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VESICULAR TRAFFICKING IN OSTEOCLASTS

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ABSTRACT

Osteoclasts are multinucleated bone-degrading cells that undergo large changes in their polarisation and vesicular trafficking during the bone resorption cycle. Rab proteins are small GTPases that offer both temporal and spatial regulation to the transport between membranous organelles. Previously the presence and function of only few of the currently known 60 Rab proteins in osteoclasts have been reported.

In this study, the expression of 26 *Rab* genes in bone-resorbing osteoclasts was demonstrated with gene-specific primer pairs. The further analysis of three *Rab* genes during human osteoclast differentiation revealed that *Rab13* gene is highly induced during osteoclastogenesis. The presence of Rab13 protein in the secretory vesicles directed towards the ruffled border and in the endocytotic or transcytotic pathways in resorbing osteoclasts was excluded. The localisation of Rab13 suggests that that it is associated with a previously unknown vesicle population travelling between the trans-Golgi network and the basolateral membrane in bone resorbing osteoclasts.

Rab proteins convey their functions by binding to specific effector proteins. We found a novel Rab13 interaction with endospanins-1 and -2 that are yet poorly characterised small transmembrane proteins. The Rab13 subfamily member Rab8 also bound to endospanins, while Rab10 and unrelated Rabs did not. Rab13 and endospanin-2 co-localised in perinuclear vesicles in transfected cells, demonstrating the interaction also *in vivo*. The inhibition of Rab13 did not interfere with the localisation of endospanin-2 nor did it affect the cell surface expression of growth hormone receptor, as has been previously described for endospanins. The physiological role of this novel protein-protein interaction thus remains to be clarified.

The analysis of the transcytotic route in bone resorbing osteoclasts revealed that multiple vesicle populations arise from the ruffled border and transport the bone degradation products for exocytosis. These vesicles are directed to the functional secretory domain that is encircled by an actin-based molecular barrier. Furthermore, the transcytotic vesicles contain abundant *Helix pomatia* lectin binding sites and represent lipid raft concentrates. Finally, autophagosomal compartments may also be involved in the transcytosis.

Keywords: osteoclast, bone resorption, vesicle traffic, transcytosis, Rab, Rab13, endospanin

Mirkka Hirvonen: KALVORAKKULALIIKENNE OSTEOKLASTEISSA.
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TIIVISTELMÄ

Osteoklastit ovat monitumaisia luuta hajottavia soluja, joiden polarisaatiossa sekä kalvorakkulaliikenteessä tapahtuu suuria muutoksia solun toiminnan aikana. Rab proteiinit ovat pieniä GTP:tä hajottavia proteiineja, jotka säätelevät kalvorakkulaliikennettä soluelinten välillä. Tällä hetkellä tunnetuista 60 Rab proteiinista vain muutaman esiintymistä ja toimintaa on tutkittu osteoklasteissa.

Tässä tutkimuksessa havaittiin 26 *Rab*-geenin ilmentyminen luuta hajottavissa osteoklasteissa. Kolmen *Rab*-geenin tarkempi tarkastelu osoitti *Rab13*-geenin ilmentymisen lisääntyvän voimakkaasti osteoklastien erilaistumisen myötä. Rab13-proteiini ei paikantunut mihinkään tällä hetkellä tunnettuun solunsisäiseen kalvoreittiin. Ei poimukalvolle suuntautuviin kalvorakenteisiin, eikä osteoklastin endosytoottiselle tai transsytoottiselle reitille. Rab13-proteiini paikantuu osteoklasteissa toistaiseksi toiminnaltaan tuntemattomttomiin kalvorakkuloihin, jota kuljetetaan trans-Golgi-alueelta kohti basolateraalista solukalvoaluetta.

Rab-proteiinit sitoutuvat niille kullekin ominaisten ns. efektori- eli vaikuttaja-proteiinien kanssa. Havaitsimme uuden Rab13 vuorovaikutuksen endospaniini-1 ja -2-proteiinien kanssa. Endospaniinit ovat pieniä solukalvon läpäiseviä proteiineja, joiden toiminta tunnetaan vielä huonosti. Niiden on aikaisemmin havaittu säätelevän kasvuhormonireseptorin esiintymistä solukalvolla. Rab8, joka on Rab13 sukulaisproteiini, sitoutui samoin endospaniineihin. Rab10 tai muut rakenteeltaan kaukaisemmat Rab-proteiinit eivät sitoutuneet endospaniineihin. Kun Rab13 ja endospaniini-2 proteiineja yli-ilmennettiin soluissa, niiden havaittiin kertyvän samoihin tuman lähistöllä sijaitseviin kooltaan pieniin kalvorakkuloihin. Rab13-proteiinin ilmentymisen estolla ei kuitenkaan ollut vaikutusta yli-ilmennetyn endospaniinin sijaintiin soluissa, eikä kasvuhormonireseptorin esiintymiseen solukalvolla. Havaitun uuden proteiini-proteiini-interaktion fysiologinen merkitys jää siten avoimeksi.

Osteklastien transsytoottisen kalvorakkulareitin tarkempi tarkastelu osoitti, että jo poimukalvoalueella muodostuu useita erilaisia kalvorakkulatyyppejä, jotka kuljetetaan solun läpi eritystä varten. Nämä luun hajotustuotteita sisältävät kalvorakkulat ohjataan ns. FSD-alueelle, jonka erottaa basolateraalikalvosta aktiinista muodostuva nauhamainen rakenne. Solun läpi kulkevat kalvorakkulat sisältävät runsaasti *Helix pomatia*-lektiinin sitoutumiskohtia sekä kolesterolikertymiä. Lisäksi havaittiin, että autofagosomit saattavat liittyä luun hajoamistuotteiden kuljetukseen.

Avainsanat: osteoklasti, luun hajotus, kalvorakkulaliikenne, transsytoosi, Rab, Rab13, endospaniini

TABLE OF CONTENTS

ABBREVIATIONS

Ac45	ATPase, H+ transporting, lysosomal accessory protein 1 (ATP6AP1)
BMP	Bone morphogenetic protein
BMU	Basic multicellular unit
CD44	Hyaluronic acid receptor
ClC-7	Chloride channel 7 (ClCN7)
EEA1	Early endosomal antigen-1
FT	Farnesyl transferase
GalNAc	N-acetylgalactosamine
GAP	GTPase activating protein
GDF	GDI displacement factor
GDI	Rab GDP dissociation inhibitor
GDP	Guanosine diphosphate
GEF	GTP/GDP exchange factor
GFP	Green fluorescent protein
GGTI	Geranylgeranyl transferase type I
GHR	Growth hormone receptor
Glut	Glucose transporter
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
GTPase	Guanosine triphospphatase
H+-ATPase	H+ adenosinetriphosphatase
HA	Influenza hemagglutinin
HPA	Helix pomatia agglutinin
HRP	Horseradish peroxidase
IGF	Insulin like growth factor
IL	Interleukin
LEPR	Leptin receptor
LRO	Lysosome-related organelle
M-CSF	Macrophage-colony stimulating factor
MBP	Maltose-binding protein
MDC	Monodansyl cadvarine
MICAL	Molecule interacting with CasL
MMP	Matrix metalloproteinase
OPG	Osteoprotegerin
PBMC	Peripheral blood monocytic cell
PDEδ	Rod cGMP phosphodiesterase subunit delta
Plekhm1	Pleckstrin homology domain-containing family M, member 1
RANK	Receptor activator of nuclear factor kappa B
RANKL	Ligand for receptor activator of nuclear factor kappa B
recChlTx-B	Recombinant cholera toxin B subunit
REP	Rab escort protein
RGGT	Rab geranylgeranyl transferase
siRNA	Small interfering ribonucleic acid

TBC-domain	Tre-2, Bub2 and Cdc16-domain
TGF-β	Transforming growth factor- beta
TGN	Trans-Golgi network
TNF-α	Tumor necrosis factor alpha
TRACP	Tartrate resistant acid phosphatase
VEGF	Vascular endothelial growth factor
VSV-G	Vesicular stomatitis virus G-protein
WGA	Wheat germ agglutinin
Zo-1	Zona occludens protein-1

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by Roman numeric (I-III).

I Hirvonen MJ, Mulari MT, Büki KG, Vihko P, Härkönen PL, Väänänen HK. Rab13 is upregulated during osteoclast differentiation and associates with small vesicles revealing polarized distribution in resorbing cells, *J Histochem Cytochem* 60(7):537-49 (2012)

II Hirvonen MJ, Büki KG, Sun Y, Mulari MT, Härkönen PL, Väänänen HK. Novel interaction of Rab13 and Rab8 with endospanins, *FEBS Open Bio* 3:83-8 (2013)

III Hirvonen MJ, Fagerlund K, Lakkakorpi P, Väänänen HK, Mulari MTK. Novel perspectives on the transcytotic route in osteoclasts, *BoneKEy Reports* 2: 306 (2013)

 In addition, some unpublished data are presented in this thesis.

1 INTRODUCTION

Bone is a living organ that is remodelled throughout life. Bone remodelling is necessary in several physiological conditions, including growth and the maintenance of the bone integrity and the calcium homeostasis in the body. The concerted actions of osteoblasts and osteoclasts, the two types of bone cells involved in bone formation and bone resorption, respectively, maintain the bone structure and strength by replacing the old, micro-damaged bone by new bone (Eriksen, 2010).

Several bone disorders are caused by imbalances in the bone remodelling, either by substantial bone degradation by osteoclasts or by impaired bone formation by osteoblasts. Postmenopausal, senile osteoporosis is the most common bone disorder affecting approximately 22 million women and 5,5 million men in the European Union in year 2010 (Svedbom *et al.*, 2013). It is defined as a decline in bone mass and strength leading to an increased tendency to fracture. Currently, the most commonly used treatments for osteoporosis are bisphosphonates, of which the nitrogen-containing bisphosphonates prevent the prenylation of proteins, including small GTPases, by inhibiting the mevalonate pathway (Das and Crockett, 2013). Due to their high avidity for bone hydroxyapatite and their release from the bone matrix primarily during bone resorption, the major target cell of the bisphosphonates is the osteoclast.

Although the major vesicle transport pathways in osteoclasts were identified almost twenty years ago, they are still rather poorly characterised and their regulators remain enigmatic. In the present study, some novel aspects of the transcytotic route in osteoclasts are described. In addition, the possible regulation of the osteoclast vesicular trafficking events by Rab proteins that are also affected by bisphosphonates, are considered with the major emphasis on the role of Rab13 in osteoclasts. Thus, the literature review will consist of the overview of bone remodelling and osteoclast function, focusing on the currently known membrane trafficking pathways in these cells, followed by a review on Rab GTPases and their identified functions in osteoclasts.

2 REVIEW OF THE LITERATURE

2.1 BONE

Human adult skeleton consists of 213 bones of different sizes and shapes. Most bones are formed of a dense outer cortical bone and an inner honeycomb-like trabecular bone. The overall ratio of cortical and trabecular bone in human adult body is 80% and 20%, respectively. Bones provide support and attachment sites for the muscles and joints, thus enabling movement and locomotion. They form cavities to protect vulnerable organs, such as brain and bone marrow. Bone regulates the mineral homeostasis, acid-base balance and acts as reservoir for growth factors and cytokines and was recently suggested to act as an endocrine organ (Clarke, 2008).

The bone extracellular matrix is formed by osteoblasts and is composed of 60% mineral (mainly calcium hydroxyapatite), 20% organic material and 20% water (Crockett *et al.*, 2011). Approximately 85% to 90% of the bone proteins are collagens, type I being most abundant. The remaining non-collagenous protein fraction consists of great variety of proteins, including osteopontin, hyaluronan, osteocalcin, and bone sialoprotein (Clarke, 2008).

2.1.1 Bone remodelling

After being initially formed, bone is remodelled continuously throughout life. This remodelling ensures the strength and rigidity of the bone through the replacement of old, micro-damaged bone with new matrix. Remodelling of the trabecular bone is also important in the maintenance of the mineral homeostasis and it is considered metabolically more active than the cortical bone. Bone remodelling occurs in cycles of bone resorption and formation of new bone, in a concerted action of basic multicellular unit (BMU) that consists of osteoclasts, osteoblasts, osteocytes and bone lining cells (Kular *et al.*, 2012). The cells of the BMU cross-talk through soluble coupling factors or direct cell-cell contacts and mediate their actions in a cascade consisting of four sequential phases: activation, resorption, reversal and formation (Figure 1).

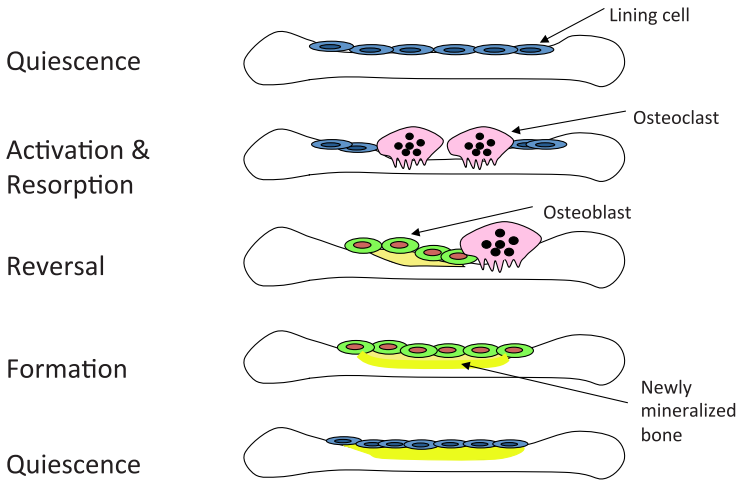

Figure 1: A schematic presentation of the bone remodelling cycle. The disruption of the bone-embedded osteocyte cell network (not shown) induces the secretion of RANKL that increases the recruitment and fusion of osteoclast precursors and thus initiates the bone resorption (activation and resorption). In the reversal phase osteoblast precursors are recruited to the resorbed area and start forming bone, while osteoclasts are inhibited and undergo apoptosis. In the formation phase, osteoblasts restore the resorbed area with new bone.

2.1.2 Bone cells

Bone remodelling begins with the recruitment of osteoclast precursors to the site of action followed by their differentiation and activation for bone resorption. It has been shown that osteocytes are the major recruiters and activators of osteoclasts precursors for targeted bone remodelling via secretion of RANKL, the inductor of osteoclastogenesis (discussed in section 2.2.1) (Nakashima *et al.*, 2011; Xiong *et al.*, 2011). Osteocyte cell death and disruption of the cellular network formed by bone-embedded osteocytes are important signals in the initiation of the bone remodelling cycle (Gu *et al.*, 2005; Kurata *et al.*, 2006; Heino *et al.*, 2009). The specific details of osteoclast differentiation and bone resorption are discussed below (see section 2.2.) After bone resorption, osteoclast activities are inhibited and they may die via apoptosis. Simultaneously, osteoblast precursors are recruited and begin to differentiate towards mature osteoblasts (reversal). Osteoclasts regulate the recruitment of osteoblasts by releasing bone-embedded cytokines and growth factors that regulate osteoblast differentiation (e.g. bone morphogenetic proteins, BMPs; insulin like growth factors, IGFs; transforming growth factor beta, TGF-β) while osteoblast-derived signals cease bone resorption (Canalis, 2009). In addition, osteoclasts and osteoblast precursors undergo bi-directional regulation via direct cell-cell contact by ephrinB2/ephrinB4 binding. These signals link the suppression of osteoclast formation to the osteoblast differentiation and the initiation of bone formation (Zhao *et al.*, 2006). Semaphorin 4D is another coupling factor expressed by osteoclasts. It binds to its receptor Plexin-B1 on osteoblasts and inhibits bone formation via suppressing IGF-1 signalling in osteoblasts (Negishi-Koga *et al.*, 2011). This coupling inhibits osteoblast recruitment and allows the complete removal of damaged bone prior to the bone formation.

Osteoblasts are of mesenchymal origin and differentiate from pluripotent mesenchymal stem cells that can give rise also to fibroblasts, muscle cells, adipocytes and chondrocytes. Osteoblast commitment and differentiation is controlled by several local and systemic stimuli that include among others; BMPs, hedgehog proteins, TGF-β, mechanical stimuli and various cell growth factors (reviewed in (Neve *et al.*, 2011)). Active bone-forming osteoblasts are cuboidal, polarised cells, that have characteristics of highly secreting cells, e.g. prominent Golgi and abundant rough endoplasmic reticulum. During bone formation, mature osteoblasts secrete the organic components of the bone matrix after which the newly formed bone matrix becomes mineralised by the local release of small hydroxyapatite crystals from the matrix vesicles shed from the osteoblast. The calcium and phosphate ions in the extracellular fluid propagate the nucleation and the slow growth of the hydroxyapatite crystals and thus bone mineralisation (formation) (Anderson, 2003). In addition to the bone formation, osteoblasts have important role in the regulation of osteoclast activity by the RANKL/OPG system (see section 2.2.1). In addition, osteoblasts inhibit locally bone resorption during the formation phase by bidirectional binding of semaphorin 3A and neuropilin-1 present on the surface of osteoclasts and osteoblasts, respectively (Hayashi *et al.*, 2012). After completing the bone formation phase, osteoblasts undergo either apoptosis, differentiate further to osteocytes or become quiescent bone lining cells (Kular *et al.*, 2012).

