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Role of vesicular trafficking in skeletal dynamics

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Vesicular trafficking is critical for the function of bone cells, exemplified by bone diseases such as osteopetrosis, which frequently results from defects in this process. Recent work has further dissected the role of the endolysosomal system in both bone formation by osteoblasts and bone resorption by osteoclasts. This pathway also plays an important role in the communication between these and other cells in bone, through trafficking and degradation of growth factors and their receptors, and microvesicle release. In addition, a crucial role for autophagy in bone remodelling and bone disease is beginning to emerge. These insights into the molecular control of bone remodelling raise the possibility of developing novel therapeutics for bone diseases designed to target specific aspects of this process.

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Introduction

Vesicular trafficking is at the basis of cellular life; it governs cell communication via secretion and uptake of signalling molecules, enzymes and adhesion molecules. Furthermore, the identity of intracellular organelles is dependent on vesicular trafficking. Seminal work by Rothman, Schekman and others beginning in the 1980s has led to the elucidation of the mechanisms underlying vesicular transport [1]. Vesicles bud from the membrane of a donor compartment (e.g. the *trans*-Golgi network) and are subsequently transported to a different, destination compartment (e.g. the plasma membrane), where membrane fusion occurs. Vesicular budding at the donor membrane is controlled by small GTPases mainly belonging to the ADP ribosylation factor (ARF) family [2]. ARFs recruit coat proteins, which select cargo proteins and shape the membrane into a bud [3]. At the target membrane, vesicles dock and cargo is delivered through membrane fusion, mediated by small membrane proteins associated with the vesicular membrane (v-SNARE) and the target membrane (t-SNARE) [4]. In addition to these proteins, the Rab family of small GTPases are master regulators of vesicular trafficking, with important roles in cargo selection, vesicle budding, cytoskeletal transport, and docking at the target membrane [5,6].

In bone, certain diseases have highlighted the importance of vesicular trafficking in bone cells. For example, most autosomal recessive cases of the bone disease osteopetrosis result from defects in endolysosomal trafficking in osteoclasts (Box 1; [7]). In osteoblasts, mutations in genes that regulate ER to Golgi traffic account for the skeletal defect in observed in patients with cranio-lenticulo-sutural dysplasia [8] and gerodermia osteodysplastica [9]. In addition, genetic disruption of post-translational modification of Rab GTPases impairs both osteoblast and osteoclast function [10].

Osteoblasts

Transport from the ER and ER stress in osteoblasts

Patients with cranio-lenticulo-sutural dysplasia have a mutation in one of the COPII coat subunits (sec23A) that regulates budding of collagen-containing vesicles from the endoplasmic reticulum [8]. Fibroblasts from patients with cranio-lenticulo-sutural dysplasia show an extended ER and reduced collagen production. A similar picture is observed in fibroblasts from mice lacking BBF2H7 (box B-binding factor-2 human homolog on chromosome 7). BBF2H7 belongs to a family of ER localised transmembrane transcription factors that are transported to the Golgi complex under ER stress [11]. In the Golgi, cleavage by the Golgi resident proteases S1P (site-1 protease) and S2P (site-2 protease) also known as SKI-1 (subtilisin kexin isozyme-1 and 2) liberates the N-terminal basic leuzine zipper transcription factor domain that then translocates to the nucleus (Figure 1). BBF2H7 is highly expressed in chondrocytes and sec23A is a transcriptional target of BBF2H7, explaining the similar phenotype observed in cranio-lenticulo-sutural dysplasia and BBF2H7$^{-/-}$ mice. BBF2H7 is not expressed in osteoblasts but ER stress activates a similar system, involving OASIS (old astrocyte specifically induced substance) [12]. OASIS gene transcription is induced by bone

