TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 866

MEDICA - ODONTOLOGICA

INTRACELLULAR MEMBRANE TRAFFICKING IN OSTEOCLASTS

THE ROLE OF DIRECT RAB PROTEIN – RAC1 INTERACTIONS IN THE TARGETING OF INTRACELLULAR VESICLES

by

Yi Sun

TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2009

Department of Cell Biology and Anatomy
Institute of Biomedicine
University of Turku
Turku, Finland

Supervised by

Professor Kalervo Väänänen
Department of Cell Biology and Anatomy
Institute of Biomedicine
University of Turku
Turku, Finland

Reviewed by

Docent Eeva-Liisa Eskelinen
University of Helsinki
Department of Biological and Environmental Sciences
Biochemistry
Helsinki, Finland

and

Senior Lecturer Arto Määttä
Durham University
School of Biological and Biomedical Sciences
Durham, United Kingdom

Opponent:

Professor Michael Horton
Department of Medicine
Bone and Mineral research and the Sackler Institute of MusculoSkeletal Research
University College London
Life Sciences in the London Centre for Nanotechnology
London, United Kingdom

ISBN 978-951-29-4035-6 (PRINT)
ISBN 978-951-29-4036-3 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2009

Yi Sun: INTRACELLULAR MEMBRANE TRAFFICKING IN OSTEOCLASTS
The role of direct Rab protein – Rac 1 interactions in the targeting of intracellular vesicles
Institute of Biomedicine, Department of Cell Biology and Anatomy, University of Turku
Annales Universitatis Turkuensis, Medica-Odontologica, Turku, Finland, 2009

ABSTRACT

Osteoclasts are cells responsible for bone resorption. These cells undergo extensive membrane re-organization during their polarization for bone resorption and form four distinct membrane domains, namely the ruffled border, the basolateral membrane, the sealing zone and the functional secretory domain. The endocytic/biosynthetic pathway and transcytotic route(s) are important for the resorption process, since the endocytic/biosynthetic pathway brings the specific vesicles to the ruffled border whereas the transcytotic flow is believed to transport the degraded bone matrix away from the resorption lacuna to the functional secretory domain.

In the present study, we found a new transcytotic route from the functional secretory domain to the ruffled border, which may compensate membrane loss from the ruffled border during the resorption process. We also found that lipid rafts are essential for the ruffled border-targeted late endosomal pathways.

A small GTP-binding protein, Rab7, has earlier been shown to regulate the late steps of the endocytic pathway. In bone-resorbing osteoclasts it is involved in the formation of the ruffled border, which displays several features of late endosomal membranes. Here we discovered a new Rab7-interacting protein, Rac1, which is another small GTP-binding protein and binds to the GTP-form of Rab7 in vitro. We demonstrated further that Rab7 colocalizes with Rac1 at the fusion zone of the ruffled border in bone-resorbing osteoclasts. In other cell types, such as fibroblast-like cells, this colocalization is mainly perinuclear. Because Rac1 is known to control the actin cytoskeleton through its effectors, we suggest that the Rab7-Rac1 interaction may mediate late endosomal transport between microtubules and microfilaments, thus enabling endosomal vesicles to switch tracks from microtubules to microfilaments before their fusion to the ruffled border.

We then studied the role of Rab-Rac1 interaction in the slow recycling pathway. We revealed that Rac1 also binds directly to Rab11 and to some other but not all Rab-proteins, suggesting that Rab-Rac1 interaction could be a general regulatory mechanism to direct the intracellular vesicles from microtubule mediated transport to actin filament mediated transport and vice versa. On the basis of our results we thus propose a new hypothesis for these GTPases in the regulation of intracellular membrane flow.

Keywords: Rab, Rac1, protein – protein interaction, membrane trafficking, osteoclast

Yi Sun: OSTEOKLASTIEN SOLUNSISÄINEN KALVOLIIKENNE. Rac 1:n ja eräiden Rab-proteiinien välisen suoran interaktion merkitys solun rakkulaliikenteessä.
Biolääketieteen laitos, Solubiologia ja anatomia, Turun yliopisto
Annales Universitatis Turkuensis, Medica-Odontologica, Turku, Suomi, 2009

YHTEENVETO

Osteoklastit ovat soluja, jotka vastaavat luun mineralisoituneen väliaineen hajotuksesta. Polarisoituessaan aktiivista toimintaa varten soluihin ilmaantuu useita spesifisiä kalvoalueita, joilla jokaisella on erillinen tehtävä solun toiminnassa. Näitä spesifisiä kalvoalueita ovat poimureunus, tarttumisvyöhyke ja eritysalue. Luuta hajottaessaan solu ylläpitää vilkasta solun sisäistä rakkulaliikennettä, jonka avulla se kuljettaa poimureunukselle luun hajottamiseen tarvittavia entsyymejä ja suolahappoa. Poimureunukselta eritysalueelle suuntautuva rakkulaliikenne puolestaan kuljettaa solun hajottamaa luun väliainetta kohti verenkiertoa.

Tässä tutkimuksessa löydettiin uusi solun sisäinen kuljetusreitti, joka suuntautuu eritysalueelta kohti solun poimureunusta. Tämä ylläpitää dynaamista tasapainoa näiden kalvoalueiden välillä ja palauttaa kalvomateriaalia kalvoalueelta toiselle. Havaittiin myös, että poimureunukselle suuntautuva rakkulaliikenne, ja siten myös luun hajotus, ovat riippuvaisia niin sanotuista lipidilautoista.

Pienen GTP:tä sitovan proteiinin, Rab7:n, on aiemmin osoitettu säätelevän poimureunukselle suuntautuvaa kalvoliikennettä osteoklasteissa. Poimureunus on solukalvon alue, joka rakenteellisesti muistuttaa solunsisäisten myöhäisten endosomien kalvoa. Tässä työssä havaittiin, että Rab7-proteiini sitoutuu suoraan erääseen toiseen pieneen GTP:tä sitovaan proteiiniin, jota kutsutaan nimellä Rac1. Osoitimme, että molemmat pienet GTP:tä sitovat proteiinit myös paikantuvat tiettyyn osaan poimureunusta, mikä vahvistaa *in vitro* havaittua suoraa proteiinien toisiinsa liittymistä. Muissa soluissa kuin osteoklasteissa proteiinien yhteisesiintyminen paikantuu pääosin tuman ympärille. Aikaisemmista tutkimuksista tiedetään Rac 1:n olevan osallisena solun aktiinisäikeiden ohjaamassa rakkulaliikenteessä. Tulokset viittaavat siihen, että Rac 1:n ja Rab 7:n välinen interaktio voisi mahdollistaa kalvorakkuloiden siirtymisen mikrotubulusvälitteisestä kuljetuksesta aktiinisäievälitteiseen kuljetukseen juuri ennen kuin rakkulat yhtyvät solukalvoon.

Työssä tutkittiin myös Rab-proteiinien ja Rac1:n välisen interaktion osuutta muissa solun sisäisissä kuljetusreiteissä. Havaittiin, että Rac 1 sitoutuu suoraan myös Rab 11 -proteiiniin solun takaisinkiertoreitillä. Tästä johtuen selvitettiin laajemmin Rac 1:n kykyä sitoutua Rab-perheen proteiineihin. Havaittiin, että Rac 1 sitoutuu useisiin, mutta ei kuitenkaan kaikkiin, Rab-proteiiniperheen jäseniin. Tulosten perusteella on mahdollista, että Rac1-Rab –proteiinien välinen interaktio on yleinen solun sisäistä rakkulaliikennettä säätelevä tapahtuma, mikä mahdollistaa kalvorakkuloiden nopean siirtymisen mikrotubulusvälitteisestä kuljetuksesta mikrofilamenttivälitteiseen kuljetukseen ja päinvastoin. Esitämme tehtyjen havaintojen perusteella kokonaan uuden tehtävän solunsisäisille pienille GTP:tä sitoville proteiineille solun sisäisessä kalvoliikenteessä.

Avainsanat: Rab, Rac1, proteiini-proteiini –sitoutuminen, kalvoliikenne, osteoklasti

TABLE OF CONTENTS

ABBREVIATIONS

BL	basolateral domain
6His/His_6	six histidin
ChlTx-B	cholera toxin subunit B
CLIP-170	cytoplasmic linker protein 170
COX 2	cyclooxygenase 2
CTX	C-terminal telopeptide of collagen type I
DIGs	detergent-insoluble glycolipid-enriched complexes
DMAPP	dimethylallyl pyrophosphate
FDS	functional secretory domain
FT	farnesyl transferase
GAP	GTPase activating proteins
GDI	Rab GDP dissociation inhibitor
GEF	GTP/GDP exchange factor
GGT-I	geranylgeranyl tranferase type I
GST	glutathione S-transferase
HA	hemagglutinin
HSC	hematopoietic stem cells
IPP	isopentenyl pyrophosphate
IQGAP1	IQ motif containing GTPase activating protein
LIF	leukemia inhibitory factor
MAPs	microtubule-associated proteins
M-CSF	macrophage-colony stimulating factor
MMP-9	matrix metalloproteinase 9
MSC	mesenchymal stem cells
MTOC	microtubule organizing center
MVB	multivesicular body
MβCD	methyl-β-cyclodextrin
N-BPs	nitrogen-containing bisposphonates
NPC	Niemann Pick disease type C
OPG	osteoprotegerin
PAK	p21-activated kinase
PAK1	$p21^{cdc42/rac}$-activated kinase
PCM	pericentriolar material
PFA	Paraformaldehyde
PGE2	prostaglandin E2
PI3K	phosphatidylinositol 3-kinase
PNRC	perinuclear recycling compartment
PTH	Parathyroid hormone

PTHrP	Parathyroid hormone-related protein
Rab11-FIP	Rab11 family of interacting proteins
Rab11Q70L	constitutive GTP-bound Rab11
Rab11S25N	constitutive GDP-bound Rab11
Rab7Q67L	constitutive GTP-bound Rab7
Rab7T22N	constitutive GDP-bound Rab7
Rac1Q61L	constitutive GTP-bound Rac1
Rac1T17N	constitutive GDP-bound Rac1
RANKL	receptor activator for nuclear factor κ b ligand
RB	ruffled border
RCP	Rab coupling protein
recVSV-GGC	construct of VSV G protein having replacement of cytoplasmic tail with that of CD4
recVSV-GCC	construct of VSV G protein having replacement of the membrane anchor with that of CD4
REP	Rab escort protein
RGGT	Rab geranylgeranyl transferase
RILP	Rab-interacting lysosomal protein
ROS	reactive oxygen species
SCV	Salmonella-containing vacuole
SNARE	soluble N-ethylmaleimide-sensitive factor receptor
SZ	sealing zone
TAK1	TGF Activated Kinase-1
Tfn	transferrin
TfR	transferrin receptor
TGF-β	transforming growth factor-β
TNF	tumor necrosis factor
TRACP	tartrate-resistant acid phosphatase
TRAF6	TNF receptor-associated factor 6
TRANCE	tumor necrosis factor (TNF)-related activation-induced cytokine
TRITC	tetramethylrhodamine isothiocyanate
Tsg101	tumor-susceptibility gene product
VSV	vesicular stomatitis virus
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

LIST OF ORIGINAL PUBLICATIONS

The present thesis is based on the following original articles, which are referred to in the text by the Roman numerals given below.

I Mulari, M.T., Nars, M., Laitala-Leinonen, T., Kaisto, T., Metsikkö, K., Sun, Y., and Väänänen, H.K. (2008). Recombinant VSV G proteins reveal a novel raft-dependent endocytic pathway in resorbing osteoclasts. Exp Cell Res *314*, 1641-1651.

II Sun, Y., Buki, K.G., Ettala, O., Vääräniemi, J.P., and Väänänen, H.K. (2005). Possible role of direct Rac1-Rab7 interaction in ruffled border formation of osteoclasts. J Biol Chem *280*, 32356-32361.

III Sun, Y., Büki, K.G., Mulari, M., Väänänen, H.K. Rab11 and Rac1 interact directly to regulate slow recycling pathway from sorting endosomes to plasma membrane. Submitted.

1 INTRODUCTION

Osteoclasts are multinucleated bone cells of hematopoietic lineage responsible for bone resorption. When the bone resorption signal is triggered, mononucleated precursor cells migrate to the site and fuse into multinucleated osteoclasts. The cells are polarized and form distinct membrane domains namely, the sealing zone, the ruffled border, the basolateral plasma membrane and the functional secretory domain. Active membrane transport is a typical feature of the resorbing osteoclast to meet the requirements of building up these membrane domains and bone resorption (*Vaananen and Laitala-Leinonen, 2008*).

Rab proteins, a family of small GTPases, are the major regulators of intracellular vesicular transport between organelles in eukaryotic cells. They are the largest family among the Ras-related small GTPases. More than 60 Rab genes have been identified in the human genome. They cycle between GTP-bound (active) and GDP-bound (inactive) forms regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), respectively. Rab proteins associate to a distinct cytoplasmic membrane compartment when they are activated and recruit a set of specific effectors to regulate vesicular targeting, docking, fusion and budding through the endocytic or secretory pathway. The GDP-bound Rab proteins in the cytosol are mainly associated with GDI (GDP dissociation inhibitor). The Rab-effector interacting complex also directs the vesicular transport by connection of either actin- or microtubule-based motor proteins to move along the cytoskeleton (*Seabra and Wasmeier, 2004*).

The regulatory roles of individual Rab proteins in the steps of endocytic and secretory pathways have been studied during the last two decades. For instance, Rab5 is involved in endocytosis, early endosome transport and vesicular sorting. Rab7 regulates trafficking between the early endosomes and late endosomes and from late endosomes to lysosomes, whereas Rab4 and Rab11 participate in endocytic membrane recycling (*Bucci et al., 1992; Daro et al., 1996; Meresse et al., 1995; Soldati et al., 1995; Sonnichsen et al., 2000; Stenmark et al., 1995; Ullrich et al., 1996; Urbe et al., 1993*).

A number of Rab proteins have been reported to be expressed in osteoclasts (*Zhao et al., 2002*). However, most of the individual function of Rabs in these unique cells still remains unclear. It has been shown that down regulation of Rab7 by antisense oligonucleotides impairs the formation of the ruffled border, thus suggesting an important role for Rab7 in the function of resorbing osteoclasts (*Zhao et al., 2001*).

Lipid rafts, an important component of cellular membranes, participate in membrane transport and protein sorting. Pathogens, such as viruses enter host cells via the portal of caveolae, a membrane domain of lipid rafts, which are composed of cholesterol, glycospingolipids and caveolin1 (*Wilflingseder and Stoiber, 2007*). Studies of vesicular stomatitis virus (VSV) G protein sorting in osteoclasts have shown it to be delivered to the basolateral membrane independent of rafts whereas the HA of influenza virus is targeted

to the functional secretory domain dependent on the presence of rafts (*Salo et al., 1996*). Interference of cholesterol transport caused by a human inherited disease NPC impairs not only the steroid transport, but also the whole endocytic pathway (*Mukherjee and Maxfield, 2004; Ory, 2000; Zhang et al., 2001*). Thus in resorbing osteoclasts, blocking the efflux of cholesterol from the late endosomes by compound U18666A mimics NPC disease and blocks the delivery of late endosomal protein V^+ - ATPase and cathepsin K to the ruffled border. The drug also interferes with the whole endocytic pathway (*Zhao and Vaananen, 2006*).

In the present study, we first discovered a new lipid raft-dependent membrane trafficking pathway from FSD (functional secretory domain) to the ruffled border in resorbing osteoclasts. Second, we sought the direct Rab7 binding proteins by screening a cDNA library derived from rat trabecular bone and bone marrow with a bacterial two-hybrid system, and found the mechanism of how Rab7 regulates the ruffled border formation in the late endocytic pathway of resorbing osteoclasts. We further found that the recycling pathway regulated by Rab11 shares the same binding protein, Rac1, with Rab7. Finally, we showed that Rab11-Rac1 interaction regulates internalized transferrin transport from sorting endosomes via perinuclear compartment to the basolateral plasma membrane in HeLa cells and osteoclasts. We propose that this interaction is involved in the regulation of cell migration.

2 REVIEW OF THE LITERATURE

2.1 Bone structure and function of bone

The adult human skeleton is comprised of 213 bones, which can be divided into two main types: flat bones, such as the skull, mandible, rib, and scapula, and long bones such as the femur, tibia and radius. Flat bones are formed by intramembranous ossification and long bones develop by a combination of endochondral bone formation and intramembranous bone formation. Morphologically, bones can be characterized as cortical bone and trabecular (cancellous, spongy) bone. Cortical bone comprises approximately 80 % of the adult skeleton. Cortical bone is on the surface of the bone, appears dense and solid and serves as "a shell" for protection of the bone marrow and trabecular bone inside. Cortical bone, especially in long bones, also supports body weight, movement and mechanical loading. In flat bones, cortical bone is thinner and more elastic than that in long bones, and thus the flat bones, such as the rib and skull, serve as armor for the vital organs that they surround. Trabecular bone is a sponge-like network of interconnected trabecular plates and bars surrounding bone marrow. It is abundant at the metaphysis and epiphysis in long bone and flat bone inside the shell of cortical bone. The metaphysis, including the epiphyseal plate (growth plate), is located between the epiphysis and the diaphysis (the long midsection of the long bone); the epiphysis is the rounded end of a long bone. Trabecular bone is generally considered to be metabolically more active than cortical bone.

Bone is a specialized form of connective tissue. It is composed of bone specific cells and the surrounding extracellular matrix including an organic and inorganic part. Approximately 95% of organic matrix is type 1 collagen. Proteoglycans and numerous noncollagenous proteins constitute the remaining 5%. Calcium and phosphate deposit in the organic matrix and form hydroxyapatite, the inorganic matrix. These organic and inorganic components of the matrix make the bone rigid as well as retain some degree of elasticity. In addition to its supportive and protective function, bone also serves as a reservoir of inorganic ions, actively participating in calcium and phosphate homeostasis of the body.

2.2 Bone cells

Bone contains four types of cells, osteoblasts, osteoclasts, bone lining cells and osteocytes. The first three types of bone cells present on the bone surface whereas the osteocytes are buried in mineralized matrix. Osteoblasts, bone lining cells and osteocytes are generated from the osteoprogenitor cells that originate from mesenchymal stem cells (MSC), whereas osteoclasts originate from hematopoietic stem cells (HSC).

2.2.1 Osteoblasts and bone formation

Osteoblasts are fully differentiated cells responsible for the production of bone matrix. They originate from local osteoprogenitor cells in bone marrow. Only 15% of MSC has

the capacity to form bone (*Wu et al., 2000*). MSCs, found in adult bone marrow can also be isolated from adult peripheral blood, fetal cord blood, fetal liver and tooth pulp (*Campagnoli et al., 2001; Erices et al., 2000; Huss et al., 2000*).

Osteoblasts are responsible for bone formation. Mature osteoblasts have a large nucleus, enlarged Golgi and extensive endoplasmic reticulum in order to synthesize type 1 collagen and other bone matrix proteins. Cells are rich in secretory vesicles. Secreted extracellular matrix (ECM) forms unmineralized osteoid towards the bone forming front. Mineral deposition around type 1 collagen is regulated by several non-collageous either calcium or phosphate binding proteins for instance, alkaline phosphatase, osteocalcin, osteopontin and bone sialoprotein that are also secreted from mature osteoblasts.

Bone formation requires osteoblast differentiation and maturation that involves a set of signaling proteins including Wnt/β-catenin (*Logan and Nusse, 2004*), bone morphogenetic proteins (BMP, members of the TGF-β family) (*ten Dijke et al., 2003*), hormones (*Xing and Boyce, 2005*), growth factors, cytokines (*Xing and Boyce, 2005*), matrix proteins, transcription factors and their co-regulatory proteins. Sequential activation, suppression and modulation of these genes represent the phenotypic, structural and functional properties of osteoblasts during the differentiation process.

Early bone formation and osteoblast differentiation are indirectly modulated by the canonical Wnt/β-catenin pathway. A high level of β-catenin commits osteoblast to differentiation, whereas low expression of β-catenin is important for chondrogenesis (*Logan and Nusse, 2004*). In addition to the canonical Wnt/β-catenin pathway, transcriptional regulators of runt homology domain factor Runx2 and Osterix are also essential for osteoblast differentiation in the early and final stages, respectively. Runx2 targets genes that are not only involved in bone formation, such as osteocalcin, osteopontin, sialoprotein (*Ducy et al., 1997*), but also genes in bone turnover (collagenase 3, RANKL, and osteoprotegerin) (*Thirunavukkarasu et al., 2000*).

Osteoblasts express α2β1-integrin which is a receptor for type I collagen, the major protein component of bone matrix. The integrin-collagen interaction in turn is required for induction of osteoblast-specific gene expression, such as osteocalcin (*Xiao et al., 1998*).

Steroid and peptide hormones, growth factors and cytokines regulate not only the growth of osteoprogenitors or their progression to mature osteoblasts, but they also regulate osteoblast activity and apoptosis. PTH (*Parathyroid hormone*) stimulates the growth of osteoprogenitor cells in the trabecular and endosteal osteoblast population, but has an opposite effect on the periosteal osteoblast population, indicating its different roles in trabecular versus cortical bone (*Calvi et al., 2001*). Endogenous glucocorticoids have been shown to promote human and mouse MSC to differentiate towards osteoblasts and have an anabolic effect in bone (*Bellows et al., 1987*). However, when used therapeutically, glucocorticoids led to negative effects on bone formation partially by inducing osteoblast and osteocyte apoptosis (*Reid, 1997; Weinstein et al., 1998*). Vitamin

D, a precursor of 1,25-dihidroxy-D3 [1,25(OH)2D3] is a potent regulator for expression of numerous osteoblast specific genes including osteocalcin. Sex hormones apparently have anabolic effects on osteoblast and bone formation. Retinoic acid is involved in the skeletal development of the embryo (*van Leeuwen et al., 2001*). Leptin, which is secreted largely by adipocytes, and also by osteoblasts at the mineralization stage, has an anabolic role in osteoblasts and stromal cells, but also induces the apoptosis of stromal cells (*Gordeladze et al., 2002*).

Cytokines, including LIF (leukemia inhibitory factor), IL6, IL-11 and oncostatin M (OSM), have been shown to play a role in osteoblast differentiation (*Malaval et al., 2005*). TGF-β (Transforming growth factor β) increases bone formation in vitro mainly by recruiting osteoprogenitors both by the means of inducing chemotaxis and by stimulating their proliferation. It has also been shown to promote the early stages of osteoblast differentiation. However, it blocks the late stages of differentiation and mineralization as well as osteoblast apoptosis (*Janssens et al., 2005*). IGF-1 (insulin-like growth factor 1) increases osteoblast function and trabecular bone volume (*Zhang et al., 2002*).

Osteoblasts synthesize and secrete two key molecules that are important for osteoclast differentiation, namely the ligand for the receptor of activator of nuclear factor κB (RANKL) and its decoy receptor osteoprotegerin (OPG) (*Kostenuik and Shalhoub, 2001*).

2.2.2 Osteocytes

Osteocytes, the most abundant cells found in bone, are star-shaped cells which may be responsible for sensing mechanical loading. They mature from osteoblasts, and once osteoblasts become trapped in the matrix they secrete and turn into osteocytes. Osteocytes are networked to each other via long cytoplasmic extensions through tiny canals called canaliculi. They are arranged around a central vascular channel to constitute Haversian systems, which are used for the exchange of nutrients and waste. The space that an osteocyte occupies is called a lacuna. Young osteocytes have a euchromatic nucleus and a large Golgi apparatus. Old osteocytes have reduced synthetic activity. However, they are actively involved in the routine turnover of bone matrix through various mechanosensory mechanisms, and destroy bone through a slow mechanism called osteocytic osteolysis (*Nijweide et al., Principle of Bone Biology 2nd edi, 2002, Academic Press, USA pp 93-96*).

2.2.3 Bone lining cells

Bone lining cells are flat, elongated and inactive cells that cover the inactive bone surface, namely neither undergoing bone formation nor bone resorption. It has been speculated that these quiescent cells could be precursors for osteoblasts (*Canalis, 2005*). However, these cells have few cytoplasmic organelles and secretory vesicles compared to osteoblasts, and they lack mature osteoblast markers, such as osteocalcin. However, little is known regarding the function of these cells.

2.2.4 Osteoclasts

Osteoclasts, which originate from hematopoietic stem cells (HSC), are responsible for bone resorption. Although some cells other than osteoclasts may also display bone resorption, such as mature osteocytes, there is no doubt that osteoclasts are the major cell type for bone resorption. It is generally agreed that the osteoclast precursors belong to the monocyte/macrophage lineage in bone marrow. Osteoclasts are fused from bone marrow mononuclear precursors and form huge multinucleated cells. Their large size is believed to amplify their resorption ability. Osteoclasts are characterized by high expression of tartrate resistant acid phosphate 5b (TRACP 5b) and cathepsin K (*Inaoka et al., 1995; Lam et al., 1982*).

2.2.4.1 Osteoclast formation and regulation

Osteoclastogenesis relies on the function of stromal cells and their derived osteoblasts. Two cytokines secreted by these cells are crucial for osteoclast differentiation. RANKL, a member of the TNF surperfamily is essential for the priming of osteoclastic precursor cells and its presence is required for osteoclast formation as well as for the function of mature osteoclasts (*Suda et al., 1999*). RANK (a TNF receptor) knockout mice exhibit a phenotype of osteopetrosis and defects in tooth eruption, along with an absence or deficiency of osteoclasts (*Dougall et al., 1999*). RANKL activates NF-κβ (nuclear factor-κβ) and NFATc1 (nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1) through RANK on the osteoclast surface (*Sharma et al., 2007*). Macrophage colony-stimulating factor (M-CSF, also called CSF-1), which is also produced by MSC/osteoblasts, is an important regulator of osteoclast formation. M-CSF acts through its receptor c-fms (colony stimulating factor 1 receptor) on osteoclasts (*Pixley and Stanley, 2004*). It is a transmembrane tyrosine kinase-receptor, leading to secondary messenger activation of tyrosine kinase Src (*Ross, 2006*). This commits HSC cells to monocyte/macrophage lineage and induces proliferation, survival, and differentiation of osteoclast precursors from monocyte/macrophage lineage cells.

Osteoprotegerin (OPG), mainly produced by MSC and osteoblasts, is a soluble decoy receptor for RANKL. It has been shown as an inhibitory factor of osteoclastogenesis by competing with RANK, the receptor on the cell membrane of osteoclast precursors and osteoclasts, to bind RANKL. Therefore, it blocks osteoclast differentiation, mature osteoclast function and bone resorption (*Kostenuik and Shalhoub, 2001*). Osteoclasts can also be differentiated from mouse spleen cells or human circulating blood when RANKL and M-CSF are administered (*Sorensen et al., 2007; Suda et al., 1999*).

Osteoclasts are regulated by several hormones, including parathyroid hormone (PTH) from the parathyroid gland, calcitonin from the thyroid gland, and growth factors and cytokines (*Martin, 2002; Selander et al., 1996*). Cytokines such as LIF/IL-6, IL-11 and oncostatin M (OSM), which transduce their signals through gp130 in MSC, induce osteoblasts to produce excess RANKL, and promote osteoclast formation (*Palmqvist et al., 2002; Sims, 2008*). However, a recent study showed the opposite role of IL-6

in osteoclastogenesis by diverting osteoclast precursors to the macrophage lineage (*Duplomb et al., 2008*). TNF-α and TGF-β have been shown to have opposite effects on osteoclastogenesis, the first induces, while the second one inhibits the proliferation of osteoclast precursors (*Chenu et al., 1988; Takahashi et al., 1986*). Hormones, PTH and 1,25(OH)2D3, stimulate osteoclast formation via PTH/ PTHrP (Parathyroid hormone-related protein) receptors (PTHR1) and 1,25(OH)2D3 receptors, respectively, and stimulate osteoclast precursor differentiation via RANKL and the MCSF-1 signal of stromal/osteoblasts. However, calcitonin which is secreted from thyroid C-cells, acts directly on osteoclasts by binding to its receptor on the cell surface to protect Ca^{2+} loss from skeleton. This effect is the opposite to the effect of PTH.

However, exogenous PTH and PTHrP have shown an anabolic action on the skeleton of mice when administered intermittently (Finkelstein et al., 2003). Indeed, PTHrP+/- mice are osteoporotic. It may suggest that PTHrP is also a crucial paracrine regulator of bone formation (*Miao et al., 2005*). Furthermore, treatment of mice with bisphosphonates, which inhibits osteoclast function, impairs the anabolic response to PTH administration, suggesting that osteoclast activity is necessary to complement the effect of PTH on the differentiation of osteoblasts. This might involve the generation of a coupling factor from osteoclasts in response to PTH (*Martin et al., 2006*) (Fig. 1).

2.2.4.2 Osteoclast polarization and plasma membrane domains

Osteoclasts polarize when they are activated. Polarized osteoclasts display four specialized membrane domains, the sealing zone, the ruffled border, the basolateral domain and the functional secretory domain, which serve different functional purposes for the cells (*Vaananen and Laitala-Leinonen, 2008*) (Fig.1).

Fig. 1. Intracellular trafficking pathways in osteoclasts

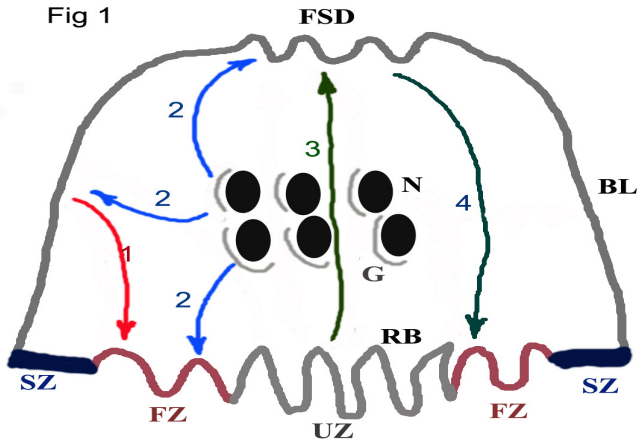

Fig. 1. 1: Endocytic pathway. 2: Biosynthetic pathway. 3: Transcytotic pathway from RB to FSD. 4: Transcytotic pathway from FSD to RB. FSD: functional secretory domain. BL: basolaterol domain. SZ: sealing zone. RB: ruffled border. FZ: fusion zone of ruffled border. UZ: uptake zone of ruffled border. N: Nuclei. G: Golgi apparatus that surround nuclei.

2.2.4.2.1 The sealing zone (SZ)

The sealing zone is a circular attachment of plasma membrane of the osteoclast to the underlying bone. This is initiated and facilitated by integrin receptors, such as αvβ3, via the specific amino acid motif Arg-Gly-Asp in bone matrix proteins, such as osteopontin. The sealing zone is free from organelles on its cytoplasmic face, and is thus also called a clear zone. Initially, the sealing zone is marked by a belt of specialized adhesion structures called podosomes, which consist of a core of actin bundles surrounded by a rosette-like structure containing αvβ3 integrin, vinculin, and α-actinin (*Marchisio et al., 1988*). Although integrin is an initial trigger for podosome formation, αvβ3 receptor is absent in the mature sealing zone (*Lakkakorpi and Vaananen, 1996*). The function of the sealing zone is to protect the diffusion of proteases and HCl from resorption lacuna to the surroundings. However, the sealing zone still retains the ability to diffuse low molecular weight molecules less than 10 KD (*Stenbeck and Horton, 2000*). MMP14, also called MT1-MMP, one of MMPs (the matrix metalloproteinases), has been shown to localize at the sealing zone, suggesting that the enzyme modifies the bone surface to facilitate the migration and attachment of osteoclasts as well as scavenging the resorption lacunae (*Irie et al., 2001*).