Osteocytes are formed via osteoblast differentiation. They become embedded into the newly formed bone matrix and reside in lacunae and represent the most abundant and long-lived cell type in bone. Although osteocytes are spatially isolated from each other, they form several dendritic processes that radiate from the cell body into small canals called the canaliculi. These cell processes form cellular contacts with neighbouring osteocytes and other types of bone cells (Bonewald, 2011; Neve *et al.*, 2012). It is currently recognised that osteocytes have a major input in the maintenance of bone structure: they work as mechano-sensory cells and secrete factors that inactivate (TGF-β) or activate (RANKL, M-CSF) osteoclasts (Heino *et al.*, 2002; Kurata *et al.*, 2006). In addition, healthy osteocytes are able to remodel their perilacunar matrix, indicating that osteocytes have a role in the maintenance of the mineral homeostasis as well (Bonewald, 2011).

A subset of osteoblasts will differentiate into bone-lining cells. These cells cover the surface of inactive bone that is not under remodelling (Menton *et al.*, 1984). This cell layer may protect the bone from inadequate activation of preosteoclasts and thus bone resorption. During bone remodelling, the BMU is sealed from the bone marrow by a continuous layer of bone lining cells generating an isolated milieu for the bone remodelling and the action of coupling factors (Hauge *et al.*, 2001). Bone lining cells may also have a role in the initiation of the remodelling cycle as their contacts with the osteocyte network are interrupted (Rochefort *et al.*, 2010). Although bone-lining cells were identified decades ago, the signals leading to their differentiation are still unknown.

2.2 OSTEOCLAST

Osteoclasts are multinucleated cells formed by the fusion of myeloid precursor cells. They were first described by Albert Kölliger in 1873 and are currently recognised as the only cell type capable of bone resorption.

2.2.1 Osteoclast formation and regulation

Mature osteoclasts originate from hematopoietic stem cells that can give rise also to dendritic cells, lymphocytes, erythrocytes, granulocytes, megakaryocytes, monocytes, and various types of tissue macrophages. The earliest marker detected during the commitment of the myeloid precursor cell towards the osteoclast lineage is the transcription factor PU.1 that, among other functions, regulates the expression of the receptor for the macrophage colony-stimulating factor (M-CSF), CSFR1 (DE Zhang *et al.*, 1994; Edwards and Mundy, 2011). M-CSF is a soluble factor essential for the differentiation of the cells of the monocyte-macrophage lineage at the early stages of differentiation, as well as a critical factor in the maturation and commitment of osteoclast precursor cells. Recently, it was demonstrated that another ligand of CSFR1, interleukin-34, is also able to support osteoclastogenesis in a similar way to M-CSF (Baud'huin *et al.*, 2010; Chen *et al.*, 2011). The main factor influencing the later stages of osteoclast differentiation is the RANK-RANKL-OPG axis. Receptor for activation of nuclear factor kappa B (RANK) ligand (RANKL) is a membrane bound factor produced by osteoblasts, osteocytes and stromal cells of the bone marrow that binds to its transmembrane signalling receptor RANK on osteoclast progenitors and mature osteoclasts. RANKL-RANK signalling is essential for osteoclast differentiation and activation as mice deficient in either one demonstrate a complete absence of osteoclasts (Kong *et al.*, 1999; Li *et al.*, 2000). RANKL and M-CSF are required to induce the expression of markers for osteoclast lineage, including tartrate-resistant acid phosphatase (TRACP), cathepsin K, calcitonin receptor and the β_3-integrin. The production of soluble recombinant form of RANKL had a great impact on the *in vitro* production of pure osteoclasts cultures without the presence of supporting stromal cells of the bone marrow and thus has allowed more detailed characterisation of the osteoclasts (Matsuzaki *et al.*, 1998). Osteoblasts and stromal cells produce a soluble factor, osteoprotegerin (OPG) that is capable of blocking osteoclast formation by competing the binding of RANK to the RANK ligand (Simonet *et al.*, 1997; Yasuda *et al.*, 1998; Burgess *et al.*, 1999). Mice lacking OPG show severe osteoporosis leading to increased mortality while mice overexpressing OPG are osteopetrotic (Simonet *et al.*, 1997; Bucay *et al.*, 1998). Osteoblasts are thus able to control bone resorption by expression of factors that affect osteoclastogenesis and osteoclast activation state.

Other signals, at least partly independent of RANKL-RANK signalling, that support osteoclastogenesis have also been described. TGF-β as well as interleukins (IL)-1, -6 and -11 are capable of inducing the formation numerous small osteoclasts in the presence of M-CSF, even in the absence of RANKL. However, these osteoclasts have lower resorptive capacity as compared with the RANKL-induced osteoclasts (Kudo *et al.*, 2003; Itonaga *et al.*, 2004). Additionally, the blockage of IL-6 receptor by a blocking antibody was shown to reduce the RANKL-induced osteoclastogenesis *in*

vitro and *in vivo* (Axmann *et al.*, 2009). The function of IL-6 may, however, be more complex; it was also shown to block RANKL-induced osteoclastogenesis and redirect osteoclast precursors into the macrophage lineage (Duplomb *et al.*, 2008). Tumor necrosis factor alpha (TNF-α) is able to induce osteoclast differentiation in the presence of M-CSF independently of RANKL signalling. Induction of the bone resorption by these cells, however, required an additional factor, namely IL-1α (Kobayashi *et al.*, 2000).

Calcitrophic humoral factors, including estrogen, parathyroid hormone, vitamin D and other members of the IL-cytokine family can modulate osteoclast differentiation and function via direct actions on the receptors present on osteoclast progenitors or indirectly by regulating the osteoblast expression of RANKL and OPG. Calcitonin, the osteoprotective hormone regulating blood Ca^{2+} homeostasis is produced by the parafollicular cells of the thyroid gland and directly binds to it its receptor on osteoclasts and supresses their resorptive activity (Chambers and Moore, 1983; Nicholson *et al.*, 1986).

Osteoclast progenitors are present in peripheral blood and bone marrow. Distinct subpopulations of peripheral blood monocytic cells (PBMCs) have different capacities to differentiate into bone-resorbing osteoclasts, the CD14-positive monocytes being the most potent (Husheem *et al.*, 2005). Upon the trigger for bone remodelling these cells travel to the site of resorption, exit the circulation, fuse and activate for resorption. Signals for extravasation of osteoclast precursors may include the direct communication of osteoclast precursors with endothelial cells via the cell surface adhesion molecules, such as the intercellular adhesion molecule 1 (ICAM-1) and the hyaluronic acid receptor (CD44) (Kindle *et al.*, 2006) and humoral factors. Sphingosine-1 (S1P) phosphate is a soluble factor that reduces the attachment of osteoclast precursors to the bone surface. RANKL stimulation in this environment reduces the expression of $S1P_1$, the receptor of S1P, in pre-osteoclasts and prevents them from re-entering the circulation (Ishii *et al.*, 2009). Stromal cell-derived factor-1 (SDF-1), produced by bone endothelium, bone marrow stromal cells and osteoblasts, binds to its receptor (CXCR4) on mononuclear cells and osteoclast precursors and promotes their recruitment to the site of resorption. In addition, SDF-1 has some role in the early stages of osteoclast differentiation and osteoclast survival (Wright *et al.*, 2005). Although the mononuclear osteoclasts are able to resorb bone to some extent, the multinucleated osteoclasts are much more potent. The microenvironment at the resorption site, consisting of high levels of RANKL and M-CSF produced by osteoblasts, osteocytes and stromal cells, supports osteoclast precursor maturation and fusion. The currently known major regulator of osteoclast precursor fusion is the seven transmembrane receptor-like molecule, dendritic cell-specific transmembrane protein (DC-STAMP). Inhibition of its function by siRNA or with a blocking antibody impairs osteoclast fusion *in vitro* and DC-STAMP-deficient mice lack multinucleated osteoclasts (Kukita *et al.*, 2004; Yagi *et al.*, 2005). At the moment however, the ligand for DC-STAMP in osteoclastogenesis is unknown (Nakahama, 2010; Zhang *et al.*, 2014).

2.2.2 Osteoclast membrane domains associated with bone resorption

Osteoclastic bone resorption occurs in cycles of cell attachment, cellular polarisation, matrix degradation and cell detachment after which osteoclasts either migrate to a new site of resorption or die via apoptosis. In culture conditions, osteoclasts can perform several rounds of bone resorption cycles. However, it is not currently known if osteoclasts can exist in quiescent state *in vivo* and thus perform multiple cycles of resorption (Väänänen and Laitala-Leinonen, 2008; Edwards and Mundy, 2011). As multinucleated osteoclast activate for bone resorption, they become strongly polarised and form four functionally distinct membrane domains: sealing zone, ruffled border, functional secretory domain and the basolateral membrane (Lakkakorpi *et al.*, 1989; Salo *et al.*, 1996; Mulari *et al.*, 2003) (Figure 2).

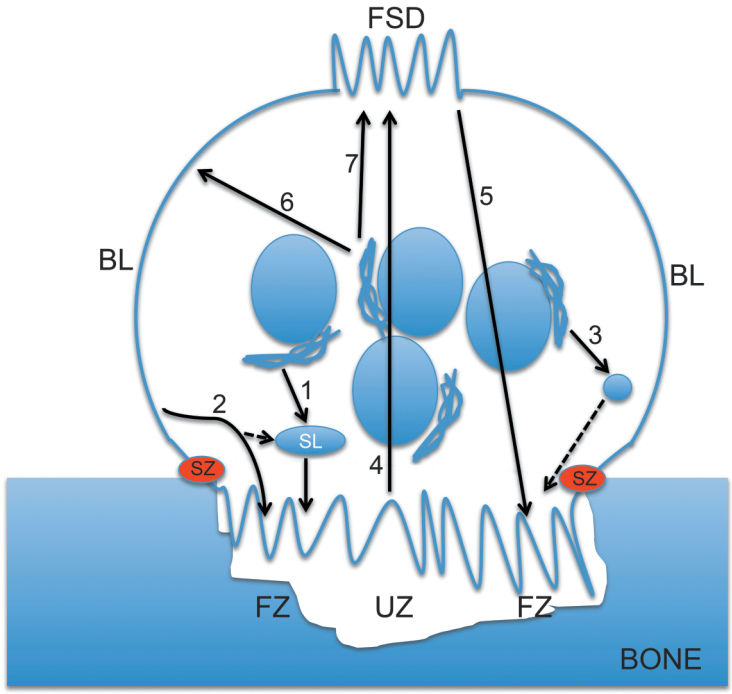

Figure 2: The known vesicular trafficking pathways in osteoclasts. The bone-resorbing osteoclast is a highly polarised cell with four distinct membrane domains: the functional secretory domain (FSD), the basolateral domain (BL), the sealing zone (SZ) and the bone facing, highly convoluted ruffled border. Bone degrading enzymes and acid are delivered to the peripheral ruffled border (fusion zone, FZ) in lysosomal vesicles (SL) from the TGN (1) and vesicles originating from the BL membrane (2). A non-lysosomal, post-Golgi vesicular pathway regulated by Rab3 is also important for ruffled border formation (3). The bone degradation products are endocytosed at the central uptake domain (UZ) of the ruffled border and transcytosed for release to the extracellular fluid (4). A recently identified reverse transcytotic pathway most likely acts as a membrane-recycling pathway (5). Biosynthetic secretory pathways towards the basolateral membrane and the functional secretory domain have been described, although their endogenous cargo is unknown (6 and 7, respectively). Arrows with dashed lines indicate pathways that have not yet been fully characterised. Modified from (Coxon and Taylor, 2008; Zhao, 2012).

2.2.2.1 Sealing zone

The sealing zone mediates the tight attachment of osteoclasts to the bone surface, encircles the actual resorptive organelle, the ruffled border and isolates the forming resorption lacuna from the rest of the extracellular space. The initial attachment of osteoclasts to the bone surface occurs via characteristic adhesion structures called podosomes that are found also in other monocyte-derived cells. Podosomes consist of central core of dense actin filaments surrounded by a cloud of filamentous actin and actin monomers. The podosome cores connect the cytoskeleton to the extracellular matrix via CD44, a hyaluronic acid receptor able to interact also with osteopontin, collagen and laminin. The surrounding actin cloud is associated with β_3-integrin that binds RGD (Arginine-Glycine-Asparagine)-containing peptides found also in bone matrix proteins (Chabadel *et al.*, 2007). In premature osteoclasts, podosomes form clusters that organise gradually into a ring-like structure and mature into the broad acting ring observed in bone resorbing osteoclasts (Lakkakorpi and Vaananen, 1991). The composition of the sealing zone remained under debate for quite some time but a novel approach using high-resolution microscopy revealed that the sealing zone indeed consists of dense array of podosome cores interconnected by high-density network of acting filaments (Luxenburg *et al.*, 2007). The structure of the podosomes is conserved in the sealing zone and the final tight sealing is mediated by CD44, the $\alpha_v\beta_3$ integrin associating with the actin cloud surrounding the dense array of actin cores (Chabadel *et al.*, 2007). The sealing zone is highly dynamic structure allowing the formation of conditions that favour bone resorption in the resorption lacuna and simultaneous migration. The isolation is not complete as negatively charged molecules with molecular weight up to 10 kDa are able to cross this barrier and diffuse into the resorption lacuna (Stenbeck and Horton, 2000).

2.2.2.2 Ruffled border

The hallmark of osteoclast activation for bone resorption is the formation of the actual resorptive organ, the ruffled border, at the bone-facing plasma membrane. It is surrounded by the actin ring generating an isolated microenvironment for bone resorption, the resorption lacuna. Ruffled border is a highly convoluted membrane domain formed by the fusion of intracellular acidic vesicles. Although the ruffled border is part of the plasma membrane, it contains several markers of the late endosomal-lysosomal compartment including Rab7, vacuolar proton pump and ClC-7 chloride channel (Vaananen *et al.*, 1990; Palokangas *et al.*, 1997).

Ruffled border has a dual function in bone resorption: secretion of acid and proteolytic enzymes for bone degradation and endocytosis of the formed bone degradation products for transcytosis. These two processes are spatially separated. The secretory vesicles are fused to the ruffled border at the peripheral parts of this membrane domain, while bone degradation products enter the transcytotic vesicles at the central parts of the ruffled border. The vesicles delivered to the fusion zone of the ruffled border switch from microtubules to microfilaments prior to the vesicle fusion as the peripheral ruffled border contains F-actin but not tubulin. The central domain however, is rich in tubulin indicating that transcytotic vesicles move along microtubules (Mulari *et al.*, 2003; Sun *et al.*, 2005).

The mechanisms of bone resorption are becoming uncovered, but rather little is known about the molecules modulating the formation and maintenance of the ruffled border. Rab7, a small GTPase regulating the endo-lysosomal trafficking is essential for ruffled border formation and thus bone resorption (Zhao *et al.*, 2001). Rab7 interacts directly and co-localises with Rac1 at the vesicle fusion zone of the ruffled border. Thus, it has been hypothesised that this interaction regulates the vesicle switch from microtubules to actin filaments prior to the secretory vesicle fusion at the ruffled border (Sun *et al.*, 2005). Rab7 also interacts with Plekhm1 and Ac45 (the regulatory subunit of the vacuolar proton pump), that are important regulators of the ruffled border formation and function (Van Wesenbeeck *et al.*, 2007; Yang *et al.*, 2012). Synaptotagmin VII, a calcium sensing protein regulating exocytosis, associates with lysosomes in resting osteoclast and redistributes to the ruffled border in resorbing cells. Genetic deletion of synaptotagmin VII inhibits cathepsin K secretion and formation of the ruffled border (Zhao *et al.*, 2008). Most recently, autophagy proteins, including autophagy proteins 5, 7, 4B and LC3 were described to regulate the lysosomal secretion at the ruffled border. Interestingly, Rab7 localisation to the ruffled border was autophagy protein 5-dependent (DeSelm *et al.*, 2011).

