

Invadopodia and basement membrane invasion in vivo

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Over 20 years ago, protrusive, F-actin-based membrane structures, termed invadopodia, were identified in highly metastatic cancer cell lines. Invadopodia penetrate artificial or explanted extracellular matrices in 2D culture conditions and have been hypothesized to facilitate the migration of cancer cells through basement membrane, a thin, dense, barrier-like matrix surrounding most tissues. Despite intensive study, the identification of invadopodia in vivo has remained elusive and until now their possible roles during invasion or even existence have remained unclear. Studies in remarkably different cellular contexts—mouse tumor models, zebrafish intestinal epithelia, and *C. elegans* organogenesis—have recently identified invadopodia structures associated with basement membrane invasion. These studies are providing the first in vivo insight into the regulation, function, and role of these fascinating sub-cellular devices with critical importance to both development and human disease.

Introduction

Basement membrane is a thin, dense, sheet-like form of extracellular matrix that underlies all epithelia and encapsulates muscle, fat, and glial cells.^{1–3} The basement membrane arose at the emergence of animal multicellularity^{4,5} and is a key regulator of many cellular and tissue-level functions, including cell polarity, differentiation, organ shape, and tissue compartmentalization.^{6–11} Despite its dense and highly cross-linked structure, leukocytes and numerous migrating cells in development traffic through basement membranes.^{12–14} For example, during the epithelial-to-mesenchymal transitions (EMTs) that occur in gastrulation and neural crest migration, cells acquire the ability to invade through the epithelial basement membrane to initiate their migration to distant sites.^{15–17} In a similar manner, cancer cells are also thought to acquire the ability to breach basement membrane to enable metastasis, the most lethal step in cancer progression.¹⁸ Largely because of its importance in cancer, there has been significant

interest in understanding the mechanisms cells utilize to invade (or transmigrate) basement membrane barriers. A better understanding of the mechanisms cells use to cross basement membrane should facilitate strategies to therapeutically modulate invasive cellular behavior.¹⁹

A considerable amount of attention has focused on the potential role of invadopodia, F-actin-based, highly protrusive, matrix-degrading membrane structures, in directing cancer cell invasion through basement membrane.^{20–23} Invadopodia depend upon integrin for their formation and are often enriched with the actin regulators Arp2/3 and Wasp, the signaling protein Src, the scaffolding proteins cortactin and Tks5, and the matrix metalloproteinase MT1-MMP.²³ Another membrane-associated structure highly similar to invadopodia, called podosomes, has also been identified. Podosomes, however, are generally associated with non-transformed cells involved with matrix remodeling events, such as osteoclasts and vascular smooth muscle cells, and not basement membrane invasion.^{23–29} The term invadosomes has recently been used to encompass both structures,^{21,30–32} recognizing that invadopodia and podosomes (and possibly other protrusive membrane structures) likely represent more of a continuum than completely distinct entities.³³

Invadopodia were first identified over 20 y ago in transformed fibroblasts and human cancer cell lines and have since been observed in primary tumor cells from human patients.^{34–37} Reflecting the growing interest in these structures, more than half of the approximately 350 scholarly articles on invadopodia have been published within the last 3 y. Although recent advances have been made in analyzing invadopodia-like protrusions in 3D culture conditions,^{19,38} most mechanistic studies on invadopodia have been performed with cancer cell lines in 2D cell culture environments. The advantages in imaging, biochemical approaches, and genetic and pharmacological perturbations in 2D conditions have led to remarkable advances in our understanding of invadopodia.^{22,39–42} These findings include the identification of different components of invadopodia,^{43–45} elucidation of the stages of invadopodia formation,^{46–49} identification of genes associated with cancer metastasis that regulate invadopodia formation,^{19,21,50–52} understanding of the trafficking of proteases to invadopodia,^{53–57} and examination of invadopodia membrane dynamics.⁵⁸ Key insights have also been gained into the regulation of their formation by growth factors, integrin activation, and the microenvironment, including hypoxia, matrix stiffness,

