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REVIEW

SUBJECT COLLECTION: INVADOPODIA AND PODOSOMES

Probing the mechanical landscape – new insights into podosome architecture and mechanics

Koen van den Dries[1,*,‡], Stefan Linder[2,*,‡], Isabelle Maridonneau-Parini[3,*,‡] and Renaud Poincloux[3,*,‡]

ABSTRACT

Podosomes are dynamic adhesion structures formed constitutively by macrophages, dendritic cells and osteoclasts and transiently in a wide variety of cells, such as endothelial cells and megakaryocytes. They mediate numerous functions, including cell–matrix adhesion, extracellular matrix degradation, mechanosensing and cell migration. Podosomes present as micron-sized F-actin cores surrounded by an adhesive ring of integrins and integrin–actin linkers, such as talin and vinculin. In this Review, we highlight recent research that has considerably advanced our understanding of the complex architecture–function relationship of podosomes by demonstrating that the podosome ring actually consists of discontinuous nano-clusters and that the actin network in between podosomes comprises two subsets of unbranched actin filaments, lateral and dorsal podosome-connecting filaments. These lateral and dorsal podosome-connecting filaments connect the core and ring of individual podosomes and adjacent podosomes, respectively. We also highlight recent insights into the podosome cap as a novel regulatory module of actomyosin-based contractility. We propose that these newly identified features are instrumental for the ability of podosomes to generate protrusion forces and to mechanically probe their environment. Furthermore, these new results point to an increasing complexity of podosome architecture and have led to our current view of podosomes as autonomous force generators that drive cell migration.

KEY WORDS: Actin, Contractility, Podosome, Mechanosensing, Cell migration

Introduction

Podosomes are structures constitutively formed in a few cell types [macrophages, dendritic cells (DCs) and osteoclasts] (Linder and Wiesner, 2015) that are involved in several functions, including adhesion, matrix degradation, mechanosensing, migration, cell–cell fusion and phagocytosis. They can also transiently be assembled by cells that become migratory and have to degrade the extracellular matrix, such as endothelial cells, neurons or megakaryocytes (Santiago-Medina et al., 2015; Schachtner et al., 2013; Seano et al., 2014). Podosomes were initially described as dot-like enrichments of F-actin at the substrate-attached side of osteoclasts and chondrocytes infected with Rous-sarcoma virus, surrounded by a ring of adhesion plaque proteins, such as vinculin and talin (which comprises talin-1 and talin-2 forms in mammals), and anchored to the extracellular matrix by integrins (Marchisio et al., 1984a,b; Zambonin-Zallone et al., 1988). Since then, a plethora of additional podosome components has been discovered, including through attempts to identify the podosome proteomes of different cell types (Cervero et al., 2012; Ezzoukhry et al., 2018), which has highlighted the unanticipated molecular complexity of these structures. It is now recognized that podosomes are composed of over 300 different components, which is comparable to other integrin-based substrate contacts, such as focal adhesions (FAs) (Humphries et al., 2009; Kuo et al., 2011; Zaidel-Bar et al., 2007). Concomitantly, novel microscopic and analytical techniques have furthered our understanding of how these components interact to give rise to an intricate molecular machinery that, in addition to cell–matrix adhesion, is also able to locally degrade extracellular matrix (Wiesner et al., 2010), actively probe the rigidity of the matrix (Labernadie et al., 2014; Luxenburg et al., 2012) and act as a sensor for substrate topography (van den Dries et al., 2012). Current efforts are focused on how these multiple, and sometimes apparently contradictory, functions are regulated in space and time. In this Review, we focus especially on the emerging features of podosome architecture and discuss how these individual elements are coordinated to induce molecular mechanotransduction events that allow local force generation. For a general introduction to the properties and function of podosomes and the related invadopodia, we refer the reader to several comprehensive reviews (Albiges-Rizo et al., 2009; Linder, 2007; Linder and Wiesner, 2015; Linder et al., 2011; Murphy and Courtneidge, 2011; van den Dries et al., 2014).

The modular architecture of podosomes

The two classical modules of podosomes are the actin-based protrusive core and the integrin-based adhesive ring. Although the exact architecture of the core actin filaments remains elusive, most of the actin in the core is probably generated by Arp2/3 complex-dependent actin nucleation (Linder et al., 2000) and thus likely consists of branched actin filaments. It is generally thought that actin polymerization in the core is one of the main drivers for podosome-mediated protrusion. The core is surrounded by the ring that contains adhesion plaque proteins and integrins (Fig. 1A,B). Although the ring was more recently shown, by super-resolution microscopy, to actually consist of several discontinuous clusters (van den Dries et al., 2013b), for convenience, we will still refer to this structure as the ‘ring’ here. Importantly, until recently, it was unclear whether and how the core and ring were connected. Therefore, it remained largely elusive how protrusion and adhesion were balanced in these micron-sized structures.

During the past decade, it has become increasingly clear that the core and ring are connected and further supported by additional modules. Two of these are the ventral lateral filaments and the dorsal connecting filaments (Fig. 1C). The ventral lateral filaments are a set

[1]Department of Cell Biology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Geert Grooteplein 26-28, 6525 GA, Nijmegen, The Netherlands. [2]Institut für medizinische Mikrobiologie, Virologie und Hygiene, Universitätsklinikum Eppendorf, Martinistr. 52, 20246 Hamburg, Germany. [3]Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, UMR5089, 205 route de Narbonne, BP64182 31077 Toulouse, France.
*All authors contributed equally to this work

‡Authors for correspondence (Koen.vandenDries@radboudumc.nl; s.linder@uke.de; Isabelle.Maridonneau-Parini@ipbs.fr; Renaud.Poincloux@ipbs.fr)

