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Review Article

Membrane trafficking in osteoclasts and implications for osteoporosis

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Osteoclasts are large multinucleated cells exquisitely adapted to resorb bone matrix. Like other eukaryotes, osteoclasts possess an elaborate ensemble of intracellular organelles through which solutes, proteins and other macromolecules are trafficked to their target destinations via membrane-bound intermediaries. During bone resorption, membrane trafficking must be tightly regulated to sustain the structural and functional polarity of the osteoclasts' membrane domains. Of these, the ruffled border (RB) is most characteristic, functioning as the osteoclasts' secretory apparatus. This highly convoluted organelle is classically considered to be formed by the targeted fusion of acidic vesicles with the bone-facing plasma membrane. Emerging findings disclose new evidence that the RB is far more complex than previously envisaged, possessing discrete subdomains that are serviced by several intersecting endocytic, secretory, transcytotic and autophagic pathways. Bone-resorbing osteoclasts therefore serve as a unique model system for studying polarized membrane trafficking. Recent advances in high-resolution microscopy together with the convergence of genetic and cell biological studies in humans and in mice have helped illuminate the major membrane trafficking pathways in osteoclasts and unmask the core molecular machinery that governs these distinct vesicle transport routes. Among these, small Rab GTPases, their binding partners and members of the endocytic sorting nexin family have emerged as critical regulators. This mini review summarizes our current understanding of membrane trafficking in osteoclasts, the key molecular participants, and discusses how these transport machinery may be exploited for the development of new therapies for metabolic disorders of bone-like osteoporosis.

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Introduction

Throughout adult life, bone undergoes cyclical removal of old or damaged bone and replacement with a new bone to replenish the skeleton. This process, termed bone remodelling, is regulated by the complementary activities of bone-residing osteoblasts and osteoclasts [1,2]. Osteoblasts synthesize and secrete new bone matrix (i.e. osteoid) whereas osteoclasts are nature's provision for bone excavation (i.e. resorption). Imbalances in bone remodelling that favour a net increase in osteoclast numbers and/or activity leads to loss of bone mass as observed in patients with osteoporosis. Conversely, impairments in osteoclast differentiation or function are associated with sclerosing (i.e. high bone mass) conditions such as osteopetrosis [3].

By definition, osteoclasts are large multinucleated (>3 nuclei/cell) bone-resorbing cells. They are derived from the asynchronous fusion of mononuclear haematopoietic progenitors of the monocyte/macrophage lineage in response to two principal cytokines: (i) macrophage colony stimulating factor (M-CSF) and (ii) receptor activator of nuclear factor κ B ligand (RANKL) [4]. Upon attachment to bone osteoclasts adopt a polarized conformation via a series of cytoskeletal reorganizations which drive the segregation of the osteoclast surface membrane into four distinct domains: (i) the sealing zone (SZ); (ii) the ruffled border (RB); (iii) the basolateral domain (BD) and; (iv) the functional secretory domain (FSD) [5] (Figure 1). Of these, the RB functions as the osteoclasts bone-resorbing

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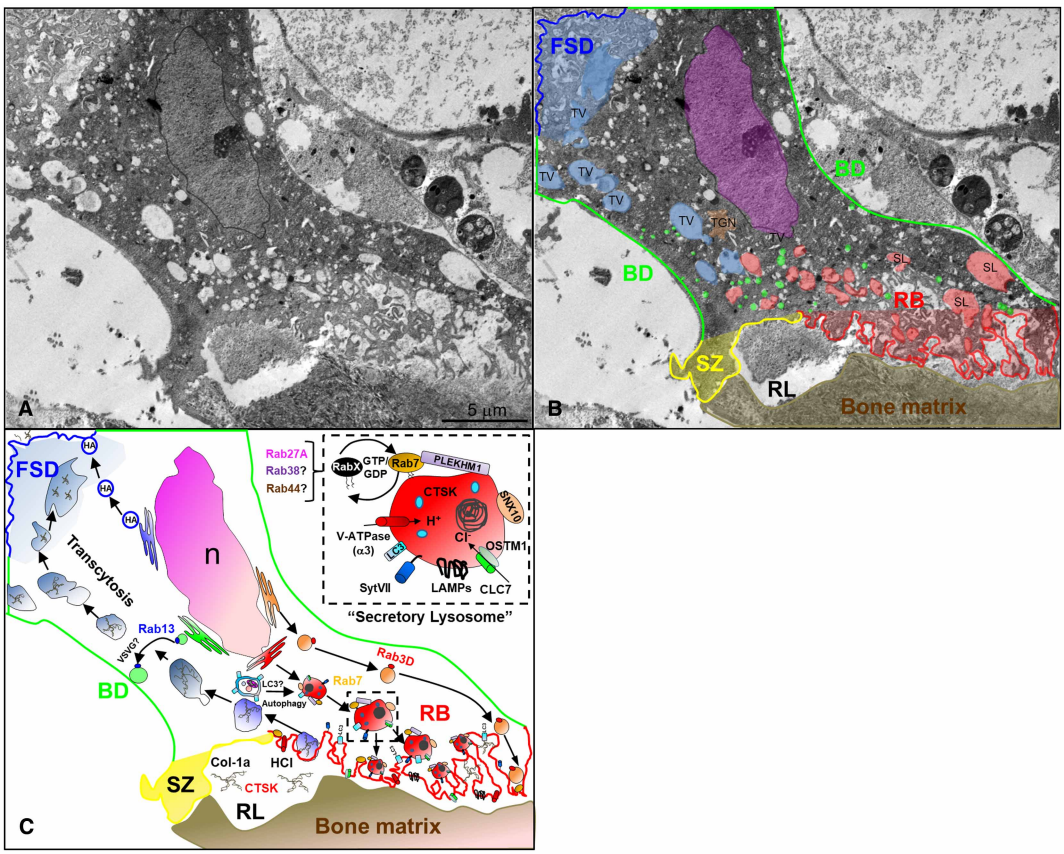

