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Invadosomes in their natural habitat

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Abstract

Podosomes and invadopodia (collectively known as invadosomes) are small, F-actin-rich protrusions that are located at points of cell-ECM contacts and endow cells with invasive capabilities. So far, they have been identified in human or murine immune (myelomonocytic), vascular and cancer cells. The overarching reason for studying invadosomes is their connection to human disease. For example, macrophages and osteoclasts lacking Wiskott-Aldrich syndrome protein (WASp) are not able to form podosomes, and this leads to altered macrophage chemotaxis and defective bone resorption by osteoclasts. In contrast, the ability of cancer cells to form invadopodia is associated with high invasive and metastatic potentials. While invadosome composition, dynamics and signalling cascades leading to their assembly can be followed easily in *in vitro* assays, studying their contribution to pathophysiological processes *in situ* remains challenging. A number of recent papers have started to address this issue and describe invadosomes *in situ* in mouse models of cancer, cardiovascular disease and angiogenesis. In addition, *in vivo* invadosome homologs have been reported in developmental model systems such as *C. elegans*, *zebrafish* and sea squirt. Comparative analyses among different invasion mechanisms as they happen in their natural habitats, i.e., *in situ*, may provide an outline of the invadosome evolutionary history, and guide our understanding of the roles of the invasion process in pathophysiology versus development.

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Keywords

invadopodia; podosomes; invadosomes; protrusions; invasion; cell motility; cell migration; cell locomotion; cancer; development; in vivo; in situ; microenvironment; microscopy; multiphoton; confocal

Introduction

Podosomes and invadopodia, collectively known as invadosomes, are specialized microdomains of the plasma membrane. They are defined by their morphology, structure and function as small F-actin-rich protrusions located at points of cell-extracellular matrix (ECM) contact that have (ECM)-degrading capability (Linder et al, 2011). Invadosomes commonly precede invasion; cell movement through obstacles into a new type of environment. Invasion occurs during both physiological and pathological processes, including different stages of embryonic and tissue development, inflammation, wound-healing and cancer metastasis. So far, invadosomes have been studied *in vitro* and reported in vascular cells, myelomonocytic cells (osteoclasts, monocytes), cancer cells and fibroblasts which are either transformed with oncogenic viruses or associated with cancer cells (cancer-associated fibroblasts) (Goicoechea et al., 2014).

Historically, the structures were discovered in chicken embryo fibroblasts transformed with the Rous Sarcoma Virus oncogene v-Src (David-Pfeuty and Singer, 1980) and named rosettes. In subsequent studies, the term podosome was preferred for describing these cell-ECM contacts containing individual actin-rich cores (Tarone et al., 1985), while a podosome rosette referred to self-organized groups of podosomes (Destaing et al., 2003). Finally, the term invadopod/invadopodium was introduced when a third team discovered that these structures were not only mediating adhesion but also invasion through their capability to degrade the ECM (Chen et al., 1989). When invadosomes were detected in other cell types, it became clear that invadosome architecture depends both on the cell type considered and on the experimental setting used for their observation. For a while, nomenclature became a major issue that complicated the interpretation and comparison of published data. Different classifications arose, including use of the term podosome when the structure extends upwards from the ventral cell surface into the cytoplasm on stiff substrata whereas long filopodia-like membrane extensions that penetrate into the ECM were referred to as invadopodia. Currently, a consensus seems to have emerged: invadosomes are referred to as podosomes when they are found in vascular and myelomonocytic cells and as invadopodia when they are found in cancer cells. The term invadosome is used when no distinction is being made between podosomes and invadopodia and also includes structures found in Src-transformed fibroblasts (which present mixed features of podosomes and invadopodia).

Invadosomes on 2D surfaces appear as punctate dynamic protrusions formed at the points of cell-ECM contact, initiated by growth factors or hypoxia (Diaz et al., 2013), highly enriched with filamentous actin (F-actin) and oriented perpendicularly to the substratum (Murphy and Courtneidge, 2011). Actin-regulatory proteins (e.g., Arp2/3, WASp/N-WASp, cortactin, dynamin, gelsolin and cofilin) are consistently found in invadosomes in close association with F-actin, together with integrins (e.g., $\beta 1$, $\beta 2$, $\beta 3$) (Beaty et al., 2013, Calle et al., 2006),

adhesion molecules (e.g., talin), lipids (e.g., PI(3,4)P2), GTPases (e.g., Cdc42) and non-receptor tyrosine kinases (e.g., Src). The scaffolding protein and Src substrate Tks5 also colocalizes with F-actin and constitutes a reliable invadosome marker (Murphy & Courtneidge, 2011). A key feature of invadosomes, which is in contrast to other cell adhesion devices (focal adhesions) or other protrusions related to motility (e.g., pseudopodia, lamellipodia), is their ECM-degrading capability enabled by resident metalloproteases (MMPs) which provide *invasive* capabilities. Such a distinction is not absolute as one study reports on low levels of ECM degradation by focal adhesions (Wang and McNiven, 2012) whereas another one describes physical displacement of the ECM as a key element enabling invasion (Hagedorn et al. 2013). In addition to the role in invasion, dynamic behavior of the organelle has now been associated with a mechanosensing function in some cell types (Collin et al., 2008; Labernardie et al, 2010; Van den Dries et al, 2013). Invadosomes are therefore expected to contribute to a wide range of biological processes. As mentioned, the structures may differ in their size and appearance, abundance, dynamics and subcellular localization and arrangement of structural components, depending on the cell type considered (for more on differences between invadopodia and podosomes, see (Murphy & Courtneidge, 2011)). Thus, the architecture, properties and subcellular distribution of invadosomes may reflect, at least in part, the cellular processes in which they are involved.

One of the main results of invadosome research is the connection of both podosomes and invadopodia with human diseases, which revealed their integral role in biological processes. Invadopodia are commonly studied in cell lines derived from solid cancers. The evidence for their existence in human tumors came from primary cells isolated from head and neck, bladder and brain tumors (Clark et al., 2007; Sutoh et al., 2010; Stylli et al., 2008). Cancer cells with invadopodia have high invasive potential *in vitro* and metastatic potential in mouse transplants (Coopman et al., 1998). Podosomes are extensively studied in cells of the myelomonocytic lineage such as macrophages, immature dendritic cells (iDCs) and osteoclasts. Pioneering studies performed by Linder and colleagues reported that macrophages from patients expressing truncated forms of WASp completely lack podosomes (Linder et al, 1999). Macrophages and iDCs devoid of podosomes show impaired chemotaxis, and WASp null osteoclasts exhibit abnormal patterns of bone resorption both *in vitro* on bone slices and *in vivo* (Calle et al, 2004). Other cell types, such as endothelial cells (ECs) or smooth muscle cells (VSMCs) in the vascular system, also have the ability to form podosomes. In these cells, podosomes are not present in quiescent cells but arise in response to certain cytokines, providing a conceptual framework to explore the role of podosomes in the pathogenesis of some vascular diseases.