2.2.4.2.2 The ruffled border (RB)

The ruffled border is the most prominent feature of a resorbing osteoclast. It is the membrane domain facing the bone. The cell membrane is highly ruffled and invaginated into the cell several micrometers from the bone surface. Underneath there is a space between the ruffled border and bone called Howship's lacuna, which is sealed by the sealing zone. Although the ruffled border is part of the plasma membrane, it displays late endosomal features; indeed, Howship's lacuna is a bone-lytic hemivacuole. The ruffled border is the cell membrane that deals with secretion of hydrogen ions and proteases to acidify and dissolve the mineralized bone as well as organic bone in this lacuna. Resorbing osteoclasts generate a considerable number of hydrogen ions ($H_2O+CO_2 \rightarrow HCO_3^- + H^+$) and release them into Howship's lacuna through the ruffled border by vacuolar-ATPase (*Vaananen et al., 1990*). Cathepsin K, a collagenolytic cysteine protease, is delivered to the ruffled border. It helps to digest the organic components of bone matrix. Meanwhile, the digested bone is endocytosed back into the cell via the central portion of the ruffled border and transcytosed across the cell, and finally released into the cell surroundings. These activities divide the ruffled border into two sub-domains: the fusion zone which is located at the periphery of the ruffled border, and the uptake zone which is in the central part of the ruffled border (*Mulari et al., 2003*).

Vacuolar-ATPase and cathepsin K inhibitors have been sought in the prevention of osteoporosis (*Niikura et al., 2004*). However, potential side-effects still remain due to nonselectivity for osteoclasts. Cathepsin K inhibitors, for instance, could impair immune function since it is also expressed in dendritic cells (*Asagiri et al., 2008*).

2.2.4.2.3 The functional secretory domain (FSD)

The functional secretory domain (FSD) is the part of the non-bone facing plasma membrane located opposite to the ruffled border. It has a slightly ruffled morphology and serves for the secretion of the digested bone matrix as well as a number of enzymes, such as TRACP 5b and cathepsin K (*Salo et al., 1997*). The mineral portion of the matrix, hydroxyaptite, including calcium and phosphate ions are released into the extracellular surroundings, thus increasing levels of the ions in blood circulation. Newly synthesized influenza HA protein is delivered to the FSD in osteoclasts (*Mulari et al., 2003; Palokangas et al., 1997; Salo et al., 1997*), but not to the basolateral surface indicating that this membrane domain is distinct.

2.2.4.2.4 The Basolateral membrane domain (BL)

A major part of the non-bone facing plasma membrane in resorbing osteoclasts is the basolateral membrane. In polarized epithelial cells, the basolateral membrane is important for internalizing nutrients, different growth factors and membrane recycling. When osteoclasts are infected with VSV (Vesicular stomatitis virus), the synthesized G protein is delivered strictly to this part of the cell surface (*Palokangas et al., 1997; Salo et al., 1997*).

2.2.5 Osteoclastic bone resorption

Osteoclasts migrate to a site where the bone needs to be resorbed (e.g. to a microfracture), possibly by chemotaxis (*Hruska and Teitelbaum, 1995*). However, which molecules govern this process is still unclear. A recent study showed that damaged ostecytes secrete M-CSF and RANKL to recruit osteoclasts, (*Kurata et al., 2006*). In contrast, healthy osteocytes show an inhibitory effect on resorption by signalling with TGF-β (*Heino et al., 2002*). MMP-9 (matrix metalloproteinase 9) is expressed by osteoclasts (*Tezuka et al., 1994*), and is known to be required for osteoclast migration and enhances prostate cancer metastasis in bone due to its powerful gelatinase activity (*Dong et al., 2005*).

Despite the specificity of osteoclasts, the migration machinery includes several general aspects. First, integrin on the leading edge of the cell binds the cell to the extracellular surface on which it is crawling (*Schmidt et al., 1993*). Second, the front cell membrane is continuously replenished from internal membrane pools (*Jones et al., 2006*). Third, actin undergoes rapid polymerization at the leading part of the plasma membrane, which pushes the front of the cell, causing it to move forward; this is a major motile force for movement (*Diez et al., 2005*). Fourth, microtubules integrate with the filamentous actin to keep the polarity of the cell for migration (*Etienne-Manneville, 2004; Kodama et al., 2004*).

Osteoclasts are capable of excavating pits of several micrometers deep underneath themselves on bone substrates after an overnight culture and the speed of excavation is about 1μm per hour (*Chambers et al., 1984; Stenbeck and Horton, 2004*). Most but not all of the degradation process of bone matrix takes place in the resorption lacuna. Indeed, the cells engulf the digested bone matrix and further degrade it in the transcytotic

vesicles before releasing it from the FSD. This is supported by the observation that TRACP, which generates reactive oxygen species (ROS), in order to destroy collagen, and cathepsin K, are located in transcytotic vesicles (*Halleen et al., 1999; Vaaraniemi et al., 2004*). Furthermore, labeled bone matrix taken up from resorption lacuna is located in the same vesicles as TRACP and cathepsin K, suggesting the bone matrix is digested further in these vesicles (*Mulari et al., 2003; Salo et al., 1997*).

Osteoclasts undergo several resorbing cycles before they undergo apoptosis. Normally, one resorption cycle takes several hours as observed by monitoring actin ring formation and disappearance in vitro (*Lakkakorpi and Vaananen, 1996*). During these cyclic changes, the cytoskeleton undergoes remarkable dynamic rearrangement. The actin ring is a bundle of F-actin which follows the shape of the sealing zone in resorbing osteoclasts. Initially, podosomes, components of the sealing zone, nucleate actin filaments in their core and are covered by vinculin -talin- β-integrin complex, which anchor actin filaments to the membrane (*Destaing et al., 2003; Ezzell et al., 1997; Xu et al., 1998*). After the osteoclast starts the resorption process, the actin ring structure is clearly visible by staining with phalloidin. The ring is surrounded by a double circle of vinculin and talin. However, αvβ3 integrin is absent from the sealing zone, although intergin is an initial trigger for podosome formation (*Lakkakorpi and Vaananen, 1996; Mulari et al., 2003*).

2.2.6 Membrane trafficking in resorbing osteoclasts

Under the electron microscope, a resorbing osteoclast displays a 'foamy' appearance. This is due to the fact that osteoclasts are highly dynamic cells with intensive membrane traffic. Active cells express a continuous process of exocytosis of acid and enzymes. At the same time there is a continuous endocytosis of digested material thus keeping the osteoclast resorbing (*Salo et al., 1997; Stenbeck and Horton, 2004*).

When osteoclasts are stimulated by integrin αvβ3 ligands, the cells start to be polarized. Intracellular membranes are transported intensively and precisely to establish membrane domains. Intracellular vesicular trafficking also plays a vital role in keeping the resorbing machinery on-going. The endocytotic /biosynthetic pathway is crucial for osteoclastic bone resorption since not only it brings protons and proteases into the resorption lacuna, but also continuously brings membranes to the ruffled border, which is the resorbing organelle of the osteoclast. It has been shown that several members of Rab small GTPases regulate these processes, for instance, Rab5 regulates endocytosis from the basolateral plasma membrane and transport to early endosomes whereas Rab7 regulates the delivery from early endosomes to late endosomes. Some late endosomes contain a lot of internal membranes invaginating from the limiting membrane. These late endosomes are also known as multivesicular bodies (MVBs). Rab7 also regulates MVB fusion to the ruffled border (*Mulari et al., 2003; Zhao et al., 2001*). However, the mechanisms by which Rab7 directs the vesicles to the ruffled border remain largely unknown. On the other hand, degraded bone matrix needs to be removed via a transcytotic route, since the sealing zone restricts the products diffusing to the surroundings. The bone debris are

removed by endocytosis at the centre of the ruffled border into vesicles that then pass through the central part of the cell and are released at the FSD. However, no Rab protein has been found to regulate the transcytotic pathway, although it seems clear that some GTPases should participate in this traffic. Since transcytosis takes away membranes from the ruffled border, it must be balanced with new membrane material that continuously fuses at the fusion zone of the ruffled border. Thus, membrane trafficking contributes to ruffled border turnover, which is vital for resorption (Fig 1).

2.3 The cytoskeleton and its organization in osteoclasts

The cytoskeleton is a cellular scaffold present not only in eukaryotic, but also in some prokaryotic cells. It undergoes highly dynamic changes for maintaining cell shape, allowing cellular locomotion, intracellular membrane transport and cytokinesis.

Each cell contains three main kinds of cytoskeletal filaments: microfilaments, intermediate filaments and microtubules. The filaments co-operate with each other in order to deal with the various tasks of the cell.

A microfilament is composed of two helical interlaced strands of actin subunits (also called F-actin or filamentous actin) which are made by the head-to-tail polymerization of actin monomers (G-actin). Microfilaments are the thinnest fibres of the cytoskeleton (measuring approximately 7 nm in diameter), which are part of the contractile apparatus in cells. Microfilaments are abundant underneath the plasma membrane, and are linked by α-actinin. Vinculin links actin filaments to plasma membraneand in epithelial cells. The head domain of vinculin associates to E-cadherin via α- or β-catenin complex (*Nelson et al, 2008; Nelson, 2008*). The tail domain of vinculin binds to membrane lipids and to actin filaments (*Goldmann and Ingber, 2002*). This network formation of microfilaments at the cortical part of a cell enables resistance of tension and maintenance of cellular shape, formation of cytoplasmatic protrusions, microvilli and distal axon in neurons, and participation in some cell-to-cell or cell-to-matrix junctions (*Izard et al., 2004*) (Fig. 2). Motor protein myosin is responsible for the motility of vesicles on actin filaments.

In resorbing osteoclasts, actin filaments are collected at the sealing zone where actin filaments are gathered into a ring-shaped adherent structure that fixes the cell on the bone and seals the resorption lacuna. The drug cytochalasin D has been used to disrupt actin cytoskeleton in osteoclasts and has been shown to impair bone resorption by inducing a complete disappearance of the ruffled border and clear zone as well as microvilli on their basolateral cell surfaces (*Sasaki et al., 1993*). Strikingly, actin is also located intensively at the periphery of the ruffled border suggesting that the release of the acid and proteases into resorption lacuna is mostly mediated by the actin cytoskeleton (*Mulari et al., 2003; Salo et al., 1997*).

Fig. 2. Actin filaments, α-actinin, catenin, vinculin and cadherin at cell adherens junction.

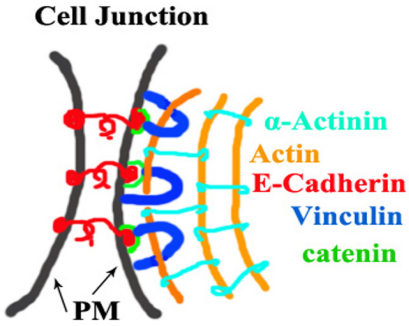

Fig. 2. Red: E cadherin. Green: catenin. Blue: vinculin. Orange: Actin filaments. Light Blue: α-actinin. PM: plasma membrane.

The intermediate filament fibres are on average 10 nm in diameter. There are several types of intermediate filaments, each constructed from one or more proteins. Intermediate filaments provide a supporting framework and function in the maintenance of cell-shape by bearing tension, and are more durable than actin filaments. Vimentin intermediate filaments provide mechanical stability and are the structural support for many cells, such as muscle cells, fibroblasts, monocytes and microphages (*Fuchs and Weber, 1994*). Lamin filaments provide structural support to the nuclear envelope in almost all cells (*Wilson et al., 2001*). A perinuclear intermediate filament network has been shown to orchestrate the microtubular cytoskeleton in resorbing osteoclast (*Mulari et al., 2003*).

Microtubules are straight hollow cylinders about 25 nm in diameter. The main constituent of microtubules is tubulin, which polymerizes as a heterodimer of α- and β-tubulin. Microtubules co-operate with other components of the cytoskeleton such as actin and are involved in a range of basic cellular processes including maintenance of cell shape, intracellular membrane transport and positioning of sub-cellular organelles (*Goldstein and Yang, 2000*). Microtubules are also involved in extracellular transport by means of cilia, and movement of cells by means of flagella and cilia (*Mimori-Kiyosue and Tsukita, 2003*). Similar to microfilaments and intermediate filaments, microtubules also provide support to cell shape. During mitosis, microtubules are organized into the mitotic spindle from centrosomes on the opposite poles of the cell. The spindle participates in the separation of the chromosomes into the two daughter cells.

Microtubules are highly dynamic structures. One end is terminated by α-tubulin anchoring on the centrosome named minus end and the other end (plus end) is terminated by the β-tubulin which is capable of binding GTP for polymerization. The plus end becomes prone to depolymerization upon hydrolyzing GTP. The rapid dynamic changes of polymerization and depolymeriation cause high instability of the microtubules, which contribute to their various cellular functions. The polarity of the microtubules is a determining fact for direction-dependent intracellular vesicular trafficking (*Unger et al., 1990*). Microtubules transport vesicles and organelles by means of association with motor proteins, such as dyneins (to the minus ends) and kinesins (to the plus ends) to

transport vesicles and organelles, such as transport of neurotransmitters to the dendrite for synaptic transmission (*Slaughter et al., 1997*). Another important characteristic of microtubules is structural dynamic change sensitive to temperature. It has been shown that a temperature of lower than 10°C disintegrates microtubules in cells whereas re-assembly occurs when a shift to 37°C in the presence of enough magnesium, GTP and MAPs (microtubule-associated proteins) *in vitro* (*Caplow et al., 1988; Shelanski et al., 1973*). Each cell has one centrosome which serves as the main microtubule organizing center (MTOC) from where microtubules array. MOTC makes a pair of centrioles which is surrounded by pericentriolar material (PCM) in which the proteins are responsible for microtubule nucleation and anchoring. These proteins are γ-tubulin, pericentrin and ninein (*Mogensen et al., 2000*). MTOC locates near the nucleus, where microtubules anchor with their minus ends. The plus end is always towards the cell periphery. Microtubule dynamics is important for cell migration (*Gotlieb et al., 1983*).

Osteoclasts are formed by fusion of mononuclear cells. The centrosome from each cell disappears when the cells are fused into mature osteoclasts in mammals. Although there is no MTOC in mammalian osteoclasts, the polarity of microtubules still exists since the MTOC pericentriolar material proteins re-distribute on the surface of the nuclear envelope resulting in the microtubule minus ends arraying from there. Microtubules are important for transport vesicles in the endocytic route before reaching the fusion zone of the ruffled border (the zone where actin filaments are rich). Microtubules and dynamin II have been discovered at the center of the ruffled border suggesting that the uptake of degrade matrix and further transcytosis are microtubule-dependent (*Mulari et al., 2003*). Interestingly, chicken osteoclasts have centrosomes, and the number of centrosomes is the same as the number of nuclei (*Mulari et al., 2003*).

Several drugs have been shown to effect microtubules, including nocodazole, vinblastine, colchicines and taxol. Nocodazole is commonly used in experiments to interfere with the stability of microtubules. Taxol (Paclitaxel**),** which was first isolated from the bark of the pacific yew tree, is widely used as an anti-cancer drug due to its binding to the β subunit of tubulin which leads to blocking of microtubule dynamics (*Kumar, 1981*). The cytoskeleton plays an important role during osteoclastic bone resorption, since either interfering with the microtubule network or disruption of the actin cytoskeleton inhibits bone resorption (*Shimizu and Sasaki, 1991; Zaidi, 1990*).

2.4 Lipid rafts and membrane trafficking

The majority of cell membrane components are lipids which are mainly composed of the three following classes: phospholipids, glycolipids and steroids. Subcellular membranes have unique compositions of these lipids. Plasma membrane, as well as early endosomes generally consist of a high level of glycosphingolipids and cholesterol, whereas late endosomes are rich in neutral phospholipids, including triglycerides and cholesterol esters (*Gruenberg, 2001; Kobayashi et al., 1998*). Lysobisphosphatidic acid (LBPA) seems to be located almost exclusively on the luminal membranes of

MVBs (*Kobayashi et al., 1998*). Membrane-anchored proteins may use different ways for membrane binding. They can be anchored on the membrane by covalently bound lipids, such as glycosylphosphatidylinositols or fatty acids (eg. palmitate or myristate). Some membrane proteins can also attach to an isoprenoid group, such as a farnesyl residue, via a thioether linkage to cysteine (*Baron and Seabra, 2008*). Phospholipids are the most abundant, and reach up to 30 % of the plasma membrane of red blood cells. Phospholipids in the cell membrane are "fluid" and exhibit rapid lateral diffusion. However, there are microdomains, known as lipid rafts and caveolae, rich in cholesterol and sphingolipids that are resistant to non-ionic detergents. Due to their insolubility in non-ionic detergents, such as Triton X-100 at low temperature, lipid rafts can be isolated and are called DIGs (detergent-insoluble glycolipid-enriched complexes) (*Jacobson and Dietrich, 1999; Simons and Ikonen, 1997*). They distribute ubiquitously in the cell membrane including the plasma membrane and vesicles in the endocytic and secretory pathways. They have been proposed as platforms for the attachment of proteins on the membrane and play an important role in signal transduction and membrane traffic (in a clathrin-independent manner) (*Parton and Richards, 2003*).

In an inherited disease, NPC (Niemann Pick disease type C, an autosomal recessive disorder), patients accumulate LDL-derived cholesterol in late endosomes and lysosomes by a blockage of efflux of cholesterol out of these vesicles. The defect is due to mutations in one of the late endosomal/lysosomal proteins NPC1 and NPC2. This interferes not only with steroid transport, but also with protein sorting and transport that results in the blockage of the endocytic pathway (*Mukherjee and Maxfield, 2004; Ory, 2000; Zhang et al., 2001*). In resorbing osteoclasts, sequestration of cholesterol in late endosomes by the hydrophobic amine U18666A, which mimics NPC disease, blocks transport of the vacuolar H+- ATPase and cathepsin K to the ruffled border. U18666A also blocks membrane trafficking pathways from the basolateral membrane to the ruffled border (*Zhao et al., 2006*).

Phosphatidylinositol is a distinct phospholipid having an inositol headgroup with hydroxyl groups that can be modified reversibly by phosphorylation at the 3', 4' or 5' position (*Katzmann et al., 2002; Odorizzi et al., 2000; Sato et al., 2001*). Phosphatidylinositol 3- phosphatase (PI3P) has an essential role in endosomal traffic. For instance, Rab5 regulates the production of PI3P by recruitment of its effectors Vps34 and PI3Kß, two PI 3-kinases that directly interact with Rab5 (*Shin et al., 2005*). PI3P locates on the endosomal membranes where it recruits cytoplasmic effectors that have either an FYVE domain (e.g. EEA1) or a PX domain and other transport factors (e.g. Vps27/Hrs which are involved in the regulation of mono-ubiquitinylated cargo protein transport) (*Katzmann et al., 2002*). PI3P-enriched membrane is eventually delivered into the lumen of the vacuole/lysosome where it is degraded (*Wurmser and Emr, 1998*). In contrast, LBPA enriched luminal membrane of MVBs is resistant to degradation (*van der Goot and Gruenberg, 2006*).

Lipid rafts are also present in osteoclasts. Increasing the expression of flotillin (a component of rafts) enhances osteoclast differentiation whereas disruption of rafts reduces the survival of osteoclasts as well as actin ring formation, resulting in impairment of bone resorption (*Ha et al., 2003*). A recent study has shown that inhibition of cholesterol transport in osteoclasts by the compound U18666A blocks the late endocytic pathway to the ruffled border (*Zhao and Vaananen, 2006*).

Studies of vesicular stomatitis virus (VSV) G protein (not associated with lipid rafts) in osteoclasts have shown it to be delivered to the basolateral membrane, whereas hemagglutinin of the influenza virus is delivered to the functional secretory domain (*Salo et al., 1996*). This raises the possibility that rafts may specifically regulate protein targeting.

2.5 General features of cell membrane trafficking

Changes in the environment influence the functional progress of cells via delivery of diverse 'commodities' such as ions, sugars, amino acids, and signal molecules, e.g. growth factors and hormones, through plasma membrane into the cells (Holsbeeks et al., 2004). Except for small molecules, most are transported through the endocytic pathway. Meanwhile, cells need to maintain their own metabolism and fulfil the functions of the tissue (organ) by the synthesizing of proteins and lipids. Some of the newly synthesized products of the cells are transported to the plasma membrane via the secretory pathway (*Seabra and Wasmeier, 2004*).

Extracellular cargoes such as proteins are invaginated into vesicles when they are delivered into the cell. In the transport route, the cargo is included in the vesicles that bud off from the donor membrane compartment. The donor vesicles then target the receiving membrane compartment and fuse with it, delivering the cargo to this compartment. These membrane trafficking events are regulated by the family of small GTPases (guanosine triphosphatase), the Rab protein family (*Bucci et al., 1992; Bucci et al., 2000; Pfeffer, 2001*).

2.5.1 Ras superfamily of small GTPases

In mammals, the Ras super family of small guanosine triphosphatases (GTPases) comprises over 150 members (*Wennerberg et al., 2005*). They are monomeric and evolutionally conserved from yeast to humans (*Colicelli, 2004*). These small GTPase proteins serve regulatory functions ranging from cell growth, cytoskeleton dynamics to membrane trafficking. They share common biochemical mechanisms and act as binary molecular switches. These small GTPases exhibit high-affinity to bind GDP or GTP (*Pfeffer, 2001*). Thus, two classes of regulatory proteins, guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) are needed to cycle them between GTP-bound (active) and GDP-bound (inactive) forms, respectively (*Bernards and Settleman, 2004; Schmidt and Hall, 2002*). With the exception of the Ran family small GTPase, Ras super family proteins undergo posttranslational modifications to direct them to specific subcellular

locations where they meet diverse proteins as effectors, and allow these small GTPases to function as sophisticated regulators in a remarkably complicated and diverse array of cellular processes (*Magee and Seabra, 2005; Seabra and Wasmeier, 2004*). The switch I and switch II regions of the Ras super family proteins mainly undergo conformational change depending on nucleotide status. These are important for the binding of regulatory proteins and effectors. Five subgroups, Rab, Rho, Ras, Arf and Ran, are defined based on their sequence similarity and functions (*Wennerberg et al., 2005*) (Fig. 3, 4, 5).

The Ras sarcoma oncoproteins (36 members) are members of the Ras family (*Wennerberg et al.2005*). They serve as signaling network regulators that respond to diverse extracellular stimuli by interacting with distinct downstream effectors, which in turn control gene expression and regulation of cell differentiation, proliferation and cell survival (*Repasky et al., 2004*).

The Rho family (20 members of Ras homologous) proteins are key regulators in several cellular processes, including cell proliferation, cell cycle procession, and cytoskeletal organization in response to extracellular stimuli. Rho proteins also regulate cell polarization, adhesion, migration, membrane ruffling, and membrane trafficking (*Ridley, 2001*).

The Rab family was named because the first Ras-like protein was found in the brain (Rab). They are major regulators of intracellular membrane trafficking. Details about Rab proteins are described in 2.5.2.

The Ran family, Ras-like nuclear proteins, is the most abundant small GTPase family in the cell and is best known for its function in nucleocytoplasmic transport of both RNA and proteins (*Weis, 2003*). Ran-specific nuclear GEF and cytoplasmic GAP regulate GTP-Ran accumulation in the nucleus and facilitate the directionality of nuclear import and export (*Weis, 2003*). The Ran GDP/GTP cycle regulates DNA replication, nuclear envelope assembly and mitotic spindle assembly (*Li et al., 2003*). Ran family proteins undergo significant conformational changes during GTP/GDP cycling. The C-terminal sequence participates in the interaction with effectors, in addition to the switch I and switch II regions of Ran (*Li et al., 2003*).

Arf family proteins belong to the ADP-ribosylation factor family. The function of this family is involved in the regulation of membrane trafficking, similar to Rab proteins. The N-terminal end of Arf contains the switch I and switch II regions. In addition, the N-terminal end participates in the conformational changes that allow the myristate group (a 14-carbon saturated fatty acid moiety) in the GTP form of Arf to interact with the membrane (*Pasqualato et al., 2002*).

2.5.2 Rab proteins and their functions in cell biology

Rab proteins control cellular events such as endocytosis, secretion, signal transduction and development. Their molecular mechanisms of action have gained increasing attention since their initial discovery in the 1980s. Rab GTPases regulate vesicular budding,

tethering and fusion in their intracellular transport and also play an important regulatory role in vesicular motility by recruiting motor proteins to organelles and transport vesicles on the actin or microtubule cytoskeleton (*Bucci et al., 2000; Jordens et al., 2001; Mellman, 1996a; Strom et al., 2002; Waters and Pfeffer, 1999*). Rabs are responsible for organization of the distribution of distinct membrane organelles (*Pfeffer, 2001; Zerial and McBride, 2001; Zerial and Stenmark, 1993*). In addition, they coordinate intracellular signalling events with membrane traffic and participate in embryo development (*Miaczynska et al., 2004; Pelissier et al., 2003; Riggs et al., 2003*).

Rab GTPases comprise the largest family of the Ras super family of small GTPases. The earliest identified Rabs were from rat brain and were named Rab1, 2, 3 and 4 (*Touchot et al., 1987*). During the last two decades, more than 60 members have been identified in mammals. Most of the Rab proteins are ubiquitously expressed although some seem to be expressed in a tissue-specific or developmental manner. For example, Rab27a is expressed only in melanocytes and secretory cell types (*Tolmachova et al., 2004*). The Rab proteins, as other Ras super family small GTPases, are evolutionarily conserved. Eleven Ypt (yeast protein for transport) genes have been identified in yeast *Saccharomyces cerevisiae.* They are similar both in sequence and function as Rab proteins in mammals. Haubruck et al. showed that mouse Rab1a can compensate for the loss of Ypt 1p in yeast (*Haubruck et al., 1989*).

2.5.3 Structure of Rab proteins

Rabs, like all Ras super family members, have conserved regions (switch I and switch II) that are important for binding guanine nucleotide. These two regions undergo significant conformational changes when GTP hydrolyzes. The Ras super family GTPases comprises of 6 β sheets, 5 α helixes and 12 loops in their structure. Five regions (RabF) are conserved only in Rab proteins. RabF1 located in the loop2/ β2 inside the switch I region (40-50 amino acids) is the effector binding domain. The other four RabF regions, RabF2 (β3), RabF3 (loop4), RabF4 (α2/loop5), and RabF5 (β4/loop6), are in or surrounding the switch II region (66-98 amino acids). These RabF regions are sensitive to the GTP/GDP bound status and have been suggested to be involved in the binding of general regulatory proteins, such as Rab GDP dissociation inhibitor (GDI) and Rab escort protein (REP). Both of them associate better with GDP-Rab than GTP-Rabs and recognize all Rab proteins (*Pereira-Leal and Seabra, 2000; Sasaki et al., 1990; Seabra, 1996*) (Fig. 3).

In addition to these five RabF regions, four Rab subfamily (RabSF) regions are noted for determining the binding between individual Rab and specific effectors. RabSF1 resides in β1, RabSF2 is in α1/loop2, RabSF3 locates in α3/loop7 and RabSF4 is in α5. Mapping of RabSF regions on the crystal structure of Rab3A shows that RabSF1, RabSF3 and RabSF4 form one contiguous patch on the protein surface, whereas RabSF2 is located on the other side (*Pereira-Leal and Seabra, 2000*). A structural study of Rab7 interacting with RILP (a binding protein of Rab7) showed that RabSF1 and RabSF4 were needed for the interaction in addition to the two switch regions (*Wu et al., 2005*) (Fig. 3).

RabGDI contacts several residues within the highly conserved Rab switch I and II regions when they are in GDP-bound form. In contrast, they fit poorly with the Rab-binding site when Rabs are in GTP-bound form. RabGDI plays a central role in the recycling of Rab proteins by mediating their dissociation from membranes. RabGDI is associated with highly hydrophobic geranylgeranyl groups in the cytosol. However, how RabGDI binds to the lipid groups remains unanswered. It is known that when RabGDI binds to Rab (on its switch regions and C-terminal), it induces conformational changes at the lipid accepting cavity, suggesting a role for RabGDI in the extraction of the first geranylgeranyl group from the membrane (*Ignatev et al., 2008; Leung et al., 2006*).

Fig. 3. Aminoacid sequence of selected small GTP-binding proteins. Adopted from Pereira-Leal and Seabra, J. Mol. Biol. (2000) 301, 1077.

Fig. 3. Residues found to be Rab specific are highlighted in red. When a position is conserved in other families, the corresponding position is also highlighted in red. Green characters denote the conserved nucleotide binding (PM/G) motifs. RabF1-5 motifs include mostly Rab-specific positions. RabF1 localizes to the effector domain in the putative switch I region. RabF2-5 regions cluster in and around switch II region. RabSF1-RabSF4 (highlighted in yellow) motifs are Rab sub-family specific regions which show higher identity within the Rab sub-family than the overall sequence. α1-5: Alpha-helices 1-5. β1-6: Beta-sheets 1-6.

Rab displacement from GDI and membrane delivery is catalysed by the GDI displacement factor (GDF). An example of this family, Ypt-interacting protein 3 (Yip3), also called prenylated Rab acceptor 1 (Pra1), acts as a GDF for Rab9. Yip3, which is also an integral membrane protein, binds to prenylated GDP-Rab proteins and displaces RabGDI. It has been shown to catalyse the dissociation of Rab9 from RabGDI and promote the recruitment of Rab9 onto membranes in an *in vitro* study, and it is specific only to Rab9 and Rab5 (*Barrowman and Novick, 2003*).

2.5.4 Rab prenylation

The most common post-translational lipid modification of proteins, prenylation, is crucially important for the association of small GTPase with the membrane. The mevalonate pathway is an important metabolic pathway synthesizing cholesterol and non-sterol isoprenoids. As illustrated inside the box in Fig. 3, the products of the mevalonate pathway, DMAPP (dimethylallyl pyrophosphate) and IPP (isopentenyl pyrophosphate), serve as building blocks for the biosynthesis of molecules used in diverse cellular processes (*Swanson and Hohl, 2006*). Protein prenylation of the Ras super family of small GTPases is the important non-sterol downstream product of this pathway, as shown in blue in Fig. 4 outside the box (*Konstantinopoulos et al., 2007*).