K.v.d.D., 0000-0002-7816-5206; S.L., 0000-0001-8226-2802; I.M.-P., 0000-0003-0189-0976; R.P., 0000-0003-2884-1744

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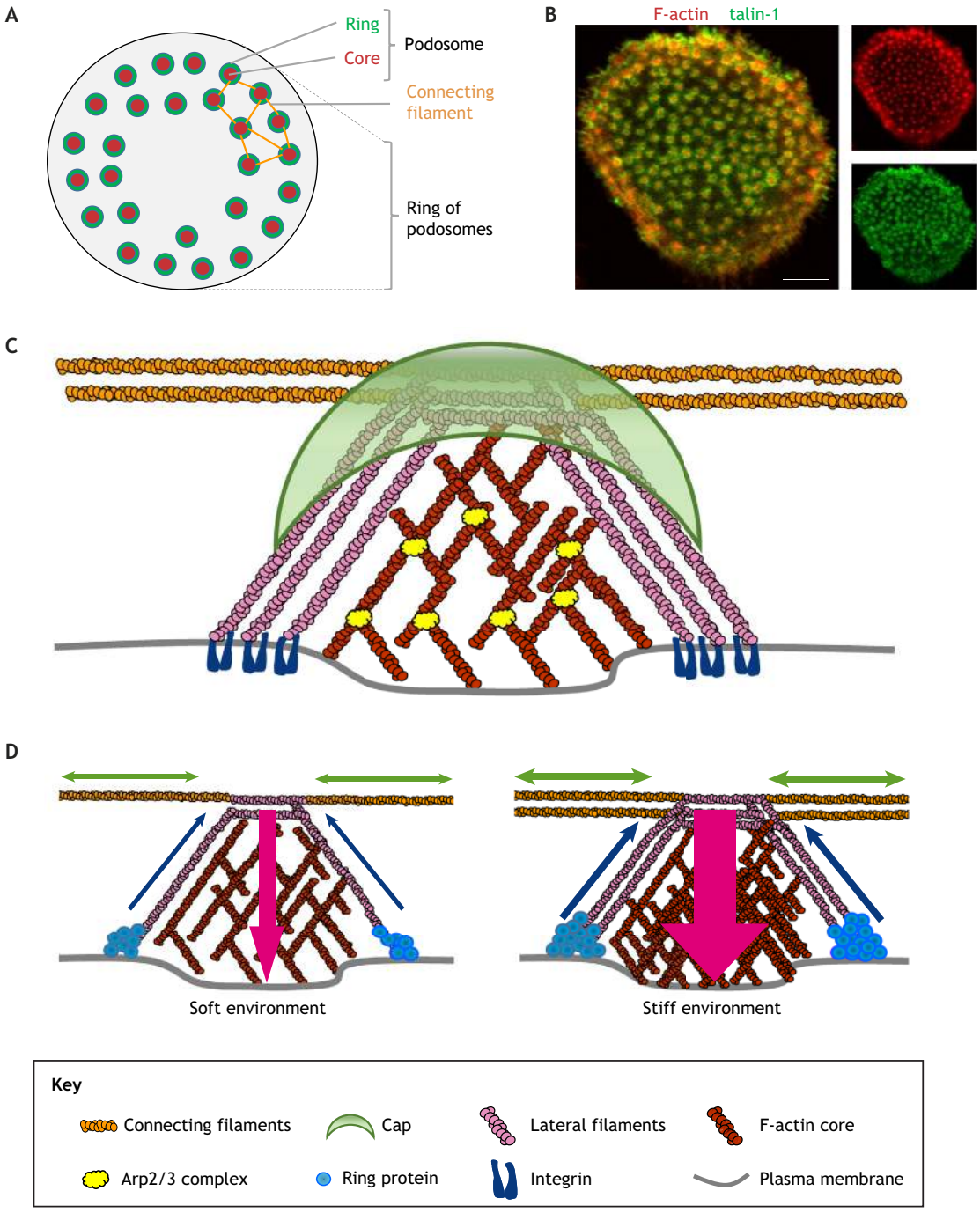

Fig. 1. Model of podosome architecture and mechanics. (A) Schematic illustration of podosomes, which are typically visualized through their F-actin core and their ring structure, comprising adhesion proteins such as talin-1. Note that individual podosomes are connected by connecting filaments into higher-ordered structures such as a ring of podosomes (not to be confused with the podosomal ring structure). (B) Confocal micrograph of a primary human macrophage stained for F-actin (red) using Alexa-Fluor-568–phalloidin and for talin-1 (green) using a specific primary antibody. Merge and single channels are shown. Scale bar: 5 µm. (C) Podosomes consist of several substructures, notably the core of Arp2/3 complex-generated (and thus likely branched) F-actin, as well as a ring structure consisting of adhesion plaque proteins, such as talin and vinculin, and a cap containing actomyosin regulatory proteins (see Fig. 2B). In addition, there are two sets of unbranched actin filaments, the connecting filaments, which link individual podosomes into higher-ordered arrays, and the lateral filaments that probably connect the cap to the ring. At the ventral membrane, podosomes are linked to the extracellular matrix by adhesion receptors such as integrins. (D) F-actin polymerization at the core of a podosome produces protrusion forces (magenta arrows) against the substrate that depend on substrate stiffness. These forces are balanced by local traction forces at the podosome ring (blue arrows) and by the contraction of dorsal connecting filaments (green arrows) (left). Podosome protrusion forces are mechanosensitive; on stiff substrates, podosomes produce stronger forces (right).

of unbranched filaments that run along the sides of the podosome core as shown by ultrastructural analysis (Luxenburg et al., 2007), and likely link the core and ring. The dorsal connecting filaments are a second set of unbranched actin filaments that link individual podosomes into higher-ordered groups (Bhuwania et al., 2012; Luxenburg et al., 2007), likely allowing the mechanical coupling of neighboring podosomes (Proag et al., 2015). Interestingly, recent evidence indicates that myosin II predominantly localizes to the

connecting filaments, suggesting that lateral filaments and connecting filaments are very different in nature (van den Dries et al., 2019).

Recently, a novel core submodule, the so-called cap, has been identified. For an overview on the gradual discovery of the cap, see Linder and Cervero (2020). The cap localizes to the top of the podosome core and extends laterally towards the ring (see Fig. 1C). Several proteins that localize to the cap have been described, all of which bind to actin or myosin, or regulate myosin activity, pointing to the cap as a podosome submodule that regulates actomyosin contractility. Cap proteins also contribute to the increasingly apparent molecular diversity of podosome subpopulations. Especially in macrophages, podosomes come in two flavors: larger, highly dynamic precursor podosomes found at the cell periphery or leading edge (Evans et al., 2003; Kopp et al., 2006), and smaller, longer-lived successor podosomes within the inner parts of the cell (Bhuwania et al., 2012). Interestingly, we could show that the precursors are enriched in lymphocyte-specific *protein* 1 (LSP1) and β-actin, whereas successors show an enrichment of supervillin and α-actin (Cervero et al., 2018). Considering that these proteins support different levels of actomyosin contractility and also differently affect the speed of actin-filament turnover (Allen et al., 1996; Bergeron et al., 2010), this likely contributes to the observed differences in the size and dynamics and possible functions of these podosome subpopulations.

