Insights and perspectives on calcium channel functions in the cockpit of cancerous space invaders

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Cell calcium Special Issue "VSI: Ca2+ Channels in Cancer"

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Highlights

- **Development of metastasis is the main cause of cancer-related death.**
- **Metastasis formation requires cancer cell invasion through extracellular matrix (ECM) remodeling.**
- **Invadosomes are metastatic cancer cell specialized structures that handle adhesion-based processes and local ECM degradation activities.**
- **Several key signaling pathways controlling invadosome dynamics/functions are Ca^{2+}-sensitive.**
- **Evidence is shedding light upon new and original Ca^{2+} channel roles in invadosome regulation.**

Graphical Abstract

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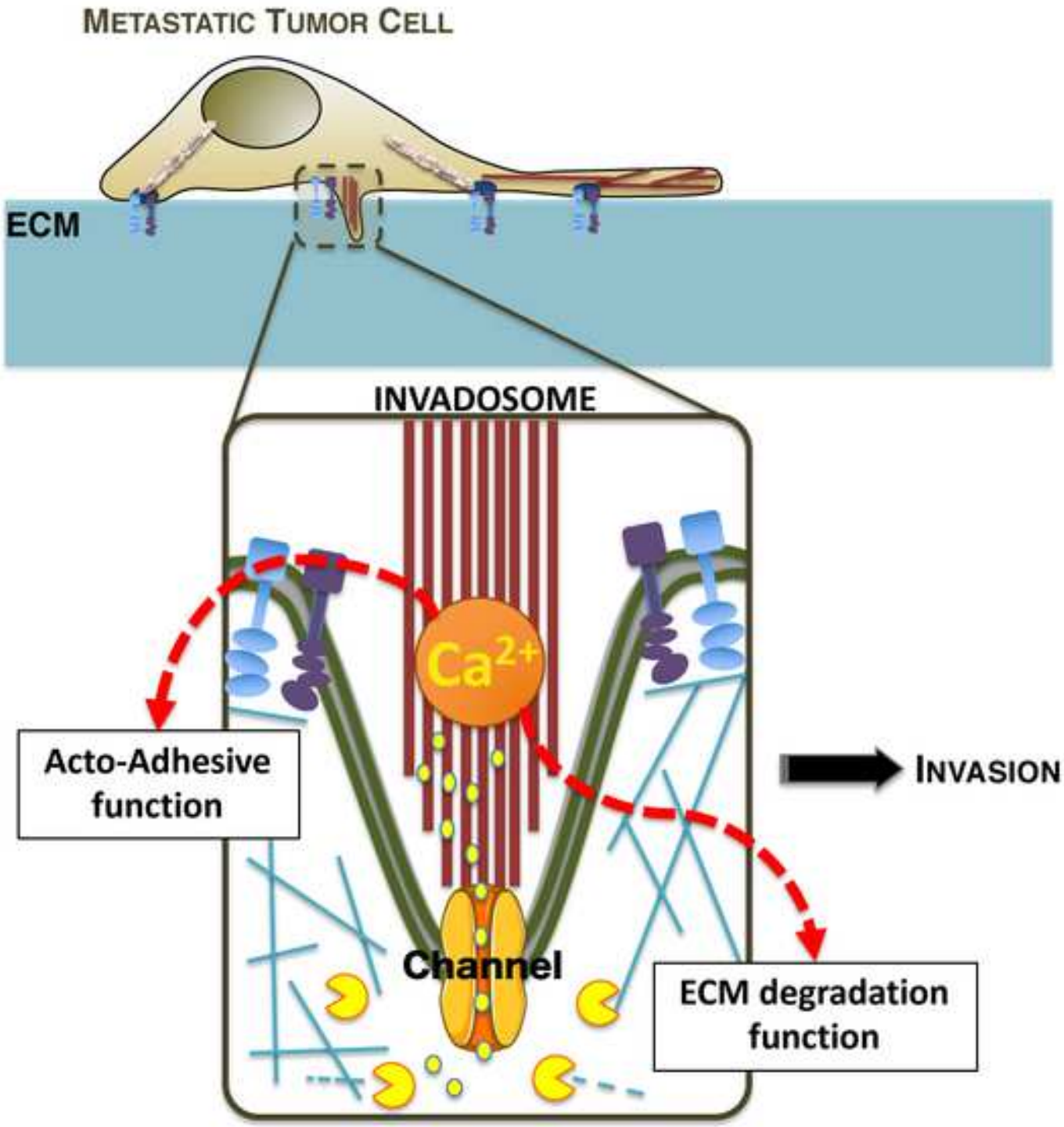

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Insights and perspectives on calcium channel functions in the cockpit of cancerous space invaders.

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Running title: Calcium channels and invadosomes

Abstract:

Development of metastasis causes the most serious clinical consequences of cancer and is responsible for over 90% of cancer-related deaths. Hence, a better understanding of the mechanisms that drive metastasis formation appears critical for drug development designed to prevent the spread of cancer and related mortality. Metastasis dissemination is a multistep process supported by the increased motility and invasiveness capacities of tumor cells. To succeed in overcoming the mechanical constraints imposed by the basement membrane and surrounding tissues, cancer cells reorganize their focal adhesions or extend acto-adhesive cellular protrusions, called invadosomes, that can both contact the extracellular matrix and tune its degradation through metalloprotease activity. Over the last decade, accumulating evidence has demonstrated that altered Ca^{2+} channel activities and/or expression promote tumor cell-specific phenotypic changes, such as exacerbated migration and invasion capacities, leading to metastasis formation. While several studies have addressed the molecular basis of Ca^{2+} channel-dependent cancer cell migration, we are still far from having a comprehensive vision of the Ca^{2+} channel-regulated mechanisms of migration/invasion. This is especially true regarding the specific context of invadosome-driven invasion. This review aims to provide an overview of the current evidence supporting a central role for Ca^{2+} channel-dependent signaling in the regulation of these dynamic degradative structures. It will present available data on the few Ca^{2+} channels that have been studied in that specific context and discuss some potential interesting actors that have not been fully explored yet.

Key words: cancer, invasion, invadosome, invadopodia, podosome, calcium channels, STIM/Orai, TRPM7, TRPV2, TRPV4, PIEZO.

Highlights

- **Development of metastasis is the main cause of cancer-related death.**
- **Metastasis formation requires cancer cell invasion through extracellular matrix (ECM) remodeling.**
- **Invadosomes are metastatic cancer cell specialized structures that handle adhesion-based processes and local ECM degradation activities.**
- **Several key signaling pathways controlling invadosome dynamics/functions are Ca^{2+}-sensitive.**
- **Evidence is shedding light upon new and original Ca^{2+} channel roles in invadosome regulation.**

Abbreviations

Ca^{2+}: Calcium ions
CaM: Calmodulin
CAM: chorioallantoic membrane
Cas9: CRISPR associated protein 9
CiB1: Calcium and integrin-binding protein 1
CRISPR: Clustered regularly interspaced short palindromic repeats
DDR: Discoidin domain receptor
ECM: Extracellular matrix
ER: Endoplasmic reticulum
FA: Focal adhesion
F-Actin: filamentous actin
FAK: Focal Adhesion Kinase
FERM Domain: F for 4.1 protein, E for ezrin, R for radixin and M for moesin
FlII: Flightless-1 homolog 1
fMLP: N-formyl-L-methionyl-L-leucyl-phenylalanine peptide
Hic-5: Transforming growth factor beta-1-induced transcript 1 protein
HUVEC: human umbilical vein endothelial cells
I_{CRAC}: calcium-release-activated Ca2+ current
IP3: Inositol trisphosphate
IP3R: IP3 Receptor
IQGAP1: IQ motif Containing GTPase activating protein 1
KCa: Calcium-activated potassium channel
LTCCs: L-type Ca^{2+} channels
MEF: Mouse embryonic fibroblast
Mg^{2+}: magnesium ions
MMP: Matrix metalloproteinase
MMTV-PyMT: mouse mammary tumor virus-polyoma middle tumor-antigen
MSC: Mechanosensitive channel
MT1-MMP: Membrane-anchored type I- matrix metalloproteinase
MYH9: myosin heavy chain 9
NHE1: sodium-hydrogen exchanger 1
NMIIA : Non-muscle myosin IIA
NSCLC : Non-small-cell lung carcinoma
P130Cas: Crk-associated substrate
PI3K: Phosphoinositide 3-kinase
Pyk2: Proline-rich tyrosine kinase 2
PLC: Phospholipase C
PM: Plasma membrane
Rac3: Rac family small GTPase 3
RANKL: Receptor activator of nuclear factor kappa-B ligand
RhoA: Ras homolog family member A
SCLC: Small-cell lung carcinoma
SH2 domain: Src Homology 2 domain
SK3: small-conductance calcium-activated potassium channel 3
SOCE: Store-operated calcium entry
Src: v-Src viral-Src and c-Src cellular-Src

SSH1: Slingshot homolog 1 phosphatase
STIM: Stromal interacting molecule
TIMPs: tissue inhibitor of metalloproteinases
TIRF microscopy: total internal reflection fluorescence microscopy
TKS5: tyrosine kinase substrate with 5 SH3 domains
TRP: Transient receptor potential
TRPM7: Transient receptor potential cation channel, subfamily M, member 7
TRPV2: Transient receptor potential cation channel, subfamily V, member 2
TRPV4: Transient receptor potential cation channel, subfamily V, member 4
VGCC: Voltage-gated calcium channel
WASP: Wiskott–Aldrich syndrome protein

1. Introduction

Cancer is the second leading cause of death globally. In 2015 alone, it was responsible for 8.8 million deaths worldwide. Due to the growth and aging of the population, the global cancer burden is growing at an alarming pace. The number of new cancer cases per year is expected to rise to more than 20 million by 2030, with 13.0 million cancer deaths expected to occur despite recent progress in the development of innovative treatments. Hence, cancer is a major health problem and, because it knows no boundaries, has a major impact on societies across the world.