Figure 1. Intracellular roadmap of polarized membrane trafficking in bone-resorbing osteoclasts.
(**A**) Transmission electron micrograph of a cross-section taken through a bone-resorbing osteoclast *in situ* from a 5-day-old mouse femur. (**B**) The osteoclast plasma membrane is segregated into four distinct subdomains: the functional secretory domain (FSD, blue), the basolateral domain (BD, green), the sealing zone (SZ, yellow) and the ruffled border (RB, red). Key intracellular organelles are highlighted. TV, transcytotic vesicle; SL, secretory lysosome; TGN, *trans*-Golgi network; RL, resorptive lacunae. Note that the pseudo-coloured organelles represent arbitrary author interpretations based on the size, morphology, position and intraluminal content of the compartments in the absence of intracellular markers. (**C**) The major vesicular transport routes in osteoclasts superimposed with key molecular participants. The ruffled border membrane is formed by the polarized trafficking and fusion of secretory lysosomes with the bone-apposed plasma membrane. The final fusion step is regulated by synaptotagmin 7 (SytVII) and expels cathepsin K (CTSK) and acid into the resorption lacunae for bone digestion. Secretory lysosome trafficking is regulated by Rab7 and its effector PLEKHM1 (Pleckstrin Homology And RUN Domain Containing M1), possibly in conjunction with other LRO-associated Rabs (RabX), i.e. Rab27A, Rab38 and Rab44 that may be recruited through GTP/GDP-dependent 'Rab-transitions'. At the same time, LC3 (Microtubule-associated protein 1A/1B-light chain 3) is recruited to the ruffled border, possibly via the fusion of autophagosomes/phagosomes with secretory lysosomes, in a process analogous to LC3-associated phagocytosis (LAP). The post-TGN-secretory vesicle trafficking route operated by Rab3D also services the ruffled border membrane but is distinct from lysosomes. During bone resorption degraded bone matrix including collagen-1a (Col-1a) is internalized at the ruffled border and transported apico-basolaterally to the FSD by transcytosis. The FSD serves as an exist site for degraded bone matrix and is distinguished from the basolateral domain via the post-Golgi trafficking of haemagglutinin (HA) and vesicular stomatitis virus G-protein (VSG-G), respectively, the latter potentially regulated by Rab13. The dashed boxed inset highlights the molecular anatomy of a secretory lysosome and its essential nanomachinery including SNX10 (Sorting nexin 10), the α3 subunit of the Vacuolar-type H^+ ATPase (V-ATPase) proton pump, chloride ion channel 7 α subunit CLC-7 and its transmembrane coregulatory protein OSTM1 (osteopetrosis-associated transmembrane protein 1). N, nucleus.

organelle. Formation and maintenance of the RB are dependent on the polarized trafficking of acidic vesicles to the bone-apposed plasmalemma. The fusion and incorporation of these carrier vesicles, in turn, furnishes the RB membrane with machinery necessary to solubilize bone mineral and digest organic bone matrix. Following

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degradation, bone matrix is engulfed by endocytic mechanisms at the RB and then transported apico-basolaterally to the opposing FSD where it is expelled into the extracellular circulation. To meet these intense membrane trafficking demands, osteoclasts acquire sets of structurally and functionally diverse trafficking protein families during RANKL-driven differentiation. Of these small Rab GTPases, their regulators and members sorting nexin family are now widely recognized as core components of osteoclasts bone-resorbing machinery.

Herein, we provide a contemporary overview of the major intracellular membrane trafficking pathways that contribute to osteoclast polarization and function. In particular, we emphasize the importance of the endomembrane system, secretory lysosomes and autophagic pathways, all which converge at the RB and contribute to its formation, maintenance and identity. Finally, we discuss how machineries that regulate these transport pathways may be targeted for next-generation anti-resorptive therapies to treat disorders of bone metabolism.