Together, these observations have led to our current understanding that podosomes have a very complex modular architecture, with each module harboring a specific set of regulatory proteins. These modules likely cooperate to enable the multitude of functions exerted by podosomes such as protrusion, adhesion and mechanosensing. Future studies should provide further insight into the integration of these functions and how the modules together facilitate cell migration.

Resolving ring nanoscale organization by super-resolution microscopy

Super-resolution microscopy experiments have provided a more detailed view of podosomes, and in particular of the adhesive ring. Indeed, resolving the localization of the integrins and cytoskeletal adaptor proteins, such as vinculin and talin, has led to a greater understanding of the structure–function relationship of podosomes.

The first high-resolution view of podosomes was achieved by using the development of a super-resolution method called Bayesian analysis of blinking and bleaching (3B-analysis) (Cox et al., 2012). This approach allowed imaging the dynamics of vinculin and talin at podosomes at a resolution of 50 nm and on a time scale of four seconds and paved the way for a nanoscale analysis of the podosome organization (Cox et al., 2012). Direct stochastic optical reconstruction microscopy (dSTORM) analyses further revealed that the ring structure is in fact discontinuous and consists of several clusters (van den Dries et al., 2013b), similar to what had been shown before for FAs (Shroff et al., 2007). αMβ2, the predominant integrin at podosomes in DCs (Gawden-Bone et al., 2014), and talin have been shown to localize to small islets that are homogeneously distributed at the plasma membrane, but excluded from the core (van den Dries et al., 2013b). By contrast, vinculin is not homogeneously distributed. A high density of vinculin staining is only observed close to the core and along the actin filaments that radiate from podosome cores. Since vinculin is a mechanosensitive protein, the current notion is that these radiating actin filaments are under tension, which is likely caused by the growth of the actin core. To note, dSTORM also allowed for the clear visualization of the radiating actin filaments as well as those that interconnect neighboring podosomes (van den Dries et al., 2013b); these confirmed scanning electron micrographs of macrophage and osteoclast ventral plasma membranes (Labernadie et al., 2014; Luxenburg et al., 2007).

More recently, we used direct optical nanoscopy axially localized detection (DONALD) to examine the three-dimensional (3D) organization of the podosome ring (Bouissou et al., 2017). DONALD combines direct dSTORM with supercritical angle fluorescence and enables the detection of single fluorophores with a three-dimensional localization precision of 15 nm (Bourg et al., 2015). This resolution enables the exploration of the stretching of mechanosensitive proteins at the podosome ring, such as talin, which, when exposed to mechanical forces transmitted by actin filaments, progressively unfolds and exposes up to 11 vinculin-binding domains (Goult et al., 2018). DONALD revealed that, at podosomes, the integrin-bound N-terminus of talin localizes in a narrow layer close to the substrate (Bouissou et al., 2017), which is at the expected vertical position of the integrin intracellular domains (Kanchanawong et al., 2010). The actin-bound C-terminus of talin, however, localizes 26 nm above the talin N-terminus, and displays an even higher localization close to the actin cores. These results demonstrated that talin is stretched in podosome adhesion rings and that the stretching intensity inversely correlates with the distance to the core, suggesting that tension is highest close to the core. Interestingly, talin stretching could be directly correlated with the generation of forces by podosomes (see below). Indeed, knockdown of vinculin or paxillin reduced both the degree of talin stretching and the forces exerted by podosomes (Fig. 2A,B) (Bouissou et al., 2017).

For FAs, it has been proposed that vinculin recruitment stabilizes the unfolded state of talin (Ciobanasu et al., 2014; Gingras et al., 2005; Yao et al., 2014). Talin stretching may also reveal vinculin-binding sites at the podosome ring, explaining the accumulation of vinculin at this position. In agreement with this notion, DONALD analyses of podosomes demonstrates that vinculin localizes on average 52 nm above the substrate, which is exactly between the N- and C-terminal domains of talin. Furthermore, close to the core, vinculin displayed an increased height (from 46 to 54 nm), which correlates with the observed increase in talin extension. These observations suggest that vinculin is recruited by stretched talin. Confirming this hypothesis, vinculin shows a diffuse localization pattern and is excluded from podosome cores in talin-depleted macrophages, (Bouissou et al., 2017). Recruitment of vinculin at the ring is also strongly reduced by treatment with cytochalasin D, which mediates disruption of the actin network surrounding podosomes (van den Dries et al., 2013b). Therefore, the actin-binding capacity of vinculin is also essential for its specific accumulation to the ring (van den Dries et al., 2013b).

Overall, these novel insights into the nanoscale organization of the ring indicate that the localization of the integrins and adapter proteins is very tightly regulated in time and space. Moreover, their localization shows clear similarities with that found in FAs, which are known to be regulated by cytoskeletal tension or forces. The nanoscale architecture of the podosome ring therefore strongly suggests that the podosome ring is also under tension, most likely generated by the filaments that radiate from the core. These aspects of force generation and coupling between the core and ring will be further discussed below.

Mechanosensitivity and protrusion force generation

Podosomes are mechanosensitive, that is, they detect and react to the mechanical properties of their environment. This feature is