A hallmark of high-grade cancers is the ability of malignant cells to move and invade unaffected tissue and spread the disease throughout the body. In solid malignancies, it is not the primary tumor but this metastatic spread and resulting systemic disease that account for approximately 90% of cancer-related deaths. Thus, to fight against the development of secondary tumors, metastasis prevention deserves to be one of the most urgent missions of cancer treatment.

Metastasis formation is a multistep process in which tumor cells have to succeed in migration/invasion, vasculature intravasation/extravasation and colonization of a distant organ. From a deterministic point of view, one essential characteristic for metastatic cells is to enhance their motility to leave the primary tumor site and spread. However, to complete the “metastatic cascade”, migrating cancer cells also require enhanced invasiveness to help them overcome barriers imposed by the basement membrane and surrounding tissues. Remodeling of this extracellular matrix (ECM) is a fundamental property of metastatic cells, achieved through the coupling of adhesion-based processes and local ECM degradation activities. This highly regulated dual coupling takes place within specific cellular structures present in invasive cancerous cells, such as specific degradative focal adhesions (FA) and invadosomes. Various studies and reviews have described the complexity of the adhesomes [1-5] and invadosomes [6]. Invadosomes in particular have been increasingly recognized as important drivers of local invasion in cancer. Their involvement in multiple steps of the metastatic cascade caused them to emerge as interesting levers to block metastasis [7, 8]. Here, we will focus our attention mainly on cancer cell invadosomes and their main regulators.

Calcium ion (Ca^{2+}) is the simplest and most versatile second messenger in biology. Cytosolic and endoplasmic reticulum (ER) Ca^{2+} levels are closely regulated in all cells by transport

mechanisms, including pumps and plasma membrane (PM) channels. This allows Ca^{2+} to flux inside the cytosol upon stimulus-induced PM channel opening and to act as an essential second messenger in a plethora of vital cellular functions [9]. Finely tuned Ca^{2+} signaling has long been known as an important regulator of normal cell-substrate adhesion, migration and invasion, but more recent studies have shown that cancer cells hijack these Ca^{2+}-dependent signaling pathways to gain motile and invasive potentials [10]. Over the last decade, accumulating evidence has demonstrated that altered Ca^{2+} permeable channel activities and/or expression promote exacerbated migration and invasion capacities of tumor cells, leading to metastasis formation [11]. Notably, members of the TRP (Transient Receptor Potential) and Orai families, have recently emerged as prognostic markers in different cancers and as important and promising classes of targets for anti-metastatic drug discovery [12]. Several studies, emphasizing the role of certain voltage-independent Ca^{2+} channels in the malignant process of tissue invasion and metastasis, have started to address the molecular basis of Ca^{2+} channel-dependent cancer cell migration/invasion. However, we are far from having reached a comprehensive and integrative view of all the mechanisms, and especially the Ca^{2+} channel-regulated ones, involved in tumor cells metastasis dissemination. This review summarizes recent data highlighting new and original roles of Ca^{2+} channels in the specific context of invadosome-driven cancer cell invasion. It will discuss the connections between Ca^{2+} signaling, Ca^{2+} channels and classical invadosome regulators in the multiple cancer-related functions of invadosomes.

2. Cellular degradative structures, cancer cell invasion and metastasis

Cell invasion relates to cell migration processes occurring within mechanically constrained environments, such as highly entangled ECM and neighboring tissues. The limiting factor for invading tumor cells is to pass their nuclei through narrow and tight spaces, which they can overcome by eroding the ECM [13]. The molecular mechanisms controlling ECM degradation in space and time have been extensively studied over the last 3 decades. Counterintuitively, it clearly appeared that cell adhesion to the ECM plays a pivotal role in the regulation of ECM degradation, suggesting the existence of a fine and precise tuning between both adhesion and degradation [14].

Two main classes of degradative structures have been described: FAs and invadosomes. FAs are adhesive structures composed of nanoclusters of ECM receptors, such as integrins, that mechanically link the ECM to the internal cytoskeleton through contractile filaments of acto-myosins. FAs were initially associated with cell migration and ECM remodeling. However, it appeared that some FAs present in cancerous cells can sustain a degradative activity that is dependent on the tyrosine kinase Src and the subsequent local activation of matrix metalloproteases (MMPs) [15]. Nonetheless, the major class of degradative structures is composed of the podosomes, the invadopodia and the linear invadosomes, collectively called "invadosomes". These structures have long been thought to be highly different based on their morphology, dynamics and the cell types where they were observed [16]. By taking into account their functional relevancies, it later became clear that they are in fact strongly related (Figure 1A). By convention, podosomes, the first to be described, refer to degradative structures present in cells overexpressing constitutively active Src mutants as well as in non-transformed cells (including dendritic cells, macrophages, endothelial cells, vascular smooth muscle cells, megakaryocytes and osteoclasts) [17]. As for invadopodia, they refer to the degradative structures present in cancer cells [18]. The last member of the invadosome family was described in 2012. These structures are specifically formed along thick bundles of collagen I and were named linear invadosomes [19].

Invadosomes have been observed *in vivo* in multiple physiological conditions (immune system scanning, vascular reorganization, bone degradation, etc.) and in different organisms, from *C. elegans* to humans [20]. Direct molecular links between the assembly of invadosomes and cancer metastasis have also been evidenced *in vivo* in the *ex ovo* chicken embryo model, as well as in mouse and human models [20-23]. An high-resolution time-lapse intravital imaging approach to visualize the dynamics of cancer cell in the *in vivo* chick chorioallantoic membrane (CAM) model has provided direct evidence of the role of invadosome during cancer cell extravasation [21, 22]. Invadosomes have also been observed *in vivo* through 2-photon imaging in a model of breast cancer cell lines xenografted in nude mice and in the breast cancer metastasis MMTV-PyMT mouse model [20]. Hence, compelling evidence exists for the role of invadosomes as mediators of invasive migration in cultured cells and in animal models of cancer. However, despite the identification of a specific tyrosine kinase substrate, TKS5, as proper invadosome marker, the lack of suitable antibodies directed against the human TKS5 protein has long prevented researchers from proving the existence of such structures inside

human tumors. It is only very recently that Chen *et al.* revealed for the first time invadosome structures in paraffin-embedded surgical specimens from human pancreatic adenocarcinoma and other organs [23], strongly supporting the clinical relevance of invadosomes in cancer progression.

Invadosome acto-adhesive and ECM degradation functions

All invadosomes share functional features, such as mechano-adhesive structures that present the ability to locally control ECM degradation. Thus, two main functions coexist in invadosomes: an acto-adhesive function implicated in ECM sensing and force production coupled to a function of local ECM degradation through the regulation of protease activities. Another striking feature of invadosomes is their recurrent collective behavior. Individual invadosomes can often present a meta-organization behavior since they can auto-assemble into clusters or rings [24, 25] that can expand in diameter, fuse with each other and disappear due to coordinated assembly/disassembly of static invadosomes [26].

Molecular basis of the invadosome acto-adhesive function

The invadosome unit is an acto-adhesive structure defined by a dense F-actin core (represented by long columns of tightly bundled F-actin filaments perpendicular to the substratum) surrounded by a ring of adhesion molecules colocalizing with a cloud of F-actin (composed of radial F-actin filaments parallel to the substratum) [24, 25, 27] (Figure 1B). By comparison, FAs are clusters of ECM receptors, adaptors and signaling proteins that are connected with more stable F-actin filaments organized into stress fibers [28].

The acto-adhesive function implies the recognition of specific components of the ECM, which is supported by the aggregation of numerous ECM receptors such as CD44, integrins (β1, β2, β3 and β5) or the discoidin domain receptors (DDRs). In addition to recognizing specific ECM components, invadosomes are also mechanosensitive structures since their formation increases together with ECM rigidity [29-31]. Linear invadosomes are also sensitive to the organization of the network of collagen I fibers [19]; however, it is not clear how the topography of collagen fibers, their mechanical properties or both affect their mechanosensitive functions. The specific 3D organization of the invadosome acto-adhesive components suggests that the forces produced in this context should be different than in FAs.

