Directed Cell Invasion and Migration During Metastasis

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Abstract

Metastasis requires tumor cell dissemination to different organs from the primary tumor. Dissemination is a complex cell motility phenomenon that requires the molecular coordination of the protrusion, chemotaxis, invasion and contractility activities of tumor cells to achieve directed cell migration. Recent studies of the spatial and temporal activities of the small GTPases have begun to elucidate how this coordination is achieved. The direct visualization of the pathways involved in actin polymerization, invasion and directed migration in dissemination competent tumor cells will help identify the molecular basis of dissemination and allow the design and testing of more specific and selective drugs to block metastasis.

Keywords

Introduction to cancer metastasis

Metastasis is a multistep process where tumor cells disseminate from the primary tumor and colonize distant organs [1]. Metastasis accounts for more than 90% of all cancer related deaths [2, 3]. The steps include tumor cell invasion of basement membranes and the surrounding tissue, intravasation into blood vessels, survival there, extravasation and/or growth at different organ sites. To achieve these steps, precise coordination of cell movement and matrix remodeling are essential [4]. At any given time, only a small proportion of tumor cells are invading and disseminating [5, 6]. Understanding the mechanisms that drive motility and invasion of these tumor cells is crucial to better understand metastasis.

Escaping the tumor: Protrusions with matrix degradation activity

EMT (Epithelial-meshenchymal transition) is believed to be an important early step in the conversion of tumor cells into a migratory population capable of systemic metastasis [7, 8]. To become migratory, the first barrier that tumor cells must overcome is their epithelial basement membrane (Figure 1). In order to cross basement membranes, tumor cells are postulated to form invadopodia. There is growing evidence that the formation of invadopodia is part of the EMT process [9[•], 10]. It is unknown the degree to which

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carcinoma cells must proteolytically digest the epithelial basement membrane as they initially invade and whether stromal cells are also involved in this process. However, there is good evidence for the involvement of invadopodium-mediated proteolysis of vascular basement membranes during intravasation and metastasis [11].

Invadopodia are defined as invasive actin polymerization dependent protrusions found specifically on cancer cells that degrade matrix. They were first described in invasive tumor cells such as the human malignant breast tumor cell MDA-MB-231[12] and human malignant melanoma cells [13]. In this review we are going to focus on describing invadopodia which, by definition, are only formed by invasive tumor cells and since they have unique characteristics that differentiate them from other matrix degrading organelles described in other cell types [14]. These other degrading organelles are called podosomes (macrophages), rosettes (fibroblasts) and more generally invadosomes (reviewed in [15••]).

When plated on 2D surfaces, tumor cells form invadopodia on their ventral surfaces which degrade extracellular matrix through the delivery of metalloproteases like MT1-MMP [16, 17]. Recent studies have shown that the formation of invadopodia is a multistep process involving actin-binding proteins and complex signaling cascades [18]. Initiation of invadopodium assembly involves "invadopodium precursor" formation prior to maturation to the degradation competent invadopodium. Cortactin phosphorylation by Arg-kinase regulates cortactin binding to other proteins including cofilin and NCK1 and this phosphorylation plays a critical role in regulating actin polymerization and precursor assembly [18-20] (reviewed in [21••]). It has been proposed that at the membrane level, caveolin regulates invadopodium formation through the regulation of membrane cholesterol levels [22], and also that extracellular matrix rigidity can influence the formation of invadopodia [23] suggesting that extra-cytoskeletal factors also regulate their formation.

Invadopodia in chemotaxis

Recent results have demonstrated that invadopodia are involved in directional polarization, signal sensing and directional protrusion during chemotaxis in 2D [24] as observed for podosomes [25]. These results raise an interesting question: are invadopodia only necessary for degradation, or do they have other functions such as orienting tumor cells towards chemotactic signals such as those associated with blood vessels *in vivo*? The importance of chemotaxis in tumor and stromal cell migration *in vivo* and in tumor cell dissemination and metastasis has been reviewed recently in detail [21••]. An important insight to come from these considerations is that invadopodia are involved in chemotaxis during migration in both 2 and 3D (Figure 2). Previous reports of human tumor cells migrating in three dimensional matrices indicate that matrix-degrading invadopodia localize to the leading edge of the invading cell where the secretory machinery and metalloproteases are located [26]. Recently, Magalhaes et al., [27] were able to identify invadopodia during cell invasion in 3D matrix. MDA-MB-231 cells in a 3D matrix contain cortactin, Tks5, MT1-MMP and matrix degradation (which together in tumor cells are unique markers of invadopodia) localized in protrusive structures at the leading front of the cell in dense native matrix consistent with studies of 3D cell migration by breast carcinoma cells [28]. Similar results implicating invadopodium-mediated proteolysis at the cell front during invasion and migration in vivo in mammary tumors have been observed [11]. Wolf and coworkers [29] have reported that tumor cells invading 3D pepsin digested collagen also exhibit pericellular degradation to widen the hole available for pseudopodium-mediated migration but did not observe proteolysis at the cell front in this pepsin treated collagen. Taken together, these 3D and *in vivo* studies indicate that proteolytic activity at the cell front is necessary for tumor cell invasion and migration in 3D and that this requirement is relaxed in pepsin treated collagens lacking telopeptides and crosslinking. Furthermore, common signaling and cytoskeletal