Fig. 4. The mevalonate pathway

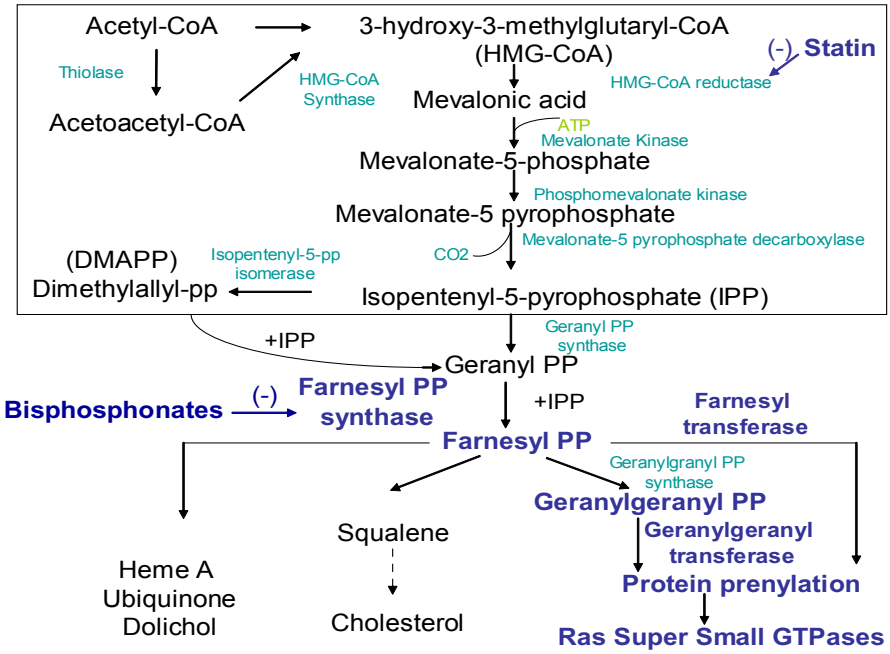

Fig. 4. The mevalonate pathway: synthesizing cholesterol and non-sterol isoprenoids. The products of the pathway synthesize dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), which serve as the basis for the biosynthesis of molecules used in diverse cellular processes (as shown in box). Protein prenylation of the Ras super family of small GTPases is the important non-sterol downstream product, as shown in blue text outside the box. Heme A, dilichol and ubiquinon are other non-sterol downstream products, whereas cholesterol is the sterol downstream product of this pathway.

Two groups of drugs inhibit the mevalonate pathway: statins and bisphosphonates. Statins, HMG-CoA reductase inhibitors, are used as pharmaceutical agents to lower cholesterol levels in people with or at risk for cardiovascular disease. Bisphosphonates are a class of drugs that inhibit bone resorption and have a high affinity to bone hydroxyapatite (*Fleisch, 1991*). Indications for bisphosphonates include the prevention and treatment of osteoporosis, osteitis deformans ("Paget's disease of bone"), bone metastasis (with

or without hypercalcemia), multiple myeloma and other conditions that feature bone fragility (*Bell and Johnson, 1997*). Nitrogen-containing bisphosphonates (N-BPs) with bulkier side-chains and a nitrogen moiety either in the alkyl chain such as alendronate and ibandronate, or with a heterocyclic structure such as risedronate and zoledronate, were identified as farnesyl diphosphate synthase inhibitors in the mevalonate pathway. This prevents the formation of two metabolites (farnesol and geranylgeraniol) that are essential for connecting small proteins to the cell membrane (*Black et al., 2007; Russell et al., 2008*) and results in the inhibition of sub-cellular membrane trafficking, thus affecting the motility and mobility of resorbing osteoclasts. The analogues of N-BPs selectively inhibit different small GTPases downstream of farnesyl diphosphate synthase, which leads to new potential treatment applications of this old drug. NE10790, an analogue of the risedronate, inhibits Rab RGGT the enzyme which geranylgeranylates ~22-26 kDa Rab proteins, showing a weak inhibition of bone resorption by interference of Rab-dependent intracellular membrane trafficking (*Coxon et al., 2001*).

Three distinct protein prenyl transferases have been identified and defined as two functional classes. CAAX prenyl transferases include farnesyl transferase (FT) that functions mainly in the prenylation of Ras GTPases, and geranylgeranyl tranferase type I (GGT-I) functions mainly in the prenylation of Rho/Rac family proteins. Rab geranylgeranyl transferase (RGGT or GGT-II) is specific only for Rab prenylation. The prenylation involves the covalent addition of either farnesyl (15 carbon) or geranylgeranyl (20 carbon) pyrophosphate to proteins via thioether linkage catalyzed by these protein prenyl transferases. RGGT was first isolated from rat brain cytosol as a multicomponent enzyme with another protein, REP (Rab escort protein) (*Seabra et al., 1992*). Further studies showed that RGGT alone does not recognize short peptides containing the Rab c-terminal prenylation motif, which is in contrast to CAAX prenyl transferases (FT and GGT-I). However, REP associates first with unprenylated Rab, which is then prenylated by RGGT by attaching the geranylgeranyl groups onto the c-terminal cysteines of the Rab (the classic prenylation pathway of Rab) (*Baron and Seabra, 2008*).

Both REP and GDI show a marked binding preference towards GDP-bound Rabs. They also share a structural similarity. Newly synthesised Rabs require REP for recognition by Rab geranylgeranyl transferase (RGGT). REP is also thought to deliver the newly prenylated Rab to the target membrane, a role similar to that of RabGDI for recycling Rabs from cytosol back to their starting compartment (*Baron and Seabra, 2008*).

Most Rab proteins contain a dicysteine prenylation motif at the C-terminus (CC or CXC). The double prenylation at the C-terminus of Rab makes the protein more hydrophobic than a single prenylation and that may be the reason why REP is required to chaperone Rab proteins in the cytosol during and after prenylation. Moreover, the double prenylation is important for targeting since mutants of Rab5 or Rab27a with only one cysteine at the C-terminal caused mislocation to the endoplasmic reticulum/Golgi region. However, some Rab proteins, such as Rab8 and Rab13, contain only a single cysteine motif (*Ali and Seabra, 2005; Gomes et al., 2003*). The CXXX motif of these Rabs seems to be a

canonical CAAX, which is similar to the Ras and Rho C-terminus. However, instead of being prenylated by class 1 FT and GGT-1, these Rab proteins are still prenylated by RGGT. The reason why most Rab proteins are dicysteine prenylated whereas some undergo single prenylation is unclear.

Postprenylation processing and methylation are another factor that regulates the proper targeting of Ras and Rho proteins in addition to some Rab proteins. Studies have shown that the CAAX motif of Ras, Rho and Rab are methylated on the C-terminal cysteine after prenylation and removal of AAX. It is believed that the carboxyl methylation of the farnesylated/geranylgeranylated cysteine increases hydrophobicity, which results in the decreased affinity of these GTPases to GDI in the cytosol and an increase of association to membranes (*Leung et al., 2007*).

2.5.5 Rab GTP/GDP cycling

Newly synthesised Rab proteins are in GDP-bound form in the cytosol. The activation of the Rab proteins is initiated by an attachment to membranes mediated by geranylgeranylation of (usually) two C-terminal cysteines by Rab geranylgeranyltransferase (RGGT) in concert with REP (Rab escort protein). Once a Rab has been geranylgeranyl-modified, it remains so for its whole lifespan. The geranylgeranyl-modified GDP-bound Rab proteins are delivered to their respective membranes and undergo a cycle of GTPase activity. This cycle is regulated by GTP/GDP exchange factors (GEFs), which accelerate the otherwise very slow rate of GDP dissociation. Rabs are activated by replacement of GDP to GTP by GEF, allowing interaction with a wide variety of effector molecules. After completion of these events and GTP hydrolysis by GTPase activating proteins (GAPs), the resulting GDP Rabs are extracted from the membrane by GDI (GDP dissociation inhibitor) into the cytosol. Then the Rabs are delivered back to their starting compartment to start a new cycle by replacing GDI with GDF (GDI displacement factor) as illustrated in Fig. 5.

2.5.6 Rab interacting proteins (effectors)

The term effector implies a protein that responds to a specific Rab and mediates at least one element of its downstream effects. The effectors bind to a specific Rab selectively in its GTP-bound state. Each Rab appears to signal through a variety of effectors that act to translate the signal from one Rab protein to several diverse aspects of membrane transport, thus contributing to specificity in membrane traffic. However, the same effector can also be shared by several Rab proteins which are functionally related, providing the concerted action of these Rabs within one pathway (*de Renzis et al., 2002*).

The first Rab effector identified was Rabphilin 3A which was purified from bovine brain crude membranes. It acts as a target protein for Rab3A (also named smg p25A), regulates neurotransmitter release in neuronal cells, and specifically binds to GTP-bound Rab3A. Rabphilin concentrates not only on the secretory vesicles of neurons but also in endocrine cells and undergoes association/ disassociation with Rab3A while it cycles from GTP to GDP (*Shirataki et al., 1993*). After this discovery, many Rab specific effectors have been

found by two-hybrid and affinity protein purification methods, for instance Rabaptin and EEA1 for Rab5, RCP (Rab coupling protein) and Rab11-Fip1-4 for Rab11, and RILP for Rab7 (*Cantalupo et al., 2001; Horiuchi et al., 1997*). The Rab-effector interactions play important regulatory roles, e.g. in motility by recruiting molecular motors to organelles and transport vesicles via microtubules and actin filaments. For instance, Rab27a recruits a specific effector called melanophilin on the melanosome, which in turn binds myosin Va (*Strom et al., 2002*).

Fig. 5. Activation cycle of Rab-GDP

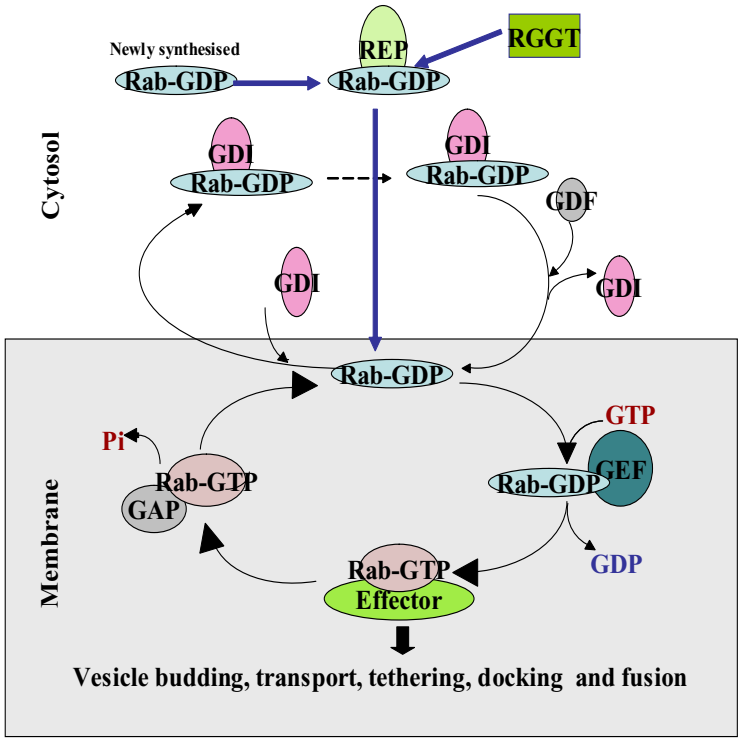

Fig.5. Rab GDP/GTP cycling: GDP-bound Rab mainly localizes in cytosol, whereas GTP-bound form Rab is on the membrane. GDP-bound Rab is delivered to the target membrane and undergoes a cycle of GTPase activity. Rab is activated by replacement of GDP to GTP by GEF, allowing interaction with a wide variety of effector molecules to carry out Rab's function. Rab is inactivated by GTP hydrolysis with GAP. GDP Rab is extracted from the membrane by GDI into the cytosol. Rab is delivered back to its starting compartment to start a new cycle by replacing GDI with GDF.

2.5.7 Regulation of Rab proteins in vesicular tethering, docking and fusion

Rabs form diverse complexes with effectors to regulate the vesicular budding, tethering, docking and fusion of membranes (*Pfeffer, 2001; Seabra and Wasmeier, 2004; Segev, 2001*). Rab proteins and their effectors participate in the generation of vesicles (*Deneka*

et al., 2003). For instance, GTP-bound Rab9 interacts with TIP47 which forms a ternary complex with Rab9 and the cytoplasmic tail of cation-independent mannose 6-phosphate receptor and draws the membrane from a donor to a budding vesicle (*Carroll et al., 2001; Diaz and Pfeffer, 1998*). During budding of COPII vesicles, Rab1 is able to recruit tethering protein p115 to 'catch' the newly formed vesicles on the target membrane, which in turn interacts with a complex of SNARE (soluble N-ethylmaleimide-sensitive factor receptor protein) proteins (*Allan et al., 2000*). The tethering step is the first level of specificity to ensure that the vesicles reach the correct target organelle. The next step, docking, requires high fidelity of the vesicular transport since the pair of SNARE proteins which are separately located on the vesicle and target organelle (v-SNARE and t-SNARE), have to be exactly matched (*Chen and Scheller, 2001; Nuoffer and Balch, 1994*). Rab proteins are generally required for transport and vesicle docking and participate in reaction catalyzing SNARE complex assembly, which also involves the Sec1 homolog Sly1 (*Sogaard et al., 1994*). GTP-bound Rab5 has been shown to mediate homotypic endosome fusion (*Jedd et al., 1995; Segev, 2001*).

2.6 Endocytosis and endocytic pathway

The process of endocytosis can be divided into two types: phagocytosis and pinocytosis. Phagocytosis (cell-eating) refers to the process by which cells ingest large solid particles (>250 nm), such as bacteria, large viruses or apoptotic cells. The process of phagocytosis is usually restricted to some cell types, including macrophages, monocytes and neutrophils. The cell membrane folds around the object, where it is invaginated and sealed off into a large vacuole, namely the phagosome (Fig. 6A). Pinocytosis (cell-drinking) refers to uptake of extracellular fluid and small molecules (<200nm) (Fig. 6B). There are three well defined types of pinocytosis: macropinocytosis, clathrin-dependent and caveolea/raft-dependent endocytosis. The latter two are special types of pinocytosis that are receptor mediated; one is via clathrin-coated pits and the other is invaginated via caveolin-coated caveola which are raft-dependent and clathrin-independent (*Oh et al., 1998*). The major route of endocytosis is receptor mediated and a more specific active event where the plasma membrane folds inward to form coated pits (Fig. 6C). The scission of these inward budding vesicles (both clathrin- and caveolin-dependent) from the inner surface of the plasma membrane is effected by dynamin, a 95 kD GTPase and its associated proteins, which form a spiral ring around the neck of the pre-vesicles and tighten the spiral with GTP hydrolysis (*McNiven, 1998; Oh et al., 1998*)**.**

The endocytic pathway plays an essential role in transport of membrane components, soluble molecules and receptor-mediated ligands to various destinations in cells, thus allowing them to communicate with the environment. Endocytosis is also important to maintain cell polarity (*Maxfield and McGraw, 2004*). Small molecules such as ions, sugars and amino acids can easily traverse the plasma membrane through various transmembrane protein complexes that are embedded in the lipid bilayer and channels, pumps and transporters. In contrast, the transport of macromolecules and e.g. viruses,

and bacteria through the plasma membrane requires a specific endocytosis (*Bonazzi and Cossart, 2006; Conner and Schmid, 2003; Pizarro-Cerda and Cossart, 2006*).

Most substances important to cells are large polar molecules and they cannot directly pass through the hydrophobic plasma membrane. The best-understood endocytic process is the internalization of receptors and their corresponding ligands by clathrin-coated pits. Endocytosed material then resides in coated vesicles. The particular cargo-containing vesicle fuses with early endosomes. The transmembrane proteins delivered to early endosomes are rapidly sorted, and then enter a pathway to late endosomes and lysosomes for degradation, or are recycled back to the plasma membrane. In this manner, the cell can re-use receptors up to several hundred times (*Maxfield and McGraw, 2004*). The receptors can be recycled directly from early/sorting endosomes to the plasma membrane. Another possibility for receptor recycling is through a perinuclear membrane cluster, namely the perinuclear recycling compartment, from which the receptors return to the plasma membrane. Some proteins can also be sent to the TNG from the perinuclear recycling compartment (*Maxfield and McGraw, 2004*).

Fig. 6. Endocytosis

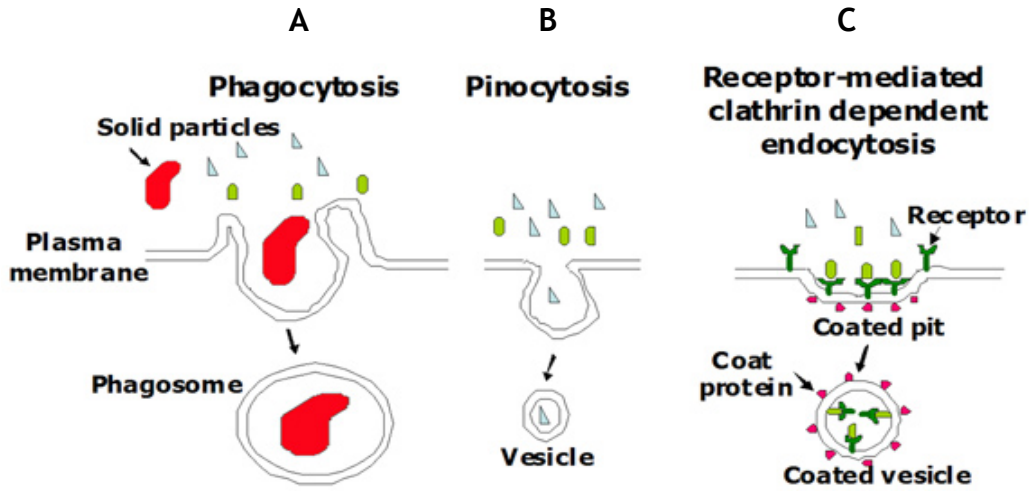

Fig. 6. Endocytosis: A. Phagocytosis (cell-eating) refers to the process by which cells ingest large solid particles. B. Pinocytosis (cell-drinking) refers to uptake of extracellular fluid and small molecules. C. Receptor mediated clathrin dependent endocytosis: a special type of pinocytosis that is receptor mediated via clathrin-coated pits on plasma membrane.

The lysosome is generally considered the end point of the endocytic pathway (*Mellman, 1996b*). It maintains low pH (about 4.5) by pumping in protons via proton pumps and contains an array of hydrolytic enzymes to destroy proteins, lipids, polysacharides and nucleic acids. Most intracellular and endocytic proteins targeted to this degradative compartment must pass through late endosomes. The biogenesis of late endosomes occurs by a process of maturation from the vacuolar elements of early endosomes. Certain regions of the endosomal membrane bud inward into the lumen and pinch off to

form small vesicles inside the original endosome. As a consequence of this distinctive morphology, late endosomes are commonly referred to as multivesicular bodies (MVBs). Late endosomes already display high efficiency in the degradation process. Surprisingly, 80 % of the proteolysis takes place in the late endosomes, although there are just 20 % of proteases of those in lysosomes (*Tjelle et al., 1996*). Proteins, lipids and carbohydrates remain in the lysosomes for complete digestion. Some membrane domains containing transmembrane receptors can bud off from early or late endosomes or lysosomes into small vesicles that are targeted to the TGN for recycling (*Allan and Balch, 1999; Maxfield and McGraw, 2004*).

Intracellular membrane transport uses the cytoskeleton, mainly microfilaments and microtubules for movement. Microtubules usually provide high-speed, long distance transport whereas the actin network manages slower and short distance local trafficking (*Jordens et al., 2005*). Motor proteins facilitate the transport of intracellular compartments on these networks by interacting with organelles and the cytoskeleton (*Tuxworth and Titus, 2000*). For instance, the myosin family of motors mediates transport of vesicles via the actin network. Their variable head domains hang on actin filaments and the tail domain mediates interaction with the cargo (*Mehta et al., 1999*). Rab27a has been shown to regulate the secretion of melansomes by interacting with its effector melanophilin, which in turn binds myosin Va and, therefore, drives vesicles along the actin cytoskeleton (*Seabra and Wasmeier, 2004*).

The transport of the recycling compartment is regulated by a direct interaction between Rab11 and myosin Vb. However, a lot of vesicular transport relies on the microtubule network, which is driven by two families of microtubule motor proteins, kinesins and dyneins (*Hirokawa, 1998; Vale, 2003*). Fourteen defined members of kinesins help to transport vesicles towards the plus end of the microtubule, the cell periphery, whereas two isoforms of dyneins drive the cargo compartments towards the minus end of microtubules, namely towards the microtubule organising centre (MTOC) (*Paschal and Vallee, 1987; Schnapp and Reese, 1989*). Kinesins have a similar structure to myosins, a motor head, a stalk and a tail domain, which is involed in binding microtubules and vesicles (*Lee et al., 2004*). Dynein is a massive multimeric complex. The heavy chains form a motor domain whereas the accessory intermediate chains bind both microtubules and regulatory proteins such as the activator dynactin, which links the complex to most cargo vesicles (*Schroer, 2004*).

2.6.1 Exocytosis and transcytosis

Exocytosis is a process by which cells secrete material by direct fusion of vesicles to the plasma membrane. Exocytosis releases soluble proteins out of the cell and incorporates membrane-bound proteins and lipids to the plasma membrane. The secretion pathway transports synthesized proteins from the endoplasmic reticulum, through the Golgi to secretory vesicles and finally to the plasma membrane for exocytosis.

There are two types of exocytosis. Constitutive exocytosis takes place in almost all cell types. It brings new membrane constituents to the plasma membrane and thus maintains a certain turnover of membrane components. The second is Ca^{2+} triggered non-constitutive exocytosis, an example of which is the releasing of neurotransmitters from synaptic vesicles in neuronal cells. In recent studies, it has been suggested that calcium at micromolar concentrations triggers SNARE complex formation and fusion between synaptic vesicles and reconstituted target membranes (*Hu et al., 2002*). Exocytosis also includes several steps of vesicular tethering and docking before fusion to the plasma membrane.

Transcytosis refers to the vesicular transport of macromolecules from one side of the cell to the other side. It is thus a method of selecting and moving material between two extracellular compartments, such as from the gut lumen to the circulation. Transcytosis is usually a process that avoids degradation of the internalized cargo. It can be clathrin-mediated or caveolae-mediated. Clathrin-mediated transcytosis of IgA by epithelial cells of the digestive tract, mammary gland and salivary gland is a well known example. Caveolae-mediated transcytosis by endothelial cells that line the microvasculature and carry circulating plasma proteins to the interstitium was discovered by Tuma et al. (*Tuma and Hubbard, 2003*). However, transcytosis is not restricted to epithelial and endothelial cells. Transcytosis has been shown to occur in M cells (microfold cells), hepatocytes, enterocytes, syncytiotrophoblasts, thyroid epithelial cells and alveolar epithelium (*Tuma and Hubbard, 2003*).

Transcytosis is also a feature of resorbing osteoclasts (*Nesbitt and Horton, 1997; Salo et al., 1997*). When the cell starts resorbing, the digested organic bone and minerals are taken up via the ruffled border in vesicles and transported to the functional secretory domain (FSD) via trancytosis. Interestingly, cathepsin K and TRACP and bone debris are present in these vesicles and these enzymes may digest the bone matrix further during transcytosis (*Vaaraniemi et al., 2004*). Thus far, little is known about the regulator(s) of this process.

2.6.2 Rab proteins and their roles in the endocytic pathway

The endocytic pathway is a highly dynamic system that coordinates multiple trafficking routes. Rabs are major regulators of intracellular vesicular transport between different organelles along the endocytic and secretory pathways. Each Rab has a specific subcellular location and regulates different steps of vesicular transport. The precise movement of the membrane to its destination organelle is managed by specific Rab proteins and their specific effectors (*Novick and Zerial, 1997; Seabra and Wasmeier, 2004; Zerial and McBride, 2001*). The regulation of endocytic vesicular trafficking of a range of molecules is involved in cooperatively and sequentially controlling the location of the vesicles, as well as vesicular tethering, docking, fusion and budding (*Mellman, 1996a*). Transport between two organelles may be governed by more than one Rab protein; thus, some Rabs may exhibit overlapping functions (*Deneka et al., 2003*). The transport of

vesicles along the cytoskeleton is done by motor proteins which are directly or indirectly regulated by Rab proteins (*Jordens et al., 2001; Strom et al., 2002*).

2.6.2.1 Role of Rab5 in the early endocytic pathway

Rab5 is a guanosine triphosphatase that is predominantly associated with early/sorting endosomes. It regulates the early endocytic pathway ranging from endocytosis to fusion of newly-formed (primary) endocytic vesicles to early endosomes. This pathway can be monitored using labeled transferrin, which reveals three sequentially displayed vesicular populations, only Rab5 occupied, Rab5 and Rab4 occupied, and Rab4 and Rab11 occupied vesicles. There is a difference between the first two populations of endosomes, namely the early and sorting endosomes. However, these two populations are generally named collectively as early/sorting endosomes (*Woodman, 2000*). The last population belongs to the recycling vesicles and will be discussed later (*Sonnichsen et al., 2000*). Rab5 has been proposed to participate in clathrin mediated endocytosis and indeed expression of wild type and GTP-bound Rab5 increases, whereas administration of anti-Rab5 antibody decreases endocytosis. However, Rab5 is rarely located on the plasma membrane (*Rauma et al., 1999; Sedej et al., 2005; van der Bliek, 2005*). A new GEF of Rab5 in C. elegans, RME-6, localizes at the plasma membrane in clathrin coated pits and provides the missing link for the function of Rab5 in endocytosis (*Sato et al., 2005; van der Bliek, 2005*). Therefore, the GEF of Rab5 that locates in the clathrin coated pits may differ from other GEFs, such as Rabex-5 which is required for Rab5 on early endosomes and Rab5 dependent membrane fusion (*Horiuchi et al., 1997*). Vps9 has similar domains to that of Rabex-5 and thus functions as a yeast homologue GEF for Rab5. Other GEFs of Rab5, Rin1 and ALS2/Alsin which also contain a Vsp9 domain, are specific for signal transduction processes (*Otomo et al., 2003; Tall et al., 2001*). For instance, the Rin1 family appears to regulate the trafficking of specific proteins to the endosome in response to external stimuli. Rin1 facilitates the degradation of activated EGF receptors and promotes the attenuation of the EGF signal transduction cascade (*Carney, D. S et al., 2006*).

Newly endocytosed vesicles are clathrin coated; however, their coats are removed before they fuse with early endosomes which are free of clathrin. GTP-bound Rab5 promotes endosome fusion by recruiting cytosolic components of the fusion apparatus (*Woodman, 2000*). The effector of Rab5, Rabaptin-5, is a homodimer with an extended structure which points to a role in membrane docking (*Stenmark et al., 1995; Vitale et al., 1998*). Rabaptin-5 is also a dual Rab protein effector, its c-terminus binds to Rab5 whereas N-terminus binds to Rab4, thus acting as a sensor to balance the inward and outward fluxes of the membrane (*Vitale et al., 1998*). EEA1 (early endosome autoantigen1) is another essential effector of Rab5 that mediates endosome docking (also called tethering factor). It contains an FYVE zinc finger that binds to lipid phosphatidylinositol-3-phosphate (PI3P) (*Simonsen et al., 1998*). Rab5 binds PI3 kinase (VPS34 (hVPS34) directly leading to the production of PI3P in situ, and recruits EEA1 for endosome fusion (*Gaullier et al., 1998; Patki et al., 1998*). Furthermore, EEA1 directly binds to syntaxin-6,

a v-SNARE for vesicular membrane fusion (*Simonsen et al., 1999*). Recently, Ohya, Zerial and colleagues found that early endosomal membrane fusion requires cooperation between Rab5, its effectors and cognate SNAREs. Rab5-SNARE complex forms a more efficient 'core machinery' than SNAREs alone (*Ohya et al., 2009*). Rab5 colocalizes with transferrin receptor, or internalized transferrin, the markers of early endosomes. For instance, endocytosis and transport of GLUT4 (insulin-responsive glucose transporter) in 3T3-1L adipocytes is controlled by Rab5. The Rab5 regulated transport of Glut4 to the minus end of the microtubules (MTOC) is dynein dependent (*Huang et al., 2001*). Infection of mammalian cells by a number of bacteria and viruses has been shown to depend on a Rab5-mediated internalization step (*Mallo et al., 2008; Jhons et al., 2009*). Rab5 RNAi is lethal in C. elegans, indicating the crucial role of Rab5 in cell function (*Grant and Hirsh, 1999*). In resorbing osteoclasts, Rab5 is located on early endosomes and colocalizes with EEA1 suggesting a similar location and function as that in other cell types (*Zhao et al., 2002*).

2.6.2.2 Rab7 in the late endocytic pathway

Two Rabs regulate late endosomal transport, namely Rab7 and Rab9 (*Chavrier et al., 1990; Lombardi et al., 1993*). Rab9 regulates late endosome–*trans*-Golgi network transport and Rab7 has been proposed to regulate early to late endosomes and late endosomes to lysosome transport (*Bucci et al., 2000; Chavrier et al., 1990; Feng et al., 1995; Meresse et al., 1995*). Feng et al. showed that GDP-bound Rab7T22N had no effect on the internalization of either HRP (horseradish peroxidase) or SV5 HN protein (paramyxovirus SV5 hemaglutinin-neuraminidase), but markedly inhibited the subsequent cleavage of the SV5 HN protein, thus supporting the role of Rab7 as a downstream regulator of Rab5 in regulating membrane transport leading from early to late endosomes (*Feng et al., 1995*).

Ypt7p, the homolog of Rab7 in yeast, works in endocytic traffic towards the vacuole (*Schimmoller and Riezman, 1993*). In HeLa cells, Rab7 is present on late endosomes, which are characterized by partial colocalization with the cation-independent mannose 6-phosphate receptor, Lamp-1 and 2 (lysosome-associated membrane protein), and cathepsin D, the markers for late endosomes (*Bucci et al., 2000; van Deurs et al., 1996*). Its location extends from the perinuclear area to the cell periphery (*Meresse et al., 1995*). Expression of wild type Rab7, mainly on the later endosomes, was partly present throughout the cytoplasm and partly concentrated in the perinuclear region (*Bucci et al., 2000; Chavrier et al., 1990*). GTP-bound Rab7 supports the transport of late endosomes to the perinuclear region. These combined data support the notion that Rab7 regulates transport from late endosomes to lysosomes.

The fate of cargo proteins which will be finally delivered to the late endosomes/lysosomes seems to have been determined at the step when they are internalized (*Bonifacino and Traub, 2003*). Mono-ubiquitinylated cargo proteins are sorted in the MVB pathway after their internalization and are eventually degraded in late endosomes/lysosomes (*Katzmann*

et al., 2002). The signal molecule mono-ubiquitin has a hydrophobic patch that contains Ile44 as a central residue. Many ubiquitin-binding proteins (several hundred in man) with 11 conserved ubiquitin-binding domains all bind ubiquitin on this patch (*Hicke et al., 2005; Raiborg et al., 2006*). When covalently attached as polymers on target lysine residues and chained up on ubiquitin (e.g. ^{29}Lys, ^{48}Lys, ^{63}Lys), cytosolic proteins are sorted into 26S proteasomal degradation in cytosol (at least covalent attachment of 4 units of the ubiquitin in a chain). Poly-ubiquitinylation is now also known to regulate a plethora of cellular activities by the transcriptional regulation of cell signaling.