Membrane trafficking in osteoclasts

Like other eukaryotic cells, the intracellular anatomy of osteoclasts can be compartmentalized into sets of membrane-delimited organelles through which proteins, solutes and other macromolecules are transported via small carrier vesicles. This process, collectively known as membrane trafficking can be operationally divided in endocytic, secretory and transcytotic pathways. Compared with other polarized cell systems (e.g. epithelial cells) that possess well-delineated apical and basolateral membrane transport routes, the nature and number of vesicle trafficking pathways governing osteoclast polarity are incompletely understood.

Much of our contemporary understanding of polarized membrane trafficking in osteoclasts has been garnered from pioneering studies in the 1990s from the laboratories of Kalervo Väänänen and Michael Horton. Using enveloped viral glycoproteins and lectins as tools Väänänen and his colleagues were the first to discover that the basolateral surface of bone-resorbing osteoclasts was discontinuous and divided into discrete membrane domains [6]. Unexpectedly, they found that the influenza haemagglutinin, which is apically targeted in epithelial cells, was trafficked to a restricted area at the top of the basal surface of osteoclasts (i.e. the FSD), while vesicular stomatitis virus G-protein (VSVG), which is basolaterally targeted in epithelia, extended across the rest of the basolateral membrane. Neither of these viral glycoproteins trafficked to the RB nor sealing zone, implying that these membrane domains were compositionally and functionally distinct. Soon after, studies by the same group [7], together with independent work arising from Steve Nesbitt and Michael Horton [8] demonstrated the utility of this denoted 'functional secretory domain'. By monitoring the intracellular fate of ingested fluorescently labelled bone, these parallel studies showed that the FSD served as an exit site for the degraded bone matrix, thus unmasking the existence of a transcytotic route connecting the RB to the basolateral membrane (Figures 1 and 2). In the same year, seminal studies by Palokangas et al. [9] employing a suite of endocytic tracers, not only confirmed that the RB shared characteristics with late endosomes but provided evidence that it is serviced by multiple endocytic pathways.

In more recent years, several laboratories have made salient contributions towards our contemporary understanding of membrane trafficking in osteoclasts including, but not limited to, work by Mulari et al. [10] that uncovered the existence of two endo-exocytic subdomains within the RB: the peripheral vesicle 'fusion' zone and the central degraded matrix 'uptake' zone; studies by DeSelm and co-workers that linked the autophagy pathway to the RB [11], endocytic tracing studies by Stenbeck and Horton [12] and; the works of the Zhao and Coxon laboratories that have defined many of the molecular participants that direct vesicular transport in osteoclasts including Rab GTPases, Rab effectors and SNARE proteins [13,14]. Together, these studies have provided the blueprints for our currently accepted intracellular roadmap of polarized membrane trafficking in osteoclasts (Figure 1).

Membrane trafficking at the RB: a paradigm for regulated lysosomal secretion

Of the membrane domains established upon osteoclast polarity, the RB is most conspicuous. This highly convoluted collation of membrane in-folds not only distinguishes osteoclasts from other multinuclear macrophage descendants (e.g. foreign-body giant cells) but also signifies their resorptive status. The RB is apically orientated and abuts the bone surface where it functions as the osteoclasts secretory organelle. It serves as an exit site for protons (H^+) and chloride ions (Cl^-), which acidify the underlying resorptive space. Generation of a low pH