pathways used by both locomotory protrusions, such as pseudopodia, and invasive protrusions, ie. invadopodia, indicate a high degree of molecular integration and cross talk between pseudopodia and invadopodia allowing efficient invasion coupled migration in both 2 and 3D (Figures 1 and 2) [21**].

Types of tumor cell motility

For some time it has been generally agreed that tumor cell motility is necessary for tumor cells to metastasize [30]. Once tumor cells have passed through their basement membrane they have to migrate through the extracellular matrix for long distances to disseminate efficiently via blood vessels and lymphatics (Figure 1). But, how do tumor cells move long distances in the tumor? The development of novel imaging techniques has illuminated these processes [31-33]. High resolution multiphoton imaging of tumors *in vivo* has shown that tumor cells use both collective and single cell motility (reviewed in references [21**, 34]). Breast carcinoma cell motility is characterized by solitary amoeboid movement. Cells undergoing amoeboid movement *in vivo* move at high speeds (~ 4 μm/min) compared to other types of cell motility described *in vivo* [31].

An important question is: why do tumor cells display these different types of motility? A common feature of cell motility *in vivo* is the formation of F-actin rich protrusions which cells use to extend forward to adhere to their surroundings, followed by contraction of the trailing end. These coordinated events, called the motility cycle, propel the cell forward. Coupled to these events is the formation of invasive protrusions used to penetrate through the extracellular matrix and to overcome matrix barriers to migration. Historically, protrusions at the leading edge of motile cells were named based on their shape: filopodia (needle shape), pseudopodia (round), lobopodia (cylindrical) and lamellipodia (flat veils) [35]. Lamellipodia are observed when cells are plated on 2D substrates. All are F-actin rich and actin polymerization is required for their protrusion [36, 37]. Invasive tumor cells form pseudopodia *in vivo* (functionally equivalent to lamellipodia but three dimensional [38]) in response to the EGF secreted by the tumor associated macrophages, as part of the tumor cell/macrophage paracrine loop described in breast tumors [21**, 39]. F-actin rich pseudopodia are the defining morphological feature of fast moving amoeboid cells and are involved during chemotaxis to direct tumor cells toward blood vessels before intravasation [11, 31] (Figure 1). Different reports have described the importance of amoeboid motility for cell migration *in vivo* [40-42]. This work identifies ROCK , and its regulator PDK1, as important mediators of cortical actomyosin contractility part of the motility cycle [41, 42], and Rac, as a central GTPase involved in the interchangeability of different modes of tumor cell motility [40, 43].

The formation of these distinct protrusive structures during the cell motility cycle of tumor cells during migration and dissemination *in vivo* highlights the fact that tumor cells adapt to different types of matrix composition, as proposed for other cell types [44]. We hypothesize that the tumor microenvironment is responsible for these different motility behaviors. The interactions with different stromal cell types, different matrix compositions and different chemoattractants further determine how tumor cells behave within, and escape from, the primary tumor. For example, the formation of invasive protrusions (invadopodia) and locomotory protrusions (pseudopodia) is regulated by stromal cells in the tumor microenvironment including fibroblasts (Squamos Cell Carcinoma) [45] and macrophages (breast carcinoma) [21**].

