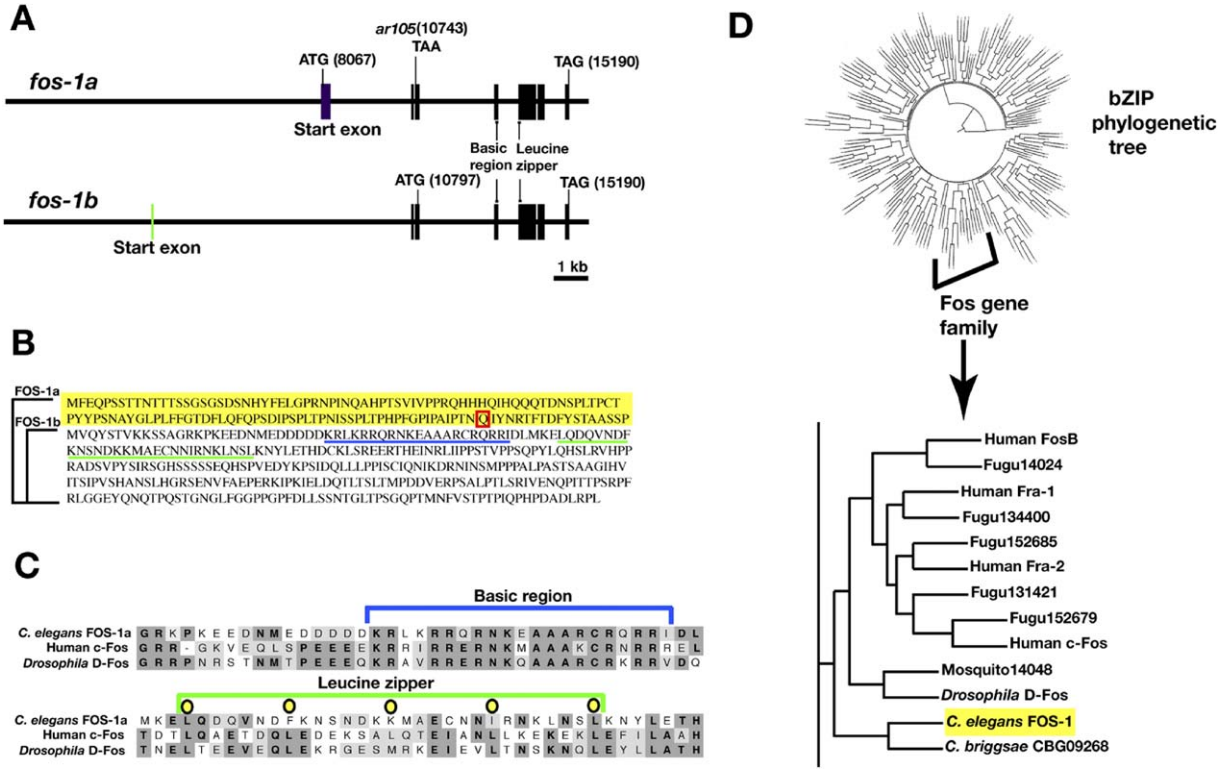

Figure 2. *fos-1* Encodes the *C. elegans fos* Ortholog

(A) Exon/intron structure of *fos-1* is shown. Start exons are shown for the *fos-1a* (purple box) and *fos-1b* (green box) transcripts. The *ar105* mutation, which inserts a stop codon in the *fos-1a* transcript, is shown. Numbers in parentheses indicate the location of these sites on the cosmid F29G9 (GenBank AF016440).

(B) Predicted translation of *fos-1a* and *fos-1b* cDNA, with the additional N-terminal sequence of FOS-1A in yellow, the basic region underlined in blue, and the leucine zipper region underlined in green. The red box indicates the location of the stop codon introduced by the *ar105* mutation.

(C) Alignment of the FOS-1 basic region and leucine zipper domain with human c-Fos and *Drosophila* D-Fos is shown. Identical amino acids are darkly shaded, similar amino acids are lightly shaded. Yellow dots indicate the position of the leucine amino acid in the leucine zipper.

(D) (top) Phylogenetic analysis of the bZIP domains from 194 bZIP proteins from *C. elegans*, *C. briggsae*, *D. melanogaster*, *A. gambiae*, *F. rubripes*, *H. sapiens*, *S. cerevisiae*, and *S. pombe* reveals that the FOS-1 bZIP protein is the only *C. elegans* protein that groups with the Fos protein family (below), and that vertebrate Fos proteins are more closely related to each other than to FOS-1.

AC-specific expression of *fos-1a* restored invasion in 72% of *fos-1* mutants at the P6.p four-cell stage (Table 1). While most of these invasions did not broadly breach the basement membrane (compare Figures 4A and 4C), by the P6.p eight-cell stage, *pAC-fos-1a*::YFP fully restored invasion in 95% of *fos-1* mutants (Table 1; Figures 4B and 4D). The delay in full rescue may be a result of a later onset of *fos-1a* expression in the AC when driven by *pAC*. *pAC* initiates expression at the L2 molt, whereas the endogenous *fos-1a* promoter begins expression 3 hr prior to the L2 molt. Since full *pAC* (*cdh-3)* expression in the AC is dependent on *fos-1a* activity (see below), it is also possible that a lower level of expression in *fos-1* mutants contributes to the delay in rescue.

