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REVIEW

SUBJECT COLLECTION: CELL BIOLOGY AND DISEASE

The osteoclast cytoskeleton – current understanding and therapeutic perspectives for osteoporosis

Anne Blangy‡, Guillaume Bompard*, David Guerit*, Pauline Marie*, Justine Maurin*, Anne Morel* and Virginie Vives*

ABSTRACT

Osteoclasts are giant multinucleated myeloid cells specialized for bone resorption, which is essential for the preservation of bone health throughout life. The activity of osteoclasts relies on the typical organization of osteoclast cytoskeleton components into a highly complex structure comprising actin, microtubules and other cytoskeletal proteins that constitutes the backbone of the bone resorption apparatus. The development of methods to differentiate osteoclasts in culture and manipulate them genetically, as well as improvements in cell imaging technologies, has shed light onto the molecular mechanisms that control the structure and dynamics of the osteoclast cytoskeleton, and thus the mechanism of bone resorption. Although essential for normal bone physiology, abnormal osteoclast activity can cause bone defects, in particular their hyper-activation is commonly associated with many pathologies, hormonal imbalance and medical treatments. Increased bone degradation by osteoclasts provokes progressive bone loss, leading to osteoporosis, with the resulting bone frailty leading to fractures, loss of autonomy and premature death. In this context, the osteoclast cytoskeleton has recently proven to be a relevant therapeutic target for controlling pathological bone resorption levels. Here, we review the present knowledge on the regulatory mechanisms of the osteoclast cytoskeleton that control their bone resorption activity in normal and pathological conditions.

KEY WORDS: Intermediate filament, Microtubule, Osteoclast, Osteoporosis, Rho GTPase, Septin

Introduction

The cytoskeleton is made of filamentous structures that belong to one of four categories: the polarized actin filaments or microtubules, or the non-polarized intermediate or septin filaments (Dogterom and Koenderink, 2019; Goldmann, 2018; Spiliotis, 2018). It fulfills essential cellular functions, in particular for cell adhesion, migration, contractility, division and vesicular transport. The cytoskeleton can adopt unique shapes to support particular functions in differentiated cells, such as phagocytosis in macrophages (Rougerie et al., 2013), myotube contractility (Henderson et al., 2017), epithelial elasticity (Latorre et al., 2018), long-distance signal transmission in neurons (Dent, 2020) and bone resorption by osteoclasts (Georgess et al., 2014a; Touaitahuata et al., 2014).

Bone is a connective tissue predominantly composed of highly organized collagen I fibrils mineralized with calcium phosphate crystals of hydroxyapatite (Reznikov et al., 2014). Although very stiff, bone is highly dynamic; old bone is removed and replaced by new bone throughout life. Specific post-mitotic differentiated cells ensure cytoskeleton remodeling: the osteoclasts resorb the bone and the osteoblasts produce and organize the new bone matrix (Florencio-Silva et al., 2015). The osteocytes, a subset of osteoblasts embedded into the bone matrix, coordinate bone remodeling by monitoring skeleton load and sensing bone damages (Tresguerres et al., 2020). Osteoclasts are multinucleated hematopoietic cells that derive from myeloid precursors of the monocyte/macrophage lineage, whose differentiation is triggered by macrophage colony-stimulating factor (M-CSF; also known as CSF1) and receptor activator of nuclear factor-κB ligand (RANKL; also known as TNFSF11) (Jacome-Galarza et al., 2019). M-CSF and RANKL induce a differentiation program that includes cell–cell fusions to generate multinucleated osteoclasts (Fig. 1A) with the ability to resorb bone (Jacome-Galarza et al., 2019). The bone resorption apparatus consists of a highly convoluted plasma membrane domain, the ruffled border, which is in contact with the bone matrix (Ng et al., 2019) (Fig. 1A). There, the ATP-dependent vacuolar proton pump V-ATPase and the chloride channel type 7 (Clcn7), respectively, secrete H^+ and Cl^- into the resorption lacuna (Fig. 1A,B), which acidifies the medium and dissolves bone hydroxyapatite (Ng et al., 2019). Mineral dissolution unmasks bone proteins, now amenable for proteolytic degradation by proteases secreted at the ruffled border, including cathepsin K, which can hydrolyze collagen fibril (Ng et al., 2019), and the matrix metalloproteinases MMP9 and MMP14 (Zhu et al., 2020) (Fig. 1A,B), thus forming a bone resorption pit (Fig. 1C). The ruffled border is confined within a unique adhesion structure made of a belt of densely packed podosomes (see Box 1), called the sealing zone or podosome belt (Fig. 1A,B, Box 2). Podosomes ensure osteoclast adhesion to the substrate via integrin αvβ3, the major osteoclast integrin (Georgess et al., 2014a). A complex network of microtubules sustains osteoclast podosome organization (Fig. 1D). Osteoclast podosomal structures are dynamic (Fig. 1E,F, Box 2); they slide, assemble and disassemble, generating a series of resorption pits on the bone surface during the 2-week lifetime of osteoclasts (Jacome-Galarza et al., 2019; Søe and Delaissé, 2017).

The osteoclast cytoskeleton is instrumental for its bone resorption function, as the sealing zone supports the ruffled border. Not surprisingly, a lot of effort has been put into understanding the dynamics and regulation of the osteoclast cytoskeleton. These studies, based on various osteoclast models (see Box 3), have established that the osteoclast cytoskeleton can also be viewed as a therapeutic target to control osteoclast activity in the context of bone diseases. This Review provides an update of our knowledge of osteoclast cytoskeleton and points to the questions that are now emerging at the beginning of the new decade.

Centre de Recherche de Biologie Cellulaire de Montpellier (CRBM), Montpellier Univ., CNRS, 34000 Montpellier, France.
*These authors contributed equally to this work

‡Author for correspondence (anne.blangy@crbm.cnrs.fr)