Regulation of actin polymerization and Metastasis

The formation of long, force-supporting membrane protrusions requires actin polymerization. Signaling pathways are altered in invasive tumor cells to increase their actin polymerization activity and motility [5, 6]. The Rho-family GTPases have been directly linked to motility and protrusion formation through their ability to activate the signaling targets that direct upstream actin cytoskeleton modifying proteins. Among the 20 members of the family, the most studied have been RhoA, Rac1 and Cdc42. The close isoforms of Rho including RhoA, RhoC and RhoB, play different roles in cancer [46-49]. RhoC expression is upregulated in metastatic tumor cells isolated from lung metastases [50•] and invasive human breast tumor cells [48••]. RhoC plays a critical role in metastasis regulating the spatial formation of invadopodia to tightly focus the matrix degradation [48••]. However, RhoA seems to play a different role in invasion, regulating MMP trafficking and Rac1 activation [47-49]. Effectors of GTPases, like the formins; and the Arp2/3 complex, have also been shown to be involved in metastasis through their roles in motility and protrusion formation (reviewed in [51]).

The cofilin activity cycle is regulated by GTPases through ROCK [52]. Cofilin plays a critical role in promoting protrusions through the formation of free actin barbed ends needed for new actin polymerization. The cofilin pathway is upregulated both at the level of gene expression and protein activity [5, 53] in metastatic tumor cells where the cofilin activity cycle is involved in actin polymerization, protrusion formation, chemotaxis, motility and invasion [48••, 54]. To explain the activation of cofilin locally, a Local Excitation Global Inhibition (LEGI) model has been proposed. Global inhibition of cofilin activity through cofilin phosphorylation mediated by RhoC, restricts cofilin activity just at the very tip of a motility supporting protrusion or within the core of an invadopodium [48••, 53] suggesting how these two types of protrusions might be coordinated at the cell front.

Rac1 GTPase, a member of the Rac GTPase subfamily, is necessary for the formation of lamellipodia. It has been proposed that the anti-capping protein Mena is involved in acting as a negative regulator of Rac1 activity [55]. In addition, it has been shown that the splicing patterns of Mena isoforms change in invasive versus non invasive tumor cells in vivo. Invasive tumor cells upregulate MENA[INV] and downregulate another Mena isoform, MENA 11a [56]. Overexpression of MENA[INV] promotes invadopodium formation and matrix degradation and also lamellipodium formation contributing to tumor cell invasion, dissemination and metastasis [57-60].

RhoGTPases and invadopodia: Spatiotemporal RhoC GTPase signaling

Different GTPases have been implicated in the formation and function of invadopodia including Cdc42, RhoA and Rac1 [17, 61, 62]. However, recent reports show that GTPases display spatiotemporal activation dynamics important for the precise control of different signaling pathways [63•]. Different regulators including GEFs, GAPs and GDIs regulate the GTPase activation cycle. To decipher such complex and dynamic activation kinetics, traditional approaches including biochemical strategies or simply tagging these molecules with fluorescent proteins, fall short in detecting these phenomena in living cells. Fluorescent biosensors for the detection of Rho GTPase activation have emerged as critical and powerful tools allowing direct interrogation of GTPase activation at subcellular resolution and in the time scale of seconds [64-66] (Figure 3). These fluorescent biosensors based on the fluorescence resonance energy transfer (FRET) rely on the conformational reorientation within the engineered molecule upon "activation" of the built-in GTPase through the guanine nucleotide exchange. This event causes the shift in the dipole-dipole coupling angle between the two fluorescent proteins within the biosensor to affect FRET; thus, through

monitoring the ratio of the FRET to donor fluorescent emissions, one can determine the relative protein activation states within a single living cell.

Using a FRET-based RhoC biosensor, a novel pathway controlling spatial restriction of invadopodium activity has been described (Figure 4). RhoC is activated surrounding the invadopodium center generating a ring pattern achieved by the spatial placement of p190RhoGAP inside the center, and p190RhoGEF outside the center. The combination of a specific GEF and GAP in these locations tightly regulates and focuses the region of RhoC activation (Figure 4). As a consequence, the activation of the RhoC/ROCK/LIMK pathway results in cofilin phosphorylation in the outer ring thereby focusing the cofilin activity to generate barbed ends and actin polymerization within the center, focusing the matrix degradation activity for efficient invasion [48••]. These results show that controlling the RhoGTPase signaling is not only achieved by the quantity of GEFs and GAPs acting on a specific GTPase, but also by the spatial and temporal placement of these components that will specifically target the activity of the GTPase and its signaling pathway.