Box 1 Vesicular trafficking defects in osteopetrosis

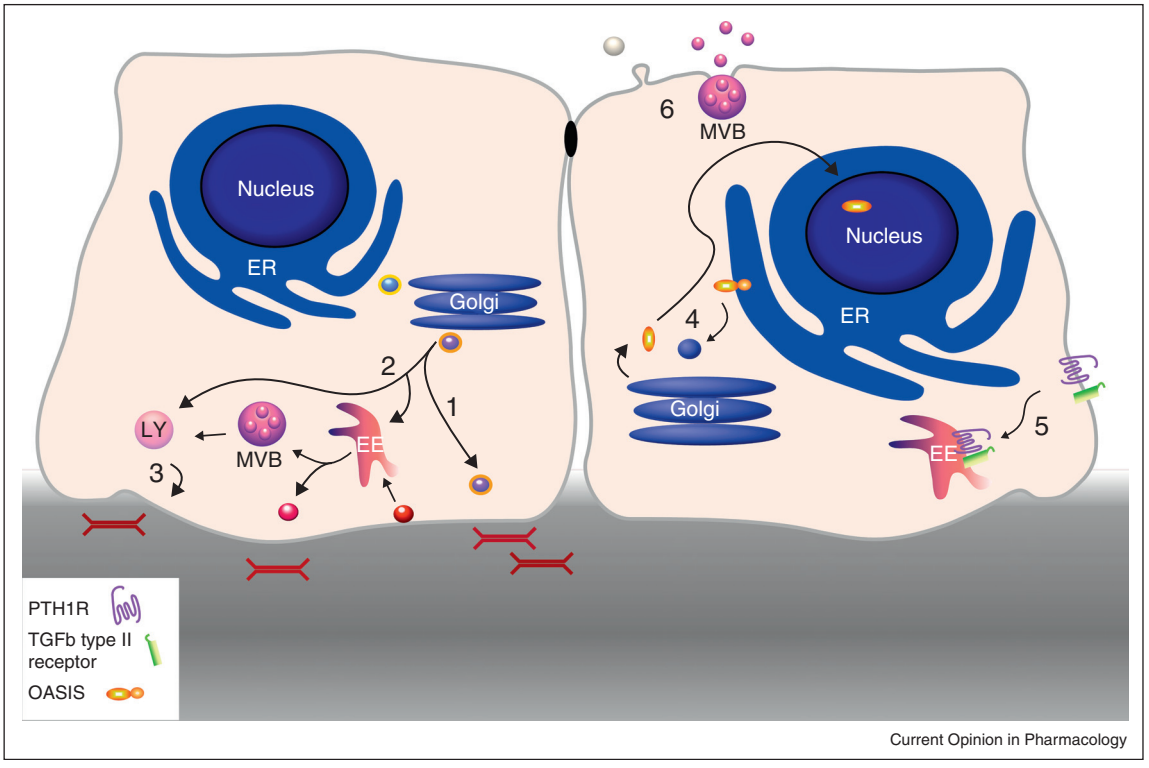
The genetic bone disease osteopetrosis is associated with high bone density due to defective resorption by osteoclasts. Over recent years it has become clear that the mutations that cause osteoclast-rich osteopetrosis (i.e. in which osteoclasts form normally, but their resorptive activity is impaired) all impair trafficking and/or fusion of lysosome-related organelles to the ruffled border [7]. The most common osteopetrosis-causing mutations are in the a3 subunit of the V-ATPase and the two subunits of the ClC-7 chloride antiporter, suggesting that the osteoclast defect in these cases results from impaired lysosomal acidification. However, osteoclasts from patients bearing these mutations completely lack ruffled borders, indicative of a defect in lysosomal trafficking. More recently, truncating mutations in Plekhm1 have been identified as a cause of osteopetrosis, with an identical osteoclast phenotype *in vitro* to the a3 and ClC-7 mutations. Plekhm1 is recruited to cathepsin K-positive lysosomes in osteoclasts by GTP-bound Rab7, and is therefore likely to be a Rab7 effector; the mutant form of Plekhm1 cannot be recruited to these vesicles and remains cytoplasmic. Finally, mutations in sorting nexin 10 (SNX10) also cause osteopetrosis. Although the function of SNX10 in osteoclasts remains unclear, SNX proteins are known to function in endosomal sorting and signalling, thus linking these cases mechanistically to the other causes of osteopetrosis [69].

