**Cdc42 and Tks5**

A minimal and universal molecular signature for functional invadosomes

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**Abbreviations:** CNF1, cytotoxic necrotizing factor 1; ECM, extracellular matrix; GEF, guanine exchange factor; HUVECs, human umbilical vein endothelial cells; IPTG, isopropyl β-D-1-thiogalactopyranoside; NaF, sodium fluoride; PAE, porcine aortic endothelial; RITC, rhodamine B isothiocianate

Invadosomes are actin-based structures involved in extracellular-matrix degradation. Invadosomes, either known as podosomes or invadopodia, are found in an increasing number of cell types. Moreover, their overall organization and molecular composition may vary from one cell type to the other. Some are constitutive such as podosomes in hematopoietic cells whereas others are inducible. However, they share the same feature, their ability to interact and to degrade the extracellular matrix. Based on the literature and our own experiments, the aim of this study was to establish a minimal molecular definition of active invadosomes. We first highlighted that Cdc42 is the key RhoGTpase involved in invadosome formation in all described models. Using different cellular models, such as NIH-3T3, HeLa, and endothelial cells, we demonstrated that overexpression of an active form of Cdc42 is sufficient to form invadosome actin cores. Therefore, active Cdc42 must be considered not only as an inducer of filopodia, but also as an inducer of invadosomes. Depending on the expression level of Tks5, these Cdc42-dependent actin cores were endowed or not with a proteolytic activity. In fact, Tks5 overexpression rescued this activity in Tks5 low expressing cells. We thus described the adaptor protein Tks5 as a major actor of the invadosomes degradation function. Surprisingly, we found that Src kinases are not always required for invadosome formation and function. These data suggest that even if Src family members are the principal kinases involved in the majority of invadosomes, it cannot be considered as a common element for all invadosome structures. We thus define a minimal and universal molecular signature of invadosome that includes Cdc42 activity and Tks5 presence in order to drive the actin machinery and the proteolytic activity of these invasive structures.

**Introduction**

Podosomes and invadopodia, formed by normal and cancer cells, respectively, are sub-cellular actin-rich structures that are endowed with a proteolytic activity. Both these structures, which share a high level of similarity, are collectively named invadosomes. As these structures are dedicated to invasion, they are found in cells that are able to migrate across tissue boundaries. Under physiological conditions, podosomes are formed in cells of the myelomonocytic lineage, such as macrophages, dendritic cells, neutrophils, and osteoclasts. Some non-hematopoietic cells including fibroblasts, endothelial, and smooth muscle cells can also form podosomes under appropriate stimulation with growth factors (PDGF, VEGF, TGFβ, TNFα) (for a review, see ref. 1) or activating components (phorbol esters, cytotoxic necrotizing factor 1 [CNF1], sodium fluoride [NaF]).²,⁵ However, VEGF, TNFα, and phorbol-12-myristate-13-acetate (PMA) were reported to promote podosome formation in human umbilical vein endothelial cells (HUVECs),²,⁶ but not in bovine aortic endothelial cells (BAECs),⁷ pointing out some different signaling pathways required for podosome formation in endothelial cells.⁸ On the other hand, invadopodia do form spontaneously in cancer cells and more specifically in metastatic cancer cells. They were also described in cancer cells that undergo epithelial-mesenchymal transition.⁹,¹⁰ Finally, we recently described a new class of invadosomes called linear invadosomes that form in a
large number of cell types, both normal and cancer cells, upon contact with fibrillar type I collagen. Even if until recently invadosomes were mainly described in vitro, an increasing number of studies tend to address their existence in vivo. Indeed, invadosomes have now been described during cell invasion in various models including mice, zebrafish, *Drosophila melanogaster*, and *Caenorhabditis elegans* (for a review, see Sherwood and collaborators, this issue). Besides their presence in a variety of cell types, invadosomes organization inside the cell is also a variable parameter. The base unit of an invadosome is an actin core observed as an actin dot by immunofluorescence. Although invadopodia are often present as individual structures in cancer cells, podosomes can assemble into mega-structures described as clusters, rosettes, belts (for a review, see ref. 1), or lines. This variability in the invadosome world is probably due to the complex combination of molecules and pathways required for their formation, organization, and function. Like focal adhesions, invadosomes are multi-protein complexes that link the extracellular matrix to the actin cytoskeleton. However, it is clear that according to the cell context and the microenvironment, the requirement of invadosome components may differ from one cell type to the other. Over the past 10 y, many components and pathways regulating invadosome formation and function have been discovered. Podosomes and invadopodia are enriched with phosphatidylinositides, which requires a tight regulation of the phosphoinositide 3-kinases (PI3K) pathway (reviewed in ref. 12). Several actin nucleators are associated with the F-actin-rich core, such as the Arp2/3 complex and its nucleation-promoting factors (N-WASP/WASP and cortactin), forms, or Spire that drive F-actin polymerization. Many actin-binding proteins such as fascin, vinculin, or cofilin are also markers of these structures. Consequently, RhoGTPases are highly required for invadopod formation and organization. RhoA, Rac1, or Cdc42 were all described to be involved, depending on the model. For example, podosomes are disrupted in osteoclast-like multinucleated cells upon inhibition of Rho using C3 transferase, whereas they still form in primary osteoclasts under the same treatment. In addition, RhoA silencing has no effect on podosome formation in fibroblasts (refs. 21–23; Table 1). Similarly, macrophages from mice that lack Rac expression (Rac1/2−/− mice) are unable to form podosomes, whereas Rac expression is dispensable for podosome formation in endothelial cells. Finally, Cdc42 appears as a consensus in the invadosome landscape as all podosomes and invadopodia are Cdc42-dependent irrespectively of the cell type (Table 1).