Podosomes and invadopodia have been extensively studied in *in vitro* models and on two-dimensional (2D) surfaces. Their similarities and differences, as well as their relationship to focal adhesions, lamellipodia and ruffles have been reviewed in detail (Murphy and Courtneidge, 2011; Hoshino et al., 2013) (Table 1). Invadosome composition as well as the extracellular cues and intracellular signalling cascades leading to their assembly, have been extensively studied in such models. However, most cells evolve within three-dimensional (3D) contexts inside living organisms, surrounded by other cells and diverse ECM components, raising the question to what extent observations made on single cells on planar

surfaces apply to *in vivo* situations. Such thinking is strengthened by reports on differences between cell migration in 2D and 3D conditions (Meyer et al., 2012; Baker et al., 2012) and motility *in vivo* (Patsialou et al., 2009). For these issues, which are intrinsically of wide interest to all biologists, there may be light at the end of the tunnel. Taking one step at a time, a number of approaches for studying invadosomes have been developed in 3D *in vitro* (Table 1), and show major differences in invadosome morphology in 3D environments as compared to 2D surfaces (Wiesner et al., 2014). Analysis of invadosomes has now been reported for *in situ* tissue explants, tissue sections and in *in vivo* models. Concerted efforts aim at tracking signs of their presence in optimized *in situ* settings to support their relevance and subsequent association with human diseases. Moreover, as invasion programs also take place during embryogenesis, invadosomes and related protrusions may be essential to developmental processes. These studies open the way to address the role of these structures in pathophysiological processes as well as their participation in developmental programs, leading towards establishment of the causative link between invadosomes and invasion.

Herein, we describe the studies where evidence for the occurrence of invadosomes or their homologs has been provided *in situ* or *in vivo*. These include mouse models of cardiovascular disease, angiogenesis and cancer. In addition, we have included several developmental models in zebrafish, *C. elegans*, and *C. intestinalis* where protrusions similar to invadosomes were recently reported. Finally, data for invadosomes in the evolutionarily lowest model organism in which F-actin rich structures exist, *Dictyostelium discoideum*, are covered.

Invadosomes *in situ* (in mouse models)

Podosomes in vascular biology

Podosome rosettes in the mouse aortic endothelium—Endothelial cells (ECs) form the monolayer lining the luminal surface of the entire circulatory system from the heart to the smallest capillaries. At the interface of flowing blood and tissues, these cells are the mediators of multiple exchanges including nutrients and oxygen, but also other gases, hormones, waste and heat (Rajendran 2013). Depending on the vessel type, ECs also fulfill more specialized functions such as controlling blood vessel tone in arteries or fluid filtration in capillaries of selective organs (Michiels, 2003). Because proper functioning of this system is vital, it is subject to constant and efficient maintenance by the ECs themselves. Another important feature of these cells resides in their intrinsic invasiveness cells. They are maintained in a quiescent state through their interactions with the BM on which they rest, through stabilization by mural cells (VSMCs or pericytes) and by signals emanating from tight junctions that inhibit proliferation, migration and apoptosis (Sato et al. 1989). Vessel injury alters vessel homeostasis and during wound healing, endothelial sprouting restores the vascular network integrity through angiogenesis. In cancer, by disturbing the pro- and anti-angiogenic factor balance, tumor microenvironment triggers the angiogenic switch that induces tumor vascularization (Gacche et al; 2013).

The development of techniques to isolate and culture ECs has allowed the exploration of their specialized functions *in vitro*. However, by doing so, the homeostasis is disturbed and cultured cells reveal the invasive potential otherwise kept silent in the quiescent vessel

(Conway et al. 2001). In this context, several types of ECs such as human umbilical or liver sinusoidal ECs (HUVECs or LSECs, respectively) display “spontaneous” podosomes (Osiak et al., 2005; Tatin et al., 2006; Juin et al., 2013). Depending on the cell types, angiogenic factors such as VEGF, TNF α (HUVECs) or TGF β (arterial ECs) stimulate the process further, indicating that, in ECs, podosome formation occurs in response to specific signals.

This research has logically progressed from cultured cells towards cultured tissues to address the role of endothelial podosomes in vascular biology. The mouse aorta is amenable to visualization through a technique called “en face” viewing. In this technique, living vessel explants are opened up, exposed to exogenous TGF β in culture conditions which resemble those used for isolated ECs, processed for immunofluorescence and imaged. This experimental setup has allowed visualization of endothelial podosome rosettes *in situ*, in the native endothelium, protruding into the VSMC layer (Figure 1). The podosome rosettes degrade collagen IV in the BM underneath the endothelium (Rottiers et al, 2009). Interestingly, there is no sign of laminin degradation. Since the laminin polymers are not covalently associated, but rather assembled together by the collagen-IV network (Yurchenco et al., 2004), it is conceivable that the disruption of the collagen bonds suffices to disrupt the laminin network, and consequently, the laminin-collagen-IV scaffold (Rowe and Weiss, 2008). The laminin may also be physically pushed away as we describe below in the *C. elegans* model (Hagedorn et al. 2013). Alternatively, by concentrating metalloprotease activities, podosomes may strip the BM, allowing the renewal of ECM components by ECM-producing cells. In this view, endothelial podosomes appear as cellular devices devoted specifically to ECM remodeling. Although the contexts in which these structures come into play remain unknown, those associated with local TGF β activation, such as vessel injury or vessel diameter enlargement, might be physiological situations favoring their emergence (Sho et al., 2002). More generally, factors that stimulate TGF β activation such as angiotensin II, mechanical stress, endothelin-1, high glucose, steroids and Reactive Oxygen Species (ROS) are likely to promote invadosome assembly in aortic ECs (Annes and Rifkin, 2003).

The endothelium that lines blood vessels is essentially attached to a 2D surface and it is therefore not surprising that podosome rosettes formed *ex vivo* are found under the same organization as those observed *in vitro*. The situation is more complex for the tiny capillaries which sprout in 3D environments. What makes the situation even more challenging is that the morphology and organization of podosomes in this context is not yet known. The current literature provides specific observations (Van Goethem et al., 2010; Van Goethem et al., 2011) (Table 1) towards dissecting the role of endothelial podosomes in angiogenesis.