To determine whether *fos-1a* and *fos-1b* have unique activities, we drove AC-specific expression of *fos-1b* in *fos-1(ar105)* mutants with the fusion transgene pAC-fos-1b::CFP, which inserted CFP at the same C-terminal site of the *fos-1b* cDNA. Unlike *fos-1a*, expression of *fos-1b* in the AC did not rescue the invasion defect in *ar105* (Table 1; Figures 4E and 4F). We conclude that *fos-1a* and *fos-1b* have distinct activities, and that *fos-1a* can function cell autonomously in the AC to promote invasion.

zmp-1, *him-4*, and *cdh-3* Are Regulated by *fos-1a* and Foster AC Invasion

To identify genes that *fos-1a* regulates in the AC, we first analyzed the expression of known genes that have AC-specific expression ($\geq$20 animals examined for each transgene). Five transgenes showed no change in AC expression in wild-type compared with *fos-1(ar105)* animals. These include the transcriptional reporters for *fos-1a* and *fos-1b*. Reporters for *sel-8*, a gene involved in LIN-12/Notch signaling (Doyle et al., 2000), *pat-3*, the β integrin subunit expressed in *C. elegans* (Plenefisch et al., 2000), and *mig-2*, a *C. elegans* Rac gene (Zipkin et al., 1997), were not altered by loss of *fos-1a* activity. By contrast, the reporter for *lin-3 (*Hwang and Sternberg, 2004) showed a 1.8-fold increase in AC expression in mutants. Expression of *zmp-1*, a predicted gly-

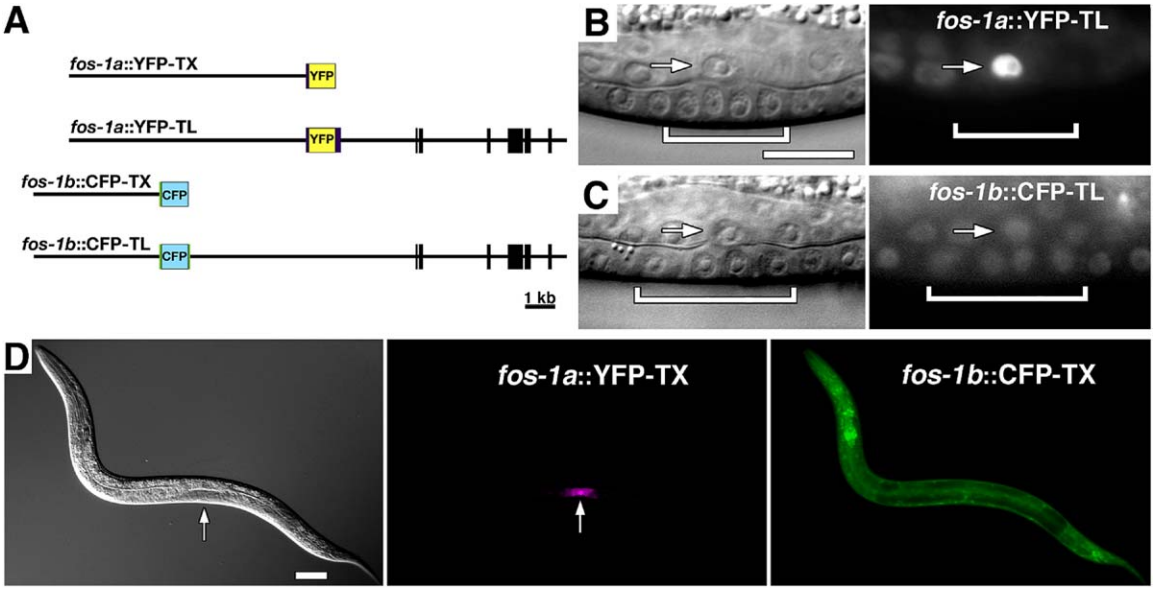

Figure 3. *fos-1a* Is Expressed at High Levels in the AC during Invasion

(A) YFP and CFP insertions into the start *fos-1a* and *fos-1b* exons, respectively, to create transcriptional (-TX) and translational (-TL) transgene reporters.

(B) Nomarski (left) and corresponding fluorescence image show that *fos-1a*::YFP-TL fusion protein (right) is localized at high levels in the nucleus of the AC (arrow) and at lower levels in the neighboring uterine cells in the late L3 (P6.p four-cell stage). The 1°-fated vulval cells (bracket) show no FOS-1A::YFP expression.

(C) The *fos-1b*::CFP-TL fusion protein is expressed at low levels in the AC (arrow) and neighboring uterine cells, as well as the underlying vulval cells.