Indeed, numerous studies have shown the ability of FAs to generate tangential forces [32, 33]. However, very few studies have detected this type of force in invadosomes [30], supporting a model in which invadosomes apply normal forces to the ECM to generate compression forces [6, 30, 34-37]. These different mechanical properties between FAs and invadosomes suggest that different mechanosensors (potentially mechanosensitive Ca^{2+} channels) could have different and specific functions in each structure.

The ECM degradative function of invadosomes

Following ECM receptor engagement, invadosome assembly is initiated in response to the activation of the non-receptor tyrosine kinase Src, which recruits the adaptor proteins TKS5 and cortactin to initiate assembly of the actin core.

Both, FA and invadosome-dependent ECM degradation are mainly driven by matrix metalloproteinases (MMPs). Two types of MMPs have been considered in ECM degradation: membrane-anchored MMPs (MT1-MMP/MMP14 to MT6-MMP) that are directly activated during their trafficking process in the secretory pathways, and proenzyme MMPs (pro-MMPs: MMP2, 9 and others) that are secreted in the pericellular environment where they undergo proteolytic activation. Interestingly, endogenously active membrane-anchored MMPs can directly activate extracellular pro-MMPs naturally associated with tissue inhibitors of metalloproteinases (TIMPs) [38-42].

Upon maturation, invadosomes recruit and/or secrete MMPs, especially MT1-MMP, MMP2 and MMP9, to locally degrade the ECM and facilitate invasion. Hence, trafficking and control of exocytosis of membrane-anchored MMPs are essential to link invadosome acto-adhesive and ECM degradation functions. Adaptor proteins, such as the calmodulin (CaM)-dependent GTPase protein IQGAP1, are particularly important in controlling membrane-anchored MMP exocytosis at adhesive sites. Indeed, IQGAP1 bridges the exocyst complex regulating MT1-MMP exocytosis with adhesive structures through interaction with the adaptor Hic-5 [43, 44].

Coupling between invadosome acto-adhesive and ECM degradation functions highlights how invadosomes are highly dynamic structures regulated by multiple layers of effectors/pathways, which need to be finely coordinated. Some of these pathways, such as kinase signaling, actin cytoskeleton dynamics, protease activation and secretion, are known

as Ca^{2+}-regulated pathways. Calcium signaling is extremely dynamic and thus particularly adapted in combining a high level of integration between functions (trafficking, signaling, actin polymerization, integrin activation, etc.) [9, 10, 45]. We will present here evidence illustrating the involvement of several calcium-sensitive elements in invadosome regulation.

3. Calcium-dependent regulation of cancer cell invadosomes

Calcium in invadosome formation

It has been clearly evidenced that Ca^{2+} oscillations and downstream Ca^{2+}-dependent signaling molecules are crucial in regulating podosome formation in healthy cells such as osteoclasts, dendritic cells or macrophages [46, 47], as well as invadosome formation in cancer cells [48, 49].

The Src kinase has been considered as a key invadosome regulator since the expression of a constitutively active mutant of this protein induces invadosome formation in several cell types. Interestingly, Sun *et al*. have shown that in melanoma cells, serum-induced cytosolic Ca^{2+} oscillation controls Src activation and invadosome formation [48]. More recently, a central role of CaM in invadosome-driven cancer cell invasion has been demonstrated. CaM is ubiquitous and represents the major intracellular Ca^{2+}-sensor, playing an essential role in Ca^{2+}-dependent signaling. It contains Ca^{2+}-binding EF-hand motifs controlling the regulation of several enzymes, or other proteins, through Ca^{2+}-binding [50]. Li *et al.* have shown that in glioblastoma multiforme cells, CaM controls invadosome formation by activating invadosome-associated proteins such as Src and the sodium/hydrogen exchanger 1 (NHE1) through direct binding [51].

In physiological conditions, Src activation is sustained by its binding to non-receptor tyrosine kinases, focal adhesion kinase (FAK) and its close relative prolin-rich tyrosine kinase 2 (Pyk2). Both kinases are activated through integrin engagements and can recruit Src through their SH2 domain. In numerous cells, such as human umbilical vein endothelial cells (HUVECs) or metastatic lung cancer cells (CL1-5), FAK silencing reduces invadosome ring (small ring-shaped adhesion structures that result from the spontaneous clustering of individual invadosomes) formation. In the RAW264.7 macrophage cell line, the metaorganization of invadosomes seems differently regulated since FAK silencing decreases invadosome rings without affecting the formation of a single invadosome unit, while Pyk2 silencing increases

their formation [52]. This suggests that FAK and Pyk2 have synergic roles in the regulation of invadosome formation. However, this functional relationship could be inversely tuned since it was recently shown that both enzymes exert antagonistic functions on invadosome formation in breast cancer cells [53], where Pyk2 silencing reduces invadosome genesis while FAK silencing increases their formation. All these data suggest that a fine equilibrium between these two kinases is important for regulating the complexity of invadosome functions and that the precise control of their molecular environment is essential for determining their action. An interesting property of FAK and Pyk2 is their sensitivity to changes in the intracellular Ca^{2+} concentration. Pyk2 is a Ca^{2+}/CaM-dependent cell adhesion kinase [54]. Indeed, the binding of Ca^{2+}/CaM to the Pyk2 auto-inhibitory FERM domain promotes Pyk2 auto-phosphorylation on tyrosine 402 and its activation. In numerous cell types, Pyk2 auto-phosphorylation preceding Src recruitment can be stimulated by different types of Ca^{2+} entry triggered in response to either agonist or integrin activation, and can be specifically blocked by Ca^{2+} chelators [55-57]. Several studies have also suggested that FAK activity could be regulated by Ca^{2+} [58] and that the activity of some Ca^{2+} channels could impact the level of FAK auto-phosphorylation [59]. Altogether, these data suggest that both kinases present a different sensitivity for Ca^{2+} modulation and/or that each kinase assembles into distinct Ca^{2+} channel signaling complexes. Interestingly, and as will be mentioned later in this review, both kinases also have the ability to regulate some Ca^{2+} channel activity through direct or indirect phosphorylation, possibly exerting feedback regulation shaping Ca^{2+} signals.

Another Ca^{2+}-sensitive signaling element crucial for invadosome formation is the calpain family. Members of this protease family present direct Ca^{2+} regulation, yet with different sensitivity, and can target several FAs and invadosome adhesion proteins, such as talin, FAK, Src, Pyk2, WASP and others. These Ca^{2+}-sensitive proteolytic events regulate FA turnover but promote invadosome formation [60, 61].

Calcium in invadosome acto-adhesive function

Integrins are the most predominant and well-characterized cell surface receptors of various ECM proteins and can be regulated by intracellular Ca^{2+}. For example, Ca^{2+} and integrin-binding protein 1 (CiB1) is a small 22-kDa intracellular Ca^{2+}-binding protein that binds multiple α-integrin chains and would rather act as a negative regulator of integrin activation [62]. In turn, Ca^{2+} regulates CiB1 functions by modulating its affinity towards integrins [63]. The

importance of CiB1 in invadosomes has been highlighted through its essential role in recruiting the small GTPase Rac3 within the structure to coordinate the coupling between acto-adhesive and ECM degradation functions [64]. Indeed, silencing of either Rac3 or CiB1 leads to invadosomes that can poorly digest the ECM.

CD44 is another ECM components receptor involved in cancer metastasis through the regulation of invadopodia activity [65, 66]. This ubiquitous transmembrane cell surface molecule is a proteoglycan able, through the indirect recruitment and activation of the phospholipase C (PLC) pathway, to mobilize Ca^{2+} and regulate Src-dependent Pyk2 phosphorylation or actin cytoskeleton modifications [67, 68]. Yet, whether CD44-induced Ca^{2+} signaling is pivotal for CD44-dependent invadosome adhesive function remain to be determined.

The recognition of the ECM fibrillar type I collagen by the DDRs shepherds linear invadosome formation [69]. DDRs are often overexpressed in cancer [70]. They display unique structural features and distinctive activation kinetics, which set them apart from other members of the receptor tyrosine kinases superfamily. Though Ca^{2+} signal does not appear as a classical element of their signal transduction cascade, a recent study has shown that DDR1 can regulate the expression of some Ca^{2+} channels to promote survival of human breast cancer cells [71]. Additionally, it was suggested that an interplay between two Ca^{2+}-sensitive signaling pathways, the DDR2/integrin–FAK–PI3K/Akt and the PIEZO1–Ca^{2+}–MLCK axis, might synergistically govern mechanotransduction of paratensile signals to regulate cytoskeletal actin polymerization in fibroblasts [72].

Actin polymerization is indeed another key process regulated by numerous Ca^{2+}-sensitive elements [73]. This is notably the case for the severing factor cofilin, which has been implicated in invadosome regulation [74]. Cofilin is regulated through a phosphorylation (inactive state)/dephosphorylation (active state) cycle by many kinases and phosphatases [75]. Although the role of Ca^{2+} in the regulation of cofilin remains ambiguous, it is known that a Ca^{2+} increase can either promote cofilin dephosphorylation/activation through a calcineurin-SSH1 pathway or induce its phosphorylation/inactivation by the Ca^{2+}-sensitive LIM kinase [76, 77]. Gelsolin, another actin filament capping and severing protein implicated in invadosome regulation, is also highly sensitive to variations in the intracellular Ca^{2+} concentration [78, 79]. Numerous other actin-regulating proteins, including talin and vinculin, are also directly or indirectly regulated by Ca^{2+} signals [80-82].