Podosome rosettes in the smooth muscle cells of the mouse aorta—

Cardiovascular diseases have become a major issue in industrialized societies. Whereas the precise nature of the initiating event is not always clear, VSMCs appear to play a major role in the progression of most of these diseases such as atherosclerosis and restenosis (Ross and Glomset 1973). These cells have thus come under intense scrutiny for their synthetic and growth capacities. Located in the medial layer of healthy arteries, VSMCs are differentiated, quiescent, highly specialized cells whose principal functions are contraction and regulation of the vessel diameter that controls blood flow distribution (Dora, 2010). In response to

vessel wall damage, VSMCs lose their contractility and acquire a proliferative, migratory and synthetic phenotype as well as the capacity to degrade the matrix components (Rensen et al. 2007). The ability to breach tissue barriers allow VSMCs to migrate into the sub-endothelial space, where they proliferate and secrete pro-inflammatory molecules causing further vessel damage. Similar to ECs, most of these studies have been carried out in tissue culture where podosomes can be induced by stimulating cells with phorbol-esters such as PdBu or PMA (Burgstaller et al., 2004; Burgstaller et al., 2005; Kaverina et al., 2003). The cellular response to these agents seems to mimic the pathological dedifferentiation process occurring *in vivo*. More physiologically, down-regulation of microRNAs (miR)-143 and -145, which are co-expressed from a single promoter in mature differentiated VSMCs, also promotes the switch from contractile to synthetic phenotype (Boettger et al., 2009; Cordes et al., 2009; Elia et al., 2009; Xin et al., 2009). Interestingly, these miRs target key podosome regulators such as PDGFR α and PKC ϵ (miR-143) and fascin (miR-145). Using the mouse model where miR-143(145) were suppressed, Quintavalle et al., showed that VSMCs from the aortas with high PDGFR α , PKC ϵ and fascin expression spontaneously assemble podosomes and that phorbol esters further stimulate the process (Quintavalle et al., 2010). Immuno-electron microscopy analysis of VSMCs in KO aortas *ex vivo* revealed the presence of circular structures similar to the ones observed *in vitro* which stained positive for cortactin and Tks5 markers. These studies brought the demonstration of the occurrence of podosomes assembled in VSMC in their natural environment (Quintavalle et al., 2010).

In summary, signals that reduce (miR)-143 and -145 expression are expected to promote the formation of VSMC podosomes. PDGF was identified as one of these signals, reducing miR-143/145 expression via a pathway involving Src, PKC and p53 (Quintavalle et al., 2010). It can be predicted that forced expression of these miRs will reverse the dedifferentiation process and repress podosome formation. This approach emerges as a promising strategy to stabilize atherosclerotic plaques and reduce neointimal hyperplasia (Albinsson et al., 2013).

Invadopodia in mouse models of breast carcinoma-from *ex vivo* to *in vivo*

Invadopodia are commonly thought to be the structure used by tumor cells to break the BM and remodel collagen during cancer cell invasion and transendothelial migration (intravasation and extravasation). Until recently, due to technological challenges, invadopodium observation was limited to cells cultured *in vitro*, a situation not representative of the tumor microenvironments encountered *in vivo* (Clark et al., 2007) and therefore, unsuitable for pinpointing the metastatic step during which invadopodia could have an active role or affect the fate of cells harboring them. A series of novel studies has recently taken research on invadopodia into the more physiological realm, shedding light on the mechanisms involved in their assembly *in situ* and on their role in metastasis:

Explants—When cancer cells are placed on top of the collagen I-based chicken chorioallantoic membrane (CAM) (Sabeh et al., 2004) or onto BM-based rat peritoneum explants, movement across membranes is initiated by invasive protrusions and depends on membrane-anchored proteases such as MT1-MMP (Hotary et al., 2006). In the latter model, invadopodium assembly leads to an initial breach of the BM via proteolysis, followed by

invadopodium extension involving microtubules and intermediate filaments, finally resulting in cancer cell migration across the BM (Schoumacher et al, 2010). In addition to CAM and rat peritoneum substrata, porcine urinary bladder matrix and pig cell-free dermis explants have also been used as native *in situ* scaffolds to visualize invadopodia (Parekh et al., 2011; Tolde et al., 2011).

Xenografts—Tumor cell polarization and migration towards blood vessels (Wyckoff et al., 2000) or formation of “pseudopodia” in tumor cells, followed by intravasation (Wang et al., 2002) were first detected *in vivo* in rat mammary carcinoma cell lines, when transplanted into mice or rats to develop into xenograft tumors (Condeelis and Segall, 2003). When human breast cancer or melanoma cells were hosted in zebrafish, the formation of “elongated protrusions” preceded transendothelial cancer cell migration (Stoletov et al., 2007). In these early studies, association of these structures with invasive capabilities was hypothesized based on the morphology and location of these protrusions.

In a more recent study, Twist 1, a transcription factor which increases breast cancer metastasis by promoting epithelial-mesenchymal transition (EMT) (Yang et al., 2004), was suggested as the molecular link between the ability of cells to form invadopodia *in vitro* and their capability to metastasize (Eckert et al., 2011). While analysis of ECM proteolysis was not done on tumors, tissue sections displayed cortactin- and Tks5-positive punctae. In Tks5-knock down (KD) tumors, both cortactin/Tks5 punctae and lung metastases were eliminated. These data suggest that the absence of a key component of the molecular machinery essential for invadopodia assembly *in vitro* abrogates invasiveness. However, this approach did not offer the possibility to determine if Tks5/cortactin punctae were *in situ* invadopodia, as it came without assessment of ECM degradation, cell morphology or relationship to tumor tissue landmarks (blood vessels, invasive edge or stromal cells).

The next step was taken by analyzing the effects of N-WASP KD in primary tumors, in order to establish a molecular link between loss of invadopodia *in vitro* and behavior of KD tumor cells *in situ*. Invadopodia elimination via N-WASP KD or overexpression of dominant negative N-WASP was first shown to result in reduced numbers of F-actin- and cortactin-rich punctae as well as in reduced ECM degradation *in vitro*, leaving lamellipodia and 2D migration speed unaffected (Yamaguchi et al, 2005). It was further shown that N-WASP and WAVE2 were mutually exclusive activators of Arp2/3-complex-dependent actin polymerization, N-WASP being specific for invadopodia and WAVE2 for lamellipodia (Sarmiento et al, 2008). In 3D *in vitro* settings, N-WASP (Yu et al., 2012) was found essential for MT1-MMP delivery to invasive protrusions and subsequent extension of the nascent structure into the ECM. In primary tumors, N-WASP KD reduced both the number of F-actin/cortactin protrusions and ECM degradation *in situ*. F-actin/cortactin-rich protrusions were commonly enriched in N-WASP and located in the perivascular niche or invasive edge. Similar to 3D invadosomes (Table 1), their morphology was small and finger-like, and they were often surrounded by pericellular collagen degradation (Gligorijevic et al., 2012, Figures 6, 7 and Supplementary Figures 6, 7). In addition, N-WASP KD *in vivo* greatly reduced the number of motile tumor cells, and the number of tumor cells circulating in blood or metastasizing to the lung. As these protrusions fulfill morphological, structural and functional requirements of invadopodia *in situ*, they can be classified as such. The

limitation of this study was that it did not provide direct *in vivo* detection of invadopodia, mainly due to the lack of *in vivo* markers for ECM degradation.