A.B., 0000-0001-7043-0784; G.B., 0000-0001-6729-4507; J.M., 0000-0001-8802-9120; V.V., 0000-0001-8502-587X

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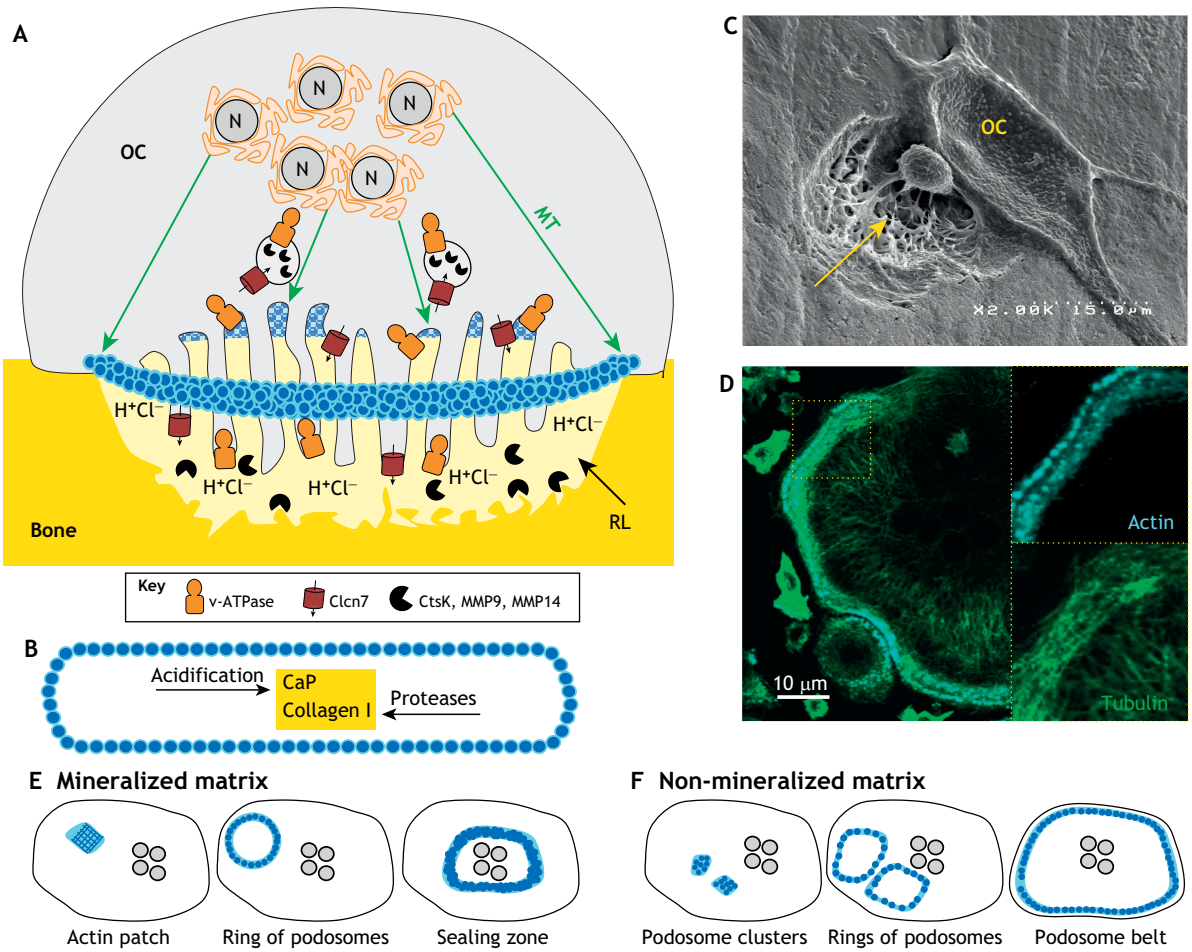

Fig. 1. General organization of the osteoclast cytoskeleton. (A,B) Schematic side view of an osteoclast (A) and top view of the sealing zone (B). Osteoclasts (OC) are multinucleated (N) cells that adhere to the bone by means of a sealing zone, consisting of a belt of densely packed podosomes (blue dots) (see Boxes 1 and 2); the belt is stabilized by microtubules (MT) that emanate from the pericentriolar material that localizes to the Golgi (light orange), which surrounds each nucleus. At the center of the sealing zone, the osteoclast membrane differentiates into a ruffled border where the v-ATPase proton pump (orange symbols) secretes protons and the Clcn7 chloride channel (red-brown symbols) secretes Cl^- ions to acidify the resorption lacuna (RL) and dissolve bone calcium phosphate crystals of hydroxyapatite (CaP). The ruffled border also secretes the proteases cathepsin K (CtsK), MMP9 and MMP14 (pacman symbols) into the RL to degrade bone proteins, mainly collagen I. (C) Scanning electron microscopy image of a resorption pit (RP, arrow) formed on a bone slice after the resorption activity of an osteoclast (OC). (D) Fluorescence microcopy image of an osteoclast plated on glass and stained for F-actin in blue and for tubulin in green to visualize the podosome belt and microtubules, respectively; images on the right show an enlargement of the boxed area in the main image. (E) Schematic view of sealing zone assembly in an osteoclast sitting on a mineralized substrate; the initial actin patch evolves into a ring of podosomes that enlarges to form a sealing zone (see Box 2). (F) Schematic view of podosome organization during osteoclast differentiation or osteoclast adhesion onto a non-mineralized substrate; initial podosome clusters evolve into rings of podosomes that expand and fuse to form the podosome belt (see Box 2 for details).

The regulation of actin dynamics in osteoclasts and its impact on bone resorption

As actin is the major component of podosomes, actin-regulating proteins have received prominent attention as potential regulators of bone resorption (Georgess et al., 2014a; Touaitahuata et al., 2014). Downstream of integrins, the tyrosine kinase Src is a key controller of the actin cytoskeleton and has been shown to control podosome dynamics and organization in osteoclasts, which express high levels of Src, and thus bone resorption (Destaing et al., 2008). Interestingly, while Src is ubiquitously expressed, its knockout (KO) in the mouse leads to one predominant phenotype: a severe osteopetrosis resulting from a defect in osteoclast function (Horne et al., 1992; Lowell and Soriano, 1996; Soriano et al., 1991). Src binds to the tyrosine kinase Pyk2 (also known as PTK2B) and they activate each other downstream of αvβ3 integrin (Fig. 2) to regulate podosome dynamics in osteoclasts, in particular through small GTPases of the Rho family (RhoGTPases), which are the major regulators of actin dynamics (Gil-Henn et al., 2007; Sanjay et al., 2001).

Accordingly, the RhoGTPases RhoA, Rac1 and Rac2 (fp130denoted generically as Rac) and Cdc42 indeed have a key role in controlling the actin cytoskeleton in osteoclasts, as detailed below (Georgess et al., 2014a; Ory et al., 2008; Touaitahuata et al., 2014) (Fig. 2). Differential gene expression has been used to identify the regulators of RhoGTPase activity necessary for osteoclast cytoskeleton organization and efficient bone resorption (Brazier et al., 2006; Georgess et al., 2014b; Maurin et al., 2018). The expression of various Rho guanine nucleotide exchange factors (GEFs), which activate the RhoGTPases by promoting their binding to GTP (Fort and Blangy, 2017), increases during osteoclast differentiation (Brazier et al., 2006). In fact, several of these control the osteoclast cytoskeleton and thus bone resorption, in particular GEFs for the RhoGTPases Rac1 and Rac2, which are both needed for podosome organization into a belt (Croke et al., 2011; Itokowa