Calcium in invadosome ECM degradative function

Plasma membrane MT-MMPs are recruited to invadosomes through the incorporation of MT-MMP-containing endocytic vesicles, and consequently MMPs are secreted at these ECM degradation sites. Intracellular Ca^{2+} is a well-known regulator of exocytic vesicle trafficking, MMP expression and secretion [11, 83].

It was specifically shown that serum-induced Ca^{2+} oscillations promote MT1-MMP recycling to the PM and invadopodia-mediated ECM degradation [48]. More recently, Pourfarhangi *et al.* have shown that ECM cross-linking induces a similar Ca^{2+} spiking signal, which in turn leads to more frequent MT1-MMP delivery to the PM and causes maximal invadopodia-mediated ECM degradation [84].

Moreover, MMPs are Ca^{2+}-dependent endopeptidases requiring Ca^{2+}-binding for their activity. Indeed, one structural calcium ion can be found within the active sites of all MMPs [85]. Hence, both intracellular and extracellular Ca^{2+} are required for the proper function of ECM remodeling enzymes involved in invadosome degradative function.

These observations clearly show that several key signaling pathways controlling invadosome dynamic/function are Ca^{2+}-sensitive, but even though a central role of Ca^{2+} signaling is certain, one component of cancer cell invadosomes, namely, Ca^{2+} ion channel proteins, has long been largely neglected.

4. Calcium channels regulating cancer cell invadosomes: Known actors and possible additional players

Ca^{2+} channel opening can be triggered by physical or mechanical clues, producing Ca^{2+} influx and leading to a sudden increase in the local intracellular concentration of free Ca^{2+} ions. In the metastatic cancer field, the current view is that the resulting variety of spatiotemporal Ca^{2+} organization patterns orchestrates the proper unfolding of the cell migration/invasion program flow. Indeed, "Ca^{2+} flickers", "Ca^{2+} spikes" or their clustering into microdomains enables the spatial and temporal encoding of complex cellular processes, in particular morphological changes or the confined regulation of enzymatic activities. This standpoint

relies on the estimation that in the tens of nanometers-confined microdomains formed within the opening mouth of activated channels, the Ca^{2+} concentration can reach tens of micromoles [86], a concentration sufficient to activate many Ca^{2+} effectors with low Ca^{2+}-binding affinity. Several studies have moreover implicated Ca^{2+} channels in the regulation of adhesion, migration and invasion of different cancer cells through the control of actin cytoskeleton remodeling, FA dynamics or ECM-degrading protease secretion. These observations have been nicely and exhaustively reviewed (see, for example, [11, 87-91]) and will not be detailed here. However, the description of the elaborate mechanisms by which Ca^{2+} channels coordinate the coupling between the acto-adhesive and ECM remodeling functions of cancer cell degradative structures, especially at the invadosome level, remains often elusive. Here, we will report recent evidence directly implicating some Ca^{2+} channels in the dynamics of cancer cell invadosomes functions. We will also explore the potential roles of other Ca^{2+} channel candidates that, in our opinion, deserve more attention.

STIM1/Orai1-mediated store-operated calcium entry

The most common route for Ca^{2+} signal generation in non-excitable cells results from the activation of the wide family of PLC-coupled cell surface receptors. Briefly, PLC activation leads to the generation of inositol 1,4,5-trisphosphate (IP_3), a second messenger that diffuses and binds to its receptors (IP_3R) at the ER membrane, inducing the release of sequestrated Ca^{2+}. The resulting decrease in the ER Ca^{2+} content is sensed by the stromal interaction molecule (STIM) proteins of the ER that aggregate and relocate at ER-PM junctions to contact, cluster and open PM Orai calcium channels into structures commonly referred to as 'puncta' [92, 93]. The resulting influx of extracellular Ca^{2+} is called store-operated Ca^{2+} entry (SOCE). The ionic current underlying STIM1/OraiI1-mediated SOCE is also known as the Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) in immune cells, where it plays fundamental functions in antigen recognition and other aspects of the immune response [94]. STIM1/Orai1 regulation of invadosomes was first evidenced in immune cells. In microglia, Orai1 has been localized in the core of podosomes, along with CaM and the small-conductance Ca^{2+}-activated SK3 potassium channel [47]. STIM1 was found to be closely associated in and around the podosomes. It was suggested that STIM1/Orai1-mediated SOCE controls microglial migration and invasion through the regulation of podosome formation or stability, although no molecular

mechanisms were provided [47]. Despite the fact that lymphocytes are highly motile immune cells not known to extend invadosomes, these cells actively probe the surface of the endothelium before initiating diapedesis by the dynamic insertion and retraction of actin-rich cylindrical protrusions related to invadosomes [95, 96]. In that specific context, it has been shown that CRAC-mediated Ca^{2+} influx can stabilize these invadosome/podosome-like protrusions in dense arrays (or so-called "podo-synapse") to delay transmigration, to gain time to complete antigen recognition and lymphocyte activation [96, 97].

SOCE acts as the principal Ca^{2+} entry pathway in numerous cellular responses outside the immune system. It is therefore not surprising that changes in the expression of key SOCE elements and the resulting Ca^{2+} homeostasis remodeling have been shown to play important and multifunctional roles in the phenotypic changes observed in transformed cells. Over the last decade, STIM1/Orai1-Ca^{2+} signaling has been reported to contribute to different aspects of cancer progression, such as tumor cell proliferation, evasion of apoptosis, migration and metastasis formation (for review, see, for example, [98, 99]). Several studies have clearly associated dysregulated SOCE with cancer spread and a poor outcome in various metastatic cancers. STIM1/Orai1-mediated molecular mechanisms for regulating cancer cell adhesion and invasive migration are diverse and include calpain-dependent FA turnover, actin cytoskeleton remodeling, actomyosin contractility and ECM degradation [100]. In 2014, Sun *et al.* revealed for the first time a major role played by STIM1/Orai1-SOCE in the regulation of cancer cell invadosomes [48]. These authors have shown that STIM1/Orai1-mediated Ca^{2+} oscillations orchestrate melanoma tumor cell invasion by inducing invadosome formation and by controlling focalized ECM degradation. Using a broad calcium chelator, pharmacological approaches and targeted overexpression/knockdown strategies, the authors unambiguously demonstrated that serum-induced persistent STIM1/Orai1-dependent oscillatory Ca^{2+} waves are required for both mediated invadosome formation and regulated individual invadosome proteolytic activity. STIM1/Orai1-mediated Ca^{2+} influx facilitates the assembly of invadosome precursors by increasing the level of activated tyrosine phosphorylated Src kinase. In addition, while STIM1/Orai1-SOCE does not affect the invadosome lifetime or the level of total or secreted MMPs, it impacts invadosome maturation by enabling MT1-MMP recycling and delivery to the plasma membrane. This study unraveled a novel mechanism for the dysregulated SOCE signals in promoting tumor invasion through the control of focalized proteolysis and ECM remodeling at the cancer cell invadosome level. Nonetheless, some

questions were left unanswered, and new questions were raised. First, as Src does not directly bind Ca^{2+}, the question remains as to how the STIM1/Orai1-mediated increase in the intracellular Ca^{2+} concentration regulates Src activity. In addition to this missing molecular link, it is also puzzling as to why melanoma cells rely on, and require, a sustained oscillatory Ca^{2+} signaling pattern to form invadosomes and invade the ECM. It is possible that melanoma cells use this signal architecture as a protection against the cytotoxicity of prolonged calcium signals, but this is probably not the main reason. Indeed, authors have shown that a prolonged global increase in the intracellular Ca^{2+} concentration is equally effective at increasing basal Src activity, yet without promoting invadosome formation or ECM invasion, thus stressing the requirement of such an oscillatory signal [48]. Paradoxically, they also demonstrated that the forced expression of constitutively active v-Src rescued invadosome formation as well as activity defects observed in STIM1 knockdown melanoma cells [48]. If this bypass defines Src activation as a key target of serum-induced STIM1/Orai1-SOCE, it argues against the need for intermittent Src activation cycles for normal invadosome development and maturation, consistent with a previous study performed using similar permanently active Src constructs in different cell types. Incidentally, these observations impose Src as an obliged hub downstream of STIM1/Orai1-mediated Ca^{2+} influx in all SOCE-regulated invadosome regulation mechanisms, including MT1-MMP recycling. Notably, the STIM1/Orai1-SOCE signal may impact the dynamic cycles of invadosome protrusion/maturation/retraction by directly affecting additional Ca^{2+}-sensitive invadosomial regulatory signaling events, such as cortactin-mediated actin cytoskeleton dynamics or the phosphatidylinositol-3 kinase (PI3K)/Akt pathway [101-105]. Hence, further studies are required to determine whether Src acts as a mandatory node in these contexts, especially in normal c-Src backgrounds. One possible explanation for the necessity of such Ca^{2+} oscillatory pattern organization is that it allows signaling through invadosomial-confined subplasmalemmal Ca^{2+} flux required to fine-tune individual invadosome formation and/or maturation, as opposed to a global and sustained increase in the intracellular Ca^{2+} concentration that would interfere with, or even disrupt, such microdomains. Finally, as noted and discussed by the authors [106], this first study did not solve how the Ca^{2+} signal and the STIM1/Orai1 proteins are organized spatially and temporally to mobilize the cancer cell invasion machinery and, in particular, whether invadosome localized (STIM1/Orai1) Ca^{2+} signals exist.