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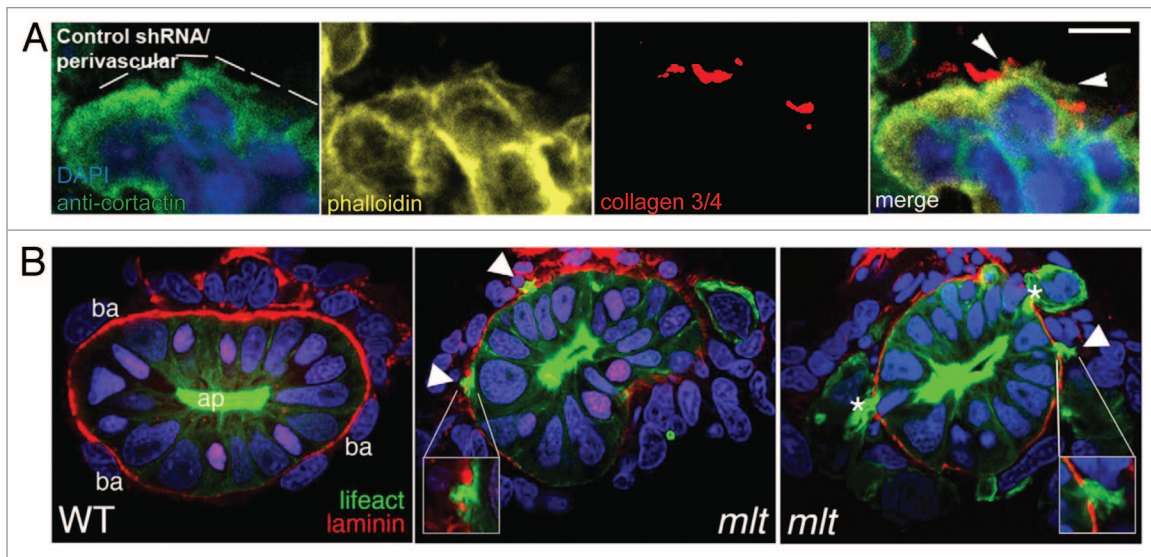


Figure 1. In vivo invadopodia in rat and zebrafish transformed cells. **(A)** Immunohistofluorescence of MTLn3-derived perivascular tumors (DAPI, blue; phalloidin, yellow; anti-cortactin, green; dotted line indicates boundary with vasculature, which is encapsulated with a basement membrane) indicate that protrusions (arrowheads) are associated with sites of collagen degradation (an antibody specific for degraded collagen I, Collagen $\frac{3}{4}$, red). **(B)** Cross sectional images of a zebrafish larvae showing intestinal epithelia in wild-type (left) and *mlt* mutants (center and right). Basement membrane is shown in red (laminin immunostain) and nuclei are stained blue (DAPI). Actin-rich (green, lifeact) protrusions in *mlt* intestine are associated with sites of basement membrane loss (arrowheads and insets). An asterisk labels an epithelial cell invading into the tissue stroma through a cleared region of the basement membrane. Scale bar is 10 μ m. Images in **(A)** reprinted with permission, ©Gligorijevic, B., et al. 2012. Originally published in *J Cell Sci*. doi#10.1242/jcs.092726. Images in **(B)** reprinted with permission, ©Sieler, C., et al. 2012. Originally published in *PLOS Biol*. doi#10.1371/journal.pbio.1001386.

and metabolism.^{28,59-61} Despite intensive study, one of the greatest gaps in the field has been establishing whether these structures actually exist in vivo, and if so, how they facilitate basement membrane transmigration.^{21,62,63} In this review, we present recent work from our lab and others that have indentified invadopodia in vivo. Although the term invadopodia is typically used to describe invasive structures in cancer cells, we propose that invadopodia are components of a normal and likely ancient invasion program utilized by cells to pass through basement membrane during development that is co-opted by transformed cells. Further, we suggest that work in model systems such as zebrafish and *C. elegans* offers new and powerful approaches to understand the role of invadopodia in basement membrane transmigration, as well as insights into the physiological cues and intrinsic programs that govern their formation and function.

The Challenge of Studying Invadopodia In Vivo

The greatest impediment to identifying invadopodia in vivo has been the difficulty of visualizing interactions at the cell–basement membrane interface during the process of invasion. In tumor models, cancer cell invasion events are highly dynamic, rare, and unpredictable; it is thus challenging to catch cells in the act of invading.⁶⁴ Further, invasion events often occur deep in complex tissues and require simultaneous visualization of the invasive cell and basement membrane. As live-cell fluorescent-based basement membrane reporters are currently not available in vertebrate models, imaging basement membrane has been

limited to ex vivo assays and fixed samples,⁶⁵⁻⁶⁸ thus hindering the ability to detect and examine the dynamics of invadopodia in native settings.