morphogenetic protein 2 (BMP2) downstream of Runx2 activation [13] and collagen type I is a direct transcriptional target of OASIS. The importance of OASIS and ER stress in bone formation is further highlighted by the phenotype of OASIS$^{-/-}$ mice that suffer from severe osteopenia akin to human osteogenesis imperfecta (OI) [13].

ER stress is induced by a number of factors such as ER-calcium depletion, oxidative stress, hypoglycaemia and expression of mutated proteins [14]. However, in chondrocytes and osteoblasts this system is activated by differentiation and involves BBF2H7 and OASIS respectively. Interestingly, expression of the ER chaperone BIP (immunoglobulin heavy-chain binding protein) is reduced in osteoblasts of osteoporosis patients and a selective inducer of BIP prevents bone loss in a murine osteoporosis model [15], indicating that regulation of ER stress plays an important role in bone formation.

Activation of transcription factors by transport from the ER to the Golgi was first described for SREBP (Sterol-Regulatory Element-Binding Protein), whose translocation

Figure 1



Schematic overview of different trafficking pathways important in osteoblast function. **(1)** Direct pathway from the Golgi apparatus to the plasma membrane for delivery of extracellular matrix proteins. **(2)** Pathway from the Golgi apparatus to the endosomal system, including early endosomes (EE), mulitvesicular bodies (MVB) and lysosomes (LY) — responsible for trafficking of RANKL and collagen. **(3)** Secretion of lysosomes. **(4)** Activation of transcription factor OASIS, endoplasmic reticulum (ER) localised OASIS is transported to the Golgi complex, cleaved by resident proteases and the N-terminal transcription factor translocates to the nucleus. **(5)** Endocytosis of growth factor receptors, important in the regulation of PTH signalling. **(6)** Microvesicle release, both MVB and plasma membrane derived.

depends on a clever system of protein–protein interactions involving the COPII coat (for review see [16]). It will be interesting to see if similar interactions between OASIS and other ER resident proteins facilitate COPII coat binding and are involved in collagen secretion in osteoblasts.

RANKL and collagen secretion

The endosomal/lysosomal compartments of cells are very plastic, receiving material from both the extracellular environment via endocytosis as well as newly synthesised material for regulated exocytosis. The endosome/lysosome thus functions as both a degradative and a secretory compartment. In osteoblasts the secretory function of this compartment appears to be important for the secretion of both receptor activator for NF-κB-ligand (RANKL) and collagen.

RANKL is the most important factor in the crosstalk between cells of the osteoblast lineage and osteoclasts, and the ratio between RANKL and osteoprotegerin (OPG) is crucial for bone homeostasis. RANKL is secreted/presented by osteoblastic cells and the interaction between RANKL and its receptor RANK on pre-osteoclasts initiates osteoclast differentiation and maturation. OPG has been identified as a decoy receptor for RANKL thereby reducing the amount of RANKL available for stimulation of pre-osteoblasts. Interestingly, in osteoblasts and osteocytes the majority of RANKL is not directly routed from the Golgi apparatus to the plasma membrane but is stored before secretion in a lysosomal compartment [17,18••] (Figure 1). Trafficking to the lysosomes is dependent on OPG, which then maintains RANKL in this compartment until secretion is promoted by RANK/RANKL interaction at the plasma membrane [17]. Therefore regulation of bone remodelling by OPG occurs through modulation of intracellular trafficking as well as extracellular binding of RANKL.