As bone resorption ceases, the ruffled border is internalised and the lysosomal enzymes destined for secretion at the ruffled border are re-routed to intracellular vacuoles as shown by the rapid inhibition of bone resorption by calcitonin (Baron *et al.*, 1990).

2.2.2.3 Non-bone facing plasma membrane

The plasma membrane domain facing the extracellular fluid is not homogenous in resorbing osteoclasts. Peripheral parts of it resemble the basolateral membranes of epithelial cells, as newly synthesised G protein of the vesicular stomatitis virus (VSV-G) is delivered to this membrane area and thus it is referred as basolateral domain (Salo *et al.*, 1996). In addition, this membrane area contains large amounts of binding sites for iron-loaded transferrin analogously to the basolateral membranes of epithelial cells (Palokangas *et al.*, 1997). Morphologically this membrane domain appears smooth.

In contrast to the peripheral parts of the non-bone facing membrane, the central, apical part is highly villous. Influenza haemagglutinin (HA), which is delivered to the apical membranes of polarised epithelial cells, is directed into this membrane area in virus-infected resorbing osteoclasts (Salo *et al.*, 1996). This functional secretory domain is the membrane area where bone degradation products are released in to the surrounding extracellular space after transcytosis (Nesbitt and Horton, 1997; Salo *et al.*, 1997). A number of enzymes, including TRACP 5b, cathepsin K, and bone-embedded hormones and growth factors such as glutamate are released to the extracellular space from this membrane area, with the release of other bone degradation products (Vaaraniemi *et al.*, 2004; Morimoto *et al.*, 2006; Zhao, 2012).

2.2.3 Vesicular trafficking in osteoclasts

2.2.3.1 Membrane transport pathways during resorption

The formation and the maintenance of the distinct membrane domains in osteoclasts during bone resorption is achieved by extensive membrane re-organisation and intracellular vesicle trafficking. The major membrane trafficking pathways in osteoclasts have been resolved (Figure 2), but much is still enigmatic. The resorptive organ of osteoclasts, the ruffled border, is formed by the delivery of vesicles containing late endosomal and lysosomal enzymes to the bone-apposing plasma membrane. These enzymes are newly synthesised enzymes from the biosynthetic route or originate from the endocytotic pathway from the basolateral membrane domain. Soluble endocytotic markers and iron-loaded transferrin that are internalised at the basolateral membrane are accumulated at the ruffled border in resorbing osteoclasts. The basolateral membrane thus serves as a membrane reservoir for the lost membrane at the ruffled border during the transcytosis (Palokangas *et al.*, 1997). Newly synthesised lysosomal enzymes are delivered to the ruffled border from the biosynthetic pathway via secretory lysosomes (Baron *et al.*, 1988; van Meel *et al.*, 2011). These two pathways may converge, as inhibition of lysosome biogenesis also disturbs the delivery of endocytosed transferrin to the ruffled border (Zhao and Väänänen, 2006). In addition, a non-lysosomal secretory pathway, partly regulated by Rab3D, contributes to the maintenance of the ruffled border (Pavlos *et al.*, 2005).

During resorption the bone degradation products are endocytosed at the central parts of the ruffled border. This area is rich in vesicle budding proteins, such as clathrin, its adaptor protein AP-2 and dynamin II (Mulari *et al.*, 2003). After the uptake, the bone degradation products are transported in transcytotic vesicles, in which they may be further digested by TRACP and cathepsin K, to the functional secretory domain for release to the extracellular space (Nesbitt and Horton, 1997; Salo *et al.*, 1997; Vaaraniemi *et al.*, 2004). An additional reverse transcytotic pathway from the functional secretory domain to the peripheral parts of the ruffled border serves as a membrane recycling pathway during bone resorption (Mulari *et al.*, 2008).

2.2.3.2 Bone resorption

Bone degradation involves dissolution of the mineral hydroxyapatite by acid and proteolytic breakdown of the organic matrix. The initial acidification is achieved by the fusion of acidic vesicles to the bone-facing plasma membrane as the ruffled border is formed. These vesicles also deliver the molecules required for further acidification to the resorption lacuna. Vacuolar H+-ATPase is a large protein complex that is responsible for the acidification of intracellular lysosomes in non-resorbing osteoclasts and other cells. In resorbing osteoclasts it is located in the ruffled border and is considered as central player in acid secretion by osteoclasts (Vaananen *et al.*, 1990). Vacuolar H+-ATPase consists of several subunits. The a3 subunit, although not strictly osteoclast-specific, has major effects on osteoclast function as shown by the impressive osteopetrotic phenotype of a3-deficient mice and human mutations in the a3 gene (Scott and Chapman, 1998; Li *et al.*, 1999; Frattini *et al.*, 2000). In addition to acid secretion, subunits of the vacuolar H+-ATPase have been described to bind filamentous actin and may be directly involved in vesicle fusion. Vacuolar H+-

ATPase could thus regulate the initial formation of the resorptive apparatus in osteoclast (Lee *et al.*, 1999; Holliday *et al.*, 2000; Zuo *et al.*, 2006). Most recently, Ac45, a V-ATPase accessory subunit, was demonstrated to be important for acid secretion, intracellular trafficking of lysosomes, exocytosis of cathepsin K and osteoclast precursor fusion (Qin *et al.*, 2011; Yang *et al.*, 2012). To sustain electroneutrality, chloride ions are secreted to the resorption lacuna mostly by ClC-7, a member of the ClC family of chloride channels abundantly expressed in osteoclasts and located mainly in the ruffled border in resorbing cells (Schaller *et al.*, 2005; Lange *et al.*, 2006; Szewczyk *et al.*, 2013). The continuous supply of protons for the vacuolar H+-ATPase is provided by cytosolic carbonic anhydrase II. It hydrates carbon dioxide to H_2CO_3 that dissociates to bicarbonate and protons (Sundquist *et al.*, 1987; Karhukorpi, 1991; Lehenkari *et al.*, 1998). The chloride ions for ClC-7 are provided by a chloride-bicarbonate exchanger, anion exchanger 2 (AE2), embedded in the basolateral membrane. This exchanger also removes excess bicarbonate from the osteoclast (Teti *et al.*, 1989).

After mineral dissolution, the organic matrix of bone is exposed to proteolytic enzymes, namely cysteine proteases and matrix metalloproteinases (MMPs). Cathepsin K is the most abundant cysteine protease in osteoclasts and is secreted to the resorption lacuna. It is activated in acidic pH and degrades type I collagen, the major organic component of bone. The importance of cathepsin K in bone resorption is demonstrated by the osteopetrotic phenotype of cathepsin K-deficient mice as well as human mutations leading to pycnodysostosis (Gelb *et al.*, 1996; Saftig *et al.*, 1998). The central role of cathepsin K in bone resorption makes it an attractive target for intervention and thus, several cathepsin K inhibitors are currently in the drug development pipeline for treatment of osteoporosis (reviewed in (Costa *et al.*, 2011)). The role of MMPs in bone degradation is not clear at the moment. Osteoclasts express at least MMP-9 and MMP-14, but their role may be more important in osteoclast migration and initiation of the resorption process than in bone matrix collagenolysis (Delaisse *et al.*, 2003).

In order to maintain the favourable acidic conditions for bone degradation in the resorption lacuna, the ions generated by mineral dissolution and collagen degradation products must be removed. Degraded matrix proteins as well as released calcium-ions are removed by endocytosis at the central ruffled border and delivered to the functional secretory domain for release to the surroundings via transcytotic vesicles (Nesbitt and Horton, 1997; Salo *et al.*, 1997; Mulari *et al.*, 2003; Yamaki *et al.*, 2005). In addition to transcytotic transport, calcium-ions are removed from the resorption lacuna by calcium transporter TRPV5 located at the ruffled border (van der Eerden *et al.*, 2005). Inside the cell, calcium ions are bound by several calcium-binding proteins and extruded from the basolateral plasma membrane that contains Na+/Ca2+ exchangers and Ca2+-ATPases (Bekker and Gay, 1990; Li *et al.*, 2007). Inorganic phosphate is removed from the resorption lacuna by Na-dependent transporters Npt2 and PiT-1 (Gupta *et al.*, 2001; Khadeer *et al.*, 2003). The electroneutral sodium-bicarbonate co-transporter, NCBn1 located at the ruffled border in active osteoclasts plays a major role in removal of the formed bicarbonate at the resorption site to keep the process ongoing (Riihonen *et al.*, 2010).

2.3 RAB PROTEINS

Eukaryotic cells are composed of several membranous organelles (endoplasmic reticulum, Golgi apparatus, plasma membrane, endosomes, lysosomes) whose functions rely on their unique protein and lipid components. However, there is continuous bi-directional membrane exchange between these organelles via vesicular transport. In addition, cells undergo continuous exchange of material (proteins, lipids, sugars) with the extracellular surroundings via secretory and endocytotic pathways. Maintaining the special characteristics of cellular organelles is essential for cell function, a fact that is even more important in cells that display polarised functions. Orchestration of the vesicle traffic is achieved by controlled selection of the cargo from the donor compartment and exact targeting of vesicles to the target organelle.

2.3.2 Introduction to Rab proteins

Rab (Ras-related proteins in brain) proteins are small guanosine nucleotide (GTP)-binding proteins that control all steps of the vesicle transport; vesicle budding and cargo selection from the donor membrane, vesicle movement along the cytoskeletal tracks to the target membrane, the initial recognition of the target membrane (tethering and docking) and the fusion of the vesicle to the target membrane (Hutagalung and Novick, 2011). In addition, the unique localisation of Rab proteins can be used to identify distinct membrane domains in organelles (Pfeffer, 2001).

Rab proteins constitute the largest subfamily of Ras superfamily of small guanosine triphosphatases (GTPases). They guide the vesicular transport by cycling between their active, GTP-bound, and inactive, GDP-bound forms. Rabs are conserved form yeast to man, but the numbers of the isoforms vary from species to species: human has more than 60 known Rab isoforms, while the budding yeast has only 11 (Pereira-Leal and Seabra, 2001). Rab proteins interact with a great variety of proteins that modify their membrane association, GTP hydrolysis or downstream effects.

Rab proteins comprise a typical GTPase fold structure, composed of a six-stranded β-sheet flanked by five α-helixes. Two highly conserved switch regions (Switch I and II) contact the γ phosphate of GTP and display large conformational changes upon nucleotide binding and hydrolysis. The cofactor Mg^{2+} is required for efficient nucleotide binding and hydrolysis. Rab protein – nucleotide interactions occur via G-motifs (G1-G5) that are highly conserved regions throughout the Ras superfamily. Motifs G2 and G3 form the switch I and II regions whose conformations change most significantly upon GTP hydrolysis. Motifs G4 and G5 recognise the nucleotide backbone and discriminate against other nucleotides except guanosine. The structural differences between the GTP- and GDP-bound forms of different Rab isoforms are small, the switch regions being the most affected. These conformational differences arise from small amino acid substitutions in the conserved switch regions as well as in the surrounding hydrophobic core (Gabe Lee *et al.*, 2009; Itzen and Goody, 2011). These differences are the key to the binding of unique set of proteins to a distinct Rab protein that determine its location, activity and downstream effects.

Identification of Rab-specific sequence motifs (RabF1-5) that lie in and around the switch regions can be used to distinguish Rabs from other small GTPases and can be used to identify novel Rabs from sequence databases (Pereira-Leal and Seabra, 2000; Diekmann *et al.*, 2011). Rab proteins can be divided into subfamilies that display similar functions and locations based on the identification of conserved Rab subfamily-specific sequences (RabSF1-4). These sequences form specific interacting surfaces on a Rab bringing the needed specificity for recognition of the Rab-interacting proteins (Pereira-Leal and Seabra, 2000).

The C-terminus of Rab proteins containing a hypervariable domain of 35 to 40 amino acids, has the highest sequence diversity among Rabs. The average identity of this region among Rabs is only 14.4% (Pereira-Leal and Seabra, 2000). The RabSF4 locates in this area and it participates in the binding of effector proteins (Pereira-Leal and Seabra, 2000). The hypervariable domain also contains conserved cysteine residues that are mono- or double prenylated and promote the membrane association of Rabs.

2.3.3 Rab protein prenylation and membrane association

2.3.3.1 Rab proteins are post-translationally modified by prenylation

Soluble protein association with cellular membranes is essential for fulfilling their tasks. Protein prenylation is the most common lipid modification of small GTPases and involves the addition of farnesyl (15-carbon) or geranylgeranyl (20-carbon) lipid moieties to the C-terminal cysteins by thioether linkage. Three enzymes are known to catalyse this process, namely farnesyl transferase, geranylgeranyl transferase I (GGTI) and Rab geranylgeranyl transferase (RGGT). The two former ones modify small GTPases with C-terminal CAAX motif (such as Ras and Rho) where C is cysteine, A aliphatic and X any amino acid. The last one modifies specifically Rab proteins that undergo double prenylation within their CC or CXC motifs (Leung *et al.*, 2006). The substrate for prenylation is provided by the mevalonate pathway (see section 2.4.3).

Rab proteins are not subject for prenylation by RGGT unless they are in complex with Rab escort protein (REP). RGGT is a heterodimeric enzyme that consists of 60kDa α-subunits and 38kDa β-subunits. RGGT binds geranylgeranyl diphospate and REP, but not Rabs. In mammals there are two REP isoforms, REP-1 and REP-2. Both isoforms are ubiquitously expressed, but expression levels vary in different tissues. The Rab binding specificity of REP isoforms may vary, making certain tissues more sensitive to their defects as in case of mutations in REP-1 leading to choroideremia in the eye (Leung *et al.*, 2006). Rab prenylation begins by the recognition of Rab GTPase domain by REP. This interaction is tightened by the binding of the Rab C-terminal hypervariable domain to REP. The resulting complex binds to RGGT via REP-RGGT interactions, which are further tightened by weak, nonspecific interactions of the Rab hypervariable domain and RGGT. These interactions also present the Rab cysteine residues for prenylation by the active site of RGGT. After sequential additions of the two isoprenoids, Rab C-terminus is dislodged from the active site of RGGT and associates with the lipid-binding site of REP (Guo *et al.*, 2008). REP then escorts the prenylated Rab protein to membrane lipid layers.

There is a subset of Rab proteins (Rab8a, Rab13, Rab18, Rab23 and Rab38) that contain only one cysteine in their C-terminal CAAX motif. These Rabs are mono-prenylated by RGGT on their single cysteine followed by the proteolytic cleavage of the AAX peptide and methylation of the terminal cysteine carboxyl group (Joberty *et al.*, 1993; Leung *et al.*, 2007). The exact role for this post-translational modification is unclear, but it was suggested that carboxy methylation would stabilise the Rab:GDI/REP binding by making the C-terminus of a Rab more hydrophobic and thus affect the membrane/cytosol partitioning of these Rabs (Leung *et al.*, 2007).

2.3.3.2 Rab recycling by RabGDI

REP proteins are structurally and functionally related to Rab GDP dissociation inhibitor (RabGDI); they both bind to prenylated GDP-bound Rabs and act as molecular chaperones in the cytosol. They have however, fundamental differences in their actions. REP binds to the GDP-bound Rab protein in its non-, mono – or diprenylated forms, the binding to the diprenylated form being the weakest. GDI does not bind to RGGT and has high affinity to diprenylated Rab proteins, while binding to unprenylated Rab proteins is weak (Wu *et al.*, 2007). This difference explains the differences of functions of these two proteins: REP escorts and presents the unprenylated Rab protein to the RGGT for prenylation, while GDI acts later in the Rab cycle by extracting the diprenylated, inactive Rab protein from lipid bilayers and recycling it back to the donor membrane. RabGDI is formed by two domains that recognise either the Rab GTPase domain in the associated Rab or the Rab hypervariable domain containing the prenyl groups. The mechanism of GDI prenylated Rab extraction from the membranes is not clear, but it has been suggested that the high affinity binding of Rab protein C-terminus and the prenyl groups to the GDI would act as a driving force and allow the extraction of prenylated Rab proteins from lipid bilayers (Goody *et al.*, 2005; Wu *et al.*, 2007).

The δ subunit of retinal rod cGMO phosphodiesterase (PDEδ) is a small 17-kDa protein that is structurally related to the dissociation inhibitors of small GTPases. However, it is lacking GTPase recognising domains and binds only to the C-terminal prenyl groups of great variety of proteins that have C-terminal CAAX-motif and thus binds to small GTPases in nucleotide-independent manner (Nancy *et al.*, 2002). PDEδ is able to exert at least Ras, Rap and Rab13 small GTPases from cellular membranes generating cytosolic pools of these proteins (Marzesco *et al.*, 1998; Nancy *et al.*, 2002). Interestingly, another CAAX-containing Rab, Rab8 did not bind to PDEδ and only Rab13 was extracted from membranes by PDEδ but not by RabGDI (Marzesco *et al.*, 1998).