Cancer Cells Generate Invadopodia Protrusions In Vivo

Despite the challenges of in vivo studies, a significant body of work has shed remarkable insight into cancer cell invasion in physiological settings.^{20,62,64,69,70} The Condeelis and Segall groups have pioneered intravital (optical live-animal) imaging techniques, as well as a tumor model where GFP-expressing MTLn3 cells (MTLn3-GFP), a highly invasive rat mammary adenocarcinoma cell line that forms invadopodia in vitro, are injected into the mammary gland of immunocompromised mice and rats and allowed to form tumors.^{20,71} Tumors derived from MTLn3 cells metastasize to lung, allowing tumor dissemination and invasion to be studied. Live-cell imaging and intravenous injection of rhodamine dextran have permitted visualization of MTLn3-GFP in the act of invading into vasculature, where these cells must pass through the vascular basement membrane.⁷² Recently, utilizing cryosectioning and immunolocalization, protrusions from MTLn3-GFP adjacent to blood vessels have been identified that contain the invadopodia markers cortactin and N-WASP (key F-actin regulators; Fig. 1A).⁷³ The formation of these protrusions also correlates with areas of collagen I degradation, an interstitial matrix component (Fig. 1A). Intriguingly, reduction of N-WASP, which regulates invadopodia in vitro,⁷⁴ resulted in reduced

protrusions, and correlated with a dramatic reduction in collagen degradation and circulating tumor cells, consistent with an inability of these cells to enter the vasculature. Mouse–tumor model studies with Ras transformed HMLE cells expressing Twist (an immortalized human mammary epithelial cell line), have also revealed punctate subcellular invadopodia-like structures within tumor cells.⁷⁵ Inhibition of invadopodia formation through targeting of the PDGF receptor α and the scaffold protein Tks5 inhibited the local invasive ability of the HMLE cells expressing Twist and the tumors remained encapsulated. Stable knockdown of Tks5 also resulted in reduced metastasis in a mouse model for metastatic lung adenocarcinoma.⁵² These results strongly support the idea that invadopodia are critical for cancer cell dissemination in vivo and suggest that these structures might be mediating basement membrane transmigration.

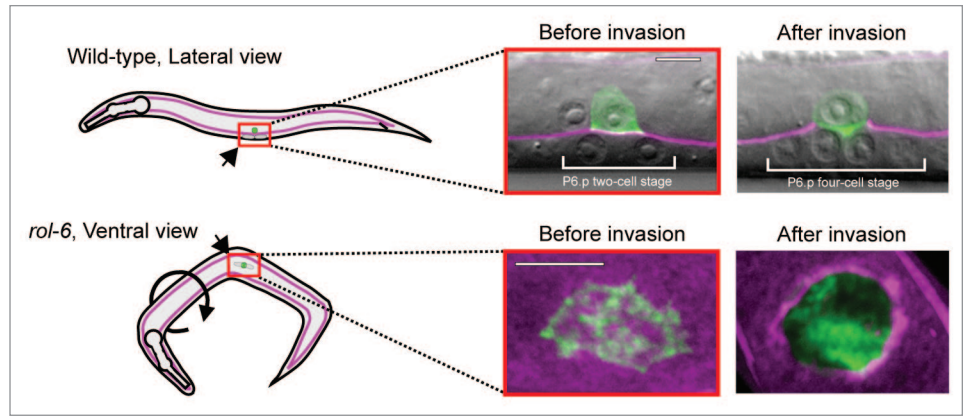


Figure 2. *C. elegans* anchor cell invasion imaged laterally or ventrally. The AC is viewed from two different perspectives for time-lapse imaging, depicted in the schematic (left), and examples of the images from these perspectives are shown (right). The basement membrane is visualized with laminin::GFP (magenta) and F-actin (green) with an AC-specific F-actin probe (*cdh-3 > mCherry::moeABD*). Fluorescence is overlaid on differential interference contrast images. Wild-type animals (top) lie on their side, resulting in lateral imaging of the AC–basement membrane interaction. Roller mutant animals (bottom) orient randomly, permitting ventral imaging within the plane of the basement membrane. Confocal slices through the AC–basement membrane interface are shown at 2X magnification relative to the lateral view panels. Subsequent panels in this review of the AC are labeled with their perspective used for imaging. Scale Bars, 5 μ m. This figure is reprinted with permission, ©Hagedorn, E.J., et al. 2013. Originally published in *J Cell Biol.* doi#10.1083/jcb.201301091.