### Table 1. RhoGTPases (Cdc42, RhoA, and Rac1) involvement in different models of invadosomes

| Cellular model       | Cdc42 | RhoA | Rac1 | References |
|----------------------|-------|------|------|------------|
| **PODOSOMES**        |       |      |      |            |
| macrophages          | +     | nd   | nd   | 1, 2, 3    |
| osteoclasts          | +     | -/+  | +    | 4, 5, 6, 7, 8, 9 |
| dendritic cells      | +     | -/+  | +    | 10, 11, 12, 13 |
| endothelial cells    | +     | -/+  | -    | 14, 15, 16, 17, 18 |
| smooth muscle cells  | +     | nd   | +    | 19, 20     |
| fibroblasts          | +     | -    | +    | 21, 22, 23  |
| Src-transformed cells| +     | -/+  | -/+  | 24, 22, 25, 26, 27, 28, 29 |
| **INVADOPODIA**      |       |      |      |            |
| MDA-MB-231           | +     | +    | nd   | 30, 31, 32 |
| A375MM               | +     | nd   | nd   | 33         |
| RPMI7951             | +     | -    | +    | 34         |
| MTh3                 | +     | +    | nd   | 35         |
| SNB19, U87           | nd    | nd   | +    | 36         |

For table legend, see following page.
Table 1 (Legend). RhoGTPases (Cdc42, RhoA, and Rac1) involvement in different models of invadosomes
nd, not determined; +, involved; - , not involved.
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we used various cellular models to challenge the functions of Cdc42 and Tks5 at invadosomes. We thus define both molecules as universal markers of invadomes in all cell types and organisms.