2.3.3.3 Rab targeting to membranes

Rab prenylation is required for their membrane association and function (Calero *et al.*, 2003). However, this signal is not sufficient to deliver Rabs to their specific locations in intracellular organelles (list of Rab localisations (Hutagalung and Novick, 2011)) and microdomains within an organelle (e.g. Rab7 and 9 are segregated in late endosomes (Barbero *et al.*, 2002)). Rab hypervariable domain displays the highest

level of sequence divergence between Rab family members and was suggested to act as a specific Rab targeting domain (Chavrier *et al.*, 1991; Stenmark *et al.*, 1994). In other studies, it was shown that swapping the hypervariable domains of Rab5a and Rab27a is not sufficient to disrupt their specific targeting to early endosomes and secretory granules, respectively, but required the mutation of the Rab subfamily specific residues (RabSFs) (Ali *et al.*, 2004). Rab targeting is thus a complex process, far from fully understood, and involves several regulatory proteins.

Recycling of the inactive Rab back to the donor membrane occurs via binding to the RabGDI. In order to be able to re-insert the prenyl groups to the lipid bilayer, this high-energy interaction must be disrupted. Small transmembrane PRA1 (Yip3 in yeast) protein was identified as GDI displacement factor (GDF), that is able to interact with several Rabs and dislodge them from RabGDI-complex and thus allow the delivery of the inactive Rab to the donor membrane (Martincic *et al.*, 1997; Hutt *et al.*, 2000; Sivars *et al.*, 2003). PRA1 has broad binding specificity (Bucci *et al.*, 1999; Figueroa *et al.*, 2001), but appears to act catalytically only on endocytotic Rabs (Rab5, 7 and 9) and Rab3 (Hutt *et al.*, 2000; Sivars *et al.*, 2003). PRA1 has one isoform in humans, PRA2, that is able to bind Rabs, but its catalytic activity has not been fully studied (Abdul-Ghani *et al.*, 2001).

An alternative model of GDI displacement includes the activation of Rabs by nucleotide exchange (GDP to GTP by GDP-GTP Exchange Factors, GEFs, discussed in detail in section 2.3.4.1), that weakens the binding affinity of the Rab:GDI/REP complex (Wu *et al.*, 2010). Targeting of Rabs to distinct membrane domains would thus be dependent on the specific array of GEFs present. Upon arrival to the membrane and activation, Rabs bind to effector proteins (discussed in section 2.3.5) that further stabilise Rabs at specific locations in membranes and act downstream of Rabs. It was also suggested that some ‘key’ effector proteins are clustered on specific membrane microdomains where they direct their specific Rabs, as in case of TIP47 and Rab9 (Ganley *et al.*, 2004; Aivazian *et al.*, 2006). Rab targeting would thus also be dependent on the specific effector proteins present.

2.3.4 The guanine nucleotide binding cycle of Rab proteins

The cycling of Rab proteins between their GTP- GDP-bound forms is essential for their function and membrane association. The GTP-bound form is considered the active with downstream effects. Small GTPases in general however, have low intrinsic GTPase activity and high-affinity binding to GDP and Rab GTPases are no exceptions. Thus, they are assisted in their activation by nucleotide exchange GDP to GTP as well as in deactivation by GTP hydrolysis by a great variety of interacting proteins termed guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), respectively. The nucleotide-binding cycle of Rabs is tightly coupled to their membrane association-dissociation cycle.

2.3.4.1 GEFs are positive regulators of Rab proteins

Small GTPases bind nucleotides with high affinity and the GDP release is generally slow. GEFs bind to GDP-bound small GTPases first by low-affinity interaction, which is tightened after the release of GDP. GEFs bind to nucleotide-free Rabs with high affinity. Finally, a binding of a new GTP-molecule to the nucleotide-binding pocket of the Rab is favoured over GDP due to the higher cytosolic content of GTP. GTP-binding then releases the GEF from the small GTPase. GEFs initiate the nucleotide release by disturbing the phosphate- or magnesium-binding site or by altering the switch motifs of the GTPase (Bos *et al.*, 2007; Gabe Lee *et al.*, 2009; Cherfils and Zeghouf, 2013). Rab GEFs are structurally diverse ranging in size and complexity. At the moment four different types of Rab GEFs are known; DENN-domain and Vps9-domain containing Rab GEFs and Sec2 and TRAPP complexes (reviewed in (Hutagalung and Novick, 2011; Cherfils and Zeghouf, 2013)). A recent family wide characterisation of the DENN (differentially expressed in normal and neoplastic cells)-domain containing proteins led to the identification of 17 DENN-domain containing Rab GEFs with unique and non-overlapping Rab targets (Yoshimura *et al.*, 2010). Vps9-domain containing GEFs, on the other hand, regulate mostly the endocytotic Rabs, including the Rab5 subfamily (Carney *et al.*, 2006). The catalytic sites of Sec2 and TRAPP complex are formed by several subunits, although they are otherwise unrelated proteins complexes, initially identified in yeast (Cherfils and Zeghouf, 2013). Mss4 (Mammalian suppressor of yeast Sec4) is a small monosubunit cytosolic protein that displays a weak GEF catalytic activity. It was first thought to act as a GEF, but is currently suggested to function as a chaperone for exocytic Rabs (Nuoffer *et al.*, 1997; Wixler *et al.*, 2011).

2.3.4.2 Rab activity is terminated by GAPs

Although able to bind GTP, most Rab proteins do not hydrolyse it with a speed required by the biological processes they are involved in. Rab GAPs are proteins that catalyse the hydrolysis of GTP and thus switch off Rab function rapidly (Cherfils and Zeghouf, 2013). Most of the known Rab GAPs contain a catalytic TBC (Tre-2, Bub2 and Cdc16) domain and they form a protein family of 44 members. The Rab substrates for approximately half of the TBC-domain GAPs are known and it appears that a single Rab can be deactivated by several TBC-domain GAPs and a TBC-GAP can have several Rabs as substrates (Itoh *et al.*, 2006; Frasa *et al.*, 2012). The mechanism by which TBC-domain GAPs inactivate Rabs differs from other GTPase GAPs; TBC-domain GAPs use arginine and glutamine fingers, that interface with the Rab nucleotide-binding pocket and substitute for a glutamine from the Rab, respectively to mediate the GTP hydrolysis by their substrate Rab (Pan *et al.*, 2006). TBC-domain GAPs can also bind to non-substrate Rabs, which may have impact on their localisation or link them into specific Rab cascades (discussed in section 2.3.5) (Kanno *et al.*, 2010).

2.3.5 Rab effector proteins and Rab cascades

Active, membrane-bound Rab proteins interact with various effector proteins to fulfil their specific functions. By definition, effector proteins bind to Rabs in their active, GTP-bound state and mediate at least one element of its downstream effects (Grosshans *et al.*, 2006). A wide array of Rab effector proteins has been identified to date (Grosshans *et al.*, 2006; Fukuda, 2008; Fukuda *et al.*, 2008; Kanno *et al.*, 2010). The interactions with the effector proteins are mediated by Rab complementary determining regions (CDRs) that locate on the Rab switch and inter-switch areas and partially overlap with the RabSF regions (Ostermeier and Brunger, 1999; Pereira-Leal and Seabra, 2000). A single Rab can interact with multiple different effector proteins and thus act on several distinct transport steps in different cell types or even within a single cell. In addition, several Rabs bind to the same effector protein, which allows these Rabs to function in a coordinated fashion. Rabs also function sequentially and monitor each other's activity by generating so-called Rab cascades. In these cases the effector of Rab A is a GEF for Rab B and activates Rab B or alternatively, Rab B effector is a GAP for Rab A terminating Rab A function (Mizuno-Yamasaki *et al.*, 2012). In the following are examples describing recently identified Rab-effector interactions and potential Rab cascades. More interactions can be found in recent review articles (Grosshans *et al.*, 2006; Stenmark, 2009; Hutagalung and Novick, 2011; Mizuno-Yamasaki *et al.*, 2012).

2.3.5.1 Rab21 sorts integrins to endosomes by direct interaction

Rab21 is an endosomal GTPase that, by direct interaction, regulates the sorting of integrin cargo to endosomes (Pellinen *et al.*, 2006). Although this interaction was recently shown to be independent of the nucleotide-binding status of Rab21, integrin endocytosis and recycling are sensitive to the Rab21 GTP-binding cycle (Pellinen *et al.*, 2006; Mai *et al.*, 2011). Interestingly, Rab21 binding to the integrin is competed by p120RasGAP. Binding to p12RabGAP dislodges the integrins from the Rab21/Rab5-positive endosomes and facilitates their recycling back to the plasma membrane (Mai *et al.*, 2011). Additional factors in this process (e.g. novel Rab21 effectors and p120RasGAP substrates) remain to be identified.

2.3.5.2 Rab6 is involved in several vesicle transport steps

Rab6 interaction with the non-muscle myosin II regulates the fission of Rab6-positive transport carriers from the Golgi. Inhibition of either of the proteins generates large tubular transport carriers that radiate to the cell periphery from the Golgi and are unable to bud off from this compartment (Miserey-Lenkei *et al.*, 2010). Rab6 also regulates the secretory vesicle movement along microtubules to the cell periphery via microtubules by indirect interaction with motor protein kinesin-1 (Grigoriev *et al.*, 2007). In addition, Rab8 recruitment to the exocytotic vesicles is Rab6 dependent, where Rab8 and its binding partner MICAL3 participate in docking and fusion of exocytotic vesicles (Grigoriev *et al.*, 2011). Rab6 is thus present during the whole lifespan of the secretory vesicle.

2.3.5.3 Endosome maturation involves Rab5 to Rab7 switch

Endocytosis is used by the cells to take up nutrients, regulate signalling receptors at the plasma membrane and is often hijacked by intracellular pathogens. Most of the endocytosed material is recycled, but some are directed for degradation in lysosomes. The journey from early endosomes to the late endosomes and lysosomes is called endosome maturation and includes homotypic and heterotypic vesicle fusions (for review see (Huotari and Helenius, 2011)). Rab5 to Rab7 conversion has been shown to play a crucial role in this maturation process and components of the mechanism have been elucidated.

The early endosomal Rab5 is activated in early endosomes by its GEF, Rabex-5. After its activation, Rab5-GTP binds its effectors out of which Rabaptin-5 is recruited to the early endosomes by direct binding to Rabex-5. Rab5 GEF and effector thus form a positive feedback loop to generate active Rab5 clusters on the endosome promoting endosome fusion (Horiuchi *et al.*, 1997). Rabex-5 interacts directly with SAND-1/Mon1 (monesin sensitivity 1), a crucial regulator of Rab7 localisation on endosomes, and recruits it to the membrane. Here, by binding to Rabex-5, SAND-1 disrupts the Rab5 positive feedback loop and recruits Rab7 to the membrane by interacting directly with components of the HOPS complex, a GEF for Rab7 (Poteryaev *et al.*, 2010). Finally, Rab5 is inactivated in late endosomes by TBC-2, a Rab5 GAP whose localisation is strongly dependent on Rab7 (Chotard *et al.*, 2010).

2.4 RAB PROTEINS IN OSTEOCLASTS

The major membrane pathways during osteoclastic bone resorption were resolved during the last decades, but the regulators still remain enigmatic. Rab proteins most likely are components of the vesicle trafficking regulatory mechanism also in the osteoclast. The importance of Rab protein function is already highlighted in osteoclasts treated with selective inhibitor of Rab prenylation (3-PEHPC or NE10790), an analogue of risedronate, that inhibits bone resorption by impairing vesicular trafficking and formation of the ruffled border in osteoclasts without affecting actin organisation (Coxon *et al.*, 2001, 2005). Furthermore, osteoclasts from mice deficient in RGGT and thus Rab geranylgeranylation, display reduced resorption capacity, due to disrupted vesicular trafficking and abnormal ruffled borders, whereas osteoclast survival and actin ring are unaffected (Taylor *et al.*, 2011). The expression of some of the Rab protein family members (Rab1B, 3D, 4B, 5C, 6, 7, 9, 10, 11B and 35) have been demonstrated, but only Rab7 and Rab3d were studied in more detail (Coxon *et al.*, 2001; Zhao *et al.*, 2001, 2002; Pavlos *et al.*, 2005; Ha *et al.*, 2008).

2.4.1 Rab7 is essential for ruffled border formation

The first Rab protein identified in osteoclasts was the late-endosomal Rab7, which is redistributed to the ruffled border upon osteoclast activation and bone resorption from the perinuclear vesicles (Palokangas *et al.*, 1997). Rab7 appears to be a key regulator of the ruffled border formation, as its down-regulation by antisense oligonucleotides

severely impairs bone resorption and osteoclast polarisation and the targeting of the endocytotic vesicles from the basolateral membrane and vacuolar H+-ATPase to the ruffled border (Zhao *et al.*, 2001). Although novel interacting proteins for Rab7 in osteoclasts has been identified, including Rac1, Plekhm1 and Ac45, the whole picture of Rab7 function in bone resorption has remained unclear. Rab7 interacts directly with Rac1, an important regulator of the actin cytoskeleton at the ruffled border area in resorbing osteoclasts. This interaction was postulated to mediate the switch of acidic vesicles from microtubules to actin filaments prior to fusion of these vesicles with the ruffled border (Sun *et al.*, 2005). Rab7 interacts directly with Plekhm1, a negative regulator on the endocytotic pathway, and recruits it to late endosomes and lysosomes in HEK293 cells, Hela cells and osteoclast precursors (Van Wesenbeeck *et al.*, 2007; Tabata *et al.*, 2010). Interestingly, mutations of *Plekhm1* in rat and human have been implicated with osteopetrosis (Van Wesenbeeck *et al.*, 2007; Del Fattore *et al.*, 2008), but the exact function of Plekhm1 in osteoclasts is not clear. Down-regulation of the regulatory subunit of the V-ATPase ATP6ap1 (Ac45) impairs osteoclast differentiation and bone resorption. In particular, the lysosomal trafficking and cathepsin K secretion to the resorption lacuna are diminished. These effects are likely to be mediated by the disrupted Ac45 -Rab7 interaction, as Ac45 was identified as a novel Rab7 effector in osteoclast (Yang *et al.*, 2012). Rab9, another late endosomal protein, localises to the ruffled border in resorbing osteoclasts, but it is distinct from Rab7 (Zhao *et al.*, 2002).

2.4.2 Rab3D is important for bone resorption

Earlier studies demonstrated the expression of Rab3A and Rab3B/C isoforms in osteoclasts, but later, more detailed analysis revealed that Rab3D is the predominant Rab3 isoform in osteoclasts (Abu-Amer *et al.*, 1999; Pavlos *et al.*, 2005). Rab3D knockout mice are osteosclerotic due to resorption defects in osteoclasts and malformed ruffled borders. Rab3D localised in a subset of post-TGN vesicles, reminiscent of secretory granules, that had no obvious polarised targeting towards the ruffled border or basolateral membrane (Pavlos *et al.*, 2005). Rab3D was recently reported to associate with microtubules in osteoclasts via its novel effector, Tctex-1, a light chain of the cytoplasmic dynein microtubule motor complex (Pavlos *et al.*, 2011). Moreover, a decrease in Tctex-1 expression disrupted Rab3D localisation in osteoclasts and resulted in a similar osteoclast phenotype with resorption defects as observed for Rab3D, indicating that Rab3D trafficking pathway is important for osteoclastic resorptive apparatus (Pavlos *et al.*, 2011).

2.4.3 Bisphosphonates and the mevalonate pathway

Bisphosphonates are one of the major antiresorptive drugs currently in clinical use. They consist of two groups; the simple pyrophosphate-resembling bisphosphonates and the more potent nitrogen containing bisphosphonates. Bisphosphonates have high affinity to bone hydroxyapatite and they are absorbed to mineral surfaces very fast after administration, which brings them to close contact with their primary target cell, the osteoclast. Bisphosphonates are released from bone surface by the secreted acid during resorption and taken up by the osteoclast via fluid-phase endocytosis. The transfer from the vesicles to the cytosol requires endosomal acidification (Thompson *et al.*, 2006; Coxon *et al.*, 2008). The ability to resorb bone and thus release the bound

bisphosphonates makes the osteoclast the primary target of these drugs, although there is some indication that other cells capable of efficient endocytosis are also able to internalise bisphosphonates (Coxon *et al.*, 2008; Roelofs *et al.*, 2010). Simple bisphosphonates act as pyrophosphate analogues and are incorporated to ATP. This leads to the accumulation of non-hydrolysable ATP metabolites that inhibit mitochondrial ADP/ATP translocase and thus mitochondrial oxygen consumption and cause osteoclast apoptosis (Selander *et al.*, 1996; Lehenkari *et al.*, 2002).