Invadopodia-Like Protrusions in Transformed Zebrafish Intestinal Epithelium

Recent work by the Pack group has modeled epithelial cell invasion in transformed cells in zebrafish.⁷⁶ Intriguingly, zebrafish harboring a mutation that results in constitutive activity of the smooth muscle myosin heavy chain gene (*mlt* mutants) show a striking invasive remodeling of the neighboring intestinal epithelium. Constitutive activation of *mlt* in the smooth muscles causes an increase in smooth muscle contractile tone. Unregulated smooth muscle contractile tone is thought to result in the production of reactive oxygen species, which non-autonomously stimulates invasive remodeling in the neighboring intestinal epithelium.⁷⁶ This invasive remodeling includes the appearance of cellular protrusions that contain the invadopodia markers cortactin and Src.⁷⁶ These protrusions extend through regions of the intestinal epithelial basement membrane that are devoid of the basement membrane protein laminin, which is consistent with a potential role of invadopodia breaching the basement membrane (Fig. 1B). Further, their formation is dependent on Tks5, as well as the non-receptor tyrosine kinase Src, an upstream regulator of invadopodia formation.^{23,77}

Recent studies have suggested that loss or disruptions of basement membrane may stimulate protrusions and invasive behavior.^{8,78,79} Thus, contractions from the smooth musculature, which is tightly associated with the intestinal epithelia, could tear the basement membrane, inducing epithelial invasive behavior and escape. While this possibility was not formally excluded in this

study, a constitutively activate form of Src expressed specifically within the intestinal epithelium in otherwise wild-type animals, led to ectopic F-actin-rich protrusions that were present in fixed samples at sites of apparent breaks in basement membrane.⁷⁶ This result adds weight to the notion that the invadopodia-like protrusions from the intestinal epithelial cells may be actively breaching the basement membrane. Interestingly, Src was not sufficient to promote escape of these cells from the epithelium, thus Src might be a specific regulator of invadopodia and does not stimulate a full EMT transition. The work in zebrafish offers further evidence that invadopodia are present in vivo and may mediate breaches in basement membrane.

Invadopodia Breach Basement Membrane During *C. elegans* Organogenesis

During uterine–vulval development in the nematode worm *C. elegans*, a specialized uterine cell called the anchor cell (AC), breaches the juxtaposed uterine and vulval epithelial basement membranes to initiate uterine–vulval attachment.^{80,81} AC invasion is highly stereotyped, occurring over a narrow developmental window. The major structural components of basement membrane (laminin and type IV collagen) as well as minor components (SPARC, hemicentin, and fibulin) have been functionally tagged with GFP or GFP-derivatives in *C. elegans*.^{82–86} Furthermore, specific proteins of interest can be tagged and expressed within the AC.⁸⁷ The ability to easily manipulate the orientation of the worm permits imaging of AC invasion from the lateral viewpoint, as well as within the plane of the cell–basement membrane interface (Fig. 2). The combination of these