(D) Nomarski image (left), *fos-1a*::YFP-TX expression (purple, center), and *fos-1b*::CFP-TX expression (green, right) of an entire late-L3 larva show that *fos-1a* expression is primarily confined to the somatic gonad (arrow indicates AC location), while *fos-1b* is expressed in most cells. The scale bars are 10 μm.

cosylphosphatidylinositol (GPI) anchored membrane-type zinc matrix metalloproteinase (Itoh et al., 1999; Wada et al., 1998), and *him-4*, which encodes an extracellular matrix fibulin family member (Vogel and Hedgecock, 2001), was undetectable in the AC of animals lacking *fos-1a* activity (Figures 5A–5D). Lastly,

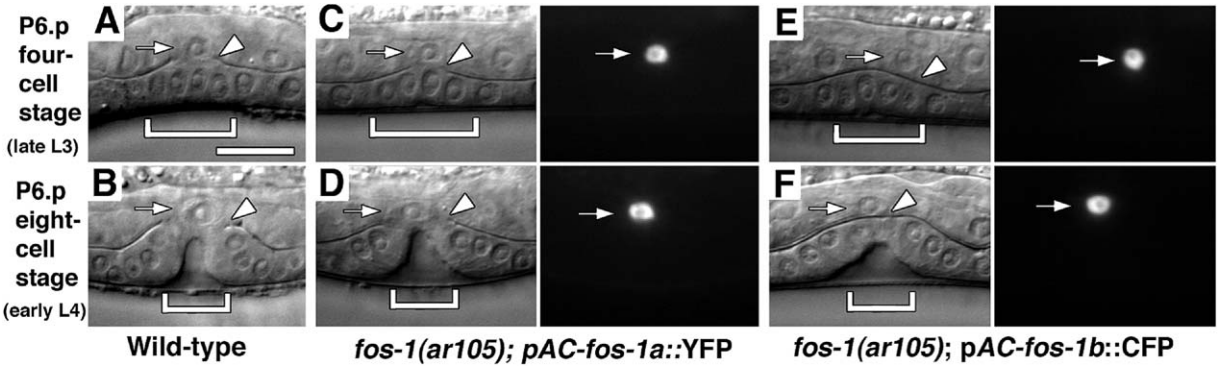

Figure 4. AC-Specific Expression of FOS-1A, but Not FOS-1B, Rescues Invasion in *fos-1(ar105)* Mutants

Arrow, AC; arrowhead, invasion area; bracket, 1°-fated vulval cells.

(A) In wild-type animals at the P6.p four-cell stage, the AC has crossed the basement membrane and is attached to the 1°-fated vulval cells and by the P6.p eight-cell stage (B) moves through the inner 1° vulval cells.

(C) Nomarski (left) and fluorescence expression (right) show that *pAC-fos-1a*::YFP fusion protein is localized to the nucleus of the AC and restores invasive activity in a *fos-1* mutant at the P6.p four-cell stage. The break in the basement membrane is not broad.

(D) By the early-L4 stage, *pAC-fos-1a*::YFP restores full AC invasion.

(E) Nomarski (left) and fluorescence (right) images of a *fos-1* mutant shows that *pAC-fos-1b*::CFP fusion protein localizes to the AC nucleus but does not rescue AC invasion at the P6.p four-cell stage or later (F) at the P6.p eight- cell stage.

The scale bar is 10 μm.

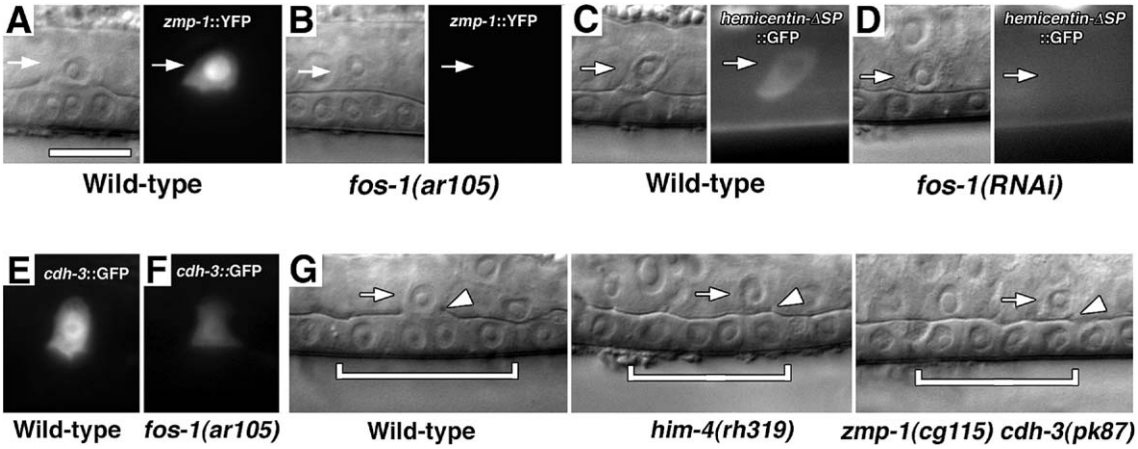

Figure 5. *zmp-1*, *cdh-3*, and *him-4* Are Targets of *fos-1a* Regulation that Promote AC Invasion

All animals were viewed at the late-L3 stage; arrow, AC.