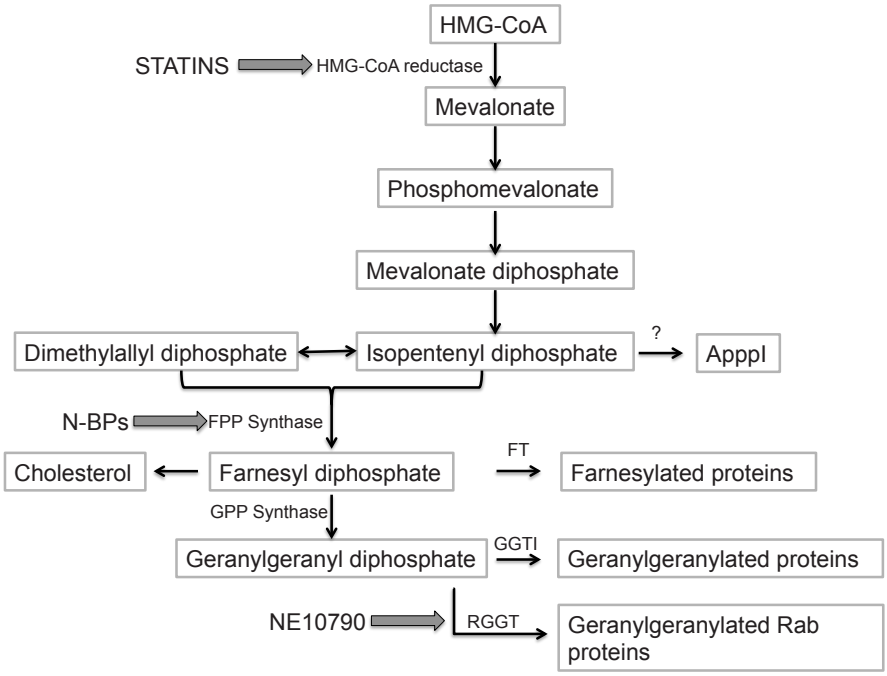

Figure 3: A schematic representation of the mevalonate pathway. The major target enzymes of statins and bisphosphonates are indicated with solid grey arrows. Bisphosphonates are able to inhibit several enzymes of this pathway, but the major target for nitrogen-containing bisphosphonates (N-BPs) is the farnesyl pyrophosphate synthase (FPP synthase). NE10790, a novel risedronate analogue, is a selective inhibitor of the Rab geranylgeranyl transferase (RGGT). Inhibition of the FPP Synthase can also cause an upstream accumulation of an ATP analogue, ApppI that inhibits the mitochondrial ADP/ATP translocase and leads to cell apoptosis. Modified from (Russell, 2011).

Nitrogen-containing bisphosphonates inhibit the mevalonate biosynthetic pathway (Figure 3) that leads to the synthesis of cholesterol and isoprenoid lipids such as farnesyl diphosphate and geranylgeranyl diphosphate that, among others, are important in post-translational modification of proteins including the small GTPases. Approximately 2% of mammalian proteins are prenylated. Statins, widely used cholesterol-lowering drugs, also inhibit the mevalonate pathway. They however, do not have potential as antiresorptive drugs, as they are selectively taken up by liver and do not accumulate in bone as bisphosphonates do (Rogers *et al.*, 2011; Russell, 2011). Osteoclasts treated with alendronate, a nitrogen-containing bisphosphonate, display impaired ruffled border and sealing zone formation and detachment from bone. The

vesicular trafficking is disrupted as demonstrated by the accumulation of vesicles inside the cell and impaired TRACP secretion. In addition, the functional secretory domain is less villous in these cells (Alakangas *et al.*, 2002). Although the major molecular target for nitrogen-containing bisphosphonates is the farnesyl pyrophosphate synthase (FPP Synthase), the loss of geranylgeranylated proteins, including the small GTPases, appear to be the major cause of their inhibitory effects on osteoclasts (Fisher *et al.*, 1999; Coxon *et al.*, 2000; Dunford *et al.*, 2001). Inhibition of the farnesyl pyrophosphate synthase also causes the accumulation Isopentenyl diphosphate, that is further metabolised to ApppI, an ATP analogue, that can cause osteoclast apoptosis (Mönkkönen *et al.*, 2006).

3 AIMS OF THE STUDY

When this study was established, the major vesicular trafficking pathways in bone resorbing osteoclasts had been described: the transcytotic route from the ruffled border to the functional secretory domain, the endocytotic pathway from the basolateral membrane towards the ruffled border and the biosynthetic pathways directed to the bone-antipodal plasma membrane. The specific details of these pathways, including their regulation, were still largely unknown. The presence of only a few members of the Rab small GTPases had been demonstrated in osteoclasts, but most of the currently known over 60 Rabs were not studied. This thesis project was initiated to increase the knowledge of vesicular trafficking events in osteoclasts with specific emphasis on Rab proteins. Better understanding of the regulation of these vesicular transport events in osteoclasts may open possibilities for developing novel therapies for osteoclast-derived disorders, such as osteoporosis. Thus, the following aims were set:

1. To catalogue the Rab GTPases expressed in osteoclasts and identify possible osteoclast-specific Rab proteins.
2. To reveal the localisation and function of Rab13, that had been shown to be highly induced during osteoclastogenesis, in mature osteoclasts.
3. To find novel Rab13 interacting partners in bone marrow environment.
4. To further characterise specific features of the transcytotic route in osteoclasts.

4 MATERIALS AND METHODS

More detailed description of the used materials and methods is available in the original publications (I-III).

4.1 Materials

4.1.1 Antibodies and fluorescent compounds

The primary and secondary antibodies used in this study are listed in Table 1.

Table 1: Antibodies and fluorescent compounds

Antibody	Source	Used in
Goat polyclonal GST	Sigma-Aldrich	II
Mouse monoclonal β-actin	Sigma-Aldrich	II
BD living colours Full-length A.v. rabbit polyclonal	Clontech Takara Bio	II
Ha.11 antibody (clone 16B12)	Covance	II
Rabbit Rab13 antibody	Atlas antibodies	II
Mouse anti-rat b_3-integrin	Dr. M.A. Horton, University College London, UK	I, II
Mouse monoclonal Growth hormone receptor mAb263	Abcam	II
Mouse monoclonal Growth hormone receptor Mab5	Santa Cruz	II
HRP-conjugated anti-mouse and anti-rabbit secondary antibodies	Dako	I, II
HRP-conjugated anti-goat secondary antibody	Zymed Laboratories	II
Alexa Fluor 488-, 546- and 647- conjugated secondary antibodies	Molecular Probes	II, III
Polyclonal rabbit anti-Rab13	Di Giovanni (Di Giovanni *et al.*, 2005)	I
Mouse anti-cathepsin K	Fuji-Chemical industries	I
Monoclonal anti-TRACP antibody DB-13Z1	Kaija (Kaija *et al.*, 1999)	I
Glucose transporter 1	Abcam	I
Glucose transporter 6	Novus Biologicals	I
Mouse monoclonal anti-TGN38	BD Transduction Laboratories	I

Mouse monoclonal anti-EEA1	BD Transduction Laboratories	I
Tetramethylrhodamine-conjugated transferrin,	Molecular Probes	I
Alexa Fluor 488- and 568- conjugated phalloidin	Molecular Probes	I
TO-PRO-3	Molecular Probes	I
FITC- and TRITC-conjugated donkey anti-mouse and anti-rabbit immunoglobulins	Jackson ImmunoResearch Laboratories	I
5-(6)-carboxyfluorescein succimidyl ester	Molecular Probes	I
Polyclonal anti-TRACP	S. Alatalo (Alatalo *et al.*, 2000)	III
Alexa Fluor® 488-conjugated recombinant cholera toxin subunit B	Molecular Probes Europe BV	III
Fluorescently conjugated Helix Pomatia lectin	Molecular Probes Europe BV	III
5-(6)-carboxytetramethylrhodamine succimidyl ester	Molecular Probes Europe BV	III
Vimentin antibody (clone V9)	Sigma-Aldrich	III
α-tubulin antibody (clone DM 1A)	Sigma-Aldrich	III
Fluorescently conjugated WGA-lectin	Sigma-Aldrich	III
Rabbit anti-human transferrin	Zymed laboratories	III
Monodansylcadaverine (MDC)	Sigma-Aldrich	III

4.2 Methods

4.2.1 Cell culture

Human osteoclasts were differentiated from peripheral blood monocytic cells (PBMCs) as previously described (Husheem *et al.*, 2005). Peripheral monocytic cells were isolated from buffy coats or heparinised blood of healthy volunteers by Ficoll-Paque Plus (Amersham Pharmacia Biotech) centrifugation. After several washes with phosphate-buffered saline the cells were plated on devitalised bovine cortical bone slices for differentiation or subjected to further purification by magnetic isolation of CD14-positive PBMCs by positive or negative selection (Miltenyi Biotech).

Primary rat osteoclasts were isolated as described (Lakkakorpi *et al.*, 1989). Osteoclasts were mechanically removed from the long bones of newborn rats and allowed to differentiate on bovine bone slices.

The culture conditions, media compositions and transfection methods of osteoclast and cell line cultures used in this study are listed in Tables 2 and 3, respectively.

Table 2: Osteoclast cultures

Parameter/Ingredient	hOC	hOC(CD14+)	rOC	Supplier
Cells per bone slice	10^6	10^5	-	-
Culture medium	α-MEM	α-MEM	α-MEM	Gibco Invitrogen
iFBS (%)	10	10	10	Gibco Invitrogen
Penicillin (IU/ml)	100	100	100	Gibco Invitrogen
Streptomycin (μg/ml)	100	100	100	Gibco Invitrogen
RANK-L (ng/ml)	20	40	-	PeproTech Inc.
M-CSF (ng/ml)	10	20	-	R&D Systems
Dexamethasone (M)	10^{-8}	-	-	Sigma-Aldrich
TNF-α (ng/ml)	10	-	-	PeproTech Inc.
Culture period (days)	14	10	2	-
Transfection	-	Nucleofection	-	Lonza

hOC, human osteoclast culture; hOC(CD14+), CD14-purified human osteoclast culture; rOC, rat osteoclast culture; α–MEM, alpha-modified minimum essential medium; iFBS, inhibited fetal bovine serum; RANK-L, Receptor activator for nuclear factor kappa-B-ligand; M-CSF, macrophage-colony stimulating factor; TNF-α, Tumor necrosis factor- alpha.

Table 3: Cell lines and transfections

Parameter/ Ingredient	Cos1	Hela	LNCaP	Supplier
Culture medium	DMEM	DMEM	RPMI	Gibco Invitrogen/ Lonza
iFCS	10%	10%	15%	Gibco Invitrogen
Penicillin (IU/ml)	100	100	100	Gibco Invitrogen
Streptomycin (μg/ml)	100	100	100	Gibco Invitrogen
Glutamax (mM)	2	2	2	Gibco Invitrogen
Na-puryvate (mmol/l)	-	-	1	Lonza
Hepes (mM)	-	-	20	Lonza
Transfection reagent	Fugene 6 (Roche)	Lipofectamine 2000 (Invitrogen)	Nucleofection (Lonza)	

DMEM, Dulbecco´s modified eagle medium; RPMI, Roswell Park memorial institute medium; iFCS, inhibited fetal calf serum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

4.2.2 Eukaryotic and bacterial expression vectors and siRNA molecules (I, II)

Eukaryotic and bacterial expression vectors were constructed by restriction cloning into the multiple cloning sites of the original vectors. The inserts were produced by PCR using gene specific primer pairs with appropriate restriction sites. After ligation with T4 ligase enzyme, the ligated vector was transformed into bacterial hosts (BL21 for protein production; JM109 for production of the plasmid) and bacteria were grown under antibiotic selection. Plasmids were isolated with commercial plasmid

purification kits (Sigma or Macherey-Nagel). Bacterial protein expression was induced with Isopropyl-b-D-1-thiogalactopyranoside (IPTG) for 1-2 hours. After collection of the cellular pellets, the cells were lysed by sonication in the binding buffer. The constructed bacterial and eukaryotic expression vectors as well as used siRNA molecules are listed in Table 4.

Table 4: Expression vectors

Plasmid construct/ siRNA molecule	**Original plasmid /target sequence**	**Supplier**	**Method**
pBT-Rab13WT	pBT	Stratagene	Bacterial 2-hybrid
pBT-Rab13Q67L	pBT	Stratagene	Bacterial 2-hybrid
pBT-Rab7	pBT	Stratagene	Bacterial 2-hybrid
pTRG-Bone marrow library	pTRG	Stratagene	Bacterial 2-hybrid
GST-Rab7	pGEX-4T-1	Amersham Biosciences	Pull-Down
GST-Rab11	pGEX-4T-1	Amersham Biosciences	Pull-Down
GST-Rab13	pGEX-4T-1	Amersham Biosciences	Pull-Down
GST-Rab32	pGEX-4T-1	Amersham Biosciences	Pull-Down
MBP-Endo-1C	pMAL-c2e	NEB	Pull-Down
MBP-Endo-2C	pMAL-c2e	NEB	Pull-Down
GFP-Rab13WT	pEGFP-Actin	Clontech Laboratories Inc.	Pull-Down, Co-IP, IF
GFP-Rab13Q67L	pEGFP-Actin	Clontech Laboratories Inc.	Co-IP
GFP-Rab13T22N	pEGFP-Actin	Clontech Laboratories Inc.	Co-IP
GFP-Rab32	pEGFP-Actin	Clontech Laboratories Inc.	Co-IP
GFP-Rab8	-	Yi Sun (Sun *et al.*, 2010)	Pull-Down
GFP-Rab10	-	Yi Sun (Sun *et al.*, 2010)	Pull-Down
Endospanin-2HA	pcDNA3.1(Neo)(+)	Invitrogen	Co-IP, IF
Human Rab13 siRNA	cagggcaaacataaatgtaaa	Qiagen	IF, FACS
Neg. control siRNA	Scrambled sequence	Qiagen	IF, FACS

4.2.3 Protein analyses (I-III)

The used protein analysis methods are listed in Table 5. Specific details can be found in the original articles (I-III).

Table 5: Protein analysis methods

Method:	Used in
Direct immunofluorescence labelling	I, III
Indirect immunofluorescence labelling	I-III
Immunohistochemistry	I
Western-transfer analysis	I, II
Bacterial two-hybrid screen	II
In vitro protein pull-down	II
Co-Immunoprecipitation	II
Flow cytometry	II
Scanning electron microscopy	III

4.2.4 Microscopy (I-III)

The immunofluorescence analysis was done by viewing more than 20 cells in at least two independent experiments. The analysis for most of the markers was made for both human and rat osteoclasts, but mostly, the results are shown for one species only. Pearson's correlation coefficient was determined with ImageJ software. Images were processed with Adobe Photoshop 7.0, CorelDRAW 9.0 and ImageJ software. Table 6 lists the microscopes used in this study.

Table 6: List of used microscopes

Microscope	Used in
Olympus 1X71 microscope with Cell R^ 2.6 software	I
Leica TCS-SP confocal microscope	I-III
Leica DMRB microscope	I
JEOL JSM-6300F scanning electron microscope	III

4.2.5 RNA analyses (I,II)

The RNA methods used in this study include RNA isolation, reverse transcription, polymerase chain reaction (PCR), real-time PCR and RNA interference. Specific details can be found in the original articles (I, II).

The gene specific primer pairs were designed to Rab mRNA sequences found in two publicly available international databanks: NCBI GeneBank and Ensembl using FastPCR primer design program (Kalendar, 1999). All PCR products with the expected size were confirmed by sequencing at the core sequencing laboratories of the Turku University Genetics Institute or the Center for Biotechnology (Turku, Finland). Real-time PCR experiments were analysed using the relative real-time quantitative PCR analysis $2^{-\Delta\Delta Ct}$-method described by Livak (Livak and Schmittgen, 2001). Greater than 2-fold changes in gene expression were considered significant.

4.2.6 Software and statistical analyses (I, II)

The densitometric analysis of co-immunoprecipitation data was done with ImageJ software. Statistical analyses were performed using two-tailed, paired Student's t-test with P-values of 0.05 or less considered statistically significant. Statistical analyses were made with Origin software (OriginLab).

Prediction of endospanin-2 transmembrane domains was made using TMHMM software (Möller *et al.*, 2001) and the schematic drawn with DOG 2.0 software (Ren *et al.*, 2009).