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Box 1. Podosomes

Podosomes are characteristic adhesion structures of myeloid cells, including osteoclasts, macrophages and immature dendritic cells (van den Dries et al., 2019b). Conversely, invadopodia designate closely related structures typical of invading tumor cells (Linder and Wiesner, 2015; Paterson and Courtneidge, 2018). The ultrastructure of podosomes has been reviewed in detail very recently (van den Dries et al., 2019b). In brief, podosomes are dot-like adhesion structures composed of a core domain, likely made of branched actin that polymerizes beneath the plasma membrane. The podosome core is surrounded by a podosome ring, usually called the podosome cloud in osteoclasts, consisting of unbranched actomyosin filaments connected to integrins; actomyosin filaments also link neighboring podosomes. In addition, a cap structure containing actin nucleators of the formin family is present on top of the podosome core, which appears to participate in the coordinated podosome oscillations through actomyosin fibers that connect adjacent podosomes (Mersich et al., 2010; Panzer et al., 2016; van den Dries et al., 2019a). In osteoclasts, the core domain of podosomes contains the adhesion protein CD44, the cloud connects to integrin αvβ3 (Georgess et al., 2014a) and the cap contains tropomyosin 4 (McMichael et al., 2006). In contrast to immature dendritic cells and macrophages, podosomes only transiently organize into clusters in osteoclasts, as visible during the early steps of osteoclastogenesis or of osteoclast adhesion (Destaing et al., 2003; Eleniste and Bruzzaniti, 2012; Saltel et al., 2004) (Fig. 1F). The typical podosome structures in osteoclasts are the sealing zone and the podosome belt (Box 2). Whereas the lifetime of an individual podosome is short, typically in the range of minutes, the sealing zone and the podosome belt, made of thousands of podosomes, can last for hours (Bhuwania et al., 2012; Destaing et al., 2003; Klapproth et al., 2019).

Box 2. Podosome belt and sealing zone

The sealing zone (Fig. 1A,B) is made of a 3–6-µm-wide belt of densely packed podosomes; the core to core distance is ∼210 nm (Luxenburg et al., 2007). The sealing zone is observed in osteoclasts on bone or placed on natural or artificial mineralized substrates, such as nacre or pure calcium phosphate coated-plates (Shemesh et al., 2017). When osteoclasts are on a non-mineralized substrate, a slightly different structure appears, called the podosome belt or sealing zone-like structure; it is 2–3 µm wide with a podosome core-to-core distance of ∼480 nm (Luxenburg et al., 2007). The podosome belt is distinct from the podosome rosettes observed in invading cells, which are related to invadopodia (Linder, 2007). Sealing zone assembly begins with an actin patch, a poorly characterized structure that evolves into a ring and then a sealing zone (McMichael et al., 2010; Saltel et al., 2004; Takito et al., 2018) (Fig. 1E). During osteoclast differentiation, which takes several days, cells initially exhibit podosome clusters that are similar to those of macrophages or immature dendritic cells; differentiating osteoclasts progressively organize rings of podosomes that expand and fuse to form a single podosome belt in mature osteoclasts (Georgess et al., 2014a; Touaitahuata et al., 2014) (Fig. 1F). During this process, podosome compaction increases: the core-to-core distance is ∼750 nm in podosome clusters, but only ∼480 nm in the podosome belt (Luxenburg et al., 2007). A similar transition is observed upon osteoclast attachment to the substrate; the formation of rings of podosomes requires about 1 h, and the podosome belt establishes after several hours (Eleniste and Bruzzaniti, 2012). Although the two structures are not exactly the same, the podosome belt formed on glass or plastic recapitulates many features of the sealing zone on bone, and the presence of the podosome belt on glass/plastic reflects the ability of the osteoclast to form a sealing zone when on a mineralized substrate (Saltel et al., 2004). The osteoclast also forms a ruffled border-like structure within the podosome belt (Fuller et al., 2010) and, as is the case on bone, the extracellular medium beneath the cell becomes highly acidic (Silver et al., 1988).

et al., 2011; Magalhaes et al., 2011; Wang et al., 2008). Among these, the Rac GEF dedicator of cytokinesis protein 5 (Dock5) localizes to the podosome belt and is required for sealing zone assembly (Vives et al., 2011). The binding to adaptor protein tensin-3 stimulates Dock5 exchange activity towards Rac (Touaitahuata et al., 2016) (Fig. 2). Dock5 controls the activating phosphorylation levels of the Src substrate p130Cas (also known as BCAR1), an adaptor protein required for the sealing zone formation (Nagai et al., 2013), through an unknown mechanism (Vives et al., 2011), Moreover, chemical inhibition of Rac activation through Dock5 confirmed its essential role for osteoclast podosome belt formation and bone resorption activity (Ferrandez et al., 2017; Vives et al., 2011). Consistent with this *Dock5* KO mice, which are perfectly viable, have increased bone mass (Vives et al., 2011). The Rac GEF VAV3 also participates in podosome belt formation (Faccio et al., 2005; Sakai et al., 2006); VAV3 is activated by the tyrosine protein kinases Src and SYK downstream of integrin αvβ3 and the M-CSF receptor c-Fms (also known as CSF1R) (Zou et al., 2007) (Fig. 2). In this way, VAV3 controls osteoclast activity, as also demonstrated by *Vav3* KO mice, which have increased bone mass (Faccio et al., 2005). FARP2 is another Rac GEF required for the localized activation of Rac1 at rings of podosomes (Box 2): FARP2 depletion impairs podosome belt formation and bone resorption, confirmed by an increased bone mass in the *Farp2* KO mouse (Takegahara et al., 2010). Thus, bone resorption involves a complex spatial and temporal regulation of Rac activity to organize the osteoclast actin cytoskeleton.

Cdc42 is also necessary for sealing zone formation (Ito et al., 2010; Steenblock et al., 2014) (Fig. 2). In contrast, the deletion of Arhgap1, a Cdc42 GTPase-activating protein (GAP), which inactivates the GTPase by stimulating its GTP hydrolysis activity (Tcherkezian and Lamarche-Vane, 2007), has been shown to favor sealing zone formation and stimulate bone resorption (Ito et al., 2010). The faciogenital dysplasia (FGD) family member FGD6 is a Cdc42 GEF required for bone resorption; it controls podosome formation and lifetime (Steenblock et al., 2014). This study in osteoclasts shows that, when it is phosphorylated by Src, FGD6 binds the Cdc42 effector IQGAP1, an adaptor protein necessary for sealing zone formation (Fig. 2), while otherwise, it binds the Cdc42 GAP Arhgap10. This switch in the binding partners of FGD6 could control the local activation of Cdc42 at the sealing zone (Steenblock et al., 2014).