Results

Cdc42 is the main RhoGTPase involved for invadosome formation

Based on their involvement in actin cytoskeleton remodeling, RhoGTPases are major regulators of invadomes. RhoGTPases involvement was sought in most of the podosomes- and invadopodia-presenting cell types. Due to the variety of cellular models combined with various stimuli, the picture obtained is not clear. However, interestingly, Cdc42 appears as a key and central molecule for invadome formation in all cell types independently of the stimuli used to form invadomes (Table 1). To confirm this observation, we used siRNA approach to knockdown Cdc42 in NIH-3T3 cells expressing a constitutively active form of Src, a classical model to study invadomes. Src-transformed NIH-3T3 (Src-3T3) cells were transfected using siRNAs targeting murine Cdc42 and formation of podosome rosettes was assayed by co-staining of F-actin and cortactin (Fig. 1A). Cdc42 expression was efficiently knocked-down using 2 distinct siRNAs (Fig. 1B). We found that Cdc42 downregulation strongly decreases the ability of cells to form invadosome rosettes, when compared with the control condition (Fig. 1A and C). This result confirms that Cdc42 is involved in invadome formation. Interestingly, whereas siRNA directed against RhoA did not affect rosette formation, Rac1 depletion altered invadome rosette organization (Fig. S1). But, in Rac1-knocked-down cells, actin dots still form (Fig. S1) and degrade the ECM (data not shown) suggesting that neither RhoA nor Rac1 silencing affects invadome formation in Src-3T3 cells. Moreover, constitutively active form of Cdc42 (V12Cdc42) was early shown to act as an inducer of invadomes in HeLa and porcine aortic endothelial (PAE) cells.1,4 We first confirmed this result in both cell types (Fig. 2A, B, and D) and also found that expression of V12Cdc42 was able to induce invadomes in additional cell types such as NIH-3T3 fibroblasts (Fig. 2C and D), HUVEC-α,2 and HuH7, a hepatocarcinoma cell line without constitutive invadopodia (data not shown). It is important to notice that V12Cdc42 protein accumulates into invadomes (Fig. 2B and C). Thus, based on the literature and our own experiments, we support the fact that Cdc42 is the critical inducer for invadome actin core formation.

Degradation activity of V12Cdc42-induced invadomes

To test the ability of V12Cdc42-induced invadomes to degrade the extracellular matrix, we used the classical gelatin matrix degradation assay. After V12Cdc42-GFP transfection, NIH-3T3 cells were seeded on rhodamine B isothiocianate (RITC)-gelatin. Twenty-four hours later, cells were fixed and stained for F-actin. Confocal analysis showed that in NIH-3T3

Figure 1. Cdc42 is involved in invadosome formation. (A) Representative confocal microscopy images of invadose rosettes in Src-3T3 cells. Control siRNA (siCT) and Cdc42 targeting siRNA (siCdc42#1)-transfected cells were seeded on glass coverslips for 24 h, fixed, and stained for F-actin (red) and cortactin (green). Scale bar: 10 μm. (B) Protein extracts of Src-3T3 cells transfected with control siRNA and 2 siRNA targeting Cdc42 (Si#1 and Si#2) were analyzed by western blot using Cdc42 and GADPH (loading control) antibodies. (C) Diagram representing the percentage of cells exhibiting podosome rosettes. Cells depleted of Cdc42 were compared with control cells. Each bar represents the mean ± SEM of 3 independent experiments. ***P < 0.001 using the t test when compared with control.
fibroblasts, V12Cdc42-induced invadosomes were able to degrade the gelatin, demonstrating the functionality of these structures (Fig. 3A and C). This result suggests that Cdc42 activity alone is sufficient to promote the formation of functional invadosomes. We aimed at confirming this result using the model of PAE cells stably expressing an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible active form of Cdc42. After 24 h IPTG stimulation, cells were placed on RITC-gelatin to visualize PAE degradation activity. Surprisingly, although many cells indeed exhibited podosomes, no degradation could be noticed (Fig. 3B and C). Thus, depending on the cellular model, active Cdc42 appears sufficient to recruit the actin machinery to form invadosomes core, but not to bring the degradation activity to the structure.