5 RESULTS

5.1 Osteoclasts express several *Rab* genes (I)

To gain more insight to the possible role of Rab proteins regulating the vesicular transport in osteoclasts, we catalogued the expression of 43 selected *Rab* genes in bone-resorbing human osteoclasts by gene specific primer pairs. We identified the expression of 26 *Rab* genes, while the expression of 17 were not detected. *Rab*7, *13* and *32* genes were chosen for further expression analysis during osteoclastogenesis due to their previously described interesting functions in other cell types. Rab7 is a late-endosome- and lysosome associated Rab protein that is essential for ruffled border formation in osteoclasts and is directed to the ruffled border in bone resorbing cells (Palokangas *et al.*, 1997; Zhao *et al.*, 2001). In our studies its expression displayed only slight changes during osteoclast differentiation from CD14-positive monocytic cells. Rab32 is a recently described Rab known to participate in melanosome transport and mitochondrial dynamics (Alto *et al.*, 2002; Wasmeier *et al.*, 2006). Since osteoclasts contain abundant mitochondria, we followed the expression of *Rab32* during osteoclastogenesis. The expression of *Rab32* was up-regulated approximately 2-fold, but the expression profile changed depending on the osteoclast differentiation stage, unlike that of *Rab7*. Rab13 is a tight junction-associated protein in polarised epithelial cells (Zahraoui *et al.*, 1994). Because we detected *Rab13* expression in osteoclasts in our initial PCR screen, we anticipated a related function for it in osteoclasts regulating the formation and maintenance of the sealing zone and thus the attachment of osteoclast to the bone surface. Interestingly, the expression levels of *Rab13* were induced 20-fold during the osteoclast differentiation. The signal was almost non-detectable at day 0, but increased already at day 3. Mature, multinucleated osteoclasts appear and start bone resorption at day 5 in this culture system.

5.2 Intracellular localisation of Rab13 in osteoclasts (I)

The subcellular localisation of Rab13 was studied using purified rabbit polyclonal anti-Rab13 antibody provided by Dr. S. Di Giovanni and Dr. Alan Faden (Di Giovanni *et al.*, 2005). This antibody detects one major band of 25 kDA in several rat tissues and recognises only bacterially expressed Rab13, but not Rab7, 11 or 32 of bacterially expressed fusion proteins. In Hela cells this antibody labelled numerous cytoplasmic vesicles as well as cell-cell contacts, which were congruent with previous reports of Rab13 localisation (Zahraoui *et al.*, 1994; Marzesco *et al.*, 2002; Wu *et al.*, 2011). In bone-resorbing osteoclasts this Rab13 antibody detected numerous small vesicles throughout the cytoplasm.

Confocal microscope analysis of Rab13 with several different known osteoclastic markers revealed that Rab13-positive vesicles are small and they are mostly located at the superior perinuclear and peripheral parts of osteoclasts. Rab13-positive vesicles did not associate with the sealing zone actin ring visualised by phalloidin staining, suggesting that it is not involved in sealing zone formation. Rab13 involvement in the

resorption process, the secretion of lysosomal enzymes to the resorption lacuna and transcytosis, was studied by double-staining experiments with cathepsin K and TRACP antibodies and in osteoclasts grown on fluorescently labelled bone. None of these markers co-localised with Rab13-positive vesicles, indicating that Rab13 is not involved in the transport of cathepsin K- or TRACP-containing lysosomal vesicles to the ruffled border or in the transcytosis of the bone degradation products.

Osteoclasts have unique endosomal route directed from the basolateral, bone-antipodal plasma membrane to the ruffled border (Palokangas *et al.*, 1997). This route can be demonstrated by incubation of osteoclasts with iron-loaded transferrin, a commonly used marker for the endosomal recycling pathway. Transferrin is endocytosed at the basolateral plasma membrane and reaches the perinuclear recycling compartment after 10 min of incubation. After 30 min of incubation, abundant transferrin is visible at the peripheral subdomain of the ruffled border, which most likely represents the transferrin directed to lysosomal degradation in other cell types. After longer incubation times, transferrin is also detected at the functional secretory domain (Palokangas *et al.*, 1997; Mulari *et al.*, 2003). To study if Rab13 is regulating one of the transferrin transport steps in osteoclasts, bone-resorbing osteoclasts were incubated with TRITC-labelled human transferrin for 60 min to cover the entire transferrin transport route and labelled with Rab13 antibody. Transferrin was detected in early endosomes close to the basolateral plasma membrane, the perinuclear recycling compartment, at the ruffled border and the functional secretory domain. However, none of these vesicular compartments were labelled with the Rab13 antibody. Moreover, Rab13-positive vesicles were not labelled with Early endosomal antigen-1 (EEA1) antibody, a marker for early phases of endosytosis and a well-known Rab5 effector protein. These results suggest that Rab13 is not associated with early or recycling endosomes in bone resorbing osteoclasts. Interestingly, EEA1-labelled early endosomes were differently distributed in resorbing and non-resorbing osteoclasts. In resorbing osteoclasts these endosomes were located peripherally, while in nonresorbing osteoclasts they were more perinuclear. Rab13-positive vesicles displayed similar distribution in active and inactive osteoclasts, but the difference was not as clear as was observed for EEA1-labelled vesicles.

Bone resorption is a highly energy consuming process and the principal energy source for osteoclasts is glucose (Williams *et al.*, 1997). Since Rab13 was recently reported to regulate the translocation of the insulin-sensitive glucose transporter Glut4 in L6 muscle cells (Sun *et al.*, 2010), we investigated if Rab13 is involved in glucose transport in osteoclasts. We were not able to detect the expression of Glut4 in osteoclasts (data not shown), but osteoclasts and osteoclast-like cells have been reported to express Glut1 (Kim *et al.*, 2007; Knowles and Athanasou, 2008; Indo *et al.*, 2013). In bone-resorbing osteoclasts Glut1 was located mainly on the plasma membrane, but was also detected on intracellular vesicular structures. These vesicles were, however, completely devoid of Rab13 labelling. We also studied the co-localisation of Rab13 with Glut6, a glucose transporter originally described in leukocytes (Doege *et al.*, 2000). Glut6 was localised in small intracellular vesicles through the osteoclasts, but these vesicles were distinct from Rab13 vesicles.

The biosynthetic route in osteoclasts can be demonstrated by TGN38-labelling. TGN38 is an integral membrane protein that predominantly locates in the trans-Golgi

network (TGN), but is also cycled to the plasma membrane in other cell types except osteoclasts (Palokangas *et al.*, 1997). Rab13 and TGN38 antibodies labelled small vesicles in the osteoclasts. A weak co-localisation of these two markers was observed in a few vesicles per cell.

Zona occludens-1 (Zo-1) is a scaffolding protein linking transmembrane proteins to the actin cytoskeleton and co-localises with Rab13 in tight junctions of polarised epithelial cells (Zahraoui *et al.*, 1994). To see if these two proteins are co-localised in polarised osteoclasts, we double labelled osteoclast grown on bone slices with antibodies against Rab13 and Zo-1 (Figure 4). Zo-1 antibody labelled small vesicles in the cytoplasm, the nuclear envelope and the sealing zone in kidney-shaped bone-resorbing osteoclasts. Rab13 and Zo-1 displayed partial co-localisation in few vesicles in these cells. Interestingly, also occludin, an integral plasma membrane protein at tight junctions had similar distribution to Zo-1 at the sealing zone and intracellular vesicles. Rab13 and occludin co-localisation was not studied due to lack of proper antibodies.

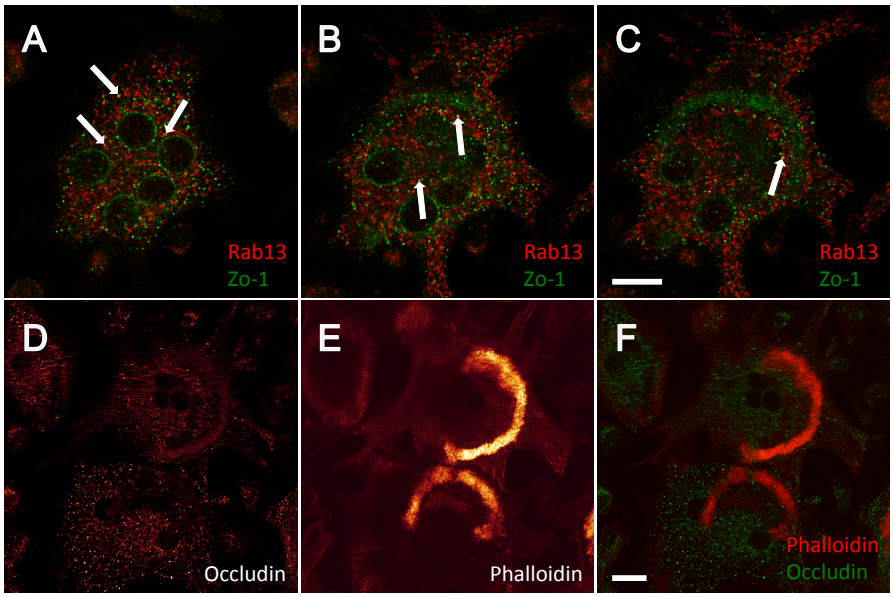

Figure 4: Human osteoclasts cultured on bovine bone and labelled for Rab13 (red) and Zo-1 (green) (A-C) or occludin (green) and phalloidin (red) (D-F). Nuclear level (A) and two sections at the sealing zone (B, C) are shown. Zo-1 localises to intracellular vesicles, nuclear envelope and sealing zone. Arrows indicate vesicles with both markers. One section at the sealing zone level of a resorbing osteoclast (D-E). Occludin (D) localises in intracellular vesicles and sealing zone marked by the dense actin bundles labelled by phalloidin staining (E). Scale bar 10μm.

5.3 Rab13 down-regulation does not inhibit osteoclastogenesis (I)

The importance of Rab13 expression in osteoclast differentiation was examined by silencing its expression in osteoclast precursors with specific siRNA molecules

introduced to cells by electroporation (Taylor *et al.*, 2007). Inhibition of Rab13 by siRNAs abolished the marked induction of *Rab13* expression observed during osteoclast differentiation whereas treatment with negative control siRNA molecules had no effect on Rab13 mRNA expression. With siRNAs, a minor tendency towards a reduced number of multinucleated osteoclast with actin rings was observed, but the difference did not reach statistical significance. On the other hand, cells transfected with Rab13 siRNAs exhibited reduced viability, which may partly explain the observed differences in osteoclast numbers. Thus, the impairment of Rab13 expression has only nominal - if any - effects on the differentiation and actin ring formation of osteoclasts.

5.4 Rab13 is expressed in osteoclasts *in vivo* (I)

Since Rab13 was detected in osteoclasts under culture conditions, it was imperative to study its expression in osteoclasts also *in vivo*. For this purpose sequential sections of newborn rat long bones were immunostained for Rab13 and cathepsin K to identify osteoclasts. Bone trabeculae were rich in osteoclasts with strong cathepsin K staining through the cell, including the ruffled border. These osteoclasts were Rab13 positive, but the labelling was not osteoclast specific; bone marrow mononuclear cells and cells lining the bone trabeculae were also positive for Rab13 staining. Congruent with confocal microscope studies, Rab13 staining was located at the superior and medial parts of the osteoclast, while the ruffled border facing the bone was almost free of Rab13. Rab13 protein was also detected in affinity-purified osteoclasts as well as in other cells of the bone marrow after immunoblotting the whole cell lysates of these cells.

5.5 Endospanin-2 is a novel Rab13-binding protein (II)

To get more insight into the possible function of Rab13 in osteoclasts, we screened a newborn rat bone marrow cDNA library (Sun *et al.*, 2005) in bacterial two-hybrid system for novel Rab13 interaction partners. The screen consisted of approximately $2x10^6$ transformants and yielded 1889 clones that demonstrated positive growth. Further validation of the interactions by retransformation of purified target plasmids with the bait plasmids, resulted in a candidate clone present in several isolates. This target plasmid demonstrated bacterial growth when co-transformed with wild type Rab13 (Rab13WT) and dominant active mutant Rab13 (Rab13Q67L), whereas co-transfectants with empty bait plasmid or plasmid encoding Rab7 did not grow, indicating that the interaction is Rab13 specific. Sequencing of the clone revealed that it contained a 353-base pair insert encoding 20 amino acids identical to the C-terminal amino acids of endospanin-2. Endospanin-2 is a small transmembrane protein with four hydrophobic transmembrane domains, intracellular N- and C-termini and short extracellular loops (Huang *et al.*, 2001; Séron *et al.*, 2011). The 20 C-terminal amino acids identified as Rab13-binding belong to the last transmembrane domain and the C-terminal intracellular tail. Endospanin-2 has one homologue in mammals, endospanin-1 sharing 67% homology in human (Huang *et al.*, 2001). Endospanin-2 and endospanin-1 were previously named as Leptin receptor overlapping transcript like-1 (LeprotL1) and Obesity gene-related protein (Ob-rgrp or Leptin receptor overlapping transcript, Leprot), respectively (Séron *et al.*, 2011).

5.6 Rab13 and Rab8 bind to endospanins *in vitro* (II)

The bacterial two-hybrid interaction of Rab13 and endospanin-2 was confirmed with bacterially expressed recombinant proteins in pull-down assays. The last 20 C-terminal amino acids of endospanin-2 and endospanin-1 (Endo-2C and Endo-1C, respectively) fused to the C-terminus of maltose binding protein (MBP) were used as baits. Glutathione-S-transferase (GST)- fused Rab13 bound to MBP-Endo-2C and MBP-Endo-1C, while no binding to the negative controls, including MBP and empty resin beads, was observed. Moreover, GST-fused Rab7, Rab11 or Rab32 did not bind to endospanin-2. Bacterially expressed MBP-endospanins also precipitated endogenous Rab13 from Hela cell lysates. Both endospanins also precipitated green fluorescent protein (GFP)- tagged Rab13 and Rab8 from transfected Cos1 or Hela cell lysates, while GFP-Rab10 did not bind to either of the bait proteins. Thus, endospanins-1 and -2 form a novel Rab13 and Rab8 interacting protein family.

5.7 Rab13-endospanin-2 interaction in the cellular environment (II)

The interaction of Rab13 with endospanin-2 was further confirmed in cellular systems. For this purpose we generated a eukaryotic expression vector for full-length endospanin-2 with a human influenza hemagglutinin (HA)-tag inserted to the first extracellular loop of endospanin-2. After transient transfection of Cos1 cells with different combinations of GFP-tagged Rab proteins together with endospanin-2HA, protein complexes were immunoprecipitated with an anti-GFP antibody. Endospanin-2HA bound to GFP-Rab13WT, but not to GFP-Rab32 or negative control. The binding of effector proteins to Rab proteins is usually nucleotide-dependent (Grosshans *et al.*, 2006). Rab13 wild type and the dominant active mutant (GFP-Rab13Q67L) co-precipitated endospanin-2HA in a similar manner. The binding of endospanin-2HA to the dominant inactive mutant of Rab13 (GFP-Rab13T22N) was observed to be weaker, suggesting that the full-length endospanin-2HA binds to Rab13 in a nucleotide dependent manner.

The intracellular localisation of endospanin-2HA and GFP-Rab13WT was studied in double transfected Cos1 cells. Endospanin-2HA demonstrated a punctate vesicular staining throughout the cell with abundant labelling at the perinuclear compartments. Minor vesicular-type labelling was also observed near the plasma membrane. GFP-Rab13WT also localised to perinuclear vesicles, but was also clearly visible at the plasma membrane. In addition, a reticular, ER-type labelling pattern was observed for both proteins. GFP-Rab13WT and endospanin-2HA co-localised in vesicular structures, although the overlap was not complete. Co-localisation was strongest at the perinuclear vesicles and weaker at the cell periphery.

To test if Rab13 knockdown by specific RNA interference has an effect on endospanin-2HA localisation, Hela cells were double transfected with Rab13 or negative control siRNA molecules and the expression vector encoding endospanin-2HA. A reduction of 80% in endogenous Rab13 protein levels was observed after Rab13 siRNA treatment. In control siRNA-treated cells endospanin-2HA was localised in vesicles across the cell, the strongest labelling found at the peripheral structures, with some vesicles visible also at the cell periphery. The Rab13 siRNA-treated cells showed similar distribution of endospanin-2HA labelling and no apparent

differences were detected compared to the negative control siRNA-transfected cells. In addition, morphological transport assays, where transfected cells were incubated at 20 degrees Celsius to block the transfer of proteins from TGN and then allowed to recover at 37 degrees for various time periods, could not differentiate Rab13 siRNA-treated cells from control siRNA-treated cells (not shown). In antibody uptake experiments, only minute amounts of endocytosed endospanin-2HA were detected (not shown).