Concluding Remarks and future directions

To date, drugs capable of blocking metastasis are not available. At least two reasons for this are 1) that such a complex process is still not well understood and 2) there are no reliable end point markers for evaluating drug performance in blocking metastasis. Invadopodia are unique protrusions with matrix degrading activity formed specifically by invasive tumor cells that have an essential role in invasion, migration and chemotaxis during metastasis, making them an attractive functional target to interfere with metastasis and as a potential marker for assessing drug performance. Defining the molecular features of invadopodia under physiological conditions will help in the design of specific drugs that could block metastasis in the future. Proteins involved in the formation of invadopodia are upregulated in the invasive and migrating population of tumor cells. The signaling pathways involved in invadopodium formation may not be present in normal cells. Understanding these signaling pathways will clarify the molecular basis of metastasis and allow the design and testing of more specific and selective drugs to block metastasis.

Acknowledgments

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•	of special interest

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Highlights

1. Invadopodium degrading protrusion facilitate degradation of basement membranes by tumor cells

2. Motility is an important feature of invasive tumor cells

3. FRET-based GTPases biosensors allow direct visualization of GTPases activation in living cells during invasion.

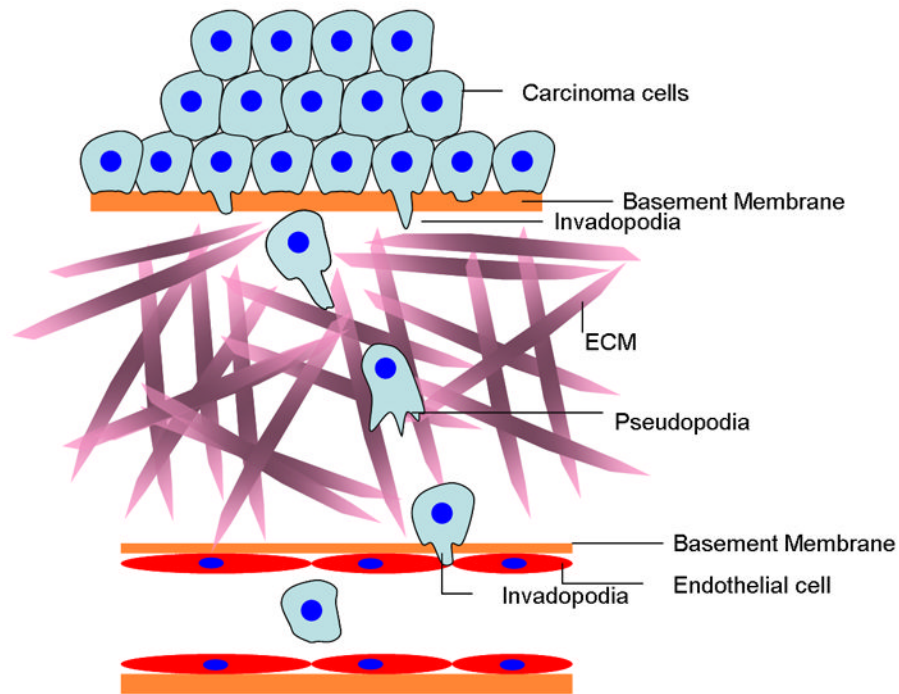4. RhoC mechanism of invadopodium protrusion confinement.

Figure 1. Invasion, migration and intravasation in tumors
Tumor cells undergoing EMT form invadopodia and acquire a migratory phenotype.
Degradation of basement membranes and extracellular matrix during migration is achieved
by invadopodia. Cells migrate on the extracellular matrix (ECM) forming pseudopodia.
There is evidence that invadopodia are involved in directional migration and chemotaxis at
this step. Once tumor cells reach a blood vessel they are believed to again use invadopodia
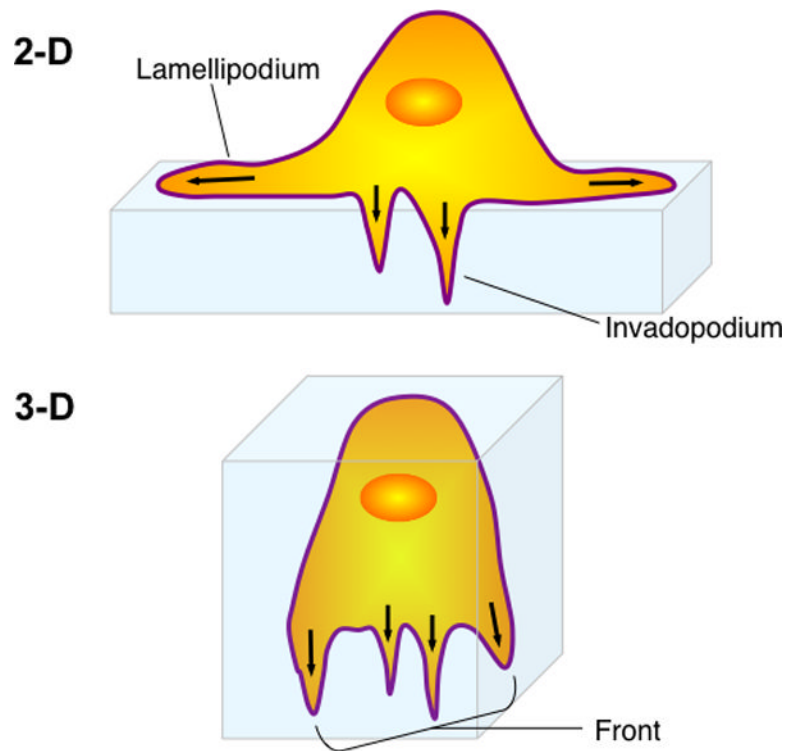to degrade the basement membrane and enter the blood stream.