However, many cellular activities are mediated by mono- rather than poly-ubiquitinylation, such as receptor down-regulation, membrane sorting and membrane trafficking (*Bonifacino and Traub, 2003; Katzmann et al., 2002*). Eps 15 [EGFR (epidermal growth factor receptor) -pathway substrate 15], which functions in clathrin-mediated endocytosis, is mono-ubiquitinylated in response to EGF stimulation. Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) is also mono-ubiquitinylated before clathrin-mediated endocytosis which is governed by Rab5. Internalization of Hrs is followed by sorting into MVBs, which is regulated by recognition of mono-ubiquitinylated proteins (*Raiborg et al., 2002; van Delft et al., 1997*). Recently, it has been shown that a GEF of Rab5, Rabex5, as well as its yeast homologue Vps21p, also binds ubiquitin, thereby regulating the early steps of mono-ubiquitinylated cargo transport towards the MVBs (*Bucci et al., 1992; Bucci et al., 2000; Davies et al., 2003; Mattera et al., 2006*). On the other hand, the transport of early endosomes to late endosomes/MVB is under the regulation of Rab7. Indeed, it is interesting to see how Rab7 takes over the mono-ubiquitinylated cargo from the control of Rab5.

We found that Rab7 directly binds ubiquitin by two hybrid experiments and confirmed the colocalization of these two molecules on the fusion zone of the ruffled border by double labeled immunofluorescent staining on the resorbing osteoclast, indicating that Rab7 may directly sort mono-ubiquitinylated cargoes into the compartment of degradation such as late endosomes and lysosomes (unpublished data).

Previous work has shown that the transport of the Rab7-positive compartment is dependent on the microtubule network since nocodazole treatment results in scattering of the compartment throughout the cytoplasm (*Meresse et al., 1995*). Recent studies revealed that Rab7 recruits the dynein/dynactin motors to the compartment when Rab7 is associated with its effector, RILP (Rab7-interacting lysosomal protein), resulting in the accumulation of these compartments at the minus end of microtubules (*Cantalupo et al., 2001; Jordens et al., 2001; Jordens et al., 2005*). However, no direct interaction exists between either Rab7 or RILP with these motors. It was proposed that a receptor for dynein, spectrin, may play a role as a potential mediator in recruiting Rab7 and the motor proteins, since spectrin recruits the Rab7 - RILP- ORP1L complex (oxysterol-binding protein-related protein 1L) to the motor and is required for the accumulation of the late endososmal compartment at MTOC (*Johansson et al., 2007; Jordens et al., 2005; Lebrand et al., 2002*).

Rab7, like other Rab proteins, fulfills its function via recruitment of its effectors and interacting proteins. In addition to the previously mentioned RILP, ORP1L, Vps34 and p150, Rab7 also interacts with other proteins such as Rab7ring with a Ring finger motif, playing roles in vesicle traffic to late endosome/lysosome and lysosome biogenesis (*Stein et al., 2003., Mizuno et al., 2003*). Rab7 also directly binds proteasome alpha-subunit XAPC7 on late endosomes (*Dong et al., 2004*).

Salmonella survives and replicates in a membrane-bound compartment, the Salmonella-containing vacuole (SCV). The maturation of invasion vacuoles of Salmonella impairs RILP recruitment to Rab7, therefore providing an additional protected space for bacterial replication (*Harrison et al., 2004*). In contrast to phagosomes containing latex beads, the SCV segregates from the normal endocytic pathway and does not contain mannose-6-phosphate receptor and lysosomal hydrolases such as cathepsin D and L (*Garcia-del Portillo and Finlay, 1995*). Rab7 locates on both the late endosomes and the SCV, but overexpression of Rab7 is not sufficient to regulate fusion between mature lysosomes and the SCV in HeLa cells. In overexpression of RILP, the effector of Rab7, SCVs were efficiently delivered to the minus ends of microtubules and fused with lysosomes (*Marsman et al., 2004*). However, recent data showed that SCVs were rapidly acidified, although there was some delay in comparison with vacuoles containing heat-killed Salmonella. The acidification was very similar to phagosome-lysosome fusion that involved Rab7 regulation (*Drecktrah et al., 2007*). RILP and the dynein motor have also been implicated in the regulation of fusion between latex-bead-containing phagosomes and lysosomes (*Harrison et al., 2003*).

2.6.2.3 Rab4 and Rab11 regulate the recycling pathway

If the endocytic pathway is divided into early and late endocytic pathways, the recycling pathway belongs to the former. The recycling pathway starts from early/sorting endosomes. Rab4 has been shown to regulate a fast route of recycling directly from early/sorting endosomes to the plasma membrane. Rab11 controls the canonical slow recycling pathway through the perinuclear recycling compartment (*Sheff et al., 1999; Ullrich et al., 1996; van der Sluijs et al., 1992*).

Transferrin, low-density lipoprotein, epidermal growth factor receptors and αvβ3 integrin can all be sorted into both the fast and slow recycling pathway by the regulation of Rab4 and Rab11, respectively (*McCaffrey et al., 2001; Roberts et al., 2001; van der Sluijs et al., 1992*). Rapid recycling of receptors such as β2AR (beta 2-adrenergic receptor) appears to occur from early/ sorting endosomes where they are dephosphorylated, and thereby allow the passing of the control of the membrane from Rab5 to Rab4 (*Seachrist et al., 2000*). Interestingly, Rab4 has also been proposed to play a role in the regulation of receptor degradation since the GDP-bound Rab4S22N leads to significant reduction in both recycling and degradation of LDL (low density lipoprotein) and EGF (epidermal growth factor) (*Daro et al., 1996*). Moreover, Rab4 also participates in the transport of receptors from sorting endosomes to the perinuclear recycling compartment in addition

to Rab11 by interacting with a dual effector (RCP) of both Rab4 and Rab11 and dynein light chain-1 (*Bielli et al., 2001; Damiani et al., 2004; Lindsay et al., 2002*). KIF3B, which belongs to the kinesin-2 family, associates with Rab4 to transport GLUT4 to the cell periphery for exocytosis via microtubules in a process mediated by insulin-induced PI3-kinase-dependent PKC-lambda (protein kinase C) activation (*Imamura et al., 2003*). Rabaptin4, an effector of Rab4, has been shown to facilitate the Rab4-mediated of delivery of cellubrevin and transferrin from early endosomes to recycling endosomes (*Nagelkerken et al., 2000*). Rab4 is also involved in the recycling of αvβ3 integrins in response to platelet-derived growth factor (PDGF) stimulation, indicating a role for Rab4 in cell adhesion and migration (*Roberts et al., 2001*). The Rab4-governed fast recycling pathway is involved in cell migration by interaction of several effectors and coupling proteins (*Jones et al., 2006*). Rabip4, an effector of Rab4, participated in the regulation for Rab4-controlled fast recycling of integrins and cell migration upon PDGF stimulation (*Vukmirica et al., 2006*).

The endocytic membrane recycling pathway regulated by Rab11 is essential for maintenance of normal membrane components, such as receptors, pumps, and channels as well as membrane lipids (*Fan et al., 2004; Grunfelder et al., 2003; Ullrich et al., 1996; Wakabayashi et al., 2004; Yoon et al., 2005*). A family of Rab11 interacting proteins (Rab11-FIPs) has been identified and shown to be involved in the regulation of the slow recycling pathway (*Cullis et al., 2002; Hales et al., 2001; Hales et al., 2002; Lindsay and McCaffrey, 2002; Peden et al., 2004; Prekeris, 2003; Wallace et al., 2002*). For instance, Rab11 takes over the vesicles from sorting endosomes to the PNRC by the interaction of RCP (Rab coupling protein) and dynein light chain-1 as mentioned previously (*Bielli et al., 2001; Damiani et al., 2004; Lindsay et al., 2002*). Class 1 Rab11-FIPs have been shown to regulate the exit of recycling vesicles from a perinuclear location to the periphery of the cell (*Lindsay and McCaffrey, 2004*). Rab11-FIP2 binds to the actin motor protein myosin Vb, which also directly binds to Rab11 and regulates membrane recycling from early endosomes to PNRC (*Hales et al., 2002*). MyosinVb is also associated with both the plasma membrane recycling system in nonpolarized cells and the apical recycling system in polarized cells (*Lapierre et al., 2001*). Masking this binding site of myosin Vb for Rab11 leads to the accumulation of receptors stalling at PNRC. Interestingly, the binding of Rab11 and myosinVb is sensitive to nocodazole, a reagent, which impairs microtubule stability, but can be restored by taxol treatment, indicating a yet undiscovered relationship between microtubules and actin filaments (*Lapierre et al., 2001*). Class 2 Rab11-FIP proteins including Rab11-FIP3/4 are known as the dual binding proteins for Arf6 and Rab11 and it has been suggested that they are involved in chemokinesis and cytokinesis (*Fielding et al., 2005; Horgan et al., 2004; Jones et al., 2006; Powelka et al., 2004; Shiba et al., 2006*). Rab11 also participates in the regulation of trafficking between the endosomal compartment and the trans-Golgi (*Chen et al., 1998; Urbe et al., 1993*). It has also been associated with the transport of post-Golgi vesicles to the plasma membrane (*Lock and Stow, 2005; Satoh et al., 2005; Wang et al., 2005*).

Interestingly, the fate of the destination of a receptor could be changed from the degradation pathway to the recycling pathway. For instance, when EGFR was unable to be mono-ubiquitinylated upon mutation of Tsg101 (tumor-susceptibility gene product), a homologue of Vps23, which has a ubiquitin-conjugating-like domain (UBC)/ubiquitin 2 variant domain (UEV), the receptor (EGFR) is unable to be sorted into late endosomes/ lysosomes. It enters the Rab11-governed recycling pathway and is delivered to the plasma membrane, therefore amplifying the signal and causing an increase of cancer metastasis (*Babst et al., 2000; Li and Cohen, 1996*).

2.6.2.3.1 The slow recycling pathway and cellular migration

Recycling of transferrin is a well defined model used for the study of the slow recycling pathway and can be successfully visualized by following transferrin (Tfn) and/or transferrin receptor (TfR) internalization (*Sonnichsen et al., 2000; Ullrich et al., 1996*). The first steps are regulated by Rab5 and the ligand-receptor (Tfn-TfR) goes to and accumulates in clathrin-coated pits on the plasma membrane. Then it is transported from early endosomes to sorting vesicles (*Sonnichsen et al., 2000*). Transferrin reaches Rab5-labelled structures soon after internalization and reaches the Rab4 positive membrane compartment at 5 min. These vesicles also stain for Rab5. After 15 min, about 80 % of transferrin has been recycled via the fast recycling route directly returning to the plasma membrane by the Rab4 dependent pathway. The remaining transferrin goes to a slow recycling pathway, which is regulated by Rab11. Interestingly, 70 % of transferrin-loaded structures are also labeled with Rab4, suggesting that the function of Rab4 might not be limited to the early stage of recycling (*Sonnichsen et al., 2000; Zerial and McBride, 2001*). Rab11 regulates Tfn and TfR positive vesicle transport from sorting vesicles to the perinuclear recycling compartment (PNRC), which is a cluster of recycling vesicles serving as a reservoir for recycling material (*Sonnichsen et al., 2000; Ullrich et al., 1996*). In addition, Rab11 also regulates the Tfn and TfR exit from PNRC to the recycling endosomes, and then to the plasma membrane (*Sonnichsen et al., 2000; Ullrich et al., 1996*). Expression of Rab11wt (wild type Rab11) and Rab11Q70L (GTP-bound Rab11), the constitutive GTP form of Rab11, causes accumulation of Rab11 and Tfn in the perinuclear recycling compartment (*Sonnichsen et al., 2000; Ullrich et al., 1996*). In contrast, expression of Rab11S25N (GDP-bound Rab11), the constitutive GDP form of Rab11, leads to an accumulation of TfR into tubulo-vesicular structures, apparently by blocking the Tfn and TfR transport in the early endosomal compartment (*Wilcke et al., 2000*). However, it is still unclear how Rab11 accumulates the recycling vesicles at PNRC and how it regulates the exit of recycling material from there. Ren et al. showed that exit from PNRC as well as exocytosis need GTP hydrolysis, indicating that the Rab11 regulation of Tfn recycling requires a GTP-bound form of Rab11 (*Ren et al., 1998*).

However, none of the identified Rab11 effectors thus far showed a direct role in Tfn and TfR exit from PNRC to the cell periphery; only myosin Vb has been suggested to play a

role in the regulation of Tfn from early/sorting endosomes to PNRC (*Hales et al., 2002; Lindsay and McCaffrey, 2002; Provance et al., 2004*).

Recently, the Rab4 and Rab11-regulated recycling pathways have emerged as regulators of cell migration. Continuous rebuilding of the lamellipodium on the leading edge of the plasma membrane is important for cell migration, and Rab11 has been shown to have a role in this vesicular exocytosis-dependent process (*Green et al., 1997; Grunfelder et al., 2003; Jones et al., 2006; Lock and Stow, 2005; Mammoto et al., 1999; Powelka et al., 2004; Savina et al., 2002; Ward et al., 2005; Yoon et al., 2005*). Disturbances of this pathway by expression of GDP-bound Rab11 or Rab11 effector negative mutant result in the impairment of migration of several cell types (*Jones et al., 2006; Powelka et al., 2004; Yoon et al., 2005*). The Rab11-regulated pathway drives haptotaxis (a kind of directional motility by an adhesion gradient or outgrowth of cells, such as axonal outgrowth) and invasive migration of cancer cells (*Jones et al., 2006*). Recycling of αvβ3, α5β1 and α6β4 integrins, but not TfR via this pathway requires PKB/Akt (protein kinase B) (*Roberts et al., 2004; Yoon et al., 2005*). It has also been suggested that Rab11 and Arf6 are master regulators of cancer cell invasiveness, which is mediated by the physical associations of Rab11 and Arf6 and/or their effectors with fibronectin-binding integrins, e.g. α5β1 (*Jones et al., 2006; Li et al., 2005*). Rab4 regulates the fast recycling route, which contributes to the spatial positioning of αvβ3 during persistent directional migration. In this fast pathway, αvβ3 must associate directly with PKC-related kinase PKD1 in order to be recycled (*Woods et al., 2004*).

3 AIMS OF THE PRESENT STUDY

Osteoclast is the only cell type that is known to be able to resorb mineralized bone. The molecular mechanism of bone resorption is based on the production and targeted secretion of acid and various proteases. This, in addition to removal of resorption products, involves intensive intracellular vesicular trafficking. Recently, studies have shown that a family of small GTPases regulates membrane transport and thus also regulates osteoclast function. However, the mechanism of how these Rab proteins regulate membrane trafficking in cells remains unclear. It is however known that lipids are major components of cell membranes. Therefore, the role of lipid rafts in membrane trafficking in osteoclasts also needs to be characterized.

The specific aims of this study were:

1. To identify the location of lipid rafts in polarized osteoclasts and the role of these rafts in osteoclast membrane trafficking.

2. To identify Rab7 specific effectors in osteoclasts.

3. To reveal the mechanisms by which Rab7 regulates ruffled border formation in resorbing osteoclasts.

4 MATERIALS AND METHODS

4.1 Antibodies and reagents

All cell culture media were from Invitrogen (Carlsbad, CA). Anti-Rab11a polyclonal antibody was from Zymed Laboratories (San Francisco, CA). The mouse monoclonal antibody against Rac1 was purchased from BD Biosciences (San Diego, CA). The rabbit polyclonal anti Rab7 antibody was from Santa Cruz. Monoclonal anti-His_6 antibody was purchased from Qiagen (Santa Clara, CA). Alexa fluor-647-transferrin (Tfn-647) and Alexa fluor-594-transferrin, Alexa fluor-488, -546 and -647 were obtained from Molecular Probes (Eugene, OR). All antibiotics, laboratory chemicals and holo-transferrin were from Sigma-Aldrich (Saint Louis, MO). DNA transfections were performed using Fugene 6 or Fugene HD from Roche Diagnostics (Indianapolis, IN).

4.2 RNA and mRNA extraction from trabecular bone and bone marrow cells

Rat primary osteoclasts were scraped from longitudinally cut long bones of 1–2-day-old rat pups and collected into α-essential medium and washed twice with PBS. Then total RNA of the cells was isolated by the GenElute Mammalian Total RNA Purification Kit (Sigma-Aldrich). Briefly, cells were lysed and homogenized in guanidine thiocyanate and 2-mercaptoethanol to release RNA and inactivate RNases. Lysates were spun through a filtration column to remove cellular debris and DNA. The filtrate was then applied to a high capacity silica membrane to bind total RNA, followed by washing and elution with DEPC-treated water.

Messenger RNA was isolated from total RNA by using the QuickPrep™ mRNA Purification Kit (Amersham Biosciences). Briefly, the total RNA solution was added with Oligo (dT)-Cellulose buffered with 20 mM Tris, pH 7.5, 1M NaCl and 2 mM EDTA. The sample was incubated for 3 min at 70°C in a heating block, and then the mixture was incubated at room temperature on a rotator for 10 min to hybridize between the oligo (dT) particle and the poly-A tail of the mRNA in the total RNA. After three washes with high salt wash buffer [10 mM Tris-HCI (pH 7.4), 1 mM EDTA, 0.5 M NaCI] and followed by three washes with low salt wash buffer [10 mM Tris-HCI (pH 7.4), 1 mM EDTA, 0.1 M NaCI] on a column, mRNA was eluted with elution buffer (10 mM Tris, pH 7.4 and 1mM EDTA). mRNA was precipitated by adding 3 volumes of absolute ethanol and 1/10 3M sodium acetate pH 5.2 in -20°C overnight followed by washing with 70% ethanol (DEPC water) two times. Then the mRNA was dissolved in DEPC-treated water for cDNA library synthesis later.

4.3 Reverse transcription and cDNA synthesis

Reverse Transcription (RT reaction), is a process in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA) from total extracted RNA or

poly(A)-RNA, with reverse transcriptase enzyme, primer, dNTPs and RNase inhibitor. RT reaction is also called first strand cDNA synthesis. Three types of primers can be used for RT reaction: oligo (dT) primers, random (hexamer) primers and gene specific primers. Usually, oligo (dT) primer and total RNA are commonly used for cDNA sythesis. One-two micrograms of total RNA are typically used for RT reaction. Total RNA was incubated with oligo (dT) primer at 70°C to denature RNA secondary structure and then quickly chilled on ice to let the primer anneal to the RNA. Then, other components of RT were added into the mixture and the RT reaction was carried out at 42°C for 1 hr.

4.4 Cloning genes into bacterial two-hybrid bait plasmid and various prokaryotic and eukaryotic expression vectors

The coding region of rat Rab11, Rab7, Rab4 and Rab9, Rac1 (GenBank™ accession number AY 491395), Rac2, RhoB, and cdc42 were obtained by PCR amplification with specific primers designed according to the sequences in the Gene Bank from trabecular bone and bone marrow cDNA. The Rab amplified reading frames were cloned into Sal1/Not1 sites in pGEX-4T-1 plasmid (Amersham Biosciences). Rac1, C-terminal Rac1, Rac2, cdc42 and RhoB reading frames were cloned into Sal1/Xho1 sites in pQE80L series vectors (Qiagen N.V., The Netherlands). The constitutive active (GTP-bound) forms, Rab7Q67L, Rab11Q70L and Rac1Q61L, and constitutive negative (GDP-bound) forms, Rab7T22N, Rab11S25N and Rac1T17N were made by using the Stratagene Quickchange Site-directed mutagenesis kit (Stratagene Europe, The Netherlands) from the wild type clones. The Rab11wt, Rab5wt and Rab7Q67L reading frames were cloned into Not1/Xho1 sites in pBT bait plasmid with a flexible linker inserted in front of each Rab. For eukaryotic expression, Rab11Q70L, Rab11wt and Rab11S25N were cloned into pEGFP C2 vector, and wild type Rac1 (Rac1wt) was cloned into Monomer DsRed-C1 vector (Clontech Laboratories, Mountain View, CA). Rac1Q61L, Rac1wt and Rac1T17N were cloned into pcDNA3.1 vector (Invitrogen, Ltd, Pailsey, UK). All constructs were confirmed by sequencing.

4.5 Mutagenesis

The constitutive GTP-bound form of Rab7Q67L, Rab11Q70L and Rac1Q61L and constitutive GDP-bound form of Rab7T22N, Rab11S25N, and Rac1T17N were made from their wild type constructs by using the QuickChange site-directed mutagenesis kit. Briefly, paired primers were designed to mutate the single amino acid into the desired one as shown above, e.g. Q changes into L at the 67th position amino acid of Rab7. The QuickChange method was done by using *PfuTurbo*® DNA Polymerase which performs replication of both plasmid strands with high fidelity using the mutated oligos as primers. Following temperature cycling, the product was treated with *Dpn* I restriction enzyme, which specifically digests methylated and hemimethylated DNA, to remove the parental DNA template keeping the synthesized DNA intact. DNA isolated from almost all *E. coli* strains was dam methylated and therefore susceptible to *Dpn* I digestion. The nicked

vector DNA incorporating the desired mutations was then transformed into the XL10-Gold *E. coil* strain. All the mutations were confirmed by sequencing.

4.6 Construction of a rat trabecular bone and bone marrow-derived cDNA library

A cDNA library derived from rat trabecular bone and bone marrow cells which were taken from the long bones of 1–2-day-old rat pups was constructed with the BacterioMatch two-hybrid system XR cDNA library construction kit, according to the protocols provided by the manufacturer (Stratagene). The information encoded by mRNAs is converted into a stable DNA duplex (cDNA) and then is inserted into the pTRG XR plasmid; thus, complementary DNA libraries represent the information encoded in the mRNA of a particular tissue or organism. Once the information is available in the form of a cDNA library, individual processed segments of the original genetic information can be isolated and examined. Based on this, a cDNA library of trabecular bone and bone marrow cells was constructed.

The total RNA was isolated by the GenElute Mammalian Total RNA Purification Kit (Sigma-Aldrich); then, mRNA from the total RNA was isolated by using the Quickprep micro mRNA purification kit (Amersham Biosciences). Briefly, about 5ug of mRNA was used for one synthesis reaction for the construction of the cDNA library. The secondary structure was diminished by heating the mRNA at 65°C for 5 min. The first strand cDNA was primed with oligo (dT) and synthesized with reverse transcriptase. The oligo (dT)18 had an Xho I cutting site. Then, reverse-transcriptase (StrataScript RT) was added into the reaction and incubated at 42°C for 1 hour for reverse transcription.

Double-stranded cDNA was synthesized by incubating the second-stand reaction at 16°C for 2.5 h. Then, the cDNA termini were blunted using *Pfu* DNA polymerase I and dNTP in the reaction, and appropriate adaptors were ligated to both ends. Then the double–stranded cDNA was digested with XhoI. The product was size fractionated with a cDNA size fractionation column (Invitrogen) packed with Sepharose CL-2B. Fractions with a cDNA size longer than 500bp were collected and pooled.

The cDNA was ligated into the linearized pTRG XR vector and transformed into XL1-Blue MRF´ Kan Library Pack Competent Cells (Stratagene). Ligation and transformation efficiency were carefully determined to obtain a cDNA library that was larger than $1x10^6$ cfu. One hundred colonies were randomly picked, and plasmids were isolated followed by digestion with the EcoRI and XhoI enzymes to determine whether they had inserts.

4.7 Bacterial Two-Hybrid library screening

The bacterial two-hybrid system was used to pull out clone proteins that interact with the full-length constantly GTP-bound form of Rab7. Rat Rab7Q67L with a flexible linker (Gly4Ser)3 in front of the start codon was inserted in the frame into the pBT bait vector

containing the phage λrepressor protein (λCI) N-terminal DNA-binding domain. The rat trabecular bone marrow-derived cDNA library in the pTRG target plasmid fused to the N-terminal domain of the α-subunit of RNA polymerase was screened by using Rab7Q67L as a bait protein in BacterioMatch two-hybrid reporter strain. The interactions were evaluated based on the promoter activation of transcription of two tandem reporter genes, an ampicillin-resistant gene and a β-galactosidase gene in the BacterioMatch two-hybrid system reporter strain. In the first round of selection, surviving colonies were selected on plates with carbenicillin, kanamycin, chloramphenicol, and tetracycline. The second round was a β-galactosidase gene expression assay using X-gal substrate. Finally, the interactions were confirmed by individual recotransformation with the bait and rescued target plasmids and evaluation of reporter gene expression.

4.8 Bacterial Two-Hybrid binding strength assay

The bacterial two hybrid system (BacterioMatch, Stratagene Europe, the Netherlands) was used to analyze the binding strength between the 2/3 C-terminal sequence of Rac1 and Rab5, Rab7 and Rab11. Briefly, rat Rab5wt, Rab7Q67L, Rab11wt were cloned into a pBT bait vector. The truncated Rac1 in the pTRG target vector, which was found with the library screening, was used as a partner. Target and bait construct pairs were co-transformed into the BacterioMatch Reporter Strain. Bacterial growth was monitored in media with carbenicillin concentrations of 250, 350, 400 and 450 ug/ml to test the resistance gene expression in the reporter strain along with a stable concentration of chloramphenicol, kanamycin and tetracycline to keep the episomes, bait and target vectors. The surviving colonies were further tested on the chloramphenicol, kanamycin and tetracycline plates with X-gal substrate to visualize the expression of the second reporter, the β-galactosidase gene, i.e. to compare the intensity of the blue color.

4.9 Introduction to *in vitro* binding

A pull-down assay is an *in vitro* binding method we used for determining putative interactions which were discovered by two-hybrid screening. It can also be used to identify novel interaction, but is less common. Normally, the bait protein is tagged and immobilized on a resin and subsequently “pulls down” a target protein which is expessed in bacteria or mammalian cells. The target proteins can also be tagged and over-expressed. The tags can be, for example, GST, His_6, and Flag.

4.9.1 Bacterial protein expression

The reading frame of Rabs (Rab4, Rab5, Rab7, Rab9, Rab11) were subcloned into bacterial expression vector pGex-4T-1 (Amersham Biosciences) and transformed into E coli strain BL21 (Stratagene). When induced by 1 mM IPTG, the cloned proteins were expressed with the fused Glutathione S transferase, a GST - tag. The Rho family reading frames (Rac1, 2/3 of C-terminal Rac1, Rac2, cdc42 and RhoB) were cloned into pQE80L series vectors (Qiagen), which express the cloned proteins with a His_6-tag.

4.9.2 His_6-tagged protein purification

The cloned His_6-tagged Rho members (Rac1, Rac2, cdc42 and RhoB) expressed in BL21 were purified from bacterial lysates on nickel-nitrilotriacetic acid (NTA)-agarose columns (Qiagen N.V., The Netherlands). Briefly, His_6-fused Rho family proteins were expressed in BL21 after being induced with 1mM IPTG for 3-4 h. The cells were harvested by centrifugation. The cells were lysed in the lysis buffer (50 mM NaH_2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0, and 1mg/ml lysozyme) and then incubated on ice for 30 min. After sonication, the lysate was centrifuged at 14,000 x *g* for 30 min at 4°C to pellet the cellular debris. The cell lysate supernatant was applied to the Ni-NTA column and washed by wash buffer three times (50 mM NaH_2PO4, 300 mM NaCl, pH 8.0, 20 mM imidazole). The bound His_6-tagged proteins were eluted with elution buffer (50 mM NaH_2PO4, 300 mM NaCl, pH 8.0, 300 mM imidazole).

4.9.3 Pull down assay

GST-fused Rab proteins (Rab7Q67L, wtRab7, Rab7T22N, Rab11Q70L, wt Rab11, Rab11S25N, wtRab4, wtRab5 and wtRab9) were prepared in crude bacterial cell lysates as described in 4.9.1. The purification of cloned His_6-tagged Rho family proteins (Rac1, Rac2, cdc42 and RhoB) was described in 4.9.2. The glutathione-Sepharose beads were bound with GST-fused proteins in binding buffer (50mM Tris-HCl, 150mM NaCl, 5mM $MgCl_2$, and 0.5% Triton-100, pH 7.4) for 1 hour at 4°C, then washed three times to remove the unbound proteins. Two μg of GST-bound proteins (assayed with Bradford reagent) were used in each binding experiment. His_6-tagged proteins were added in excess, and the beads were further incubated for 3 hours at 4°C. After three washes with binding buffer, the bound complexes were separated on 12% SDS-PAGE. The separated proteins were blotted to PVDF membrane and incubated with monoclonal antibody against His_6, followed by horseradish peroxidase conjugated anti-mouse secondary antibody. Visualization was done with the ECL system (Amersham BioSciences UK, Little Chalfont, England). The same amount of GST protein alone was used as a negative control for each pull down experiment. All experiments were performed in the presence of 100 μM GTP (or GDP for Rab7T22N and Rab11S25N), and repeated at least three times.

4.10 Osteoclast culture

Rat primary osteoclasts were scraped from longitudinally cut long bones of 1–2-day-old rat pups and collected into α-essential medium with 20 mM Hepes, 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μM/ml streptomycin pH 6.8. Subsequently, they were plated on bovine bone slices or on glass coverslips. After a 30-min incubation at 37°C in 5% CO_2 and 95% air atmosphere for attachment, non-adherent cells were washed away, and attached cells were cultured further for 48 h in the same conditions.

4.11 HeLa cell culture and transient trasfection

HeLa cells were maintained in DMEM buffered with 20mM HEPES, 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin and 100 μM/ml streptomycin. The transfections were performed in 24 well plates. Briefly, the cells were grown overnight in DMEM and then transiently transfected with expression constructs with different combinations and controls. When Fugene 6 was used, the reagent was mixed into DMEM and incubated for 5 min. Then the plasmids were added into the mixture and incubated for another 40 min. Finally, the mixture was added into the cell culture and kept 24-48 hours for further analysis. When Fugene HD reagent was administered, the plasmids were mixed with medium, and the reagent was added into the mixture. For confocal microscopy and stably expressing cell line construction, we used Fugene 6 transfection reagent, whereas for biochemical assays, we chose Fugene HD transfection reagents to obtain higher transfection efficiency.

4.11.1 HeLa stable cell line selection

Wild type, GTP- and GDP- Rab11 in pEGFP-C2 (clontech) constructs were stably transfected into HeLa cells. Briefly, all constructs were digested with ApaI restriction enzyme to linearize the vectors. Then the transfections were performed as described in 4.11. After two days, the media were replaced with growth media containing G418 for the selection of stable transfections. Single colonies were examined under the reverse UV microscope and the colonies with shining green fluorescence were picked and maintained in the growth medium containing G418.

4.12 Co-immunoprecipitation

HeLa cells stably expressing GFP-Rab11Q70L, Rab11wt or Rab11S25N were transiently transfected with DsRed-Rac1 construct. After 48 hours, cells were lysed in RIPA buffer. Anti-Rac1 antibody was pre-bound to Protein G-Sepharose beads (Amersham BioSciences) for one hour at 4˚C; then each lysate containing approximately the same amount of GFP-Rab11 variants was incubated with an equal amount of anti-Rac1 antibody conjugated protein G-Sepharose beads for 3 hours at 4˚C. After three washes with the wash buffer (150mM NaCl, 10mM Tris pH7.4, 0.5% Triton TX), the proteins bound to the protein G-Sepharose beads were eluted with SDS sample buffer and western blots were made with anti Rab11 antibody to visualize the co-immunoprecipitated GFP Rab11 protein and ECL. The same amount of mouse IgG in the place of anti Rac1 antibody was used as a negative control for each co-immunoprecipitation experiment. All experiments were performed in the presence of 100 μM GTP (or GDP for Rab11-S25N) and repeated at least three times.