(A) Nomarski (left) and fluorescence images (right) of a *zmp-1*::YFP transgenic animal shows that *zmp-1* is strongly expressed in the AC during invasion.

(B) AC *zmp-1*::YFP expression is undetectable in *fos-1(ar105)* mutants.

(C) Unsecreted hemicentin (ΔSP::GFP) is retained within the cells in which it is expressed and is present in the AC at the time of invasion.

(D) In *fos-1(RNAi)* animals, hemicentin-ΔSP::GFP is not detected in the AC.

(E) A wild-type animal with expression of *cdh-3*::GFP in the AC during invasion is shown.

(F) Reduced *cdh-3*::GFP expression in a *fos-1* mutant is shown.

(G) 100% of wild-type animals (left panel) exhibit AC invasion through the basement membrane (arrowhead) and attachment to the 1°-fated vulval cells at the P6.p four-cell stage (bracket). A *him-4* mutant (center panel) and a *zmp-1 cdh-3* double mutant (right panel) in which the ACs have failed to invade (arrowhead) at this time are shown. Both ACs have slid posterior to the central 1° vulval cells (bracket).

The scale bar is 10 μm.

cdh-3, a Fat-like protocadherin (Pettitt et al., 1996), was expressed at approximately one-third of normal levels in *fos-1* mutants (Figures 5E and 5F).

The modest increase in *lin-3* expression in *fos-1* mutants appears to be functionally negligible, as it neither increased VPC induction (50/50 animals) nor altered 1° fate specification (Figure 1B). We thus further analyzed the regulation of *zmp-1*, *him-4*, and *cdh-3* by *fos-1a*. We first found that pAC-fos-1a::YFP can increase the level of expression of *cdh-3* and restore detectable expression of *zmp-1* and *him-4* in the AC of *fos-1* mutants (≥20 animals examined for each transgene). Overexpression of fos-1a::YFP driven by a heat-shock promoter, however, did not result in ectopic expression of *cdh-3*, *zmp-1*, or *him-4*, indicating that *fos-1a* is required for normal expression of these targets in the AC but is not sufficient on its own to drive their expression. Consistent with this notion, loss of *fos-1a* activity did not alter the expression of these genes in cells other than the AC.

We next examined whether *zmp-1*, *him-4*, and *cdh-3* promote AC invasion. A putative null allele in *zmp-1*, *cg115,* was created by generating a deletion mutant that removes the catalytic domain of this metalloproteinase (see Supplemental Data). We also analyzed putative null alleles for *him-4 (rh319*; Vogel and Hedgecock, 2001) and *cdh-3* (*pk87*; Pettitt et al., 1996*)*. No defects in AC invasion were observed in *zmp-1* mutants, while 2% of *cdh-3* and 17% of *him-4* mutants showed delays in AC invasion (Table 1; Figure 5G). To test whether these genes function together to promote invasion, double- and triple-mutant strains were constructed. Seven percent of *zmp-1 cdh-3* double and 25% of *zmp-1 cdh-3; him-4* triple-mutant animals invaded in a delayed manner (Table 1; Figure 5G). In addition, 2% of *zmp-1 cdh-3; him-4* triple-mutant animals failed to complete invasion by the P6.p eight-cell stage (Table 1). Thus animals with mutations in *zmp-1*, *cdh-3*, and *him-4* together impede AC invasion more than animals with any single mutant alone. To confirm that double- or triple-mutant strains do not impede invasion nonspecifically, we examined invasion in two other triple-mutant strains containing defects in genes that have no apparent role in AC invasion (see Table 1). No defects were observed in these triple-mutant strains. We thus infer that *zmp-1*, *cdh-3*, and *him-4* are functional targets of *fos-1a* in the AC that function together to promote invasion.

FOS-1A Expression and Activity Are Not Regulated by the 1° Vulval Cell Cue

The 1° vulval cells stimulate AC invasion by generating a cue that promotes invasive activity toward these cells. We thus examined whether this signal stimulates invasion by regulating *fos-1a*. The level of expression of *fos-1a*::YFP-TL fusion protein and the localization of this protein to the nucleus was normal in vulvaless animals. Also, transgene reporters for *zmp-1* and *cdh-3* showed no expression differences in wild-type animals compared with vulvaless animals (≥20 animals examined for each transgene). We infer that the cue from the 1° vulval cells does not stimulate AC invasion by controlling *fos-1a* expression, localization, or activity.