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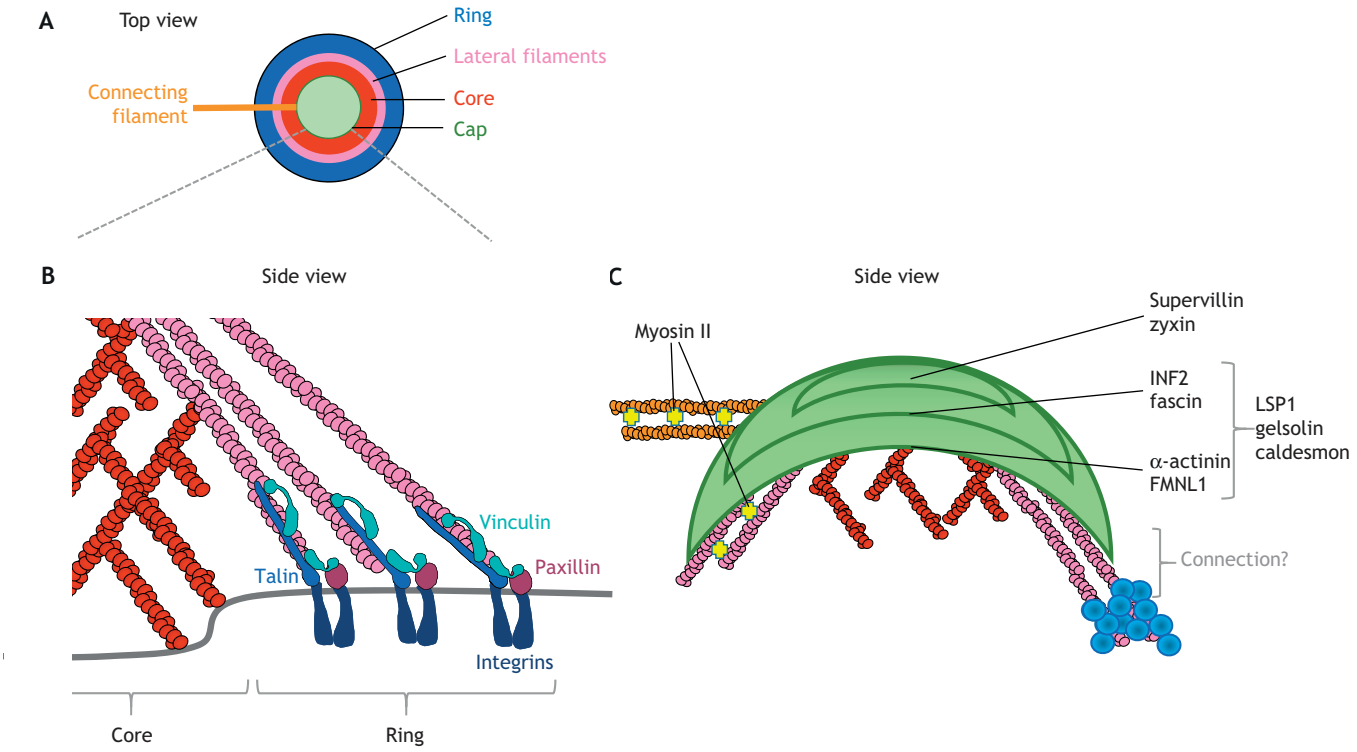

Fig. 2. Interaction between podosome substructures. (A) Top-view model of podosome substructures with the cap structure (light green) on top of the F-actin core (red), which is connected by lateral filaments (pink) to the ring structure (blue). Individual podosomes are linked by connecting filaments (orange). (B,C) Detailed side view models of podosome substructures. (B) Podosome protrusion forces are balanced by local traction forces at the podosome ring. These traction forces are transmitted to the substrate through the coordinated action of talin, vinculin and paxillin. Under traction, which is probably mediated by lateral actin filaments, talin assumes a stretched, open conformation. This tension-dependent stretching of talin uncovers multiple vinculin-binding sites and triggers vinculin recruitment to the podosome ring, as well as stabilizes the extended form of talin. (C) The dorsal connecting filaments contain myosin II, which supports filament bundling and actomyosin contractility. These filaments also connect to the cap structure on top of the F-actin core. Based on the cap components identified in different cell types, the cap is likely a layered structure (illustrated with the green shapes), with subdomains that are arranged according to function and actin-binding properties. Presented here is a suggested model and not an actual description of these subdomains, which still awaits closer investigation. Furthermore, as some cap components also extend to the ring structure, a connection between both substructures is possible, but not yet formally proven.

particularly interesting considering the wide range of mechanical tissue environments encountered by podosome-bearing cells, such as macrophages and dendritic cells. Mechanosensitivity has originally been described for podosomes in fibroblasts transformed by v-Src (Collin et al., 2008, 2006) and has subsequently been demonstrated for cancer cell invadopodia (Alexander et al., 2008), as well as macrophage podosomes (Labernadie et al., 2014). In these experiments, the formation and dynamics of podosomes was shown to positively correlate with substrate stiffness in the range of 1–100 kPa, which corresponds to the physiological stiffness range of most tissues in the body.

Mechanosensing has been well documented for FAs, which sense the environment by pulling on the matrix. In FAs, actomyosin-dependent traction forces are applied laterally to the substrate. FAs grow in response to their mechanical environment, that is, upon an increase of internal contractile forces by actin filaments or an increase in external forces applied to the cell (Riveline et al., 2001; Tan et al., 2003). Traction force microscopy (TFM) experiments have been instrumental in dissecting the molecular mechanisms underlying mechanosensing by FAs (Kraning-Rush et al., 2012; Plotnikov et al., 2012; Schwarz and Gardel, 2012; Zhang et al., 2008).

In contrast to FAs, podosomes are perpendicular to the matrix and are able to protrude into it. In cells plated on glass coverslips, such protruding activity translates into a close contact of the basal membrane with the glass coverslip beneath the podosome core, as, for example, determined in DCs by internal reflection microscopy (IRM), which can highlight sites of close contact between the basal membrane and the glass coverslip beneath the core (Evans et al., 2003; van den Dries et al., 2013a). However, detecting the forces that podosomes apply on the substrate has been challenging, as TFM, which has been used to measure FA forces, can only detect lateral forces. To determine the actual forces that podosomes apply on the extracellular environment, we developed a method called protrusion force microscopy (PFM). This technique uses podosome-forming cells plated on a compliant sheet of polyvinyl formal resin, which allows to measure podosome-induced deformations by atomic force microscopy (AFM). PFM revealed that podosomes induce the formation of bulges of a few nanometers into the substrate. The forces responsible for such deformations could be evaluated owing to finite element-based mechanical simulations and analytical modelling, and were shown to be in the 10 nN range (Labernadie et al., 2014; Proag et al., 2015, 2016). How actin filaments, which individually only produce forces in the picoNewton (pN) range, collectively generate these larger, nanoscale forces is still an open issue. One of our studies also addressed mechanosensing in the context of protrusion force generation. When the stiffness sensed by macrophages was increased (by augmenting the thickness of the film), podosomes generated higher forces, as determined by PFM (Fig. 1D) (Labernadie et al., 2014).