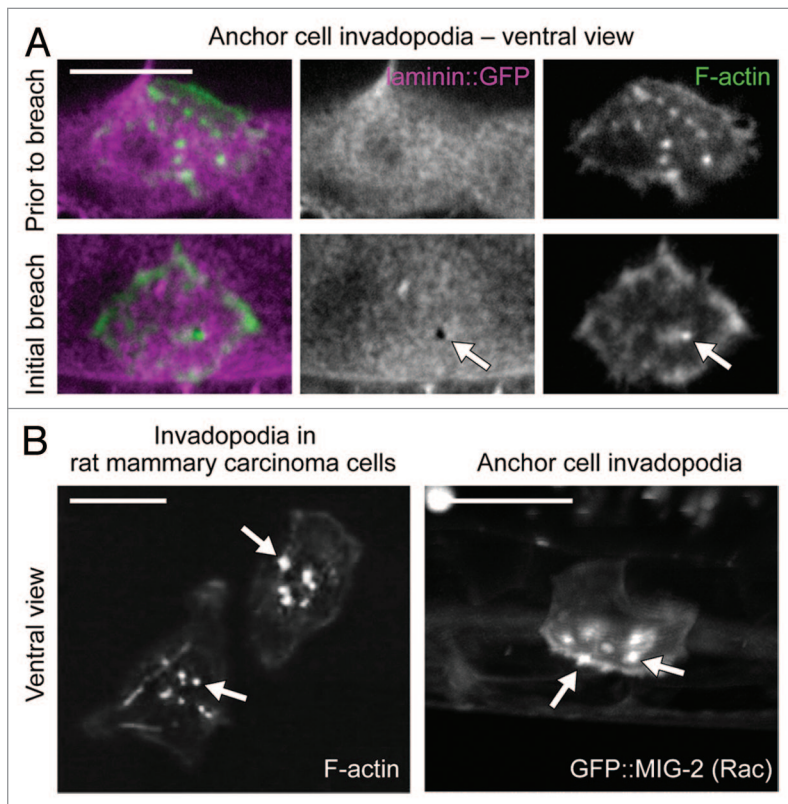


Figure 3. AC invadopodia breach the basement membrane and are similar to cancer cell invadopodia. **(A)** A ventral view time-lapse series of AC invadopodia (actin, green) and the basement membrane (laminin, magenta). Prior to a detectable breach of the basement membrane (top), the AC has many punctate F-actin structures. Upon breach of the basement membrane (bottom), a single F-actin-rich structure occupies the gap in the basement membrane (arrow). **(B)** Phalloidin staining of F-actin in MtLn3 plated on fibronectin- and gelatin-coated coverslips labels invadopodia (left). An AC-specific translational reporter for a *C. elegans* Rac ortholog (right), GFP::MIG-2, labels invadopodia in a lateral image tilted 45 degrees back. These two models allow for resolution of individual invadopodia. AC scale bars, 5 μ m; cancer cell scale bar, 20 μ m. Left panel of **(B)** is reprinted with permission**, ©Yamaguchi, H., et al. 2005. Originally published in *J Cell Biol.* 168(3):441–52.

perspectives has facilitated the first real-time, high-resolution imaging of cell–basement membrane interactions during invasion in vivo.¹²

Using live-cell imaging of F-actin and actin regulators, we have recently found that the AC breaches the basement membrane with invadopodia (Fig. 3A).⁸⁸ Prior to invasion, these F-actin-rich foci, which form at the cell–basement membrane interface, look strikingly similar to invadopodia from cancer cells generated upon contact with gelatin and other artificial matrices plated on glass slides (Fig. 3B). The *C. elegans* invadopodia also have molecular markers shared by cancer cell invadopodia, including the membrane anchored Rac GTPases, the actin regulatory protein Ena/VASP, and the phospholipid PI(4,5)P₂.^{44,89} Similar to cancer cell invadopodia, formation of these structures in the AC is dependent on integrin function.^{88,90,91} Validating their ability to breach basement membrane, time-lapse analysis indicated that these structures depress basement membrane during turnover, and one always presages and then occupies the first visible breach in the

basement membrane (Fig. 3A). These studies confirm that invadopodia breach basement membrane in vivo and strongly suggest that invadopodia are a conserved subcellular device utilized in normal development to penetrate basement membrane barriers.

One of the more interesting aspects of AC invadopodia is how rapidly they turnover and puncture basement membrane. Whereas invadopodia in cancer cells can persist over an hour,²¹ the average lifetime of AC invadopodia is approximately 1 min, with the invadopodium that breaches the basement membrane persisting for approximately 5 min.⁸⁸ The reasons and significance of these dynamics between in vitro and AC invadopodia are unclear, and may reflect differences in the microenvironment (signaling or matrix properties) or intrinsic factors within the cells. It is notable that the entire process of AC invasion through the juxtaposed uterine and vulval basement membranes is completed in only 90 min.^{80,87} In contrast, breast and colon cancer cells plated on native basement membranes in ex vivo assays take days to complete invasion.^{65,68} It seems likely that these ex vivo assays may not recapitulate the in vivo signaling environment that mediates rapid invasion. Alternatively, cancer cells might be less efficient in their ability to invade basement membrane.