**Tks5 is required to assure degradation activity of invadosomes**

In the literature, Tks5 appears as an early marker during invadosome formation. However, depending on the cellular model, Tks5 depletion could promote invadosome destabilization or only inhibition of the degradation activity. It was shown that Tks5 allows metalloproteinase activation at invadosomes, and consequently, matrix degradation as described in macrophages. Considering this, we hypothesized that Cdc42 could promote formation of the invadosome F-actin architecture, and Tks5 could control and regulate the degradation activity. Interestingly, we demonstrated previously that PAE cells express very low level of Tks5 protein, when compared with other cell lines. In addition, we found that, in PAE cells, V12Cdc42-induced podosomes...
are devoid of Tks5 staining whereas Tks5 clearly accumulates in V12Cdc42-induced podosomes of NIH-3T3 cells (Fig. 4A). We then asked whether Tks5 overexpression could rescue the degradation phenotype of PAE-V12Cdc42 cells. After Tks5-GFP transfection, PAE-V12Cdc42 cells were stimulated with IPTG to promote invadosome formation and seeded on RITC-gelatin to investigate their degradation activity. Using confocal microscopy, we showed that Tks5-GFP co-localizes with F-actin in invadosomes (Fig. 4B). Strikingly, even if Tks5-GFP overexpression does not affect the number of cells exhibiting podosomes (Fig. 4C), this overexpression is associated with a degradation activity of V12-Cdc42-induced invadosomes (Fig. 4B and D). We can notice a perfect co-localization of F-actin, Tks5-GFP, and gelatin degradation (Fig. 4B). This result prompted us to check for the expression of MT1-MMP in GFP control condition vs. Tks5-GFP in PAE cells. As expected, we were able to visualize MT1-MMP staining only upon Tks5-GFP transfection (Fig. 4E and F). Thus, these data suggest that Tks5 is a limiting factor in PAE cells, which allows us to dissect the molecular requirements to build a functional invadosome. Our results demonstrate the molecular complementary between Cdc42 and Tks5 to form functional invadosomes.

Src kinase involvement varies according to the invadosome model

As Src family kinases are thought to be required for invadosome formation, we checked their involvement in V12Cdc42-expressing PAE cells that form individual podosomes. We found that Src inhibitors such as SU6656 and PP2 were not able to inhibit podosome formation in PAE cells (Fig. 5A and C). As expected, both inhibitors abolished the formation of rosettes in Src-3T3 fibroblasts (Fig. 5B and D). However, V12Cdc42-induced invadosomes in NIH-3T3 cells were also resistant to Src inhibitors (Fig. 5E). These data confirm results that we previously observed in V12Cdc42-induced podosomes in HUVECs. To strengthen this observation, we further screened for other invadosome models that were resistant to Src inhibition. We previously demonstrated that NIH-3T3 fibroblasts form linear invadosomes upon contact with collagen fibrils. Treatment of NIH-3T3 cells with either SU6656 or PP2 did not affect their ability to form linear invadosomes (Fig. 5F). Altogether, these data demonstrate that Src activation is not a prerequisite for invadosome formation in all models.

We further tested whether Src kinases were required for the degradation activity of these latter invadosomes. Thus, V12Cdc42-transformed NIH-3T3 cells were seeded on fluorescent gelatin and treated using Src inhibitors. Whereas invadosomes were still present, inhibition of Src kinases completely blocked their degradation activity (Fig. 6A). However, when invadosomes were induced upon contact with collagen fibrils in the same cellular background, we found that neither PP2 nor SU6656 treatment abolished the degradation activity of the linear invadosomes (Fig. 6B). These results suggest that depending on the inducer, Src kinases are or not required for invadosome formation and function. Thus, these data demonstrate that even if Src is required in a large number of invadosomes, it is not a common element of all invadosome models.