The data from the Twist1 and N-WASP KDs suggest a molecular link between the ability of tumor cells to form invadopodia (both in culture and in tumors) and their potential to metastasize (invade, intravasate and colonize lung).

Most recently, human cell xenografts in mice were analyzed for single tumor cell motility (Gligorijevic et al, undergoing minor revisions) using high-resolution 4-channel multiphoton microscopy (Entenberg et al, 2011). This study revealed that locomotion in primary tumors occurs at two different speeds, where slow (but not fast-) locomotion is preceded by the formation of small protrusions. Small protrusions were defined as dynamic compartments via motion analysis (Figure 2A) (Gaggioli et al., 2007) and then tested for invadosome markers (Gligorijevic et al., undergoing minor revisions). In particular, small protrusions were analyzed for essential components (cortactin, Tks5, MMPs) and functionality (ECM degradation). Enrichment in cortactin-GFP (Figure 2B) and Tks5 (Figure 2C), elimination by Tks5KD or MMP inhibitors as well as active degradation of the surrounding matrix (Figure 2B, C) classified these protrusions as *in vivo* invadopodia. While the orientation of fast-locomoting cells was determined by collagen fibers, cells with invadopodia were oriented towards blood vessels and surrounding ECM. Fast- and slow-locomoting cells appeared under different tumor microenvironment conditions (e.g., collagen levels, macrophage numbers, blood vessel size) and in turn, manipulation of the microenvironment cues was able to induce switching between locomotion modes.

The ability to identify invadopodia *in vivo*, in real-time, opened the possibility of determining the fate of tumor cells which assemble such structures in primary tumors. By photoconverting tumor cells that display invadopodia and monitoring them for 48h, they are directly preceding tumor cell intravasation and metastasis to lungs. In summary, this work suggests that the tumor microenvironment controls invadopodium assembly and that treatments which specifically inhibit invadopodium formation may result in significant reduction in metastasis.

Transgenic animals—Based on xenograft animal models of late stage carcinoma, strong evidence was gathered that cancer cells assemble invadopodia in primary tumors to actively move across the blood vessel wall during intravasation. Such findings open a new set of questions, regarding the tumor progression stage(s) in which invadopodia can be detected, the frequency at which they assemble in different stages of tumor progression as well as their possible contribution to processes other than intravasation. Towards addressing these questions, future studies will likely turn to transgenic mouse models which have the capability of reproducing different stages of human cancer.

One such model is the PyMT-MMTV model (Lin et al., 2003), where polyoma middle T (PyMT) oncoprotein expression is under the control of the mouse mammary tumor virus (MMTV) promoter, making it specific to mammary gland. The result is tumor progression in four stages: hyperplasia, adenoma, early- and late carcinoma and metastasis to lymph nodes and lungs. In the late carcinoma stage of PyMT-MMTV animals expressing Dendra2 in

tumor cells (Figure 3) assemble small, dynamic protrusions at the blood vessel borders (Supplementary Video 1) which can be visualized via motion analysis, in the same way as done for xenografts (Figure 2A; Gligorić et al., undergoing minor revisions). Namely, protrusion extension over 30 minutes can be detected by subtraction of images taken at 0 and 30 min; the difference is marked in red (Figure 3B). While ECM degradation and structural components remain to be tested in this model, small perivascular protrusions in PyMT-MMTV tumors present similarities with the invadopodia identified in xenografts in terms of morphology and tissue location—they appear in tumor regions of high collagen density, that are also abundant in blood vessels, infiltrated with macrophages and devoid of fast-locomoting cells (Figure 3A). The near future may bring evidence for invadopodia in the PyMT-MMTV model, allowing us to identify all stages of tumor progression in which the structures are involved.

Intravasation versus extravasation—While the intravasation step seems, based on current data, to require invadopodia assembly and ECM degradation, it is not clear if invadopodia have a role in the extravasation step of metastasis. Although blood vessels in primary tumors and secondary organs consist of the same basic components, transendothelial migration during blood vessel entry or exit may involve different strategies. Probably the first visualization of colon cancer cells extravasating *in situ* was done by electron microscopy of mouse lung sections, detecting small protrusions that extended between EC junctions into the BM (Weis et al., 2004). However, structural components and degrading capabilities of such protrusions were not tested in this model. Later on, extravasation studies which visualized cancer cells in real-time, in zebrafish and mouse models, quantified the fraction of cells crossing the endothelium. However, possibly due to the lack of spatial and temporal resolution, there was no mention of small protrusions with invadopodial features (Kienast et al., 2010; Stoletov et al., 2010). Arguing against a role for invadopodia in extravasation is the work performed in mouse mammary carcinoma cells and focusing on the Arg non-receptor tyrosine kinase (Gil-Henn et al., 2013). Arg is essential for invadopodia functionality *in vitro* (Mader et al., 2011), and elimination of Arg in tumors stops the invasion, intravasation and spontaneous lung metastasis (Gil-Henn et al., 2013). In this model, the extravasation process was not affected and experimental lung metastases were actually increased in Arg KD tumors, due to the major increase in cell proliferation.

Invadosome homologs in development

Gonadal anchor cell protrusion in *Caenorhabditis elegans*

Caenorhabditis elegans is often used as a model system in studies of developmental processes. An important feature of *C. elegans* is that they provide the opportunity to directly visualize invasive protrusions *in vivo*, how they assemble and lead to invasion within their native context, a quest much more technically challenging to observe in higher organisms with slower physiology. The adult body plan is anatomically simple with about 1000 somatic cells and the nematode is particularly amenable to live imaging studies because of its short developmental time and transparent body. The development of fluorescently labeled tags and the application of sophisticated imaging techniques have allowed the visualization of specific cellular structures and proteins in the developing embryo. The availability of a

wide range of mutants make this organism a powerful tool for revealing the gene networks that specify the interactions that occur at the cell-basement membrane interface followed by the invasive behavior (Hagedorn et al, 2009).

In *C. elegans*, a single cell in the embryonic gonad, the anchor cell (AC), initiates a cascade of signals that establishes the fate of the vulval precursor cells. Nascent uterine and vulval tissues are initially delimited by their respective basement membranes (BM). As larval development proceeds, the specialized uterine cell elaborates numerous protrusions which resemble 2D podosomes and invadopodia in punctate morphology, structure (enriched in F-actin) and function (remodeling ECM to allow invasion) (Figure 4) (courtesy of Elliott J. Hagedorn and David R. Sherwood).