The exact origin of podosome protrusion force was unclear for a long time, but evidence collected in the past decade strongly indicates that continuous actin polymerization in the core is the main driver of this phenomenon. Podosomes have a lifetime of 2 to 20 min, but they exhibit an even faster internal dynamic of actin, which is completely turned over approximately three times during the lifetime of a podosome (Destaing et al., 2003). AFM has revealed further internal dynamics, by detecting periodic oscillations of the stiffness of the podosome core that are dependent upon actomyosin contractility (Labernadie et al., 2010). Although it is not entirely clear which factors contribute to these oscillations in stiffness, it is likely that the actin density in the core is one of the main determinants for changes in podosome core stiffness. This notion is supported by PFM experiments and analyses of podosome fluorescence dynamics, which revealed that the protrusion oscillations closely correlate with the actin content of podosomes, with both exhibiting a periodicity of 30 to 40 s (Labernadie et al., 2014, 2010; Proag et al., 2015, 2016; van den Dries et al., 2013a). Interestingly, treatment of cells with a low dose (2 µM) of cytochalasin D, to reduce, but not abolish, actin polymerization, eliminated both podosome oscillations and podosome-mediated protrusion (Labernadie et al., 2014; van den Dries et al., 2013a), further suggesting that actin polymerization is the major force generator at podosomes.

Coupled regulation between podosome core and ring modules

To allow the deformation of the underlying substrate, protrusion forces exerted by a cell need to be balanced with those pulling towards the cell. The architecture of podosomes suggests that the podosome machinery relies on a local balance of a central protrusion force exerted beneath the core with a peripheral traction force at the ring, as suggested originally in the model by Gawden-Bone et al., who proposed that two opposing forces exist at the podosome: a protrusive force at the core driven by actin treadmilling and an opposing (toward the core) myosin II-mediated force at the lateral filaments surrounding the core (Fig. 1D) (Gawden-Bone et al., 2010). Likewise, Luxenburg et al. also proposed subsequently that treadmilling of core actin is the underlying reason for the radial filaments being under tension (Luxenburg et al., 2012). Such a coupling between the ring and the core could explain, at least in part, the observed podosome oscillations (Labernadie et al., 2014; Proag et al., 2015, 2016; van den Dries et al., 2013a). Indeed, our theoretical modeling of the force balance between polymerizing core actin filaments and lateral contractile filaments predicts that, if the protrusive force is close to the maximal load that can be sustained by the core actin filaments, the coupling of the core and the ring becomes unstable, and force generation at the podosome acquires an oscillatory behavior (Labernadie et al., 2014).

The two-component model presented in Fig. 1D implies that podosome ring components experience tension. Several proteins that localize to the ring, such as talin and vinculin, are known to sustain and transmit tension in FAs (Atherton et al., 2015; Calderwood et al., 2013; Case et al., 2015; Ciobanasu et al., 2014; del Rio et al., 2009; Yao et al., 2014). Interestingly, the analysis of fluorescence intensity fluctuations of podosome components revealed a positive correlation between the amount of actin in the core and the presence of tension-sensitive adhesion components in the ring, such as vinculin and zyxin (Proag et al., 2016; van den Dries et al., 2013a). These results strongly suggest that the growth of the core drives the recruitment of tension-sensitive ring components and so strengthens the mechanical coupling between the core and the ring. In agreement with this, measurements of a vinculin tension sensor using Förster resonance energy transfer by fluorescence lifetime imaging (FRET-FLIM) have shown that vinculin is under tension at podosomes (van den Dries et al., 2013a). Furthermore, the direct measurement of forces on integrins in the ring using DNA-based FRET-FLIM probes has recently revealed that the integrins sustain picoNewton forces at podosomes, further supporting the notion that the ring experiences tension (Glazier et al., 2019). The importance of the ring for counterbalancing the protrusive forces of podosomes has also been assessed by PFM measurements. Here, siRNA-mediated depletion of ring proteins, such as talin, vinculin and paxillin, in macrophages has shown that each of them plays a critical role in podosome force generation and force transmission to the substrate (Bouissou et al., 2017). Altogether, these results suggest that, in contrast to what is found at FAs, where forces are counterbalanced at the scale of the whole cell, the protrusive forces at podosomes are locally counterbalanced by pulling forces on adhesion complexes, thus making podosomes autonomous force generators.

Although no direct evidence has been provided so far, it is likely that the lateral actin filaments facilitate the local balance of forces at podosomes. One of the next challenges will be to decipher how the protrusive forces that are produced by Arp2/3-mediated branched actin filaments at the core are transmitted to the unbranched lateral filaments, which are most likely nucleated by formins. Such a transmission of forces would be necessary to enable the mechanical coupling between these podosome modules (Linder et al., 1999; Panzer et al., 2016). In this respect, filamin A could be a potentially interesting candidate to perform this coupling as it localizes to podosomes (Guiet et al., 2012) and has been shown to interconnect different types of actin networks in fibroblasts (Kumar et al., 2019). Interestingly, for cancer cell invadopodia, a model for mechanical coupling has already been proposed based on the fact that invadopodia, as opposed to podosomes, are often located beneath the nucleus and able to indent it (Revach et al., 2015). The authors suggest that this nuclear connection could have a role in the transmission of forces that are required for invadopodia to protrude through an extracellular matrix (Revach et al., 2015) and, as such, would negate the necessity for a cap and lateral filaments to transmit forces. However, the relevance of this model for podosomes is unclear. Although less common than for podosomes, the transient formation of adhesion plaque rings at nascent invadopodia has been described. Analogous to the ring structure of podosomes, these rings apparently connect growing invadopodia precursors to the underlying matrix and act as sensors of matrix rigidity that could regulate invadopodia maturation (Branch et al., 2012). However, it is currently unclear how these rings are coupled to the actin core, and whether there also exists an invadopodia analog of the podosome cap, which connects core and ring (Rottner et al., 2017; Rottner and Schaks, 2019).

Myosin II as a master regulator of podosome mechanics and dynamics

Non-muscle myosin II is an actin crosslinker that controls the contractility of actin filaments. Although podosomes are classically associated with low contractility (Burgstaller and Gimona, 2004), non-muscle myosin II has been identified at the site of podosomes, first, in mouse osteoclasts (Chabadel et al., 2007) and later also in human dendritic cells (Gawden-Bone et al., 2010; van den Dries et al., 2013a; van Helden et al., 2008), human macrophages (Bhuwania et al., 2012), transformed baby hamster kidney cells (Collin et al., 2008), and human monocytic THP-1 cells (Rafiq

et al., 2019a). In all these cell types, myosin II is not located within the podosome core, but rather localizes in between podosome cores, where the filamentous actin network is also located. More recently, dual-color structured illumination microscopy (SIM) of intact dendritic cells (Meddens et al., 2016) and immunogold labeling of unroofed macrophages (Labernadie et al., 2014) has revealed that myosin II is in fact closely associates with the unbranched actin filaments that surround the podosome core. A recent study further assessed the axial position of myosin II with respect to the plasma membrane in dendritic cells; this suggests that the majority of myosin II is located to the connecting filaments (van den Dries et al., 2019).