In a recently published study, the same authors started to address the spatiotemporal dynamics of SOCE in orchestrating complex cell behaviors such as invadosome-driven cell invasion as well as to tackle other unresolved issues [107]. To that end, they developed plasmalemmal-targeted biosensors, including an Orai1 channel N-terminally fused to the genetically encoded Ca^{2+} indicator GCaMP6f, to preferentially report and directly visualize Ca^{2+} influx near the mouth of Orai1 channels. High-resolution imaging of invadosomial Ca^{2+} signals in melanoma cells revealed the organization of open Orai1 channels in a ring-like structure wrapped around the actin-rich invadosomial core or surrounding the pits and holes in the ECM [107]. Time-lapse imaging revealed discrete (average spatial size of 3.4 μm^2), sudden, highly variable-shaped and long-lasting (11.5 s on average) Ca^{2+} transients confined to a single invadosome as well as to discrete sites at the cell periphery of invading melanoma cells. Hence, such local Ca^{2+} transients, dubbed 'Ca^{2+} glows' due to their exceptionally long duration, appear to constitute elementary SOCE events originating from STIM1-Orai1 clusters. Interestingly, the repetitive activity of Ca^{2+} glows could be recorded at the level of a single invadosome indicative of STIM1-Orai1 clusters operating in a discrete and intermittent fashion. However, adjacent invadosomes can exhibit distinctly different temporal patterns of activity. Thus, in invading melanoma cells, SOCE does not seem to operate in a continuous mode but rather to comprise spatiotemporally discrete and independent elementary events in the form of invadosome-confined repetitive and asynchronous Ca^{2+} glows. It is not clear, however, how this relates to the global serum-induced and STIM1/Orai1-mediated Ca^{2+} oscillatory waves observed previously in the same cellular model [48] and what dynamically triggers/controls STIM1/Orai1 activity at the level of individual invadosomes. Interestingly, serum stimulation was shown to induce STIM1/Orai1-SOCE, STIM1/Orai1-mediated invadosome formation, and CaM/Pyk2 association [48, 107]. In addition, SOCE can promote the physical interaction between Pyk2 and Src and thus Src activity [48, 107]. Hence, as the active form of the Ca^{2+}-regulated cytoplasmic kinase Pyk2 (pY402) and CaM were enriched in invadosomes, SOCE-STIM1/Orai1-mediated Ca^{2+} glows seem to orchestrate invadosome formation and melanoma cell invasion through Ca^{2+}/CaM and Pyk2-dependent Src activation at the invadosome level [48, 107].

While signifying the importance of spatiotemporal Ca^{2+} signal coding during confined cell invasion, all these data were obtained in a single cellular model, the WM793 melanoma cell line, raising the question of whether the identified role and mechanisms of SOCE in cancer

cell invadosome regulation could be extended to all metastatic melanoma tumors and to other metastatic cancers. Arguing in favor of that possibility, STIM1 is required for invadosome formation in the MDA-MB-231 breast cancer and U2OS osteosarcoma cell lines [108], and STIM1-dependent SOCE promotes cervical cancer as well as glioma migration, invasion and metastasis formation through the modulation of Pyk2 phosphorylation [109, 110]. Notably, an intensive crosstalk exists between Pyk2, the nonreceptor Src-family kinases and Ca^{2+} transients, including SOCE [111, 112]. Activation of these kinases can mediate Ca^{2+} mobilization by regulating Ca^{2+} channels. For example, in endothelial cells (ECs), ER Ca^{2+} store depletion rapidly induces Y361 STIM1 phosphorylation *via* Pyk2. This critical phospho-switch enables the recruitment of Orai1 into STIM1 puncta, leading to SOCE [112]. Conversely, the generated Ca^{2+} signals can in turn exert control on the kinase activities *via* CaM [107, 111]. This points toward a possible invadosome-confined Pyk2 (and Src)-dependent SOCE (and possibly other types of influxes) amplification loop.

In addition to cancer, the STIM1/Orai1-Ca^{2+} signal could be a regulator of invadosome dynamics in a broad variety of cells, including healthy cells. v-Src-transformed mouse embryonic fibroblast (MEF) cells can be used as a generic invadosome model encompassing podosome and invadopodia properties [113]. Chen *et al.* used this model to investigate the role of SOCE in the formation of individual invadosomes (or podosome dots) and invadosome rings (also known as podosome rings or rosettes) altogether referred to as invadosome-like structures [108]. In that experimental setting, they demonstrated that SOCE pharmacological inhibition, or either STIM1 or Orai1 knockdown/knockout, strongly altered the organization of invadosome rings, leading to disrupted and fragmented structures [108]. Hence, SOCE-mediated Ca^{2+} signaling seems to be essential to invadosome ring formation dynamics by promoting the clustering of individual invadosomes. STIM1-mediated SOCE Ca^{2+} influx also appeared to be required during the maintenance phase of v-Src-induced invadosome rings and their degradative function, as it was important for secreted MMP activity, invadosome-mediated matrix degradation, and cell invasion [108]. This is in apparent contrast with Sun *et al.'s* results in melanoma cells where v-Src forced expression was able to override the need for STIM1-mediated SOCE to complete cell invasion [48]. Nevertheless, by bypassing SOCE-induced Src-dependent individual invadosome formation [48], these results unveiled an additional SOCE function in regulating invadosome superstructure organization and possibly function.

Additionally, confocal microscopic analyses revealed that STIM1 colocalized with invadosome rings in v-Src–transformed MEFs. This is consistent with the recent observation of ER close apposition to invadosome ring centers in v-Src-expressing NIH-3T3 cells [114]. ER presence in such a structure may serve multiple functions by allowing invadosome-localized STIM1-Orai1 puncta formation and SOCE Ca^{2+} glows, as well as intrinsic and specific active translational machinery activity, both controlling invadosome formation [107, 114].

Taken together, these observations suggest a role for STIM1/Orai1-SOCE as a universal invadosome regulatory mechanism in cancer and in healthy cells. To clarify the exact roles, as well as the complexity and the ubiquity of SOCE-mediated invadosome regulation, it appears important to further evaluate *i)* STIM1/Orai1 localization in invadosomes in a broader diversity of metastatic cancer cells, *ii)* STIM1/Orai1 involvement in their serum-induced Ca^{2+} oscillations and invadosome formation, *iii)* if invadosome-confined STIM1/Orai1-dependent Ca^{2+} glow activity could be recorded in all these cells, and finally, *iv)* whether and how STIM1/Orai1-SOCE impacts the Ca^{2+}/CaM–Pyk2–Src pathway and/or other signaling events controlling invadosome formation and maturation. From a therapeutic standpoint, however, the ubiquitous nature of SOCE in various physiologic functions, especially in the immune and cardiovascular systems, raises concern about probable compromised immunity or cardiovascular complication-related severe side effects of the systemic administration of SOCE channel inhibitors for cancer therapeutics. Therefore, perhaps a more localized and specific approach would be to target other Ca^{2+} conductances involved in fine-tuning the invadosome acto-adhesive and/or degradative activities.

Stretch-activated/mechanosensitive calcium channels

Mechanical cues regulate, or even promote, the metastatic spread. During confined migration, cancer cells must respond to mechanical constraints and changes in tissue stiffness to complete invasion. Mechanosensing occurs at the invadosome level, and both ECM rigidity and cellular traction stresses favor invadosome formation and ECM remodeling through mechanotransduction signaling [115, 116]. Plasma membrane mechanosensitive ion channels are a particularly efficient way to translate mechanical changes in the microenvironment, such as matrix stiffness or constraints applied to cell protrusions, into intracellular biochemical signals for a fast adaptive response (outside-in mechanosensation) [117, 118]. Consequently, mechanosensing by Ca^{2+} channels is likely to play fundamental roles in invadosome biology,

and some evidence point toward the possible involvement of the recently discovered PIEZO channel family as well as some TRP channels displaying mechanosensitive properties such as TRPM7, TRPV2 or TRPV4 [118].