4.13 Immunofluorescent straining of resorbing osteoclasts

Osteoclasts were grown on bone slices or on glass coverslips and washed with PBS. Permeabilization was performed either by pre-treatment before fixation with 0.01%

saponin in 80 mM Pipes, pH 6.8, 5 mM EGTA, 1 mM $MgCl_2$ or 0.01% saponin in PBS for 5 min at room temperature after fixation. The cells were fixed in 3% paraformaldehyde in PBS for 20 min and quenched with 50 mM NH_4Cl for 10 min. The cells were then incubated in blocking buffer (2% bovine serum albumin with 0.01% saponin in PBS) for 30 min to block nonspecific binding. Primary antibodies in blocking buffer were incubated with the permeabilized cells for 1 h at room temperature. After washing three times with the washing buffer (0.2% bovine serum albumin with 0.01% saponin in PBS), the cells were incubated with Alexa Fluor 488-, TRITC/Alexa 546- and Alexa Fluor 647-conjugated secondary antibodies in blocking buffer for 1 h at room temperature. In some cases the samples were incubated with recombinant cholera toxin subunit B or phalloidin for 30 minutes at 37°C. Nuclei were stained with Hoechst 33258. Cells were washed and then mounted with 80% glycerol in PBS. Confocal laser scanning microscopy was performed to obtain and analyze the immunofluorescence images with a Leica TCS-SP unit equipped with argon-krypton lasers (Leica Microsystems Heidelberg GmbH) and a x40 oil immersion lens.

4.14 Transferrin internalization in resorbing osteoclasts followed by Methyl-β-Cyclodextrin treatment

Human holo-transferrin (0.1 mg/ml) was added into the culture medium for 5 minutes in a 48 h osteoclast culture. After this, 10 mM, 15 mM, or 20 mM Methyl-β-Cyclodextrin (for cholesterol depletion) was added for 25 minutes. After a quick wash in pre-warmed PBS and fixation with 3% PFA (Paraformaldehyde) for 15 minutes, cells were permeabilized with 0.1% Triton X-100 in PBS and finally immunolabeled.

4.15 Fluorescent transferrin internalization, location and transferrin recycling assay

Rat primary osteoclasts cultured on bone slides or HeLa cells on glass cover slips 24-48 hours after transfection were treated with transferrin as follows. Cells were serum starved for 1 hour in DMEM containing 0.2% BSA to deplete the endogenous transferrin. Then the cells were incubated with Alexa fluor Tfn-647 (in some experiments, Alexa fluor Tfn-594) at a concentration of 40μg/ml in serum free medium. After Tfn internalization for 1, 5 or 30 min, the cells were washed quickly with pre-warmed PBS three times and fixed with 3% paraformaldehyde in PBS for 20 min. For the transferrin recycling assay, serum depleted HeLa cells were pre-incubated with 40μg/ml Tfn-647 in DMEM containing 0.2% BSA at 16°C for 90 min, and then the internalized Tfn-647 was chased using 200μg/ml holo-Tfn (Sigma) DMEM at 37°C for different times. Cells were washed 3 times with pre-warmed PBS and fixed in 3% PFA. At zero time point cells were fixed directly after 16°C pre-incubation. For quantitation of total internalized Tfn-647 in cells, the Tfn-associated fluorescence was summed from all confocal planes (0.5μm/plane, with unchanged setting for acquiring Tfn-647 signal for each cell) in Leica program and imported into Image J analysis software (NIH, Bethesda, MD) to quantitate in 20 cells

(background subtracted). The data were expressed in arbitrary units/cell area. For 0 time point, 40 cells were processed. For time course experiments, the results are expressed as percentages of fluorescence/cell area before chasing (0 time point being 100%).

4.16 Quantification of colocalization

HeLa cells transfected with different combinations of DNA constructs or primary rat osteoclasts that were cultured on bone were further examined by indirect immunofluorescent labeling and confocal microscopy. The cells were treated with or without transferrin and were incubated for predetermined times. The cells were immunofluorescent strained as described in 4.13. Then, the cells were mounted with mowiol (Sigma). Finally, confocal microscopy was performed to obtain images by sequential scanning in each channel with 40X Plan Apo object oil immersion and a Leica laser microscope. For study of colocalization, confocal images were processed using Adobe Photoshop 6.0 and saved as TIFF files. Colocalizations were analyzed using 510 LSM software (Carl Zeiss) on TIFF images of a single section from at least 10 cells for each time point on the selected area and scored. Data were expressed as the average of the colocalization score of the cell area of each cell type and time point ± SD.

4.17 Biotinylated transferrin ELISA assays

Endogenous Tfn-depleted cells were incubated with 5μg/ml biotinylated Tfn (B-Tfn, Molecular Probes) in DMEM containing 0.2% BSA for 90 min at 16°C. Non-internalized B-Tfn was removed by three alternate washes with ice-cold 0.1% BSA in PBS, pH 4.2 (set to 25mM glacial acid), and PBS containing 0.5% BSA. The cells were chased at 37°C using 100-fold holo-Tfn in DMEM. Media were exchanged and sampled at various time points and the amount of B-Tfn recycled into the media at each time point was quantified by ELISA using the Human Tfn Elisa kit (Bethyl, TX) (Strick and Elferink, 2005). Bound B-Tfn was detected using 0.1μg/ml streptavidin–horseradish peroxidase and QuantaBlue fluorogenic peroxidase substrate (Pierce) and then measured in a Tecan Infinite M200 monochromator multimode microplate reader (Tecan, Switzerland) and expressed as fluorescent units (Rfu)±SD per microgram of protein. Data presented are from 3 independent experiments.

4.18 Viral infections

The cytoplasmic tail (recVSV-GGC) and both the tail and the membrane anchor (recVSV-GCC) of VSV constructs were kind gifts from Dr. John Rose (Yale University School of Medicine, New Haven, Connecticut). These viral stocks were produced in BHK cells. The influenza virus stocks were produced in MDBK cells. Viral stocks were diluted to obtain about $1x10^8$ pfu/ml. Osteoclasts were cultured on bone for 48h, and then were infected with VSV wild type, recVSV-GGC, recVSV-GCC or with influenza virus for 60 minutes in the conditioned medium obtained from infected cell cultures of the same

cells. After a quick rinse in warm PBS (pH 7.3), the cultures were placed back into the initial culture medium. Wild type VSV infections were propagated for 3 hrs, influenza infections 5 hrs, and recVSV-GGC and recVSV-GCC infections were propagated for 4 hrs. The cells were then quickly rinsed in PBS and fixed in 3% PFA and processed further for immunolabeling. Methyl-β-cyclodextrin (MβCD), when used, was applied for three hours before fixation.

4.19 Quantification of viral glycoprotein association with rafts

BHK cells were infected with influenza virus, VSV, recVSV-GCC, or recVSV-GGC for 6 h (10 pfu/cell, 1 h adsorption time) and pulse-labeled with ^{35}S-methionine for 10 minutes, followed by a 90 min chase. The cells were then scraped at 0°C in PBS, pH 6.5, containing 1% Triton X-100. After centrifugation for 5 min at 10000g, the pellet was extracted with PBS, pH 7.0, containing 1% deoxycholate and 1% Triton X-100. 50 µl of both the Triton-soluble and insoluble fractions were subjected to SDS-PAGE. The corresponding viral glycoprotein bands were then visualized by phosphoimaging (Fuji Phofilm Co. Ltd.) and quantified using Fujifilm Science Lab 99 Image Gauge software.

4.20 Bi-phasic labeling and measuring the area of resorption lacunae and immunoassays of bone resorption markers

Rat osteoclasts were cultured on bovine bone slices for 24 hours. The cultures were then transferred into PBS containing sulpho-NHS-biotin (2mg/ml) for two minutes to biotinylate and visualize the organic fringe at the bottom of the resorption pits formed at this time point. After a quick rinse in fresh culture medium, cultures were transferred to MβCD-containing culture medium for 30 minutes and finally back to the original culture medium for another 24 hours. Finally, after fixation with 3% paraformaldehyde/PBS for 15 minutes, we detected biotin with FITC-streptavidin (1:150, DAKO) and visualized all the lacunae with TRITC-wheat germ agglutinin (WGA) -lectin (1:50). The lacunae labelled with WGA-lectin (red) but not with sulpho-NHS-biotin (green) were considered as those formed after MβCD treatment.

The levels of C-terminal telopeptides of type I collagen (CTX) and tartrate resistant acid phosphatase 5b (TRACP 5b) were measured using an enzyme-linked immunosorbent assay (ELISA) according to the protocol recommended by the supplier (Nordic Bioscience Diagnostics) and Alatalo et al. (*Alatalo et al., 2000*), respectively.

5 RESULTS

5.1 Identification of a lipid raft-dependent transcytosis route in resorbing osteoclasts (I)

Influenza HA and recombinant VSV G were transported to FSD in a lipid raft dependent manner in resorbing osteoclasts----Previous studies have shown that the transcytotic route in resorbing osteoclasts goes from the central uptake zone of the ruffled border to the functional secretory domain (FSD). This vesicular pathway contains the degraded bone matrix taken up from the resorption lacuna. In this study, we identified a new transcytotic route from FSD to the fusion zone of the ruffled border by using recombinant VSV G-proteins. We found that this transcytosis process is lipid raft dependent.

Newly synthesized influenza HA is delivered from Golgi to FSD, whereas VSV G protein is targeted to the basolateral membrane but not to FSD in resorbing osteoclasts (*Salo et al., 1997*). In the present study, we confirmed that influenza HA is a raft-associated protein in osteoclasts as in other cell types (*Keller and Simons, 1998; Scheiffele et al., 1997*). First, influenza HA mainly localizes to the FSD and vesicles between the nuclei and basolateral membrane. At these sites it shows an intensive colocalization with recChlTx-B, suggesting the presence of rafts. Second, cholesterol depletion by methyl-β-cyclodextrin (MβCD) blocks its delivery from the Golgi apparatus to the FSD. Third, with 0.5% cold Triton-100 (<+4°C) incubation before fixation of resorbing osteoclasts, influenza HA remained at the FSD, whereas under treatment of the cells at room temperature, nearly all HA was removed from the FSD. In contrast, wild type VSV G protein can be removed by incubation of cold Triton X-100 from the basolateral plasma membrane, suggesting a non-raft binding mechanism of wild-type VSV G protein transport (I, Fig.1).

We hypothesized that if the cytoplasmic tail (recVSV-GGC) or both the tail and the membrane anchor (recVSV-GCC) of the VSV G protein were replaced by those of the CD4 protein which has been reported to associate with rafts (*Fragoso et al., 2003; Percherancier et al., 2003*), the recombinant VSVs would be retargeted to the FSD. Indeed, the majority of recombinant VSVs followed the raft-dependent transport as HA did to the FSD in resorbing osteoclasts. Both recombinant VSVs colocalized with recChlTx-B, αvβ3 integrin and ganglioside G_{M1} at FSD (I, Fig.2).

Recombinant VSVs were transcytosed from FSD to the peripherial fusion zone of the ruffled border---RecVSV-GGC and -GCC appeared not only on the FSD but also at the fusion zone of the ruffled border, indicating a potential transport route of the proteins from the FSD to the ruffled border or vice versa. We thus followed the time course of VSV translocation after viral infection by anti-G protein antibody. After 60, 90, 120, 180, and 240 min, 28.2%, 60.0%, 62.8%, 77.3% and 90.2% of the infected resorbing osteoclasts had an intense plasma membrane staining, while 0.0%, 6.3%, 18.2%, 34.1% and 34.3%

showed GCC at the ruffled border, respectively. The recVSV-GCC or GGC were observed on the ruffled border only after their appearance at the FSD, suggesting that the transport of the proteins were from the FSD to the ruffled border. In another experiment, the infected osteoclasts were treated with anti G protein antibodies for 30 min before fixation. After permeabilization of the cells, the internalized antibodies were observed in intracellular vesicles both at the nuclear level and at the periphery of the ruffled border. This also strongly suggested the active uptake and transcytosis of antibody the recVSV-GCC complex from the FSD to the fusion zone of the ruffled border (I, Fig.3).

5.2 Identification of the role of lipid rafts in late endosomal transport in resorbing osteoclasts

Cholesterol depletion disturbs the endocytic pathway to the ruffled border---Previous studies have shown that internalized holo-transferrin is delivered to the peripheral area of the ruffled border (*Mulari et al., 2003; Palokangas et al., 1997*). Interestingly, a raft marker, cholera toxin, specifically accumulates in the same area. We thus analyzed whether the presence of cholesterol is critical for the delivery of transferrin to the ruffled border by depletion of cholesterol with MβCD (methyl-β-cyclodextrin). Osteoclast cultures were incubated in the presence of human transferrin for 5 min and then incubated for 25 min in the absence of the ligand. Upon addition of 10 mM, 15 mM, and 20 mM MβCD into the culture medium after the transferrin pulse, transferrin was seen only in 56.1%, 41.5%, and 34.6% of the cells, respectively, at the ruffled border. Transferrin accumulated intracellularly above the ruffled border and in circumnuclear structures indicating a membrane trafficking block before reaching the ruffled border. A previous study showed that the peripheral fusion zone of the ruffled border right inside the actin attachment ring is highly concentrated with filamentous actin and β_3-integrin (*Mulari et al., 2003*). MβCD disrupted the association of β_3-integrin with the ruffled border actin and inhibited accumulation of actin to the ruffled border, suggesting that cholesterol is essential for maintaining ruffled border structure (I, Fig.4).

Cholesterol extraction impairs osteoclastic bone resorption---Previously, studies have been shown that raft disruption blocks actin ring and ruffled border formation (*Ha et al., 2003; Zhao and Väänänen, 2006*). However, we could not observe disturbances of actin rings or increased apoptosis during the short 25-min exposure period to MβCD, although the ruffled border architecture and vesicular trafficking were clearly affected. In fact, using sulpho-NHS-biotinylated resorption pit staining and MβCD treatment and additional cultivation of the cells for 24 h, we found that MβCD inhibited bone resorption dose-dependently. Although actin ring-containing osteoclasts were still present, fewer lacunae were formed after MβCD treatment in a dose-dependent manner. 20 mM MβCD blocked bone resorption entirely. The number of actin rings was, however, only slightly but dose-depently decreased after MβCD treatment (I, Fig.5).

Finally, we measured resorption markers CTX and TRACP-5b from the culture medium of similarly treated samples after 24 and 48 h, and observed again a dose-dependent

decrease in the content of both CTX and TRACP-5b, suggesting that cholesterol depletion results in osteoclast inactivation via mechanisms that leave sealing zone attachment structures intact (I, Fig.5).

5.3 The discovery of a novel Rab7 interacting protein Rac1 (II)

Previous studies have shown that Rab7 locates at the ruffled border in resorbing osteoclasts, whereas in the non-resorbing osteoclast, Rab7 is localized around the nuclei where late endosomes and lysosomes are found (*Mulari et al., 2003; Palokangas et al., 1997; Zhao et al., 2001*). Down-regulation of Rab7 by antisense oligonucleotides prevented the formation of the ruffled border and consequently bone resorption, suggesting that Rab7 is a regulator of transport of the late endosomes to the ruffled border and/or their fusion with the ruffled border (*Zhao et al., 2001*). To reveal the mechanism of how Rab7 regulates ruffled border formation, we sought down stream effectors/direct interacting proteins that participate in this process.

Identification of the New Rab-interacting Proteins Using the Bacterial Two-Hybrid system---A cDNA library derived from rat trabecular bone and bone marrow was constructed from 1–2-day-old rat pups as they contain a relatively high number of osteoclasts. About 5 μg of mRNA was collected and used for the library construction. After second strand synthesis, we used a cDNA size fractionation column to eliminate cDNA shorter than 500 bp. Gel electrophoresis of the cDNA showed a smear ranging from 500 to 10,000 bp, the most dense part being between 1,000 and 3,000 bp. The library represents $2x10^6$ colonies/ml. One hundred colonies were analyzed randomly by isolating plasmids followed by digestion with the EcoRI and XhoI enzymes. Ninety-five colonies showed inserts, all longer than 500 bp (II).

To identify proteins important for Rab7-regulated vesicle traffic, we constructed a constitutively active, GTPase-deficient mutant, Rab7Q67L as molecular bait to screen the rat trabecular bone marrow-derived cDNA library in pTRG plasmids via the bacterial two-hybrid selection. The constantly active form of the protein (Rab7Q67L mutant) was chosen, because we anticipated that Rab effectors would bind mainly to this form. Target proteins, which interacted with the bait, were selected by the activation of ampicillin resistance and next by induced β-galactosidase. Approximately 2×10^6 transformants were screened. The first screening led to isolation of 2,800 colonies that activated the upstream Amp^r reporter gene. Here we used carbenicillin instead of ampicillin to reduce the background, because carbenicillin is a more stable analog of ampicillin. Of the above, 104 colonies turned strongly blue on X-gal plates also containing chloramphenicol, kanamycin, and tetracycline, which meant that the bait-target interaction had also been strong enough to trigger the downstream reporter gene. Finally, the interactions were confirmed by cotransformation of the bait and the rescued target plasmid pairs individually back into the reporter strain. Isolates that survived the first screen were subjected to a higher antibiotic concentration to reduce their number.

We finally obtained a limited number of colonies for further studies. One of the strong positive colonies contained a 658-bp long insert, consisting of a 445-bp open-reading frame, a stop codon, and a 213-bp 3'-untranslated region, and a poly(A) tail. A search in the Ensembl rat genome data bank produced a 100% sequence match on the p11 band of chromosome 12 (ENSRNOG00000001068 and predicted transcript: ENSRNOT00000001417). The gene on this site codes for Rac1, which is a member of the Rho family small GTPase proteins. Thus, we had isolated the C-terminal 149 residues moiety of Rac1 with the two-hybrid method. To get the full reading frame, we designed a primer pair based on the predicted sequence and PCR-amplified the full-length reading frame and the 3'-untranslated region from rat bone cDNA. We then cloned and sequenced it and deposited the sequence in the GenBank™ (accession number AY491395 (II – Fig. 1). Comparisons with the Rac1 transcript of mouse (GenBank™ accession number BC003828), and human (GenBank™ accession number BC004247) revealed a more than 30-base-pair completely conserved sequence starting with the translation start codon. Rat and mouse sequences showed 97% identity, whereas between rat and human it was 90% in the coding region. At the protein level, Rac1 sequences of rat and mouse are identical, only one amino acid is different in man at position 135, having isoleucine instead of threonine.

In vitro assays verified the specific binding of GTP/wild-type Rab7 and Rac1---After we found that Rac1 strongly interacted with Rab7Q67L in the bacterial two-hybrid screening, we confirmed the direct interaction between Rab7 and Rac1 also with *in vitro* binding assays using recombinant proteins. The His-tagged full-length Rac1 and the isolated 445-bp C-terminal of Rac1 both selectively bound to the GST-fused Rab7Q67L and wild type Rab7, but less so with the GST-fused Rab7T22N, the GDP-bound form of Rab7 (II – Fig. 2A). We then examined the specificity of the binding. We cloned GST-fused Rab9, another Rab protein that is also located in the late endosomal compartment. Rac1 did not bind to Rab9 in the same assay. Next, we studied interactions of Rab7 with Rac2, CDC42, and Rho B, other Rho family proteins. Rac2 is an isoform of Rac1 and shares 93% similarity in protein sequence, and the cellular functions of Rac1, Rac2, and CDC42 are overlapping, although the tissue distribution of them may be different (Hall, 1998; Moll et al., 1991; Shirsat et al., 1990). We found that none of the three types of Rab7 bound to these Rho family members in our *in vitro* binding experiments (II – Fig. 2B). GST protein alone was also included in the assays as a negative control and showed no binding to Rac1 or to the C-terminal Rac1. These experiments indicated that Rab7 specifically interacted with Rac1.

Rac1 colocalizes with Rab7 in Rat osteoclasts and fibroblast-like cells---To visualize the subcellular localization of endogenous Rab7 and Rac1, we used indirect immunofluorescence staining and laser confocal microscopy. In non-resorbing osteoclasts, we found that Rab7-positive vesicles were distributed around the nuclei. As also in other cells, Rab7 colocalized with the late endosomal/lysosomal marker LAMP2, which was in agreement with a previous study (*Zhao et al., 2001*), whereas Rac1 displayed a rather uniform distribution in the cytoplasm. We found that Rab7 colocalized with Rac1 in some

but not all vesicles around the nuclei of osteoclasts where late endosomes and lysosomes were located (data not shown). Next, we examined the location of these two proteins in resorbing osteoclasts on bone. As has been shown previously, Rab7 was redistributed to the fusion zone of the ruffled border just inside the sealing zone (actin ring). We also found a large amount of Rac1 present at the same area, clearly colocalizing with Rab7 (II – Fig. 3 A-F). In the same rat osteoclast culture on the bovine bone slice, we also saw Rab7 clearly colocalizing with Rac1 in numerous vesicles of fibroblast-like cells (II – Fig. 3 G-I). These data suggest that Rab7-Rac1 interaction contributes to the formation of the fusion zone of the ruffled border.

5.4 Rab11 and Rac1 interact directly to regulate the slow recycling pathway from the sorting endosomes to the plasma membrane (III)

We found that Rac1 could selectively interact with some, but not all Rab proteins in our pull-down experiments and bacterial two-hybrid assays (II, III). We showed that Rab11 directly binds to Rac1 and proved that this interaction accelerates the internalized Tfn transport from the sorting endosomes to PNRC. The interaction complex also regulates PNRC segregation into recycling vesicles, allowing Tfn the exit from the PNRC and translocation to the plasma membrane both in HeLa cells and in primary osteoclasts. We also suggest that this interaction is involved in the regulation of cell migration, since we saw an intensive triple colocalization of Rab11, Rac1 and recycled transferrin mainly on the leading edge of the migrating osteoclasts.

Pull-down assay and co-immunoprecipitation demonstrate the direct binding of Rab11 to Rac1---In study III, we used the same assay system of bacterial two-hybrid as in study II to determine the binding strength between various Rab bait proteins and the Rac1 target protein. The interaction induces expression of two tandem genes: the ampicillin resistance gene and β-galactosidase gene. We found that the carbenicillin concentration tolerated was a better measure of the strength of interaction, X-gal hydrolysis being less so, since all survived transformants could make an equally strong blue colour from X-gal overnight. While Rab7Q67L plus Rac1 cotransformants survived up to 350 μg/ml carbenicillin on the selection plates, Rab11-Rac1 cotransformants remarkably survived at 450 μg/ml. Rab5 and Rac1 constructs grew only on 250 μg/ml carbenicillin plates. These data suggested that Rab11 binding to Rac1 was the strongest among these Rabs (III - Fig. S1).

We utilized pull-down assays using recombinant proteins to confirm the direct interaction between Rab11 and Rac1. GST-Rab11wt, Rab11Q70L and Rab11S25N all bound to 6His-Rac1 protein, but Rab11S25N showed the weakest binding. GST protein alone showed no affinity to Rac1 at all (III - Fig 1A, row a). To address the question whether other Rab proteins bind to Rac1, we examined GST-Rab5 and Rab9 with 6His-Rac1 and found no binding (III - Fig 1A row b).

We also found that Rab11wt, like all other tested Rab proteins including Rab4, Rab5, Rab9, had no affinity to Rac2, cdc42, or RhoB (Rho family members) in our *in vitro*

binding assays (III - Fig 1A rows c-d). This is in agreement with our previous findings with Rab7, as described in study II.

To confirm that specific binding between Rac1 and Rab11 also exists in cells, we co-immunoprecipitated Rab11 and its recombinant mutants by anti-Rac1 antibody from HeLa cell lysates. Cells stably expressing GFP-Rab11Q70L, Rab11S25N and Rab11wt, respectively, were transiently transfected with DsRed-Rac1 expressing construct. As demonstrated in III- Fig 1B row e, GFP-Rab11Q70L can be precipitated with anti-Rac1 antibody. GFP-Rab11wt and Rab11S25N can also be precipitated with Rac1 antibody, but Rab11S25N showed the weakest binding (III - Fig 1B row f). Thus, these results further confirmed our pull down data (III).

Wild type and Rab11Q70L colocalizes with Rac1 in HeLa cells---In non-polarized HeLa cells, endogenous Rac1 was located in the juxtanuclear area, in some vesicular structures scattered in the cytosol, and in some cellular protrusions. Similar localization was observed when expressing Rac1 in HeLa cells (data not shown). In GFP-Rab11Q70L/ Rab11wt and DsRed-Rac1 co-expressing HeLa cells, the location of Rab11wt and Rab11Q70L were in the PNRC and recycling vesicles, as well as at the plasma membrane, in agreement with previous studies (Sonnichsen et al., 2000; Ullrich et al., 1996). Interestingly, Rac1 showed some colocalization with Rab11 at the PNRC and was also concentrated perinuclearly. Some colocalization was also found in punctuate recycling vesicles scattered in the cytoplasm and at the plasma membrane. The PNRC was much more intensive and condensed in Rab11Q70L plus Rac1 expressing cells (III - Fig. 2 a-f), whereas the colocalization of Rab11 and Rac1 on recycling vesicles and the plasma membrane was much more intensive in the Rab11wt plus Rac1 cells (III - Fig. 2 f). Colocalization of these two molecules along the recycling pathway suggested that Rab11 works closely with Rac1. In cells co-expressing GFP-Rab11S25N and DsRed-Rac1, part of Rab11S25N was in the cytoplasm. However, some coincidence of Rab11S25N and Rac1 was observed at the perinuclear membrane clusters where Rab11S25N and Rac1 both accumulated. No colocalization was observed on plasma membrane where Rac1 was present and Rab11S25N was absent (III - Fig. 2 g-i).

5.5 Rab11 colocalizes with Rac1 along the transferrin-recycling pathway (III)

We utilized Tfn to study the functional role of Rab11-Rac1 interaction along the slow recycling pathway. In serum-deprived HeLa cells co-expressing GFP-Rab11wt and DsRed-Rac1, interalized Tfn-647 was clearly observed entering the perinuclear compartment after 5 min of internalization at 37°C, and part of Rac1 colocalized with Tfn in this compartment with some colocalization with Rab11. After 30 min, the PNRC became non-condensed and dispersed into vesicles. As a result, the triple colocalization of Rab11, Rac1 and Tfn increased on punctuate vesicles in the cytoplasm and on the plasma membrane. This indicated that Rab11-Rac1 interaction is not only involved in centrifugal movement of Tfn, but may also be involved in the actual exocytic process of Tfn. Similar results were obtained with the GFP-Rab11Q70L plus DsRed-Rac1 expressing cells. The

sequence of events, as we observed, was clearly faster than previous studies described - possibly due to co-expression of these Rab11-Rac1 GTPases in a pair wise manner (Wilcke et al., 2000) (III – Fig3).

In contrast, in the cells co-expressing GFP-Rab11S25N with DsRed-Rac1, Tfn did not enter the perinuclear membrane cluster at the 5 min time point. This membrane cluster was re-organized as big spot-like clusters where Rac1, Rab11 and Tfn accumulated at the 30 min time point. These spot-like membrane clusters were EEA1 positive (data not shown), which is in agreement with the study by Wilcke et al., indicating that the internalized Tfn was restricted in the early/sorting endosomal compartment (*Wilcke et al., 2000*). As a result, very little Tfn was recycled back to the plasma membrane, where we did not see any colocalization with either Rab11S25N or Rac1 (III - Fig. 4).

We next examined in which steps of recycling Rab11-Rac1 interaction takes place. Previous studies have shown that Tfn is loaded in early/sorting endosomes when internalized at 16°C (*Ren et al., 1998; Sheff et al., 1999; Strick and Elferink, 2005*). Surprisingly, in Rab11wt and Rab11Q70L plus Rac1 co-expressing cells, Tfn-647 reached PNRC already at 16°C. Rab11 and Rac1 were also present there, indicating that the block of transferrin transport to the PNRC at a low temperature was overcome by Rab11-Rac1 pair-wise co-expression. Quantitative data collected from confocal images showed that Tfn reached PNRC when internalized at 16°C and colocalized well with GTP/wt - Rab11 and Rac1. After 5 min of incubation at 37°C, Tfn-647 kept loading into the PNRC where Rab11 and Rac1 were still present. Simultaneously, colocalizations were observed and quantified in vesicles. The total Tfn-647 fluorescence of whole cells gradually weakened, indicating that Tfn was moving through the pathway and leaving the cell. After 10 min, the total in-cell Tfn-647 signal decreased further and the PNRC structure was no longer fluoresced. Colocalization of Rab11-Rac1, Rac1-Tfn and Rab11-Tfn were still clearly observed on plasma membrane in Rab11wt plus Rac1 cells (III-Fig. 5 and S2).

In Rab11S25N and Rac1 expressing cells, internalized Tfn-647 distributed in punctuate vesicles at 0 and 5 min time points without colocalization to Rab11 and Rac1. At 10 min, Tfn-647 accumulated perinuclearly as clusters where Rac1 was also present and colocalized with Rab11S25N (III S2). In mock-transfected cells, Tfn-647 started to load into the PNRC at the 10 min time point and increased at 20min, although the total fluorescence of Tfn-647 in-cell was much weaker at 20 min, which is due to both fast and slow recycling (data not shown).

5.6 Quantification of the internalized Tfn-647 remaining in-cell in HeLa cells

In order to gain quantitative data we measured the internalized Tfn-647 remaining in-cell at different time points. In Rab11wt or Rab11Q70L and Rac1 co-expressing cells, Tfn-647 recycled fast, after 5 min chasing about 60% of the originally internalized Tfn-647 (0 time point) remained in-cell. At the 10 min time point, the Tfn-647 signal dropped

to 47.1% and 26.7% in Rab11wt and Rab11Q67L cells, respectively. Twenty minutes later, the Tfn signal dropped to about 8%, and at 30 min to about 2.5%. In Rab11S25N and Rac1 cells the kinetics was very different: fluorescence of Tfn-647 did not decrease remarkably (about 95% of the original at 5 and 10 min) during the first 10 min, and decreased to 18% by 20 min but was still 18 % at the 30 min time point. All time points showed statistically significant differences compared to the Rab11wt or Rab11Q70L and Rac1 co-expressing cells. In mock transfected cells, the Tfn-647 signal decreased to 60% of the original at 10 min, suggesting that the recycling speed was clearly slower than that in Rab11wt or Rab11Q70L and Rac1 co-expressing cells (data not included in the figure); however, Rab11S25N caused the slowest clearance (III - Fig. 6A).

The initial fluorescence of internalized Tfn-647 in cells at 0 time point was determined by incubating the HeLa cells with Tfn-647 at 16°C for 90 min. The fluorescence was then quantified on confocal microscopy images as described in 4.15. We also noticed that the amount of Tfn-647 uptake in cells expressing Rab11 and Rac1 was as follows: Rab11wt or Rab11Q70L and Rac1 co-expressing cells > mock transfected > Rab11S25N and Rac1 co-expressing cells. This indicates that expression of Rac1 in Rab11wt or Rab11Q70L expressing cells enhances Tfn uptake (III - Fig. 6B).