Discussion

Recently, our group described a novel class of invadosomes, named linear invadosomes, which form upon contact with fibrillar type-I collagen in either normal or cancer cells. These structures belong to the invadosome family as they are F-actin-based...
Figure 4. Tks5 overexpression rescues degradation activity of V12Cdc42-induced podosomes in PAE cells. (A) Immunofluorescence analysis was performed to control Tks5 localization on V12Cdc42-induced podosomes in NIH-3T3 and PAE cells. Cells were stained for F-actin (red), Tks5 (Green). Scale bar: 5 µm. (B) Tks5-GFP was overexpressed in PAE-V12Cdc42 cells. Cells were seeded on fluorescent gelatin and stained for F-actin (red), Tks5-GFP (green), gelatin (gray), and nuclei (blue). Dark areas on gelatin show the degradative activity of podosomes in the Tks5 overexpression condition. Scale bar: 10 µm. (C) Quantification of the percentage of PAE-V12Cdc42 cells exhibiting invadosomes after GFP or Tks5-GFP transfection. ns, non-significant from t test when compared with control. (D) Quantification of the percentage of gelatin degrading PAE-V12Cdc42 cells after or not IPTG treatment and GFP or Tks5-GFP transfection. Each bar represents the mean ± SEM of 3 independent experiments. ns, non-significant from the t test when compared with control (-IPTG); ***P < 0.01 from the t test when compared with control (GFp-transfected PAE-V12Cdc42). (E and F) MT1-MMP staining was performed on PAE-V12Cdc42 transfected respectively with GFp or Tks5-GFP. Cells were stained for F-actin (red), GFp or Tks5-GFP (green), gelatin (gray), and MT1-MMP (gray). Tks5-GFP overexpression promotes a concentration of MT1-MMP on podosomes. Scale bar: 5 µm.
structures endowed with a proteolytic activity. However, these structures challenged our view of the minimal molecular composition of the invadosome unit. Indeed, linear invadosomes form in an integrin and Src-independent manner. This led us to propose that a limited number of elements might be enough to define an invadosome. Based on our own data and data available in the

![Figure 5. Src involvement in invadosome formation. (A, B, E, F) PAE-V12Cdc42 cells stimulated with IPTG (A), Src-3T3 cells (B), GFP-V12Cdc42-expressing NIH-3T3 cells (E), and NIH-3T3 cells seeded on type I collagen fibrils for 12 h (F) that form invadosomes in the control condition (DMSO), were treated or not with Src inhibitors, SU6656, or PP2. Cells were treated with inhibitors at 5 µM for 1 h. After fixation, cells were stained for F-actin (red), cortactin (green), and nuclei (blue). Scale bars: 10 µm. (C and D) Quantification of the percentage of PAE-V12Cdc42 and Src-3T3 cells exhibiting invadosomes after Src inhibitor treatments, when compared with control (DMSO) condition. Each bar represents the mean ± SEM of 3 independent experiments where approx. ns, non significant, ***P = 0.0004 in ANOVA, when compared with control. This analysis reveals that even in presence of Src inhibitors, V12Cdc42- and type I collagen-induced invadosomes are still present, whereas formation of Src-induced rosettes is strongly inhibited.](image-url)
Cdc42 is a universal regulator of invadosomes