While the association of myosin II with these actin filaments argues for a role of myosin II-mediated contractility in the interpodosomal network, the exact role of myosin II in regulating the function and organization of podosomes is still unclear. Myosin II activity appears not to be essential for podosome maintenance, since myosin inhibition by treatment with blebbistatin (Gawden-Bone et al., 2010; Labernadie et al., 2014, 2010; van den Dries et al., 2013a; van Helden et al., 2008) or knockdown of the myosin heavy chain (Bhuwania et al., 2012; Labernadie et al., 2014; Rafiq et al., 2017) did not inhibit podosome formation in various cell types. Moreover, the presence and recruitment of podosome components that are typically associated with actomyosin contraction, such as vinculin and zyxin, are apparently unchanged after inhibition of myosin II (van den Dries et al., 2013a).

By contrast, myosin II inhibition has been shown to decrease the periodicity of the oscillations of actin intensity (van den Dries et al., 2013a) and oscillations of podosome stiffness (Labernadie et al., 2010), two features that are associated with podosome protrusiveness. In human dendritic cells, myosin II inhibition results in a decreased length of podosome protrusions into filter pores (Gawden-Bone et al., 2010). More recently, our AFM study showed that the protrusive force of podosomes depends on myosin activity (Labernadie et al., 2014). Thus, while myosin II activity is redundant for podosome assembly itself, it does appear to control their protrusive force and dynamics. How exactly myosin II contributes to generating this protrusive force remains an open question.

Intriguingly, several reports suggest that podosomes can only assemble under conditions where cellular myosin II activity is low. For example, a global increase in myosin II activity by stimulation with prostaglandin E2 (PGE_2) results in complete loss of podosomes in human dendritic cells (van Helden et al., 2008). This effect is entirely dependent on myosin II contractility, since incubation with the myosin II inhibitor blebbistatin completely prevents PGE_2-mediated podosome loss (van Helden et al., 2008). Similarly, increased contractility due to the loss of the small GTPase Arf1 induces podosome loss in THP-1 cells, dependent on the activity of Rho-associated protein kinases (ROCK1 and ROCK2), which directly activate myosin II (Rafiq et al., 2017). Interestingly, a recent report demonstrates that loss of podosomes that is induced by microtubule disruption is also dependent on myosin II activation through the Rho guanine nucleotide exchange factor H1 (GEF-H1) pathway (Rafiq et al., 2019b). This work demonstrated that GEF-H1 is normally trapped on microtubules and that the disruption of microtubules releases GEF-H1, resulting in a global cellular increase in RhoA activity, which activates myosin II and, in turn, results in podosome loss. Notably, microtubule disruption had already been shown to induce RhoA-dependent podosome dissolution in multinucleated giant cells (Ory et al., 2002, 2000), suggesting that global myosin II activation is a general signal for podosome dissolution independent of cell type. Together, these reports highlight that a tightly controlled level of myosin II-mediated contractility is important for the ability of cells to form and maintain podosomes. Myosin II is locally activated to stimulate protrusion, but cells need to prevent the overactivation of myosin II to avert podosome dissolution. Such a tight control of myosin II activity may be regulated by the newly identified components of the podosome cap discussed in the next section.

The cap as a regulator of podosome mechanics

As mentioned above, several proteins that regulate actomyosin have been shown to be cap components, including the formins INF2 (Panzer et al., 2016) and FMNL1 (Mersich et al., 2010), supervillin (Bhuwania et al., 2012) and LSP-1 (Cervero et al., 2018). Recently, the actin-binding protein zyxin, which has previously been associated with the podosome ring, has been identified as a cap component in dendritic cells (Joosten et al., 2018). This apparent discrepancy is most likely due to the fact that cap-localized proteins can appear in a dot- or a ring-like distribution, depending on the plane of focus (see below). Moreover, cap localizations have been described for tropomyosin-4 in osteoclasts (McMichael et al., 2006), caldesmon in smooth muscle cells (Gu et al., 2007), gelsolin in dendritic cells (Gawden-Bone et al., 2010), and for fascin in THP-1 macrophages and dendritic cells (Van Audenhove et al., 2015), pointing to the existence of a cap as a more widespread phenomenon among podosome-forming cells. All of the cap proteins identified so far localize to a specific region that covers the upper part of the podosome core and extends laterally downwards to the podosome ring (Fig. 2A,C). Depending on the plane of imaging, the cap can thus appear as either dot- or ring-like, and only the use of optical *Z*-stacks enables its unambiguous identification. Considering that all known cap components are associated with contractile actomyosin filaments, it is likely that the cap represents a subset of the lateral unbranched actin filaments that connect the top of the core with the ring structure (Luxenburg et al., 2007) and are enriched with a specific subset of actin-associated proteins. It is therefore to be expected that more of the known or any thus far unidentified podosome components linked to actomyosin regulation turn out to be cap constituents. As most cap proteins have been identified individually in different cell models, it is currently unclear whether the same set of proteins is present at the cap of several, if not all, podosome-forming cells. However, as the cap functions as an actomyosin regulatory module in all monocytic cells, it is likely that the composition of the cap is conserved in these cells. Clearly, a systematic effort is needed to address this question. For a more in-depth discussion of this question, see (Linder and Cervero, 2019).

In terms of function, both supervillin and LSP1 have been shown to affect actomyosin contractility at the cap. In particular, LSP1-induced actomyosin contractility regulates the oscillatory protrusion of podosomes, with LSP1 overexpression resulting in a higher frequency of oscillations, whereas siRNA-mediated knockdown leads to a more irregular amplitude, as well as reduced protrusive force of individual podosomes (Cervero et al., 2018). Intriguingly, LSP1 and supervillin are differentially distributed in macrophages, with LSP1 being more enriched at precursors (Cervero et al., 2018) and supervillin at successors (Bhuwania et al., 2012). In part, this is based on the preferential binding of LSP1 to β-actin, which is enriched in precursors, and thus restricts the access of supervillin to this podosome subpopulation.