F-actin in punctate structures of the AC present invadosome characteristics, including similarities in morphology, composition and dynamics (Hagedorn et al., 2013), as well as the requirement of integrin engagement for their formation (Hagedorn et al., 2009). The *C. elegans* genome encodes orthologs of most components implicated in invadosome formation or function including Src, the master regulator of invadosome formation which may play a similar role in the AC as it does in cancer cells (Saltel et al., 2011; Shaye and Greenwald, 2011) and proteases such as *zmp-1* (Sherwood et al., 2005). One exception in structural components is cortactin, a key regulator of invadosome formation in cultured primary tumor cells or derived cell lines (Artym et al., 2006; Ayala et al., 2008; Shaye and Greenwald, 2011). Although it is not yet clear whether BM breach in *C. elegans*' AC involves proteolytic degradation or simply a mechanical displacement, accumulating evidence suggests that the described anchor cell protrusions are invadosome homologs. Initial breach of the BM, caused by one such protrusion, leads to the recruitment of the netrin receptor UNC-40 and the subsequent protrusion growth by F-actin polymerization at this site. In response to UNC-6 (ortholog of vertebrate netrin), the protrusion enlarges and eventually crosses both BM to invaginate in between underlying dividing vulval precursor cells. The AC remodels the BM components, laminin and type IV collagen, during BM gap formation (Hagedorn et al., 2013). This model opens the possibility that invasion in *C. elegans* may not be strictly dependent on protease activities and matrix dissolution, a mechanism that may be specific to this relatively simple model organism. Namely, *C. elegans* exhibits an extremely short life cycle with physiological processes that are faster and simpler compared to more complex species. It is possible that under such a different kinetic regime and simpler ECM context, remodeling of ECM and displacement of cells may result from forces generated by actin polymerization without the need for prior ECM degradation.

Similarities of behaviors between AC in *C. elegans* and cells with invadopodia in culture suggest that findings from the *C. elegans* model system may be useful as initial hypotheses for mechanisms of human cancer invasion. Supporting this proposition, the sequence of events leading to AC invasion, involving the occurrence of multiple protrusions, only one of which evolves into invasive protrusion which penetrates the underlying tissue, mirrors findings obtained in human cancer cell lines, where only some of many invadopodia elongate, mature and lead to transmigration of BM (Schoumacher et al., 2011). Another outcome of *C. elegans* studies is the emergence of a novel mechanism of cell invasion through BM barriers, where the physical forces (Hagedorn, 2013), possibly combined with

action of proteases (Sherwood et al., 2005; Morrissey et al., 2013) coordinate BM removal. Last, these studies reveal the involvement of netrin signaling at the cell-BM interface that directs invading cells across BM barriers. Although some studies have started addressing the role of netrin signaling in cancer cell invasion (Rodrigues et al., 2007; Kaufmann et al., 2009; Dumartin et al., 2010; Shimizu et al., 2012) or endothelial cell invasion (Heissig et al., 2003; Wilson et al., 2006), the role of this pathway has not yet been explored in cancer cell invadosomes.

Neural crest and intestinal protrusions in *Danio rerio*

Similar to *C. elegans*, the zebrafish model system is robust, transparent and easy to manipulate and image. Traditionally, it has been used in developmental studies but due to the low cost and incentives to reduce the number of mammal-based studies, the zebrafish model has also been paving the way in cancer research. As zebrafish larvae do not develop an immune system in the first two weeks post-fertilization, they are convenient for studies of both growth and metastasis of human cancer cells. A growing number of reports have shown that cancer cells maintain their phenotype within the zebrafish, making this model useful for metastasis assays, genetic and chemical screens as well as for imaging at single-cell levels (Teng et al, 2013, Marques et al., 2009, Tat et al., 2013).

Murphy et al., studied neural crest migration during the embryonic development of zebrafish. Neural crest cells are multipotent cells which arise in the dorsal neural tube. When stimulated by TGF β , they undergo EMT and migrate into different tissues, similar to metastatic tumor cells. Neural crest cell migration presents elegant examples of directional, streaming single cell motility and homotypic contact-inhibition of locomotion (Carmona-Fontaine et al., 2008). Persistence and directionality of migration are dependent on Src and Tks5 (Murphy et al., 2011), two proteins essential for invadopodium assembly in murine and human metastatic cancer cells. The dependence of neural crest protrusions on the Src-Tks5 pathway was established by Tks5 KD which eliminated both actin/cortactin-rich punctae in 2D neural crest stem cell cultures and elongated protrusions, both in 3D cultures and *in vivo* situations (Figure 5A). While the morphology of protrusions seen in neural crest stem cells in 3D or *in vivo* is long and thin, resembling invadopodia in 3D (Schoumacher et al., 2011), it is not yet clear if the punctae visualized in 2D represent equivalents of the elongated protrusions seen in 3D and *in vivo* in terms of structure or if any of the protrusions (2D, 3D, *in vivo*) are capable of degrading the ECM. However, this work makes an important contribution towards linking the mechanisms of cell invasion during development or cancer.

In the zebrafish developing intestine, actin-rich protrusions were observed in meltdown (*mlt*) mutant embryos, which carry a mutation in the contractile protein smooth muscle myosin (myosin heavy chain 11), making it constitutively active. Meltdown mutants exhibit heightened smooth muscle contractility, which leads to overexpression of metalloproteases MT1-MMP and MMP2 (Wallace et al., 2005) followed by the invasion of epithelial cells into the surrounding tissue (Figure 5B, C) (Seiler et al., 2012). Detailed characterization of the invasion process demonstrated its initiation via small, long-lived protrusions, which are enriched in actin and cortactin and can be eliminated by Tks5 KD or induced by

constitutively active Src (Figure 3 in Seiler et al., 2012). While the images supporting the hypothesis that actin-rich protrusions are the site of the BM breach are quite convincing (Figure 5C), the MT1-MMP fluorescent chimera shows only a slight concentration of protein to the basal area of the cell in *mlt* mutants. Hence, similarly to the example AC invasion in *C. elegans*, it is not clear whether the underlying mechanism of BM breach is based on mechanical forces or proteolytic degradation or both.

Similarly to invadopodia in human cancer cells, where ROS were shown to contribute to invadopodium formation and function (Diaz et al., 2009), intestinal protrusions require ROS to effectively breach the BM (Figure 9 in Seiler et al., 2012). There are however, important mechanistic differences between intestinal protrusion and invadopodium formation. While intestinal protrusions are induced by muscle contraction through a redox signaling pathway, without modulation of ECM stiffness, invadopodium assembly and degradation in human cancer cells is stimulated by external factors such as ECM stiffness through a mechanosensing pathway involving myosin II-FAK/Cas (Alexander et al., 2008). From these two examples, we see, in the same model system, at least two developmental processes where invadosome homologs play a role. While both types of protrusions share the main structural components, it is not clear if their function involves proteolytic ECM degradation and it is not certain if they are part of two completely separate phenomena. They also appear morphologically distinct; the protrusions reported in neural crest migration are long and thin whereas those in intestines are round and short. Systematic investigation of other invadosome structural components, their ECM-degrading function, relationship to cell migration and activation of signaling pathways known to associate with invadosome biogenesis is needed before proceeding to categorizing such protrusions as invadosomes.