Endospanins are reported as negative regulators of cell surface expression of growth hormone receptor (GHR) and Leptin receptor (LEPR) (Couturier *et al.*, 2007; Touvier *et al.*, 2009; Séron *et al.*, 2011). To test if Rab13 is involved in the regulation of GHR, the cell surface expression of GHR was studied in LNCaP prostatic cancer cell line that has functional endogenous GHR and growth hormone signalling pathways (Weiss-Messer *et al.*, 2004). Immunoprecipitation of GHR first confirmed the expression of the receptor in these cells. Rab13 siRNA treatment downregulated Rab13 protein expression by 80% in LNCaP cells. Labelling with GHR antibody (mAb263) and flow cytometry analysis of the cell surface GHR was not able to differentiate Rab13 siRNA-treated cells from control siRNA-treated cells, indicating that the Rab13-endospanin-2 interaction may have functions other than regulating the cell surface GHR. Similar results were obtained from growth hormone-treated LNCaP cells (data not shown). The observed fluorescence intensities were similar to the intensities reported previously in HEK293 cells overexpressing GHR (Milward *et al.*, 2004).

5.8 Osteoclasts express *endospanin-2* (II)

Since the Rab13-endospanin-2 interaction was initially found in a mixed cell population of the bone marrow, we next studied the possibility of its presence also in osteoclasts. Due to the lack of functional antibodies against endogenous endospanin-2, we analysed its expression at mRNA level in affinity purified primary rat osteoclasts with gene specific primer pairs. The isolation of rat osteoclasts was successful as indicated by the enrichment of cathepsin K mRNA. Endospanin-2 mRNA was expressed in osteoclasts; but its expression was not restricted to them.

5.9 Osteoclast transcytotic vesicles contain HPA-binding sites (III)

Helix Pomatia (HPA) lectin binds to terminal alpha- N-acetylgalactosamine (GalNAc)-residues that are increased in cancer cells with metastatic competence (Brooks, 2000). Osteoclasts, highly invasive cells, penetrating to the bone matrix during physiological bone resorption are also labelled with HPA-lectin (CZ Zhang *et al.*, 1994). Since the role of GalNAc-residues in bone resorption is unknown at the moment, the distribution of HPA-binding sites in bone-resorbing osteoclasts was analysed. In rat and human osteoclasts, HPA bound to numerous vesicular structures across the cells. In human osteoclasts HPA-labelling was observed also at the ruffled border, while in rat osteoclasts the ruffled border was almost devoid of HPA-labelling. In osteoclasts cultured on pre-coated bone, HPA-labelling was observed in vesicles containing fluorescently labelled bone and TRACP-5b, indicating that osteoclast transcytotic vesicles contain GalNAc-binding sites. HPA-labelling was also present in vesicles containing cathepsin K and TRACP, two established transcytotic

markers (Vaaraniemi *et al.*, 2004). The association of used markers, TRACP, cathepsin K and HPA with the vesicles containing labelled bone showed no vertical polarisation and they could be detected at all levels of the cell. However, part of the vesicles containing labelled bone particles were devoid of the other markers used, TRACP, cathepsin K and HPA. This may suggest the existence of multiple transcytotic routes.

To study the presence of HPA-binding sites on the endocytotic route directed to the ruffled border from the bone-antipodal basolateral plasma membrane (Salo *et al.*, 1996; Palokangas *et al.*, 1997), osteoclast were incubated with human transferrin for 60 min prior to their fixation and labelling for HPA. Only minimal HPA-labelling of transferrin-containing vesicles was observed, indicating that HPA-binding sites dissociate from the transferrin recycling vesicles travelling between the ruffled border and the basolateral plasma membrane.

5.10 TRACP on the ruffled border (I, III)

TRACP, the acid phosphatase used as an osteoclastic marker, is typically located in the large transcytotic vesicles containing the bone degradation products (Halleen *et al.*, 1999; Vaaraniemi *et al.*, 2004). TRACP has also been repeatedly reported to localise in the lower parts of the osteoclasts and to be secreted to the resorption lacuna (Reinholt *et al.*, 1990; Nakayama *et al.*, 2011; van Meel *et al.*, 2011). In our studies, we observed the labelling of the osteoclast ruffled border with two distinct TRACP antibodies. Moreover, TRACP co-localised with the recombinant cholera toxin B-subunit (recChlTx-B) and β_3-integrin, two previously described markers of the ruffled border fusion zone (Mulari *et al.*, 2003), further supporting its secretion to the resorption lacuna via the ruffled border.

5.11 Association of the lipid rafts and TRACP along the transcytotic route (III)

Since osteoclast-mediated bone resorption and the biosynthetic delivery of HA from the Golgi to the functional secretory domain are sensitive to the integrity of the lipid raft domains (Mulari *et al.*, 2008), we speculated that lipid rafts might also be important in the transcytosis of bone degradation products. Osteoclasts were cultured on rhodamine-coated bone slices, fixed and labelled with antibodies against TRACP and recChlTx-B. Eleven randomly selected osteoclasts were analysed by scanning 10 sections with equal intervals across the cells with confocal microscope. Out of the 723 analysed bone-containing transcytotic vesicles 44.3% bound recChlTx-B but not TRACP, 17.6% were TRACP positive but devoid of recChlTx-B and 17.0% contained both markers. 21.2% of the bone-containing vesicles co-localised with neither of the used markers. The vertical analysis of these transcytotic vesicles revealed no departure from this variation, and all vesicle populations were detected at all levels of the cells. These results do not support the commonly held idea of vesicle maturation along the transcytotic pathway; instead they may suggest that several distinct vesicle populations rise from the ruffled border.

5.12 The role of $\alpha_v\beta_3$–integrin in the transcytosis of bone degradation products (III)

Since the α_vb_3-integrin localises, among others, to the ruffled border and intracellular vesicles in bone resorbing osteoclasts and has been demonstrated to bind to denatured collagen (Lakkakorpi *et al.*, 1991; Helfrich *et al.*, 1996; Faccio *et al.*, 2002), we studied its presence in the transcytotic vesicles. Rat osteoclasts were cultured on bone slices pre-coated with tetramethylrodamine or recombinant osteopontin and labelled for $\alpha_v\beta_3$-integrin. We found that osteopontin was transcytosed from the ruffled border to the functional secretory domain in $\alpha_v\beta_3$-positive vesicles, whereas tetrametylrhodamine-labelled bone vesicles were separate from $\alpha_v\beta_3$-integrin vesicles. These results may suggest that the selection of cargo molecules into distinct vesicular pathways occurs already at the ruffled border.

5.13 Autophagosomal machinery along the transcytotic pathway (III)

Autophagosomal proteins were recently reported to regulate the formation of the ruffled border in osteoclasts (DeSelm *et al.*, 2011). Although osteoclasts were rich in autolysosomal structures, these structures were not detected in the vicinity of the ruffled border but at the medial and superior levels of the cell (DeSelm *et al.*, 2011). The co-localisation of autophagosomes with the transcytotic vesicles was studied in osteoclasts cultured on fluorescein-coated bone slices and labelled with monodansylcadaverine (MDC), a marker for autophagosomes (Biederbick *et al.*, 1995), and antibodies against TRACP and cathepsin K. MDC labelled vesicular structures that also contained degraded bone, TRACP and cathepsin K. MDC and TRACP co-localised in vesicles at the medial and superior parts of the cell, while the ruffled border was absent from MDC-labelling. This finding suggests that the osteoclast transcytotic route and autophagic vacuoles may converge.

5.14 A molecular barrier between the functional secretory domain and the basolateral plasma membrane (III)

Previous studies have described a strictly demarcated border between the HA-labelled functional secretory domain and the peripheral bone-antipodal plasma membrane in 66.7% (n=120) of the resorbing osteoclasts (Mulari *et al.*, 2008). In 31.7% of these resorbing cells, no clear borderline is observed, but the budding virions are directed towards the functional secretory domain. In the remaining part of the cells (1.6%), HA is scattered throughout the bone-antipodal plasma membrane. Similar subsets of osteoclasts have been also reported when the villous morphology of the functional secretory domain was analysed with scanning electron microscope (Salo *et al.*, 1996). In several independent experiments, we observed a thin actin circle structure at the top of a subset of resorbing osteoclasts. WGA-lectin, that binds to the transcytotic degraded bone matrix (Salo *et al.*, 1997) accumulated inside this actin structure. Only few vimentin filaments were present at this area, but microtubules were abundant. These findings suggest that bone-resorbing osteoclasts have a molecular barrier encircling the functional secretory domain. This barrier is formed by actin filaments and other – yet unidentified – components.

6 DISCUSSION

6.1 Expression of *Rab* genes in osteoclasts

Osteoclasts are polarised cells with highly specialised membrane domains that undergo continuous exchange of material during bone resorption. Careful balancing of the vesicular trafficking is essential to preserve the specialised membrane domains and osteoclast function. Rab proteins are attractive candidates for providing such regulation due to their role as molecular switches during vesicular transport in other cell types. The presence of some Rabs has already been described in osteoclasts, namely Rab1B, Rab3A/B, Rab3D, Rab4B, Rab5C, Rab6, RAb7, Rab9, Rab10, Rab11B and Rab35 (Abu-Amer *et al.*, 1999; Coxon *et al.*, 2001; Zhao *et al.*, 2001, 2002; Pavlos *et al.*, 2005; Ha *et al.*, 2008). Our screen with gene specific primer pairs for 43 Rab genes demonstrated the expression of 26 *Rab* genes in human osteoclasts differentiated from peripheral blood monocytes. Among these, 23 were novel, previously unidentified in osteoclastst, while the detection of the remaining three, *Rab7, Rab9* and *Rab10*, confirmed the previous descriptions. Later, the expression of *Rab38* in osteoclasts was reported, congruent with our results (Charles *et al.*, 2012). Further expressional profiling of selected Rabs during osteoclast differentiation revealed that the expression of *Rab7*, a Rab essential for ruffled border formation and bone resorption (Zhao *et al.*, 2002), remained stable during the differentiation, indicating that Rab7 is re-targeted to the ruffled border from late endosomes upon osteoclast activation (Palokangas *et al.*, 1997). The expression of *Rab32* and *Rab13*, however, were induced two- and twentyfold, respectively. A computer-based analysis of the promoter region of *Rab13* revealed that it contained a highly conserved binding site for the transcription factor NFATc1, a master regulator of osteoclast differentiation, induced by RANKL (data not shown).

6.2 Rab13 in osteoclasts

Rab13 is previously described to localise to and regulate the formation and maintenance of tight junctions in polarised epithelial cells (Zahraoui *et al.*, 1994; Marzesco *et al.*, 2002), but was recently reported to have more versatile functions in different cell types. The Rab13-mediated regulation of tight junctions in epithelial cells is comprised of several different mechanisms, including endocytotic recycling of the tight junctional proteins claudin-1 and occludin as well as remodelling of the actin cytoskeleton via direct interactions with JRAB/MICAL-L2 and the catalytic subdomain of protein kinase A that associate with actin remodelling proteins Actinin-4 and vasodilator-stimulated phosphoprotein (VASP), respectively (Yamamoto *et al.*, 2003; Kohler *et al.*, 2004; Morimoto *et al.*, 2005; Nakatsuji *et al.*, 2008; Nokes *et al.*, 2008). Rab13 also regulates the neurite outgrowth in regenerating neurons (Di Giovanni *et al.*, 2005), VEGF- induced directional migration in endothelial cells (Wu *et al.*, 2011) and promotes glucose transporter 4 (GLUT4) trafficking to the cell surface in L6-myoblasts upon insulin stimulus (Sun *et al.*, 2010).

We detected the expression of Rab13 in human osteoclasts at mRNA and protein level *in vitro* and in rat osteoclasts also *in vivo*. The expression was not, however restricted to osteoclasts in the bone environment; *Rab13* is also expressed in osteoblasts, adipocytes, macrophages, endothelial cells and dentritic cells (data not shown; Vuorikoski H, unpublished data). Since osteoclasts and epithelial cells are highly polarised cells, we speculated an analogous function for Rab13 in these two cell types and the possibility of Rab13 to regulate the sealing zone and thus the tight attachment to bone surface in osteoclasts. However, we observed no localisation of Rab13 to the sealing zone and the observed changes in the actin ring numbers in Rab13-depleted cells were only minimal. Instead, Rab13 localised to a distinct vesicle population in the vicinity of the nuclei at the superior and middle parts of the osteoclast, but it did not co-localise with the tested resorption markers (TRACP, cathepsin K, or FITC-labelled bone). Also early endosomes and the transferrin-labelled recycling compartment were devoid of Rab13 labelling. A significant co-distribution of Rab13 with Rab7 was recently described in non-polarised epithelial cells (Abou-Zeid *et al.*, 2011). In the active, bone resorbing osteoclasts, it is highly unlikely that these two proteins reside in the same compartments, as Rab7 is almost exclusively located at the ruffled border in these cells (Palokangas *et al.*, 1997; Zhao *et al.*, 2001), whereas very little of Rab13 could be detected at the lower parts of the resorbing osteoclasts. Down-regulation of Rab13 expression by electroporation of specific siRNA molecules into the osteoclast precursor cells did not produce significant differences in osteoclast differentiation or actin ring formation. More detailed analysis is needed to reveal the role of Rab13 in bone resorption and possible defects in vesicular trafficking in Rab13-depleted osteoclasts.

Rab13 was described to regulate the biosynthetic transport of cell surface proteins from TGN via transferrin receptor positive recycling endosomes in polarised MDCK cells (Nokes *et al.*, 2008). In our study, we observed no co-localisation of Rab13 with the transferrin-labelled compartments, indicating that in osteoclasts Rab13-positive vesicles are distinct from the transferrin recycling. However, we detected a weak co-localisation of Rab13 with TGN38-labelled trans-Golgi, suggesting that Rab13 indeed could regulate some biosynthetic route in osteoclasts. Based on the distribution of the Rab13-positive vesicles at the peripheral, superior parts of the resorbing osteoclast and its absence in the endosomal compartments, secretory vesicles directed towards the ruffled border and the transcytotic vesicles, we postulate that Rab13 resides in a novel vesicle type, possibly directed towards the basolateral membrane domain from the TGN in bone resorbing osteoclasts.

6.3 Tight junction proteins in osteoclasts

While trying to identify possible function for Rab13 in osteoclasts, it was hypothesised that tight junction proteins, occludin and Zo-1, may be present also in osteoclasts and regulated by Rab13. Indeed, we observed a vesicular staining of Zo-1 and occludin throughout the cytoplasm in resorbing osteoclasts. In addition, both tight junctional proteins displayed staining at the sealing zone (Figure 4). Interestingly, Zo-1 was recently described to modulate the podosome formation in various cell types including marcophages and non-resorbing osteoclast (Kremerskothen *et al.*, 2011). In addition, both Zo-1 and occludin regulate the directional migration and are localised to the leading edge in migrating cells (Taliana *et al.*, 2005; Du *et al.*, 2010). Rab13

was recently described to translocate to the leading edge of migrating endothelial cells under VEGF gradient (Wu *et al.*, 2011). Since Rab13 regulates the recruitment of occludin and Zo-1 to the tight junctions (Marzesco *et al.*, 2002; Kohler *et al.*, 2004; Morimoto *et al.*, 2005), a similar function is possible in the recruitment of these proteins to the lamellipodia in migrating cells. We observed a weak vesicular co-localisation of Rab13 and Zo-1 in bone resorbing kidney-shaped osteoclasts, but did not detect any polarisation of Rab13, Zo-1 or occludin labelling to the leading edge of these migrating osteoclasts. It is, however, possible that a specific signal is needed to facilitate the recruitment of these proteins for cell migration also in osteoclasts. This and the possible role of Zo-1 and occludin in the sealing zone of osteoclasts remain to be clarified by further studies.

6.4 Novel Rab13 interaction with endospanins

Several Rab13 interaction partners have been described, including the MICAL-like (Molecule Interacting with CasL-like) protein family members and the catalytic subdomain of protein kinase A and PDEδ (δ subunit of retinal rod cGMO phosphodiesterase) (Nancy *et al.*, 2002; Kohler *et al.*, 2004; Terai *et al.*, 2006; Yamamura *et al.*, 2008; Abou-Zeid *et al.*, 2011). We employed the bacterial two-hybrid screen to find novel Rab13 interaction partners in bone marrow environment, and identified a new highly conserved Rab13-interacting protein family consisting of two members, endospanin-2 and endospanin-1. The comparison of the yeast- and bacterial two-hybrid systems has been shown to produce differing interaction profiles (Serebriiskii *et al.*, 2005), which most likely explains why previously described Rab13-binding partners, determined by yeast two-hybrid screens, were not identified in our screen.