LSP1 and supervillin are also involved in the recruitment of myosin II to podosomes. siRNA-mediated knockdown of LSP1 leads to a general reduction of myosin II levels at podosomes (Cervero et al., 2018), while supervillin-dependent recruitment of

myosin II to successor podosomes precedes their dissolution (Bhuwania et al., 2012). This is probably owing to the fact that LSP1 and supervillin support different levels of myosin II activity. LSP1 is associated with moderate levels of myosin activity and binds myosin II in an F-actin-dependent manner (Cervero et al., 2018), whereas supervillin is a myosin II hyperactivator that binds directly and preferentially to activated myosin and also induces its further activation, which leads to a feed-forward cycle of myosin activity (Bhuwania et al., 2012). Interestingly, myosin II activity has been shown to contribute to the differential localization of supervillin to successors (Bhuwania et al., 2012), suggesting a reciprocal control between the recruitment of myosin II and its interactors.

In addition, cap-associated formins are likely to be involved in F-actin regulation at podosomes. Particularly INF2, which is both able to polymerize and depolymerize linear actin filaments (Chhabra and Higgs, 2006), might enable the swift adaptability of the actomyosin system that couples core and ring, and is likely to be crucial for the mechanosensitivity of podosomes. However, it is still unclear how the cap-associated unbranched actin filaments are anchored to the branched F-actin core of podosomes. Furthermore, thus far, no clear functions have been attributed to tropomyosin-4, caldesmon, gelsolin or fascin at the respective cap-like structures they localize to. However, because all of these factors are involved in either actin filament binding, bundling or turnover, their presence at the cap further points to it also being an acto(myosin) regulatory module in these cell types.

Dynamics and organization of podosomes at the mesoscale

A feature that distinguishes podosomes from other actomyosin-based structures, such as focal adhesions and invadopodia, is their organization in higher-ordered mesoscale superstructures (Fig. 3A). Dozens and sometimes hundreds of individual podosomes organize into clusters in human macrophages and dendritic cells (Burns et al., 2001; Linder et al., 1999), in so-called rosettes in endothelial cells (Seano et al., 2014) and smooth muscle cells (Mukhopadhyay et al., 2009), as well as in rings, belts and sealing zones in osteoclasts (Jurdic et al., 2006).

During osteoclast differentiation, podosomes organize sequentially into clusters, rings and belts (Destaing et al., 2003) and, during the final stage of maturation on bone, they form the sealing zone. It is unclear whether the sealing zone derives from a maturation of podosome belts (Jurdic et al., 2006; Saltel et al., 2004) or is formed *de novo*. Nevertheless, ultrastructural studies clearly indicate that the sealing zone consists of densely packed podosome cores (Luxenburg et al., 2007). Absence of the typical ring structure (Chabadel et al., 2007), which has been proposed as a hub for protease-containing vesicles (Linder et al., 2011), may explain why sealing zone podosomes are not degradative themselves (Saltel et al., 2004). Also, the exocytosis of secretory lysosomes in osteoclasts, which is required for ruffled border formation, appears to be mechanistically unique (Fujiwara et al., 2016; Ng et al., 2019). Further work is necessary to elucidate the mechanisms distinguishing lysosome fusion at podosomes in macrophages from that at the ruffled border in osteoclasts. Integrity of the podosome belt or sealing zone is dependent on microtubules (Destaing et al., 2003), RhoA (Saltel et al., 2004) and Src (Destaing et al., 2008). Loss of any of these signals leads to an unstable sealing zone, which hampers efficient bone resorption. Interestingly, the sealing zone only forms on mineralized matrices, while podosome rings form on glass or matrix proteins, indicating that, in osteoclasts, podosome organization is also controlled by extracellular signals, and podosome rings on glass are also more mobile and discontinuous compared to podosomes in mouse osteoclasts seeded on bone or calcite (Geblinger et al., 2009; Saltel et al., 2004).

While podosomes in osteoclasts appear to require a higher-order organization to function properly, much less is known about podosome cluster organization in dendritic cells and macrophages. Evidence is emerging that such clusters in these cells are very well organized, both hierarchically and spatially. First, by combining scanning electron microscopy and super-resolution light microscopy, it was recently demonstrated that podosome clusters in dendritic cells are characterized by a layer of cortical actin that is much thicker than for the rest of the cell, suggesting that the podosome cluster is indeed a separate cellular superstructure (Joosten et al., 2018). Second, the discovery that some components are differentially recruited to podosome subsets in human macrophages (Bhuwania et al., 2012; Cervero et al., 2018) suggests a hierarchical organization of macrophage podosomes, where the functionality of individual podosomes depends on their location. Third, two recent studies have reported that there is communication between neighboring podosomes, both in macrophages (Proag et al., 2015) and dendritic cells (Meddens et al., 2016). Our AFM-based PFM study showed that podosomes close to each other (first neighbors) tend to protrude in-phase synchrony (Proag et al., 2015). The second study utilized image correlation spectroscopy to show that F-actin, vinculin and talin exhibit directional and correlated flow patterns throughout podosome clusters, even beyond nearest podosome neighbours (Meddens et al., 2016). Based on computational modeling (Proag et al., 2015) and actin polymerization perturbation experiments (Meddens et al., 2016), both these studies point to the importance of the network of podosome-connecting filaments in regulating inter-podosomal communication.

Finally, when macrophages and immature dendritic cells migrate inside 3D matrices, another level of podosome complexity is apparent. In contrast to podosomes organized in ventral arrays in 2D cultures, cells in a 3D environment appear to organize podosome components as globular structures at the tip of cell protrusions. As these structures are also sites of MT1-MMP (also known as MMP14) surface accumulation and extracellular matrix degradation, they were accordingly denominated 3D podosomes (Cougoule et al., 2018; Van Goethem et al., 2011, 2010; Wiesner et al., 2013). However, the architecture of these structures, and in particular the position of ring, cap and core proteins, is still unknown (Wiesner et al., 2014).

In summary, the podosome superstructures formed by migrating cells could act as a mesoscale mechanosensing apparatus that scans basement membranes or dense interstitial tissue for weak spots in order to breach tissue boundaries and migrate through peripheral tissue. Interpodosomal communication could greatly enhance the efficacy of this process (Fig. 3B). Future studies should be directed at further elucidating the mechanisms that control interpodosomal communication, as well as the role of podosome clustering and 3D podosomes in macrophages and dendritic cells.