Polarized protrusions in *Ciona intestinalis*

The sea squirt is another model organism established for its simplicity and used increasingly in developmental biology for several reasons. First, the full *C. intestinalis* genome was recently sequenced and assembled, yielding a genome size of approximately 5% that of *Homo sapiens*. Second, cell lineages are robust and have been mapped. Third, these species have very low cell numbers at the gastrulation stage (110 cells) and larval stage (3,000 cells in tadpole larvae) as compared to millions of cells in vertebrate species (Davidson, 2007) and they quickly reach adult form. Fourth, despite its simplicity, the embryonic development of *C. intestinalis* is surprisingly conserved, making it a useful model system for cardiogenesis studies.

Cooley et al. focused their work on two symmetrical pairs of founder cells which divide asymmetrically at the gastrulation stage. After the division, the smaller daughter cell proceeds to form the heart lineage while the larger cell forms the muscle. During mitosis, founder cells extend transient, polarized protrusions into the underlying epidermis (Cooley et al, 2011). The activity of Cdc42 is enhanced locally, within the protrusions and can be disrupted by expressing mutants of Rac, Rho, Arp2/3 or WASP. Protrusion formation leads to differential activation of receptor tyrosine kinase (RTK) and, downstream, to differential activation of MAPK in smaller daughter cells. While polarized protrusions observed in *C. intestinalis* presents major similarities with invadosomes in terms of morphology,

composition and signaling pathways, their function is not invasion but localized signal transduction. Such findings suggest a common ancestor pathway which may have regulated cell invasion (to transfer cells into a new environment) as well as asymmetric cell division (to create new cell lineages).

Are there invadosomes in single cell organisms?

Eupodia in *Dictyostelium discoideum*

The social amoeba *Dictyostelium discoideum* is one of the simplest eukaryotic models commonly used to study motility and chemotaxis (Van Haastert & Devreotes, 2004). This cellular slime mold has a strategic phylogenetic position. *Dictyostelium* seems to have emerged in evolution about one billion years ago, after divergence of the kingdom Plantae and before individualization of the kingdoms Animalia and Fungi. Typically, cells grow as individual amoebae in rich environments. However, when challenged by adverse conditions such as starvation, cells signal each other by releasing 3,5-cyclic adenosine monophosphate (cAMP) and coalesce by chemotaxis to form an aggregate that becomes surrounded by an extracellular matrix before differentiating into a fruiting body.

The amoeboid movements of *Dictyostelium* toward cAMP are based on the extension of pseudopodia. Chemoattractants activate G-protein-coupled receptors (GPCRs), resulting in the recruitment of the cytosolic enzyme phosphatidylinositol 3-kinase (PI3K) to the plasma membrane, where it phosphorylates phosphatidylinositol biphosphate (PIP2) into PIP3. PIP3 accumulation toward the high side of the gradient leads to pseudopodium extension at the leading edge of cells, which is thought to be driven by localized Rac-mediated actin polymerization. Work performed in the Yoshio-Fukui lab has studied cortical structures (referred to as actin-rich knobby feet or eupodia) that are responsible for cell-substratum anchorage during amoeboid movement (Fukui & Inoue, 1997). These eupodia, each about 1 μm in diameter, appear transiently in multiple units at the base of certain pseudopodia where the amoeba contacts a partially deformable substrate. The feet were discovered, and their spatial and temporal behavior relative to pseudopodial anchorage and invasive locomotion were observed, by examining the amoebae using a differential interference contrast (DIC) video microscope providing a 0.3 μm depth of field. Key evidence for the anchoring role of the knobby feet was obtained by investigating amoebae, flattened in a specially devised observation chamber, and attracted by chemotaxis towards cAMP. Immunofluorescence studies revealed that eupodia contain a high concentration of filamentous (F-actin), myoB (a member of *Dictyostelium* myosin-I family), α -actinin (an actin-binding protein) and coronin (Fukui et al, 1997; Fukui & Inoue, 1997). Interestingly, myoB exhibits a circular disposition around each foot. In terms of signalling, *Dictyostelium* does not have Cdc42, but 14 Rac proteins have been found (Rivero et al, 2001).

Eupodia seem to provide anchorage to the substratum needed by cells to exert projectile forces enabling the pseudopod to invade narrow spaces. They may also enable the flattened amoeba to secure itself to a deformable substratum. Although the proteolytic activities of eupodia have not been investigated, such structures are highly reminiscent of podosomes found in mammalian cells in terms of morphology, structure and anchorage function, which may thus come into play in a context with similar mechanical constraints. In addition, the

signalling pathways and proteins that regulate cell movement in *Dictyostelium* are highly conserved with those found in mammals. Further studies exploring the similarities in the regulatory mechanisms of eupodium and podosome formation may test the hypothesis that eupodia may be invadosome homologs in lower organisms.

Conclusions

By recreating physiologic environments in tissue explants or in *in vivo* models, we are now slowly establishing platforms for studying invadosomes in their natural habitat. Such technological advancement opens the possibility of tackling numerous open questions on the role of invadosomes in cell invasion. One of the major roles of invadosomes is in cancer, where multiple types of invadosome forming cells come in contact. For example, macrophages and cancer cells are known to mutually stimulate each other via EGF-CSF-1 paracrine loop (Wyckoff et al., 2004). Hence, macrophage podosomes and cancer cell invadopodia may degrade the ECM in concert (Yamaguchi et al., 2006). Taking this hypothesis further, as ECs may be induced to assemble podosomes in response to TGF β present in the tumor microenvironment (Rottiers et al, 2009), all three cell types which come in contact during the intravasation process, may contribute to ECM degradation simultaneously. Moreover, fibroblasts were previously suggested to lead the way during tumor cell invasion and interstitial migration and remodel the ECM, forming tunnels through which tumor cells can move (Gaggioli et al., 2007; Sabeh et al., 2004). As cancer associated fibroblasts were recently shown to harbor invadosomes (Goicoechea et al., 2014), this opens the possibility that fibroblasts remodel the ECM and enable tumor cell invasion and metastasis via invadosomes. This issue can be addressed by combining invadosome and cell type markers in mouse models such as PyMT-MMTV. Moreover, application of invadosome markers to patient samples may lead towards utilization of the invadopodium density in primary tumors as a predictor of metastasis and test of treatment performance.

There is accumulating evidence that invadosomes are present in a number of physiological or pathological processes, including development, inflammation and cancer. Recent development studies report on the presences of invadosomes in human placenta cells (extravillous trophoblasts) (Patel and Dash, 2012) as well as invadosome homologs in model systems *in vivo*. Invadosome homologs seem to be diverse in terms of morphology and signaling pathways involved in their formation. Hence, establishment of standards of detection and classification of invadosome homologs such as that proposed in Table 1 are important. As many of the invadosome features have not yet been tested in development, the descriptions made are still confined to specific model organisms. However, once the presence of invadosome markers is systematically tested in several developmental models, both phenomenological and mechanistic comparisons should be made across models and/or different conditions, shifting our focus from differences to similarities and constructing an evolutionary model for invadosomes. Evolutionary analysis of invadosomes involved in different processes may take us to the discovery of a common underlying mechanism.