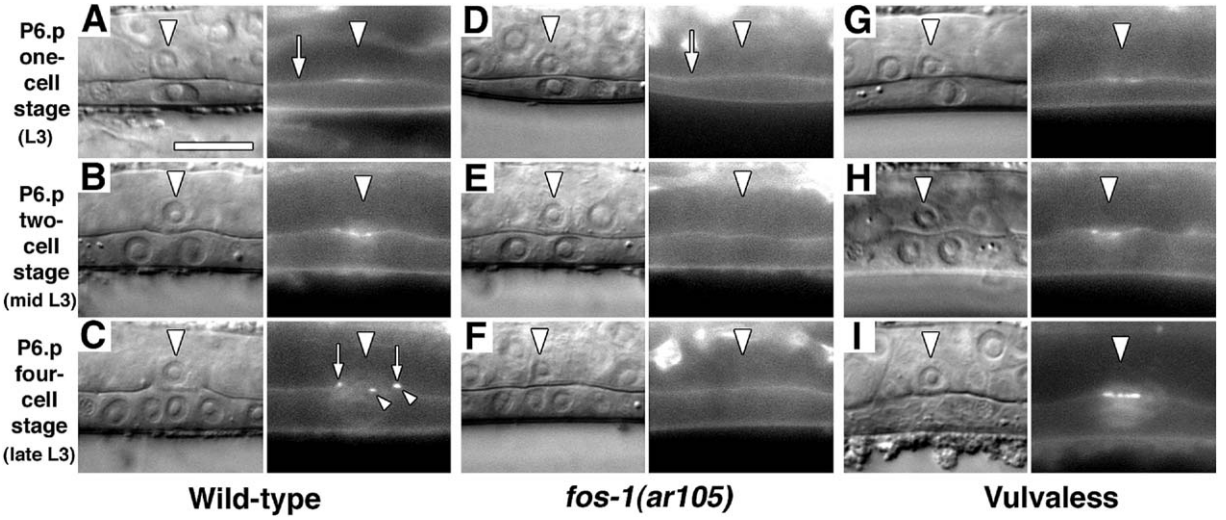

Figure 6. Hemicentin::GFP Presages the Site of Invasion

Left, Nomarski; right, corresponding fluorescence; arrowhead, site of invasion.

(A–C) show expression patterns of hemicentin::GFP in wild-type animals. (A) At the P6.p one-cell stage, higher levels of hemicentin::GFP begin to accumulate in the basement membrane under the AC compared with the adjacent basement membrane (arrow). (B) By the early-to-mid P6.p two-cell stage, more hemicentin::GFP is deposited under the AC. (C) By the P6.p four-cell stage, the AC has removed the underlying basement membrane and hemicentin::GFP is cleared under the AC (area between arrows) and found in large aggregates surrounding the site of invasion (small arrowheads).

(D–F) In *fos-1(ar105)* mutants, the low level of basement-membrane localization of hemicentin::GFP is normal (arrow); however, hemicentin::GFP is not deposited at increased levels under the AC nor is it cleared under the AC by the P6.p four-cell stage. The bright staining on the top of the fluorescence images is autofluorescence from gut tissue.

(G–I) In vulvaless animals, hemicentin::GFP shows increased accumulation under the AC in the L3 and mid-L3 stages (G and H) but is not cleared under the AC when invasion fails at the late L3 (I).

The scale bar is 10 μm.

Targets of FOS-1A Are Expressed at the Site of Invasion

CDH-3 and ZMP-1 appear to play subtle roles in promoting AC invasion. The vertebrate Fat1 protocadherin localizes to leading edges of cellular protrusions (Moeller et al., 2004). We have similarly found that a YFP-tagged version of CDH-3 (CDH-3::YFP-TL) localizes to the protrusive invasive basolateral membrane of the AC (Figures S1A and S1B). Also, a FLAG epitope-tagged version of ZMP-1 (ZMP-1-FLAG) localizes to various sized puncta in the AC (Figures S1C–S1F). These puncta may be microdomains on the cell surface formed by lipid rafts where GPI-anchored proteins localize. A similar expression pattern has been observed with MT4-MMP, the vertebrate GPI-linked MMP most similar to ZMP-1 (Itoh et al., 1999). Notably, there often appears to be more ZMP-1 localization near the invasive membrane of the AC. These results suggest that CDH-3 and ZMP-1 proteins may directly promote basement-membrane removal.

The *him-4* (hemicentin) mutant affected AC invasion the most severely of the identified targets of *fos-1a*. We thus examined the expression of a full-length hemicentin::GFP transgene (Vogel and Hedgecock, 2001) during the course of AC invasion. Two and a half hours prior to invasion, increased levels of hemicentin::GFP first accumulate in the basement membrane under the AC, presaging the site of invasion (Figure 6A). Hemicentin::GFP levels continue to increase at this location until the time of invasion (Figure 6B), when hemicentin::GFP is cleared, leaving large aggregates surrounding the invasion site (Figure 6C). In *fos-1(ar105)* mutants, increased levels of hemicentin::GFP localization did not occur under the AC (Figures 6D–6F), indicating that this accumulation is controlled by *fos-1a* activity. In vulvaless animals, hemicentin::GFP accumulated under the AC in a similar manner to wild-type animals (Figures 6G and 6H). At the normal time of AC invasion, however, the accumulated hemicentin was not removed in vulvaless animals in which AC invasion failed to occur (Figure 6I). Taken together, these results indicate that hemicentin accumulates in the basement membrane under the AC to promote basement-membrane removal and enable invasion. During successful invasion, hemicentin is then itself removed.