RhoA has a crucial role in podosome organization in osteoclasts; both increasing and decreasing its activity disturbs the podosome belt (Chellaiah et al., 2000; Ory et al., 2000). RhoA activity decreases when podosomes organize from clusters into rings and then belts, and RhoA inhibition accelerates podosome belt formation (Destaing et al., 2005). Conversely, increasing the activity of RhoA collapses the belt into rings and clusters of podosomes (Destaing et al., 2005; Ory et al., 2000). However, on a mineralized substrate, the inhibition of RhoA leads to a relaxation of the sealing zone and the formation of a podosome belt (Saltel et al., 2004), in which podosomes are less densely packed (Box 2). Owing to its crucial role in podosome organization in osteoclasts, the activity of RhoA is tightly balanced (Fig. 2). For instance, the RhoA GAP myosin 9b (Myo9b) (Post et al., 1998) accumulates at the podosome belt, where RhoA activity is low, whereas it is absent at the sealing zone, where RhoA activity is high (McMichael et al., 2014). Depletion of Myo9b increases the levels of active RhoA and perturbs sealing zone formation, as well as bone resorption, which can be counteracted through treatment with the RhoA inhibitor C3

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Box 3. Historical overview of model systems used to study osteoclast biology

Until the late 1990s, osteoclasts were either isolated directly from rat, rabbit, human or chicken bones, which were very fragile and few in numbers, allowing only limited studies (Teti et al., 1991), or they were obtained from co-cultures of bone marrow or spleen cells with osteoblastic cells, which led to non-homogeneous cellular cultures (Akatsu et al., 1992). Then, RANKL was identified as the key cytokine that, together with M-CSF, can support the differentiation of primary hematopoietic progenitors into bone-resorbing osteoclasts in culture (Lacey et al., 1998). Hence, osteoclast differentiation became possible, either by *ex vivo* culture of mouse bone marrow or human peripheral blood cells in the presence of RANKL and M-CSF, or culturing the mouse monocyte/macrophage-derived cell line RAW264.7 in the presence of RANKL (Hsu et al., 1999). To study the role of a gene of interest, one can either differentiate osteoclasts from a relevant KO mouse model, or transfect siRNAs or transduce shRNAs in wild-type osteoclasts or osteoclast precursors. Very recently, mouse myeloid progenitors have been immortalized by the ectopic expression of the homeodomain containing transcription factor Hoxb8; these cells differentiate into functional osteoclasts in the presence of RANKL and M-CSF and are amenable for CRISP/Cas9-mediated gene deletion (Di Ceglie et al., 2017). These different systems have paved the way for the genetic manipulation of osteoclasts in culture and shed light on the molecular mechanisms controlling osteoclast cytoskeleton and then bone resorption. Of note, the different osteoclast models may lead to non-consistent findings, as for instance regarding Myo10 function (see main text).

(McMichael et al., 2014). Furthermore, the expression of the atypical RhoGTPase RhoE (also known as Rnd3) increases during osteoclast differentiation (Georgess et al., 2014b). RhoE antagonizes RhoA activity: it both binds and activates the RhoA GAPs p190ARhoGAP (ARHGAP35) and p190BRhoGAP (ARHGAP5) (Wennerberg et al., 2003), whereas it binds to but inhibits the RhoA effector Rho-associated protein kinase 1 (ROCK1) (Oinuma et al., 2012). RhoE KO osteoclasts have fewer podosomes, and they are defective in podosome belt and sealing zone formation and have a low bone resorption activity (Georgess et al., 2014b). RhoE prevents the ROCK1-mediated inhibitory phosphorylation of the actin-severing protein cofilin (herein referring to cofilin 1) on serine 3 (Georgess et al., 2014b) (Fig. 2), which is needed for podosome belt formation (Zalli et al., 2016). Cofilin activity, as monitored by serine 3 dephosphorylation, increases during the differentiation of osteoclasts (Blangy et al., 2012). Furthermore, cofilin serine 3 dephosphorylation is required for its association with cortactin (Zalli et al., 2016), an actin-nucleation promoting factor also needed for podosome organization and bone resorption (Tehrani et al., 2006). The cofilin–cortactin complex is required for belt formation (Zalli et al., 2016), as detailed below. Finally, the RhoA GAP Arap1 also localizes to osteoclast podosomal structures and its RhoA GAP activity is needed for podosome belt and sealing zone formation (Segeletz et al., 2018).

Although the spatiotemporal regulation of RhoGTPase signaling pathways by specific GEFs and GAPs is clearly crucial for controlling the formation of osteoclast podosome superstructures, how they are coordinated in time and space remains far from being understood. Besides controlling RhoGTPase activity, GEFs and GAPs are multimodal proteins that are able to couple the actin cytoskeleton to other cellular features. For instance, Arap1 has two GAP domains, one for Rho and one for Arf GTPases (Miura et al., 2002), which are essential regulators of intracellular trafficking (Sztul et al., 2019). Thus, Arap1 couples the regulation of actin through its RhoA GAP domain to that of membrane trafficking to the ruffled border through its Arf GAP domain (Segeletz et al., 2018). Similarly, Dock5 is likely to participate in actin and microtubule crosstalk, as this GEF for Rac also controls microtubule