TRPM7: The ubiquitously expressed transient receptor potential melastatin-related 7 (TRPM7) is an atypical cationic channel because it is a bifunctional protein encoding a nonselective cation TRP channel fused to a functional COOH-terminal serine/threonine kinase domain [119, 120]. Physiologically, this TRPM7 "chanzyme" is implicated in cellular and systemic magnesium (Mg^{2+}) homeostasis but can also conduct Ca^{2+} and relay signals by phosphorylating downstream effector molecules. TRPM7 can be activated by mechanical stimuli such as membrane stretch. This channel controls polarized migration by mediating high-Ca^{2+} microdomains, or "Ca^{2+} flickers", at the leading lamella of migrating cells (thus, opposite to the global Ca^{2+} gradient), steering directional cell migration in response to membrane tension and chemoattractant signal transduction [121, 122]. TRPM7 also acts as a crucial regulator of matrix rigidity-dependent Ca^{2+} mobilization during the adhesion process. TRPM7 is locally associated with FAK and the integrin/FA complex and controls normal and pathological migration through the regulation of Ca^{2+}-sensitive calpain activity and acto-myosin contractility [49, 120, 123-125]. TRPM7-mediated increases in intracellular Ca^{2+} levels enhance cell spreading and cell–matrix adhesion independently of TRPM7 kinase activity. TRPM7-mediated Ca^{2+} influx also triggers the direct association of TRPM7 with cytoskeleton proteins such as non-muscle myosin IIA (NMIIA), yet an active kinase domain is required for both this interaction and the regulation of acto-myosin contractility by directly phosphorylating the NMIIA heavy chain [49, 125]. However, some studies have uncovered that TRPM7-dependent changes in Mg^{2+} intracellular homeostasis could be responsible for TRPM7's control over the cytoskeleton and regulation of polarized cell movements, independent of Ca^{2+} permeation or kinase signaling [126-128]. Thus, the regulation of cell adhesion and migration by the TRPM7 channel is complex, combining the effects of kinase and/or Ca^{2+}/Mg^{2+}-dependent and -independent pathways on acto-myosin contractility and other targets. In addition, TRPM7 promotes the invasive behavior of some cancers, such as hepatocellular carcinoma or pancreatic ductal adenocarcinoma, by regulating the secretion of matrix proteolytic proteases and possibly through invadosome regulation [120, 125, 127, 129].

Acto-myosin contractility plays a central role in regulating the assembly and disassembly of adhesive contacts and invadosome formation [130, 131]. Notably, the formation of invadosomes requires actin cytoskeleton remodeling *via* local inhibition of acto-myosin contractility, and invadosome protrusive force depends on acto-myosin contractility [34, 131]. In TRPM7-overexpressing mouse N1E-115 neuroblastoma cells, Clark *et al.* have shown that TRPM7 is enriched in invadosome ring structures together with NMIIA [49]. Activation of overexpressed TRPM7 furthermore induces the transformation of pre-existing FAs into invadosome-like structures through the TRPM7 kinase-dependent inhibition of NMIIA function, resulting in the acto-myosin cytoskeleton remodeling [49]. Their observations are in line with the hypothesis that TRPM7 may regulate invadosome assembly by locally promoting the relaxation of the acto-myosin cytoskeleton. In the same model, using a combination of Total Internal Reflection Fluorescence (TIRF), Ca^{2+}-indicator-based fluorometry and loaded excess intracellular Ca^{2+} chelators, they also searched for Ca^{2+} sparks or flickers similar to invadosome-localized TRPM7-dependent Ca^{2+} signals [132]. Although by inducing TRPM7 overexpression, they did observe a highly repetitive and localized Ca^{2+} rise into microdomains (which they called "Ca^{2+} sparking hotspots") at the ventral plasma membrane, this signal seems kinetically distinctive compared to other known elementary Ca^{2+} signaling events. As opposed to melanoma WM793 cell invadosomial SOCE-dependent Ca^{2+} glows, it did not localize specifically at invadosomes [107, 132]. First and foremost, they did not observe any invadosomes that formed or disappeared at Ca^{2+} hotspots during, prior to, or following Ca^{2+} spark ignition [132]. Hence, invadosome formation appeared to be functionally and spatially dissociated from TRPM7-mediated Ca^{2+} sparks, ruling out the possibility that TRPM7 controls acto-myosin contractility-dependent invadosome formation and dynamics *via* localized Ca^{2+} signals. Conforming to that hypothesis, while TRPM7 is expressed and required for microglial migration and invasion [47, 133], TRPM7 ion transport activity inhibition by spermine had no impact on invadosome formation in this cellular context [47]. Incidentally, this channel was not enriched in microglial cell invadosomes, questioning a universal role of TRPM7 in invadosome regulation [47]. Indeed, while studying Ca^{2+} glows in WM793 melanoma cells, Lu *et al.* demonstrated that total suppression of TRPM7 expression using CRISPR-Cas9 had little effect on the frequency, amplitude and kinetics of the glows and did not significantly affect invadosome formation [107]. Accordingly, any TRPM7 involvement in invadosome biogenesis seems highly dependent on the cellular context.

Hence, it seems clear that TRPM7 is part of a large cytoskeletal complex universally affecting the malignant potential of tumor cells by regulating acto-myosin dynamics and cell-matrix interactions [120, 129]. However, whether endogenous TRPM7 channels regulate invadosome dynamics through kinase-dependent signaling locally impacting acto-myosin contractility (and possibly SOCE [134, 135]) and in which tissue remained to be clarified.

TRPV2 and TRPV4: Transient receptor potential vanilloid 2 (TRPV2) and 4 (TRPV4) are two highly polymodal members of the TRP vanilloid subfamily whose gating is highly sensitive to the membrane environment, including membrane stretch induced by changes in osmotic pressure or mechanical stress [136]. As both these nonselective cationic channels with permeability for Ca^{2+} can respond to mechanical clues, they can act as mechanosensors in different physiological or pathological settings. Engagement of various PM receptors (such as growth factor receptors) or mechanical stimuli promotes TRPV2 activity by triggering its dynamic translocation from the endosomal compartment to the PM *via* a PI3K-dependent pathway [137]. This regulated PM targeting of constitutively active TRPV2 channels has been described for a broad range of stimuli and seems to be the major regulatory mechanism accounting for TRPV2 activation in nonexcitable cells. Nonetheless, additional levels of regulation directly affecting TRPV2 gating are probably involved in fine-tuning its activity [138]. Among all TRPVs, focal stress-induced dynamic trafficking and confined accumulation are mechanisms that only TRPV2 and TRPV4 subunit seem to endorse [139]. Channel clustering at the site of mechanical stimulation strikes as an interesting way to temporally and spatially control TRPV2/V4-dependent signaling in response to the mechanical constraints of the microenvironment.

Regarding TRPV2, changes in the actin cytoskeleton appear to be required for its dynamic trafficking, while TRPV2 activity can in return alter actin cytoskeleton dynamics [140]. Despite cloning of the TRPV2 gene exactly 20 years ago, little is known about the precise cellular functions of this channel [137]. TRPV2 has been associated to the innate immune response, where it regulates cytokine secretion, phagocytosis or chemoattractant-elicited motility [141, 142]. In the mouse TtT/M87 macrophage cell line, TRPV2 is enriched in invadosome dots and ring structures along with ß2-integrin, paxillin, vinculin, Pyk2 and active PI3K signaling [143]. Chemotactic peptide formyl-Met-Leu-Phe (fMLP) stimulation of these cells induces TRPV2-dependent Ca^{2+} entry, promotes cell migration and recruits TRPV2 at the PM, especially in invadosomes [143, 144]. In this study, Nagasawa *et al.* used a Yellow

Chameleon-based PM-targeted Ca^{2+} biosensor to record a TRPV2-dependent increase in the subplasmalemmal Ca^{2+} concentration, concentrated around the invadosomes [144]. These Ca^{2+} microdomains seem to stimulate Pyk2 phosphorylation and to trigger invadosome disassembly. Indeed, TRPV2 genetic ablation or overexpression of a trafficking-defective dominant-negative TRPV2 variant reduced global phospho-Pyk2 expression and increased the number of invadosomes possibly by preventing their breakdown [144]. Thus, TRPV2-dependent Ca^{2+} signaling appears to be required for the dynamics of invadosome adhesive structures by promoting their turnover to allow cell migration and invasion [143, 144]. Although this first and unique study suggests an essential role of TRPV2 in invadosome dynamics, several questions remain: does TRPV2 specifically accumulate in invadosomes and (as for other channels such as Orai1 or TRPM7) by which mechanism(s)? How does Pyk2 switch between promoting invadosome assembly to inducing disassembly under the control of TRPV2-dependent Ca^{2+} signaling? Does TRPV2 regulate other molecular pathways influencing invadosome dynamics or functions? Finally, does TRPV2 play a role in the specific context of cancer cell invadosomes? There is no evidence of such involvement, but TRPV2 has been found to be overexpressed in samples from patients with metastatic disease compared to solid primary tumors [145]. This aberrant TRPV2 expression has been associated with a poor prognosis in tumors of different origins, such as esophageal squamous cell, breast, prostate and urothelial carcinomas [146-151]. Despite evidence that TRPV2 activity promotes cancer cell migration and invasion, the molecular mechanisms involved are still elusive but might likely include effects on ECM degradation enzyme secretion/activity (such as MMP2, MMP9 or cathepsins [150, 152]), actin cytoskeleton dynamics or Pyk2 regulation. Determining whether such TRPV2-mediated downstream pathways occur at the invadosome level and impact their dynamics or functions deserves further study. Mechanistic studies on TRPV2 control of cell invasion should be facilitated by the recent discovery of potent and specific TRPV2 antagonists that were sorely lacking until now [153, 154].