Discussion

The ability of cells to invade through basement membranes and enter new tissues is crucial to normal morphogenetic processes and the spread of cancer. Our results show that the *C. elegans fos* transcription factor ortholog *fos-1a* is a key player in this regulatory network, promoting basement-membrane removal during AC invasion. We have also demonstrated that *zmp-1*, *cdh-3*, and *him-4* are downstream targets of *fos-1a* that have subtle roles in promoting this process (Figure 7).

fos-1a Functions in the AC to Promote Basement-Membrane Removal during Invasion

We have found that the *fos-1* gene specifically promotes basement-membrane removal during AC inva-

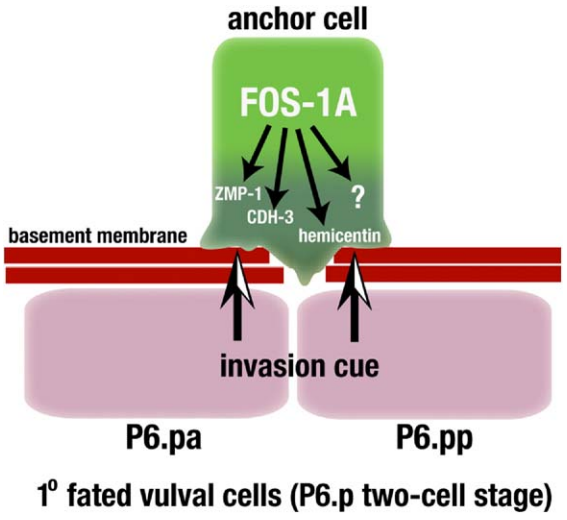

Figure 7. FOS-1A Promotes Removal of the Basement Membrane during AC Invasion

Schematic diagram showing the basolateral portion of the AC (dark green) breaching the underlying gonadal and ventral epidermal basement membranes and moving toward the vulval cells (1°-fated P6.pa and P6.pp). FOS-1A controls basement-membrane removal, in part through activating the expression of the matrix metalloproteinase ZMP-1, the Fat-like protocadherin CDH-3, and the extracellular matrix protein hemicentin. Additional targets of FOS-1A must also contribute to basement-membrane removal, as animals lacking ZMP-1, CDH-3, and hemicentin have only a modest invasion defect compared with animals deficient for FOS-1A. The diffusible cue from the 1°-vulval cells that stimulates invasion does not regulate FOS-1A. The 1° vulval cue and FOS-1A thus coordinately regulate AC invasion. FOS-1A controls the expression of components that mediate breaking down the basement membrane, while the vulval cue may direct invasive basolateral processes through this barrier.

sion. In *fos-1* mutants, the AC associates normally with the underlying gonadal basement membrane. At the late-L3 stage, however, the basement membrane is not removed, and the AC fails to execute invasion. In *fos-1* mutants, the 1° vulval cells still generate the invasion-promoting cue and the AC can still respond and extend normal mesenchymal-like processes toward these cells. These processes, however, accumulate at the basement membrane, demonstrating a requirement for *fos-1* to break down this barrier.

Our results further demonstrate that a specific isoform of the *fos-1* gene, *fos-1a*, is expressed at high levels in the AC during invasion and functions cell autonomously to regulate basement-membrane removal. The production of multiple isoforms from a single *fos* gene is not unique to *C. elegans*. An alternative splicing event in the mouse *fosB* gene generates two transcripts that have antagonistic activities (Nakabeppu and Nathans, 1991). Also, the *Drosophila D-fos* gene uses two start exons and generates differentially expressed transcripts encoding proteins with unique N termini (Souid and Yanicostas, 2003). The production of multiple isoforms from individual *fos* genes may be a common strategy for regulating the activity of genes in this family.