The importance of secretory lysosomes in bone homeostasis has been highlighted by the bone phenotype (osteopenia/increased bone volume respectively) of two mouse strains, synaptotagmin VII$^{-/-}$ and jinx (loss of function mutation in Munc 13-4) mice [19,20]. Both synaptotagmin VII and Munc 13-4 are components of the docking and fusion machinery necessary for lysosome and multivesicular body (MVB) secretion. Munc 13-4 is an effector of Rab27, a small GTPase that regulates secretory lysosome release [21], whereas synaptotagmin VII modulates the fusion event itself [22]. The differences in the mouse phenotypes are likely due to the specific functions these proteins play in osteoblasts and osteoclasts. The role of Rab27 appears to be restricted to osteoblasts, where it may regulate plasma membrane expression of RANKL [19], which could explain the jinx mouse phenotype. Rab27 also appears to regulate collagen secretion most likely through a Munc 13-4-independent mechanism [23]. By contrast synaptotagmin VII is essential for both osteoblast and osteoclast function, due to the requirement for lysosome secretion in both these cell types [20]. In osteoclasts, trafficking of these lysosomes is under the control of Rab7 rather than Rab27.

Microvesicle release

Endosomes mature into lysosomes via MVBs, which contain numerous small intra-luminal vesicles [24]. Similar to lysosomes, under certain circumstances MVBs can fuse with the plasma membrane, resulting in the extracellular release of these 40–100 nm microvesicles, which are then termed exosomes. Alternatively, microvesicles can form directly from the plasma membrane [25] (Figure 1). In osteoblasts, a type of microvesicle known as a matrix vesicle is crucial for mineralisation of bone [26] and the release of intracellular calcium [27•]. The importance of the actin cytoskeleton in matrix vesicle release has recently been highlighted by several studies [28,29]. In recent years it has become clear that microvesicles are also important for cell to cell communication, as they contain RNAs, including microRNAs, DNA and enzymes [30]. They can act locally or at distant sites as they are released into body fluids, such as blood, saliva and urine. At target cells they bind to receptors or are taken up by endocytosis, the released components then altering gene expression. Furthermore, microvesicles can also release nucleotides, which work as autocrine/paracrine signalling molecules via purinergic receptors [31] and the purinergic P2X7 receptor itself has been shown to induce microvesicle shedding in a number of cell types [32]. In bone, it has recently been shown that monocytes use exosomes to stimulate osteogenic gene expression in mesenchymal stem cells [33•]. Importantly, bone marrow-mesenchymal stem cells (BM-MSC) communicate with multiple myeloma (MM) cells via exosomes; exosomes derived from normal BM-MSC inhibit the growth of MM cells whereas MM BM-MSC derived exosomes promote it [34••].

Endocytosis regulates differential signalling in osteoblasts

Steady state levels of a number of proteins, especially cytokine and growth factor receptors, are controlled by selective internalisation. Endocytosed proteins first reach the endosome where cargo sorting takes place; internalised proteins may then recycle back to the plasma membrane or are delivered to the lysosome for degradation (Figure 1). This selective internalisation fine-tunes receptor signalling [35]. A striking example of this mechanism is the parathyroid hormone/parathyroid hormone related protein (PTH/PTHrP) receptor type I (PTH1R). PTH regulates whole body calcium homeostasis whereas PTHrP is a paracrine factor that has an anabolic effect on bone by controlling osteoblast differentiation and proliferation. Both proteins bind to the same receptor (PTH1R) but elicit different responses. PTH binding to the receptor triggers endocytosis and a sustained cAMP