Endospanins are small, 131-amino acid proteins that contain four transmembrane domains with short intra- and extracellular loops and intracellular N- and C-termini (Huang *et al.*, 2001; Séron *et al.*, 2011). The yeast genome has only one endospanin homology, Vps55, which localises mainly in late endosomes, but is suggested to cycle between the late endosomes and the Golgi (Belgareh-Touzé *et al.*, 2002). In mammalian cells the two endospanins have distinct distributions; endospanin-1 is mostly found in late endosomes at the cell periphery, while the majority of endospanin-2 localises perinuclearily in TGN and to lesser extent in early endosomes (Séron *et al.*, 2011). These differing localisations may indicate that endospanins have divergent functions in higher organisms. Interestingly, Vps55 forms a complex with Vps68, another tetraspanning protein represented by two isoforms, Tmem50a (or SMP1, small membrane protein 1) and Tmem50b (or c21orf4), in humans. The Vps55/68 complex regulates the transit of biosynthetic and endocytotic cargo proteins through multivesicular bodies in yeast (Schluter *et al.*, 2008). The mammalian homologs of Vps68 are poorly characterised and it is not known whether they interact with the endospanins.

6.5 Rab13 and growth hormone receptor

Endospanins are implicated as negative regulators of cell surface leptin receptor (LEPR) and growth hormone receptors (GHR) by regulating the lysosomal degradation of the endocytosed receptors (Couturier *et al.*, 2007; Touvier *et al.*, 2009;

Séron *et al.*, 2011). Both endospanin-1 and endospanin-2 physically interact with leptin receptor, while the binding to growth hormone receptor has not been studied (Couturier *et al.*, 2007; Séron *et al.*, 2011). In our experiments, we assessed the effect of *Rab13* knockdown on cell surface GHR in LNCaP cells, and observed no apparent differences in cell surface GHR in growth hormone treated (not shown) or non-treated cells. This suggests that Rab13-endospanin interaction may have additional functions unrelated to the GHR cell surface trafficking. Alternatively, as only small fraction of the GHR (and LEPR) reaches the cell surface while the main bulk is retained in intracellular membranes such as ER, Golgi and TGN (Barr *et al.*, 1999; Belouzard *et al.*, 2004; Milward *et al.*, 2004; Landsman and Waxman, 2005), it is possible that this novel interaction is more important in the regulation of intracellular trafficking of these receptors. Previously described intracellular locations of endospanin-2 and Rab13 to the TGN (Nokes *et al.*, 2008; Séron *et al.*, 2011) and our observations may support this idea. Insulin treatment induces the GTP-loading of Rab13 and Rab8, but not Rab10 in L6 muscle cells. The activation of these Rab proteins promotes translocation of the insulin sensitive glucose transporter 4 to the cell surface (Sun *et al.*, 2010). Insulin treatment also reduces the cell surface growth hormone receptor by impairing the receptor translocation from the intracellular pool in hepatoma and osteoblast-like cell lines without affecting receptor internalisation (Leung *et al.*, 1996, 1997, 2000; Ji *et al.*, 1999). In addition, endospanin mRNA expression levels are regulated by insulin and changes in nutritional status (Touvier *et al.*, 2009; Wu *et al.*, 2013). Future studies will reveal if Glut4 and growth hormone receptor intracellular trafficking pathways are coordinated by Rab13 and Rab8 interactions with endospanins.

6.6 Endospanin-2 in bone and bone marrow

Endospanins are previously described as ubiquitously expressed proteins in human or mouse tissues (Huang *et al.*, 2001; Séron *et al.*, 2011), but their distribution in different cell types displays differences in two publicly available microarray databases (www.biogps.org and www.nextbio.com). In both databases *endospanin-2* (*LeprotL1*) displays highest mRNA expression levels in the cells of the immune system, including T-lymphocytes, eosinophils, hematopoietic stem cells and natural killer cells while *endospanin-1* (*Leprot*) is highly present in platelets, epithelial cells, adipocytes and some cells of the immune system. These expression differences may indicate that endospanins have some cell type-specific functions. We detected the expression of endospanin-2 mRNA in cells of the bone marrow and osteoclasts in rats *ex vivo*. The interaction of Rab13 with endospanin-2 in osteoclasts is thus possible, but is to be demonstrated by further studies at protein level.

Both, leptin and growth hormone are important regulators of bone homeostasis. Their effects on cells are conveyed via binding to LEPR and GHR that belong to the type I cytokine receptor superfamily (Liongue and Ward, 2007). LEPR is expressed in osteoblast precursors and mature osteoblasts (Cornish *et al.*, 2002) as well as in CD14-positive osteoclast precursors (Holloway *et al.*, 2002), while its expression in mature osteoclasts has not been demonstrated. Thus, leptin actions on osteoclasts may be at least partly mediated by the RANKL-OPG axis (Holloway *et al.*, 2002). The effects of leptin on bone appear to be anabolic, increasing bone formation by augmenting osteoblast proliferation and differentiation (Idelevich *et al.*, 2013). *In*

vitro studies suggest that leptin has inhibitory functions on osteoclast differentiation, while their resorption activity is unaffected (Cornish *et al.*, 2002; Holloway *et al.*, 2002). However, a recent *In vivo* study demonstrates that leptin signalling is an important regulator of osteoclast activity and leptin signalling-deficient mice display mild osteopetrotic phenotype due to overall suppression of bone turnover with reduced bone formation and even more affected bone resorption (Turner *et al.*, 2013). In addition to direct actions on bone cells, leptin may also have indirect actions on bone mediated by the central nervous system (Ducy *et al.*, 2000; Elefteriou *et al.*, 2005). Growth hormone is an important regulator of longitudinal bone growth but exerts also anabolic functions on bone as demonstrated by the bone loss in conditions with growth hormone deficiency in humans and GHR knockout animals (Sjögren *et al.*, 2000; Andreassen and Oxlund, 2001; Bonkowski *et al.*, 2006). In histological samples GHR expression has been demonstrated on the cell surface of osteoblasts and osteoclasts, but not on mature osteocytes (Lobie *et al.*, 1992; Zhang *et al.*, 1992). Part of the growth hormone actions on bone, however, may be mediated by IGF-I that is secreted systemically by liver and locally by many cell types, including osteoblasts (Andreassen and Oxlund, 2001; Wang *et al.*, 2004). Future studies will show if the anabolic effects of leptin and growth hormone on bone can be exploited by selectively disrupting the negative regulation of the cell surface expression of LEPR and GHR by endospanins in bone cells.

6.7 Transcytotic vesicles in osteoclasts

After the dissolution of mineral and organic components of the bone, they are removed from the resorption lacuna by the osteoclasts. The released matrix proteins and calcium ions are endocytosed at the central ruffled border of the osteoclast and delivered towards the functional secretory domain in vesicular structures at the bone-antipodal plasma membrane and finally expelled from the cell to the blood stream (Nesbitt and Horton, 1997; Salo *et al.*, 1997; Mulari *et al.*, 2003; Yamaki *et al.*, 2005). Although the transcytotic route in osteoclasts has been known for years, its special features and regulation are largely unknown. Our studies demonstrate multiple types of transcytotic vesicles in osteoclasts and that the cargo to these vesicles is selected at the ruffled border level. Moreover, integrin $\alpha_v\beta_3$ may serve as a receptor for some components of the degraded bone matrix, as suggested previously (Helfrich *et al.*, 1996).

HPA epitopes are abundant in cancer cells that have high invasion and metastasising capacity (Brooks, 2000; Valentiner *et al.*, 2005; Saint-Guirons *et al.*, 2007; Parameswaran *et al.*, 2011; Rambaruth *et al.*, 2012). Thus, HPA binding is strongly associated with the presence of metastasis in local lymph nodes (Brooks, 2000). Ultrastructurally, HPA positive cancer cells typically display intense HPA binding at the cell surface and perinuclear vesicles at least partly representing the Golgi area (Brooks, 2000; Saint-Guirons *et al.*, 2007; Rambaruth *et al.*, 2012). Proteomic analyses of breast and colorectal cancer cells reveal integrin alpha 6 and alpha v, annexin A2/A4, as well as some heat shock proteins and transcription proteins as HPA-binding glycoproteins (Saint-Guirons *et al.*, 2007; Rambaruth *et al.*, 2012). We analysed the presence of HPA-binding sites in another physiologically relevant invasive cell, the osteoclast that penetrates the bone matrix during resorption. HPA-binding sites were abundant in intracellular vesicular structures along the transcytotic

pathway in osteoclast. Moreover, in human osteoclasts also the ruffled border area was positive for HPA binding. Future studies identifying the HPA-binding glycoproteins in osteoclasts will reveal the role of GalNAc glycogonjugates in transcytosis and bone resorption.

The transcytotic vesicles are delivered and their contents are released to the extracellular space at the central domain of the bone-antipodal plasma membrane, the functional secretory domain (Nesbitt and Horton, 1997; Salo *et al.*, 1997). This central domain is strictly demarcated from the peripheral basolateral plasma membrane in resorbing osteoclast, but the mechanisms determining the strict borderline remain uncharacterised (Salo *et al.*, 1996; Mulari *et al.*, 2008). Neurons have no cell contacts supporting their membrane polarity, but their free lipid diffusion is hindered at the axon hillock and axonal initial segment by an obstacle formed by transmembrane proteins anchored to the actin-based membrane skeleton (Winckler *et al.*, 1999; Nakada *et al.*, 2003). We observed a thin actin-based circular structure at the border of the functional secretory domain and the basolateral membrane in osteoclasts. This actin structure could act as a visible part of a molecular barrier preventing free diffusion across the two membrane domains and thus formation of the functional secretory domain may be regulated by cytoskeletal organisation. However, in contrast to neurons and epithelial cells, osteoclasts are motile cells whose polarisation changes during the resorption cycle. Osteoclasts may thus have several mechanisms for creating and maintaining the functional secretory domain.

6.8 Possible transcytotic Rab proteins in osteoclasts

The bone degradation products and calcium are transported through the osteoclasts in vesicular structures prior to their release to the extracellular space, but the regulation of these vesicles is unknown (Nesbitt and Horton, 1997; Salo *et al.*, 1997; Yamaki *et al.*, 2005). Rab proteins that have been reported to regulate the transcytotic transport in epithelial or endothelial cells include Rabs 5, 11, 17 and 25 (Hunziker and Peters, 1998; Wang *et al.*, 2000; Predescu *et al.*, 2001; Xu *et al.*, 2011). Out of these, however, only Rab5 and Rab11 isoforms are expressed in osteoclasts (Zhao *et al.*, 2002). The distribution of these two Rabs in osteoclasts does not support their participation in transcytosis, since they are typically localised to small vesicles at the supranuclear and nuclear level, Rab5 being present in the early endocytotic vesicles at the proximity of the basolateral membrane and Rab11 in perinuclear recycling compartment (Zhao *et al.*, 2002).

The osteoclast ruffled border is a highly specialised membrane domain with several features of late endosomes and lysosomes rather than plasma membrane and could thus be regarded as one gigantic late endosome (or lysosome). Rab9 is a protein regulating the retrograde transport of proteins from the late endosomes to the trans-Golgi (Lombardi *et al.*, 1993; Barbero *et al.*, 2002). In osteoclasts, Rab9 resides in late endosomal vesicles around the nuclei with strongest labelling observed at the lower parts of the cell. In addition, Rab9 localises to the ruffled border, but its labelling pattern is complementary to that of Rab7 (Zhao *et al.*, 2002; Vaaraniemi *et al.*, 2004). Rab9 is suggested to regulate the traffic between the ruffled border and transcytotic vesicles and indeed, it is co-localised with TRACP in resorbing osteoclasts (Vaaraniemi *et al.*, 2004). It is thus possible that bone-resorbing

osteoclasts have adopted this retrograde transport pathway for the early steps of transcytosis of bone degradation products.

Rab9 was recently reported to regulate the formation of alternative, autophagy protein (Atg) 5 and 7-independent, autophagosomes (Nishida *et al.*, 2009). In osteoclasts, the formation of the ruffled border is dependent on proteins essential for classical autophagy, including Atg5 and Atg7, and these proteins also localise to the ruffled border. However, although autophagosomes were apparent in osteoclasts, they did not associate with the ruffled border (DeSelm *et al.*, 2011). In our study, we observed the co-localisation of MDC-labelled autophagosomes with transcytotic vesicles in bone-resorbing osteoclasts. It remains to be elucidated if these autophagosomes belong to the newly described alternative autophagosomal pathway.

Lysosome related organelles (LRO) are specialised compartments that have many similarities with late endosomes and lysosomes but harbour specialised cargo that is designated for secretion. LROs include among others, the lytic granules in cytolytic T cells, dense granules in platelets and megakaryocytes, MH II compartments in antigen presenting cells and melanosomes in pigment cells (Raposo *et al.*, 2007). It has been suggested that the transcytotic route of osteoclasts is related to the antigen-presenting pathway in cells such as macrophages (Vaananen *et al.*, 2000). LROs develop from precursors that gradually mature by acquiring their specific cargo via vesicular transport. Although the origin of LRO precursors varies from early endosomes to TGN and late endosomes, their maturation shares common features and regulators (Marks *et al.*, 2013). The process of LRO precursor segregation from lysosomes is still not fully understood, but there is evidence that Rab9 may regulate the early steps of LRO biogenesis. In early LROs, Rab9 interacts with BLOC-3 (Biogenesis of Lysosome-related Organelle Complex-3) components that functions as a GEF for Rab32 and Rab38 and thus may recruit these two Rabs to the developing LRO (Kloer *et al.*, 2010; Gerondopoulos *et al.*, 2012). Rab32 and 38 are important in the biogenesis of LROs, including the platelet dense granules and melanosomes (Wasmeier *et al.*, 2006; Ambrosio *et al.*, 2012). These Rabs directly bind to BLOC-2 complex and adaptor protein complex-1 and -3 (AP-1 and AP-3), proteins that regulate the delivery of crucial proteins to the maturing melanosome (Bultema *et al.*, 2012). Rab32 and Rab38 are closely related and their functions are at least partly redundant, however Rab32 has additional, yet poorly characterised, functions that cannot be done by Rab38 (Bultema *et al.*, 2012). Rab27 isoforms are important in the transport of melanosomes along actin tracts, while in platelets they are less important in vesicle movement but regulate the secretion of dense granules (Fukuda and Kuroda, 2002; Wu *et al.*, 2002; Tolmachova *et al.*, 2007; Harper *et al.*, 2013). Interestingly, Rab32 is also required for the formation of autophagic vacuoles (Hirota and Tanaka, 2009). Since all the Rab components of the LRO biogenesis described above are expressed also in osteoclasts, it is tempting to speculate that the Rab cascade is recruited to remove the bone degradation products from the resorption lacuna through the osteoclast.

7 CONCLUSIONS

The regulators of membrane transport pathways in bone-degrading osteoclasts are still mostly unknown. We performed a family-wide screen for the expression of Rab small GTPases in osteoclasts and found that osteoclasts express at least 26 *Rab* genes. *Rab13* gene expression was induced during osteoclastogenesis and thus it was chosen for further studies. Rab13 was present in bone-resorbing human osteoclast *in vitro* and in rat osteoclasts also *in vivo*. We identified a novel vesicle type, rich in Rab13, in osteoclasts. The transcytotic vesicles and the endocytotic route directed towards the ruffled border were devoid of Rab13. Our results also indicate that Rab13 was not involved in glucose transport or actin dynamics in bone resorbing osteoclasts. Although we were unable to directly demonstrate the precise function of Rab13 in osteoclasts, our results suggest that it is present in small vesicles travelling between the TGN and the basolateral membrane. The observed minimal vesicular co-localisation with Zo-1 may also suggest that it has a role in cell migration.

The function of a Rab in a specific cell type is determined by the presence of a variety of interacting proteins, including the effector proteins. We found novel interaction of Rab13 and Rab8 with endospanin-2 and its homologue endospanin-1. Endospanin-2 binding to Rab13 was nucleotide-dependent suggesting it is an effector for Rab13. Interestingly, Rab10, a closely related Rab of Rab13 and Rab8, did not bind endospanins, nor did Rab7, Rab11 or Rab32. This novel interaction was originally identified in the mixed hematopoietic cell population of the rat bone marrow. Thus its physiological function in osteoclasts and other cell types remains to be clarified.

The transcytotic route from the ruffled border to the functional secretory domain was described almost two decades ago, but is still incompletely characterised. Our studies demonstrate that the cargo designated for transcytotic delivery at the functional secretory domain is selected at the ruffled border to distinct vesicle populations that persist along the transcytotic pathway. These vesicles are targeted to the functional secretory domain that is circumscribed and segregated from the peripheral bone-antipodal plasma membrane by an actin-supported molecular barrier.

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