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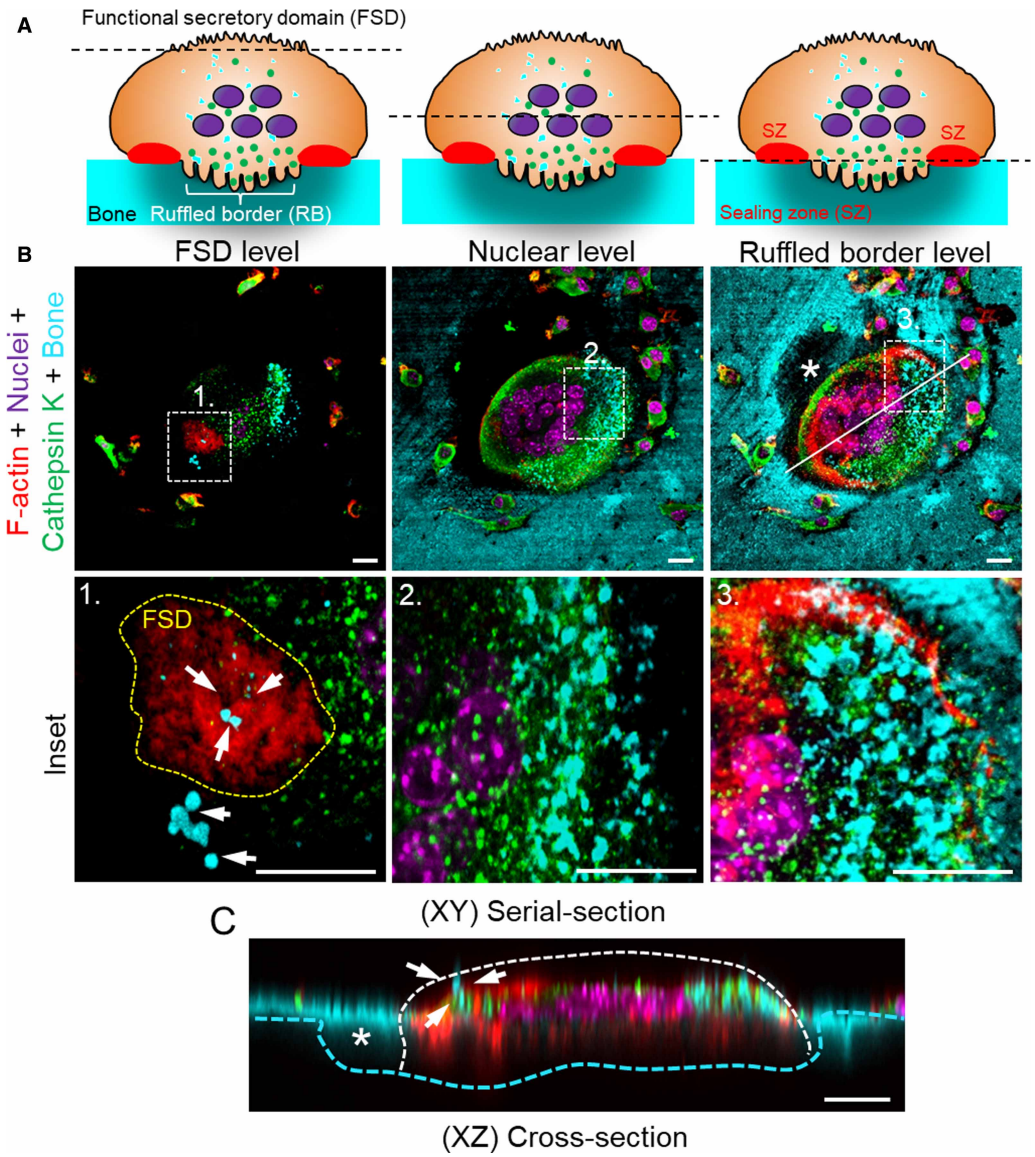

Figure 2. Apicobasolateral transcytosis of degraded bone matrix from the ruffled border to the FSD in osteoclasts. (**A**) Cartoon depicting a cross-section of a polarized bone-resorbing osteoclast to orientate the respective imaging planes. (**B**,**C**) Fluorescent confocal images of a bone-resorbing osteoclast derived from mouse bone marrow monocytes cultured in the presence of M-CSF and RANKL. Osteoclasts were cultured on devitalized bovine bone discs labelled with fluorescently conjugated bisphosphonates (cyan), fixed with 4% paraformaldehyde and then stained with specific markers against cathepsin K (green), F-actin (Rhodamine-conjugated Phalloidin, red) and nuclei (Hoechst 33256, magenta). Images were obtained using a confocal microscope (Nikon A1) equipped with a 60× oil objective (NA = 1.4). Images represent serial confocal planes of individual pseudo-coloured fluorescent channels merged together. XY = top view (**B**), XZ = side view (**C**). Representative views of a bone-resorbing osteoclast are taken from the top (FSD), mid-point (nuclear level) and bottom (ruffled border/bone surface). Insets correspond to the magnification of boxed regions 1–3. Yellow dashed line in Inset 1 demarcates the FSD. White arrows denote degraded bone matrix delivered apciobasolaterally from the ruffled border to the FSD, presumably via transcytotic carrier vesicles. Solid white line indicates the orientation of XZ plane shown in (**C**). Asterisks correspond to the trailing resorptive pit. Scale bar = 10 μm.

environment (pH ~ 4.5) is required for the dissolution of the mineral component (inorganic phase) of bone (i.e. hydroxyapatite) and the activation of lysosomal hydrolases (e.g. cathepsin K) that are released synchronously at the RB to digest the exposed organic matrix (i.e. collagen type1a).

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The long-accepted model for RB formation involves the fusion of intracellular acidic vesicles with the bone-apposed plasma membrane *en masse* [15]. Unlike most mammalian cell types that possess conventional lysosomes, osteoclasts (and other haematopoietic lineage cells, e.g. melanocytes) have evolved specialized lysosome-related organelles (LROs) [16], termed 'secretory lysosomes', that undergo regulated exocytosis, i.e. capable of fusing with the plasma membrane in response to external stimuli.