production whereas PTHrP rapidly dissociates from the receptor so that only a transient cAMP increase is induced at the plasma membrane [36]. The two ligands thus stabilise different conformations of the receptor leading to changes in signal strength, which is dependent on receptor endocytosis and sustained signalling on the endosome. This extended signalling after internalisation might explain why bone anabolic effects are seen with intermittent PTH treatment only, whereas continued PTH treatment results in bone loss [37,38]. Another example of signal modulation by selective internalisation is the recently described interaction between transforming growth factor (TGF)-β type II receptor and PTH1R. TGF-β is a local factor deposited in the bone matrix that plays an important role in bone maintenance [39]. It is activated by bone resorbing osteoclasts and attenuates further bone resorption by impairing osteoclastogenesis. TGF-β promotes bone formation through chemotactic attraction of osteoblasts and their precursors, whose proliferation and differentiation is stimulated. TGF-β interacts with a large number other growth factors and hormones, one of which is PTH. In response to PTH, PTH1R forms a complex with TGF-β type II receptor, one of the three TGF-β receptors. Subsequent phosphorylation of PTH1R by the constitutively active TGF-β type II receptor triggers formation of an endocytic complex [40••]. Both receptors are removed from the plasma membrane and signalling is attenuated. Interaction between PTH1R and TGF-β type II receptor is reduced by TGF-β binding to its receptor so that endocytosis rates modulate signalling strength of both pathways.

Osteoclasts

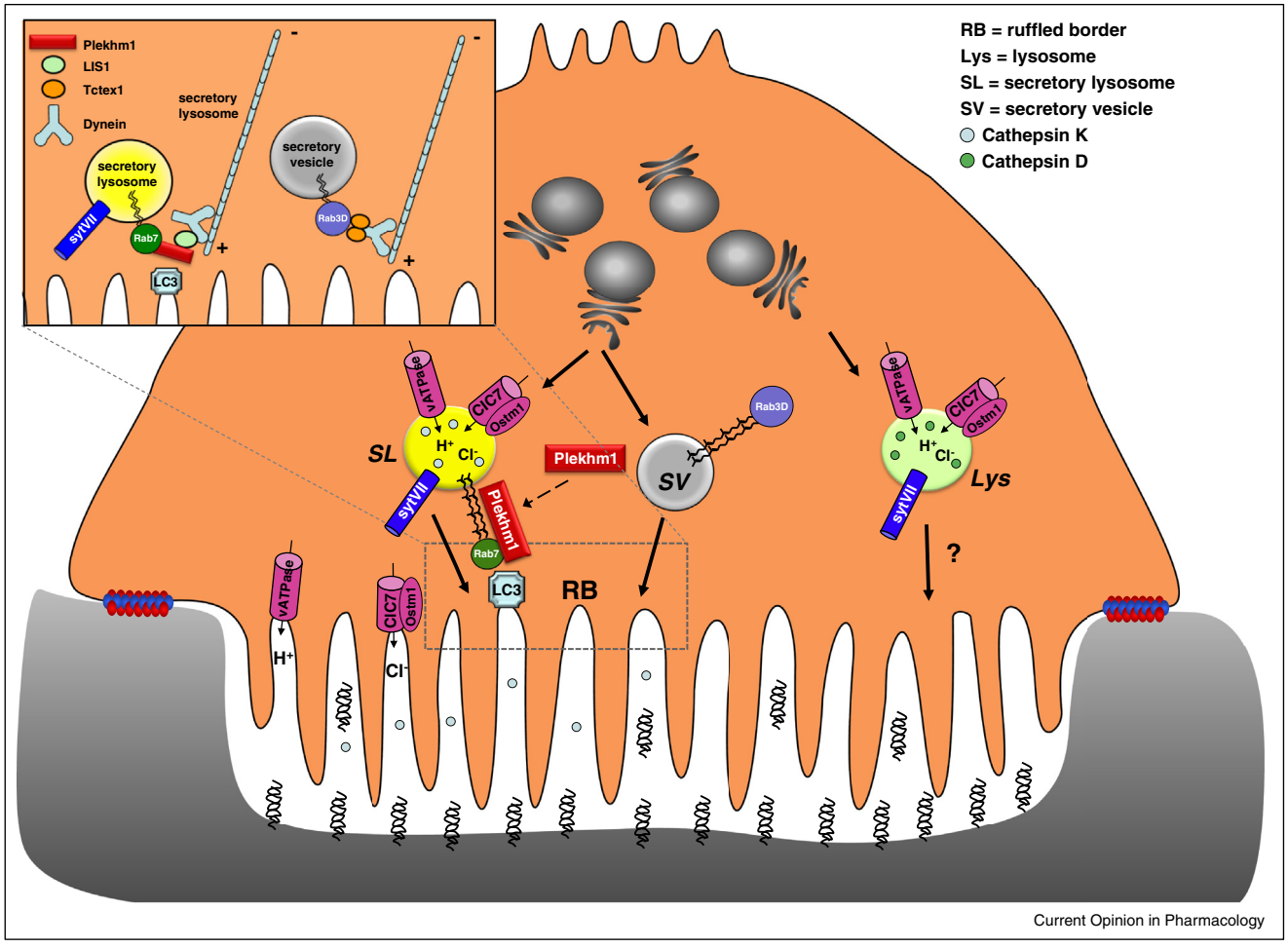
Vesicular trafficking to the ruffled border