The presence of lysine in the third leucine repeat of FOS-1 where the hydrophobic residue leucine typically resides suggests that FOS-1 may have distinct dimerization properties. This position is involved in dimerization stability (Moitra et al., 1997), and charged residues such as lysine reduce the stability of dimerization (Tripet et al., 2000). Suggesting that this may be a conserved aspect of Fos activity within nematodes, a lysine is present at the same position in the predicted Fos proteins in *C. briggsae* and *C. remanei*, which diverged from *C. elegans* approximately 100 million years ago (Coghlan and Wolfe, 2002).

zmp-1, *cdh-3*, and *him-4* Are Targets of *fos-1a* that Promote AC Invasion

We have found that *zmp-1*, *him-4*, and *cdh-3* are targets of *fos-1a* that function together to promote basement-membrane removal during AC invasion. *fos-1a* is first expressed in the AC precursor approximately 3 hr prior to the initiation of *zmp-1*, *cdh-3*, and *him-4* expression during the L2 molt, consistent with *fos-1a* directly regulating the expression of these genes. In mammals, Fos proteins function as dimeric transcription factors with Jun proteins that bind to AP-1 regulatory elements. Fos/Jun dimers bind to the recognition sequence TGA(C/G)TCA (AP-1) most strongly in vitro (Nakabeppu et al., 1988). In vivo studies, however, have indicated that direct regulatory targets of Fos proteins often contain binding sites that deviate from this optimal recognition sequence (Chinenov and Kerppola, 2001). The *zmp-1* and *cdh-3* promoter regions that direct AC expression have been determined (Kirouac and Sternberg, 2003). The *cdh-3* AC element contains two degenerate AP-1 binding sites that differ from AP-1 by one nucleotide, while the *zmp-1* AC element has a single degenerate site. Thus, if FOS-1A directly regulates these genes, it is not through a consensus AP-1 binding site.

Animals lacking *zmp-1*, *cdh-3*, and *him-4* function showed modest defects in invasion; there was a delay in AC invasion in 25% of mutant animals compared with absence of invasion in most *fos-1* mutants. We have found that increased AC-specific expression of *zmp-1*, *cdh-3*, as well as increased levels of hemicentin under the AC in *fos-1* mutants does not rescue invasion. Thus, other targets of *fos-1a* must make critical contributions to basement-membrane removal.

zmp-1 encodes a functional MMP predicted to be GPI linked (Wada et al., 1998; Itoh et al., 1999). Human MMPs are strongly associated with metastasis and in vitro studies have shown that they stimulate cell-invasive activity in numerous cell lines (Egeblad and Werb, 2002). The function of MMPs in regulating cell invasion, however, has been difficult to establish because of the multiple cellular functions that MMPs regulate (Stetler-Stevenson and Yu, 2001). A *zmp-1(cg115)* null mutant alone does not cause a defect in AC invasion but does enhance the delay in AC invasion observed in *cdh-3* null mutants. The ZMP-1 protein often shows increased localization to the invasive membrane domain of the AC. Thus, *zmp-1* may play a subtle role in contributing to basement-membrane removal during AC invasion.

The *cdh-3* gene encodes a Fat-like protocadherin required for the proper morphogenesis of the epithelial hyp10 cell (Pettitt et al., 1996; Hill et al., 2001). In *Dro-*

sophila and mice, Fat-like protocadherins regulate epithelial morphogenesis, as well as cell polarity (Ciani et al., 2003; Castillejo-Lopez et al., 2004; Moeller et al., 2004; Yang et al., 2002). Our work suggests that Fat-like cadherins can also promote cell-invasive behavior. The mouse Fat1 protein localizes to the leading edge of cellular protrusions and regulates the dynamics of these extensions (Moeller et al., 2004). We have found that CDH-3 localizes to the protrusive invasive membrane domain of the AC. CDH-3 may thus promote invasion by regulating aspects of the invasive cell membrane important for basement-membrane removal.

The *him-4* gene encodes hemicentin, an extracellular matrix protein of the six-member fibulin family of proteins (Vogel and Hedgecock, 2001; Argraves et al., 2003). Fibulins are thought to stabilize the organization of extracellular matrix structures, and some members promote integrin-mediated cell adhesion (Timpl et al., 2003). Human fibulin 2 is overexpressed in adenocarcinoma metastases of diverse origins (Ramaswamy et al., 2003), indicating that members of this family are associated with metastasis in solid tumors. In *C. elegans*, hemicentin regulates cell migration, cellularization of the germline, and the anchorage of cells to the epidermis (Vogel and Hedgecock, 2001; Emtage et al., 2004). Our work now extends these functions to include the regulation of basement-membrane removal during invasion. Mutants lacking functional hemicentin showed the greatest defect in AC invasion of all targets of *fos-1a* that we have identified. A full-length hemicentin::GFP transgene indicated that *fos-1a* controls the accumulation of high levels of hemicentin in the basement membrane under the AC just prior to its removal during invasion. Thus, hemicentin might promote basement-membrane removal either through a structural modification of the basement membrane or by fostering adhesive interactions with the AC.

The 1° Vulval Cells and *fos-1a* Function Together to Mediate Basement-Membrane Removal

The 1° vulval cells generate a cue that stimulates and directs AC invasion through the basement membrane toward these cells (Sherwood and Sternberg, 2003). We have found that *fos-1a* is expressed and localized to the AC nucleus normally in vulvaless animals. Also, the expression of the downstream *fos-1a* targets *zmp-1* and *cdh-3* are not altered, and hemicentin accumulates normally in the basement membrane under the AC. Thus, the 1° vulval signal does not stimulate AC invasion by regulating *fos-1a*. These results also indicate that *fos-1a* activity in the AC is not sufficient to specify full basement-membrane removal. Even though the AC initiates invasion, as evidenced by hemicentin deposition in the underlying basement membrane, it requires the vulval cue to complete invasion. The 1° vulval signal and *fos-1a* thus function together to promote basement-membrane removal (Figure 7). The 1° vulval cue may direct invasive protrusions from the AC into the basement membrane that are required to remove this barrier or could provide a component(s) necessary to break down the basement membrane.