Secretory lysosomes represent the major storehouse and activation sites of acidic hydrolases such as cathepsin K (Figures 1 and 2), the most abundant collagenase expressed in osteoclasts [17]. Upon delivery to the *trans*-Golgi network (TGN), newly synthesized cathepsin K is sorted via mannose-6-phosphate receptors into secretory lysosomes that are destined for fusion with the RB membrane [18,19]. The precise trigger eliciting secretory lysosome exocytosis remains incompletely understood but the final fusion step requires calcium and is regulated by the calcium-sensing SNARE fusiogen synaptotagmin VII [20]. Fusion and incorporation of secretory lysosomes enrich the RB membrane with cholesterol [21]. At the same time equips it the RB with sets of nanoscale biomachinery that are indispensable for bone resorption. These include: (i) active macromolecule membrane transporters (most notably V-ATPase proton pumps) [22] and ion channels (CLC-7) required for acidification and the maintenance of electroneutrality across the RB membrane; (ii) lysosomal membrane proteins such as OSTM1 (osteopetrosis-associated transmembrane protein 1) and LAMP1/2 (lysosome-associated membrane protein); (iii) members of membrane trafficking protein families (e.g. small Rab GTPases and Sorting Nexins (SNX) described in detail below) and; (iv) components of the autophagy pathway. Hence, the lipid and protein composition of the RB shares close analogies with late endosomal and lysosomal membranes [10,18,23]. Together with the underlying resorptive lacunae, the RB is considered a 'giant extracellular lysosome' [23] and serves as a useful paradigm for regulated lysosomal secretion. It is unsurprising therefore that most of the known forms osteoclast-rich osteopetrosis (>70%) in humans are associated with mutations in genes encoding proteins that function in either the (i) trafficking (e.g. SNX10), (ii) maturation (e.g. PHEKHM1) and/or (ii) function (e.g. TCIRG1, CLCN7, OSTM1) of secretory lysosomes [24,25].

Autophagy in osteoclasts

The lysosomal network has traditionally been viewed as the default pathway for the degradation of extracellular proteins and receptors but also intersects with accessory pathways including those which regulate 'self-catabolism' i.e. autophagy. While the term 'autophagy' encompasses a broad class of intracellular digestion processes, in its simplest form, autophagy can be considered a dynamic process that comprises three sequential steps: (i) formation of autophagosomes, (ii) the fusion of autophagosomes with lysosomes and (iii) degradation [26]. The entrapped cargo materials within autolysosomes are degraded by hydrolases, most notably, cathepsins [27]. In recent years, the importance of the autophagy (i.e. macroautophagy) during osteoclastic bone resorption and skeletal disease has been increasingly appreciated [28–30]. Indeed, several key components of the autophagic conjugation cascade (including ATG5, ATG7, ATG4B, LC3 and PLEKHM1) have been implicated in osteoclast secretory action and the maturation of the RB during bone resorption [12]. In particular, ATG5 has been shown to be a prerequisite for the targeting of lipidated LC3, Rab7, cathepsin K and LAMP1 to the RB membrane. Similarly, loss of ATG5 leads to deregulated autophagic flux in osteoclasts, impaired bone resorption and elevated bone mass in mice [12]. More recently, LC3 has been shown to traffic to the RB through an autophagy-independent process akin to LC3-associated phagocytosis (LAP), in which LC3 is acquired by phagosomes (instead of autophagosomes) which converge with lysosomes at the RB [31]. Accordingly, LC3 targeting to the RB and bone resorptive function was unaltered in osteoclasts derived from an autophagy-deficient mouse model (i.e. FIP200-null: focal adhesion kinase family interacting protein of 200 kD) implying that a process analogous to LAP may contribute to RB formation [31]. At present, the molecular link for this LAP-RB convergence in osteoclasts remains unknown but is potentially mediated by the Rab7-effector protein PLEKHM1 (Pleckstrin Homology And RUN Domain Containing M1), an endolysosomal adaptor protein housing an LC3-binding domain that has been implicated in both autophagy and osteopetrosis (described in detail below). Regardless of the exact molecular mechanism, these recent findings further highlight the intimate relationship that exists between lysosomal and autophagic pathways at the osteoclast RB membrane.

Rab GTPases and their regulators in osteoclasts

Of the molecular participants known to orchestrate membrane trafficking in eukaryotes, members of the small Rab (ras-like in rat brain) GTPase superfamily are best recognized [32,33]. Over 70 Rab proteins (including

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isoforms) have been identified in mammalian cells, each localized to a distinct subcellular compartment. Organelle selectivity and specificity are encoded within the far C-terminal of each Rab. This region contains cysteines (usually two) that undergo post-translational modification (i.e. isoprenylation) resulting in the addition of geranylgeranyl (GGPP) groups, requisite for membrane binding. Rabs are thus considered 'deciphers of organelle identity', with each Rab regulating at a distinct stage of membrane trafficking along the endocytic and secretory pathways. Like other *ras*-related GTPases, Rabs function as molecular switches, oscillating between 'GTP' (active) and 'GDP' (inactive) bound-conformations. This functional GTPase cycle is governed by cytosolic factors that facilitate (i) GTP/GDP exchange (Rab GEFs); (ii) GTP-hydrolysis (Rab GAPs) and/or (iii) Rab retrieval (Rab GDI) and coincides with Rab membrane association/dissociation. Upon activation, GTP-Rabs recruit effector molecules from the cytosol to organelle membrane where they are organized within discrete micro-domains ('Rab-domains'). Rab effectors constitute a highly pleiotropic group of structurally and functionally diverse proteins and include multiprotein scaffolding adaptors, cytoskeletal motor proteins and tethering/docking complexes, each required for a specific stage of membrane transport ranging from vesicle budding to membrane fusion.