Here we focus on vesicular trafficking to the ruffled border, the resorptive organelle of the osteoclast; other trafficking routes in osteoclasts have been reviewed elsewhere [41,42]. The ruffled border forms as a result of extensive vesicular fusion within the area bounded by the actin-rich sealing zone, where the osteoclast adheres to the bone matrix (Figure 2). This process creates a huge surface area for the continuing delivery of acid and proteases responsible for degrading type I collagen, principally cathepsin K, to the resorption lacuna. Trafficking to this domain occurs through at least two independent pathways, a Rab7-regulated lysosomal pathway and a Rab3D-regulated non-lysosomal pathway [41] (Figure 2). An effector of Rab7 in osteoclasts, Plekhm1, is recruited to the secretory lysosomes by Rab7 and plays a crucial role in their trafficking to the ruffled border [43]. The function of this protein appears to be largely restricted to osteoclasts, since mutations in the gene encoding this protein are responsible for rare cases of osteopetrosis (see Box 1). Rab3D, which is known to regulate exocytosis in cells with high secretory requirements, localises to a subpopulation of post-TGN vesicles in osteoclasts, rather than lysosomes, and appears to regulate the trafficking of these vesicles to the ruffled border [44].

Both these types of vesicles are trafficked to the plus end of microtubules at the ruffled border, that is, the same orientation as the plasma membrane of other cell types [45]. However, it has become apparent that the dynein–dynactin motor complex, which regulates trafficking of vesicles towards microtubule minus ends, is essential for targeting of both vesicular populations to the ruffled border. Tctex-1, one of many different light chains of the dynein complex, interacts with GTP-bound Rab3D, and RNA interference studies have implicated it in the trafficking of Rab3D-containing vesicles in osteoclasts [46]. In addition, trafficking of cathepsin K-containing lysosomes to the RB is prevented by disruption of the dynein–dynactin complex, presumably involving a different dynein light chain that has yet to be identified [45]. The dynein motor adapter Lissencephaly-1 (LIS1) plays a role in this process; LIS1 binds to dynein in osteoclasts and also interacts with Plekhm1 [47], thereby potentially linking the dynein motor to lysosomes via Plekhm1 and Rab7 in osteoclasts, shedding light on the role of Plekhm1 in lysosomal trafficking (Figure 2, inset). Decreasing LIS1 expression causes perinuclear accumulation of lysosomes, and impairs cathepsin K secretion [47]. Although it seems counterintuitive that a minus-end motor is essential for trafficking to the plus-end ruffled border, dynein is known to be able to act as a molecular anchor at the plus ends of microtubules [48], and may therefore tether secretory lysosomes here before membrane fusion [45].