As a polymodal channel, TRPV4 that can be activated as much by chemical as by physical stimuli, is involved in multiple physiological processes including osmoregulation, thermoregulation and mechanosensation in various tissues [155, 156]. For instance, in osteoclasts, TRPV4 contributes to bone mass regulation through the RANKL-evoked Ca^{2+} entry, sealing the invadosome actin ring structure formation and osteoclast terminal differentiation, [157-159]. Interestingly, TRPV4 activity signaling likely occurs downstream of the Ca^{2+}/CaM–

Pyk2–Src pathway, either *via* Ca^{2+}/CaM direct binding or possibly *via* Src phosphorylation [157, 159, 160]. In these cells, TRPV4 physically interacts with the myosin heavy chain MYH9 subunit of the NMIIA isoform, a protein enriched within the dynamic cytoskeletal compartments, including the sealing zone of osteoclasts [157].

Importantly, TRPV4 is the only other member of the TRPV subfamily for which a mechanical sensitivity was clearly established. It can form supramolecular complexes with ß1-integrins (and Src) [161, 162]. Stretch activation of integrin requires Ca^{2+} entry through TRPV4, and reciprocally, mechanical forces applied to integrins elicit TRPV4 gating [163, 164]. Further strengthening the link between TRPV4-dependent Ca^{2+} signaling and mechanotransduction, TRPV4 is capable of sensing matrix stiffness [165, 166] and shaping the ECM. Indeed, integrin-stimulated TRPV4 Ca^{2+} influx can trigger the association of the actin-binding protein flightless-1 (FlII) with NMIIA at cell-matrix adhesion sites, stimulating the number and length of cell extensions that remodel ECM collagen [162]. TRPV4 activity is also thought to control aligned collagen fibril assembly at these cell-matrix adhesion sites through force regulation by dynamic unloading and reloading of vinculin [167]. Altogether, it is therefore not surprising that TRPV4 was recently reported as an interesting emerging element in cancer cell migration, invasion and metastasis formation [168]. Invadosomes are ß1-integrin-regulated acto-adhesive structures regulated by mechanical cues [29, 115, 116]. Thus, in disseminating cancer cells, the TRPV4 (and TRPV2) mechano-TRP channels could be part of molecular complexes that orchestrate ECM rigidity and cellular traction stress mechanosensing of invadosomes, regulating their formation and/or function. In line with this hypothesis, it has been demonstrated over the last few years that this channel participates in the establishment of trailing adhesions and directional persistence of migrating cells [169]. In breast, gastric and glioma cancer cells, TRPV4 can further promote migration and invasion through actin cytoskeleton remodeling, regulation of FA dynamics or extracellular protease secretion [170-173].

PIEZO CHANNELS: True mechanosensitive ion channels (MSCs) are to date the best characterized biological force-sensing systems. They represent the foremost example of coupling protein conformations to the mechanics of the surrounding cell membrane. PIEZO1 and PIEZO2 channels were discovered in 2010 [174]. They constitute a *bona fide* family of excitatory ion channels directly gated by mechanical forces and are believed to embody the long-sought mammalian MSCs [175-177]. The intrinsically mechanosensitive PIEZO proteins

play critical roles in various mammalian physiological processes, including touch sensation, proprioception and regulation of vascular development and blood pressure [176, 177], but also in pathological conditions such as cancer [118].

PIEZO1 (formerly known as Fam38A) can regulate integrin activation [178] and mediates confinement sensing, enabling cells to sense and adapt to different physical microenvironments [179]. During wall shear stress or proangiogenic mediator-induced endothelial cell angiogenesis, PIEZO1 activity is required for MT1-MMP PM translocation and MMP2 activation [180]. In small cell lung cancer (SCLC), loss of PIEZO1 promotes a switch to the reduced integrin-dependent amoeboid mode of cell migration, suggesting a possible role for this channel in invadosome-driven integrin-based mesenchymal migration [181]. Of note, a study made available as bioRxiv preprint during the writing of this review suggests that compressive stress enhanced invasion, matrix degradation and invadopodia formation of breast cancer cells through Piezo1 mechanoactivation [182]. In that context, PIEZO1-$Ca2^{+}$ signal would trigger activation of RhoA, Src, FAK and ERK signaling, as well as MMP-9 expression.

As for PIEZO2, it is required by breast cancer brain metastatic cells to probe their physical environment as they anchor and pull on their surroundings or when confronted with confined migration through narrow pores [183]. In these cells, PIEZO2-mediated Ca^{2+} influx impacts actin cytoskeleton dynamics and FA orientation through RhoA activation. This channel also controls cancer cell invasion by regulating the matrix degradative function of invadosomes [183]. Hence, the direct regulation of cancer cell invadosome by PIEZO channel family members would deserve in-depth studies. Especially, defining their involvement in invadosome mechanobiological responses to both changes in ECM rigidity and cellular traction stresses, would determine if they locally contribute to the fine-tuning of invadosome biogenesis or activity [115].

Voltage-gated calcium channels

In addition to non-voltage-gated Ca^{2+} channels from the Orai and TRP families, some cells express members of another distinct channel family, the voltage-gated Ca^{2+} channels (VGCCs, also known as the Ca_v family). These structurally distinct channels mediate fast Ca^{2+} influxes in response to membrane depolarization and play fundamental roles in electrically excitable cells such as neurons, muscle or cardiac cells. Indeed, these cells can respond to

stimuli by transmembrane potential changes that are able to activate VGCC-mediated currents.

VGCC expression was also detected in nonexcitable cells, such as immune cells [184, 185], leading to controversies and debates especially about their activation mechanism in that specific context [186]. As they are probably not voltage-operated in physiological conditions, how they are regulated in non-excitable cells has to be fully explained. Thirty years ago, a study suggested that VGCCs could control invadosome-mediated cell adhesion through resting intracellular Ca^{2+} levels in osteoclasts [46]. VGCCs appeared inhibited during the invadosome-dependent bone attachment of osteoclasts, allowing invadosome assembly and bone resorptive activity [46].

More recently, studies have shown a remodeling of VGCC expression in various cancers. A loss of VGCC expression was detected in certain cancers [187]. As in osteolasts, this loss might facilitate invadosome formation and invasiveness of these cancer cells. On the contrary, VGCC overexpression was associated with a worse prognostic in other types of cancers such as breast cancer, non-small cell lung cancer, prostate cancer, colon and esophageal cancer, glioma or melanoma, compared to that in their healthy counterparts [187-194]. In these contexts, aberrant expression of VGCC promotes cancer cell proliferation, migration or invasion [190, 191]. Several L-type Ca^{2+} channels (LTCCs) blockers were also identified in a screen for novel regulators of filopodia dynamics as compounds that consistently inhibit filopodia formation. Jacquemet *et al.* further demonstrated that LTCCs drive MDA-MB-231 breast and pancreatic cancer cell invasion through regulation of the filopodia adhesive cellular protrusions [195]. Within these structures, LTCCs are activated by integrin inside-out signaling, integrin activation and Src [195]. Moreover, LTCCs promote filopodia stability and maturation into talin-rich adhesions through the spatially restricted regulation of Ca^{2+} entry and subsequent calpain activation [195]. Considering that i) LTCC activity can be regulated *via* direct binding and phosphorylation by Src and Pyk2 [196], ii) Src regulates filopodia formation and LTCC-mediated Ca^{2+} entry at filopodia tips [195] and iii) Src is a master regulator of invadosome dynamics (see above), it would be pertinent to determine if Src-induced L-type-mediated Ca^{2+} entry could promote invadosome formation in cancer cells.

As mentioned before, the voltage-sensitive nature of VGCC activation could raise the questions of how these channels are activated and enhance the migration and invasiveness of

non-excitable cells such as cancer cells (as do voltage-gated sodium channels [197, 198]), or if they are even functional. In fact, forced membrane depolarization of cancer cells does not always induce channel activity despite VGCC expression [191]. Interestingly, a large number of VGCCs have been endorsed with noncanonical functions. They can act as scaffolds for the assembly of signaling complexes involved in transcriptional regulation, indirectly regulating Ca^{2+} signaling or possibly achieving other functions [189, 191].

Calcium-activated potassium channels

Despite not being Ca^{2+} channels *per se*, Ca^{2+}-activated potassium channels (KCas) play fundamental roles in Ca^{2+} signaling by hyperpolarizing the PM and maintaining the driving force for Ca^{2+} entry by Ca^{2+}-permeant channels. As such, we thought they deserved to be mentioned here, especially considering that KCa2.3/SK3 was enriched in microglial podosomes [47] and that some KCa members are aberrantly expressed in cancer where they promote metastasis formation by forming physical and/or functional complexes with Ca^{2+} channel subunits such as Orai1 or TRPV2 [199-201].

5. Conclusion and perspectives

Due to the central role they play in the metastatic cascade, interfering with invadosomes appears as an interesting way to block cancer spreading and related mortality [7].
Invadosome biogenesis and turnover are modulated by cellular tension, mechanical cues and PM receptor signaling, but the molecular mechanisms that coordinate invadosome dynamics are not well understood. A better understanding of these regulation pathways would certainly reveal innovative targets for therapeutic intervention aiming at fighting against cancer metastasis.