Cdc42 is a well-studied RhoGTPase involved in actin cytoskeleton remodeling and in the establishment of cell polarity from yeast to mammalian cells. Since the pioneer experiments demonstrating that expression of V12Cdc42 in Swiss 3T3 fibroblasts induced the formation of finger-like protrusions called filopodia involved in probing the environment during cell migration, it has been a dogma that expression of constitutively active Cdc42 promotes filopodia formation. However, activation of Cdc42 is also able to induce podosomes in a large number of cell types including HeLa, NIH-3T3, PAE, and HUVEC cells (refs. 4 and 41; Fig. 1). In addition, knockdown of Cdc42 inhibits podosomes and invadopodia formation (Table 1; Fig. 1). Accordingly, Cdc42 should not be restricted to its role as filopodia inducer but instead should be presented as a filopodia and invadosome inducer. In contrast to a recent publication showing that vSrc-induced invadosomes in NIH-3T3 fibroblasts were highly sensitive to the expression of a dominant-negative mutant of Rac1 and only moderately sensitive to the expression of a dominant-negative mutant of Cdc42, we found that Cdc42 knockdown completely abolished podosome rosettes in this cell type. In addition, in our conditions, Rac1 knockdown did alter rosette organization but invadosomes still formed. The dominant-negative form of Rho GTases acts by sequestering the upstream Rho guanine exchange factors (GEFs), which can lead to non-specific effects since the same GEF can activate several Rho GTases. Due to this possible “sponge” effect of overexpressed RhoGTPase mutants, it is highly recommended to use knockdown approach to address GTase requirement in cells. Thus, we can safely conclude that Cdc42 is both necessary and sufficient to promote the formation of invadopodia and podosomes. For the initiation of both kinds of structures, the best-described pathway regulated by Cdc42 is the activation of Arp2/3 complex-dependent actin polymerization. This leads to the generation of a branched actin network via the activation of WASP/N-WASP proteins. Activation of this machinery will engage many additional components involved in regulating actin polymerization. However, invadosomes induced by active Cdc42 exhibit a lack of dynamics, as they appear abnormally stable (data not shown). This may probably be due to the incapacity of V12Cdc42 protein to cycle between an active and an inactive form. Indeed, wild-type Cdc42 is activated by GEFs. Cdc42-activating GEFs involved in invadosome formation include α-PIX/ArhGEF6, β-PIX/ArhGEF7, Fgd1, and Vav1. However, as more than 70 Rho-GEFs are encoded in the human genome and as most are tissue-specific, the identity of the Cdc42-GEF may differ according to the cell type. Also, recently, other molecules such as palladin were shown to regulate Cdc42 activation. More generally, upstream stimuli known to induce podosome formation such as TGFβ, phorbol ester, CNF1, NaF were all described to induce Cdc42 activation. Altogether, these observations lead to the fact that all pathways promoting invadosome formation converge to Cdc42 activation.

It has to be noted that many other RhoGTases including, RhoA, Rac1, Rac2, RhoC, RhoE, RhoG, and RhoU (for a review, see refs. 53–58) were also involved in invadosome formation or function, and they can contribute to control invadosome organization and dynamics; but their requirement is not as universal as Cdc42 one’s is. Moreover, Cdc42 is the only RhoGTPase to act as an invadosome inducer.
Tks5 is a marker of all invadosomes

Tks5 encoded by the human *SH3PXD2A* gene is a large scaffolding protein with an Nter phox homology (PX) domain and 5 SH3 domains. Tks5 localized to invadosomes in normal and cancer cells. Tks5 was indeed found in podosomes of Src-transformed cells, macrophages, osteoclasts, PDBu-treated A7r5 rat smooth muscle cells, myoblasts, TGFβ-stimulated neural crest stem cells, microvascular cells, in invadopodia of breast, melanoma, and prostate cancer cell lines, and in linear invadosomes found in endothelial cells, macrophages, fibroblasts, and cancer cells. Thus, Tks5 appears as an universal marker of invadosomes and is particularly useful as it lacks specific localization in the absence of invadosomes. Indeed, Tks5 seems dedicated to invadosome formation and function.

As described above, Cdc42 activation in cells may lead to podosome/invadopodia formation. However, depending on the cellular background, the structures may not be functional. Indeed, in PAE cells, V12Cdc42-induced podosomes were devoid of proteolytic activity. Interestingly, we found that expression of Tks5 rescued the function of these invadosomes. This result suggests a key role of Tks5 in the proteolytic activity of invadosomes as previously described in macrophages and in prostate cancer cells. We cannot exclude a role of Tks5 in invadosome formation as demonstrated in Src-transformed fibroblasts, in breast cancer cells, and in bovine aortic endothelial cells. However, a threshold of Tks5 is necessary in PAE cells to localize MT1-MMP and functionalize invadosomes.

Given that Tks5 has no catalytic activity, its function at invadosomes should come from its interaction with other molecules. Indeed, as a scaffolding protein, Tks5 interacts with multiple proteins found in invadosomes, including the SH3/SH2 adaptors, Grb2, Nck1/2, the nucleation-promoting factor, N-WASP, and ADAM (a disintegrin and metalloproteinase) family metalloproteases. Together, these data suggest that Tks5 is an essential scaffold protein of invadosomes.