Conclusions

Based on the recent findings discussed above, we propose an updated model for podosome architecture, mechanics and function, which further develops that proposed previously (Linder and Wiesner, 2016). In particular, this model explains how the protrusive activity of podosomes may feedback on the degradative capacity of these structures as podosomes undergo oscillatory cycles of actin-based growth and shrinkage. During the growing phase, the podosome core triggers the protrusion into a compliant

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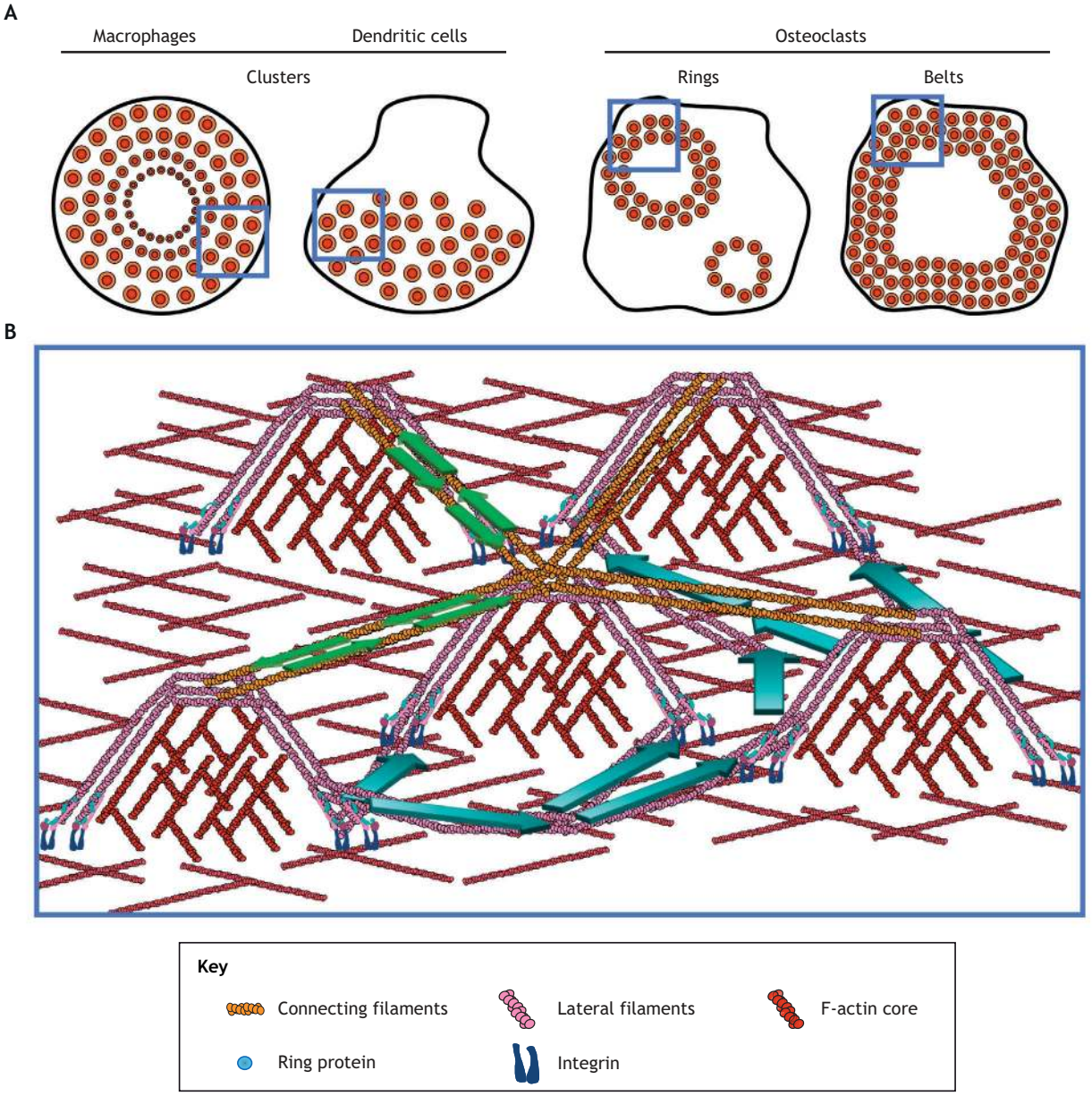

Fig. 3. Mesoscale organization of podosomes and their components. (A) Depending on cell type and differentiation stage, podosomes cluster into differently shaped mesoscale superstructures. Four of the most frequently observed shapes are depicted here. In general, macrophages and dendritic cells form clusters, while osteoclasts form rings and belts. (B) Independent from the actual mesoscale organization of podosome superstructures, the local organization of podosomes within such a superstructure appears to be highly similar. Podosomes are typically embedded in a layer of cortical actin (pink) that is thicker than that of the surrounding non-podosomal areas. Furthermore, podosomes are linked by dorsal connecting filaments (orange) that contract and likely facilitate interpodosomal contraction and force transmission (green arrows). Finally, within such a superstructure, there is a constant exchange of podosome components between neighboring podosomes, resulting in dynamic spatial patterns at the mesoscale (light blue arrows). Together, interpodosomal contractions and dynamic spatial patterns are likely to enable the podosome superstructure to function as a mesoscale mechanosensing apparatus that scans basement membranes or dense interstitial tissue for weak spots. Please note that the podosome cap and the oscillations of individual podosomes are not shown for the sake of clarity.

substratum, which, in contrast, is impeded on a rigid matrix. Concomitantly to protrusion of the podosome core, traction forces are generated and transmitted by the lateral actin filaments that connect podosome core and ring. As a result, mechanosensitive proteins in the ring such as talin are stretched, leading to the exposure of cryptic binding sites, for example, for vinculin, in turn eliciting intracellular signaling events. Mechanosensing of substrate rigidity by podosomes could thus be linked to their ability to degrade matrix. The cap structure and its ability to regulate actomyosin activity are likely to participate in these phenomena. It will be extremely interesting to test the different aspects of this model and also to further investigate molecular mechanisms that help to coordinate the multiple functions of these fascinating cell structures in space and time. Finally, considering that immune cells are mostly migrating within body tissues, research efforts should next be directed towards deciphering the nanoscale architecture of 3D podosomes and evaluating podosome forces in 3D environments.

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