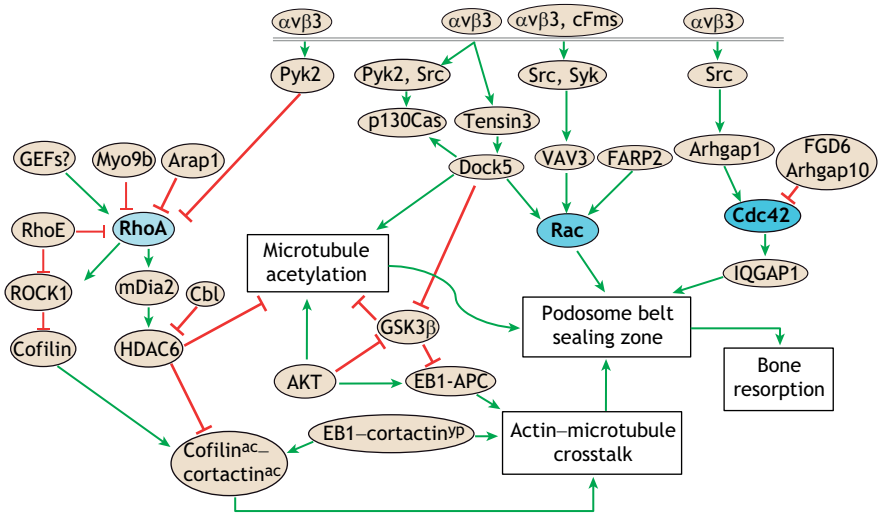

Fig. 2. Signaling pathways involved in the control of the osteoclast cytoskeleton and bone resorption. Downstream of integrin αvβ3 and the M-CSF receptor c-Fms, the non-receptor tyrosine kinases Src, Pyk2 and Syk control the activity of various GEFs that activate the GTPases Rac and Cdc42. Dock5, Vav3 and Farp2 activate Rac, whereas FGD6 activates Cdc42 and allows the binding of the GTPase to its effector IQGAP1. The GAPs Arhgap1 and Arhgap10 negatively regulate Cdc42 activity, whereas the GAPs Myo9b and Arap1 negatively regulate RhoA activity. The GTPase RhoE also acts as a negative regulator of RhoA, and it also inactivates the RhoA effector ROCK1, which inactivates cofilin by phosphorylation on serine 3. Microtubule acetylation is under the control of the RhoA effector mDia2, which activates the α-tubulin deacetylase HDAC6, and of Cbl, which inhibits HDAC6. The cofilin–cortactin complex participates in actin–microtubule crosstalk in osteoclasts. The formation of this complex requires the acetylation (ac) of the both cofilin and cortactin; it is counteracted by the deacetylase HDAC6 and by ROCK1-mediated cofilin phosphorylation. When cortactin is not acetylated, but is tyrosine-phosphorylated by Src (yp), it binds the microtubule +TIP protein EB1; the cortactin–EB1 complex is involved in targeting microtubules to podosome clusters. The GSK3β kinase is another inhibitor of microtubule acetylation in osteoclasts; AKT kinase and Dock5 negatively regulate the activity of GSK3β. GSK3β inhibits, whereas AKT favors, the formation of a complex between EB1 and APC, two proteins involved in actin–microtubule crosstalk.

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dynamics and acetylation in osteoclasts (Guimbal et al., 2019), as detailed below.

Interplay between actin and microtubules in osteoclasts

It has become clear that microtubules and the actin cytoskeleton are intimately connected and work together to fulfill many cellular processes (Dogterom and Koenderink, 2019). In osteoclasts in fact, depolymerization of microtubules destabilizes both the podosome belt, which breaks down into rings and clusters of podosomes (Babb et al., 1997; Destaing et al., 2003), and the sealing zone, which prevents bone resorption (Okumura et al., 2006).

Complexity of the osteoclast microtubule network

Actin and microtubules are intimately connected at the podosome belt and at the sealing zone (Babb et al., 1997; Batsir et al., 2017; Destaing et al., 2005) (Fig. 1A,D). Mammalian osteoclasts are devoid of centrosomes; instead, microtubules emanate from the pericentriolar material that localizes to the Golgi, which surrounds each nucleus (Mulari et al., 2003; Yamamoto et al., 2019). Some microtubules can be observed growing until they reach a podosome, while others are observed to bend back toward the cell center when they reach the podosome belt (Batsir et al., 2017; Biosse Duplan et al., 2014) or grow towards the top of the podosomes where they form a dense circular network over the podosomal belt (Fig. 3). Microtubules also associate with the actin-rich area of the sealing zone; the network there is even denser than at the podosome belt, therefore, hampering further insights into its structure (Okumura et al., 2006).

Actin and microtubule crosstalk at the podosome belt

As discussed above, microtubules are necessary for the formation of the podosome belt and the sealing zone. Accordingly, treatment with high concentrations of nocodazole in order to disrupt microtubules induces rapid podosome belt disorganization, while individual podosomes are maintained but reorganize into rings and clusters (Babb et al., 1997; Destaing et al., 2003). Depolymerization of microtubules also precludes stabilization of the sealing zone (Okumura et al., 2006). The perturbation of microtubule dynamic instability with low doses of nocodazole or paclitaxel similarly destabilizes the podosome belt (Biosse Duplan et al., 2014).

Various mechanisms control the crosstalk between actin and microtubule networks in osteoclasts (Fig. 2). In fact, microtubules at the podosome belt and the sealing zone are highly acetylated as compared to those at rings and clusters of podosomes (Akisaka et al., 2011; Destaing et al., 2005), and tubulin acetylation is important for osteoclast function (Destaing et al., 2005). RhoA activity has a negative effect on microtubule acetylation: in osteoclasts, RhoA counteracts microtubule acetylation through its effector mDia2 (also known as DIAPH3), a formin that binds and activates the microtubule deacetylase HDAC6 (Destaing et al., 2005) (Fig. 2). By preventing the interaction of HDAC6 with microtubules, the adaptor proteins c-Cbl and Cbl-b protect microtubules against deacetylation (Fig. 2). Suppression of c-Cbl and Cbl-b prevents bone resorption by disorganizing the cytoskeleton (Purev et al., 2009). The kinase Pyk2 also participates in preserving tubulin acetylation by maintaining low RhoA activity through an unknown mechanism (Fig. 2; Gil-Henn et al., 2007). Thus, understanding the precise functions of RhoA in controlling actin and microtubule crosstalk still requires further investigations.

The kinases AKT1 and AKT2 (collectively known as AKT kinases) and the kinase GSK3β also control microtubule acetylation in osteoclasts (Matsumoto et al., 2013) (Fig. 2). GSK3β activity inhibits osteoclast microtubule acetylation, but its phosphorylation