Osteoclasts express more than half the complement of mammalian Rab proteins [34,35,36]. Despite their abundance and well-established roles in other cell systems, very few Rabs have been functionally characterized in osteoclasts. Rab7, a late endosomal/lysosomal resident, is best recognized in osteoclasts and ranks among the most intensively studied member of all Rab GTPases. Rab7 localizes to secretory lysosomes and redistributes to the RB membrane during bone resorption [37]. Accordingly, down-regulation of Rab7 expression impairs osteoclast polarization, cathepsin K secretion and bone resorption *in vitro*. The complement of Rab GEFs, GAPs and effectors through which Rab7 operates during bone resorption has only just begun to emerge. To date, only two GTP-dependent Rab7 effectors have been characterized in osteoclasts, namely Rac1 and PLEKHM1. Rac1, a small GTPase of the Rho family, was identified during a bacterial two-hybrid screen for Rab7-binding partners [38]. This association was validated by recombinant protein–protein interaction studies which showed that Rac1 preferentially bound Rab7 in its GTP-bound state. In addition, Rab7 and Rac1 colocalized, albeit partially, at the RB. Based on these findings the authors speculated that Rab7–Rac1 interaction may mediate late endosomal transport between microtubules and microfilaments during RB formation. While the precise structural and functional implications of this interaction are currently lacking, it is noteworthy that conditional deletion of either Rac [39] or Rab7 [40] in osteoclasts corresponds with osteopetrosis and osteoclast dysfunction. Moreover, the recent observation that the Rac1 effector (ARMUS/TBC1D2A) moonlights as a Rab7 RabGAP offers a physical basis through which GTPase inter-switching in osteoclasts might occur [41,42].

PLEKHM1, by comparison, is a multi-domain containing cytosolic protein that functions as a molecular adaptor to integrate endolysosomes with the autophagy pathway [43,44]. It possesses a RUN domain and two pleckstrin homology (PH1/2) domains, the former an established interaction moiety of ras-like GTPases. Accordingly, PLEKHM1 is recruited directly by GTP-Rab7 to late endosomes and lysosomes [45,46]. Mutations in *PLEKHM1* correspond with an intermediate form of osteopetrosis in humans and underscore the osteoporotic phenotype observed in the naturally occurring incisor absence (*ia*) rats *Plekhm1*$^{ia/ia}$ [45,47]. Osteoclasts derived from patients harbouring mutations in *PLEKHM1* fail to develop mature RBs, exhibit impaired cathepsin K secretion and have a reduced capacity to resorb bone [45]. This phenotype is recapitulated in mice conditionally or globally lacking *Plekhm1* [48]. Here, deletion of *Plekhm1* correlates with bone resorption defects and altered lysosomal distribution in osteoclasts owing to a loss of connectivity between lysosomes and microtubules. Moreover, PLEKHM1 acts as a molecular platform upon which microtubule-associated proteins FAM98A, NDEL1 and LIS1 assemble a molecular complex that links lysosomes to dynein/dynactin and the underlying cytoskeleton, all whilst under the aegis of Rab7 [48,49].

Whereas the vesicular transport route governed by Rab7 in osteoclasts is now well defined, the intracellular trafficking pathway regulated by Rab3D is less so. Rab3D is a non-neuronal member of the Rab3 subfamily (Rab3A,-B,-C and -D) of exocytic-related GTPases which are expressed in osteoclasts [50,51] and other secretion competent cells [52]. In osteoclasts, Rab3D localizes to a subset of post-TGN vesicles that are required to sustain membrane equilibrium at the RB [51]. In keeping with this position, inhibition of Rab3D activity by either genetic ablation or expression of a dominant-negative mutant (N135I) impairs osteoclast bone resorption. Moreover, mice lacking Rab3D develop osteosclerosis [51]. Although the precise cargo trafficked by Rab3D-bearing vesicles remains unclear, like Rab7, their directionality is coupled to microtubules, in this instance, via the GTP-dependent recruitment of Tctex-1, a light chain of the minus-end directed dynein motor complex [53,54].