Other than the value of fundamental understanding of the invasion process, drawing parallels among models of development and cancer has a clear motivation in the mounting evidence for developmental processes being repurposed during cancer progression. Stem

cell maintenance, resistance to apoptosis and EMT (Thiery, 2003) are good examples of such processes. The first steps towards establishing a link between invadopodia in cancer and their homologs in development were recently taken (Matus et al, 2010; Murphy et al, 2011). In Matus et al., a number of genes associated with AC invasion in a *C. elegans* screen were mapped to human orthologs. In turn, KDs of human orthologs led to a decrease in human breast and colon cancer cell line invasiveness. In Murphy et al., KD genes identified as essential for invadopodia formation in cancer cells eliminated the formation of protrusions in neural crest cells and the migration of these cells in zebrafish embryos *in vivo*.

Some *in vivo* model systems useful for studying invasion appear promising for functional studies of invadosomes and/or their homologs. Some of them are relatively easy to manipulate and suitable for acquiring high-throughput imaging data. For example, CAM assays have proved useful for cell invasion, intravasation and extravasation studies (Deryugina & Quigley, 2008; Sabeh et al., 2004). In addition, chicken gastrulation is a well-established model for EMT of mesodermal and endodermal cells and BM disassembly (Nakaya et al., 2008) in which high-resolution microscopy for testing invadosomes could be made. Next, *Drosophila melanogaster* (fruit fly) is known to have a high genetic homology to mammals, short generation times and can easily be utilized for automated microscopy. An advantage of *Drosophila* is that it comes with established libraries of thousands of mutants and genome-wide RNAi, making it a good model system for screening. The downside of this model organism is a lack of vasculature and immune system. Recent studies have shown that larval imaginal wing disc and eye in *Drosophila* flies could represent powerful models for invasion during metamorphosis (Singh et al., 2010, Vidal et al, 2007). For instance, in the wing disc, the overexpression of Src or Abl, tyrosine kinases known to be important for functional invadopodium assembly was demonstrated to induce MMP expression (*Drosophila* has only two MMPs), invasion through ECM and loss of apical/basal polarity. The near future will likely bring imaging of invasion and presumably, invadosomes, at subcellular resolution in this model. The African clawed frog *Xenopus laevis* is yet another developmental model easy to image which may also offer good options for tracking invadosome and ECM remodeling during morphogenesis *in vivo*. Finally, a recent model of mouse embryonic development has demonstrated the breaching of BM initiated by mechanical forces (Hiramatsu et al., 2013). The growing embryo is spatially restricted by maternal uterine tissue, posing mechanical stress at the distal tip of the embryo. Early epiblast cells migrate through gaps in the BM, which may be induced directly by mechanical stress or alternatively, by invasive cell behavior. Although MMP activity was not found at the distal tip, morphologies typical of invadosomes were demonstrated in tissue sections. Future live cell, high-resolution, time-resolved imaging in this model may potentially demonstrate more similarities between intestinal protrusions induced by mechanical forces in zebrafish (Seiler et al., 2012) and epiblast migration (Hiramatsu et al., 2013).

In summary, invadosomes have been studied mainly in human and mouse cell lines and related to pathological processes involving invasion. Recently, a number of developmental models, including *C. elegans*, *D. rerio* and *C. intestinalis*, have been shown to assemble invadosome homologs. We can soon expect an even broader spectrum of *in vivo* model systems for studying invadosomes and their homologs. Systematic characterization of all

structures followed by phenotypic evolution analysis may provide us with deeper insights into the origin of the invasion program and similarities of invasion mechanisms in development, physiology and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2D	two-dimensional
3D	three-dimensional
cAMP	3, 5-cyclic adenosine monophosphate
Ab	antibody
AC	anchor cell
Arp2/3	complex containing actin regulators 2 and 3
BM	basement membrane
CAM	chicken chorioallantoic membrane
Cdc42	Cell division control protein 42 homolog, small protein of RhoGTPase family
DIC	differential interference contrast
EC	endothelial cells
ECM	extracellular matrix
EMT	Epithelial-mesenchymal transition
GPCR	G-protein-coupled receptors
hpf	hours post-fertilization
HUVEC	human umbilical vascular endothelial cells
KD	knock down
KO	knock out
LSEC	liver sinusoidal endothelial cells
(N)-WASp	(Neural)-Wiskott-Aldrich syndrome protein

MMP	matrix metalloproteases
miR	microRNAs
myoB	myosin family protein
PDGFR	platelet-derived growth factor receptor
ROS	reactive oxygen species
Rac	small signaling G proteins, subfamily of Rho-GTPases
RTK	receptor tyrosine kinases
TGFβ	transforming growth factor
Tks5	Src substrate otherwise known as Fish
TNFα	tumor necrosis factor
UNC-6	netrin receptor
VSMC	vascular smooth muscle cells
Src	short for sarcoma, a non-receptor tyrosine kinase
PI3K	phosphatidylinositol 3-kinase
PIP2/PIP3	phosphatidylinositol bi/triphosphate
PKC	protein kinase C
p53	a tumor suppressor protein
PdBU	Phorbol (ester) 12,13-dibutyrate
PMA	phorbol myristate acetate

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Figure 1. Endothelial cells in the murine aorta form podosome rosettes upon exposure to TGF β *ex vivo*

A living aortic segment was explanted from an anesthetized mouse, opened up along its long axis, was treated with TGF β for 20 h, then processed for fluorescence staining and analyzed by confocal microscopy for “en face” viewing. Serial optical sections are shown and presented from the apical (top) to the basal (bottom) regions of the endothelial monolayer. F-actin (red) and Hoechst 33342 (blue) distinguish between ECs with large round nuclei and vascular VSMCs with thin and elongated nuclei. The merged staining for F-actin (red) and cortactin (green) shows the rosette located at the basal surface of the ECs, extending towards the VSMC along the z-axis. Bar, 5 μ m. Courtesy of Patricia Rottiers, (University of Bordeaux).

Figure 2. Presence of invadosome markers in xenografts of MDA-MB-231 human breast carcinoma

(A) A tumor cell (green) forms a small protrusion (arrow) oriented towards a blood vessel (red). Dendra2 is stably expressed in tumor cells (green); flowing blood vessels are labeled with Texas Red 70kDa dextran; collagen fibers are visualized via second harmonic generation (purple) with a multiphoton microscope. In the insert below, a small protrusion was identified using motion analysis: Images from a time lapse series taken at 30min and 0min were subtracted and the difference in the images was marked in red, revealing dynamic pixels. (B) Small protrusions *in vivo* contain cortactin colocalized with ECM degradation. Cortactin-GFP is stably expressed in MDA-MB-231 cells (green); ECM degradation is transiently labeled *in vivo* by injection of MMPsense680 (cyan); collagen fibers are shown in purple. Scale bar 20 μ m. Arrow in overlay image and zoomed-in insert points to colocalization of cortactin and degradation. (C) Tks5-rich punctae colocalize with ECM degradation *in situ*, in tumor cryosections. Green-cytoplasmic Dendra2; purple-Tks5 mAb; cyan-degraded ECM visualized using antibody against fragments of collagen I (collagen I-3/4 C, (Gligorijevic et al., 2012)). Arrows in overlay image and zoomed-in inserts point to specific points of colocalization of Tks5 and degradation. White lines delineate blood

vessels. Scale bar 20 μm . Courtesy of Bojana Gligorić and John Condeelis (Albert Einstein College of Medicine).