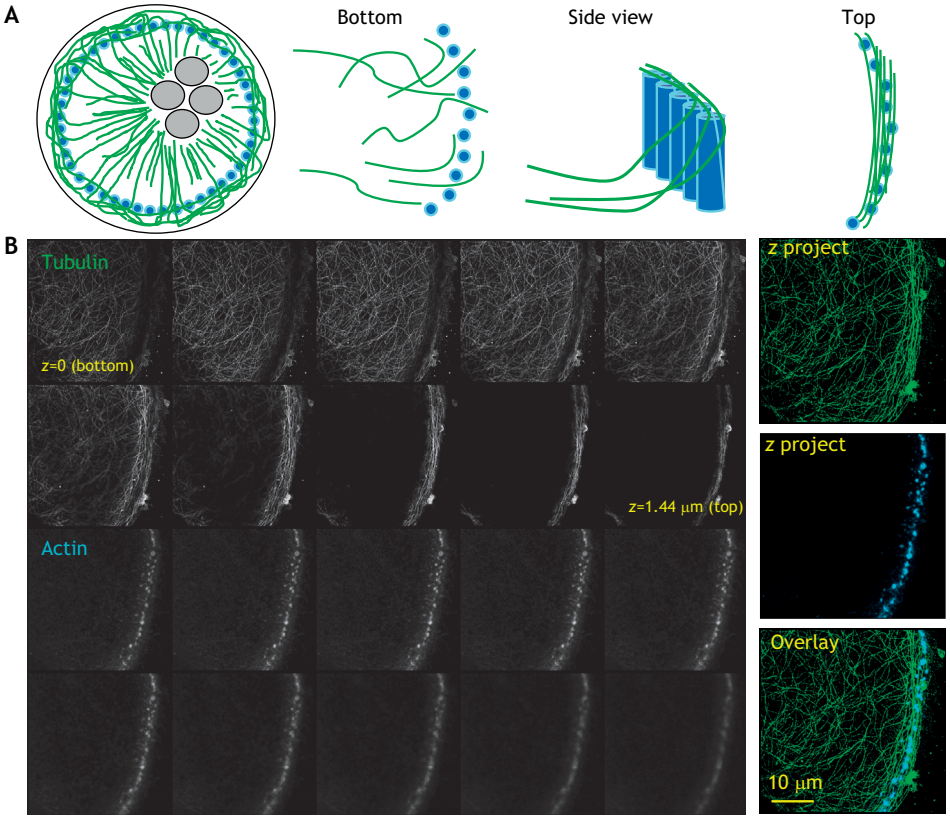

Fig. 3. Microtubule network in osteoclasts. (A) Schematic illustration of the microtubules at the podosome belt. Microtubules (in green) radiating from the perinuclear area extend towards the podosomes (blue dots). When they reach the podosome belt, they can stop or bend back toward the cell center, or cross the podosome belt (bottom). Some microtubules also bend and extend upwards (side view) and form a circular network above the podosome belt (top). (B) Airyscan imaging of tubulin (green) and F-actin (blue) staining at the podosome belt of an osteoclast plated on glass. Shown here is a gallery of 10 equally distant planes between the bottom of the cell and the top of the podosome belt, as schematized in A. The images on the right show the *z* projection of the 10 actin and microtubule planes in the galleries, with the merged images at the bottom. Image courtesy of Julien Cau and Amélie Sarrazin, Montpellier Ressources Imagerie (MRI) platform.

by AKT inhibits GSK3β and thus favors microtubule acetylation; accordingly, GSK3β inhibition can compensate for the KO of AKT and rescue the formation of the sealing zone (Matsumoto et al., 2013). AKT kinases are also needed for the formation of a complex between the microtubule plus-end tracking protein (+TIP) EB1 (also known as MAPRE1) and adenomatosous polyposis coli protein (APC) in osteoclasts and for APC localization at the podosome belt (Matsumoto et al., 2013). Both EB1 and APC participate in actin–microtubule crosstalk (Dogterom and Koenderink, 2019). In osteoclasts, EB1 is required for podosome belt formation (Biosse Duplan et al., 2014), and indirect evidence suggests that APC has an important role at the podosome belt as well as in bone resorption (Guo et al., 2018). Besides AKT, Dock5 also regulates microtubule acetylation and GSK3β activity in osteoclasts (Fig. 2). In the absence of Dock5, GSK3β activity increases and microtubules acetylation is reduced, with a concomitant reduction in the length of microtubules and duration of their growth (Guimbal et al., 2019).

Taken together, the above data suggest that RhoA functions as an inhibitor of microtubule acetylation through its effect on the activation of HDAC6, whereas AKT, by inhibiting GSK3β, favors microtubule acetylation and the recruitment of the microtubule +TIP complex EB1–APC (Fig. 2). APC is an important mediator of actin–microtubule crosstalk in cell adhesion (Juanes et al., 2019), and APC phosphorylation by GSK3β inhibits its binding to microtubules (Zumbrunn et al., 2001). Taken together, this suggests that APC could be an important factor for the actin–microtubule crosstalk in osteoclasts, and that is under the control of AKT and GSK3β kinases, although this remains to be established. In addition, the targeting of microtubules to podosomes has been found to involve a dynamic balance between acetylation and phosphorylation of cortactin and cofilin (Fig. 2), which together with EB1 are required for podosome belt formation and bone resorption (Biosse Duplan et al., 2014; Zalli et al., 2016). These studies show that in osteoclasts, EB1 is present in a complex with cortactin phosphorylated by Src on Y421, to target EB1-decorated microtubules to podosome clusters. The formation of the EB1–cortactin complex is inhibited by cortactin acetylation, which itself is counteracted by HDAC6 (Biosse Duplan et al., 2014). Upon its acetylation, cortactin can then interact with cofilin that is acetylated and not phosphorylated on serine 3 (Fig. 2) to allow podosome belt formation (Zalli et al., 2016). To date, the enzymes involved in cofilin and cortactin acetylation are unknown.

The actin motor protein unconventional myosin X (Myo10) can bind actin, microtubules and integrins (Lee, 2018). Localized at the interface between actin and microtubules during the formation of the podosome belt and the sealing zone, Myo10 has been proposed to crosslink actin cytoskeleton and microtubules in osteoclasts, (McMichael et al., 2010). Targeting Myo10 expression with siRNAs in osteoclasts derived from RAW264.7 cells (see Box 3) reduced sealing zone size and bone resorption activity; conversely, Myo10 overexpression increased sealing zone size (McMichael et al., 2010). Moreover, podosome belt reformation after microtubule disassembly was defective upon Myo10 suppression (McMichael et al., 2010). These results were confirmed by shRNA-mediated Myo10 silencing in primary mouse osteoclasts (Box 3), which also affected osteoclast differentiation (Tasca et al., 2017). In contrast to these reports, a recent study found that Myo10 KO osteoclasts differentiated more efficiently (Wang et al., 2019). The actual bone resorption activity of was not assessed, but the serum of the mice contained high levels of bone resorption marker deoxypyridinoline, suggesting Myo10 KO osteoclasts were active *in vivo* (Wang et al., 2019). Of note, Myo10 KO osteoclasts were derived from *Myo10*$^{tm1a(KOMP)Wtsi}$ mice, which have a LacZ cassette insertion between exons 26 and 27 of *Myo10* (Heimsath et al., 2017). The inconsistencies could result from the expression of truncated forms of myosin X in the *Myo10* KO osteoclasts, as suggested by western blots on mouse brain lysates of the *Myo10*$^{tm1a(KOMP)Wtsi}$ mice (Heimsath et al., 2017). In fact, Myo10 deleted after exon 26 contains the motor domain but lacks the C-terminal region, which functions as an intra-molecular inhibitor of the motor domain (Kerber and Cheney, 2011) and indeed suppressed formation of the podosome belt and the sealing zone when expressed in osteoclasts (McMichael et al., 2010). These truncated Myo10 proteins could lead to gain-of-function effects in osteoclasts derived from *Myo10*$^{tm1a(KOMP)Wtsi}$ mice. Thus, the exact role of Myo10 in osteoclasts remains unclear.

The function of kinesins, microtubule-associated motors, in osteoclasts has not been highly studied to date. The only published report shows that KIF1C is essential for the formation of the podosome belt (Kobayakawa et al., 2019). This study shows that in osteoclasts, KIF1C, which is in a complex with Src and p130Cas and localized at the podosome belt, is necessary for podosome belt formation. Moreover, KIF1C overexpression increases the resorption activity of osteoclasts and partially compensates for the KO of p130Cas, but not that of Src with regard to podosome belt organization and bone resorption (Kobayakawa et al., 2019). It is not known whether KIF1C is important for the activating phosphorylation of p130Cas and the activation of Rac, as shown for Dock5 (Vives et al., 2011), or it is needed for the interaction between Dock5 and the kinases Src and Pyk2, as shown for p130Cas (Nagai et al., 2013).