5.7 Quantification the amount of internalized B-Tfn recycled to the cell culture media (III)

We next examined whether the amount of Tfn recycled to the cell culture media was proportional to the data we obtained with the Tfn-647 chasing experiments described above. To study this we used biotinylated-Tfn and assayed it with ELISA in cell culture media (*Strick and Elferink, 2005*). In the cultures of HeLa cells with Tfn-647 pre-incubation at 16°C for 90 min, we observed a marked increase of B-Tfn recycling (17 and 11.6-fold) in Rab11wt/ Rab11Q70L plus Rac1 co-expressing cells 5min after chasing as compared to Rab11S25N and Rac1 co-expressing cells. At 10 min, B-Tfn release was still increased to about 5-fold and 2.6-fold in Rab11wt/ Rab11Q70L plus Rac1 co-expressing cells. B-Tfn release was about 1.9-fold at 20 min in both cell types (III – Fig. 7A). Methods are detailed in section 4.17.

We also quantified B-Tfn recycling in the media of HeLa cells stably expressing GFP-fused constructs of Rab11Q70L, Rab11wt or Rab11S25N. The early peak at (5 min) of B-Tfn recycling was totally missing from Rab11wt or Rab11Q70L expressing cell, indicating that only simultaneous Rab11 and Rac1 expression accelerated transport of B-Tfn (III - Fig. 7A and B). At the 5 and 10 min time points, B-Tfn recycling was significantly lower in Rab11S25N and Rac1 co-expressing cells compared to only Rab11S25N expressing cells, indicating that Rac1 co-expression with GDP-bound Rab11 inhibited Tfn recycling during the first 10 min (III – Fig. 7B).

We next examined whether expressing Rac1 alone has an effect on transferrin recycling. HeLa cells transiently expressing Rac1Q61L and Rac1wt showed a similar pattern of

B-Tfn recycling. Rac1S25N showed a similar increase of B-Tfn release at 5 and 20 min, but with a severe drop at 10 and 20 min compared to Rac1wt and Rac1Q61L cells ($p<0.01$). The mock-transfected cells recycled the least B-Tfn into the media, indicating participation of Rac1 in Tfn recycling (III - Fig. 7C).

5.8 Rab11-Rac1 interaction regulates Tfn recycling in osteoclasts and is involved in cellular migration (III)

We studied the role of Rab11-Rac1 interaction in primary osteoclasts. Endogenous Rab11 is located mostly around nuclei showing typical Golgi-like staining and, in addition, a more diffuse circumnuclear staining. Some plasma membrane staining was also observed. Endogenous Rac1 showed a strong staining at the fusion zone of the ruffled border. There was also less intensive staining around the nuclei and at the plasma membrane. We found some colocalization on the basolateral plasma membrane of resorbing osteoclasts.

In resorbing osteoclasts, internalized transferrin has been shown to be delivered to the ruffled border. It also enters into the resorption lacuna, which is considered to be analogous to the late endosomal compartment (*Palokangas et al., 1997*). When osteoclasts were serum-deprived in culture they stop resorbing, as expected. When Tfn was administered to these osteoclasts, they recycled it to the basolateral plasma membrane instead of targeting it to the ruffled border (*Mulari et al., 2003; Palokangas et al., 1997*). After 30 min of Tfn-594 internalization, we visualized a clear triple colocalization of Rab11, Tfn-594 and Rac1 in the peripheral vesicles and on the basolateral plasma membrane. This further supported our observations that in HeLa cells the molecular interaction between Rab11-Rac1 is involved in the regulation of Tfn and TfR recycling to plasma membrane. Interestingly, migrating osteoclasts showed intensive Rab11, Tfn-594 and Rac1 triple colocalization also at the leading edge. This observation suggests that Rab11-Rac1 interaction is not only involved in Tfn recycling to the plasma membrane, but could also regulate cell migration (III – Fig. 8 and S3).

6 DISCUSSION

6.1 Lipid rafts participate in transcytosis and the late endocytic pathway and determine the internalized transferrin trafficking route in osteoclasts

Previous studies have revealed that there is a transcytotic route by which digested bone is carried from the resorption lacuna and ruffled border through the cell via the perinuclear region to the apical functional secretory domain (*Nesbitt and Horton, 1997; Salo et al., 1997*). It is clear that this trafficking transports membrane material from the ruffled border to the FSD. In the present study, we identified a novel transcytotic route from the FSD to the fusion zone of the ruffled border, which could compensate the membrane loss from the RB. Wild-type VSV G protein has been shown to be delivered to the basolateral membrane (*Salo et al., 1996*). The replacement of its cytoplasmic tail by that of the CD4 protein targets it to lipid rafts (*Fragoso et al., 2003; Percherancier et al., 2003*). We found that lipid rafts were highly enriched in the ruffled border and especially concentrated at the peripheral fusion zone, both localization of recChlTx-B and VSV-GGC and GCC with this area suggesting the presence of rafts. This is also in agreement with, for example, $\alpha v\beta_3$ integrin distribution into this same membrane area.

Indeed, recent studies have shown that rafts are present on late endosomes in particular cell types (*Fivaz et al., 2002; Nydegger et al., 2003*). The ruffled border shows the structural and functional characteristics of late endosomal/lysosomal membranes. The observation that MβCD (methyl-beta-cyclodextrin) disrupted the co-organization of β_3-integrin and ruffled border actin in a concentration-dependent manner suggests that rafts are essential for the maintenance of ruffled border structure. This is in agreement with the earlier study (*Zhao et al., 2007*). Thus, it can be suggested that certain membrane proteins are recruited to the fusion zone by the lipid rafts, whereas other proteins are excluded and placed on the central ruffled border.

Internalized transferrin is delivered to the ruffled border in resorbing osteoclasts. MβCD blocked transferrin delivery to the ruffled border suggesting that rafts are essential for ruffled border membrane supply. Although the transferrin receptor has generally been considered to be a non-raft protein, recently, cell type-dependent compositions of rafts have been described. It was found that the transferrin receptor was resistant to extraction with Lubrol WX in MDCK cells suggesting its possible association with rafts in these cells (*Schuck et al., 2003*), which is in agreement with our findings of association with rafts on the ruffled border.

On the other hand, it should also be noted that the recycling transferrin pool may not be associated with lipid microdomains and therefore, cholesterol depletion might shift all transferrin from the late endocytic route which is regulated by Rab7, to the recycling route which is regulated by Rab11. This may explain why the internalized transferrin was recycled to the basolateral membrane when serum was depleted in the culture. In

these conditions, the resorbing osteoclasts cease resorption but retain their functional domains (I & III).

Cholesterol depletion disturbed vesicular trafficking and ruffled border structure while only a minor effect on actin ring attachment structures was observed. However, timing of cholesterol depletion played an important role on the results obtained, since massive cell death was induced when we performed MβCD treatment after 30 min of primary attachment. In the experiments reported in this thesis, MβCD incubation time was 30 min., followed by an incubation in fresh medium for 3 h before fixation. This treatment did not harm the cells.

6.2 The role of Rab7-Rac1 interaction in membrane trafficking

The formation of four functional domains involves rapid and intensive membrane rearrangements to create resorbing osteoclasts. The majority of the membrane targeting to the peripheral fusion zone is contributed by late endosomal transport. *Cantalupo et al. (2001) and Jordens et al. (2001)* found an effector of Rab7, RILP, which controls lysosomal transport by inducing the recruitment of dynein-dynactin motors to the minus ends of microtubules. RILP has also been reported to be the Rab34 effector involved in spatial positioning of lysosomes in high RILP expressing cells (*Wang et al., 2004*). We examined the endogenous RILP in resorbing osteoclasts, but we could not find Rab7 colocalization with RILP (antibody kindly provided by Dr. J. Neefjes). The absence of RILP-Rab7 in osteoclasts prompted us to look for other Rab7-interacting proteins/ effectors.

The two-hybrid system is widely used for seeking novel interacting proteins of a bait protein such as Rab7. We chose the relatively new bacterial two-hybrid system for the following reasons. The yeast two-hybrid system has been used by many others, and we judged that its potential for Rab7 may be exhausted. Yeast has its own Vps small GTP-binding proteins that might confound the finding of further effectors. *Cantalupo et al. (2001)* reported that prenylation of Rab7 in yeast can cause a strong background, so they deleted the last three (prenylable) amino acid residues from the bait sequence, although they might be important in effector recognition. In bacteria, there are no Rab-like proteins, and there is no prenylation. Thus full-length Rab7 can be used as a bait.

Making a cDNA library for the target constructs from isolated rat osteoclasts would have been labourious, considering the scarcity of these cells. Therefore, we chose newborn rat bone marrow, which is a rich source of osteoclasts. For the bait construct, full-length Rab7 was chosen with a mutation in its GTP-binding region. The Q67L mutation renders the protein unable to hydrolyze GTP, keeping it in a constantly activated state (*Cantalupo et al., 2001*).

We found three new Rab7-interacting peptide sequences with the bacterial two-hybrid assay, and one of these was Rac1. The direct protein-protein interaction between Rab7

and Rac1 was confirmed by GST-fused protein pull-down experiments. Apparently, the interaction is specific for Rac1, because other Rho GTPases such as RhoB, Rac2, or cdc42 have no affinity to Rab7 in these pull-down assays. As far as we know this is first time direct interaction between two sub-families of small GTPase has been found.

Rab7 and Rac1 colocalize in resorbing osteoclasts. The bulk of the colocalization was on the periphery of the ruffled border, suggesting that the function of the interaction is the delivery of late endosomal compartments to the ruffled border, such as transferrin containing vesicles (II). Therefore, this late endosomal transport that is regulated by Rab7-Rac1 interaction is also raft sensitive. One may thus speculate that the rafts on the fusion zone could recruit Rab7 driven vesicles.

Fluorescent images showed punctate staining of the colocalization of Rab7 and Rac1, which indicates that they stain the same intracellular vesicles in osteoclasts. The overlap is far from 100%; this also corroborates the notion that Rab7 does not need Rac1 for vesicular transport at every step. The observation that the partial colocalization of Rab7 and Rac1 is not confined to resorbing osteoclasts only, suggests that the phenomenon is more general. In fibroblast-like cells present in the culture of rat primary osteoclasts, the co-stained structures are mostly perinuclear, where the late endosomes usually reside. It is thus possible that Rab7-Rac1 interaction is a general mechanism of late endosomal trafficking in eukaryotic cells.

Knock out of the Rac1 gene is lethal (*Pierre et al., 1992*). Conditional knock out of Rac1 has been done by the cre-LoxP system with either interferon-inducible (*Sugihara et al., 1998*) or cell line-specific cre expression (with lysozyme promoter-cre) (*Wells et al., 2004*). The analysis of macrophages and leukocytes in Rac1 null mice showed anomalies in cell-dependent immunity. It may well be that some of these defects are caused by the absence of functional Rab7-Rac1 complex. In a Chlamydia-induced arthritis model, it was found that the clearance of pathogens in Rac1-deficient animals was defective (*Zhang et al., 2005*). All these findings point in the direction of Rac1 being an integral component of the endocytic/recycling pathway.

6.3 Rab11 and Rac1 interaction regulates the slow recycling pathway

It is well known that Rab11 delivers Tfn and TfR-containing recycling vesicles to the plasma membrane via a slow recycling pathway. Retention of Tfn-TfR in the PNRC was observed in both GTP and Rab11wt expressing cells after 30 minutes of Tfn uptake when the recycling pathway was stably maintained (*Hales et al., 2002; Sonnichsen et al., 2000; Ullrich et al., 1996*). A previous study showed that exit of Tfn-TfR from the PNRC and exocytosis required GTP hydrolysis. However, exit from sorting endosomes was dependent on GTP-bound Rab11 (*Ren et al., 1998*). We speculate that these steps may be promoted by a similar molecular machinery, e.g. Rab11 may recruit some additional binding proteins/effectors in its GTP form, thus allowing movement from sorting endosomes to the PNRC, and from the PNRC to the plasma membrane.

It has been shown earlier that although Rab11-FIP2 (Rab11-Family of Interacting Protein 2) forms a strong bond with Rab11S25N or Rab11Q70L in pull-down assays, affinity of Rab11-FIP2 towards Rab11Q70L is almost 20 times higher than to Rab11S25N (*Junutula et al., 2004*). On the other hand, it has also been shown that Rab11 may undergo its GTP/GDP cycle without dissociating from the membrane (*Pasqualato et al., 2004*). Thus, this may explain why we can not see a 'cutting off' binding difference between GTP/GDP-bound Rab11 and Rac1 in pull down and co-immunoprecipitation experiments. Since Rac1 dissociates from Rab11S25N, part of Rab11S25N may still be co-immunoprecipited in a membrane protein complex with Rac1 by the anti-Rac1 antibody. However, at this moment we cannot rule out the possibility that Rac1 stays attached to GDP-bound Rab11 inside the cell. Since primary osteoclasts are difficult to be transfected we decided to use HeLa cells for further experiments.

We present functional evidence that Rab11 interacting protein, Rac1, participates in Tfn-TfR recycling, when it interacts with Rab11wt or Rab11Q70L. Balanced co-expression of wild type or Rab11Q70L with Rac1 enhances Tfn uptake at 16°C, whereas uptake was inhibited in cells overexpressing Rab11S25N with Rac1, when compared to that of mock-transfected cells. Moreover, the block of Tfn in sorting endosomes at 16°C was overcome in the wild type or Rab11Q70L plus Rac1 expressing cells, indicating an acceleration of Tfn transport from the sorting endosomes to the PNRC. This was supported by following the time course of Tfn-647 localization during internalization at 37°C, since we observed that the bulk of Tfn was loaded into the PNRC already at 5 min. In non-transfected cells, this requires 10- 20 minutes. Impressively, there was a quick secretion of Tfn-647 and B-Tfn (16°C pre-incubation) after 5 min chasing, since we observed a sharp drop of fluorescent Tfn-647 in-cell and a peak of B-Tfn released to the medium at this time point. We saw that all forms of Rac1 expressed enhanced B-Tfn recycling compared to mock transfected cells. However, the value was much lower than that in Rab11wt or Rab11Q70L and Rac1 co-expressing cells. We also saw that Rab11 alone was unable to promote the transport during the first 10 min of chasing, indicating that the effects we observed were dependent on Rab11-Rac1 interaction, and not on Rab11 or Rac1 function alone.

The peak of B-Tfn released from cells at 5 min is evidence that Rab11-Rac1 interaction enhances Tfn exit from the PNRC to the plasma membrane. At 5 and 10 min time points, B-Tfn recycling was significantly lower in Rab11S25N and Rac1 co-expressing cells compared to only Rab11S25N expressing cells, indicating that Rac1 co-expression with Rab11S25N inhibited Tfn recycling. This is supported by the observations of accumulation of Tfn-647 in perinuclear membrane clusters where it colocalized with Rac1 and Rab11S25N.

Indeed, we observed Rac1, Rab11 and Tfn colocalization in the vesicles, PNRC and plasma membrane during 5-30 min of Tfn-647 internalization at 37°C in Rab11wt plus Rac1 cells. We also observed that the bulk of the PNRC dispersed into Rab11 and Rac1 positive vesicles, which transported Tfn to the plasma membrane in these cells.

We conclude that Rab11-Rac1 interaction accelerates Tfn recycling from the sorting endosomes to the PNRC and its exit from the PNRC to the plasma membrane.

Data obtained from primary cells, osteoclasts, further suggested that Rab11-Rac1 interaction regulates the internalized Tfn recycling back to the basolateral plasma membrane. Thus, these results were in good agreement with our observations in HeLa cells, suggesting that Rac1 interaction with Rab11 could be a regulator of the recycling pathway.

Rac1 has been suggested to be a key molecule in cellular migration. The interaction between PI3K and Rac1 regulates cell polarization and directional migration (*Srinivasan et al., 2003*). We observed an intensive triple colocalization of internalized Tfn, Rab11 and Rac1 on the leading edge of migrating osteoclasts. This strongly suggests that Rab11-Rac1 interaction is involved in normal cellular migration. Indeed, the Rab11 regulated recycling pathway has recently emerged as an important component of cell migration machinery (*Jones et al., 2006*). Cell migration needs continuous replenishment of the leading edge membrane and other important components, such as integrins (*Jones et al., 2006; Yoon et al., 2005*). Internalized Tfn has earlier been shown to traffic to the tips of the lamellipodium and to exocytose at the leading edge of fibroblasts (*Hopkins et al., 1994*).

6.4 Rab-Rac1 interaction is a crucial step in intracellular vesicle trafficking

Microtubules are used to transport different types of vesicles inside the cell. On the other hand, when vesicles are delivered near acceptor membrane in the perinuclear area or underneath of the plasma membrane, they are transferred to actin filaments. Our results suggested that Rac1-Rab interaction could be a key for this transition from microtubules to microfilaments.

The functions of the Rho family of GTPases are well known. They organize the actin cytoskeleton for cell shape, polarization, and migration through their effectors near the cell cortex (*Kaibuchi et al., 1999; Knaus et al., 1998; Nomura et al., 1994; Weernink et al., 2004; Weissbach et al., 1994*). Recent studies have shown that one of the Rac1/Cdc42 effectors, IQGAP1 is also an actin-binding protein (*Fukata et al., 2002*; *Erickson et al., 1997; Fukata et al., 1997*), and directly interacts with CLIP-170 (*Fukata et al., 2002*) that specifically accumulates at the plus ends of growing microtubules and tethers endosomes to the microtubules (*Perez et al., 1999; Pierre et al., 1992*). Thus, these three molecules (Rac1/IQGAP1/CLIP-170) make a complex that bridges the plus ends of MT and the cortical actin meshwork (*Fukata et al., 2002*).

We speculate that in resorbing osteoclasts, activated Rab7 binds Rac1 to regulate the acidic compartments dragging along microtubules to the actin filaments before their fusion to the fusion zone of the ruffled border. Thus this interaction could control ruffled border formation.

We have here confirmed by several different techniques that Rab7 and Ra11 are able to interact directly with Rac1. With a bacterial two-hybrid assay, we also found that other but not all Rab proteins show affinity towards Rac1. Therefore, on the basis of our findings we suggest that direct Rac1-Rab protein interaction may represent a general mechanism in the regulation of vesicular trafficking (Fig. 7).

Fig. 7. The model for the role of Rac1-Rab interaction

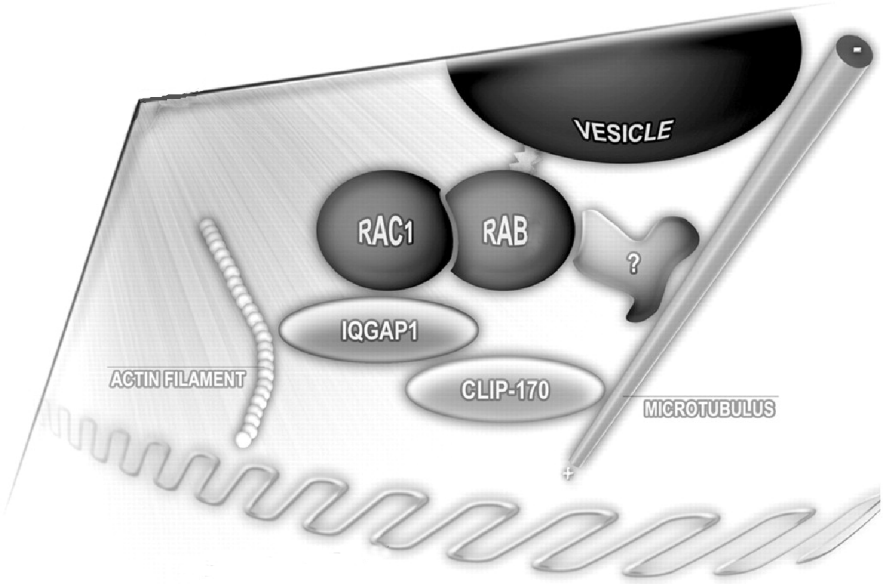

Fig. 7. Workng model: Certain Rab proteins bind Rac1 directly. Rac1 binds two other molecules, IQGAP1 and CLIP-170, which interacts with microfilaments and microtubules, respectively (previouse studies). We suggest that the direct binding between Rac1 and specific Rabs could be used to switch vesicular trafficking from microtubules to microfilaments or vice versa.

7 CONCLUSIONS

We have shown that Rac1 directly interacts with different Rab proteins in the different pathways of intracellular vesicle transport. First, we revealed that Rac1 directly binds to Rab7 and demonstrated that this molecular interaction is a crucial step in the transport of late endosomes to the fusion zone of the ruffled border in bone resorbing osteoclasts. Second, we revealed that Rac1 also directly binds to some other Rabs, such as Rab11. We demonstrated that direct Rac1-Rab11 interaction positively regulates transferrin and its receptor recycling from sorting endosomes to the PNRC and from the PNRC to the plasma membrane in the slow recycling pathway. This interaction also regulates transferrin receptor recycling in osteoclasts, since we found that the internalized transferrin recycled to the plasma membrane where it colocalized with both Rab11 and Rac1. Importantly, in migrating osteoclasts, Rac1, Rab11 and transferrin were all colocalized at the leading edge of the cell, suggesting that the interaction may be involved in cell migration.

On the basis of our earlier work and the present results, we suggest that certain Rab proteins (at least Rab7 and Rab11), but not all, bind Rac1 directly. It is known from previous studies that Rac1 binds two other molecules, IQGAP1 and CLIP-170, which interacts with microfilaments and microtubules, respectively. We suggest that the direct binding between Rac1 and specific Rabs could be used to switch vesicular trafficking from microtubules to microfilaments or vice versa.

In resorbing osteoclasts the transcytotic route from the central zone of the ruffled border to the functional secretory domain removes degraded bone matrix from resorption lacuna. In this study, a new transcytotic route from the FSD to the fusion zone of the ruffled border was identified and shown to be lipid raft dependent. We suggest that a role of this transcytosis is to compensate the membrane loss from the ruffled border during resorption. We also showed that the depletion of cholesterol inhibited accumulation of cortical actin at the ruffled border and prevented the fusion of endosomal vesicles to the ruffled border.

8 ACKNOWLEDGEMENTS

The work for this thesis was carried out at the Department of Cell Biology and Anatomy, Institute of Biomedicine, University of Turku.

I express my deepest gratitude to my supervisor, Professor Kalervo Väänänen, M.D., Ph.D., for giving me the opportunity to work in the Department of Cell Biology and Anatomy and for providing me with excellent research facilities and scientific atmosphere. Thank you so much for your guiding me to this brilliant research field. I am astonished at your ever-enthusiastic attitude towards science, tremendous experience, and profound and intense knowledge of the field of bone biology, cell biology as well as molecular biology. I am deeply grateful for your careful guidance, supervision, never-ending support, encouragement, faith and understanding throughout these years. Without these, it would not have been possible for me to accomplish this thesis.

I thank Dr. Kalman Büki, M.D., Ph.D. You offered me your enormous knowledge, invaluable and educational comments, and your enthusiastic and supportive attitude. You were always ready to help and discuss with me during these years. You are so important for me and my thesis. I am deeply grateful to Professor Pirkko Härkönen, M.D., Ph.D., and Dr. Mika Mulari, M.D., Ph.D. for sharing their knowledge in various research topics. I am impressed by your great personalities, helpful advice and inspiring scientific discussions. Dr. Mika Mulari is also acknowledged for his cooperation in two studies.

I also thank my co-authors Dr. Jukka Vääräniemi, Ph.D., Otto Ettala, M.D., Dr. Kalervo Metsikkö, Ph.D., and Martin Nars, M.Sc. Your work and knowledge were important for the publications as well as this thesis. I warmly thank Dr. Hannele Ylipahkala and Dr. Kaisa Ivaska for their friendship when I joined the group. I deeply thank my dear Ph.D. student fellows Mirkka Hirvonen, Johanna Saarimäki, Katja Fagerlund, Jonas Nyman, Heikki Vuorikoski, Salla Suomi and Anne Seppänen, M.Sc., for your understanding and friendship. We shared times when we were happy and depressed during our Ph.D. work. I wish the best for all of you in the future. Dr. Terhi Heino and Pirkko Muhonen are acknowledged for working with me during these years.

Mrs. Iris Dunder, Mrs. Soili Huhta and Mrs. Outi Irjala are acknowledged for their fine organization of the department and great help. You are the best secretaries. Dr. Tiina Laitala-Leinonen, Dr. Teuvo Hentunen and Dr. Harri Härmä are warmly acknowledged for sharing their knowledge and valuable discussions. I warmly acknowledge every one in the Department of Cell Biology and Anatomy. It was a great pleasure to work with you.

I express my deep acknowledgement to my Chinese friends in our department, especially to Dr. Guoliang Gu, Dr. Zhiqi Peng as well as Dr. Zhi Chen and Dr. Min Jiang. I also express my special thanks to all my Chinese friends and their families in Turku, for their

company and sharing happiness together in our leisure time. The friendship, help and encouragement of each other are important and indispensable.

I am thankful to the reviewers of my thesis, Docent Eeva-Liisa Eskelinen and Lecturer Arto Määttä, Ph.D., for their comments and criticism, which helped to improve the quality of this work. Michael Nelson, Ph.D., is acknowledged for the revision of the language of the thesis. I express my sincere thanks to Professor *Michael Horton,* Ph.D., for being the opponent.

I also thank my parents and my brother deeply for their never-ending love and encouragement and support during these years. Finally, I devote my heart-felt thanks to my son ShiHong Shen and my husband ZhongYi Shen. You have shared all my good and bad moments during these years. Your encouragement and support has been essential for me, and without you, I do not believe that I would have been able to finish this thesis.

Turku, August 2009

Yi Sun

9 REFERENCES

Alatalo, S.L., Halleen, J.M., Hentunen, T.A., Mönkkönen, J., and Väänänen, H.K. (2000). Rapid screening method for osteoclast differentiation *in vitro* that measures tartrate-resistant acid phosphatase 5b activity secreted into the culture medium. Clin Chem *46*, 1751-1754.

Ali, B.R., and Seabra, M.C. (2005). Targeting of Rab GTPases to cellular membranes. Biochem Soc Trans *33*, 652-656.

Allan, B.B., and Balch, W.E. (1999). Protein sorting by directed maturation of Golgi compartments. Science *285*, 63-66.

Allan, B.B., Moyer, B.D., and Balch, W.E. (2000). Rab1 recruitment of p115 into a cis-SNARE complex: programming budding COPII vesicles for fusion. Science *289*, 444-448.

Asagiri, M., Hirai, T., Kunigami, T., Kamano, S., Gober, H.J., Okamoto, K., Nishikawa, K., Latz, E., Golenbock, D.T., Aoki, K., *et al.* (2008). Cathepsin K-dependent toll-like receptor 9 signaling revealed in experimental arthritis. Science *319*, 624-627.

Babst, M., Odorizzi, G., Estepa, E.J., and Emr, S.D. (2000). Mammalian tumor susceptibility gene 101 (TSG101) and the yeast homologue, Vps23p, both function in late endosomal trafficking. Traffic *1*, 248-258.

Baron, R.A., and Seabra, M.C. (2008). Rab geranylgeranylation occurs preferentially via the pre-formed REP-RGGT complex and is regulated by geranylgeranyl pyrophosphate. Biochem J *415*, 67-75.

Barrowman, J., and Novick, P. (2003). Three Yips for Rab recruitment. Nat Cell Biol *5*, 955-956.

Bell, N.H., and Johnson, R.H. (1997). Bisphosphonates in the treatment of osteoporosis. Endocrine *6*, 203-206.

Bellows, C.G., Aubin, J.E., and Heersche, J.N. (1987). Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells *in vitro*. Endocrinology *121*, 1985-1992.

Bernards, A., and Settleman, J. (2004). GAP control: regulating the regulators of small GTPases. Trends Cell Biol *14*, 377-385.

Bielli, A., Thornqvist, P.O., Hendrick, A.G., Finn, R., Fitzgerald, K., and McCaffrey, M.W. (2001). The small GTPase Rab4A interacts with the central region of cytoplasmic dynein light intermediate chain-1. Biochem Biophys Res Commun *281*, 1141-1153.

Black, D.M., Delmas, P.D., Eastell, R., Reid, I.R., Boonen, S., Cauley, J.A., Cosman, F., Lakatos, P., Leung, P.C., Man, Z., *et al.* (2007). Once-yearly zoledronic acid for treatment of postmenopausal osteoporosis. N Engl J Med *356*, 1809-1822.

Bonazzi, M., and Cossart, P. (2006). Bacterial entry into cells: a role for the endocytic machinery. FEBS Lett *580*, 2962-2967.

Bonifacino, J.S., and Traub, L.M. (2003). Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem *72*, 395-447.

Bucci, C., Parton, R.G., Mather, I.H., Stunnenberg, H., Simons, K., Hoflack, B., and Zerial, M. (1992). The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. Cell *70*, 715-728.

Bucci, C., Thomsen, P., Nicoziani, P., McCarthy, J., and van Deurs, B. (2000). Rab7: a key to lysosome biogenesis. Mol Biol Cell *11*, 467-480.

Calvi, L.M., Sims, N.A., Hunzelman, J.L., Knight, M.C., Giovannetti, A., Saxton, J.M., Kronenberg, H.M., Baron, R., and Schipani, E. (2001). Activated parathyroid hormone/parathyroid hormone-related protein receptor in osteoblastic cells differentially affects cortical and trabecular bone. J Clin Invest *107*, 277-286.

Campagnoli, C., Roberts, I.A., Kumar, S., Bennett, P.R., Bellantuono, I., and Fisk, N.M. (2001). Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood *98*, 2396-2402.

Canalis, E. (2005). The fate of circulating osteoblasts. N Engl J Med *352*, 2014-2016.

Cantalupo, G., Alifano, P., Roberti, V., Bruni, C.B., and Bucci, C. (2001). Rab-interacting lysosomal protein (RILP): the Rab7 effector required for transport to lysosomes. Embo J *20*, 683-693.

Caplow, M., Shanks, J., and Ruhlen, R.L. (1988). Temperature-jump studies of microtubule dynamic instability. J Biol Chem *263*, 10344-10352.

Carroll, K.S., Hanna, J., Simon, I., Krise, J., Barbero, P., and Pfeffer, S.R. (2001). Role of Rab9 GTPase in facilitating receptor recruitment by TIP47. Science *292*, 1373-1376.

Chambers, T.J., Revell, P.A., Fuller, K., and Athanasou, N.A. (1984). Resorption of bone by isolated rabbit osteoclasts. J Cell Sci *66*, 383-399.

Chavrier, P., Parton, R.G., Hauri, H.P., Simons, K., and Zerial, M. (1990). Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. Cell *62*, 317-329.

Chen, W., Feng, Y., Chen, D., and Wandinger-Ness, A. (1998). Rab11 is required for trans-golgi network-to-plasma membrane transport and a preferential target for GDP dissociation inhibitor. Mol Biol Cell *9*, 3241-3257.

Chen, Y.A., and Scheller, R.H. (2001). SNARE-mediated membrane fusion. Nat Rev Mol Cell Biol *2*, 98-106.