The signalling pathways that stimulate secretion of lysosomes in osteoclasts are not well understood. However, it has recently been demonstrated that osteoclast-specific impairment of the class IA phosphatidylinositide 3-kinase (PI3K) by deletion of the p85 regulatory subunit results in an osteopetrotic (high bone mass) phenotype due to defective ruffled border formation and secretion of cathepsin K [49]. The sealing zone remains intact, supporting a direct role in lysosomal trafficking, most likely through activation of Akt [49]. Moreover, an isoform of protein kinase C that is highly expressed in osteoclasts, PKCδ, also plays a role in lysosomal trafficking [50]. PKCδ phosphorylates the actin bundling protein MARCKS, displacing it from the membrane and impairing its actin binding ability, resulting in a local loss of actin filaments, which enables fusion of cathepsin K-containing lysosomes at the membrane [51•]. Interestingly, trafficking of the V-ATPase and formation of the ruffled border are unaffected by PKCδ deficiency, suggesting that cathepsin K and the V-ATPase may reach the ruffled border through distinct trafficking mechanisms [51•], which could also include polarised mRNA transport [52,53]. In support of this model, cathepsin K has been localised to F-actin free regions of the ruffled border, distinct from those occupied

Figure 2



Schematic overview of trafficking pathways to the ruffled border in osteoclasts. Cathepsin K is trafficked in 'secretory lysosomes', whereas V-ATPase can also traffic to the ruffled border through an independent pathway, most likely a cathepsin D-positive lysosomal compartment, but also the Rab3D-positive 'secretory vesicles'. Both secretory lysosomes and secretory vesicles are trafficked on the microtubules towards plus ends at the ruffled border (inset). The dynein motor complex is crucial for this process, possibly by tethering the cargo at the plus ends before fusion. The molecular interactions here, however, are different; for rab3D-expressing vesicles involving the dynein light chain Tctex1, and for Rab7-expressing lysosomes the adapter LIS1, possibly through binding to Plekhm1 (inset). Fusion at the ruffled border is poorly understood, but in the case of the lysosomal compartment involves synaptotagmin VII and most likely LC3.

by the V-ATPase [54]. Furthermore, disruption of dynein does not impair acidification of the resorption lacuna [45] suggesting that it is not involved in V-ATPase trafficking.

Further support for the existence of two distinct lysosomal subpopulations in osteoclasts is provided by a mouse model with defects in the mannose-6-phosphate targeting pathway [55]. Secretory lysosomes containing cathepsin K and TRAP are dependent on the conventional mannose-6-phosphate targeting pathway, whereas a distinct population of cathepsin D-positive lysosomes are formed through a mannose-6-phosphate-independent pathway and likely carry out general lysosomal functions within the osteoclast [55]. However, it is possible that these vesicles may also fuse with the plasma membrane (and potentially also with secretory lysosomes), which could represent the means by which V-ATPase is able to traffic to the RB independently of cathepsin K, although cathepsin D has not been shown to localise to the ruffled border [51•]. In mannose-6-phosphate defective osteoclasts, cathepsin K is mistargeted and constitutively secreted from the TGN; this trafficking route is likely to be the Rab3D-regulated pathway that has been shown to be important for RB formation [45], although evidence for this is currently lacking.

Autophagy and bone

Autophagy is the vesicular trafficking process by which cells degrade and recycle misfolded proteins/damaged organelles; this recycling process is also crucial for survival

under stressful conditions such as nutrient starvation [56]. It begins with *de novo* formation of double-membraned autophagosomes around the cargo selected for degradation, which then fuse with lysosomes to form the autolysosome, thereby enabling degradation of cargo to occur. The importance of autophagy in bone cells is only just becoming clear. Autophagy-related proteins, including lipidated Atg8/LC3 (which is frequently used as a marker of autophagy), are essential for bone resorption by osteoclasts, but this may be through a non-autophagic role for LC3 in controlling the fusion of secretory lysosomes at the ruffled border [57•] (Figure 2). This would help to explain the finding that levels of lipidated LC3 increase during differentiation, apparently independently from levels of autophagy [58]. In support of this, it has recently become clear that autophagy-related proteins possess a range of non-autophagic roles in many other cell types, including participation in exocytotic processes [59].