In this review, we have presented several lines of evidence showing that invadosomes are hubs for subcellular Ca^{2+} signaling to regulate cell adhesion-ECM degradation coupling and invasion (Figure 2). Despite recent progress, the fundamental question is now determining the origin and routes of the Ca^{2+} ions controlling these signaling hubs. In other words, the current challenge is to identify all the Ca^{2+} channels involved in invadosome regulation, to decipher the spatiotemporal dynamics of the Ca^{2+} signal they mediate and to decode their entanglement in regulating specific downstream pathways. As discussed above, STIM1/Orai1, TRPM7, TRPV2, PIEZO2 or VGCCs/LTCCs can all regulate invadosomes in different ways and in different

cell types (Figure 2), underscoring the intricacy of this question. Indeed, cancer cell invadosome formation, adhesion and ECM degradation activities, as well as their turnover, likely require the concerted activity of multiple Ca^{2+} channels, some probably having not been identified yet, such as additional mechano-TRPs or members of other channel families like the purinoreceptor P2X channels [136, 168]. The diversity of the multiple Ca^{2+} channels implicated in invadosomes raised the question of the specific molecular complexes associated with each of them and the impact of Ca^{2+} on their specific downstream signaling events and functions.
To gain a thorough understanding of these invadosome-associated mechanisms, it would certainly help to define the triggering element(s) for each channel and to simultaneously record their spatiotemporal activity during the unfolding of a single invadosome life. Moreover, nonselective cation channels in the PM can conduct Ca^{2+} and also alter the membrane potential, which in turn tightly regulates other Ca^{2+} channels, such as STIM1/Orai1 or VGCCs. Similarly, Ca^{2+} signals regulate Ca^{2+}/CaM and Src/Pyk2 signaling that reciprocally can modulate some channel activities. Hence, further studies should address the interconnection between each Ca^{2+} influx pathway in shaping Ca^{2+} signals, such as Ca^{2+} oscillations, Ca^{2+} flickers, Ca^{2+} sparks or Ca^{2+} glows, and the functional consequences in a single cellular model or across different cancers. Determine how these Ca^{2+} signals are integrated, sometimes at the level of the same effectors such as Pyk2, to mediate opposite effects (e.g., promoting invadosome assembly *versus* disassembly) would be decisive.

Ion channels are very attractive and extremely promising therapeutic targets in cancerology due to their ability to switch on or off specific phenotypic aspects of tumor cells (including invasion) and due to their accessibility to small molecule inhibitors or to extracellular antagonistic antibodies [12, 202]. Thus, from a therapeutic perspective, identifying the specific regulation and function of ion channels within the cancer tissue in general, or in cancer cell invadosomes in particular, is one of today's challenges that will likely lead to the development of new antimetastatic treatments in the future. Indeed, integrating Ca^{2+} channel functions in invasion should reveal new therapeutic strategies to modulate pathological invasive processes and prevent the spread of cancer cells. However, as illustrated in this review, there is a long path ahead to fully understand how Ca^{2+} signaling dysregulation promotes cancer metastasis.

FIGURE LEGEND

Figure 1: Functional and structural characterization of the invadosomes.

A. Multiples physio-pathological situations where podosomes, invadopodia and linear invadosomes are implicated. **B - Left panel:** Example of an invadosome unit in a macrophage expressing the filamentous actin (F-actin) marker LifeAct-GFP. An invadosome unit is defined by a core of F-actin surrounded by a ring of F-actin and adhesion molecules. **Right panel:** Both F-actin networks are supporting the ability of invadosome to couple protrusive forces and adhesion forces to local degradation of the extracellular matrix (ECM) through the delivery of transmembranous matrix metalloproteases (MMPs). Scale bar: 4 micrometer.

Figure 2: Ca^{2+}-dependent signaling pathways and channels implicated in the acto-adhesive structures that can present ECM degradative activity.

Despite presenting different mechanical properties, focal adhesion (FA) and invadosomes share numerous molecular components that connect the acto-adhesive function to the regulation of the local ECM degradation mediated by the exocytosis of membrane-anchored metalloproteases (MT-MMPs) (red shaded oval). The coupling of these functions relies on the specific expression and accumulation of a large diversity of Ca^{2+} channels at the plasma membrane (PM) and the fine-tuned integration of Ca^{2+}-triggered signaling events. Briefly, the acto-adhesive function implies the recognition of components of the ECM, which is supported by receptors such as integrins or DDRs. Reciprocally, invadosomes formation correlates with ECM rigidity, which makes them mechanosensitive structures. This function is sustained by several mechanosensors, including Ca^{2+} channels, enabling a spatiotemporal encoding through the formation of submembrane Ca^{2+} microdomains (yellow shaded ovals). Here we highlighted the roles of original Ca^{2+} channels, such as STIM/Orai1, TRPM7, TRPV2/V4, PIEZO and VGCCs, initiating distinct Ca^{2+}-sensitive molecular cascades. These specific dynamics in turn generate different compression forces, tangential in FA or protusive in invadosomes. Among the molecular components identified in these signaling pathways, some are common to both FAs and invadosomes, like FAK or the calpains, whereas some are specific to each structure. For instance, the degradative function of some FAs is under the control of a FAK/p130Cas signaling axis, which does not seem involved in invadosome formation. By

contrast, a Ca^{2+}/CaM-dependent cell adhesion kinases, Pyk2, play a unique synergic role with FAK in the regulation of invadosome formation.

Acknowledgments

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Figure1

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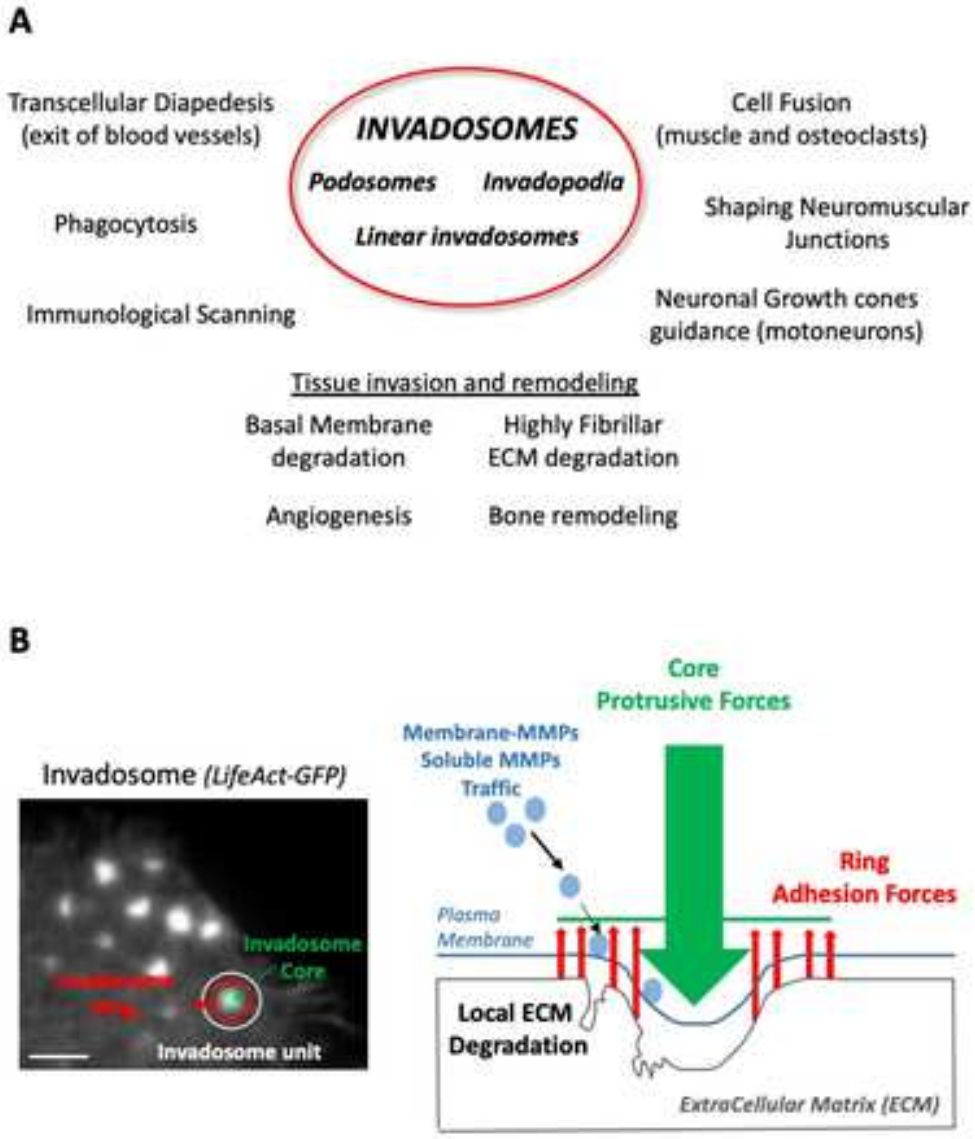

Figure2

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