Diseases associated with osteoclast function

Under physiological conditions, bone homeostasis is maintained by the coupling between resorption and formation: the amount of bone removed by osteoclasts is identical to the amount of new bone deposited by osteoblasts (Florencio-Silva et al., 2015). Bone modeling and remodeling are necessary for fracture healing and the adaptation of the skeleton to load and to mechanical use, as well as for Ca^{2+} and phosphorus homeostasis. An imbalance between bone resorption and bone formation results in several bone diseases.

Osteoclast-related diseases

A few and rare genetic diseases prevent osteoclast activity, thereby increasing bone mass and causing osteopetrosis (Palagano et al., 2018); casual mutations include mutations in genes encoding the a3 subunit of the v-ATPase, chloride channel 7 and cathespin K (Fig. 1A,B), whose mutation causes the severe human bone disorder pycnodysostosis. Mutations in kindlin-3, a hematopoietic protein regulating integrins and osteoclast cytoskeleton (Schmidt et al., 2011), also cause osteopetrosis in association with leukocyte adhesion deficiency type III. Conversely, rare genetic diseases, such as juvenile Paget's disease of bone, cause osteoclasts to be hyperactive, leading to osteoporosis (Masi et al., 2015; Palagano et al., 2018). However, the most common bone remodeling disorder is an excessive resorption activity of osteoclasts, resulting in bone loss and eventually osteoporosis (Compston et al., 2019). Indeed, a number of physiopathological conditions are accompanied by excessive osteoclast activity. These include hormone deficiency after menopause (Compston et al., 2019), inflammatory diseases such as rheumatoid arthritis (Coury et al., 2019; Madel et al., 2019), cancer metastasis to the bone and multiple myeloma (Croucher et al., 2016) and corticotherapy (Buckley and Humphrey, 2018), as well as lack of mechanical forces applied to the bone, for instance

due to prolonged bed rest, spinal cord injury or space flights (Bettis et al., 2018). Finally, abnormal bone loss also accompanies some infectious diseases (Raynaud-Messina et al., 2019). For instance, HIV-1 infection targets osteoclasts where the viral protein Nef favors sealing zone formation and increases bone resorption activity (Raynaud-Messina et al., 2018). Osteoclasts are the obvious therapeutic target to prevent osteoporosis and the associated increased risk of fracture, which cause pain, disabilities and premature death. Therefore, understanding osteoclast biology and development of strategies to target their activity can lead to important medical applications.

The osteoclast cytoskeleton as a therapeutic target

Molecules of the bisphosphonate family are the most widely used drugs to control osteoclast activity; these pyrophosphate analogs incorporate into the bone matrix and are ingested by the bone-degrading osteoclasts, causing their apoptosis (Cremers et al., 2019). More recently, a human monoclonal antibody against RANKL, called Denosumab, was developed; it prevents osteoclast differentiation by inhibiting the key function of RANKL in this process (Deeks, 2018). Through the killing of osteoclasts or the inhibition of their differentiation, both types of drugs efficiently protect against pathological bone loss. However, these drugs suffer limitations, including counter-indications and undesirable side effects (Vargas-Franco et al., 2018). In particular, because Denosumab and bisphosphonates prevent the differentiation or cause the apoptosis of osteoclasts, they inhibit the stimulatory activity of osteoclasts on osteoblast differentiation and bone formation activity and, as a consequence, patients receiving these drugs suffer from a blockade of *de novo* bone formation (Drissi and Sanjay, 2016; Teitelbaum, 2016). Active research is ongoing to tackle osteoclast activity with a different approach and better fulfill patient needs. Specifically targeting the bone-resorption function of osteoclasts appears an attractive strategy to control bone resorption while preserving bone formation. One promising approach is to inhibit cathepsin K. Indeed, the cathepsin K inhibitor Odanacatib efficiently prevents pathological bone loss while preserving bone formation in patients, but unfortunately, it recently failed in clinical phase III trials due to increased risk of stroke (Stone et al., 2019).

Targeting the unique cytoskeletal organization of osteoclasts appears as a tempting approach, and is still in preclinical or early clinical exploration. One strategy to control osteoclast activity is to perturb the function of integrin αvβ3. Indeed, a selective inhibitor of αvβ3, which does not target platelet integrin αIIbβ3 (Pickarski et al., 2015), allowed reducing osteoclast activity and proved efficient in protecting women from postmenopausal bone loss in a phase I study (Murphy et al., 2005). More compounds were recently developed to target αvβ3 selectively, or much more efficiently than αIIbβ3, and block osteoclast activity in culture or in the mouse (Li et al., 2019; Lin et al., 2017). However, to our knowledge, no clinical trial is ongoing with αvβ3 inhibitors. Another approach is to aim at actin regulatory proteins. For instance, two classes of small chemical compounds were developed to inhibit Dock5 exchange activity towards Rac (Ferrandez et al., 2017; patent WO2019197659A1). When administered to the mouse, Dock5 inhibitors prevented pathological bone loss, while bone formation by osteoblasts was maintained, making this a promising strategy to prevent pathological bone loss (patent WO2019197659A1; Vives et al., 2015). Better understanding of osteoclast cytoskeleton regulation is likely to provide information on novel molecular targets to control osteoclast activity and prevent osteoporosis.

Emerging questions and research perspectives

The osteoclast cytoskeleton has a key role in establishing the bone resorption apparatus and represents a promising target to control osteoclast activity in pathological situations. However, we are far from a full understanding of how it is organized and regulated. Below, we highlight several aspects that have recently emerged as potentially important for the regulation of osteoclast cytoskeleton and bone resorption: actin isoforms, tubulin isotypes, intermediate filaments and septins.

Beyond generic actin and tubulin

Post-translational modifications of both actin and tubulin participate in cytoskeleton complexity (Janke and Magiera, 2020; Terman and Kashina, 2013), with microtubule acetylation being crucial for osteoclast function. Furthermore, although actin filaments and microtubules are often considered as dynamic polymers of simply ‘actin’ and ‘α- and β-tubulin dimers’, respectively, these proteins have in fact several isoforms with specific properties, both at the biochemical and the biological level, which are only just beginning to emerge (Janke and Magiera, 2020; Vedula and Kashina, 2018).