In addition, it has been suggested that the pathogenesis of Paget's disease of bone (PBD) may be at least partly due to alterations in autophagy. PDB is a late-onset disorder characterised by focal areas of increased bone turnover containing enlarged, hyperactive osteoclasts. The disease has a strong genetic predisposition and has been associated with mutations in the ubiquitin-binding domain of p62 (SQSTM1), a protein that plays a crucial role in the recruitment to autophagosomes of material to be degraded. These mutations result in the formation of intracellular aggregates that are clearly visible by electron microscopy, possibly due to defective autophagic clearance. However, how such disturbances may contribute to the pathogenesis of the disease remain unclear [60]. Furthermore, defects in autophagy have been detected in a mouse model of a disease with a similar bone phenotype, inclusion body myopathy associated with PDB and frontotemporal dementia (IBMPFD), which is caused by mutations in valosin-containing protein (VCP). However, osteoclasts from these mice have yet to be studied [61].

Recent work has linked the age-related decline in bone mass to alterations in autophagy in osteocytes [62••]. Impairment of autophagy in osteocytes by conditional deletion of the autophagy gene Atg7 decreased bone mass in 6-month-old mice. This was associated with decreased osteoclast and osteoblast number, reduced bone formation rate, and increased oxidative stress, alterations that are all characteristic of changes in ageing mice. The mechanism underlying these changes remains unclear, but this raises the possibility that stimulating autophagy in osteocytes may be able to reverse age-related bone loss.

Vesicle trafficking pathways as pharmacological targets

Bisphosphonates, which are currently the most widely used class of drugs for the treatment of osteoporosis and other disease associated with excessive bone resorption, disrupt osteoclast-mediated bone resorption by inhibiting the function of Rab GTPases, thereby impairing vesicular trafficking, as well as impairing other small GTPase-dependent processes [63]. Interestingly, bisphosphonates may have additional effects on vesicular trafficking in osteoblasts. In Oasis$^{-/-}$ mice, which are characterised by osteopenia due to defective type I collagen secretion, bisphosphonates inhibit osteoclastic resorption and the resulting low bone turnover reduces osteoblastic ER expansion contributing to the observed increase in bone volume [64]. Modulators of ER stress themselves have shown potential as treatment options in osteoporosis [15].

The highly osteoclast-specific nature of osteopetrosis-causing mutations has identified the proteins encoded by these genes as promising novel anti-resorptive targets. One potential advantage of this approach is that it would result in increased numbers of inactive osteoclasts, and consequently increased bone formation as a result of the coupling process, whereby osteoclasts (active or inactive) are able to promote the activity of osteoblasts [65]. Other potential targets include the class IA PI3K, which plays a role in formation of the ruffled border. Established inhibitors of the p100β and p100δ catalytic subunits of this enzyme, TGX-221 and GS-9820, respectively, have recently been shown to inhibit osteoclast activity *in vitro*, but with differing effects on cytoskeletal organisation [66].

Finally, microvesicles have therapeutic relevance as they have been shown to not only be of prognostic value but have potential as gene therapeutic and drug delivery tools [67]. Furthermore, they might hold the clue to the osteoclast derived coupling factor that regulates osteoblast activity possibly through the stimulation of canopy cells that are situated above the osteoclast *in vivo* [68].

In conclusion, significant advances in our understanding of the role of vesicular trafficking in bone homeostasis have been made in recent years, identifying a plethora of possible drug targets. It remains to be seen how many novel drugs that target these pathways are ultimately translated into clinical use for bone diseases.

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