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Outside of Rab7 and Rab3D, the functional contribution of other osteoclast Rabs remains surprisingly scant. Nonetheless, many Rab proteins have recently emerged whose expression is up-regulated in during RANKL-induced osteoclast differentiation and thus are predicted to modulate osteoclast formation and/or function. For example, Rab27A, which occupies LROs in other haematopoietic cells (e.g. melanosomes in melanocytes and platelet dense granules), has been recently implicated in osteoclast differentiation and lysosomal function [55]. By combining siRNA knockdown studies in RAW264.7 macrophages with osteoclasts derived from ashen mice, which possess a naturally occurring mutation in *RAB27A,* the authors demonstrated that Rab27A modulates the trafficking of key surface receptors that drive both multinucleation (i.e. c-fms and RANK) and lysosome-associated functions required for osteoclast polarization and resorption. Curiously, these findings diverge from the osteoclast phenotype observed from *gunmetal (gm/gm)* mice that bear a mutation in the catalytic subunit of Rab geranylgeranyl transferase (RGGT), which results in widespread Rab prenylation deficiency but primarily targets Rab27A [56]. In this setting, osteoclast formation and polarization are normal but *gm/gm* osteoclasts exhibit reduced bone resorptive capacity *in vitro* [36]. The exact reason for this discrepancy is unclear but may reflect differences in the genetic strains of the mice bearing the respective mutations (i.e. ashen:C3H/HeSnJ vs *gm/gm*: C57BL/6J) or point to differences in the residual levels of Rab27A in each model. More recently, inactive GDP-Rab27A has been shown to be recruited to secretory lysosomes via a direct association with the a3 subunit of the V-ATPase complex [57]. This finding is somewhat unexpected when considering that Rab27A does not undergo the classical REP1 and/or GDI-mediated membrane delivery [58] and remains largely constitutively active on membranes during vesicle trafficking [59,60]. Notwithstanding, a similar phenomenon was observed for GDP-bound Rab7 in the same study [57]. Based on these findings, the authors posit a model whereby the V-ATPase proton pump directs secretory lysosome trafficking via recruitment of Rab7 and Rab27A. Future studies will be required, however, to determine the structural basis for this association as well as the identity of the putative RabGEF/GAP catalysing this 'Rab-transition'.

Rab38 is another LRO-associated GTPase whose expression in osteoclasts is dependent on the RANKL-induced NFATc1 transactivation [61]. Despite this, characterization of chocolate mice ($Rab38^{cht/ch}$) which house a naturally Gly to Val substitution in the GTP-binding pocket of Rab38 failed to yield any overt bone phenotypes. Reflecting this, *in vitro* osteoclast formation and function is unaltered in $Rab38^{cht/ch}$ mice implying an accessory or redundant role for this GTPase. Similarly, the function of Rab13, another transcript that is up-regulated during osteoclast differentiation remains unknown [35]. Rab13 localizes to small carrier vesicles distinct from early or recycling endosomes but positioned between the TGN and basolateral membrane domain of bone-resorbing osteoclasts. Together with Rab8, Rab13 interacts with small transmembrane endospanins [62]; however, the physiological relevance of this interaction in osteoclast remains to be elucidated. Lastly, Rab44, a large atypical Rab GTPase has been recently identified in RAW-D derived osteoclast-like cells [63]. Rab44 is up-regulated during osteoclast differentiation, localizes to the Golgi and lysosomes and prompts early endosome enlargement upon exogenous expression. By combining siRNA knockdown studies together with a series of structural deletion mutants, the authors showed that Rab44 modulates osteoclast differentiation, acting as a negative regulator via the modulation of intracellular calcium and, in turn, influencing NFATc1 activation.

Sorting nexins in osteoclasts: the emerging role of SNX10

Sorting nexins (SNXs) are a family of structurally and functionally diverse proteins (34 members in total) unified by the presence of a PX (phox-homology) domain, responsible for membrane lipid attachment [64]. In particular, SNX10 binds to phosphoinositide-3-phosphate (PI(3)P)-enriched endosomes and has recently emerged as a crucial regulator of osteoclast function. SNX10 expression is robustly induced in response to RANKL-signalling and is a critical component of the osteoclasts bone resorptive machinery [65]. This is exemplified in humans with patients harbouring mutations in SNX10 manifesting severe Autosomal Recessive Osteopetrosis attributed to osteoclast dysfunction [66–71]. Osteoclasts derived from these patients exhibit defective RBs and fail to resorb bone [70]. This is phenocopied in mice conditionally lacking SNX10 in osteoclasts but deviates in germline-deficient animals where osteopetrosis is superimposed with rickets 'osteopetrorickets', thus extending SNX10 function to gastrointestinal calcium absorption [71]. Despite its established importance, the exact localization and function of SNX10 in osteoclasts remain ill-defined. Based on analogies with studies performed in zebrafish and immortalized HeLa cells [72], SNX10 is thought to localize to endosomes and has been speculated to facilitate in the post-TGN sorting and/or trafficking of V-ATPases to the RB [66]. More recently, the function of SNX10 has been expanded to the trafficking and secretion of MMP9 [73]

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and chaperone-mediated autophagy [74]. Precisely how SNX10 modulates these diverse function(s) in osteoclasts warrants further investigation.

Membrane trafficking machinery: targets for anti-osteoporosis therapy?