Src is not always required for invadosome formation and function

Src is the funding member of a small family of structurally related non-receptor tyrosine kinases, called the Src family kinases (SK) that includes Src, Yes, Fyn, and the hematopoietic restricted members Hck, Lyn, and Fgr. Src, which plays a key role in regulating various signal transduction pathways, is described as a central hub of the invadoposome network. Pioneer work by Marchisio and collaborators demonstrated the ability of Rous-sarcoma virus encoding the v-Src oncogene to induce the formation of F-actin dots and rosettes at the ventral membrane in BHK cells. Cellular Src was required for mitogenesis initiated by multiple growth factor receptors, including the receptors for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and colony-stimulating factor-1 (CSF1), which were all described to promote invadosome formation. Moreover, many proteins of the invadosome core are Src-substrates, such as cortactin, p190RhoGAP, Tks4/5, Grb2, FAK, and CIP4. Consequently, many models of invadosomes are Src inhibitor sensitive, suggesting that Src kinase is necessary and sufficient for the induction of invadopodia and podosome formation. However, there is evidence that Src kinases are not required for all invadosome formation. Indeed, we described herein that V12Cdc42 and type-I collagen-induced invadosomes are resistant to Src-family kinase inhibitors such as PP2 and SU6656. These results are reminiscent of the fact that actin cores still formed in e-src-deficient osteoclasts. Interestingly, Mader et al. also demonstrated that Src is not required for the actin core formation of invadopodia in breast cancer cells. Podosome-like structures formed in non-transformed fibroblasts plated on an Arg-Gly-Asp peptide-lipid surface were also found to be resistant to a high concentration of the PP2 inhibitor. Another example comes from the in vivo description of invadopodia-like protrusions in the zebrafish intestine epithelium. Indeed, matrix-degrading protrusions observed in invasive cancer cells of the zebrafish *meltdown* mutant, are not disrupted upon Src inhibition. Thus, Src kinases are clearly not always required for invadosome formation. On the other hand, in most of the models described above, Src activity was required for invadosome maturation and matrix degradation. This is also true for V12Cdc42-induced podosomes in NIH-3T3 fibroblasts, which appear to require Src kinase for their proteolysis activity. However, we described here for the first time Src kinase-independent invadosomes as formation and function of linear invadosomes are insensitive to Src kinase inhibitors. Altogether these data suggest that Src kinases, depending on the cellular context, are involved either in invadosome formation or only in later stages for matrix degradation, or not involved at all.

In conclusion, even if many components such as kinases, phosphatases, integrins, MMPs are required to regulate invadosome dynamics, supra-organization, and function, we propose a minimal definition of invadosomes based on Cdc42 dependence and Tks5 presence. We thereby reconcile podosomes and invadopodia, structures that indeed share the same molecular basis. We invite you to challenge our findings: is there one model where Cdc42 and Tks5 are not involved?

**Material and Methods**

**Cells and culture conditions**

NIH-3T3 cells and their derivatives, Src-3T3 cells, which stably express a mutant form of chicken Src with an activating Y527F substitution, were generous gifts from Dr Sara A Courtneidge (Burnham Institute for Medical Research). HeLa cells were purchased from ATCC. Porcine aortic endothelial (PAE) cells (clone p23) and their derivatives PAE-V12Cdc42 cells expressing V12Cdc42 under the control of an isopropyl-β-d-thiogalactopyranoside (IPTG)-inducible promoter were described previously. NIH-3T3, Src-3T3, and HeLa cells were maintained in DMEM Glutamax 4.5 g/L glucose medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (FCS; PAN-Biotech GmbH) and 100 U/mL penicillin–streptomycin (PS; GIBCO). PAE cells were cultured as published previously. Expression of V12Cdc42 was achieved by using 0.1 mM IPTG. All cell lines were maintained at 37 °C in 5% CO₂ humidified atmosphere.
Transfection