Figure 2. Invadopodia and locomotory protrusions are functionally coupled during both 2 and 3-D migration

In 2-D invadopodia and pseudopodia (lamellipodia) are separated in space. In 3-D both types of protrusions are found together at the cell front. Common signaling and cytoskeletal pathways used by both motility supporting protrusions such as pseudopodia and invasion specific protrusions such as invadopodia indicate a high degree of molecular integration and cross talk between pseudopodia and invadopodia allowing efficient invasion coupled migration in 2 and 3D.
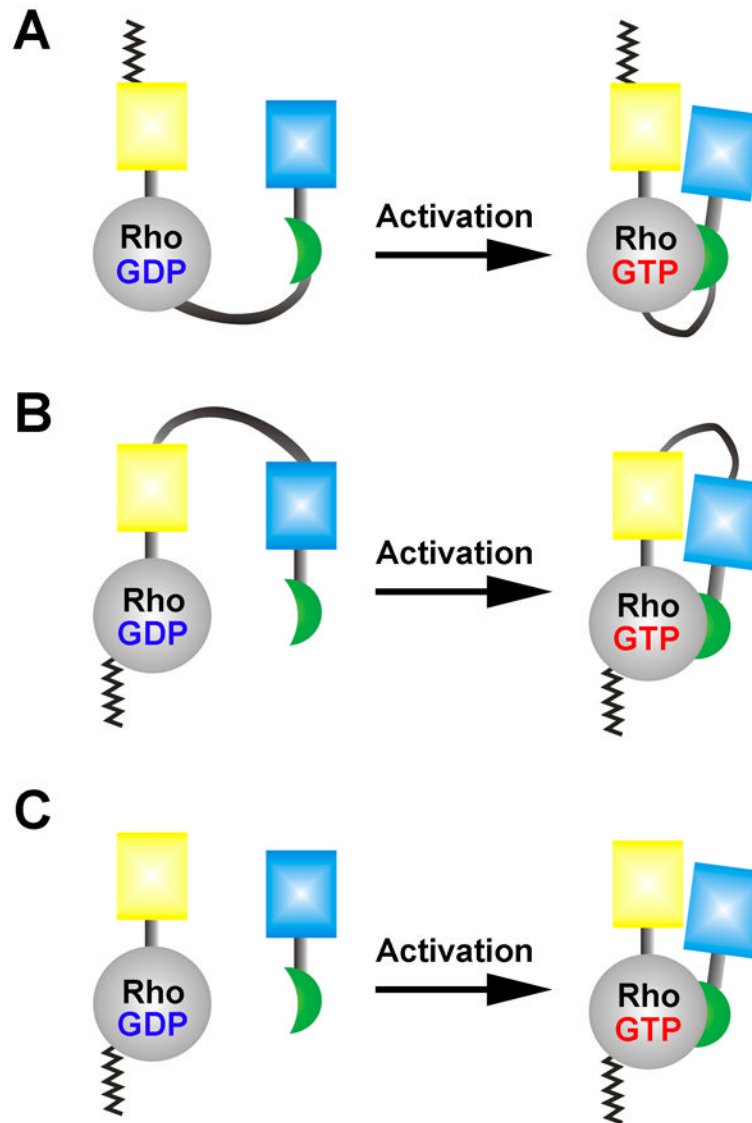
Figure 3. Examples of Rho-family GTPase biosensors based on FRET