Chenu, C., Pfeilschifter, J., Mundy, G.R., and Roodman, G.D. (1988). Transforming growth factor beta inhibits formation of osteoclast-like cells in long-term human marrow cultures. Proc Natl Acad Sci U S A *85*, 5683-5687.

Colicelli, J. (2004). Human RAS superfamily proteins and related GTPases. Sci STKE *2004*, RE13.

Conner, S.D., and Schmid, S.L. (2003). Regulated portals of entry into the cell. Nature *422*, 37-44.

Coxon, F.P., Helfrich, M.H., Larijani, B., Muzylak, M., Dunford, J.E., Marshall, D., McKinnon, A.D., Nesbitt, S.A., Horton, M.A., Seabra, M.C., *et al.* (2001). Identification of a novel phosphonocarboxylate inhibitor of Rab geranylgeranyl transferase that specifically prevents Rab prenylation in osteoclasts and macrophages. J Biol Chem *276*, 48213-48222.

Cullis, D.N., Philip, B., Baleja, J.D., and Feig, L.A. (2002). Rab11-FIP2, an adaptor protein connecting cellular components involved in internalization and recycling of epidermal growth factor receptors. J Biol Chem *277*, 49158-49166.

Damiani, M.T., Pavarotti, M., Leiva, N., Lindsay, A.J., McCaffrey, M.W., and Colombo, M.I. (2004). Rab coupling protein associates with phagosomes and regulates recycling from the phagosomal compartment. Traffic *5*, 785-797.

Daro, E., van der Sluijs, P., Galli, T., and Mellman, I. (1996). Rab4 and cellubrevin define different early endosome populations on the pathway of transferrin receptor recycling. Proc Natl Acad Sci U S A *93*, 9559-9564.

Davies, B.A., Topp, J.D., Sfeir, A.J., Katzmann, D.J., Carney, D.S., Tall, G.G., Friedberg, A.S., Deng, L., Chen, Z., and Horazdovsky, B.F. (2003). Vps9p CUE domain ubiquitin binding is required for efficient endocytic protein traffic. J Biol Chem *278*, 19826-19833.

de Renzis, S., Sonnichsen, B., and Zerial, M. (2002). Divalent Rab effectors regulate the sub-compartmental organization and sorting of early endosomes. Nat Cell Biol *4*, 124-133.

Deneka, M., Neeft, M., and van der Sluijs, P. (2003). Regulation of membrane transport by rab GTPases. Crit Rev Biochem Mol Biol *38*, 121-142.

Destaing, O., Saltel, F., Geminard, J.C., Jurdic, P., and Bard, F. (2003). Podosomes display actin turnover and dynamic self-organization in osteoclasts expressing actin-green fluorescent protein. Mol Biol Cell *14*, 407-416.

Diaz, E., and Pfeffer, S.R. (1998). TIP47: a cargo selection device for mannose 6-phosphate receptor trafficking. Cell *93*, 433-443.

Diez, S., Gerisch, G., Anderson, K., Muller-Taubenberger, A., and Bretschneider, T. (2005). Subsecond reorganization of the actin network in cell motility and chemotaxis. Proc Natl Acad Sci U S A *102*, 7601-7606.

Dong, J., Chen, W., Welford, A., and Wandinger-Ness, A. (2004). The proteasome alpha-subunit XAPC7 interacts specifically with Rab7 and late endosomes. J Biol Chem *279*, 21334-21342.

Dong, Z., Bonfil, R.D., Chinni, S., Deng, X., Trindade Filho, J.C., Bernardo, M., Vaishampayan, U., Che, M., Sloane, B.F., Sheng, S., *et al.* (2005). Matrix metalloproteinase activity and osteoclasts in experimental prostate cancer bone metastasis tissue. Am J Pathol *166*, 1173-1186.

Dougall, W.C., Glaccum, M., Charrier, K., Rohrbach, K., Brasel, K., De Smedt, T., Daro, E., Smith, J., Tometsko, M.E., Maliszewski, C.R., *et al.* (1999). RANK is essential for osteoclast and lymph node development. Genes Dev *13*, 2412-2424.

Ducy, P., Zhang, R., Geoffroy, V., Ridall, A.L., and Karsenty, G. (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell *89*, 747-754.

Duplomb, L., Baud'huin, M., Charrier, C., Berreur, M., Trichet, V., Blanchard, F., and Heymann, D. (2008). IL-6 inhibits RANKL-induced osteoclastogenesis by diverting cells into the macrophage lineage: key role of Serine727 phosphorylation of STAT3. Endocrinology.

Erices, A., Conget, P., and Minguell, J.J. (2000). Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol *109*, 235-242.

Erickson, J.W., Cerione, R.A., and Hart, M.J. (1997). Identification of an actin cytoskeletal complex that includes IQGAP and the Cdc42 GTPase. J Biol Chem *272*, 24443-24447.

Etienne-Manneville, S. (2004). Actin and microtubules in cell motility: which one is in control? Traffic *5*, 470-477.

Ezzell, R.M., Goldmann, W.H., Wang, N., Parasharama, N., and Ingber, D.E. (1997). Vinculin promotes cell spreading by mechanically coupling integrins to the cytoskeleton. Exp Cell Res *231*, 14-26.

Fan, G.H., Lapierre, L.A., Goldenring, J.R., Sai, J., and Richmond, A. (2004). Rab11-family interacting protein 2 and myosin Vb are required for CXCR2 recycling and receptor-mediated chemotaxis. Mol Biol Cell *15*, 2456-2469.

Feng, Y., Press, B., and Wandinger-Ness, A. (1995). Rab 7: an important regulator of late endocytic membrane traffic. J Cell Biol *131*, 1435-1452.

Fielding, A.B., Schonteich, E., Matheson, J., Wilson, G., Yu, X., Hickson, G.R., Srivastava, S., Baldwin, S.A., Prekeris, R., and Gould, G.W. (2005). Rab11-FIP3 and FIP4 interact with Arf6 and the exocyst to control membrane traffic in cytokinesis. Embo J *24*, 3389-3399.

Finkelstein, J.S., Hayes, A., Hunzelman, J.L., Wyland, J.J., Lee, H., and Neer, R.M. (2003). The effects of parathyroid hormone, alendronate, or both in men with osteoporosis. N Engl J Med *349*, 1216-1226.

Fivaz, M., Vilbois, F., Thurnheer, S., Pasquali, C., Abrami, L., Bickel, P.E., Parton, R.G., and van der Goot, F.G. (2002). Differential sorting and fate of endocytosed GPI-anchored proteins. Embo J *21*, 3989-4000.

Fleisch, H. (1991). Bisphosphonates. Pharmacology and use in the treatment of tumour-induced hypercalcaemic and metastatic bone disease. Drugs *42*, 919-944.

Fragoso, R., Ren, D., Zhang, X., Su, M.W., Burakoff, S.J., and Jin, Y.J. (2003). Lipid raft distribution of CD4 depends on its palmitoylation and association with Lck, and evidence for CD4-induced lipid raft aggregation as an additional mechanism to enhance CD3 signaling. J Immunol *170*, 913-921.

Fuchs, E., and Weber, K. (1994). Intermediate filaments: structure, dynamics, function, and disease. Annu Rev Biochem *63*, 345-382.

Fukata, M., Kuroda, S., Fujii, K., Nakamura, T., Shoji, I., Matsuura, Y., Okawa, K., Iwamatsu, A., Kikuchi, A., and Kaibuchi, K. (1997). Regulation of cross-linking of actin filament by IQGAP1, a target for Cdc42. J Biol Chem *272*, 29579-29583.

Fukata, M., Watanabe, T., Noritake, J., Nakagawa, M., Yamaga, M., Kuroda, S., Matsuura, Y., Iwamatsu, A., Perez, F., and Kaibuchi, K. (2002). Rac1 and Cdc42 capture microtubules through IQGAP1 and CLIP-170. Cell *109*, 873-885.

Garcia-del Portillo, F., and Finlay, B.B. (1995). The varied lifestyles of intracellular pathogens within eukaryotic vacuolar compartments. Trends Microbiol *3*, 373-380.

Gaullier, J.M., Simonsen, A., D'Arrigo, A., Bremnes, B., Stenmark, H., and Aasland, R. (1998). FYVE fingers bind PtdIns(3)P. Nature *394*, 432-433.

Goldmann, W.H., and Ingber, D.E. (2002). Intact vinculin protein is required for control of cell shape, cell mechanics, and rac-dependent lamellipodia formation. Biochem Biophys Res Commun *290*, 749-755.

Goldstein, L.S., and Yang, Z. (2000). Microtubule-based transport systems in neurons: the roles of kinesins and dyneins. Annu Rev Neurosci *23*, 39-71.

Gomes, A.Q., Ali, B.R., Ramalho, J.S., Godfrey, R.F., Barral, D.C., Hume, A.N., and Seabra, M.C. (2003). Membrane targeting of Rab GTPases is influenced by the prenylation motif. Mol Biol Cell *14*, 1882-1899.

Gordeladze, J.O., Drevon, C.A., Syversen, U., and Reseland, J.E. (2002). Leptin stimulates human osteoblastic cell proliferation, de novo collagen synthesis, and mineralization: Impact on differentiation markers, apoptosis, and osteoclastic signaling. J Cell Biochem *85*, 825-836.

Gotlieb, A.I., Subrahmanyan, L., and Kalnins, V.I. (1983). Microtubule-organizing centers and cell migration: effect of inhibition of migration and microtubule disruption in endothelial cells. J Cell Biol *96*, 1266-1272.

Grant, B., and Hirsh, D. (1999). Receptor-mediated endocytosis in the Caenorhabditis elegans oocyte. Mol Biol Cell *10*, 4311-4326.

Green, E.G., Ramm, E., Riley, N.M., Spiro, D.J., Goldenring, J.R., and Wessling-Resnick, M. (1997). Rab11 is associated with transferrin-containing recycling compartments in K562 cells. Biochem Biophys Res Commun *239*, 612-616.

Gruenberg, J. (2001). The endocytic pathway: a mosaic of domains. Nat Rev Mol Cell Biol *2*, 721-730.

Grunfelder, C.G., Engstler, M., Weise, F., Schwarz, H., Stierhof, Y.D., Morgan, G.W., Field, M.C., and Overath, P. (2003). Endocytosis of a glycosylphosphatidylinositol-anchored protein via clathrin-coated vesicles, sorting by default in endosomes, and exocytosis via RAB11-positive carriers. Mol Biol Cell *14*, 2029-2040.

Ha, H., Kwak, H.B., Lee, S.K., Na, D.S., Rudd, C.E., Lee, Z.H., and Kim, H.H. (2003). Membrane rafts play a crucial role in receptor activator of nuclear

factor kappaB signaling and osteoclast function. J Biol Chem *278*, 18573-18580.

Hales, C.M., Griner, R., Hobdy-Henderson, K.C., Dorn, M.C., Hardy, D., Kumar, R., Navarre, J., Chan, E.K., Lapierre, L.A., and Goldenring, J.R. (2001). Identification and characterization of a family of Rab11-interacting proteins. J Biol Chem *276*, 39067-39075.

Hales, C.M., Vaerman, J.P., and Goldenring, J.R. (2002). Rab11 family interacting protein 2 associates with Myosin Vb and regulates plasma membrane recycling. J Biol Chem *277*, 50415-50421.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. Science *279*, 509-514.

Halleen, J.M., Raisanen, S., Salo, J.J., Reddy, S.V., Roodman, G.D., Hentunen, T.A., Lehenkari, P.P., Kaija, H., Vihko, P., and Vaananen, H.K. (1999). Intracellular fragmentation of bone resorption products by reactive oxygen species generated by osteoclastic tartrate-resistant acid phosphatase. J Biol Chem *274*, 22907-22910.

Harrison, R.E., Brumell, J.H., Khandani, A., Bucci, C., Scott, C.C., Jiang, X., Finlay, B.B., and Grinstein, S. (2004). Salmonella impairs RILP recruitment to Rab7 during maturation of invasion vacuoles. Mol Biol Cell *15*, 3146-3154.

Harrison, R.E., Bucci, C., Vieira, O.V., Schroer, T.A., and Grinstein, S. (2003). Phagosomes fuse with late endosomes and/or lysosomes by extension of membrane protrusions along microtubules: role of Rab7 and RILP. Mol Cell Biol *23*, 6494-6506.

Haubruck, H., Prange, R., Vorgias, C., and Gallwitz, D. (1989). The ras-related mouse ypt1 protein can functionally replace the YPT1 gene product in yeast. Embo J *8*, 1427-1432.

Heino, T.J., Hentunen, T.A., and Vaananen, H.K. (2002). Osteocytes inhibit osteoclastic bone resorption through transforming growth factor-beta: enhancement by estrogen. J Cell Biochem *85*, 185-197.

Hicke, L., Schubert, H.L., and Hill, C.P. (2005). Ubiquitin-binding domains. Nat Rev Mol Cell Biol *6*, 610-621.

Hirokawa, N. (1998). Kinesin and dynein superfamily proteins and the mechanism of organelle transport. Science *279*, 519-526.

Holsbeeks, I., Lagatie, O., Van Nuland, A., Van de Velde, S., and Thevelein, J.M. (2004). The eukaryotic plasma membrane as a nutrient-sensing device. Trends Biochem Sci *29*, 556-564.

Hopkins, C.R., Gibson, A., Shipman, M., Strickland, D.K., and Trowbridge, I.S. (1994). In migrating fibroblasts, recycling receptors are concentrated in narrow tubules in the pericentriolar area, and then routed to the plasma membrane of the leading lamella. J Cell Biol *125*, 1265-1274.

Horgan, C.P., Walsh, M., Zurawski, T.H., and McCaffrey, M.W. (2004). Rab11-FIP3 localises to a Rab11-positive pericentrosomal compartment during interphase and to the cleavage furrow during cytokinesis. Biochem Biophys Res Commun *319*, 83-94.

Hu, K., Carroll, J., Fedorovich, S., Rickman, C., Sukhodub, A., Davletov, B. (2002) Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion. Nature *415*, 646-50.

Horiuchi, H., Lippe, R., McBride, H.M., Rubino, M., Woodman, P., Stenmark, H., Rybin, V., Wilm, M., Ashman, K., Mann, M., and Zerial, M. (1997). A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function. Cell *90*, 1149-1159.

Hruska, K.A., and Teitelbaum, S.L. (1995). Renal osteodystrophy. N Engl J Med *333*, 166-174.

Huang, J., Imamura, T., and Olefsky, J.M. (2001). Insulin can regulate GLUT4 internalization by signaling to Rab5 and the motor protein dynein. Proc Natl Acad Sci U S A *98*, 13084-13089.

Huss, R., Lange, C., Weissinger, E.M., Kolb, H.J., and Thalmeier, K. (2000). Evidence of peripheral blood-derived, plastic-adherent CD34(-/low) hematopoietic stem cell clones with mesenchymal stem cell characteristics. Stem Cells *18*, 252-260.

Ignatev, A., Kravchenko, S., Rak, A., Goody, R.S., and Pylypenko, O. (2008). A structural model of the GDP dissociation inhibitor rab membrane extraction mechanism. J Biol Chem *283*, 18377-18384.

Imamura, T., Huang, J., Usui, I., Satoh, H., Bever, J., and Olefsky, J.M. (2003). Insulin-induced GLUT4 translocation involves protein kinase C-lambda-mediated functional coupling between Rab4 and the motor protein kinesin. Mol Cell Biol *23*, 4892-4900.

Inaoka, T., Bilbe, G., Ishibashi, O., Tezuka, K., Kumegawa, M., and Kokubo, T. (1995). Molecular cloning of human cDNA for cathepsin K: novel cysteine proteinase predominantly expressed in bone. Biochem Biophys Res Commun *206*, 89-96.

Irie, K., Tsuruga, E., Sakakura, Y., Muto, T., and Yajima, T. (2001). Immunohistochemical localization of membrane type 1-matrix metalloproteinase (MT1-MMP) in osteoclasts in vivo. Tissue Cell *33*, 478-482.

Izard, T., Evans, G., Borgon, R.A., Rush, C.L., Bricogne, G., and Bois, P.R. (2004). Vinculin activation by talin through helical bundle conversion. Nature *427*, 171-175.

Jacobson, K., and Dietrich, C. (1999). Looking at lipid rafts? Trends Cell Biol *9*, 87-91.

Janssens, K., ten Dijke, P., Janssens, S., and Van Hul, W. (2005). Transforming growth factor-beta1 to the bone. Endocr Rev *26*, 743-774.

Jedd, G., Richardson, C., Litt, R., and Segev, N. (1995). The Ypt1 GTPase is essential for the first two steps of the yeast secretory pathway. J Cell Biol *131*, 583-590.

Johns, H.L., Berryman, S., Monaghan, P., Belsham, G.J., Jackson, T. (2009). A dominant-negative mutant of rab5 inhibits infection of cells by foot-and-mouth disease virus: implications for virus entry. J Virol. *83(12)*, 6247-56.

Johansson, M., Rocha, N., Zwart, W., Jordens, I., Janssen, L., Kuijl, C., Olkkonen, V.M., and Neefjes, J. (2007). Activation of endosomal dynein motors by stepwise assembly of Rab7-RILP-p150Glued, ORP1L, and the receptor betalll spectrin. J Cell Biol *176*, 459-471.

Jones, M.C., Caswell, P.T., and Norman, J.C. (2006). Endocytic recycling pathways: emerging regulators of cell migration. Curr Opin Cell Biol *18*, 549-557.

Jordens, I., Fernandez-Borja, M., Marsman, M., Dusseljee, S., Janssen, L., Calafat, J., Janssen, H., Wubbolts, R., and Neefjes, J. (2001). The Rab7 effector protein RILP controls lysosomal transport by inducing the recruitment of dynein-dynactin motors. Curr Biol *11*, 1680-1685.

Jordens, I., Marsman, M., Kuijl, C., and Neefjes, J. (2005). Rab proteins, connecting transport and vesicle fusion. Traffic *6*, 1070-1077.

Junutula, J.R., Schonteich, E., Wilson, G.M., Peden, A.A., Scheller, R.H., and Prekeris, R. (2004). Molecular characterization of Rab11 interactions with members of the family of Rab11-interacting proteins. J Biol Chem *279*, 33430-33437.

Kaibuchi, K., Kuroda, S., and Amano, M. (1999). Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. Annu Rev Biochem *68*, 459-486.

Katzmann, D.J., Odorizzi, G., and Emr, S.D. (2002). Receptor downregulation and multivesicular-body sorting. Nat Rev Mol Cell Biol *3*, 893-905.

Keller, P., and Simons, K. (1998). Cholesterol is required for surface transport of influenza virus hemagglutinin. J Cell Biol *140*, 1357-1367.

Knaus, U.G., Wang, Y., Reilly, A.M., Warnock, D., and Jackson, J.H. (1998). Structural requirements for PAK activation by Rac GTPases. J Biol Chem *273*, 21512-21518.

Kobayashi, T., Stang, E., Fang, K.S., de Moerloose, P., Parton, R.G., and Gruenberg, J. (1998). A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. Nature *392*, 193-197.

Kodama, A., Lechler, T., and Fuchs, E. (2004). Coordinating cytoskeletal tracks to polarize cellular movements. J Cell Biol *167*, 203-207.

Konstantinopoulos, P.A., Karamouzis, M.V., and Papavassiliou, A.G. (2007). Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets. Nat Rev Drug Discov *6*, 541-555.

Kostenuik, P.J., and Shalhoub, V. (2001). Osteoprotegerin: a physiological and pharmacological inhibitor of bone resorption. Curr Pharm Des *7*, 613-635.

Kumar, N. (1981). Taxol-induced polymerization of purified tubulin. Mechanism of action. J Biol Chem *256*, 10435-10441.

Kurata, K., Heino, T.J., Higaki, H., and Vaananen, H.K. (2006). Bone marrow cell differentiation induced by mechanically damaged osteocytes in 3D gel-embedded culture. J Bone Miner Res *21*, 616-625.

Lakkakorpi, P.T., and Vaananen, H.K. (1996). Cytoskeletal changes in osteoclasts during the resorption cycle. Microsc Res Tech *33*, 171-181.

Lam, K.W., Li, C.Y., Yam, L.T., Smith, R.S., and Hacker, B. (1982). Comparison of prostatic and nonprostatic acid phosphatase. Ann N Y Acad Sci *390*, 1-15.

Lapierre, L.A., Kumar, R., Hales, C.M., Navarre, J., Bhartur, S.G., Burnette, J.O., Provance, D.W., Jr., Mercer, J.A., Bahler, M., and Goldenring, J.R. (2001). Myosin vb is associated with plasma membrane recycling systems. Mol Biol Cell *12*, 1843-1857.

Lebrand, C., Corti, M., Goodson, H., Cosson, P., Cavalli, V., Mayran, N., Faure, J., and Gruenberg, J. (2002). Late endosome motility depends on lipids via the small GTPase Rab7. Embo J *21*, 1289-1300.

Lee, J.R., Shin, H., Choi, J., Ko, J., Kim, S., Lee, H.W., Kim, K., Rho, S.H., Lee, J.H., Song, H.E., *et al.* (2004). An intramolecular interaction between the FHA domain and a coiled coil negatively regulates the kinesin motor KIF1A. Embo J *23*, 1506-1515.

Leung, K.F., Baron, R., Ali, B.R., Magee, A.I., and Seabra, M.C. (2007). Rab GTPases containing a CAAX motif are processed

post-geranylgeranylation by proteolysis and methylation. J Biol Chem *282*, 1487-1497.

Leung, K.F., Baron, R., and Seabra, M.C. (2006). Thematic review series: lipid posttranslational modifications. geranylgeranylation of Rab GTPases. J Lipid Res *47*, 467-475.

Li, H.Y., Cao, K., and Zheng, Y. (2003). Ran in the spindle checkpoint: a new function for a versatile GTPase. Trends Cell Biol *13*, 553-557.

Li, J., Ballif, B.A., Powelka, A.M., Dai, J., Gygi, S.P., and Hsu, V.W. (2005). Phosphorylation of ACAP1 by Akt regulates the stimulation-dependent recycling of integrin beta1 to control cell migration. Dev Cell *9*, 663-673.

Li, L., and Cohen, S.N. (1996). Tsg101: a novel tumor susceptibility gene isolated by controlled homozygous functional knockout of allelic loci in mammalian cells. Cell *85*, 319-329.

Lindsay, A.J., Hendrick, A.G., Cantalupo, G., Senic-Matuglia, F., Goud, B., Bucci, C., and McCaffrey, M.W. (2002). Rab coupling protein (RCP), a novel Rab4 and Rab11 effector protein. J Biol Chem *277*, 12190-12199.

Lindsay, A.J., and McCaffrey, M.W. (2002). Rab11-FIP2 functions in transferrin recycling and associates with endosomal membranes via its COOH-terminal domain. J Biol Chem *277*, 27193-27199.

Lindsay, A.J., and McCaffrey, M.W. (2004). The C2 domains of the class I Rab11 family of interacting proteins target recycling vesicles to the plasma membrane. J Cell Sci *117*, 4365-4375.

Lock, J.G., and Stow, J.L. (2005). Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin. Mol Biol Cell *16*, 1744-1755.

Logan, C.Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol *20*, 781-810.

Lombardi, D., Soldati, T., Riederer, M.A., Goda, Y., Zerial, M., and Pfeffer, S.R. (1993). Rab9 functions in transport between late endosomes and the trans Golgi network. Embo J *12*, 677-682.

Magee, T., and Seabra, M.C. (2005). Fatty acylation and prenylation of proteins: what's hot in fat. Curr Opin Cell Biol *17*, 190-196.

Malaval, L., Liu, F., Vernallis, A.B., and Aubin, J.E. (2005). GP130/OSMR is the only LIF/IL-6 family receptor complex to promote osteoblast differentiation of calvaria progenitors. J Cell Physiol *204*, 585-593.

Mallo, G.V., Espina, M., Smith, A.C., Terebiznik, M.R., Aleman, A., Finlay, B.B., Rameh, L.E., Grinstein, S., Brumell, J.H. (2008). SopB promotes phosphatidylinositol 3-phosphate formation on Salmonella vacuoles by recruiting Rab5 and Vps34. J Cell Biol. *25*, 741-52.

Mammoto, A., Ohtsuka, T., Hotta, I., Sasaki, T., and Takai, Y. (1999). Rab11BP/Rabphilin-11, a downstream target of rab11 small G protein implicated in vesicle recycling. J Biol Chem *274*, 25517-25524.

Marchisio, P.C., Bergui, L., Corbascio, G.C., Cremona, O., D'Urso, N., Schena, M., Tesio, L., and Caligaris-Cappio, F. (1988). Vinculin, talin, and integrins are localized at specific adhesion sites of malignant B lymphocytes. Blood *72*, 830-833.

Marsman, M., Jordens, I., Kuijl, C., Janssen, L., and Neefjes, J. (2004). Dynein-mediated vesicle transport controls intracellular Salmonella replication. Mol Biol Cell *15*, 2954-2964.

Martin, T.J. (2002). Manipulating the environment of cancer cells in bone: a novel therapeutic approach. J Clin Invest *110*, 1399-1401.

Martin, T.J., Quinn, J.M., Gillespie, M.T., Ng, K.W., Karsdal, M.A., and Sims, N.A. (2006). Mechanisms involved in skeletal anabolic therapies. Ann N Y Acad Sci *1068*, 458-470.

Mattera, R., Tsai, Y.C., Weissman, A.M., and Bonifacino, J.S. (2006). The Rab5 guanine nucleotide exchange factor Rabex-5 binds ubiquitin (Ub) and functions as a Ub ligase through an atypical Ub-interacting motif and a zinc finger domain. J Biol Chem *281*, 6874-6883.

Maxfield, F.R., and McGraw, T.E. (2004). Endocytic recycling. Nat Rev Mol Cell Biol *5*, 121-132.

McCaffrey, M.W., Bielli, A., Cantalupo, G., Mora, S., Roberti, V., Santillo, M., Drummond, F., and Bucci, C. (2001). Rab4 affects both recycling and degradative endosomal trafficking. FEBS Lett *495*, 21-30.

McNiven, M.A. (1998). Dynamin: a molecular motor with pinchase action. Cell *94*, 151-154.

Mehta, A.D., Rock, R.S., Rief, M., Spudich, J.A., Mooseker, M.S., and Cheney, R.E. (1999). Myosin-V is a processive actin-based motor. Nature *400*, 590-593.

Mellman, I. (1996a). Endocytosis and molecular sorting. Annu Rev Cell Dev Biol *12*, 575-625.

Mellman, I. (1996b). Membranes and sorting. Curr Opin Cell Biol *8*, 497-498.

Meresse, S., Gorvel, J.P., and Chavrier, P. (1995). The rab7 GTPase resides on a vesicular compartment connected to lysosomes. J Cell Sci *108 (Pt 11)*, 3349-3358.

Miaczynska, M., Pelkmans, L., and Zerial, M. (2004). Not just a sink: endosomes in control of signal transduction. Curr Opin Cell Biol *16*, 400-406.

Miao, D., He, B., Jiang, Y., Kobayashi, T., Soroceanu, M.A., Zhao, J., Su, H., Tong, X., Amizuka, N., Gupta, A.*, et al.* (2005). Osteoblast-derived PTHrP is a potent endogenous bone anabolic agent that modifies the therapeutic efficacy of administered PTH 1-34. J Clin Invest *115*, 2402-2411.

Mimori-Kiyosue, Y., and Tsukita, S. (2003). "Search-and-capture" of microtubules through plus-end-binding proteins (+TIPs). J Biochem *134*, 321-326.

Mizuno, K., Kitamura, A., and Sasaki, T. (2003). Rabring7, a novel Rab7 target protein with a RING finger motif. Mol Biol Cell *14*, 3741-3752.

Mogensen, M.M., Malik, A., Piel, M., Bouckson-Castaing, V., and Bornens, M. (2000). Microtubule minus-end anchorage at centrosomal and non-centrosomal sites: the role of ninein. J Cell Sci *113 (Pt 17)*, 3013-3023.

Moll, J., Sansig, G., Fattori, E., and van der Putten, H. (1991). The murine rac1 gene: cDNA cloning, tissue distribution and regulated expression of rac1 mRNA by disassembly of actin microfilaments. Oncogene *6*, 863-866.

Mukherjee, S., and Maxfield, F.R. (2004). Lipid and cholesterol trafficking in NPC. Biochim Biophys Acta *1685*, 28-37.

Mulari, M.T., Zhao, H., Lakkakorpi, P.T., and Vaananen, H.K. (2003). Osteoclast ruffled border has distinct subdomains for secretion and degraded matrix uptake. Traffic *4*, 113-125.

Nagelkerken, B., Van Anken, E., Van Raak, M., Gerez, L., Mohrmann, K., Van Uden, N., Holthuizen, J., Pelkmans, L., and Van Der Sluijs, P. (2000). Rabaptin4, a novel effector of the small GTPase rab4a, is recruited to perinuclear recycling vesicles. Biochem J *346 Pt 3*, 593-601.

Nelson, W.J, (2008). Regulation of cell-cell adhesion by the cadherin-catenin complex. Biochem Soc Trans. *36(Pt 2)*, 149-55.

Nesbitt, S.A., and Horton, M.A. (1997). Trafficking of matrix collagens through bone-resorbing osteoclasts. Science *276*, 266-269.

Niikura, K., Takano, M., and Sawada, M. (2004). A novel inhibitor of vacuolar ATPase, FR167356, which can discriminate between osteoclast vacuolar ATPase and lysosomal vacuolar ATPase. Br J Pharmacol *142*, 558-566.

Nijweide et al., Principle of Bone Biology 2nd edi, 2002, Academic Press, USA pp 93-96

Nomura, N., Nagase, T., Miyajima, N., Sazuka, T., Tanaka, A., Sato, S., Seki, N., Kawarabayasi, Y., Ishikawa, K., and Tabata, S. (1994). Prediction of the coding sequences of unidentified human genes. II. The coding sequences of 40 new genes (KIAA0041-KIAA0080) deduced by analysis of cDNA clones from human cell line KG-1. DNA Res *1*, 223-229.

Novick, P., and Zerial, M. (1997). The diversity of Rab proteins in vesicle transport. Curr Opin Cell Biol *9*, 496-504.

Nuoffer, C., and Balch, W.E. (1994). GTPases: multifunctional molecular switches regulating vesicular traffic. Annu Rev Biochem *63*, 949-990.

Nydegger, S., Foti, M., Derdowski, A., Spearman, P., and Thali, M. (2003). HIV-1 egress is gated through late endosomal membranes. Traffic *4*, 902-910.

Odorizzi, G., Babst, M., and Emr, S.D. (2000). Phosphoinositide signaling and the regulation of membrane trafficking in yeast. Trends Biochem Sci *25*, 229-235.

Oh, P., McIntosh, D.P., and Schnitzer, J.E. (1998). Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. J Cell Biol *141*, 101-114.

Ohya, T., Miaczynska, M., Coskun, U., Lommer, B., Runge, A., Drechsel, D., Kalaidzidis, Y., Zerial, M. (2009) Reconstitution of Rab- and SNARE-dependent membrane fusion by synthetic endosomes. Nature *25*, 1091-7.