siRNAs were purchased from Eurofins MWG Operons. Cdc42-specific siRNA duplexes are directed against the target sequence 5′-AAGATACTACCACCTGTCCA-3′ for siRNA#1 and 5′-GAGATGACCC CTCTACTATT G-3′ for siRNA#2. Rac1 and RhoA-specific siRNA duplexes are directed against the target sequences 5′-AAGTCTTAA TTTGCTTTTC C-3′ and 5′-GAAGTCAAGC ATTTCTGTC-3′, respectively. The control siRNA is targeted against luciferase 5′-CGTACGCGGA ATACTTCGA-3′. Cells were transfected in 2 rounds of transfection (one reverse and one forward transfection both 24 h spaced) with 50 nM siRNA using lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocol. Experiments were performed 48 h or 72 h after transfection. For DNA transfection, according to the manufacturer’s protocol. Experiments were performed 24 h after transfection. Tks5-GFP plasmid was kindly donated by Dr Sara A Courtneidge. Cdc42-GFP and Cdc42-Myc plasmids were generous gifts from Dr Philippe Fort.

Reagents and antibodies

Puromycin, hygromycin B, and IPTG were from Calbiochem. FCS was from PAN-Biotech GmbH, and culture medium and antibiotics were purchased from Gibco. Fluorescent Gelatin was homemade using the following protocol: 10 µg/mL 5-(and-6)-Carboxy-X-Rhodamine, Succinimidyl Ester (5[6]-ROX, SE) mixed isoesters (Molecular Probes®) was hybridized with 10 mg/mL gelatin solution (Sigma), 10 min at room temperature, and finally used at 1 mg/mL on coverslip. Collagen type I from rat tail was purchased from BD Biosciences. Phalloidin-FluorProbes® and FluorProbes® secondary antibodies were purchased from Interchim and Hoescht from Sigma. Monoclonal anti-cortactin (4F11), anti-Cdc42, and anti-MT1-MMP antibodies were purchased from Millipore, BD transduction Laboratories and Invitrogen, respectively. Monoclonal anti-GAPDH (D-6) and anti-actin antibodies were from Santa Cruz Biotechnology. Src inhibitors PP2 and SU6656 were purchased from Millipore, BD transduction Laboratories and Hoescht from Sigma. Monoclonal anti-cortactin (4F11), anti-Cdc42, and anti-MT1-MMP antibodies were purchased from Millipore, BD transduction Laboratories and Chemicon, respectively. Monoclonal anti-GAPDH (D-6) and polyclonal anti-Tks5 (M300) antibodies were from Santa Cruz Biotechnology. Sαcplinin antibodies PP2 and SU6656 were purchased from Abcam and Santa Cruz Biotechnology, respectively.

Immunofluorescence microscopy

For matrix degradation assay, glass coverslips were coated with fluorescent gelatin and crosslinked with glutaraldehyde 0.5% before cell seeding. 3.5 x 10^5 cells were seeded on each coverslip. For type-I collagen stimulation, glass coverslips were coated as described previously.11 Twenty-four hours after seeding, cells were fixed with 4% paraformaldehyde prepared in PBS for 10 min at room temperature and permeabilized with 0.2% Triton X-100 for 10 min. After 3 washes in PBS, the cells were incubated successively with blocking solution (1% bovine serum albumin, 2% FCS in Tris-buffered saline, 20 mM Tris, 150 mM NaCl, 2 mM EGTA, 2 mM MgCl_2 [pH 7.5]) for 10 min, with primary antibody diluted in blocking solution for 40 min, and then with fluorescently labeled secondary diluted in blocking solution antibody for 30 min. Between each step, cells were washed 3 times with Tris-buffered saline. The coverslips were washed in water and mounted on microscope slides using Fluoromount-G mounting medium (FluorProbes® Interchim). Confocal images were captured on a Leica SP5 microscope using a 63X oil immersion objective at the Bordeaux Imaging Center. The images were processed with Adobe Photoshop 5.5. Quantitation of cells showing invadosomes was assessed in 3 independent experiments in which at least 200 cells were counted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/celladhesion/article/28833/
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