Fos and Cell-Invasive Behavior

Many studies have suggested a link between Fos protein function and cell-invasive behavior. For example, Fra-1 is required for vessels of the chorio-allantoic plate to enter into the labyrinth trophoblast during mouse placentation, a morphogenetic event that depends on cell-invasive behavior (Schreiber et al., 2000). Moreover, overexpression of c-Fos and Fra-1 proteins, as well as the v-Fos oncogene, stimulates invasive behavior in cell lines (Hennigan et al., 1994; Reichmann et al., 1992; Kustikova et al., 1998). Human Fos proteins are overexpressed in many metastatic cancers (e.g., Gamberi et al., 1998; Zajchowski et al., 2001; Kataki et al., 2003). In mice, c-Fos is required for the progression of benign papillomas to malignant skin carcinomas and appears to be a key activator for expression of several MMPs (Saez et al., 1995). Despite these important observations, the cellular mechanisms by which Fos proteins promote placentation and possibly metastasis in vivo are not known. Our findings demonstrate a crucial in vivo requirement for the *C. elegans* Fos ortholog FOS-1A in controlling AC invasion into the vulval epithelium and indicate a function for this Fos protein in regulating basement-membrane removal during this process. We also demonstrate that FOS-1A promotes AC invasion in part through the activation of a *C. elegans* MMP, ZMP-1. Taken together, these results suggest that the Fos transcription factor is a conserved component of a genetic pathway underlying cell-invasive behavior, and that the mechanisms directing AC invasion may be widely utilized by invasive cells.

Experimental Procedures

General Methods and Strains

C. elegans strains were cultured at 20°C as in Brenner (1974). Some of the genes, alleles, and integrated transgenes used in this work were as follows: *evl-5(ar105)V*, which we have renamed *fos-1* (Seydoux et al., 1993); *rhIs23*(hemicentin::GFP)*III*, *him-4*(*rh319*)*X* (Vogel and Hedgecock, 2001); *rhIs2*(β^{PAT-3}::GFP)*I* (Plenefisch et al., 2000); *arIs50*(SEL-8::GFP)*II* (Doyle et al., 2000); *muIs27*(MIG-2::GFP) (Zipkin et al., 1997); *cdh-3*(*pk87*)*III* (Pettitt et al., 1996). See Supplemental Data for additional strains. Vulvaless animals were created as previously described (Sherwood and Sternberg, 2003). RNA-mediated interference (RNAi) was targeted against the complete *fos-1a* cDNA through ingestion of *fos-1a* dsRNA-expressing bacteria as described previously (Kamath et al., 2001). While this targeted both the *fos-1a* and *fos-1b* transcripts, no obvious *fos-1b* phenotypes were detected. See Supplemental Data for gene expression constructs, transgenic animals, and molecular methods used in this study.

Phylogenetic Analysis

Phylogenetic analyis was performed using the PHYLIP 3.6a3 program (Felsenstein, 1989).

Fluorescence-Intensity Measurements and Imaging

Fluorescence and Nomarski images were captured using a Hamamatsu ORCA-ER on a Zeiss Axioplan compound microscope and images were overlaid using Adobe Photoshop 7.0. Mean fluorescence intensity of AC expression of GFP, CFP, and YFP from transgenes in *fos-1(ar105)* mutants, vulvaless animals, and wild-type worms was calculated using the Openlab software package (Improvision).

Antibody and Basement-Membrane Staining

Staged L3 hermaphrodites were fixed with methanol and stained (Sherwood and Sternberg, 2003). Basement membranes were

stained with MitoTracker Red CMXRos (Molecular Probes) by placing worms in a solution of 10 μM MitoTracker Red in M9 buffer (Brenner, 1974) at 25°C for 2 hr. The worms were then allowed to recover for 30 min on an NGM agar plate. The MitoTracker Red mitochondrial staining was photobleached using a 30 s exposure to fluorescence through a TRITC filter set, leaving the basement membrane staining, which is resistant to photobleaching.

Scoring of AC Invasion

AC invasion was evaluated in relation to the timing of P6.p descendant divisions, the L3 molt, gonad reflection, and ventral uterine (VU) cell division (see Table S1).

Supplemental Data

Supplemental data include Experimental Procedures, one figure, and one table and can be found with this article online at http://www.cell.com/cgi/content/full/121/6/951/DC1/.

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fos-1a and *fos-1b* sequences were deposited in GenBank with accession numbers AY83542 and AY835433, respectively.