In all examples, the guanine nucleotide loading state (GDP versus GTP) confers either a conformational change within the molecule or to bring the binding pairs together to affect FRET between the cyan and yellow fluorescent proteins. **A:** The fluorescent protein pair for FRET is placed at the terminal ends of the molecule, necessitating an attachment of a CAAX-box at the C-terminus for the plasma membrane insertion [67•]. This design compromises the GDI-GTPase interaction that requires the C-terminal lipid-moiety in addition to the Switch I/II accessibility by the GDI. **B:** The Rho GTPase biosensor based on a design that maintains the GDI-GTPase interaction by placing a full-length RhoA at the C-terminus of the molecule [65•]. The RhoC GTPase biosensor is based on a similar design as **B** [48] . **C:** Bimolecular FRET biosensor for Rho GTPases [63]. This approach maximizes the total dynamic range of FRET while making the data processing and interpretation more challenging due to the non-equimolar distribution of the biosensor components in living cells.
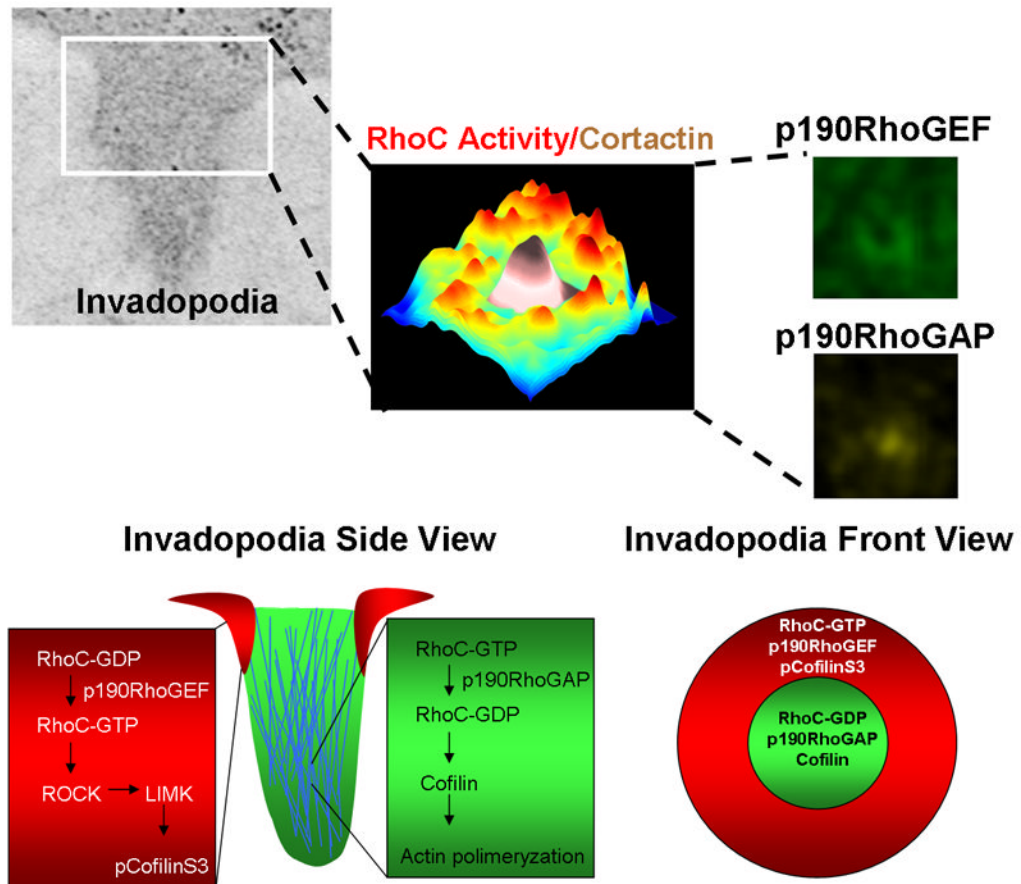
Figure 4. A model for the spatial regulation of RhoC activity during invadopodium protrusion
Formation of a focused invadopodium is mediated by the spatiotemporal localization of
RhoC activity outside invadopodia. RhoC activity increases around the invadopodial core
structure, as shown by this plot of the maximum projection over time of RhoC activity.
Pseudo-color shows low RhoC activity levels (blue) to high RhoC activity levels (red) in
relation to low (white) and high (brown) cortactin intensity where cortactin marks the central
core of the invadopodium. This activation pattern is achieved by activation of p190RhoGEF
(green) outside and p190RhoGAP inside (yellow), restricting RhoC activity just outside the
invadopodium core. This spatial restriction localizes active cofilin to the core of the
structure and focuses actin polymerization so as to achieve optimum protrusion elongation
and invasion (bottom part adapted from [68]).