Ory, D.S. (2000). Niemann-Pick type C: a disorder of cellular cholesterol trafficking. Biochim Biophys Acta *1529*, 331-339.

Otomo, A., Hadano, S., Okada, T., Mizumura, H., Kunita, R., Nishijima, H., Showguchi-Miyata, J., Yanagisawa, Y., Kohiki, E., Suga, E.*, et al.* (2003). ALS2, a novel guanine nucleotide exchange factor for the small GTPase Rab5, is implicated in endosomal dynamics. Hum Mol Genet *12*, 1671-1687.

Palmqvist, P., Persson, E., Conaway, H.H., and Lerner, U.H. (2002). IL-6, leukemia inhibitory factor, and oncostatin M stimulate bone resorption and regulate the expression of receptor activator of NF-kappa B ligand, osteoprotegerin, and receptor activator of NF-kappa B in mouse calvariae. J Immunol *169*, 3353-3362.

Palokangas, H., Mulari, M., and Vaananen, H.K. (1997). Endocytic pathway from the basal plasma membrane to the ruffled border membrane in

bone-resorbing osteoclasts. J Cell Sci *110 (Pt 15)*, 1767-1780.

Parton, R.G., and Richards, A.A. (2003). Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms. Traffic *4*, 724-738.

Paschal, B.M., and Vallee, R.B. (1987). Retrograde transport by the microtubule-associated protein MAP 1C. Nature *330*, 181-183.

Pasqualato, S., Renault, L., and Cherfils, J. (2002). Arf, Arl, Arp and Sar proteins: a family of GTP-binding proteins with a structural device for 'front-back' communication. EMBO Rep *3*, 1035-1041.

Pasqualato, S., Senic-Matuglia, F., Renault, L., Goud, B., Salamero, J., and Cherfils, J. (2004). The structural GDP/GTP cycle of Rab11 reveals a novel interface involved in the dynamics of recycling endosomes. J Biol Chem *279*, 11480-11488.

Patki, V., Lawe, D.C., Corvera, S., Virbasius, J.V., and Chawla, A. (1998). A functional PtdIns(3) P-binding motif. Nature *394*, 433-434.

Peden, A.A., Schonteich, E., Chun, J., Junutula, J.R., Scheller, R.H., and Prekeris, R. (2004). The RCP-Rab11 complex regulates endocytic protein sorting. Mol Biol Cell *15*, 3530-3541.

Pelissier, A., Chauvin, J.P., and Lecuit, T. (2003). Trafficking through Rab11 endosomes is required for cellularization during Drosophila embryogenesis. Curr Biol *13*, 1848-1857.

Percherancier, Y., Lagane, B., Planchenault, T., Staropoli, I., Altmeyer, R., Virelizier, J.L., Arenzana-Seisdedos, F., Hoessli, D.C., and Bachelerie, F. (2003). HIV-1 entry into T-cells is not dependent on CD4 and CCR5 localization to sphingolipid-enriched, detergent-resistant, raft membrane domains. J Biol Chem *278*, 3153-3161.

Pereira-Leal, J.B., and Seabra, M.C. (2000). The mammalian Rab family of small GTPases: definition of family and subfamily sequence motifs suggests a mechanism for functional specificity in the Ras superfamily. J Mol Biol *301*, 1077-1087.

Perez, F., Diamantopoulos, G.S., Stalder, R., and Kreis, T.E. (1999). CLIP-170 highlights growing microtubule ends in vivo. Cell *96*, 517-527.

Pfeffer, S.R. (2001). Rab GTPases: specifying and deciphering organelle identity and function. Trends Cell Biol *11*, 487-491.

Pierre, P., Scheel, J., Rickard, J.E., and Kreis, T.E. (1992). CLIP-170 links endocytic vesicles to microtubules. Cell *70*, 887-900.

Pixley, F.J., and Stanley, E.R. (2004). CSF-1 regulation of the wandering macrophage: complexity in action. Trends Cell Biol *14*, 628-638.

Pizarro-Cerda, J., and Cossart, P. (2006). Bacterial adhesion and entry into host cells. Cell *124*, 715-727.

Powelka, A.M., Sun, J., Li, J., Gao, M., Shaw, L.M., Sonnenberg, A., and Hsu, V.W. (2004). Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. Traffic *5*, 20-36.

Prekeris, R. (2003). Rabs, Rips, FIPs, and endocytic membrane traffic. Scientific World Journal *3*, 870-880.

Provance, D.W., Jr., Gourley, C.R., Silan, C.M., Cameron, L.C., Shokat, K.M., Goldenring, J.R., Shah, K., Gillespie, P.G., and Mercer, J.A. (2004). Chemical-genetic inhibition of a sensitized mutant myosin Vb demonstrates a role in peripheral-pericentriolar membrane traffic. Proc Natl Acad Sci U S A *101*, 1868-1873.

Raiborg, C., Bache, K.G., Gillooly, D.J., Madshus, I.H., Stang, E., and Stenmark, H. (2002). Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. Nat Cell Biol *4*, 394-398.

Raiborg, C., Slagsvold, T., and Stenmark, H. (2006). A new side to ubiquitin. Trends Biochem Sci *31*, 541-544.

Rauma, T., Tuukkanen, J., Bergelson, J.M., Denning, G., and Hautala, T. (1999). rab5 GTPase regulates adenovirus endocytosis. J Virol *73*, 9664-9668.

Reid, I.R. (1997). Glucocorticoid osteoporosis--mechanisms and management. Eur J Endocrinol *137*, 209-217.

Ren, M., Xu, G., Zeng, J., De Lemos-Chiarandini, C., Adesnik, M., and Sabatini, D.D. (1998). Hydrolysis of GTP on rab11 is required for the direct delivery of transferrin from the pericentriolar recycling compartment to the cell surface but not from sorting endosomes. Proc Natl Acad Sci U S A *95*, 6187-6192.

Repasky, G.A., Chenette, E.J., and Der, C.J. (2004). Renewing the conspiracy theory debate: does Raf function alone to mediate Ras oncogenesis? Trends Cell Biol *14*, 639-647.

Ridley, A. J., (2001). Rho proteins: linking signaling with membrane trafficking. Traffic *2(5)*, 3003-10

Riggs, B., Rothwell, W., Mische, S., Hickson, G.R., Matheson, J., Hays, T.S., Gould, G.W., and Sullivan, W. (2003). Actin cytoskeleton remodeling during early Drosophila furrow formation requires recycling endosomal

components Nuclear-fallout and Rab11. J Cell Biol *163*, 143-154.

Roberts, M., Barry, S., Woods, A., van der Sluijs, P., and Norman, J. (2001). PDGF-regulated rab4-dependent recycling of alphavbeta3 integrin from early endosomes is necessary for cell adhesion and spreading. Curr Biol *11*, 1392-1402.

Roberts, M.S., Woods, A.J., Dale, T.C., Van Der Sluijs, P., and Norman, J.C. (2004). Protein kinase B/Akt acts via glycogen synthase kinase 3 to regulate recycling of alpha v beta 3 and alpha 5 beta 1 integrins. Mol Cell Biol *24*, 1505-1515.

Ross, F.P. (2006). M-CSF, c-Fms, and signaling in osteoclasts and their precursors. Ann N Y Acad Sci *1068*, 110-116.

Russell, R.G., Watts, N.B., Ebetino, F.H., and Rogers, M.J. (2008). Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporos Int *19*, 733-759.

Salo, J., Lehenkari, P., Mulari, M., Metsikko, K., and Vaananen, H.K. (1997). Removal of osteoclast bone resorption products by transcytosis. Science *276*, 270-273.

Salo, J., Metsikko, K., Palokangas, H., Lehenkari, P., and Vaananen, H.K. (1996). Bone-resorbing osteoclasts reveal a dynamic division of basal plasma membrane into two different domains. J Cell Sci *109 (Pt 2)*, 301-307.

Sasaki, T., Debari, K., and Udagawa, N. (1993). Cytochalasin D reduces osteoclastic bone resorption by inhibiting development of ruffled border-clear zone complex. Calcif Tissue Int *53*, 217-221.

Sasaki, T., Kikuchi, A., Araki, S., Hata, Y., Isomura, M., Kuroda, S., and Takai, Y. (1990). Purification and characterization from bovine brain cytosol of a protein that inhibits the dissociation of GDP from and the subsequent binding of GTP to smg p25A, a ras p21-like GTP-binding protein. J Biol Chem *265*, 2333-2337.

Sato, M., Sato, K., Fonarev, P., Huang, C.J., Liou, W., and Grant, B.D. (2005). Caenorhabditis elegans RME-6 is a novel regulator of RAB-5 at the clathrin-coated pit. Nat Cell Biol *7*, 559-569.

Sato, T.K., Overduin, M., and Emr, S.D. (2001). Location, location, location: membrane targeting directed by PX domains. Science *294*, 1881-1885.

Satoh, A.K., O'Tousa, J.E., Ozaki, K., and Ready, D.F. (2005). Rab11 mediates post-Golgi trafficking of rhodopsin to the photosensitive apical membrane of Drosophila photoreceptors. Development *132*, 1487-1497.

Savina, A., Vidal, M., and Colombo, M.I. (2002). The exosome pathway in K562 cells is regulated by Rab11. J Cell Sci *115*, 2505-2515.

Scheiffele, P., Roth, M.G., and Simons, K. (1997). Interaction of influenza virus haemagglutinin with sphingolipid-cholesterol membrane domains via its transmembrane domain. Embo J *16*, 5501-5508.

Schimmoller, F., and Riezman, H. (1993). Involvement of Ypt7p, a small GTPase, in traffic from late endosome to the vacuole in yeast. J Cell Sci *106 (Pt 3)*, 823-830.

Schmidt, A., and Hall, A. (2002). Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev *16*, 1587-1609.

Schmidt, C.E., Horwitz, A.F., Lauffenburger, D.A., and Sheetz, M.P. (1993). Integrin-cytoskeletal interactions in migrating fibroblasts are dynamic, asymmetric, and regulated. J Cell Biol *123*, 977-991.

Schnapp, B.J., and Reese, T.S. (1989). Dynein is the motor for retrograde axonal transport of organelles. Proc Natl Acad Sci U S A *86*, 1548-1552.

Schroer, T.A. (2004). Dynactin. Annu Rev Cell Dev Biol *20*, 759-779.

Schuck, S., Honsho, M., Ekroos, K., Shevchenko, A., and Simons, K. (2003). Resistance of cell membranes to different detergents. Proc Natl Acad Sci U S A *100*, 5795-5800.

Seabra, M.C. (1996). Nucleotide dependence of Rab geranylgeranylation. Rab escort protein interacts preferentially with GDP-bound Rab. J Biol Chem *271*, 14398-14404.

Seabra, M.C., Brown, M.S., Slaughter, C.A., Sudhof, T.C., and Goldstein, J.L. (1992). Purification of component A of Rab geranylgeranyl transferase: possible identity with the choroideremia gene product. Cell *70*, 1049-1057.

Seabra, M.C., and Wasmeier, C. (2004). Controlling the location and activation of Rab GTPases. Curr Opin Cell Biol *16*, 451-457.

Seachrist, J.L., Anborgh, P.H., and Ferguson, S.S. (2000). beta 2-adrenergic receptor internalization, endosomal sorting, and plasma membrane recycling are regulated by rab GTPases. J Biol Chem *275*, 27221-27228.

Sedej, S., Rupnik, M., and Zorec, R. (2005). Endocytosis-dominated membrane area decrease requires Rab5 protein in rat melanotrophs. Ann N Y Acad Sci *1048*, 272-280.

Segev, N. (2001). Ypt and Rab GTPases: insight into functions through novel interactions. Curr Opin Cell Biol *13*, 500-511.

Selander, K.S., Harkonen, P.L., Valve, E., Monkkonen, J., Hannuniemi, R., and Vaananen, H.K. (1996). Calcitonin promotes osteoclast survival *in vitro*. Mol Cell Endocrinol *122*, 119-129.

Sharma, S.M., Bronisz, A., Hu, R., Patel, K., Mansky, K.C., Sif, S., and Ostrowski, M.C. (2007). MITF and PU.1 recruit p38 MAPK and NFATc1 to target genes during osteoclast differentiation. J Biol Chem *282*, 15921-15929.

Sheff, D.R., Daro, E.A., Hull, M., and Mellman, I. (1999). The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions. J Cell Biol *145*, 123-139.

Shelanski, M.L., Gaskin, F., and Cantor, C.R. (1973). Microtubule assembly in the absence of added nucleotides. Proc Natl Acad Sci U S A *70*, 765-768.

Shiba, T., Koga, H., Shin, H.W., Kawasaki, M., Kato, R., Nakayama, K., and Wakatsuki, S. (2006). Structural basis for Rab11-dependent membrane recruitment of a family of Rab11-interacting protein 3 (FIP3)/Arfophilin-1. Proc Natl Acad Sci U S A *103*, 15416-15421.

Shimizu, T., and Sasaki, T. (1991). Ultrastructural study of the effects of cytochalasin D administration on the structure and acid trimetaphosphatase activity of osteoclast. J Electron Microsc (Tokyo) *40*, 346-355.

Shin, H.W., Hayashi, M., Christoforidis, S., Lacas-Gervais, S., Hoepfner, S., Wenk, M.R., Modregger, J., Uttenweiler-Joseph, S., Wilm, M., Nystuen, A.*, et al.* (2005). An enzymatic cascade of Rab5 effectors regulates phosphoinositide turnover in the endocytic pathway. J Cell Biol *170*, 607-618.

Shirataki, H., Kaibuchi, K., Sakoda, T., Kishida, S., Yamaguchi, T., Wada, K., Miyazaki, M., and Takai, Y. (1993). Rabphilin-3A, a putative target protein for smg p25A/rab3A p25 small GTP-binding protein related to synaptotagmin. Mol Cell Biol *13*, 2061-2068.

Shirsat, N.V., Pignolo, R.J., Kreider, B.L., and Rovera, G. (1990). A member of the ras gene superfamily is expressed specifically in T, B and myeloid hemopoietic cells. Oncogene *5*, 769-772.

Simons, K., and Ikonen, E. (1997). Functional rafts in cell membranes. Nature *387*, 569-572.

Simonsen, A., Gaullier, J.M., D'Arrigo, A., and Stenmark, H. (1999). The Rab5 effector EEA1 interacts directly with syntaxin-6. J Biol Chem *274*, 28857-28860.

Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J.M., Brech, A., Callaghan, J., Toh, B.H., Murphy, C., Zerial, M., and Stenmark, H. (1998). EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature *394*, 494-498.

Sims, N.A. (2008). gp130 signaling in bone cell biology: Multiple roles revealed by analysis of genetically altered mice. Mol Cell Endocrinol.

Slaughter, T., Wang, J., and Black, M.M. (1997). Microtubule transport from the cell body into the axons of growing neurons. J Neurosci *17*, 5807-5819.

Sogaard, M., Tani, K., Ye, R.R., Geromanos, S., Tempst, P., Kirchhausen, T., Rothman, J.E., and Sollner, T. (1994). A rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. Cell *78*, 937-948.

Soldati, T., Rancano, C., Geissler, H., and Pfeffer, S.R. (1995). Rab7 and Rab9 are recruited onto late endosomes by biochemically distinguishable processes. J Biol Chem *270*, 25541-25548.

Sonnichsen, B., De Renzis, S., Nielsen, E., Rietdorf, J., and Zerial, M. (2000). Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. J Cell Biol *149*, 901-914.

Sorensen, M.G., Henriksen, K., Schaller, S., Henriksen, D.B., Nielsen, F.C., Dziegiel, M.H., and Karsdal, M.A. (2007). Characterization of osteoclasts derived from CD14+ monocytes isolated from peripheral blood. J Bone Miner Metab *25*, 36-45.

Srinivasan, S., Wang, F., Glavas, S., Ott, A., Hofmann, F., Aktories, K., Kalman, D., and Bourne, H.R. (2003). Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis. J Cell Biol *160*, 375-385.

Stein, M.P., Feng, Y., Cooper, K.L., Welford, A.M., and Wandinger-Ness, A. (2003). Human VPS34 and p150 are Rab7 interacting partners. Traffic *4*, 754-771.

Stenbeck, G., and Horton, M.A. (2004). Endocytic trafficking in actively resorbing osteoclasts. J Cell Sci *117*, 827-836.

Stenmark, H., Vitale, G., Ullrich, O., and Zerial, M. (1995). Rabaptin-5 is a direct effector of the small GTPase Rab5 in endocytic membrane fusion. Cell *83*, 423-432.

Strick, D.J., and Elferink, L.A. (2005). Rab15 effector protein: a novel protein for receptor recycling from the endocytic recycling compartment. Mol Biol Cell *16*, 5699-5709.

Strom, M., Hume, A.N., Tarafder, A.K., Barkagianni, E., and Seabra, M.C. (2002). A family of Rab27-binding proteins. Melanophilin links Rab27a and

myosin Va function in melanosome transport. J Biol Chem *277*, 25423-25430.

Suda, T., Takahashi, N., Udagawa, N., Jimi, E., Gillespie, M.T., and Martin, T.J. (1999). Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev *20*, 345-357.

Sugihara, K., Nakatsuji, N., Nakamura, K., Nakao, K., Hashimoto, R., Otani, H., Sakagami, H., Kondo, H., Nozawa, S., Aiba, A., and Katsuki, M. (1998). Rac1 is required for the formation of three germ layers during gastrulation. Oncogene *17*, 3427-3433.

Swanson, K.M., and Hohl, R.J. (2006). Anti-cancer therapy: targeting the mevalonate pathway. Curr Cancer Drug Targets *6*, 15-37.

Takahashi, N., MacDonald, B.R., Hon, J., Winkler, M.E., Derynck, R., Mundy, G.R., and Roodman, G.D. (1986). Recombinant human transforming growth factor-alpha stimulates the formation of osteoclast-like cells in long-term human marrow cultures. J Clin Invest *78*, 894-898.

Tall, G.G., Barbieri, M.A., Stahl, P.D., and Horazdovsky, B.F. (2001). Ras-activated endocytosis is mediated by the Rab5 guanine nucleotide exchange activity of RIN1. Dev Cell *1*, 73-82.

ten Dijke, P., Fu, J., Schaap, P., and Roelen, B.A. (2003). Signal transduction of bone morphogenetic proteins in osteoblast differentiation. J Bone Joint Surg Am *85-A Suppl 3*, 34-38.

Tezuka, K., Nemoto, K., Tezuka, Y., Sato, T., Ikeda, Y., Kobori, M., Kawashima, H., Eguchi, H., Hakeda, Y., and Kumegawa, M. (1994). Identification of matrix metalloproteinase 9 in rabbit osteoclasts. J Biol Chem *269*, 15006-15009.

Thirunavukkarasu, K., Halladay, D.L., Miles, R.R., Yang, X., Galvin, R.J., Chandrasekhar, S., Martin, T.J., and Onyia, J.E. (2000). The osteoblast-specific transcription factor Cbfa1 contributes to the expression of osteoprotegerin, a potent inhibitor of osteoclast differentiation and function. J Biol Chem *275*, 25163-25172.

Tjelle, T.E., Brech, A., Juvet, L.K., Griffiths, G., and Berg, T. (1996). Isolation and characterization of early endosomes, late endosomes and terminal lysosomes: their role in protein degradation. J Cell Sci *109 (Pt 12)*, 2905-2914.

Tolmachova, T., Anders, R., Stinchcombe, J., Bossi, G., Griffiths, G.M., Huxley, C., and Seabra, M.C. (2004). A general role for Rab27a in secretory cells. Mol Biol Cell *15*, 332-344.

Touchot, N., Chardin, P., and Tavitian, A. (1987). Four additional members of the ras gene superfamily isolated by an oligonucleotide strategy: molecular cloning of YPT-related cDNAs from a rat brain library. Proc Natl Acad Sci U S A *84*, 8210-8214.

Tuma, P.L., and Hubbard, A.L. (2003). Transcytosis: crossing cellular barriers. Physiol Rev *83*, 871-932.

Tuxworth, R.I., and Titus, M.A. (2000). Unconventional myosins: anchors in the membrane traffic relay. Traffic *1*, 11-18.

Ullrich, O., Reinsch, S., Urbe, S., Zerial, M., and Parton, R.G. (1996). Rab11 regulates recycling through the pericentriolar recycling endosome. J Cell Biol *135*, 913-924.

Unger, E., Bohm, K.J., and Vater, W. (1990). Structural diversity and dynamics of microtubules and polymorphic tubulin assemblies. Electron Microsc Rev *3*, 355-395.

Urbe, S., Huber, L.A., Zerial, M., Tooze, S.A., and Parton, R.G. (1993). Rab11, a small GTPase associated with both constitutive and regulated secretory pathways in PC12 cells. FEBS Lett *334*, 175-182.

Vaananen, H.K., Karhukorpi, E.K., Sundquist, K., Wallmark, B., Roininen, I., Hentunen, T., Tuukkanen, J., and Lakkakorpi, P. (1990). Evidence for the presence of a proton pump of the vacuolar H(+)-ATPase type in the ruffled borders of osteoclasts. J Cell Biol *111*, 1305-1311.

Vaananen, H.K., and Laitala-Leinonen, T. (2008). Osteoclast lineage and function. Arch Biochem Biophys *473*, 132-138.

Vaaraniemi, J., Halleen, J.M., Kaarlonen, K., Ylipahkala, H., Alatalo, S.L., Andersson, G., Kaija, H., Vihko, P., and Vaananen, H.K. (2004). Intracellular machinery for matrix degradation in bone-resorbing osteoclasts. J Bone Miner Res *19*, 1432-1440.

Vale, R.D. (2003). The molecular motor toolbox for intracellular transport. Cell *112*, 467-480.

van Delft, S., Govers, R., Strous, G.J., Verkleij, A.J., and van Bergen en Henegouwen, P.M. (1997). Epidermal growth factor induces ubiquitination of Eps15. J Biol Chem *272*, 14013-14016.

van der Bliek, A.M. (2005). A sixth sense for Rab5. Nat Cell Biol *7*, 548-550.

van der Goot, F.G., and Gruenberg, J. (2006). Intra-endosomal membrane traffic. Trends Cell Biol *16*, 514-521.

van der Sluijs, P., Hull, M., Webster, P., Male, P., Goud, B., and Mellman, I. (1992). The small GTP-binding protein rab4 controls an early

sorting event on the endocytic pathway. Cell *70*, 729-740.

van Deurs, B., Holm, P.K., and Sandvig, K. (1996). Inhibition of the vacuolar H(+)-ATPase with bafilomycin reduces delivery of internalized molecules from mature multivesicular endosomes to lysosomes in HEp-2 cells. Eur J Cell Biol *69*, 343-350.

van Leeuwen, J.P., van Driel, M., van den Bemd, G.J., and Pols, H.A. (2001). Vitamin D control of osteoblast function and bone extracellular matrix mineralization. Crit Rev Eukaryot Gene Expr *11*, 199-226.

Vitale, G., Rybin, V., Christoforidis, S., Thornqvist, P., McCaffrey, M., Stenmark, H., and Zerial, M. (1998). Distinct Rab-binding domains mediate the interaction of Rabaptin-5 with GTP-bound Rab4 and Rab5. Embo J *17*, 1941-1951.

Vukmirica, J., Monzo, P., Le Marchand-Brustel, Y., and Cormont, M. (2006). The Rab4A effector protein Rabip4 is involved in migration of NIH 3T3 fibroblasts. J Biol Chem *281*, 36360-36368.

Wakabayashi, Y., Lippincott-Schwartz, J., and Arias, I.M. (2004). Intracellular trafficking of bile salt export pump (ABCB11) in polarized hepatic cells: constitutive cycling between the canalicular membrane and rab11-positive endosomes. Mol Biol Cell *15*, 3485-3496.

Wallace, D.M., Lindsay, A.J., Hendrick, A.G., and McCaffrey, M.W. (2002). Rab11-FIP4 interacts with Rab11 in a GTP-dependent manner and its overexpression condenses the Rab11 positive compartment in HeLa cells. Biochem Biophys Res Commun *299*, 770-779.

Wang, B., Wylie, F.G., Teasdale, R.D., and Stow, J.L. (2005). Polarized trafficking of E-cadherin is regulated by Rac1 and Cdc42 in Madin-Darby canine kidney cells. Am J Physiol Cell Physiol *288*, C1411-1419.

Wang, T., Wong, K.K., and Hong, W. (2004). A unique region of RILP distinguishes it from its related proteins in its regulation of lysosomal morphology and interaction with Rab7 and Rab34. Mol Biol Cell *15*, 815-826.

Ward, E.S., Martinez, C., Vaccaro, C., Zhou, J., Tang, Q., and Ober, R.J. (2005). From sorting endosomes to exocytosis: association of Rab4 and Rab11 GTPases with the Fc receptor, FcRn, during recycling. Mol Biol Cell *16*, 2028-2038.

Waters, M.G., and Pfeffer, S.R. (1999). Membrane tethering in intracellular transport. Curr Opin Cell Biol *11*, 453-459.

Weernink, P.A., Meletiadis, K., Hommeltenberg, S., Hinz, M., Ishihara, H., Schmidt, M., and Jakobs, K.H. (2004). Activation of type I phosphatidylinositol 4-phosphate 5-kinase isoforms by the Rho GTPases, RhoA, Rac1, and Cdc42. J Biol Chem *279*, 7840-7849.

Weinstein, R.S., Jilka, R.L., Parfitt, A.M., and Manolagas, S.C. (1998). Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Invest *102*, 274-282.

Weis, K. (2003). Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle. Cell *112*, 441-451.

Weissbach, L., Settleman, J., Kalady, M.F., Snijders, A.J., Murthy, A.E., Yan, Y.X., and Bernards, A. (1994). Identification of a human rasGAP-related protein containing calmodulin-binding motifs. J Biol Chem *269*, 20517-20521.

Wells, C.M., Walmsley, M., Ooi, S., Tybulewicz, V., and Ridley, A.J. (2004). Rac1-deficient macrophages exhibit defects in cell spreading and membrane ruffling but not migration. J Cell Sci *117*, 1259-1268.

Wennerberg, K., Rossman, K.L., and Der, C.J. (2005). The Ras superfamily at a glance. J Cell Sci *118*, 843-846.

Wilcke, M., Johannes, L., Galli, T., Mayau, V., Goud, B., and Salamero, J. (2000). Rab11 regulates the compartmentalization of early endosomes required for efficient transport from early endosomes to the trans-golgi network. J Cell Biol *151*, 1207-1220.

Wilflingseder, D., and Stoiber, H. (2007). Float on: lipid rafts in the lifecycle of HIV. Front Biosci *12*, 2124-2135.

Wilson, K.L., Zastrow, M.S., and Lee, K.K. (2001). Lamins and disease: insights into nuclear infrastructure. Cell *104*, 647-650.

Woodman, P.G. (2000). Biogenesis of the sorting endosome: the role of Rab5. Traffic *1*, 695-701.

Woods, A.J., White, D.P., Caswell, P.T., and Norman, J.C. (2004). PKD1/PKCmu promotes alphavbeta3 integrin recycling and delivery to nascent focal adhesions. Embo J *23*, 2531-2543.

Wu, M., Wang, T., Loh, E., Hong, W., and Song, H. (2005). Structural basis for recruitment of RILP by small GTPase Rab7. Embo J *24*, 1491-1501.

Wu, X., Peters, J.M., Gonzalez, F.J., Prasad, H.S., Rohrer, M.D., and Gimble, J.M. (2000). Frequency of stromal lineage colony forming units in bone marrow of peroxisome proliferator-activated receptor-alpha-null mice. Bone *26*, 21-26.

Wurmser, A.E., and Emr, S.D. (1998). Phosphoinositide signaling and turnover: PtdIns(3)P, a regulator of membrane traffic, is transported to the vacuole and degraded by a process that requires lumenal vacuolar hydrolase activities. Embo J *17*, 4930-4942.

Xiao, G., Wang, D., Benson, M.D., Karsenty, G., and Franceschi, R.T. (1998). Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. J Biol Chem *273*, 32988-32994.

Xing, L., and Boyce, B.F. (2005). Regulation of apoptosis in osteoclasts and osteoblastic cells. Biochem Biophys Res Commun *328*, 709-720.

Xu, W., Baribault, H., and Adamson, E.D. (1998). Vinculin knockout results in heart and brain defects during embryonic development. Development *125*, 327-337.

Yamada, S., Pokutta, S., Drees, F., Weis, W.I., and Nelson, W.J. (2005). Deconstructing the cadherin-catenin-actin complex. Cell *123*, 889-901.

Yoon, S.O., Shin, S., and Mercurio, A.M. (2005). Hypoxia stimulates carcinoma invasion by stabilizing microtubules and promoting the Rab11 trafficking of the alpha6beta4 integrin. Cancer Res *65*, 2761-2769.

Zaidi, M. (1990). Modularity of osteoclast behaviour and "mode-specific" inhibition of osteoclast function. Biosci Rep *10*, 547-556.

Zerial, M., and McBride, H. (2001). Rab proteins as membrane organizers. Nat Rev Mol Cell Biol *2*, 107-117.

Zerial, M., and Stenmark, H. (1993). Rab GTPases in vesicular transport. Curr Opin Cell Biol *5*, 613-620.

Zhang, J., Pekosz, A., and Lamb, R.A. (2000). Influenza virus assembly and lipid raft microdomains: a role for the cytoplasmic tails of the spike glycoproteins. J Virol *74*, 4634-4644.

Zhang, M., Dwyer, N.K., Love, D.C., Cooney, A., Comly, M., Neufeld, E., Pentchev, P.G., Blanchette-Mackie, E.J., and Hanover, J.A. (2001). Cessation of rapid late endosomal tubulovesicular trafficking in Niemann-Pick type C1 disease. Proc Natl Acad Sci U S A *98*, 4466-4471.

Zhang, M., Xuan, S., Bouxsein, M.L., von Stechow, D., Akeno, N., Faugere, M.C., Malluche, H., Zhao, G., Rosen, C.J., Efstratiadis, A., and Clemens, T.L. (2002). Osteoblast-specific knockout of the insulin-like growth factor (IGF) receptor gene reveals an essential role of IGF signaling in bone matrix mineralization. J Biol Chem *277*, 44005-44012.

Zhang, X., Glogauer, M., Zhu, F., Kim, T.H., Chiu, B., and Inman, R.D. (2005). Innate immunity and arthritis: neutrophil Rac and toll-like receptor 4 expression define outcomes in infection-triggered arthritis. Arthritis Rheum *52*, 1297-1304.

Zhao, H., Ettala, O., and Vaananen, H.K. (2002). Intracellular membrane trafficking pathways in bone-resorbing osteoclasts revealed by cloning and subcellular localization studies of small GTP-binding rab proteins. Biochem Biophys Res Commun *293*, 1060-1065.

Zhao, H., Laitala-Leinonen, T., Parikka, V., and Vaananen, H.K. (2001). Downregulation of small GTPase Rab7 impairs osteoclast polarization and bone resorption. J Biol Chem *276*, 39295-39302.

Zhao, H., and Vaananen, H.K. (2006). Pharmacological sequestration of intracellular cholesterol in late endosomes disrupts ruffled border formation in osteoclasts. J Bone Miner Res *21*, 456-465.