Current treatments for osteoclast-mediated diseases such as osteoporosis focus on reducing further bone loss using pharmacological bone mimetics such as bisphosphonates or anti-resorptive RANKL antibodies (Denosumab) [75]. Approved treatments to help build new bone (anabolics) such as parathyroid hormone are (i) expensive; (ii) require daily subcutaneous injection; and thus (iii) are currently restricted to patients with the most severe osteoporosis (i.e. sustained at least one osteoporosis-associated vertebral fracture). Despite several anti-resorptive drugs on the market, issues remain concerning compliance and unwanted side effects such as osteonecrosis (i.e. bone death) of the jaw or atypical sub-trochanteric femoral fractures following prolonged use [76]. Moreover, both bisphosphonates and anti-RANKL antibodies (Denosumab/Prolia) suppress osteoclast differentiation and survival. This leads to low turnover with low bone formation, as osteclasts help regulate bone remodelling by promoting recruitment of bone-forming osteoblasts ('coupling') [77]. Therefore, unravelling the molecular machinery that regulates membrane trafficking in osteoclasts offers unique opportunities to identify new targets for the development of next-generation anti-osteoporosis therapies that inhibit osteoclast activity while maintaining 'coupling' with osteoblasts, thereby preserving bone formation and quality. Indeed, Rab proteins (and other ras-related GTPases) are already well-established molecular targets of nitrogen-containing bisphosphonates (N-BPs) [78], which inhibit post-translational prenylation and, in turn, membrane attachment [79]. Within the last decade, significant progress has been made towards the development and characterization of N-BP analogues that selectively target Rabs such as phosphonocarboxylate, which blocks RGGT and inhibits bone resorption *in vitro* and *in vivo* [80,81]. Similar pharmacological interventions may be envisaged to target other membrane trafficking machinery. Regardless of the pharmacological strategy employed, development of future inhibitors targeting machinery or cargo related to secretory lysosomes must err on the side of caution following the discontinuation of the Merck & Co Inc. cathepsin K inhibitor Odanacatib, especially if the expression of the intended target extends beyond the osteoclast and the skeleton.

Conclusions and perspectives

Over the past few decades, significant advances in fluorescence microscopy together with the establishment of reproducible and reliable *in vitro* osteoclast culture methods have greatly accelerated our understanding of membrane trafficking in osteoclasts. Once considered meagre bone eaters, there is now rejuvenated interest and appreciation for these specialized polykaryons as a unique model system in which to study polarized membrane trafficking. Traditionally, most of our knowledge of the vesicular transport pathways that contribute to the formation of the osteoclast's functional membrane domains has been gleaned from static snapshots taken from *in vitro* settings. A future challenge remains, therefore, to illuminate and recapitulate these pathways in intact living systems where the dynamics and complexities can be monitored in real-time [82]. In particular, despite its unveiling more than two decades ago, our knowledge of the molecular participants governing the transcytosis of degraded bone matrix from the RB to the FSD remains in its infancy. This is, in part, due to the notorious technical limitations encountered when interrogating osteoclasts, i.e. they are not readily amenable to conventional manipulations such as transfection and must be studied on their native substrate in order to induce membrane polarity. Furthermore, the immense heterogeneity encountered when imaging bone-resorbing osteoclast *in vitro* makes it challenging to distinguish its discrete polarized membrane domains. Indeed, the existence and segregation of the FSD from the basolateral surface has long been contentious as no group other than that of Väänänen has, to our knowledge, replicated this observation. However, the FSD can be readily identified in bone-resorbing osteoclasts using fluorescently conjugated actin (Figure 2) or lectin-based stains (N. Pavlos et al., unpublished).

A final outstanding knowledge gap is how the vast volume of secretion activities through the osteoclastic RB and FSD may influence the behaviour of other cells within the bone marrow microenvironment (i.e. via intercellular 'coupling'). For instance, the bone matrix embedded growth factor TGF-β may be released from the FSD in discrete quanta to stimulate osteoblast recruitment during bone remodelling. A similar scenario might be envisaged for the trafficking and release of other bone-tropic factors including insulin-like growth factors or cytokines secreted by mature osteoclasts (i.e. clastokines) such as Sphingosine-1-phosphate (S1P), collagen

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triple helix repeat containing 1 (Cthrc1), complement component 3a (C3a), BMP-6, Wnt10b and semaphorin4D (Sema4D) [83].

In closing, only by studying osteoclasts on bone and in four dimensions (i.e. three dimensions over time) can the true intricacy of these dynamic membrane networks be fully realized. The recent technological advances in super-resolution and electron microscopy coupled with the application of new fluorescent protein tags, organelle and bone-specific probes [84], biochemical organelle isolation methods and quantitative proteomics will undoubtedly yield unprecedented insights into the inner-workings of these enigmatic bone-resorbing polykaryons and unmask potential new drug targets for the treatment and alleviation of metabolic bone diseases.

Abbreviations

BD, basolateral domain; FSD, functional secretory domain; LAP, LC3-associated phagocytosis; LRO, lysosome-related organelle; M-CSF, macrophage colony stimulating factor; N-BPs, nitrogen-containing bisphosphonates; RANKL, receptor activator of nuclear factor κ B ligand; RB, ruffled border; RGGT, Rab geranylgeranyl transferase; RL, resorptive lacunae; SL, secretory lysosome; SNX, sorting nexins; SZ, sealing zone; *TGN*, *trans*-Golgi network; TV, transcytotic vesicle; V-ATPase, vacuolar-type H^+ ATPase; VSG-G, vesicular stomatitis virus G-protein; VSVG, vesicular stomatitis virus G-protein.

Author Contribution

P.Y.N., A.B.R. and N.J.P. collectively contributed to the conceptualization, writing and editing of the manuscript and accompanying figures.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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