Over 50 proteins as well as specific phosphoinositide lipids are known to be associated with invadopodia in cancer cells.^{43,44} Using genetic, expression, and RNAi based screens, we have identified over 100 genes that regulate AC invasion or are expressed specifically in the AC.^{87,92–95} It will be important to determine their possible connection to AC invadopodia, as this will likely increase our understanding of the regulation of invadopodia as well as identify pathways that function in parallel with invadopodia to promote invasion. Most proteins strongly associated with invadopodia in cancer cells are encoded in the *C. elegans* genome and many of these have been identified in screens for genes promoting AC invasion (Table 1). Several important regulators, however, are absent. These include the actin regulator and adaptor protein cortactin, the Tks4/5 adaptor proteins, and the transmembrane matrix metalloproteinase MT1-MMP (Table 1).^{42,44,96} Other proteins may functionally compensate for these absences. For example, similar to cortactin, the highly conserved Abp1 actin-binding protein also activates the Arp2/3 complex.^{97,98} In addition, *C. elegans* encodes a predicted GPI membrane anchored matrix metalloproteinase, *zmp-1*, which is specifically expressed in the AC at the time of invasion. Although loss of *zmp-1* does not disrupt invasion in isolation, it is possible that one or more of the five other matrix metalloproteinases in *C. elegans* function redundantly with ZMP-1 during invasion.⁹⁹ Finally, it will be important to understand the potential role of PI(4,5)P₂ and other phosphoinositides in AC invadopodia. In cancer cells, these lipids have been proposed to play important roles in recruiting and activating actin regulators within

Table 1. Major cancer cell invadopodia proteins and *C. elegans* orthologs

Human protein	<i>C. elegans</i> ortholog	Category	Known role in AC invasion
Src ¹²⁷	SRC-1/2	Tyrosine kinase	
Tks4/5 ¹²⁸	none	Src associated adaptor protein	
Cortactin ¹²⁹	none	Cytoskeleton regulation	
Abl family kinases ^{130,131}	ABL-1	Tyrosine kinase	
MMPs ^{56,132}	ZMP-1–6	Matrix degradation	+ ⁸⁷
β1 and β3 integrins ^{133,134}	PAT-3	Cell adhesion	+ ⁹¹
Actin ¹³⁵	ACT-1	Cell structure and signaling scaffold	+ ⁹¹
Fascin ¹³⁶	FASN-1	Cytoskeleton regulation	
Arp2/3 ⁷⁴	ARX-1–7	Cytoskeleton regulation	+ ⁹²
Cofilin ⁷⁴	UNC-60	Cytoskeleton regulation	+ ⁹²
WASp family ¹³⁷	WSP-1	Cytoskeleton regulation	
Ena/VASP/Mena ¹³⁸	UNC-34	Cytoskeleton regulation	+ ⁹³
PKC ¹³⁹	PKC-1/2	Serine/Threonine kinase	
Fak/Pyk2 ^{140,141}	KIN-32	Tyrosine kinase	
Paxilin ^{139,142}	PXL-1	Cytoskeleton regulation	
Nck ⁷⁴	NCK-1	Scaffold protein	
PI3K ¹⁴³	AAP-1	Kinase	
Rho family GTPases ¹⁴⁴⁻¹⁴⁶	RHO-1	Cytoskeleton regulation	
	RAC-2, MIG-2, CED-10	Cytoskeleton regulation	+ ⁹³
	CDC-42	Cytoskeleton regulation	+ ⁹²

invadopodia, as well as linking the F-actin-based invadopodium core to the plasma membrane.^{47,100}

Invadopodia Are Only One Component of the Invasion Process In Vivo

One of the advantages of examining invadopodia at high resolution in vivo is elucidating the role they play in the context of the complete process of invasion. By following the fate of breached AC invadopodia in *C. elegans*, we have also discovered that soon after penetrating the basement membrane, a large protrusion forms at the site of breach, crosses the basement membrane, and intercalates between cells of the underlying vulval epithelium (Fig. 4). Formation of this protrusion correlates with cessation of invadopodia formation, which likely prevents or reduces multiple breaching events. The *C. elegans* ortholog of the vertebrate netrin receptor DCC (UNC-40) plays a critical role in mediating the morphogenetic transition from invadopodia-driven basement membrane breaching to the formation of a protrusion that extends through the basement membrane.^{88,93} During BM breaching by invadopodia, UNC-40 traffics to the initial breach and directs the formation of the invasive protrusion by recruiting and concentrating the same F-actin effectors that generate invadopodia (Fig. 4).⁸⁸ UNC-40 accumulation at the breach site appears to act as a molecular sink that depletes F-actin regulators at invadopodia—in the absence of UNC-40, invadopodia persist, multiple breaching events occur, and