With regard to actin, there are six isoforms, which have been shown to fulfill distinct biological functions; the β- and γ-cytoplasmic actins are ubiquitous, whereas other actins have a more restricted expression pattern, such as α-skeletal and α-cardiac actin (Vedula and Kashina, 2018). Interestingly, it was found recently in macrophages that there is a differential actin isoform distribution between podosomes near the cortex and those located inside the cell (Cervero et al., 2018). This, in fact, dictates the subcellular distribution of two podosome-regulatory proteins; leukocyte-specific protein 1 (LSP1), an actin-bundling protein, preferentially localizes at podosomes near the cell cortex, whereas supervillin, which connects actin filaments to the plasma membrane, associates with podosomes in the center of the macrophage (Cervero et al., 2018). Such distribution relies on the distinct binding affinities of LSP1 and supervillin for specific actin isoforms. As supervillin can bind to myosin IIa (Bhuwania et al., 2012), this generates gradients of podosome actomyosin contractility from the cortex to the macrophage center (Cervero et al., 2018). Furthermore, an analysis of podosome ultrastructure in immature dendritic cells has revealed that cytoplasmic β- and γ-actin show a different distribution within the podosome (van den Dries et al., 2019a). The central part of the core is enriched in branched β-actin filaments and contains cortactin; it is surrounded by linear filaments containing γ-actin and crosslinked by α-actinin (van den Dries et al., 2019a). Thus far, there are no reports addressing actin isoforms in osteoclasts; it would thus be very interesting to examine the distribution and function of the different actin isoforms at the podosome belt and their possible role in bone resorption.

Microtubules consist of dimers of one α- and one β-tubulin, with each of them occurring in several isotypes: eight α- and nine β-tubulin-encoding genes are present in the human genome (Janke and Magiera, 2020). Recent reports highlighted that tubulin isotypes confer distinct dynamic properties to microtubules and dictate the differential binding of microtubule-associated proteins (Janke and Magiera, 2020). Interestingly, a recent study shows that osteoclasts express four α- and four β-tubulin-encoding genes, with a remarkably high amount of tubulin β6 protein (Guerit et al., 2020). In fact, tubulin β6 levels appeared to control microtubule shaping at the osteoclast periphery, as well as podosome patterning. Interfering with tubulin β6 expression impaired podosome belt formation, led to smaller sealing zones and inhibited bone resorption (Guerit et al., 2020). However, it remains to establish

whether tubulin β6 levels affect microtubule dynamics or the binding of specific proteins to microtubules in osteoclasts.

Intermediate filaments and septins

In contrast to actin filaments and microtubules, intermediate filaments are non-polarized filaments that connect the nuclear and plasma membranes with microtubules and actin filaments (Leduc and Etienne Manneville, 2015). There is little literature about intermediate filaments in osteoclasts. Vimentin filaments are present at the podosome belt and along osteoclast microtubules (Akisaka et al., 2008; Babb et al., 1997). In addition, plectin, which connects vimentin to actin filaments and to microtubules (Leduc and Etienne Manneville, 2015), is a podosomal protein (Gad et al., 2008). Plectin is required for actin organization and resorption activity in osteoclasts derived from RAW264.7 cells (Box 3), where it appears to control microtubule acetylation as well as Src and Pyk2 activities (Matsubara et al., 2017, 2020). Fimbrin also connects vimentin to actin filaments (Leduc and Etienne-Manneville, 2015). T- and L-fimbrin are present at the osteoclast podosome core (Babb et al., 1997). L-fimbrin (also known as L-plastin) associates with osteoclast podosomes and with the actin patch of the nascent sealing zone (Chellaiah et al., 2018, 2020). L-fimbrin recruitment to the actin patch requires phosphorylation by the leucine rich repeat kinase 1 (LRRK1) (Chellaiah et al., 2018; Si et al., 2018) and binding to the membrane-tethering complex protein TRAPPC9 (Hussein et al., 2019). Interestingly, mutations in *LRRK1* give rise to a severe osteopetrotic phenotype in the mouse and cause human osteosclerotic metaphyseal dysplasia that is associated with osteoclast dysfunction (Miryounesi et al., 2019; Xing et al., 2013). Conversely, mutations in T-fimbrin cause X-linked primary osteoporosis in men and a variable bone phenotype in women (van Dijk et al., 2013). In fact, T-fimbrin is an inhibitor of sealing zone formation and its deletion increases bone resorption (Neugebauer et al., 2018). Finally, APC is involved in the association between vimentin intermediate filaments and microtubules (Leduc and Etienne-Manneville, 2015), and, as described above, APC appears important in organizing the podosome belt and for bone resorption (Guo et al., 2018; Matsumoto et al., 2013). However, the specific functions of APC and vimentin in osteoclasts are unknown. In conclusion, how intermediate filaments and associated proteins participate in the organization of actin and microtubules to control bone resorption remains an open question.

The 13 septins form heteromeric GTP-dependent dynamic apolar filaments, and septin-9 has been shown to link septin filaments to other cytoskeletal elements and membranes (Valadares et al., 2017) and also to bundle microtubules and affect their dynamics (Bai et al., 2013; Nakos et al., 2019). Furthermore, septin-9 also binds to actin filaments, and inhibits the activity of myosin and cofilin (Smith et al., 2015). Currently, the only study about septins in osteoclasts shows that septin-9 is associated with actin and microtubules in the area of the sealing zone (Møller et al., 2018). The authors show that the inhibition of septin filament dynamics is detrimental for bone resorption and that targeted deletion of septin-9 in osteoclasts increased bone mass in the mouse, suggesting that osteoclasts are deficient for bone resorption in the absence of septin-9 (Møller et al., 2018). Nevertheless, the actual activity and cytoskeleton organization of septin-9 KO osteoclasts were not examined. The precise roles of septin filaments in osteoclasts, therefore, remain to be established.

How intermediate filaments and septins, together with the proteins that connect them to actin filaments and microtubules, participate in the organization of osteoclast cytoskeleton for efficient bone resorption is still an emerging field of research.

Conclusion

Understanding the functional crosstalk between the different elements of the cytoskeleton is an important challenge in cell biology. Osteoclasts offer an excellent cellular model to tackle this question as the cytoskeleton plays a major role in their physiological function of bone resorption. It is clear that actin and microtubules are intimately linked in osteoclasts to organize podosomes. The activity of RhoA is key to coordinate the two elements of the cytoskeleton, but how it is regulated remains poorly understood, in particular, no RhoA GEF has been described so far as having a role in podosome belt formation. It has been shown recently in macrophages that microtubules sequester GEF-H1 to prevent excessive activity of RhoA, which is detrimental for podosome stability (Rafiq et al., 2019). Whether such a mechanism also underlies podosome organization in osteoclasts would be interesting to examine. Microtubules guide the delivery of regulatory molecules to cell adhesion structures and they regulate podosome dynamics (Seetharaman and Etienne-Manneville, 2019). Thus, examining the role of kinesins in osteoclasts and identifying their cargoes should also bring valuable information on the mechanisms driving the organization of the sealing zone. Moreover, intermediate filament and septins are integral components of the cytoskeleton interacting with actin and microtubules; although they have not been highly studied in osteoclasts, they appear important in controlling bone resorption. Finally, the osteoclast cytoskeleton also emerged as a potential therapeutic target, and better understanding of the molecular mechanisms controlling cytoskeleton dynamics in osteoclasts could pave the way for novel treatments against pathological bone loss.

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Competing interests

A.B. is inventor of patent WO2019197659 referenced in this review. The other authors declare they have no competing or financial interests.

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