Figure 3. Transgenic mouse model MMTV-PyMT exhibits small protrusions in perivascular regions

Tumor cells (green) in a region rich in collagen fibers (purple), macrophages (white) and blood vessels (red) in transgenic animals MMTV-PyMT $\times$ MMTV-iCre/CAG-CAC-Dendra2 at carcinoma stage (15 w). The white box is enlarged in (B), focusing on a single 5 μm -thick slice in the green channel. (B) During a 30-minute time-lapse, tumor cells next to blood vessels do not move their body but extend small protrusions (B, red overlay). All small protrusions are located at the tumor cell-blood vessel contacts (white line). Scale bars 75 μm in (A), 20 μm in (B). Protrusion extension tracked over 30 minutes is overlaid in red (B). Also see Supplementary Video 1. Courtesy of Bojana Gligorijevic and John Condeelis (Albert Einstein College of Medicine).

Figure 4. An F-actin-rich, invasive protrusion initiates the breach of the basement membrane during anchor cell invasion in *C. elegans*.

Panel is a single confocal section of the anchor cell's surface in contact with the basement membrane (top-down-view). An F-actin probe (middle; moeABD::mCherry) specifically expressed in the anchor cell reveals a puncta located in the center of the anchor cell's invasive cell membrane breaching the BM (right, small gap in laminin::GFP; left, overlay). Scale bar = 5 μ m. Courtesy of Elliott J. Hagedorn and David R. Sherwood (Duke University).

Figure 5. Zebrafish embryonic development of neural crest and intestine requires Tks5-dependent protrusions

(A) Neural crest cells labeled with transgenic (*sox10:RFP*) in control (T5 MM) and Tks5 morphant (T5 MO) (30 hours post-fertilization, hpf). Arrows mark individual cells which migrate over the 1.5 hours duration. * = protrusions emanating from neural crest cells. Image reproduced from Murphy et al, 2011. (B) Actin-rich protrusions in epithelial cells of the intestine in meltdown mutant (*mlt*). 3D rendering of sagittal confocal sections through the intestine of 78 hpf wild type (WT) and *mlt* larvae. Actin is labeled by transgenic Lifeact-GFP expression (green). In WT, actin is mainly in the epithelial cell apical brush border (bracket). In *mlt*, actin-rich protrusions are detected (arrows), in addition to brush border actin (bracket). (C) Actin-rich protrusions in *mlt* co-localize with sites missing basal lamina (lower panel, arrowhead and inset). Histological cross-sections through the intestine of 74 hpf immunostained *mlt* larvae. BM is labeled red (laminin Ab), actin is green (GFP Ab) and nuclei are blue (DAPI). ap, apical epithelial cell borders; ba, basal cell epithelial cell border. Images (B, C) reproduced from Seiler et al., 2012.

Table 1

Main features used as markers for classical invadosomes (in 2D) and their performance in 3D or *in situ* conditions

Main features useful as markers	Invadosomes <i>in vitro</i>				Invadosomes <i>in situ</i>	
	Classical invadosomes in 2D ¹		Invadosomes in 3D		Invadopodia ^{12,13}	Podosomes ^{14,15}
	Invadopodia	Podosomes	Invadopodia	Podosomes	Location in cell: Leading edge or side of migrating cell Location in tissue: Perivascular ^{12,13} , vascular ^{14,15} , invasive edge ¹²	
Location in cell/tissue	Ventral cell surface		Leading edge or side of migrating cell			
Main molecular components localized to invadosomes²	<ul style="list-style-type: none"> F-Actin Actin regulators-Cortactin, N-WASP, Arp2/3, cofilin, fascin MMPs eg. MT1-MMP Integrins-cell type dependent P-Tyr Tks5 		<ul style="list-style-type: none"> F-Actin Cortactin, N-WASP, Arp2/3, cofilin, fascin MT1-MMP Integrins P-Tyr Microtubules (when elongated)⁴ Others not tested 		<ul style="list-style-type: none"> F-Actin Cortactin, N-WASP, Arp2/3 MT1-MMP Integrins P-Tyr Tks5 Others not tested 	
Morphology	F-actin rich punctae		<ul style="list-style-type: none"> F-actin rich punctae at the tip or side of (leading) pseudopod⁵⁻⁷ Filopodia on cell side^{7,8} 	F-actin-rich sphere at the tip of pseudopod ^{10,11}	Small finger-like protrusions, rich in cortactin and F-actin ^{12, 13}	Rosettes ^{14,15}
Length	~1 µm		5–25 µm	5 µm	5–10 µm	Rosette diameter ~5 µm
Superstructure	No	Rosettes, clusters	No	Rosettes	No	Rosettes
Number per cell	++	+++	+	+	+	Not tested
Functionality	ECM degradation colocalized with F-actin or cortactin	ECM degradation colocalized with F-actin, mechano-sensing ⁴	ECM degradation <ul style="list-style-type: none"> Colocalized with or surrounding cortactin-rich puncta & protrusion tips^{4,8} Base of protrusion^{7,9} 	Degradation, pushing, ingestion and degradation of ECM adjacent to protrusion tip	ECM degradation colocalized or adjacent to cortactin-rich puncta & protrusion tips	Degradation of collagen IV colocalized with rosette
Lifetime	minutes to hours	1–5min	minutes to hours	~5min	Not tested	

Main features useful as markers	Invadosomes <i>in vitro</i>						Invadosomes <i>in situ</i>	
	Classical invadosomes in 2D ¹			Invadosomes in 3D			Invadopodia ^{12,13}	Podosomes ^{14,15}
	Invadopodia	Podosomes		Invadopodia	Podosomes			
References	1, 2. Reviewed in Murphy and Courtneidge, 2011 and Beatty and Condeelis, 2014; 3. Van den Dries et al., 2013; 4. Schoumacher et al., 2011; 6. Beatty et al., 2013; 7. Yu et al., 2012; 8. Li et al., 2010; 9. Tolde et al., 2010; 10. Van Goethem et al., 2011; 11. Wiesner et al., 2014; 12. Gligorijevic et al., 2012; 13. Gligorijevic et al., undergoing minor revisions; 14. Rottiers et al., 2009; 15. Quintavalle et al., 2010							