a large invasive protrusion fails to form (Fig. 4). This elegant mechanism shuts down invadopodia and focuses invasion through a single basement breach. The switch from invadopodia to invasive protrusion-driven changes probably involves mechanisms to dynamically add membrane and actin regulators that facilitate extension of the actin network. Furthermore, in cell culture, microtubules and the intermediate filament protein vimentin are required to extend invasive protrusions.⁶⁸ Mechanisms regulating an invadopodia-to-invasive protrusions switch likely occur in other invasive cells as electron microscopy and immunofluorescence studies have observed single protrusions from cancer cells crossing basement membrane in ex vivo invasion assays.^{65,68} Whether these are also regulated by the netrin receptor DCC is unknown, but is possible given the strong association of netrin with metastatic cancers and invasive behavior in cell culture.¹⁰¹⁻¹⁰⁴ Importantly, after breaching basement membrane (a 2D flat, sheet-like surface), cancer cells often enter interstitial tissue, a largely acellular environment dominated by a less dense 3D network of fibrillar collagens.¹⁰⁵ This environment appears to trigger the formation of invadopodia-like structures that promote removal of interstitial matrix at points of cell restriction.^{38,106-108} Thus, invadopodia dynamics are likely rapidly tuned to the matrix environment encountered by invasive cells.

The AC also specifically modifies the composition of the basement membrane targeted for invasion. It has been known for sometime that cancer cells secrete normal as well as novel basement membrane proteins and matrix-modifying enzymes.¹⁰⁹⁻¹¹³

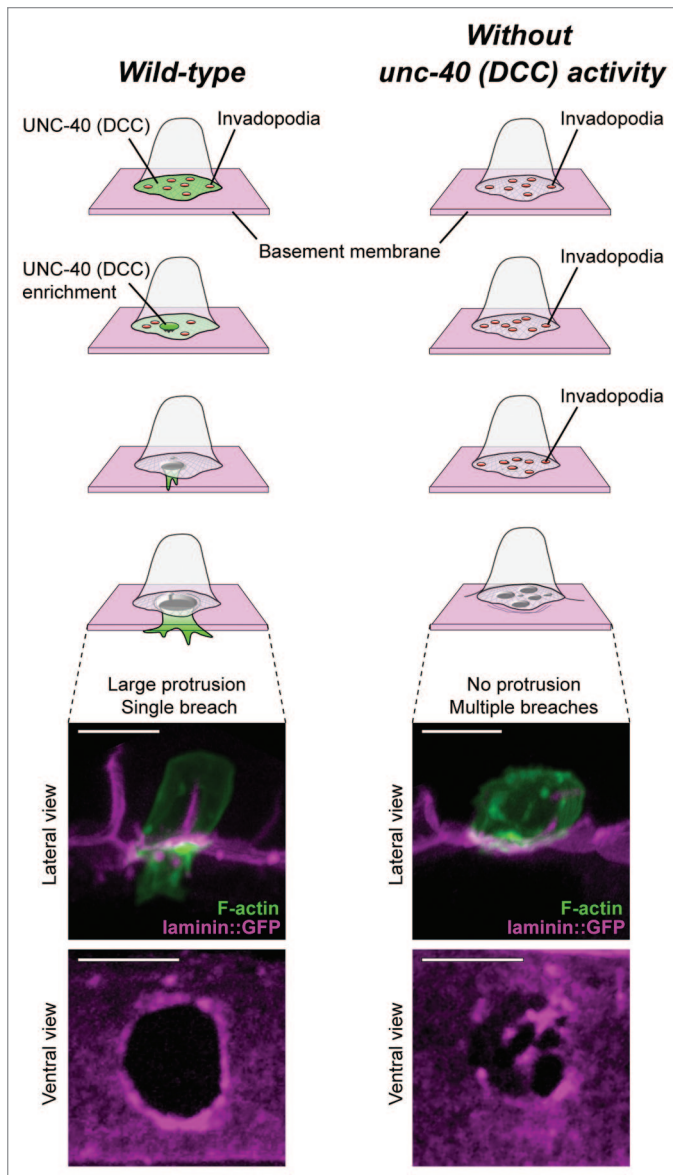


Figure 4. The netrin receptor DCC (UNC-40) mediates invadopodia to invasive protrusion transition. In wild-type animals (left), invadopodia (red circles) are dynamic and rapidly turn over until one breaches the basement membrane. UNC-40 (green) localizes to the initial breach and mediates the transition from many invadopodia to a single invasive protrusion that extends through the single gap in the basement membrane. The invasive protrusion opens a single gap in the basement membrane, allowing the basal membrane of the AC to contact the underlying vulval cells. A lateral view image shows the invasive protrusion extending (actin, green) through a single gap in the basement membrane (laminin, magenta). A ventral view image shows the widened gap through the basement membrane. In the absence of UNC-40 (right), invadopodia persist after the initial breach in the basement membrane, an invasive protrusion fails to form, and multiple breaches through the basement membrane occur. A lateral view image shows the lack of invasive protrusion formation in the AC and a ventral view images shows the multiple breaches through the basement membrane. Scale bars, 5 μ m. This figure is adapted from and reprinted with permission, ©Morrissey, M.A., et al., 2013. Originally published in *Worm*. doi#20.4262/worm.26169 and ©Hagedorn, E.J., et al. 2013. Originally published in *J Cell Biol*. doi#10.1083/jcb.201301091.

How this may specifically contribute to invasion and tumor metastasis is poorly understood. Just prior to invasion, the AC secretes the conserved extracellular matrix protein hemicentin specifically under the AC in the basement membrane that will be crossed.^{84,87} Although the precise function of hemicentin is unclear, deposition of hemicentin assists basement removal during invasion.⁸⁷ Active modification of the basement membrane might change its structural properties to facilitate invadopodia penetration and precise removal.

One possible function for hemicentin is to promote physical displacement of the basement membrane. Although it has been generally assumed that basement membrane is dissolved by proteases during invasion,¹¹⁴ optical highlighting of basement membrane components using a photoconvertible Dendra tag^{83,115} has revealed that the basement membrane under the AC is removed, in part, by physical means.⁸⁸ The protrusion generated by the AC appears to generate forces that physically shift the basement membrane. Given that the protease *zmp-1* is expressed throughout AC invasion, *zmp-1* and other proteases might assist invasion by weakening the structural make-up of the basement membrane to facilitate physical displacement. Further sensitized screening, as well as profiling gene expression in the AC using single cell isolation techniques,¹¹⁶ will help reveal mechanisms of how basement membrane is modified to assist in cell invasion events.

Summary and Outlook

The recent studies highlighted here have established that invadopodia and invadopodia-like protrusions exist in vivo. Although examination of invadopodia and cell invasion in natural settings are inherently more challenging due to limitations in visual analysis, experimental manipulations, and examination of large numbers of events, these studies will be essential in establishing how invadopodia are regulated and function during invasion. It is already clear from in vivo studies in zebrafish and *C. elegans* that while invadopodia mediate basement membrane breaching, they are not sufficient to facilitate basement membrane crossing or full removal of this barrier. The use of model systems such as zebrafish and *C. elegans*, with their visual accessibility, simple tissue architecture, transgenics, and amenability to genetic analysis, offers a potent complementary approach to tumor models to answer some of the most pressing questions concerning invadopodia and cell invasion. These include the identities of the signaling pathways that control their formation and regulation, and the cell-intrinsic factors, including transcriptional networks that facilitate invadopodia generation in cells. Identification of other models of invadopodia-basement membrane breaching events in vivo will also be valuable in elucidating the range of invadopodia dynamics and activity in breaching basement membrane. Perhaps most importantly, in vivo studies will be critical in determining how widespread the use of invadopodia are in breaching basement membrane. For example, are invadopodia used in EMT events in development and cancer to facilitate basement membrane crossing? While several studies

have implicated invadopodia with EMT,^{75,117} direct visualization of their activity and formation is lacking. Moreover, there is evidence that cells cross basement membrane through a variety of mechanisms, including downregulation of basement membrane receptors,¹¹⁸ physical forces that shift or breach the basement membrane,^{79,83,119} entry through preformed portals,^{66,67,120,121} and remodeling events that create absences or holes in the basement membrane.^{62,122-124} These studies will be important in evaluating whether targeting invadopodia would be an efficient approach to blocking cancer metastasis. The observation of invadopodia in nematodes, which diverged from the lineage that gave rise to vertebrates approximately 600 million y ago,^{125,126} argues strongly that invadopodia are ancient and likely widely utilized structures for basement membrane transmigration. We expect

the expansion of *in vivo* studies will be essential in clarifying the utilization of invadopodia in invasive cell contexts and reveal novel aspects of the regulation and function of these fascinating basement membrane-piercing structures.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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